UNCOVERING THE MECHANISMS OF *TRANS*-ARACHIDONIC ACIDS: FUNCTION AND IMPLICATIONS FOR CEREBRAL ISCHEMIA AND BEYOND

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I dedicate this thesis to my parents, Slah and Najoua, my sisters Zeineb and Yosr, my late grandmother bibi Jnina, my beloved grandmother bibi Nefissa, to my extended family who always showed support and last but not least, to Kais for always being there.

Mamma, Papa, j'espère que cet aboutissement est digne de votre fierté et qu'il comble les rêves et les projets que vous aviez pour moi. Cette thèse, du début jusqu'à la fin, n'aurait pas été possible sans votre présence continue, vos encouragements incessants et votre amour inconditionnel. Cette thèse est faite en partie pour vous et surtout grâce à vous. Merci de m'avoir donné des bases d'éducation solides surlesquelles j'ai pu grandir et m'améliorer, d'être présents dans ma vie aussi bien dans les moments difficiles de ce parcours que les moments de reconnaissance et surtout de m'avoir appris tout ce que je sais.

TABLE OF CONTENTS

ABSTRACT

Cerebral ischemia is the principal cause of morbidity and mortality worldwide. In addition to neuronal loss associated with hypoxic-ischemic damage, cerebral ischemia is characterized by a neuromicrovascular injury. Nitrative stress and lipid peroxidation increase in hypoxic-ischemic damages and play an essential role in neuromicrovascular injury leading to cerebral ischemia. We hypothesized that newly described lipid peroxidation products, termed *trans*-arachidonic acids (*T*AA), could be implicated in the pathogenesis of hypoxia-ischemia by affecting the cerebral vasomotricity and microvascular integrity.

The effects of *T*AA on neuromicrovascular tone were tested *ex vivo* by monitoring the changes in vascular diameter of rat cerebral pial microvessels. Four isomers of *T*AA, namely 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA induced an endothelium-dependent vasorelaxation. Possible mechanisms involved in *T*AA-induced vasorelaxation were thoroughly investigated. Collectively, data enclosed revealed that *T*AA induce cerebral vasorelaxation through the interactive activation of BK_{Ca} channels with heme oxygenase-2. This interaction leads to generation of carbon monoxide which in turn activates soluble guanylate cyclase and triggers vasorelaxation.

Chronic effects of *T*AA on microvascular integrity were examined by generating a unilateral hypoxic-ischemic (HI) model of cerebral ischemia on newborn rat pups. Our HI model showed microvascular degeneration as early as 24h post-HI, preceded by an increase in cerebral *T*AA levels. HI-induced microvascular lesions were dependent on nitric oxide synthase activation and ensued *T*AA formation. Although the molecular mechanisms leading to *T*AA-induced microvascular degeneration were, in part uncovered for the retina, the primary site of action of *T*AA remains unknown. We demonstrated that *T*AA binds and activates GPR40 receptor, a newly described free fatty acid receptor. Importantly, GPR40 receptor knock-out prevents *T*AA-induced reduction in cerebral microvascular density and limits HI-induced brain infarct.

These novel data depict a new biological role for *T*AA, reveal for the first time the primary target of non-enzymatically produced lipid peroxidation products (*T*AA) and identify a novel function for GPR40 receptor in the brain. These data impart several interesting avenues for future therapeutic research involving *T*AA and GPR40 antagonism to limit the extent of ischemic brain injuries.

RÉSUMÉ

Les ischémies cérébrales constituent la principale cause de morbi-mortalité dans le monde. En plus de la mort neuronale associée aux dommages hypoxiques-ischémiques, les ischémies cérébrales sont caractérisées par une lésion neuromicrovasculaire. Les stress nitratif et peroxydation lipidique augmentent dans les dommages hypoxiques-ischémiques en jouant un rôle essentiel dans l'induction des lésions neuromicrovasculaires conduisant à une ischémie cérébrale. Nous avons donc émis l'hypothèse, que les produits de peroxidation lipidiques nouvellement décrits, appelés acides *trans*-arachidoniques (*T*AA) pourraient être impliqués dans la pathogénèse des hypoxies-ischémies, en affectant la vasomotricité cérébrale et l'intégrité microvasculaire.

Les effets des *T*AA sur le tonus neuromicrovasculaire ont été testés *ex vivo*, en contrôlant les changements du diamètre artériel des microvaisseaux piaux cérébraux, chez le rat. Les quatre isomères des *T*AA nommés 5*E*-AA, 8*E*-AA, 11*E*-AA et 14 *E*-AA exercent une vasodilatation dépendante de l'endothélium. Les mécanismes possibles impliqués dans cette vasorelaxation induite par les *T*AA ont été investigués de manière approfondie. Les données incluses révèlent que les *T*AA induisent une vasorelaxation à travers une activation interactive des canaux BK_{Ca} avec l'hème oxygénase-2. Cette interaction génère du monoxyde de carbone qui, à son tour active la guanylate cyclase responsable de la vasorelaxation cérébrale.

Les effets chroniques des *T*AA sur l'intégrité neuromicrovasculaire ont été examinés en générant un modèle animal d'hypoxie-ischémie (HI) unilatérale sur des ratons nouveaux nés. Notre modèle d'HI a montré une dégénérescence microvasculaire aussi tôt que 24h après l'induction du modèle, précédée par une augmentation des niveaux cérébraux des *T*AA. Les lésions microvasculaires induites par le modèle de HI sont dépendantes de l'activation de l'oxyde nitrique synthase et par conséquent de la formation des *T*AA. Alors que les mécanismes moléculaires amenant à la dégénérescence microvasculaire induite par les *T*AA

ont déjà été démontré au niveau de la rétine, la cible primaire des *T*AA demeure inconnue. Nous avons démontré que les *T*AA lient et activent le récepteur GPR40, nouvellement décrit comme récepteur des acides gras libres. De plus, l'ablation génique de GPR40 prévient la réduction de la densité vasculaire cérébrale induite par les *T*AA et limite l'infarctus cérébral induit par l'HI.

Ces nouvelles données illustrent un nouveau rôle biologique des *T*AA, révèlent pour la première fois une cible primaire pour des produits non enzymatiques de peroxydation lipidique (les *T*AA) et identifient une nouvelle fonction pour le récepteur GPR40 au niveau du cerveau. Ces données confèrent plusieurs axes intéressants pour la recherche thérapeutique impliquant les *T*AA et l'antagonisme de GPR40 afin de limiter les dommages ischémiques cérébraux.

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LIST OF TABLES

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF ABBREVIATIONS AND SYMBOLS

1-ABT	1-aminobenzotriazole
4-HNE	4-hydroxynonenal
8-OH-dG	8-hydroxy-2'-deoxyguanosine
AA	arachidonic acid
mAb	monoclonal antibody
cAMP	cyclic adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BBB	blood brain barrier
BHT	butylated hydroxytoluene
BK_{Ca}	large conductance calcium-dependent potassium channels
CBF	cerebral blood flow
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]- 1-propanesulphonate
CNS	central nervous system
CO	carbon monoxide
CORM-2	carbon monoxide releasing molecule-2
COX	cyclooxygenase
aCSF	artificial cerebrospinal fluid
DHA	docosahexaenoic acid
DiHETEs	dihydroxyeicosatetraenoic acid
EBM	endothelial basal media
ECM	extracellular matrix
EDHF	endothelium derived hyperpolarizing factor

EDTA	ethylenediaminetetraacetic acid
EET	eicosatrienoic acid
ET-1	endothelin-1
FA	fatty acid
FAD	falvin adenine dinucleotide
FDA	food and drug administration
FFAR1	free fatty acid receptor 1
FR	free radicals
GABA	gamma aminobutyric acid
sGC	soluble guanylate cyclise
GPCR	G protein coupled receptors
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H_2O	water
HBMEC	human brain microvascular endothelial cells
HETE	hydroxyeicosatetraenoic acid
HI	hypoxia-ischemia
HIF-1α	hypoxia inducible factor 1 alpha
HMEC-1	human dermal microvascular endothelial cells
HO	heme oxygenase
HT	haemorrhage transformation
Ibx	iberiotoxin
IBMX	3-isobutyl-1-methylxanthine

ICAM	endothelial intercellular adhesion molecule
IL	interleukin
IsoF	isofurans
IsoP	isoprostanes
IUPAC	international union of pure and applied chemistry
KO	knock out
LA	linoleic acid
oxLDL	oxidized low-density lipoprotein
LOX	lipoxygenase
LT	leukotrienes
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
MEM	minimum essential medium
MEK	mitogen activated protein kinase
MMP	matrix metallopeptidase
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NDGA	nordihydroguaiaretic acid
NeuroP	neuroprostanes
NFκB	nuclear factor-kappa B
NMDA	n-methyl-d-aspartic acid
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OL	oligodendrocytes

tPA	tissue plasminogen activator
PFA	paraformaldehyde
PG	prostaglandins
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator-activated receptors
PKC	protein kinase C
PUFA	polyunsaturated fatty acids
PVL	periventricular leukomalecia
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNP	sodium nitroprusside
SOD	superoxide dismutase
*T*AA	*trans*-arachidonic acids
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TSP	thrombospondin
TTC	2,3,5-Triphenyltetrazolium chloride
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor
WT	Wild type
ZnPPIX	zinc protoporphyrin IX

CLAIMS OF ORIGINALITY

The following are original findings:

1) Demonstration of the vasomotor effects of *T*AA on cerebral microvasculature via a novel mechanism involving the interaction of BK_{Ca} channels and heme oxygenase (HO)-2 and not HO-1. *T*AA exert a vasodilatory effect on cerebral microvessels independent of all classic vasorelaxation pathways. The *T*AA-induced vasorelaxation requires the physical interaction of both BK_{Ca} channels and HO-2 responsible for carbon monoxide production which in turn, activates soluble guanylate cyclase.

2) Demonstration of the implication of *T*AA in the genesis of hypoxia-ischemia. During HI, increased levels of *T*AA induce microvascular degeneration and brain injury, hallmarks of cerebral ischemic damage. These latter could be prevented using nitric oxide inhibitor L-name which also abrogates *T*AA increase *in vivo*.

3) Demonstration of the first receptor that binds lipid peroxidation product, namely *T*AA. *T*AA bind and activate GPR40 receptor leading to the activation of a rather specific pathway. *T*AA/GPR40 complex induces MAPK activation and the overexpression of the anti-angiogenic factor thrombospondin-1 (TSP-1).

4) Demonstration of the first new role of GPR40 in the central nervous system. GPR40 mRNA is present in rodent cerebral microvessels. GPR40 knock-out mice show reduced microvascular degeneration when exposed to *T*AA and limited infarct in response to HI.

PUBLICATIONS AND CONTRIBUTION OF AUTHORS

This thesis is written in manuscript form as permitted by the McGill University Faculty of Graduate Studies and Research. It is composed of two separate manuscripts, as listed below:

1) **Kooli A**, Kermorvant-Duchemin E, Sennlaub F, Bossolasco M, Hou X, Honoré JC, Dennery PA, Varma D, Hardy S, Jain K, Balazy M, Chemtob S (2008). *Trans*-arachidonic acids induce a heme oxygenase dependant vasorelaxation of cerebral microvasculature. *Free Radical Biology and Medicine* 44 (5):815-825.

2) **Kooli A,** Honoré JC, Alquier T, Bossolasco M, Hou X, Poitout V, Hardy P, Jain K, Balazy M, Sylvain Chemtob (2008). *Trans*-arachidonic acids and cerebral microvascular degeneration: Implication of GPR40 receptor. To be submitted.

Contribution of authors

In these studies, the candidate conducted the majority of the experiments and wrote the manuscripts. Preliminary experimental evidences of *T*AA-induced cytotoxicity were obtained by Dr Elsa Kermorvant-Duchemin and Dr Florian

Sennlaub (Kermorvant-Duchemin et al. 2005). Dr Jean-Claude Honoré and Dr Michela Bossolasco provided technical advice and experimental and editorial guidance. Dr Michael Balazy provided us with *trans*-arachidonic acids and Kavita Jain performed the MS/MS measurements of *trans*-arachidonic acids, prostaglandins and HETEs in Dr Balazy's laboratory. Thierry Alquier assisted with the *in vivo* experiments on *gpr40* knockout mice in the laboratory of Dr Vincent Poitout. Dr Pierre Hardy and Dr Pierre Lachapelle assisted in the interpretation of data. Dr Xin Hou provided assistance with the *in vivo* experiments. Dr Daya Varma provided guidance during manuscript preparation. These studies were undertaken under the supervision of Dr Sylvain Chemtob.

Other publications

Kooli A, Seshadri S, Mwaikambo B, Yang C, Gagnon C, Chemtob S and Hardy P. PGJ2 induce cerebral microvascular degeneration in streptozotocin-induced diabetes. To be submitted.

Quniou C, **Kooli A**, Joyal JS, Sapieha P, Sennlaub F, Lahaie I, Hou X, Hardy P, Lubell W, Chemtob S. Interleukin-1 and ischemic brain injury in the newborn: Development of a small molecule inhibitor of IL-1 receptor. *Semin Perinatol* 32 (5): 325-33 (2008)

Brault S, Gobeil F Jr, Fortier A, Honoré JC, Joyal JS, Sapieha PS, **Kooli A**, Martin E, Hardy P, Ribeiro-da-Silva A, Peri K, Lachapelle P, Varma D, Chemtob S. Lysophosphatidic acid induces endothelial cell death by modulating the redox

environment. *Am J Physiol Regul Integr Comp Physiology* 292 (3): R1174-83 (2007).

Leduc M, Kermorvant-Duchemin E, Checchin D, Sennlaub F, Sirinyan M, **Kooli A**, Lachappelle P, Chemtob S. Hypercapnia and trans-arachidonic acids-induced retinal microvascular degeneration: implication in the genesis of retinopathy of prematurity. *Semin Perinatol* 30 (3): 129-38 (2006).

Kermorvant-Duchemin E, Sennlaub F, Sirinyan M, Brault S, Andelfinger G, **Kooli A**, Germain S, Ong H, d'Orleans-Juste P, Gobeil F Jr, Zhu T, Boisvert C, Hardy P, Jain K, Falck Jr, Balazy M, Chemtob S. *Trans*-arachidonic acids generated during nitrative stress induce a thrombospondin-1 dependent microvascular degeneration. *Nature Med* 11(12):1339-45 (2005)

Quiniou C, Sennlaub F, Beauchamp MH, Checchin D, Lahaie I, Brault S, Gobeil F Jr, Sirinyan M, **Kooli A**, Hardy P, Pshezhetsky A, Chemtob S. Dominant role for calpain in thromboxane-induced neuromicrovascular endothelial cytotoxicity. *J Pharmacol Exp Ther* 316 (2): 618-27 (2005)

CHAPTER 1: Introduction

Rationale and objectives of research

Cerebral ischemia is the most common cause of acute stroke worldwide. Considered as the third leading cause of death in the United States, cerebral ischemia is the number one cause of permanent disabilities. In recent years, advances in medical and therapeutic interventions have significantly improved ischemic patients' survival rate in developed countries. It is estimated that approximately 700,000 Americans suffer brain ischemic episodes every year and 75% of them survive beyond 5 years (Sarti et al. 2000; Thom et al. 2006). More than half of those survivors must cope with complications and long-term disabilities: 62% will exhibit motor impairments (Bonita et al. 1988) and 43% will exhibit moderate to severe neurological deficits (Kelly-Hayes et al. 2003). Thus, yearly, around 300,000 Americans live with irreversible deficits due to brain damage.

Cerebral ischemia greatly affects premature infants as well. From 90% of premature newborns who survive in the United States (Hoyert et al. 2001), 5-15% will manifest major spastic motor deficits and an additional 25-50% will reveal developmental cognitive and behavioural impairments resulting from brain injury (Volpe 1997). Hence, approximately 25,000 prematures will show, at different degree and age, permanent deficits due to brain injury. Understanding the fundamental mechanisms underlying a cerebral ischemic-induced brain injury is of foremost importance.

Two major forms of cerebral ischemia exist, namely global ischemia which involves the entire brain (Martin et al. 1998) and focal ischemia which affects restricted brain regions and occurs in a wide variety of clinical settings affecting both adults and premature infants (Volpe 2001; Phillips 2008). Over the past three decades, substantial progress has been made in elucidating the mechanisms by which cerebral ischemia leads to brain injury. However, prevention of brain damage has not similarly advanced due to an incomplete comprehension of the pathogenesis and to increased survival rates over the years (Volpe 1997; Caplan 2004). Hence, cerebral ischemia remains the principal cause of morbidity.

A cerebral ischemic condition is associated with a shortage of oxygen supply and blood flow to the brain caused by blockage or obstruction of the supplying blood vessels (Inder et al. 2000; Durukan et al. 2007). The ensued reduction in cerebral blood flow will initiate a cascade of distinct and complex pathophysiological events including energy depletion, severe metabolic failure, generation of reactive oxygen species (ROS) and cell death (Choi et al. 1990; Lo et al. 2003). These events occur within minutes (Siesjo et al. 1998) and are responsible for multiple hemodynamic, metabolic and ionic changes resulting in the disruption of the blood brain barrier, dysregulation of vascular tonus and irreversible neuronal and endothelial cell death. If prolonged, cerebral oedema and hemorrhagic transformation will ensue (Dirnagl et al. 1999; Fagan et al. 2004). Thus, the pathogenesis of cerebral ischemia presents both a vascular and neurological component.

Upon ischemia and along with the subsequent reperfusion of the brain tissue, there is an increased production of free radicals (FR) and reactive oxygen and nitrogen species (ROS/RNS) (Kontos et al. 1992; Cuzzocrea et al. 2001; Kontos 2001). The excessive generation of FR can exceed the antioxidant capacity of the brain and hence lead to brain injury (Dorrepaal et al. 1996; Fellman et al. 1997; Chan 2001). This oxidative and nitrative stress is even more important in the immature brain where the antioxidant defences are not fully developed (Braughler et al. 1989; Hall et al. 1989; Ferriero 2001; Warner et al. 2004). The brain tissue constitutes a reservoir of poly-unsaturated fatty acids (PUFA) which content increases during cerebral ischemia (Bazan et al. 1970; Siesjo et al. 1983). This latter renders the brain tissue very susceptible to FR attack and generation of ensued lipid peroxidation products (Imaizumi et al. 1986). Whereas the fundamental contribution of FR and ROS/RNS formation is widely accepted in cerebral ischemia-induced injury, the specific role of lipid peroxidation products and possible mediators in the pathogenesis remains unclear and poorly defined.

Trans-arachidonic acids are newly described lipid peroxidation products formed via the *cis-trans*-isomerization of arachidonic acid by the free radical $NO_2^{\bullet}$ (Balazy 2000). This non-enzymatic reaction produces four stable isomers of *trans*-arachidonic acids (*T*AA), namely 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA where 5*E*-AA and 8*E*-AA are endogenous (Balazy 2000) and not found in diet (Ferreri et al. 2002). Levels of *T*AA were found increased during nitrooxidative stress, in the retina of both newborn and adult rodents (Kermorvant-Duchemin, Sennlaub et al. 2005; Xu et al. 2008). *T*AA lead to selective retinal endothelial

cell death and microvascular degeneration via the overexpression of the anti-angiogenic factor thrombospondin-1 (TSP-1) (Kermorvant-Duchemin, Sennlaub et al. 2005) in an endothelial nitric oxide synthase (eNOS)-dependent manner. Despite these few advances in identifying a biological role for *T*AA, a full understanding of their mode of action, as possible mediators of nitrative stress, is yet to be investigated.

Based on these considerations, we hypothesized that *trans*-arachidonic acids, produced upon nitrative stress, are major players in the genesis of a cerebral ischemic condition by affecting the vasomotor tone and survival of cerebral microvasculature.

In order to address this hypothesis, the following objectives were established:

1) Determine the acute effect of *cis*-arachidonic acid and *trans*-arachidonic acids isomers on cerebral vasomotor tone in a concentration-dependent manner. Vasomotor studies were performed on adult rat brain pial microvessels. The vascular diameter was measured before and after each topical application of increasing concentrations of *cis-/trans*-AA.

2) Identify plausible mechanisms involved in *T*AA-induced vasomotor changes of cerebral microvessels using several pharmacological inhibitors and performing various biochemical assays and measuring levels of vasodilator second messengers (NO, CO, cGMP, cAMP, EDHF).

3) Examine the involvement of nitric oxide synthase (NOS), lipid peroxidation and nitrative stress in the hypoxic-ischemic animal model of

cerebral ischemia by determining their effect on the brain vasculature and infarct size following injury.

4) Examine the direct role of *T*AA in the hypoxia-ischemia model. Brain *T*AA levels were measured *in vivo* in a time dependent manner by tandem MS/MS. The effect of *T*AA on brain vasculature was assessed *in vivo* by intracerebral ventricular (ICV) injections and *ex vivo* by treatment of brain explants. Vascular density was measured using a microvascular specific marker staining (lectin).

5) Study the involvement of free fatty acid receptor 1 (FFAR1/GPR40), a G_q coupled receptor, as a possible target for *T*AA by performing binding assays. Assessing the functionality of this receptor was done by measuring the release of calcium by FURA-2AM and evaluating the role of MAPK by western blot.

6) Finally, the role of GPR40 in the hypoxic-ischemic model was looked at using *gpr40* knockout mice. Vascular density and infarct size were assessed using lectin and TTC staining, respectively.

The literature review included in the following chapter describes the current concepts of a cerebral ischemic pathogenesis, the cellular and molecular mechanisms implicated in hypoxic-ischemic brain injuries, the importance of free radicals, ROS and RNS in cellular responses, as well as, the synthesis, metabolism and biological role of *T*AA. Data showing the role of TAA in the cerebral vasomotor response by a mechanism involving heme oxygenase 2

and carbon monoxide is presented in Chapter 3 (Kooli et al., 2008). Evidences that *T*AA exert a chronic severe effect on cerebral vasculature in HI injury via the activation of GPR40 (Kooli et al., 2008) are presented in Chapter 4. A global discussion of the data obtained, a conclusion and perspectives can be found in Chapter 5.

CHAPTER 2: Literature review

2.1 Cerebral ischemia and Hypoxic-ischemic brain injuries

Cerebral ischemia refers to neurological symptoms that result from the obstruction of one of the major cerebral arteries, leading to a severe reduction in cerebral blood flow (CBF) (Figure1). Ischemic brain injuries, also referred to as hypoxic-ischemic injuries, occur in several clinical settings. The most common is focal brain ischemia characterized by a localized disruption to one part of the brain. Less commonly, cerebral ischemia results from the absence of CBF to the entire brain as occurs during cardiac arrest (global ischemia). In premature infants, however, a different form of brain injury occurs, named periventricular leukomalacia (PVL) sharing some pathophysiological similarities with the latter clinical settings.

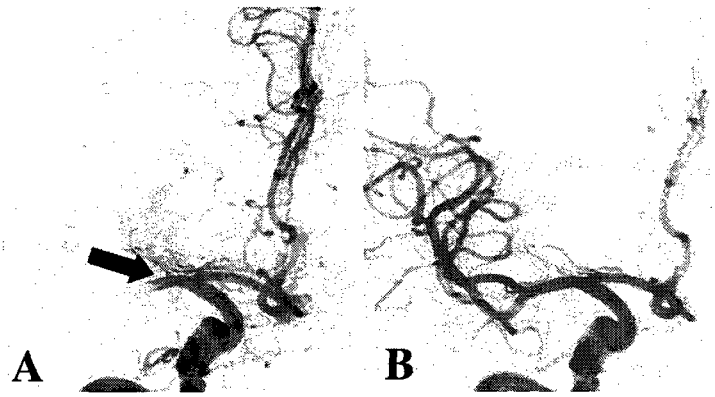

Figure 1| Angiography (A-B) shows an occluded right carotid artery (arrow) in A. (B) After treatment, carotid artery shows complete clot lysis and restored blood flow. Adapted from Furlan A, 1999 (Furlan et al. 1999).

In adults, normal CBF is about 50 to 55 mL/100g/min. If the CBF drops below 16 to 18 mL/100mg/min, electrical activity ceases (Sharbrough et al.

1973; Heiss et al. 1976) and if it dips below 12 to 10 mL/100mg/min, neuronal metabolism stops (Harris et al. 1981; Mies et al. 1991). The brain vulnerability following cerebral ischemia depends on both, the severity of the ischemic insult (reduction of blood flow) and the duration of blood flow loss. In global ischemia, the CA1 sector and dentate hilus of the hippocampus, cortical projection neurons in layer 3, dorsolateral striatum and Purkinje neurons in the cerebellum are most vulnerable. In contrast, focal ischemia forms a core of severe hypoperfusion when flow is reduced to 18-10 mL/100g/min (Mies, Ishimaru et al. 1991). Adjacent to the core is the penumbra, a peripheral zone of moderate to mild ischemia where residual blood flow may be only mildly depressed and transiently sustain tissue viability (Hossmann 1994) (Figure 2). If not perfused early, the ischemic core evolves into an ischemic infarct where all cellular components are necrotic and salvage of rapidly dying brain cells become unfeasible.

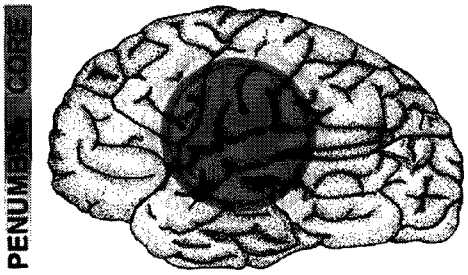

Figure 2| The ischemic penumbra. Note that from the onset of ischemia-reperfusion, the core and penumbra are very dynamic in space and time. Taken from (Hossmann 1994)

Focal ischemia is characterized by lesions in the gray matter as well as the white matter (Bramlett et al. 2004). More specifically, the gray matter regions affected by hypoxia-ischemia consist of the cerebral cortex, thalamus,

hippocampus and cerebellum (Vite et al. 2008). These structures suffer atrophy where their respective volume is prominently reduced (Stebbins et al. 2008). A necrotic lesion occurring in these sites could have a great effect on cognitive functions and depending on the precise localization of the injury, it may influence the type of cognitive deficit observed. Among the cognitive domains affected, the most vulnerable are: orientation, attention, language, visuospatial skills and psychomotor speed (Stebbins, Nyenhuis et al. 2008). In addition to damage to gray matter structures, white matter vulnerability is also observed in hypoxic-ischemic conditions. Because the white matter blood flow is lower than in gray matter due to little collateral blood supply in white matter deep regions (Nonaka et al. 2003) (Figure 3), structures are more susceptible to hypoxia-ischemia and suffer rapid cell swelling and severe tissue oedema (Lo, Dalkara et al. 2003). Due to the extraordinarily richness of the white matter in fibres that ensure connectivity within, and to all brain regions, damages to its structures result in myelin damage/loss and could lead to severe consequences on behavioural neurology (Filley 2005) (Figure 4). In the perinatal period, HI-induced damage to subcortical fiber tracts and

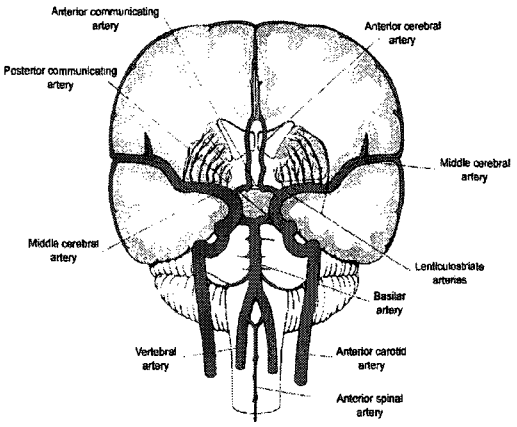

Figure 3| Arterial supply to an adult human brain demonstrated from a cross section of the cerebrum. Taken from Oechmichen M, 2006 (Oechmichen et al. 2006).

oligodendrocytes progenitor cells result in long term demyelination which is a major etiology of cerebral palsy (Volpe 2001). The diffuse component of hypoxic-ischemic insults are not easily detected by current imaging techniques (Kohrmann et al. 2006), although, recently developed techniques named diffusion weighted magnetic resonance imaging and voxel-based morphometry seem to greatly facilitate the diagnosis (Barth et al. 2000; Stebbins, Nyenhuis et al. 2008).

The most dramatic outcome of cerebral ischemia is neuromicrovascular injury which affects the integrity of the cerebral microvasculature resulting in the disruption of the blood brain barrier (BBB) and dysregulation of the vascular tonus (Fagan, Hess et al. 2004). These events will eventually lead to cerebral oedema and hemorrhagic transformation. Progressive brain oedema results in neurological deterioration caused by tissue shifts compressing the midline structures and leading to herniation (Qureshi et al. 2003). While cerebral oedema could only be triggered by the disintegration of BBB or malfunctioning of ion pumps,

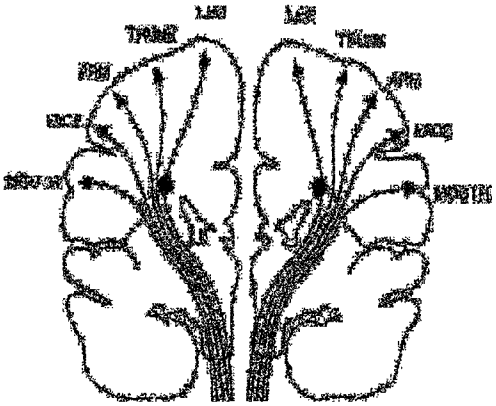

Figure 4| Schematic diagram of corticospinal tracts. Fibers are descending from the motor cortex, past the periventricular region (Square-shaped areas), to the spinal cord. Taken from (Filley 2005)

hemorrhagic transformation may occur spontaneously due to the increase of use of thrombolytic stroke therapy (Passero et al. 2001; Vahedi et al. 2002). These severe complications have a fatal outcome with a high mortality rate of up to 80% (Hacke et al. 1996; Dzialowski et al. 2007).

Incidence of cerebral ischemia is greatest in people over the age of 55 and in the most premature infants (younger than 32 weeks gestation at birth). The major determinant factor in perinatal period, for the development of PVL, is prematurity (Volpe 1997; Back et al. 2004). In adults, several factors could intervene such as an elevated arterial blood pressure, arteriosclerosis, diabetes and atrial fibrillation (Manchev et al. 2001). Two common characteristics predispose, either premature infants or adults, to brain injury: failure in blood flow autoregulation and intrinsic vulnerability of brain cells.

Failure of cerebral blood flow autoregulation is associated, in part, to vascular immaturity in premature infants (Kuban et al. 1985; Nelson et al. 1991) and high blood pressure in adults (Paulson et al. 1990). Failure of cerebral autoregulation results in a fall of both systemic blood pressure and CBF reflecting an exhausted vasodilatation leading to ischemia. Finally, brain cells in general are very susceptible to brain ischemia and die irreversibly. In the developing brain, oligodendrocytes are the most vulnerable due to their immaturity and hence, their lack of anti-oxidative defences.

2.1.1 Accepted molecular mechanisms of hypoxic-ischemic injury

In order to understand the molecular mechanisms involved in the pathogenesis of cerebral ischemia, it is particularly key to appreciate the normal function of the brain.

The human brain consists of only 2% of the total body mass but, it consumes over 15% of the total energy generated in the body (Drubach 2000). Most of this energy is used by the extensive and continuous ion pumping to maintain ion gradients necessary for electrical and synaptic functions in neurons (Erecinska et al. 1994). The rest of the energy is used to support catabolic activity in neurons and glial cells (Attwell et al. 2001). Thus, more than 80% of the energy is devoted to neuronal excitatory signalling.

Because the brain has no energy stores of its own, most neuronal activity depends on second-by-second delivery of glucose and oxygen from the blood (Guyton AC 2000). Thus, glucose is an obligatory energy substrate of the brain (Pellerin et al. 2002). Oxidative phosphorylation of glucose (glycolysis) forms adenosine triphosphate (ATP) which is essential to fuel active ion pumps. Hydrolysis of ATP is critical to maintain mainly (1) the sodium/potassium-ATPase; the most important transporter for keeping high intracellular concentrations of K^+ (about 155 mmol/L) and low intracellular concentrations of Na^+ (about 12 mmol/L) (Keynes 1979) (2) the ATP-dependent Ca^{2+} pumps which exports calcium outside the cell in a 1:10,000 ratio (Carafoli et al. 1978) (3) Na^+-Ca^{2+} exchange pump and (4) sequestration of intracellular calcium at the endoplasmic reticulum level (Blaustein et al.

1978). A schema summarizing the pathological events of cerebral ischemia is displayed in figure 5.

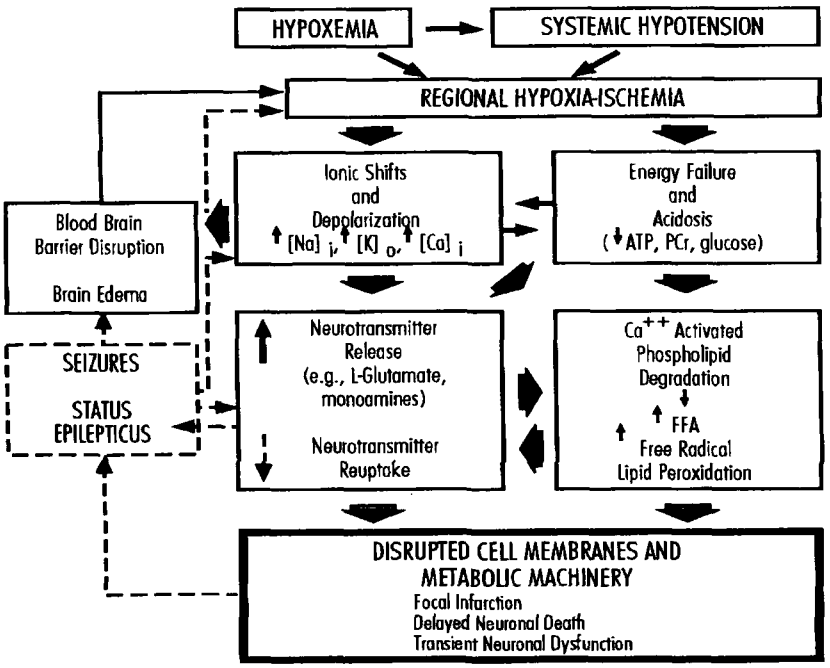

Figure 5| Pathophysiology of Hypoxia-Ischemia. PCr = phosphocreatinine. FFA = free fatty acids. Taken from Siesjo BK 1981.

a) Energy depletion and ionic pump failure

Focal impairment of CBF restricts the delivery of both glucose and oxygen within 10 seconds (Martin et al. 1994). In absence of glucose, oxidative phosphorylation ceases and anaerobic glycolysis becomes the only source of ATP producing less than 10% of the ATP needed (Yamane et al. 2000). Moreover, without incoming blood supply,

neurons and glial cells rely on pre-existing stores of glucose and glycogen which are to be depleted within 3 minutes during ischemia (Nestler et al. 2001). Consequently to these events, ion pumps fail. The cessation of the Na^+-K^+-ATPase pump activity creates cellular depolarization and action potential firing; thus activating voltage-dependent Ca^{2+} channels; responsible for the release of excitatory amino acids (Katsura et al. 1994). At the same time, the energy dependent processes, such as reuptake of excitatory amino acids, are impeded, which further increases the accumulation of glutamate in the extracellular space (Dirnagl, Iadecola et al. 1999).

b) Glutamate excitotoxicity

Glutamate excessive release is considered as the major excitatory transmitter in the brain and trigger of enzymatic cascades in hypoxic-ischemic brain injuries (Paschen 1996). Subsequently, binding of glutamate to ionotropic NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors promote excessive influx of Ca^{2+} (Park et al. 1989). Calcium overload initiates an array of downstream phospholipases and proteases that degrade membranes and proteins essential for cellular integrity (Chen et al. 1997; Furukawa et al. 1997). In addition, ionotropic glutamate receptors promote an excessive influx of sodium and chloride with concomitant cell swelling and oedema (Bringmann et al. 2005). More

recently, metabotropic glutamate receptors have been implicated in ischemic cell death (Bruno et al. 2001).

c) Oxidative and nitrative stresses

Activation of phospholipases and cyclooxygenases by calcium engenders prostanoid formation and increase in oxygen radicals, especially upon reperfusion. This phenomenon is particularly harmful to the injured brain because levels of endogenous antioxidant enzymes (i.e. superoxide dismutase, catalase and glutathione) and antioxidant vitamins (for example; α-tocopherol and ascorbic acid) are normally not high enough to match excess radical formation (Makar et al. 1994). A hypoxic-ischemic episode is followed by a reperfusion phase (return to normal blood flow and oxygen concentration) named ischemia-reperfusion which contributes to more tissue damage (Pachori et al. 2004). During this phase, production of free radicals including superoxide and hydroxyl radicals overwhelms endogenous scavenging mechanisms and directly damage DNA, proteins, lipids and carbohydrates. Moreover, activation of nitric oxide synthase (NOS), during ischemia, leads to nitric oxide increase which combines with superoxide to form a strong oxidant, peroxynitrite (Beckman et al. 1990) that promotes tissue damage (Beckman et al. 1996; Iadecola 1997). FR, ROS and RNS are known mediators of brain injury and fundamental contributors to tissue damage (Beckman 1990; Makar, Nedergaard et al. 1994; Kondo et al. 1997; Kroemer et al. 2000; Chan

2001). Importantly, FR facilitate the disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport and ATP production (Dugan et al. 1994; Kroemer and Reed 2000). Subsequently, the mitochondrial membrane becomes leaky due to the formation of the mitochondrial transition pore which promotes mitochondrial swelling and a secondary oxygen radical burst (Kristian et al. 1998). Due to the brain richness in polyunsaturated fatty acids (Sastry 1985), presence of an excess in FR will facilitate their attack on lipid membranes resulting in a significant formation of lipid peroxidation products. These products influence the cellular membrane fluidity, cell signalling and survival (Halliwell et al. 1993; Acworth et al. 1997). In addition, FR can also serve as important signalling molecules triggering inflammation.

d) Inflammation and cytokine production

Inflammation, a host defence mechanism that is initiated by injury or infection, is a process through which blood/leukocytes and circulatory factors attempt to restore tissue homeostasis (Bethea et al. 2002). Following HI and endothelial damage, activated platelets interact with the endothelium leading to a pro-inflammatory response (del Zoppo et al. 2001). There is increasing evidence that inflammatory cytokines are involved in the pathogenesis of cerebral ischemia (Feuerstein et al. 1994) ((Stoll et al. 1998). Most cells in the ischemic brain, including endothelial cells, perivascular macrophages, microglia and astrocytes

are able to produce the three major pro-inflammatory cytokines: interleukin-1β (IL-1 β), tumor necrosis factor-1α (TNF- α) and interleukin-6 (IL-6). These cytokines are found to be highly elevated early after ischemic onset (Pringle et al. 2001; Hu et al. 2005; Aly et al. 2006) and may lead to brain injury (Hagberg et al. 1996; Martin-Ancel et al. 1997) and cell death (Lipton 1999). Among the proposed mechanisms for the cytotoxic action, the most attractive ones being: disturbance in glutamate transport (Liao et al. 2001) and nuclear factor κ B (NFκB) activation (Hu, Nesic-Taylor et al. 2005). In addition to their effect on BBB permeability, these cytokines also exert vasomotor and vaso-occlusive effects (Vila et al. 2005).

e) Cortical spreading depression

As previously mentioned, reduction in CBF resulting in metabolic distress can lead to a variety of ionic imbalance and cellular depolarization. Experimentally, spreading depression waves will spontaneously radiate concentrically out of an area of massive depolarization. These ionic events contribute to neuronal damage in vulnerable brain regions such as the penumbra (Mies et al. 1993; Back et al. 1996; Busch et al. 1996). Although documented massively in various injury models until recently, limited clinical evidence was available to support the presence of these deleterious events (McLachlan et al. 1994; Mayevsky et al. 1996; Back et al. 2003). In this regard, the first evidence of this slow moving depression of

electrical activity has been demonstrated in the human neocortex following HI (Strong et al. 2002).

2.1.2 Cellular vulnerability in cerebral ischemic injuries.

Most studies have focused primarily on neurons and to a lesser extent, oligodendrocytes with little emphasis on endothelial cells and astrocytes.

a) Oligodendrocytes

Oligodendroglia cells are abundant in both gray and white matter of the brain. Because they are the only cells in the central nervous system (CNS) capable of forming myelin, oligodendrocyte (OL) damage has most profound consequences for function in the CNS myelinated tracts (Dewar et al. 2003). In the perinatal period, damage to the immature OLs, particularly susceptible to HI, will cease the initiation of the myelination process, necessary for acquisition of skilled motor behaviour during development (Rivkin et al. 1995; Volpe 2001). The differentiation of OLs proceeds through several stages by their antigenic and morphologic characteristics (Raff 1989). Cerebral oligodendrocytes arise from progenitor OLs that originate in the subventricular zone and migrate into gray and white matter to differentiate into mature OLs (Goldman 1995; Miller 1996). After induction of ischemia, OLs display a swollen appearance with pyknosis features as early as 3h post injury (Dewar, Underhill et al. 2003). Their acute vulnerability to ischemia is accompanied with a

variety of structural abnormalities, including alteration in the Golgi apparatus and endoplasmic reticulum and increases in perikaryal size (Petito 1986; Migheli et al. 1988; LoPresti et al. 1995; Richter-Landsberg 2000). Several pathogenic mechanisms have been shown to initiate OLs injury including a decreased ability to cope with a concomitant increase in ROS due to their high lipid and iron content and low antioxidant defences (Kim et al. 1991; Oka et al. 1993; Husain et al. 1995; Thorburne et al. 1996; Juurlink et al. 1998) and an overactivation of glutamate receptors (Liu et al. 1995; Yoshioka et al. 1995; Matute et al. 1997; McDonald et al. 1998). In the developing brain, OL survival requires sequential activation of trophic factors and sustained contact with axons and astrocytes (Noble et al. 1984; Barres et al. 1993; Barres et al. 1994).

b) Microvascular endothelial cells

Microvascular endothelial cells are part of the cerebral vascular endothelium, which has classically been considered to be resistant to cerebral ischemia (Garcia et al. 1971; Petito et al. 1982). However, a series of strong evidence reported recently in the literature, demonstrate the importance and vulnerability of microvascular endothelial cells in HI injury (Petito et al. 1987; Cipolla et al. 1997; Morris et al. 2003; Foroutan et al. 2005; Bastide et al. 2007). During focal ischemia, cerebral endothelial cell damage occurs early and in a progressive fashion (Cipolla, McCall et al. 1997). Accompanied with

endothelial cell death, damage to the vasculature contributes to post-ischemic injury, exacerbating the initial brain damage (Pesonen et al. 1981; Yang et al. 1994; Legos et al. 2002). Mechanisms of endothelial cell dysfunction and death implicate essentially increased ROS/RNS formation as endothelial cell are an important source of FR (El Kossi et al. 2000; Alexandrova et al. 2005; Bazan et al. 2005). Despite their heterogeneity, cerebral microvascular endothelial cells form a tight monolayer separating the blood from the parenchyma and protecting the CNS (Pasqualini et al. 2002; Trepel et al. 2002). At this blood-vascular-parenchymal interface, endothelial cells exert several important functions: they regulate the blood flow through liberation of vasoactive compounds, supply a non-fibrinergic surface and control inflammation via the expression of adhesion molecules (Owman et al. 1988; Frijns et al. 2002; Atwood et al. 2003; Andresen et al. 2006). Being in an anatomic relationship with the foot processes of astrocytes, they form together the BBB (Goldstein 1988; Faraci et al. 1998; Utsumi et al. 2000; Abbott 2002) which constitutes the primary barrier to permeability (Risau et al. 1990). Thus, upon a HI insult, alterations in the fine structure of microvascular endothelial cell would have a severe impact on brain injury by disrupting the extracellular matrix, activating coagulation factors, provoking leakage and breakdown of the BBB and increasing the adhesiveness of inflammatory cells (Petty et al. 2001; del Zoppo et al. 2003). These events result in brain oedema and haemorrhagic complications by causing microvascular

degeneration, regional ischemia and compromising cerebral hemodynamic regulation (del Zoppo et al. 1993; de Vries et al. 1997; Barone et al. 1999; Morris, Yeich et al. 2003).

c) Neurons

The neuropathology of neuronal cell death in HI injuries has been extensively studied and intensely looked at. In fact, neurons are considered to be the most vulnerable cells to ischemia even if it occurs for a short period (Wieloch 1985; Ogawa et al. 2007). Neuron susceptibility and resistance to HI varies depending on their location in the brain. For example, hippocampal and Purkinje neurons show enhanced vulnerability following global ischemia, due to latent deficiency in blood flow, lapping of cerebral blood vessels and metabolic disparity (Matsumoto et al. 1987). Neuronal damage occurs shortly after onset of ischemia with potentially reversible changes accompanied by distinct morphological alterations to post-synaptic structures (Garcia et al. 1993; Garcia et al. 1995) determined by electron microscopy (Bonnekoh et al. 1990). Neuronal cell death, however, precedes 1 to 3 days after insult, a process referred to as delayed neuronal death characterized by cell shrinkage and nuclear fragmentation; hallmarks of apoptosis (Kirino 1982; Kirino et al. 1984; Wang et al. 2002). The pathophysiological mechanisms leading to neuronal injury and degeneration are complex and multifactorial. There are substantial experimental evidences that excitotoxicity,

calcium-mediated events, free radical generation, mitochondrial damage and inflammation, all lead to neuronal cell death (Chauhan et al. 2003; Rodrigo et al. 2005).

d) Glia cells

Astrocytes exert many functions in the brain such as the modulation of neuronal excitability, pH homeostasis and stabilization of synaptic connections (Koehler et al. 2006). Thus, they are essential for neuronal survival and synapse function, as well as for neurogenesis and neural repair (Aschner et al. 2002; Swanson et al. 2004). Few studies have focused on the fate of astrocytes in cerebral ischemia. Although, many researchers have thought astrocytes to be resistant to ischemia *in vitro*, recent studies *in vivo*, showed they are more susceptible than neurons to ischemic insults (Martin et al. 1997; Lukaszevicz et al. 2002). In fact, astrocytes exhibit cell swelling and fragmentation of processes consisting with rapid cell necrosis, rapidly after insult (Penfield 1928; Hulse et al. 2001; Lukaszevicz, Sampaio et al. 2002). Interestingly, these ultrastructural changes preceded alterations observed in neighbouring neurons (Giffard et al. 2005). Moreover, astrocyte death was shown to be apoptotic as early as 10 minutes after ischemic onset (Petito et al. 1998; Sugawara et al. 2002).

Microglial cells, unlike astrocytes, derive from stem cells and have a ramified morphology with multiple processes, in the normal brain

(Giulian et al. 1996). Following cerebral ischemia, these cells retract their processes and display an amoeboid appearance typical of activated microglia (Morioka et al. 1993; Giulian, Corpuz et al. 1996). However, their role in HI is not understood yet.

2.1.3 Current therapies

Several experimental therapies exist for cerebral ischemic insults. The only effective therapy for acute stroke, approved by the United States Food and Drug Administration (FDA), is thrombolysis which consists of administration of recombinant tPA in a 3 hour-period following vascular occlusion (Hacke et al. 1999). tPA is the abbreviation for tissue plasminogen activator: protease normally present on the surface of endothelial cells and secreted after vascular injury. tPA converts the pro-enzyme plasminogen into plasmin, known for its fibrinolytic properties (1995; Frankel 1997; Demchuk et al. 1999). Therefore, administration of recombinant tPA helps blood clot lysis and treatment is currently limited to 3h period. Beyond this time limit, it presents a risk of intracerebral haemorrhage and brain oedema (Hacke, Brott et al. 1999).

A large number of preclinical observations indicate a synergistic effect when combining neuroprotective drugs with tPA. This combination reduces the reperfusion injury, post-ischemic damage and inhibits downstream targets in cell death cascades. Synergistic or additive effects were reported when thrombolysis was used in conjuncture with neuroprotectants such as oxygen radical scavengers (Asahi et al. 2000), AMPA (Meden et al. 1993) and NMDA receptor antagonists

(Zivin et al. 1991), citicoline (Andersen et al. 1999), topiramate (Yang et al. 2000) and anti-leukotic adhesion antibodies (Bowes et al. 1995). Citicoline is an intermediate in the biosynthesis of phosphatidylcholine, shown to have several beneficial effects in various CNS injury models (Adibhatla et al. 2002; Adibhatla et al. 2002). Topiramate is a GABA agonist which acts as an anticonvulsant drug used to prevent epilepsy seizures shown to have therapeutic effects in HI injuries (Edmonds et al. 2001). The advantage of combinational therapy would be to decrease dosages for each agent, thereby reducing the occurrence of adverse effects. Two recent clinical trials reported feasibility and safety of treatments with intravenous tPA followed by neuroprotectants, clomethiazole which is a GABA agonist acting as a sedative (Wahlgren et al. 2000; Lyden et al. 2001) or lubeluzole; blocker of the voltage-activated Ca^{2+}-channels (Diener et al. 2000; Marrannes et al. 2000; Grotta 2001; Grotta 2001).

Another effective therapy is hypothermia. Prolonged submersion in icy water showed surprisingly favourable outcome, particularly in children. Hypothermia was first used in 1940s to treat head trauma (Lo, Dalkara et al. 2003). Several non randomized trials followed, but were hindered by serious complications owing to low core temperatures (24-33°C), thereby making this treatment lethal (Inamasu et al. 2002). In the past decade, however, it was recognized that a small drop in temperature, from 37°C to 33-35°C, was sufficient to reduced neuronal death, in experimental models of HI injury (Busto et al. 1987; 2002; Bernard et al. 2002). Moreover, neuroprotective effects of certain drugs such as MK-801 (glutamate

receptor antagonist), partially correspond to lowering brain temperature (Minard et al. 1982).

2.2 Neuromicrovascular injury

Despite an immense interest in neurons and neuronal cell death in cerebral ischemia, a few studies have focused on the capital importance of the cerebral microvasculature and endothelial cell dysfunction.

The cerebral vascular endothelium is a complex and metabolically active organ. The microvasculature is defined as vascular elements less than 100 μm diameter, which include the capillaries and their afferent/efferent networks (del Zoppo and Mabuchi 2003). The endothelium was regarded, for decades, as inert and inactive conduit for blood transport (George 2003). Today, these early views have evolved and the endothelium has become a major focus in many research disciplines.

