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# ISOPROSTANES IN BRAIN ENDOTHELIAL CELL DEATH.

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## ABSTRACT

Oxygen free radicals have been implicated in several diseases including ischemic stroke, and myocardial infarction. They can trigger chain reactions like peroxidation of membrane phospholipids, leading to osmotic imbalance and cell death. Isoprostanes are stable products of lipid peroxidation that have a constrictor effect on the vasculature and bronchii. As isoprostanes are abundantly generated in tissues under oxidant stress, we have hypothesized that they could be related to endothelial dysfunction observed during ischemia/reperfusion by affecting endothelial cell survival. The effects of 8-iso-$PGE_2$ and 8-iso-$PGF_{2\alpha}$, two abundantly produced isoprostanes, were studied on porcine endothelial cultures and isolated brain microvessels. Cell survival was evaluated by MTT reduction, double staining with DNA-binding fluorochromes and *in situ* DNA fragmentation labeling,

8-Iso-$PGF_{2\alpha}$ (1-10 nM) induced 20-25% cell death in endothelial cultures after 24 h coincident with similar increase in the number of cells that become permeable to PI. On the contrary, 8-iso-$PGE_2$ did not affect endothelial cell survival. Approximately 9% of the cells suffered apoptosis. This percentage remained unchanged regardless the treatment. Several observations indicate a role for thromboxane$A_2$ to mediate 8-iso-$PGF_{2\alpha}$-induced death: 1) the levels of thromboxane $A_2$ increased dramatically in endothelial cultures after 8-iso-$PGF_{2\alpha}$-treatment; 2) inhibitors of thromboxane synthase, CGS12970 and U6355A and Ibuprofen, a non-selective inhibitor of cyclooxygenases, reverted the effect of the isoprostane. 3) analogs of thromboxane $A_2$ U46619 and IBOP, reproduce the effect of 8-iso-$PGF_{2\alpha}$ after 24 h. 8-Iso-$PGF_{2\alpha}$ also decreased endothelial viability on isolated brain

microvessels. These results suggest, that 8-iso-$PGF_{2\alpha}$, might be a direct contributor to ischemia/reperfusion injury.

## RÉSUMÉ.

Les radicaux libres de l'oxygène ont été impliqués dans plusieurs maladies d'origine ischémiques. Ils peuvent déclencher des réactions en chaîne comme la péroxydation des phospholipides membranaires, conduisant à la perte de l'équilibre osmotique et la mort cellulaire. Les isoprostanes, produits stables de la péroxydation des lipides, ont un effet constricteur sur les vaisseaux sanguins et les bronches. Leur abondance dans les tissus soumis à un stress oxydatif nous a conduit à formuler l'hypothèse selon laquelle ils pourraient intervenir dans le dysfonctionnement de l'endothélium durant l'ischémie/reperfusion en modulant la survie des cellules endothéliales. Les effets des deux abondants isoprostanes, 8-iso-$PGE_2$ et 8-iso-$PGF_{2\alpha}$, ont été étudiés sur des cultures de cellules endothéliales de porc et sur des micro-vaisseaux sanguins isolés du cerveau. Le taus de survie des cellules fut évalué par le test MTT, la double coloration avec des ligands fluorescents de l'ADN et le marquage *in situ* de l'ADN fragmenté. Après 24 h de traitement sur des cellules endothéliales en culture, 8-iso-$PGF_{2\alpha}$ (1-10 nM) induit une diminution de 20 à 25% de la population cellulaire qui coïncide avec une augmentation de la même amplitude du nombre de cellules devenues perméables au IP. En revanche, 8-iso-$PGE_2$ n'altère pas le taux de survie cellulaire. Environ 9% des cellules meurent par apoptose. Ce pourcentage demeure inchangé quel que soit le traitement. Plusieurs observations indiquent que le thrombaxane $A_2$ joue un rôle d'intermédiaire dans la mort cellulaire induite par 8-iso-$PGE_2$: 1) la quantité de thrombaxane $A_2$ augmente de façon dramatique dans les cultures de cellules endothéliales traitées par 8-iso-$PGF_{2\alpha}$; 2) les inhibiteurs de la thrombaxane synthase, CGS12970, U6355 et l'ibuprofène, un inhibiteur

inhibiteur non-sélectif des cyclooxygénases, renversent l'effet des isoprostanes; 3) les analogues du thrombaxane $A_2$, U46619 et IBOP, reproduisent les effets de 8-iso-$PGF_{2\alpha}$ après 24 h. Ce dernier diminue également la viabilité des micro-vaisseaux sanguins isolés du cerveau. Ces résultats suggèrent que 8-iso-$PGF_{2\alpha}$ contribue directement aux dommages associés à l'ischémie/reperfusion.

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## LIST OF ABBREVIATIONS

AD -adenine dinucleotide

ANOVA -analysis of variances

Apaf-1 -protease activating factor

ATP-adenosine triphosphate

ATPase-adenosine triphosphatase

$C_7$, $C_{10}$, $C_{13}$ -carbon atom #

$Ca^{2+}$-calcium ion

CAT -catalase

CoA- coenzyme A

COX-1 -constitutive cyclooxygenase

COX-2 -inducible cyclooxygenase

CSF -cerebrospinal fluid

Cu-Zn SOD -supper-zinc superoxide dismutase (SOD1)

DAPI- 4'-6 -diamidino-2-phenylindole dihydrochloride

DMEM -Dulbeco's modified essential medium

DNA -deoxyribonucleic acid

DUTP -deoxyuridine triphosphate

$EC_{50}$ -concentration of drug that produces 50% of maximal effect

EGM -Endothelial growth medium

ET-1 -endothelin-1

Factor VIII -von Willebrand Factor

$Fe^{2+}$-ferrous ion

$Fe^{3+}$-ferric ion

FBS -Fetal bovine serum

FITC -fluoresceine iso-thiocyanate

GFAP -glial fibrillary acidic protein

GPx -glutathione peroxidase

GSH -reduced glutathione

$H_2O_2$ -hydrogen peroxide

HBSS -Hank's balanced salt solution

$HOO^{\bullet}$ -hydroperoxyl radical

HHTrE -hydroxylated fatty acids

HRP -horseradish peroxidase

ICE -interleukin1-α converting enzyme

IgG- immunoglobulin G

$IP_3$ -inositol triphosphate

$K^{+}$-potassium ion

KCL -potassium chloride

$KH_2PO_4$ -potassium dihydrogen orthophosphate

LDL -low density lipoprotein

$LO^{\bullet}$ -lipid radical

LOX -lipoxygenase

$Mg^{2+}$-magnesium ion

MAPK -mitogen activated protein kinase

MDA -malondialdehyde

Mn SOD -manganese superoxide dismutase (SOD2)

MTT- 3-(4,5- dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

$Na^+$-sodium ion

NaCl -sodium chloride

$Na_2HPO_4$ -disodium hydrogen orthophosphate dibasic

NADH -reduced nicotinamide adenine denucleotide

NADPH -reduced nicotinamide adenine denucleotide phosphate

μ M -micromolar

nM -nanomolar

$NO^\bullet$ -nitric oxide

$NO_2$-nitrogen dioxide

$NO_2^-$ -nitrite

$NO_3^-$ -nitrate

NOS -nitric oxide synthase

$O_2$ -oxygen (triplet oxygen)

$O_2^\bullet$-superoxide anion

$^1O_2$ -singlet oxygen

$OH^\bullet$-hydroxyl radical

$ONOO^\bullet$-peroxinitrite acid

ONOOH -peroxinitrous acid

PAF -platelet activating factor

PBS -phosphate buffer saline

PCD -programmed cell death

$PGF_{2\alpha}$ -prostaglandin F

$PGE_2$ -prostaglandin E

$PGG_2$ -prostaglandin $G_2$

$PGH_2$ -prostaglandin $H_2$

$PGI_2$ -prostacyclin

PGHS -prostaglandin H synthase or cyclooxygenase

PI -propidium iodide

$PLA_2$ -phospholipase $A_2$

PLC -phospholipase C

$R^{\bullet}$-free radical

RNA -ribonucleic acid

Rnase -ribonuclease

SOD -superoxide dismutase

TP -thromboxane $A_2$ receptor

tRNase -transfer ribonuclease

$TxA_2$ -tromboxane $A_2$

Vitamin C -ascorbic acid

Vitamin E -α-tocopherol

XO -xanthine oxidase

# 1. INTRODUCTION.

Hypoxic/ischemic injury is related to several pathological conditions such as myocardial infarction, cerebral vascular accidents and retinopathies, leading causes of death and chronic disability. Experimental studies have suggested that the insult resulting from ischemia be amplified during tissue reperfusion. Decreased oxygen pressure resulting from ischemia, leads to depletion of energy stores in form of ATP. In addition, an incremented generation of oxygen free radicals occurs. Free radicals interact with critical biological molecules such as DNA, proteins and membrane phospholipids eventually leading to cell death. The vascular endothelium, in direct contact with changing oxygen tension in the blood, becomes the first potential target to free radical deleterious effects, however, the mechanisms mediating endothelia dysfuction following ischemia /reperfusion are not totally understood.

Peroxidation of membrane phospholipids by oxygen free radicals produces prostaglandin isomers known as isoprostanes among other compounds. The best known property of isoprostanes is their vasoconstrictor effect in heart, lung, kidney, brain and retina. In this work, the involvement of isoprostanes in cell death is discussed and a possible mechanism by which these compounds could mediate brain endothelial damage is presented.

## *1.1 The free radicals.*

Free radicals are atoms or molecules that contain one or more unpaired electrons in their orbitals (Halliwell and Gutteridge, 1984). In general, free radicals are very reactive

molecules and may extract electrons from neighboring compounds in order to pair their odd electrons. Molecular oxygen is a di-radical that in its ground state contains two unpaired electrons, each located in a separate orbital and having an identical spin. Direct reaction of oxygen with other compounds whose paired electrons rotate in opposite directions has a "spin restriction", since two electrons can not occupy the same orbital having different spins. Electrons in the oxygen molecule may invert their spin by absorbing energy resulting in the formation of singlet oxygen ($^1O_2$). Also the oxygen can accept single electrons from transition metals like iron, which promote free radical formation (Fig. 1)(Grisham, 1992; Olanow, 1993; Jacobson, 1996).

Formation of free radicals is not restricted to oxygen derivatives. There are reactive metabolites of nitrogen, specially nitric oxide ($NO^{\bullet}$) that have been implicated in physiological and pathological processes (Furchgott and Zawadzki, 1980; Moncada ET al., 1991.)

### *1.2 The non-lipidic free radicals.*

#### A) The superoxide anion ($O_2^{\cdot -}$)

The superoxide anion is a relatively unstable free radical produced by the addition of one electron to the oxygen molecule (Fig.1). This anion exists in equilibrium with its conjugate acid, the hydroperoxyl radical ($HOO^{\bullet}$). At physiological pH, practically all superoxyde is unprotonated (Halliwel and Gutteridge, 1984). However at low pH occurring in phagocytes leukocyte, phospholipid bilayers and the ischemic tissue for example, the concentrations of hydroperoxyl radical can increase significantly. In

aqueous environment and physiologic pH, the superoxide anion is rapidly and spontaneously dismutated yielding hydrogen peroxide ($H_2O_2$) and $O_2$:

$$O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$$

This reaction seems to be important in several pathological processes because $H_2O_2$ passes through biological membranes and converts to more reactive and harmful oxidants like hydroxyl radical ($OH^{\bullet}$). Superoxide may act as both oxidant and reductant. In addition, it may inactivate several enzymes such as tRNase, RNase, glyceraldehyde-3-phosphate dehydrogenase and aconitase (Klebanoff, 1985; Gardner and Fridovich, 1991).

Several enzymes produce superoxide anion, among them, nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), found in leukocytes, macrophages, platelets and endothelial cells (Babior, 1978). Cyclooxygenases, lipoxygenases (Kukreja et al., 1986), nitric oxide synthases (Pou et al., 1992) and xanthine oxidase also produce superoxide (Fridovich, 1970). Not less important sources of superoxide anion are several dehydrogenase and peroxidase activities (McCord and Fridovich, 1968), the auto-oxidation of catecholamines (Misra and Fridovich, 1972) and the ionizing radiation when traversing oxygen-containing aqueous solutions (Marklund, 1985). In addition, superoxide is produced by cytochrome $P_{450}$ (Estabrook and Werringloer, 1976).

### B) Hydrogen Peroxide ($H_2O_2$)

Hydrogen peroxide is not properly a free radical because it has not unpaired electrons, however it is stable oxidant that may react with practically all biorganic molecules. It is formed by direct two-electron reduction of oxygen in the presence of protons or by

dismutation of superoxide anion. Hydrogen peroxide is lipid soluble thus traversing biological membranes. Hydrogen peroxide reacts with transition metals such as iron or copper producing hydroxyl radical or ferryl derivatives. $H_2O_2$ inactivates directly some enzymes at slow rate by oxidizing sulfhydryl groups, but its deleterious effects seem to be mediated mainly by formation of more reactive radicals (Grisham, 1992).

C) Hydroxyl Radical ($OH^{\bullet}$)

As previously mentioned, the hydroxyl radical is an extremely unstable and reactive molecule formed by the reaction of $H_2O_2$ and $O_2^{\cdot-}$ catalyzed by transition metals like iron and copper or their low molecular chelates such as ADP-$Fe^{3+}$ and citrate-$Fe^{3+}$, via the Fenton and the Haber-Weiss reactions (Halliwel and Gutteridge, 1984).

$$\mathbf{O_2^{\cdot-} + Fe3^{+} \rightarrow O_2 + Fe^{2+}} \quad \textbf{( Haber-Weiss)}$$

$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + O_2} \quad \textbf{(Fenton)}$$

Hydroxyl radical may also be produced by interaction of nitric oxide ($NO^{\bullet}$) and superoxide forming the peroxinitrite anion ($ONOO^{\bullet}$) which at physiological pH gets transformed to peroxinitrous acid (ONOOH) (Beckman et al., 1990). This acid is very unstable and will subsequently decompose to hydroxyl radical and nitrogen dioxide ($NO_2$):

$$\mathbf{O2^{\cdot-} + NO^{\bullet} \rightarrow ONOO^{\bullet} + H^{+} \rightarrow ONOOH \rightarrow OH^{\bullet} + NO_2}$$

Water decomposition by ionizing radiation also yields hydroxyl radical (Southorn and Powis, 1988).

