EFFECTS OF ESTROGEN ON ANGIOTENSIN II RECEPTORS

by

© PAUL-DANIEL CARRIERE

D.V.M., M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Department of Medicine
Division of Experimental Medicine
McGill University, Montréal

Supervisor: Marc Cantin, M.D., Ph.D.
Director of the laboratory of pathobiology
and of the Multidisciplinary Research Group
on Hypertension of the Clinical Research
Institute of Montréal

EFFECTS OF ESTROGEN ON ANGIOTENSIN II RECEPTORS

by Paul Daniel Carrière

ARSTRACT: The effect of estradiol treatment on angiotensin II receptors was studied in the anterior pituitary, adrenal cortex and mesenteric artery of the female rat. Estradiol treatment for 7 days caused a marked reduction in angiotensin II receptor density in the anterior pituitary, a moderate reduction in the adrenal cortex and no apparent effect on receptor density in the mesenteric artery. These effects did not appear to be mediated by alterations in circulating levels of angiotensin II. Estrogen-induced down-regulation of angiotensin II receptors in the adrenal cortex was also correlated in vivo with the failure of angiotensin II infusion to stimulate aldosterone release. In the anterior pituitary, down-regulation of angiotensin II receptors by estradiol was not accompanied by decreased prolactin cell responsiveness to angiotensin II when studied both in vivo and in vitro. Down-regulation of angiotensin II receptors was also observed in cultured anterior pituitary cells treated with estradiol for 48 hours. This thesis suggests that estrogens are important modulators of the angiotensin II receptor and may act directly to alter target cell responsiveness to angiotensin II.

EFFETS DES OESTROGÈNES SUR LES RÉCEPTEURS DE L'ANGIOTENSINE II

par Paul Daniel Carrière

RÉSUMÉ: L'effet du traitement à l'oestradiol sur les récepteurs de l'angiotensine II a été étudié chez la rate au niveau de l'hypophyse antérieure, du cortex surrénalien et de l'artère mésentérique. Le traitement à l'oestradiol durant 7 jours produisit une diminution marquée de la densité des récepteurs à l'angiotensine II au niveau de l'hypophyse antérieure, une diminution modérée au niveau du cortex surrénalien et ne sembla avoir aucun effet sur la densité des récepteurs de l'artère mésentérique. Ces effets ne semblaient pas dus à des changements dans le taux plasmatique de l'angiotensine II. Dans le cortex surrénalien, la "down-regulation" des récepteurs à l'angiotensine II par l'oestradiol correspondait *in vivo* à l'absence de stimulation de l'aldostérone par une infusion d'angiotensine II. Dans l'hypophyse antérieure, la "down-regulation" des récepteurs à l'angiotensine II par l'oestradiol n'était pas accompagnée d'une diminution de la sensibilité des cellules lactotropes à l'angiotensine II tel qu'observée *in vivo* et *in vitro*. Une diminution de la densité des récepteurs à l'angiotensine II a aussi été observée dans des cultures de cellules adénohypophysaires traitées à l'oestradiol pendant 48 heures. Cette thèse suggère que les oestrogènes sont impliqués dans la modulation des récepteurs à l'angiotensine II et que ces stéroïdes pourraient agir directement afin d'altérer la réactivité cellulaire à l'angiotensine II.

Cette thèse est dédiée

A mon épouse Micheline pour son courage et son amour

A mes filles Marie-Eve, Stéphanie et Emilie pour leur innocence

A mes parents pour leur exemple

A mes beaux-parents pour leur dévouement

A Dieu pour la vie, la santé et l'inspiration

Mahatma Gandhi's seven deadly evils:

"Wealth without work

Pleasure without conscience

Commerce without morality

Politics without principles

Worship without sacrifice

Knowledge without character

Science without humility"

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. Marc Cantin for his advice, help, encouragement and criticism in the completion of this Ph.D. research project. He has communicated to me some of his vitality, energy and experience which were extremely valuable in the pursuit of this research project.

I also wish to thank Dr. André De Léan of the Clinical Research Institute of Montréal who has co-supervised this project and has allowed me to perform the receptor binding studies in his laboratory. Special thanks are also expressed to Dr. Jolanta Gutkowska for her contributions and Dr. Jacques Drouin who has shown me the anterior pituitary cell culture technique. I also thank Dr. Harry L. Goldsmith, director of the Division of Experimental Medicine, Department of Medicine, McGill University for his support.

I would also like to express my appreciation to the technical staff of Dr. Cantin and Dr. De Léan particularly to Christian Charbonneau.

I am also extremely grateful to the Medical Research Council of Canada for granting me a Fellowship to support me throughout my 3 years of training at McGill University and at the Clinical Research Institute of Montréal.

Last but not least I thank Vivianne Jodoin for her advice, expeditiousness and professionalism in the typing of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

This thesis describes the effects of estrogen treatment on ANG II receptors in several target tissues with particular emphasis on the anterior pituitary gland. I have chosen the option provided in section 7 of the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University which reads as follows: "The candidate has the option, subject to the approval of the Department of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported. Abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted (end of citation)". This provision allowed me to include, as chapters of this thesis, the texts of two manuscripts which I wrote for publication concerning data obtained during my Ph.D. research project. In the first manuscript (chapter 3) minor changes in the text were performed for uniformity and new figures were included for additional graphical representation of the data taken from the tables. Minor editorial changes were also performed for the second manuscript (chapter 4).

Co-authorship of these manuscripts include: Dr. André De Léan who has taught me the principles of radio-receptor assays and has shown me how to use his computer programs for the analysis of binding data from radio-receptor assays (SCATFIT, ALLFIT) and radioimmunoassays (RIA) (RIAFIT); Dr. Jolanta Gutkowska who has helped me with iodination procedures and RIA techniques and Dr. Jacques Genest as previous director of the Multidisciplinary Research Group on Hypertension. The use of the terms "we" and "our" in chapters 3 and 4 refers to myself, my supervisor and the co-authors mentioned above.

Each of the following chapters consist of a separate unit with its own figures, tables and references. References are grouped in alphabetical order at the end of each chapter with the corresponding number appearing in the text. The only abbreviation appearing consistently throughout this thesis is ANG II used for angiotensin II. The two first chapters consist of a review of the literature dealing with the general properties of ANG II receptors in different target tissues (chapter 1) and a review of the current knowledge on estrogens and the renin-angiotensin system (chapter 2). Chapter 5 consists of the general discussion and conclusion including a few working hypotheses on the mechanism of action of estrogens on ANG II receptors and is followed by the claims to originality. Appendix I describes the original observations of <u>in vivo</u> uptake studies which have led us to the study of the effects of estrogen on ANG II receptors. Appendix II describes a preliminary study which may very well complement results described in chapter 3 on the effect of estradiol on vascular ANG II receptors.

GENERAL INTRODUCTION

The physiology, biochemistry and methods of measurements of the renin-angiotensin system will be briefly reviewed in this general introduction to familiarize the reader with this system. This will be followed by a description of the objectives and rationale that have led to the present thesis.

Angiotensin II is generally considered to be circulating hormone whose peripheral actions are centered around the regulation of arterial pressure and circulating blood volume. The octapeptide ANG II (Asp^1-Arg-Val-Tyr-Ile-His-Pro-Phe^8-OH) is a potent vasoconstrictor and causes renal sodium retention by stimulating aldosterone secretion from the adrenal cortex. The effects of centrally administered ANG II (in the brain) are complementary to the peripheral actions of the octapeptide. Acting directly on certain regions of the central nervous system, ANG II causes a rise in arterial blood pressure via activation of the sympatho-adrenal axis, a stimulation of corticotropin (ACTH) and antidiuretic hormone(ADH or vasopressin) release and volume repletion via induction of drinking behavior and salt appetite (106).

For a comprehensive review of the biochemistry of the renin-angiotensin system, the reader is referred to previous review articles (106,137). The special aspect of the "classical" renin-angiotensin system is that the hormone itself ANG II instead of being synthesized intracellularly is generated in the blood following enzymatic cleavage of ANG II precursors. The first-proteolytic enzyme in this cascade is renin which is synthesized as a precursor molecule "prorenin" and is converted to the enzymatically active renin inside

the cell and stored within secretory granules. Although renin is probably synthesized in many organs, it was first discovered in modified smooth muscle cells of the afferent arteriole of the renal glomerulus which is part of the so-called juxtaglomerular apparatus. Renin then acts in the blood on the larger precursor protein angiotensinogen (also called renin substrate) to produce another inactive hormone precursor, ANG I. The decapeptide ANG I is converted to the active octapeptide ANG II by a dipeptidyl-carboxyhydrolase converting enzyme which is bound to membranes on the surface of the vascular endothelial cells of the lung and probably also in other capillary beds. Angiotensin II has a short plasma half-life of less than 1 minute and is metabolized by action of the aminopeptidase angiotensinase A, to generate the heptapeptide ANG III, which possesses only a weak pressor activity but retains aldosterone stimulatory effects. Other angiotensinases degrade ANG II into inactive fragments.

The tetradecapeptide angiotensinogen is the natural protein substrate for renin, it is synthesized in the liver and, without demonstrable storage is released into the circulation. Renin's only known function is to catalyze the production of ANG I. The enzyme activity has a broad pH optimum between 5.5 and 7.0 which is in contrast to acid proteases such as pepsin and cathepsin D which have lower pH optima. Converting enzyme is dependent on chloride or other halide ions and can be inhibited by EDTA. Converting enzyme removes the dipeptide His-Leu from ANG I, but has also other functions: it inactivates, for example, bradykinin, and is identical with the enzyme kininase II.

Several methods of measurements of the different components of the renin-angiotensin system have been developed. The measurement of the circulating concentrations of ANG I and II presents special problems. Their concentration in plasma is very low (10-100 pM), and they differ little from one another and their metabolites in amino acid sequence which leads invariably to cross-reactivity of ANG II antisera with these products. The most widely applied and useful assay both in the clinical and investigative areas is the measurement of plasma renin activity. Plasma renin activity is determined by incubating diluted plasma at 4°C and 37°C at an optimal pH with converting enzyme inhibitors and then measuring generated ANG I levels by RIA. The result reflects the concentration of both active renin and its substrate angiotensinogen and does not permit full dissection of component concentration and activities. Therefore the value that is obtained is termed "renin activity". Using a similar approach, renin concentration can also be estimated indirectly by measuring generated ANG I in the presence of an excess of exogenous substrate.

In the early 1980's, specific binding sites for ANG II were characterized pharmacologically in homogenates of anterior pituitary glands. The first objectives of this Ph.D. project were to study the cellular localization of anterior pituitary ANG II binding sites (by light and electron microscope autoradiography) in order to gain a better understanding of the physiological relevance of these receptors in the pituitary gland. As a first approach, <u>in vivo</u> uptake studies were performed using intact cycling female rats to determine the presence of specific uptake of radiolabeled ANG II. A summary of these preliminary studies is shown in appendix I.

The most consistent finding in these uptake studies was the demonstration of specific uptake only in the adrenal gland (75-80% uptake inhibition by cold hormone). In the pituitary gland and other target tissues, specific uptake was not observed except for occasional experiments which were not reproducible. The impossibility to demonstrate specific uptake in ANG II target tissues other than the adrenal gland remained unexplained. Female rats sampled randomly with respect to their estrous cycle were used in these uptake studies. One possibility might be that ANG II receptors are regulated by cycle variations in circulating sex steroids possibly estrogens. These alterations in circulating levels of sex steroids might prevent the demonstration of in vivo specific uptake of [^{125}I] ANG II or [^{125}I]SARILE in ANG II target tissues containing a low density of ANG II receptors. Although this hypothesis was not verified by using male rats, it did serve as an incentive to study the effect of estrogen treatment on ANG II receptors in at least three target tissues namely the adrenal cortex, mesenteric artery and anterior pituitary gland.

The two following chapters consist of a review of the literature. In chapter 1 the characteristics and regulation of ANG II receptors in the hypothalamo-hypophyseal system, adrenal gland and mesenteric artery will be reviewed. Chapter 2 consists of a review of the effects of estrogens on the renin-angiotensin system with particular emphasis on estrogen modulation of ANG II receptors in the uterus, brain and vasculature.

CHAPTER 1 CHARACTERIZATION OF THE RENIN-ANGIOTENSIN SYSTEM IN THE HYPOTHALAMO-HYPOPHYSEAL SYSTEM, ADRENAL CORTEX AND MESENTERIC ARTERY

TABLE I IDENTIFICATION OF THE COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM IN THE MEDIAN EMINENCE AND PITUITARY GLAND

	MEDIAN EMINENCE	PITUITARY ANTERIOR	PITUITARY INTERMEDIATE	PITUITARY POSTERIOR	TECHNIQUE	SPECIES	REFERENCE
ANGIOTENSIN (ANG II)	+ (EZ)				IF	R	68
	+ (EZ)	+	+	-	IF	R	71
	+ (IZ, EZ)	+	-	+ (PIT)	IC	R	38
	+ (IZ, EZ)			+	IC	R	103
		+ (GON)	-	+ (FEW)	IC	R	177
	+ (IZ, EZ)	+	-	+	IC, IF	R	24
	+ (IZ, EZ)NPX	+	+ (NPX)	-	IC	R	92
		+ (GON)			IC	R	49
	+ (IZ, EZ)	+			IC	R	113
	+ (IZ, EZ)				IC	R	139
	+ (IZ, EZ)				IC	R	111
	+				BIOCH.	R	134
RENIN		+		-	BIOCH.	H,R,B	94,95
		+	-	-	IC	M	35
		+			IC	R,M	98,99
		+		+ (PIT)	IC	H	168
				+ (FEW)	IC, IF	R,M	69
		+ (GON)	-	-	IC	R	125
	+ (IZ)	-	-	+	IC	R	92
ANGIOTENSIN I		+ (FEW)	+	+	IC	R	37
	+ (IZ)NPX	- (NPX)	- (NPX)	+ (FEW, NPX)	IC	R	92
ANGIOTENSINOGEN	+	+		+	BIOCH.	R	110
		+		+	BIOCH.	M	90
	- (EZ)NPX	-	-	-, + (NPX)	IC	R	92
CONVERTING ENZYME ACTIVITY		+		+	BIOCH.	R	199
		+		+	BIOCH.	R	156
	+ (ENDOTH)				IC	R	21
	+		+ (*)		BIOCH.	R	42,43
	+	+	+	+	BIOCH.	R	158
		+		+	BIOCH.	R	140

EZ = External zone
IZ = Internal zone
GON = Gonadotroph cell

PIT = Pituicyte
NPX = Nephrectomized
ENDOTH = Endothelium

IF = Immunofluorescence
IC = Immunocytochemistry
BIOCH. = Biochemistry

R = Rat
H = Human
M = Mouse
B = Bovine

(*) Whole pituitary gland

A. HYPOTHALAMO-HYPOPHYSEAL SYSTEM

a) Introduction

Although the renin-angiotensin system is still usually considered to be a circulating blood hormone system, an increasing number of studies suggest the existence of local angiotensin generating systems within different tissues including the brain (66,74,92,97,113,117, 131,142), kidney (28,36,109,117,124,186), adrenal (8,72.75), testis (136), arterial wall (25,75,182,183) and anterior pituitary gland (ref. Table I). With the possible exception of the brain where increasing evidence strongly suggests de novo synthesis of angiotensin II (ANG II) it is still unclear in other tissues whether all or part of the components of the renin-angiotensin system are synthesized locally or are picked up from the peripheral circulation via specific or nonspecific uptake mechanisms.

In the central nervous system (CNS), immunocytochemical studies have localized peptides related to the renin-angiotensin system in a unique topographical distribution in the brain, brain stem and spinal cord of several animal species including man (21,35,67-69,92,98,99,103, 111-113,156,168,195). Many of these areas are situated within the blood-brain barrier suggesting local production of ANG II rather than uptake from the peripheral circulation. Several biochemical studies have also identified the different components of the renin-angiotensin system in brain tissue (40,90,93,95,110,140,144,156,158,200). Receptors for ANG II have also been characterized by techniques in physiology, pharmacology and autoradiography in several different areas of the brain, particularly in the hypothalamus and in the circumventricular organs (9,15,16,78,100,119,130,159,174,190). Furthermore, components of the renin-angiotensin system including the

ANG II receptor have been identified in the anterior pituitary gland (Table I, 88,100,174).

Taken together these studies showed that in the CNS, components of the renin-angiotensin system are preferentially localized to the brain stem and hypothalamic structures which have been implicated throughout evolution in the control of homeostatic functions related to body fluid balance. These studies strongly suggest a functional role for the renin-angiotensin system in the hypothalamo-hypophyseal axis which will now be reviewed in more detail.

b) Hypothalamus, median eminence and neuro-intermediate pituitary lobes

i) Hypothalamus

Many neuropeptides are synthesized by central nervous system neurons and are involved in the control of body fluid homeostasis. These neuropeptides are present in large numbers and in fairly high concentrations in all those areas which are important in the control of body fluid homeostasis such as the circumventricular organs, the primary baroreceptor centre in the medulla oblongata and certain hypothalamic nuclei (134).

In the hypothalamus, neurons of the supraoptic and paraventricular nuclei would appear to be the most important in body fluid homeostasis. Both hypothalamic nuclei possess large "magnocellular" neurons which contain oxytocin or vasopressin (also called antidiuretic hormone) and which project their axons through the internal zone of the median eminence "en route" to the neurohypophysis where these neuropeptides are released into the circulation. In addition, smaller "parvocellular" oxytocinergic and vasopressinergic neurons are also

present in these hypothalamic nuclei and project to a wide variety of extra-hypothalamic areas (for review see 201). There is increasing evidence that parvocellular neurons from the paraventricular nucleus are also involved in anterior pituitary cell function since they contain vasopressin (161), corticotropin-releasing factor (CRF) (161,184) and ANG II (71,92,111,113) and project their axons to the pituitary portal system in the external lamina of the median eminence (201).

Angiotensin immunoreactivity has thus been identified in cell bodies and nerve terminals in paraventricular parvocellular neurons as well as in magnocellular neurons of both hypothalamic nuclei by light and electron microscopy immunocytochemistry (21,92,103,111,113,134, 139,195,197). Immunocytochemical co-localization of ANG II with vasopressin in the same perikarya has also been demonstrated in these two hypothalamic nuclei (103). It was even suggested that this co-localization could reflect staining of an ANG II sequence homology within a pro-pressophysin precursor (103). This hypothesis seems unlikely however since ANG II immunostaining was found in several different areas of the brain of the vasopressin-deficient Brattleboro rat including the paraventricular parvocellular neurons whereas decreased but nevertheless persistent staining was found in the magnocellular neurons (111). Renin-like immunocytochemical reactivity has also been reported in the paraventricular and supraoptic nuclei of the rat and mouse (69,92) and appears to be associated with oxytocinergic neurons (24,103) whereas ANG II appears to be restricted to the vasopressinergic neurons.

ii) Median eminence

Several studies have also reported the immunocytochemical localization of ANG II in the internal and external lamina of the median eminence (Table I). Experimental manipulations suggest that ANG II stained fibers in the external and internal lamina of the median eminence arise from essentially separate groups of neurons. The source of ANG II immunoreactivity in the internal zone is likely from the magnocellular neurons of the paraventricular and supraoptic nuclei whereas a recent study has provided evidence that ANG II in the external zone is derived from the parvocellular neurons of the paraventricular nucleus (111).

Following bilateral lesions of the paraventricular nucleus, ANG II staining of the external zone disappeared whereas decreased but persistent staining remained in the internal zone undoubtedly arising from ANG II stained magnocellular neurons in the supraoptic nucleus (111). Adrenalectomy was also shown to specifically enhance ANG II staining in the external zone of the median eminence as well as in the parvocellular part of the paraventricular nucleus where extensive triple co-localization of immunoreactive CRF, vasopressin and ANG II was also shown (111). This finding is similar to previous reports showing that adrenalectomy also enhanced CRF, oxytocin and vasopressin staining in the external zone of the median eminence (103,111,184). Furthermore water deprivation decreased ANG II immunostaining in the external lamina while at the same time staining in the internal lamina increased (111). This latter finding is consistent with evidence for increased vasopressin synthesis and release in the same pathway (through the internal lamina) following water deprivation (192).

Following nephrectomy, one study reports no change in the distribution of ANG II immunostaining in the median eminence (111) whereas in another study, staining of the internal layer and magnocellular neurons of the hypothalamic nuclei was observed only after such treatment (92) which is in agreement with the proposed different afferent ANG II connections of the median eminence. Alternatively, in the median eminence where a blood-brain barrier is absent, ANG II as well as the other components of the renin-angiotensin system could possibly be picked up from the peripheral circulation through specific or nonspecific uptake mechanisms and selectively compartmentalized in the different zones following experimental manipulations. In support of the hypothesis of specific uptake of ANG II, receptors for ANG II have been identified in the median eminence by autoradiography (174,190). Biochemical studies have also reported significant levels of angiotensinogen and converting enzyme in the supraoptic nucleus as well as in the median eminence suggesting possible local ANG II production at either site (110,158).

iii) Neuro-intermediate pituitary lobes

In the neuro-intermediate pituitary lobes, biochemical and immunocytochemical investigations have identified angiotensinogen, renin, ANG I, converting enzyme and ANG II even though divergent results have been reported (Table I). Receptors for ANG II have never been found in the neuro-intermediate pituitary (88,100,174) except for one study (173).

iv) Hypothalamic angiotensin II receptors

Specific binding sites for ANG II have been identified in membrane fractions of the rat hypothalamus (15,16). In vitro autoradiography of

frozen sections of rat brain has revealed the presence of a very high density of ANG II receptors in the paraventricular nucleus (78,100,119) in both the magnocellular and parvocellular regions (119). Angiotensin II has also been shown to increase the discharge rate of supraoptic and paraventricular neurosecretory cells when applied iontophoretically (9,130) or when present in the medium of in vitro preparation of the supraoptic nucleus (159).

These studies suggest the existence of ANG II receptors in several strategic areas of the hypothalamus and median eminence where native ANG II has also been identified. The presence of ANG II in these hypothalamic nuclei as well as in other brain areas situated within the blood-brain barrier suggests local production of ANG II rather than uptake from the peripheral circulation.

v) Endogenous brain renin-angiotensin system?

More definitive direct evidence for a functional endogenous renin-angiotensin system within the same cell has been reported by several in vitro studies using culture systems of fetal rat brain cells as well as neuroblastoma cell lines. Immunoreactive ANG II has been identified immunocytochemically in both culture systems supplemented either with serum (169,196) or defined serum-free medium (194). In neuroblastoma cells, immunocytochemistry revealed the presence of immunoreactive renin (169) whereas radioimmunoassays (RIA) showed the presence of renin and angiotensins (131,169) and enzymatic assays revealed that they contained angiotensinogen and converting enzyme as well (143,169). Large proportions of renin, ANG II and converting enzyme were found to be present in pellet fractions and were not released by repeated washing without detergent suggesting their

localization in particulate organelles or bound to plasma membranes (131). Furthermore in neuroblastoma cells, renin appears to be present in an enzymatically inactive form that can be activated by trypsin, and renin concentration and activity have been shown to increase with serum withdrawal (169). Angiotensin II synthesis was also shown by the ability of primary cultures from fetal rat brain to incorporate [^{3}H]-Isoleucine and [^{3}H]-Valine into immunoprecipitable ANG II (145). Primary fetal rat brain cells cultured in serum-free medium were also shown to incorporate [^{3}H]-Proline in newly synthesized ANG II characterized by RIA and high performance liquid chromatography (HPLC) and shown to be biologically active (194). Different secretory rates of endogenous brain ANG II have been shown by different ANG II immunoreactivity levels between neuronal cultures from normotensive and hypertensive rats (146).

Receptors for ANG II have also been characterized in both neuroblastoma and fetal rat brain cell cultures (143,147,193) suggesting the existence of autoregulatory pre-synaptic ANG II receptors. Direct in vivo biochemical evidence comes from work demonstrating the synthesis of ANG II in the brain of intact and nephrectomized rats as well as the increased turnover rate in hypertensive animals (74). Finally the discovery of rat brain angiotensinogen mRNA in a cell-free translation system has dissipated any doubts about blood contamination or peripheral uptake of the precursor into the brain (26).

In summary several studies suggest the existence of an endogenous ANG II-generating system in the mammalian brain. There is good evidence that in a particular cell or cell type of the mammalian CNS a complete ANG II biosynthetic pathway appears to be functional. This

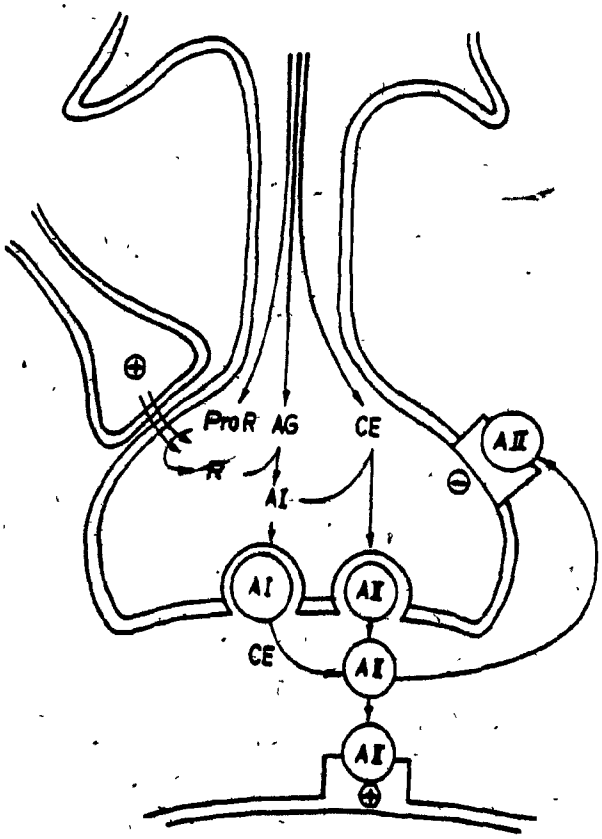


Figure 1 Hypothetical model of an endogenous angiotensin II-generating system in the CNS. Component localization and possible angiotensin II receptor function.

In this model, pro-renin (ProR), angiotensinogen (AG) and converting enzyme (CE) are synthesized by the pre-synaptic neuron. Activation of ProR to renin (R) could be the cascade initiator which might be mediated by an afferent neuronal signal. Active R would then cleave AG to produce angiotensin I (AI). AI could be cleaved intracellularly by CE to produce angiotensin II (AII). AII released directly (or by extracellular CE activity on AI) would then activate a post-synaptic AII receptor (+). In addition an ultra-short negative feedback loop acting through a hypothetical pre-synaptic AII receptor (-) may serve to attenuate this system.

ANG II-generating system could possibly by regulated through activation of prorenin or via the intracellular release of compartmentalized components of the renin-angiotensin cascade (Fig. 1). Auto-regulation of these ANG II producing neurons could perhaps also occur through a negative feed-back mechanism via pre-synaptic ANG II receptors in a manner analogous to that described for catecholaminergic neurons. Cellular co-localization of ~~all the~~ components of the renin-angiotensin system does not always occur however in the CNS nor in other tissues, suggesting that local ANG II could be generated extracellularly in the vicinity of interacting cells each possessing certain components presumably synthesized or picked up from the peripheral circulation (Fig. 1).

Thus in several areas of the brain such as the paraventricular and supraoptic nuclei where ANG II and its receptor have been identified, ANG II could serve as a neurotransmitter or neuromodulator acting on pre- and post-synaptic ANG II receptors. The presence of ANG II in these nuclei could represent ANG II synthesized intracellularly or internalized from afferent ANG II-containing nerve terminals. In addition, ANG II in the parvocellular neurons of the paraventricular nucleus could be linked to vasopressin secretion or might be released as a neurohormone in the pituitary portal system in the external zone of the median eminence. Recent evidence supporting the existence of a functional renin-angiotensin system in the anterior pituitary gland is also emerging and gives further support to an integrated system in the hypothalamo-pituitary axis.

c) Anterior pituitary gland

i) Anterior pituitary angiotensin II receptors

Recent receptor binding studies have demonstrated the existence of specific ANG II binding sites in membrane-rich particles of anterior pituitary glands of several animal species (30,41,88,121). These receptor saturation studies revealed the presence of a single class of saturable high-affinity binding sites for [^{125}I] ANG II in the anterior pituitary of mature and immature animals of both sexes with an equilibrium dissociation constant (K_D) in the nanomolar range (0.5 to 4 x 10 nM). Comparisons between the density of ANG II binding sites from different target tissues in the rat revealed that ANG II receptor density in the anterior pituitary (300 fmol/mg protein) was less than in purified adrenal zona glomerulosa (2000 fmol/mg) but much greater than in uterine muscle homogenates (135 fmol/mg) (88).

Quantitative autoradiography studies performed *in vitro* on anterior pituitary tissue slices of rats and dogs also revealed the presence of specific ANG II binding sites (91,100,174).

The physiological significance of these newly discovered anterior pituitary ANG II receptors was demonstrated in several *in vivo* and *in vitro* studies. The association of ANG II with prolactin and adrenocorticotropin (ACTH) release has been confirmed by many authors. In several animal species, ANG II has been shown to affect the release of prolactin *in vivo*. In the rat, high dose (5 nmol) intravenous bolus injections of ANG II increased plasma prolactin levels while intracerebroventricular microinjections depressed prolactin release (179,180). In the Rhesus monkey, systemic as well as central injections of µg doses of ANG II have also been shown to increase plasma prolactin levels (62). More direct evidence suggesting the

existence of ANG II receptors in lactotrophs has been provided from several *in vitro* studies using nanomolar concentrations of ANG II (5,165,166,180). Furthermore, partial purification of anterior pituitary cell types by elutriation revealed a larger prolactin response to ANG II as well as the preferential location of ANG II binding sites in lactotroph-containing fractions (5). The presence of specific binding sites for [^{125}I] ANG II in dispersed rat lactotrophs was also confirmed by autoradiography (133).

Corticotrophs are the only other anterior pituitary cell type where ANG II receptors have been demonstrated repeatedly. Angiotensin II has been shown to increase plasma ACTH and/or glucocorticoid release in rats (17,45,175,176), dogs (148,154) and primates (149,164). *In vitro* studies have also confirmed that ANG II directly stimulates ACTH release (30,70,87,123,170-172,175,176). Physiological concentrations of corticosterone *in vitro* (170,171) as well as dexamethasone *in vivo* (17) have also been shown to inhibit ANG II-mediated ACTH release. Direct evidence for corticotroph ANG II receptors has also been shown by autoradiography (133).

Since ACTH, β-endorphin and β-lipotropin (β-LPH) are derived from a common precursor molecule and are released concomitantly in response to several stimuli it is not surprising that ANG II has also been shown to stimulate the release of β-endorphin and β-LPH when given either *in vivo* (12,17) or *in vitro* (104,171). These studies also report glucocorticoid inhibition of β-endorphin and β-LPH release by ANG II.

Several *in vitro* studies have failed to show a stimulatory effect of physiological doses of ANG II on the release of growth hormone (GH),

luteinizing hormone (LH) or thyroid-stimulating hormone (TSH) (5,70,88,105,172). Modest increases in GH, LH and TSH were reported only in one study where hemipituitaries were incubated with supraphysiological micromolar concentrations of ANG II (180). Further evidence against the existence of ANG II receptors in thyrotrophs was shown in two in vivo studies where intravenous or intracerebroventricular injections of ANG II had no effect on TSH release in conscious ovariectomized rats (179,180). In these same two studies, it was also shown that ANG II injection tended to decrease GH and LH release in ovariectomized rats whereas an inverse effect on LH release was noticed when rats were treated with estrogens and/or progesterone (178,179). Taken together, these studies suggest that the effect of ANG II on GH and LH release may very well be indirectly mediated via the CNS rather than acting through somatotroph or gonadotroph ANG II receptors.

ii) Endogenous pituitary renin-angiotensin system?

In addition to the identification of ANG II receptors, angiotensinogen, renin, ANG I, converting enzyme and ANG II have also been identified in the anterior pituitary gland by biochemical and immunocytochemical techniques (Table I). Immunocytochemistry has revealed that renin (125) as well as ANG II-like immunoreactivity (49,177) is found exclusively in gonadotrophs. Interestingly, morphological studies have revealed that lactotrophs and gonadotrophs are found in close apposition (160). Since lactotrophs possess ANG II receptors it is tempting to speculate that ANG II possibly generated in gonadotrophs could serve as a paracrine hormone to release prolactin. Medium from gonadotroph-rich cell aggregates has been shown to

stimulate prolactin release (48). This effect was not due to follicle-stimulating hormone (FSH) or LH (48) but could perhaps be due to gonadotroph-derived ANG II. Furthermore, gonadotrophs have been shown to potentiate the prolactin response to ANG II when co-cultured with lactotrophs suggesting a yet undefined priming effect of gonadotrophs on lactotroph sensitivity (47). The increasing amount of newly discovered paracrine interactions in the anterior pituitary might also reveal an important regulatory function for the putative renin-angiotensin system of the anterior pituitary.