2.2.1 Anatomy of the cerebral circulation

The microvascular structure of CNS is remarkably well preserved among species (Edvinsson 1993). About 18% of the total blood volume of the body circulates in the brain and is driven by two internal carotids and two vertebral arteries (Edvinsson 2002). Only a small amount of blood is brought by the anterior spinal artery to brainstem. In their final tract, vertebral arteries converge to form the basilar artery. The conjunction of the basilar artery and internal carotids, form the Circle of Willis (Figure 6); which represents the great vessels that perfuse the cerebral cortex. This organization allows reversal of flow and cross filling of deep brain structures and provides protection against occlusion of one of these arteries. Below the Circle of Willis, the right

and left carotid arteries do not have communicating branches (anastosomes) while the vertebral arteries have more developed ones. Among cerebral arterioles, small anastosomes are present but blood flow is normally inadequate to maintain perfusion if cerebral occlusion occurs. Pial arteries, connected by anastomotic branches, penetrate the cortex perpendicularly to its

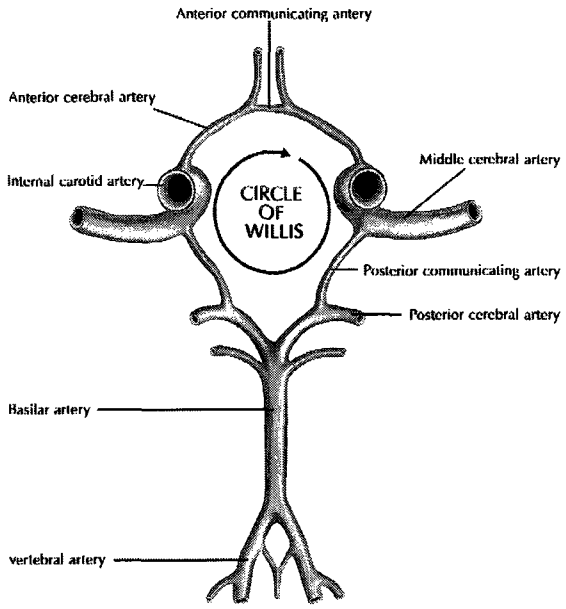

Figure 6| Circle of Willis. The Circle of Willis represents the most important communication system among the main arteries directed to the brain. Taken from Folino AF, 2007 (Franco Folino 2007).

surface and divide into microvascular arterioles and capillaries, serving columns of cortical gray matter (Bar 1978; Bar 1983). At the gray matter-white matter junction, these connections are less abundant (Bar 1980) and the microvascular density is significantly decreased. In contrast, microvascular networks of the striatum and thalamus have not yet been formally mapped (Fenstermacher et al. 1991; Peters 1991).

The most unique feature of cerebral microvessels corresponds to its remarkable limited permeability. This is represented by tight junctions, or *zonulae occludentes*, between two adjoining endothelial cells or of an endothelial cell upon itself, as in capillaries (Risau and Wolburg 1990; Ge et al. 2005). At the brain capillaries, the endothelial layer is intimately associated with different cells. Thus, pericytes form a meshwork of the outer surface sharing basal lamina with endothelial cells (Allt et al. 2001) and the end-feet of perivascular astrocytes form a further covering of fine processes and lamellae punctuated by gaps (Kacem et al. 1998). The interaction between these different cell types constitutes the blood brain barrier (Abbott 2005). In addition, a second permeability barrier is formed with basal lamina, which includes elements of the extracellular matrix (ECM) such as laminin, fibronectin and collagen IV, limiting the movement between blood and parenchyma (Hamann et al. 1995; del Zoppo and Mabuchi 2003). It is now widely accepted that the permeability of both the BBB and the basal lamina derive from a concerted interaction between endothelial cells and astrocytes (Bernstein et al. 1985; Kusaka et al. 1985; Janzer et al. 1987; Tagami et al. 1992; Webersinke et al. 1992; Nagano et al. 1993).

Cellular proximity within the BBB emphasizes the dynamic interactions between endothelial cells, astrocytes and microglia, neurons, vascular smooth muscle cells and components of the ECM in both the gray and white matter; forming together the neurovascular unit (Lo, Dalkara et al. 2003). This interface provides a key element to understand injury as an integrated

response where all cellular components are players in the evolution of tissue damage. Thus, disturbances in one of these cell types will eventually cause perturbations among the neurovascular unit.

The endothelium alone performs a number of highly specialized biological functions: it provides a compatible interface to facilitate blood circulation, it inhibits platelet aggregation and leukocyte adhesion where it acts as a gatekeeper and it produces a balance of vasorelaxant and vasoconstrictive molecules that regulate the vascular tone (Abraham et al. 2007).

2.2.2 Cerebral autoregulation

Regulation of the cerebral circulation has to be considered as an integrated mechanism where intracranial volume should be maintained constant. This complex mechanism induces modifications of cerebral vascular resistance by changing arteriole diameter, in response to systemic blood pressure. Preservation of an adequate CBF during changes in the systemic blood pressure requires an autoregulation system aimed to protect the brain from drastic changes in arterial pressure. Therefore, within 60 and 140 mm Hg (lower and higher limits of blood pressure), CBF remains somewhat constant (Figure 7). The main arterioles involved in cerebral autoregulation are the ones less than 100 μm diameter, namely cerebral microvessels.

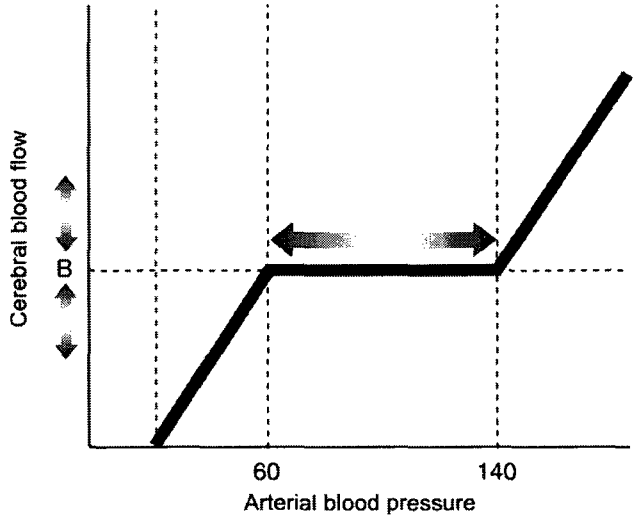

Figure 7| Typical cerebral autoregulation curve. Note that a drop in arterial blood pressure corresponds to decreased blood flow. Taken from Folino AF, 2007.

An efficient autoregulation system necessitates coordinated vasoactive processes in pial arteries and intracerebral arterioles. In fact, an increase in the resistance of the pial arteries prevents an effective vasodilation of the downstream vascular beds. Cerebral regulation is maintained by three different control pathways: myogenic, metabolic and neurogenic. The myogenic process involves the intrinsic ability of smooth muscle cells to induce vasoconstriction in response to a direct increase in blood pressure (Bayliss 1902; Folkow 1964; MacKenzie et al. 1979). Among the proposed mechanisms, the most plausible and accepted one is a change in membrane polarization via an alteration in the activation state of K^+ and Ca^{2+} channels (Harder 1984). The metabolic mechanism is based on modification of arterial resistance, induced by release of elements such as adenosine and oxygen (Paulson, Strandgaard et al. 1990) or carbon dioxide (CO_2) concentration

(Ursino et al. 1998). An elevated concentration of (CO_2) leads to vasodilation and constant rise in blood flow, supposedly via prostaglandin production (Markus et al. 1994). Finally, the neurogenic mechanism is based on the rich innervations of cerebral microvessels by both sympathetic and parasympathetic nerves (Goadsby et al. 2000). The latter do not play a significant role in cerebral autoregulation despite its potential vasodilatory effect (Hamel 2006). On the contrary, the sympathetic system is believed to elicit a vasoconstriction mediated by norepinephrine and neuropeptide Y (Gulbenkian et al. 2001), causing a shift of the autoregulation curve towards high limits, thus protecting the brain from systemic high blood pressure (Umeyama et al. 1995; Visocchi et al. 1996; Zhang et al. 2002). Hence, it is clear that the basal cerebrovascular tonus favors a partial vasoconstriction and plays a major role in the regulation of CBF.

2.2.3 Microvascular response upon injury

As stated above, cerebral microvessels and more specifically microvascular endothelial cells play a significant role in cerebral autoregulation. During cerebral ischemia, microvascular endothelial cells are not only easily damageable but once injured; they greatly contribute to tissue damage during the reperfusion period. Thus, many studies have tried to identify an interesting target in order to develop vascular protection.

Microvascular injury occurs in three separate phases: acute (hours), subacute (hours to days) and chronic (days to month), as depicted in Figure 8.

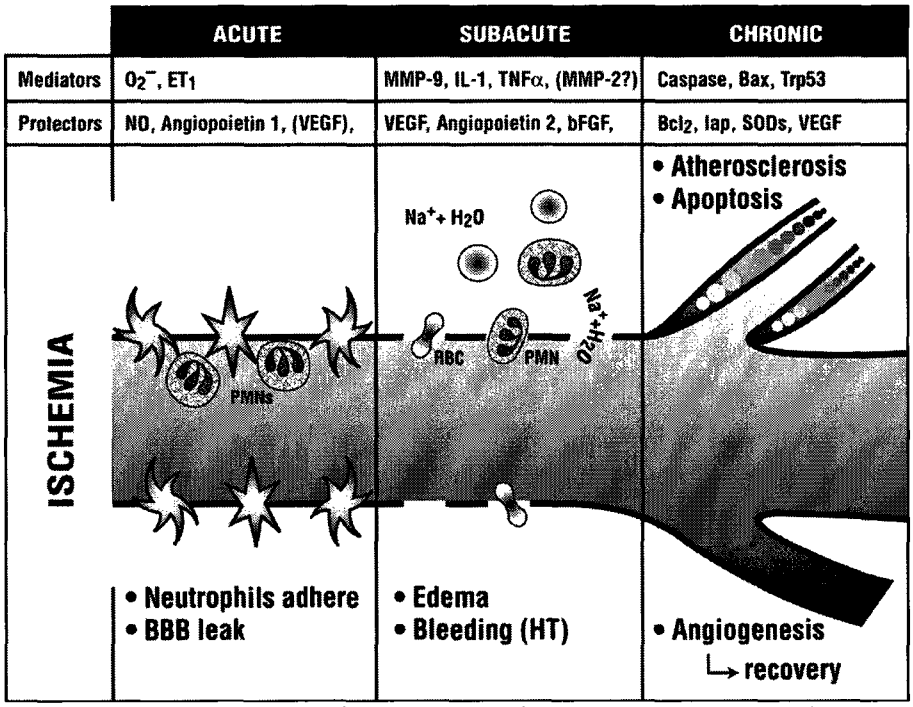

Figure 8| Time course of microvascular injury following cerebral ischemia and reperfusion. MMP-9 = metalloprotease 9, MMP-2 = metalloprotease 2, bFGF = basic fibroblast growth factor, HT = haemorrhage transformation, SOD = superoxide dismutase. Caspase, bax and Trp53 are pro-apoptotic proteins which predominate on the anti-apoptotic Bcl2, and Iap. PMN indicates polymorphonuclear leukocytes and RBC are red blood cells. Taken from Fagan SC, 2004.

a) Acute phase

The acute phase usually lasts several hours. During this phase, numerous hemodynamic and metabolic changes occur resulting in BBB leakage and dysregulation of the vascular tonus. The latter is regulated by many different factors such as oxygen radicals as well as vasoactive factors including nitric oxide (NO) and endothelin-1 (ET-1) (Dirnagl, Iadecola et

al. 1999; Lipton 1999). It has been reported that vasodilation occurs during the early phases and parallels the production of ROS/RNS (Nelson et al. 1992). In fact, endothelium-dependent vasorelaxation of cerebral microvessels could occur through a number of mechanisms. Nitric oxide (NO) (Toda et al. 1996), carbon monoxide (CO) (Leffler et al. 2006), prostaglandin E_2 (PGE_2) (Davis et al. 2004), Prostacyclin (PGI_2) (Faraci 1993) and endothelium-derived hyperpolarizing factors (EDHF) including hydroxyeicosotetraenoic acids (HETEs) (Alonso-Galicia et al. 1999) and eicosotrienoic acids (EETs) (Kotlikoff 2005) have all been described to mediate cerebral relaxation. The interaction between oxygen radicals and NO form peroxynitrite, (Beckman, Beckman et al. 1990; Huie et al. 1993) which acts as a vasorelaxant of cerebral microvasculature (Wei et al. 1996). Ensued lipid peroxidation is also responsible for the generation of vasorelaxant products such as 4-hydroxynonenal (4-HNE) (Martinez et al. 1994) and acrolein (Tsakadze et al. 2003).

Following cerebral vasorelaxation, a number of other vasoactive factors are synthesized or released, including ET-1 which acts as a potent vasoconstrictor. Several studies have demonstrated increased levels of ET-1 in stroke patient and animal models of hypoxic-ischemic injuries (Tsang et al. 2001; Lo et al. 2005). In addition to its effect on vascular tone, ET-1 increases BBB permeability and neuronal damage (Macrae et al. 1993; Stanimirovic et al. 1994; Matsuo et al. 2001). BBB integrity could be also altered by other important factors such as vascular endothelial growth

factor (VEGF) and angiopoietin-1, generally essential for angiogenesis (Yancopoulos et al. 2000; Zhang et al. 2002). In fact, VEGF-induced NO production increases peroxynitrite formation causing further tissue damage (Gursoy-Ozdemir et al. 2000) while decreased levels of angiopoietin-1 coincide with advanced instability of BBB (Thurston et al. 2000; Zhang et al. 2002). More specifically, BBB permeability is due to disrupted tight junctions attacked by metalloproteases and reactive oxygen species, released upon a hypoxic-ischemic insult (Rosenberg et al. 2007; Schreibelt et al. 2007).

b) Subacute phase

The subacute phase lasts hours to several days and, in continuing with the chronic phase of neuromicrovascular injury, is characterized by gene activation. In fact, a number of pro-inflammatory genes including, IL-1β and TNF-α (Rothwell et al. 1995; Vila et al. 2000; Sairanen et al. 2001) as well transcription factors such as NFκB, hypoxia-inducible factor-1 (HIF-1α) are activated in response to hypoxia, FR formation and intracellular Ca^{2+} influx that occur earlier in the pathogenesis (Forsythe et al. 1996). These pro-inflammatory mediators influence the expression of adhesion molecules on endothelial cells. First, decreased $\alpha_1\beta_1$ integrin reactivity is observable in response to HI. These subunits play an important role in maintaining cell viability and are responsible for endothelial cell-matrix interactions (Strater et al. 1996). Their downregulation is due in part to a shut-down in their gene expression (Tagaya et al. 2001) caused by IL-1β

and TNF-α which ultimately leads to endothelial cell detachment and oedema formation (Defilippi et al. 1992; Defilippi et al. 1992; Reed et al. 1992; Yamasaki et al. 1992; Rodt et al. 1994). Second, along with TGF-β, IL-1β and TNF-α, are associated with the differential upregulation of multiple proteases which degrade ECM components and thus contribute to vascular and parenchymal matrix proteolysis (Schleef et al. 1988; van Hinsbergh et al. 1990; Docagne et al. 1999). Third, an increase in leukocyte adhesion receptors on endothelial cells occurs in response to HI and corresponds to the secondary injury process. The expression of P-selectin, intercellular adhesion molecule-1 (ICAM-1), and E-selectin on microvessel endothelium, together with their counter-receptors on polymorphonuclear (PMN) leukocytes results in adherence of activated PMN leukocytes to capillaries and their transmigration to the neuropil (Okada et al. 1994; Haring et al. 1996; Abumiya et al. 1999). The accumulation of activated platelets and fibrin also due to cellular interactions promotes capillary occlusion, microvascular obstruction and increased filtration of large molecules (albumin, IgG) (Nordborg et al. 1991; Okada et al. 1994; Mabuchi et al. 2000). Interestingly, the described alterations in microvascular-matrix interactions are confined to the regions of documented neuronal damage (Wagner et al. 1997). In addition to increased pro-inflammatory genes as stated above, inducible nitric oxide synthase (iNOS) is upregulated causing production of toxic amounts of NO (Iadecola 1997; Lerouet et al. 2002; Zhu et al. 2002). Overall, these

changes confirm the relevance of the microvascular wall in the pathogenesis of HI.

c) Chronic phase

The mechanisms involved in the chronic phase implicate induction of genes that participate in the regulation of apoptosis, as well as stimulation of angiogenic factors in endothelial cells. Programmed cell death is triggered by apoptogenic factors including activation of cell surface receptors via IL-1β, TNF-α (Martin-Villalba et al. 1999; Nicotera et al. 1999), excessive oxygen radical formation (Budd et al. 2000), DNA damage (Chopp et al. 1996) and possibly lysosomal protease activation (Salvesen 2001) (Stoka et al. 2001). In response to these stimuli a cascade of proteolytic enzymes known as caspases and other pro-apoptotic proteins such as Bax and Bad are activated (Chen et al. 1996; Hara et al. 1996; Sairanen et al. 1997). Losses of membrane integrity and organelle failure are the most prominent mechanisms of cell death in ischemic conditions. In the arena of cell death, the mitochondria and the nucleus occupy the centre stage. The mitochondria, with their complement of pro-apoptotic proteins (such as cytochrome c), transition pore formation and their role in oxidative phosphorylation, are uniquely positioned to detect and amplify cell death signalling processes (Fiskum 2000). Until recently, the nucleus was viewed as a target of pro-death cytosolic proteins. Recent data indicate that the nucleus also release molecules that recruit organelles in cell death processes, especially in caspase-independent apoptosis (Leist et

al. 2001). It is also known that angiogenesis takes place a month after ischemia-reperfusion (Wei et al. 2001), particularly in the penumbral region (Plate 1999). It is often considered as a compensatory neovascularisation to meet the metabolic demand in consequence to the severe reduction in cerebral blood flow. Interestingly, the extent of angiogenesis has been correlated with survival in stroke patients (Krupinski et al. 1994; Plate 1999). The identification of key angiogenic molecules that positively regulate the vascular growth has captivated all the attention on this neovascularisation phase. Angiogenic activity reflects a balance between angiogenic and angiostatic drives (Folkman 1995; Pepper 1996; Rosenberg et al. 1996; O'Reilly et al. 1997; Risau 1997; Detmar 2000), thus, it is expected that the expression of angiostatic factors would contribute to the resolution of post-ischemic damage. However, only a couple of recent studies have set interest in understanding the fate of endothelial cell death upon hypoxia-ischemia and the possible relationship with anti-angiogenic factors. Among major angiostatic factors, thrombospondins have gained increasing importance in recent studies. Both, thrombospondin-1 and -2 (TSP-1, TSP-2) have been shown to increase in the ischemic brain, notably in the endothelial cells (Manoonkitiwongsa et al. 2001; Lin et al. 2003). Despite a regression in vascular sprouting after HI, thrombospondins are thought to inhibit vascular growth without mediating endothelial cell apoptosis (Manoonkitiwongsa, Jackson-Friedman et al. 2001; Lin, Kim et al. 2003).

2.3 Free radicals, reactive species and lipid peroxidation

A free radical is an atom or group of atoms with one or more impaired electrons, usually symbolized with a dot ($^{\bullet}$). This chemical configuration renders the molecule very unstable, therefore highly reactive and short-lived (Simic et al. 1988). There is a continuous production of free radicals (FR) and reactive species providing a balance between pro-oxidants and anti-oxidant defence systems, also termed redox state. Transient instabilities in the redox state via limited formation of superoxide anion ($O_2^{\bullet-}$), nitric oxide and ensued reactive oxygen and nitrogen species (ROS/RNS), can transduce cellular signals involved in several physiological functions (Droge 2002). These include immunological host defence, hormone action and secretion, iron transport, gene transcription, apoptosis, vasomotor tone regulation, and neuromodulation (Lander 1997). FR, ROS and RNS are typically generated in a tightly regulated mechanism via the activation of nitric oxide synthase and NADPH oxidase isoforms (Droge 2002). In hypoxic-ischemic conditions however, a perturbation occurs affecting the production and/or metabolism of these molecules and disrupting the balance between oxidants and anti-oxidants, in favour of the formers (Alexandrova and Bochev 2005). As a result of oxidative stress, nitrative stress and accumulated FR, lipids, proteins and nucleic acids are damaged and cellular injury occurs. An excessive and/or sustained increase in ROS/RNS is believed to be a critical causative factor in the development of endothelial dysfunction, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases and cancer (Wolff 1993; Simic 1994; Love 1999; Giasson et al. 2002; Warner, Sheng et al. 2004).

2.3.1 Reactive oxygen and nitrogen species

Free radicals result from the breakdown of a covalent bond, between two atoms, leaving them each with an unpaired electron. Because of their high instability, FR tend to react quickly with the nearest molecule, donating (reduction) or extracting (oxidation) electrons, in order to pair their lone electron and gain stability. The neighbour molecule becomes a FR itself and will begin a chain reaction, leading to a cascade of events resulting in cell disruption (Sies 1991). Reactive oxygen species are derived from an oxygen free radical and include superoxide ($O_2^{\bullet -}$), oxide ($O^{\bullet -}$), singlet oxygen ($^1O_2$; excited form of oxygen), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), hydrogen dioxide (HO_2^-) and alkylperoxyl radicals ($ROO^{\bullet}$; where R could be a long carbon chain or lipids). Reactive nitrogen species derive from nitric oxide and include nitric oxide itself ($NO^{\bullet}$), peroxynitrite (ONOO-), nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3). The chemical reactions that lead to the formation of these reactive species are presented in Figure 9.

a) Reactive oxygen species

Most of the ROS named in the previous section are formed in biological systems. Superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are the two most contributors ROS in the genesis of hypoxic-ischemic damage (Love 1999). Superoxide anion is formed by a number of oxidases including NADPH oxidase (Figure 9, Reaction 1), xanthine oxidase, lipoxygenases,

cytochrome P450 and uncoupling of the mitochondrial respiratory chain, via electron leakage (Miller et al. 2006) (Piantadosi et al. 1996). Superoxide is a long-lived free radical which does not cause much cellular damage by itself (Magder 2006). However, when generated in great amounts, superoxide is converted rather quickly to hydrogen peroxide (H_2O_2), either in a spontaneous reaction called dismutation or by superoxide dismutase (Figure 9, Reaction 2) (Fridovich 1995). (H_2O_2) is not a radical but is considered ROS that is more stable than superoxide and capable of diffusing across cellular membranes. In presence of ferrous iron, (H_2O_2) can be reduced to the highly reactive hydroxyl radical ($OH^{\bullet}$) via the Fenton reaction (Figure 9, Reaction 3) or the iron-catalyzed Haber-Weiss reaction (Figure 9, Reaction 4 and 5) (Kehrer 2000). The reactivity of ($OH^{\bullet}$) is so high and non specific that it reacts with any molecular target nearby. Thus, toxicity is limited by this radical's diffusion rate (Magder 2006).

b) Reactive nitrogen species

The radical ($NO^{\bullet}$) is an important pleiotropic gas produced in higher organisms by the oxidation of L-arginine; catalyzed by nitric oxide synthase (NOS) (Bruckdorfer 2005). Due to its short half-life, ($NO^{\bullet}$) is rapidly oxidized to nitrates (NO_3^-) and nitrites (NO_2^-) which were thought to be inert and stable but recently shown to be recycled, *in vivo*, to form ($NO^{\bullet}$) (Lundberg et al. 2008). In

pathological settings, ($NO^{\bullet}$) easily reacts with ($O_2^{\bullet-}$) to form stable and highly reactive compounds namely peroxynitrite ($ONOO^-$) and its peroxynitrious acid (ONOOH) (Figure 9, Reaction 6). This protonated form of peroxynitrite readily decays, at physiological pH, into nitrogen dioxide ($NO_2^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$), two potent oxidants (Beckman, Beckman et al. 1990) (Figure 9, Reaction 6). Peroxynitrite is also able to react with ($NO^{\bullet}$) to form nitrogen dioxide ($NO_2^{\bullet}$) (Figure 9, Reaction 7). The latter is able to react with ($NO^{\bullet}$) to form (N_2O_3), which can also be formed when ($NO^{\bullet}$) reacts with oxygen (Figure 9, Reaction 8). These species are exceedingly dominant cytotoxic RNS (Pryor et al. 2006).

(1) O_2 + electron $O_2^{\bullet-}$ (catalyzed by oxidases)

(2) 2 $O_2^{\bullet-}$ + 2H^+ H_2O_2 + O_2 (catalyzed by SOD)

(3) H_2O_2 + Fe^{2+} Fe^{3+} + $OH^{\bullet}$ + OH^- (Fenton's reaction)

(4) $O_2^{\bullet-}$ + Fe^{3+} Fe^{2+} + O_2 (Haber-Weiss reaction)

(5) H_2O_2 + $O_2^{\bullet-}$ O_2 + $OH^{\bullet}$ + OH^- (Haber-Weiss reaction)

(6) $O_2^{\bullet-}$ + $NO^{\bullet}$ $ONOO^-$ ONOOH $OH^{\bullet}$ + $NO_2^{\bullet}$

(7) $ONOO^-$ + $NO^{\bullet}$ $NO_2^{\bullet}$

(8) $NO^{\bullet}$ + O_2 N_2O_3

Figure 9| Chemical reactions involved in the formation of ROS and RNS. SOD = superoxide dismutase

2.3.2 Sources of ROS/RNS

The cumulative production of ROS and RNS in living organisms is initiated from various exogenous and endogenous sources. Some of these reactive species are generated as undesired secondary reactions between biomolecules or alternatively

in the detoxification of xenobiotics. Other species, however, are generated *in vivo* in a controlled and continuous manner (Frei 1994).

a) Exogenous sources of ROS and RNS

Exogenous sources of ROS and RNS are varied and include majorly environmental pollutants. Such pollutants include inhaled toxic gaseous such as ozone, nitrogen dioxide ($NO_2^{\bullet}$) and cigarette smoke. Other sources include ionizing and UV irradiation as well as exposure to a hyperoxic environment. A large variety of xenobiotics, chemicals (e.g. quinines, nitroaromatics, ferritin) and food (quinoid-derived lipids) also promote the generation of reactive species as by-products of their metabolism *in vivo* (Mantell 2006). However, in disease states, most of the ROS/RNS production is endogenous.

b) Endogenous sources of ROS and RNS

There are several sources of endogenous free radicals that are worth mentioning. These sources involve different enzymes and processes which contribute to form reactive oxygen and nitrogen species, especially in pathological settings such as cerebral ischemia.

- Mitochondrial electron transport chain

  In aerobic organisms, O_2 is reduced into H_2O at the end of the mitochondrial respiratory chain. However, further upstream in this chain (Complex I, II and/or III), there is ''leakage'' of single

electrons, mainly from non ferrous proteins, that will lead to partial reduction of oxygen into superoxide ($O_2^{\bullet -}$) (Kehrer 2000). It is estimated that 1-2% of the oxygen consumed is converted to superoxide ($O_2^{\bullet -}$). During hypoxia-ischemia, the components of electron-transport chain are reduced, increasing electron ''leakage'', thus enhancing ROS production (Love 1999).

- Enzymatic production of ROS/RNS

Enzymatic production of ROS/RNS could be cytoplasmic or microsomal, depending on their intracellular location. Cytoplasmic enzymes include xanthine oxidase (XO) and nitric oxide synthases (NOS). XO is an iron sulfur flavoprotein with multiple functions comprising induction of superoxide generation (McCord 1985). During ischemic conditions, the enzyme xanthine dehydrogenase is converted to XO. The latter, by using oxygen, catalyzes the conversion of xanthine and hypoxanthine to uric acid, delivering superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) which contribute to brain injury (Beetsch et al. 1998; Mink et al. 2007). On the other hand, nitric oxide synthases generate nitric oxide from the amino acid L-arginine. The overall reaction requires a multitude of co-factors for which there are specific binding sites on NOS enzymes (Palmer et al. 1988). The NOS are complex enzymes, composed of at least three different isoforms; the neuronal NOS or nNOS (NOS I), the endothelial NOS or eNOS (NOS III) which are constitutive

and the inducible NOS or iNOS (NOS II) (Papapetropoulos et al. 1999). Between 51% and 57% of homology exists between these enzymes at the amino acid level (Bruckdorfer 2005). nNOS and eNOS constitutively form ($NO^{\bullet}$) in response to a stimulus and in some cases, they are activated by calcium, liberating ($NO^{\bullet}$) in small quantity for signal transduction (Love 1999). iNOS isoform however, is expressed after induction by external stimuli and produces ($NO^{\bullet}$) in large amounts (20 times or more the levels of nNOS and eNOS) (Berdeaux 1993; Bonmann et al. 1997). These isoforms could be found in different cell types and the amounts of NO are able to dictate their effects whether it has beneficial or deleterious influences on the cellular system. Although it was hard to believe when discovered (Ignarro et al. 1987; Ignarro et al. 1987; Palmer et al. 1987; Radomski et al. 1987), it is now widely accepted that ($NO^{\bullet}$) plays various physiological functions where itmodulates synaptic plasticity and neurotransmission, regulates blood flow and inhibits platelet aggregation (Ignarro 1989; Calabrese et al. 2007). In hypoxia-ischemia, following iNOS induction, massive amounts of ($NO^{\bullet}$) are found to be harmful indirectly via the production of RNS and hydrogen peroxide (H_2O_2) (Figure 8, reaction 6) (Moro et al. 2004). Though, NOS enzymes are known to form predominantly ($NO^{\bullet}$), they are also able to produce other ROS. In fact, in order to produce ($NO^{\bullet}$) from L-arginine, NOS needs to be fully coupled with tetrahydrobiopterin

(BH_4). During hypoxia, a deficiency in BH_4 will lead to the formation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) by the reductase domain of NOS isoforms (Bruckdorfer 2005).

- Neutrophils

  Neutrophils play a key role in host defence and inflammation by releasing a large amount of superoxide ($O_2^{\bullet-}$) and other ROS in a phenomenon known as respiratory burst (Babior 1984). ROS production is dependent on the activation of NADPH oxidase; which catalyzes the reduction of oxygen to superoxide ($O_2^{\bullet-}$) (Figure 8, reaction 1) (Quinn et al. 2004). NADPH oxidase is also found in brain cells, notably glia and microvascular endothelial cells (Vallet et al. 2005; Miller et al. 2006). The cerebral NADPH oxidase promotes ROS generation which participate in BBB disruption and brain oedema formation (Tang et al. 2005; Kahles et al. 2007).

- Other sources of ROS/RNS

  Although their contribution to reactive species generation is considered minor; cytochrome P450 (CYP_{450})-, cyclooxygenases (COX)- and lipoxygenases (LOX)-derived ROS are essential in the cerebrovascular regulation (Puntarulo et al. 1998; Kang et al. 2001; Bazan et al. 2002). The catalytic activation of CYP_{450} is responsible for superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and

hydrogen peroxide (H_2O_2) production (Porter et al. 1991; Terashvili et al. 2006).

Cyclooxygenases and lipoxygenases mediate the oxidative metabolism of arachidonic acid (AA) and liberate reactive oxygen species. Production of ROS and RNS during brain ischemia reperfusion was inhibited by administration of COX or LOX specific inhibitors (Pourcyrous et al. 1993; Faraci et al. 2001). COX exists in two isoforms namely COX-1 (constitutive) and COX-2 (inducible), which convert AA into endoperoxide (PGH_2); precursor of prostanoids. Though inducible, COX-2 is found in a constitutive manner in cerebral endothelial cells (Parfenova et al. 2001; LaManna et al. 2006). In cerebral ischemic animal models, ROS production was decreased when blocking COX-2 activation (Wang et al. 2006). LOX enzymes are also found in various isoforms producing leukotrienes and hydroperoxides. These products are highly increased in cerebral ischemic conditions taking part in brain injury (van Leyen et al. 2006).

2.3.3 Lipid peroxidation

Lipid peroxidation can be defined as the oxidative degradation and deterioration of membrane lipids containing any carbon-carbon double bonds (Farooqui et al. 1998). These lipids are referred to as unsaturated fatty acids and when containing more than one double bond, they are named polyunsaturated fatty acids (PUFA)

such as linoleic acid (LA; C18:2n-6), arachidonic acid (AA; C20:4n-6) and docosahexaenoic acid (DHA; C22:6n-3). The phospholipids of the brain are uniquely rich in PUFA (Farooqui and Horrocks 1998). Not only they are essential to maintain cellular function integrity conferring membrane fluidity and selective permeability of cellular membranes, but these lipids are also a target of choice of oxidative stress (Choe et al. 1995; Simopoulos et al. 1999). On the contrary, monounsaturated (one double bond) and saturated fatty acids (no double bonds) are much less reactive and do not usually participate in lipid peroxidation. When oxidized, membrane lipids can disrupt the organization of the cellular membrane, causing rigidity, inhibition of metabolic processes, increased ion permeability (Nigam et al. 2000), covalent cross-linking between lipids and proteins and inactivation of membrane proteins and receptors (Burns et al. 2002). Lipid peroxidation gives rise to lipid hydroperoxides which formation occurs through two distinctive mechanisms, either enzymatic or non enzymatic.

Enzymatic production of oxidized lipids occurs via the activation of lipoxygenases (LOX), cyclooxygenases (COX) and cytochrome P450 enzymes (CYP_{450}) (Figure 10). These enzymes generate distinctively stereo- and regio-specific products (O'Donnell 2003). During cerebral ischemia, the calcium influx will activate the enzyme phospholipase A2 (PLA_2) which cleaves fatty acids from the membrane (Muralikrishna Adibhatla et al. 2006). In fact, the most abundant fatty acid in the brain is arachidonic acid which levels are found to be highly increased in ischemic conditions (Bazan and Rakowski 1970). Once released, AA is oxidatively metabolized by LOX, COX or CYP_{450} (Figure 10). Three major

isoforms of LOX exist, with respect to the position preference of arachidonic acid oxygenation: 5-LOX, 12-LOX and 15-LOX (Catala 2006). The most characterized lipoxygenases in the brain are the 5-LOX and 12-LOX where the 5-LOX generates leukotrienes and the 12-LOX produces hydroxy-peroxy-eicosatetraenoic acids (HpETEs). These latter metabolites are the most abundant eicosanoids found in neurons, glia and cerebral endothelial cells (Rossi et al. 2007). Cyclooxygenase catalyze the stepwise conversion of AA into prostaglandin G (PGG_2) and then prostaglandin H (PGH_2); which is in turn metabolized into various prostaglandins (PGs); prostacyclin (PGI_2), prostaglandin E (PGE_2), prostaglandin D (PGD_2), prostaglandin F ($PGF_{2\alpha}$) and thromboxane (TXA_2). All of these PGs exert distinctive functions via the activation of their specific receptors; essentially involved in the regulation of vasomotor tone and inflammation (Kadowitz et al. 1988; Murray et al. 1988). The microsomal cytochrome P450 complex may also act on free endogenous AA, catalyzing its conversion into epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs).

Non enzymatic lipid peroxidation is a free radical-driven chain reaction (Imaizumi, Tominaga et al. 1986). A critically important aspect of lipid peroxidation is that it is self-propagating and will proceed until substrate is consumed or termination occurs (Montine et al. 2004) (Figure 11A). Such a chain reaction is initiated by the abstraction of a hydrogen atom from a *cis* methylen group of PUFA by hydroxyl radical ($OH^{\bullet}$). This reaction induces the formation of

a lipid alkyl radical ($L^{\bullet}$) (Figure 11B, Reaction 1) that combines rapidly with oxygen to generate lipid peroxyl radical ($LOO^{\bullet}$) (Figure 11B, Reaction 2).

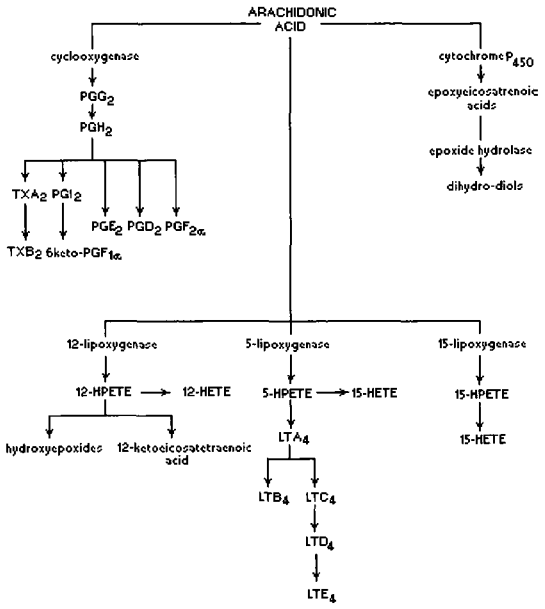

Figure 10| Pathways of arachidonic acid metabolism. LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4 = leukotrienes. TXB_2 = thromboxane B_2; stable metabolite of TXA_2. 6-keto-$PGF_{1\alpha}$ = stable metabolite of PGI_2.

Lipid peroxides can in turn, abstract a hydrogen from another PUFA, and so set in motion a propagating chain reaction (Porter et al. 1995) (Figure 11B, Reaction 3). Under conditions where lipid peroxidation is continuously initiated, a termination reaction limits the process yielding non radical products.

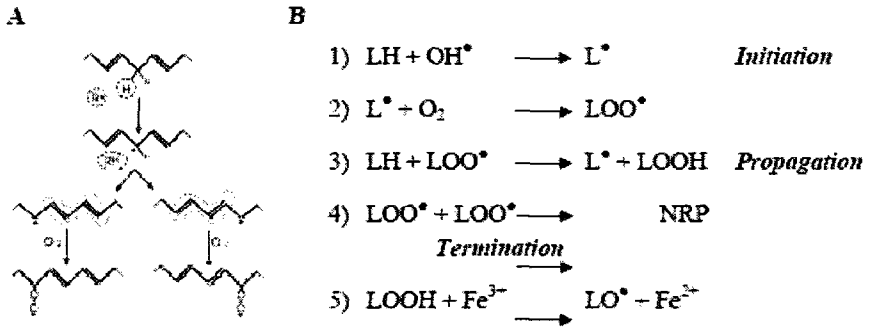

Figure 11| Lipid peroxidation. A) Mechanism of hydrogen abstraction by a free radical ($R^{\bullet}$) and formation of lipid peroxyl radical ($LOO^{\bullet}$). B) Cascade of non enzymatic lipid peroxidation. LH = PUFA. LOOH = lipid hydroperoxide; relatively stable product. NRP = non radical products.

In the presence of transition metal ions such as iron and copper, which levels are highly increased in cerebral ischemia, lipid hydroperoxide (LOOH) can be decomposed into peroxyl ($LOO^{\bullet}$) and alkoxyl radicals ($LO^{\bullet}$) that are able to reinitiate lipid peroxidation (Halliwell 1994); thus participating in amplifying this phenomenon (Marnett et al. 1995) (Figure 11B, Reactions 5 and 6). Lipid hydroperoxides are able to generate a large variety of products including short and long chain aldehydes as well as phospholipid and ester core aldehydes, many of which are used to assess the degree of lipid peroxidation (Porter 1990). Some of these aldehydes are highly reactive and may be considered as toxic second messengers increasing initial free radical events. The most intensively studied aldehydes are 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), both of which are the main aldehydes formed from linoleic and arachidonic acids (Pryor et al. 1990; Esterbauer et al. 1991). Compared to free radicals, aldehydic molecules are more stable and diffuse within the cell or escape from it, attacking targets far from the site of origin (Uchida 2003). Though extensively used as

biomarkers of oxidative stress, 4-HNE and MDA show a high reactivity with other biomolecules and a rapid metabolism, thus limiting their use as *in vivo* markers (Cheng et al. 2001; Montine, Quinn et al. 2004). For these reasons, the attention was driven towards other products of lipid peroxidation that are chemically and metabolically stable and therefore suitable as *in vivo* biomarkers. These compounds include the isoprostanes (IsoPs) (Morrow et al. 1990), isofurans (IsoFs) (Fessel et al. 2002) and neuroprostanes (NeuroPs) (Roberts et al. 1998).

Isoprostanes are formed *in situ* by peroxidation of AA (C20:n4-6) independently from COX (Morrow, Hill et al. 1990). After abstraction of a hydrogen and addition of an oxygen molecule to form a peroxyl radical, endocyclization occurs and an additional molecule of oxygen is added to form PGG_2-like compounds (Morrow et al. 1990). These instable compounds are then converted to IsoPs. Based on this mechanism of formation, four region-isomers of IsoPs are generated; namely 5-, 8-, 12- or 15-series depending on the carbon atom to which the side chain hydroxyl is attached (Taber et al. 1997). One of the major differences between IsoPs and COX-derived eicosanoids is that the formers are produced within lipid membranes from esterified AA and are subsequently released by phospholipases (Liu et al. 1999). On the contrary, prostaglandins are generated only from free arachidonic acid (Morrow et al. 2002). The most abundant isoprostane in the brain is the 8-iso-PGF_2 which is a promising marker of oxidative injury due to its specificity as an index of lipid peroxidation, its stability, its detectability in normal biological fluids and its insensitivity to the diet content of lipids (Roberts et al. 2000). In fact, serum levels of 8-iso-PGF_2 were found increased in stroke patients, a few days after the event (Sanchez-Moreno et

al. 2004) reaching concentrations 10 times over the baseline (Hoffman et al. 1996). Since their discovery, many studies have focused on the role of IsoPs as index of lipid peroxidation. Furthermore, IsoPs are also bioactive compounds. Both, the 8- and 15-series IsoPs have been attributed a thromboxane- dependent vasoconstrictor effect on various vascular beds, notably the kidney, the lung, the aorta and coronary arteries, the retina and the brain (Wagner et al. 1997; Lahaie et al. 1998; Salahudeen et al. 1998; Wilson et al. 1999; Hou et al. 2000; Janssen et al. 2002). Recent evidence has implicated the isoprostanes as potent cytotoxic compounds to both cerebral microvascular endothelial cells and oligodendrocytes progenitor cells (Brault et al. 2003; Brault et al. 2004).

Isofurans (IsoFs) are also non enzymatic products of arachidonic acid discovered only very recently (Fessel, Porter et al. 2002). IsoFs and IsoPs have the same carbon-centered radical intermediate. However, an oxygen insertion diverts this intermediate of the IsoPs pathway, to form instead tetrahydrofuran ring-containing compounds, termed the IsoFs. Due to this particular formation pathway, IsoFs generation is favoured to IsoPs', in high oxygen tension conditions. Whereas levels of IsoPs reach a plateau at 21% O_2 (normal oxygen tension), IsoFs levels increase linearly with increasing oxygen tension. IsoFs are stable compounds and present in normal tissues and biological fluids at readily measurable concentrations (Fessel, Porter et al. 2002). IsoFs are now being considered as more sensitive and robust indicators of oxidative injury compared to IsoPs, as they provide unique information about the severity of lipid peroxidation (Montine, Quinn et al. 2004; Patel et al. 2008). No biological activity has been

assigned to IsoFs until today thus considered only as biomarkers of oxidative damage.

Neuroprostanes are DHA-derived lipid peroxidation products. NeuroPs have the same nomenclature as IsoPs (Roberts, Montine et al. 1998). In contrast to AA which is widely distributed in all cell types of all brain regions, DHA is very highly concentrated in neuronal membranes (Salem 1986). Thus, NeuroPs could provide an interesting window through which quantification of oxidative damage is related to neuronal membranes *in vivo*. This approach could be extremely useful when considering neurodegenerative diseases (Montine, Quinn et al. 2004).

Although not extensively documented, nitrogen dioxide ($NO_2^{\bullet}$) also participates in lipid peroxidation. Formed from peroxynitrite ($ONOO^-$) (Figure 8, Reaction 6) along with hydroxyl radical ($OH^{\bullet}$); ($NO_2^{\bullet}$) is also capable to attack fatty acids by hydrogen abstraction as well as triggers secondary free radical reactions (Szabo et al. 2007). Two mechanisms have been proposed to elucidate the formation of nitrogen dioxide ($NO_2^{\bullet}$)-derived lipid hydroperoxides.

First, a homolytic addition of ($NO_2^{\bullet}$) to the double bond of PUFA yields a β-nitroalkyl radical, which fate depends on the ionic environment (Baker et al. 2005). In a hydrophobic environment, the radical intermediate will revert and the slow but irreversible hydrogen abstraction eventually prevails and initiates chain peroxidation reactions (Freeman et al. 2008). Under hypoxic-ischemic conditions, the balance between peroxyl radical ($LOO^{\bullet}$) formation and coupling with ($NO_2^{\bullet}$) will shift towards nitration reaction and formation of nitro-fatty acids (NO_2-FA). With regard to stereochemistry, the nitro group preferentially constrains the double bond of (NO_2-FA) in the *cis* configuration, with only a minor proportion

present as *trans* isomers (Freeman, Baker et al. 2008). Among the nitrated fatty acids identified *in vivo*, to date, the most studied are nitro-linoleic and nitro-oleic acids. These (NO_2-FA) display an NO-dependent vasorelaxation (Lim et al. 2002; Lima et al. 2005) and receptor-mediated reactions to exert adaptive and anti-inflammatory cellular responses (Balazy et al. 2001; Batthyany et al. 2006).

The second mechanism involves a *cis-trans* isomerisation reaction where ($NO_2^{\bullet}$) will most likely bind to one of the PUFA double bonds, forming a nitro-radical followed by the elimination of ($NO_2^{\bullet}$) and generation of the *trans* bond (Balazy 2000). The only PUFA for which this mechanism has been described is arachidonic acid (C20:4-n6); forming 4 different *trans*-isomers: the *trans*-arachidonic acids (*T*AA). Since their discovery, *T*AA were used as markers of ($NO_2^{\bullet}$) mediated injury due to their stability and availability in biological fluids and plasma. *T*AA levels were found increased in a septic shock model of experimental inflammation and in hypercapnic and hyperoxic settings (Balazy 2000; Kermorvant-Duchemin, Sennlaub et al. 2005; Checchin et al. 2006).

2.3.4 Deleterious effects of ROS/RNS

Excessive production of reactive species can overwhelm protective mechanisms and initiate structural changes in the cell. Proteins, lipids and nucleic acids can be modified by ROS/RNS (Floyd et al. 1989). Damages to macromolecules lead to formation of a plethora of products; some of which were stated in the previous section with reference to lipids.

a) Effects on nucleic acids and DNA

Reactive species damage nucleic acids producing three types of oxidative lesions: strand breakage, nucleic acid-protein cross-linking and nucleic acid base modifications (Breen et al. 1995). In fact, nucleic acids are very reactive with strong oxidants such as superoxide and hydroxyl radical, which are able to attack sugars, purines and pyrimidines in DNA (Breen and Murphy 1995). Single strand breaks can be detected within minutes of reperfusion after hypoxic-ischemic (HI) injury, whereas double strand breaks are detected within 1 hour (Li et al. 1995). Hydroxyl radical, superoxide, peroxynitrite and to a lesser extent nitric oxide attack the sugar portion of DNA and cause strand breaks (Brawn et al. 1981; Floyd et al. 1990; Nguyen et al. 1992; Epe et al. 1996). Strand breaks have important implications in pathological conditions and repair becomes essential for the cellular function. Because enzymes repairing DNA have decreased fidelity, there is a high probability of misincorporation of DNA bases, providing further damage. There are at least three base modifications that result from reaction with DNA which are 5-hydroxymethyluracil, thymine glycol and 8-hydroxy-2-deoxy-guanosine (8-OHdG) (Inoue et al. 1995). These modifications are carried out by hydroxyl radical and singlet oxygen attacks on DNA (Floyd et al. 1988; Floyd, West et al. 1989). The 8-OHdG is the most studied biomarker of oxidative DNA damage due to its specificity and abundance in DNA (Cui et al. 2000). Plasma levels of 8-OHdG were found increased in animal models subjected to HI injury (Nagayama et al. 2000).

b) Effects on proteins

Reactive species can also affect proteins giving rise to different derivatives via a variety of mechanisms that include amino acid oxidation and fragmentation. Oxidation of proteins can lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and conversion of some amino groups to carbonyl derivatives. Oxidation can also lead to cleavage of the polypeptide chain and formation of cross-linked protein aggregates (Stadtman et al. 2003). Such modifications can alter the structure of the protein causing a loss of function or its degradation and proteolysis (Dean et al. 1997). The most susceptible amino acids to be attacked by free radicals are cysteine and tyrosine and other aromatic amino acids (Davies et al. 1987). Some of the modifications stated above are reversible and such reversal may lead to reactivation of the protein function. For instance, modifications of thiols on cysteine residues such as nitrososthiol (S-NO) and sulfenic acids (S-OH) by the action of peroxynitrite and hydroxyl radical, respectively, occur by abstraction of an electron from the thiol group (Davies 2001). Some antioxidants cofactors such as glutathione exploit their reactive thiol group (GSH) as an electron acceptor where it cycles from oxidized (GSSG) to reduced state (GSH), converting hydrogen peroxide (H_2O_2) to water, in a reaction catalyzed by glutathione peroxidase (Figure 13). This particular modification of

cysteine residues may have a dual role of protection from irreversible and terminal oxidation and modulation of the redox regulation function (Giustarini et al. 2004). However, under pathophysiological settings, oxidised thiol groups could undergo further oxidation and form irreversibly, sulphinic (SO_2H) and sulphonic acids (SO_3H) (Giustarini, Rossi et al. 2004). Reactive species are also responsible for the formation of disulphide bonds within a protein (intra-molecular cross-linking) or between two proteins (inter-moelcular cross-linking) which may greatly affect the protein conformation. Inappropriate protein conformation leads to improper protein structure and increases the susceptibility of their degradation. The specific process of bridge formation between a protein thiol cysteine group and glutathione is called S-glutathionylation and this process is reversibly catalyzed by disulphide isomerases (Klatt et al. 2000).

Nitric oxide and its derived reactive nitrogen species catalyze three different reactions with proteins, namely nitration, nitrosylation and nitrosation. Nitrosation reactions can be defined as the addition of nitrosonium equivalent (NO^+) to a nucleophile amine, thiol or hydroxyl residues (Williams 1998). This process is also referred to as *S*-nitrosylation (Ischiropoulos et al. 2005). Primarily, *S*-nitrosylation is a chemical reaction which takes place without the requirement of any enzymatic activity, leading to the formation of *S*-nitrosocysteine. Though all NOS isoforms are able to form *S*-nitrosocysteine; the secondary

structure of the target protein dictates the specificity and selectivity of this modification (Stamler et al. 1997). Two specific structural motifs have been identified as putative *S*-nitrosylation sites; (1) the location of a cysteine residue within a pattern of acidic and basic flanking residues (Choi et al. 2000) and (2) a cysteine contained in a hydrophobic region (Mannick et al. 1999). The functional consequence depends on the protein that is nitrosated. For instance, nitrosated caspases or NMDA receptors result in their inactivation which might procure a protective effect (Mannick, Hausladen et al. 1999; Choi, Tenneti et al. 2000) during HI, whereas nitrosated matrix metalloproteinases increase enzyme activity and may result in enhanced damage to the blood brain barrier (Gu et al. 2002). Nitrosylation is often mistakenly used interchangeably with *S*-nitrosylation (nitrosation). Nitrosylation is defined as the addition of (NO) without change in the formal charge of the substrate. Examples of nitrosylation include addition of NO to heme proteins and formation of iron nitrosyl complexes (Espey et al. 2002). Unlike nitrosation and nitrosylation, nitration reactions; addition of a nitro ($-NO_2$) group, is facilitated by the formation of higher oxides of nitrogen. Protein nitration is a common characteristic of nitrative injury involving mostly aromatic amino acids such as tyrosine and tryptophan (Gow et al. 2004). The most recurrent modification occurs on tyrosine residues which leads the formation of the protein-bound 3-nitrotyrosine, considered as a molecular footprint of nitrative injury (Gursoy-Ozdemir et al. 2004). The two mostly invoked mechanisms of biological nitration namely, peroxynitrite and heme

peroxidase pathways, lead to the concomitant formation of tyrosyl radicals and ($NO_2^{\bullet}$), which combine together to form 3-nitrotyrosine (Figure 12). In fact, ($NO_2^{\bullet}$) alone is inefficient to promote nitration because of its slow reaction rate with a tyrosine residue compared to other processes such as carbonate radical ($CO3^{\bullet-}$) or oxo-metal complexes.