D) Nitric oxide ($NO^{\bullet}$)

The nitric oxide radical is involved in several physiological processes including vasodilatation (Furchgott and Zawadzki, 1980), synaptic transmission (Garthwaite et al., 1988), platelet aggregation (Azuma et al. 1986) and cytotoxicity mediated by macrophages (Hibbs et al., 1987). $NO^{\bullet}$ is produced from L-arginine by nitric oxide synthase (NOS). The enzyme catalyzes the conversion of L-arginine to L-citruline in presence of oxygen and NADPH (Palmer et al., 1988). In the vascular endothelium, $NO^{\bullet}$ is released by changes in blood flow, hypoxia, or following receptor activation by ligands such as acetylcholine, thrombin and bradykinine (Moncada et al., 1991). Under physiological conditions, $NO^{\bullet}$ may bind hemoglobin or guanylate cyclase. The reaction of $NO^{\bullet}$ with another oxygen molecule gives rise to nitrogen dioxide ($NO_2$) that in aqueouse solution gets transformed into nitrite ($NO_2^-$) and nitrate ($NO_3^-$) (Moncada et al, 1991). $NO^{\bullet}$ also reacts with $O_2$ to form peroxinitrite anion (Blough and Zafiriou, 1985).

## *1.3 The biological sources of free radicals*

A) Mitochondria

The mitochondrial respiratory chain transforms the $O_2$ in $H_2O$ by a sequence of oxidation-reduction reactions in order to produce the energy in form of ATP. This energy is required for the cell metabolism. There are three components in the chain in which the univalent reduction of $O_2$ may take place (Freedman and Crapo, 1982): the NADH dehydrogenase complex, the ubiquinone-cytochrome b region (Turrens et al., 1982) and

the dihydroorotate dehydrogenase (Forman and Kennedy, 1976). Formation of free radicals is proportional to the $O_2$ concentration. Their basal levels may increase from 1-2%, to a 25% in hyperoxia (Turrens et al., 1982).

### B) Cytosol

Thiol group-containing proteins, hydroquinones, cathecholamines and flavines found in the cytosol, may auto-oxidize producing important amounts of intracellular $O_2^{\cdot}$ and $H_2O_2$. Besides, several cytosolic and microsomal enzymes like xanthine oxidase, aldehyde oxydase, flavoprotein dehydrogenase, tryptophane dioxygenase and nitric oxide synthase also produce free radicals (Freeman and Crapo, 1982).

### C) Peroxysomes

Peroxisomal aminoacid oxidases and uric acid oxidase are important in the formation of $H_2O_2$, transformed in $H_2O$ by catalase (Jones, 1982).

### D) Plasma membrane

The high abundance of polyunsaturated fatty acids present in biological membranes makes them very susceptible to be attacked by oxygen free radicals, a situation that may compromise the cell integrity. Cyclooxygenases and lipoxygenases, the enzymes that participate in the arachidonic acid transformation into prostaglandins and leukotrienes

respectively, may form lipidic and non-lipidic radicals like hydroxyl radical (Freeman and Crapo, 1982).

### E) Cytochromes

The cytochromes $P_{450}$ and b5 present in the endoplasmic reticulum, microsomes and the nuclear membrane participate in auto-oxidations becoming sources of superoxide and hydroxyl radicals, hydrogen peroxide and peroxyl radical (Estabrook et Werringloer, 1976; Freeman and Crapo, 1982).

### F) Leukocytes

Several stimuli activate NADPH oxydase present in leukocytes and macrophages. This enzyme converts molecular oxygen in superoxide and hydrogen peroxide for bactericidal function (Babior, 1984).

### G) Other biological sources of free radicals

Endothelial cells, macrophages and neutrophils can produce superoxide, NO and peroxinitrite by their combination (Moncada et al., 1988). Moreover, degradation of hemoglobin and myoglobine by excessive $H_2O_2$ production may release iron that contributes to the formation of hydroxyl radical.

## *1.4 The antioxidant systems*

In aerobic organisms, there is a continuos production of oxygen reactive species derived from metabolic reactions. This low proportion of oxidants is neutralized by several antioxidant systems. There are two categories of antioxidants: the enzymatic antioxidants, that inactivate free radicals that can initiate peroxidations, and the non-enzymatic antioxidants that can inactivate free radicals that may start reduction processess.

### 1.4.1 Enzymatic antioxidants

Several enzymes accomplish an antioxidant function, formed by superoxide dismutase (SOD), catalase (CAT), and gluthatione peroxidase (Gpx).

**A) Superoxide Dismutases (SOD's).**

There are two main metalloproteins with superoxide dismutase activity in the cells. Both transform superoxide anion in hydrogen peroxide and molecular oxygen (Fridovich, 1978):

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

These metalloproteines are the copper-zinc cytosolic super oxide dismutase (Cu-Zn SOD or SOD1), and the manganese (MnSOD or SOD2) superoxide dismutase, found in mitochondria. A third form of SOD is present in the extracellular space (plasma, lymph and cerebrospinal fluid (McCord, 1974).

### B) Catalase (CAT)

Catalase is a heme containing protein found in peroxisomes that eliminates hydrogen peroxide and transforms it in water through the following reaction:

$$\text{Catalase-Fe}^{3+} + H_2O_2 \rightarrow \text{Compound I}$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase} + Fe^{3+} + H_2O + O_2$$

The affinity of this hemoprotein for hydrogen peroxide is low, thus is very efficacious at high concentrations of its substrate (Southorn and Powis, 1988).

### C) Glutathione peroxidase (GPx)

This enzyme eliminates low concentrations of hydrogen peroxide, since it has a high affinity for this substrate. In addition GPx plays a role in elimination of peroxides that proceed from the propagation of lipid peroxidation. The enzyme requires reduced glutathione as a cofactor. Glutahione is a tripeptide constituted by glutamic acid, glycine and cysteine. Glutathione gets oxidized and is regenerated by the enzyme glutathione reductase (Southorn and Powis, 1988. GPx is found in mitochondria and cytosol (Flaherty and Weisfeldt, 1988).

Recent experiments performed in transgenic animals have shown that overexpression of GPx and Cu-ZnSOD gene decreases the adverse effects of ischemia /reperfusion in brain and myocardium respectively. In the first study it was observed that the size of infarction decreased in mice overexpressing GPx after a focal ischemic. A second study revealed that SOD1 overexpression improved the heart contractile function and decreases the size of infarction. Also it was observed a smaller generation of hydroxyl radical

during reperfusion and a better recovery of the level of high energy phosphates such as ATP and phosphocreatinine (Wang et al., 1998 and Weisbrot-Lefkowits et al., 1998)

### 1.4.2 Non-enzymatic antioxidants

The vitamins E and C, the carotenoids, the thiol-containing molecules, the ceruloplasmine, the transition metal binding compounds and others compose this group of antioxidants.

#### A) Vitamin E (α-tocopherol)

This vitamin may inactivate superoxide, hydroxyl, singlet oxygen, peroxyl and alkoxyl radicals. It has a liposoluble structure that allow it to intercalate into lipidic membranes protecting them from peroxidation (Ozawa et al., 1978)

#### B) Vitamin C (ascorbic acid)

Vitamine C may eliminate superoxide, hydroperoxyde, hydroxyl singlet oxygen, alkoxyl radicals and hypochlorous acid. It possesses a hydrosoluble structure, and can be found in extracellular fluids and in certain organs like the eye (Varma ET al., 1984; Halliwel and Gutteridge, 1990). It may inhibit peroxidation by interfering with myoglobine and hemoglobine degradation and also to protect plasmatic membrane from

free radicals released by activated neutrophils. Vitamin C may regenerate oxidized α-tocopherol, contributing to the antioxidant function of the latter.

### C) Carotenoids

Carotenoids terminate peroxidation reactions and also eliminate singlet oxygen originating from photochemical reactions. The most important carotenoids are β-carotene, lycopene and luteine (Halliwell, 1990).

### D) Thiol-containig compounds

Some thiol-contaning compounds such as cysteine, gluthathione and some proteins, may neutralize free radicals by donating electrons from their sulfhydryl bonds (Freeman and Crapo, 1982).

### E) Ceruloplasmine

This plasma protein plays an important role in iron metabolism by its ferroxydase activity that avoids formation of oxygen free radicals during $Fe^{3+}$ oxidation to $Fe^{2+}$. Also it may bind inespecifically copper ions. Due to its properties ceruloplasmine prevents the formation of hydroxyl radical and thus lipid peroxidation (Gutteridge and Stokcs, 1981).

### F) The metal-binding compounds

Several plasmatic proteins like transferrine, lactoferrine, albumin and ferritine may bind metals such as iron and copper thus inhibiting the free radical formation and peroxidation. In addition, bilirubine that is bound to albumin may avoid peroxidation of fatty acids transported by albumin (Halliwell and Gutteridge, 1990).

### G) Other antioxidants

Other compounds found both inside the cell and in plasma may possess antioxidant properties. This is the case of uric acid, haptoglobine/hemopexine and glucose and the ubiquinol-10 (Halliwel and Gutteridge, 1990; Frei ET al., 1990).

The antioxidant systems in general neutralize most free radicals generated in the organism. If production of reactive species overcomes the antioxidant capacity, the organism suffers oxidant stress. This situation may follow hyperoxia, for example in new born individuals, where the antioxidant defenses are not totally developed, and has been proposed as the causes of retinopathies of prematurity and periventricular leukomalacia (Nielsen et al., 1988; Oliver and Newsome, 1992).

## *1.5 Reaction of free radicals with cellular components*

Free radicals react with proteins, nucleic acids and membrane phopholipids altering their structure, which may cause loss of ionic balance and cell death. In this section some of these effects will be described.

### A) Proteins

Sulfhydryl and aromatic groups present in proteins are susceptible to be oxidized by free radicals. These changes produce chain fragmentation, alteration of secondary and tertiary structure, inactivation of catalytic sites and decrease in protein solubility by exposition of hydrophobic regions. Loss of protein solubility is believed to be directly associated to cataractogenesis (Taylor and Davies, 1987).

### B) Nucleic acids and DNA

DNA damage induced by free radicals has been observed in several systems. Hydrogen peroxide, may induce DNA damage and apoptosis probably through generation of hydroxyl radical (Gardner et al., 1997). DNA purine and pyrimidine bases and as well as desoxyribose are substrates for hydroxylation which may increment mutations and chromosomic aberrations. In addition the adverse effects of free radicals eventually may induce separation of DNA chains with a consequent alteration in protein synthesis (Teoule, 1987; Ryter et al., 1990).

### C) Peroxidation of membrane phospholipids

Peroxidation is a free radical auto-oxidation with the polyunsaturated fatty acids (Fig. 2). The reaction is initiated when a free radical ($R^{\bullet}$) attracts a hydrogen atom from a carbon

atom on the fatty acids forming in this manner an alkyl radical that will undergo a rearrangement in its double bonds resulting in the formation of a conjugated diene (Fig. 2A). The reaction of a conjugated diene with an oxygen molecule will form a peroxyl radical and a hydroperoxide. A chain reaction can be then established in which peroxyl radical in turn may extract hydrogen another carbon atom forming a conjugated diene. In this manner, the reaction will be propagated until an antioxidant molecule will react with a free lipidic radical (Fig.2 B) (Flaherty and Weisfeldt, 1988, Southorn and Powis, 1988). Hydroperoxides formed in presence of metallic ions produce aldehydes such as malondialdehyde (MDA), also alkanes and alcoxyl may contribute to the propagation phase will be formed.

Lipid peroxidation products are implicated in inflammation, edema, and changes in vascular permeability, modification in function of membrane receptors, ionic channels and enzymes (Del Maestro, 1980). There is also a direct implication in cell death, as illustrated in the case of 4-hydroxynonenal, an aldehyde product involved in neuronal apoptosis (Kruman et al., 1997).

### *1.6 The isoprostanes*

The isoprostanes were discovered by Morrows and Roberts early in this decade (Morrow et al., 1990a). They are prostaglandin isomers formed by free radical-mediated peroxidation of arachidonic acid. It is known that this type of reaction may also give rise to D2, $E_2$, $F_2$ isoprostanes, isothromboxanes and isoleukotrienes (Morrow et al., 1994;

Harrison and Murphy, 1995; Serhan, 1996). So far, the best-characterized compounds in terms of biological activity are the isoprostanes.

### 1.6.1. Formation of isoprostanes

Isoprostanes are formed *in vitro* and *in vivo* by a process in which a free radical ($R^{\bullet}$) extracts one electron from a carbon-hydrogen bond possibly at $C_7$, $C_{10}$ or $C_{13}$ on the arachidonic acid structure (Fig. 3A) when it is still esterified on the membrane glycerophospholipids (Morrow and Roberts, 1996). A lipid radical ($LO^{\bullet}$) is formed and upon its reaction with an oxygen atom, will form a peroxyl radical ($LOO^{\bullet}$) (Fig. 3 B). According to the carbon atom that is attacked, four isomers of peroxyl radicals may form. A second oxygen molecule is added, and a biclyclic endoperoxide is formed possessing a cyclopentane ring (Fig 3. C). This bicyclic endoperoxide may be reduced forming $F_2$-isoprostanes. On the other hand, if an spontaneous rearrangement of the structure occurs, isoprostanes $E_2$, $D_2$ or isothromboxanes might form (Morrow et al., 1994; 1996). Isoprostane lateral chains have a *cis* orientation with respect to the cyclopentane ring, contrary to cyclooxygenase-formed prostaglandins that possess a *trans* orientation. Cyclooxygenase enzymes are in not required to form isoprostanes. It Only a very small proportion of isoprostanes is formed by cyclooxygenase activity in platelets and serum (Pratico ET al., 1995; Wang et al., 1995). Isoprostanes are stable molecules found in plasma, urine, CSF, bile, lymph, bronchoalveolar and sinovial liquid (Morrow and Roberts, 1996). Therefore, they are very reliable indicators of oxidant stress. It is

believed that phospholipase $A_2$ may release isoprostanes in free form after their formation (Morrow et al., 1992a).