Biochemical characterization of ANG II receptors in the anterior pituitary gland revealed the presence of specific, high-affinity binding sites, with properties generally similar to those in the adrenal zona glomerulosa. However, in contrast to the marked regulatory changes of ANG II receptors observed in adrenal zona glomerulosa and vascular smooth muscle during changes in sodium intake and ANG II infusion, pituitary ANG II receptors were unchanged by these manipulations (118). This suggests that anterior pituitary ANG II receptors are not sensitive to alterations in circulating ANG II. On the other hand, water-deprivation has been shown to upregulate ANG II receptors in the anterior pituitary gland (101) whereas chronic estradiol treatment has an opposite effect (41). Clarification of the significance of these alterations awaits a better understanding of the physiological relevance of the renin-angiotensin system in the anterior pituitary.

B. ADRENAL CORTEX

a) Introduction

The role of ANG II as a major regulator of aldosterone secretion has been demonstrated by several early studies in man and other species (for review 7). Specific binding sites for ANG II have been identified mainly in the adrenal cortex and also in the medulla by several studies using both tritiated and monoiodinated ANG II (for review 7,29). These studies showed predominant location of ANG II binding sites in the plasma membrane with high specificity and affinity for ANG II and related peptides. Contrary to other species where ANG II is also known to stimulate cortisol production, likely through specific binding sites identified in the zona fasciculata, in the rat adrenal, ANG II does not stimulate corticosterone secretion and receptors are concentrated in the zona glomerulosa of the adrenal cortex (29).

This brief review will focus on ANG II receptor regulation in the adrenal cortex with particular emphasis on the effect of sodium and will provide some evidence in support of an endogenous renin-angiotensin system in the adrenal gland.

b) Adrenal angiotensin II receptor regulation

i) Angiotensin II receptor binding-properties and requirements

Among the several target tissues for ANG II, receptors in the adrenal cortex were the best characterized in terms of their properties and requirements for binding of the octapeptide. When compared to other target tissues, the adrenal cortex possesses the highest concentration of ANG II binding sites (57,88,118).

Binding of radiolabeled ANG II to adrenal receptors in classical pharmacological tissue preparations is dependent upon time, temperature and tracer concentration and degradation. Steady-state binding is usually reached within 10 to 60 minutes of incubation at temperatures ranging from 20° to 37°C (29). The addition of proteolytic enzyme inhibitors such as EDTA is necessary to inhibit degradation of the free tracer. Binding of ANG II to the adrenal receptor is also affected by cation concentration of the incubation mixture. Sodium and potassium significantly increase the binding of [^{125}I] ANG II reaching a maximum at about 250 mM (81). In the presence of sodium, increased uptake of ANG II by adrenal receptors is associated with the appearance of high-affinity binding sites with an association constant (K_D) in the nanomolar range (81). The high affinity form of the adrenal ANG II receptor is agonist specific and appears to include a guanine-nucleotide binding protein involved in the regulation of receptor affinity and possibly of its function (46,82). Although the lower-affinity sites are not always apparent, the presence of two populations of ANG II binding sites of high and low affinity have often been reported using adrenal glands of several different animal species (29). The physiological significance of different affinity states of the adrenal ANG II receptors awaits further understanding of the molecular interactions between ANG II and its plasma membrane receptor.

ii) Effect of sodium intake

In the adrenal gland, several early studies have demonstrated a relationship between peptide binding activity and biological response as expressed by steroidogenesis (29). Regulation of aldosterone secretion during altered sodium intake is a good example of this direct

correlation between modulation of adrenal ANG II receptors and the corresponding aldosterone response. The sensitivity of the adrenal response to ANG II is increased during sodium restriction in several animals species (29). Measurement of [^{125}I] ANG II binding to adrenal zona glomerulosa cells has shown an increase in ANG II receptors at early stages of sodium restriction. Increased binding was detected within 36 hours of the onset of sodium restriction due predominantly to an increase in receptor affinity (decrease in the dissociation constant: K_D) with a minor increase in receptor density (4). These acute 36-hour changes were accompanied by a corresponding increase in sensitivity of the aldosterone response to ANG II measured both in vivo and in suspensions of collagenase- dispersed glomerulosa cells (4). The increased sensitivity of isolated glomerulosa cells to ANG II during short term sodium restriction was based on the marked reduction of the ANG II concentration required to achieve a half-maximal aldosterone response (ED_{50}) and on the enhancement of the maximal aldosterone response to ANG II. In contrast, high sodium intake for 36 to 96 hours had an opposite effect on each of these parameters (4,61). Prolongation of sodium restriction from 4 days to 6 weeks has been shown to cause a more prominent elevation in adrenal ANG II receptor density with maximal aldosterone responses in vivo and in vitro without altering receptor affinity or ED_{50} (4,7,29). Therefore during acute sodium restriction, rapid changes in receptor affinity and glomerulosa sensitivity would appear to contribute to the rapid regulation of aldosterone secretion. On the other hand, when sodium deficiency is

prolonged, increases in receptor density as well as enhanced activity of the early and late steps of the aldosterone biosynthetic pathway (2,33) would appear to be more relevant in maintaining the elevated aldosterone secretion.

Sodium restriction induces similar changes in hypophysectomized rats although the aldosterone response to sodium deficiency is usually reduced in the absence of the pituitary gland (135). This attenuated aldosterone response is probably due to the absence of ACTH which is necessary to maintain the enzymes of the early aldosterone pathway.

iii) Effect of infused angiotensin II

Since circulating levels of ANG II are elevated during sodium restriction, enhanced activity of the aldosterone biosynthetic pathway as well as increases in ANG II receptor affinity and/or density could be caused by the trophic effect of the octapeptide on the adrenal glomerulosa cells. Evidence supporting this mechanism has been shown in rats fed with a normal diet and infused with ANG II at 10-100 pmol per minute for periods ranging from 36 hours to 6 days (2,6,7,89).

Prolonged infusion of ANG II (50 pmol/minute/rat) for 6 days in normal and hypophysectomized rats has been shown to reproduce the effects of sodium restriction on the enzymes of the aldosterone biosynthetic pathway. Angiotensin II infusion exerts marked stimulating effects on the last steps of aldosterone biosynthesis (conversion of corticosterone to aldosterone by 18-hydroxylase activity) and can also enhance the early biosynthetic pathway by increasing the enzymes leading to corticosterone production (7).

Acute or chronic ANG II infusion increased adrenal ANG II receptor density without affecting receptor affinity and increased the magnitude of glomerulosa cell responses to ANG II administered both in vivo and in isolated cells (2,6,7,89). This rate of infusion increased blood ANG II levels to those observed in rats on a low sodium diet. In contrast, infusion of higher doses of ANG II ($\geq$ 250 pmol/minute for 36 hours) which increased circulating levels 30-fold, decreased the density of ANG II receptors in the adrenal glomerulosa below control values (7). This "biphasic" action of ANG II may contribute to the transient nature of the aldosterone responses observed during prolonged infusion of the peptide in some animal species (115). Since aldosterone infusion has been shown to decrease the density of ANG II receptors in the adrenal cortex (60), the biphasic effect of ANG II on adrenal cortical ANG II receptors might be due to enhanced aldosterone secretion with high infusion rates of ANG II.

Suppression of endogenous ANG II by converting enzyme inhibition has provided further support for a role of circulating ANG II in the regulation of adrenal cortical ANG II receptors. Blockade of ANG II formation by infusion of the converting enzyme inhibitors (SQ 14,225 and SQ 20,881) has been shown to prevent the increase in adrenal receptors and aldosterone secretion that normally accompany sodium restriction (7). In animals on normal sodium intake, inhibition of converting enzyme activity also caused a fall in blood ANG II, adrenal ANG II receptors, and plasma aldosterone. The effect of converting enzyme inhibition was reversed by simultaneous infusion of ANG II, supporting the contention that the decrease in ANG II receptor density is due mostly to suppression of ANG II and not to other actions of the inhibitor.

These studies clearly demonstrate the absolute dependence of adrenal glomerulosa cell responses upon ANG II levels and indicate that the renin-angiotensin system is the primary regulator of aldosterone secretion during physiological changes in sodium intake. The fact that tonic infusions of physiological doses of ANG II only partially reproduce the elevation in plasma aldosterone that occurs during sodium restriction (2) suggests that other factors may be involved or that sustained tonic levels of the regulator may be less effective than intermittent elevations in stimulating target cell responsiveness. The presence of diurnal variations and episodic secretion of renin in man (102) could reflect the importance of pulsatile changes of ANG II on adrenal glomerulosa function and aldosterone secretion. However, other factors are likely involved in the regulation of aldosterone secretion and possibly interact with ANG II to modify the action of the octapeptide on the adrenal glomerulosa cell. One of these factors could be extracellular potassium since minor increases in serum potassium are frequently seen in sodium deficiency (19) but not during ANG II infusion.

iv) Effect of potassium

Elevation of extracellular potassium is known to cause a direct and marked trophic action on the zona glomerulosa by increasing ANG II receptors and aldosterone secretion (58). After treatment of rats with a high potassium diet for 3 days to 6 weeks, adrenal receptors for ANG II are markedly increased while low potassium intake has the opposite effect (58,59). Potassium would appear to have a direct action upon the zona glomerulosa since potassium loading is characterized by a reduction in plasma renin and ANG II levels (167). The combined effect

of several factors such as the increased circulating ANG II and slightly elevated potassium and ACTH could possibly explain the enhanced sensitivity of the adrenal cortex during sodium deficiency.

v) Effect of anesthesia

A number of earlier reports indicated that the rat did not respond to infusion of renin or ANG II with an appropriate rise in aldosterone production (for review 57). It was even suggested that the renin-angiotensin axis did not control the release of aldosterone from the rat adrenal cortex. This was not supported by others who have shown prominent aldosterone responses to infusion of low concentrations of ANG II in conscious, unstressed rats (27,44). The erroneous conclusions of previous studies could have been due to the use of supraphysiological doses of ANG II, the use of anesthetized rats, and surgical or handling stress. Pentobarbital anesthesia in the rat has been shown to produce marked increases in plasma renin activity (58,138) and plasma renin concentration (31) despite lowered blood pressure (31,138) and similar or slightly elevated aldosterone levels (44,58). During pentobarbital anesthesia the pressor response to intravenous ANG II has been shown to remain the same when compared to conscious rats whereas the aldosterone response to ANG II appears to be blunted during pentobarbital anesthesia (44). Nevertheless, acute ANG II infusions (200 pmol/kg/minute, i.v. for 20 minutes) during pentobarbital anesthesia was shown to reduce the density of ANG II receptors in the adrenal cortex (58). The half-life of circulating ANG II has been shown to increase (39) whereas angiotensinogen levels remained the same (32) during barbiturate anesthesia.

vi) Effect of ACTH

Several studies have also shown that ACTH causes a transient increase in aldosterone secretion (57,107,122,129) followed by a decrease when treatment is prolonged. In cultured glomerulosa cells, ANG II receptor density has also been shown to decrease within hours of exposure to ACTH (11). Therefore the relatively small responses of the rat adrenal to ANG II *in vivo* could be obscured by the overriding effects of ACTH release induced by stress or by pentobarbital anesthesia (132).

c) Endogenous adrenal renin-angiotensin system?

Analogous to the endogenous renin-angiotensin system of the brain, the possibility of *in situ* formation of ANG II in the adrenal gland has been suggested by several studies. Renin activity has been reported in the adrenal gland and among the various extrarenal tissues, the adrenal gland possesses the highest true renin activity per unit weight of protein (72,128). Increased adrenal capsular renin activity has been demonstrated in nephrectomized rats (8). Renin has also been shown to exist in human adrenal tissue (127). The lack of correlation between plasma renin activity (PRA) and the adrenal renin, and the subcellular distribution of adrenal renin suggested its local origin rather than contamination or uptake from plasma renin. Renin-like activity was present in adrenal cells from zona glomerulosa and zona fasciculata maintained in monolayer tissue cultures for several generations (77). Angiotensinogen was also present in adrenal gland homogenates (77). Renin mRNA has recently been identified in mouse adrenal glands (65). Marked elevated specific renin levels were found mostly in the adrenal cortex but also in the medulla in genetically hypertensive rats (126).

As is often the case in biological systems, the interaction of several factors appears to modulate ANG II receptors in the adrenal cortex. Among these regulating factors, sodium intake appears to exert its effects mostly via alterations in circulating levels of ANG II, whereas ACTH, potassium and aldosterone appear to have a more direct effect at the level of the adrenal cortical ANG II receptor. The potential role of an endogenous renin-angiotensin system and its regulation in the adrenal cortex must also be considered in future investigations.

C. MESENTERIC ARTERY

a) Introduction

Angiotensin II is the most potent natural pressor substance known. Normal circulating levels of ANG II in the rat vary between 10 and 100 pmol/liter (141) which is several orders of magnitude less than most circulating hormones (nmol/liter). Injection of the octapeptide causes an immediate sharp increase in blood pressure which is most likely a result of its direct action on vascular smooth muscle contraction and its central neurogenic effect (29,56). In vitro, ANG II provokes contraction of smooth muscle preparations from both vascular (arteries) and visceral (uterus, ileum, urinary bladder) tissues in a peptide specific manner. Saturable specific binding of radiolabeled ANG II was first identified in the aorta (13,108). However the affinity of the receptor was found to be low (15-50 nM) and since only a small amount of smooth muscle is present in the fibroelastic aorta, ANG II receptors have been preferentially

characterized in muscular resistance-type vessels such as the mesenteric artery.

b) Vascular angiotensin II receptor regulation

i) Angiotensin II receptor binding properties and requirements

Specific sites of high affinity and low capacity for [^{125}I] ANG II have been characterized in membrane particulate fractions derived from arterial arcades of the rat mesenteric artery (3,83-85,86,116). Divalent cations (manganese, magnesium, calcium) and sodium increase binding of [^{125}I] ANG II to mesenteric artery particulate fractions whereas potassium, sulfhydryl-reducing agents (dithiotreitol), chelating agents (EDTA, EGTA), and guanine nucleotides inhibit receptor binding (83,85,116). These alterations in receptor binding reflect modulation of the affinity of the receptor rather than an effect on receptor density. It was suggested that cations and guanine nucleotides interact in one or more receptor-associated membrane sites, probably including a guanine nucleotide-regulatory protein, to modulate the affinity of the vascular receptor for ANG II (198). This complex interplay may be involved in the modulation of physiological smooth muscle responses to ANG II. Sodium stimulates binding to ANG II receptors in both the adrenal cortex (81,82) and mesenteric artery (116,198) suggesting the existence of a membrane monovalent cation site closely related to the receptor. Cation requirements for the binding of [^{125}I] ANG II vary between vascular tissues since Mg^{2+} and Ca^{2+} at concentrations lower than 10-20 mM enhance binding in the mesenteric artery and inhibit binding to the rabbit aorta (52).

Initial studies indicated the presence of a single class of binding sites in the mesenteric artery (K_D;0.9 nM, receptor density; 50 fmol per mg of protein) without any detectable degradation of [^{125}I] ANG II (85). In contrast heterogeneity of binding sites (high and low affinity sites) (116) and extreme degradation (83,116) in tracer ANG II has also been reported in mesenteric artery particulate fractions. Degradation of [^{125}I] ANG II has been prevented by washing the membranes with EDTA and including EGTA in the assay buffer (83). The subcellular distribution of the specific binding of [^{125}I] ANG II was shown to occur in plasma membrane enriched fractions of rat mesenteric arteries where enhanced receptor density was observed (420 fmol per mg protein) with a similar affinity (K_D;2.2 nM) (83).

ii) Angiotensin II receptors and their physiological relevance to blood pressure response.

Even though ANG II receptors have been well characterized in the mesenteric artery, the physiological relevance of these receptors in relation to pressor response to ANG II is much less clear. In the same study, pressor responses of vascular tissues to exogenous ANG II have been shown to vary depending upon the tissue of origin (18). In most vascular preparations such as the isolated dog mesenteric artery, ANG II produced a transient contraction and showed prominent desensitization whereas the aorta showed persistent contraction and little desensitization. In the rat, a small increase in superior mesenteric artery perfusion pressure was observed after bolus injections (0.1 - 1 nmol) of ANG II which was much less than that observed after injection of arginine-vasopressin or noradrenaline

(96). Isolated rat mesenteric arteries have generally been shown to be insensitive to ANG II-pressor response (51,63).

A possible explanation for the lack of consistent marked ANG II-pressor responses of isolated rat mesenteric artery preparations might be due to concomitant release of prostaglandins. Angiotensin II infusion in the mesenteric artery has been shown to cause the release of the potent vasodilator prostacyclin (PGI_2) or its metabolite (6 keto-$PGF_{1\alpha}$) which is a potent vasodilator and also to a lesser extent the release of the vasoconstrictor prostaglandin PGE_2 (51,63,64,96). Angiotensin II stimulation of PGE-production from cultured human umbilical vein endothelial cells has also been reported (80). There is evidence that in the vascular beds of the mesenteric artery and other blood vessels, vasoactive ANG II and prostaglandins may have local regulatory roles. Conversion of ANG I to ANG II occurs in vascular endothelial cells where converting enzyme has been localized (23,186). The biosynthetic capacity for PGI_2 in vascular walls is also concentrated in the endothelium (120). Therefore exogenous or locally produced ANG II could stimulate endothelial PGI_2 release which would serve to counteract the pressor effect of ANG II on vascular smooth muscle receptors. In addition to a direct effect on mesenteric artery ANG II receptors, ANG II may also act indirectly since facilitation of adrenergic vasoconstrictor response by locally generated ANG II has been demonstrated in the rat mesenteric artery (114).

iii) Effect of sodium intake

The pressor activity of ANG II is often attenuated by the development of hyporesponsiveness to the vasoconstrictor effect of ANG II in clinical disorders associated with activation of the renin-angiotensin system. This has been shown to occur during

pregnancy, hepatic cirrhosis, Bartter's syndrome, adrenal insufficiency and sodium depletion; conditions in which high plasma-ANG II levels are not accompanied by corresponding elevations of blood pressure (34). Alterations in vascular responsiveness to ANG II during changes in sodium and potassium balance (1,155,157) suggest that factors including circulating ANG II levels must act at or beyond the ANG II receptors of the smooth muscle in the control of vascular sensitivity.

Vascular hyporesponsiveness to ANG II during sodium restriction has been attributed to changes in affinity (22) or number (3) of ANG II receptors and also to prior occupancy of receptors by increased circulating ANG II resulting in fewer "free" receptors available to respond to ANG II (188). More recent studies have shown that sodium restriction decreases and sodium loading increases smooth muscle ANG II receptor density of the uterus (4,53,54), urinary bladder (3) and mesenteric artery (3,86). Although the properties of ANG II receptors in the non-vascular smooth muscle tissues have been shown to be generally similar (3,60), direct extrapolations with vascular smooth muscle receptors should be applied with caution as shown by discrepancies following ANG II infusion.

iv) Effect of angiotensin II and mineralocorticoid infusion

Alterations in ANG II receptor density during ANG II infusion are biphasic and dose-dependent in the mesenteric artery (163). High doses appear to up-regulate whereas low dose infusions of ANG II down-regulate ANG II receptors in the mesenteric artery (163). This biphasic effect on smooth muscle ANG II receptors was not observed in non-vascular tissue where persistent down-regulation of urinary bladder ANG II receptors has been reported following increased ANG II infusion dose (3). A reciprocal inverse biphasic effect has also been observed in the adrenal cortex following high infusion rates of ANG II (7).

The effect of low sodium diet in smooth muscle ANG II receptor can be reproduced by infusion of ANG II which reduces ANG II receptor densities in the urinary bladder (3), uterus (55) and mesenteric artery (163). However, the sensitivity of the adrenal gland and blood vessels to ANG II has been shown to vary with minor continuous elevations of ANG II in a direction "opposite" to that produced by sodium depletion (14,20). In these latter studies, the adrenal gland aldosterone response appears to be less sensitive to ANG II after exposure to chronic low dose infusions of this agent whereas enhanced pressor responses to ANG II and hypertension develop under identical conditions. These effects on target tissues contrast with the enhanced aldosterone secretory response and blunted pressor response to ANG II produced by sodium depletion in spite of similar directional changes in plasma ANG II. These findings further emphasize that ANG II receptor regulation by changes in sodium balance may not be solely related to ANG II concentration or that continuous infusion of ANG II may not reflect adequately the situation encountered during sodium restriction where fluctuations in the enhanced levels of ANG II are likely to occur.

Reciprocal effects on number, but not affinity, of smooth muscle and adrenal ANG II receptor number without any effect on receptor affinity have generally been reported with changes in sodium balance and ANG II infusion. However, using lower dose infusions of ANG II for 7 days in intact female rats, it was shown that in addition to the increase in ANG II receptor density, receptor affinity was slightly

decreased (K_D from 1 to 2 nM) in the adrenal cortex whereas only the affinity increased in uterine smooth muscle preparations (60). It was suggested that these reciprocal tissue changes in receptor affinity could possibly explain the previously reported blunted aldosterone response of the adrenal gland and the enhanced pressor response to ANG II following chronic infusion of ANG II (14,20).

Insight into the complex interactions following ANG II infusion was provided by the observation that alterations in vascular ANG II receptors are dose dependent. Mesenteric artery ANG II receptor density decreases during low doses (60 pmol/kg/minute i.v.) of ANG II, but at higher infusion rates (200 pmol/kg/minute i.v.) when aldosterone levels are elevated, this effect was lost, suggesting that endogenous mineralocorticoid activity might antagonize the down-regulatory action of ANG II on vascular receptors (163). This hypothesis was supported by the observation that mineralocorticoid (DOCA or aldosterone) administration *in vivo* or *in vitro* increase the density of ANG II receptors in the mesenteric artery and mineralocorticoids (aldosterone) given *in vivo* decrease the density of ANG II receptors in the adrenal cortex (60). It is interesting to note that mineralocorticoids appear to regulate ANG II receptors in the mesenteric artery and adrenal cortex in an opposite direction. These reciprocal alterations of ANG II receptors appear to be a general regulatory phenomenon for these two target tissues.

Functional ANG II receptors have also been characterized in cultured vascular smooth muscle cells of the mesenteric artery (84). Receptor affinity and density was similar to that reported in particulate fractions and variable proportions of cells (30%) elicited rapid, reversible and specific contractile response to nanomolar concentrations of ANG II. Exposure of these cells to elevated levels of ANG II has also been shown to induce time-dependent reversible decreases in receptor number (10). Direct up-regulation of vascular ANG II receptors by mineralocorticoids has also been demonstrated in cultured mesenteric artery cells (162).

c) Endogenous vascular renin-angiotensin system?

Evidence supporting the existence of an endogenous renin-angiotensin system in the arterial wall has also been reported. The presence of renin-like activity in the arterial wall tissue of several different animal species has been confirmed biochemically and by immunohistochemical techniques (73,79,185). Concentrations of renin in the arterial wall tend to vary in the same direction as altered plasma renin levels suggesting that renin in the arterial wall might be picked up from the circulation (181,182,189). However renin in mesenteric arteries could be stimulated in nephrectomized dogs (73) and endogenous synthesis of renin has been demonstrated in cultured aortic smooth muscle cells by specific immunoprecipitation of newly synthesized protein and by histochemical techniques (150,151). Renin substrate has been identified in the mesenteric arterial wall of the rat and variations in vascular substrate concentrations after nephrectomy and after adrenalectomy have also been reported (50). Angiotensin II-like

activity in kidney arteries and in resistance vessels of several organs has also been detected in the medial smooth muscle layer by immunocytochemistry (185). Converting-enzyme activity has been localized in endothelial cells of several vascular beds (23,186) and in the media of the aorta in rats (191). The hypotensive effect of converting-enzyme inhibitors in low renin hypertension is also suggestive of a functional local renin-angiotensin system in the vascular wall.

The presence of components of the renin-angiotensin system in the arterial wall of several types of resistance vessels is not all that surprising if one considers the vascular nature of the epithelioid cells of the media of the afferent glomerular arteriole where renin is principally synthesized and coexists with ANG II (84,186,187).

Irrespective of the site of its generation, the intracellular localization of ANG II raises the question whether ANG II has intracellular actions as well. It has been shown in the liver that ANG II increases DNA, RNA and protein synthesis (152) and specifically binds to rat liver and spleen nuclei (153). It has also been reported that ANG II is mitogenic for arterial smooth muscle cells in culture (76). From such observations the possibility of ANG II-induced effects on vascular smooth muscle functions other than vasoconstriction must also be considered.

D. REFERENCES TO CHAPTER 1

1. ADAMIK R, GOLD ME, HAYES S, COLEMAN R, McCREARY JT, SABATINI S, ARRUDA JAL, KURTZMAN NA: Factors influencing vascular hyporesponsiveness to angiotensin II. Circ. Res. 1981, 49:932-939.
2. AGUILERA G, CATT KJ: Regulation of aldosterone secretion during altered sodium intake. J. Steroid Biochem. 1983, 19:525-530.
3. AGUILERA G, CATT KJ: Regulation of vascular angiotensin II receptors in the rat during altered sodium intake. Circ. Res. 1981, 49:751-758.
4. AGUILERA G, HAUGER RL, CATT KJ: Control of aldosterone secretion during sodium restriction: adrenal receptor regulation and increased sensitivity to angiotensin II. Proc. Natl. Acad. Sci. 1978, 75:975-979.
5. AGUILERA G, HYDE CL, CATT KJ: Angiotensin II receptors and prolactin release in pituitary lactotrophs. Endocrinology 1982, 111:1045-1050.
6. AGUILERA G, MENARD RH, CATT KJ: Regulatory actions of angiotensin II on receptors and steroidogenic enzymes in adrenal glomerulosa cells. Endocrinology 1980, 107:55-59.
7. AGUILERA G, SHIRAR A, BAUKAL A, CATT KJ: Angiotensin II receptors. Properties and regulation in adrenal glomerulosa cells. Circ. Res. 1980, 46 (Suppl. 2):I-118-I-125.
8. AGUILERA G, SHIRAR A, BAUKAL A, CATT KJ: Circulating angiotensin II and adrenal receptors after nephrectomy. Nature 1981, 289: 507-509.

9. AKAISHI T, NEGORO H, KOBAYASI S: Electrophysiological evidence for multiple sites of action of angiotensin II for stimulating paraventricular neurosecretory cells in the rat. Brain Res. 1981, 220:386-390.

10. ALEXANDER RW, HYMAN S, ATKINSON W, GIMBRONE MA: Regulation of angiotensin II receptors in cultured vascular smooth muscle cells. Circulation (abstracts) 1980, 62:III-90.

11. ANDOKA G, CHAUVIN MA, MARIE J, SAEZ JM, MORERA AM: Adrenocorticotropin regulates angiotensin II receptors in bovine adrenal cells in vitro. Biochem. Biophys. Res. Comm. 1984, 121:441-447.

12. ANHUT H, KNEPEL W, HOLLAND A, MEYER DK: β-Endorphin release by angiotensin II: studies on the mechanism of action. Regulatory Peptides 1982, 4:83-90.

13. BAUDOUIN M, MEYER P, WORCEL M: Specific binding of ^{3}H-angiotensin II in rabbit aorta. Biochem. Biophys. Res. Comm. 1971, 42:435-440.

14. BEAN BL, BROWN JJ, CASALS-STENZEL J, FRASER R, LEVER AJ, MILLAR JA, MORTON JJ, PETCH B, RIEGER AJF, ROBERTSON JIS, FREE M: The relation of arterial pressure and plasma angiotensin II concentration. Circ. Res. 1979, 44:452-458.

15. BENNETT JP, SNYDER SH: Angiotensin II binding to mammalian brain membranes. J. Biol. Chem. 1976, 251:7423-7430.

16. BENNETT JP, SNYDER SH: Receptor binding interactions of the angiotensin II antagonist, ^{125}I-[sarcosine^{1} leucine^{8}] angiotensin II, with mammalian brain and peripheral tissues. Eur. J. Pharmacol. 1980, 67:11-25.

17. BEUERS U, HERTTING G, KNEPEL W: Release of β-lipotropin- and β-endorphin-like material induced by angiotensin in the conscious rat. Br. J. Pharmacol. 1982, 76:579-585.

18. BOHR DF, UCHIDA E: Individualities of vascular smooth muscles in response to angiotensin. Circ. Res. 1967, (Suppl. to 20-21):II-135-II-143.

19. BOYD JE, PALMORE WP, MULROW PJ: Role of potassium in the control of aldosterone secretion in the rat. Endocrinology 1971, 88:556-565.

20. BROWN AJ, CASALS-STENZEL J, GOFFORD S, LEVER AF, MORTON JJ: Comparison of fast and slow pressor effects of angiotensin II in the conscious rat. Am. J. Physiol. 1981, 241:H381-H388.

21. BROWNFIELD MS, REID IA, GANTEN D, GANONG WF: Differential distribution of immunoreactive angiotensin and angiotensin-converting enzyme in rat brain. Neurosc. 1982, 7:1759-1769.

22. BRUNNER BR, CHANG P, WALLACH R, SEALEY JE, LARAGH JH: Angiotensin II vascular receptors: their avidity in relationship to sodium balance, the autonomic nervous system, and hypertension. J. Clin. Invest. 1972, 51:58-67.

23. CALDWELL PRB, SEEGAL BC, HSU KC, DAS M, SOFFER RL: Angiotensin-converting enzyme: vascular endothelial localization. Science 1976, 191:1050-1051.

24. CALZA L, FUXE K, AGNATI LF, ZINI I, GANTEN D, LANG RE, POULSEN K, HOKFELT T: Presence of renin-like immunoreactivity in oxytocin immunoreactive nerve cells of the paraventricular and supraoptic nuclei in the rat hypothalamus. Acta Physiol. Scand. 1982, 116:313-316.

25. CAMPBELL DJ: The site of angiotensin production. J. Hypertension 1985, 3:199-207.

26. CAMPBELL DJ, ROUHNIK J, MENARD J, CORVOL P: Identity of angiotensin precursors of rat brain and liver. Nature Lond. 1984, 308:206-208.

27. CAMPBELL WH, BROOK SN, PETTINGER WA: Angiotensin II and angiotensin III-induced aldosterone releases in the rat. Science 1974, 184:994-996.

28. CANTIN M, GUTKOWSKA J, LACASSE J, BALLAK M, LEDOUX S, INAGAMI T, BFUZERON J, GENEST J: Ultrastructural immunocytochemical localization of renin and angiotensin II in the juxtaglomerular cells of the ischemic kidney in experimental renal hypertension. Am. J. Path. 1984, 115:212-224.

29. CAPPONI AM, AGUILERA G, FAKUNDING JL, CATT KJ. Angiotensin II receptors and mechanisms of action. In: Soffer RL (ed) Biochemical Regulation of Blood Pressure. 1981, John Wiley & Sons, New York, pp. 207-255.

30. CAPPONI AM, FAVROD-COUNE CA, GAILLARD RC, MULLER AF: Binding and activation properties of angiotensin II in dispersed rat anterior pituitary cells. Endocrinology 1982, 110:1043-1044.

31. CARVALHO JS, CHERKES JK: Renin release by pentobarbital anesthesia in the rat: a role for vascular mechanisms. Life Sci. 1982, 30:887-897.

32. CARVALHO JS, SHAPIRO R, HOPPER P, PAGE LB: Methods for serial study of renin-angiotensin system in the unanesthetized rat. Am. J. Physiol. 1975, 228:369-375.

33. CATT KJ, AGUILERA G, CAPPONI A, FUJITA K, SCHIRAR A, FAKUNDING J: Angiotensin II receptors and aldosterone secretion. J. Endocrinol. 1979, 81:37P-48P.

34. CATT KJ, MENDELSOHN FAC, MILLAN MA, AGUILERA G: The role of angiotensin II receptors in vascular regulation. J. Card. Pharmacol. 1984, 6:5575-5586.

35. CELIO MR, CLEMENS DL, INAGAMI T: Renin in anterior pituitary, pineal, and neuronal cells of mouse brain. Immunohistochemical localization. Biomed. Res. 1980, 1:427-431.

36. CELIO MR, INAGAMI T: Angiotensin II immunoreactivity coexists with renin in the juxtaglomerular cells of the kidney. Proc. Natl. Acad. Sci. 1981, 78:3897-3900.

37. CHANGARIS DG, DEMERS LM, KEIL LC, SEVERS WB: Identification of angiotensin I in the rat and human pituitary gland. Exp. Neurol. 1976, 51:699-704.

38. CHANGARIS DG, SEVERS WB, KEIL LC: Localization of angiotensin in rat brain. J. Histochem. Cytochem. 1978, 26:593-607.

39. CHAPMAN BJ, BROOKS DP, MUNDAY KA: Half-life of angiotensin II in the conscious and barbiturate-anaesthetized rat. Br. J. Anaesth. 1980, 52:389-393.