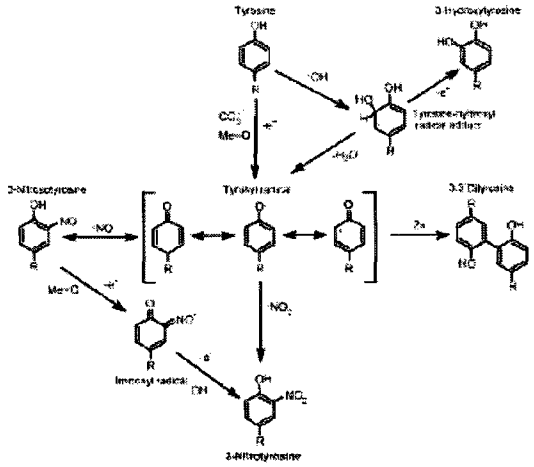

Figure 12| Free radical pathway of 3-nitrotyrosine formation. (Taken from Radi PNAS 2004) (Radi 2004).

The addition of an –NO2 group on tyrosine constitutes a bulky substituent and, if placed on biologically important tyrosine, nitration can impose steric restrictions and inhibits tyrosine phosophorylation. However, to have a biological significance, nitration has to induce either a gain or loss of function. The gain of function scenario has been shown for a few proteins such as nitrated cytochrome P_{450} which acquires a strong

peroxidase activity (Cassina et al. 2000) and Protein Kinase C (PKC) which becomes active and translocates upon nitration (Balafanova et al. 2002). A notable example of enzyme loss of activity linked to nitration *in vivo* is the anti-oxidant mitochondrial manganese superoxide dismutase (MnSOD) or PGI_2 synthase (MacMillan-Crow et al. 1996; Pearce et al. 1999). Following ischemia-reperfusion, nitrotyrosine immunoreactivity was majorly found in the infarcted area of the human brain (Forster et al. 1999). Site-specific protein nitration is associated to acute and chronic vascular disease states (Radi 2004) where extensive nitration has been observed in cerebral vessels following focal ischemia (Coeroli et al. 1998) and might mediate the disruption of blood brain barrier (Han et al. 2006).

2.3.5 Major defences against oxidative and nitrative damage

The ROS and RNS levels are controlled through an intricate network of enzymes that control their production as well as their consumption. Consumption of free radicals occurs in two ways, namely chain breaking or preventive, driven by a plethora of anti-oxidants. By definition, anti-oxidants are substances that are able, at relatively low concentrations, to neutralize, stabilize, inhibit or significantly delay oxidation (Halliwell et al. 1989). Chain breaking anti-oxidants are able to terminate the self-propagating chain reactions driven by free radicals. Preventive anti-oxidants scavenge initiating free radicals or stabilize transition metal radicals (e.g. iron, copper), thus reducing the rate of chain initiation or thwarting a chain from setting in motion (Halliwell 1993). Anti-oxidants defences in biological

systems play an important role in the detoxification process where they are generally categorized as non enzymatic and enzymatic lines.

2.3.5.1 Enzymatic antioxidant defences

Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx) and heme oxygenase (HO) which serve as the primary line of defence in destroying free radicals (Figure 13).

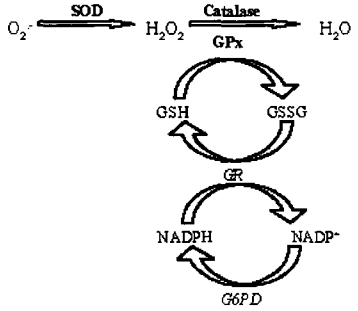

Figure 13| Detoxification pathway of free radicals. Enzymatic antioxidants involved in the degradation of superoxide. GR = glutathione reductase. G6PD = glucose-6-phosphate dehydrogenase. GSH = reduced glutathione. GSSG = oxidized glutathione. (Modified from (Janssen et al. 1993)).

a) Superoxide dismutases

Superoxide dismutases are a class of enzymes that catalyze the conversion of superoxide ($O_2^{\bullet-}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Figure 9, Reaction 2). Superoxide enzymes contain metal ion cofactors that, depending on the isoenzyme, can be copper, zinc or manganese (Zelko et al. 2002). There are three major isoforms of SOD in the human brain. The copper/zinc SOD (Cu/Zn SOD) or SOD1 is principally found in cytosolic and lysosomal fractions but could also be present in the mitochondrial intermembrane space. The manganese SOD (MnSOD) or SOD2 is only found in the mitochondrial matrix (Bannister et al. 1987). The cerebral extracellular SOD (ECSOD) or SOD3 is secreted in the

extracellular matrix with substantially lower concentrations (Marklund 1984; Nozik-Grayck et al. 2005). Both Cu/ZnSOD and MnSOD play a crucial role against the overproduction of superoxide in ischemia-reperfusion (Fujimura et al. 1999). Decreased activity of one or the other exacerbates brain damage and increases the infarct volume (Yang et al. 1994; Kim et al. 2002).

b) Catalases

Catalases are responsible for the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). Localized in peroxisomes, their levels and activity decrease in the brain following injury (Homi et al. 2002). These changes are associated with enhanced susceptibility of certain brain regions and prolonged free radical-induced damage.

c) Glutathione peroxidases

GPx contain four selenium cofactors that are capable of reducing organic and inorganic hydroperoxides to the corresponding hydroxyl compounds (water in the case of H_2O_2) utilizing glutathione (Brigelius-Flohe 1999) and with a much higher affinity for (H_2O_2) than catalase (Sies 1991). GPx exist in at least 5 isoforms, two of them are present in the brain (Schweizer et al. 2004). While the cytosolic GPx1 isoform is ubiquitously found in cerebral cells, the phospholipid hydroperoxide GPx4 is exclusively present in neurons (Lindenau et al. 1998; Borchert et al. 2003). However, only the GPx1 isoform is known to reduce (H_2O_2) into water (Thomas et al. 1990).

On the other hand, GPx4 is unique among antioxidative enzymes as it reduces directly phospholipid hydroperoxides within lipid membranes (Sattler et al. 1994; Savaskan et al. 2007).

d) Heme oxygenases

Heme oxygenases are a component of the endogenous cell defence against oxidative injury (Otterbein et al. 2000). HO catalyzes the first and rate limiting step reaction of heme degradation. In the HO reaction, the oxidation of heme generates equimolar concentrations of ferrous iron, carbon monoxide and biliverdin IXα which is then reduced into bilirubin by biliverdin reductase (Tenhunen et al. 1969). In hypoxic cellular environments, the bile pigments have antioxidant properties by scavenging peroxyl radicals (Stocker et al. 1987). On the other hand, the liberated free iron is readily detoxified by sequestration by ferritin, an intracellular iron-storage molecule with potential cytoprotective function, particularly in endothelial cells (Oberle et al. 1999). Of the two main isoforms of HO, only the inducible isoform HO-1 responds to xenobiotics (Maines 1992).

2.3.5.2 Non enzymatic antioxidant defences

Non enzymatic antioxidants are natural small molecules that act as free radical scavengers. By donating one of their electrons, these molecules neutralize ROS and RNS and remain stable in their oxidized form. Natural antioxidants include Vitamin C, Vitamin E, glutathione, carotenes and melatonin. Briefly, vitamin C or ascorbic acid acts in the water-soluble compartment, whereas Vitamin E (α-tocopherol) intervenes exclusively in lipid-soluble membranes and is a major

chain-breaking antioxidant (Padayatty et al. 2003; Traber et al. 2007). Melatonin is a powerful antioxidant able to cross cellular membranes as well as the blood brain barrier. It is also known as a suicidal antioxidant because it does not undergo the redox cycling (repeated reduction-oxidation reactions). Once oxidized, melatonin forms different stable end products (Tan et al. 2000). Carotenes are part of the carotenoid family containing specific hydrocarbon end groups. The antioxidant actions of carotenes relay on their ability to scavenge peroxyl radicals and quenching singlet oxygen (Stahl et al. 1996). This results in an excited cartone which has the ability of returning to its former state by a series of rotational and vibrational reactions with the solvent, thus allowing itself to be reused for further cycles of singlet oxygen quenching (Paiva et al. 1999). The most document carotene is β-carotene which scavenges peroxyl radicals at low oxygen tension (Burton et al. 1984).

2.3.6 Potential antioxidant therapies in the ischemic brain

Increased expression of antioxidant enzymes can occur shortly in response to ischemia, however, endogenous antioxidant capacity can be overwhelmed, leading to increased ROS and RNS products (Fukui et al. 2002). Because of the deleterious and serious irreversible cellular and tissue damage induced by ROS and RNS, an urgent need to counteract theses species in a more efficient way, is needed. Many studies over the years have focused their interest in finding inhibitors for pro-oxidative enzymes and free radical scavengers. In this section, only drugs related to the ischemic brain are mentioned.

a) Inhibition of lipid peroxidation

In the 1990's, a novel group of compounds, the 21-amino-steroids (lazroids), have been designed as potent inhibitors of FR-induced iron-catalyzed lipid peroxidation (Braughler and Hall 1989; Hall et al. 1994). One of these compounds, tirilazad mesylate, has been selected for clinical evaluation as a cerebroprotective agent. Tirilazad mesylate (Tirilazad) is a non-glucocorticoid steroid that localizes within cell membranes with high affinity for vascular endothelium (Audus et al. 1991). The compound possesses many antioxidant mechanisms including: (1) Scavenging of peroxyl lipids similar to the action of Vitamin E (2) Scavenging of hydroxyl radicals with decrease in membrane fluidity (3) Restricting the movement of peroxidized lipids within the membrane disabling them to attack other PUFAs with potential inhibition of the propagation of chain reactions (Braughler and Hall 1989; Hall, McCall et al. 1994). In hypoxic ischemic animal models, tirilazad was very effective against endothelium-dependent relaxation, increased infarct size, neuronal necrosis, brain injury and cerebral oedema (Hall et al. 1988; Park et al. 1994; McKenna et al. 1995). As a result of these animal studies, clinical trials were undertaken to assess the safety and efficacy of tirilazad in the treatment of acute ischemic stroke. A total of six trials concluded that, contrary to animal studies outcome, tirilazad increased death and disability in humans (1994; 1996; Johnston et al. 1998; 2000).

b) Inhibition of xanthine oxidase

The most documented inhibitor of XO is allopurinol (1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one) which is widely used to treat gout and hyperurecimia (Pea 2005; Pacher et al. 2006). Allopurinol is rapidly oxidized by XO in vivo to its active metabolite oxypurinol, which also inhibits XO. At low concentrations, allopurinol is a substrate for and competitive inhibitor of the enzyme, whereas oxypurinol is a non competitive inhibitor. The formation of oxypurinol, together with its long persistence in tissues, is responsible for much of the pharmacological activity of allopurinol (Pacher, Nivorozhkin et al. 2006). Both compounds were tested in various animal models of ischemic and reperfusion brain. Allopurinol was found to be protective against mortality and neurological deficits (Itoh et al. 1986). Pre-treatment with this compounds reduced infarct size, even if its administration was delayed to the beginning of the reperfusion period (Martz et al. 1989; Palmer et al. 1990; Palmer et al. 1993; Shadid et al. 1998). On the other hand, oxypurinol reduced the ischemic cerebral damage and attenuated neurological deficits by improving cellular ATP levels (Phillis et al. 1991; Lin et al. 1992; Phillis et al. 1995). However, it is necessary to mention that oxypurinol was without significant protective effects in some studies (Arai et al. 1998; Nakashima et al. 1999). So far, the human therapeutic experience with allupurinol is limited to one study conducted on severly asphyxiated infants. In this study, allopurinol tented to improve survival, exerted beneficial effects on FR formation, cerebral blood flow volume and electrical brain activity (Van Bel et al. 1998). During the past decade,

definite progress has been achieved to develop new powerful XO inhibitors. Two compounds were found very potent, namely febuxostat and Y-700 (Okamoto et al. 2003; Becker et al. 2004). These are reported to have a favourable toxicology profile, a high bioavailability and a more potent and longer-lasting hypourecemic action than allopurinol (Hoshide et al. 2004). These compounds are currently in human clinical trials for the treatment of hyperurecimia and gout (Mayer et al. 2005).

c) Inhibition of nitric oxide synthases

Since the original suggestion that nitric oxide plays a role in cerebral ischemia (Marshall et al. 1990); extensive studies have addressed this issue. NOS inhibitors were soon tested on animal models but given in large doses with little selectivity. Some studies found improved outcome, while others found worsened outcome. It soon became apparent that the effect of NOS inhibition was dependent on which isoform was being inhibited. Pharmacological inhibition of eNOS will worsen the outcome, secondary to vasoconstriction and reduced blood flow (Lo et al. 1996). Use of selective nNOS antagonists confirmed neuronal production of NO which contributes to ischemic cell death (O'Neill et al. 2000). iNOS has been associated with oxidative stress and modifying its activity may have therapeutic potential (Parmentier et al. 1999). However, little has been reported on efforts to bring nitric oxide inhibitors to clinical investigation (1999; Legido 2000).

d) Superoxide dismutase mimetics

SOD is a key constituent of scavenging reactive species. Recent pharmacological advances have allowed the advent of potent SOD mimetics, capable of crossing the blood-brain barrier (Sheng et al. 2002). Several major classes of SOD mimetics have been reported including the Mn(II) cyclic polyamines, Mn(III) salen derivatives, Mn(III) porphyrins and stable cyclic nitroxides (Riley 2000; Batinic-Haberle 2002; Kwon et al. 2003). All of them eliminate superoxide with various selective SOD-like properties (Salvemini et al. 1999), modest catalase-like activity (Baker et al. 1998) and ability to oxidize nitric oxide (Sharpe et al. 2002) and eliminate peroxynitrite (Ferrer-Sueta et al. 2003) or peroxynitrite-derived products such as nitrogen dioxide (Goldstein et al. 2004). Due to their reactivity towards a wide range of ROS/RNS, these antioxidants are considered versatile and could be protective in different cellular environments. The most documented class of mimetics used in models of hypoxia-ischemia is the Mn(III) porphyrins. When given, up to 6 hours after the onset, these compounds provided potent protection against infarct formation (Mackensen et al. 2001). However, long-term outcome studies have not yet been reported.

e) Spin traps

Spin traps were developed by chemist to "capture" reactive species allowing their detection and quantification. A classic application of this

technology in the study of ischemic brain is the use of salicylate, which reacts with hydroxyl radicals to form a relatively stable adduct, 2,3-DHBA, useful in microdialysis studies (Globus et al. 1995). Other spin traps from the nitrones spin trap family, such as PBN (α-phenyl-*N*-t-butyl-nitrone was found to have therapeutic effects against both global and focal cerebral ischemic insults (Yue et al. 1992; Zhao et al. 1994). More interesting, a second generation of spin traps, NXY-059, has been found to improve ischemic outcome in primates when measured 10 weeks after injury, even when treatment started 4h after onset of ischemia (Marshall et al. 2003). After being proved to be tolerated at proposed therapeutic concentrations in humans, this compound went in Phase III clinical trials where it improved primary outcome (reduced disability at 90 days) but did not alter the neurological deficits (Lees et al. 2006). However, a second attempt of this trail failed and NXY-059 was found ineffective (Shuaib et al. 2007).

2.4 *Trans*-arachidonic acids

Since their discovery in the year 1999 as lipid peroxidation products, *trans*-arachidonic acids (*T*AA) have gained increase interest (Jiang et al. 1999). However, only a few studies have been published since.

2.4.1 Biosynthesis and sources of *TAA*

Arachidonic acid is the most abundant polyunsaturated fatty acid (PUFA) within lipid membranes in all tissues, including the brain. Arachidonic acid is a carboxylic acid with a 20-carbon chain containing four *cis*-double bonds, located between C5=C6, C8=C9, C11=C12 and C14=C15. According to the International Union of Pure and Applied Chemistry Nomenclature (IUPAC), arachidonic acid is termed all-*cis*-5,8,11,14-eicosatetraenoic acid. Because of the location of the first double bond on carbon 6 from the omega end, arachidonic acid is considered as an ω-6 fatty acid (Figure 14). Abstraction of one hydrogen from these methylen groups via a homolytic cleavage of a C-H bond is a fundamental process of arachidonic acid metabolism by enzymatic as well as non enzymatic reactions. Enzymatic processes lead to the formation of biologically active prostaglandins and leukotrienes (Figure 10) known as the eicosanoids (Samuelsson 1991), while known non-enzymatic processes could be led by ROS and RNS (Morrow et al. 1997).

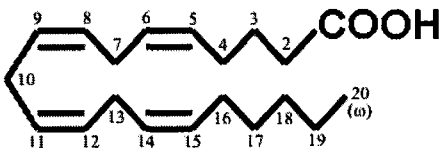

Figure 14| Structure of arachidonic acid.

The non enzymatic oxidation of arachidonic acid by nitrogen dioxide ($NO2^{\bullet}$) occurs within the lipid bilayer in a *cis-trans* isomerisation reaction leading to the potential formation of 4 isomers, named *trans*-arachidonic acids (Jiang, Kruger et al. 1999). Two putative mechanisms of the *cis-trans* isomerisation reaction were proposed as depicted in figure 15.

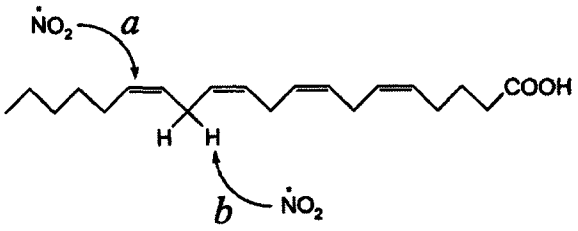

Figure 15| Two mechanism of *cis-trans* isomerisation by ($NO2^{\bullet}$). a) Addition of ($NO2^{\bullet}$) on one of the double bonds and transient formation of a nitroarachidonyl radical. b) Hydrogen abstraction and transient formation of arachidonyl radical. (Taken from (Balazy 2000))

The first mechanism consists of ($NO2^{\bullet}$) binding on arachidonic acid and formation of nitroarachidonyl radical followed by elimination of ($NO2^{\bullet}$) and generation of a *trans* bond (Figure 15, reaction a). The nitroarachidonyl radical is able to react with oxygen to produce 8 isomers of nitrohydroxyeicosatrienoic acids (NO_2OHAA). The second mechanism involves a hydrogen abstraction and formation of arachidonyl radical (Figure 15, reaction b). The arachidonyl radical could either lead to a *trans* bond or react with oxygen to form oxime arachidonic acid and hydroxyeicosatrienoic acids (Balazy 2000). However, this latter mechanism is not fully understood. Some studies have described an iron-catalyzed non enzymatic *cis-trans* isomerisation reaction on PUFA where iron transforms the *cis* bond from an sp2 level to an sp3 level followed by a

reconformation and a rotation, thus generating a *trans* bond (Selzer 1972). The *cis-trans* isomerisation reaction on arachidonic acid mediated by ($NO2^{\bullet}$), described *in vitro*, give rise to 4 different isomers of *trans*-arachidonic acids, named 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA, where *E* stands for the *trans* configuration (Figure 16).

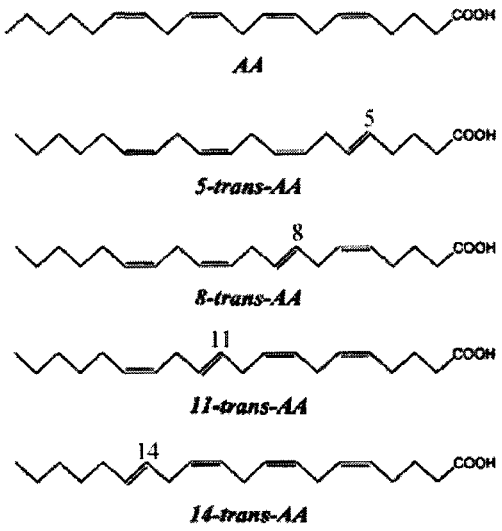

Figure 16| Arachidonic acid and its four *trans*-isomers. Taken from (Balazy 2000).

Although it has been described that a *cis* bond configuration could be converted to a *trans* bond geometry, the presence of *trans* isomers of PUFA is exclusively attributed to exogenous sources. *Trans* fatty acids come from diet, particularly from meat, milk and partially hydrogenated fats and oils (Aro 1998). It is worth noting that dietary *cis* and *trans* fatty acids can be incorporated into cellular membranes (Reichwald-Hacker et al. 1979; Wolff et al. 1994). The double bond angle of the *trans* fatty acids is smaller than the *cis* isomeric configuration and the acyl chain is more linear, resulting in a more stable and rigid molecule which decrease permeability and fluidity of the lipid bilayer (Schofield 1979; Ferreri,

Faraone Mennella et al. 2002). Due to the efficiency of FR-catalyzed isomerisation process, and although the 4 mono-*trans* isomers of arachidonic acid have been described *in vitro*, it is possible to distinguish between the *trans* fatty acids derived from an exogenous supplementation and those formed *in vivo* (Carlson et al. 1997). Such a distinction is crucial to understand the biochemical meaning of *trans* lipids and their relationship with health concerns (Ip et al. 1996; Koletzko et al. 1997; Sebedio et al. 2000; van de Vijver et al. 2000). In the case of arachidonic acid, the *trans* double bonds in positions C5 and C8 (i.e. 5*E*-AA and 8*E*-AA) of the hydrocarbon chain are indicative of endogenous isomerisation, since such isomers cannot derive from dietary precursors. These latter include linoleic acid (C18:2n) which contains 2 *cis* double bonds at position C9 and C12. Through the enzymatic pathways of elongation and desaturation, linoleic acid is metabolized *in vivo* into arachidonic acid. The former double bonds 9 *cis* and 12 *cis* of LA become 11 *cis* and 14 *cis* then leading to 11 *trans* and 14 *trans* respectively (Privett et al. 1967; Beyers et al. 1991). For the past 10 years, accumulating evidence suggests that all 4 *trans* isomers of arachidonic acids are detected *in vivo* under various pathological processes. Due to their stability, *T*AA are measurable in tissues, plasma and biological fluids. Plasma levels from healthy humans showed that all four isomers of *T*AA are present with concentrations reaching around 12 ng/ml for 5*E*-AA and 11*E*-AA isomers and around 4 ng/ml for 8*E*-AA and 14*E*-AA isomers. No significant difference was detected in these levels between males and females (Zghibeh et al. 2004). When measured in various pathological settings, such as septic shock, breast cancer, hyperoxia- and hypercapnia-induced vascular obliteration and diabetes, *T*AA

levels tended to increase, approaching micromolar concentrations in a few hours after injury onset (Balazy 2000; Ferreri, Faraone Mennella et al. 2002; Kermorvant-Duchemin, Sennlaub et al. 2005; Checchin, Sennlaub et al. 2006; Xu, Xun et al. 2008).

Several methods have been proposed to synthesize *T*AA *in vitro*, in order to evaluate their effects on biological tissues. These methods include a gamma-irradiation of methyl arachidonate with thiyl radical as a catalyst (Ferreri, Faraone Mennella et al. 2002) or by flushing ($NO2^{\bullet}$) on arachidonic acid (Jiang, Kruger et al. 1999). However, these methods do not allow for separation of individual isomers. A more convenient method was developed where pure *T*AA isomers could be generated from corresponding *cis*-epoxyeicosatrienoic acids (EETs) (Krishna et al. 2001).

2.4.2 Metabolism of *T*AA isomers

Metabolism of arachidonic acid shows a very distinctive profile as shown in Figure 10. *T*AA isomers however failed to show a similar metabolic profile. *T*AA metabolism requires only oxygen and reduced NADP, indicative of CYP_{450} epoxygenase involvement (Roy et al. 2004; Kermorvant-Duchemin, Sennlaub et al. 2005). Though very little is known about the epoxidation of *trans* fatty acids, 14*E*-AA isomer was proposed to be metabolized first by CYP_{450}/NADPH forming *trans*-epoxides (*trans*-EETs), then converted into dihydoxyeicosatrienoic acids (DiHETEs) by epoxide hydrolase (Figure 17). This process has only been proven *in vitro* by incubating *T*AA with liver microsomal fractions (Roy, Loreau et al. 2004).

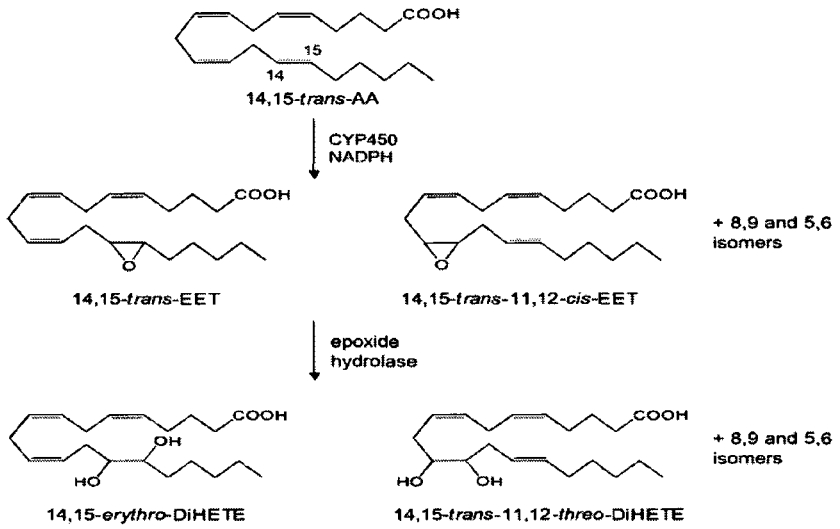

Figure 17| Microsomal epoxidation of 14*E*-AA. CYP450 metabolizes 14E-AA into 4 epoxides which are then converted by epoxide hydrolase into diols (DiHETEs). Taken from (Roy, Loreau et al. 2004).

Another study showed that the 5*E*-AA isomer generates an array of *trans*-EET, DiHETEs and *cis*-HETEs metabolites. Some of these metabolites are bioactive and exert a vasorelaxation effect on various vascular beds (Balazy, Iesaki et al. 2001; Falck et al. 2003; Roy et al. 2005; Oliw et al. 2007).

2.4.3 Biological actions of *T*AA

Although first considered as markers of nitrative stress, *T*AA are also bioactive. All four *T*AA isomers have potent and specific cytotoxic effects. *T*AA induce a selective time- and dose-dependent apoptosis of microvascular endothelial cells resulting in retinal microvascular obliteration. These effects require the upregulation of the anti-angiogenic factor thrombospondin-1 (TSP-1) which increase depends on the transient activation of ERK1/2 (Kermorvant-Duchemin,

Sennlaub et al. 2005). The signalling pathway leading to *T*AA-induced activation of MAPK to upregulation of TSP-1 remains undefined. Furthermore, there is evidence that each isomer of *T*AA might have a distinct function. 5*E*-AA was shown to distinctively induce an arrest of cancer cell growth followed by apoptosis (Jain et al. 2005). The specific mechanisms by which 5-*E*AA mediate these effects is not identified.

2.4.4 Putative receptors for *T*AA

The era of eicosanoid research began in the 1930s with two seminal, though seemingly unrelated observations. The first study found that exclusion of fat from diet in the rats led to growth retardation, reproductive disorders, scaly skin and kidney injuries which led to the discovery of essential fatty acids (Burr et al. 1930). The second study identified a factor with fatty acid properties and smooth muscle stimulating activity termed prostaglandin (US 1934). Some 30 years later, Bergström and Samuelsson have linked both these observations after elucidating the structures of classical prostaglandins and demonstrated that they are produced from the same essential fatty acid: arachidonic acid (Bergstrom et al. 1964). This Nobel Prize winner discovery has stimulated research in the field leading to the discovery of all prostaglandins and leukotrienes as products of AA metabolism, the elucidation of their biosynthesis and characterization of their mechanisms of action (Bergstrom et al. 1982). The main biological action of both prostaglandins and leukotrienes is exerted via disctinct G protein coupled receptors (GPCR) listed in Table 1.

Prostaglandins	Receptors	Roles
PGE_2	EP1	Pain response
	EP2	Ovulation / fertilization
	EP3	Fever
	EP4	Bone resorption
PGI_2 (prostacyclin)	IP	Vasorelaxation
PGD_2	DP1	Allergic asthma
	DP2	Chemotaxis
$PGF_{2\alpha}$	FP	Contraction / Parturition
TXA_2 (thromboxane)	TP_α	Aggregation
	TP_β	Vasoconstriction
Leukotrienes	**Receptors**	**Roles**
LTB_4	B-LT1	Chemotaxis
	B-LT2	?
LTC_4	Cys-LT2	?
LTD_4	Cys-LT1	Bronchoconstriction
	Cys-LT2	?

Table 1. Prostaglandins and leukotrienes receptors and actions. Each synthesized prostaglandin and leukotriene will exert autocrine or paracrine function on a distinct receptor, depending on targeted cell type. Only a few actions are described in this table. B-LT2 and CysLT2 receptors evoke functions that are yet unknown despite their presence in various tissues. (Adapted from Funk CD 2001 (Funk 2001))

Nonetheless, prostaglandins and leukotrienes do not seem to exert their effects solely on GPCRs. Peroxisomal proliferator-activated receptors (PPARs) are able to bind and be activated by various eicosanoids; PPARα by LTB_4 and 8-HETE, PPARγ by 15-deoxy-12,14-PGJ_2 (dehydration product of PGD_2) and PPARδ by prostacyclin analogs (Forman et al. 1995; Kliewer et al. 1995; Yu et al. 1995; Devchand et al. 1996; Gupta et al. 2000). Prostaglandins and leukotrienes are not the only constituents of the eicosanoid family with bioactive properties. Advances

in the eicosanoid field extend beyond the enzymatic products of AA to a variety of non enzymatic derivatives of AA. As mentioned in the previous section, lipid peroxidation products of AA present a vast array of biological actions in many different cell types, paradoxically acting as both friend and foe. Isoprostanes (IsoPs) have intrigued for many years until nowadays all scientists in the field have failed to assign a specific receptor for their bioactive functions. Because some of these effects could be abrogated using a thromboxane receptor antagonist, many have speculated IsoPs to act on TP receptor (Takahashi et al. 1992; Audoly et al. 2000; Sametz et al. 2000). However, evidences against it indicated that IsoPs may not interact with TP (Fukunaga et al. 1993; Longmire et al. 1994; Lahaie, Hardy et al. 1998; Pratico et al. 2001) hence suggesting the existence of a distinct receptor for IsoPs. On another hand, nitrolinoleic acid (LNO_2), nitrooleic acid and other nitrated fatty acids are considered to be endogenous ligands of PPARγ acting within physiological concentrations (Baker, Lin et al. 2005; Schopfer et al. 2005; Alexander et al. 2006). Conversly, other ($NO_2^{\bullet}$)-derived lipid peroxidation products and free fatty acids such as *T*AA mediate their effects via PPARγ-independent mechanisms (Sauer et al. 2000; Louet et al. 2001; Kermorvant-Duchemin, Sennlaub et al. 2005) and are rather characteristic of cell surface receptors.

Recent phylogenetic findings have categorized new nonchemosensory orphan GPCRs to a subfamily of receptors termed "Orphan A5", most closely related to the nucleotide, eicosanoid, protease-activated and lipid receptors (Vassilatis et al. 2003; Brown et al. 2005). These receptors named GPR40, GPR41, GPR42,

GPR43 and GPR120 were found to be activated by free fatty acids via a so-called reverse pharmacology strategy (Kotarsky et al. 2003), previously successful in identifying ligands for other orphan GPCRs (Stadel et al. 1997). The receptors GPR41 and GPR43 are only activated by short chain fatty acids such as formic acid (HCOOH) (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003) while GPR40 and GPR120 are activated by medium or long chain fatty acids including linoleic acid (C18), arachidonic acid (C20) and docosahexaenoic acid (C22) (Briscoe et al. 2003; Itoh et al. 2003; Kotarsky, Nilsson et al. 2003; Hirasawa et al. 2005). Though GPR40 is preferentially activated by chain lengths 12 to 18 carbons among saturated fatty acid and chain lengths 20 to 22 carbons among unsaturated fatty acids (Brown, Jupe et al. 2005), GPR120 functions as a receptor for only unsaturated long chain fatty acids of 16 to 22 carbons (Hirasawa, Tsumaya et al. 2005; Katsuma et al. 2005). However, it is worth mentioning that the nature of ligands for a given receptor differs from one tissue to another depending on the cell type (Stewart et al. 2006). GPR40 receptor is mainly enriched in human and rodent pancreatic islets (where its role in diabetes is being investigated) but only found in the human or primate brain (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003). GPR120 shows a different distribution profile, where it highly concentrated in the human lungs, stomach and slightly in the brain (Fredriksson et al. 2003; Hirasawa, Tsumaya et al. 2005). Both GPR40 and GPR120 activation result in a transient elevation of intracellular calcium and downstream MAP kinase activation, characteristic of $G\alpha_q$ coupled receptors (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003; Hirasawa, Tsumaya et al.

2005). Moreover, GPR40 activation requires the binding of a carboxylate group (COOH) which coordinates with positively charged arginine residue. The regiochemistry of the carboxylate group is also an important factor to determine the receptor affinity (Brown, Goldsworthy et al. 2003; Sum et al. 2007; Tikhonova et al. 2007). In fact, replacement of the carboxylate by a nitro group will elicit antagonistic properties (Tikhonova et al. 2007). However, it is noteworthy to mention that ligands' affinities to these receptors is low as indicated by high EC_{50} values which range from 5 to 50 μM (Briscoe, Tadayyon et al. 2003; Kotarsky, Nilsson et al. 2003; Katsuma, Hatae et al. 2005), depending on the cell type. Due to this low affinity, no binding studies demonstrating the binding of any ligand to these receptors have been reported (Kotarsky, Nilsson et al. 2003). Although these studies have included arachidonic acid in their investigation, no evidence was provided for *T*AA. Nevertheless, the structural resemblance between AA and *T*AA (Figure 18) as well as the specificity of the effect of *T*AA on expression of TSP-1 suggests that *T*AA might exert their effect through an unknown receptor, possibly GPR40 and/or GPR120.

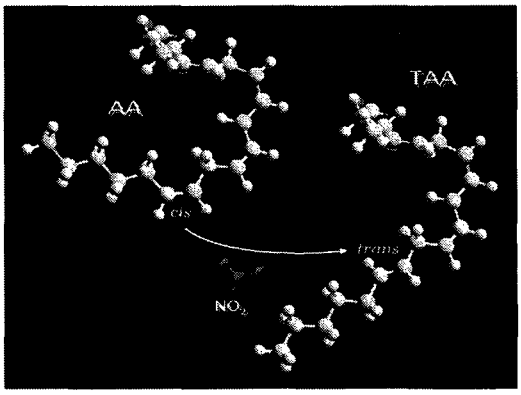

Figure 18| 3-dimensional structures of arachidonic acid (AA) and *t*rans-arachidonic acids (*T*AA). The *T*AA depicted in the picture is the 14*E*-AA: Note the *trans* bond configuration at carbon 14 which alters only the ω-end of the fatty acid leaving intact the carboxylic end of the molecule. The requirement for an acid group seems absolute for GPR40 activation (Brown, Goldsworthy et al. 2003).

CHAPTER 3: *Trans*-arachidonic acids induce a heme oxygenase-dependent vasorelaxation of cerebral microvasculature

3.1 PREAMBULE

In the following pages are represented data demonstrating the role of *trans*-arachidonic acid in the acute phase of cerebral ischemia and dissecting the mechanisms involved. *T*AA caused a rapid endothelium-dependent vasorelaxation of rat brain pial microvessels. This vasorelaxation was independent of all classical vasodilatory pathways (nitric oxide, prostaglandins and endothelium-derived hyperpolarizing factors). Instead, *T*AA-induced vasorelaxation was depenent on a rather unusual pathway necessiting the interaction of heme oxygenase-2 and large conductance calcium-dependent potassium channels. These data demonstrate that *T*AA may participate early in the onset of brain injury by compromising the vascular tone resulting in further oxygen supply and peroxidation. These data are represented in the following publication:

Kooli A, Kermorvant-Duchemin E, Sennlaub F, Bossolasco M, Hou X, Honoré JC, Dennery PA, Varma D, Hardy S, Jain K, Balazy M, Chemtob S (2007). *Trans*-arachidonic acids induce a heme oxygenase dependant vasorelaxation of cerebral microvasculature. *Free Radical Biology and Medicine* 44 (5):815-825.

***Trans*-arachidonic acids induce a heme oxygenase-dependent vasorelaxation of cerebral microvasculature**

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Running title: *Trans*-arachidonic acids and cerebral vasorelaxation

3.2 ABSTRACT

Nitrative stress is an important regulator of vascular tone. We have recently described that *trans*-arachidonic acids (*T*AA) are major products of $NO_2^{\bullet}$–mediated isomerization of arachidonic acid in cell membranes and that nitrative stress increases *T*AA levels leading to neural microvascular degeneration. In the present study, we explored whether *T*AA exert acute effects on neuromicrovascular tone and investigated potential mechanisms thereof. *T*AA induced an endothelium-dependent vasorelaxation of rat brain pial microvasculature. This vasorelaxation was independent of nitric oxide, prostanoids, lipoxygenase products, and CYP_{450} metabolites *trans*-hydroxyeicosatetraenoic acids. However, inhibition of heme oxygenase (using zinc protoporphyrin IX) and of dependent soluble guanylate cyclase (sGC; using ODQ), significantly diminished (by ~70%) the *T*AA-induced vasorelaxation. Consistent with these findings, *T*AA stimulated heme oxygenase (HO)-2-dependent bilirubin (using siRNA HO-2) and cGMP formation, and the HO product carbon monoxide (using CO releasing CORM-2) reproduced the sGC-dependent cGMP formation and vasorelaxation. Further exploration revealed that *T*AA-induced vasorelaxation and bilirubin formation (HO activation) were nearly abrogated by large-conductance calcium-dependent potassium channels BK_{Ca} (using TEA and iberiotoxin); opening of BK_{Ca} channels with the selective activator, NS1619, induced a concentration-dependent vasorelaxation, which was inhibited by HO and sGC inhibitors. Co-immunoprecipitation suggested molecular complex interaction between BK_{Ca} and HO-2 (but not HO-1).

Collectively, findings identify new properties for *T*AA, specifically cerebral vasorelaxation through interactive activation of BK_{Ca} with HO-2, and in turn sGC. Our findings provide new insights into the characterization of nitrative stress-derived *T*AA products, by acting as acute mediators of nitrative stress on neurovascular tone.

Keywords: *trans*-arachidonic acids, nitrative stress, cerebral vasorelaxation, heme oxygenase and carbon monoxide, cyclic GMP, large conductance calcium-dependent potassium channels.

3.3 INTRODUCTION

Nitrative stress derivatives and lipid peroxidation products are endogenous molecules that exert diverse biological effects. Nitrative stress leads to the formation of reactive nitrogen species generated *in vivo* by the reactivity of nitric oxide (NO) with a superoxide anion (O_2^-) giving rise to peroxynitrite, which has been implicated to participate in various pathologies (Ronson et al. 1999; Wattanapitayakul et al. 2000; Dickhout et al. 2005). Oxygen and nitrogen free radicals can target a variety of classes of molecules, such as proteins, nucleic acids, carbohydrates, and notably unsaturated fatty acids, including those in the cell membrane resulting in lipid peroxidation and cellular injury (Radi et al. 1991; Salgo et al. 1995; Szabo et al. 1996). Some of the generated products can act as mediators of the oxidative and nitroxidative stresses; this has clearly been shown for isoprostanes (Lahaie, Hardy et al. 1998; Hou, Gobeil et al. 2000; Hou et al. 2001; Hou et al. 2004).

In brain, peroxidation is believed to play an important role in the genesis of various pathologies such as Parkinson's (Selley 1998), Alzheimer's (Butterfield et al. 2001; Lovell et al. 2001), and particularly hypoxic-ischemic cerebral insult (Perlman 2006). Several studies have focused on the identification of NO-derived reactive nitrogen species and their ensued chronic effects. We have recently described a novel peroxidation process mediated by $NO_2^{\bullet}$ which results in a *cis* to *trans* isomerization of arachidonic acid. This non enzymatic reaction produces four stable *trans*-arachidonic acid (*T*AA) isomers namely 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA, where 5*E*-AA and 8*E*-AA are endogenous (Balazy 2000) and not found in diet (Ferreri, Faraone Mennella et al. 2002). Along with other peroxidation products such as isoprostanes, *T*AA are markedly increased during nitroxidative stress and mediate selective endothelial cell death and microvascular degeneration (Brault, Martinez-Bermudez et al. 2003; Kermorvant-Duchemin, Sennlaub et al. 2005). These severe consequences require a sustained and relatively prolonged (minimum 6 h) exposure of endothelium to *T*AA. However, acute hemodynamic changes secondary to generation of *T*AA may also participate to the ongoing injury, either by compromising blood flow or conversely augmenting it and resulting in increased O_2 supply and further fueling peroxidation. For instance, isoprostanes cause significant cerebral vasoconstriction (Lahaie, Hardy et al. 1998; Hou, Gobeil et al. 2000; Hou, Roberts et al. 2001; Hou, Roberts et al. 2004), while reactive nitrogen species, such as peroxynitrite, are potent vasorelaxants (Villa et al. 1994; Graves et al. 1998). To further elucidate the biological functions of *T*AA (as potential mediators of nitroxidative stress) we proceeded to characterize their acute

vasomotor properties specifically as it applies to neural vasculature, in line with previously reported longer-term effects on neural microvascular integrity (Brault, Martinez-Bermudez et al. 2003; Kermorvant-Duchemin, Sennlaub et al. 2005); in this process we investigated their mechanisms of action. We hereby describe that *T*AA elicit pial vasorelaxation via a less conventional pathway involving heme oxygenase-2, through an apparent interaction with the large conductance Ca^{++}-dependent potassium channel (BK_{Ca}).

3.4 MATERIALS AND METHODS

Animals and Tissue Preparation

All animals were used according to a protocol of Hôpital Ste-Justine Animal Care Committee in accordance with the principles of the Guide to the Care and Use of Experimental Animals and guidelines of the Canadian Council on Animal Care. Young adult Sprague-Dawley rats (~2-3 months old [250-300 g]) were purchased from Charles River. Animals were anesthesized by isoflurane (2.5-5%) and killed by intracardiac injection of saline or CHAPS (to remove endothelium; 5 mg/l for 3 min) plus India ink-containing heparin (to visualize brain microvessels). Brains were quickly removed and placed in ice-cold Krebs buffer (pH 7.4) of the following composition (mM): NaCl 120, KCl 4.5, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 27, KH_2PO_4 1.0 and glucose 10.

Synthesis of *Trans*-arachidonic acids

Four *trans*-arachidonic acids were obtained from Dr JR Falck laboratory. All four mono-*trans* isomers of arachidonic acids were synthesized from corresponding epoxides as previously described (Krishna, Reddy et al. 2001). These isomers were 100% pure and did not contain *cis*-arachidonic acid. Their purity and structure were confirmed by mass spectrometry, NMR, IR, HPLC and TLC. The *T*AA are stable as sodium salts in buffered solutions; *T*AA remain stable when stored refrigerated (-80°C) in ethanol (no degradation or peroxidation products detected by mass spectrometry, and biological effects are highly reproducible in aliquots frozen for variable durations)(Jiang, Kruger et al. 1999; Balazy 2000; Krishna, Reddy et al. 2001; Kermorvant-Duchemin, Sennlaub et al. 2005; Roy, Joshua et al. 2005).

Vasomotor Responses of Cerebral Pial Microvessels

Brains were sliced and pinned to a wax support in a bath containing 20 ml Krebs buffer equilibrated in 95% O_2 and 5% CO_2 and maintained at 37°C, as previously described in detail by us (Hou, Gobeil et al. 2000). Sliced brains were allowed to equilibrate for 30 to 45 min before starting the experiment. The effects of *trans*-arachidonic acid (*T*AA) isomers, NS1619 a selective activator of large conductance calcium-dependent potassium channels (BK_{Ca}) (Holland et al. 1996), and the CO releasing molecule ($[Ru(CO)_3Cl_2]_2$ [CORM-2] releases 0.7 mole of CO per mole of CORM-2 (Motterlini et al. 2003)), were studied on the diameter of brain pial microvessels. Since pilot experiments revealed profound vasorelaxation of intact vessels in response to *T*AA, all vasomotor experiments were conducted without need to preconstrict (Nagaoka et al. 2007). Integrity of

smooth muscle vasomotor activity on CHAPS-treated tissues was confirmed by preservation of full contractility to U46619 and relaxation to sodium nitroprusside (SNP), as previously described (Hardy et al. 1998). Microvessels (50-75 μm) were visualized using a video camera mounted on a microscope (Zeiss M-400) and vascular diameter was measured using a digital image analyzer (Sigma Scan Software). Measurements are repeated three times (<1% variability). Vascular diameter was recorded before and after topical administration of increasing concentrations of *trans*-arachidonic acids following inhibitor application. Cumulative dose-responses (10^{-14} to 10^{-6} M for *T*AAs and 10^{-9} to 10^{-5} M for NS1619) were studied on cerebral pial microvessels from brains of different animals in the absence or presence of the nitric oxide synthase inhibitor L-nitro-arginine methyl ester (L-NAME; 1 mM) (Kozma et al. 1999), the cyclooxygenase inhibitor ibuprofen (10 μM) (Wang et al. 2002), the soluble guanylyl cyclase inhibitors 1H-[1,2,4]Oxadiazolo[4,3]quinoxalin-1-one (ODQ, 0.1 mM) (del Zoppo, Becker et al. 2001) and NS2028 (1 μM) (Olesen et al. 1998), the calcium-dependent potassium channel blocker tetraethylammonium chloride (TEA, 1 mM) (Hardy, Abran et al. 1998), the specific BK_{Ca} channel blocker iberiotoxin (0.1 μM) (Hardy, Abran et al. 1998; Gobeil et al. 2002), the cytochrome P_{450} blockers 1-aminobenzotriazole (1-ABT; 1 mM) (Mathews et al. 1985) and miconazole (10 μM) (Pelligrino et al. 1999), the lipoxygenase inhibitor nordihydroguairetic acid (NDGA, 3 μM) (Chen et al. 2005), the heme oxygenase inhibitor zinc protoporphyrin IX (ZnPPIX, 0.5 μM) (Appleton et al. 1999). Concentrations of all

blockers are consistent with those reported to inhibit targeted enzymes and channels.

Cell Culture

Brain microvascular endothelial cells were cultured from newborn pig brains as previously described (Lahaie, Hardy et al. 1998). Human microvascular endothelial cells (HMEC-1) were purchased from ATCC and cultured in EBM media containing 10% FBS, 10 ng/mL EGF and 1 μg/mL hydrocortisone.

Measurements of Nitrite production in porcine endothelial cells

Production of nitrite (major oxidation product of NO) was measured by chemiluminescence (Sievers NO analyzer) as described (Brault, Martinez-Bermudez et al. 2003; Kermorvant-Duchemin, Sennlaub et al. 2005). In brief, porcine brain microvascular endothelial cells were placed in an incubation buffer of the following composition: Tris-HCl (10 mM), pH 7.5, KCl (10 mM), $MgCl_2$ (3 mM), $CaCl_2$ (100 nM). L-Arginine (100 μM), NADPH (1 mM), tetrahydrobiopterin (15 μM), calmodulin (1 μM), FAD (1 μM), and nitrate reductase (80 units/liter) were added and bubbled with O_2 (reaction medium volume of 500 μl) in sealed containers. Cells were incubated 10 min with vehicle (buffer alone), 8*E*-AA (0.1 μM) or SNP (1 mM). Protein concentration was determined by Bradford protein assay using bovine serum albumin as standard. Nitrite production was measured and normalized to protein content.

Measurement of eicosanoids

Brain microvascular endothelial cells were exposed to 8*E*-AA isomer (0.1 μM) for 10 min, and then immediately collected. Cell media was frozen after adding 0.001% of the antioxidant butylated hydroxytoluene (BHT). Lipids were extracted from cell media and analyzed by tandem mass spectrometry (LC/MS/MS) using MRM (multiple reaction monitoring) technique as we reported (Jiang, Kruger et al. 1999; Roy, Joshua et al. 2005). Briefly, the samples were analyzed on a Zorbax Eclipse XDB-C18 column (2.1×50 mm, 1.8μm) and eluted isocratically with 60% solvent A (water/acetonitrile/acetic acid 75:25:0.05 v/v/v) and 40% solvent B (100% acetonitrile) at a flow rate of 0.300 ml/min using an Agilent 1100 HPLC system. The mass spectrometer (API 2000, Sciex, Canada) was set to detect prostaglandins (PGE_2, PGD_2, and 6-keto-$PGF_{1\alpha}$), lipoxygenase-derived HETEs (5-, 8-, 11-, 12-, 15-HETE) and cytochrome P_{450} metabolites of AA (20-HETE, 19-HETE and epoxides: 5,6-, 8,9-, 11,12-, 14,15-EET). The eicosanoids' peaks were detected and quantified using two deuterium-labeled internal standards: 8-iso-$PGF_{2\alpha}$-d_4 and 12-HETE-d_8 (obtained from Biomol, Inc.), which were added during sample extraction. The eicosanoids were quantified using standard curves and the nanogram amounts were normalized to protein content.

Determination of cGMP accumulation

Sliced rat brains or porcine cerebral microvessels were used to determine cGMP production and guanylate cyclase activity as described (Hardy, Abran et al. 1998). Sliced brains and cerebral microvessels were pre-incubated in Krebs buffer in the presence of phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX; 0.5 mM) for 10 min. Pre-incubation period was followed by pre-treatement with

vehicle, ZnPPIX (0.5 μM, 30 min) or ODQ (0.1 mM, 20 min) followed by the addition of sodium nitroprusside (SNP; 1 mM, 30 min), NS1619 (10 μM, 30 min) or iberiotoxin (IBX, 0.1 μM, 30 min) or CORM-2 (0.3 mM, 2 min) or 8*E*-AA (0.1 μM, 15 min). Enzymatic reactions were stopped by addition of 1 mL acidified ethanol to extract cGMP. Slices were then homogenized and washed with ethanol-water (2:1), centrifuged for 10 min at 1000*g*; cGMP was assayed on supernatant (Amersham, TRK500).

Determination of cAMP production

Porcine cerebral microvascular endothelial cells were used to determine cAMP production and adenylate cyclase activity. Endothelial cells were pre-incubated with cell media in the presence of IBMX (1 mM). Pre-incubation period was followed by treatment with vehicle, *T*AA (1 μM) or adenylate cyclase activator forskolin (100 μM). The reaction was terminated with 200 μl of acidic ethanol. After centrifugation, cAMP was measured by radioimmunoassay as described by the manufacturer (Amersham, TRK432). Levels of cAMP produced were normalized to protein content.