In basal conditions, the amount of isoprostanes formed in humans exceeds the amount of cyclooxygenase formed-prostaglandins (Morrow and Roberts, 1996). Oxidant stress produced by smoking or intoxication may increase up to 250-fold the amounts of isoprostanes found in tissues (Salahudeen et al., 1995; Longmire et al., 1994b; Morrow et al., 1992b; Morrow et al., 1990b).

The type of isoprostanes formed varies in different tissues: $F_2$-isoprostanes are more abundant in some tissues, and in others, all $F_2$, $E_2$ and $D_2$ can be found in equivalent amounts. Generally, the $F_2$-isoprostanes are the most abundantly produced. The possible presence of an enzyme and the relative amount of glutathione in the tissue have been proposed to play a role in this preferential formation (Morrow, 1996).

### 1.6.2. Biological activity of isoprostanes

Type $F_2$ and $E_2$ isoprostanes exert a potent vasoconstriction with an $EC_{50}$ at the nanomolar range in the rat kidney (Takahashi et al, 1992). These two isoprostanes are also potent vasoconstrictors in lungs, bronchial smooth muscle (Banerjee et al, 1992; Kang et al, 1993; Wagner et al, 1997), aorta, coronaries (Wagner et al., 1997; Kromer and Tippins, 1996) and brain arterioles (Hoffman et al, 1997). Isoprostanes induce formation of inositol triphosphate increasing free cytosolic calcium. Increments in thromboxane $A_2$ formation and DNA synthesis in aortic smooth muscle cells and weak aggregation of platelets have also been reported as a result of isoprostanes treatment

(Fukunaga et al., 1993; Pratico, et al., 1996; Wagner et al., 1997). Recently, it has been shown that 8-iso-$PGF_{2\alpha}$ stimulates secretion of endothelin-1 in bovine aorta endothelium (Fukunaga et al., 1995). The effects of $D_2$ and isothromboxanes have not been elucidated.

Experiments have been directed to study the mechanisms of action of these stable oxidants. The vasomotor effects of isoprostanes may be inhibited by the thromboxane $A_2$ (TP) receptor antagonist SQ29548, suggesting that isoprostanes could act via TP receptors (Takahashi et al., 1992; Kang et al., 1993; Kromer and Tippins, 1996; Hoffman et al., 1997). Other studies have demonstrated that isoprostane effects are more important that those produced by U46619, a thromboxane $A_2$ mimetic, on its TP receptor (Fukunaga et al., 1993; Fukunaga et al., 1997). In addition 8-iso-$PGF_{2\alpha}$ acts mostly as an antagonist on platelet TP receptors (Morrow et al., 1992c). In HEK cells transfected with TP receptors, it has been observed that 8-iso-$PGF_{2\alpha}$ does not bind these receptors (Pratico et al., 1996). Therefore it has been proposed that isoprostanes might act through a receptor structurally similar but different to TP (Fukunaga et al., 1993; Longmire et al., 1994a). Recently it was found that 8-iso-$PGF_{2\alpha}$ activates two important intracellular signaling pathways in carotid arteries via TP receptors: the MAPK (mitogen activated protein kinase) pathway and the phosphorylation of the myosin light chains (Mohler et al., 1996).

In bronchii, 8-iso-$PGF_{2\alpha}$ induces constriction and plasma exudation that are abolished by thromboxane synthase inhibitors (Okazawa et al., 1997). In the retina, 8-iso-$PGF_{2\alpha}$ induces vasoconstriction possibly via a receptor operated calcium channel. This constriction involves secondary release of thromboxane $A_2$ and endothelin-1 in endothelial cells (Lahaie, et al., 1998).

Isoprostanes are proposed to directly mediate cellular damage, but there are not studies concerning toxicity so far. A recent report has shown derangement in the barrier function of endothelial cell monolayers in pulmonary artery induced by 8-iso-$PGF_{2\alpha}$, however, a brief two hour-exposure to the isoprostane, did not have any adverse effect on endothelial cell survival (Hart, et al., 1998). This is an interesting finding, since 8-iso-$PGF_{2\alpha}$, increases during LDL oxidation and some products found in oxidized LDL may induce a delayed process of death in several cell types including endothelium. The process of cell death induced by products found in oxidized LDL may take in some cases 48 to 72 h (Sevanian et al., 1995; Lizard et al., 1996; Chang, 1998a). It could suggest that a longer time lapse might be necessary to observe the adverse effects of isoprostanes on cell viability.

### 1.6.3. The role of Thromboxane $A_2$ in biological effects of isoprostanes.

Thromboxane $A_2$, the prostanoid that mediates most of the 8-iso-$PGF_{2\alpha}$ biological effects of isoprostanes, is a 20-carbon fatty acid produced by thromboxane synthase activity on arachidonic acid released from the membrane and transformed into $PGG_2$ and $PGH_2$ via cyclooxygenase enzymes (Fig. 4). The steps of thromboxane $A_2$ formation will be described in more detail in the following paragraphs.

#### A) The arachidonic acid mobilization

Arachidonic acid is found in membrane phospholipids. After a stimulus, arachidonic acid is released by phospholipases. Phospholipase $A_2$ ($PLA_2$) cleaves arachidonic acid

from phosphatidylcholine, whereas phospholipase C (PLC) releases it from phosphatidyl inositol. In this pathway two more lipase activities are required: diacylglycerol lipase and monoacyl glycerol lipase. The most important pathway seems to be the one mediated by $PLA_2$ (Dennis, 1987).

### B) The cyclooxygenase pathway

Cyclooxygenase (COX) or prostaglandin $H_2$ synthase is the enzyme that catalyzes the conversion of arachidonic acid into $PGG_2$ (Fig. 4). The enzyme has two activities: an oxygenase activity that converts arachidonic acid into $PGG_2$ and a peroxydase activity that transforms $PGG_2$ into $PGH_2$ (Miyamoto et al., 1976; Van Der Ouderra et al., 1977). These two activities require a heme group to be present (Van Der Uderra et al., 1977; Roth et al, 1981).

During the conversion of arachidonic acid to $PGG_2$, two oxygen molecules are required. One hydrogen atom is attracted from $C_{13}$ of arachidonic acid and a lipidic radical is produced. This lipidic radical reacts quickly with an oxygen molecule and suffers a rearrangement that forms a bicyclic peroxide with *trans*-oriented lateral chains. Following, a second oxygen molecule reacts with a lipidic radical in $C_{15}$ and by reduction of an electron on the 15-hydroperoxyl radical, $PGG_2$ is formed (Hamberg and Samuelsson, 1967). The cyclooxygenase is activated by a lipoperoxides (Hemler et al., 1979; Kulmacz and Lands, 1983).

$PGH_2$ is formed by the simultaneous reduction of a $PGG_2$ hydroperoxyl group by two electrons, catalyzed by the COX peroxidase activity (Smith et al., 1991). This peroxidase

has the same spectral properties as other heme-dependent peroxidases, such as catalase, cytochrome c, peroxidase and horseradish peroxidase (Yamazaki, 1974; Dunford and Stillman, 1976); but it exhibits a high affinity by fatty acid-derived hydroperoxydes in comparison to hydrogen peroxide or tertiary hydroperoxydes like t-butyl-hydroperoxide and cumene hydroperoxide (Ohki et al., 1979; Kulmacz and Lands, 1983).

There are two cyclooxygenase isoforms: a constitutive one or COX-1 that is present in all cells, and an inducible one or COX-2 that is expressed in certain cell types after proper stimulation. COX-2 is expressed in macrophages, monocytes, fibroblasts, epithelial cells, endothelial and smooth muscle cells in blood vessels, neurons and glia in CNS, osteoblasts and female reproductor system (Herschman, 1996). Many extracellular and intracellular stimuli may induce COX-2, including mitogens, cytokines, tumour promoting and growth factors, and even synaptic stimulation (Hershman, 1996; Williams and DuBois, 1996).

COX-1 is mostly found on the endoplasmic reticulum. On the contrary, COX-2 is located on the endoplasmic reticulum and the nuclear envelope (Morita et al., 1995). It might suggest that prostaglandins formed by COX-2 could be distributed into the nuclear compartiment to modulate gene transcription (Williams and DuBois, 1996) and also explain the existence of two prostaglandin $H_2$ synthase isoforms.

### C) The thromboxane synthase reaction.

Once formed, $PGH_2$ can be metabolized to prostacyclin ($PGI_2$) via prostacyclin synthase, prostaglandins, via prostaglandin synthases ($D_2$ or $F_2$) or isomerase ($E_2$), or in

thromboxane $A_2$ through thromboxane synthase (Fig. 4). Thromboxane synthase is found in platelets, macrophages and lungs (Hamberg et al., 1975; Smith, 1987) and has some similarities to cytochrome $P_{450}$ (Ulrich and Haurand, 1983). In the conversion of $PGH_2$ to thromboxane $A_2$, the first step involves the formation of one electropositive oxygen on C-11 of $PGH_2$ followed by the separation of a peroxide group (Pace-Asciak and Smith, 1983). Malondialdehyde (MDA) and hydroxylated fatty acids are formed (HHTrE) as byproducts of this reaction (Smith et al., 1991).

### 1.6.4. Biological effects of Thromboxane $A_2$

The half -life of thromboxane is 30 sec at 37° C, and it is quickly hydrolyzed to its stable metabolite, thromboxane $B_2$ (Smith et al., 1991). Thromboxane $A_2$ acts locally in an autocrine (on the same cell) or a paracrine manner (on the neighbouring cells), on its TP receptor. Thromboxane $A_2$ is a vasoconstrictor and a stimulator of platelet aggregation and secretion of platelet granules (Smith et al, 1991). It has been suggested that thromboxane $A_2$ may play a role in ischemia/reperfusion injury since it is involved in free radical production during ischemia/ reperfusion (Matsuo et al., 1996). In addition, thromboxane synthase inhibitors have been found to prevent the structural damage in the brain after an ischemic insult (Iijima, et al., 1996).

## *1.7. Cell death and its role in development and disease*

Cell death is the loss of vital cellular structure and function. It is a fundamental phenomenon since the number of cells in a multicellular organism equals the rate of cell proliferation minus the rate of cell elimination. Cell death occurs as a physiological process during organogenesis in embryos, in cell turnover in adults or in response to various injuries (Buja et al., 1993). Depending on the context and cause cells die by one of two mechanisms: necrosis and apoptosis also known as programmed cell death (PCD) (Kerr, et al., 1972)

### 1.7.1 Necrosis

Necrosis occurs in acute non-physiological injury (e.g. in the center of an infarction or at the site of toxin action). Necrotic cells swell and lyse releasing their cytoplasmic and nuclear contents into the intercellular milieu (Fig.5A, B), thus spreading inflammation. Necrosis is important in acute injury and certain severe inflamatory responses, but it is not the mechanism by which cells normally die (Hetts, 1998). Several patterns of necrosis have been described (Buja et al., 1993).

#### A) Coagulation necrosis

Coagulation necrosis results from denaturation and coagulation of cellular proteins, and it is the basic pattern of pathologic cell death that develops in response to severe injuries like ischemia, hypoxia, chemical toxins, infections and trauma (Farber, 1982). Morphologic changes associated to this type of necrosis are cytoplasmic

hypereosinophilia and nuclar pycnosis, karyorrhexis and karyolysis. Necrosis involves cell swelling rather than shrinkage, depending on the rapidity of the injury. A late phase of necrosis consists of cell fragmentation that is the result of degradative changes of autolysis (from activation and release of lysosomal enzymes), or heterolysis, due to the action of inflammatory cells invading the necrotic tissue following cell death (Fig.5A-B) (Buja et al., 1993).

Early metabolic changes such as depletion of cellular ATP are accompanied by a series of morphological alterations that can be reversible according to the insult intensity. Some reversible changes may include condensation and clumping of chromatin (early picnosis) and intracellular edema, characterized by swelling of organelles (endoplasmic reticulum, mitochondria, and lysosomes). Some changes in nucleoli with or without changes in the chromatin have been observed.

Irreversible changes are associated with advanced changes in nuclear chromatin, mitochondrial lesions, including amorphous matrix densities (formed by osmiophilic aggregates of lipoproteins), damage in the mitochondrial cristae, and formation of calcium deposits. Finally, physical defects in plasma and organelle membranes develop (Searle et al., 1982; Trump et al., 1973).

### B) Colliquative necrosis

Colliquative necrosis is characterized by rapid liquefaction in tissues with a low content in proteins (brain tissue, for example) and/or abundance in hydrolytic enzyme activation.

### C) The membrane injury

Necrosis implicates loss of membrane integrity. There are three stages in membrane damage:

1) Discrete alterations in ionic transport systems;

2) Non specific increase in membrane permeability; and

3) Physical membrane disruption;

Early electrolyte changes associated with potentially reversible injury consist of increases in phosphate and hydrogen ion (in the form of phosphoric acid) and transient increase followed by decrease in magnesium $Mg^{2+}$. These electrolyte changes together with lactate accumulation contributing to derangement of energetic metabolism and decreasing cytosolic pH (from 7,2 to 6.5 or lower). In some cell types there is an early efflux of $K^+$ without a change in $Na^+$. In addition, early electrolyte changes lead to an increment in free cytosolic calcium, responsible for the deposits observed in mitochondria of tissue affected by ischemia/reperfusion. The late phase is characterized by changes in calcium homeostasis that is accompanied by further deterioration of $K^+$, $Na^+$, $Cl^-$ balance and $Mg^{2+}$ loss.