40. CHEN FM, HAWKINS R, PRINTZ MP: Evidence for a functional, independent brain-angiotensin system: correlation between regional distribution of brain angiotensin receptors, brain angiotensinogen and drinking during the estrous cycle of rats. In: Ganten D, Printz MP, Philipps MF, Scholkens BA. (eds) Experimental Brain Research, 1982, Suppl. 4, Springer-Verlag, Heidelberg, pp. 157-168.

41. CHEN FM, PRINTZ MP: Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 1983, 113:1503-1510.

42. CHEVILLARD C, NIWA M, SAAVEDRA JM: Angiotensin-converting enzyme in discrete forebrain areas of spontaneous hypertensive rats. Brain Res. 1984, 309:389-392.

43. CHEVILLARD C, SAAVEDRA JM: Angiotensin-converting enzyme (kinase II) in pituitary gland of spontaneously hypertensive rats. Regul. Peptides 1983, 5:333-341.

44. COLEMAN TG, McCAA KE, McCAA CS: Effect of angiotensin II on aldosterone secretion in the conscious rat. J. Endocrinol. 1974, 60:421-427.

45. DANIELS-SEVERS A, OGDEN E, VERNIKOS-DANELLIS J: Centrally mediated effects of angiotensin II in the unanesthetized rat. Physiol. and Behavior 1971, 7:785-787.

46. DE LEAN A, ONG H, GUTKOWSKA J, SCHILLER PW, McNICOLL N: Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. Mol. Pharmacol. 1984, 26:498-508.

47. DENEF C: Paracrine interaction in anterior pituitary. Abstract # S93, 7th International Congress of Endocrinology 1984, p. 107.

48. DENEF C, ANDRIES M: Evidence for paracrine interaction between gonadotrophs and lactotrophs in pituitary cell aggregates. Endocrinology 1983, 112:813-822.

49. DESCHEPPER CF, SEIDLER CD, STEELE MK, GANONG WF: Further studies on the localization of angiotensin II-like immunoreactivity in the anterior pituitary gland of the male rat, comparing various antisera to pituitary hormones and their specificity. Neuroendocrinology 1985, 40:471-475.

50. DESJARDINS-GIASSON S, GUTKOWSKA J, GARCIA R, GENEST J: Renin substrate in rat mesenteric artery. J. Physiol. Pharmacol. 1981, 59:528-532.

51. DESJARDINS-GIASSON S, GUTKOWSKA J, GARCIA R, GENEST J: Effect of angiotensin II and norepinephrine on release of prostaglandins E_2 and I_2 by the perfused rat mesenteric artery. Prostaglandins 1982, 24:105-114.

52. DEVYNCK MA, MEYER P: Angiotensin receptors in vascular tissue. Am. J. Med. 1976, 61:758-767.

53. DEVYNCK MA, PERNOLLET MG, MacDONALD GJ, MATTHEWS PG, RAISMAN RS, MEYER P: Alterations of adrenal and uterine angiotensin II receptors during variation of sodium intake and/or experimental hypertension. Clin. Sci. Mol. Med. 1978, 55 (Suppl. 4):171S-174S.

54. DEVYNCK MA, PERNOLLET MG, MATTHEWS PG, MacDONALD GJ, RASIMAN RS, MEYER P: Sodium intake and plasma angiotensin levels as modulators of adrenal and uterine angiotensin II receptors in the rat. J. Cardiovasc. Pharmacol. 1979, 1:163-179.

55. DEVYNCK MA, ROUZAIRE-DUBOIS B, CHEVILLOTTE E, MEYER P: Variations in the number of uterine angiotensin receptors following changes in plasma angiotensin levels. Eur. J. Pharmacol. 1976, 40:27-37.

56. DICKINSON CJ, FERRARIO CM: Central neurogenic effects of angiotensin. In: Page IH, Bumpus FM (eds) Angiotensin. 1974, Springer-Verlag, Berlin, pp. 408-416

57. DOUGLAS J, AGUILERA G, KONDO T, CATT K: Angiotensin II receptors and aldosterone production in rat adrenal glomerulosa cells. Endocrinology 1978, 102:685-696.

58. DOUGLAS J, CATT KJ: Regulation of angiotensin II receptors in the rat adrenal cortex by dietary electrolytes. J. Clin. Invest. 1976, 58:834-843.

59. DOUGLAS JG: Changes in potassium balance: inverse relationship between number and affinity of angiotensin II receptors of smooth and adrenal target tissues. Am. J. Physiol. 1979, 237:E519-E523.

60. DOUGLAS JG, BROWN GP: Effect of prolonged low dose infusion of angiotensin II and aldosterone on rat smooth muscle and adrenal angiotensin II receptors. Endocrinology 1982, 111:988-992.

61. DUFAU ML, CRAWFORD JD, KLIMAN E: Effect of high sodium intake on the response of the rat adrenal to angiotensin II. Endocrinology 1969, 84:462-463.

62. DUFY-BARBE L, RODRIGUEZ F, ARSAUT J, VERRIER D, VINCENT JD: Angiotensin II stimulates prolactin release in the Rhesus monkey. Neuroendocrinology 1982, 35:242-247.

63. DUSTING GJ, MULLINS EM: Stimulation by angiotensin of prostacyclin biosynthesis in rats and dogs. Clin. Exp. Pharmacol. & Physiol. 1980, 7:545-550.

64. DUSTING GJ, MULLINS EM, NOLAN RD: Prostacyclin (PGI_2) release accompanying angiotensin conversion in rat mesenteric vasculature. Eur. J. Pharmacol. 1981, 70:129-137.

65. FIELD LJ, McGOWAN RA, DICKINSON DP, GROSS KW: Tissue and gene specificity of mouse renin expression. Hypertension 1984, 6:597-603.

66. FISHMAN MC, ZIMMERMAN EA, SLATER EE: Renin and angiotensin: the complete system within the neuroblastoma glioma cell. Science 1981, 214:921-923.

67. FUXE K, AGNATI LF, GANTEN D, LANG RE, CALZA L, POULSEN K, INFANTELLINA F: Morphometric evaluation of the coexistence of renin-like and oxytocin-like immunoreactivity in nerve cells of the paraventricular hypothalamic nucleus of the rat. Neurosc. Lett. 1982, 33:19-24.

68. FUXE K, GANTEN D, HOKFELT T, BOLME P: Immunocytochemical evidence for the existence of angiotensin II-containing nerve terminals in the brain and spinal cord in the rat. Neurosc. Lett. 1976, 2: 224-234.

69. FUXE K, GANTEN D, HOKFELT T, LOCATELLI V, POULSEN K, STOCK G, RIX E, TAUGNER R: Renin-like immunocytochemical activity in the rat and mouse brain. Neurosc. Lett. 1980, 18:245-250.

70. GAILLARD RC, GROSSMAN A, GILLIES G, REES LH, BESSER GM: Angiotensin II stimulates the release of ACTH from dispersed rat anterior pituitary cells. Clin. Endocrinol. 1981, 15:573-578.

71. GANTEN D, FUXE K, PHILLIPS MI., MANN JFE, GANTEN U: The brain isorenin-angiotensin system: biochemistry, localization, and possible role in drinking and blood pressure regulation. In: Ganong WF, Martini L (eds) Frontiers in Neuroendocrinology. 1978, vol. 5, Raven Press, New York, pp. 61-100.

72. GANTEN D, GANTEN U, KUBO S, GRANGER P, NOWACZYNSKI W, BOUCHER R, GENEST J: Iso-renin in rat adrenal glands: influence of sodium, potassium and pituitary hormones. Am. J. Physiol. 1974, 227: 224-229.

73. GANTEN D, HAYDUK K, BRECHT HM, BOUCHER R, GENEST J: Evidence of renin release or production in splanchnic territory. Nature 1970, 226:551-552.

74. GANTEN D, HERMANN K, BAYER C, UNGER T, LANG RE: Angiotensin synthesis in the brain and increased turnover in hypertensive rats. Science 1983, 221:869-871.

75. GANTEN D, HERMANN K, UNGER T, LANG RE: The tissue renin-angiotensin systems: focus on brain angiotensin, adrenal gland and arterial wall. Clin. Exp. Hypertens. [A] 1983, 5: 1099-1118.

76. GANTEN D, SCHELLING P, FLUGEL RM, FISCHER H: Effect of angiotensin and the angiotensin antagonist P113 on iso-renin and cell growth in 3T3 mouse cells. Int. Res. Comm. Med. Sci. 1975, 3:327.

77. GANTEN D, SCHELLING P, VECSEI P, GANTEN U: Iso-renin of extrarenal origin: the tissue angiotensinogenase systems. Am. J. Med. 1976, 60:760-772.

78. GEHLERT DR, SPETH RC, WAMSLEY JK: Autoradiographic localization of angiotensin II receptors in the rat brain and kidney. Eur. J. Pharmacol. 1984, 98:145-146.

79. GENEST J, SIMARD S, ROSENTHAL J, ROUCHER R: Norepinephrine and renin content in arterial tissue from different vascular beds. Can. J. Physiol. Pharmacol. 1969, 47:87-91.

80. GIMBRONE MA Jr, ALEXANDER RW: Angiotensin II stimulation of prostaglandin production in cultured human vascular endothelium. Science 1975, 189:219-220.

81. GLOSSMAN H, BAUKAL A, CATT KJ: Cation dependence of high-affinity angiotensin II binding to adrenal cortex receptors. Science 1974, 185:281-283.

82. GLOSSMAN H, BAUKAL A, CATT KJ: Angiotensin II receptors in bovine adrenal cortex. Modification of angiotensin II binding by guanyl nucleotides. J. Biol. Chem. 1974, 249:664-666.

83. GROVER AK, KWAN CY, KOSTKA P, DANIEL EE: Binding and degradation of angiotensin II by mesenteric artery subcellular membranes. Eur. J. Pharmacol. 1985, 112:137-143.

84. GUNTHER S, ALEXANDER RW, ATKINSON WJ, GIMBRONE MA: Functional angiotensin II receptors in cultured vascular smooth muscle cells. J. Cell Biol. 1982, 92:289-298.

85. GUNTHER S, GIMBRONE MA, ALEXANDER RW: Identification and characterization of the high affinity vascular angiotensin II receptor in rat mesenteric artery. Circ. Res. 1980, 47:278-286.

86. GUNTHER S, GIMBRONE MA, ALEXANDER RW: Regulation by angiotensin II of its receptors in resistance blood vessels. Nature 1980, 287:113-117.

87. HASHIMOTO K, YUNOKI S, TAKAHARA J, OFUJI T: ACTH release in pituitary cell cultures. Effect of neurogenic peptides and neurotransmitter substances on ACTH release induced by hypothalamic corticotropin releasing factor (CRF). Endocrinol. Jpn 1979, 26:103-109.

88. HAUGER RL, AGUILERA G, BAUKAL AJ, CATT KJ: Characterization of angiotensin II receptors in the anterior pituitary gland. Mol. Cell. Endocrinol. 1982, 25:203-212.

89. HAUGER RL, AGUILERA G, CATT KJ: Angiotensin II regulates its receptor sites in the adrenal glomerulosa zone. Nature 1978, 271: 176-178.

90. HAWKINS RL, PRINTZ MP: Distribution of angiotensinogen in Brattleboro rat brain. Brain Res. Bull. 1983, 10:163-166.

91. HEALY DP, PRINTZ MP: Autoradiographic localization of angiotensin II binding sites in the brain, pituitary, kidney and adrenal gland of the rat. J. Hypert. 1984, 2 (Suppl. 3):57-61.

92. HEALY DP, PRINTZ MP: Distribution of immunoreactive angiotensin II, angiotensin I, angiotensinogen, and renin in the central nervous system of intact and nephrectomized rats. Hypertension 1984, 6 (Suppl. I):I-130-I-136.

93. HERMANN K, GANTEN D, BAYER C, UNGER T, LANG RE, RASCHER W: Definitive evidence for the presence of [Ile^5]-angiotensin I and [Ile^5]-angiotensin II in the brain of rats. In: Ganten D, Printz MP, Philipps MF, Scholkens BA. (eds) Experimental Brain Research, 1982, Suppl. 4, Springer-Verlag, Heidelberg, pp. 192-207.

94. HIROSE S, OHSAWA T, INAGAMI T, MURAKAMI K: Brain renin from bovine anterior pituitary. Isolation and properties. J. Biol. Chem. 1982, 257:6316-6321.

95. HIROSE S, YOKOSAWA H, INAGAMI T, WORKMAN J: Renin and prorenin in hog brain: ubiquitous distribution and high concentration in the pituitary and pineal. Brain Res. 1980, 191:489-499.

96. IIJIMA F, MALIK KU: Mesenteric vascular reactivity in dexamethasone-treated hypertensive rats. Hypertension 1985, 7:783-790.

97. INAGAMI T: Renin in the brain and neuroblastoma cells: an endogenous intracellular system. Neuroendocrinology 1982, 35: 475-482.

98. INAGAMI T, CELIO MR, CLEMENS DL, LAU D, TAKII Y, KASSELBERG AG, HIROSE S: Renin in rat and mouse brain: immunohistochemical identification and localization. Clin. Sci. 1980, 59:49s-51s.

99. INAGAMI T, CLEMENS DL, CELIO MR, BROWN A, SANDRU L, HERSHCKOWITZ N, HOFFMAN LH, KASSELBERG AG: Immunohistochemical localization of renin in mouse brain. Neurosc. Lett. 1980, 18:91-98.

100. ISRAEL A, CORREA FMA, NIWA M, SAAVEDRA JM: Quantitative determination of angiotensin II binding sites in rat brain and pituitary gland by autoradiography. Brain Res. 1984, 322:341-345.

101. ISRAEL A, SAAVEDRA JM, PLUNKETT L: Water deprivation upregulates angiotensin II receptors in rat anterior pituitary. Am. J. Physiol. 1985, 248:E264-E267.

102. KATZ FH, ROMF R, SMITH JA: Normal variation of plasma aldosterone, cortisol and renin activity in supine man. J. Clin. Endocrinol. Metab. 1975, 40:125-134.

103. KILCOYNE MM, HOFFMAN DL, ZIMMERMAN EA: Immunocytochemical localization of angiotensin II and vasopressin in rat hypothalamus: evidence for production in the same neuron. Clin. Sci. 1980, 59:57s-60s.

104. KRAFT K, LANG RR, GAIDA W, UNGER T, GANTEN D: Angiotensin stimulates β-endorphin release from anterior pituitary gland cell cultures of rats. Neurosci. Lett. 1984, 46:25-29.

105. KRAMER J, FEUERSTEIN G, BEN-DAVID M: The effect of angiotensin II on in vitro prolactin and LH release from the rat pituitary. Neuropharmacology 1979, 18:709-710.

106. LANG RE, UNGER T, RASCHER W, GANTEN D: Brain Angiotensin. In: Iversen LL, Iversen SD, Snyder SH (eds) Handbook of Psychopharmacology. 1983, vol 16, Plenum Press, New York, pp. 307-361.

107. LEGROS F, LEHOUX JG: Changes in characteristics of rat adrenal glomerulosa cells under acute and chronic treatment with ACTH. Can. J. Biochem. Cell. Biol. 1982, 61:538-546.

108. LE MORVAN P, PALAIC D: Characterization of angiotensin II receptors in guinea pig aorta. J. Pharmacol. Exp. Ther. 1975, 195:167-175.

109. LEVENS NR, PEACH MJ, CAREY RM: Role of the intrarenal renin-angiotensin system in the control of renal function. Circ. Res. 1981, 48:157-167.

110. LEWICKI JA, FALLON JH, PRINTZ MP: Regional distribution of angiotensinogen in rat brain. Brain Res. 1978, 158:359-371.

111. LIND RW, SWANSON LW, BRUHN TO, GANTEN D: The distribution of angiotensin II-immunoreactive cells and fibers in the paraventriculo-hypophysial system of the rat. Brain Res. 1985, 338:81-89.

112. LIND RW, SWANSON LW, GANTEN D: Angiotensin II immunoreactivity in the neural afferents and efferents of the subfornical organ of the rat. Brain Res. 1984, 321:209-215.

113. LIND RW, SWANSON LW, GANTEN D: Organization of angiotensin II immunoreactive cells and fibers in the rat central nervous system. Neuroendocrinology 1985, 40:2-24.

114. MALIK KU, NASJLETTI A: Facilitation of adrenergic transmission by locally generated angiotensin II in rat mesenteric arteries. Circ. Res. 1976, 38:26-30.

115. McCAA RE, McCAA CS, GUYTON AC: Role of angiotensin II and potassium in the long term regulation of aldosterone secretion in intact conscious dogs. Circ. Res. 1975, 36 (Suppl. I):57-67.

116. McQUEEN J, MURRAY GD, SEMPLE PF: Identification of the angiotensin II receptor in rat mesenteric artery. Biochem. J. 1984, 223:659-671.

117. MENDELSOHN FAO: Evidence for the local occurence of angiotensin II in rat kidney and its modulation by dietary sodium intake and converting enzyme blockade. Clin. Sci. Mol. Med. 1979, 57: 173-179.

118. MENDELSOHN FAO, AGUILERA G, SAAVEDRA JM, QUIRION R, CATT KJ: Characteristics and regulation of angiotensin II receptors in pituitary, circumventricular organs and kidney. Clin. Exp. Hypertens. 1983, 5:1081-1097.

119. MENDELSOHN FAO, QUIRION R, SAAVEDRA JM, AGUILERA G, CATT KJ: Autoradiographic localization of angiotensin II receptors in rat brain. Proc. Natl. Acad. Sci. 1984, 81:1575-1579.

120. MONCADA S, HERMAN AG, HIGGS EA, VANE JR: Differential formation of prostacyclin (PGX or PGI_2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. Thrombosis Res. 1977, 11:323-344.

121. MUKHERJEE A, KULKARNI P, McCANN SM, NEGRO-VILAR A: Evidence for the presence and characterization of angiotensin II receptors in rat anterior pituitary membranes. Endocrinology 1982, 110:665-667.

122. MULLER J: Supression of aldosterone biosynthesis by treatment of rats with adrenocorticotropin: comparison with glucocorticoid effects. Endocrinology 1978, 103:2061-2068.

123. MURAKAMI K, HASHIMOTO K, OTA Z: Effect of angiotensin II, catecholamines and glucocorticoid on corticotropin releasing factor (CRF)-induced ACTH release in pituitary cell cultures. Acta Med. Okayama 1984, 38:349-358.

124. NARUSE K, INAGAMI T, CELIO MR, WORKMAN RJ, TAKII Y: Immunocytochemical evidence that angiotensins I and II are formed by intracellular mechanism in juxtaglomerular cells. Hypertension 1982, 4 (Suppl. II):II-70-II-74.

125. NARUSE K, TAKII Y, INAGAMI T: Immunohistochemical localization of renin in luteinizing hormone-producing cells of rat pituitary. Proc. Natl. Acad. Sci. 1981, 78:7579-7583.

126. NARUSE M, INAGAMI T: Markedly elevated specific renin levels in the adrenal in genetically hypertensive rats. Proc. Natl. Acad. Sci. 1982, 79:3295-3299.

127. NARUSE M, SUSSMAN CR, NARUSE K, JACKSON RV, INAGAMI T: Renin exists in human adrenal tissue. J. Clin. Endocrinol. Metab. 1983, 57:482-486.

128. NARUSE M, TAKII Y, INAGAMI T: Renin exists in high concentration in the adrenal gland of the rat. Biomed. Res. 1981, 2 (5):583-586.

129. NEWTON MA, LARAGH JH: Effect of corticotropin on aldosterone excretion and plasma renin in normal subjects in essential hypertension and primary hyperaldosteronism. J. Clin. Endocrinol. Metab. 1968, 28:1006-1013.

130. NICOLL RA, BARKER JL: Excitation of supraoptic neurosecretory cells by angiotensin II. Nature New Biol. 1971, 233:172-174.

131. OKAMURA T, CLEMENS DL, INAGAMI T: Renin, angiotensins and angiotensin-converting enzyme in neuroblastoma cells: evidence for intracellular formation of angiotensins. Proc. Natl. Acad. Sci. 1981, 78:6940-6943.

132. OYAMA T: Endocrine responses to anaesthetic agents. Br. J. Anaesth. 1973, 45:276-281.

133. PAGLIN S, STUKENBROK H, JAMIESON JD: Interaction of angiotensin II with dispersed cells from the anterior pituitary of the male rat. Endocrinology 1984, 114:2284-2292.

134. PALKOVITS M: Role of the central nervous system neuropeptides in body fluid homeostasis. J. Physiol. 1984, 79:428-431.

135. PALMORE WP, ANDERSON R, MULROW PJ: Role of the pituitary in controlling aldosterone production in sodium depleted rats. Endocrinology 1970, 86:728-734.

136. PANDEY KN, MISONO KS, INAGAMI T: Evidence for intracellular formation of angiotensins: coexistence of renin and angiotensin-converting enzyme in Leydig cells of rat testis. Biochem. Biophys. Res. Commun. 1984, 122:1337-1343.

137. PEACH MJ: Renin-angiotensin system: biochemistry and mechanisms of action. Physiol. Rev. 1977, 57:313-370.

138. PETTINGER WA, TANAKA K, KEETON K, CAMPBELL WB, BROOKS SN: Renin release, an artifact of anesthesia and its implications in rats. Proc. Soc. Exp. Biol. & Med. 1975, 148:625-630.

139. PHILLIPS MI, WEYENMEYER J, FELIX D, GANTEN D, HOFFMAN WE: Evidence for an endogenous brain renin-angiotensin system. Fed. Proc. 1979, 38:2260-2266.

140. POLSKY-CYNKIN R, REICHLIN S, FANBURG BL: Angiotensin I-converting enzyme activity in the spontaneously hypertensive rat (40855). Proc. Soc. Exp. Biol. Med. 1980, 164:242-247.

141. POWELL-JACKSON JD, MacGREGOR J: Radioimmunoassay of angiotensin II in the rat. J. Endocrinol. 1976, 68:175-176.

142. PRINTZ MP, GANTEN D, UNGER T, PHILLIPS MI: Minireview: The brain renin angiotensin system. In: Ganten D, Printz MP, Philipps MI, Scholkens BA (eds) Experimental Brain Research. 1982, Suppl. 4, Springer Verlag, Heidelberg, pp. 3-52.

143. PRINTZ MP, HAWKINS R, GIPE D, KALTER V, HEALY D: Angiotensin receptors and angiotensinogen in cultured neural and neuroendocrine cells. Clin. Exp. Hypertens. 1984, A6 (10-11):2113-2116.

144. PRINTZ MP, PRINTZ JM, GREGORY TJ: Identification of angiotensinogen in animal brain homogenates. Relationship to a possible angiotensin I-generating pathway. Circ. Res. 1978, 43 (Suppl. I):I-21-I-27.

145. RAIZADA MK, PHILLIPS MI, GERNDT JS: Primary cultures from fetal rat brain incorporate [^{3}H]-isoleucine and [^{3}H]-valine into immunoprecipitable angiotensin II. Neuroendocrinology 1983, 36:64-67.

146. RAIZADA MK, STENSTROM B, PHILLIPS MI, SUMNERS C: Angiotensin II in neuronal cultures from brains of normotensive and hypertensive rats. Am. J. Physiol. 1984, 247:115-119.

147. RAIZADA MK, YANG JW, PHILLIPS MI, FELLOWS RE: Rat brain cells in primary culture: characterization of angiotensin II binding sites. Brain Res. 1981, 207:343-355.

148. RAMSAY DJ, KEIL LC, SHARPE MC, SHINSAKO J: Angiotensin II infusion increases vasopressin, ACTH, and 11-hydroxycorticosteroid secretion. Am. J. Physiol. 1978, 234:R66-R71.

149. RAYYIS SS, HORTON R: Effect of angiotensin II on adrenal and pituitary function in man. J. Clin. Endocrinol. Metab. 1971, 32:539-546.

150. RE R, FALLON JT, DZAU V, QUAY S, HABER E: Renin synthesis by arterial smooth muscle cells. (Abstract) Circulation 1979, 60 (Suppl. II):II-10.

151. RE R, FALLON JT, DZAU V, QUAY S, HABER E: Renin synthesis by canine aortic smooth muscle cells in culture. Life Sci. 1982, 30:99-106.

152. RE RN: Changes in nuclear initiation sites after the treatment of isolated nuclei with angiotensin II. Clin. Sci. 1982, 63:191s-193s.

153. RE RN, MACPHEE A, FALLON J: Specific nuclear binding of angiotensin II by rat liver and spleen nuclei. Clin. Sci. 1981, 61:245s-247s.

154. REID JA, BROOKS VL, RUDOLPH CD, KEIL LC: Analysis of the actions of angiotensin on the central nervous system of conscious dogs. Am. J. Physiol, 1982, 243:R82-R91.

155. REID WD, LARAGH JH: Sodium and potassium intake, blood pressure and pressor response to angiotensin. Proc. Soc. Exp. Biol, Med. 1965, 120:26-29.

156. RIX E, GANTEN D, SCHULL B, UNGER T, TAUGNER R: Converting-enzyme in the choroid plexus, brain, and kidney: immunocytochemical and biochemical studies in rats. Neurosc. Lett. 1981, 22:125-130.

157. ROSENMAN RH, FREED C, FRIEDMAN M: The peripheral vascular reactivity of potassium deficient rats. Circulation 1952, 5:412-414.

158. SAAVEDRA JM, FERNANDEZ-PARDAL J, CHEVILLARD C: Angiotensin-converting enzyme in discrete areas of the rat forebrain and pituitary gland. Brain Res. 1982, 245:317-325.

159. SAKAI KK, MARKS BH, GEORGE J, KOESTNER A: Specific angiotensin II receptors in organ-cultured canine supraoptic nucleus cells. Life Sci. 1974, 14:1337-1344.

160. SATO S: Postnatal development, sexual difference and sexual cyclic variation of prolactin cells in rats: special reference to the topographic affinity to a gonadotroph. Endocrinol. Jpn. 1980, 5:573-583.

161. SAWCHENKO PE, SWANSON LW, VALE WW: Coexpression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. Proc. Natl. Acad. Sci. 1984, 81:1883-1887.

162. SCHIFFRIN EL, FRANKS DJ, GUTKOWSKA J: Aldosterone up-regulates vascular angiotensin II receptors in vivo and in cultured vascular smooth muscle cells in vitro (Abstract). Clin. Res. 1983, 31:688A.

163. SCHIFFRIN EL, GUTKOWSKA J, GENEST J: Effect of angiotensin II and deoxycorticosterone infusion on vascular angiotensin II receptors in rats. Am. J. Physiol. 1984, 246:H608-H614.

164. SCHOLKENS BA, JUNG W, RASCHER W, DIETZ R, GANTEN D: Intracerebroventricular angiotensin II increases arterial blood pressure in rhesus monkeys by stimulation of pituitary hormones and the sympathetic nervous system. Experientia 1982, 38:469-470.

165. SCHRAMME C, DENEF C: Stimulation of prolactin by angiotensin II in superfused rat anterior pituitary cell aggregates. Neuroendocrinology 1983, 36:483-485.

166. SCHRAMME C, DENEF C: Stimulation of spontaneous and dopamine-inhibited prolactin release from anterior pituitary reaggregate cell cultures by angiotensin peptides. Life Sciences 1984, 34:1651-1658.

167. SEALY JE, CLARK I, BULL MR, LARAGH JH: Potassium balance and the control of renin secretion. J. Clin. Invest. 1972, 49:2119-2127.

168. SLATER EE, DEFENDINI R, ZIMMERMAN EA: Wide distribution of immunoreactive renin in nerve cells of human brain. Proc. Natl. Acad. Sci. 1980, 77:5458-5460.

169. SLATER EE, ZIMMERMAN EA, FISHMAN MC: A complete renin-angiotensin system within clonal neuroblastoma cell lines. In: Costa E and Trabucchi M (eds) Regulatory Peptides: From Molecular Biology to Function. 1982, Raven Press, New York, pp. 487-496.

170. SOREL DO: Characterization of angiotensin-mediated ACTH release. Neuroendocrinology 1983, 36:249-253.

171. SOBEL DO: Angiotensin II mediates β-endorphin like immunoactivity release in rat pituitary cell culture. Peptides 1984, 5:47-51.

172. SOBEL DO, VAGNUCCI A: Angiotensin II mediated ACTH release in rat pituitary cell culture. Life Sci. 1982, 30:1281-1286.

173. SPETH RC, VALLOTTON MB, WAMSLEY JK, KHOSLA MC, CHERNICKY CL, BUMPUS FM, FERRARIO CM: Localization of angiotensin receptors in the canine CNS. Clin. Exp. Hypertens. 1984, A6 (10-11):1749-1753.

174. SPETH RC, WAMSLEY JK, GEHLERT DR, CHERNICKY CL, BARNES KL, FERRARIO CM: Angiotensin II receptor localization in the canine CNS. Brain Res. 1985, 326:137-143.

175. SPINEDI E, NEGRO-VILAR A: Angiotensin II and ACTH release: site of action and potency relative to corticotropin releasing factor and vasopressin. Neuroendocrinology 1983, 37:446-453.

176. SPINEDI E, NEGRO-VILAR A: Angiotensin II increases ACTH release in the absence of endogenous arginine-vasopressin. Life Sciences 1983, 34:721-729.

177. STEELE MK, BROWNFIELD MS, GANONG WF: Immunocytochemical localization of angiotensin immunoreactivity in gonadotrops and lactotrops of the rat anterior pituitary gland. Neuroendocrinology 1982, 35:155-158.

178. STEELE MK, GALLO RV, GANONG WF: Stimulatory or inhibitory effects of angiotensin II upon LH secretion in ovariectomized rats: a function of gonadal steroids. Neuroendocrinology 1985, 40:210-216.

179. STEELE MK, McCANN SM, NEGRO-VILAR A: Modulation by dopamine and estradiol of the central effects of angiotensin II on anterior pituitary hormone release. Endocrinology 1982, 111:722-729.

180. STEELE MK, NEGRO-VILAR A, McCANN SM: Effect of angiotensin II on in vivo and in vitro release of anterior pituitary hormones. Endocrinology 1981, 109:893-899.

181. SWALES JD: Arterial wall or plasma renin in hypertension. Clin. Sci. 1979, 56:293-298.

182. SWALES JD, ABRAMOVICI A, BECK F, BING RF, LOUDON M, THURSTON H: Arterial wall renin. J. Hypert. 1983, 1:I-17-I-22.

183. SWALES JD, ABRAMOVICI A, BECK F, BING RF, LOUDON M, THURSTON H: Arterial wall renin. J. Hypertension 1983, 1 (Suppl. 1):17-22.

184. SWANSON LW, SAWCHENKO PE, RIVIER J, VALE WW: Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 1983, 36:165-186.

185. TAUGNER R, BUHRLE CP, GANTEN D, HACKENTHAL E, HARDEGG C, HARDEGG G, NOBILING R, UNGER T: Angiotensin-like activity in resistance vessels. Histochemistry 1983, 78:71-70.

186. TAUGNER R, HACKENTHAL E, HELMCHEN U, GANTEN D, KUGLER P, MARIN-GREZ M, NOBILING R, UNGER T, LOCKWALD I, KEILBACH R: The intrarenal renin-angiotensin system. An immunocytochemical study on the localization of renin, angiotensinogen, converting enzyme and the angiotensins in the kidney of mouse and rat. Klin. Wochenschr. 1982, 60:1218-1222.

187. TAUGNER R, MANNECK E, NOBILING R, RUHRLE CP, HACKENTHAL E, GANTEN D, INAGAMI T, SCHRODER H: Coexistence of renin and angiotensin II in epitheloid cell secretory granules of rat kidney. Histochemistry 1984, 81:39-45.

188. THURSTON H, LARAGH JH: Prior receptor occupancy as a determinant of the pressor activity of infused angiotensin II in the rat. Circ. Res. 1975, 36:113-117.

189. THURSTON H, SWALES JD, BIND RF, HURST BC, MARKS ES: Vascular renin-like activity and blood pressure maintenance in the rat. Hypertension 1979, 1:643-649.

190. VAN HOUTEN M, SCHIFFRIN EL, MANN JFE, POSNER BI, BOUCHER R: Radioautographic localization of specific binding sites for blood-borne angiotensin II in the rat brain. Brain Res. 1980, 186:480-485.

191. VELLETRI P, BEAN BL: The effects of captopril on rat aortic angiotensin-converting enzyme. J. Cardiovasc. Pharmacol. 1982, 4:315-325.

192. VILHARDT LH: Vasopressin content and neurosecretory material in the hypothalamo-neurohypophysial system of rats under different states of water metabolism. Acta Endocrinol. 1970, 63:585-594.

193. WEYHENMEYER JA, HWANG CJ: Characterization of angiotensin II binding sites on neuroblastoma X glioma hybrid cells. Brain Res. 1985, 14:409-414.

194. WEYHENMEYER JA, MEYER JM, WATKINS JE: Angiotensin II synthesis studies in dissociated brain cell cultures. J. Neurochemistry 1984, 43:716-723.

195. WEYHENMEYER JA, PHILLIPS MI: Angiotensin-like immunoreactivity in the brain of the spontaneously hypertensive rat. Hypertension 1982, 4:514-523.