Heme oxygenase activity (bilirubin assay)

Heme oxygenases (HO) catalyze the equimolar production of biliverdin and carbon monoxide (Maines 1997); biliverdin is then converted into bilirubin. HO activity was measured in neuromicrovascular endothelial cells (microsomal fraction) by assaying the levels of bilirubin formation as previously described (Motterlini et al. 1996). Endothelial cells were treated with 8*E*-AA (0.1 μM, 10

min), vehicle (10 min), or pre-treated with ZnPPIX (0.5 μM, 30 min), iberiotoxin (10 μM, 30 min) or ODQ (0.1 mM, 20 min) followed by 8*E*-AA isomer (0.1 μM, 10 min). Cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4), suspended and centrifuged at 400*g* for 10 min at 4°C. The cell pellet was homogenized in potassium phosphate buffer (pH 7.4) and centrifuged at 100,000*g* for 60 min. Microsomal fractions were resuspended in 0.1 M potassium phosphate buffer containing 2 mM $MgCl_2$. Microsomes (500 μg protein) were incubated with hemin (30 μM), rat liver cytosol (2 mg/mL), glucose-6-phosphate dehydrogenase (0.2 U), glucose-6-phosphate (2 mM), and NADPH (0.8 mM) in 0.5 ml for 1 h at 37°C in the dark. Bilirubin was extracted with chloroform, and its absorbance at 464 nm measured against a baseline absorbance at 528 nm (excitation coefficient, 40 $mM^{-1}cm^{-1}$ for bilirubin). Heme oxygenase activity was expressed as % of control where 100% is equal to 112.5 nmol of bilirubin formed/mg protein/60 min as percentage of control.

Heme oxygenase immunohistochemistry

Rats were anesthetised with isoflurane 5% and perfused either with saline or CHAPS. Brains were collected and immediately placed in 4% formalin for 24 h at room temperature followed by immersion in 30% sucrose in 0.1 M phosphate buffer. Brain sections (10 μm) were cut with a cryostat (Microm International, HM500 O), mounted on a Supersoft Plus slides. Immunohistochemistry was performed using HO-1 or -2 antibodies (1:500, Stressgen) then double-labelled with endothelial specific cell marker CD31 antibody (1:50, Oxford Biotechnologies), as previously described (Sennlaub et al. 2003).

Immunoprecipitation of BK_{Ca} and HO-1/HO-2

Rats were anesthesized with 5% isoflurane and perfused with saline. Brains were collected, homogenized and then centrifuged for 10 min at 10,000 rpm, followed by a second centrifugation for 1 h at 65,000 rpm to isolate microsomal fraction. Latter was resuspended in 500 μl lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% NP40 in addition to a cocktail of protease inhibitors). To each suspension was added 5 μg of BKα (Transduction laboratories), or 1:100 HO-2 (Stressgen), or 1:100 HO-1 (Stressgen) antibodies and the mixtures were incubated at 4°C overnight with gentle rotation before addition of 50 μL Protein G beads. The mixtures were further incubated for 3 h at 4°C. The beads were pelleted at 7,000*g* for 5 min and washed 3 times with lysis buffer, then resuspended in lameli buffer and loaded onto 10% resolving SDS-polyacrylamide gels. After the transfer, polyvinylidene diflouride membranes were blocked in 2% albumin bovine serum in PBS-tween (Sigma) for 2 h at room temperature and transferred to primary antibody diluted 1:250 in blocking buffer for BKα or 1:5000 for HO-2.

Downregulation of Heme oxygenase-2 with RNA interference.

HMEC-1 cells were grown to 70% confluence and transfected with 100 nM pre-designed, ready to transfect, siRNA sequences targeting HO-2 (Ambion) or a scrambled negative control, using lipofectamine 2000 reagent (invitrogen); the sequence targeting HO-2 is the following: 5'-ggacauggaguauuucuuutt-3' (forward) and 5'-aaagaaauacuccaugucctt-3' (reverse). Silencer® Negative Control

#1 siRNA (Ambion) was used as a negative control siRNA for our control experiment. Cells were incubated with the HO-2 siRNA for 48 h after which they were either subjected to heme oxygenase assay or mRNA determination.

Quantitative Real-time Reverse Transcription-PCR.

Total RNA was isolated from confluent HMEC-1 cells using TRIzol reagent (Invitrogen) and cDNA was generated via a reverse transcriptase reaction from 2.5 μg of RNA using the MMLV-RT (invitrogen). Quantitative real-time PCR was performed using the Mx3005P® QPCR system and iTaq SYBR green (Biorad). The primers used were as follows: HO-2 5'-aagcacacgaccgggcagaaaac-3'(forward) and 5'-tgttcaggtccagggcgttcatc-3' (reverse), HO-1 5'-cccctacacaccagccatgcagc-3'(forward) and 5'-atcggagaagcggagcctgggag-3'(reverse), β-actin 5'-catcgagcacggcatcgtca-3'(forward) and 5'-tagcacagcctggatagcaac-3'(reverse).

Chemicals

Four isomers of *T*AA were individually synthesized via stereospecifc synthesis from corresponding arachidonic acid epoxides (EETs) in a pure form as described (Krishna, Reddy et al. 2001) and were kind gift from Dr. Camille Falck (University of Texas Southwestern Medical Center, Dallas, TX). The following agents were purchased: NS1619, L-NMMA, 1H-[1,2,4]Oxadiazolo[4,3-a]quioxalin-1-one (ODQ), NS2028, iberiotoxin, 1-ABT, tricarbonyldichlororuthenium (II) dimer (CORM-2), hemin, D-glucose 6-phosphate sodium salt, sodium nitroprusside, ibuprofen, nordihydroguaiaretic acid

(NDGA), glucose 6-phosphate dehydrogenase, IBMX (Sigma-Aldrich), NAPDH (Roche), zinc protoporphyrin IX (Frontier Scientific), TEA (Calbiochem), cGMP assay kit (TRK500, Amersham), anti-HO1 and anti-HO2 (Stressgen Bioreagents), anti-CD31 (Oxford Biotechnologies), anti-BKα (Transduction Laboratories), Albumin bovine serum (Sigma Aldrich).

Data analysis

Results were analyzed by two-way analysis of variance by factoring for concentration and treatments; post-hoc analysis was performed by Bonferroni method. Statistical significance was set at $p<0.05$.

3.5 RESULTS

Effects of *Cis*-arachidonic acid and corresponding *Trans*-isomers on Rat Brain Pial Microvessels.

Cis-arachidonic acid and corresponding *trans*-isomers, namely 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA, produced a concentration-dependent vasorelaxation of brain pial microvessels (Figure 1). 5- and 8*E*-AA were the most potent *trans*-arachidonic acids, and 8*E*-AA was also the most effective vasorelaxant, nearly equivalent to *cis*-arachidonic acid (Table 1). Accordingly, we opted to utilize 8*E*-AA to characterize *T*AA mode of action in vasorelaxation.

*T*AA-induced Vasorelaxation is Endothelium-Dependent

To examine the involvement of the endothelium, we tested the effects of *T*AA on perfused pial microvessels denuded or not of the endothelial cell layer using 3-

[(3-cholamidopropyl) dimethylammonio]-1-propansulfonate (CHAPS). Removal of endothelium was functionally successful since vasorelaxation to endothelium-dependent substance P (1 mM) (Hardy, Abran et al. 1998) was abolished, whereas constriction and relaxation respectively to U46619 (0.3 μM) and NO donor SNP (1 μM) were unaffected (data not shown). 8*E*-AA-dependent vasorelaxation was abrogated by CHAPS-treated tissues (Figure 1B) ($P<0.001$), indicating endothelial-dependence.

Effects of *T*AA on Endothelial Cell-Dependent Vasodilatory Pathways

The endothelium is responsible for various vasodilatory mechanisms. Major vasorelaxant pathways contained within the endothelium are NO synthase (NOS), prostaglandin (mainly PGI_2) synthase, and endothelium-derived hyperpolarizing factors for which the chemical identity(ies) remains elusive, albeit seems to be participated by cytochrome P_{450} metabolites (Triggle et al. 1999; Sandow 2004), namely epoxyeicosatrienoic acids and (EETs) and hydroxyeicosatetraenoic acids (HETEs) which have been shown to exert significant vasorelaxation (Harder et al. 1995; Fang et al. 2006). We tested whether these pathways contributed to 8*E*-AA -induced vasodilation. NOS and cyclooxygenase inhibitors, namely L-NAME and ibuprofen, negligibly affected *T*AA-induced vasorelaxation (Figure 2A and 2C); inhibitors did not affect basal microvessel diameter. Consistent with these observations, *T*AA stimulation of brain microvascular endothelium did not elicit nitrite or (total) prostaglandin production (Figure 2B and 2D); the latter consisted predominantly of PGE_2 (~80%) and PGI_2 (~20%, measured by its stable metabolite 6-keto-$PGF_{1\alpha}$), both of which did not change. Moreover, 8*E*-AA did

not evoke formation of cAMP, a second messenger generally associated with vasorelaxation including in response to PGE_2 and PGI_2 receptors associated with vasorelaxation (Wright et al. 2001) (Figure 2D).

Interestingly, we have previously described that *T*AA can be metabolized by CYP_{450} monooxygenases into 11-HETEs and 12-HETEs (Roy, Loreau et al. 2004). Blockade of cytochrome P_{450} enzyme with 1-ABT partly inhibited *T*AA-evoked dilation (Figure 2E), while another inhibitor, miconazole, did not affect the vasodilation. Consistent with the latter observations, HETE levels did not increase upon exposure of endothelial cells to 8*E*-AA (Figure 2 F). Furthermore, analysis of lipid extracts from pial microvessels by tandem mass spectrometry using MRM LC/MS/MS technique revealed basal levels of PGE_2, 11-HETE, 12-HETE and minor amounts of lipoxygenase metabolites 15-HETE and 5-HETE, all of which were unaltered by 8*E*-AA; CYP_{450} metabolites of arachidonic acid, EETs and 20-HETE, were not detectable. Correspondingly, inhibition of lipoxygenase using the non-selective inhibitor namely nordihydroguaiaretic acid (NDGA) also did not affect *T*AA-induced pial microvessel relaxation (Figure 2E). Hence, 8*E*-AA failed to induce changes in levels of cyclooxygenase, lipoxygenase and CYP_{450} metabolites in both pial microvessels and endothelial cells (Figure 2D and 2F), and inhibitors of these pathways hardly affect 8*E*-AA-evoked vasorelaxation (Figure 2C and 2E).

Although NO is a major regulator of soluble guanylate cyclase (sGC), it is not an exclusive source of cGMP generation. Brain vasculature also contains membrane-bound guanylate cyclase and heme oxygenase (Mori et al. 1997); the latter

catalyzes the formation of carbon monoxide (CO), a potent activator of sGC (Koneru et al. 2004). We investigated if sGC activity was involved in *T*AA-mediated vasorelaxation. 8*E*-AA caused a robust increase in cGMP production, comparably to that seen with CORM-2 (Figure 3A). This *T*AA-induced cGMP generation as well as vasorelaxation were markedly diminished (~70%) by the sGC blockers, ODQ and NS2028 (Figure 3A,B) ($P<0.001$); similar results were obtained using a pharmacologically distinct inhibitor of sGC namely methylene blue (data not shown). As expected, CORM-2 also elicited vasorelaxation inhibited by ODQ (Figure 3C) (Leffler et al. 1999); effects were similar in endothelium-denuded and intact vasculature.

_T_AA induce a Heme Oxygenase-Dependent vasorelaxation

We investigated if HO could contribute to *T*AA-elicited vasorelaxation. 8*E*-AA-triggered cGMP formation was abolished by the HO inhibitor zinc protoporphyrin IX (ZnPPIX, at heme oxygenase specific concentrations (Appleton, Chretien et al. 1999)) (Figure 3A). Since *T*AA effects were endothelium-dependent, we surmised HO presence on brain endothelium. Indeed, immunohistochemical analysis revealed predominant localization of inducible and constitutive heme oxygenase isoforms (respectively, HO-1 and HO-2) in brain endothelium (CD31 positive) (Figure 4A and B); endothelium denudation virtually abolished HO-1/-2 immunoreactivity (data not shown). Effects of *T*AA on HO activity were assessed by measuring bilirubin (Motterlini, Foresti et al. 1996). Stimulation of neuromicrovascular endothelial cells with 8*E*-AA caused a significant increase in bilirubin concentration, which was fully inhibited by ZnPPIX (Figure 4D). In line

with these observations, ZnPPIX diminished 8*E*-AA-induced vasorelaxation to the same extent seen with sGC blockade (Figures 3B and 4C); ZnPPIX did not inhibit SNP-induced dilation pointing out its selectivity (data not shown). Attempt to use constitutive HO gene knockout mice to determine the role of this factor on the vasorelaxation induced by *T*AA, failed to reveal attenuated vasodilation (data not shown), likely due to the compensatory overexpression of HO-1 in these animals (Dennery et al. 1998; Ding et al. 2006). We therefore proceeded to study *T*AA-induced effects after knockdown of constitutive HO-2 using siRNA. HO-2 siRNA markedly downregulated HO-2 (but not HO-1) mRNA expression in endothelial cells (Figure 4E); negative control was ineffective as anticipated. 8*E*-AA-induced bilirubin production was virtually abolished by HO-2 siRNA (Figure 4 F), underlining the dominant role of HO-2 in relation to acute cerebrovascular effects of *T*AA.

*T*AA-Induced Vasorelaxation Is Dependent on BK_{Ca} Channels Activation

Because *T*AA-induced vasorelaxation could only partially be attributed to the HO/sGC pathway (Figures 3B, 4C) we investigated the role of other mechanisms. Large conductance calcium-activated potassium (BK_{Ca}) channels play a prominent role in the regulation of vascular tone (Nelson et al. 1995; Knot et al. 1998). Blockade of these channels using tetraethyl ammonium sulfate (TEA) or the selective iberiotoxin inhibited >80% of the 8*E*-AA-induced vasorelaxation (Figure 5A) ($P<0.001$). Conversely, the BK_{Ca} activator NS1619 evoked a concentration-dependent relaxation of pial brain microvessels. Interestingly, this relaxation was strongly diminished by ZnPPIX and ODQ (Figure 5B); in line with

these observations, NS1619 stimulated cGMP generation (Figure 5C). Furthermore, 8*E*-AA-induced cGMP and bilirubin production was completely inhibited by iberiotoxin (Figures 3A, 4D). Collectively these findings underscore the importance of BK_{Ca} in 8*E*-AA-induced vasorelaxation and suggest a HO/sGC activation by *T*AA downstream of BK_{Ca}; on the other hand, as previously reported CO can also lead to BK_{Ca} activation (Williams et al. 2004), as inferred by the observed BK_{Ca}-dependent CO-induced relaxation (inhibited by iberiotoxin; Figure 5D).

To explain this 2-way interplay between HO (activity) and BK_{Ca} we surmised these proteins to modulate each other's activities as physical partners of the same molecular complex. Immunoprecipitates of BK_{Ca} or HO-2, but not HO-1, from brain microsomal fractions were immunoreactive to both BK_{Ca} and HO-2 (Figure 5E).

3.6 DISCUSSION

Nitrative and oxidative stress exert a number of biological effects relevant to physiology and pathology. However, in many instances the mechanisms of actions of nitrative and oxidative stress remain not well elucidated. Nitrative stress derivatives and lipid peroxidation products, respectively such as *trans*-arachidonic acids (Kermorvant-Duchemin, Sennlaub et al. 2005) and isoprostanes (Fessel, Porter et al. 2002), are generated abundantly in tissues including brain, retina and lung, during pathological conditions (Reich et al. 2001; Kermorvant-Duchemin, Sennlaub et al. 2005; Musiek et al. 2006). Recent studies have

demonstrated that these products can act as mediators of the nitrative and oxidative stresses, by reproducing biological effects of relevance to disease states. Lately, we have shown that the formation of peroxynitrite/$NO_2^{\bullet}$-dependent *trans*-arachidonic acids *in vivo* can contribute to a significant extent to the microvascular degeneration seen in models of ischemic retinopathy (Kermorvant-Duchemin, Sennlaub et al. 2005). Because peroxynitrite can also affect acute vasomotor tone by eliciting vasorelaxation (Graves, Lewis et al. 1998), we proceeded to further characterize the properties of *T*AA on neurovascular tissue by examining their vasomotor effects, and thereon investigated their mechanisms of action. Our findings reveal that *T*AA exert an endothelium-dependent pial vasodilation. Although the primary site of action of *T*AA remains unknown, as is the case for non-enzymatically-derived products of oxidation such as isoprostanes and isoketals, the mode of action of *T*AA involves nonetheless a less conventional vasorelaxant pathway, notably dependent on HO-2 by interacting with BK_{Ca}.

Major endothelium-dependent vasorelaxant mediators include NO, PGI_2, and endothelium-derived hyperpolarizing factors which is contributed by CYP_{450} metabolites (Sandow 2004), namely epoxyeicosatrienoic acids and (EETs) and hydroxyeicosatetraenoic acids (HETEs), reported to cause vasorelaxation (Harder, Campbell et al. 1995; Fang, Faraci et al. 2006). However, none of these factors seem to participate in *T*AA-induced pial vasorelaxation based on concordance of lack of effects of inhibitors and failure of mediators to rise upon stimulation with *T*AA (for NO and prostaglandins; Figure 2A-D), or lack of concordance between function and concentrations of mediators (for CYP_{450} metabolites; Figure 2E,F);

in this regard 1-ABT may exert non-specific effects on K channels (Yuan et al. 1995). On the other hand, a significant role for HO in *T*AA-induced vasorelaxation was uncovered based on the following evidence. 1) Both HO-1 and HO-2 are present in brain vascular endothelium (Figure 4A,B), as reported (Parfenova et al. 2001). 2) 8*E*-AA stimulated HO activity (revealed by measuring formation of bilirubin (Maines 1997) inhibited by the HO inhibitor ZnPPIX), and this effect is associated with a vasorelaxation significantly blunted by ZnPPIX (Figure 4C,D). HO-2 mRNA knockdown substantiated the role of HO-2 in *T*AA-induced acute brain endothelial effects (Figure 4E,F). Our findings are consistent with an important contribution of constitutive HO in pial vasorelaxation during conditions associated with oxidative/nitrative stresses such as excess glutamate stimulation and seizures (Parfenova et al. 2006). 3) The HO product CO exhibits a prominent role in neural vasodilatation (Leffler, Nasjletti et al. 1999), by activating sGC (Taskiran et al. 2003; Ryter et al. 2004). This ensued cGMP-dependent vasorelaxation was reproduced using the CO-releasing molecule CORM-2 $[Ru(CO)_3Cl_2]_2$, (Motterlini, Mann et al. 2003) (Figure 3C). 4) Finally, the HO-dependent 8*E*-AA-evoked cGMP production and (in turn) vasorelaxation (Figure 4C,D) were markedly attenuated by the sGC inhibitor ODQ (Figure 3A,B), to the same extent seen with ZnPPIX (Figure 4C,D).

A major feature of *T*AA-induced cerebral vasorelaxation is the significant contribution of BK_{Ca} channels, which interplay with HO. Evidence for this inference is provided by various observations. 1) First, general and selective BK_{Ca} channel blockers, respectively TEA and iberiotoxin, virtually abolished pial

vasorelaxation in response to *T*AA (Figure 5A), as observed with de-endothelialization (Figure 1B). 2) Secondly, although the activation of BK_{Ca} by HO/CO is documented (Knot and Nelson 1998; Jaggar et al. 2002), our observations also point to the reverse, specifically a BK_{Ca}-dependent activation of HO and sGC upon stimulation with *T*AA (Figures 3A, 4D); concordantly, activation of BK_{Ca} increased cGMP formation and HO- as well as sGC-dependent vasorelaxation (Figure 5B,C). Findings infer a hyperpolarization-induced activation of HO and sGC; these observations are consistent with an increase in cGMP levels in endothelial cells by hyperpolarization (Kuhlmann et al. 2005), but underlying mechanisms have yet to be understood. 3) Because of the dominant role of BK_{Ca} relative to HO alone in *T*AA-induced cerebral vasodilation, and the 2-way interplay between HO/sGC and BK_{Ca}, we surmised that BK_{Ca} channels and HO may modulate each other's activities as partners of the same molecular complex, in line with the recently described physical interaction between BK_{Ca} channels and HO-2 (Williams, Wootton et al. 2004). BK_{Ca} and HO-2, but not HO-1, were found to co-immunoprecipitate (Figure 5E), suggesting that the first two were indeed part of the same protein complex, consistent with the virtually exclusive role of HO-2 in acute *T*AA-induced effects on brain endothelium (Figure 4F). This inferred physical interaction of proteins is fundamental to signaling pathways (Chanrion et al. 2007); we propose that conformational changes in one partner may affect activity of the other and vice versa (Knot and Nelson 1998; Jaggar, Leffler et al. 2002; Williams, Wootton et al. 2004).

In summary, findings unveil new biological properties for *T*AA, notably endothelium-dependent relaxation of neural microvasculature mediated through a less predictable pathway involving an HO-2-BK_{Ca} interaction; a model depicting the mode of action of *T*AA in eliciting vasorelaxation is presented in Figure 6. Interestingly, both neurovascular effects of *T*AA reported to date, specifically vasorelaxation (present study) and microvascular obliteration (Brault, Martinez-Bermudez et al. 2003; Kermorvant-Duchemin, Sennlaub et al. 2005), concur with reported effects of highly reactive nitrogen species (Graves, Lewis et al. 1998), from which they are generated (Balazy 2000). Hence, *T*AA are not only markers but also act as mediators of nitrative stress. In a pathophysiological setting associated with nitrative stress, such as ischemia, septic shock and reperfusion injury, an acute vasorelaxation would appropriately attempt to counter an impending curtailment in local neural hemodynamics; on the other hand, a sustained increase in oxygen delivery under such conditions may amplify the formation of reactive nitrogen and oxygen species, and ultimately lead to vascular and ensued neural degeneration, especially in a young developing subject (Hardy et al. 1996; Sirinyan et al. 2006).

3.7 ACKNOWLEGMENTS

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3.8 LIST OF ABBREVIATIONS

CYP_{450}: Cytochrome P 450

NOS: Nitric oxide synthase

COX: cyclooxygenase

HO: Heme oxygenase

PG: prostaglandins

*T*AA: *trans*-arachidonic acids

$NO_2^{\bullet}$: nitrogen dioxide

cGMP: cyclic 3':5'-guanosine monophosphate

CO: carbon monoxide

EDHF: endothelium-derived hyperpolarizing factor

sGC: soluble guanylyl cyclase

BK_{Ca}: Large conductance calcium-dependent potassium channels

BKα: α subunit of large conductance potassium channels

CORM-2: CO releasing molecule -2

HETEs: Hydroxyeicosatetraenoic acids

EETs: Epoxyeicosatrienoic acids

SNP: sodium nitroprusside

NDGA: nordihydroguairetic acid

3.9 REFERENCES

Appleton, S. D., M. L. Chretien, et al. (1999). "Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations." Drug Metab Dispos **27**(10): 1214-9.

Balazy, M. (2000). "Trans-arachidonic acids: new mediators of inflammation." J Physiol Pharmacol **51**(4 Pt 1): 597-607.

Becker, E. M., C. Alonso-Alija, et al. (2001). "NO-independent regulatory site of direct sGC stimulators like YC-1 and BAY 41-2272." BMC Pharmacol **1**: 13.

Brault, S., A. K. Martinez-Bermudez, et al. (2003). "Selective neuromicrovascular endothelial cell death by 8-Iso-prostaglandin F2alpha: possible role in ischemic brain injury." Stroke **34**(3): 776-82.

Butterfield, D. A., J. Drake, et al. (2001). "Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide." Trends Mol Med **7**(12): 548-54.

Chanrion, B., C. Mannoury la Cour, et al. (2007). "Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity." Proc Natl Acad Sci U S A **104**(19): 8119-24.

Chen, T. Y., M. S. Shiao, et al. (2005). "Inhibition of 12- and 15-lipoxygenase activities and protection of human and tilapia low density lipoprotein oxidation by I-Tiao-Gung (Glycine tomentella)." Lipids **40**(11): 1171-7.

Dennery, P. A., D. R. Spitz, et al. (1998). "Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2." J Clin Invest **101**(5): 1001-11.

Dickhout, J. G., G. S. Hossain, et al. (2005). "Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human vascular endothelium: implications in atherogenesis." Arterioscler Thromb Vasc Biol **25**(12): 2623-9.

Ding, Y., Y. Z. Zhang, et al. (2006). "Down-regulation of heme oxygenase-2 is associated with the increased expression of heme oxygenase-1 in human cell lines." Febs J **273**(23): 5333-46.

Fang, X., F. M. Faraci, et al. (2006). "20-Hydroxyeicosatetraenoic acid is a potent dilator of mouse basilar artery: role of cyclooxygenase." Am J Physiol Heart Circ Physiol **291**(5): H2301-7.

Ferreri, C., M. R. Faraone Mennella, et al. (2002). "Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples." Free Radic Biol Med **33**(11): 1516-26.

Fessel, J. P., N. A. Porter, et al. (2002). "Discovery of lipid peroxidation products formed in vivo with a substituted tetrahydrofuran ring (isofurans) that are favored by increased oxygen tension." Proc Natl Acad Sci U S A **99**(26): 16713-8.

Gobeil, F., Jr., I. Dumont, et al. (2002). "Regulation of eNOS expression in brain endothelial cells by perinuclear EP(3) receptors." Circ Res **90**(6): 682-9.

Graves, J. E., S. J. Lewis, et al. (1998). "Peroxynitrite-mediated vasorelaxation: evidence against the formation of circulating S-nitrosothiols." Am J Physiol **274**(3 Pt 2): H1001-8.

Harder, D. R., W. B. Campbell, et al. (1995). "Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone." J Vasc Res **32**(2): 79-92.

Hardy, P., D. Abran, et al. (1998). "A major role for prostacyclin in nitric oxide-induced ocular vasorelaxation in the piglet." Circ Res **83**(7): 721-9.

Hardy, P., K. G. Peri, et al. (1996). "Increased nitric oxide synthesis and action preclude choroidal vasoconstriction to hyperoxia in newborn pigs." Circ Res **79**(3): 504-11.

Holland, M., P. D. Langton, et al. (1996). "Effects of the BKCa channel activator, NS1619, on rat cerebral artery smooth muscle." Br J Pharmacol **117**(1): 119-29.

Hou, X., F. Gobeil, Jr., et al. (2000). "Augmented vasoconstriction and thromboxane formation by 15-F(2t)-isoprostane (8-iso-prostaglandin F(2alpha)) in immature pig periventricular brain microvessels." Stroke **31**(2): 516-24; discussion 525.

Hou, X., L. J. Roberts, 2nd, et al. (2004). "Isomer-specific contractile effects of a series of synthetic f2-isoprostanes on retinal and cerebral microvasculature." Free Radic Biol Med **36**(2): 163-72.

Hou, X., L. J. Roberts, 2nd, et al. (2001). "2,3-Dinor-5,6-dihydro-15-F(2t)-isoprostane: a bioactive prostanoid metabolite." Am J Physiol Regul Integr Comp Physiol **281**(2): R391-400.

Imuta, N., O. Hori, et al. (2007). "Hypoxia-mediated induction of heme oxygenase type I and carbon monoxide release from astrocytes protects nearby cerebral neurons from hypoxia-mediated apoptosis." Antioxid Redox Signal **9**(5): 543-52.

Jaggar, J. H., C. W. Leffler, et al. (2002). "Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca2+ sparks to Ca2+-activated K+ channels." Circ Res **91**(7): 610-7.

Jiang, H., N. Kruger, et al. (1999). "Nitrogen dioxide induces cis-trans-isomerization of arachidonic acid within cellular phospholipids. Detection of trans-arachidonic acids in vivo." J Biol Chem **274**(23): 16235-41.

Kermorvant-Duchemin, E., F. Sennlaub, et al. (2005). "Trans-arachidonic acids generated during nitrative stress induce a thrombospondin-1-dependent microvascular degeneration." Nat Med **11**(12): 1339-45.

Knot, H. J. and M. T. Nelson (1998). "Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure." J Physiol **508 (Pt 1)**: 199-209.

Koneru, P. and C. W. Leffler (2004). "Role of cGMP in carbon monoxide-induced cerebral vasodilation in piglets." Am J Physiol Heart Circ Physiol **286**(1): H304-9.

Kozma, F., R. A. Johnson, et al. (1999). "Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels." Am J Physiol **276**(4 Pt 2): R1087-94.

Krishna, U. M., M. M. Reddy, et al. (2001). "Stereospecific synthesis of trans-arachidonic acids." Bioorg Med Chem Lett **11**(18): 2415-8.

Kuhlmann, C. R., J. R. Trumper, et al. (2005). "Nicotine inhibits large conductance Ca(2+)-activated K(+) channels and the NO/-cGMP signaling pathway in cultured human endothelial cells." Scand Cardiovasc J **39**(6): 348-52.

Lahaie, I., P. Hardy, et al. (1998). "A novel mechanism for vasoconstrictor action of 8-isoprostaglandin F2 alpha on retinal vessels." Am J Physiol **274**(5 Pt 2): R1406-16.

Leffler, C. W., A. Nasjletti, et al. (1999). "Carbon monoxide and cerebral microvascular tone in newborn pigs." Am J Physiol **276**(5 Pt 2): H1641-6.

Lovell, M. A., C. Xie, et al. (2001). "Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures." Neurobiol Aging **22**(2): 187-94.

Maines, M. D. (1997). "The heme oxygenase system: a regulator of second messenger gases." Annu Rev Pharmacol Toxicol **37**: 517-54.

Mathews, J. M., L. A. Dostal, et al. (1985). "Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole." J Pharmacol Exp Ther **235**(1): 186-90.

Mori, Y., M. Takayasu, et al. (1997). "Vasodilator effects of C-type natriuretic peptide on cerebral arterioles in rats." Eur J Pharmacol **320**(2-3): 183-6.

Motterlini, R., R. Foresti, et al. (1996). "NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium." Am J Physiol **270**(1 Pt 2): H107-14.

Motterlini, R., B. E. Mann, et al. (2003). "Bioactivity and pharmacological actions of carbon monoxide-releasing molecules." Curr Pharm Des **9**(30): 2525-39.

Musiek, E. S., R. S. Breeding, et al. (2006). "Cyclopentenone isoprostanes are novel bioactive products of lipid oxidation which enhance neurodegeneration." J Neurochem **97**(5): 1301-13.

Nagaoka, T., T. W. Hein, et al. (2007). "Simvastatin elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and mevalonate-rho kinase pathways." Invest Ophthalmol Vis Sci **48**(2): 825-32.

Nelson, M. T., H. Cheng, et al. (1995). "Relaxation of arterial smooth muscle by calcium sparks." Science **270**(5236): 633-7.

Olesen, S. P., J. Drejer, et al. (1998). "Characterization of NS 2028 as a specific inhibitor of soluble guanylyl cyclase." Br J Pharmacol **123**(2): 299-309.

Parfenova, H., S. Basuroy, et al. (2006). "Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection." Am J Physiol Cell Physiol **290**(5): C1399-410.

Parfenova, H., R. A. Neff, 3rd, et al. (2001). "Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate." Am J Physiol Cell Physiol **281**(6): C1954-63.

Pelligrino, D. A., R. A. Santizo, et al. (1999). "Miconazole represses CO(2)-induced pial arteriolar dilation only under selected circumstances." Am J Physiol **277**(4 Pt 2): H1484-90.

Perlman, J. M. (2006). "Intervention strategies for neonatal hypoxic-ischemic cerebral injury." Clin Ther **28**(9): 1353-65.

Radi, R., J. S. Beckman, et al. (1991). "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide." Arch Biochem Biophys **288**(2): 481-7.

Reich, E. E., W. R. Markesbery, et al. (2001). "Brain regional quantification of F-ring and D-/E-ring isoprostanes and neuroprostanes in Alzheimer's disease." Am J Pathol **158**(1): 293-7.

Ronson, R. S., M. Nakamura, et al. (1999). "The cardiovascular effects and implications of peroxynitrite." Cardiovasc Res **44**(1): 47-59.

Roy, U., R. Joshua, et al. (2005). "Cytochrome P450/NADPH-dependent biosynthesis of 5,6-trans-epoxyeicosatrienoic acid from 5,6-trans-arachidonic acid." Biochem J **390**(Pt 3): 719-27.

Roy, U., O. Loreau, et al. (2004). "Cytochrome P450/NADPH-dependent formation of trans epoxides from trans-arachidonic acids." Bioorg Med Chem Lett **14**(4): 1019-22.

Ryter, S. W., D. Morse, et al. (2004). "Carbon monoxide: to boldly go where NO has gone before." Sci STKE **2004**(230): RE6.

Salgo, M. G., G. L. Squadrito, et al. (1995). "Peroxynitrite causes apoptosis in rat thymocytes." Biochem Biophys Res Commun **215**(3): 1111-8.

Sandow, S. L. (2004). "Factors, fiction and endothelium-derived hyperpolarizing factor." Clin Exp Pharmacol Physiol **31**(9): 563-70.

Selley, M. L. (1998). "(E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease." Free Radic Biol Med **25**(2): 169-74.

Sennlaub, F., F. Valamanesh, et al. (2003). "Cyclooxygenase-2 in human and experimental ischemic proliferative retinopathy." Circulation **108**(2): 198-204.

Sirinyan, M., F. Sennlaub, et al. (2006). "Hyperoxic exposure leads to nitrative stress and ensuing microvascular degeneration and diminished brain mass and function in the immature subject." Stroke **37**(11): 2807-15.

Szabo, C., B. Zingarelli, et al. (1996). "DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite." Proc Natl Acad Sci U S A **93**(5): 1753-8.

Taskiran, D., F. Z. Kutay, et al. (2003). "Effect of carbon monoxide on dopamine and glutamate uptake and cGMP levels in rat brain." Neuropsychopharmacology **28**(6): 1176-81.

Triggle, C. R., H. Dong, et al. (1999). "Endothelium-derived hyperpolarizing factor(s): species and tissue heterogeneity." Clin Exp Pharmacol Physiol **26**(2): 176-9.

Villa, L. M., E. Salas, et al. (1994). "Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart." Proc Natl Acad Sci U S A **91**(26): 12383-7.

Wang, X. and R. Loutzenhiser (2002). "Determinants of renal microvascular response to ACh: afferent and efferent arteriolar actions of EDHF." Am J Physiol Renal Physiol **282**(1): F124-32.

Wattanapitayakul, S. K., D. M. Weinstein, et al. (2000). "Endothelial dysfunction and peroxynitrite formation are early events in angiotensin-induced cardiovascular disorders." Faseb J **14**(2): 271-8.

Williams, S. E., P. Wootton, et al. (2004). "Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel." Science **306**(5704): 2093-7.

Wright, D. H., D. Abran, et al. (2001). "Prostanoid receptors: ontogeny and implications in vascular physiology." Am J Physiol Regul Integr Comp Physiol **281**(5): R1343-60.

Yuan, X. J., M. L. Tod, et al. (1995). "Inhibition of cytochrome P-450 reduces voltage-gated K+ currents in pulmonary arterial myocytes." Am J Physiol **268**(1 Pt 1): C259-70.

3.10 TABLES

Table 1. Maximum vasorelaxation and EC_{50} of *cis*-arachidonic acid and four *trans*-isomers of arachidonic acid measured in pial microvessels of young adult rat brain.

Agents	Maximum vasorelaxation (%)	EC50 (nM)
Cis-Arachidonic acid	35±9.82	6.3 ±0.8
5*E*-AA	18 ±2.53	0.32 ±0.05
8*E*-AA	24 ± 1.96	0.46 ±0.05
11*E*-AA	20 ±1.32	3.04 ±0.07
14*E*-AA	15 ±0.77	1.8 ±0.05

3.11 FIGURES

Figure 1

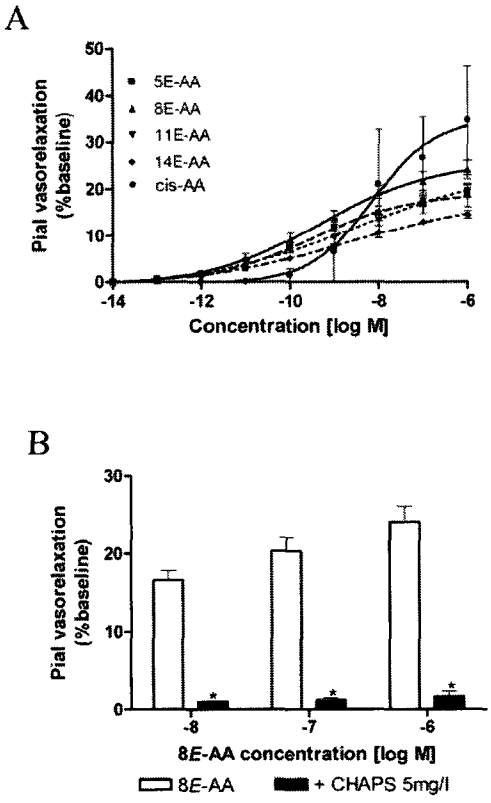
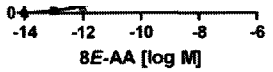
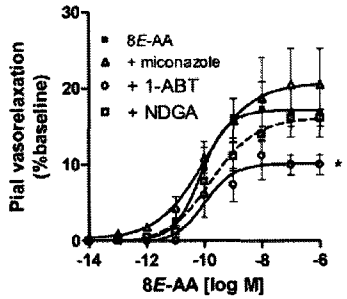


Figure 1.

Vasomotor effects of *trans*- and *cis*-arachidonic acids on intact (A) and de-endothelialized brain pial microvessels (B); de-endothelialization of brain vasculature was performed by intracarotid perfusion of CHAPS (see Materials and Methods). Values are means ± SEM of 3-11 separate experiments. *P<0.001 compared to 8*E*-AA (two-way ANOVA).


0
-14
-12
-10
-8
-6
8E-AA [log M]

E


30
20
10
0
8E-AA
+ miconazole
+ 1-ABT
+ NDGA
Pial vasorelaxation (%baseline)
*
-14
-12
-10
-8
-6
8E-AA [log M]

F

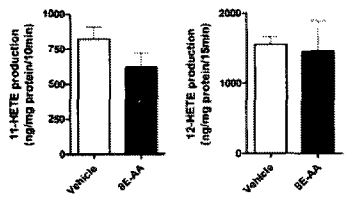

11-HETE production (ng/mg protein/10min)
1000
750
500
250
0
Vehicle
8E-AA
12-HETE production (ng/mg protein/15min)
2000
1000
0
Vehicle
8E-AA

Figure 2

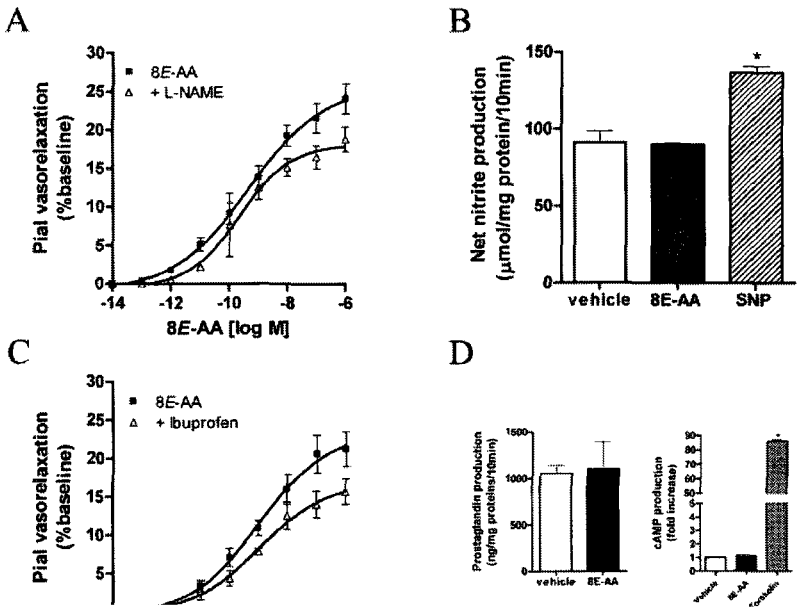

A
8E-AA
+ L-NAME
Pial vasorelaxation (%baseline)
8E-AA [log M]
B
Net nitrite production (µmol/mg protein/10min)
vehicle
8E-AA
SNP
C
8E-AA
+ Ibuprofen
Pial vasorelaxation (%baseline)
D
Prostaglandin production (ng/mg proteins/10min)
vehicle
8E-AA
cAMP production (fold increase)

Figure 2.

Role of (A,B) NO, (C,D) prostaglandins, and (E,F) CYP_{450} monooxygenase and lipoxygenase products on 8*E*-AA-induced brain pial microvessel relaxation. Tissues were pretreated (30 min) with NOS inhibitor L-NAME (1 mM), cyclooxygenase inhibitor ibuprofen (10 μM), CYP_{450} monooxygenase 1-ABT (1 mM), miconazole (10 μM) or lipoxygenase inhibitor NDGA (3 μM). Data are mean ± SEM of 3-7 separate experiments, expressed as % baseline. *$p<0.05$ compared to TAA-treated preparations (two-way ANOVA).

A) Participation of nitric oxide synthase on 8*E*-AA-induced pial vasorelaxation. B) Effects of 8*E*-AA (1 μM) and SNP (1 mM; positive control) on nitrite measurements in brain microvascular endothelial cells stimulated for 10 min. Nitrites were measured by chemiluminescence (see Methods). Values are ± SEM of 3 independent experiments. * $p<0.01$ compared to other values without asterisks. C) Participation of cyclooxygenase products on 8*E*-AA-induced pial vasorelaxation. D) Effects of 8*E*-AA (1 μM; 10 min) on prostaglandin and cAMP production; the adenylate cyclase activator forskolin (100 μM) was used as a positive control. Values are ± SEM of 3 independent experiments. * $p<0.01$ compared to other values without asterisks. E) Participation of CYP_{450} monooxygenase and lipoxygenase products on 8*E*-AA-induced pial vasorelaxation. F) Effects of 8*E*-AA (1 μM; 15 min) on production of *T*AA metabolites 11-HETEs and 12-HETEs; HETEs were measured by tandem mass spectrometry. Values are ± SEM of 3 independent experiments.

Figure 3

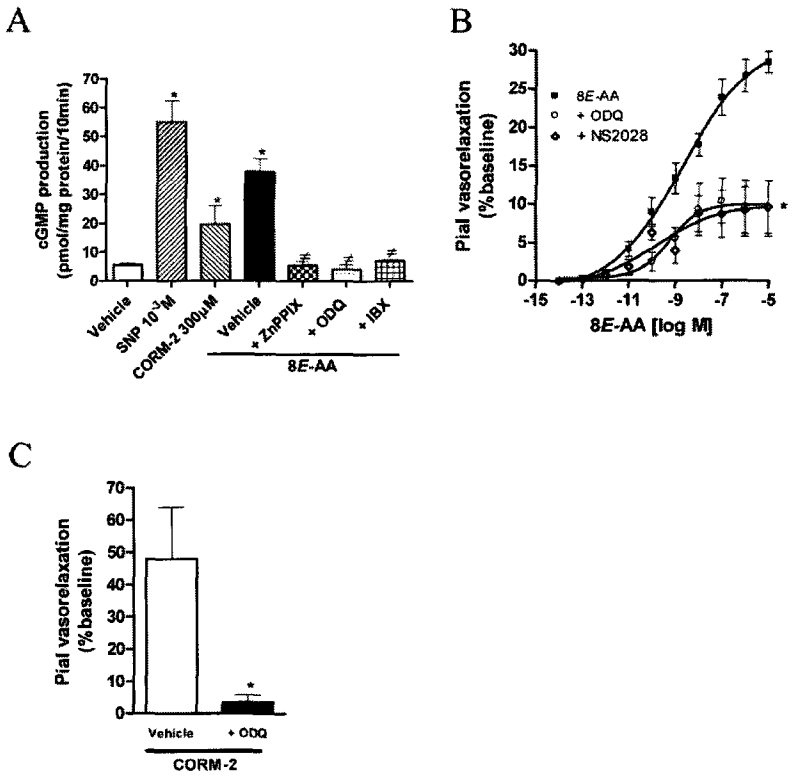

A
cGMP production (pmol/mg protein/10min)
Vehicle
SNP 10⁻³M
CORM-2 300µM
Vehicle
+ ZnPPIX
+ ODQ
+ IBX
8E-AA
B
Pial vasorelaxation (%baseline)
8E-AA
+ ODQ
+ NS2028
8E-AA [log M]
C
Pial vasorelaxation (%baseline)
Vehicle
+ ODQ
CORM-2

Figure 3.

*T*AA-induced vasorelaxation is sGC/cGMP-dependent. A) Effects of 8*E*-AA on brain cGMP production. Tissues were pre-treated (30 min) with sGC inhibitor ODQ (0.1 mM) or heme oxygenase inhibitor ZnPPIX (0.5 μM) or saline before the addition of either vehicle, the NO donor sodium nitroprusside SNP (1 mM), 8*E*-AA (0.1 μM) or CO donor CORM-2 (222 μM). B) Vasorelaxant response to 8*E*-AA in the presence or absence of ODQ (0.1 mM) or NS2028 1 μM. C) Effects of CORM-2 on brain pial vasodilation. Brains were pre-perfused or not with CHAPS to remove endothelium (see Methods); vasorelaxation to CORM-2 was similar in both cases. Values are mean ± SEM of 4 independent experiments. * $P<0.001$ compared to vehicle-treated. ≠ $P<0.001$ compared to 8*E*-AA-treated (alone).

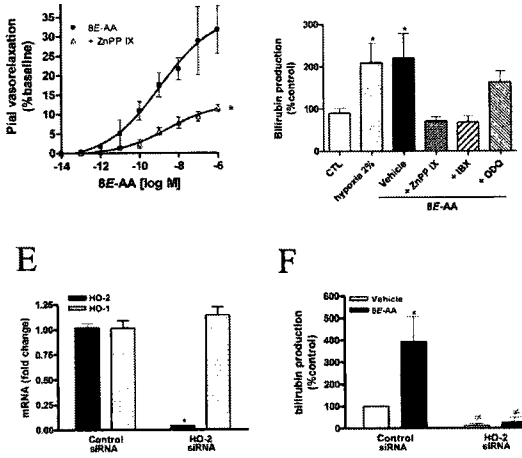

Pial vasorelaxation (%baseline)
8E-AA
+ ZnPP IX
8E-AA [log M]
Bilirubin production (%control)
CTL
hypoxia 2%
Vehicle
+ ZnPP IX
+ IBX
+ QVQ
8E-AA
E
HO-2
HO-1
mRNA (fold change)
Control siRNA
HO-2 siRNA
F
Vehicle
8E-AA
bilirubin production (%control)
Control siRNA
HO-2 siRNA

Figure 4

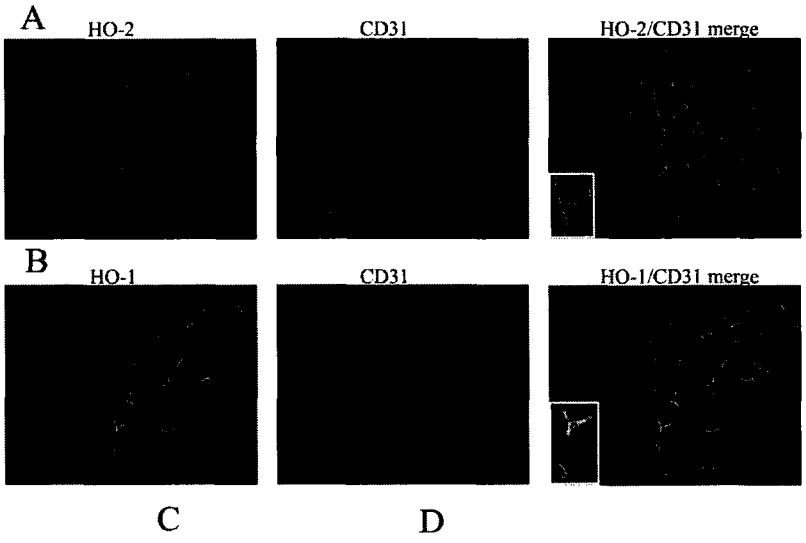


Figure 4.

Localization of heme oxygenase in brain. Brain tissue from young adult rat was sectioned (10 μm) and stained with (A) anti-HO2 or (B) anti-HO1 (green), and anti-CD31 (red [endothelial cell marker]). Note immuno-localization of both HO isoforms (constitutive and inducible) in brain endothelium (highlighted in inset panels of merged figures) taken at 20X magnification. Panel is representative of 3 independent experiments. C) Effects of HO inhibitor on 8*E*-AA-induced vasorelaxation. Brain slices were pre-treated (30 min) with zinc protoporphyrin IX (ZnPPIX; 0.5 μM) before addition of increasing concentrations of 8*E*-AA. Data are means ± SEM of 4 separate experiments. * $P<0.01$ compared to 8*E*-AA alone treated preparations (two-way ANOVA). D) Effects of 8*E*-AA on HO activity (see Methods); 2% hypoxia exposure for 4 h induces HO-1 expression (Imuta, et al., 2007) and was utilized as a positive control. Endothelial cells were pre-treated with ZnPPIX (0.5 μM), or iberiotoxin (IBX; 0.1 μM), ODQ (0.1 mM) or saline before addition of 8*E*-AA (0.1 μM) for 10 min. Values are mean ± SEM of 4 independent experiments. * $P<0.05$ compared to values without asterisks. E) Fold increase in mRNA levels of HO-1 and HO-2 by qPCR. Data were normalized to β-actin mRNA and presented as mean +/- SEM of 3 independent experiments. * $P<0.05$ compared to values without asterisks. F) Effects of 8*E*-AA on HO activity (measured as bilirubin formation) in microvascular endothelial cells treated with siRNA HO-2 or control siRNA (see Methods). Cells were pre-treated with 100 nM Silencer® Negative control #1 (Control) or HO-2 siRNA for 48 h before addition of 8*E*-AA (0.1 μM) for 10 min. Data are normalized to cells

untreated with siRNA (equivalent to negative control siRNA), and presented as mean ± SEM of 4 independent experiments. * $P<0.05$ compared to values without asterisks.

Figure 5

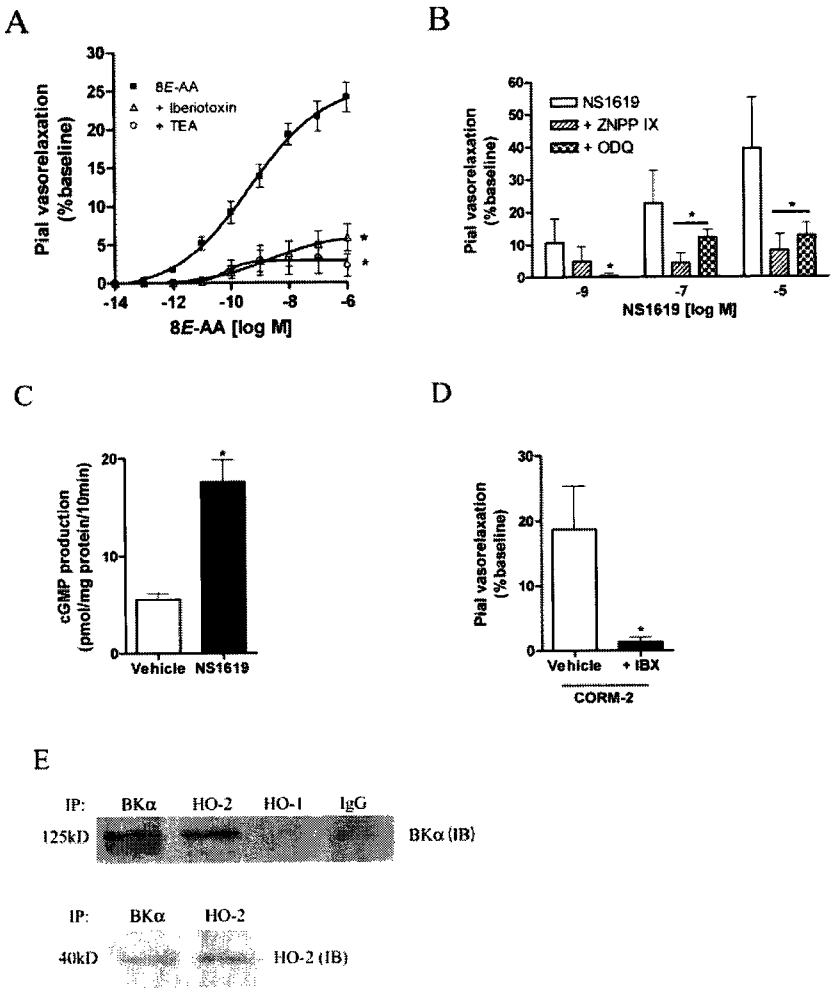

A
Pial vasorelaxation (%baseline)
8E-AA
+ Iberiotoxin
+ TEA
8E-AA [log M]
B
Pial vasorelaxation (%baseline)
NS1619
+ ZNPP IX
+ ODQ
NS1619 [log M]
C
cGMP production (pmol/mg protein/10min)
Vehicle
NS1619
D
Pial vasorelaxation (%baseline)
Vehicle
+ tBX
CORM-2
E
IP: BKα HO-2 HO-1 IgG
125kD
BKα (IB)
IP: BKα HO-2
40kD
HO-2 (IB)

Figure 5.