Membrane injury is also related to decreased oxidation of fatty acids in mitochondria and enhanced degradation of phospholipids by activated phospholipases. Arachidonic acid and lysophospholipids are released that together with other fatty acids such as long-chain fatty acyl CoA and acyl carnitines may accumulate and exert a detergent-like action, altering fluidity and function of the membranes.

Another phenomenon related to membrane injury is deterioration of cytoskeletal filaments that normally stabilize and modulate the function of plasma membrane (Burridge et al., 1988) for example loss of vinculin has been observed in the ischemic heart (Steenbergen et al, 1987). It is possible that cytoskeletal changes further spell the metabolically altered plasma membrane and predispose it to its physical disruption.

### D) Role of $Ca^{2+}$ in cell death

There are two phases of calcium alterations in the progression of cell injury: an initial increase in free cytosolic $Ca^{2+}$, and a subsequent increase in total calcium. Potential mechanisms for the early increase in free $Ca^{2+}$ include the net influx of $Ca^{2+}$ across the plasma membrane, due to altered function of the slow calcium channel and other $Ca^{2+}$ systems, and the release of calcium by mitochondria due to energy depletion of the membranes. Also $Mg^{2+}$ depletion promotes $Ca^{2+}$ accumulation. The late phase of $Ca^{2+}$ loading is due to non-specific increase in membrane permeability. Progressive increase in cytosolic free $Ca^{2+}$ may have several deleterious effects, including activation of phospholipases and proteases, activation of ATP-ases that will exhaust ATP, and mitochondrial $Ca^{2+}$ overload (inhibiting ATP production) (Buja et al., 1988; Farber, 1982; Braunwald, 1982; Clague et al., 1993).

## 1.7.2. Apoptosis

In 1972 Kerr et al, described the physiological process of apoptosis (from the Greek word for "falling off", also known as programmed cell death: PCD). Apoptosis is characterized by chromatin condensation, DNA fragmentation due to endonuclease activation, cytoplasmic blebbing, and cellular fragmentation into small apoptotic bodies that are quickly phagocytosed and digested by neighboring cells and macrophages (Fig. 5C-E) (Hetts, 1998). Apoptosis plays a role during development and contributes to the homeostasis in the adult tissue.

Ionizing radiation, hyperthermia and viral infections (< biblio >) can actively trigger Apoptosis. In addition to its developmental role, apoptosis is important in disease. Defective apoptosis has been implicated in tumorigenesis, autoimmune disorders like systemic lupus erythematosus (defective apoptosis) and infection by some viruses (Thompson, 1995). Increase in apoptosis has been related to neurodegenerative diseases, AIDS alcohol-induced liver damage and ischemic reperfusion injury, suggesting that cells far from the ischemic lesion site may die by these mechanisms.

Apoptotic cell death is an evolutionary conserved process. Much of the knowledge about apoptosis obtained so far, is due to developmental studies on the nematode *C. elegans*. In this organism, 1090 cells are born, of which 131 die by apoptosis (Ellis et al., 1991; Hetts, 1998). These studies have revealed that apoptosis consists of 4 sequential stages:

A) Commitment to death, induced by intracellular or extracellular triggers;

B) Cell killing executed by activated proteases;

C) Engulfment of the cell corpse by other cells; and

D) Degradation of the corpse into the lysosomes of phagocytic cells.

### A) Commitment to death

Several stimuli may commit cells to die. For example, DNA damage may arrest cell cycle that can be resumed if the damage is repaired. If it is not the case, apoptosis will be triggered. The gene product *p53*, involved in this mechanism is required to initiate apoptosis in response to genotoxic damage. Cells with deficient apoptotic triggering mechanisms in response to DNA damage are prone to transformation. It has been observed that the *p53* gene is mutated in several human tumors. Other mechanisms leading to apoptosis are present only in specific cell types and at certain times (e.g. in the central nervous system, growth factor withdrawal-induced apoptosis during development). In other cases, commitment to death can be triggered by extracellular death promoting ligands that bind cell surface-death receptors which intracellular portions will bind cytosolic adaptor proteins that activate proteases (Hsu et al., 1995).

### B) Execution and regulation of apoptosis

Activated caspases and nucleases execute apoptotic cell death. In C. *elegans* two genes have been found, *ced-3* and *ced-4* (Yuan and Horvitz, 1990) that promote apoptosis. Another gene, *ced-9* acts to suppress *ced-3/ced-4* mediated apoptosis (Hengartner et al, 1992). The *ced-3* gene codifies for a cysteine aspartyl protease (caspase) that when activated cleaves several proteins activating some and inactivating others. Among these substrates is DNA repairing enzymes such as polyadenosine

diphosphate ribose polymerase (PARP), components of the nuclear membrane and DNA-cleaving endonucleases responsible for DNA degradation during the apoptotic process. Caspases exist as inactive forms (procaspases) in all cells. Activation of caspases breaks the normal barriers between organelles inside the cells, but leaves the plasma membrane intact. The *ced-4* product acts upstream of *ced-3*, receiving the commitment signal to death. Following, *ced-4* binds to pro-*ced-3* causing its release and activation.

The gene product of *ced-9* is a protein similar to cytochrome b 560 of complex II of mitochondrial respiratory chain found in the outer mitochondrial membrane, that binds to *ced-4* to prevent pro-*ced-3* activation (Chinnaiyan et al., 1997).

There are mammalian homologues for some of these genes: the human protoconcogene *bcl-2* is homologous of *ced-9*, whereas the nematode gene *ced*-3 caspase is similar to the interleukin-1$\alpha$ converting enzyme (ICE). At least 10 different caspases have been isolated from human cells. Activated caspases cleave each other's precursor into mature proteolytic enzymes. Human death substrates for human caspases are nuclear proteins involved in DNA repair and replication, in RNA splicing, and in cell division. These enzymes also act on cytoskeletal components.

The human homologous for *ced*-4 is known as protease activating factor or Apaf-1 (Zou et al., 1997). When cytochrome c protein found in mitochondria binds Apaf-1, it is able to bind and activate caspase-3 initiating the apoptotic cascade. Apaf-1 has also a binding site for 5'-triphosphate (ATP), which probably will decide the mechanism of cell death that takes place, according to the cellular energetic status (energy-independent necrosis Vs energy dependent apoptosis). Apaf-1 also binds antiapoptotic members of the *Bcl-2* family, which may sequester it from caspase-3, suppressing apoptosis.

The *bcl-2* family is member of a family of proteins with different regulatory functions. *Bax* and *bad* are apoptosis-promoting genes, whereas *bcl-2* and bcl-$_{XL}$ inhibit apoptosis. The *Bcl-2* family is involved in apoptosis induced by growth factors withdrawal. There are several theories to explain the function of these proteins: *bcl-2*-like proteins may inhibit activation of caspases by binding directly to Apaf-1, by preventing release of cytochrome c from mitochondria, or both. (Wu et al., 1997; Spector, 1997). *Bcl-2* and *bax* are located in the outer membranes of mitochondria and may bind to each other to form dimers: *bcl-2: bax* heterodimers have antiapoptotic function. In contrast, *bax* homodimers promote apoptosis. These protein dimers form ion-conducting channels in the membrane: the anti-apoptotic members of the family form pores that allow ionic homeostasis in cell organelles and pro-apoptotic members of the family may interfere with channel formation (Antonsson et al., 1997).

### C) Degradation of apoptotic bodies

The last phases of apoptosis are not very well understood. Changes in the external cellular localization of proteins and lipids (like phosphatidylserine) allow apoptotic bodies to be recognized for other cells for fagocytosis. Apoptotic bodies have intact membranes and there is no leak of proinflammatory components. It is believed that defects in the final degradation of apoptotic bodies are implicated in some autoimmune disorders (Casciola-Rosen et al., 1994).

## *1.8. The structure of blood vessels*

Blood vessels are composed of three layers of tissue: an internal layer constituted by the endothelial cells that cover the vessel lumen or *tunica intima*, and depending to the vessel type, some elastic fibers, an intermediate layer or *tunica media*, formed predominantly by circular smooth muscle cells, and a external layer or *tunica adventitia*, formed by connective tissue, small blood vessels and peripheral nerves.

Endothelial cells are functionally and morphologically heterogeneous in different tissues, however, they possess common properties like a non thrombogenic luminal surface, and abluminal basement membrane and von Willebrand-Factor production (Risau, 1991; Dorovini-Zis et al., 1991). The endothelium contributes to the control of blood movement and the vessels resistance by secreting substances like endothelin and nitric oxide, that influence the smooth muscle contraction (Sabry et al., 1995; Riedel, et al., 1995).

### 1.8.1 Vascular damage induced by ischemia/reperfusion

Experiments performed early in this century show that reperfusion following cardiac ischemia, produced arrythmias not seen with ischemia alone (Tennant and Wiggers, 1935). This observation led to the idea that the effects of the ischemic insult are different and often amplified by the injury of subsequent reperfusion. During reperfusion there is a decrease in ATP content and the free radicals formation increases due to activation of enzymes, including xanthine oxydase and phospholipases. In addition, neutrophils sequestered in small vessels release proteases, elastases, and prostanoids such as platelet

activating factor (PAF) and leukotrienes. Iron, released by hemolysis may also form potent oxidants via Fenton reaction. Endothelial damage induced by reperfusion leads to increase in vascular permeability to macromolecules and loss of endothelium-mediated vasodilation. The reperfusion injury may extend to several organs, probably by release of humoral mediators and activation of circulating neutrophils (Lefer, 1987; Conger and Weil, 1994).

## HYPOTHESIS

The mechanisms that mediate vascular dysfuction following ischemia are still not totally elucidated. Excessive free radical formation has been related to this process. Isoprostanes are stable prostaglandin isomers formed by free radical-mediated peroxidation of membrane phospholipids. As levels of isoprostanes increase in tissues exposed to oxidant stress, it has been thought that these compounds are direct mediators of oxydant injury. However, the best studied biological effect of isoprostanes is constriction of smooth muscle in blood vessels and bronchi and there are not reports concerning isoprostane-induced cytotoxicity so far. Since peroxides *per se* are cytotoxic to the endothelium, we hypothesize that the products of peroxidation isoprostanes might be implicated in ischemia/reperfusion injury by inducing endothelial cell death.

## OBJECTIVES

1. To test whether isoprostanes may affect survival of brain endothelial cells.
2. To assess the nature of cell death elicited by isoprostanes.
3. To determine the possible mechanism that mediates isoprostane-induced cell death.

## 2. MATERIALS AND METHODS

### *2.1 Endothelial cell primary cultures:*

Newborn piglets were obtained from Fermes Ménard Inc (L'Ange-Gardien, QC, Canada) and used according to a protocol of the Animal Care Committee of Hôpital Sainte Justine. The animals were anesthetized with halothane (2,5%) and sacrificed with an intracardial injection of sodium pentobarbital (120mg/kg) to collect their brains. The tissue (except the cerebellum) was collected and dissected into small pieces (2-3$mm^3$) in Hank's Balanced Salt Solution (HBSS pH 7.4) that contains (mM): KCl 2.8, $KH_2PO_4$ 0.2, NaCl 68, $Na_2HPO_4$ 0.16, glucose 2.8, HEPES 100 and Phenol Red 0.01. Following, the brain tissue was centrifuged at 20 000 x g for 20 min at 4°C, in a 1:1 (v/v) proportion with a 40 % Ficoll 400 solution. The pellet that contains the microvessels was washed three times in cold HBSS. The microvessels were seeded in 75 $cm^2$ flasks in Endothelial Growth Medium (EGM, Clonetics, CA, USA) containing 5% fetal bovine serum (FBS), gentamycin (10units/ml) and penicillin (50units/ml) plus streptomycin (50 mg/ml). The cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ for several days, until a confluent monolayer of endothelial cells was observed (Dorovini-Zis et al., 1991). Purity of the endothelial cultures was evaluated by positive reactivity to Factor VIII antibody and negative reaction to the glial fibrillary acidic protein (GFAP) antibody, a marker for astroglial cells. Cell survival was studied in growth-arrested cultures (between $5^{th}$ and $10^{th}$ passage) which had been maintained in DMEM alone for 48 h before the experiments.

### *2.2 Immunocytochemistry:*

Endothelial cells were seeded on glass coverslips for 24h, washed twice with cold phosphate buffer (PBS, pH 7.2) and fixed with acetone for 10 min at -20°C. After rinsing twice in PBS, incubation with (1:100) monoclonal antihuman Factor VIII or GFAP antibodies (Dako, Carpinteria, CA) was performed at room temperature for 1h. The cells were subsequently washed for 5 minutes (three times in PBS) and goat anti mouse-FITC conjugated IgG1 (Jackson Immunoresearch Laboratories, West Grove, PA) (1:50) was applied as a secondary antibody for 1 h. Both antibodies were diluted in a buffer containing 0.1% Triton X, 5% FBS, 5% rabbit serum and 0.02% sodium azide. Cell nuclei were counterstained for 15 minutes with 1 μg/ml 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, Polysciences, Inc., Warrington, PA). Afterwards, the coverslips were washed twice in distilled water, and mounted with Immu-Mount (Shandon, PA, USA). Fluorescence was observed with a Leitz Diaplan microscope.

### *2.3 MTT assay of cell viability:*

Cell viability was estimated by reduction of 3-(4,5- dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., S. Louis, Mo, USA). The MTT soluble yellow salt is reduced to an insoluble blue formazan product by mitochondrial dehydrogenases in living cells. The amount of formazan produced is proportional to the amount of cells present (Denizot and Lang, 1986). Endothelial cells,

growing in 24-well dishes were incubated with different concentrations of the isoprostanes 8-iso-$PGE_2$ and 8-iso-$PGF_{2\alpha}$, the thromboxane $A_2$ analogs U46619 and IBOP (Cayman, Chemical, MI,USA) or hydrogen peroxide for different time periods. In other experiments, the cultures were pretreated for 30 min with two thromboxane synthase inhibitors, U6355A (Cayman, Chemical, MI, USA) and CGS12970 (Ciba-Geigy, Summit, NJ), or with ibuprofen, a non-specific inhibitor of cyclooxygenases (Sigma Chemical Co., S. Louis, Mo, USA). Once the experiments concluded, MTT (0.5 mg/ml in PBS, pH 7.2) was added to the cultures that were incubated for 2-3 h more at 37°C. Afterwards, the medium was removed, and the formazan product was dissolved with acidified isopropanol (0.04 M HCl). Optic density was determined at 600 nm. Cell viability was expressed as a percentage of values obtained from untreated cultures. Every condition was studied four times in five different cell preparations.