196. WEYHENMEYER JA, RAIZADA MK, PHILLIPS MI, FELLOWS RE: Presence of angiotensin II in neurons cultured from fetal rat brain. Neurosc. Lett. 1980, 16:41-46.

197. WILLIAMS TH, JEW JY, GANTEN D: Brain angiotensin-like localization at the ultrastructural level. Clin. Exp. Hypertens. 1984, A6 (10-11):1897-1914.

198. WRIGHT GB, ALEXANDER RW, EKSTEIN LS, GIMBRONE MA: Sodium, divalent cations, and guanine nucleotides regulate the affinity of the rat mesenteric artery angiotensin II receptor. Circ. Res. 1982, 50:462-469.

199. YANG HYT, NEFF NH: Differential distribution of angiotensin converting enzyme in the anterior and posterior lobe of the rat pituitary. J. Neurochem. 1973, 21:1035-1036.

200. YANG HYT, NEFF NH: Distribution and properties of angiotensin converting enzyme of rat brain. J. Neurochem. 1972, 19:2443-2450.

201. ZIMMERMANN EA: Oxytocin, vasopressin and neurophysins. In: Krieger DT, Brownstein MJ, Martin RJB (eds) Brain Peptides. 1983, John Wiley & Sons, New York, pp. 597-609.

CHAPTER 2 ESTROGENS AND THE RENIN-ANGIOTENSIN SYSTEM

A) INTRODUCTION

An increasing amount of physiological and pharmacological investigations suggest that estrogens are important modulators of the renin-angiotensin-aldosterone system. Estrogens appear to have profound effects on the complex ANG II-mediated mechanisms regulating uterine contractility, thirst, sodium appetite, blood pressure control, vascular reactivity and anterior pituitary function. Several components of the renin-angiotensin system fluctuate during the estrous and menstrual cycle and also during pregnancy. At estrus, plasma renin substrate has been shown to increase in rats whereas, in several areas of the brain renin substrate decreases (8,54). In women, during the pre-ovulatory and luteal phase of the menstrual cycle the activity of the renin-angiotensin-aldosterone system is increased (32,73).

Evidence for a physiological role of estrogens has been suggested in the retention of sodium and water with occasional edema that occurs during the luteal phase of the menstrual cycle (which is a period of increased estrogen secretion and excretion) (3,65,79).

Modulation of ANG II receptors in several target tissues also occurs during the estrous cycle. When compared to diestrus, ANG II receptor density increases in the uterus at estrus (8) and proestrus (62) and decreases at estrus in the pituitary gland (8). Several studies have indicated that female sex steroids, particularly estrogens alter the circulating components of the renin-angiotensin system. Increasing evidence also suggests that estrogens may be involved in the modulation of ANG II receptors. Target cell

responsiveness to ANG II appears to be particularly affected by estrogens suggesting direct or indirect actions with the receptor for ANG II.

B) EFFECTS OF ESTROGENS AND PROGESTOGENS ON THE CIRCULATING COMPONENTS OF THE RENIN-ANGIOTENSIN SYSTEM

a) Effects of estrogens

It has been known for more than 30 years that administration of estrogens in rats and in several other species causes a marked sustained elevation of plasma renin-substrate (previously called "hypertensinogen" and also referred to now as angiotensinogen) (25). Several studies using isolated perfused rat livers have established that estrogens increase hepatic plasma renin substrate synthesis and release (48,49). Following estrogen treatment, the most consistent alteration in the circulating components of the renin-angiotensin system in every animal species studied so far, including man, is a rapid, usually within 24 to 48 hours, and persistent increase in plasma renin substrate (25,33,34,45,46). It has also been shown that estrogens induce the synthesis and release of several forms of renin substrate which are electrophoretically and immunologically distinct from the predominant form of plasma renin substrate (14). In gel filtration studies, a high molecular weight form of plasma renin substrate has been shown to increase in absolute quantity and relative percent in high estrogen states such as pregnancy and in women treated with estrogens (63,77). Statistically significant elevations of this high molecular weight form have been associated with estrogenic hypertension, normotensive pregnancy and pregnancy-induced hypertension (63,77).

Several investigators have suggested that estrogen-induced increases in plasma renin substrate results in an increase in the rate of generation of ANG II within the body (5,39) which may be a causal factor in certain forms of estrogen-induced hypertension. Even though conflicting results have been published (for review 55), it can be generally concluded that in humans, estrogens increase plasma renin activity (33,34) and circulating levels of ANG II (6) which both return towards normal with chronic usage as plasma renin concentrations are being suppressed (34). Most of these studies were performed on women taking oral contraceptives and therefore several factors could explain conflicting results on circulating parameters of the renin-angiotensin system. Among these factors are the dose of estrogen, the relative proportion of estrogen vs progestogen content in the pill, the duration of contraceptive treatment and the preceding sex hormone status of the women used in different studies.

In rats, where larger doses of estrogens have generally been used, there appears to be a more rapid resetting of the renin-angiotensin system. Estradiol treatment in this species produces a rapid increase within 6 hours of plasma renin substrate which leads to a transient 48-hour increase in plasma renin activity, which gradually returns below normal levels by day 6 when the plasma renin concentration is reduced by half (45). These results further substantiate the efficiency of the compensatory regulation of circulating ANG II in rats chronically treated with estradiol. It must be emphasized however that pharmacological doses of estrogens have been used in rats compared to much smaller therapeutic doses used in women. Furthermore, in rats

chronically treated with mg doses of estrogens to induce hypertension (36,60) plasma renin activity was increased while plasma renin concentration was unchanged or slightly decreased again indicating the importance of dose effect on these parameters.

In addition to effects on renin substrate and possible inhibitors and activators which tend to increase plasma renin activity, oral contraceptives also have direct renal effects which modify renin release. Studies in humans and animals indicate that natural and synthetic estrogens have mineralocorticoid activity leading to sodium retention, expansion of plasma volume and subsequent suppression of renal renin release (29,78). Variable elevations of plasma and urinary aldosterone have also been reported in women taking oral contraceptives or only estrogens (12,34,82). Although variable dose- and time-dependent stimulation of the adrenal cortex by estrogens has been reported (35,41,81,84), the endogenous mineralocorticoid activity of estradiol has been suggested by its sodium retaining properties in adrenalectomized dogs (29).

Various estrogens differ in their qualitative and quantitative ability to activate the different parameters of the renin-aldosterone axis. Plasma renin substrate is the most stimulated parameter following administration of commonly used non-esterified polar steroids such as ethinyl estradiol. Esterified conjugated estrogens like estradiol benzoate have a more prolonged effect and delayed absorption causing a greater increase in plasma renin activity with elevations of plasma renin concentrations preceding increases in plasma renin substrate (33,34).

b) Effects of progestogens

The effects of the progestogenic component is more complex. Natural progesterone counteracts the effect of estrogens on aldosterone and sodium metabolism. Progesterone is an aldosterone antagonist causing natriuresis and thus increasing renin release (38). Progesterone causes an increase in plasma renin activity and aldosterone excretion rate but has no effect on renin substrate (11). On the other hand, several of the "synthetic" progestins used in oral contraceptives have a direct mineralocorticoid effect and act with the estrogenic component to suppress renin release (52). Furthermore, because of suppression of the pituitary gonadotropins, endogenous progesterone is not available to exert its anti-aldosterone effect during the administration of the synthetic progestins of oral contraceptives. The net effect of estrogens and oral contraceptives upon the components of the renin-angiotensin system is probably due to the balance of stimulatory effects of estrogens upon plasma renin substrate and an inhibitory effect of estrogen and synthetic progestins due to their mineralocorticoid activity.

The classical mechanism of action of estrogens as well as for steroid hormones in general, consists of hormone binding to a specific cytosolic receptor followed by translocation of the receptor-estrogen complex to the nucleus where it binds to specific sites on the genome (DNA) and activates transcription of new RNA leading to new protein synthesis. In the liver this sequence of events appears to reflect a more general alteration in the rate of synthesis of several specific proteins. During estrogen treatment or pregnancy the increase in renin substrate is accompanied by the increase of several steroid binding

proteins such as corticosteroid-binding globulin (transcortin), testosterone- and estradiol-binding globulin, and also thyroxine-binding globulin, ceruloplasmin and transferrin (copper and iron binding globulins) (47). Apart from this classical anabolic steroid effect, more recent evidence suggests that estrogens may also regulate peptide hormone receptors.

C) EFFECTS OF ESTROGENS ON ANGIOTENSIN II RECEPTORS

a) Effects on uterine angiotensin II receptors

It has been known for quite some time that uterine contractility is increased by estrogen treatment (42) and depressed by progesterone (37). Several *in vivo* studies have shown that estrogen treatment increases α-adrenergic and oxytocin receptors in rat and rabbit uterus (61,67,83), effects which likely contribute to the increased sensitivity of the estrogen-primed uterus to the contractile actions of these compounds. Receptors for ANG II have been identified in uterine smooth muscle (40,59). Although the functional significance of these receptors in the uterus is still unclear, sex steroids have been shown to regulate ANG II receptors in the rat and rabbit uterus and ANG II receptor density varies during the estrous cycle (62). During the ovarian cycle, the density of rat uterine ANG II receptors was about 4 times higher at proestrus than at diestrus II. Ovariectomy caused a progressive decrease in uterine ANG II receptor density. This regulation of uterine ANG II receptors appears to depend upon the relative proportions of estradiol and progesterone (62). In rats, a single injection of unconjugated 17β-estradiol (0.1-10 μg) has been shown to cause a dose-dependent increase in uterine ANG II receptors

measured 24 hours after steroid treatment. This effect is transient however since 48 hours after a single injection (or ? consecutive daily injections) of 17β-estradiol, uterine ANG II receptors return to or below control levels. Infusion of sustained high doses of 17β-estradiol exerted a biphasic effect on uterine ANG II receptors. Short term infusion caused an increase in uterine ANG II receptors, which was detectable after 12 hours and reached a maximum of 4- to 6-fold above control values at 36 hours. Long term infusion for 5-8 days produced an opposite effect as the density of ANG II receptors returned to or below control values. The same short term and long term effects of 17β-estradiol infusion were observed in immature, adult or ovariectomized rats, and in no case did estrogen treatment alter the affinity of the receptors. These observations are consistent with previous studies showing enhanced sensitivity to ANG II of uteri from rats from diestrus I to proestrus or pretreated with estrogens (4,7).

In the rat isolated uterus, contraction-sensitivity to ANG II appears to be mediated by prostaglandins. Indomethacin (a prostaglandin synthesis inhibitor) was shown to suppress the increased uterine sensitivity to ANG II in diestrus and proestrus rats (4) suggesting that increased prostaglandin synthesis could be the mechanism of action of estrogens on enhanced uterine sensitivity to ANG II. However, uteri from estrogen-treated animals have been shown to produce less PGE (72) and indomethacin did not displace the leftward shift of ANG II dose-response curves observed in ovariectomized rats treated with 17β-estradiol (7). The implications of tissue

prostaglandins in estrogen-induced enhancement of uterine sensitivity to ANG II remains equivocal.

Progesterone infusion for up to 2 days has been shown to have no effect on rat uterine ANG II receptors whereas a chronic 7-day infusion decreased receptor density in a dose-dependent manner (62). These results are consistent with the inhibitory effect of progesterone on myometrial contractility (53). Furthermore, progesterone has been shown to block the estradiol-induced increase in uterine ANG II receptor density as demonstrated by persistent receptor down-regulation in uteri of progesterone-treated, estrogen-primed rabbits (62). Alterations in rat uterine ANG II receptor density during pregnancy have also been reported (61). In the implantation area, low binding capacity was observed during the second half of pregnancy until one day after delivery. These changes could be due to high circulating levels of progesterone which may counteract the effect of elevated estrogen levels or might be related to the deciduation process itself. Infusion of the converting enzyme inhibitor (SQ 14 225) between days 4 and 9 of pregnancy had no effect on uterine ANG II receptor density suggesting that the changes observed were not related to altered plasma ANG II levels. The significance of uterine ANG II receptor modulation during pregnancy remains unclear, the extremely low levels of ANG II receptor density in the uterus at the end of pregnancy appear to exclude a major role for ANG II in the process of parturition in the rat (61).

b) Effects on the brain mechanism of thirst

Ingestive behaviors such as food, salt and water intake are affected by the estrous cycle (13,15,76). Ad libitum, ANG II-induced and isoproterenol (β-adrenergic agonist) induced water intake are all decreased at estrus, (15,76). Ovariectomy abolishes fluctuations in drinking (15). Recently, there has been increasing evidence to suggest that elevated estrogen levels are responsible for decreased fluid intake. A single intramuscular dose of estrogen decreases ad libitum drinking after 24 hours (15). Chronic subcutaneous treatment (8-23 weeks) with either conjugated or unconjugated estradiol attenuated the drinking responses of intact female rats to acute peripheral administration of either isoproterenol or graded doses of ANG I or ANG II (17-19). The drinking response of intact female rats to intracerebroventricular administration of ANG II was also attenuated by chronic peripheral estrogen treatment (18), although one study reports no change (64). Acute intracerebroventricular administration of estradiol benzoate to ovariectomized rats was also shown to decrease ad libitum and central ANG II-induced drinking and pressor response after 26 hours followed by a full recovery by 50 hours after estrogen administration (30). This attenuated effect was shown to be specific since water intake and pressor response induced by hypertonic NaCl or carbachol was unaffected by steroid treatment. Therefore chronic or more acute administration of estrogens in the periphery or directly in brain ventricles attenuated ad libitum as well as ANG II- and β-adrenergic-induced thirst in intact and ovariectomized rats. These studies suggested that estrogens could affect the central receptors mediating thirst.

At estrus, brain ANG II receptor density "tends" to decrease in several discrete areas of the brain (8,54). Chronic (10 weeks), and more acute (48 hours) systemic administration of estradiol benzoate was shown to decrease ANG II receptor density in discrete areas of the brain namely the preoptic area and the thalamus/septum (18,31). Interestingly, localized application of estrogens in the brain using crystalline implants of estradiol benzoate inserted in the medial preoptic area also decreased central ANG II-induced drinking whereas estrogen implants in the ventromedial hypothalamus (which is the presumed site for the estrogen effect on decreased food intake) had no effect on ANG II-induced drinking (31). Therefore receptors for both estrogen and ANG II are found together in several discrete areas of the brain. Binding studies suggest that this steroid could alter brain ANG II receptor density by a mechanism that remains to be discovered.

The effect of estrogens on attenuation of drinking response appears to be sex specific and depends upon sexual differentiation of the brain. In intact and castrated male rats, estrogen treatment failed to decrease ad libitum and ANG II-induced drinking and also failed to elicit attenuation of ANG II-induced pressor response (30). The brain of a neonatal rat can be altered by androgens or castration during the so-called critical period which occurs during the first 4-5 days of life (23). In a genetic male endogenous testosterone becomes aromatized to estrogen in the brain and causes differentiation to a "male" brain. A female is not normally exposed to testosterone (or estrogen at this early stage) and so her brain remains undifferentiated and "female". Neonatal castration of a male rat eliminates the source of androgen and therefore the brain remains undifferentiated

("feminization") just like an intact female. Similar to females, "feminized" males tended to decrease drinking following estrogen administration (31). Reciprocally, neonatal "androgenization" of females resulted in animals which as adults no longer respond to estrogens with a decreased drinking response to ANG II (31). These findings could possibly reflect modulation of brain estrogen receptors occurring in the neonatal period which might persist throughout adult life.

In summary, high circulating estrogen levels appear to act at the level of the brain to attenuate the drinking-response of several dypsogenic stimuli particularly ANG II. This effect appears to involve down-regulation of brain ANG II receptors. Furthermore, glucocorticoid or estrogen treatment (24-72 hours) has been shown to decrease brain renin substrate levels in the medial basal hypothalamus as well as in several other discrete areas of the brain (54). A similar trend was also observed in rats at estrus (8). Therefore the possibility that estrogen depletion of brain renin substrate reflects increased local production of ANG II must be considered as a possible mechanism for down-regulation of brain ANG II receptors. Whether or not these effects of estrogens in the brain or elsewhere represent direct alterations at the ANG II receptor level or is mediated by other events remains to be established.

c) Effects on vascular responsiveness to angiotensin II

Normal pregnancy in women as well as in several animal species is associated with significant vascular refractoriness to the pressor effects of infused ANG II (21,22,56,75). This refractoriness to infused ANG II is lost in pregnancies complicated by pregnancy-induced

hypertension (26,74). In pregnant women, this refractoriness to infused ANG II is not altered by volume expansion with physiologic saline or dextran solutions which suppress plasma renin activity (21). Similarly, ANG II refractoriness was not affected by volume expansion in the pregnant ewe whereas this treatment increased vascular reactivity in the nonpregnant animal (43). In this latter study, patterns of changes in plasma renin activity and heart rate due to volume load were similar between nonpregnant and pregnant animals suggesting that pregnancy-induced refractoriness to ANG II is not due to changes in circulating ANG II nor to a baroreceptor response but are more likely the result of alterations at the level of the vessel wall.

This refractoriness to ANG II pressor response can be seen as early as mid pregnancy (22). Since plasma concentrations of sex steroid hormones gradually increase during pregnancy (80) it was suggested that estrogens and/or progesterone could be involved in this attenuated pressor response. Estrogens which increase 20- to 40-fold during pregnancy appear to be important in the increase and maintenance of uteroplacental blood flow as well as in vasodilation of nonreproductive tissues such as the skin (58). Acute estrogen treatment also appears to induce refractoriness to the systemic pressor effects of infused ANG II.

Attenuation of the pressor response to graded intravenous (i.v.)doses of ANG II has been reported following i.v. infusion of either high-dose or low-dose of unconjugated 17β-estradiol for 90 to 100 minutes into unanesthetized, ovariectomized nonpregnant sheep (57). In that study, estrogen infusion was also shown to decrease systemic vascular resistance and to increase cardiac output and plasma

renin activity. The decreased vascular resitance was likely compensated by the increased cardiac output since mean arterial pressure did not change during estrogen infusion. Thus, acutely "estrogenized"nonpregnant sheep develop significant alterations in both the cardiovascular and the renin-angiotensin systems in addition to decreased pressor responsiveness to infused ANG II.

In a similar study, again using nonpregnant sheep, pressor response to i.v. infusion of ANG II was also suppressed by a 60 minute infusion of 17β-estradiol, whereas infusion of progesterone or 5 α-dihydroprogesterone did not affect pressor responses (75). In this latter study however, plasma renin activity did not change nor did acid-base status or serum electrolytes following acute administration of estrogens suggesting that estrogens might have a direct effect on the vascular wall. In this respect, estrogen treatment has been shown to affect arterial connective tissue components (10,16) and alter vascular tone (2). Estradiol will reduce the accumulation of aortic collagen and elastin so that the vessel is more distensible (16).

In ovariectomized rats, acute intracerebroventricular administration of estradiol benzoate was shown to reduce central ANG II-induced pressor response after 26 hours followed by full recovery by 50 hours after estrogen administration (30). Similar to the drinking response, this attenuated pressor response by estrogens was restricted to female rats. On the other hand, in intact female rats anesthetized with sodium pentobarbitone, acute subcutaneous administration of estradiol sulfate for 24 hours had no effect on i.v. ANG II pressor response whereas progesterone treatment diminished the response to ANG II (26). Similarly, in unanesthetized ovariectomized rats, receiving 0.1 mg

of diethylstilbestrol during 5 days, there was no difference in the pressor response to 5 pmol bolus injections of ANG II between control and treated rats (50).

Chronic subcutaneous injections of estradiol for 5 to 11 days to female ovariectomized rats was shown to reduce the duration but not the peak pressor response to central administration of ANG II when monitored (30 minutes) in the absence of drinking water (64). Chronic subcutaneous administration of estrogens for several weeks was also shown to attenuate the development of hypertension in spontaneously hypertensive rats (27,28).

Discrepancies in pressor response between all of these studies are difficult to interpret due to differences in experimental design and the complexity of interactions occurring in vivo.

d) Effects on angiotensin II-induced pituitary hormone release

Estrogens have been shown to modulate the response of anterior pituitary hormone release to exogenous ANG II. Intraventricular (i.v.t.) injection and to a lesser extent i.v. injection of a high dose of ANG II (5 nmol) has been shown to increase plasma levels of luteinizing hormone (LH) in ovariectomized rats pretreated for 48 hours with a subcutaneous (s.c.) injection of estradiol benzoate (70). Further evidence supporting a permissive role for estrogens in ANG II-induced LH release was shown in another in vivo study where a lower dose of ANG II (50 pmol) injected i.v.t. (but not when injected i.v.) increased plasma LH levels in intact rats on the morning of proestrus when endogenous estrogen levels are high (69). In this latter study, i.v.t. but not i.v. administration of either the ANG II antagonist saralasin or the converting enzyme inhibitor enalapril diacid,

inhibited the proestrus LH surge and blocked ovulation. These studies suggest that ANG II likely generated within the brain may play an important role in the regulation of LH secretion and ovulation on the day of proestrus. In contrast to estrogen-primed rats, LH levels in untreated ovariectomized rats were not significantly affected by acute i.v.t. or i.v. administration of ANG II (70,71). However, since LH release in ovariectomized animals is pulsatile with wide fluctuations in blood LH levels occurring over short time intervals it was concluded by the same authors that previous studies (70,71) did not detect individual hormone pulses and therefore mean circulating LH measurements could be misleading (68).

The authors therefore used a continuous blood sampling protocol to fully characterize the pulsatile nature of LH secretion in ovariectomized animals before and during i.v.t. infusion of ANG II. Their results showed that i.v.t. infusion of graded low doses of ANG II into untreated ovariectomized rats reduced the mean whole blood concentration of LH, pulse frequency, amplitude and nadir in a dose dependent manner (68). Estradiol treatment of these ovariectomized animals for 48 hours prevented the i.v.t. ANG II-induced suppression of LH release observed in untreated animals. On the other hand, in ovariectomized rats pretreated with both estradiol and progesterone, infusions of doses of ANG II that depressed LH release in untreated animals, were shown to increase blood LH levels in a dose dependent manner. Therefore, i.v.t. infusion of ANG II can suppress or facilitate the release of LH from the anterior pituitary gland of the rat, with the direction of the LH response determined by the gonadal steroid background of the animal.

The mechanism by which ANG II affects LH secretion in vivo is presently unknown. It is unlikely that ANG II affects LH release by a direct action on the pituitary gland since several studies have failed to show that ANG II modifies LH secretion when added to anterior pituitary cells in culture (1,24,66). Angiotensin II could act directly upon LHRH neurons in the brain to release LHRH from the endings of these neurons in the median eminence. The presence of receptors for both ANG II (8,54) and estrogens (31,44) in the preoptic area where LHRH neurons are situated suggests that estrogens might interact with the postulated brain renin-angiotensin system to alter LHRH release and subsequently lead to altered LH release from the anterior pituitary. Angiotensin II could also be acting via brain catecholamines to modify LHRH secretion since the effects of i.v.t. infusion of ANG II on LH secretion in ovariectomized rats in the presence or absence of estrogens are the same as those shown after i.v.t. administration of norepinephrine (20).

Injection of high doses of ANG II (5 nmol) i.v.t. depressed both prolactin and growth hormone levels in ovariectomized rats with and without estradiol treatment (70,71). The central effect of ANG II suppression of prolactin release could involve dopamine release since the dopamine antagonist domperidone abolished this effect (70). In the presence or absence of estrogens, the suppression of GH release by i.v.t. ANG II does not appear to involve dopamine release and is likely mediated by another central nervous system transmitter system since ANG II does not directly affect GH release as shown in vitro (1).

ANG II receptor density of the rat anterior pituitary gland was also shown to fluctuate during the estrous cycle with the highest level found in diestrus and the lowest found at estrus (8). This effect could be mediated by estrogens since chronic estradiol treatment has been shown to down-regulate ANG II receptor density in the anterior pituitary (9).

D. REFERENCES TO CHAPTER 2

1. AGUILERA G, HYDE CL, CATT KJ: Angiotensin II receptors and prolactin release in pituitary lactotrophs. Endocrinology 1982, 111:1045-1050.
2. ALTURA BM: Sex as a factor influencing the responsiveness of arterioles to catecholamines. Eur. J. Pharmacol. 1972, 20:261-266.
3. BARLOW JJ, LOGAN CM. Estrogen secretion, biosynthesis and metabolism; their relationship to the menstrual cycle. Steroids 1966, 7:309-320.
4. BAUDOUIN-LEGROS M, MEYER MP, WORCEL M. Effects of prostaglandin inhibitors on angiotensin, oxytocin and prostaglandin $F_{2\alpha}$ contractile effects on the rat uterus during oestrous cycle. Br. J. Pharmacol. 1974, 52:393-399.
5. CAIN MD, WALTERS WA, CATT KJ. Effects of oral contraceptive therapy on the renin-angiotensin system. J. Clin. Endocrinol. Metab. 1971, 33:671-676.
6. CATT KJ, CRAN E, ZIMMET PZ, REST JB, CAIN MD, COGHLAN JP. Angiotensin II blood-levels in human hypertension. Lancet 1971, i:459-464.
7. CHAUD M, VIGGIANO M, GIMENO MF, GIMENO AL. Prostaglandins and the contractile effect of angiotensin I and II in the uterus isolated from ovariectomized rats. Influences of indomethacin and 17-β - estradiol. Prostaglandins Leuk. Med. 1984, 16:225-234.

8. CHEN FM, HAWKINS R, PRINTZ MP: Evidence for functional, independent brain-angiotensin system: correlation between regional distribution of brain angiotensin receptors, brain angiotensinogen and drinking during the estrous cycle of rats. In: Ganten D, Printz M, Phillips MI, Scholkens BA (eds) Experimental Brain Research, 1982, Suppl. 4, pp. 157-168.

9. CHEN F-CM, PRINTZ MP: Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 1983, 115:2026-2028.

10. COX RH, FISCHER GM: Effect of sex hormones on the passive mechanical properties of rat carotid artery. Blood Vessels 1978, 15:266-276.

11. CRANE MG, HARRIS JJ. Effects of estrogens and gestagen on the renin-aldosterone system. In: Fregly MJ, Fregly MS (eds) Oral Contraceptives and High Blood Pressure. 1974, Gainesville, Fla., p. 100.

12. CRANE MG, HARRIS JJ. Plasma renin activity and aldosterone excretion rate in normal subjects. I. Effect of ethinyl oestradiol and medroxy-progesterone acetate. J. Clin. Endocrinol. Metab. 1969, 29:550-557.

13. DANIELSEN J, BUGGY J. Depression of ad lib and angiotensin-induced sodium intake at oestrus. Brain Res. Bull. 1980, 5:501-504.

14. EGGENA P, HIDAKA H, BARRETT JD, SAMBHI MP. Multiple forms of human plasma renin substrate. J. Clin. Invest. 1978, 62:367-372.

15. FINDLAY ALR. FITZSIMONS JT, KUCHARCZYK J. Dependence of spontaneous and angiotensin-induced drinking upon the estrous cycle and ovarian hormones. J. Endocrinol 1979, 82:215-225.

16. FISCHER GM, SWAIN ML: Effect of sex hormones on blood pressure and vascular connective tissue in castrated and noncastrated male rats. Am. J. Physiol. 1977, 232:H617-H621.

17. FREGLY MJ. Fffect of chronic treatment with estrogen on the dipsogenic response of rats to angiotensin. Pharmacol. Biochem. Behav. 1980, 12:131-136.

18. FREGLY MJ, ROWLAND NE, SUMNERS C, GORDON DR. Reduced dipsogenic responsiveness to intracerebroventricularly administered angiotensin II in estrogen-treated rats. Brain Res. 1985, 338:115-121.

19. FREGLY MJ, THRASHER TN. Attenuation of angiotensin-induced water intake in estrogen-treated rats. Pharmacol. Biochem. Behav. 1978, 9:509-514.

20. GALLO RV, DROUVA SV. Effect of intraventricular infusion of catecholamines on LH release in ovariectomized and ovariectomized steroid-primed rats. Neuroendocrinology 1979, 29:149-162.

21. GANT NF, CHAND S, WHALLEY PJ, MACDONALD PC. The nature of pressor responsiveness to angiotensin II in human pregnancy. Obstet. Gynecol. 1974, 43:854-860.

22. GANT NF, DALEY GL, CHAND S, WHALLEY PJ, MACDONALD PC. A study of angiotensin II pressor response throughout primigravid pregnancy. J. Clin. Invest. 1973, 52:2682-2689.

23. GORSKI RA. Sexual differentiation of the brain. In: Krieger DT, Hughes JC (eds) Neuroendocrinology. 1980, Sinauers Associates Inc., Sunderland-Massachusetts, pp. 215-222.

24. HAUGER RL, AGUILERA G, BAUKAL AJ, CATT KJ: Characterization of angiotensin II receptors in the anterior pituitary gland. Mol. Cell. Endocrinol. 1982, 25:203-212.

25. HELMER OM, GRIFFITH RS. The effect of the administration of estrogens on the renin-substrate (hypertensinogen) content of rat plasma. Endocrinology 1952, 51:421-426.

26. HETTIARATCHI ESG, PICKFORD M. The effect of oestrogen and progesterone on the pressor action of angiotensin in the rat. J. Physiol. 1968, 196:447-451.

27. HOEG JM, WILLIS LR, WEINBERGER MH. Estrogen attenuation of the development of hypertension in spontaneously hypertensive rats. Am. J. Physiol. 1977, 233:H-369-H-373.

28. IAMS SG, WEXLER BC. Inhibition of the development of spontaneous hypertension in SH rats by gonadectomy or estradiol. J. Lab. Clin. Med. 1979, 94:608-616.

29. JOHNSON JA, DAVIS JO, BROWN PR, WHEELER PD, WITTY RT. Effects of estradiol on sodium and potassium balances in adrenalectomized dogs. Am. J. Physiol. 1972, 223:194-197.

30. JONKLAAS J, BUGGY J. Angiotensin-estrogen interaction in female brain reduces drinking and pressor responses. Am. J. Physiol. 1984, 247:R167-R172.

31. JONKLAAS J, BUGGY J. Angiotensin-estrogen control interaction: localization and mechanism. Brain Res. 1985, 326:239-249.

32. KAULHAUSEN H, LEYENDECKER G, BENKER G, BREUER H: The relationship of the renin-angiotensin-aldosterone-system to plasma gonadotropin, prolactin and ovarian steroid patterns during the menstrual cycle. Arch. Gynaekol. 1978, 225:179-200.

33. KAULHAUSEN H, VENN HJ, MUHLBAUER W, BECK KJ, BREUER H. Effect of ethynyl estradiol on renin activity and on the concentrations of renin substrate and aldosterone in plasma of ovariectomized women. Arch. Gynecol. 1980, 229:29-36.

34. KAULHAUSEN H, WEYAND C. Evidence for two independent effects of oestradiol benzoate on the renin-angiotensin-aldosterone system. Acta Endocrinol. 1982, 100:77-84.

35. KITAY JI, COYNE MD, SWYGERT NH. Influence of gonadectomy and replacement with estradiol or testosterone on formation of 5α-reduced metabolites of corticosterone by the adrenal gland of the rat. Endocrinology 1970, 87:1257-1265.

36. KONDO K, MISUMI J, NAKAMURA R, SAITO I, SARUTA J. Effect of estrogen upon the juxtaglomerular apparatus and the renin-angiotensin system in rats. Tohoku J. Exp. Med. 1978, 126:267-272.

37. KURIYAMA H. The effects of progesterone and oxytocin on mouse myometrium. J. Physiol. 1961, 159:26-29.

38. LANDAU RL, LUGIBIHL K: The catabolic and natriuretic effects of progesterone in man. Recent Prog. Horm. Res. 1961, 17:249-292.

39. LARAGH JH, SEALY JE, LEDINGHAM JJG, NEWTON MA. Oral contraceptives: renin, aldosterone and high blood pressure. JAMA 1967, 201:918-922.

40. LIN SY, GOODFRIEND TL. Angiotensin receptors. Am. J. Physiol. 1970, 218:1319-1328.

41. MALENDOWICZ LK, JACHIMOWICZ B. Sex differences in adrenocortical structure and function. XI. Autoradiographic studies on cell proliferation and turnover in the adrenal cortex of the male and female rat and its dependence on testosterone and estradiol. Cell Tiss. Res. 1982, 227:651-657.

42. MARSHALL JM. Effects of estrogen and progesterone on a single uterine muscle fiber in the rat. Am. J. Physiol. 1959, 197:935-942.

43. MATSUURA S, NADEN RP, GANT NF, PARKER CR, ROSENFELD CR. Effect of volume expansion on pressor response to angiotensin II in pregnant ewes. Am. J. Physiol. 1981, 240:H908-H913.

44. McEWEN BS, GERLACH JL, LUINE VN, LIEBERBURG I.: Neural steroid hormone receptor. Psychoendocrinology 1979, 3:249-255.

45. MENARD J, CATT KJ. Effects of oestrogen treatment on plasma renin parameters in the rat. Endocrinology 1973, 92:1382-1388.