Involvement of BK_{Ca} channel in 8*E*-AA-induced vasorelaxation. A) Effect of BK_{Ca} blockers tetraethylammonium chloride (TEA; 1 mM) and highly selective iberiotoxin (0.1 μM) in 8*E*-AA -induced vasorelaxation; tissues were pretreated with blockers 20-30 min prior to addition of cumulative 8*E*-AA. Data are mean ± SEM of 4 independent experiments. * $P<0.001$ compared to 8*E*-AA alone treated preparations (two-way ANOVA). B) Effect of BK_{Ca} activator NS1619 on brain pial vasorelaxation in absence or presence of pre-treatment (20-30 min) with ZnPPIX (0.5 μM), guanylate cylcase inhibitor ODQ (0.1 mM). Values are mean ± SEM of 3-5 independent experiments. * $P<0.05$ compared to NS1619 alone (two-way ANOVA). C) Effect of NS1619 (10 μM) on cGMP production. Values are means ± SEM of 3 independent experiments. * $P<0.001$ compared to vehicle treated preparations. D) Effects of CORM-2 (222 μM) on brain pial microvessel relaxation in tissues pre-treated (30 min) or not with iberiotoxin (IBX; 0.1 μM). Data are means ± SEM of 3 independent experiments. * $P<0.001$ compared to CORM-2 alone. E) BKα and HO-2 immunoblots (IB) of immunoprecipitates (IP) of BKα, HO-2 and HO-1 (see details in Materials and Methods); non specific IgG mouse antibody served as negative control.

Figure 6

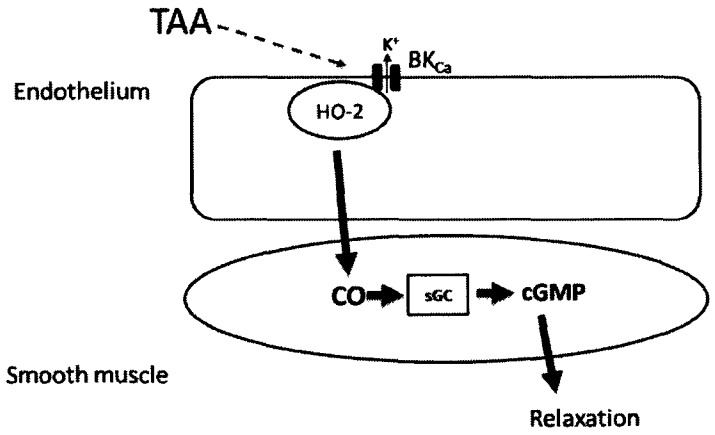


Figure 6.

Model based on data presented, depicting mode of *T*AA-evoked relaxation on brain pial microvessels. BK_{Ca}, HO-2, CO, sGC refer respectively to large conductance calcium-dependent potassium channels, heme oxygenase-2, carbon monoxide, and soluble guanylate cyclase.

CHAPTER 4: TRANS-ARACHIDONIC ACIDS AND CEREBRAL MICROVASCULAR DEGENERATION: IMPLICATIONS OF GPR40 RECEPTOR

4.1 PREAMBULE

The previous chapter has identified a new function for *T*AA demonstrating that their acute effects are vasodilatory. This following chapter focuses on chronic effects of *T*AA. Previous data have shown that *T*AA mediate retinal microvascular damage in a thrombospondin-1 dependent pathway. Data represented in the following pages demonstrate that *T*AA are highly increased in a hypoxia-ischemia model where they act as mediators of microvascular degeneration. For the first time, our data identify a primary site of action for non-enzymatically generated lipid peroxidation products. These data are submitted for publication in the following manuscript:

Kooli A, Honoré JC, Alquier T, Bossolasco M, Hou X, Hardy P, Poitout V, Balazy M, Chemtob S. Trans-arachidonic acids and cerebral microvascular degeneration: Implications of GPR40 receptor. To be submitted.

TRANS-ARACHIDONIC ACID AND CEREBRAL MICROVASCULAR DEGENERATION: IMPLICATIONS OF GPR40 RECEPTOR

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List of abbreviations: *Trans*-arachidonic acids (*T*AA), Human Brain Microvascular endothelial cells (HBMEC), Hypoxia-Ischemia (HI), Thrombospondin-1 (TSP-1), Linoleic Acid (LA), Arachidonic Acid (AA), 3-NitroTyrosine (3-NT)

4.2 ABSTRACT

Nitrative stress plays a crucial role in microvascular injury following brain ischemia. We recently described novel mediators of nitrative stress named *trans*-arachidonic acids (*T*AA) as major contributors of microvascular degeneration in the retina. Although the molecular mechanisms involved in *T*AA-induced endothelial cell death and microvascular damage are now unravelled, the primary site of action of *T*AA remains unknown. In this study, we explored whether *T*AA act via the recently described orphan receptor GPR40 shown to be activated by long chain unsaturated fatty acids. Here, we show that hypoxic-ischemic-induced microvascular degeneration begins as early as 24 hours post-injury, preceded by an increase in *T*AA levels. Interestingly, *T*AA-caused upregulation of the anti-angiogenic factor thrombospondin-1 and resulted cell death is dependent on *T*AA specific binding and activation of the GPR40 receptor. These effects are significantly prevented in GPR40-deficient mice which show more resistance to hypoxia-ischemia. These results strongly suggest an angiostatic role for GPR40 in the brain. Our findings uncover for the first time the primary target of a non-enzymatically generated nitroxidative product and identify a new function for GPR40 in the central nervous system. GPR40 represent an important target to limit the extent of ischemic brain injuries.

Keywords: *Trans*-arachidonic acids, brain injury, GPR40 receptor, neuromicrovascular degeneration, free fatty acids

4.3 ACKNOWLEDGMENTS

We acknowledge Dr Anne Monique Nuyt for kindly providing us with the stereotaxic apparatus. We wish to thank Melanie Ethier and Grace Fergusson for their technical skills and help. This study was supported in part by grants from the Canadian Institute of Health Research (CIHR) and the Heart and Stroke Foundation of Quebec. S Chemtob is a recipient of a Canada Research Chair in perinatology. V Poitout holds a Canada Research Chair in Diabetes and β cell function. A Kooli, JC Honoré and T Alquier are recipients of the Fonds de la Recherche en Santé du Québec, the "Focus on Stroke" CIHR/Rx&D and the Canadian Diabetes Association fellowships, respectively.

4.4 INTRODUCTION

Hypoxic-ischemic insults, cerebral ischemia and other ischemic-induced brain injuries are characterized by a severe reduction in cerebral blood flow leading to an irreversible neuronal cell death and ultimately to a disruption of the blood brain barrier (BBB), responsible for ensued brain oedema (Hamann, Okada et al. 1995; Brott et al. 1997). The most dramatic outcome of brain ischemic pathologies consists of neuromicrovascular injuries (Fagan, Hess et al. 2004) characterized by endothelial cell dysfunction and death. The mechanisms involved implicate essentially free radical generation. Indeed, increased reactive oxygen and nitrogen species (ROS/RNS) formation greatly contributes to microvascular injury (Palmer 1995; Beetsch, Park et al. 1998; Brault, Martinez-Bermudez et al. 2003; Gursoy-Ozdemir, Can et al. 2004). Conversely, antioxidants and free radical scavengers

reveal strong effectiveness against brain damage in experimental hypoxia-ischemia (Audus, Guillot et al. 1991; Sheng, Batinc-Haberle et al. 2002). Yet little is known on the mediators of oxidative and nitrative stresses. We have recently described a novel lipid peroxidation process mediated by $NO2^{\bullet}$ resulting in a *cis-trans*-isomerisation of arachidonic acid (Jiang, Kruger et al. 1999). This non enzymatic reaction produces four stable free fatty acids: the *trans*-arachidonic acids (*T*AA) namely 5*E*-AA, 8*E*-AA, 11*E*-AA and 14*E*-AA where 5*E*-AA and 8*E*-AA are endogenous (Balazy 2000) and not found in diet (Ferreri, Faraone Mennella et al. 2002). We further reported that in a hyperoxic model of retinopathy of prematurity, *T*AA levels rise and mediate a thrombospondin-1-dependent microvascular degeneration (Kermorvant-Duchemin, Sennlaub et al. 2005). While the molecular mechanisms behind *T*AA effects in the retina have been unveiled, the challenging task ahead remains to identify the primary site of action of *T*AA.

The main biological actions of prostaglandins and leukotrienes are exerted via diverse GPCR and in some cases by distinct PPARs (Funk 2001). Paradoxically, the selective bioactive functions exerted by non enzymatically produced eicosanoids have intrigued scientists for many years without assigning a receptor for a specific action; as is the case for isoprostanes and isoketals. On the other hand, nitrated fatty acids such as nitro-linoleic acid or nitro-oleic acid, were shown to exert their effects via PPARγ activation (Baker, Lin et al. 2005; Schopfer, Lin et al. 2005; Alexander, Bates et al. 2006). On the contrary, *T*AA which are non enzymatically produced lipid peroxidation products, seem to exert

their cytotoxic effects independent of classical major arachidonic acid metabolic pathways and PPARγ (Kermorvant-Duchemin, Sennlaub et al. 2005). The primary target of *T*AA is rather characteristic of a cell surface receptor.

Three independent groups have reported that GPR40, a previously described orphan receptor (Sawzdargo et al. 1997), is activated by long chain unsaturated free fatty acids (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003). GPR40 was identified as a putative receptor for linoleic acid (LA, C18:2n), arachidonic acid (AA, C20:4n) and docosahexaenoic acid (DHA, C22:6n). Highly expressed in pancreatic β cells, its role in diabetes and insulin regulation is currently heavily investigated (Latour et al. 2007; Winzell et al. 2007; Meidute Abaraviciene et al. 2008; Tan et al. 2008). Interestingly, increasing evidences suggests the expression of GPR40 in the brain, however its functions are yet unidentified (Ma et al. 2007; Ma et al. 2008; Yamashima 2008). Therefore, we proceeded to investigate the role of *T*AA in a hypoxic-ischemic model of cerebral ischemia and examined the putative relationship that may exist between *T*AA and GPR40 receptor.

4.5 MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River, Quebec) were used according to a protocol approved by the Ste-Justine Hospital Animal Care Committee. Female GPR40 knock out (KO) mice were generated as described (Latour, Alquier et al. 2007) and backcrossed to the C57Bl/6 strain for more than 7 generations. All

procedures using GPR40 KO mice were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal. GPR40 wild type (WT) littermates were used as controls.

Animals and induction of unilateral hypoxia-ischemia

P7 (post-natal day 7) Sprague Dawley rat pups and *gpr40* +/+ and *gpr40* -/- C57Bl/6 mice (6-8 weeks of age) were anesthetised with isoflurane (2.5% for induction and 1.5-2% for maintenance) and subjected to a unilateral carotid ligation as previously described (Rice et al. 1981; Brault, Martinez-Bermudez et al. 2003). The left common carotid artery was ligatured with silk (5-0) and animals were allowed to recover for 1-2 hours with full access to food and water after the surgical procedure. Animals were then placed in a hypoxic chamber (8% O_2, balanced with nitrogen, Oxycycler) on a heated pad, for 90 minutes. Animals were then allowed to recover for 3h, 18h, 24 or 48 hours. Control animals were either subjected to hypoxia or carotid ligature alone or sham. Brains were extracted for immunohistochemistry or TTC (2,3,5-Triphenyltetrazolium chloride) staining (see below for details).

Brain intracerebroventricular infusions and injections

P7 rat pups were infused in the cerebral lateral ventricles with Alzet micro-osmotic pumps (Alzet) as described previously (Brault, Martinez-Bermudez et al. 2003). Briefly, animals were anesthetised with isoflurane (3% for induction, 1.5-2% maintenance) and osmotic pumps (infusion rate: 0.5 μL/h) were implanted subcutaneously in the nuchal region. Pumps were connected to a cannula for drug

delivery in the lateral ventricle of the left hemisphere (stereotaxic coordinates: PA-1.0 mm, lateral-1.0 mm from bregma and ventral-2.0 mm relative to dura). Rat pups were randomly selected to receive either artificial cerebrospinal fluid (aCSF, vehicle) or L-Name (2 mg/kg) for 24 hours.

For intracerebroventricular (ICV) injections, P7 rat pups or young *gpr40*+/+ and *gpr40*-/- mice were injected (Stereotaxic coordinates for young adult mice: PA-0.5 mm, lateral-1.0 mm from bregma and ventral-2.0 mm relative to dura) with 5 μL of either vehicle or *T*AA ($5x10^{-5}$M) over 30 seconds, using a 10 μL Hamilton syringe and the needle was left in place for another minute. At the end of the 24 or 48 hour period, animals were sacrificed and brains were removed and subjected to fixation (immunohistochemistry) or TTC staining correspondingly, as described below.

Evaluation of brain infarct size (TTC staining)

Animals were anesthesized and perfused through the left ventricle with 4% TTC (2,3,5-Triphenyltetrazolium chloride). Animals were then wrapped in aluminium foil for 30 minutes on a heated pad (37°C). After decapitation, the brain was removed and placed in 10% formalin for 48 hours, after which, the brain was cut into 2 mm slices. The infarct volume (mm^3), appearing as a white colour, was calculated by multiplying the measured area by the slice thickness and was expressed as a percentage of infarct size.

Brain explants and quantification of vascular density

Brain explants of rat pups (postnatal days 3 to 6) were cultured *in vitro* based on a modification of a retinal explant protocol (Caffe et al. 2001; Quiniou et al. 2006). Brains were sectioned in ice-cold culture medium using a vibratome. Sections obtained (150 μm thick) were delicately placed in six-well dishes on top of a free-floating membrane (Nuclepore polycarbonate Track Etch, pore size 0.03 μm; Whatman, Brentford, UK). Brain explants were cultured for 3 days in EBM-2 medium, without FBS, containing vehicle (ethanol 100%, control) or 14*E*-AA (5 μmol/L) and pre-treated with vehicle, anti-CD36 mAb against the TSP-1 binding site (clone FA6-152 IgG_1 mouse; Beckman Coulter, Fullerton, CA, 200 μg/mL, ON treatment) or anti-CD36 mAb against the ox-LDL binding site (clone JC63.1 IgA mouse; Cayman Chemical, Ann Arbor, MI, 100 μg/mL, ON treatment) or the MEK-1 inhibitor PD98059 (10 μmol/L). *Ex vivo* monitoring of vascular degeneration was visualized by live-staining the endothelium using FITC-conjugated lectin (*Griffonia simplicifolia*; Sigma), and vascular density was quantified using a software program (ImagePro Plus 4.1). Control explants did not show signs of vascular degeneration for up to 5 days of culture.

Aortic ring angiogenesis assay

This assay was performed as described previously by us and others (Masson et al. 2002; Kermorvant-Duchemin, Sennlaub et al. 2005). In brief, thoracic aortas were removed from 6 to 8-week-old *gpr40*+/+ and *gpr40*-/- mice and immediately transferred to a culture dish containing ice-cold endothelial cell medium (EGM-2; Cambrex Bio Science, Walkersville, MD). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and scissors, paying special

attention not to damage the aortic wall. One millimeter–long aortic rings (12 per aorta) were sectioned and rinsed extensively in eight consecutive washes of EGM-2. The rings were then individually embedded in 48-well plates previously coated with 50 μL synthetic basement membrane (Matrigel; BD Biosciences, Bedford, MA) per well. Next, an additional 50 μL of Matrigel was placed over each ring. After 1 hour, 500 μL EGM-2 was added to each well, and the cultures were incubated at 37°C for 5 days. The culture medium was changed on day 3 and the test compounds added: vehicle or 14*E*-AA (5 μmol/L). The aortic rings were photographed on day 3 to 5 at 4X magnification with an inverted microscope (Eclipse TE300; Nikon). The angiogenic response was determined by measuring the area of neovessel formation on computer (Image Pro Plus software; Media Cybernetics, Inc.).

Immunohistochemistry

Mice and rats were anesthetised with isoflurane 5% and perfused with 4% paraformaldehyde (PFA). Brains were collected and immediately placed in 4% PFA for 24 h at 4°C followed by immersion in 30% sucrose in 0.1 M phosphate buffer. Brain sections (10 μm) were cut with a cryostat (Microm International, HM500 O), mounted on Supersoft Plus slides. Immunohistochemistry was performed using 3-nitrotyrosine (1:200, Upstate) or TSP-1 antibodies (1:50, Calbiochem) then double-labelled with lectin (1:200, Sigma; microvascular specific marker), as previously described (Sennlaub, Valamanesh et al. 2003).

Microvessels / Islets isolation and RNA extraction

Cerebral microvessels were prepared as previously described (Hou, Gobeil et al.

2000). Briefly, rat brains were gently homogenized with a Wheaton pestle in MEM media containing 6.3 mmol/L HEPES and 2.5 % dextran. The homogenates were then filtered through a 200 μm followed by a second filtration through a 100 μm nylon mesh and the resulting filtrates were mixed to an equal volume of 40 % dextran. The mixture was then centrifuged at 7000g for 15 minutes at 4°C. The resulting pellet was washed twice with PBS then resuspended with TRIzol reagent (invitrogen) for RNA extraction, according to the manufacturer's instructions.

Pancreatic islets were isolated from adult male Wistar rats (250-300 g) as previously described (Kelpe et al. 2003). Briefly, rats were anesthetized by intraperitoneal injection of a 100 mg/ml Ketamine Hydrochloride (Bimeda-MTC Animal Health, Cambridge, ON, Canada)/20 mg/ml Xylazine (Bayer, Toronto, ON, Canada) mixture, and islets were isolated by collagenase digestion (Sigma) and dextran density gradient centrifugation. Total RNA was extracted from aliquots of 100 islets using Qiagen RNeasy Micro-RNA Extraction kit according to the manufacturer's instructions.

Quantitative Real time PCR

Primer sequences were designed using the DNAMAN software (Lynnon Inc., Pointe-Claire, QC, Canada).The following primers were used for qPCR experiments: Rat (r) primers had the following forward (F) and reverse (R) sequences: rGPR40 (F) 5'-CTATATGGGCCCTTGTCC TTT-3' and (R) 5'-AATCGAGAAACTGAGTCGGG-3', rTSP-1 (F) 5'-GGCCCAGCTCTACA TTGACT-3' and (R) 5'-TGACATCTCCCTTTGCGAC-3', rCD36 (F) 5'-GTGGCAAAGAAT AGCAGCAA-3' and (R) 5'-

GTGAAGGCTCAAAGATGGC T-3'. Human (h) GPR40 primers were selected according to Katsuma S *et al.* (Katsuma, Hatae et al. 2005) whereas hTSP-1 and hCD36 primers were designed as described above with the following sequences: hTSP-1 (F) 5'-GCTG CAGAATGTGAGGTTTG-3' and (R) 5'-TGGCCAATGTAGTTAGTGCG-3', hCD36 (F) 5'-CACTGATCCTGCAAATGGAC-3' and (R) 5'-TTCTGAAGATGCCAAGCGT-3'. Specificity of the primers designed using the DNAMAN software has been evaluated according to the nucleotide BLASTN program as well as the size of the amplified products on 2% agarose gels.

Cell culture and transfection

Human brain microvascular endothelial cells (HBMEC) were purchased from ScienCell Research laboratories and cultured in ECM media containing FBS and endothelial growth supplement. HEK 293E cells were stably transfected with 2 μg of either pIRES-puro-GPR40 expressing human GPR40 receptor (provided by Marc Prentki, Montreal Diabetes Research Center, Montreal, Canada) or a control plasmid pIRES-puro using home-made prepared PEI transfection reagent (Polyethylenimine; Polysciences, Inc. Warrington, PA).

Radioligand binding assay

For ligand binding assay, 100,000 cells were seeded in 24-well dishes per well, coated with 0.1 % poly-D-lysine. After 24 hours, specific binding of [^{14}C]14*E*-AA (230 nmol/L) was determined in the presence of cold 14*E*-AA (40 μM). This radioligand assay was carried out on attached cells for 90 minutes on ice in binding buffer (10 mmol/L PBS/0.05 % BSA). The amount of bound radioactivity

was determined after washing twice with cold binding buffer and lysing cells in 0.1N NaOH/0.1 % Triton X-100.

For competition assay, attached HEK cells transfected with pIRES-puro-GPR40 or pIRES-puro were used. Briefly, cells were incubated in a total amount of 200 μL in a buffer containing 50 mmol/L Tris and 0.01% BSA, and 20 nmol/L [^{3}H]Arachidonic acid. Non specific binding was estimated in the presence of increased concentrations of cold *cis*-arachidonic acid and *trans*-arachidonic acids. Binding reactions were incubated on ice for 45 minutes and were terminated by rapid filtration through glass fibers (GF/C) filters with ice-cold 50 mmol/L Tris buffer, pH 7.4.

Calcium mobilization assay

Intracellular Ca^{2+} ($[Ca^{2+}]i$) levels were measured using the fluorescent indicator fura 2-AM as we have reported (Lahaie, Hardy et al. 1998; Hou, Gobeil et al. 2000) with slight modifications. Briefly, HEK 293E cells transfected with pIRES-puro-GPR40 or pIRES-puro (control plasmid) were seeded on glass cover slips in a 6-well plate until reaching 80-90 % confluence. Cells were washed and then kept in a HBSS/Calcium buffer (HEPES 0.02 mol/L, $CaCl_2$ 0.1 mol/L, NaCl 5 mol/L, KCl 2 mol/L). FURA 2 -AM (1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′N′-tetraacetic Acid Pentaacetoxymethyl Ester; 4 µmol/L)was added to each well and the plate was incubated at 37°C for 60 min. The cover slips were then washed twice and transferred to an acrylic cuvette for stimulation with vehicle (ethanol 100%), *trans*-arachidonic acids (10^{-5} mol/L) or linoleic acid (10^{-5} mol/L). The

$[Ca^{2+}]i$ was measured with a spectrofluorometer (Model LS 50; Perkin–Elmer, Beaconsfield, UK) by using excitation wavelengths of 340 and 380 nm and emission at 510 nm. Calibration of the fluorescent signal was determined using 0.2% Triton X-100 and 20 mmol/L EGTA to obtain a maximal and minimal fluorescence signal, respectively. $[Ca^{2+}]i$ was calculated as reported (Grynkiewicz et al. 1985).

Cell viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to assess cell viability as described (Beauchamp et al. 2001). Briefly, quiescent cells were first starved for 18h and then treated with either vehicle (Ethanol 100%), *T*AA or linoleic acid for 24 hours, after which they were added 50 μL of MTT in each well. Following a 3hour-incubation at 37°C, media was removed and cells were solubilised with an acidifed isopropanol solution (40 mM HCl). Optical density was then measured at 560 nm with 690 nm as a reference read out. Cell viability was expressed as a percentage of optical density relative to control.

Western blotting

Standard SDS-PAGE techniques were followed as previously described (Beauchamp et al. 2004). Primary antibodies were used according to the following conditions: p-erk1/2 (1:1000 dilution, Cell Signaling) and TSP-1 (1:200, Calbiochem). Equal protein loading was insured by probing with 1:200 total erk antibody (Santa Cruz) and 1:50,000 ß-actin antibody (Novus Biologicals),

correspondingly. Densitometry was measured in pixel intensity by Image-Pro Plus.

Synthesis of *trans*-arachidonic acids

Four *trans*-arachidonic acids were obtained from Dr JR Falck laboratory. All four mono-*trans* isomers of arachidonic acids were synthesized from corresponding epoxides as previously described (Roy, Loreau et al. 2004). These isomers were 100% pure and did not contain *cis*-arachidonic acid. Their purity and structure were confirmed by mass spectrometry, NMR, IR, HPLC and TLC. The *T*AA are stable as sodium salts in buffered solutions; *T*AA remain stable when stored refrigerated (-80°C) in ethanol. No degradation or peroxidation products were detected by mass spectrometry, and biological effects were highly reproducible in aliquots frozen for variable durations (Jiang, Kruger et al. 1999; Balazy 2000; Krishna, Reddy et al. 2001; Kermorvant-Duchemin, Sennlaub et al. 2005).

Measurement of *trans*-arachidonic acids

Lipids were extracted from brain cortex and analyzed by tandem mass spectrometry (LC/MS/MS) using MRM (multiple reaction monitoring) technique as we previously reported (Jiang, Kruger et al. 1999; Roy, Joshua et al. 2005; Kooli et al. 2008). Briefly, the samples were analyzed on a Zorbax Eclipse XDB-C18 column (2.1×50 mm, 1.8 μm) and eluted isocratically with 60% solvent A (water/acetonitrile/acetic acid 75:25:0.05 v/v/v) and 40% solvent B (100% acetonitrile) at a flow rate of 0.300 mL/min using an Agilent 1100 HPLC system. *T*AA levels were quantified using standard curves and nanogram amounts were

normalized to protein content. *T*AA levels are expressed in fold increase compared to sham animals and basal levels (24.98±4.13 ng/mg proteins).

Chemicals and Materials

Trans-arachidonic acids and [^{14}C]*14*E-AA were a kind gift from Dr JR Falck and Michael Balazy (New York Medical College, NY, USA). Linoleic acid, *cis*-arachidonic acid, arachidonic acid-5,6,8,9,11,12,14,15-^{3}H(N), lectin FITC-coupled and TRITC-coupled were purchased from Sigma Aldrich Inc.

Statistical analysis

Data were analyzed by two-way ANOVA followed by Bonferroni post test. Values are presented as means±S.E.M. Statistical significance was set at $p<0.05$.

4.6 RESULTS

Hypoxia-ischemia-induced brain damage is NO$^{\bullet}$-dependent.

Exposure of rat pups to a combination of both ischemic and hypoxic environments led to an increase in the infarcted area by 4 fold at 24 hours post-injury (Fig. 1 a-b). Moreover, hypoxia-ischemia (HI) was accompanied by a severe reduction of the cerebral microvascular density (Fig. 1 d). In order to evaluate the contribution of nitrative stress in the HI model, we first surmised the presence of nitrated proteins in the brain endothelium. The most recurrent modification following nitrative injury is the addition of $-NO_2$ group on tyrosine residues leading to the formation of 3-nitrotyrosine (3-NT) (Gursoy-Ozdemir, Can et al. 2004). Indeed, immunohistochemical analysis revealed predominant

localization of 3-NT within the microvasculature (lectin staining) in the HI hemisphere at 18 hours post-injury (Fig 1 c). All other controls of the HI model, namely sham, hypoxic alone or ischemic alone exposed animals showed little or no lectin staining at 3, 18 or 24h post-HI and no vascular degeneration (Fig. 1 d and data not shown) through 24 hours post-injury. Second, to ascertain that HI-induced brain injury is $NO^{\bullet}$-dependent, we tested the non selective nitric oxide synthase (NOS) inhibitor (l-name). Continuous cerebral intraventricular infusion of l-name completely reduced the infarct size observed in HI (Fig. 1 a-b) and partially preserved the cerebral microvascular degeneration (Fig. 1 g-h) highlighting the involvement of nitrative stress in HI-induced brain injury.

Trans-arachidonic acids levels rise in the hypoxic-ischemic rat pup model

To examine the possibility that HI and the concurrent nitrative stress favour the *cis-trans* isomerisation reaction of arachidonic acid and formation of *T*AA isomers, we measured *in vivo* brain levels of *T*AA at various time points following HI. *T*AA levels rise at 3h and peak at 18h post-injury reaching a 4 fold increase corresponding to a cerebral concentration of 0.1 μM (Fig. 1e, calculated from estimated volume). Because *T*AA formation requires NOS activation in a hyperoxia model affecting the retina (Kermorvant-Duchemin, Sennlaub et al. 2005), and due to the currently demonstrated protective effects of l-name (Figure 1 a-b), we measured *T*AA levels in the HI brain upon l-name treatment. Inhibition of NOS enzymes abolished the increased *T*AA formation in HI (Figure 1 f), indicative of a $NO^{\bullet}$-dependent pathway with an ensuing nitrative stress in our experimental model.

***T*AA induced cerebral vaso-obliteration is TSP-1 dependent**

We then assessed the hypothesis that the nitrative stress-evoked increase in *T*AA levels could be involved in the cerebral microvascular degeneration observed in HI. Using concentrations of *T*AA in the same range as those measured *in vivo*, we determined their effects on normal angiogenesis of rat cerebral vasculature *in vivo* and rat brain explants *ex vivo*. ICV injections of two different isomers of *T*AA (8*E*-AA and 14*E*-AA) showed a 40 to 50% reduction in vascular density (Fig. 2 a-b) respectively, similar to the changes observed in our HI model (Figure 1 d). We have previously demonstrated that *T*AA-induced retinal vasoobliteration and microvascular endothelial cell death depends on the overexpression of the anti-angiogenic factor thrombospondin-1 (TSP-1) (Kermorvant-Duchemin, Sennlaub et al. 2005). TSP-1 is a large matricellular protein known to inhibit angiogenesis and induce endothelial cell apoptosis (Nor et al. 2000; Armstrong et al. 2003). The ability of TSP-1 to mediate its angiostatic effects is dependent on its transmembrane receptor, CD36 (Dawson et al. 1997; Jimenez et al. 2000). In our HI model, we observed that the decreased vascular density was preceded by a strong immunoreactivity for TSP-1, particularly within cerebral microvessels (Figure 2c). Moreover, only *T*AA-injected brains displayed a remarkable staining for TSP-1 compared to vehicle treated animals (Figure 2 c). Interestingly, TSP-1 immunoreactivity was solely specific to the endothelium (co-immunostaining with lectin). When investigating the effect of *T*AA *ex vivo* by exposing brain explants to either *T*AA or vehicle, we demonstrate that *T*AA exposure induces vascular degeneration in a time-dependent manner (data not shown). To confirm

the cause and effect relationship between *T*AA and the TSP-1/CD36 complex in the brain, we used function blocking antibodies directed against CD36. The CD36 antibody that specifically blocks the binding site of TSP-1 fully prevented *T*AA-induced vascular degeneration. In contrast, abrogated vascular density was not altered when a CD36 antibody specifically blocking the binding site for low density lipoproteins was used (Figure 2 d-e). We have previously reported that TSP-1 upregulation along with endothelial cell death is dependent on *T*AA-induced transient activation of ERK1/2 (Kermorvant-Duchemin, Sennlaub et al. 2005). We, therefore, treated brain explants with the MEK inhibitor PD98059 and showed that inhibition of the MAPK pathway prevented *T*AA-induced vascular degeneration (Figure 2 d-e) *ex vivo*.

_T_AA bind and activate GPR40 receptor

Along with our previous data, our results suggest that *T*AA might act on a specific receptor to mediate its cytotoxic and angiostatic effects in the brain. A recently described orphan G protein coupled receptor, namely GPR40, was identified as a putative receptor for long chain unsaturated free fatty acids, including linoleic acid (LA, C18: 2n), arachidonic acid (AA, C20: 4n) and docasahexaenoic acid (DHA, C22:6n). However, this receptor has not been studied in the rodent brain. Furthermore, GPR40 activation necessitates a carboxyl group within its ligands (Brown, Goldsworthy et al. 2003). Because of the structural resemblance between AA and *T*AA at the carboxylic end of both molecules (Figure 3 a) as well as the specificity for MAPK activation and ensued TSP-1 upregulation, we hypothesized that *T*AA might act via GPR40. GPR40 is a $G_{\alpha q}$-coupled receptor, enriched in

both human and rodent pancreatic islets but appears to be present only in human and primate central nervous system (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003). Thus, we first determined the levels of GPR40 receptor in the brain from P7 rat pups. Although we failed to detect GPR40 receptor mRNA in rat whole brain lysates, we were able to measure its expression in isolated cerebral microvessels (Figure 3 b) with fairly low levels as compared to rat pancreatic islets which mRNA is used as a positive control. Because the cerebral microvasculature corresponds to only 2% of the brain mass (Goehlert et al. 1981), we decided to conduct part of the experiments *in vitro*. Similarly to what we have observed *in vivo*, human brain endothelial cells, *in vitro*, show significant levels of GPR40 mRNA (Figure 3 c) thus suggesting that *T*AA produced in our HI model may interact with the GPR40 receptor located on microvascular endothelial cells in the rat pup. Along this hypothesis, we then looked at GPR40 receptor and *T*AA binding interactions and functions *in vitro*. First, we showed that [^{14}C]14*E*-AA binding revealed specific binding to only HEK-transfected cells with GPR40 receptor, termed GPR40 [+]. On the contrary, 14-*E*AA did not bind to HEK293 cells lacking GPR40 receptor (Flodgren et al. 2007), termed GPR40 [-] (Figure 3 d). In the competition binding assay, unlabeled 14*E*-AA displaced bound [^{3}H]AA as successfully as unlabeled AA with an IC50 of approximately 10^{-5}M in presence of 20 nM of [^{3}H]AA (figure 3 e). In addition to binding to GPR40 receptor, we showed that *T*AA exposure on GPR40 [+], but not GPR40 [-] cells, elicit strong calcium influx, suggestive of GPR40 activation by *T*AA (Figure 3 f).

T*AA induce a GPR40 ensuing cell death *in vitro* and vasoobliteration *in vivo

To further corroborate the role of GPR40 in response to *T*AA, we tested the effects of *T*AA-induced cell signalling on GPR40 [+], GPR40 [-] Hek293 cells and human brain microvascular endothelial cells (HBMEC). Incubation of either 14*E*-AA or LA (positive control) induced a strong and transient MAPK activation only in GPR40 [+] cells and HBMEC, two cell lines that express GPR40 receptor (Figure 3 b and 4 c). In addition, a 5 fold increase in TSP-1 protein expression level was noted upon treatment of GPR40 [+] cells with *T*AA (Figure 4 d). Following TSP-1 upregulation, we tested *T*AA potency on the survival of different cell types. Here we show that *T*AA induced a concentration-dependent cell death of GPR40 [+] and HBMEC only. Since GPR40 [-] cells were insensitive to either *T*AA (Figure 4 e) for a period up to 48 hours (data not shown), all data presented so far are suggestive of the anti-angiogenic function of GPR40 in the brain.

To further confirm this angiostatic role of GPR40 in response to *T*AA, we tested the effects of *T*AA on vascular sprouting from Matrigel-embedded aortic rings isolated from GPR40-KO and WT animals. As expected, *T*AA interfered with vascular growth in aortas of WT mice, whereas this effect was partially prevented in mice with disrupted GPR40 receptor (Figure 5 a). Furthermore, we corroborated these results in the brain where ICV injections of *T*AA failed to induce vascular degeneration in GPR40 KO mice compared to a 26% reduction of vascular density in WT littermates (Figure 5 b).

HI induced brain damage is GPR40-dependent

Finally, to verify that GPR40 activation by *T*AA is pathologically relevant and examine the putative relationship between microvascular obliteration and brain damage, we induced the HI model in GPR40 WT and KO mice. GPR40 deficient mice showed a significantly attenuated HI-induced infarct compared to WT littermates (Figure 5 c) suggestive of a strong resistance to HI insult.

4.7 DISCUSSION

Nitrative stress derivatives and lipid peroxidation products perform a number of biological actions. The newly described products of $NO_2^{\bullet}$-mediated isomerisation of arachidonic acid and markers of nitrative stress, *T*AA, represent a new aspect in ischemia-induced microvascular injuries. Lately, we have uncovered the molecular mechanisms involved in *T*AA-induced retinal microvascular obliteration in an oxygen-induced model of retinopathy of prematurity (Kermorvant-Duchemin, Sennlaub et al. 2005). However, as other non enzymatically formed lipid peroxidation products, such as isoprostanes, the primary target of *T*AA remains unknown. Our data demonstrate that HI is associated with vascular obliteration resulting in part from nitrative stress (Figure 1 g-h) which increases formation of *T*AA (Balazy 2000; Balazy et al. 2004) (Figure 1 e-f). *T*AA in turn, caused cerebral microvascular damage *in vivo*, *ex-vivo* and *in vitro* (Figures 2 a-b, e-f, 4e and 5a-b) through activation of GPR40 receptor (Figures 3e and 4c). We therefore investigated the involvement of GPR40 receptor in HI-induced brain damage. We first showed that *T*AA

specifically bind to GPR40 receptor (Figure 3 d) which is present in cerebral microvessels as shown by qPCR (Figure 3 b). *T*AA binding to GPR40 elicited its activation as demonstrated by measurement of calcium influx, ERK1/2 phosphorylation and TSP-1 overexpression (Figures 3f and 4c-d). On the other hand, a TSP-1 expression (by immunohistochemistry) was detected in HI brains along with a rise in *T*AA levels (Figures 1c and 2c) followed by a *T*AA-dependent decrease in vascular density (Figure 1 g-h). Correspondingly, vascular degeneration was significantly attenuated in *gpr40-/-* mice upon treatment with *T*AA (Figure 5a-b). Finally, *gpr40-/-* mice showed resistance to HI-induced brain damage (Figure 5 c). By showing that *T*AA are increased in our HI model in an NO-dependent pathway and induce cerebral microvascular damage, similar to what we have previously shown in the retina (Kermorvant-Duchemin, Sennlaub et al. 2005) via TSP-1 upregulation, our results confirm that *T*AA are strong mediators of nitrative stress *in vivo* and possess a powerful anti-angiogenic effect. On another hand, our results unveil the importance of microvascular integrity in hypoxic-ischemic injuries. Indeed, the endothelium is continuously emerging as an important component of the neurovascular unit, which disturbances exacerbate the brain damage (Pesonen, Kaprio et al. 1981; Yang and Betz 1994; Legos, Tuma et al. 2002; Lo, Dalkara et al. 2003). However, brain damage is usually associated with an irreversible neuronal death (Wieloch 1985; Ogawa, Kitao et al. 2007) whereas endothelium was regarded for decades as an inert conduit; hence few studies have focused on the importance of the microvascular integrity in HI injuries. Here we provide supplementary evidence on the major contribution of endothelium dysregulation, here microvascular degeneration to HI-induced brain

damage and provide new insight on GPR40 activation. First, the prevention of microvascular degeneration (Figure 1 g-h) is associated with decreased brain infarct upon HI insult (Figure 1 a-b); where *T*AA seem to be the connecting bond between both damages. Moreover, deletion of GPR40 receptor, activated by *T*AA, shows a considerable resistance to HI-mediated microvascular injury and brain damage (Figure 5 b-c). Although, according to the literature, GPR40 appears to be activated by an array of distinct free fatty acids including DHA and linoleic acid, none of them were described to possess a role in angiogenesis (Bourre et al. 1992; Bourre et al. 1993; Abbott 2000; Bazan 2006). Therefore, the anti-angiogenic action of GPR40 in the brain depends on its activation by *T*AA which may suggest that GPR40 activation by the neuroprotective DHA may elicit a rather protective effect in the brain.

Interestingly, previous studies have failed to identify GPR40 receptor in rodent brain and therefore concentrated their efforts in understanding its role in β-cell function, where it is predominantly expressed (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Kotarsky, Nilsson et al. 2003). Nonetheless, GPR40 profile distribution in human and primate brains (Ma, Tao et al. 2007; Hirasawa et al. 2008; Ma, Lu et al. 2008) is suggested to play a role in memory, however, it is not yet fully investigated (Yamashima 2008). Due to the fact that *T*AA are unsaturated fatty acids, we investigated the involvement of GPR40 receptor, shown to be activated by long chain fatty acids, in mediating the *T*AA-induced cytotoxic effects. Our findings reveal that GPR40 receptor is present within rodent cerebral microvessels. Because the microvasculature only represents 2% of

the brain mass (Goehlert, Ng Ying Kin et al. 1981), it could explain why it is very difficult to observe the presence of GPR40 mRNA in rodent whole brain lysates. Moreover, in the present study, we identified for the first time a new function for GPR40 in the brain where it acts as an anti-angiogenic receptor when activated by *T*AA. Our data provide strong evidence that *T*AA bind to GPR40 receptor with respect to other studies which show only activation of GPR40 by various FFA, demonstrated by calcium mobilization assay (Briscoe, Tadayyon et al. 2003; Brown, Goldsworthy et al. 2003; Itoh, Kawamata et al. 2003; Itoh et al. 2005; Briscoe et al. 2006; Garrido et al. 2006). This inference is supported in our study by binding assays, performed on both GPR40 expressing and lacking cells, clearly demonstrating the specific interaction of *T*AA to GPR40 receptor (Figure 3 d). This binding is revealed to be active generating significant calcium influx upon *T*AA exposure and shortly leading to MAPK transient activation; another evidence for GPR40 activation (Figures 3 f and 4 c). This exact pathway has been recently shown to be crucial for normal stimulation of insulin secretion in humans (Vettor et al. 2008) which reinforces the role of GPR40 receptor in the progression of diabetes and as shown here in microvascular degeneration.

Furthermore, MAPK phosphorylation is followed by TSP-1 upregulation and subsequent cell death, all dependent on GPR40 presence and activation, including in cerebral microvascular endothelial cells (Figure 4 d-e). Thus, our data confirm the relationship of *T*AA/GPR40 *in vivo* and reinforce the involvement of GPR40 in angiogenesis. An additional surprising result was to observe an extended role to GPR40 in hypoxic-ischemic-induced brain damage; *gpr40-/-* mice are resistant to

HI compared to their wild type littermates. These data strongly suggest a very tight relationship between microvascular obliteration and brain damage in HI insults. More importantly, our data demonstrate that GPR40 receptor is the responsible moiety for this relationship.

In this study, we have identified a new function to GPR40 and unravelled a new mechanism by which GPR40 could mediate cell cytotoxicity and microvascular degeneration. However, GPR40 deletion does not fully explain the vascular degeneration mediated by *T*AA. Participation of other possible receptors for *T*AA is currently under investigation as well as further characterization of the effects of *T*AA on the β-cell function. In addition, our results open an enticing debate on whether to develop an antagonist for GPR40 receptor which limits its damaging effects in the brain or an agonist needed for insulin regulation. In both cases, development of these drugs might need elaborate and long term studies on the cardiovascular effects according to the latest FDA decision concerning diabetes drugs.

Taken together, our data indicate that a *T*AA/GPR40 interaction is responsible for endothelial cell death and subsequent microvascular degeneration in hypoxic-ischemic damages and provide new evidence for a previously undescribed link between GPR40 and CNS associated injuries.

4.8 REFERENCES

Abbott, N. J. (2000). "Inflammatory mediators and modulation of blood-brain barrier permeability." Cell Mol Neurobiol **20**(2): 131-47.

Alexander, R. L., D. J. Bates, et al. (2006). "Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPARgamma-dependent transcription activation." Biochemistry **45**(25): 7889-96.

Armstrong, L. C. and P. Bornstein (2003). "Thrombospondins 1 and 2 function as inhibitors of angiogenesis." Matrix Biol **22**(1): 63-71.

Audus, K. L., F. L. Guillot, et al. (1991). "Evidence for 21-aminosteroid association with the hydrophobic domains of brain microvessel endothelial cells." Free Radic Biol Med **11**(4): 361-71.

Baker, P. R., Y. Lin, et al. (2005). "Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands." J Biol Chem **280**(51): 42464-75.

Balazy, M. (2000). "Trans-arachidonic acids: new mediators of inflammation." J Physiol Pharmacol **51**(4 Pt 1): 597-607.

Balazy, M. and C. D. Poff (2004). "Biological nitration of arachidonic acid." Curr Vasc Pharmacol **2**(1): 81-93.

Bazan, N. G. (2006). "The onset of brain injury and neurodegeneration triggers the synthesis of docosanoid neuroprotective signaling." Cell Mol Neurobiol **26**(4-6): 901-13.

Beauchamp, M. H., A. K. Martinez-Bermudez, et al. (2001). "Role of thromboxane in retinal microvascular degeneration in oxygen-induced retinopathy." J Appl Physiol **90**(6): 2279-88.

Beauchamp, M. H., F. Sennlaub, et al. (2004). "Redox-dependent effects of nitric oxide on microvascular integrity in oxygen-induced retinopathy." Free Radic Biol Med **37**(11): 1885-94.

Beetsch, J. W., T. S. Park, et al. (1998). "Xanthine oxidase-derived superoxide causes reoxygenation injury of ischemic cerebral endothelial cells." Brain Res **786**(1-2): 89-95.

Bourre, J. M., M. Bonneil, et al. (1992). "Structural and functional importance of dietary polyunsaturated fatty acids in the nervous system." Adv Exp Med Biol **318**: 211-29.

Bourre, J. M., M. Bonneil, et al. (1993). "Function of dietary polyunsaturated fatty acids in the nervous system." Prostaglandins Leukot Essent Fatty Acids **48**(1): 5-15.

Brault, S., A. K. Martinez-Bermudez, et al. (2003). "Selective neuromicrovascular endothelial cell death by 8-Iso-prostaglandin F2alpha: possible role in ischemic brain injury." Stroke **34**(3): 776-82.

Briscoe, C. P., A. J. Peat, et al. (2006). "Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules." Br J Pharmacol **148**(5): 619-28.

Briscoe, C. P., M. Tadayyon, et al. (2003). "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids." J Biol Chem **278**(13): 11303-11.

Brott, T., J. P. Broderick, et al. (1997). Thrombolysis for acute ischemic stroke. Primer on Cerebrovascular disease. K. M. A. Welch, L. R. Caplan, D. J. Reis, B. K. Siesjo and W. Bryce. San Diego, CA, Academic Press: pp 728:731.

Brown, A. J., S. M. Goldsworthy, et al. (2003). "The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids." J Biol Chem **278**(13): 11312-9.

Caffe, A. R., P. Ahuja, et al. (2001). "Mouse retina explants after long-term culture in serum free medium." J Chem Neuroanat **22**(4): 263-73.

Dawson, D. W., S. F. Pearce, et al. (1997). "CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells." J Cell Biol **138**(3): 707-17.

Fagan, S. C., D. C. Hess, et al. (2004). "Targets for vascular protection after acute ischemic stroke." Stroke **35**(9): 2220-5.

Ferreri, C., M. R. Faraone Mennella, et al. (2002). "Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples." Free Radic Biol Med **33**(11): 1516-26.

Flodgren, E., B. Olde, et al. (2007). "GPR40 is expressed in glucagon producing cells and affects glucagon secretion." Biochem Biophys Res Commun **354**(1): 240-5.

Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." Science **294**(5548): 1871-5.

Garrido, D. M., D. F. Corbett, et al. (2006). "Synthesis and activity of small molecule GPR40 agonists." Bioorg Med Chem Lett **16**(7): 1840-5.

Goehlert, U. G., N. M. Ng Ying Kin, et al. (1981). "Biosynthesis of prostacyclin in rat cerebral microvessels and the choroid plexus." J Neurochem **36**(3): 1192-201.

Grynkiewicz, G., M. Poenie, et al. (1985). "A new generation of Ca2+ indicators with greatly improved fluorescence properties." J Biol Chem **260**(6): 3440-50.

Gursoy-Ozdemir, Y., A. Can, et al. (2004). "Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia." Stroke **35**(6): 1449-53.

Hamann, G. F., Y. Okada, et al. (1995). "Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion." Stroke **26**(11): 2120-6.

Hirasawa, A., C. Itsubo, et al. (2008). "Production and characterization of a monoclonal antibody against GPR40 (FFAR1; free fatty acid receptor 1)." Biochem Biophys Res Commun **365**(1): 22-8.

Hou, X., F. Gobeil, Jr., et al. (2000). "Augmented vasoconstriction and thromboxane formation by 15-F(2t)-isoprostane (8-iso-prostaglandin F(2alpha)) in immature pig periventricular brain microvessels." Stroke **31**(2): 516-24; discussion 525.

Itoh, Y. and S. Hinuma (2005). "GPR40, a free fatty acid receptor on pancreatic beta cells, regulates insulin secretion." Hepatol Res **33**(2): 171-3.

Itoh, Y., Y. Kawamata, et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-6.

Jiang, H., N. Kruger, et al. (1999). "Nitrogen dioxide induces cis-trans-isomerization of arachidonic acid within cellular phospholipids. Detection of trans-arachidonic acids in vivo." J Biol Chem **274**(23): 16235-41.

Jimenez, B., O. V. Volpert, et al. (2000). "Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1." Nat Med **6**(1): 41-8.

Katsuma, S., N. Hatae, et al. (2005). "Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1." J Biol Chem **280**(20): 19507-15.

Kelpe, C. L., P. C. Moore, et al. (2003). "Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis." J Biol Chem **278**(32): 30015-21.

Kermorvant-Duchemin, E., F. Sennlaub, et al. (2005). "Trans-arachidonic acids generated during nitrative stress induce a thrombospondin-1-dependent microvascular degeneration." Nat Med **11**(12): 1339-45.

Kooli, A., E. Kermorvant-Duchemin, et al. (2008). "trans-Arachidonic acids induce a heme oxygenase-dependent vasorelaxation of cerebral microvasculature." Free Radic Biol Med **44**(5): 815-25.

Kotarsky, K., N. E. Nilsson, et al. (2003). "A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs." Biochem Biophys Res Commun **301**(2): 406-10.

Krishna, U. M., M. M. Reddy, et al. (2001). "Stereospecific synthesis of trans-arachidonic acids." Bioorg Med Chem Lett **11**(18): 2415-8.

Lahaie, I., P. Hardy, et al. (1998). "A novel mechanism for vasoconstrictor action of 8-isoprostaglandin F2 alpha on retinal vessels." Am J Physiol **274**(5 Pt 2): R1406-16.

Latour, M. G., T. Alquier, et al. (2007). "GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo." Diabetes **56**(4): 1087-94.

Legos, J. J., R. F. Tuma, et al. (2002). "Pharmacological interventions for stroke: failures and future." Expert Opin Investig Drugs **11**(5): 603-14.

Lo, E. H., T. Dalkara, et al. (2003). "Mechanisms, challenges and opportunities in stroke." Nat Rev Neurosci **4**(5): 399-415.

Ma, D., L. Lu, et al. (2008). "Expression of free fatty acid receptor GPR40 in the neurogenic niche of adult monkey hippocampus." Hippocampus **18**(3): 326-33.

Ma, D., B. Tao, et al. (2007). "Expression of free fatty acid receptor GPR40 in the central nervous system of adult monkeys." Neurosci Res **58**(4): 394-401.