### *2.4 Cell counting.*

To characterize the type of cell death (necrosis or apoptosis) elicited by isoprostanes, incorporation of the DNA-binding dye propidium iodide (PI) and Hoechst 33342 was studied. Nonviable cells lose their membrane integrity and become permeable to PI that emits red flourescence. The combination of PI with Hoechst 33342, that emits blue fluorescence and binds DNA in all cells regardless their membrane state, allowed to differentiate between viable and death cells. Viable cells exhibit low blue fluorescence and also exclude PI. Early apoptotic cells exclude PI but exhibit high blue fluorescence in their condensed or fragmented nuclei. Dead cells (with damaged membranes) fluoresce

both red and blue (McGahon et al., 1995).

Endothelial cells were seeded in 6-well culture plates and incubated with different concentrations of 8-iso-$PGF_{2\alpha}$ for 6, 12, 24, 36 and 48 h. Following, cultures were incubated for 15 min at 37°C with PI (5μg/ml) and Hoechst 33342 (5μg/ml). Cells were observed with at 400X with a water immersion objective placed directly into the culture medium, using the rhodamine and UV filters. The percentage of PI (necrotic) positive cells and cells with fragmented or condensed nuclei and intact membrane (apoptotic) was determined in 5 fields per well. Each experimental condition was studied in triplicates performed in three different cell preparations.

### 2.5 In situ *labeling of fragmented DNA*

To identify cells undergoing apoptosis, *in situ* DNA labeling was performed using a comercial kit (Apoptag Direct Fluorescein Kit, Intergen, Gaithersburg, MD, USA). Endothelial cells were grown on coverslips and treated with 100 nM 8-iso-$PGF_{2\alpha}$ for different time periods. Cells were washed twice in cold PBS, fixed with 4% paraformaldehyde at room temperture for 10 minutes, washed twice in PBS and postfixed in ethanol:acetic acid (2:1) for 5 minutes at –20°C. After another washing step, equilibration buffer (supplied by the kit) was applied for 5 minutes. The cells were incubated with terminal transferase and FITC-conjugated dUTP forl h at 37°C in a humid chamber. Subsequently, the reaction was stopped by incubation with Stop/Wash buffer for 10 minutes at room temperature. Nuclei were counterstained with PI (5 μg/ml), the slides were washed in distilled water and mounted with Immu-mount. Apoptotic cells,

stained in green, were observed under a fluorescence microscope.

### *2.6 Effect of 8-iso-$PGF_{2\alpha}$ on brain microvessels*

Porcine brain microvessels were isolated as previously reported (Li et al., 1997) and allowed to attach to culture dishes overnight. Following, the microvessels were incubated for 48 h with 100 nM 8-iso-$PGF_{2\alpha}$. Endothelial cell death was assessed by PI incorporation and Hoechst 33342 staining. In order to minimize the sample variability, cell counts were performed only in microvessels formed by more than 20 endothelial cells. The experiment was performed twice and six microvessels were studied per condition.

### *2.7 Thromboxane $B_2$ assay:*

Production of thromboxane $B_2$, was determined in the culture media of endothelial cells that were seeded in 6-well culture dishes and treated with 100nM 8-iso-$PGF_{2\alpha}$ for 6, 18, 24 and 48 h. Measurements were performed by radioimmunoassay using a comercial kit (Cayman Chemical, MI, U.S.A.) as previously described (Lahaie et al., 1998). The interassay variability was $< 5\%$.

### *2.8 Statistical analysis:*

Mean values were calculated for every experimental condition. Differences between

group means were established by one-way ANOVA with post hoc analysis by Tukey-Kramer method. Statistical significance was set at $P < 0.05$. Values are presented as means $\pm$ SEM.

# 3. RESULTS

## *3.1 Effect of isoprostanes on brain endothelial cell survival:*

To determine whether isoprostanes affect survival of cerebrovascular endothelial cells, cultures originated from newborn pigs were exposed from 6 to 48 h to increasing concentrations of 8-iso-$PGE_2$ and 8-iso-$PGF_{2\alpha}$. A decrease ($EC_{50}$=1nM) in cell viability was produced by 8-iso-$PGF_{2\alpha}$ reaching a maximal 20-25% with 10 nM. In contrast, 8-iso-$PGE_2$ did not have any effect (Fig. 6A). 8-Iso-$PGF_{2\alpha}$-induced cell death was statistically significant ($p< 0.01$) after 24 h (Fig 6B).

## *3.2 Type of death elicited by 8-iso-$PGF_{2\alpha}$ on endothelial cells.*

In order to study the possible mechanism of cell death elicited by 8-iso-$PGF_{2\alpha}$, the cultures were incubated for 15 minutes with PI, a DNA- intercalating agent that can only penetrate cells with compromised membrane integrity. The state of nuclear chromatin was evaluated in endothelial cells by Hoechst 33342, that penetrates all cells regardless their membrane condition, and allows to observe DNA fragmentation o condensation, characteristics of apoptosis. In addition, apoptotic cells were identified by positive labeling of 3'OH DNA fragments. This labeling was obtained through a terminal nucleotide transferase reaction (using the APOPTAG kit). Treatment with 100 nM 8-iso-$PGF_{2\alpha}$ for 24 h increased the number of cells that incorporate PI by 20-30% with respect to control (Fig 7B, D). In contrast, the number of apoptotic cells with fragmented or

condensed chromatin, positive DNA *in situ* staining an intact membranes, was not altered by the treatment and never exceeded 8% of total (Fig.7A, D).

### *3.3 Role for thromboxane $A_2$ in 8-iso-$PGF_{2\alpha}$-induced cell death:*

To test the possibility that tromboxane $A_2$ mediates the 8-iso-$PGF_{2\alpha}$-induced cytotoxicity on endothelial cells, the content of thromboxane $B_2$, a stable metabolite of thromboxane $A_2$, was measured in the culture media after stimulation with 100 nM 8-iso-$PGF_{2\alpha}$. An 18-fold increase in the levels of this eicosanoid was registered after 6 hours treatment. A 3-fold increase was found after 48 h at the latest time point studied (Fig. 8A). In addition, pretreatment of cultures with different inhibitors of thromboxane $A_2$ synthesis for 30 minutes was performed. It was found that with 1μM Ibuprofen, a nonspecific inhibitor of cyclooxygenases or 100 nM CGS12970 and U63557A respectively, two specific inhibitors of thromboxane synthase, prevented the effect of 1nM 8-iso-$PGF_{2\alpha}$, on endothelial survival (Fig. 8B). To corroborate the previous findings, the possible cytotoxic effect of thromboxane $A_2$ was assessed. U46619 and IBOP, two analogs of thromboxane $A_2$, elicited a 15-20% decrease in endothelial cell viability 24h after exposure. This effect was maximal at 1-10 nM for IBOP and 100 nM for U46619 (Fig.8C).

### *3.4 Role of Thromboxane $A_2$ in $H_2O_2$-induced endothelial cell cytotoxicity*

As $H_2O_2$, known to cause cell damage in various cell types, also release both

isoprostanes and cyclooxygenase-derived prostanoids including thromboxane A2 (Lahaie, et al., 1997; Abran et al., 1995), we tested the possibility that the cytotoxic effect of $H_2O_2$ on endothelial cells, could be mediated to some extent by thromboxane $A_2$. Concentrations from 10 to 100 μM hydrogen peroxide produced a concentration-dependent decline in the endothelial cell population observed after 18 h exposure (Fig. 9A). Ibuprofen, U6355A and CGS12970 significantly diminished cytotoxicity elicited by hydrogen peroxide (Fig. 9B).

### *3.5 Effects of 8-iso-PGF2$_\alpha$ on isolated brain microvessels.*

In order to assess the toxic effect of 8-iso- $PGF_{2\alpha}$ on the vascular endothelium in conditions that resemble the *in vivo* situation, brain microvessels were isolated as previously described (Li, et al., 1997) and incubated with this isoprostane for 48h. In agreement with the previous results, 30 % of the cells included PI in the microvessels. Moreover, the treatment did not increase apoptosis, since there was not difference in the number of cells with intact membranes and fragmented nuclei in the microvessels exposed to the isoprostane (Fig 10).