46. MENARD J, MALMEJAC A, MILLIEZ P. Influence of diethylstilbestrol on the renin-angiotensin system of male rats. Endocrinology 1970, 86:774-780.

47. MUSA BU, SEAL US, DOE RP. Elevation of certain plasma proteins in man following estrogen administration. A dose-response relationship. J. Clin. Endocr. Metab. 1965, 25:1163-1166.

48. NASJLETTI A, MASSON GMC: Hepatic origin of renin substrate. Canad. J. Physiol. Pharmacol. 1971, 49:931-932.

49. NASJLETTI A, MASSON GMC: Studies on angiotensinogen formation in a liver perfusion system. Circ. Res. 1972, 31 (Suppl. II):II-187-II-198.

50. NASJLETTI A, MATSUNAGA M, MASSON GMC: Effects of estrogens on pressor responses to angiotensin and renin. Proc. Soc. Exp. Biol. Med. 1970, 133:407-409.

51. NISSENSON R, FLOURET G, HECHTER O. Opposing effects of estradiol and progesterone on oxytocin receptors in rabbit uterus. Proc. Natl. Acad. Sci. USA 1978, 75:2044-2048.

52. OELKERS W, SCHONESHOFER M, BLUMEL A. Effects of progesterone and four synthetic progestogens on sodium balance and the renin-aldosterone system in man. J. Clin. Endocr. Metab. 1974, 39:882-890.

53. O'MALLEY BW, STROTT CA. The mechanism of action of progesterone. In: Greep RO (ed) Handbook of Physiology, 1973, vol. 2, section 7, part 1, American Physiological Society, Washington DC, pp. 591-602.

54. PRINTZ MP, HAWKINS RL, WALLIS CJ, CHEN FM. Steroid hormones as feedback regulators of brain angiotensinogen and catecholamines. Chest 1983, 2:308-311.

55. ROBERTS JM. Oestrogens and hypertension. Clin. Endocrinol. Metab. 1981, 10:489-512.

56. ROSENFELD CR, GANT NF. The chronically instrumented ewe. a model for studying vascular reactivity to angiotensin II in pregnancy. J. Clin. Invest. 1981, 67:486-492.

57. ROSENFELD CR, JACKSON GM. Estrogen-induced refractoriness to the pressor effects of infused angiotensin II. Am. J. Obstet. Gynecol. 1984, 148:429-435.

58. ROSENFELD CR, MORRISS FH Jr, BATTAGLIA FC, MAKOWSKI EL, MESCHIA G. Effect of estradiol-17β on blood flow to reproductive and nonreproductive tissues in pregnant ewes. Am. J. Obstet. Gynecol. 1976, 124:618-629.

59. ROUZAIRE-DUBOIS E, DEVYNCK MA, CHEVILLOTTE E, MEYER P. Angiotensin receptors in rat uterine membranes. FEBS Lett 1975, 55:168-172.

60. SARUTA T, NAKAMURA R, SAITO I, KONDO K, MATUKI S. Oestrogen hypertension in rats. Clin. Sci. 1975, 48:457-460.

61. SCHIRAR A, CAPPONI A, CATT KJ. Elevation of uterine angiotensin II receptors during early pregnancy in the rat. Endocrinology 1980, 106:1521-1527.

62. SCHIAVI A, CAPPONI A, CATT KJ. Regulation of uterine angiotensin II receptors by estrogen and progesterone. Endocrinology 1980, 106:5-12.

63. SHIONOIRI H, EGGENA P, BARRETT JD, THAMANOPAVARN C, GOLUB MS, EGGENA Z, NAKAMURA R, JUDD HL, SAMBHI MP. An increase in high-molecular weight renin substrate associated with estrogenic hypertension. Biochem. Med. 1983, 29:14-22.

64. SKOOG KM, KENNEY NJ: Pressor responses to central angiotensin II, prostaglandin E_1 and prostaglandin E_2. Effects of ovarian steroids. Neuroendocrinology 1983, 36:144-151.

65. SMITH OW, SMITH GV, GAVIAN NG. Urinary estrogens in women. Am. J. Obstet. Gynecol. 1959, 78:1028-1047.

66. SOBEL DO, VAGNUCCI A: Angiotensin II mediated ACTH release in rat pituitary cell culture. Life Sci. 1982, 30:1281-1286.

67. SOLOFF MS. Uterine receptors for oxytocin: effects of estrogen. Biochem. Biophys. Res. Comm. 1975, 65:205-212.

68. STEELE MK, GALLO RV, GANONG WF. Stimulatory or inhibitory effects of angiotensin upon LH secretion in ovariectomized rats: a function of gonadal steroids. Neuroendocrinology 1985, 40:210-216.

69. STEELE MK, GALLO RV, GANONG WF. A possible role for the brain renin-angiotensin system in the regulation of LH secretion. Am. J. Physiol. 1983, 245:R805-R810.

70. STEELE MK, McCANN SM, NEGRO-VILLAR A: Modulation by dopamine and estradiol of the central effects opf angiotensin II on anterior pituitary hormone release. Endocrinology 1982, 111:722-729.

71. STEELE MK, NEGRO-VILLAR A, McCANN SM: Effect of angiotensin II on in vivo and in vitro release of anterior pituitary hormones in the female rat. Endocrinology 1981, 109:893-899.

72. STERIN-SPEZIALE N, GIMENO MF, BONACOSSA A, GIMENO AL. The effect of estradiol on isolated rat uterine motility and on prostaglandin generation. Prostaglandins 1980, 20:233-243.

73. SUNDSFJORD JA, AAKVAAG A: Variations in plasma aldosterone and plasma renin activity throughout the menstrual cycle with special reference to the pre-ovulatory period. Acta Endocrinol. 1973, 73:449-508.

74. TALLEDO OE, CHESLEY LC, ZUSPAN FP. Renin-angiotensin system in normal and toxemic pregnancies. III. Differential sensitivity to angiotensin II and norepinephrine in toxemia of pregnancy. Am. J. Obstet. Gynecol. 1968, 100:218-221.

75. TAMAI T, MATSUURA S, TATSUMI N, NUNOTANI T, SAGAWA N. Role of sex steroid hormones in relative refractoriness to angiotensin II during pregnancy. Am. J. Obstet. Gynecol. 1984, 149:177-183.

76. TARTTELIN MF, GORSKI RA. Variations in food and water intake in the normal and acyclic female rat. Physiol. Behav. 1971, 7:847-852.

77. TEWKSBURY DA, DART RA. High molecular weight angiotensinogen levels in hypertensive pregnant women. Hypertension 1982, 4:729-734.

78. THORN GW, ENGEL LL. The effect of sex hormones on the renal excretion of electrolytes. J. Exp. Med. 1938, 68:299-312.

79. THORN GW, NELSON KR, THORN DW. A study of the mechanism of edema associated with menstruation. Endocrinology 1938, 22:155-163.

80. TULCHINSKY D, HOBEL CJ, YEAGER E, MARCHALL JR. Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy. Am. J. Obstet. Gynecol. 1972, 112:1095-1100.

81. UEBERBERG H, MUFF H-G, TRIEB G. Autoradiographic investigation of cell proliferation in the adrenal cortex of castrated female rats under the influence of oestradiol. Experientia 1984, 40:1384-1385.

82. VALLOTON MB, MERKELBACH U, GAILLARD RC. Studies of the factors modulating antidiuretic hormone excretion in man in response to the osmolar stimulus: effects of oestrogen and angiotensin. Acta Endocrinol. 1983, 104:295-302.

83. WILLIAMS LT, LEFKOWITZ RJ. Regulation of rabbit myometrium alpha adrenergic receptors by estrogen and progesterone. J. Clin. Invest. 1977, 60:815-818.

84. ZAKI K, RIZK M, KIRA L, NOUR H, GUIRGUIS R. Studies on the effects of ethinyl estradiol and norethisterone acetate on the adrenal cortex and some other tissues in the rat. Endokrinologie 1979, 73:66-76.

ORIGINAL PAPERS

CHAPTER 3 : CHRONIC ESTRADIOL TREATMENT DECREASES ANGIOTENSIN II RECEPTOR DENSITY IN THE ANTERIOR PITUITARY GLAND AND ADRENAL CORTEX BUT NOT IN THE MESENTERIC ARTERY

A. PREFACE

From the preceding literature review, it appears quite clear that ANG II receptors are widely distributed in several target tissues throughout the mammalian organism. In addition to the classical adrenal and vascular receptors for ANG II there is increasing evidence in support of a functional role for the newly discovered ANG II receptors in discrete areas of the brain, hypothalamo-hypophyseal system, myocardium, kidney, liver, urinary bladder and uterus (for review see 33). Binding sites for ANG II have also been found in platelets and leukocytes although, their physiological role in these cells remains to be clarified.

In earlier studies, it was believed that regulation of circulating levels of ANG II was the most important if not the only means by which the renin-angiotensin system operated to preserve body fluid homeostasis. More recent studies suggest that ANG II receptor regulation is also an important means of controling target cell responsiveness. The study of the effect of estrogens on the renin-angiotensin system is a good example of the evolution of this concept. Early studies have shown that estrogens alter the circulating components of the renin-angiotensin system particularly by increasing renin substrate levels. More recent studies have shown that during the high estrogenic phase of the estrous cycle or following estrogen administration to ovariectomized animals, the physiological response to ANG II in the uterine smooth muscle and, the central mechanisms regulating thirst, blood pressure and ANG II-induced pituitary hormone release appear to be markedly affected by this steroid.

Although this compelling evidence strongly suggests that estrogens are important modulators of the response of several target tissues to ANG II, the mechanisms by which estrogens exert these effects are virtually unknown. Separate studies have shown that estrogens in vivo can up-regulate receptors for ANG II in the uterus and down-regulate them in the anterior pituitary gland and in certain discrete areas of the brain. It has not been established however, if these effects of estrogens are indirectly mediated by alterations in the components of the renin-angiotensin system (or any other substances)which would then act at the cellular level to modulate receptors for ANG II. Another possibility which has not yet been explored is whether estrogens themselves act directly on target cells to modulate ANG II receptors.

Furthermore, there has been no comprehensive study looking at the effect of estrogens on the well characterized ANG II receptors of the adrenal cortex and mesenteric artery. The understanding of the effect of estrogens on ANG II receptors in these two target tissues is of particular interest since ANG II receptors in the adrenal and arterial tissue appear to be regulated in an opposite direction after such treatments as sodium restriction, sodium loading or ANG II infusion.

In several target tissues, there appears to be a close correlation between regulation of ANG II receptor density and/or affinity with altered physiological response. Parallel changes in receptor density and physiological response to ANG II have been documented in studies looking at the effect of estrogens on uterine contractility and thirst mechanisms of the brain. It is not known however whether this correlation applies only to some or all ANG II target tissues under the influence of estrogens.

To answer some of these questions the effect of chronic estradiol treatment on ANG II receptor density and affinity was studied in the anterior pituitary gland, adrenal cortex and mesenteric artery. The effect of estrogen treatment was studied simultaneously from all three target tissues and plasma renin activity (PRA) and circulating ANG II levels were measured in order to determine the possible involvement of this peptide in the observed estrogen-induced down-regulation of ANG II receptors. In addition the effect of estrogen treatment on the aldosterone response to ANG II was also examined. These findings are described in the following paper which has been accepted for publication in: "Neuroendocrinology".

CHRONIC ESTRADIOL TREATMENT DECREASES ANGIOTENSIN II RECEPTOR DENSITY IN THE ANTERIOR PITUITARY GLAND AND ADRENAL CORTEX BUT NOT IN THE MESENTERIC ARTERY.

This paper has been accepted for publication and will appear in: Neuroendocrinology, 1986.

Received: February 6, 1985.

Accepted after revision: June 28, 1985.

B. ABSTRACT

Chronic estrogen treatment has been shown to produce a marked reduction in anterior pituitary angiotensin II (ANG II) receptor density. In order to determine whether this effect is generalized, we studied the effect of chronic estradiol treatment on ANG II receptor density in the anterior pituitary gland, adrenal cortex and mesenteric artery of ovariectomized (OVX) rats. Treated rats were injected daily with 25 µg of estradiol valerate while controls received only the vehicle. Binding affinity and density of ANG II receptors were measured using the ANG II antagonist: [^{125}I]Sar^{1}Ile^{8} ANG II ([^{125}I] SARILE). Following 7-, 14- or 28-day treatments, ANG II receptor density decreased by approximately 80% in the anterior pituitary; 30% in the adrenal cortex and remained the same in mesenteric artery particulate fractions. In all 3 target tissues, dissociation constants (K_D) for binding of [^{125}I]SARILE were in the nanomolar range and were the same between control and treated rats. Using conscious rats, estradiol treatment for 7 days was also shown to block the release of aldosterone by a low dose infusion of ANG II (10 pmol/minute, 30 minutes). Plasma ANG II and plasma renin activity were also the same or slightly decreased following estradiol treatments. This study suggests that estrogens may be important modulators of the ANG II receptor and may be directly involved in modulating target cell responsiveness to ANG II as expressed through differential down-regulation of ANG II receptors.

C. INTRODUCTION

There is increasing evidence that several components of the renin-angiotensin system fluctuate during the estrous cycle. During early estrus, ANG II receptor density increases in the uterus (9,42) and decreases in the pituitary gland (9). At estrus, plasma renin substrate increases whereas in several areas of the brain renin substrate decreases (9,36). Estrogens appear to be important effectors involved in some of these changes since the most consistent alteration in the circulating components of the renin-angiotensin system is a rapid (usually within 24 to 48 hours) and persistent increase in plasma renin substrate (22,25,26,31).

Chronic estradiol treatment has also been shown to decrease ANG II receptor density in the anterior pituitary gland (8), whereas acute but not chronic estradiol treatment increases the density of uterine ANG II receptors (42). The objective of this study was to further characterize the effect of prolonged 7-, 14- and 28- day estrogen treatments on ANG II receptors in the anterior pituitary gland as well as in two physiologically important target tissues: the adrenal cortex and the mesenteric artery. We chose to study the binding affinity and density of ANG II receptor sites with the potent antagonist [^{125}I] (Sarcosine ^{1}-Isoleucine^{8}) ANG II ([^{125}I]SARILE). The antagonist [^{125}I] SARILE was preferred over the agonist [^{125}I]ANG II because it identifies a single uniform class of ANG II receptors with high affinity, stability and a slow dissociation rate (11). In the present study, down-regulation of the ANG II receptor by estrogen was shown in the anterior pituitary gland, and was also shown to occur in the adrenal cortex but not in the mesenteric artery. Down-regulation of

ANG II receptors in the adrenal cortex was also suggested by the effect of estradiol in blocking the release of aldosterone by infused ANG II. These effects of estradiol did not appear to be due to alterations in circulating levels of ANG II.

D. MATERIALS AND METHODS

a) Animals

Female 200-250 g Sprague-Dawley rats (Charles River Labs, St-Constant, Que.) were used for this study. Rats were kept at 22°C and exposed to a 12 hour light 12 hour dark cycle with free access to normal Purina rat chow (152 mmol Na per kg) and water.

b) Experimental protocol

Rats were ovariectomized (OVX) under ether anesthesia, 3-4 days after acclimatization. Two days following ovariectomy they received 25 µg of estradiol valerate (Delestrogen, Squibb Canada, Montréal, Que.) diluted in 0.2 ml of sesame oil vehicle (0-154, Fisher Scientific, Fairlawn, N.J.) injected subcutaneously once a day between 08.00 and 10.00 hour for 7, 14 or 28 days. Equal numbers of OVX-control rats were treated identically with the vehicle only.

For binding studies, control and treated animals were sacrificed in the morning by decapitation and 2-3 ml of trunk blood was collected within 10 seconds in EDTA-coated glass tubes (Vacutainer No. 6452, Becton Dickinson, Rutherford, N.J.) kept on ice. Plasma was then rapidly collected after centrifugation and kept at -20°C until assayed for plasma renin activity and ANG II by radioimmunoassay (RIA).

The biological response study was similar to that previously reported using intact male rats (7). Control and estradiol-treated

OVX-rats were anesthetized with ether and cannulated with a polyethylene catheter (PE-50, Intramedic, Clay Adams, N.J.) filled with 0.9% NaCl in water containing 100 U/ml of heparin (Hepalean 100 U/ml, Harris Lab., Toronto, Ont.) and inserted 2.5 cm into the right jugular vein near its junction with the right atrium. The catheter was then passed under the skin and brought out at the scruff of the neck. After 24 hours, the conscious rats were studied between 09.00 and 13.00 hours. OVX and OVX-estradiol-treated rats were studied separately during two consecutive days. ANG II (Peninsula, Palo Alto, Calif.) was dissolved in 5% dextrose in water and infused in a total volume of 0.3 ml over 30 minutes at a rate of 10 pmol ANG II/minute with a Sage pump (model 220-1, Sage Instruments, White Plains, N.Y.). Control rats were infused with 5% dextrose in water. At the end of the infusion period, rats were decapitated and trunk blood was collected in cold EDTA-coated glass tubes. Blood from the first 5 seconds was used for plasma renin activity and the rest was collected for aldosterone determinations. Blood was immediately centrifuged at 4°C and plasma was separated and stored at -20°C until assayed.

c) Iodination of SARILE

The iodination of SARILE was slightly modified from the previously described technique (11) using 5 µg of SARILE (Peninsula) and 1 mCi of carrier-free ^{125}I (IMS-30, Amersham, Toronto, Ont.) diluted in 200 µl of 0.05 M sodium phosphate buffer, pH 7.4, kept on ice at 4°C and incubated for 15 minutes with one Iodo-Bead (No. 28666, Pierce Chem., Rockford, Ill.). Separation of [^{125}I]SARILE was performed on a DEAE-A-25 Sephadex column (1.5 x 50 cm, Pharmacia, Montreal, Que) (11). The specific activity of [^{125}I]SARILE was determined by comparing SARILE with [^{125}I]SARILE in receptor binding competition curves and provided an estimated specific activity of 300-500 Ci/mmol.

d) Tissue preparation

i) Anterior pituitary gland

A modification of the technique used by Hauger et al (20) was employed. The anterior pituitary lobes (10 per group) were homogenized at 4°C in 5 ml of sodium bicarbonate washing buffer and centrifuged at 10 g for 10 minutes. The resulting supernatant was centrifuged at 40,000 g at 4°C for 10 minutes. The pellet was then washed and recentrifuged for 15 minutes and finally resuspended in 4 ml of assay buffer without BSA. The final assay buffer concentration was 50 mM Tris-HCl, pH 7.4,at 25°C, 10 mM $MgCl_2$, 1 mM Na-EDTA in 0.2% heat-inactivated BSA.

ii) Adrenal cortex

The adrenal medulla was gently squeezed from the adrenal cortex through a small lateral incision. Six to 20 adrenal cortical capsules per group were collected and stored on ice, then processed as described for the anterior pituitary gland. The final assay buffer concentration consisted of 50 mM Tris-HCl, pH 7.4, at 25°C, 1 mM $MgCl_2$, 0.1 mM Na-EDTA, 150 mM NaCl in 0.2% heat-inactivated BSA.

iii) Mesenteric artery

A modification of the technique used by Wei et al (45) and similar to that described by Gunther et al (14) and Schiffrin et al (41) was employed. Three rats were used per group. The cleaned mesenteric arteries were finely minced with scissors in 30 ml of 0.25 M sucrose, and homogenized in a Polytron (setting 8; 2x10 seconds; Kinematica, Lucerne, Switzerland). The crude homogenate was then filtered through 4 layers of cheesecloth and centrifuged at 1500 g for 10 minutes at 4°C, and the supernatant was decanted and recentrifuged. The

supernatant was then centrifuged at 100,000 g for 30 minutes, and the pellet was homogenized in 5 ml of 0.25 M sucrose and centrifuged again at 1500 g for 10 minutes at 4°C. The supernatant was made up to 7 ml with 0.25 M sucrose and was then used for binding in a final concentration of 25 mM Tris-HCl, pH 7.4, at 25°C, 0.5 mM $MgCl_2$, 0.05 mM Na-EDTA, 75 mM NaCl, 125 mM sucrose in 0.1% heat-inactivated BSA. For each time period the following number of experiments were performed: 7 days n=3, 14 days n=3, 28 days n=2.

e) Binding Assay

Saturation binding was always performed on fresh mesenteric artery particulate fractions. When necessary anterior pituitary and adrenal cortical membranes were frozen in liquid nitrogen and kept at -70°C in their respective assay buffer to which 0.25 M sucrose was added. The binding assay was performed in duplicate as follows: 100 µl of homogenized tissue membranes were incubated with varying concentrations of [^{125}I]SARILE in borosilicate glass tubes in a final volume of 200 µl of assay buffer. Nonspecific binding was determined at 4 concentrations by finally adding 1 µM of unlabeled SARILE. Incubation was carried out in a shaker bath for 60 minutes at 25°C, terminated by the addition of 4 ml of cold assay buffer without BSA and filtered through glass-fiber filters (Whatman GF/B, Maidstone, UK) prewetted with assay buffer. The incubation tubes were rinsed with an additional 4-ml of assay buffer followed by two rapid 4 ml rinses of filter wells. Filters were counted in a gamma counter (LKB-Wallak 1274 Quatro, LKB, Finland). Data were analysed by computer-assisted least squares nonlinear regression analysis (11). Proteins were measured according to the technique described by Lowry et al. (29).

f) Plasma angiotensin II, plasma renin activity, and aldosterone measurements

Plasma ANG II levels and plasma renin activity were measured directly as described (16,17). Briefly ANG II was determined by RIA using [^{125}I] ANG II prepared by the chloramine-T method (23) and purified by partition chromatography on Sephadex G-25. Plasma renin activity was determined by incubating 1 ml of diluted plasma at 4°C and 37°C for 2 hours at pH 6.5 and then measuring generated ANG I levels by RIA. Plasma aldosterone concentration was measured by RIA after extraction and chromatography (44).

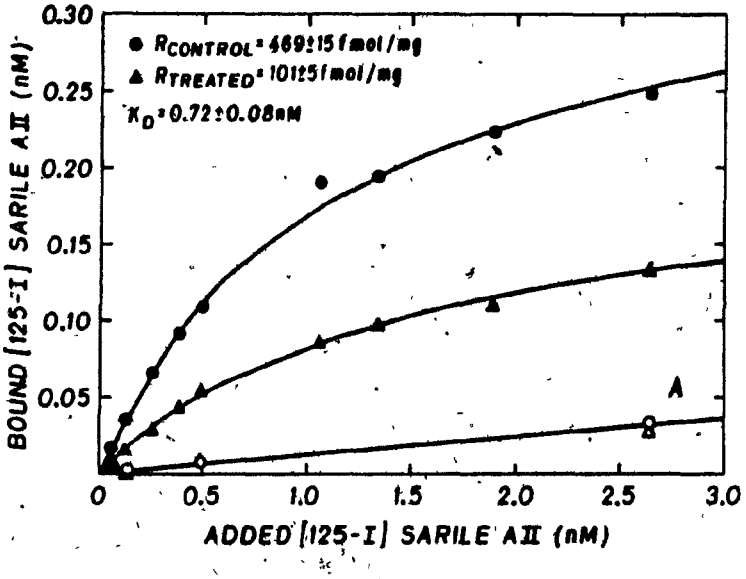
g) Statistical analysis

Two-way analysis of variance (ANOVA) was performed for each of the 4 variables shown in tables I and II using as factors: (a) the type of treatment and (b) the duration of treatment. Differences of the mean from data of tables III and IV were analysed by two-tailed unpaired Student's t test.

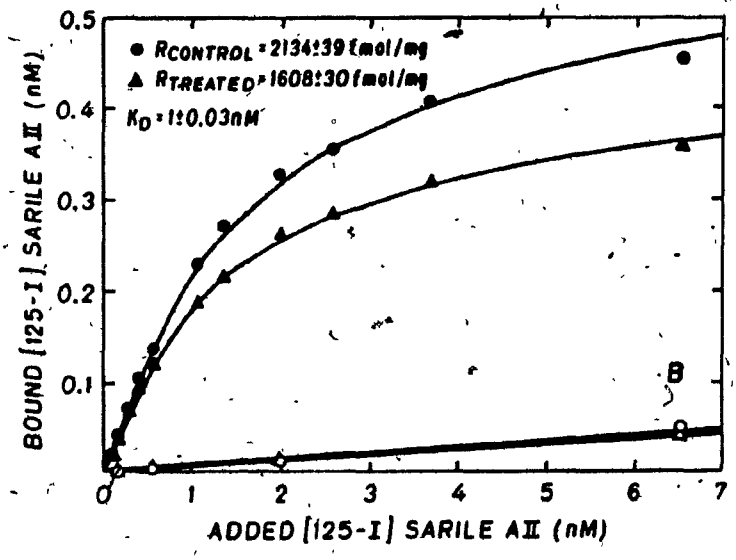
E. RESULTS

Representative receptor saturation experiments using membranes from all 3 target tissues are shown in figure 1, where each point represents bound [^{125}I]SARILE as a function of added increasing concentrations of radioligand and the lines are the best fit of the data as analysed simultaneously by computer-assisted least squares nonlinear regression analysis (11). The 2 top curves of each graph represent total binding of radioligand from control and treated animals, and the 2 lower lines represent the corresponding nonspecific

A. ANTERIOR PITUITARY [125-I] SARILE AII BINDING
(14 DAYS OVARIECTOMIZED RATS)



B. ADRENAL CORTEX [125-I] SARILE AII BINDING
(14 DAYS OVARIECTOMIZED RATS)



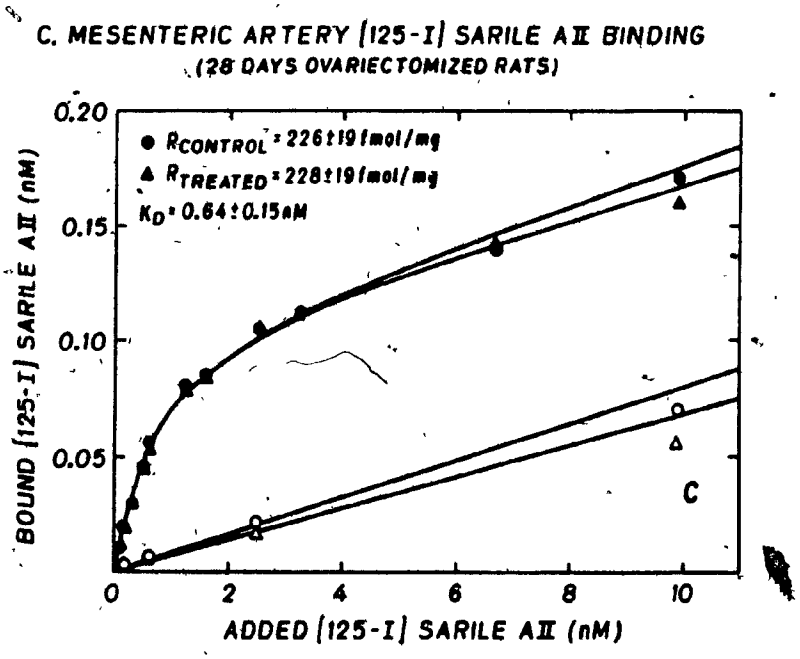


Figure 1

Representative [^{125}I]SARILE receptor saturation experiments using membranes from (A) anterior pituitary gland, (B) adrenal cortex, and (C) mesenteric artery (A and B are from 14-day OVX control and estradiol-treated rats, and C is from a 28-day experiment). The black symbols represent total binding: ● = controls ▲ = treated, whereas open symbols (o, Δ) represent corresponding nonspecific binding. (AII=ANG II).

Table I

PARAMETERS MEASURED FROM ANTERIOR PITUITAPY MEMBRANES OF OVARIECTOMIZED RATS

(MEANS ± SEM)

		R		K_D	P	
		(fmol/mg)*	(fmol/gland)*	(nM)	(µg/gland)*	
7 days	Control	348 ± 36	111 ± 40	0.48 ± 0.08	270 ± 108	n = 4
	Treated	113 ± 33	60 ± 26	0.60 ± 0.34	487 ± 142	n = 4
14 days	Control	375 ± 64	143 ± 30	0.55 ± 0.10	367 ± 33	n = 6
	Treated	71 ± 16	53 ± 11	0.65 ± 0.12	754 ± 63	n = 6
28 days	Control	278 ± 91	102 ± 16	0.72 ± 0.15	410 ± 68	n = 3
	Treated	49 ± 16	38 ± 6	0.73 ± 0.14	876 ± 163	n = 3

R = receptor density, K_D = dissociation constant, P = anterior pituitary membrane protein

* = ANOVA (type of treatment): $p < 0.01$

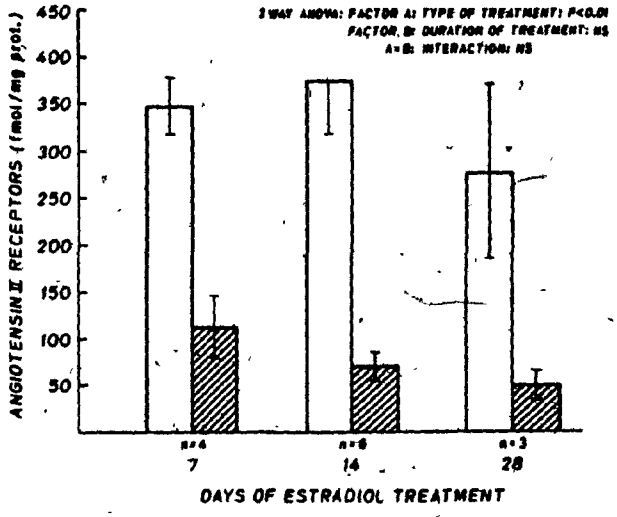


Figure 2

Anterior pituitary gland ANG II receptor density following chronic estradiol treatment. Bars represent a summary of all experiments ± 1 SEM. Data are from Table I.

binding established in the presence of an excess of unlabeled SARILE (1 μM). Receptor concentration (nM) was estimated at saturation of specific binding (total minus nonspecific) and corrected for tissue protein concentration (fmol/mg). The equilibrium dissociation constant (K_D) is calculated as the concentration of radioligand at which receptor binding is half-maximal.

a) Anterior pituitary gland

In the anterior pituitary gland, estradiol decreased ($p < 0.01$) ANG II receptor density throughout treatment (table I, figs. 1A and 2). There was no significant difference between groups with time and no interaction between the duration and type of treatment. The estradiol-induced decrease in ANG II receptor density in the anterior pituitary gland was completed after 7 days of treatment (control: 348 ± 36 vs treated 113 ± 33 fmol/mg protein). Decrease in ANG II receptor density in the anterior pituitary gland was not an artifact due to estrogen-induced increase in tissue protein. This is demonstrated in table I where ANG II receptor density is expressed in fmol/gland and indicates an absolute decrease after 7 days (from 111 ± 40 to 60 ± 26 fmol/gland). Table I also indicates that the K_D for [^{125}I]SARILE was not significantly different between control and treated rats at any time period. The measure of protein from homogenized tissue expressed in μg/gland also confirmed that estradiol treatment approximately doubled the amount of anterior pituitary membrane protein (table I) and that these values significantly increased with time ($p < 0.02$).