Masson, V. V., L. Devy, et al. (2002). "Mouse aortic ring assay: a new approach of the molecular genetics of angiogenesis." Biol. Proc. Online **4**: pp 24-31.

Meidute Abaraviciene, S., I. Lundquist, et al. (2008). "Palmitate-induced beta-cell dysfunction is associated with excessive NO production and is reversed by

thiazolidinedione-mediated inhibition of GPR40 transduction mechanisms." PLoS ONE **3**(5): e2182.

Nor, J. E., R. S. Mitra, et al. (2000). "Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway." J Vasc Res **37**(3): 209-18.

Ogawa, S., Y. Kitao, et al. (2007). "Ischemia-induced neuronal cell death and stress response." Antioxid Redox Signal **9**(5): 573-87.

Palmer, C. (1995). "Hypoxic-ischemic encephalopathy. Therapeutic approaches against microvascular injury, and role of neutrophils, PAF, and free radicals." Clin Perinatol **22**(2): 481-517.

Pesonen, E., E. Kaprio, et al. (1981). "Endothelial cell damage in piglet coronary artery after intravenous administration of E. coli endotoxin. A scanning and transmission electron-microscopic study." Atherosclerosis **40**(1): 65-73.

Quiniou, C., F. Sennlaub, et al. (2006). "Dominant role for calpain in thromboxane-induced neuromicrovascular endothelial cytotoxicity." J Pharmacol Exp Ther **316**(2): 618-27.

Rice, J. E., 3rd, R. C. Vannucci, et al. (1981). "The influence of immaturity on hypoxic-ischemic brain damage in the rat." Ann Neurol **9**(2): 131-41.

Roy, U., R. Joshua, et al. (2005). "Cytochrome P450/NADPH-dependent biosynthesis of 5,6-trans-epoxyeicosatrienoic acid from 5,6-trans-arachidonic acid." Biochem J **390**(Pt 3): 719-27.

Roy, U., O. Loreau, et al. (2004). "Cytochrome P450/NADPH-dependent formation of trans epoxides from trans-arachidonic acids." Bioorg Med Chem Lett **14**(4): 1019-22.

Sawzdargo, M., S. R. George, et al. (1997). "A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1." Biochem Biophys Res Commun **239**(2): 543-7.

Schopfer, F. J., Y. Lin, et al. (2005). "Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand." Proc Natl Acad Sci U S A **102**(7): 2340-5.

Sennlaub, F., F. Valamanesh, et al. (2003). "Cyclooxygenase-2 in human and experimental ischemic proliferative retinopathy." Circulation **108**(2): 198-204.

Sheng, H., I. Batinc-Haberle, et al. (2002). "Catalytic Antioxidants as Novel Pharmacologic Approaches to Treatment of Ischemic Brain Injury." Drug News Perspect **15**(10): 654-665.

Tan, C. P., Y. Feng, et al. (2008). "Selective Small-Molecule Agonists of G protein-coupled Receptor 40 Promote Glucose-Dependent Insulin Secretion and Reduce Blood Glucose in Mice." Diabetes.

Vettor, R., M. Granzotto, et al. (2008). "Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization." J Clin Endocrinol Metab.

Wieloch, T. (1985). "Neurochemical correlates to selective neuronal vulnerability." Prog Brain Res **63**: 69-85.

Winzell, M. S. and B. Ahren (2007). "G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes." Pharmacol Ther **116**(3): 437-48.

Yamashima, T. (2008). "A putative link of PUFA, GPR40 and adult-born hippocampal neurons for memory." Prog Neurobiol **84**(2): 105-15.

Yang, G. Y. and A. L. Betz (1994). "Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats." Stroke **25**(8): 1658-64; discussion 1664-5.

4.9 FIGURES

Figure1

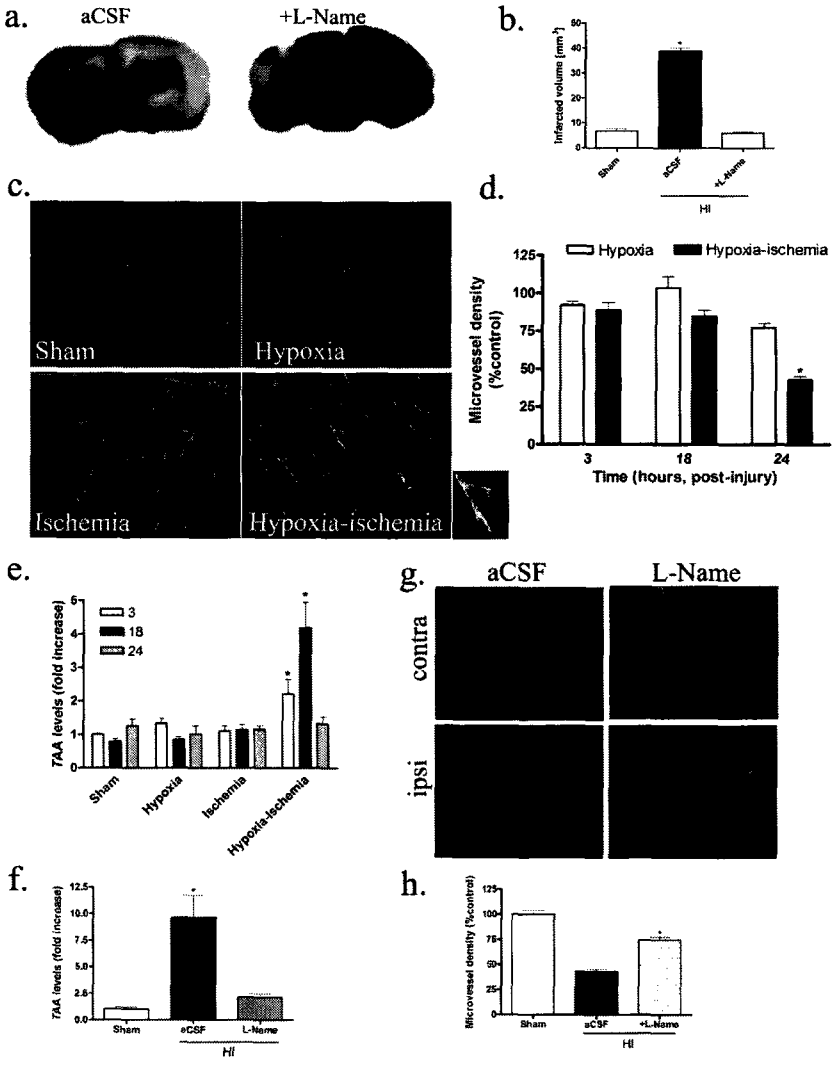
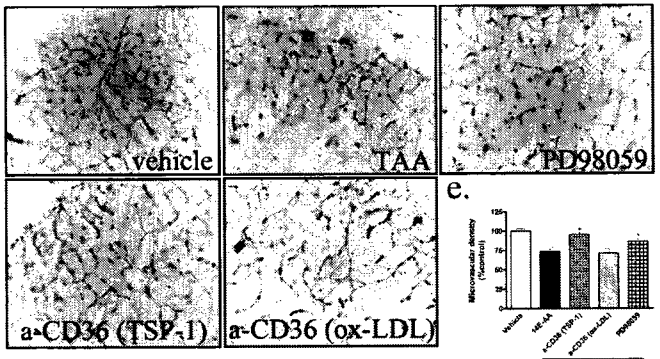


Figure 1

*T*AA levels rise secondary to nitrative stress in a hypoxia-ischemia (HI)-induced brain injury model in rat pups. (a) Brain infarctus evaluated by TTC staining as detailed in materials and methods. The white area is distinctive of an infarct as seen in artificial cerebrospinal fluid (aCSF) treated HI-subjected rat pups and significantly attenuated in l-name treated animals. (b) Quantification of the infarcted volume in (mm^3). *$P \leq 0.01$ compared to aCSF treated animals and subjected to HI. (c) Presence of nitrative stress within rat cerebral microvessels. Co-staining of 3-NT (green) and lectin (red) at 18 hours post- HI. Sham, hypoxia and ischemia serve as controls for the model. (d) Quantification of cerebral vascular density in HI exposed P7 rat pups at 3h, 18h and 24 hours post-HI. *$P \leq 0.01$ compared to 3h sham vascular density(e) Total (free + bound (phospholipid-esterified)) brain *T*AA levels in HI-induced animals at 3h, 18h and 24h post-injury. (f) Total brain *T*AA levels in HI-induced rat pups treated with aCSF or l-name (2mg/kg) at 18 hours post-injury. (g) Effect of NOS inhibitor l-name on vascular density in HI-exposed P7 pups. (h) Quantification of vascular density as described in materials and methods. *$P \leq 0.01$ compared to aCSF treated animals and subjected to HI. Values in histogram are mean ± s.e.m of 3-8 separate experiments.

d.



e.

Figure 2

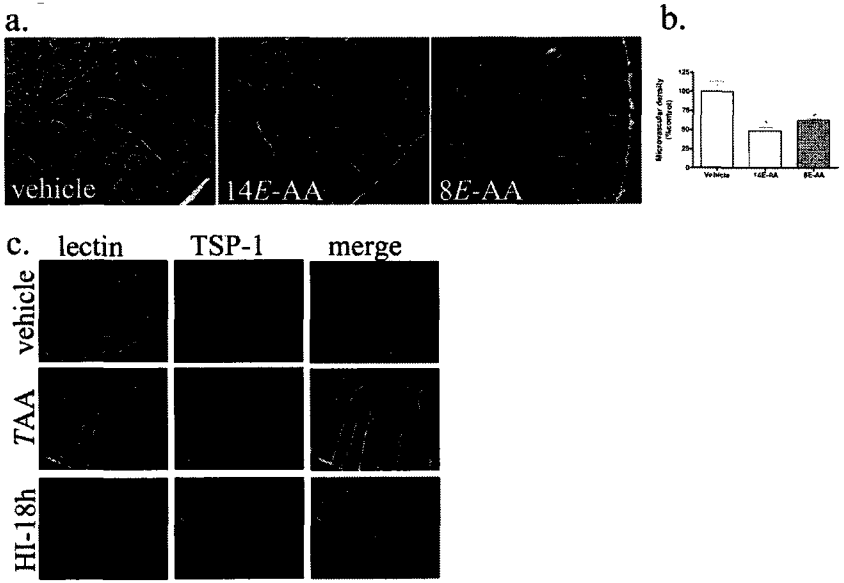
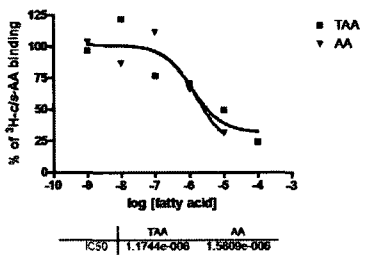


Figure 2

*T*AA induce a TSP-1/CD36 cerebral microvascular degeneration. (a) In vivo effects of 14*E*-AA and 8*E*-AA on rat cerebral vascular density injected in the lateral ventricules. (b) Quantification of cerebral vascular density 24 hours post-ICV injections. *P≤0.01 compared to vehicle injected P1 pups. (c) Microvascular localization of TSP-1 in rat pups exposed to HI (18h post-HI) or injected with either vehicle or *T*AA (24h post-ICV injections); representative of 5 pups at each group and time. (d) Ex vivo effects of 14*E*-AA on vascular density of rat brain explants in presence or absence of CD36 mAb (specific for TSP-1 binding site, 200 μg/mL), CD36 mAb (specific for ox-LDL binding site, 100 μg/mL) or a MEK inhibitor PD98059 (10 μmo/L). (e) Quantification of cerebral vascular density. *P≤0.01 compared to 14*E*-AA treated explants. Values in histogram are mean ± s.e.m of 4 separate experiments.


% of ^{3}H-*cis*-AA binding
log [fatty acid]
TAA
AA
IC50
TAA
AA
1.1744e-006
1.5809e-006

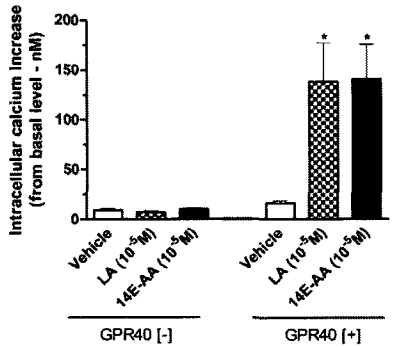

Intracellular calcium increase
(from basal level - nM)
Vehicle
LA (10^{-5}M)
14E-AA (10^{-5}M)
Vehicle
LA (10^{-5}M)
14E-AA (10^{-5}M)
GPR40 [-]
GPR40 [+]

Figure 3

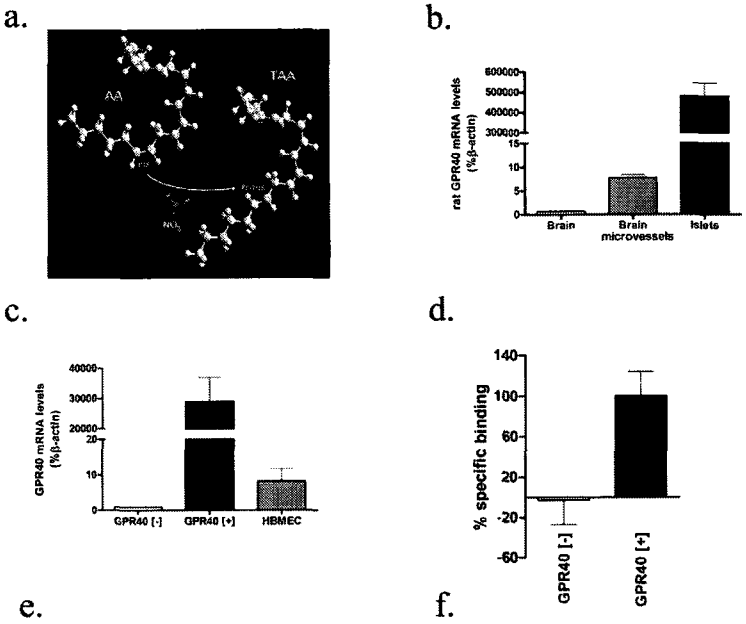

a.
AA
TAA
b.
rat GPR40 mRNA levels (%β-actin)
Brain
Brain microvessels
Islets
c.
GPR40 mRNA levels (%β-actin)
GPR40 [-]
GPR40 [+]
HBMEC
d.
% specific binding
GPR40 [-]
GPR40 [+]
e.
f.

Figure 3

*T*AA bind and activate GPR40 receptor. (a) Three-dimensional structures of arachidonic acid and 14*E*-AA. Note the *trans* bond configuration at carbon 14 which alters only the w-end of the molecule leaving intact the carboxylic end. (b) GPR40 mRNA expression corrected for β-actin from whole rat brain lysates (brain), rat cerebral microvessels or rat pancreatic islets (islets) which serve as a positive control. (c) GPR40 mRNA expression corrected for β-actin from HEK 293 cells transfected with puro-IRES vector (GPR40 [-]), HEK293 cells transfected with puro-IRES-GPR40 vector (GPR40[+]) and human brain microvascular endothelial cells (HBMEC). (d) Representative histogram of binding [^{14}C]14*E*-AA to GPR40 [-] or GPR40 [+] cells. Values are in percentage of specific binding. (e) Competitive binding assay using increasing concentrations of unlabeled 14*E*-AA or AA in the presence of 20 nM of [^{3}H]AA on GPR40 [+] cells. (f) Effect of 14*E*-AA on calcium mobilization in both GPR40 [-] and GPR40 [+] cells. Linoleic acid (LA) serves as a positive control. *P≤0.01 compared to vehicle treated cells. Values are mean ± s.e.m of 3 separate experiments.

e.

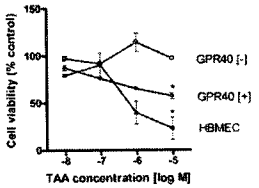

Cell viability (% control)
150
100
50
0
-8
-7
-6
-5
TAA concentration [log M]
GPR40 [-]
GPR40 [+]
HBMEC

Figure 4

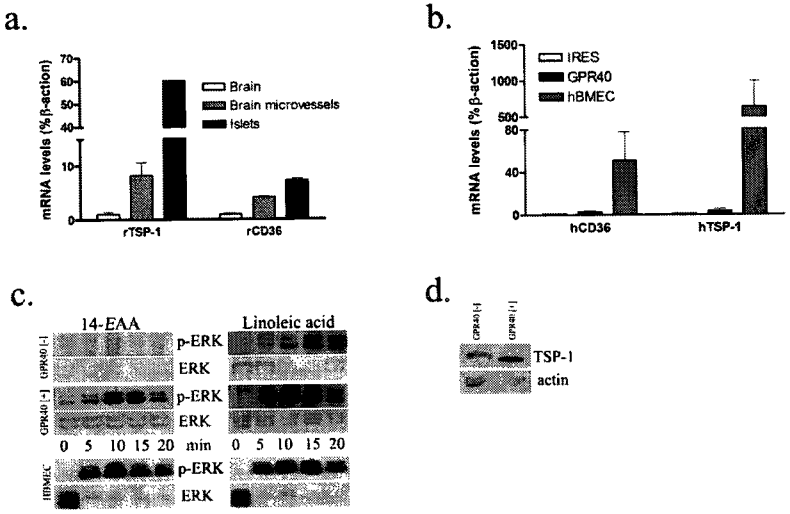

a.
mRNA levels (% β-action)
Brain
Brain microvessels
Islets
rTSP-1
rCD36
b.
mRNA levels (% β-action)
IRES
GPR40
hBMEC
hCD36
hTSP-1
c.
14-EAA
Linoleic acid
p-ERK
ERK
0 5 10 15 20 min
d.
TSP-1
actin

Figure 4

*T*AA-induced cell death is GPR40-dependent. (a) mRNA expression of CD36 and TSP-1 corrected for β-actin , in rat whole brain lysate (brain), rat cerebral microvessels and rat pancreatic islets (islet). (b) mRNA expression of CD36 and TSP-1 corrected for β-actin, in GPR40 [-], GPR40 [+] and HBME cells. (c) Representative western blots of ERK1/2 phosphorylation in GPR40 [-], GPR40 [+] and HBME cells exposed to 14*E*-AA and LA. (d) Representative western blot of TSP-1 expression in GPR40 [-] and GPR40 [+] cells exposed to 14*E*-AA ($5\ 10^{-6}$M) for 21 hours. Immunoblots are representative of three independent experiments. (e) Effect of 14*E*-AA on cell viability of GPR40 [-], GPR40 [+] and HBME cells. Values are mean ± s.e.m of 3 separate experiments.

Figure 5

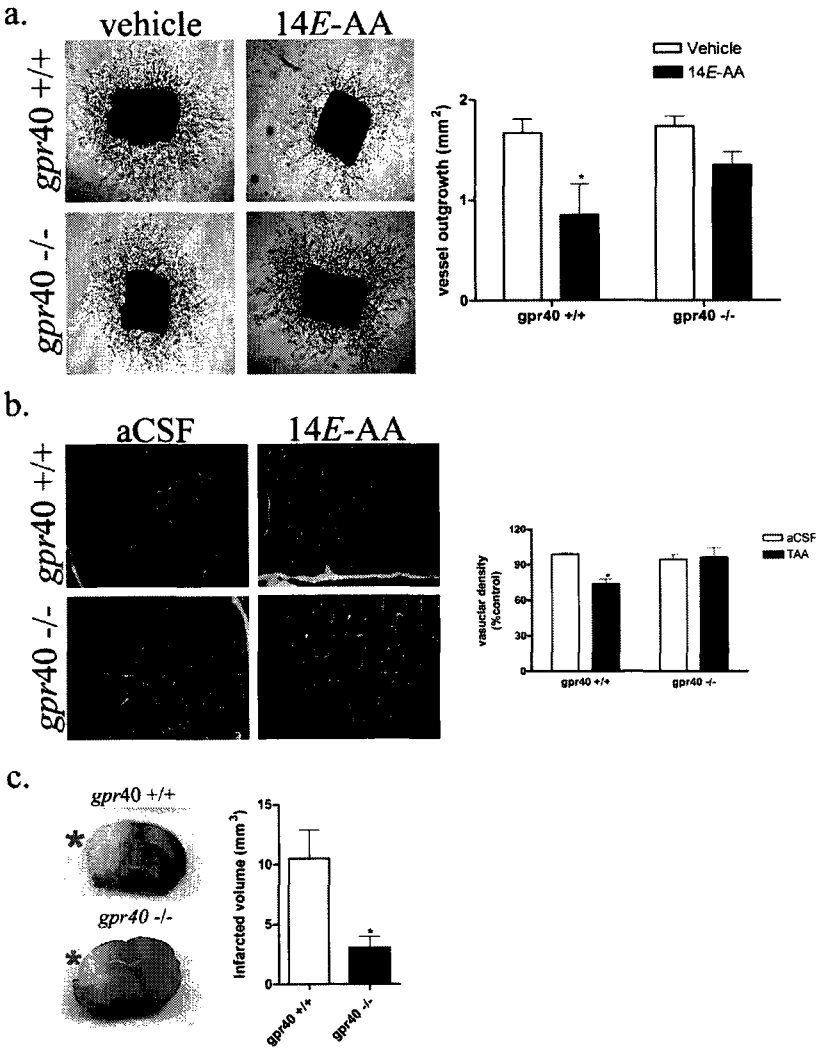

a.
vehicle
14E-AA
gpr40 +/+
gpr40 -/-
Vehicle
14E-AA
vessel outgrowth (mm2)
gpr40 +/+
gpr40 -/-
b.
aCSF
14E-AA
gpr40 +/+
gpr40 -/-
aCSF
TAA
vascular density (%control)
gpr40 +/+
gpr40 -/-
c.
gpr40 +/+
gpr40 -/-
Infarcted volume (mm3)
gpr40 +/+
gpr40 -/-

Figure 5

*T*AA-induced vasoobliteration and HI-induced brain damage in mice are GPR40 dependent. (a) Effect of ICV injections of vehicle or 14*E*-AA (510^{-5}M) on wild type and *gpr40-/-* cerebral vascular density, marked with lectin (green). Values in histogram are mean ± s.e.m of 4 independent experiments. *P≤0.01 compared to vehicle injected wild type mice. (b) Representative microvascular sprouting from matrigel-embedded aortic rings from wild type and *gpr40-/-* mice exposed to vehicle or 14*E*-AA (510^{-6}M, left panel). Values in histogram are mean ± s.e.m of 6 separate experiments. *P≤0.01 compared to vehicle wild type treated aortic rings. (c) TTC staining in *gpr40-/-* and *gpr40+/+* mice subjected to HI. Infarcted volume was assessed as described in materials and methods. Values in histogram are mean ± s.e.m of 3-7 separate experiments. *P≤0.01 compared to *gpr40+/+* infarct.

CHAPTER 5: DISCUSSION, CONCLUSION AND PERSPECTIVES

5.1 DISCUSSION

Cerebral ischemia is characterized by a reduction in CBF initiating a number of molecular and enzymatic cascades leading to disrupted brain function (Siesjo, Kristian et al. 1998). The most dramatic outcome of cerebral ischemia consists of neuromicrovascular injury (del Zoppo 1997; del Zoppo et al. 1997). Although most studies have extensively reported on neuronal damage and neuronal cell death following HI injuries, only a few have focused on examining the cerebral microvasculature. Several mechanisms involved in microvascular damage, BBB leakage, and endothelial cell death were proposed with great emphasis on increased ROS/RNS and lipid peroxidation products (Siesjo and Wieloch 1983; Traystman et al. 1991; Choi et al. 1998; Janardhan et al. 2004). However, the exact mediators of oxidative and nitrative stresses involved in neuromicrovascular damage are not well defined. Moreover, the mechanisms by which these mediators participate in microvascular injury are not fully characterized. We hypothesized that the newly described *trans*-arachidonic acids could be direct mediators of nitrative stress causing cerebral microvascular injury and brain damage, since they are largely produced in other ischemic pathologies (Kermorvant-Duchemin, Sennlaub et al. 2005; Checchin, Sennlaub et al. 2006). Data represented in this thesis support this hypothesis and demonstrate that:

1) *T*AA act as mediators of nitrative stress in a hypoxic-ischemic model of cerebral ischemia. *T*AA levels are increased upon HI as early as 3h post injury and peak at 18 hours post-injury. *T*AA concentration levels reach

the micromolar range which consists of a very high concentration for damageable lipid peroxidation products.

2) Early in the injury, *T*AA induce a concentration dependent vasorelaxation of brain pial microvessels. This vasorelaxation occurs within 15 minutes of *T*AA exposure but is independent of all classical vasodilation pathways. *T*AA-induced vasorelaxation is rather dependent on heme oxygenase-2 and carbon monoxide production whereas heme oxygenase-1 is not involved. Heme oxygenase-2 activation by *T*AA requires opening of large conductance calcium-dependent potassium channels. This vasorelaxation could entirely be prevented when using siRNA against heme oxygenase-2 only, not affecting heme oxygenase-1 expression.

3) *T*AA-mediated vasorelaxation could participate in enhancing lipid peroxidation and ROS/RNS generation due to the continuous formation of *T*AA at 18 hours post-onset of the injury. *T*AA formation coincides with increased nitrative stress within cerebral microvessels in hypoxic-ischemic brains. Ultimately, *T*AA lead to microvascular degeneration and brain damage. Inhibition of NOS isoforms using l-name, which also inhibit *T*AA formation, partially prevents microvascular degeneration and completely reduces brain infarct induced by HI.

4) *T*AA-mediated microvascular degeneration involves a very specific pathway requiring the upregulation of the anti-angiogenic factor thrombospondin-1 (TSP-1). TSP-1 overexpression is dependent on the

transient activation of ERK1/2 and lead specifically to microvascular endothelial cell death. Inhibition of MAPK phosphorylation or blockade of TSP-1 binding to its receptor CD36 fully prevents *T*AA-induced microvascular degeneration.

5) TSP-1 overexpression and ensued endothelial cell death implicate the activation of GPR40 receptor. *T*AA bind specifically to GPR40 and its activation leads to transient phosphorylation of ERK1/2 and TSP-1 upregulation in cells overexpressing the receptor GPR40 and endothelial cells but not in cells devoid of GPR40 receptor. *T*AA also induce a GPR40-dependent cell death.

6) GPR40 receptor is expressed within rat brain microvessels. *T*AA fail to induce microvascular degeneration in GPR40-deficient mice, conferring a role for GPR40 in angiogenesis. More importantly, GPR40-deficient mice showed more resistance to HI-induced brain damage than their wild type littermates.

5.1.1 Treatment limitations in HI injury

Despite the large number of clinical trials and experimental interventions that lower brain damage, a number of them have failed. A few suggestions could explain such discrepancies between bench and bedside studies:

a) Timing

While in experimental animal models the onset of ischemia and reperfusion can be precisely determined, in humans this is not always possible. Therefore, it is difficult to accurately define the time window in which a certain drug is effective for each patient (1992). Treatment strategies are needed to prevent post-ischemic injuries.

b) Age and associated illness

Experimental studies are mostly conducted on healthy, young animals under rigorously controlled laboratory conditions. The typical patient, however, is either older or younger and could present other complications such as diabetes, hypertension or heart diseases (Dirnagl, Iadecola et al. 1999).

c) Functional differences between human brain and animal brains

The basic biology and biochemical processes of cerebral ischemia are similar between different species. Nonetheless, we cannot ignore the interspecies difference in brain structure, function and vascular anatomy. For example, in rodents, glucose and oxygen metabolism is three times higher than in humans. Moreover, neuronal and glial densities vary between mammals. The human brain is more gyrated and present a different vascular anatomy (Luginbuhl 1973). Some rodents, such as gerbils, do not have a complete circle of Willis, while others, such as rats, could have more effective collaterals between large vessels. On another

note, the effect of neuroprotective drugs has been shown to be species dependent (Dirnagl, Iadecola et al. 1999).

d) Drug side effects

Anti-excitotoxic drugs display several psychomimetic and cardiovascular side effects (Prass et al. 1998; Lee et al. 2000; Volbracht et al. 2006). This limits the tolerated dose such that in humans, only a fifth is reached compared to rodents. Hence, it is important to design safer drugs with more favourable pharmacokinetics and fewer side effects.

5.1.2 Role of nitrative stress in HI injuries.

The crucial role of nitrative stress and their mediators in the pathogenesis of ischemic pathologies was emphasised in this thesis with the help of *T*AA. While numerous molecules have been identified to contribute to nitrative-stress induced HI damage, the blockade of their formation or site of action was ineffective in humans (De Keyser et al. 1999). Most free radical scavengers and lipid peroxidation inhibitors were very effective against brain damage for up to two weeks of treatment. No improvement of functional outcome and no reduction in mortality were observed after 3 months (1996; Grotta 1997; Diener 1998; Yamaguchi et al. 1998) in patients with cerebral ischemia. This was the case for tirilazad (lipid peroxidation inhibitor), lubeluzole (NO pathway modulator) and Ebselen (free radical scavenger). Unfortunately, the reasons for such inconsistencies between pre-clinical and clinical data are not yet understood. The difficulty to achieve favourable outcome using lipid peroxidation inhibitors or

free radical scavengers is mostly due to our lack of knowledge in the area of free radical-mediated processes. Based on this precedence, significant efforts in elucidating the underlying molecular mechanisms of hypoxic-ischemic brain injuries may lead to new treatments following the lead of nitrative stress inhibition.

Data presented in this thesis shed the light on novel mediators of nitrative stress; *T*AA and their mode of action. Direct detection of reactive oxygen and nitrogen species has always presented a major technical challenge which makes it difficult to quantitatively measure these species *in vivo*, in addition to their rapid reactivity and short lifespan (Freitas et al. 2002; Kohen et al. 2002). On the contrary, *T*AA are more stable molecules that can be measured in various tissues including retina (Kermorvant-Duchemin, Sennlaub et al. 2005; Checchin, Sennlaub et al. 2006) and brain and can also be found in plasma and biological fluids. Formation of *T*AA is completely dependent on NOS activation; such that inhibition of NOS isoforms prevents *T*AA-induced cytotoxic effects. Moreover, *T*AA activate two specific yet different metabolic pathways namely BK_{Ca}/HO-2 and GPR40/TSP-1 that are associated with brain damage. Thus, *T*AA present a reliable quantitative indication on the extent of nitrative-stress injury. Furthermore, the identification and dissection of these molecular mechanisms by which *T*AA act, allow us to define novel targets to treat HI injury.

5.1.3 Pivotal role of *T*AA.

Data presented in this thesis have unveiled two distinct yet specific mechanisms by which *T*AA mediate their effects in the cerebral vasculature. The first pathway

implicated the activation of the BK_{Ca}/HO-2 complex, through which, *T*AA mediate vasorelaxation of cerebral microvessels and hence regulate the vascular tonus. The second pathway involved the activation of GPR40 receptor and ensued TSP-1 upregulation and consequenced endothelial cell death; implicated in cerebral microvascular degeneration and brain damage. These data suggest a pivotal role for *T*AA and bring one particular question: Are *T*AA friend or foe? This same question was before put with regard to eicosanoids, nitric oxide and carbon monoxide which act as both. Accordingly, eicosanoids possess multifaceted roles in prominent areas of research including inflammation (FitzGerald et al. 2001; Rocha et al. 2001), vasodilation (Levy et al. 2001; Tilley et al. 2001), fever (Ushikubi et al. 1998; Sugimoto et al. 2000) and pain (Ushikubi, Segi et al. 1998; Narumiya et al. 2001) which specific mechanisms are still heavily investigated. On the other hand, both nitric oxide and carbon monoxide were long thought to be extremely poisonous gaseous molecules that mediate cytotoxic effects when inhaled (Buckley et al. 2005; Vanin et al. 2007). Subsequent to these widely accepted detrimental statements, controversial data have demonstrated the total opposite. In fact, both gases are produced *in vivo* and small amounts of each are involved in various physiological processes, which do not mediate toxicity, including vasorelaxation, neurotransmission, penile erection, memory, hypertension, transplantation and more (Xu et al. 1998; Mariotto et al. 2004; Mannaioni et al. 2006; Nakao et al. 2006; Mann et al. 2007). On this similar note, *T*AA measurements *in vivo* present very low levels which correspond to 10^{-11}M to 10^{-9}M in the control animal groups. Theses concentrations, though considered low and physiological, are associated with a biological function when

tested on pial brain microvessels. In fact, low levels of *T*AA mediate a significant vasorelaxation of cerebral microvessels compared to basal vascular tonus but are ineffective in inducing cell death. Increasing concentrations of *T*AA, however, mediate a stronger vasorelaxation. Longer exposure of endothelial cells with high concentrations of *T*AA implicates the activation of GPR40 and subsequent cell death. Thus, the pivotal role of *T*AA, like NO or CO, appear to be dependent on the concentration. Further in-depth research is required to fully appreciate and understand these provocative data.

It is also noteworthy to mention that these data provide additional evidence to the important vasodilatory role of CO in the brain. Although it is already reported, many could argue the affinity of CO with soluble guanylate cyclase as very weak compared to NO's affinity. Data presented in this thesis exclude the implication of NO or NO products and demonstrate clearly that CO is highly generated upon *T*AA-mediated activation of heme oxygenase-2. These CO levels are sufficient to mediate a strong vasodilation of pial microvessels dependent on the activation of soluble guanylate cyclase.

In order to exclude or include the possibility that both pathways, activated by *T*AA, might be related; a simple experiment is ought to be performed. It is important to test the vasomotor effects of *T*AA on GPR40-deficient mice which will allow us to understand if BK_{Ca}/HO-2 activation is dependent on *T*AA binding to GPR40 receptor. How *T*AA activate BK_{Ca} channels is still unclear. Some hypotheses arose but haven't been investigated yet. *T*AA binding to GPR40 will elicit a calcium influx, may be sufficient to activate BK_{Ca} channels. However, due

to the low excitability of endothelial cells, we were unable to register any calcium mobilization in two different microvascular endothelial cells: human dermal microvascular endothelial cells (HMEC-1) and porcine cerebral microvascular endothelial cells. The alternative hypothesis is that due to the rigidity of *T*AA structure and the increased content of *T*AA within or across the lipid bilayer, membrane fluidity could be altered which may activate the potassium channels as previously reported for arachidonic acid and other free fatty acids (Ordway et al. 1989; Ordway et al. 1991; Kirber et al. 1992; Meves 1994; Zou et al. 1996).

5.1.4 GPR40, a receptor for *T*AA.

Previously published data on the elucidation of *T*AA-induced cytotoxic effects on endothelial cells strongly suggested that *T*AA act on a cell surface receptor. The current data have identified this receptor and demonstrated its mode of action. This particular finding is exceptionally important as it presents irrefutable evidences that a receptor is assigned for non-enzymatically produced eicosanoids for the first time. Many researchers have struggled to identify a precise receptor for isoprostanes, also generated non-enzymatically. Although most of the molecular mechanisms have already been discovered for each isoprostane, the primary target of each remains unknown. All four isomers of *T*AA however, appear to mediate the same effect with a slightly different efficacy. All isomers of *T*AA mediate a vasorelaxation of brain pial microvessels and mediate endothelial cell toxicity. These findings suggest that all isomers of *T*AA may act on the same receptor.

The detection of GPR40 receptor mRNA within rodent brain microvessels is also a first. GPR40 was only shown to be present at the mRNA and protein level in the human and primate brains. Commercially available antibodies against GPR40 receptor were unfortunately not specific when tested in our models and in the pancreas. Newly developed antibodies however used by the same group demonstrate the presence of GPR40 in almost all brain structures in primates (Brown, Jupe et al. 2005; Ma, Tao et al. 2007; Hirasawa, Itsubo et al. 2008; Ma, Lu et al. 2008; Yamashima 2008); including the microvasculature. This antibody is not commercially available yet and therefore, we were not able to test it. The novelty of our data reside in the fact that not only GPR40 mRNA was detected in the micrvoasculature of rodents but also that it possesses an anti-angiogenic function which has not yet been described. Most importantly, GPR40 appear to be a key mediator of brain damage. Deficiency in GPR40 limits the extent of brain damage induced by HI. What was previously thought as two independent mechanisms, namely microvascular degeneration and brain infarct in HI injuries, appear here to be strongly linked via GPR40 and its activation by *T*AA.

Most studies on GPR40 have focused on its role in insulin regulation and β-cell function and its profile expression in both pancreas and brain. Our study remains the first that identified a new function to GPR40 in the brain and elucidated a mechanism for GPR40 anti-angiogenic effect. It is also noteworthy to notice that GPR40 is not only activated by *T*AA but also by an array of free fatty acids including linoleic acid, oleic acid, docosahexanoic acid and arachidonic acid. These molecules however are not known to have any direct anti-angiogenic or

toxic effects in the cerebral microvasculature (Bourre, Bonneil et al. 1992; Bourre, Bonneil et al. 1993; Abbott 2000; Lauritzen et al. 2001; Bazan 2006) which suggests that GPR40 anti-angiogenic activity is dependent on its activation by *T*AA. Hence, these findings may also suggest that GPR40 activation by DHA (or any other ω-3 fatty acid) may elicit a rather protective effect in the brain. It is still unknown whether GPR40 may present detrimental or protective effects in the brain depending on its ligands. This particular query could also be extrapolated to the pancreatic tissue where *T*AA is yet to be tested. Further investigation is needed to examine these hypothesises.

Unfortunately, we are unable to conclude that GPR40 is the only receptor for *T*AA. GPR40 deficiency is not entirely sufficient in inhibiting *T*AA-induced microvascular degeneration in our aortic ring model. This result could be explained by: (1) A conundrum compensation or mechanistic adaptation for the missing receptor that might occur in the GPR40 deficient mice; which may elicit *T*AA-mediated cytotoxic effects (2) The participation of a second receptor activated by *T*AA. In fact, GPR120 presents an attractive target which is currently under study.

Another implicit finding of this work is the capital role of slight modifications in the geometrical configuration of a molecule. *Trans*-arachidonic acids differ from their *cis*-isomer by only one double bond. This one double bond difference makes *T*AA act on different targets, activate distinct molecular pathways and modulate the cell signalling outcomes unsimilarly than AA. Therefore, it is fascinating to be

able to appreciate the complexity of such a subtle yet fate determining molecular modification.

5.2 CONCLUSION

It is widely accepted and acknowledged that ROS/RNS and lipid peroxidation products are major contributors to the pathogenesis of hypoxic-ischemic injuries. Despite the tremendous efforts in drug development which interfere with the different biochemical processes involved, most clinical trials have failed to demonstrate efficacy. Data presented in this thesis have identified novel mediators of nitrative stress that are highly generated during HI, named *T*AA. This work demonstrates that these lipid peroxidation products possess two major effects on cerebral microvasculature. Their acute effects consist of a strong vasorelaxation dependent on the activation of the complex BK_{Ca}/HO-2 which lead to carbon monoxide production and hence vasorelaxation. On the other hand, *T*AA chronic effects present rather deleterious effects on the cerebral microvasculature where they upregulate the most damaging anti-angiogenic factor; thrombospondin-1. In addition, this work constitutes the very first reference for the involvement of GPR40 receptor in microvascular rarefaction and modulation of brain damage. These data demonstrate that GPR40 is a receptor for *T*AA mediating TSP-1 overexpression, subsequent endothelial cell death and microvascular degeneration. Hence, the current work has identified one major player, *T*AA, suspected to actively partake in the microvascular degeneration induced by HI by, first, inducing a vasorelaxation which enhances the lipid peroxidation process and

second, binding to GPR40 receptor and activating a specific molecular cascade involved in endothelial cell death and microvascular obliteration.

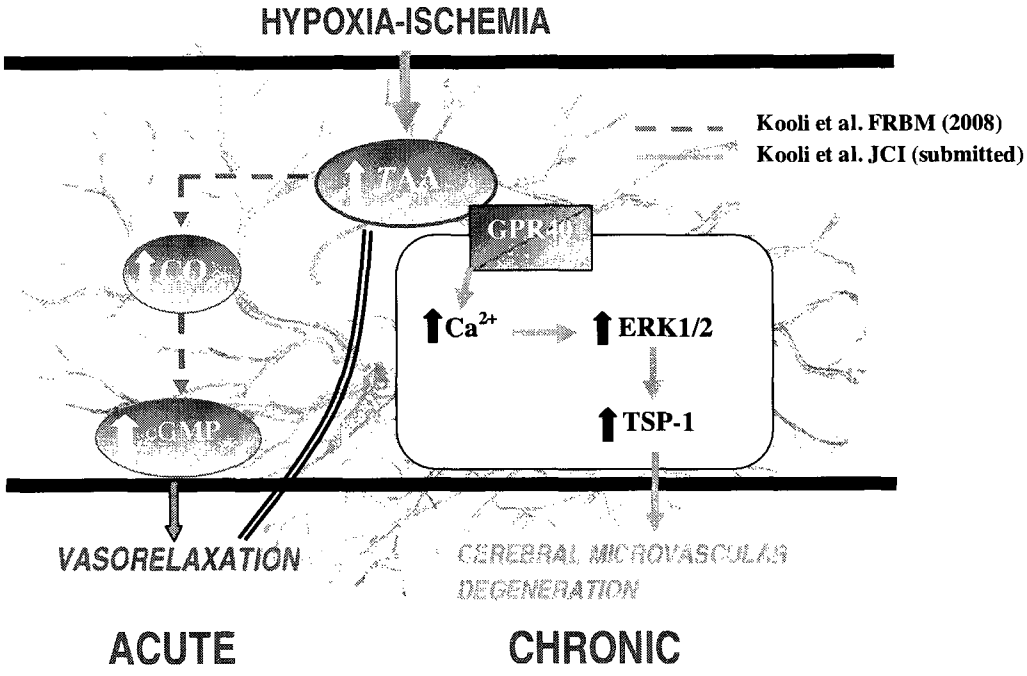


Figure 1| Schematic overview of data presented. Under hypoxic-ischemic injury, increased free radical generation causes peroxidation of polyunsaturated fatty acids leading to the formation of *T*AA. Released *T*AA will then act on two distinctive pathways. Acutely, *T*AA are responsible for CO production and subsequent cGMP release which will exert a strong vasorelaxation (dashed arrows) on cerebral microvessels. This vasorelaxation might be responsible for further lipid peroxidation, due to a strong reoxygenation, therefore leading to increased *T*AA production. When *T*AA reach micromolar concentrations *in vivo*, they bind and activate GPR40 receptor, present on cerebral microvessels, initiating a molecular cascade which leads to TSP-1 overexpression. Ultimately, endothelial cell death ensues resulting in a cerebral microvascular rarefaction and brain damage (lines).

5.3 PERSPECTIVES

There are no effective treatments available for hypoxic-ischemic injuries beside tPA administration which has a very limited therapeutic window. As mentioned earlier, most developed drugs which showed promising results in pre-clinical studies, have failed when tested in stroke patients. The current findings identified novel therapeutic targets which limit two major consequences of HI insult: microvascular degeneration and brain damage. Having shown that activation of GPR40 by *T*AA induces blood vessel regression leading to an ischemic brain infarction, a possible avenue of research would be to identify possible antagonists or allosteric modulators that could inhibit GPR40-induced signalling cascade. One could argue the necessity to antagonize GPR40 due to its role in insulin regulation which opens the doors to an enticing debate whether to develop antagonists or agonists to GPR40 receptor. Accordingly, an additional query that could be explored is the blockade of TSP-1 or CD36 as they constitute the only complex by which *T*AA mediate cerebral microvascular damage.

A further potentially appealing area of research concerns the role of *T*AA/GPR40 in inducing vascular damage in ischemic pathologies. Previous work on *T*AA's effect in the ischemic retina (Kermorvant-Duchemin, Sennlaub et al. 2005) and continued studies on GPR40-deficient mice may shed valuable insight into the precise contribution of *T*AA/GPR40 including characterization of in-depth mechanisms involved in these processes. Because the BBB leakage constitutes one major trigger of brain oedema and haemorrhage transformation (Fagan, Hess et al. 2004), future research could also address whether *T*AA/GPR40 alters

expression of endothelial tight junctions or promote the extravasation of inflammatory cells into the neuropil.

Vascular growth and vascular degeneration are hallmarks of various injuries including cancer, neurodegenerative diseases and ischemic pathologies. Therefore, it would be of great interest to evaluate the expression profile of GPR40 in vascular tissues and determine whether it is able to regulate or play a role in any of these processes.

CHAPTER 6: REFERENCES

(1992). "Clinical trial of nimodipine in acute ischemic stroke. The American Nimodipine Study Group." Stroke **23**(1): 3-8.

(1994). "Safety study of tirilazad mesylate in patients with acute ischemic stroke (STIPAS)." Stroke **25**(2): 418-23.

(1995). "Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group." N Engl J Med **333**(24): 1581-7.

(1996). "A randomized trial of tirilazad mesylate in patients with acute stroke (RANTTAS). The RANTTAS Investigators." Stroke **27**(9): 1453-8.

(1999). "Recommendations for standards regarding preclinical neuroprotective and restorative drug development." Stroke **30**(12): 2752-8.

(2000). "Tirilazad mesylate in acute ischemic stroke: A systematic review. Tirilazad International Steering Committee." Stroke **31**(9): 2257-65.

(2002). "Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest." N Engl J Med **346**(8): 549-56.

Abbott, N. J. (2000). "Inflammatory mediators and modulation of blood-brain barrier permeability." Cell Mol Neurobiol **20**(2): 131-47.

Abbott, N. J. (2002). "Astrocyte-endothelial interactions and blood-brain barrier permeability." J Anat **200**(6): 629-38.

Abbott, N. J. (2005). "Dynamics of CNS barriers: evolution, differentiation, and modulation." Cell Mol Neurobiol **25**(1): 5-23.

Abraham, D., et al. (2007). "How does endothelial cell injury start? The role of endothelin in systemic sclerosis." Arthritis Res Ther **9 Suppl 2**: S2.

Abumiya, T., et al. (1999). "Activated microvessels express vascular endothelial growth factor and integrin alpha(v)beta3 during focal cerebral ischemia." J Cereb Blood Flow Metab **19**(9): 1038-50.

Acworth, I. N., et al. (1997). Reactive oxygen species. The handbook of oxidative metabolism. Massachussetts, ESA Inc.: pp 1-1 to 4-4.

Adibhatla, R. M., et al. (2002). "Citicoline mechanisms and clinical efficacy in cerebral ischemia." J Neurosci Res **70**(2): 133-9.

Adibhatla, R. M., et al. (2002). "Citicoline: neuroprotective mechanisms in cerebral ischemia." J Neurochem **80**(1): 12-23.

Alexander, R. L., et al. (2006). "Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPARgamma-dependent transcription activation." Biochemistry **45**(25): 7889-96.

Alexandrova, M. L., et al. (2005). "Oxidative stress during the chronic phase after stroke." Free Radic Biol Med **39**(3): 297-316.

Allt, G., et al. (2001). "Pericytes: cell biology and pathology." Cells Tissues Organs **169**(1): 1-11.

Alonso-Galicia, M., et al. (1999). "Contribution of 20-HETE to vasodilator actions of nitric oxide in the cerebral microcirculation." Stroke **30**(12): 2727-34; discussion 2734.

Aly, H., et al. (2006). "IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy." Brain Dev **28**(3): 178-82.

Andersen, M., et al. (1999). "Effects of citicoline combined with thrombolytic therapy in a rat embolic stroke model." Stroke **30**(7): 1464-71.

Andresen, J., et al. (2006). "Endothelial influences on cerebrovascular tone." J Appl Physiol **100**(1): 318-27.

Appleton, S. D., et al. (1999). "Selective inhibition of heme oxygenase, without inhibition of nitric oxide synthase or soluble guanylyl cyclase, by metalloporphyrins at low concentrations." Drug Metab Dispos **27**(10): 1214-9.

Arai, T., et al. (1998). "Oxypurinol, a xanthine oxidase inhibitor and a superoxide scavenger, did not attenuate ischemic neuronal damage in gerbils." Life Sci **63**(7): PL 107-12.

Armstrong, L. C., et al. (2003). "Thrombospondins 1 and 2 function as inhibitors of angiogenesis." Matrix Biol **22**(1): 63-71.

Aro, A. (1998). Epidemiological of trans fatty acids and coronary heart disease. Trans Fatty Acids in Human Nutrition. J. L. Sébédio and W. W. e. Christie. Dundee, Scotland, The Oily Press: pp 235-260.

Asahi, M., et al. (2000). "Reduction of tissue plasminogen activator-induced hemorrhage and brain injury by free radical spin trapping after embolic focal cerebral ischemia in rats." J Cereb Blood Flow Metab **20**(3): 452-7.

Aschner, M., et al. (2002). "Astrocyte modulation of neurotoxic injury." Brain Pathol **12**(4): 475-81.

Attwell, D., et al. (2001). "An energy budget for signaling in the grey matter of the brain." J Cereb Blood Flow Metab **21**(10): 1133-45.

Atwood, C. S., et al. (2003). "Cerebrovascular requirement for sealant, anti-coagulant and remodeling molecules that allow for the maintenance of

vascular integrity and blood supply." Brain Res Brain Res Rev **43**(1): 164-78.

Audoly, L. P., et al. (2000). "Cardiovascular responses to the isoprostanes iPF(2alpha)-III and iPE(2)-III are mediated via the thromboxane A(2) receptor in vivo." Circulation **101**(24): 2833-40.

Audus, K. L., et al. (1991). "Evidence for 21-aminosteroid association with the hydrophobic domains of brain microvessel endothelial cells." Free Radic Biol Med **11**(4): 361-71.

Babior, B. M. (1984). "Oxidants from phagocytes: agents of defense and destruction." Blood **64**(5): 959-66.

Back, S. A., et al. (2004). "Emerging concepts in periventricular white matter injury." Semin Perinatol **28**(6): 405-14.

Back, T., et al. (1996). "Induction of spreading depression in the ischemic hemisphere following experimental middle cerebral artery occlusion: effect on infarct morphology." J Cereb Blood Flow Metab **16**(2): 202-13.

Back, T., et al. (2003). "Combined MR measurements of magnetization transfer, tissue diffusion and proton spectroscopy. A feasibility study with neurological cases." Neurol Res **25**(3): 292-300.

Baker, K., et al. (1998). "Synthetic combined superoxide dismutase/catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury." J Pharmacol Exp Ther **284**(1): 215-21.

Baker, P. R., et al. (2005). "Fatty acid transduction of nitric oxide signaling: multiple nitrated unsaturated fatty acid derivatives exist in human blood

and urine and serve as endogenous peroxisome proliferator-activated receptor ligands." J Biol Chem **280**(51): 42464-75.

Balafanova, Z., et al. (2002). "Nitric oxide (NO) induces nitration of protein kinase Cepsilon (PKCepsilon), facilitating PKCepsilon translocation via enhanced PKCepsilon -RACK2 interactions: a novel mechanism of no-triggered activation of PKCepsilon." J Biol Chem **277**(17): 15021-7.

Balazy, M. (2000). "Trans-arachidonic acids: new mediators of inflammation." J Physiol Pharmacol **51**(4 Pt 1): 597-607.

Balazy, M., et al. (2001). "Vicinal nitrohydroxyeicosatrienoic acids: vasodilator lipids formed by reaction of nitrogen dioxide with arachidonic acid." J Pharmacol Exp Ther **299**(2): 611-9.

Balazy, M., et al. (2004). "Biological nitration of arachidonic acid." Curr Vasc Pharmacol **2**(1): 81-93.

Bannister, J. V., et al. (1987). "Aspects of the structure, function, and applications of superoxide dismutase." CRC Crit Rev Biochem **22**(2): 111-80.