# FIGURES

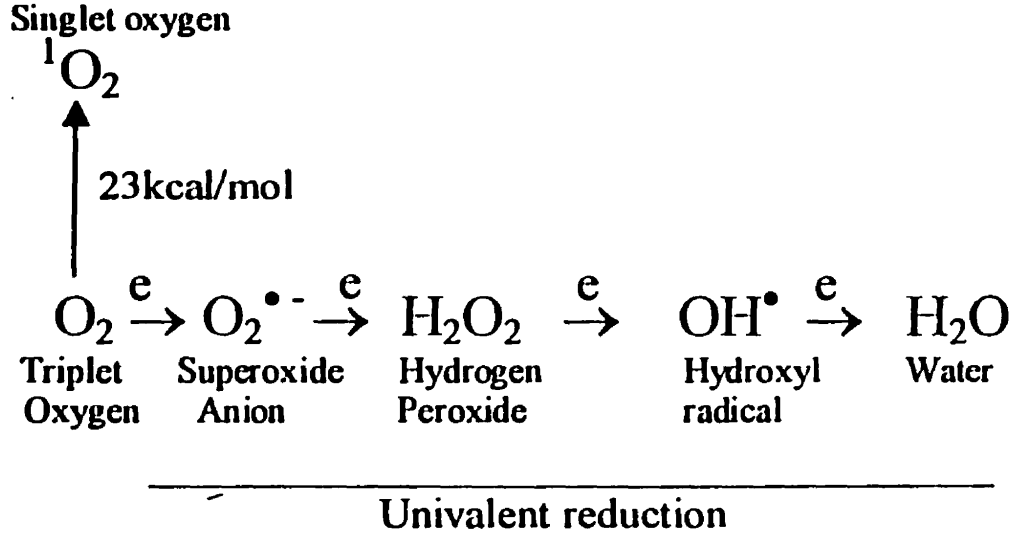



**Figure 1.** The oxygen free radicals (modified from Grisham, 1992)

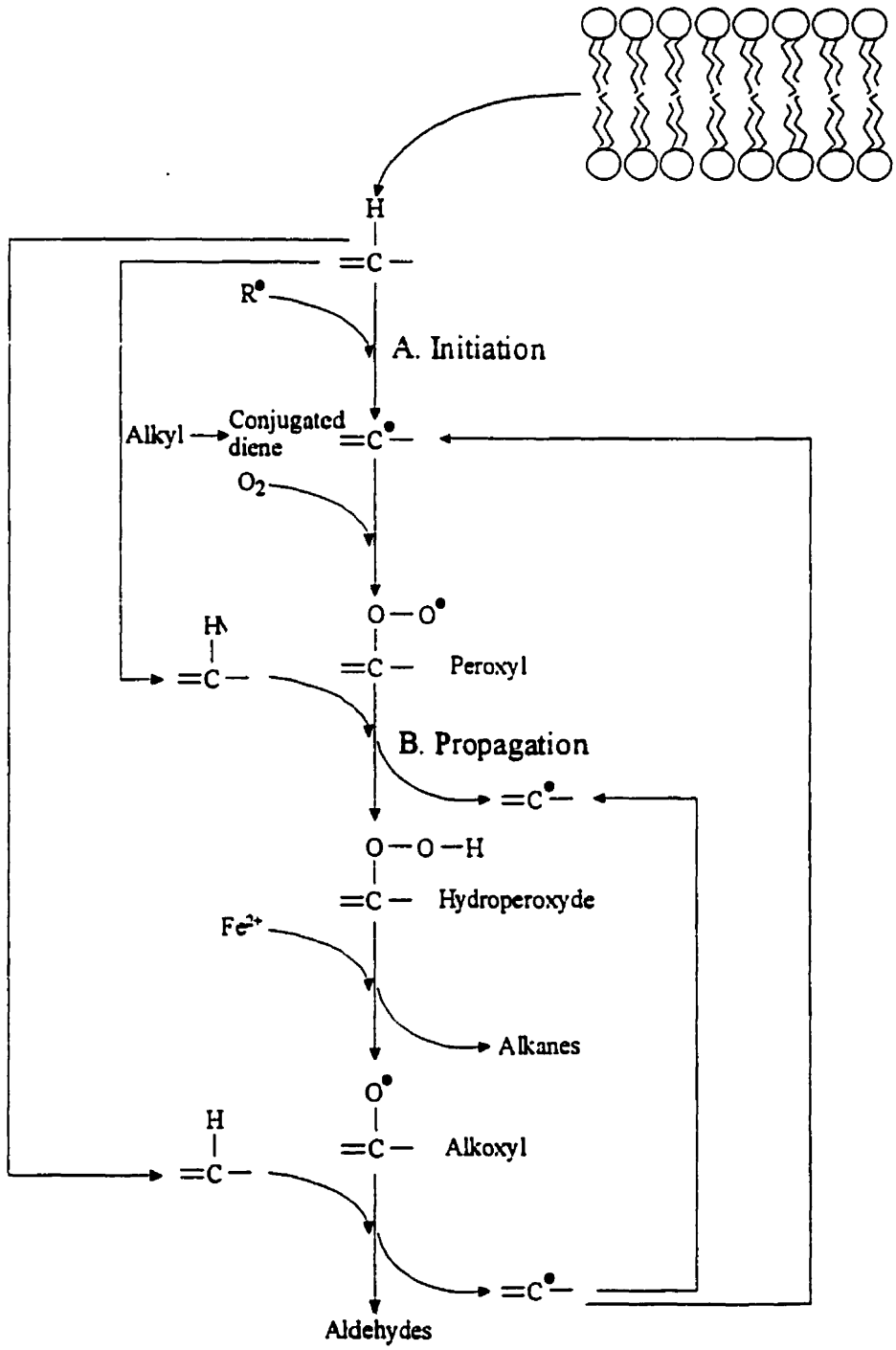



**Figure 2. Peroxidation of membrane phospholipids**

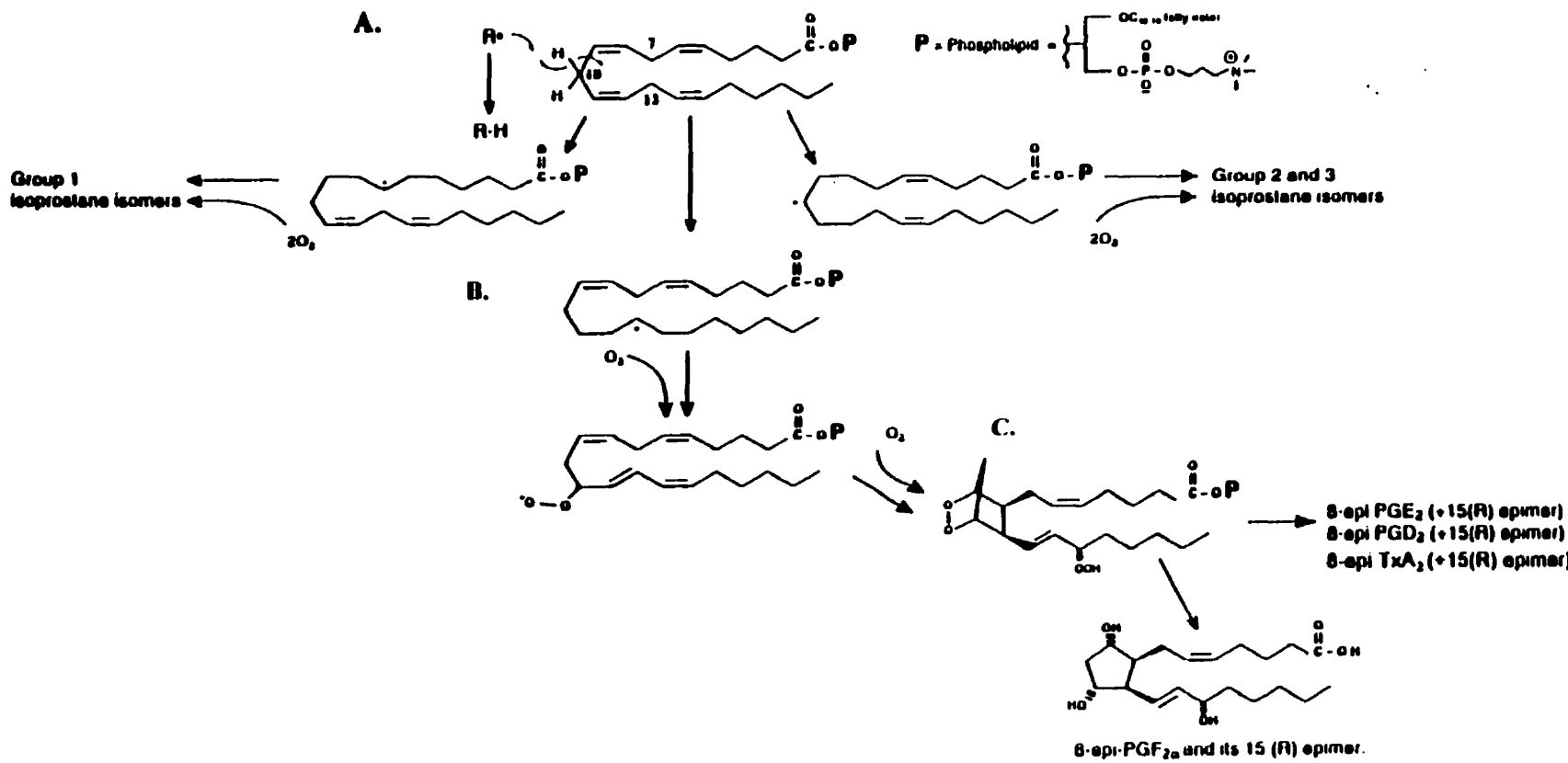



**Figure 3**. The formation of isoprostanes (modified from Lawson and Maxey, 1996)

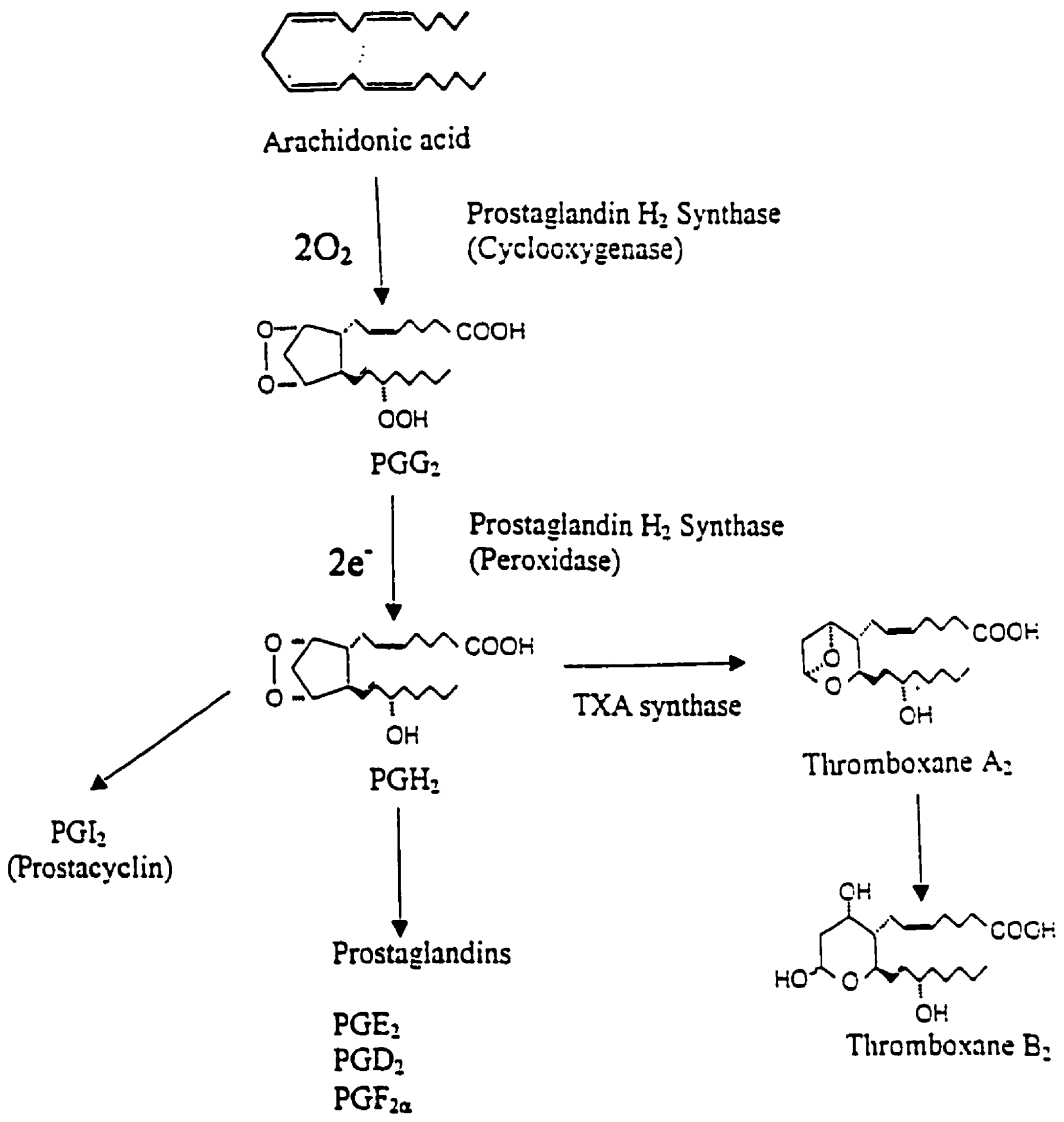



**Figure 4. The cyclooxygenase pathway.**

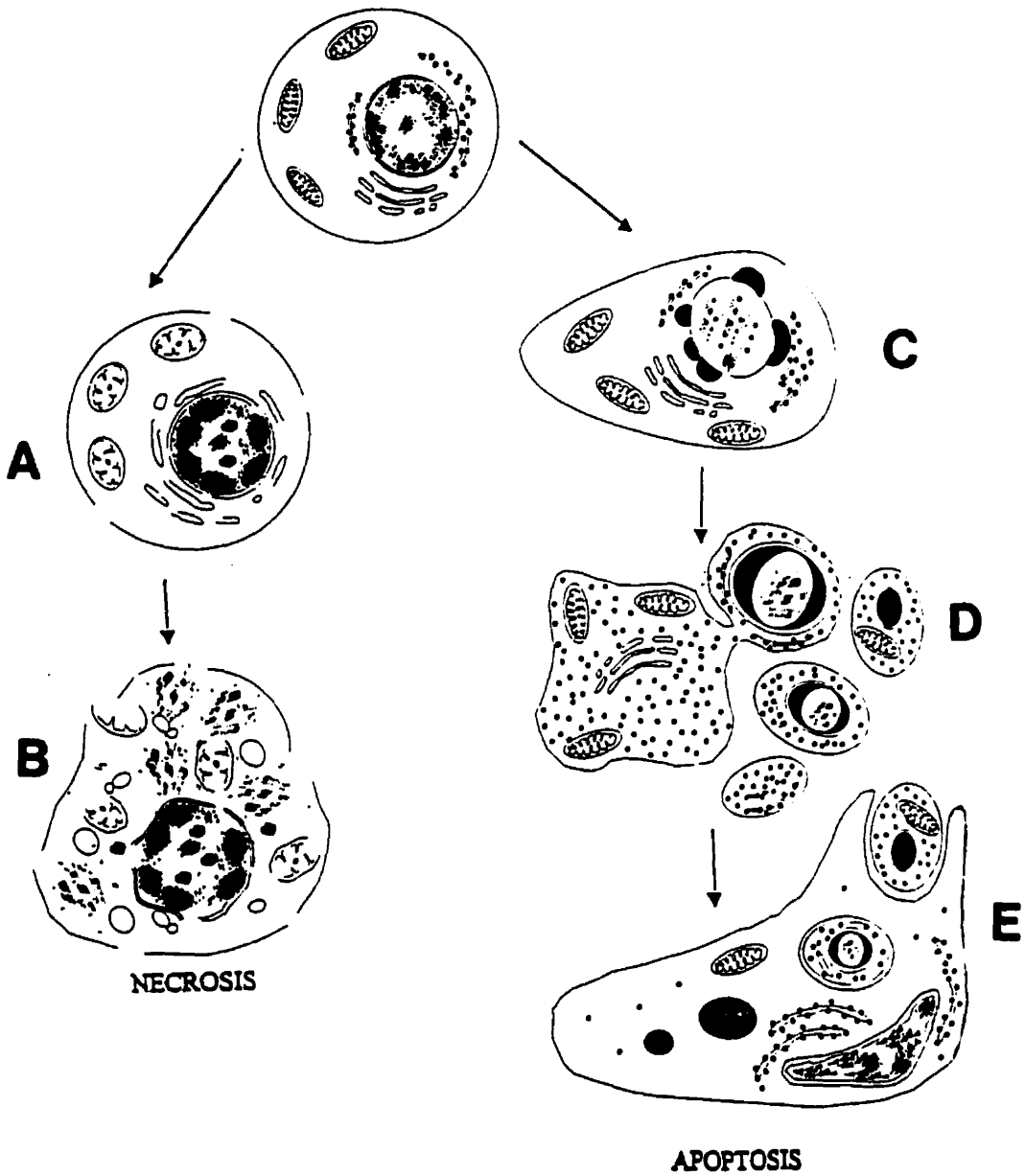
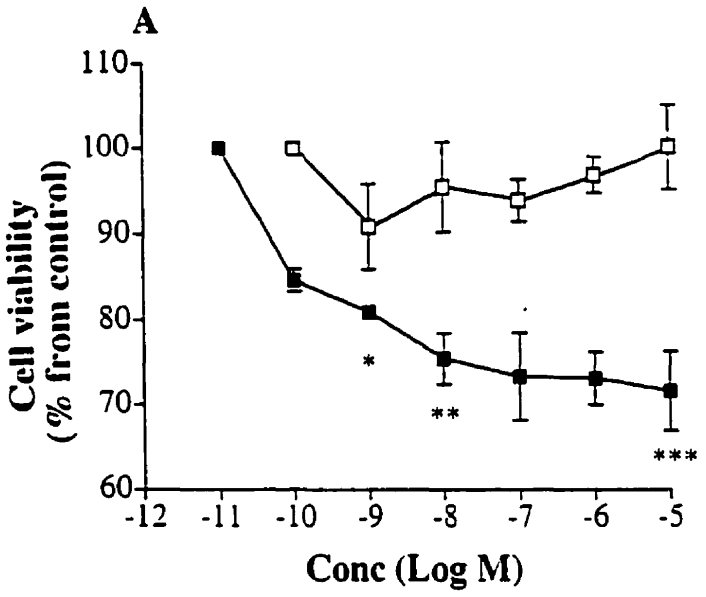



**Figure 5** Structural changes in apoptosis and necrosis (modified from Kerr et al. 1995)


A
110
100
90
80
70
60
Cell viability
(% from control)
*
**
***
-12
-11
-10
-9
-8
-7
-6
-5
Conc (Log M)

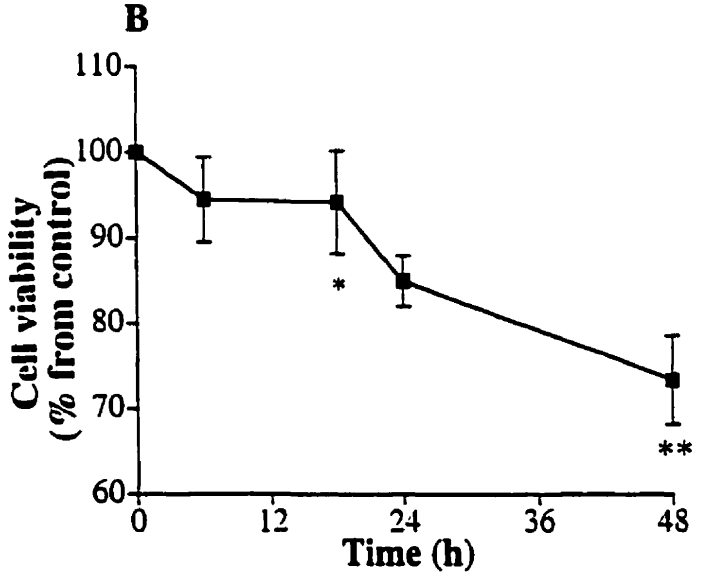

B
110
100
90
80
70
60
Cell viability
(% from control)
*
**
0
12
24
36
48
Time (h)

**Figure 6. Effect of isoprostanes on brain endothelial cell survival.**

Endothelial cells were treated for 48 h with increasing concentrations of 8-iso-$PGE_2$ (□) and 8-iso-$PGF_{2\alpha}$ (■) (A) or 100 nM 8-iso-$PGF_{2\alpha}$ for 6-48 h (B) and cell viability was assessed by the MTT assay. Values are mean ± SEM of 4 experiments each performed in quadruplicate * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with values for untreated control cultures.