Table II

PARAMETERS MEASURED FROM ADRENAL CORTICAL MEMBRANES OF OVARIECTOMIZED RATS

(MEANS ± SEM)

	R		K_D	P	
	(fmol/mg)*	(fmol/gland)*	(nM)	(µg/gland)	
7 days Control	2428 ± 278	596 ± 85	2.40 ± 0.47	248 ± 21	n = 7
Treated	1848 ± 297	404 ± 77	2.13 ± 0.56	218 ± 25	n = 7
14 days Control	1726 ± 283	396 ± 83	1.27 ± 0.42	225 ± 33	n = 6
Treated	1111 ± 248	281 ± 75	1.23 ± 0.43	237 ± 20	n = 6
28 days Control	2056 ± 177	287 ± 2	0.83 ± 0.005	141 ± 13	n = 2
Treated	1247 ± 80	186 ± 21	0.83 ± 0.005	151 ± 26	n = 2

R = receptor density, K_D = dissociation constant, P = adrenal cortical membrane protein

* = ANOVA (type of treatment): $p < 0.05$

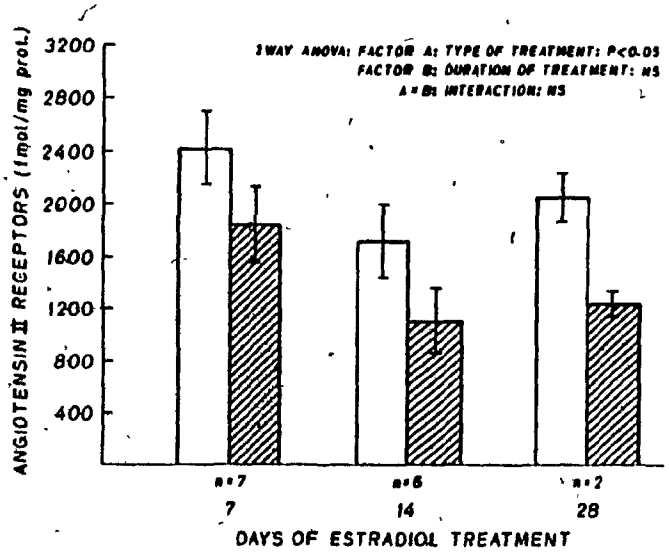


Figure 3

Adrenal cortex ANG II receptor density following chronic estradiol treatment. Bars represent a summary of all experiments ± 1 SEM. Data are from Table II.

b) Adrenal cortex

Estradiol treatment significantly decreased ($p < 0.05$) ANG II receptor density in the adrenal cortex and the extent of down-regulation was not statistically significant ($p > 0.05$) between 7-, 14- or 28- day treatments (table II, figs. 1B and 3). Table II also indicates that the K_D for [^{125}I]SARILE as well as tissue protein measurements were not significantly different between control and treated rats from any time period. Decreases in ANG II receptor density in the adrenal cortex was less pronounced however than in the anterior pituitary gland when expressed either in fmol/mg protein (~30% vs ~80% decrease) or in fmol/gland (~30% vs ~60% decrease) (tables I, II). As shown in table III, low dose infusion of ANG II into freely-moving conscious ovariectomized rats significantly increased ($p < 0.05$) circulating aldosterone levels from 15.6 ± 1.1 to 22.9 ± 2.2 ng/dl and suppressed plasma renin activity from 2.23 ± 0.13 to 0.67 ± 0.14 ng ANG I/ml/hour. In 7-day estradiol-treated rats however, aldosterone levels did not significantly increase ($p > 0.05$) from 17.6 ± 1.8 to 19.1 ± 1.6 ng/dl despite a significant decrease ($p < 0.01$) in plasma renin activity from 1.65 ± 0.15 to 0.19 ± 0.03 ng ANG I/ml/hour.

c) Mesenteric artery

In the mesenteric artery, estradiol treatment produced no significant effect on ANG II receptor density nor on the affinity of [^{125}I]SARILE for its receptor sites (fig. 1C) nor did it alter tissue protein. Two-way ANOVA confirmed that there was no significant difference between control and treated rats at any time period and no difference with time. The mean values for mesenteric artery ANG II

Table III

EFFECT OF 7 DAY-ESTRADIOL TREATMENT ON THE RELEASE OF ALDOSTERONE BY EXOGENOUS ANG II IN CONSCIOUS OVARIECTOMIZED RATS (MEANS ± SEM)

	Aldosterone (ng/dl)[1]	Plasma renin activity (ng ANG I/ml/h)[2]	
a) Control: 5% dextrose	15.6 ± 1.1	2.23 ± 0.13	n = 10
b) Control: ANG II 10 ng/min/30 min	22.9 ± 2.2*	0.67 ± 0.14**	n = 10
c) Estradiol: 5% dextrose	17.6 ± 1.8	1.65 ± 0.15	n = 11
d) Estradiol: ANG II 10 ng/min/30 min	19.1 ± 1.6	0.19 ± 0.03**	n = 9

* $p < 0.05$ Two-tailed unpaired Student's t-test comparing a & b.

** $p < 0.01$ Two-tailed unpaired Student's t-test comparing a & b, c & d

---

Table IV

EFFECT OF CHRONIC ESTRADIOL TREATMENT ON CIRCULATING ANG II, PRA, TOTAL BODY WEIGHT AND ANTERIOR PITUITARY GLAND WEIGHT IN OVARIECTOMIZED RATS (MEANS ± SEM)

	ANG II (pg/ml)[3]	PRA (ng ANG I/ml/h)[2]	BW (g)	AP (mg)
7 days Control	56.8 ± 6.2 n = 46	2.99 ± 0.27 n = 30	245 ± 3	12.8 ± 0.6
Treated	48.6 ± 6.3 n = 43	2.46 ± 0.31 n = 29	223 ± 3**	21.4 ± 1.1**
14 days Control	59.7 ± 3.3 n = 21	2.34 ± 0.28 n = 21	261 ± 4	16.9 ± 0.3
Treated	43.8 ± 6.6* n = 20	1.78 ± 0.23 n = 22	215 ± 4**	33.1 ± 1.4**
28 days Control	-	1.13 ± 0.20 n = 8	293 ± 9	14.8 ± 0.4
Treated	-	0.73 ± 0.21 n = 7	235 ± 4**	25.6 ± 1.2**

ANG II = plasma angiotensin II, PRA = plasma renin activity, BW = total body weight, AP = anterior pituitary gland weight.

*,** Significantly different from OVX-control at $p < 0.05$ (*) or $p < 0.01$ (**) (Two-tailed unpaired Student's t test).

SI unit conversion factor (1) ÷ 0.36045 = pmol/dl
(2) ÷ 1.3247 = pmol ANG I/ml/h
(3) ÷ 1 = pmol/l

receptor density of pooled control vs treated rats were: 206 ± 19 vs 184 ± 15 fmol/mg and K_D values of 0.62 ± 0.06 vs 0.59 ± 0.07 nM respectively (means ± SEM).

d) Other parameters

In rats used for binding studies, estradiol treatment produced a significant decrease in circulating ANG II levels measured from individual rats only after 2 weeks of treatment ($p < 0.05$, table IV). Plasma renin activity also appeared to decrease following estradiol treatment (tables III, IV). Estradiol treatment also significantly reduced ($p < 0.01$) total body weights whereas the anterior pituitary gland approximately doubled in size (table IV). These latter results are similar to those previously reported (8).

F. DISCUSSION

A novel approach in this study was the use of the potent antagonist [^{125}I]SARILE to quantify ANG II receptor densities in the anterior pituitary gland, adrenal cortex and mesenteric artery. The use of radiolabeled antagonists for hormone receptor quantifications has been widely validated in several other receptor systems (10, 27). Validation of the use of [^{125}I]SARILE to characterize ANG II receptors has been recently demonstrated using adrenal zona glomerulosa membranes (11). In that study, binding of [^{125}I]ANG II was shown to be sensitive to the addition of guanine nucleotides and competition curves revealed the presence of two apparent classes of binding sites of high and low affinity. Similar findings have also been reported for the β-adrenergic receptor when using radiolabeled β-adrenergic agonists

(27). In contrast to the binding of agonists, the binding of [^{125}I]SARILE (11) was shown to be insensitive to the addition of guanine nucleotides and did not discriminate between these different affinity states of the receptor. Therefore, the antagonist [^{125}I]SARILE measured a more uniform class of ANG II receptors with high affinity, specificity, stability, and a slow dissociation rate (11). Another advantage in using [^{125}I]SARILE over [^{125}I]ANG II is that the sarcosine substitution in position 1 stabilizes the octapeptide by decreasing enzymatic degradation (19). In a recent published abstract, another group has also used [^{125}I]SARILE to localize ANG II receptors in rat brain homogenates and in brain slices by autoradiography (21).

Furthermore the potency order of agonists competing for [^{125}I]SARILE was also shown to be the same as the potency order of agonists on aldosterone release (11). Although the use of either [^{125}I]SARILE or [^{125}I]ANG II measures the same total number of adrenal ANG II receptors (11), the use of the radiolabeled antagonist should allow for better quantitative estimations of receptor density and affinity. In the present study, ANG II receptor density values were similar to those previously reported for the rat anterior pituitary gland and purified adrenal zona glomerulosa (20, 34). However, for the mesenteric artery, we report a much higher density than previously reported (14, 41) which may be due to differences in membrane preparation or ionic strength of the buffers or possibly to the use of [^{125}I]SARILE.

In this study, chronic estradiol treatment has been shown for the first time to produce different effects on ANG II receptor density depending upon the target tissue examined. This differential effect does not appear to be mediated via altered circulating levels of ANG II since plasma ANG II levels were not increased following sustained administration of a large dose of estradiol (table IV). This finding is in agreement with Ménard and Catt (31) who have shown that estradiol treatment in rats produced a rapid increase within 6 hours of plasma renin substrate and that following a transient 48-hour increase, plasma renin activity fell slightly below normal levels by day 6 when plasma renin concentrations were reduced by half. Although conflicting results have been published (for review see 38 & chapter 2), it can be generally concluded that in women estrogens increase circulating ANG II (6) and plasma renin activity (25,26). These values approach normal levels with chronic treatment as plasma renin concentrations are being suppressed (26,31,32,35). Several reports (26,35) have suggested that in rats chronically treated with estradiol, homeostatic mechanisms are more efficient in compensating for increased plasma renin substrate than in women taking oral contraceptives or estrogens. It must be emphasized however, that discrepant results may be due to the use of pharmacological doses of estrogens in rats compared to much smaller therapeutic doses used in women. Furthermore, in rats chronically treated with mg doses of estrogens to induce hypertension (28,40) plasma renin activity was increased while plasma renin concentration was unchanged or slightly decreased again indicating the importance of the dose effect on these parameters.

Decreased circulating levels of ANG II have been shown to down-regulate ANG II receptors in the adrenal gland and to up-regulate them in vascular smooth muscle (5,15). Elevated ANG II levels produce an opposite effect. Since estrogens exert a mineralocorticoid effect in addition to their effect on the renin-aldosterone system (24) it might be argued that in this study a positive sodium balance may be responsible for the observed down-regulation of the adrenal ANG II receptor via lowered circulating ANG II (table IV, ANG II at 14 days). This does not appear to be the predominant mechanism of action since up-regulation of the mesenteric artery ANG II receptor was not observed at any time period during the present study.

In the anterior pituitary gland however, down-regulation of the ANG II receptor may be due to release of ANG II into the portal circulation. Neurons which display uptake of estradiol have been localized in neurophysin-containing magnocellular neurons of the paraventricular and supraoptic nuclei (37). These neurons have also been shown to contain native ANG II (13). These hypothalamic nuclei project to a wide variety of extrahypothalamic CNS regions which also contain ANG II (13). Since one of these ANG II containing regions is the external zone of the median eminence, it is possible that circulating estrogens may act directly on the paraventricular and supraoptic nuclei to cause the release of ANG II into the hypothalamo-hypophyseal portal system, leading to ANG II-induced down-regulation of ANG II receptors. This hypothesis does not agree however with the results of Mendelsohn et al. (34) who have shown that in contrast to adrenal and vascular ANG II receptors, those in the anterior pituitary gland were not affected by changes in salt balance

or ANG II infusion. In a limited number of observations, we did not see any difference between the density of ANG II receptors in the anterior pituitary of ovariectomized and intact female rats sampled randomly with respect to their estrous cycle. This finding has also been reported in another study where only a small apparent decrease (not significant) in anterior pituitary ANG II receptor density was observed from intact cycling females when compared to ovariectomized controls (8). These findings suggest that significant down-regulation of anterior pituitary ANG II receptors may occur only during the high estrogenic phase of the cycle, and may not be detected in pooled pituitary glands obtained randomly from cycling female rats, since about half of these rats are in the diestrous stage (characterized by low circulating estrogen levels). Estrogen treatment also produces several well known alterations in peptide hormone secretion such as a decrease in gonadotropins and an increase in prolactin. To our knowledge the possible indirect implications of these peptide hormones or other estrogen-induced alterations on ANG II receptors have not been documented.

It appears unlikely that the estradiol-induced down-regulation of ANG II receptors is due to a direct effect on the plasma membrane leading to allosteric alterations of the ANG II receptor or to alterations in membrane fluidity analogous to the effect of cholesteryl hemisuccinate on adrenal ANG II receptors reported by Carroll et al. (4). In separate studies, when estradiol was added into test tubes containing homogenized anterior pituitary membranes (8) or adrenal cortical membranes (3), there was no effect upon [^{125}I]ANG II binding. Nevertheless, since specific estradiol binding sites have been

identified in the anterior pituitary gland (12), adrenal cortex (30) and in vascular smooth muscle (18) it is possible to speculate that estradiol may act directly in vivo on these target tissues and alter ANG II receptor density and/or sensitivity to ANG II. This study cannot exclude that other ovarian products may also be involved in ANG II receptor down-regulation since chronic estradiol treatment has also been shown to reduce ANG II receptor density in the uterus of intact female rats (42).

In the adrenal cortex and vascular smooth muscle, reciprocal alterations in ANG II receptor density are followed by parallel changes in sensitivity to ANG II (1, 2). Our biological response study confirms that estradiol-induced down-regulation of adrenal cortical ANG II receptor density blocks the release of aldosterone by low dose infusion of ANG II. It is noteworthy that in a similar experiment using intact male rats (7), the aldosterone response to the same dose of infused ANG II was much more pronounced than the response observed in the present study using OVX-female rats. These findings may indicate important sex differences in the aldosterone response to ANG II.

In contrast with the adrenal cortex, estrogen-treated rats appear to be more sensitive to ANG II stimulation of prolactin release despite decreased pituitary ANG II receptor density (see chapter 4). This latter finding suggests that the effect of estrogen as a factor regulating prolactin responsiveness may overcome down-regulation of anterior pituitary ANG II receptor density.

Furthermore, contrary to the anterior pituitary gland, and similar to the adrenal cortex, estrogen treatment has been shown to induce refractoriness to the pressor effects of infused ANG II (39, 43). Our results on the mesenteric artery suggest that the previously reported estrogen-induced refractoriness (39, 43) may not be due to down-regulation of vascular ANG II receptor density.

The present observations bring to mind at least three fundamental questions: (1) What is the mechanism of action of estradiol on ANG II receptors? (2) What is the significance of differential modulation of ANG II receptors in different target tissues? (3) How relevant is down-regulation of ANG II receptors to altered physiological response? Definite answers on the mechanism of action of estradiol-induced down-regulation of ANG, II receptors in such tissues as the anterior pituitary gland could best be solved by using a cell culture system. Further investigations looking at estrogen regulation of target cell responsiveness to exogenous ANG II in addition to quantifying ANG II receptor density may shed some light on the physiological significance of differential modulation of ANG II receptors in different target tissues.

G. REFERENCES TO CHAPTER 3

1. AGUILERA G, CATT KJ: Regulation of vascular angiotensin II receptors in the rat during altered sodium intake. Circ. Res. 1981, 49:751-758.
2. AGUILERA G, SCHIRAR A, BAUKAL A, CATT KJ: Angiotensin II receptors: properties and regulation in adrenal glomerulosa cells. Circ. Res. 1980, 46 (Suppl. I):I-118-I-127.
3. CAMPANILE CP, GOODFRIEND TL: Steroids as potential modulators of angiotensin receptors in bovine adrenal glomerulosa and kidney. Steroids 1981, 37:681-700.
4. CARROLL JE, LANDRY AS, ELLIOTT ME, YATVIN MB, VORPHAL J, GOODFRIEND TL: Cholesteryl hemisuccinate alters membrane fluidity, angiotensin receptors and responses in adrenal glomerulosa cells. Life Sci. 1983, 32:1573-1581.
5. CATT KJ, AGUILERA G, CAPPONI A, FUJITA K, SCHIRAR A, FAKUNDING J: Angiotensin II receptors and aldosterone secretion. J. Endocrin. 1979, 81:37p-48p.
6. CATT KJ, CRAN E, ZIMMET PZ, BEST JB, CAIN, MD, COGHLAN JP: Angiotensin II blood levels in human hypertension. Lancet 1971, i:459-464.
7. CHARTIER L, SCHIFFRIN E, THIBAULT G, GARCIA R: Atrial natriuretic factor inhibits the stimulation of aldosterone secretion by angiotensin II, ACTH and potassium in vitro and angiotensin II-induced steroidogenesis in vivo. Endocrinology
8. CHEN F-CH, PRINTZ MP: Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 1983, 113:1503-1510.
1984, 115:2026-2028.

9. CHEN FM, HAWKINS R, PRINTZ MP: Evidence for a functional, independent brain-angiotensin system: correlation between regional distribution of brain angiotensin receptors, brain angiotensinogen and drinking during the estrous cycle of rats. In: Ganten D, Printz MP, Phillips MI, Scholkens BA, (eds) Experimental Brain Research. Suppl. 4, 1982, Springer-Verlag, Berlin pp. 157-168.

10. DE LEAN A, KILPATRICK RF, CARON M: Dopamine receptor of the porcine anterior pituitary gland: evidence for two affinity states of the receptor discriminating by both agonists and antagonists. Mol. Pharmacol. 1982, 22:290-297.

11. DE LEAN A, ONG H, GUTKOWSKA J, SCHILLER PW, MCNICOLL N: Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. Mol. Pharmacol. 1984, 26:498-508.

12. EISENFELD AJ: ^{3}H-estradiol: In vitro binding to macromolecules from the rat hypothalamus, anterior pituitary and uterus. Endocrinology 1970, 86:1313-1318.

13. FUXE K, GANTEN D, ANDERSON K, CALZA L, AGNATI LF, LANG RE, POULSEN K, HOKFELT T, BERNARDI P: Immunocytochemical demonstration of angiotensin II and renin-like immunoreactive nerve cells in the hypothalamus. Angiotensin peptides as comodulators in vasopressin and oxytocin neurons and their regulation of various types of central catecholamine nerve terminal systems. In: Ganten D, Printz MP, Phillips MI, Scholkens BA, (eds) Experimental Brain Research, Suppl. 4, 1982, Springer-Verlag, Berlin, pp. 208-232.

14. GUNTER S, GIMBRONE MA Jr, ALEXANDER RW: Identification and characterization of the high affinity vascular angiotensin II receptor in rat mesenteric artery. Circ. Res. 1980, 47: 278-286.

15. GUNTER S, GIMBRONE MA Jr, ALEXANDER RW: Regulation by angiotensin II of its receptors in resistance blood vessels. Nature 1980, 287:230-232.

16. GUTKOWSKA J, BOUCHER R, GENEST J: Dosage radioimmunologique de l'activité de la rénine plasmatique. Union Méd. Can, 1977, 106:446-450.

17. GUTKOWSKA J, CORVOL P, FIGUEIREDO AF, INAGAMI T, BOUHNIK J, GENEST J: Kinetic studies of rat renin and tonin on purified rat angiotensinogen. Can. J. Biochem. Cell. Biol. 1984, 62: 137-142.

18. HARDER DR, COULSON PB: Estrogen receptors and effects of estrogen on membrane electrical properties of coronary vascular smooth muscle. J. Cell Physiol. 1979, 100:375-382.

19. HALL MM, KHOSLA MC, KHAIRALLAH PA, BUMPUS FM: Angiotensin analogs: the influence of sarcosine substituted in position 1. J. Pharmacol. Exp. Ther. 1974, 188:222-228.

20. HAUGER RL, AGUILERA G, BAUKAL AJ, CATT KJ: Characterization of angiotensin II receptors in the anterior pituitary gland. Mol. Cell. Endocrinol. 1982, 25:203-212.

21. HEALY DP, PRINTZ MP: Localization of angiotensin II receptors in rat brain with [^{125}]-Sar 1, Ile 8-angiotensin II. (Abstract) Fed. Proc. 1985, 44:720.

22. HELMER OM, GRIFFITH RS: The effect of the administration of estrogens on the renin-substrate (Hypertensinogen) content of rat plasma. Endocrinology 1952, 51:421-426.

23. HUNTER WM, GREENWOOD FC: Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 1962, 194:495-496.

24. JOHNSON JA, DAVIS JO, BROWN PR, WHEELER PD, WITTY RT. Effects of estradiol on sodium and potassium balances in adrenalectomized dogs. Am. J. Physiol. 1972, 223:194-197.

25. KAULHAUSEN H, VENN HJ, MUHLBAUER W, BECK KJ, BREUER H: Effect of ethinyl estradiol on renin activity and on the concentrations of renin substrate and aldostrone in plasma of ovarjectomized women. Arch. Gynecol. 1980, 229:29-36.

26. KAULHAUSEN H, WEYAND C: Evidence for two independent effects of oestradiol benzoate on the renin-angiotensin-aldosterone system. Acta. Endocrinol. 1982, 100:77-84.

27. KENT RS, DE LEAN A, LEFKOWITZ RJ: A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. Mol. Pharmacol. 1979, 17:14-23.

28. KONDO K, MISUMI J, NAKAMURA R, SAITO I, SARUTA T: Effect of estrogen upon the juxtaglomerular apparatus and the renin-angiotensin system in rats. Tohoku J. Exp. Med. 1978, 126:267-272.

29. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951, 193:265-275.

30. MARTIN PM, COCHET C, ROLLAND PH, CHAMBAZ EM: Demonstration of high-affinity binding of estradiol in adrenal cortex tissue. C.R. Acad. Sci. Paris 4 Apr. 1977, 284:1305-1307.

31. MENARD J, CATT KJ: Effects of estrogen treatment on plasma renin parameters in the rat. Endocrinology 1973, 92:1382-1388.

32. MENARD J, MALMEJAC A, MILLIEZ P: Influence of diethylstilbestrol on the renin-angiotensin system of male rats. Endocrinology 1970, 86:774-780.

33. MENDELSOHN FAO: Localization and properties of angiotensin receptors. J. Hypert. 1985, 3:307-316.

34. MENDELSOHN FAO, AGUILERA G, SAAVEDRA JM, QUIRION R, CATT KJ: Characteristics and regulation of angiotensin II receptors in pituitary, circumventricular organs and kidney. Clin. Exp. Hypertens. 1983, 5:1081-1097.

35. NASJLETTI A, MATSUNAGA M, TATEISHI H, MASSON GMC: Effects of stilbestrol on the renal pressor system as modified by adrenalectomy and secondary aldosteronism. J. Lab. Clin. Med. 1971, 78:30-38.

36. PRINTZ MP, HAWKINS RL, WALLIS CJ, CHEN FM: Steroid hormones as feedback regulators of brain angiotensinogen and catecholamines. Chest 1983, 83:308-311.

37. RHODES CH, MORPELL JI, PFAFF DW: Distribution of estrogen-concentrating, neurophysin-containing magnocellular neurons in the rat hypothalamus as demonstrated by a technique combining steroid autoradiography and immunohistology in the same tissue. Neuroendocrinology 1981, 33:18-23.

38. ROBERTS JM: Oestrogens and hypertension. Clin. Endocrinol. Metab. 1981, 10:489-512.

39. ROSENFELD CR, JACKSON FM: Estrogen-induced refractoriness to the pressor effects of infused angiotensin II. Am. J. Obstet. Gynecol. 1984, 148:429-435.

40. SARUTA T, NAKAMURA R, SAITA I, KONDO K, MATUKI S: Oestrogen hypertension in rats. Clin. Sci. 1975, 48:457-460.

41. SCHIFFRIN EL, THOME FS, GENEST J: Vascular angiotensin II receptors in renal and DOCA-salt hypertensive rat. Hypertension 1983, 5:V-16-V-21.

42. SCHIRAR A, CAPPONI A, CATT KJ: Regulation of uterine angiotensin II receptors by estrogen and progesterone. Endocrinology 1980, 106:5-12.

43. TAMAI T, MATSUURA S, TATSUMI N, NUNOTANI T, SAGAWA N: Role of sex steroid hormones in relative refractoriness to angiotensin II during pregnancy. Am. J. Obst. Gynec. 1984, 149:177-183.

44. UNDERWOOD R, WILLIAMS GH: The simultaneous measurment of aldosterone, cortisol and corticosterone in human peripheral plasma by displacement analysis. J. Lab. Clin. Med. 1972, 79: 48-862.

45. WEI JW, JANIS RA, DANIEL EL: Isolation and characterization of plasma membrane from rat mesenteric arteries. Blood Vessels 1976, 13:279-292.

CHAPTER 4 ESTROGENS DIRECTLY DOWN-REGULATE RECEPTORS FOR ANGIOTENSIN II IN THE ANTERIOR PITUITARY GLAND WITHOUT DECREASING TARGET CELL RESPONSIVENESS

A. PREFACE

In chapter 3, chronic estradiol treatment was shown to cause a marked reduction in ANG II receptor density in the anterior pituitary gland (∿80%), a moderate reduction in the adrenal cortex (∿ 30%) and no apparent effect on receptor density in the mesenteric artery. The down-regulation of ANG II receptors in the adrenal cortex was also shown in a physiological experiment when a low-dose infusion of ANG II failed to stimulate aldosterone release in ovariectomized rats treated with estradiol for 7 days. These estrogen effects did not appear to be mediated by alterations in circulating levels of ANG II.

Despite these *in vivo* findings, direct evidence in support of a role for estrogens in the down-regulation of ANG II receptor density was still lacking. To gain a better understanding of the mechanism of action of estradiol, it was decided to use cultured anterior pituitary cells, since the most pronounced effect of estrogens on ANG II receptor density was observed in this tissue. In the preceding chapter, it was also postulated that the effect of estrogens in the anterior pituitary gland might be indirectly mediated by the release of ANG II into the portal circulation. Hence, the use of cultured pituitary cells removed from any hypothalamic influence would either support or rule out a possible contribution of the hypothalamus in the effect of estradiol on anterior pituitary ANG II receptors.

In addition to clarifying the mechanism of action of estrogens, the study described in the following chapter questions the relationship between estrogen-induced down-regulation of anterior pituitary ANG II receptors and altered target cell responsiveness to ANG II. This question was addressed by looking at the effect of estradiol on ANG II-induced prolactin release both *in vitro* and *in vivo*.

ESTROGENS DIRECTLY DOWN-REGULATE RECEPTORS FOR ANGIOTENSIN II IN THE ANTERIOR PITUITARY GLAND WITHOUT DECREASING TARGET CELL RESPONSIVENESS

This paper has been submitted for publication.

B. ABSTRACT

Chronic estradiol treatment *in vivo* has been shown to reduce the density of receptors for ANG II in the anterior pituitary gland. We studied whether estradiol is directly involved in the down-regulation of ANG II receptors using anterior pituitary cells in culture. Binding affinity and density of ANG II receptors were measured in disrupted anterior pituitary cells using the radiolabeled ANG II antagonist [^{125}I]Sar^{1},Ile^{8}-ANG II ([^{125}I]SARILE). Estradiol treatment (10 nM) for either 48 or 96 hours caused a marked reduction ($\sim$ 70%) in the density of receptors for ANG II in cultured anterior pituitary cells with no change in the dissociation constant of [^{125}I]SARILE (K_D, 0.5 ± 0.1 nM). In the anterior pituitary specific binding sites for ANG II are present in lactotrophs and ANG II has been shown to release prolactin both *in vivo* and *in vitro*. We therefore studied the effect of estradiol on ANG II-induced prolactin release. In anterior pituitary cells treated with estradiol for 48 hours, dose-response curves revealed that ANG II still increased prolactin release ($p < 0.01$). The average net prolactin release (ANG II-stimulated minus basal) was greater in estradiol-treated cells than in controls whereas the half maximal stimulation dose (ED_{50}) of ANG II was the same (0.07 ± 0.04 nM). A greater prolactin response to a 5 nmol bolus injection of ANG II was also observed in anesthetized-ovariectomized rats treated with estradiol for 7 days. These results suggest that estrogens are directly involved in the modulation of ANG II receptors in the anterior pituitary causing marked receptor down-regulation without decreasing target cell responsiveness.

C. INTRODUCTION

In several animal species, ANG II has been shown to stimulate the release of prolactin in vivo (13,32,33) and in vitro (30,33) as well as the release of ACTH (3,16,28,31). Specific ANG II binding sites have been identified in the anterior pituitary gland (18,25) and are predominantly associated with lactotrophs and corticotrophs (1,27). Prolactin appears to have an important role in vertebrate osmoregulation (22). In sub-mammalian vertebrates, prolactin seems to be a key hormone in the regulation of body fluid and electrolytes (14,24) whereas in mammals its physiological importance in body fluid homeostasis remains unclear. During early estrus, the density of ANG II receptors tends to decrease in certain areas of the brain and in the anterior pituitary gland (5) but increases in the uterus (5,29). Estrogens appear to be important effectors involved in these changes since estradiol treatment in vivo decreases ANG II density in the anterior pituitary (chap 3,6) adrenal cortex (chap 3), and in discrete areas of the brain (15,19). Acute but not chronic estradiol treatment has also been shown to increase the density of uterine ANG II receptors (29).

In this study, we addressed the following questions: Are estrogens directly involved in the down-regulation of anterior pituitary receptors for ANG II? Secondly the significance of this down-regulation was assessed by looking at the effect of 17β-estradiol on ANG II-induced prolactin release both in vitro and in vivo.

D. MATERIAL AND METHODS

a) Animals

Adult female Sprague-Dawley rats (200-225 g) (Charles River Labs, St-Constant, Que.) were used for this study and were sampled randomly with respect to their estrous cycle. Rats were kept at 22°C and exposed to a 12 hour-light, 12 hour-dark cycle with free access to normal Purina rat chow (152 mmol Na per kg) and water.

b) Anterior pituitary cell culture technique

Female rats were used for the preparation of primary cultures as described (12). For prolactin release studies, 5×10^5 cells were plated per well in quadruplicate (Primaria 3847, Oxnard, Calif.) in 1 ml of medium and for binding studies 4 to 5×10^6 cells in 5 ml were plated in triplicate Petri dishes (Primaria 3802). The medium consisted of DMEM with 2.5% dextran-coated charcoal adsorbed (DCC-adsorbed) (12) fetal calf serum and 10% DCC-adsorbed horse serum with antibiotics (Gibco, Grand Island, N.Y.). After 3 days of incubation at 37°C in 5% CO_2 water saturated atmosphere, media were replaced with fresh medium in the presence or absence of 10 nM 17β-estradiol (Steraloids, Wilton, N.H.: stock solution 10^{-4}M in 100% ethanol) for 48 or 96 hours (changing media once after 2 days).

The ANG II dose-response study was performed as follows; after 48 hours, cells were washed twice before incubating for 3½ hours at 37°C with increasing concentrations of ANG II diluted in DMEM containing 0.02% lysosyme chloride (Sigma, St-Louis, MO.) in the presence or absence of 10 nM estradiol. Culture medium was then collected, centrifuged (100 g, 4°C, 10 minutes) and the supernatant stored at -20°C until assayed for prolactin.

c) [^{125}I]SARILE binding technique

Iodination of (Sar^1,Ile^8) ANG II (SARILE) was performed using Iodo-Beads (Pierce Chem., Rockford, Ill.) and radioligand was purified on HPLC using an ODS column with a gradient of acetonitrile. Specific activity of [^{125}I]SARILE was determined by comparing SARILE with [^{125}I]SARILE in receptor binding competition curves and provided an estimated specific activity of 2000 Ci/mmole. After 48 or 96 hours of incubation, triplicate Petri dishes were rinsed with assay buffer consisting of 50 mM Tris-HCl, pH 7.4 at 25°C, 1 mM $MgCl_2$, 150 mM NaCl and 0.1 mM Na-EDTA. The cells were then scraped with a rubber policeman disrupted with a glass homogenizer at 4°C, washed at 40,000 g for 15 minutes and the pellet resuspended in assay buffer. Angiotensin II receptor density was calculated from competition binding curves performed as follows: 100 µl of freshly homogenized tissue membranes were incubated for 60 minutes in a shaker bath at 25°C with 50 µl of increasing doses of unlabeled SARILE and 50 µl of [^{125}I]SARILE (250 pM) both prepared in assay buffer with 0.5% heat-inactivated BSA. The reaction was stopped by filtration through glass-fiber filters (Whatman, GF/B, Maidstone, U.K.) followed by four washings with 5 ml of ice-cold assay buffer. Proteins were measured as described by Lowry et al. (23).

d) In vivo angiotensin II-induced prolactin release study

Two days following ovariectomy, 10 rats received 25 µg of estradiol valerate (Delestrogen, Squibb Canada, Montréal, Que.) diluted in 0.2 ml of sesame oil (90-154, Fisher Sci., Fairlawn, N.J.) and injected subcutaneously every day for 7 days. Control OVX-rats received only the vehicle. On day 7, rats were anesthetized with

sodium pentobarbital (Somnotol, MTC Pharm. 50 mg/kg i.p.) and a polyethylene tubing (PE-50, Intramedic, Clay Adams, N.J.) was inserted through the right jugular vein and positioned near the junction with the right atrium. Five nmol of ANG II (Peninsula, Palo Alto, Calif.) were injected as a bolus in 100 µl of 0.9% NaCl and serial blood samples (1 ml) were collected 5 minutes prior to, as well as 2, 5, 15 and 30 minutes after ANG II injection. Each blood sample was immediately replaced with equal volumes of 0.9% NaCl containing 200 U/ml of heparin. Blood samples were collected in tubes containing 100 U of heparin with 10 mM EDTA and after centrifugation plasma was stored at -20°C until assayed for prolactin by RIA. Prolactin was measured using RIA kits from NIADDK, Pituitary Hormone Distribution Program using [^{125}I]-prolactin from New England Nuclear (NEX 108, specific activity 40 µCi/ug). At the end of each experiment, anterior pituitary glands were removed and weighed.

e) Morphological technique

Additional control and estradiol-treated rats were also perfused with 50 ml of 0.9% NaCl injected into the left ventricle followed by 10 minutes of *in toto* fixation with 1% glutaraldehyde buffered with 0.1 M cacodylate pH 7.4. Anterior pituitary glands were then removed, sectioned, fixed for one additional hour in 1% glutaraldehyde buffered with cacodylate HCl rinsed in the same buffer before dehydration and embedding in Araldite. Fine sections were cut with a Reichert OMU_2 ultramicrotome, stained with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope.