Bar, T. (1978). morphometric evaluation. Advances in Neurology. New York, Raven Press: pp 1-9.

Bar, T. (1980). "The vascular system of the cerebral cortex." Adv Anat Embryol Cell Biol **59**: I-VI,1-62.

Bar, T. (1983). "Patterns of vascularization in the developing cerebral cortex." Ciba Found Symp **100**: 20-36.

Barone, F. C., et al. (1999). "Inflammatory mediators and stroke: new opportunities for novel therapeutics." J Cereb Blood Flow Metab **19**(8): 819-34.

Barres, B. A., et al. (1993). "Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons." Nature **361**(6409): 258-60.

Barres, B. A., et al. (1994). "Control of oligodendrocyte number in the developing rat optic nerve." Neuron **12**(5): 935-42.

Barth, A., et al. (2000). "Silent cerebral ischemia detected by diffusion-weighted MRI after carotid endarterectomy." Stroke **31**(8): 1824-8.

Bastide, M., et al. (2007). "Neurogliovascular unit after cerebral ischemia: is the vascular wall a pharmacological target." Psychoneuroendocrinology **32 Suppl 1**: S36-9.

Batinic-Haberle, I. (2002). "Manganese porphyrins and related compounds as mimics of superoxide dismutase." Methods Enzymol **349**: 223-33.

Batthyany, C., et al. (2006). "Reversible post-translational modification of proteins by nitrated fatty acids in vivo." J Biol Chem **281**(29): 20450-63.

Bayliss, W. M. (1902). "On the local reactions of the arterial wall to changes of internal pressure." J Physiol **28**(3): 220-31.

Bazan, N. G. (2006). "The onset of brain injury and neurodegeneration triggers the synthesis of docosanoid neuroprotective signaling." Cell Mol Neurobiol **26**(4-6): 901-13.

Bazan, N. G., et al. (2002). "Prostaglandins and other lipid mediators in Alzheimer's disease." Prostaglandins Other Lipid Mediat **68-69**: 197-210.

Bazan, N. G., Jr., et al. (1970). "Increased levels of brain free fatty acids after electroconvulsive shock." Life Sci **9**(9): 501-7.

Bazan, N. G., et al. (2005). "Brain response to injury and neurodegeneration: endogenous neuroprotective signaling." Ann N Y Acad Sci **1053**: 137-47.

Beauchamp, M. H., et al. (2001). "Role of thromboxane in retinal microvascular degeneration in oxygen-induced retinopathy." J Appl Physiol **90**(6): 2279-88.

Beauchamp, M. H., et al. (2004). "Redox-dependent effects of nitric oxide on microvascular integrity in oxygen-induced retinopathy." Free Radic Biol Med **37**(11): 1885-94.

Becker, M. A., et al. (2004). "Febuxostat (TMX-67), a novel, non-purine, selective inhibitor of xanthine oxidase, is safe and decreases serum urate in healthy volunteers." Nucleosides Nucleotides Nucleic Acids **23**(8-9): 1111-6.

Beckman, J. S. (1990). "Ischaemic injury mediator." Nature **345**(6270): 27-8.

Beckman, J. S., et al. (1990). "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide." Proc Natl Acad Sci U S A **87**(4): 1620-4.

Beckman, J. S., et al. (1996). "Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly." Am J Physiol **271**(5 Pt 1): C1424-37.

Beetsch, J. W., et al. (1998). "Xanthine oxidase-derived superoxide causes reoxygenation injury of ischemic cerebral endothelial cells." Brain Res **786**(1-2): 89-95.

Berdeaux, A. (1993). "Nitric oxide: an ubiquitous messenger." Fundam Clin Pharmacol **7**(8): 401-11.

Bergstrom, S., et al. (1964). "The enzymatic formation of Prostaglandin E2 from arachidonic acid." Biochim Biophys Acta **90**: pp 207-210.

Bergstrom, S., et al. (1982). "Discoveries concerning prostanglandins and related biologically active substances." Nobel Prize in Physiology and Medicine, from http://nobelprize.org/nobel_prizes/medicine/laureates/1982/index.html.

Bernard, S. A., et al. (2002). "Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia." N Engl J Med **346**(8): 557-63.

Bernstein, J. J., et al. (1985). "Astrocytes secrete basal lamina after hemisection of rat spinal cord." Brain Res **327**(1-2): 135-41.

Bethea, J. R., et al. (2002). "Targeting the host inflammatory response in traumatic spinal cord injury." Curr Opin Neurol **15**(3): 355-60.

Beyers, E. C., et al. (1991). "Metabolites of cis,trans, and trans,cis isomers of linoleic acid in mice and incorporation into tissue lipids." Biochim Biophys Acta **1082**(3): 275-84.

Blaustein, M. P., et al. (1978). "The regulation of intracellular calcium in presynaptic nerve terminals." Ann N Y Acad Sci **307**: 195-212.

Bonita, R., et al. (1988). "Recovery of motor function after stroke." Stroke **19**(12): 1497-500.

Bonmann, E., et al. (1997). "The dominant role of exogenous or endogenous interleukin-1 beta on expression and activity of inducible nitric oxide synthase in rat microvascular brain endothelial cells." Neurosci Lett **230**(2): 109-12.

Bonnekoh, P., et al. (1990). "Selective vulnerability in the gerbil hippocampus: morphological changes after 5-min ischemia and long survival times." Acta Neuropathol **80**(1): 18-25.

Borchert, A., et al. (2003). "Regulation of expression of the phospholipid hydroperoxide/sperm nucleus glutathione peroxidase gene. Tissue-specific expression pattern and identification of functional cis- and trans-regulatory elements." J Biol Chem **278**(4): 2571-80.

Bourre, J. M., et al. (1992). "Structural and functional importance of dietary polyunsaturated fatty acids in the nervous system." Adv Exp Med Biol **318**: 211-29.

Bourre, J. M., et al. (1993). "Function of dietary polyunsaturated fatty acids in the nervous system." Prostaglandins Leukot Essent Fatty Acids **48**(1): 5-15.

Bowes, M. P., et al. (1995). "Monoclonal antibodies preventing leukocyte activation reduce experimental neurologic injury and enhance efficacy of thrombolytic therapy." Neurology **45**(4): 815-9.

Bramlett, H. M., et al. (2004). "Pathophysiology of cerebral ischemia and brain trauma: similarities and differences." J Cereb Blood Flow Metab **24**(2): 133-50.

Braughler, J. M., et al. (1989). "Central nervous system trauma and stroke. I. Biochemical considerations for oxygen radical formation and lipid peroxidation." Free Radic Biol Med **6**(3): 289-301.

Brault, S., et al. (2003). "Selective neuromicrovascular endothelial cell death by 8-Iso-prostaglandin F2alpha: possible role in ischemic brain injury." Stroke **34**(3): 776-82.

Brault, S., et al. (2004). "Cytotoxicity of the E(2)-isoprostane 15-E(2t)-IsoP on oligodendrocyte progenitors." Free Radic Biol Med **37**(3): 358-66.

Brawn, K., et al. (1981). "DNA strand scission by enzymically generated oxygen radicals." Arch Biochem Biophys **206**(2): 414-9.

Breen, A. P., et al. (1995). "Reactions of oxyl radicals with DNA." Free Radic Biol Med **18**(6): 1033-77.

Brigelius-Flohe, R. (1999). "Tissue-specific functions of individual glutathione peroxidases." Free Radic Biol Med **27**(9-10): 951-65.

Bringmann, A., et al. (2005). "Neuronal versus glial cell swelling in the ischaemic retina." Acta Ophthalmol Scand **83**(5): 528-38.

Briscoe, C. P., et al. (2006). "Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules." Br J Pharmacol **148**(5): 619-28.

Briscoe, C. P., et al. (2003). "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids." J Biol Chem **278**(13): 11303-11.

Brott, T., et al. (1997). Thrombolysis for acute ischemic stroke. Primer on Cerebrovascular disease. K. M. A. Welch, L. R. Caplan, D. J. Reis, B. K. Siesjo and W. Bryce. San Diego, CA, Academic Press: pp 728:731.

Brown, A. J., et al. (2003). "The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids." J Biol Chem **278**(13): 11312-9.

Brown, A. J., et al. (2005). "A family of fatty acid binding receptors." DNA Cell Biol **24**(1): 54-61.

Bruckdorfer, R. (2005). "The basics about nitric oxide." Mol Aspects Med **26**(1-2): 3-31.

Bruno, V., et al. (2001). "Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs." J Cereb Blood Flow Metab **21**(9): 1013-33.

Buckley, N. A., et al. (2005). "Hyperbaric oxygen for carbon monoxide poisoning : a systematic review and critical analysis of the evidence." Toxicol Rev **24**(2): 75-92.

Budd, S. L., et al. (2000). "Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons." Proc Natl Acad Sci U S A **97**(11): 6161-6.

Burns, C. P., et al. (2002). "Role of nitric oxide and membrane phospholipid polyunsaturation in oxidative cell death." Subcell Biochem **36**: 97-121.

Burr, G. O., et al. (1930). "the nature and role of fatty acids essential in nutrition." J Biol Chem **86**: 587-621.

Burton, G. W., et al. (1984). "beta-Carotene: an unusual type of lipid antioxidant." Science **224**(4649): 569-73.

Busch, E., et al. (1996). "Potassium-induced cortical spreading depressions during focal cerebral ischemia in rats: contribution to lesion growth assessed by diffusion-weighted NMR and biochemical imaging." J Cereb Blood Flow Metab **16**(6): 1090-9.

Busto, R., et al. (1987). "Small differences in intraischemic brain temperature critically determine the extent of ischemic neuronal injury." J Cereb Blood Flow Metab **7**(6): 729-38.

Butterfield, D. A., et al. (2001). "Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide." Trends Mol Med **7**(12): 548-54.

Caffe, A. R., et al. (2001). "Mouse retina explants after long-term culture in serum free medium." J Chem Neuroanat **22**(4): 263-73.

Calabrese, V., et al. (2007). "Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity." Nat Rev Neurosci **8**(10): 766-75.

Caplan, L. R. (2004). "Treatment of acute stroke: still struggling." Jama **292**(15): 1883-5.

Carafoli, E., et al. (1978). "The regulation of intracellular calcium by mitochondria." Ann N Y Acad Sci **307**: 269-84.

Carlson, S. E., et al. (1997). "trans Fatty acids: infant and fetal development." Am J Clin Nutr **66**(3): 715S-36S.

Cassina, A. M., et al. (2000). "Cytochrome c nitration by peroxynitrite." J Biol Chem **275**(28): 21409-15.

Catala, A. (2006). "An overview of lipid peroxidation with emphasis in outer segments of photoreceptors and the chemiluminescence assay." Int J Biochem Cell Biol **38**(9): 1482-95.

Chan, P. H. (2001). "Reactive oxygen radicals in signaling and damage in the ischemic brain." J Cereb Blood Flow Metab **21**(1): 2-14.

Chanrion, B., et al. (2007). "Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity." Proc Natl Acad Sci U S A **104**(19): 8119-24.

Chauhan, N., et al. (2003). "Lessons in experimental ischemia for clinical stroke medicine." Curr Opin Neurol **16**(1): 65-71.

Checchin, D., et al. (2006). "Hypercapnia prevents neovascularization via nitrative stress." Free Radic Biol Med **40**(3): 543-53.

Chen, J., et al. (1996). "Expression of the apoptosis-effector gene, Bax, is up-regulated in vulnerable hippocampal CA1 neurons following global ischemia." J Neurochem **67**(1): 64-71.

Chen, T. Y., et al. (2005). "Inhibition of 12- and 15-lipoxygenase activities and protection of human and tilapia low density lipoprotein oxidation by I-Tiao-Gung (Glycine tomentella)." Lipids **40**(11): 1171-7.

Chen, Z. L., et al. (1997). "Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin." Cell **91**(7): 917-25.

Cheng, J. Z., et al. (2001). "Accelerated metabolism and exclusion of 4-hydroxynonenal through induction of RLIP76 and hGST5.8 is an early adaptive response of cells to heat and oxidative stress." J Biol Chem **276**(44): 41213-23.

Choe, M., et al. (1995). "Lipid peroxidation contributes to age-related membrane rigidity." Free Radic Biol Med **18**(6): 977-84.

Choi, D. W., et al. (1998). Glutamate receptor-mediated neuronal death in the ischemic brain. Ischemic stroke: From basic mechanisms to new drug development. C. Y. Hsu. Basel, Karger. **16:** pp1-13.

Choi, D. W., et al. (1990). "The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death." Annu Rev Neurosci **13**: 171-82.

Choi, Y. B., et al. (2000). "Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation." Nat Neurosci **3**(1): 15-21.

Chopp, M., et al. (1996). "DNA damage and repair in central nervous system injury: National Institute of Neurological Disorders and Stroke Workshop Summary." Stroke **27**(3): 363-9.

Cipolla, M. J., et al. (1997). "Reperfusion decreases myogenic reactivity and alters middle cerebral artery function after focal cerebral ischemia in rats." Stroke **28**(1): 176-80.

Coeroli, L., et al. (1998). "Nitric oxide production and perivascular tyrosine nitration following focal ischemia in neonatal rat." J Neurochem **70**(6): 2516-25.

Cui, J., et al. (2000). "Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain." Faseb J **14**(7): 955-67.

Cuzzocrea, S., et al. (2001). "Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury." Pharmacol Rev **53**(1): 135-59.

Davies, K. J. (2001). "Degradation of oxidized proteins by the 20S proteasome." Biochimie **83**(3-4): 301-10.

Davies, K. J., et al. (1987). "Protein damage and degradation by oxygen radicals. II. Modification of amino acids." J Biol Chem **262**(20): 9902-7.

Davis, R. J., et al. (2004). "EP4 prostanoid receptor-mediated vasodilatation of human middle cerebral arteries." Br J Pharmacol **141**(4): 580-5.

Dawson, D. W., et al. (1997). "CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells." J Cell Biol **138**(3): 707-17.

De Keyser, J., et al. (1999). "Clinical trials with neuroprotective drugs in acute ischaemic stroke: are we doing the right thing?" Trends Neurosci **22**(12): 535-40.

de Vries, H. E., et al. (1997). "The blood-brain barrier in neuroinflammatory diseases." Pharmacol Rev **49**(2): 143-55.

Dean, R. T., et al. (1997). "Biochemistry and pathology of radical-mediated protein oxidation." Biochem J **324 (Pt 1)**: 1-18.

Defilippi, P., et al. (1992). "Modulation of extracellular matrix receptors (integrins) on human endothelial cells by cytokines." Exs **61**: 193-7.

Defilippi, P., et al. (1992). "Alpha 6.beta 1 integrin (laminin receptor) is down-regulated by tumor necrosis factor alpha and interleukin-1 beta in human endothelial cells." J Biol Chem **267**(26): 18303-7.

del Zoppo, G. J. (1997). "Microvascular responses to cerebral ischemia/inflammation." Ann N Y Acad Sci **823**: 132-47.

del Zoppo, G. J., et al. (2001). "Inflammation after stroke: is it harmful?" Arch Neurol **58**(4): 669-72.

del Zoppo, G. J., et al. (2003). "Cerebral microvessel responses to focal ischemia." J Cereb Blood Flow Metab **23**(8): 879-94.

del Zoppo, G. J., et al. (1993). "Thrombolytic intervention in acute thrombotic and embolic stroke." Z Kardiol **82 Suppl 2**: 89-104.

del Zoppo, G. J., et al. (1997). "Trends and future developments in the pharmacological treatment of acute ischaemic stroke." Drugs **54**(1): 9-38.

Demchuk, A. M., et al. (1999). "Clinical recovery from acute ischemic stroke after early reperfusion of the brain with intravenous thrombolysis." N Engl J Med **340**(11): 894-5.

Dennery, P. A., et al. (1998). "Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2." J Clin Invest **101**(5): 1001-11.

Detmar, M. (2000). "The role of VEGF and thrombospondins in skin angiogenesis." J Dermatol Sci **24 Suppl 1**: S78-84.

Devchand, P. R., et al. (1996). "The PPARalpha-leukotriene B4 pathway to inflammation control." Nature **384**(6604): 39-43.

Dewar, D., et al. (2003). "Oligodendrocytes and ischemic brain injury." J Cereb Blood Flow Metab **23**(3): 263-74.

Dickhout, J. G., et al. (2005). "Peroxynitrite causes endoplasmic reticulum stress and apoptosis in human vascular endothelium: implications in atherogenesis." Arterioscler Thromb Vasc Biol **25**(12): 2623-9.

Diener, H. C. (1998). "Multinational randomised controlled trial of lubeluzole in acute ischaemic stroke. European and Australian Lubeluzole Ischaemic Stroke Study Group." Cerebrovasc Dis **8**(3): 172-81.

Diener, H. C., et al. (2000). "Lubeluzole in acute ischemic stroke treatment: A double-blind study with an 8-hour inclusion window comparing a 10-mg daily dose of lubeluzole with placebo." Stroke **31**(11): 2543-51.

Ding, Y., et al. (2006). "Down-regulation of heme oxygenase-2 is associated with the increased expression of heme oxygenase-1 in human cell lines." Febs J **273**(23): 5333-46.

Dirnagl, U., et al. (1999). "Pathobiology of ischaemic stroke: an integrated view." Trends Neurosci **22**(9): 391-7.

Docagne, F., et al. (1999). "Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia." Faseb J **13**(11): 1315-24.

Dorrepaal, C. A., et al. (1996). "Nonprotein-bound iron in postasphyxial reperfusion injury of the newborn." Pediatrics **98**(5): 883-9.

Droge, W. (2002). "Free radicals in the physiological control of cell function." Physiol Rev **82**(1): 47-95.

Drubach, D. (2000). The brain explained. New Jersey, Prentice Hall.

Dugan, L. L., et al. (1994). "Excitotoxicity, free radicals, and cell membrane changes." Ann Neurol **35 Suppl**: S17-21.

Durukan, A., et al. (2007). "Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia." Pharmacol Biochem Behav **87**(1): 179-97.

Dzialowski, I., et al. (2007). "Asymptomatic hemorrhage after thrombolysis may not be benign: prognosis by hemorrhage type in the Canadian alteplase for stroke effectiveness study registry." Stroke **38**(1): 75-9.

Edmonds, H. L., Jr., et al. (2001). "Topiramate as a neuroprotectant in a rat model of global ischemia-induced neurodegeneration." Life Sci **69**(19): 2265-77.

Edvinsson, L. (1993). General and comparative anatomy. Cerebral blood flow and metabolism. New York, Raven Press: pp 3-39.

Edvinsson, L. (2002). Cerebral blood flow and metabolism. Philadelphia, PA, Lippincott Williams and Wilkins.

El Kossi, M. M., et al. (2000). "Oxidative stress in the context of acute cerebrovascular stroke." Stroke **31**(8): 1889-92.

Epe, B., et al. (1996). "DNA damage by peroxynitrite characterized with DNA repair enzymes." Nucleic Acids Res **24**(21): 4105-10.

Erecinska, M., et al. (1994). "Ions and energy in mammalian brain." Prog Neurobiol **43**(1): 37-71.

Espey, M. G., et al. (2002). "A chemical perspective on the interplay between NO, reactive oxygen species, and reactive nitrogen oxide species." Ann N Y Acad Sci **962**: 195-206.

Esterbauer, H., et al. (1991). "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes." Free Radic Biol Med **11**(1): 81-128.

Fagan, S. C., et al. (2004). "Targets for vascular protection after acute ischemic stroke." Stroke **35**(9): 2220-5.

Falck, J. R., et al. (2003). "Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation." Am J Physiol Heart Circ Physiol **284**(1): H337-49.

Fang, X., et al. (2006). "20-Hydroxyeicosatetraenoic acid is a potent dilator of mouse basilar artery: role of cyclooxygenase." Am J Physiol Heart Circ Physiol **291**(5): H2301-7.

Faraci, F. M. (1993). "Endothelium-derived vasoactive factors and regulation of the cerebral circulation." Neurosurgery **33**(4): 648-58; discussion 658-9.

Faraci, F. M., et al. (1998). "Regulation of the cerebral circulation: role of endothelium and potassium channels." Physiol Rev **78**(1): 53-97.

Faraci, F. M., et al. (2001). "Arachidonate dilates basilar artery by lipoxygenase-dependent mechanism and activation of K(+) channels." Am J Physiol Regul Integr Comp Physiol **281**(1): R246-53.

Farooqui, A. A., et al. (1998). "Lipid peroxides in the free radical pathophysiology of brain diseases." Cell Mol Neurobiol **18**(6): 599-608.

Fellman, V., et al. (1997). "Reperfusion injury as the mechanism of brain damage after perinatal asphyxia." Pediatr Res **41**(5): 599-606.

Fenstermacher, J., et al. (1991). "Functional variations in parenchymal microvascular systems within the brain." Magn Reson Med **19**(2): 217-20.

Ferrer-Sueta, G., et al. (2003). "Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion." J Biol Chem **278**(30): 27432-8.

Ferreri, C., et al. (2002). "Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples." Free Radic Biol Med **33**(11): 1516-26.

Ferriero, D. M. (2001). "Oxidant mechanisms in neonatal hypoxia-ischemia." Dev Neurosci **23**(3): 198-202.

Fessel, J. P., et al. (2002). "Discovery of lipid peroxidation products formed in vivo with a substituted tetrahydrofuran ring (isofurans) that are favored by increased oxygen tension." Proc Natl Acad Sci U S A **99**(26): 16713-8.

Feuerstein, G. Z., et al. (1994). "Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha." Cerebrovasc Brain Metab Rev **6**(4): 341-60.

Filley, C. M. (2005). "White matter and behavioral neurology." Ann N Y Acad Sci **1064**: 162-83.

Fiskum, G. (2000). "Mitochondrial participation in ischemic and traumatic neural cell death." J Neurotrauma **17**(10): 843-55.

FitzGerald, G. A., et al. (2001). "The coxibs, selective inhibitors of cyclooxygenase-2." N Engl J Med **345**(6): 433-42.

Flodgren, E., et al. (2007). "GPR40 is expressed in glucagon producing cells and affects glucagon secretion." Biochem Biophys Res Commun **354**(1): 240-5.

Floyd, R. A., et al. (1990). Hydroxy free radical damage to DNA. Membrane Lipid Oxidation, Vol I. C. Vigo-Pelfrey. Boca Raton, FL, CRC Press.

Floyd, R. A., et al. (1988). "Hydroxyl free radical mediated formation of 8-hydroxyguanine in isolated DNA." Arch Biochem Biophys **262**(1): 266-72.

Floyd, R. A., et al. (1989). "Methylene blue plus light mediates 8-hydroxyguanine formation in DNA." Arch Biochem Biophys **273**(1): 106-11.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nat Med **1**(1): 27-31.

Folkow, B. (1964). "Description of the Myogenic Hypothesis." Circ Res **15**: SUPPL:279-87.

Forman, B. M., et al. (1995). "15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma." Cell **83**(5): 803-12.

Foroutan, S., et al. (2005). "Moderate-to-severe ischemic conditions increase activity and phosphorylation of the cerebral microvascular endothelial cell Na+-K+-Cl- cotransporter." Am J Physiol Cell Physiol **289**(6): C1492-501.

Forster, C., et al. (1999). "Inducible nitric oxide synthase expression in human cerebral infarcts." Acta Neuropathol **97**(3): 215-20.

Forsythe, J. A., et al. (1996). "Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1." Mol Cell Biol **16**(9): 4604-13.

Franco Folino, A. (2007). "Cerebral autoregulation and syncope." Prog Cardiovasc Dis **50**(1): 49-80.

Frankel, M. R. (1997). "Thrombolytic therapy in acute ischemic stroke." N Engl J Med **336**(1): 65; author reply 66-7.

Fredriksson, R., et al. (2003). "Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives." FEBS Lett **554**(3): 381-8.

Freeman, B. A., et al. (2008). "Nitro-fatty acid formation and signaling." J Biol Chem.

Frei, B. (1994). "Reactive oxygen species and antioxidant vitamins: mechanisms of action." Am J Med **97**(3A): 5S-13S; discussion 22S-28S.

Freitas, I., et al. (2002). "In situ detection of reactive oxygen species and nitric oxide production in normal and pathological tissues: improvement by differential interference contrast." Exp Gerontol **37**(4): 591-602.

Fridovich, I. (1995). "Superoxide radical and superoxide dismutases." Annu Rev Biochem **64**: 97-112.

Frijns, C. J., et al. (2002). "Inflammatory cell adhesion molecules in ischemic cerebrovascular disease." Stroke **33**(8): 2115-22.

Fujimura, M., et al. (1999). "Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice." J Neurosci **19**(9): 3414-22.

Fukui, S., et al. (2002). "Post-ischemic transcriptional and translational responses of EC-SOD in mouse brain and serum." Free Radic Biol Med **32**(3): 289-98.

Fukunaga, M., et al. (1993). "Vascular smooth muscle actions and receptor interactions of 8-iso-prostaglandin E2, an E2-isoprostane." Biochem Biophys Res Commun **195**(2): 507-15.

Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." Science **294**(5548): 1871-5.

Furlan, A., et al. (1999). "Intra-arterial prourokinase for acute ischemic stroke. The PROACT II study: a randomized controlled trial. Prolyse in Acute Cerebral Thromboembolism." Jama **282**(21): 2003-11.

Furukawa, K., et al. (1997). "The actin-severing protein gelsolin modulates calcium channel and NMDA receptor activities and vulnerability to excitotoxicity in hippocampal neurons." J Neurosci **17**(21): 8178-86.

Garcia, J. F., et al. (1971). "Dissociation of erythropoietin from erythropoietin-antierythropoietin complex." Proc Soc Exp Biol Med **138**(1): 213-5.

Garcia, J. H., et al. (1995). "Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex." Stroke **26**(4): 636-42; discussion 643.

Garcia, J. H., et al. (1993). "Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat." Am J Pathol **142**(2): 623-35.

Garrido, D. M., et al. (2006). "Synthesis and activity of small molecule GPR40 agonists." Bioorg Med Chem Lett **16**(7): 1840-5.

Ge, S., et al. (2005). "Where is the blood-brain barrier ... really?" J Neurosci Res **79**(4): 421-7.

George, C. R. (2003). "The cellular history of the glomerulus." J Nephrol **16**(6): 949-57.

Giasson, B. I., et al. (2002). "The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases." Free Radic Biol Med **32**(12): 1264-75.

Giffard, R. G., et al. (2005). "Ischemia-induced programmed cell death in astrocytes." Glia **50**(4): 299-306.

Giulian, D., et al. (1996). "Activated microglia are the principal glial source of thromboxane in the central nervous system." Neurochem Int **29**(1): 65-76.

Giustarini, D., et al. (2004). "S-glutathionylation: from redox regulation of protein functions to human diseases." J Cell Mol Med **8**(2): 201-12.

Globus, M. Y., et al. (1995). "Detection of free radical activity during transient global ischemia and recirculation: effects of intraischemic brain temperature modulation." J Neurochem **65**(3): 1250-6.

Goadsby, P. J., et al. (2000). "Glutamatergic transmission in the trigeminal nucleus assessed with local blood flow." Brain Res **875**(1-2): 119-24.

Gobeil, F., Jr., et al. (2002). "Regulation of eNOS expression in brain endothelial cells by perinuclear EP(3) receptors." Circ Res **90**(6): 682-9.

Goehlert, U. G., et al. (1981). "Biosynthesis of prostacyclin in rat cerebral microvessels and the choroid plexus." J Neurochem **36**(3): 1192-201.

Goldman, J. E. (1995). "Lineage, migration, and fate determination of postnatal subventricular zone cells in the mammalian CNS." J Neurooncol **24**(1): 61-4.

Goldstein, G. W. (1988). "Endothelial cell-astrocyte interactions. A cellular model of the blood-brain barrier." Ann N Y Acad Sci **529**: 31-9.

Goldstein, S., et al. (2004). "Reactions of nitric oxide, peroxynitrite, and carbonate radicals with nitroxides and their corresponding oxoammonium cations." Chem Res Toxicol **17**(2): 250-7.

Gow, A. J., et al. (2004). "Biological significance of nitric oxide-mediated protein modifications." Am J Physiol Lung Cell Mol Physiol **287**(2): L262-8.

Graves, J. E., et al. (1998). "Peroxynitrite-mediated vasorelaxation: evidence against the formation of circulating S-nitrosothiols." Am J Physiol **274**(3 Pt 2): H1001-8.

Grotta, J. (1997). "Lubeluzole treatment of acute ischemic stroke. The US and Canadian Lubeluzole Ischemic Stroke Study Group." Stroke **28**(12): 2338-46.

Grotta, J. (2001). "Combination therapy stroke trial. rt-PA +/- lubeluzole." Ann N Y Acad Sci **939**: 309-10.

Grotta, J. (2001). "Combination Therapy Stroke Trial: recombinant tissue-type plasminogen activator with/without lubeluzole." Cerebrovasc Dis **12**(3): 258-63.

Grynkiewicz, G., et al. (1985). "A new generation of Ca2+ indicators with greatly improved fluorescence properties." J Biol Chem **260**(6): 3440-50.

Gu, Z., et al. (2002). "S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death." Science **297**(5584): 1186-90.

Gulbenkian, S., et al. (2001). "Neuronal messengers in the human cerebral circulation." Peptides **22**(6): 995-1007.

Gupta, R. A., et al. (2000). "Prostacyclin-mediated activation of peroxisome proliferator-activated receptor delta in colorectal cancer." Proc Natl Acad Sci U S A **97**(24): 13275-80.

Gursoy-Ozdemir, Y., et al. (2000). "Role of endothelial nitric oxide generation and peroxynitrite formation in reperfusion injury after focal cerebral ischemia." Stroke **31**(8): 1974-80; discussion 1981.

Gursoy-Ozdemir, Y., et al. (2004). "Reperfusion-induced oxidative/nitrative injury to neurovascular unit after focal cerebral ischemia." Stroke **35**(6): 1449-53.

Guyton AC, H. J. (2000). Textbook of Medical Physiology. London, UK, W.B Saunders Compagny.

Hacke, W., et al. (1999). "Thrombolysis in acute ischemic stroke: controlled trials and clinical experience." Neurology **53**(7 Suppl 4): S3-14.

Hacke, W., et al. (1996). "'Malignant' middle cerebral artery territory infarction: clinical course and prognostic signs." Arch Neurol **53**(4): 309-15.

Hagberg, H., et al. (1996). "Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats." Pediatr Res **40**(4): 603-9.

Hall, E. D., et al. (1989). "Central nervous system trauma and stroke. II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation." Free Radic Biol Med **6**(3): 303-13.

Hall, E. D., et al. (1994). "Therapeutic potential of the lazaroids (21-aminosteroids) in acute central nervous system trauma, ischemia and subarachnoid hemorrhage." Adv Pharmacol **28**: 221-68.

Hall, E. D., et al. (1988). "21-Aminosteroid lipid peroxidation inhibitor U74006F protects against cerebral ischemia in gerbils." Stroke **19**(8): 997-1002.

Halliwell, B. (1993). "The role of oxygen radicals in human disease, with particular reference to the vascular system." Haemostasis **23 Suppl 1**: 118-26.

Halliwell, B. (1994). "Free radicals, antioxidants, and human disease: curiosity, cause, or consequence?" Lancet **344**(8924): 721-4.

Halliwell, B., et al. (1993). "Lipid peroxidation: its mechanism, measurement, and significance." Am J Clin Nutr **57**(5 Suppl): 715S-724S; discussion 724S-725S.

Halliwell, B., et al. (1989). Free Radicals in Biology and Medecine (2nd edition). Oxford, UK, Clarendon.

Hamann, G. F., et al. (1995). "Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion." Stroke **26**(11): 2120-6.

Hamel, E. (2006). "Perivascular nerves and the regulation of cerebrovascular tone." J Appl Physiol **100**(3): 1059-64.

Han, F., et al. (2006). "Microsphere embolism-induced endothelial nitric oxide synthase expression mediates disruption of the blood-brain barrier in rat brain." J Neurochem **99**(1): 97-106.

Hara, A., et al. (1996). "Immunohistochemical detection of Bax and Bcl-2 proteins in gerbil hippocampus following transient forebrain ischemia." Brain Res **711**(1-2): 249-53.

Harder, D. R. (1984). "Pressure-dependent membrane depolarization in cat middle cerebral artery." Circ Res **55**(2): 197-202.

Harder, D. R., et al. (1995). "Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone." J Vasc Res **32**(2): 79-92.

Hardy, P., et al. (1998). "A major role for prostacyclin in nitric oxide-induced ocular vasorelaxation in the piglet." Circ Res **83**(7): 721-9.

Hardy, P., et al. (1996). "Increased nitric oxide synthesis and action preclude choroidal vasoconstriction to hyperoxia in newborn pigs." Circ Res **79**(3): 504-11.

Haring, H. P., et al. (1996). "E-selectin appears in nonischemic tissue during experimental focal cerebral ischemia." Stroke **27**(8): 1386-91; discussion 1391-2.

Harris, R. J., et al. (1981). "Changes in extracellular calcium activity in cerebral ischaemia." J Cereb Blood Flow Metab **1**(2): 203-9.

Heiss, W. D., et al. (1976). "Cortical neuronal function during ischemia. Effects of occlusion of one middle cerebral artery on single-unit activity in cats." Arch Neurol **33**(12): 813-20.

Hirasawa, A., et al. (2008). "Production and characterization of a monoclonal antibody against GPR40 (FFAR1; free fatty acid receptor 1)." Biochem Biophys Res Commun **365**(1): 22-8.

Hirasawa, A., et al. (2005). "Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120." Nat Med **11**(1): 90-4.

Hoffman, S. W., et al. (1996). "A reliable and sensitive enzyme immunoassay method for measuring 8-isoprostaglandin F2 alpha: a marker for lipid

peroxidation after experimental brain injury." J Neurosci Methods **68**(2): 133-6.

Holland, M., et al. (1996). "Effects of the BKCa channel activator, NS1619, on rat cerebral artery smooth muscle." Br J Pharmacol **117**(1): 119-29.

Homi, H. M., et al. (2002). "Changes in superoxide dismutase and catalase activities of rat brain regions during early global transient ischemia/reperfusion." Neurosci Lett **333**(1): 37-40.

Hoshide, S., et al. (2004). "PK/PD and safety of a single dose of TMX-67 (febuxostat) in subjects with mild and moderate renal impairment." Nucleosides Nucleotides Nucleic Acids **23**(8-9): 1117-8.

Hossmann, K. A. (1994). "Viability thresholds and the penumbra of focal ischemia." Ann Neurol **36**(4): 557-65.

Hou, X., et al. (2000). "Augmented vasoconstriction and thromboxane formation by 15-F(2t)-isoprostane (8-iso-prostaglandin F(2alpha)) in immature pig periventricular brain microvessels." Stroke **31**(2): 516-24; discussion 525.

Hou, X., et al. (2004). "Isomer-specific contractile effects of a series of synthetic f2-isoprostanes on retinal and cerebral microvasculature." Free Radic Biol Med **36**(2): 163-72.

Hou, X., et al. (2001). "2,3-Dinor-5,6-dihydro-15-F(2t)-isoprostane: a bioactive prostanoid metabolite." Am J Physiol Regul Integr Comp Physiol **281**(2): R391-400.

Hoyert, D. L., et al. (2001). "Annual summary of vital statistics: 2000." Pediatrics **108**(6): 1241-55.

Hu, X., et al. (2005). "Activation of nuclear factor-kappaB signaling pathway by interleukin-1 after hypoxia/ischemia in neonatal rat hippocampus and cortex." J Neurochem **93**(1): 26-37.

Huie, R. E., et al. (1993). "The reaction of no with superoxide." Free Radic Res Commun **18**(4): 195-9.

Hulse, R. E., et al. (2001). "Astrocytic clasmatodendrosis in hippocampal organ culture." Glia **33**(2): 169-79.

Husain, J., et al. (1995). "Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species." Brain Res **698**(1-2): 86-94.

Iadecola, C. (1997). "Bright and dark sides of nitric oxide in ischemic brain injury." Trends Neurosci **20**(3): 132-9.

Ignarro, L. J. (1989). "Endothelium-derived nitric oxide: actions and properties." Faseb J **3**(1): 31-6.

Ignarro, L. J., et al. (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A **84**(24): 9265-9.

Ignarro, L. J., et al. (1987). "Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical." Circ Res **61**(6): 866-79.

Imaizumi, S., et al. (1986). "Initiation and propagation of lipid peroxidation in cerebral infarction models. Experimental studies." Neurol Res **8**(4): 214-20.

Inamasu, J., et al. (2002). "Mild hypothermia in neurologic emergency: an update." Ann Emerg Med **40**(2): 220-30.

Inder, T. E., et al. (2000). "Mechanisms of perinatal brain injury." Semin Neonatol **5**(1): 3-16.

Inoue, S., et al. (1995). "Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide." FEBS Lett **371**(1): 86-8.

Ip, C., et al. (1996). "Trans fatty acids and cancer." Nutr Rev **54**(5): 138-45.

Ischiropoulos, H., et al. (2005). "Pathophysiological functions of nitric oxide-mediated protein modifications." Toxicology **208**(2): 299-303.

Itoh, T., et al. (1986). "Effect of allopurinol on ischemia and reperfusion-induced cerebral injury in spontaneously hypertensive rats." Stroke **17**(6): 1284-7.

Itoh, Y., et al. (2005). "GPR40, a free fatty acid receptor on pancreatic beta cells, regulates insulin secretion." Hepatol Res **33**(2): 171-3.

Itoh, Y., et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-6.

Jaggar, J. H., et al. (2002). "Carbon monoxide dilates cerebral arterioles by enhancing the coupling of Ca2+ sparks to Ca2+-activated K+ channels." Circ Res **91**(7): 610-7.

Jain, K., et al. (2005). "5E, 8Z, 11Z, 14Z-eicosatetraenoic acid, a novel trans isomer of arachidonic acid, causes G1 phase arrest and induces apoptosis of HL-60 cells." Int J Oncol **27**(5): 1177-85.

Janardhan, V., et al. (2004). "Mechanisms of ischemic brain injury." Curr Cardiol Rep **6**(2): 117-23.

Janssen, L. J., et al. (2002). "Involvement of TP and EP3 receptors in vasoconstrictor responses to isoprostanes in pulmonary vasculature." J Pharmacol Exp Ther **301**(3): 1060-6.

Janssen, Y. M., et al. (1993). "Cell and tissue responses to oxidative damage." Lab Invest **69**(3): 261-74.

Janzer, R. C., et al. (1987). "Astrocytes induce blood-brain barrier properties in endothelial cells." Nature **325**(6101): 253-7.

Jiang, H., et al. (1999). "Nitrogen dioxide induces cis-trans-isomerization of arachidonic acid within cellular phospholipids. Detection of trans-arachidonic acids in vivo." J Biol Chem **274**(23): 16235-41.

Jimenez, B., et al. (2000). "Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1." Nat Med **6**(1): 41-8.

Johnston, K. C., et al. (1998). "Medical and neurological complications of ischemic stroke: experience from the RANTTAS trial. RANTTAS Investigators." Stroke **29**(2): 447-53.

Juurlink, B. H., et al. (1998). "Peroxide-scavenging deficit underlies oligodendrocyte susceptibility to oxidative stress." Glia **22**(4): 371-8.

Kacem, K., et al. (1998). "Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study." Glia **23**(1): 1-10.

Kadowitz, P. J., et al. (1988). Circulatory system. The eicosanoids. P. Curtis-Pryor, Wiler: pp 387-392.

Kahles, T., et al. (2007). "NADPH oxidase plays a central role in blood-brain barrier damage in experimental stroke." Stroke **38**(11): 3000-6.

Kang, J., et al. (2001). "Reactive oxygen species mediate A beta(25-35)-induced activation of BV-2 microglia." Neuroreport **12**(7): 1449-52.

Katsuma, S., et al. (2005). "Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1." J Biol Chem **280**(20): 19507-15.

Katsura, K., et al. (1994). "Energy metabolism, ion homeostasis, and cell damage in the brain." Biochem Soc Trans **22**(4): 991-6.

Kehrer, J. P. (2000). "The Haber-Weiss reaction and mechanisms of toxicity." Toxicology **149**(1): 43-50.

Kelly-Hayes, M., et al. (2003). "The influence of gender and age on disability following ischemic stroke: the Framingham study." J Stroke Cerebrovasc Dis **12**(3): 119-26.

Kelpe, C. L., et al. (2003). "Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis." J Biol Chem **278**(32): 30015-21.

Kermorvant-Duchemin, E., et al. (2005). "Trans-arachidonic acids generated during nitrative stress induce a thrombospondin-1-dependent microvascular degeneration." Nat Med **11**(12): 1339-45.

Keynes, R. D. (1979). "Ion channels in the nerve-cell membrane." Sci Am **240**(3): 126-32, 134-5.

Kim, G. W., et al. (2002). "Manganese superoxide dismutase deficiency exacerbates cerebral infarction after focal cerebral ischemia/reperfusion in mice: implications for the production and role of superoxide radicals." Stroke **33**(3): 809-15.

Kim, Y. S., et al. (1991). "Oligodendroglial cell death induced by oxygen radicals and its protection by catalase." J Neurosci Res **29**(1): 100-6.

Kirber, M. T., et al. (1992). "Both membrane stretch and fatty acids directly activate large conductance Ca(2+)-activated K+ channels in vascular smooth muscle cells." FEBS Lett **297**(1-2): 24-8.

Kirino, T. (1982). "Delayed neuronal death in the gerbil hippocampus following ischemia." Brain Res **239**(1): 57-69.

Kirino, T., et al. (1984). "Delayed neuronal death in the rat hippocampus following transient forebrain ischemia." Acta Neuropathol **64**(2): 139-47.

Klatt, P., et al. (2000). "Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress." Eur J Biochem **267**(16): 4928-44.

Kliewer, S. A., et al. (1995). "A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation." Cell **83**(5): 813-9.

Knot, H. J., et al. (1998). "Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure." J Physiol **508 (Pt 1)**: 199-209.

Koehler, R. C., et al. (2006). "Role of astrocytes in cerebrovascular regulation." J Appl Physiol **100**(1): 307-17.

Kohen, R., et al. (2002). "Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification." Toxicol Pathol **30**(6): 620-50.

Kohrmann, M., et al. (2006). "MRI versus CT-based thrombolysis treatment within and beyond the 3 h time window after stroke onset: a cohort study." Lancet Neurol **5**(8): 661-7.

Koletzko, B., et al. (1997). "Metabolic aspects of trans fatty acids." Clin Nutr **16**(5): 229-37.

Kondo, T., et al. (1997). "Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia." J Neurosci **17**(11): 4180-9.

Koneru, P., et al. (2004). "Role of cGMP in carbon monoxide-induced cerebral vasodilation in piglets." Am J Physiol Heart Circ Physiol **286**(1): H304-9.

Kontos, C. D., et al. (1992). "Cytochemical detection of superoxide in cerebral inflammation and ischemia in vivo." Am J Physiol **263**(4 Pt 2): H1234-42.

Kontos, H. A. (2001). "Oxygen radicals in cerebral ischemia: the 2001 Willis lecture." Stroke **32**(11): 2712-6.

Kooli, A., et al. (2008). "trans-Arachidonic acids induce a heme oxygenase-dependent vasorelaxation of cerebral microvasculature." Free Radic Biol Med **44**(5): 815-25.

Kotarsky, K., et al. (2003). "A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs." Biochem Biophys Res Commun **301**(2): 406-10.

Kotlikoff, M. I. (2005). "EDHF redux: EETs, TRPV4, and Ca2+ sparks." Circ Res **97**(12): 1209-10.

Kozma, F., et al. (1999). "Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels." Am J Physiol **276**(4 Pt 2): R1087-94.

Krishna, U. M., et al. (2001). "Stereospecific synthesis of trans-arachidonic acids." Bioorg Med Chem Lett **11**(18): 2415-8.

Kristian, T., et al. (1998). "Calcium in ischemic cell death." Stroke **29**(3): 705-18.

Kroemer, G., et al. (2000). "Mitochondrial control of cell death." Nat Med **6**(5): 513-9.

Krupinski, J., et al. (1994). "Role of angiogenesis in patients with cerebral ischemic stroke." Stroke **25**(9): 1794-8.

Kuban, K. C., et al. (1985). "Human telencephalic angiogenesis." Ann Neurol **17**(6): 539-48.

Kuhlmann, C. R., et al. (2005). "Nicotine inhibits large conductance Ca(2+)-activated K(+) channels and the NO/-cGMP signaling pathway in cultured human endothelial cells." Scand Cardiovasc J **39**(6): 348-52.

Kusaka, H., et al. (1985). "Basal lamina formation by astrocytes in organotypic cultures of mouse spinal cord tissue." J Neuropathol Exp Neurol **44**(3): 295-303.

Kwon, T. H., et al. (2003). "Tempol, a novel stable nitroxide, reduces brain damage and free radical production, after acute subdural hematoma in the rat." J Neurotrauma **20**(4): 337-45.

Lahaie, I., et al. (1998). "A novel mechanism for vasoconstrictor action of 8-isoprostaglandin F2 alpha on retinal vessels." Am J Physiol **274**(5 Pt 2): R1406-16.

LaManna, J. C., et al. (2006). "Is cycloxygenase-2 (COX-2) a major component of the mechanism responsible for microvascular remodeling in the brain?" Adv Exp Med Biol **578**: 297-303.

Lander, H. M. (1997). "An essential role for free radicals and derived species in signal transduction." Faseb J **11**(2): 118-24.

Latour, M. G., et al. (2007). "GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo." Diabetes **56**(4): 1087-94.

Lauritzen, L., et al. (2001). "The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina." Prog Lipid Res **40**(1-2): 1-94.

Le Poul, E., et al. (2003). "Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation." J Biol Chem **278**(28): 25481-9.

Lee, J. M., et al. (2000). "Brain tissue responses to ischemia." J Clin Invest **106**(6): 723-31.

Lees, K. R., et al. (2006). "NXY-059 for acute ischemic stroke." N Engl J Med **354**(6): 588-600.

Leffler, C. W., et al. (1999). "Carbon monoxide and cerebral microvascular tone in newborn pigs." Am J Physiol **276**(5 Pt 2): H1641-6.

Leffler, C. W., et al. (2006). "Carbon monoxide and hydrogen sulfide: gaseous messengers in cerebrovascular circulation." J Appl Physiol **100**(3): 1065-76.

Legido, M. (2000). "Perinatal Hypoxic-Ischemic Encephalopathy: Current and Future Treatments." International Pediatrics **15**(3): 143-151.

Legos, J. J., et al. (2002). "Pharmacological interventions for stroke: failures and future." Expert Opin Investig Drugs **11**(5): 603-14.

Leist, M., et al. (2001). "Four deaths and a funeral: from caspases to alternative mechanisms." Nat Rev Mol Cell Biol **2**(8): 589-98.

Lerouet, D., et al. (2002). "Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats." Brain Res **958**(1): 166-75.

Levy, B. D., et al. (2001). "Lipid mediator class switching during acute inflammation: signals in resolution." Nat Immunol **2**(7): 612-9.

Li, Y., et al. (1995). "Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats." Stroke **26**(7): 1252-7; discussion 1257-8.

Liao, S. L., et al. (2001). "Differential effects of cytokines and redox potential on glutamate uptake in rat cortical glial cultures." Neurosci Lett **299**(1-2): 113-6.

Lim, D. G., et al. (2002). "Nitrolinoleate, a nitric oxide-derived mediator of cell function: synthesis, characterization, and vasomotor activity." Proc Natl Acad Sci U S A **99**(25): 15941-6.

Lima, E. S., et al. (2005). "Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation." Free Radic Biol Med **39**(4): 532-9.

Lin, T. N., et al. (2003). "Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion." Stroke **34**(1): 177-86.

Lin, Y., et al. (1992). "Deoxycoformycin and oxypurinol: protection against focal ischemic brain injury in the rat." Brain Res **571**(2): 272-80.

Lindenau, J., et al. (1998). "Enhanced cellular glutathione peroxidase immunoreactivity in activated astrocytes and in microglia during excitotoxin induced neurodegeneration." Glia **24**(2): 252-6.

Lipton, P. (1999). "Ischemic cell death in brain neurons." Physiol Rev **79**(4): 1431-568.

Liu, H. N., et al. (1995). "Glutamate induces c-fos proto-oncogene expression and inhibits proliferation in oligodendrocyte progenitors: receptor characterization." Eur J Neurosci **7**(12): 2355-63.

Liu, T., et al. (1999). "The isoprostanes: novel prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid." J Biomed Sci **6**(4): 226-35.

Lo, A. C., et al. (2005). "Endothelin-1 overexpression leads to further water accumulation and brain edema after middle cerebral artery occlusion via aquaporin 4 expression in astrocytic end-feet." J Cereb Blood Flow Metab **25**(8): 998-1011.

Lo, E. H., et al. (2003). "Mechanisms, challenges and opportunities in stroke." Nat Rev Neurosci **4**(5): 399-415.

Lo, E. H., et al. (1996). "Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene expression." Stroke **27**(8): 1381-5.

Longmire, A. W., et al. (1994). "Actions of the E2-isoprostane, 8-ISO-PGE2, on the platelet thromboxane/endoperoxide receptor in humans and rats: additional evidence for the existence of a unique isoprostane receptor." Prostaglandins **48**(4): 247-56.

LoPresti, P., et al. (1995). "Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes." Proc Natl Acad Sci U S A **92**(22): 10369-73.

Louet, J. F., et al. (2001). "Long-chain fatty acids regulate liver carnitine palmitoyltransferase I gene (L-CPT I) expression through a peroxisome-proliferator-activated receptor alpha (PPARalpha)-independent pathway." Biochem J **354**(Pt 1): 189-97.

Love, S. (1999). "Oxidative stress in brain ischemia." Brain Pathol **9**(1): 119-31.

Lovell, M. A., et al. (2001). "Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures." Neurobiol Aging **22**(2): 187-94.

Luginbuhl, H. (1973). "[Comparative studies on cerebral vessel pathology in animals and man]." Schweiz Arch Neurol Neurochir Psychiatr **112**(2): 323-8.

Lukaszevicz, A. C., et al. (2002). "High sensitivity of protoplasmic cortical astroglia to focal ischemia." J Cereb Blood Flow Metab **22**(3): 289-98.

Lundberg, J. O., et al. (2008). "The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics." Nat Rev Drug Discov **7**(2): 156-67.

Lyden, P., et al. (2001). "The Clomethiazole Acute Stroke Study in tissue-type plasminogen activator-treated stroke (CLASS-T): final results." Neurology **57**(7): 1199-205.

Ma, D., et al. (2008). "Expression of free fatty acid receptor GPR40 in the neurogenic niche of adult monkey hippocampus." Hippocampus **18**(3): 326-33.

Ma, D., et al. (2007). "Expression of free fatty acid receptor GPR40 in the central nervous system of adult monkeys." Neurosci Res **58**(4): 394-401.

Mabuchi, T., et al. (2000). "Contribution of microglia/macrophages to expansion of infarction and response of oligodendrocytes after focal cerebral ischemia in rats." Stroke **31**(7): 1735-43.

Mackensen, G. B., et al. (2001). "Neuroprotection from delayed postischemic administration of a metalloporphyrin catalytic antioxidant." J Neurosci **21**(13): 4582-92.