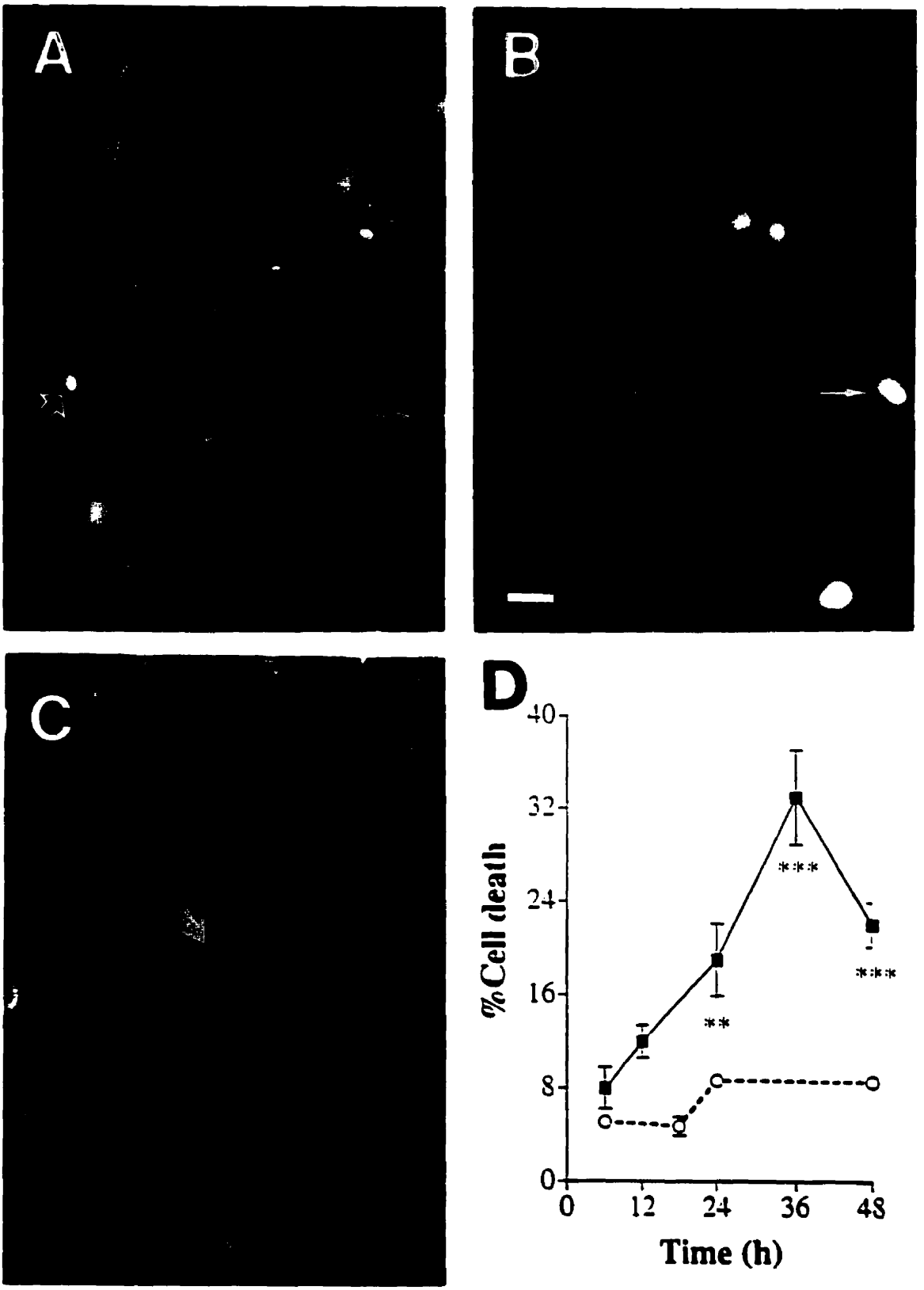

A
B
C
D
40
32
24
16
8
0
%Cell death
0
12
24
36
48
Time (h)
**
***
***

**Figure 7. Fluorescent labeling of endothelial nuclei after treatment with 8-iso-$PGF_{2\alpha}$.**

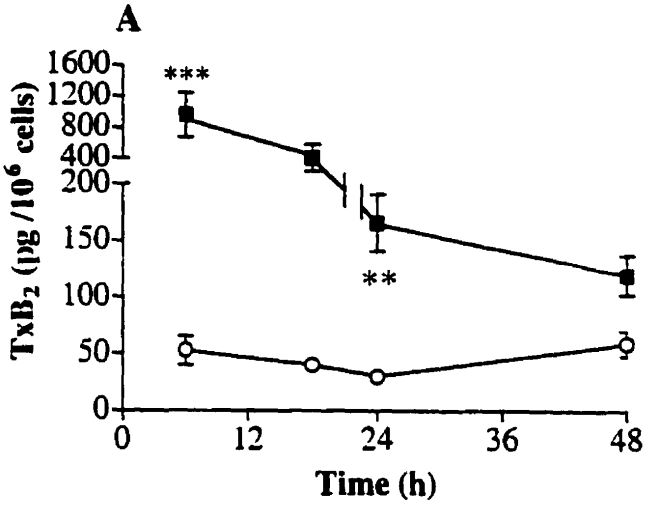
Endothelial cultures were treated with 100 nM 8-iso-$PGF_{2\alpha}$ for different periods of time.

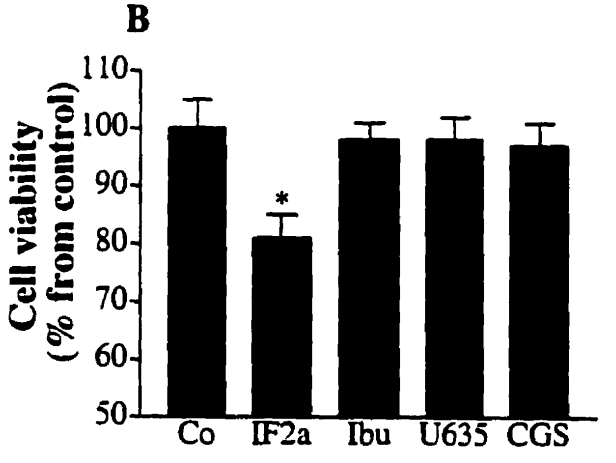
(A) Hoechst 33342 stained nuclei. Apoptotic nuclei with condensed chromatin are depicted by open arrows (bar = 40 μM).

(B) PI positive nuclei (necrotic cells) observed in the same field. These cells fluoresce both in blue and red (thin arrows).

(C) In situ labeling of 3' OH DNA fragments by dUTP addition through a terminal transferase reaction. The arrow shows three apoptotic bodies, two of them included in phagosomes into neighbor cells.

(D) Time course of PI incorporation (■) and *in situ* DNA labeling (o). Values are mean ± SEM percentages of positive cells found in 5 fields / coverslip in 3 different cell preparations, each condition was studied in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with values for untreated control cultures.


A
1600
1200
800
400
200
150
100
50
0
$TxB_2$ (pg /$10^6$ cells)
***
**
0
12
24
36
48
Time (h)


B
110
100
90
80
70
60
50
Cell viability
(% from control)
*
Co
IF2a
Ibu
U635
CGS

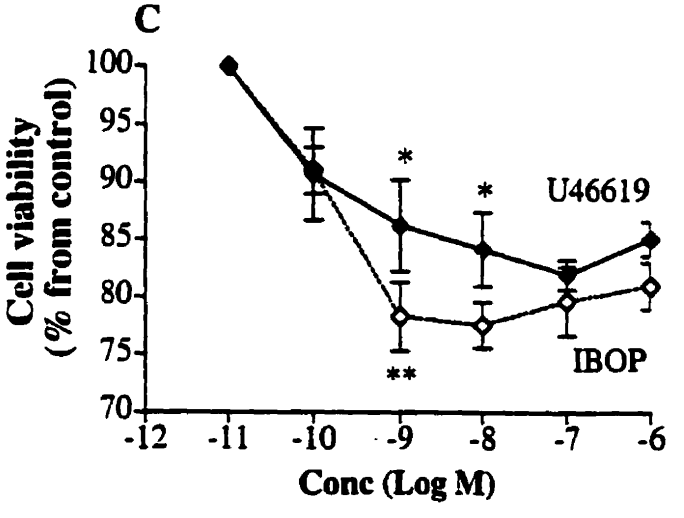
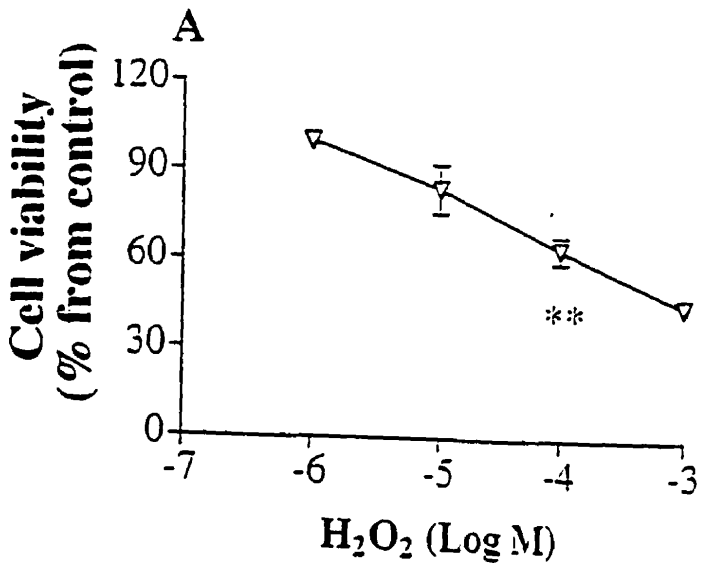

C
100
95
90
85
80
75
70
Cell viability
(% from control)
*
*
**
U46619
IBOP
-12
-11
-10
-9
-8
-7
-6
Conc (Log M)

**Figure 8. Role of thromboxane $A_2$ in endothelial cell death induced by 8-iso-$PGF_{2\alpha}$.**

(A) Thromboxane $B_2$ levels in endothelial cultures treated with 100 nM 8-iso-$PGF_{2\alpha}$ for different periods of time (■) or matching control cultures (o). Values are means ± SEM of three determinations. ** $P < 0.01$ and *** $P < 0.001$ compared to untreated cultures.

(B) Effects of cyclooxygenase and thromboxane synthase inhibitors on isoprostane-induced cell death values for endothelial cultures pretreated for 30 minutes with 1μM Ibuprofen or 100 nM CGS12970 and U6355A respectively, following by 1nM 8-iso-$PGF_{2\alpha}$ treatment for 48 h. Cell viability was assessed by MTT assay. Values are mean ± SEM of two experiments * $P < 0.05$. The experiment was performed in quadruplicate.

(C) Dose response curve of survival for U46619 and IBOP on cultures treated for 48 h. The effect was evaluated in quadruplicate in three different cellular preparations. * $P < 0.05$, ** $P < 0.01$.


A
Cell viability
(% from control)
120
90
60
30
0
-7
-6
-5
-4
-3
**
H2O2 (Log M)

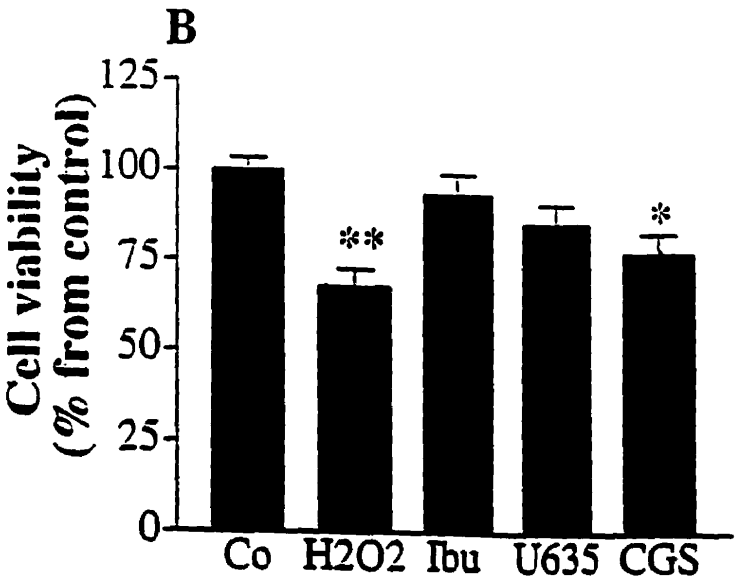

B
Cell viability
(% from control)
125
100
75
50
25
0
**
*
Co
H2O2
Ibu
U635
CGS

**Figure 9. Role of thromboxane $A_2$ in $H_2O_2$–mediated toxicity in endothelial cells.**

(A) $H_2O_2$ -induced toxicity in porcine brain endothelial cells. MTT values of cultures treated for 18h. The experiment was performed in quadruplicate in three different cell preparations.