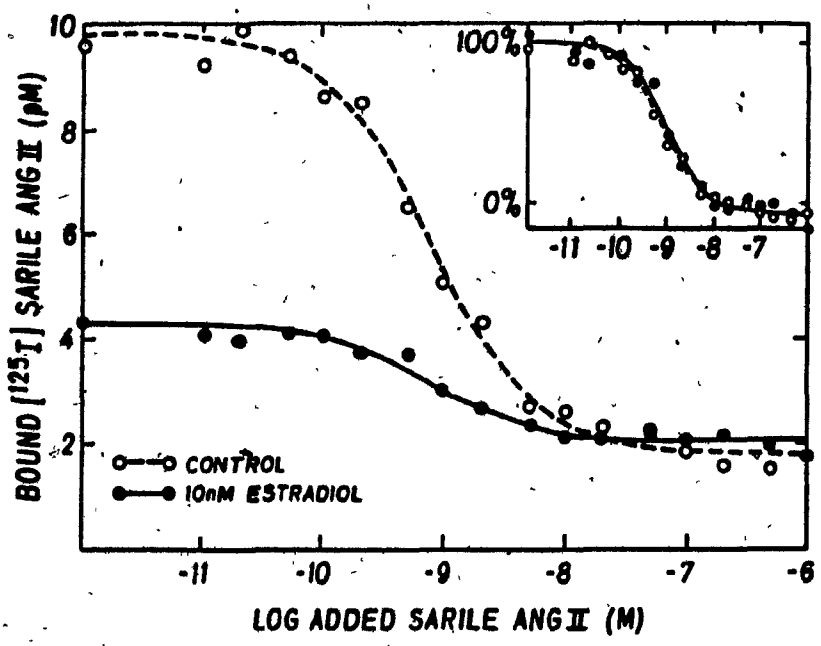


Figure 1

Competition binding curve of [^{125}I]SARILE versus added increasing concentrations of unlabeled SARILE. Each curve represents analysis of binding data from cells pooled from 3 Petri dishes treated with or without 10 nM estradiol for 48 hours. Estradiol treatment decreased ANG II receptor density from 81 ± 6 to 24 ± 3 fmol/mg protein ($p < 0.01$) with no effect on the K_D (0.5 ± 0.1 nM). Inset represents 0 to 100% normalized data. Protein concentrations are the same between control and treated cells.

f) Statistical analysis

Dose-response curves on prolactin release and binding data were analysed by computer-assisted non linear least squares regression analysis and the F-test (9) (Figs. 1, 2). Two and 3-way ANOVA with Dunnett's test were used respectively for the analysis of other parameters and for comparing net prolactin release (see results). For *in vivo* experiments (Fig. 4), differences across time within each of the two groups were tested using analysis of variance (ANOVA) with the repeated measures design followed by Dunnett's test. Treatment effect on total body weight, anterior pituitary weight and anterior pituitary cell protein was analysed by two-tailed unpaired Student's t test.

E. RESULTS

a) In vitro [^{125}I]SARILE binding study

The effect of estradiol treatment on the density and affinity of anterior pituitary ANG II receptors was calculated from competition binding curves (8,20) (Fig. 1). Each point represents bound [^{125}I]SARILE as a function of added increasing concentrations of unlabeled SARILE and the lines are the best fit of the data as determined by computer analysis (8,20). Estradiol treatment for either 48 or 96 hours markedly reduced the density of anterior pituitary ANG II receptors by approximately 70%. Values decreased from 81 ± 6 to 24 ± 3 fmol/mg protein ($p < 0.01$, n=4) after 48 hours of estradiol treatment and from 63 ± 9 to 23 ± 4 fmol/mg protein ($p < 0.01$, n=5) after 96 hours. Normalized data from competition curves showed no statistical difference ($p > 0.05$) between the binding characteristics of control and estradiol-treated cells (Fig. 1, inset). The equilibrium

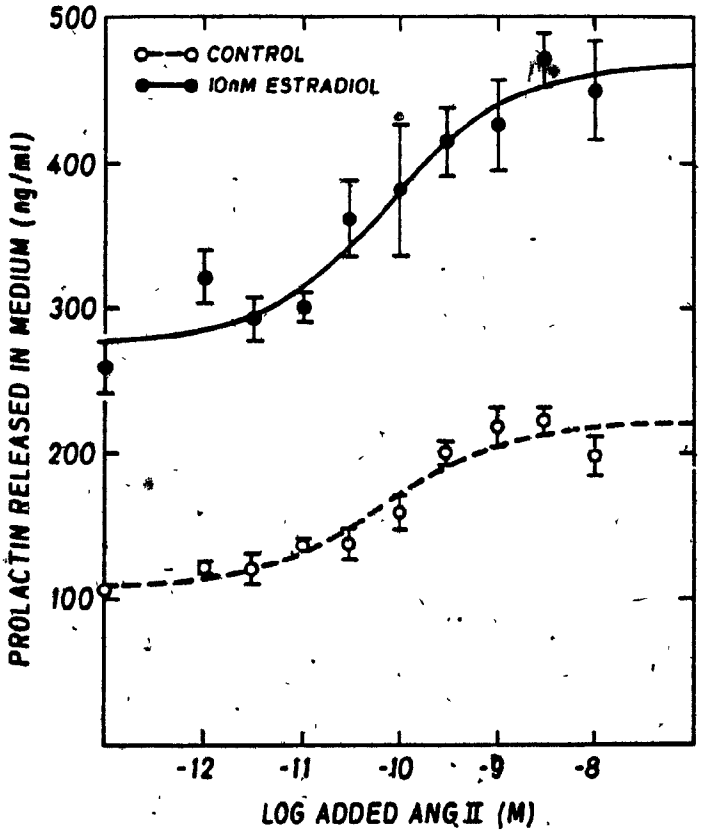


Figure 2

Dose-response curves of increasing concentrations of ANG II versus prolactin release measured in the medium of control and 48-hour estradiol-treated anterior pituitary cells. Angiotensin II increased ($p < 0.01$) the release of prolactin in medium of both control and 10 nM estradiol-treated cells. ED_{50} values were the same at 0.07 ± 0.04 nM. Values are the means ± SEM of triplicate experiments.

SI unit conversion factor ng/ml x 43.5 = pmol/l.

dissociation constant (K_D) was calculated by computer analysis of competition curves according to the mass action law (8,20) and showed no difference ($p > 0.05$) between control and treated cells with the same K_D value of 0.5 ± 0.1 nM. Membrane protein concentrations between control cells (652 ± 72 µg/dish) and cells treated with estradiol for 48 hours (680 ± 59 µg/dish) or 96 hours (626 ± 89 µg/dish) were not significantly different ($p > 0.05$). No difference was observed when competition curves were performed using untreated anterior pituitary cell membranes in the presence or absence of 10 nM estradiol added directly in the binding assay tubes (data not shown). In a limited number of observations, the specificity of SARILE for ANG II binding sites was confirmed by the use of ANG II as the competing ligand for [^{125}I]SARILE and resulted in the measurement of the same total number of ANG II binding sites in anterior pituitary cell cultures (data not shown).

b) <u>In vitro angiotensin II-induced prolactin release study</u>

Dose-response curves revealed that ANG II increased ($p < 0.01$) the release of prolactin into the medium of both control and 48-hour estradiol treated cells (Fig. 2). The average net prolactin release (10 nM ANG II-stimulated minus basal levels) was greater in estradiol-treated cells (450 ± 34 minus 260 ± 18 = Δ 190 ng/ml) than in control cells (197 ± 14 minus 105 ± 3 = Δ 92 ng/ml) ($p < 0.01$). However the half-maximal stimulation dose (ED_{50}) of ANG II on prolactin release was 0.07 ± 0.04 nM and was not statistically different between control and treated cells ($p > 0.05$). Confirming a well documented effect of estrogens, basal prolactin levels were also significantly increased ($p < 0.01$) following estradiol treatment (Fig. 2). Under low

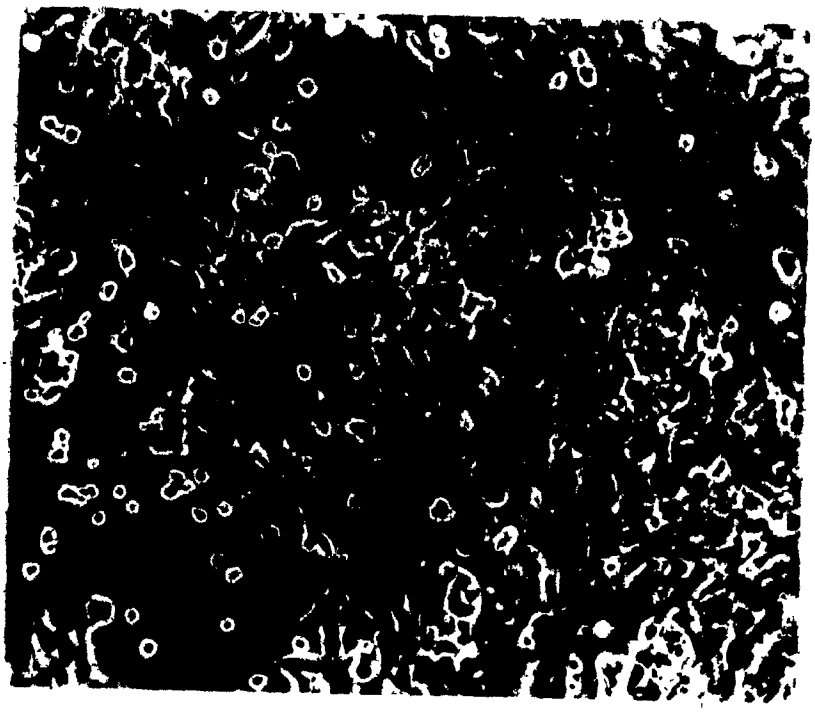


Figure 3

Morphology of anterior pituitary cells cultured for 5 days in the absence of estradiol. Secretory epithelial cells are spherical. Note the presence of secretory granules in their cytoplasm. (X 400).

magnification, no obvious difference was observed in the morphological appearance of cultured anterior pituitary cells in the presence or absence of estradiol (Fig. 3).

c) In vivo angiotensin II-induced prolactin release study

Pooled plasma prolactin values from 8 OVX-controls and 8 OVX-estradiol treated rats are shown in Figure 4. In OVX-controls, maximum plasma prolactin release appeared 5 minutes after administration of ANG II (1.6-fold increase from the basal level of 3.8±1.0 to 6.1±1.2 ng/ml); however this increase was not significant ($p > 0.05$). In estradiol-treated rats prolactin levels increased 2.7-fold ($p < 0.05$) from the basal level of 51±20 to 140±36 ng/ml, 2 minutes after ANG II injection. Overall prolactin values from estradiol-treated rats were higher ($p < 0.01$) than in OVX-controls reflecting much lower pituitary levels in the latter group. Similar to previous reports (chapter 3,6), total body weights decreased ($p < 0.01$) from 218±2 g in OVX-controls to 202±4 g in estradiol-treated rats while anterior pituitary glands approximately doubled in size weighing 18.4±1.0 mg compared to 11.1±0.6 mg in controls ($p < 0.01$). Figure 5 is an electron micrograph of a hypertrophic lactotroph taken from a mid-section of the anterior pituitary gland of a female rat treated with estradiol for 7 days.

F. DISCUSSION

The present study demonstrates for the first time that estrogens act directly on anterior pituitary cells to decrease ANG II receptor density. Furthermore, this estrogen-induced down-regulation is not accompanied by decreased prolactin cell responsiveness to ANG II.

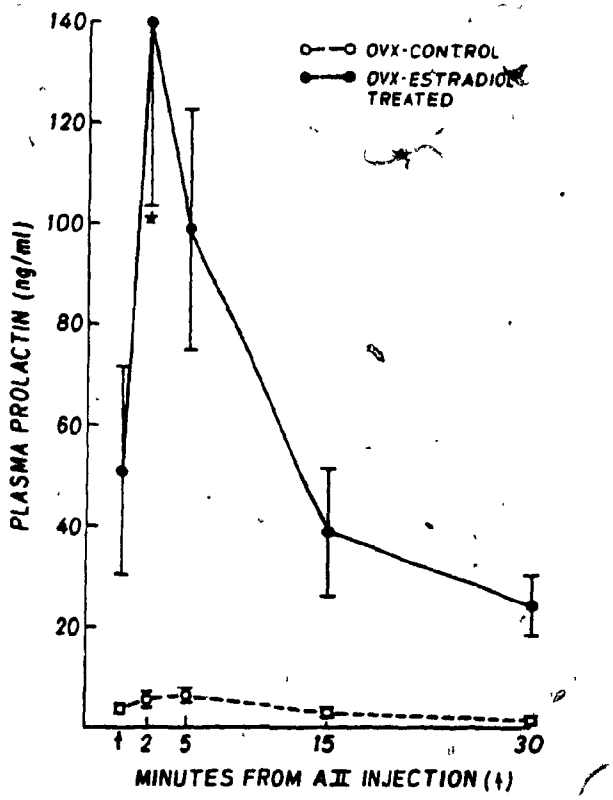


Figure 4

Stimulation of prolactin release in vivo by 5 nmol of ANG II injected i.v. to OVX-control and OVX-estradiol treated rats. In OVX-controls (n=8) the 1.6-fold increase is not statistically significant ($p > 0.05$). In OVX-estradiol treated rats (n=8), a significant 2.7-fold increase was noticed after 2 minutes. * $p < 0.05$. Values are the means ± SEM (AII=ANG II). SI unit conversion factor ng/ml x 43.5 = pmol/l.

Figure 5

Represents an electron micrograph of a lactotroph from a 7-day estradiol treated rat. The amount of rough endoplasmic reticulum and the size of the Golgi complex are markedly increased. Typical pleomorphic secretory granules are scattered throughout the cytoplasm. (X 16,000).

In a similar *in vivo* study, Steele et al. (32) report that in 2-day estrogen primed conscious OVX-rats, ANG II induced a 1.8-fold increase in plasma prolactin after 15 minutes when compared to saline-injected controls whereas in OVX-only rats they report in another study (33) a 2.7-fold increase after 5 minutes. Taken together, both studies from Steele et al. (32,33) did not establish whether estrogen treatment *in vivo* had any effect on the sensitivity of response to ANG II, but merely indicated that ANG II could still elicit prolactin release in estrogen-treated rats. In the present study, we found that in anesthetized OVX-rats previously treated with estradiol for 7 days, plasma prolactin increased 2.7-fold after 2 minutes of ANG II injection whereas in OVX-only rats, the apparent 1.4 and 1.6-fold increases at 2 and 5 minutes respectively were not statistically significant (Fig. 4).We have no explanation for the discrepancies between both studies concerning OVX-only rats other than speculation on differences in experimental approach such as the state of anesthesia. We have thus observed a greater *in vivo* prolactin response to ANG II in anesthetized OVX-rats chronically treated with estrogens than in OVX-controls. However, interpretations of our *in vivo* experiment are difficult since we have used anesthetized rats injected with supraphysiological doses of estradiol and ANG II. This treatment may have caused a nonspecific prolactin response due to the stress of our *in vivo* study. To overcome these difficulties we elected to do a dose-response study and to quantify ANG II receptors in cultured anterior pituitary cells.

The use of radiolabeled antagonists for hormone receptor quantifications has been widely recognized. Validation of the use of [^{125}I]SARILE to characterize ANG II receptors has been previously

demonstrated (chapter 3,10). A parallel study using OVX-rats treated identically as in the present study showed that estradiol treatment for 7 days caused an 80% reduction in ANG II receptor density in the anterior pituitary gland (chapter 3). This has also been confirmed in the present study using cultured anterior pituitary cells and tends to rule out the hypothesis that estrogen-induced down-regulation of anterior pituitary ANG II receptors might be mediated by the release of ANG II into the portal circulation.

The observed effect of estrogen may well reflect down-regulation of lactotroph ANG II receptor density. Lactotrophs represent a large proportion (~40%) of the total anterior pituitary cell population in the normal female rat (7). Estrogens have been shown to induce hypertrophy and hyperplasia of prolactin cells in vivo (17) whereas estrogens increase the synthesis of prolactin in cultured cells without causing any apparent hyperplasia of lactotrophs (2,21). The association of ANG II receptors with lactotrophs has been suggested in several studies (1,13,27,30,33) particularly by Aguilera et al. (1) who have demonstrated larger prolactin responses to ANG II as well as the preferential location of ANG II binding sites in lactotroph-enriched fractions. Several in vitro studies except for one (33), have failed to show a direct effect of ANG II on the release of GH, FSH, LH or TSH (1,16). Corticotrophs are the only other anterior pituitary cell type where ANG II receptors have been demonstrated repeatedly (3,16,28,31). However, in normal rats, corticotrophs represent less than 5% of the anterior pituitary cell population (7). Contrary to lactotroph-containing fractions, corticotroph fractions did not display maximal binding of ANG II (1). Several studies therefore suggest that

the majority of anterior pituitary ANG II receptors are situated in lactotrophs and that this cell type is likely to be the target for the estrogen effect observed in the present study.

The significance of higher net prolactin release by ANG II in estrogen-treated anterior pituitary cells remains to be clarified with respect to down-regulation of ANG II receptors. Since 48 hour estradiol treatment in vitro did not alter the ED_{50} of ANG II for prolactin-release, the observed increase in prolactin-release by ANG II might be due to stimulation of a greater releasable pool of prolactin under estrogenic activition regardless of alterations in plasma membrane receptors for ANG II. Alternatively, estrogens could possibly increase ANG II-induced prolactin release by enhancing the coupling of the ANG II receptors to its yet unresolved effector and bring about a negative feed-back on ANG II receptor density. Estrogens might down-regulate ANG II receptors by promoting the internalization with sequestration or increased degradation of the receptor or by interfering with receptor synthesis. The accumulation of a putative estrogen-induced product that may inhibit ANG II receptors should also be considered. It appears unlikely that estrogen-induced down-regulation could be due to a direct allosteric effect on the plasma membrane since estradiol added to disrupted anterior pituitary cell membranes in the present study did not interfere with the binding of [^{125}I]SARILE. In light of the increasing evidence supporting the existence of a paracrine endogenous renin-angiotensin system in the anterior pituitary gland (11,26), estrogens might stimulate the release of ANG II produced in gonadotrophs, resulting in down-regulation of the

nearby lactotroph ANG II receptor. Prolactin itself may also be involved in the down-regulation of ANG II receptors.

Further studies aimed at determining the mechanism of action of ANG II on lactotrophs and how estrogens might interfere with this mechanism are needed to verify the hypothesis put forward in the present study. This study also cautions against the assumption that down-regulation of hormone receptor density always leads to decreased target cell responsiveness.

G. REFERENCES TO CHAPTER 4

1. AGUILERA G, HYDE CL, CATT KJ: Angiotensin II receptors and prolactin release in pituitary lactotrophs. Endocrinology 1982, 111:1045-1050.
2. ANTAKLY T, PELLETIER G, ZEYTINOGLU F, LABRIE F: Changes of cell morphology and prolactin secretion-induced by 2-Br-α-ergocryptine, estradiol, and thyrotropin-relasing hormone in rat anterior pituitary cells in culture. J. Cell Biol. 1980, 86:377-387.
3. CAPPONI AM, FAVROD-COUNE CA, GAILLARD RC, MULLER AF: Binding and activation properties of angiotensin II in dispersed rat anterior pituitary cells. Endocrinology 1982, 110:1043-1045.
4. CARRIERE PD, DE LEAN A, GUTKOWSKA J, GENEST J, CANTIN M: Chronic estradiol treatment decreases angiotensin II receptor density in the anterior pituitary gland and adrenal cortex but not in the mesenteric artery. Neuroendocrinology 1986 (in press).
5. CHEN FM, HAWKINS R, PRINTZ MP: Evidence for a functional, independent brain-angiotensin system: correlation between regional distribution of brain angiotensin receptors, brain angiotensinogen and drinking during the estrous cycle of rats. In: Ganten D, Printz MP, Philipps MF, Scholkens BA (eds) Experimental Brain Research, 1982, Suppl. 4, Springer-Verlag, Heidelberg, pp. 157-168.
6. CHEN FM, PRINTZ MP: Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 1983, 113:1503-1510.

7. COSTOFF A: Ultrastructure of Rat Adenohypophysis. Correlation with Function. 1982, Academic Press, New York and London, pp. 70-115.

8. DE LEAN A, HANCOCK AA, LEFKOWITZ RJ: Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. Mol. Pharmacol. 1981, 21:5-16.

9. DE LEAN A, MUNSON PJ, RODBARD D: Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 1978, 235:E97-E102.

10. DE LEAN A, ONG H, GUTKOWSKA J, SCHILLER PW, MCNICOLL N: Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. Mol. Pharmacol. 1984, 26:498-508.

11. DESCHEPPER CF, SEIDLER CD, STEELE MK, GANONG WF: Further studies on the localizaiton of angiotensin II-like immunoreactivity in the anterior pituitary gland of the male rat, comparing various antisera to pituitary hormones and their specificity. Neuroendocrinology 1985, 40:471-475.

12. DROUIN J, LABRIE F: Selective effect of androgens on LH and FSH release in anterior pituitary cells in culture. Endocrinology 1976, 98:1528-1534.

13. DUFY-BARBE L, RODRIGUEZ F, ARSANT J, VERRIER D, VINCENT JD: Angiotensin II stimulates prolactin release in the Rhesus monkey. Neuroendocrinology 1982, 35:242-247.

14. ENSOR DM, PHILLIPS JG: The effect of environmental stimuli on the circadian rhythm of prolactin production in the duck (Anas platyrhynchos). J. Endocrinol. 1970, 48:167-172.

15. FREGLY MJ, ROWLAND NE, SUMNERS C, GORDON DB. Reduced dipsogenic responsiveness to intracerebroventricularly administered angiotensin II in estrogen-treated rats. Brain Res. 1985, 338:115-121.

16. GAILLARD RL, GROSSMAN A, GILLIES G, REES LH. Angiotensin II stimulates the release of ACTH from dispersed rat anterior pituitary cells. Clin. Endocrinol. 1981, 15:573-578.

17. GERSTEN BE, BAKER BL: Local action of intrahypophyseal implants of estrogen as revealed by staining with peroxidase-labeled antibody. Am. J. Anat. 1970, 128:1-19.

18. HAUGER RL, AGUILERA G, BAUKAL AJ, CATT KJ. Characterization of angiotensin II receptors in the anterior pituitary gland. Mol. Cell. Endocrinol. 1982, 25:203-212.

19. JONKLAAS J, BUGGY J. Angiotensin-estrogen central interaction: localization and mechanism. Brain Res. 1985, 326:239-249.

20. KENT RS, DE LEAN A, LEFKOWITZ RJ. A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. Mol. Pharmacol. 1979, 17:14-23.

21. LIEBERMAN ME, MAURER RA, GORSKI J. Estrogen control of prolactin synthesis *in vitro*. Proc. Natl. Acad. Sci. 1978, 75:5946-5949.

22. LORETZ CA, BERN HA. Prolactin and osmoregulation in vertebrates. Neuroendocrinology 1982, 35:292-304.

23. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951, 193:265-275.

24. MEITES J, NICOLL CS. Adenohypophysis: prolactin. Ann. Rev. Physiol. 1966, 28:57-88.

25. MUKHERJEE A, KULKARNI P, MCCANN M, NEGRO-VILLAR A. Evidence for the presence and characterization of angiotensin II receptors in rat anterior pituitary membranes. Endocrinology 1982, 110:665-667.

26. NARUSE K, TAKII Y, INAGAMI T. Immunohistochemical localization of renin in luteinizing hormone-producing cells of rat pituitary. Proc. Natl. Acad. Sci. 1981, 78:7579-7583.

27. PAGLIN S, STUKENBROK H, JAMIESON JD. Interaction of angiotensin II with dispersed cells from the anterior pituitary of the male rat. Endocrinology 1984, 114:2284-2292.

28. RAMSAY DJ, KEIL LC, SHARPE MC, SHINSAKO J. Angiotensin II increases vasopressin, ACTH, and 11-hydroxycorticoid secretion. Am. J. Physiol. 1978, 234:R66-R71.

29. SCHIRAR A, CAPPONI A, CATT KJ. Regulation of uterine angiotensin II receptors by estrogen and progesterone. Endocrinology 1980, 106:5-12.

30. SCHRAMME C, DENEF C. Stimulation of spontaneous and dopamine-inhibited prolactin release from anterior pituitary reaggregate cell cultures by angiotensin peptides. Life Sciences 1984, 34:1651-1658.

31. SOBEL DO. Characterization of angiotensin-mediated ACTH release. Neuroendocrinology 1983, 36:249-253.

32. STEELE MK, McCANN SM, NEGRO-VILLAR A. Modulation by dopamine and estradiol of the central effects of angiotensin II on anterior pituitary hormone release. Endocrinology 1982, 111:722-729.

33. STEELE MK, NEGRO-VILLAR A, MCCANN SM. Effect of angiotensin II on in vivo and in vitro release of anterior pituitary hormones in the female rat. Endocrinology 1981, 109:893-899.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

From this thesis, it appears quite clear that estrogens exert profound effects on the renin-angiotensin system, particularly at the level of the ANG II receptor. From our first study (chapter 3) the effects of estrogens on ANG II receptors were shown to be quite different depending upon the target tissue examined. Chronic estrogen treatment (7, 14 and 28 days) in vivo was shown to cause a marked reduction in the density of ANG II receptors in the anterior pituitary gland, a moderate reduction in the adrenal cortex and no apparent effect in receptor density in the mesenteric artery. This down-regulation of ANG II receptors was observed after 7 days of estrogen treatment and persisted throughout prolonged treatment. Similarly, estrogen treatment in vivo for 48 hours (33) or for several weeks (25) has been shown to decrease ANG II receptor density in certain discrete areas of the brain namely in the thalamus/septum and preoptic areas. These brain areas are implicated in the ANG II-induced drinking response. Therefore it appears that chronic estrogen treatment in vivo for periods exceeding 48 hours could down-regulate ANG II receptor in several target tissues. It is not clear to what extent this estrogen effect is dependent upon the duration of estrogen treatment. Estrogen treatment in vivo for less than 48 hours has been shown to up-regulate ANG II receptor density in uterine smooth muscle whereas more prolonged treatment decreased receptor density (54). There has been no studies to date reporting early effects of estrogen treatment (less than 48 hours) on ANG II receptors in other target tissues.

A. EFFECTS OF ESTROGEN ON ANGIOTENSIN II RECEPTORS IN THE ADRENAL CORTEX

The physiological significance of this estrogen-induced down-regulation of ANG II receptors in different target tissues must be interpreted with caution. In this thesis, chronic estrogen treatment was shown to reduce ANG II receptor density in the adrenal cortex and to attenuate the aldosterone response to a low dose infusion of ANG II. This finding fits well with the classical scheme correlating alterations in receptor number with altered physiological response. A fundamental question is whether or not estrogens act directly on the adrenal cortex to cause down-regulation of ANG II receptors. Several studies have indicated that chronic estrogen treatment has a trophic effect on the adrenal by increasing adrenal gland weight (4,31,65). Female rats normally have larger adrenal glands than males and concomitantly produce more corticosterone under both quiescent and stressful conditions (31). Ovariectomy is associated with a reduction in pituitary and adrenal gland weight and a concomitant decrease in ACTH, corticosterone and 11-deoxycorticosterone (DOC) levels (9,31). Sex differences in adrenocortical structure and function have also been reported (40). The exact mechanism of action of this trophic effect of estrogens on the adrenal gland is unclear. High affinity binding sites for estradiol have been identified in cytosolic soluble fractions of the adrenal cortex (43) which suggests that the trophic effect of estrogens may be mediated directly via these receptors. Micromolar concentrations of estradiol have been shown to stimulate 11β-hydroxylation of DOC leading to increased corticosterone production in rat adrenal homogenates and tissue slices (24,39). Increased corticosterone levels have also been shown in vivo following chronic estradiol treatment (23,31).

It was suggested that the apparent increase in corticosterone production by estradiol might be due to estrogen-induced inhibition of adrenal 5α-reductase activity, resulting in attenuation of corticosterone metabolism (39). Conflicting data concerning the effects of estrogen on adrenal steroidogenesis have also been reported in the literature and probably relate to differences of dosage and/or of the type of estrogenic compound used (23,39). It would appear that here again estradiol has a biphasic effect; small amounts stimulate whereas large amounts inhibit adrenal steroidogenesis (39).

Morphological studies have revealed that chronic estrogen treatment for several weeks causes regressive transformation (atrophy) in the zona glomerulosa of young male rats but not in adult animals (56). The fasciculata cells of young rats only, were also shown to be enlarged following estrogen treatment (56). Massive fat deposits and cellular hypertrophy in the zona reticularis occurs following chronic estrogen treatment of both young and adult male rats (56) as well as in adult female gerbils (47). Fluctuations in the number of DNA-synthesizing cells in the adrenal cortex of female rats have been observed during the estrous cycle (49). Ovariectomy and subsequent treatment of rats with a single high dose of estradiol produced a transient 48-hour increase in DNA synthesizing cells in the zona glomerulosa which returned to normal within 5 days (60). These findings suggest that estrogen treatment in vivo produces divergent effects on the different adrenal cortical zones. The significance of these effects remains to be clarified.

The effect of estrogens on the adrenal cortex might also be mediated indirectly through the pituitary-adrenal axis. Previous

studies on the effects of ovariectomy and replacement with estradiol on pituitary secretion of ACTH have suggested that removal of the ovaries results in decreased synthesis and release of ACTH and that this effect could be reversed by estrogen administration (38). Whether estrogens act directly on anterior pituitary corticotrophs or through the release of corticotropin-releasing factor (CRF) from the hypothalamus has not been clearly established (9).

From these studies it would seem likely that estrogens have a direct and/or indirect trophic effect on one or several zones of the adrenal cortex to increase DOC and corticosterone synthesis and release. There is no evidence that estrogens directly stimulate the zona glomerulosa to increase aldosterone release. In this thesis, plasma aldosterone levels did not significantly increase in ovariectomized rats treated with estradiol for 7 days (chapter 3, Table III). Perhaps estrogens act directly on the zona glomerulosa to decrease ANG II receptor density. Estrogens might also interfere with the late aldosterone biosynthetic pathway (conversion of corticosterone to aldosterone by 18-hydroxylation) (52). Another possibility is that a transient estrogen-induced increase in circulating ANG II would stimulate aldosterone release and that increased aldosterone levels by negative feedback would decrease adrenal ANG II receptor density (18). These hypotheses could perhaps explain the blunted aldosterone response to ANG II despite the presumed increase in aldosterone precursors (DOC and corticosterone) that have been reported in other studies following estrogen treatment. Alternatively since ACTH has been shown to decrease ANG II receptor density in cultured glomerulosa cells (3), estrogen-induced down-regulation of adrenal cortical ANG II receptor might be mediated indirectly by *in vivo* stimulation of ACTH.

The exact mechanism of action of estrogens on ANG II receptors in the adrenal cortex cannot be clearly established from the present in vivo study and would require studying the effect of 17β-estradiol in cultured glomerulosa cells.

B. EFFECTS OF ESTROGEN ON ANGIOTENSIN II RECEPTORS IN THE MESENTERIC ARTERY

In the mesenteric artery, chronic estradiol treatment was not shown to affect the density of ANG II receptors (chapter 3). A similar effect of chronic estrogen treatment on smooth muscle ANG II receptors has also been reported in the rat uterus. Infusion of sustained high doses of estrogens has been shown to exert a biphasic effect on uterine smooth muscle ANG II receptors (54). Short term infusion caused a transient increase in ANG II receptor density which was detectable after 12 hours, reaching its maximum at 36 hours and declining to or below control values after chronic treatment for 5 to 8 days. Following infusions of ANG II, dose-dependent up- and down-regulation of ANG II receptors was observed in the adrenal cortex (1) whereas an inverse biphasic effect was observed in the mesenteric artery (53). Although the significance of these biphasic effects is still unknown, these findings further emphasize the dynamic nature of ANG II receptor regulation.

Interestingly, estrogens have been shown to have both stimulatory and inhibitory effects on uterine proteins and DNA synthesis (57). Contrary to a single injection or several intermittent injections of estradiol, prolonged exposure to this estrogen for 3 consecutive days causes uterine cells to become metabolically refractory to the well known genomic actions of this steroid. The estrogen receptor itself

does not appear to be affected and the accumulation of an inhibitory product has been suggested as a possible explanation for this phenomenon (57).