MacKenzie, E. T., et al. (1979). "Effects of hemorrhagic hypotension on the cerebral circulation. I. Cerebral blood flow and pial arteriolar caliber." Stroke **10**(6): 711-8.

MacMillan-Crow, L. A., et al. (1996). "Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts." Proc Natl Acad Sci U S A **93**(21): 11853-8.

Macrae, I. M., et al. (1993). "Endothelin-1-induced reductions in cerebral blood flow: dose dependency, time course, and neuropathological consequences." J Cereb Blood Flow Metab **13**(2): 276-84.

Magder, S. (2006). "Reactive oxygen species: toxic molecules or spark of life?" Crit Care **10**(1): 208.

Maines, M. D. (1992). Heme oxygenase: Clinical applications and functions. Boca Raton, FL, CRC Press.

Maines, M. D. (1997). "The heme oxygenase system: a regulator of second messenger gases." Annu Rev Pharmacol Toxicol **37**: 517-54.

Makar, T. K., et al. (1994). "Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain." J Neurochem **62**(1): 45-53.

Manchev, I. C., et al. (2001). "Prevalence of stroke risk factors and their outcomes. A population-based longitudinal epidemiological study." Cerebrovasc Dis **12**(4): 303-7.

Mann, B. E., et al. (2007). "CO and NO in medicine." Chem Commun (Camb)(41): 4197-208.

Mannaioni, P. F., et al. (2006). "Carbon monoxide: the bad and the good side of the coin, from neuronal death to anti-inflammatory activity." Inflamm Res **55**(7): 261-73.

Mannick, J. B., et al. (1999). "Fas-induced caspase denitrosylation." Science **284**(5414): 651-4.

Manoonkitiwongsa, P. S., et al. (2001). "Angiogenesis after stroke is correlated with increased numbers of macrophages: the clean-up hypothesis." J Cereb Blood Flow Metab **21**(10): 1223-31.

Mantell, L. L. (2006). "Introduction to serial reviews on redox signaling in immune function and cellular responses in lung injury and diseases." Free Radic Biol Med **41**(1): 1-3.

Mariotto, S., et al. (2004). "Biochemical aspects of nitric oxide." Curr Pharm Des **10**(14): 1627-45.

Marklund, S. L. (1984). "Extracellular superoxide dismutase in human tissues and human cell lines." J Clin Invest **74**(4): 1398-403.

Markus, H. S., et al. (1994). "Differential effect of three cyclooxygenase inhibitors on human cerebral blood flow velocity and carbon dioxide reactivity." Stroke **25**(9): 1760-4.

Marnett, L. J., et al. (1995). "The chemistry of lipid alkoxyl radicals and their role in metal-amplified lipid peroxidation." Biochem Soc Symp **61**: 65-72.

Marrannes, R., et al. (2000). "Site of action of lubeluzole on voltage-sensitive Ca(2+) channels in isolated dorsal root ganglion cells of the rat: influence of pH." J Pharmacol Exp Ther **295**(2): 531-45.

Marshall, J. J., et al. (1990). "Endothelium-derived relaxing factors. A perspective from in vivo data." Hypertension **16**(4): 371-86.

Marshall, J. W., et al. (2003). "Functional and histological evidence for the protective effect of NXY-059 in a primate model of stroke when given 4 hours after occlusion." Stroke **34**(9): 2228-33.

Martin-Ancel, A., et al. (1997). "Interleukin-6 in the cerebrospinal fluid after perinatal asphyxia is related to early and late neurological manifestations." Pediatrics **100**(5): 789-94.

Martin-Villalba, A., et al. (1999). "CD95 ligand (Fas-L/APO-1L) and tumor necrosis factor-related apoptosis-inducing ligand mediate ischemia-induced apoptosis in neurons." J Neurosci **19**(10): 3809-17.

Martin, L. J., et al. (1998). "Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis." Brain Res Bull **46**(4): 281-309.

Martin, L. J., et al. (1997). "Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum." Ann Neurol **42**(3): 335-48.

Martin, R. L., et al. (1994). "The early events of oxygen and glucose deprivation: setting the scene for neuronal death?" Trends Neurosci **17**(6): 251-7.

Martinez, M. C., et al. (1994). "4-Hydroxynonenal, a lipid peroxidation product, induces relaxation of human cerebral arteries." J Cereb Blood Flow Metab **14**(4): 693-6.

Martz, D., et al. (1989). "Allopurinol and dimethylthiourea reduce brain infarction following middle cerebral artery occlusion in rats." Stroke **20**(4): 488-94.

Masson, V. V., et al. (2002). "Mouse aortic ring assay: a new approach of the molecular genetics of angiogenesis." Biol. Proc. Online **4**: pp 24-31.

Mathews, J. M., et al. (1985). "Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole." J Pharmacol Exp Ther **235**(1): 186-90.

Matsumoto, M., et al. (1987). "A combined method for measurement of cerebral blood flow and immunohistochemistry for investigation of cerebral ischemia." Brain Res **424**(2): 231-8.

Matsuo, Y., et al. (2001). "Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats." Stroke **32**(9): 2143-8.

Matute, C., et al. (1997). "Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes." Proc Natl Acad Sci U S A **94**(16): 8830-5.

Mayer, M. D., et al. (2005). "Pharmacokinetics and pharmacodynamics of febuxostat, a new non-purine selective inhibitor of xanthine oxidase in subjects with renal impairment." Am J Ther **12**(1): 22-34.

Mayevsky, A., et al. (1996). "Cortical spreading depression recorded from the human brain using a multiparametric monitoring system." Brain Res **740**(1-2): 268-74.

McCord, J. M. (1985). "Oxygen-derived free radicals in postischemic tissue injury." N Engl J Med **312**(3): 159-63.

McDonald, J. W., et al. (1998). "Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity." Nat Med **4**(3): 291-7.

McKenna, R., et al. (1995). "Vascular effects of the 21-aminosteroid tirilazad mesylate: protection against oxidant stress." Methods Find Exp Clin Pharmacol **17**(4): 279-92.

McLachlan, R. S., et al. (1994). "Spreading depression of Leao in rodent and human cortex." Brain Res **666**(1): 133-6.

Meden, P., et al. (1993). "Enhancing the efficacy of thrombolysis by AMPA receptor blockade with NBQX in a rat embolic stroke model." J Neurol Sci **119**(2): 209-16.

Meidute Abaraviciene, S., et al. (2008). "Palmitate-induced beta-cell dysfunction is associated with excessive NO production and is reversed by thiazolidinedione-mediated inhibition of GPR40 transduction mechanisms." PLoS ONE **3**(5): e2182.

Meves, H. (1994). "Modulation of ion channels by arachidonic acid." Prog Neurobiol **43**(2): 175-86.

Mies, G., et al. (1993). "Correlation between peri-infarct DC shifts and ischaemic neuronal damage in rat." Neuroreport **4**(6): 709-11.

Mies, G., et al. (1991). "Ischemic thresholds of cerebral protein synthesis and energy state following middle cerebral artery occlusion in rat." J Cereb Blood Flow Metab **11**(5): 753-61.

Migheli, A., et al. (1988). "Light and electron microscope localization of the microtubule-associated tau protein in rat brain." J Neurosci **8**(6): 1846-51.

Miller, A. A., et al. (2006). "Reactive oxygen species in the cerebral circulation: are they all bad?" Antioxid Redox Signal **8**(7-8): 1113-20.

Miller, A. A., et al. (2006). "NADPH-oxidase activity is elevated in penumbral and non-ischemic cerebral arteries following stroke." Brain Res **1111**(1): 111-6.

Miller, R. H. (1996). "Oligodendrocyte origins." Trends Neurosci **19**(3): 92-6.

Minard, F. N., et al. (1982). "Hypothermia as a mechanism for drug-induced resistance to hypoxia." Biochem Pharmacol **31**(7): 1197-203.

Mink, R., et al. (2007). "The effect of infusing hypoxanthine or xanthine on hypoxic-ischemic brain injury in rabbits." Brain Res **1147**: 256-64.

Montine, K. S., et al. (2004). "Isoprostanes and related products of lipid peroxidation in neurodegenerative diseases." Chem Phys Lipids **128**(1-2): 117-24.

Mori, Y., et al. (1997). "Vasodilator effects of C-type natriuretic peptide on cerebral arterioles in rats." Eur J Pharmacol **320**(2-3): 183-6.

Morioka, T., et al. (1993). "Characterization of microglial reaction after middle cerebral artery occlusion in rat brain." J Comp Neurol **327**(1): 123-32.

Moro, M. A., et al. (2004). "Role of nitric oxide after brain ischaemia." Cell Calcium **36**(3-4): 265-75.

Morris, D. C., et al. (2003). "Microvascular structure after embolic focal cerebral ischemia in the rat." Brain Res **972**(1-2): 31-7.

Morrow, J. D., et al. (1990). "Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids." Anal Biochem **184**(1): 1-10.

Morrow, J. D., et al. (1990). "A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism." Proc Natl Acad Sci U S A **87**(23): 9383-7.

Morrow, J. D., et al. (1997). "The isoprostanes: unique bioactive products of lipid peroxidation." Prog Lipid Res **36**(1): 1-21.

Morrow, J. D., et al. (2002). "The isoprostanes: their role as an index of oxidant stress status in human pulmonary disease." Am J Respir Crit Care Med **166**(12 Pt 2): S25-30.

Motterlini, R., et al. (1996). "NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium." Am J Physiol **270**(1 Pt 2): H107-14.

Motterlini, R., et al. (2003). "Bioactivity and pharmacological actions of carbon monoxide-releasing molecules." Curr Pharm Des **9**(30): 2525-39.

Muralikrishna Adibhatla, R., et al. (2006). "Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia." Free Radic Biol Med **40**(3): 376-87.

Murray, L., et al. (1988). Inflammation. The eicosanoids. P. Curtis-Pryor, Wiler: pp 333-340.

Musiek, E. S., et al. (2006). "Cyclopentenone isoprostanes are novel bioactive products of lipid oxidation which enhance neurodegeneration." J Neurochem **97**(5): 1301-13.

Nagano, N., et al. (1993). "Extracellular matrix modulates the proliferation of rat astrocytes in serum-free culture." Glia **8**(2): 71-6.

Nagaoka, T., et al. (2007). "Simvastatin elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and mevalonate-rho kinase pathways." Invest Ophthalmol Vis Sci **48**(2): 825-32.

Nagayama, T., et al. (2000). "Induction of oxidative DNA damage in the peri-infarct region after permanent focal cerebral ischemia." J Neurochem **75**(4): 1716-28.

Nakao, A., et al. (2006). "Protective effect of carbon monoxide in transplantation." J Cell Mol Med **10**(3): 650-71.

Nakashima, M., et al. (1999). "Involvement of free radicals in cerebral vascular reperfusion injury evaluated in a transient focal cerebral ischemia model of rat." Free Radic Biol Med **26**(5-6): 722-9.

Narumiya, S., et al. (2001). "Genetic and pharmacological analysis of prostanoid receptor function." J Clin Invest **108**(1): 25-30.

Nelson, C. W., et al. (1992). "Oxygen radicals in cerebral ischemia." Am J Physiol **263**(5 Pt 2): H1356-62.

Nelson, M. D., Jr., et al. (1991). "Dyke Award. The search for human telencephalic ventriculofugal arteries." AJNR Am J Neuroradiol **12**(2): 215-22.

Nelson, M. T., et al. (1995). "Relaxation of arterial smooth muscle by calcium sparks." Science **270**(5236): 633-7.

Nestler, E. J., et al. (2001). Molecular neuropharmacology: A foundation for clinical neuroscience. McGraw-Hill, Medical Pub. Div.

Nguyen, T., et al. (1992). "DNA damage and mutation in human cells exposed to nitric oxide in vitro." Proc Natl Acad Sci U S A **89**(7): 3030-4.

Nicotera, P., et al. (1999). "Excitotoxins in neuronal apoptosis and necrosis." J Cereb Blood Flow Metab **19**(6): 583-91.

Nigam, S., et al. (2000). "Phospholipase A(2)s and lipid peroxidation." Biochim Biophys Acta **1488**(1-2): 167-81.

Nilsson, N. E., et al. (2003). "Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids." Biochem Biophys Res Commun **303**(4): 1047-52.

Noble, M., et al. (1984). "Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell." Embo J **3**(10): 2243-7.

Nonaka, H., et al. (2003). "Microvasculature of the human cerebral white matter: arteries of the deep white matter." Neuropathology **23**(2): 111-8.

Nor, J. E., et al. (2000). "Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway." J Vasc Res **37**(3): 209-18.

Nordborg, C., et al. (1991). "The relationship between plasma protein extravasation and remote tissue changes after experimental brain infarction." Acta Neuropathol **82**(2): 118-26.

Nozik-Grayck, E., et al. (2005). "Extracellular superoxide dismutase." Int J Biochem Cell Biol **37**(12): 2466-71.

O'Donnell, V. B. (2003). "Free radicals and lipid signaling in endothelial cells." Antioxid Redox Signal **5**(2): 195-203.

O'Neill, M. J., et al. (2000). "ARL 17477, a selective nitric oxide synthase inhibitor, with neuroprotective effects in animal models of global and focal cerebral ischaemia." Brain Res **871**(2): 234-44.

O'Reilly, M. S., et al. (1997). "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth." Cell **88**(2): 277-85.

Oberle, S., et al. (1999). "The antioxidant defense protein ferritin is a novel and specific target for pentaerithrityl tetranitrate in endothelial cells." Biochem Biophys Res Commun **261**(1): 28-34.

Oechmichen, M., et al. (2006). "Cerebral hypoxia and ischemia: the forensic point of view: a review." J Forensic Sci **51**(4): 880-7.

Ogawa, S., et al. (2007). "Ischemia-induced neuronal cell death and stress response." Antioxid Redox Signal **9**(5): 573-87.

Oka, A., et al. (1993). "Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention." J Neurosci **13**(4): 1441-53.

Okada, Y., et al. (1994). "Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion." Stroke **25**(9): 1847-53; discussion 1853-4.

Okada, Y., et al. (1994). "P-selectin and intercellular adhesion molecule-1 expression after focal brain ischemia and reperfusion." Stroke **25**(1): 202-11.

Okamoto, K., et al. (2003). "An extremely potent inhibitor of xanthine oxidoreductase. Crystal structure of the enzyme-inhibitor complex and mechanism of inhibition." J Biol Chem **278**(3): 1848-55.

Olesen, S. P., et al. (1998). "Characterization of NS 2028 as a specific inhibitor of soluble guanylyl cyclase." Br J Pharmacol **123**(2): 299-309.

Oliw, E. H., et al. (2007). Oxygenation of arachidonic acid by cytochromes P-450. Prostaglandins, Leukotrienes and other eicosanoids: From Biogenesis to Clinical application. F. Marks and G. Furstenburger**:** pp 142-168.

Ordway, R. W., et al. (1991). "Direct regulation of ion channels by fatty acids." Trends Neurosci **14**(3): 96-100.

Ordway, R. W., et al. (1989). "Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells." Science **244**(4909): 1176-9.

Otterbein, L. E., et al. (2000). "Heme oxygenase: colors of defense against cellular stress." Am J Physiol Lung Cell Mol Physiol **279**(6): L1029-37.

Owman, C., et al. (1988). "Functional heterogeneity of the cerebrovascular endothelium." Brain Behav Evol **32**(2): 65-75.

Pacher, P., et al. (2006). "Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol." Pharmacol Rev **58**(1): 87-114.

Pachori, A. S., et al. (2004). "Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury." Proc Natl Acad Sci U S A **101**(33): 12282-7.

Padayatty, S. J., et al. (2003). "Vitamin C as an antioxidant: evaluation of its role in disease prevention." J Am Coll Nutr **22**(1): 18-35.

Paiva, S. A., et al. (1999). "Beta-carotene and other carotenoids as antioxidants." J Am Coll Nutr **18**(5): 426-33.

Palmer, C. (1995). "Hypoxic-ischemic encephalopathy. Therapeutic approaches against microvascular injury, and role of neutrophils, PAF, and free radicals." Clin Perinatol **22**(2): 481-517.

Palmer, C., et al. (1993). "Allopurinol administered after inducing hypoxia-ischemia reduces brain injury in 7-day-old rats." Pediatr Res **33**(4 Pt 1): 405-11.

Palmer, C., et al. (1990). "Reduction of perinatal hypoxic-ischemic brain damage with allopurinol." Pediatr Res **27**(4 Pt 1): 332-6.

Palmer, R. M., et al. (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." Nature **327**(6122): 524-6.

Palmer, R. M., et al. (1988). "L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation." Biochem Biophys Res Commun **153**(3): 1251-6.

Papapetropoulos, A., et al. (1999). "Molecular control of nitric oxide synthases in the cardiovascular system." Cardiovasc Res **43**(3): 509-20.

Parfenova, H., et al. (2006). "Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection." Am J Physiol Cell Physiol **290**(5): C1399-410.

Parfenova, H., et al. (2001). "Cerebral vascular endothelial heme oxygenase: expression, localization, and activation by glutamate." Am J Physiol Cell Physiol **281**(6): C1954-63.

Parfenova, H., et al. (2001). "Dynamics of nuclear localization sites for COX-2 in vascular endothelial cells." Am J Physiol Cell Physiol **281**(1): C166-78.

Park, C. K., et al. (1994). "Dose-response analysis of the effect of 21-aminosteroid tirilazad mesylate (U-74006F) upon neurological outcome and ischemic brain damage in permanent focal cerebral ischemia." Brain Res **645**(1-2): 157-63.

Park, C. K., et al. (1989). "Effect of the NMDA antagonist MK-801 on local cerebral blood flow in focal cerebral ischaemia in the rat." J Cereb Blood Flow Metab **9**(5): 617-22.

Parmentier, S., et al. (1999). "Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury." Br J Pharmacol **127**(2): 546-52.

Paschen, W. (1996). "Glutamate excitotoxicity in transient global cerebral ischemia." Acta Neurobiol Exp (Wars) **56**(1): 313-22.

Pasqualini, R., et al. (2002). "Probing the structural and molecular diversity of tumor vasculature." Trends Mol Med **8**(12): 563-71.

Passero, S., et al. (2001). "Potential triggering factors of intracerebral hemorrhage." Cerebrovasc Dis **12**(3): 220-7.

Patel, M., et al. (2008). "Seizure-induced formation of isofurans: novel products of lipid peroxidation whose formation is positively modulated by oxygen tension." J Neurochem **104**(1): 264-70.

Paulson, O. B., et al. (1990). "Cerebral autoregulation." Cerebrovasc Brain Metab Rev **2**(2): 161-92.

Pea, F. (2005). "Pharmacology of drugs for hyperuricemia. Mechanisms, kinetics and interactions." Contrib Nephrol **147**: 35-46.

Pearce, L. L., et al. (1999). "The peroxynitrite reductase activity of cytochrome c oxidase involves a two-electron redox reaction at the heme a(3)-Cu(B) site." J Biol Chem **274**(50): 35763-7.

Pellerin, L., et al. (2002). "Role of neuron-glia interaction in the regulation of brain glucose utilization." Diabetes Nutr Metab **15**(5): 268-73; discussion 273.

Pelligrino, D. A., et al. (1999). "Miconazole represses CO(2)-induced pial arteriolar dilation only under selected circumstances." Am J Physiol **277**(4 Pt 2): H1484-90.

Penfield, W. (1928). Neuroglia and Microglia - The interstial tissue of the central nervous system. E. Cowdry, editor. New York, Hoeber. **Special cytology - the form and function of the cell in health and disease:** 1033-1068

Pepper, M. S. (1996). "Positive and negative regulation of angiogenesis: from cell biology to the clinic." Vasc Med **1**(4): 259-66.

Perlman, J. M. (2006). "Intervention strategies for neonatal hypoxic-ischemic cerebral injury." Clin Ther **28**(9): 1353-65.

Pesonen, E., et al. (1981). "Endothelial cell damage in piglet coronary artery after intravenous administration of E. coli endotoxin. A scanning and transmission electron-microscopic study." Atherosclerosis **40**(1): 65-73.

Peters, A. (1991). The fine structure of the nervous system. New York, Oxford University Press.

Petito, C. K. (1986). "Transformation of postischemic perineuronal glial cells. I. Electron microscopic studies." J Cereb Blood Flow Metab **6**(5): 616-24.

Petito, C. K., et al. (1987). "Delayed hippocampal damage in humans following cardiorespiratory arrest." Neurology **37**(8): 1281-6.

Petito, C. K., et al. (1998). "Selective glial vulnerability following transient global ischemia in rat brain." J Neuropathol Exp Neurol **57**(3): 231-8.

Petito, C. K., et al. (1982). "Edema and vascular permeability in cerebral ischemia: comparison between ischemic neuronal damage and infarction." J Neuropathol Exp Neurol **41**(4): 423-36.

Petty, M. A., et al. (2001). "Elements of cerebral microvascular ischaemia." Brain Res Brain Res Rev **36**(1): 23-34.

Phillips, R. A. (2008). "A review of therapeutic strategies for risk reduction of recurrent stroke." Prog Cardiovasc Dis **50**(4): 264-73.

Phillis, J. W., et al. (1991). "Oxypurinol reduces ischemic brain injury in the gerbil and rat." Adv Exp Med Biol **309A**: 343-6.

Phillis, J. W., et al. (1995). "Oxypurinol-enhanced postischemic recovery of the rat brain involves preservation of adenine nucleotides." J Neurochem **64**(5): 2177-84.

Piantadosi, C. A., et al. (1996). "Mitochondrial generation of reactive oxygen species after brain ischemia in the rat." Stroke **27**(2): 327-31; discussion 332.

Plate, K. H. (1999). "Mechanisms of angiogenesis in the brain." J Neuropathol Exp Neurol **58**(4): 313-20.

Porter, N. A. (1990). Autoxidation of polyunsaturated fatty acids: Initiation, propagation and product distribution (Basic chemistry). Membrane lipid oxidation: vol. I. C. V.-P. (Ed.). Boca Raton, FL, CRC Press: pp 33-62.

Porter, N. A., et al. (1995). "Mechanisms of free radical oxidation of unsaturated lipids." Lipids **30**(4): 277-90.

Porter, T. D., et al. (1991). "Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms." J Biol Chem **266**(21): 13469-72.

Pourcyrous, M., et al. (1993). "Brain superoxide anion generation in asphyxiated piglets and the effect of indomethacin at therapeutic dose." Pediatr Res **34**(3): 366-9.

Prass, K., et al. (1998). "Glutamate antagonists in therapy of stroke." Restor Neurol Neurosci **13**(1-2): 3-10.

Pratico, D., et al. (2001). "The isoprostanes in biology and medicine." Trends Endocrinol Metab **12**(6): 243-7.

Pringle, A. K., et al. (2001). "Interleukin-1beta exacerbates hypoxia-induced neuronal damage, but attenuates toxicity produced by simulated ischaemia and excitotoxicity in rat organotypic hippocampal slice cultures." Neurosci Lett **305**(1): 29-32.

Privett, O. S., et al. (1967). "Metabolism of the geometric isomers of linoleic acid in the rat." J Nutr **92**(3): 303-10.

Pryor, W. A., et al. (2006). "Free radical biology and medicine: it's a gas, man!" Am J Physiol Regul Integr Comp Physiol **291**(3): R491-511.

Pryor, W. A., et al. (1990). "Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids." Free Radic Biol Med **8**(6): 541-3.

Puntarulo, S., et al. (1998). "Production of reactive oxygen species by microsomes enriched in specific human cytochrome P450 enzymes." Free Radic Biol Med **24**(7-8): 1324-30.

Quiniou, C., et al. (2006). "Dominant role for calpain in thromboxane-induced neuromicrovascular endothelial cytotoxicity." J Pharmacol Exp Ther **316**(2): 618-27.

Quinn, M. T., et al. (2004). "Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases." J Leukoc Biol **76**(4): 760-81.

Qureshi, A. I., et al. (2003). "Timing of neurologic deterioration in massive middle cerebral artery infarction: a multicenter review." Crit Care Med **31**(1): 272-7.

Radi, R. (2004). "Nitric oxide, oxidants, and protein tyrosine nitration." Proc Natl Acad Sci U S A **101**(12): 4003-8.

Radi, R., et al. (1991). "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide." Arch Biochem Biophys **288**(2): 481-7.

Radomski, M. W., et al. (1987). "Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium." Lancet **2**(8567): 1057-8.

Raff, M. C. (1989). "Glial cell diversification in the rat optic nerve." Science **243**(4897): 1450-5.

Reed, R. K., et al. (1992). "Blockade of beta 1-integrins in skin causes edema through lowering of interstitial fluid pressure." Circ Res **71**(4): 978-83.

Reich, E. E., et al. (2001). "Brain regional quantification of F-ring and D-/E-ring isoprostanes and neuroprostanes in Alzheimer's disease." Am J Pathol **158**(1): 293-7.

Reichwald-Hacker, I., et al. (1979). "Incorporation of positional isomers of cis- and trans-octadecenoic acids into acyl moieties of rat tissue lipids." Biochim Biophys Acta **575**(3): 327-34.

Rice, J. E., 3rd, et al. (1981). "The influence of immaturity on hypoxic-ischemic brain damage in the rat." Ann Neurol **9**(2): 131-41.

Richter-Landsberg, C. (2000). "The oligodendroglia cytoskeleton in health and disease." J Neurosci Res **59**(1): 11-8.

Riley, D. P. (2000). "Rational design of synthetic enzymes and their potential utility as human pharmaceuticals." Adv Supramol Chem **6**: 217-244.

Risau, W. (1997). "Mechanisms of angiogenesis." Nature **386**(6626): 671-4.

Risau, W., et al. (1990). "Development of the blood-brain barrier." Trends Neurosci **13**(5): 174-8.

Rivkin, M. J., et al. (1995). "Oligodendroglial development in human fetal cerebrum." Ann Neurol **38**(1): 92-101.

Roberts, L. J., 2nd, et al. (1998). "Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid." J Biol Chem **273**(22): 13605-12.

Roberts, L. J., et al. (2000). "Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo." Free Radic Biol Med **28**(4): 505-13.

Rocha, J. L., et al. (2001). "Acute tubulointerstitial nephritis associated with the selective COX-2 enzyme inhibitor, rofecoxib." Lancet **357**(9272): 1946-7.

Rodrigo, J., et al. (2005). "The role of free radicals in cerebral hypoxia and ischemia." Free Radic Biol Med **39**(1): 26-50.

Rodt, S. A., et al. (1994). "The anti-inflammatory agent alpha-trinositol exerts its edema-preventing effects through modulation of beta 1 integrin function." Circ Res **75**(5): 942-8.

Ronson, R. S., et al. (1999). "The cardiovascular effects and implications of peroxynitrite." Cardiovasc Res **44**(1): 47-59.

Rosenberg, G. A., et al. (1996). "Proteolytic cascade enzymes increase in focal cerebral ischemia in rat." J Cereb Blood Flow Metab **16**(3): 360-6.

Rosenberg, G. A., et al. (2007). "Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia." Neurosurg Focus **22**(5): E4.

Rossi, A., et al. (2007). "The role of 5-lipoxygenase and leukotrienes in shock and ischemia-reperfusion injury." ScientificWorldJournal **7**: 56-74.

Rothwell, N. J., et al. (1995). "Cytokines and the nervous system II: Actions and mechanisms of action." Trends Neurosci **18**(3): 130-6.

Roy, U., et al. (2005). "Cytochrome P450/NADPH-dependent biosynthesis of 5,6-trans-epoxyeicosatrienoic acid from 5,6-trans-arachidonic acid." Biochem J **390**(Pt 3): 719-27.

Roy, U., et al. (2004). "Cytochrome P450/NADPH-dependent formation of trans epoxides from trans-arachidonic acids." Bioorg Med Chem Lett **14**(4): 1019-22.

Ryter, S. W., et al. (2004). "Carbon monoxide: to boldly go where NO has gone before." Sci STKE **2004**(230): RE6.

Sairanen, T., et al. (2001). "Evolution of cerebral tumor necrosis factor-alpha production during human ischemic stroke." Stroke **32**(8): 1750-8.

Sairanen, T. R., et al. (1997). "Global forebrain ischemia results in differential cellular expression of interleukin-1beta (IL-1beta) and its receptor at mRNA and protein level." J Cereb Blood Flow Metab **17**(10): 1107-20.

Salahudeen, A. K., et al. (1998). "F2-isoprostanes and the kidney." Drug News Perspect **11**(5): 287-90.

Salem, N., Kim, H. Y., Yergey, J. A. (1986). Docosahexaenoic acid: membrane function and metabolism. Docosahexaenoic Acid: Membrane Function and Metabolism. A. P. Simopoulos, Kifer, R. R., Martin, R. E. (Eds). New York, Academic Press: pp 263-317.

Salgo, M. G., et al. (1995). "Peroxynitrite causes apoptosis in rat thymocytes." Biochem Biophys Res Commun **215**(3): 1111-8.

Salvemini, D., et al. (1999). "A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats." Science **286**(5438): 304-6.

Salvesen, G. S. (2001). "A lysosomal protease enters the death scene." J Clin Invest **107**(1): 21-2.

Sametz, W., et al. (2000). "Characterization of prostanoid receptors mediating actions of the isoprostanes, 8-iso-PGE(2) and 8-iso-PGF(2alpha), in some isolated smooth muscle preparations." Br J Pharmacol **130**(8): 1903-10.

Samuelsson, B. (1991). "Arachidonic acid metabolism: role in inflammation." Z Rheumatol **50 Suppl 1**: 3-6.

Sanchez-Moreno, C., et al. (2004). "Decreased levels of plasma vitamin C and increased concentrations of inflammatory and oxidative stress markers after stroke." Stroke **35**(1): 163-8.

Sandow, S. L. (2004). "Factors, fiction and endothelium-derived hyperpolarizing factor." Clin Exp Pharmacol Physiol **31**(9): 563-70.

Sarti, C., et al. (2000). "International trends in mortality from stroke, 1968 to 1994." Stroke **31**(7): 1588-601.

Sastry, P. S. (1985). "Lipids of nervous tissue: composition and metabolism." Prog Lipid Res **24**(2): 69-176.

Sattler, W., et al. (1994). "Reduction of HDL- and LDL-associated cholesterylester and phospholipid hydroperoxides by phospholipid hydroperoxide glutathione peroxidase and Ebselen (PZ 51)." Arch Biochem Biophys **309**(2): 214-21.

Sauer, L. A., et al. (2000). "Mechanism for the antitumor and anticachectic effects of n-3 fatty acids." Cancer Res **60**(18): 5289-95.

Savaskan, N. E., et al. (2007). "Molecular biology of glutathione peroxidase 4: from genomic structure to developmental expression and neural function." Biol Chem **388**(10): 1007-17.

Sawzdargo, M., et al. (1997). "A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1." Biochem Biophys Res Commun **239**(2): 543-7.

Schleef, R. R., et al. (1988). "Cytokine activation of vascular endothelium. Effects on tissue-type plasminogen activator and type 1 plasminogen activator inhibitor." J Biol Chem **263**(12): 5797-803.

Schofield, C. (1979). Analysis and physical properties of isomeric fatty acids.

Schopfer, F. J., et al. (2005). "Nitrolinoleic acid: an endogenous peroxisome proliferator-activated receptor gamma ligand." Proc Natl Acad Sci U S A **102**(7): 2340-5.

Schreibelt, G., et al. (2007). "Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling." Faseb J **21**(13): 3666-76.

Schweizer, U., et al. (2004). "Selenium and brain function: a poorly recognized liaison." Brain Res Brain Res Rev **45**(3): 164-78.

Sebedio, J. L., et al. (2000). "The effect of dietary trans alpha-linolenic acid on plasma lipids and platelet fatty acid composition: the TransLinE study." Eur J Clin Nutr **54**(2): 104-13.

Selley, M. L. (1998). "(E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease." Free Radic Biol Med **25**(2): 169-74.

Selzer, S. (1972). cis-trans. The enzymes, vol. 6. P. D. e. Boyer. New York, Academic Press: pp 381-405.

Sennlaub, F., et al. (2003). "Cyclooxygenase-2 in human and experimental ischemic proliferative retinopathy." Circulation **108**(2): 198-204.

Shadid, M., et al. (1998). "The effect of antioxidative combination therapy on post hypoxic-ischemic perfusion, metabolism, and electrical activity of the newborn brain." Pediatr Res **44**(1): 119-24.

Sharbrough, F. W., et al. (1973). "Correlation of continuous electroencephalograms with cerebral blood flow measurements during carotid endarterectomy." Stroke **4**(4): 674-83.

Sharpe, M. A., et al. (2002). "Oxidation of nitric oxide by oxomanganese-salen complexes: a new mechanism for cellular protection by superoxide dismutase/catalase mimetics." Biochem J **366**(Pt 1): 97-107.

Sheng, H., et al. (2002). "Catalytic Antioxidants as Novel Pharmacologic Approaches to Treatment of Ischemic Brain Injury." Drug News Perspect **15**(10): 654-665.

Shuaib, A., et al. (2007). "NXY-059 for the treatment of acute ischemic stroke." N Engl J Med **357**(6): 562-71.

Sies, H. (1991). Oxidative Stress II: oxidants and antioxidants. London, Academic Press.

Siesjo, B. K., et al. (1998). Cerebrovascular disease. Cerebrovascular disease. M. D. Ginsberg and J. Bogousslavsky, Blackwell Science: p1-13.

Siesjo, B. K., et al. (1983). Fatty acid metabolism and the mechanisms of ischemic brain damage. Cerebrovascular diseases. M. Rievich and H. Hurtig. New York, Raven Press: 251-268.

Simic, M. G. (1994). "DNA markers of oxidative processes in vivo: relevance to carcinogenesis and anticarcinogenesis." Cancer Res **54**(7 Suppl): 1918s-1923s.

Simic, M. G., et al. (1988). "Introduction to peroxidation and antioxidation mechanisms." Basic Life Sci **49**: 1-10.

Simopoulos, A. P., et al. (1999). "Workshop on the Essentiality of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids." J Am Coll Nutr **18**(5): 487-9.

Sirinyan, M., et al. (2006). "Hyperoxic exposure leads to nitrative stress and ensuing microvascular degeneration and diminished brain mass and function in the immature subject." Stroke **37**(11): 2807-15.

Stadel, J. M., et al. (1997). "Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery." Trends Pharmacol Sci **18**(11): 430-7.

Stadtman, E. R., et al. (2003). "Free radical-mediated oxidation of free amino acids and amino acid residues in proteins." Amino Acids **25**(3-4): 207-18.

Stahl, W., et al. (1996). "Lycopene: a biologically important carotenoid for humans?" Arch Biochem Biophys **336**(1): 1-9.

Stamler, J. S., et al. (1997). "(S)NO signals: translocation, regulation, and a consensus motif." Neuron **18**(5): 691-6.

Stanimirovic, D. B., et al. (1994). "Arachidonic acid release and permeability changes induced by endothelins in human cerebromicrovascular endothelium." Acta Neurochir Suppl (Wien) **60**: 71-5.

Stebbins, G. T., et al. (2008). "Gray matter atrophy in patients with ischemic stroke with cognitive impairment." Stroke **39**(3): 785-93.

Stewart, G., et al. (2006). "Mouse GPR40 heterologously expressed in Xenopus oocytes is activated by short-, medium-, and long-chain fatty acids." Am J Physiol Cell Physiol **290**(3): C785-92.

Stocker, R., et al. (1987). "Bilirubin is an antioxidant of possible physiological importance." Science **235**(4792): 1043-6.

Stoka, V., et al. (2001). "Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route." J Biol Chem **276**(5): 3149-57.

Stoll, G., et al. (1998). "Inflammation and glial responses in ischemic brain lesions." Prog Neurobiol **56**(2): 149-71.

Strater, J., et al. (1996). "Rapid onset of apoptosis in vitro follows disruption of beta 1-integrin/matrix interactions in human colonic crypt cells." Gastroenterology **110**(6): 1776-84.

Strong, A. J., et al. (2002). "Spreading and synchronous depressions of cortical activity in acutely injured human brain." Stroke **33**(12): 2738-43.

Sugawara, T., et al. (2002). "Effects of global ischemia duration on neuronal, astroglial, oligodendroglial, and microglial reactions in the vulnerable hippocampal CA1 subregion in rats." J Neurotrauma **19**(1): 85-98.

Sugimoto, Y., et al. (2000). "Distribution and function of prostanoid receptors: studies from knockout mice." Prog Lipid Res **39**(4): 289-314.

Sum, C. S., et al. (2007). "Identification of residues important for agonist recognition and activation in GPR40." J Biol Chem **282**(40): 29248-55.

Swanson, R. A., et al. (2004). "Astrocyte influences on ischemic neuronal death." Curr Mol Med **4**(2): 193-205.

Szabo, C., et al. (2007). "Peroxynitrite: biochemistry, pathophysiology and development of therapeutics." Nat Rev Drug Discov **6**(8): 662-80.

Szabo, C., et al. (1996). "DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite." Proc Natl Acad Sci U S A **93**(5): 1753-8.

Taber, D. F., et al. (1997). "A nomenclature system for the isoprostanes." Prostaglandins **53**(2): 63-7.

Tagami, M., et al. (1992). "Morphological differentiation of endothelial cells co-cultured with astrocytes on type-I or type-IV collagen." Cell Tissue Res **268**(2): 225-32.

Tagaya, M., et al. (2001). "Rapid loss of microvascular integrin expression during focal brain ischemia reflects neuron injury." J Cereb Blood Flow Metab **21**(7): 835-46.

Takahashi, K., et al. (1992). "Glomerular actions of a free radical-generated novel prostaglandin, 8-epi-prostaglandin F2 alpha, in the rat. Evidence for interaction with thromboxane A2 receptors." J Clin Invest **90**(1): 136-41.

Tan, C. P., et al. (2008). "Selective Small-Molecule Agonists of G protein-coupled Receptor 40 Promote Glucose-Dependent Insulin Secretion and Reduce Blood Glucose in Mice." Diabetes.

Tan, D. X., et al. (2000). "Significance of melatonin in antioxidative defense system: reactions and products." Biol Signals Recept **9**(3-4): 137-59.

Tang, J., et al. (2005). "Role of NADPH oxidase in the brain injury of intracerebral hemorrhage." J Neurochem **94**(5): 1342-50.

Taskiran, D., et al. (2003). "Effect of carbon monoxide on dopamine and glutamate uptake and cGMP levels in rat brain." Neuropsychopharmacology **28**(6): 1176-81.

Tenhunen, R., et al. (1969). "Microsomal heme oxygenase. Characterization of the enzyme." J Biol Chem **244**(23): 6388-94.

Terashvili, M., et al. (2006). "Reactive oxygen species cerebral autoregulation in health and disease." Pediatr Clin North Am **53**(5): 1029-37, xi.

Thom, T., et al. (2006). "Heart disease and stroke statistics--2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee." Circulation **113**(6): e85-151.

Thomas, J. P., et al. (1990). "Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides." J Biol Chem **265**(1): 454-61.

Thorburne, S. K., et al. (1996). "Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress." J Neurochem **67**(3): 1014-22.

Thurston, G., et al. (2000). "Angiopoietin-1 protects the adult vasculature against plasma leakage." Nat Med **6**(4): 460-3.

Tikhonova, I. G., et al. (2007). "Validated ligand binding sites in CCK receptors. next step: computer-aided design of novel CCK ligands." Curr Top Med Chem **7**(12): 1243-7.

Tikhonova, I. G., et al. (2007). "Bidirectional, Iterative Approach to the Structural Delineation of the Functional "Chemoprint" in GPR40 for Agonist Recognition." J Med Chem **50**(13): 2981-9.

Tilley, S. L., et al. (2001). "Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes." J Clin Invest **108**(1): 15-23.

Toda, N., et al. (1996). "Neurogenic nitric oxide (NO) in the regulation of cerebroarterial tone." J Chem Neuroanat **10**(3-4): 259-65.

Traber, M. G., et al. (2007). "Vitamin E, antioxidant and nothing more." Free Radic Biol Med **43**(1): 4-15.

Traystman, R. J., et al. (1991). "Oxygen radical mechanisms of brain injury following ischemia and reperfusion." J Appl Physiol **71**(4): 1185-95.

Trepel, M., et al. (2002). "In vivo phage display and vascular heterogeneity: implications for targeted medicine." Curr Opin Chem Biol **6**(3): 399-404.

Triggle, C. R., et al. (1999). "Endothelium-derived hyperpolarizing factor(s): species and tissue heterogeneity." Clin Exp Pharmacol Physiol **26**(2): 176-9.

Tsakadze, N. L., et al. (2003). "Acrolein-induced vasomotor responses of rat aorta." Am J Physiol Heart Circ Physiol **285**(2): H727-34.

Tsang, M. C., et al. (2001). "Perinatal hypoxia-/ischemia-induced endothelin-1 mRNA in astrocyte-like and endothelial cells." Neuroreport **12**(10): 2265-70.

Uchida, K. (2003). "4-Hydroxy-2-nonenal: a product and mediator of oxidative stress." Prog Lipid Res **42**(4): 318-43.

Umeyama, T., et al. (1995). "Changes in cerebral blood flow estimated after stellate ganglion block by single photon emission computed tomography." J Auton Nerv Syst **50**(3): 339-46.

Ursino, M., et al. (1998). "Interaction among autoregulation, CO2 reactivity, and intracranial pressure: a mathematical model." Am J Physiol **274**(5 Pt 2): H1715-28.

US, V. E. (1934). "An adrenaline-like action in extracts from the prostatic and related glands." J Physiol **81**(1): 102-12.

Ushikubi, F., et al. (1998). "Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3." Nature **395**(6699): 281-4.

Utsumi, H., et al. (2000). "Expression of GFRalpha-1, receptor for GDNF, in rat brain capillary during postnatal development of the BBB." Am J Physiol Cell Physiol **279**(2): C361-8.

Vahedi, K., et al. (2002). "Thrombolysis in stroke." Curr Opin Hematol **9**(5): 443-7.

Vallet, P., et al. (2005). "Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia." Neuroscience **132**(2): 233-8.

Van Bel, F., et al. (1998). "Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electrical brain activity." Pediatrics **101**(2): 185-93.

van de Vijver, L. P., et al. (2000). "Association between trans fatty acid intake and cardiovascular risk factors in Europe: the TRANSFAIR study." Eur J Clin Nutr **54**(2): 126-35.

van Hinsbergh, V. W., et al. (1990). "Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells." Blood **75**(10): 1991-8.

van Leyen, K., et al. (2006). "Baicalein and 12/15-lipoxygenase in the ischemic brain." Stroke **37**(12): 3014-8.

Vanin, A., et al. (2007). Radicals for life. Amsterdam, Elsevier.

Vassilatis, D. K., et al. (2003). "The G protein-coupled receptor repertoires of human and mouse." Proc Natl Acad Sci U S A **100**(8): 4903-8.

Vettor, R., et al. (2008). "Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization." J Clin Endocrinol Metab.

Vila, E., et al. (2005). "Cytokines and vascular reactivity in resistance arteries." Am J Physiol Heart Circ Physiol **288**(3): H1016-21.

Vila, N., et al. (2000). "Proinflammatory cytokines and early neurological worsening in ischemic stroke." Stroke **31**(10): 2325-9.

Villa, L. M., et al. (1994). "Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart." Proc Natl Acad Sci U S A **91**(26): 12383-7.

Visocchi, M., et al. (1996). "Cerebral blood flow velocities and trigeminal ganglion stimulation. A transcranial Doppler study." Stereotact Funct Neurosurg **66**(4): 184-92.

Vite, C. H., et al. (2008). "Apparent diffusion coefficient reveals gray and white matter disease, and t2 mapping detects white matter disease in the brain in feline alpha-mannosidosis." AJNR Am J Neuroradiol **29**(2): 308-13.

Volbracht, C., et al. (2006). "Neuroprotective properties of memantine in different in vitro and in vivo models of excitotoxicity." Eur J Neurosci **23**(10): 2611-22.

Volpe, J. J. (1997). "Brain injury in the premature infant--from pathogenesis to prevention." Brain Dev **19**(8): 519-34.

Volpe, J. J. (2001). "Neurobiology of periventricular leukomalacia in the premature infant." Pediatr Res **50**(5): 553-62.

Volpe, J. J. (2001). "Perinatal brain injury: from pathogenesis to neuroprotection." Ment Retard Dev Disabil Res Rev **7**(1): 56-64.

Wagner, R. S., et al. (1997). "Characterization of signal transduction events stimulated by 8-epi-prostaglandin(PG)F2 alpha in rat aortic rings." Prostaglandins **54**(2): 581-99.

Wagner, S., et al. (1997). "Rapid disruption of an astrocyte interaction with the extracellular matrix mediated by integrin alpha 6 beta 4 during focal cerebral ischemia/reperfusion." Stroke **28**(4): 858-65.

Wahlgren, N. G., et al. (2000). "Neuroprotectants in the treatment of stroke--an overview." J Stroke Cerebrovasc Dis **9**(6 Pt 2): 32-5.

Wang, Q., et al. (2002). "Resveratrol protects against global cerebral ischemic injury in gerbils." Brain Res **958**(2): 439-47.

Wang, X., et al. (2002). "Determinants of renal microvascular response to ACh: afferent and efferent arteriolar actions of EDHF." Am J Physiol Renal Physiol **282**(1): F124-32.

Wang, Y. H., et al. (2006). "Taxifolin ameliorates cerebral ischemia-reperfusion injury in rats through its anti-oxidative effect and modulation of NF-kappa B activation." J Biomed Sci **13**(1): 127-41.

Warner, D. S., et al. (2004). "Oxidants, antioxidants and the ischemic brain." J Exp Biol **207**(Pt 18): 3221-31.

Wattanapitayakul, S. K., et al. (2000). "Endothelial dysfunction and peroxynitrite formation are early events in angiotensin-induced cardiovascular disorders." Faseb J **14**(2): 271-8.

Webersinke, G., et al. (1992). "Comparison of gene expression of extracellular matrix molecules in brain microvascular endothelial cells and astrocytes." Biochem Biophys Res Commun **189**(2): 877-84.

Wei, E. P., et al. (1996). "Mechanisms of cerebral vasodilation by superoxide, hydrogen peroxide, and peroxynitrite." Am J Physiol **271**(3 Pt 2): H1262-6.

Wei, L., et al. (2001). "Collateral growth and angiogenesis around cortical stroke." Stroke **32**(9): 2179-84.

Wieloch, T. (1985). "Neurochemical correlates to selective neuronal vulnerability." Prog Brain Res **63**: 69-85.

Williams, D. L. H. (1998). Nitrosation. Oxford, Cambridge Press.

Williams, S. E., et al. (2004). "Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel." Science **306**(5704): 2093-7.

Wilson, S. H., et al. (1999). "Enhanced coronary vasoconstriction to oxidative stress product, 8-epi-prostaglandinF2 alpha, in experimental hypercholesterolemia." Cardiovasc Res **44**(3): 601-7.

Winzell, M. S., et al. (2007). "G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes." Pharmacol Ther **116**(3): 437-48.

Wolff, R. L., et al. (1994). "Steady-state fluorescence polarization study of structurally defined phospholipids from liver mitochondria of rats fed elaidic acid." Biochim Biophys Acta **1211**(2): 198-206.

Wolff, S. P. (1993). "Diabetes mellitus and free radicals. Free radicals, transition metals and oxidative stress in the aetiology of diabetes mellitus and complications." Br Med Bull **49**(3): 642-52.

Wright, D. H., et al. (2001). "Prostanoid receptors: ontogeny and implications in vascular physiology." Am J Physiol Regul Integr Comp Physiol **281**(5): R1343-60.

Xu, L., et al. (2008). "Effects of generated trans-arachidonic acids on retinal capillary during nitrative stress in diabetic rats." Ophthalmologica **222**(1): 37-41.

Xu, W. M., et al. (1998). "Nitric oxide: from a mysterious labile factor to the molecule of the Nobel Prize. Recent progress in nitric oxide research." Cell Res **8**(4): 251-8.

Yamaguchi, T., et al. (1998). "Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group." Stroke **29**(1): 12-7.

Yamane, K., et al. (2000). "Anaerobic glycolysis is crucial for the maintenance of neural activity in guinea pig hippocampal slices." J Neurosci Methods **103**(2): 163-71.

Yamasaki, Y., et al. (1992). "Possible involvement of interleukin-1 in ischemic brain edema formation." Neurosci Lett **142**(1): 45-7.

Yamashima, T. (2008). "A putative link of PUFA, GPR40 and adult-born hippocampal neurons for memory." Prog Neurobiol **84**(2): 105-15.

Yancopoulos, G. D., et al. (2000). "Vascular-specific growth factors and blood vessel formation." Nature **407**(6801): 242-8.

Yang, G., et al. (1994). "Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia." Stroke **25**(1): 165-70.

Yang, G. Y., et al. (1994). "Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats." Stroke **25**(8): 1658-64; discussion 1664-5.

Yang, Y., et al. (2000). "Enhanced neuroprotection and reduced hemorrhagic incidence in focal cerebral ischemia of rat by low dose combination therapy of urokinase and topiramate." Neuropharmacology **39**(5): 881-8.

Yoshioka, A., et al. (1995). "Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage." J Neurochem **64**(6): 2442-8.

Yu, K., et al. (1995). "Differential activation of peroxisome proliferator-activated receptors by eicosanoids." J Biol Chem **270**(41): 23975-83.

Yuan, X. J., et al. (1995). "Inhibition of cytochrome P-450 reduces voltage-gated K+ currents in pulmonary arterial myocytes." Am J Physiol **268**(1 Pt 1): C259-70.

Yue, T. L., et al. (1992). "Neuroprotective effects of phenyl-t-butyl-nitrone in gerbil global brain ischemia and in cultured rat cerebellar neurons." Brain Res **574**(1-2): 193-7.

Zelko, I. N., et al. (2002). "Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression." Free Radic Biol Med **33**(3): 337-49.

Zghibeh, C. M., et al. (2004). "Determination of trans-arachidonic acid isomers in human blood plasma." Anal Biochem **332**(1): 137-44.

Zhang, R., et al. (2002). "Autonomic neural control of dynamic cerebral autoregulation in humans." Circulation **106**(14): 1814-20.

Zhang, Z., et al. (2002). "Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia." Trends Cardiovasc Med **12**(2): 62-6.

Zhang, Z. G., et al. (2002). "Angiopoietin-1 reduces cerebral blood vessel leakage and ischemic lesion volume after focal cerebral embolic ischemia in mice." Neuroscience **113**(3): 683-7.

Zhao, Q., et al. (1994). "Delayed treatment with the spin trap alpha-phenyl-N-tert-butyl nitrone (PBN) reduces infarct size following transient middle cerebral artery occlusion in rats." Acta Physiol Scand **152**(3): 349-50.

Zhu, D. Y., et al. (2002). "Inducible nitric oxide synthase expression in the ischemic core and penumbra after transient focal cerebral ischemia in mice." Life Sci **71**(17): 1985-96.

Zivin, J. A., et al. (1991). "Tissue plasminogen activator plus glutamate antagonist improves outcome after embolic stroke." Arch Neurol **48**(12): 1235-8.

Zou, A. P., et al. (1996). "Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K(+)-channel activity." Am J Physiol **270**(5 Pt 2): F822-32.

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3	Chrome 51	4 MBq	s/o	s/o
4	Hydrogène 3	100 GBq	s/o	s/o
5	Iode 125	10 GBq	s/o	s/o
6	Sodium 22	83 MBq	s/o	s/o
7	Niobium 95	83 MBq	s/o	s/o
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