(B) Effect of 30 minutes pretreatment with 1μM Ibuprofen, CGS12970 and U6355A on cytotoxicity induced by 18 h exposure to O.1 mM $H_2O_2$.. ** $P < 0.01$ compared to control.

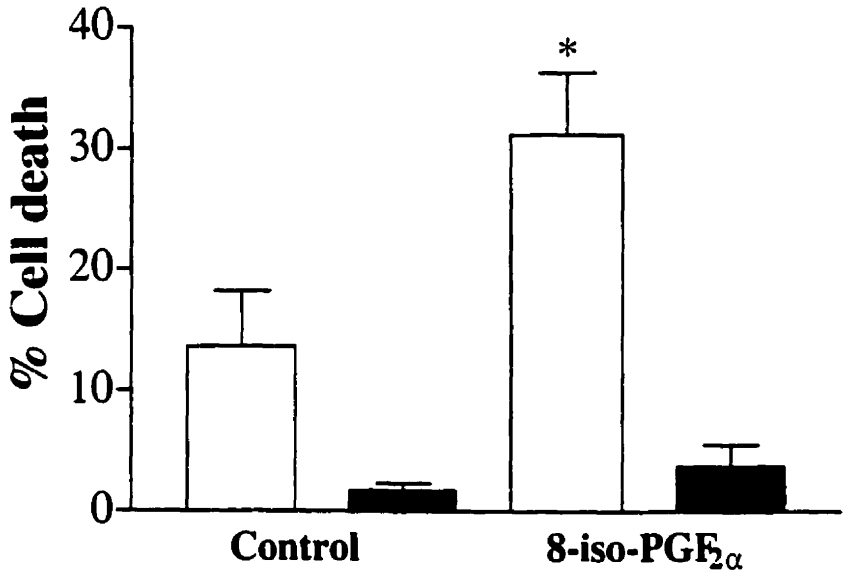

40
30
20
10
0
% Cell death
*
Control
8-iso-PGF2α

**Figure 10. Effect of 48 h treatment with 8-iso-$PGF_{2\alpha}$ on endothelial survival in isolated brain microvessels.**

Porcine brain microvessels were treated with 100 nM 8-iso-$PGF_{2\alpha}$ for 48 h and nuclei were stained with Hoechst 33342 and propidium iodide. The open bars represent the percentage of PI positive cells and the closed bars the percentage of cells with condensed or fragmented nuclei, observed by Hoechst 33342 staining. * $P < 0.05$ compared to untreated microvessels. Six microvessels were studied per experimental condition in two different preparations.

# 4. DISCUSSION.

The vascular endothelium is the first target for ischemia/reperfusion injury. Damaged endothelial cells get activated attracting neutrophils and macrophages, which leads to edema and tissue destruction in infarction areas (Hsu et al., 1997). Ischemia decreases oxygen supply which results in decreased production of high-energy phosphate compounds. During reperfusion, damage in mitochondria, calcium overload and activation of several enzymes increase oxygen free radical production implicated in cytotoxicity. Exposure of endothelial cells to simulated hypoxia (KCN poisoning and low pH) produces mitochondrial depolarization and rapid necrosis after 4 hours that is prevented by addition of glucose (Nishimura, 1998). Hydrogen peroxide, an oxidant abundantly produced by endothelial cells, impairs activity of key metabolic enzymes such as glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, even before loss of cellular ATP (Ciolino, 1996). In addition, low concentrations of hydrogen peroxide produce DNA fragmentation and apoptosis (De Bono, 1995).

Arachidonic acid on membrane phospholipids is a target for free radical-induced peroxidation that yields stable prostaglandin isomers termed isoprostanes (Morrow, et al., 1990a). Levels of isoprostanes increase in several tissues under oxidative stress including brain and retina (Morrow et al., 1993; Morrow et al., 1992b; Morrow et al., 1995; Lahaie, et al., 1998). These compounds induce constriction of vascular and bronchial smooth muscle. It has also been proposed that they might cause alterations in structure and fluidity of membranes, since their formation occurs when arachidonic acid is still esterified on phospholipids until their release in free form probably by phospholipases

(Morrow and Roberts, 1996). No evidence has been provided for any cytotoxic effect of isoprostanes. However, it is known that 8-iso-$PGF_{2\alpha}$ alters the barrier function on pulmonary artery endothelium (Hart et al., 1998). Therefore, we have hypothesized that isoprostanes, as peroxides, could be mediators of ischemic damage and produce direct cytotoxic effect on endothelial cells.

We have assessed the effects of two isoprostanes, 8-iso-$PGE_2$ and 8-iso-$PGF_{2\alpha}$, on survival of brain endothelial cell cultures, and on isolated brain microvessels *ex vivo*. We have evaluated cell survival by combining the study of mitochondrial oxidative capacity (MTT assay), with the morphological evaluation of membrane and nuclear chromatin integrity by double fluorescence. Other researchers have reported previously that values of oxidative capacity correlate well with numbers of viable endothelial cells assessed by counting (De Bono, 1995).

We found that nanomolar concentrations of 8-iso-$PGF_{2\alpha}$ decreased viability of endothelial cell cultures by 25-30% after 24h hours exposure, demonstrated by a decrement in mitochondrial oxidative function shown by MTT assay (Fig. 6A,B) and increased permeability to the DNA-binding dye propidium iodide (Fig.7 A, B). In contrast, 8-iso-$PGE_2$ did not have any adverse effect on endothelial cell viability (Fig. 5A).

As apoptosis or programmed cell death (PCD) has been related to ischemic injury, (Thompson, 1995; Hetts, 1998), we also investigated the possibility that 8-iso-$PGF_{2\alpha}$ could elicit apoptosis on endothelial cells. Only a small percentage of the treated cells showed nuclear condensation, intact membranes and DNA fragmentation, as demonstrated by an *in situ* labeling technique based in the addition of fluoresceinated

cytidine by a terminal transferase reaction that allows to label 3' OH ends on the DNA structure (APOPTAG method). In addition, to assess the membrane integrity on apoptotic cells, double fluorescence with the DNA-binding dyes propidium iodide and Hoechst 33324 was performed. The percentage of apoptotic cells in 8-iso-$PGF_{2\alpha}$- treated cultures was not significantly different from control (Fig. 7 C, D).

In order to investigate the effect of 8-iso-$PGF_{2\alpha}$ under conditions that better resemble those found *in vivo*, we performed a cell survival study on purified brain microvessels (Li et al., 1997) which were incubated for 48 h in the presence of 8-iso-$PGF_{2\alpha}$. The results of these experiments were similar both in terms of cell numbers affected and time course to the observations recorded on endothelial cultures: 8-iso-$PGF_{2\alpha}$ induced membrane damage on 30% of the cells whereas only 5% of the cells had condensed chromatin and intact membranes (Fig. 10).

Cell death occurs principally by two mechanisms: necrosis, characterized by loss of osmotic balance that leads to cytoplasmic disintegration and membrane disruption, and apoptosis, a genetically controlled process in which the cell is fragmented in apoptotic bodies and there is absence of inflammatory reaction (Kerr, et al., 1972; Hetts, 1998). The intensity of the insult is important in triggering one mechanism versus another, and in general, the less intense insults elicit apoptosis rather that necrosis.

It appears that both mechanisms of cell death occur after ischemia/reperfusion injury (Buja et al., 1993; Thompson, 1995; Hetts, 1998). At the center of the infarction area, necrosis of parenchymal cells and blood vessels results over a few hours. In the surrounding area or "ischemic penumbra", cells die over a more prolonged period of time, either by necrosis or apoptosis (Thompson, 1995). Although it has been reported

that agents that prevent apoptosis *in vitro* may limit the infarction size when utilized in experimental animals, (Uyama, 1992) several studies support the general notion that most of the cells affected by ischemia die by necrosis (Li et al, 1998; Van Lookeren and Gill, 1996).

Our results suggest that, 8-iso-$PGF_{2\alpha}$ elicits a necrotic-like type of cell death in endothelial cells, characterized by loss of membrane integrity and absence of DNA condensation or fragmentation. A similar kind of cytotoxicity is observed after prolonged exposures to low concentrations of oxidants (Gardner, et al. 1996). Hence, it could be possible that 8-iso-$PGF_{2\alpha}$ might be related to delay cell death observed at the ischemic penumbra.

In this study, the role of thromboxane $A_2$ in cell death was assessed. Thromboxane $A_2$ is a vasoconstrictor and promotor of platelet aggregation that contributes to cerebral blood flow autoregulation (Chemtob, et al., 1991). It has been observed that thromboxane and other prostanoids accumulate after ischemia and that the treatment with inhibitors of thromboxane synthesis diminishes postischemic structural damage in the brain (Iijima, et al., 1996).

Previous research has shown that the constrictor effects of 8-iso-$PGF_{2\alpha}$ are mediated by a secondary generation of thromboxane $A_2$ (Morrow and Roberts, 1996). Therefore, we investigated if thromboxane could also be related to the effect of 8-iso-$PGF_{2\alpha}$ endothelial cell survival. Certainly, 8-iso-$PGF_{2\alpha}$ increased the levels of thromboxane $B_2$ (a stable metabolite of thromboxane $A_2$) in the endothelial cultures up to 18-fold after 6 h exposure (Fig. 8A). Also pretreatment with CGS12970 and U63557A, two inhibitors of thromboxane synthase abrogated 8-iso-$PGF_{2\alpha}$-induced cytotoxicity. Similar protection

was achieved when cultures were pretreated with micromolar concentrations of Ibuprofen, a non-selective inhibitor of cyclooxygenases (Fig. 8B). This result is not surprising, since cyclooxygenase activity is necessary for thromboxane $A_2$ synthesis (Smith et al., 1991). Finally, we found that thromboxane $A_2$ *per se* is cytotoxic to endothelial cells: exposure to U46619 and IBOP, two thromboxane $A_2$ analogs decreased survival in the cultures by 25%.

As hydrogen peroxide may trigger both isoprostane formation and cyclooxygenase derived prostanoid synthesis, (Salaudeen et al., 1995; Lahaie, et al., 1998; Abran, et al., 1995), we investigated if the toxic effect of hydrogen peroxide could be mediated, at least in part, by the release of thromboxane $A_2$. As expected, significant decrease in endothelial cell viability was observed after hydrogen peroxide treatment, with an $EC_{50}$= 0.1mM (Fig. 9A). Furthermore, it was found that thromboxane synthase inhibitors like CGS12970, U63557A, and Ibuprofen, reduced necrotic cell death induced by 18 h exposure to 0.1 mM hydrogen peroxide, confirming our prediction (Fig. 9B).

There are only a few studies implicating thromboxane $A_2$ as a direct cause of cell death. It has been reported that thromboxane may induce apoptosis in thymocytes (Ushikubi, et al., 1993) and also that it can act as a mediator in cysplatin-induced death in kidney cells (Jariyawat, et al., 1997). Thromboxane increases intracellular $Ca^{2+}$ in several vascular smooth muscle cells (Lahaie, et al, 1998), accordingly, it could be expected that this prostanoid could be related to disruption of mitochondrial function and intracellular $Ca^{2+}$ overload eventually leading to cell death and vascular dysfunction. Thromboxane $A_2$ has also been observed to increase free radical production in the brain

during reperfusion (Matsuo et al., 1995). Here we demonstrate that thromboxane $A_2$ is a mediator of 8-iso-$PGF_{2\alpha}$-induced cytotoxicity. However, the events following TP receptor activation leading to endothelial cell death will have to be addressed in future studies.

In conclusion, the present work provides evidence for a new function of isoprostanes, which relate directly these products of peroxidation to cell death. 8-iso-$PGF_{2\alpha}$ elicits death of brain endothelial cells and microcapillaries at a concentration and time course attainable *in vivo*, since isoprostanes are stable compounds continuously produced under oxidant stress. The cytotoxic effect of 8-iso-$PGF_{2\alpha}$ requires synthesis of thromboxane A2 that may act in an autocrine or paracrine manner. For these reasons we speculate that isoprostanes are mediators of vascular dysfuction found in ischemic or diabetic neuropathies, retinopathies and atherosclerosis.

## 5. SUMMARY AND FUTURE DIRECTIONS.

We have demonstrated that 8-iso-$PGF_{2\alpha}$ a product of arachidonic acid peroxidation by free radicals, elicits death on brain endothelium; and that thromboxane $A_2$ is directly involved in this process. Further research will be necessary to the test if isoprostanes elicit cytotoxicity on other cell types *in vitro* and eventually *in vivo* in specific areas of the brain and retina. To further comprehend the isoprostane biological effects, molecular cloning of it receptor and studies on their secondary messenger systems will have to be performed. Another interesting question is whether isoprostanes may act on intracellular or extracellular sites, since isoprostanes can be transported through the cell membrane using the prostaglandin transporter (Itoh et al., 1996). Finally studies on the mechanism mediating thromboxane A2-induced cell death might contribute to development of more appropriated therapies for the treatment of vascular dysfunction associated to ischemia/reperfusion damage.

## 6. CLAIMS OF ORIGINALITY.

The following findings presented in this thesis represent original contributions to knowledge:

1. The isoprostane 8-iso-$PGF_{2\alpha}$ elicits death of brain endothelial cells.
2. Thromboxane $A_2$ is the mediator of 8-iso-$PGF_{2\alpha}$-induced endothelial cell death.

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