The absence of an effect of chronic 7-day estradiol treatment on ANG II receptor density in the mesenteric artery does not correlate with the previously reported estrogen-induced refractoriness to the systemic pressor effects of infused ANG II (51,58). However, extrapolations from these physiological studies to the present ANG II receptor quantification study on the mesenteric artery are somewhat tenuous since there are marked differences in the experimental approach. In the physiological studies, ovariectomized sheep were used and they were acutely infused i.v. with estradiol given over a period of 1 to 2 hours. Chronic subcutaneous injections of estradiol for 5 to 11 days to female ovariectomized rats was shown to reduce the duration but not the peak pressor response to central administration of ANG II when monitored (30 minutes) in the absence of available water (55). In a preliminary study described in appendix II, duration and peak pressor responses to infused ANG II (40 pmol/200 g/minute/30 minutes) were not significantly different between control ovariectomized rats and rats treated with 17β-estradiol for 7 days. Similar findings have also been reported in rats treated with synthetic estrogens for 5 days (46). These latter findings (Appendix II and ref. 46) are in agreement with the absence of an effect of chronic estradiol treatment on vascular ANG II receptors as observed in the mesenteric artery (chapter 3). Nevertheless it is uncertain whether alterations in ANG II receptor density and/or affinity in the mesenteric artery correspond to changes in systemic pressor response to ANG II. Further <u>in vitro</u> studies using

arterial strips or cultured vascular smooth muscle cells would allow a better assessment of the effect of estrogens on vascular responsiveness to ANG II and how these findings relate to possible alterations in receptors for ANG II.

C. EFFECTS OF ESTROGEN ON ANGIOTENSIN II RECEPTORS IN THE ANTERIOR PITUITARY GLAND

a) Hypotheses on the mechanism of action of estrogen

i) Calcium mobilization and inhibition of adenylate cyclase

Down-regulation of ANG II receptor density in anterior pituitary gland following chronic estradiol treatment is most intriguing since this effect is not accompanied by a loss of prolactin responsiveness to ANG II. To formulate a reasonable hypothesis on this apparent paradoxical effect requires a better understanding of the mechanism of action of ANG II. Many different laboratories have reported inhibition of adenylate cyclase by ANG II in membranes from several target tissues, including the liver (32), kidney (62), adrenal glands (42), smooth muscle (2) and anterior pituitary gland (41). In the anterior pituitary gland, a close correlation between the respective potencies of a series of ANG II analogs to inhibit adenylate cyclase and the potencies of these analogs to elicit prolactin or ACTH release has been observed (41). Dopamine and ANG II have opposite effects on prolactin secretion even though they both inhibit the same adenylate cyclase. Marie et al (41) proposed a model to reconcile this apparent discrepancy in the mechanism of action of dopamine and ANG II on prolactin secretion. This model implies that prolactin release can be elicited through either a "calcium mobilization pathway" or a "cAMP production pathway". According to this model, ANG II could stimulate

prolactin release through a calcium pathway as has been suggested in several other ANG II target tissues such as the adrenals (20,21), smooth muscle (11) and liver (36). Concomitantly with stimulation of the calcium pathway, ANG II could attenuate the cAMP pathway by a direct inhibition of the adenylate cyclase. One could reason teleologically that while ANG II stimulates prolactin release through calcium mobilization, ANG II might also regulate its stimulatory effect by decreasing the activity of the cAMP pathway. This would render the pituitary less sensitive to other prolactin secretagogues such as VIP (vaso-intestinal polypeptide) and TRH (thyrotropin releasing hormone) which act through this cAMP pathway. Prolactin regulating hormones such as VIP, and dopamine could therefore modulate prolactin release by stimulating or inhibiting the cAMP pathway respectively (41) whereas ANG II would act by a different mechanism possibly by increasing phosphoinositol turnover (6,30). Although several studies indicate that intracellular calcium mobilization appears to be important in ANG II receptor transduction (mediation of cellular response); what actually triggers this calcium mobilization is still unclear. For instance, it is not known whether calcium is brought in from the outside of the cell or is mobilized intracellularly. In the anterior pituitary gland, ANG II-induced calcium mobilization might involve the "opening" of plasma membrane calcium channels giving rise to an influx of calcium into the cell leading to stimulation of prolactin release. Such a mechanism of action suggests a working hypothesis to explain why prolactin release is still increased by ANG II in estrogen-treated cells despite lowered numbers of ANG II receptors. Since estrogen-primed lactotrophs synthesize greater amounts of prolactin, enhancement of prolactin

release by ANG II might occur due to a calcium channel gating effect induced by the stimulation of even just a few (20%) remaining ANG II receptors (chapter 3). This hypothesis would be compatible with the apparent absence of an effect of estrogens on lactotroph sensitivity to ANG II as determined by the similarity of the ED_{50} for ANG II between control and treated cells (chapter 3). On the other hand, direct stimulatory effects of estrogens on basal as well as TRH-induced prolactin release have also been reported (50). This latter finding suggests that estrogens might also interact with the TRH-cAMP coupled system likely exerting its effect at a step after cAMP formation (26).

ii) Nucleotide-binding regulatory protein

Another possible hypothesis is that estrogens might enhance pituitary ANG II receptor sensitivity and that down-regulation of lactotroph ANG II receptors is a secondary compensatory event. This hypothesis implies that estrogens act primarily to enhance the coupling of the ANG II receptor to its, as yet, unresolved effector. By analogy, glucocorticoids have been shown to increase the responsiveness of rat peritoneal mast cells to β-adrenergic agonists without changing the number of β-adrenergic receptors (59). Another study has shown evidence for estrogen-induced "attenuation" of a stimulatory nucleotide-binding regulatory component (N-component) involved in the coupling of the LH and β-adrenergic corpora luteal receptors to the catalytic component of adenylate cyclase (37). This N-component of adenylate cyclase systems is the protein that serves as the interface between hormone receptors and the catalytic unit of the system (37). This decrease in N-component activity suggested that estrogens in addition to causing a decrease in luteal LH receptor number, could also

cause a receptor-independent decrease in the responsiveness of the adenylate cyclase system to LH (37). It is still an open question as to whether similar studies on the anterior pituitary gland ANG II receptor would reveal "enhanced" rather than "suppressed" estrogen-induced "coupling" of the ANG II receptor to its yet unknown effector.

Several studies suggest that the ANG II receptor may very well be linked to a nucleotide-binding protein which may be regulating agonist-induced post-receptor events. [^{125}]ANG II binding is decreased by guanine nucleotides (GTP) (10,27,63). High and low affinity binding sites for the agonist ANG II (which are sensitive to GTP) have been reported for ANG II receptors in the adrenal (13), liver (10), myocardium (64) and mesenteric artery (44) uterus (17) and brain (5). Other studies have reported only a single uniform class of ANG II receptors (28,29,45,54). These discrepancies might be due to methodological differences in radio-receptor assays or in the analysis of binding data. Nevertheless, it appears that the ANG II agonist-specific high affinity receptor complex includes a guanine nucleotide-binding protein which is involved in the regulation of receptor affinity and possibly of its function. In adenylate cyclase coupled systems such as the β-adrenergic receptor, high and low affinity sites sensitive to GTP have been previously characterized (35). Interestingly, the ability of β-adrenergic agonists to activate adenylate cyclase (intrinsic activity) correlated closely with the amount of high affinity state formed in the presence of the agonist (35). There is an apparent impairment of high affinity state formation in membranes derived from "desensitized" cells which may be responsible

for the decreased activation of adenylate cyclase observed after desensitization (35).

Extrapolating these findings from the β-adrenergic receptor to the present study, it might be speculated that estrogens interact with a guanine nucleotide regulatory protein which may be involved in the enhanced pituitary cellular response to exogenous ANG II. This hypothesis suggests that following estrogen treatment less receptors are available for stimulation however the "transduction signal"following ANG II binding to the receptor is enhanced. Further studies will be needed to determine whether or not different affinity states of the ANG II receptor exist in the anterior pituitary gland. This could be followed by a study on the effect of estrogen treatment on these presumed different affinity states of pituitary ANG II receptors which might further substantiate this hypothesis.

iii) Endogenous renin-angiotensin system

In view of the increasing evidence suggesting the existence of local endogenous ANG II producing systems in several target tissues, one might also speculate that estrogens could alter the rate of production of ANG II and that this locally produced ANG II would act as a paracrine hormone to stimulate and eventually to desensitize and down-regulate the nearby ANG II receptor. In the anterior pituitary gland, all elements supporting such a concept are present. Estrogen receptors, renin substrate, renin, ANG I, converting enzyme and ANG II are all present in the anterior pituitary (chapter 1, table I). More precisely, estrogen receptors (34) and native ANG II (16) are found in gonadotrophs and ANG II receptors are situated in the nearby lactotrophs. Medium from gonadotroph-rich cell aggregates has been

shown to stimulate prolactin release (15). This effect was not due to FSH or LH (15) but might be due to gonadotroph-derived ANG II. Although this hypothesis could explain how locally produced ANG II could cause down-regulation of lactotroph ANG II receptors, the absence of desensitization of lactotrophs to ANG II following estrogen treatment remains unexplained. Perhaps another intervening factor is involved in estrogen sensitization of lactotrophs to ANG II. In this respect gonadotrophs have been shown to potentiate the prolactin response to ANG II when co-cultured with lactotrophs (14) suggesting a yet undefined priming effect of gonadotrophs on lactotroph sensitivity. Another possibility for lack of correspondence between enhanced prolactin responsiveness to ANG II and estrogen-induced down-regulation of pituitary ANG II receptors might signify that the lactotroph cell is not the proper target cell for the observed estrogen effect. This possibility would seem unlikely however as previously discussed in chapter 4.

D. EFFECTS OF ESTROGEN ON OTHER ANTERIOR PITUITARY PEPTIDE HORMONE RECEPTORS.

Analogous to the present study, 17β-estradiol treatment in vivo has been shown to produce dissociated changes of pituitary luteinizing hormone releasing hormone (LHRH) receptors and responsiveness to LHRH (22). In this instance a single injection of 17β-estradiol to ovariectomized rats caused a biphasic effect on the LH response to LHRH (from hypo- to hyperresponsiveness without any change in [^{125}I] LHRH binding. Moreover, the self-priming effect of LHRH (10-fold increase in the LH response to a second injection of LHRH) was accompanied by a 40% loss of pituitary LHRH receptors (22). In anterior pituitary cell

culture, the stimulatory effect of estrogens on LH responsiveness to LHRH appeared to be due to changes in the sensitivity of the release mechanisms in gonadotrophs, since total LH content (release plus cell content) was not affected by 17β-estradiol treatment (19). Therefore these findings on the modulation of gonadotropin secretion also suggest that post-receptor events play a predominant role in the control of pituitary hormone secretion by estrogens. On the other hand, direct correlations between estrogen-induced effects on receptor density and cell responsiveness have also been reported in the anterior pituitary. Estradiol treatment of rats for 2 days has been shown to increase TRH receptor density and is accompanied by enhanced sensitivity of the TSH response to TRH as well as a larger prolactin response to TRH (12). This further emphasizes the complexity of the effect of estrogens on different receptor systems and precludes a common mechanism of action for this steroid.

E. EFFECTS OF OTHER SEX STEROIDS ON ANGIOTENSIN II RECEPTORS

Sex steroids other than estrogens have also been shown to produce marked effects on several different hormone systems. In vivo studies have shown that estrogen treatment increases uterine α-adrenergic and oxytocin receptors whereas progesterone appears to have an opposite effect (48,61). Progesterone has also been shown to block the transient estradiol-induced increase in uterine ANG II receptor density (54). Using collagenase-dispersed adrenal glomerulosa cells, it was also shown that androgens (testosterone hemisuccinate) increased the aldosterone response to ANG II and displayed rapid increases within minutes in whole cell ANG II binding that was not seen when the steroid was present during the binding assay (7). In fact when testosterone

was added in medium during binding determinations with the dispersed cells, binding of ANG II was inhibited rather than enhanced. This apparent allosteric effect of androgen steroids on adrenal ANG II receptors was not observed in this thesis when studying the effects of estrogen (chapter 3,8). This finding may be peculiar to the use of testosterone or to the study of ANG II binding in intact cells.

This thesis therefore suggests that the effect of estrogens on ANG II receptors may differ depending upon the target tissue examined. This may imply that there are important differences between ANG II receptors in different target tissues. Although the nature of these differences is still unknown, one possible locus may involve the post-receptor transduction system (possibly a nucleotide-binding regulatory protein). How estrogens might interfere with these transduction systems and how estrogens affect receptor turnover awaits further investigation using cell biology techniques.

The present study might contribute to a new field of investigation on the mechanism of action of estrogen on ANG II receptors. An increasing number of studies indicate that steroid hormones modulate peptide and protein hormone receptors. In addition to its classical anabolic mechanism of action, estrogen might also have other effects as suggested by estradiol-induced modulation of ANG II receptors.

F. REFERENCES TO CHAPTER 5

1. AGUILERA G, SHIRAR A, BAUKAL A, CATT KJ: Angiotensin II receptors. Properties and regulation in adrenal glomerulosa cells. Circ. Res. 1980, 46 (Suppl. 2):I-118-I-125.
2. ANAND-SRIVASTAVA MB: Angiotensin II receptors negatively coupled to adenylate cyclase in rat aorta. Biochem. Biophys. Res. Comm. 1983, 117:420-428.
3. ANDOKA G, CHAUVIN MA, MARIE J, SAEZ JM, MORERA AM: Adrenocorticotropin regulates angiotensin II receptors in bovine adrenal cells in vitro. Biochem. Biophys. Res. Comm. 1984, 121:441-447.
4. BENAS A: Serum proteins in rats treated with large doses of stilboestrol. J. Endocrinol. 1959, 19:203-206.
5. BENNETT JP Jr, SNYDER SH: Receptor binding interactions of the angiotensin II antagonist, ^{125}I-[Sarcosine^{1},leucine^{8}] angiotensin II, with mammalian brain and peripheral tissues. Eur. J. Pharmacol. 1980, 67:11-25.
6. BOCCKINO SB, BLACKMORE PF, EXTON JH: Stimulation of 1,2-diacylglycerol accumulation in hepatocytes by vasopressin epinephrine and angiotensin II. J. Biol. Chem. 1985, 260:14201-14207.
7. CARROLL JE, GOODFRIEND TL: Androgen modulation of adrenal angiotensin receptors. Science 1984, 224:1009-1011.
8. CHEN FM, PRINTZ MP: Chronic estrogen treatment reduces angiotensin II receptors in the anterior pituitary. Endocrinology 1983, 113:1503-1510.

9. COYNE MD, KITAY J: Effect of ovariectomy on pituitary secretion of ACTH. Endocrinology 1969, 85:1097-1102.

10. CRANE JK, CAMPANILE CP, GARRISON JC: The hepatic angiotensin II receptor. II. Effect of guanine nucleotides and interaction with cyclic AMP production. J. Biol. Chem. 1982, 257:4959-4965.

11. D'AURIAC AG, BAUDOIN M, MEYER P: Mechanism of action of angiotensin II in the smooth muscle cell. Circ. Res. 1972, 30 (Suppl.II):II-151-II-157.

12. DE LEAN A, LABRIE F: Sensitizing effect of treatment with estrogens on TSH response to TRH in male rats. Am. J. Physiol. 1977, 233:E235-E239.

13. DE LEAN A, ONG H, GUTKOWSKA J, SCHILLER PW, McNICOLL N: Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. Mol. Pharmacol. 1984, 26:498-508.

14. DENEF C: Paracrine interaction in anterior pituitary. Abstract # S93, 7th International congress of Endocrinology 1984, p. 107.

15. DENEF C, ANDRIES M: Evidence for paracrine interaction between gonadotrophs and lactotrophs in pituitary cell aggregates. Endocrinology 1983, 112:813-822.

16. DESCHEPPER CF, SEIDLER CD, STEELE MK, GANONG WF: Further studies on the localization of angiotensin II-like immunoreactivity in the anterior pituitary gland of the male rat, comparing various antisera to pituitary hormones and their specificity. Neuroendocrinology 1985, 40:471-475.

17. DEVYNCK MA, ROUZAIRE-DUBOIS B, CHEVILLOTTE E, MEYER P: Variations in the number of uterine angiotensin receptors following changes in plasma angiotensin levels. Eur. J. Pharmacol. 1976, 40:27-37.

18. DOUGLAS JG, BROWN GP: Effect of prolonged low dose infusion of angiotensin II and aldosterone on rat smooth muscle and adrenal angiotensin II receptors. Endocrinology 1982, 111:988-992.

19. DROUIN J, LABRIE F: Interactions between 17β-estradiol and progesterone in the control of luteinizing hormone and follicle-stimulating hormone release in rat anterior pituitary cells in culture. Endocrinology 1981, 108:52-57.

20. FAKUNDING JL, CATT KJ: Calcium-dependent regulation of aldosterone production in isolated adrenal glomerulosa cells: effects of the ionophore A-23187. Endocrinology 1982, 110:2006-2010.

21. FAKUNDING JL, CHOW R, CATT KJ: The role of calcium in the stimulation of aldosterone production by adrenocorticotropin, angiotensin II, and potassium in isolated glomerulosa cells. Endocrinology 1979, 105:327-333.

22. FERLAND L, MARCHETTI B, SEGUIN C, LEFEBVRE FA, REEVES JJ, LABRIE F: Dissociated changes of pituitary luteinizing hormone-releasing hormone (LHRH) receptors and responsiveness to the neurohormone induced by 17β-estradiol and LHRH in vivo in the rat. Endocrinology 1981, 109:87-95.

23. FONZO D, MIMS RB, NELSON DH: Estrogen influence on adrenal and pituitary function in the rats. Endocrinology 1967, 81:29-33.

24. FONZO D, NELSON DH: In vitro influence of oestradiol on succinate-supported steroid 11β-hydroxylation by rat adrenal homogenates. Acta Endocrinol. 1970, 64:59-64.

25. FREGLY MJ, ROWLAND NE, SUMNERS C, GORDON DR: Reduced dipsogenic responsiveness to intracerebroventricularly administered angiotensin II in estrogen-treated rats. Brain Res. 1985, 338:115-121.

26. GIGUERE V, MEUNIER H, VEILLEUX R, LARRIE F: Direct effects of sex steroids on prolactin release at the anterior pituitary level: interactions with dopamine, thyrotropin-releasing hormone, and isobutylmethylxanthine. Endocrinology 1982, 111:857-862.

27. GLOSSMANN H, BAUKAL A, CATT KJ: Angiotensin II receptors in bovine adrenal cortex: modification of angiotensin II binding by guanyl nucleotides. Communication J. Biol. Chem. 1974, 249:664-666.

28. GUNTHER S, GIMBRONE MA Jr, ALEXANDER RW: Identification and characterization of the high affinity vascular angiotensin II receptor in rat mesenteric artery. Circ. Res. 1980, 47:278-286.

29. GURCHINOFF S, KHAIRALLAH PA, DEVYNCK MA, MEYER P: Angiotensin II binding to zona glomerulosa cells from rabbit adrenal glands. Biochem. Pharmacol. 1976, 25:1031-1034.

30. HADJIAN AJ, CULTY M, CHAMBAZ EM: Stimulation of phosphatidylinositol turnover by acetylcholine, angiotensin II and ACTH in bovine adrenal fasciculata cells. Biochem. Biophys. Acta 1984, 804:427-433.

31. IAMS SG, WEXLER BC: Inhibition of the development of spontaneous hypertension in SH rats by gonadectomy or estradiol. J. Lab. Clin. Med. 1979, 94:608-616.

32. JARD S, CANTAU B, JAKOBS KH: Angiotensin II and α-adrenergic agonists inhibit rat liver adenylate cyclase. J. Biol. Chem. 1981, 256:2603-2606.

33. JONKLAAS J, BUGGY J: Angiotensin-estrogen control interaction: localization and mechanism. Brain Res. 1985, 326:239-249.

34. KEEFER DA, STUMPF WE, PETRUZ P: Quantitative autoradiographic assessment of ^{3}H-estradiol uptake in immunocytochemically characterized pituitary cells. Cell Tiss. Res. 1976, 166:25-35.

35. KENT RS, DE LEAN A, LEFKOWITZ RJ: A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. Mol. Pharmacol. 1979, 17:14-23.

36. KEPPENS S, VENDENHEEDE JR, DE WULF H: On the role of calcium as a second messenger in liver for the hormonally induced activation of glycogen phosphorylase. Biochim. Biophys. Acta 1977, 496:448-457.

37. KIRCHICK HJ, BIRNBAUMER L: Effects of estradiol treatment on rabbit luteal adenylyl cyclase: loss of luteinizing hormone receptors and attenuation of the regulatory N component activity. Endocrinology 1983, 113:1629-1637.

38. KITAY JI: Pituitary-adrenal function in the rat after gonadectomy and gonadal hormone replacement. Endocrinology 1963, 73:253-260.

39. KITAY JI, COYNE MD, SWYGERT NH: Influence of gonadectomy and replacement with estradiol or testosterone on formation of 5 α-reduced metabolites of corticosterone by the adrenal gland of the rat. Endocrinology 1970, 87:1257-1265.

40. MALENDOWICZ LK, JACHIMOWICZ B: Sex differences in adrenocortical structure and function. XI. Autoradiographic studies on cell proliferation and turnover in the adrenal cortex of the male and female rat and its dependence on testosterone and estradiol. Cell Tiss. Res. 1982, 227:651-657.

41. MARIE J, GAILLARD RC, SCHOENENBERG P, JARD S, ROCKAERT J: Pharmacological characterization of the angiotensin receptor negatively coupled with adenylate cyclase in rat anterior pituitary gland. Endocrinology 1985, 116:1044-1050.

42. MARIE J, JARD S: Angiotensin II inhibits adenylate cyclase from adrenal cortex glomerulosa zone. FEBS Lett. 1983, 159:97-101.

43. MARTIN PM, COCHET C, ROLLAND PH, CHAMBAZ EM: Demonstration of high affinity binding of estradiol in adrenal cortex tissue. C.r. hebd. Séanc. Acad. Sci. Paris 1977, 284:1305-1307.

44. McQUEEN J, MURRAY GD, SEMPLE PF: Identification of the angiotensin II receptor in rat mesenteric artery. Biochem. J. 1984, 223:659-671.

45. MUKHERJEE A, KULKARNI P, McCANN SM, NEGRO-VILLAR A: Evidence for the presence and characterization of angiotensin II receptors in rat anterior pituitary membranes. Endocrinology 1982, 110:665-667.

46. NASJLETTI A, MATSUNAGA M, MASSON GMC: Effects of estrogens on pressor responses to angiotensin and renin. Proc. Soc. Exp. Biol. Med. 1970, 133:407-409.

47. NICKERSON PA: Fat accumulation in the adrenal gland: effect of estradiol benzoate on the zona reticularis of the Mongolian gerbil adrenal gland. Path. Eur. 1975, 10:287-297.

48. NISSENSON R, FLOURET G, HECHTER O: Opposing effects of estradiol and progesterone on oxytocin receptors in rabbit uterus. Proc. Natl. Acad. Sci. 1978, 75:2044-2048.

49. PAPPRITZ G, KEAZOR H, UEBERBERG H: Estrous cycle-controlled cell proliferation in the adrenal cortex of female rats. Cell Tiss. Res. 1977, 184:269-279.

50. RAYMOND V, BEAULIEU M, LARRIF F, BOISSIER JR: Potent antidopaminergic activity of estradiol at the pituitary level on prolactin release. Science 1978, 200:1173-1175.

51. ROSENFELD CR, JACKSON FM: Estrogen-induced refractoriness to the pressor effects of infused angiotensin II. Am. J. Obstet. Gynecol. 1984, 148:429-435.

52. RUHMANN-WENNHOLD A, LAUPO R, NELSON DH: An acute effect of estradiol on steroid hydroxylation by the rat adrenal. Endocrinology 1970, 86:175-179.

53. SCHIFFRIN EL, GUTKOWSKA J, GENEST J: Effect of angiotensin II and deoxycorticosterone infusion on vascular angiotensin II receptors in rats. Am. J. Physiol. 1984, 246:H608-H614.

54. SHIRAR A, CAPPONI A, CATT KJ: Regulation of uterine angiotensin II receptors by estrogen and progesterone. Endocrinology 1980, 106:5-12.

55. SKOOG KM, KENNEY NJ: Pressor responses to central angiotensin II, prostaglandin E_1 and prostaglandin E_2. Effects of ovarian steroids. Neuroendocrinology 1983, 36:144-151.

56. STADTLER FAW, BLACKERT KWA: The effect of cyproterone and estradiol benzoate on the adrenal gland of juvenile and adult rats. Path. Res. Pract. 1982, 173:325-333.

57. STORMSHAK F, LEAKE R, WERTZ N, GORSKI J: Stimulatory and inhibitory effects of estrogens on uterine DNA synthesis. Endocrinology 1976, 99:1501-1511.

58. TAMAI T, MATSUURA S, TATSUMI N, NUMOTANI T, SAGAWA N: Role of sex steroid hormones in relative refractoriness to angiotensin II during pregnancy. Am. J. Obstet. Gynec. 1984, 149:177-183.

59. TOLONE G, BONASERA L, SAJEVA R: Hydrocortisone increases the responsiveness of mast cells to beta-adrenergic agonists by an action distal to the beta-adrenoreceptors. Br. J. Exp. Pathol. 1979, 60:269-275.

60. UEBERGERG H, MUFF H-G, TRIEB G: Autoradiographic investigation of cell proliferation in the adrenal cortex of castrated female rats under the influence of oestradiol. Experientia 1984, 40: 1384-1385.

61. WILLIAMS LT, LEFKOWITZ RJ: Regulation of rabbit myometrial alpha adrenergic receptors by estrogen and progesterone. J. Clin. Invest. 1977, 60:815-818.

62. WOODCOCK EA, JOHNSTON CI: Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. Endocrinology 1982, 111:1687-1691.

63. WRIGHT GB, ALEXANDER RW, EKSTEIN LS, GIMBRONE MA: Sodium, divalent cations, and guanine nucleotides regulate the affinity of the rat mesenteric artery angiotensin II receptor. Circ. Res. 1982, 50:467-467.

64. WRIGHT GB, ALEXANDER RW, EKSTEIN LS, GIMBRONE MA Jr: Characterization of the rabbit ventricular myocardial receptor for angiotensin II. Evidence for two sites of different affinities and specificities. Mol. Pharmacol. 1983, 24:213-221.

65. ZAKI K, RIZK M, KIRA L, NOUR H, GUIRGUIS R: Studies on the effects of ethinyl estradiol and norethisterone acetate on the adrenal cortex and some other tissues in the rat. Endokrinologie 1979, 73:66-76.

CLAIMS TO ORIGINALITY

1) A novel approach in this thesis was the use of the radiolabeled angiotensin II antagonist: [^{125}I]SARILE to quantify angiotensin II receptors in several target tissues. Validation of the use of angiotensin II antagonists to quantify adrenal angiotensin II receptors has thus been extended to the anterior pituitary gland and mesenteric artery.

2) This thesis demonstrates for the first time that estrogen treatment modulates angiotensin II receptors differently depending upon the target tissue examined:

a) <u>In the adrenal cortex</u>:

Chronic estradiol treatment has been shown to suppress angiotensin II-mediated aldosterone release. As shown in chapter 3 this effect might be mediated by estrogen-induced down-regulation of angiotensin II receptor density in the adrenal cortex.

b) <u>In the mesenteric artery</u>:

Several studies report that estrogens induce refractoriness to the pressor effects of angiotensin II. This is the first study that attempts to correlate these findings with alteration of angiotensin II receptors in vascular arterial smooth muscle cells. From this thesis it would appear that mesenteric artery angiotensin II receptors are not affected by chronic estradiol treatment.

c) In the anterior pituitary gland:

This is the first report that chronic estrogen treatment for 7, 14 or 28 days decreases angiotensin II receptor density in the anterior pituitary gland. Furthermore, in this tissue, dissociated changes between pituitary angiotensin II receptor number and target cell responsiveness to angiotensin II have been demonstrated both *in vivo* and in cultured anterior pituitary cells following estradiol treatment. Results from the anterior pituitary gland also caution against the assumption that receptor down-regulation necessarily leads to attenuation in hormone physiological response.

3) This thesis also shows for the first time that estrogen acts directly on anterior pituitary cells (within 48 hours) to decrease angiotensin II receptor density. This new finding opens up a new field of investigation on the mechanism of action of estrogens on angiotensin II receptors and how target cell responsiveness is affected by this steroid.

APPENDIX I

In vivo [^{125}I]ANG II and [^{125}I]SARILE uptake studies

In these uptake studies, variable doses of [^{125}I]ANG II or [^{125}I]SARILE (ANG II antagonist) were injected as a bolus into pentobarbital-anesthetized female rats and allowed to circulate for a certain period of time before intracardiac perfusion with saline and *in toto* fixation with glutaraldehyde (see morphological technique in chapter 4). Selected target tissues for ANG II, namely the adrenal glands, thoracic aorta, mesenteric artery, atria and ventricles from the heart, median eminence/hypothalamus, and the whole pituitary gland were removed after fixation, weighed, and wet tissue radioactivity was measured with a gamma counter (total uptake). Specific uptake was determined using separate animals injected with a large excess of different unlabeled ANG II antagonists administered simultaneously or prior to the injection of [^{125}I] ANG II or [^{125}I]SARILE. Specific uptake was calculated as the percent of inhibition of radioactivity counts for each target tissue in "cold hormone-injected" animals compared with animals injected only with the radioligand. Variations in the dose, route of injection and circulation time of the radioligand and unlabeled peptide were tested as shown in tables I and II. Table I summarizes experiments using [^{125}I] ANG II and studies using [^{125}I]SARILE are shown in table II.

TABLE I IN VIVO [^{125}I]ANG II UPTAKE STUDIES IN INTACT CYCLING FEMALE RATS

Total uptake		Uptake inhibition		
[^{125}I]ANG II(a) bolus dose	Time in circulation (minutes)	Unlabeled ANG II antagonist dose (1) or (2)	Time in circulation of unlabeled ANG II antagonist before [^{125}I] ANG II injection (minutes)	Time in circulation of [^{125}I]ANG II + ANG II antagonist together (minutes)
0.09 nmol*	2' i.c. desc. bolus (b)	5 nmol	30' i.j. infusion (d)	2'
0.09 nmol*	2' i.c. desc. bolus (b)	50 nmol	30' i.j. infusion (d)	2'
0.18 nmol	2' i.j. bolus (c)	50 nmol	30' i.j. infusion (d)	2'
0.04 nmol	2' i.j. bolus (c)	120 nmol	30' i.j. infusion (d)	2'
0.18 nmol	5' i.j. bolus (c)	50 nmol	30' i.j. infusion (d)	5'

(1) Sar^1Ile^8 (SARILE)
(2) Sar^1Ala^8 (Saralazin)
(a) [^{125}I] ANG II: 300-500 Ci/mmol
(b) i.c. desc. bolus: 5 second bolus injection (0.2-0.5 ml) into the right carotid artery ligated proximaly and injected at counter current towards the heart.
(c) i.j. bolus: 5 second bolus injection (0.2-0.5 ml) through the non-ligated right jugular vein.
(d) i.j. infusion: left jugular vein infusion (1 ml) through PE 50 polyethylene tubing inserted into the vein near its junction with the left atrium.
*: 40 g female rat (all other studies were performed with 200 g rats).

TABLE II IN VIVO [^{125}I]SARILE UPTAKE STUDIES IN INTACT CYCLING FEMALE RATS

Total uptake		Uptake inhibition		
[^{125}I]SARILE(a) us dose	Time in circulation (minutes)	Unlabeled SARILE dose	Time in circulation of unlabeled SARILE before [^{125}I]SARILE injection (minutes)	Time in circulation of [^{125}]SARILE + SARILE together (minutes)
18 nmol	2' (i.j. bolus) (c)	80 nmol	-	2'
18 nmol	2' (i.j. bolus) (c)	80 nmol	30' i.j. infusion (d)	2'
04 nmol	2' (i.j. bolus) (c)	300 nmol	-	2'
04 nmol	2' (i.j. bolus) (c)	300 nmol	30' i.j. infusion (d)	2'
03 nmol	2' (i.j. bolus) (c)	300 nmol	2' i.j. infusion (d)	2'
.01 nmol	2' (i.c. asc. bolus) (b)	130 nmol	-	2'
.06 nmol	2' (i.c. asc. bolus) (b)	130 nmol	-	2'
.04 nmol	30' (i.j. bolus) (c)	300 nmol	2' i.j. infusion (d)	30'
.02 nmol	30' (i.c. asc. bolus) (d)	10 nmol	2' i.j. infusion (d)	30'
.02 nmol	30' (i.c. asc. bolus) (d)	160 nmol	2' i.j. infusion (d)	30'
.02 nmol	30' (i.c. asc. bolus) (d)	300 nmol	2' i.j. infusion (d)	30'

(a) [^{125}I]SARILE: 300-500 Ci/mmol
(b) i.c. asc. bolus: 5 second bolus injection (0.2-0.5 ml) into the right carotid artery ligated distally and injected towards the brain.
(c) i.j. bolus: 5 second bolus injection (0.2-0.5 ml) through the non-ligated right jugular vein.
(d) i.j. infusion: left jugular vein infusion (1 ml) through PE 50 polyethylene tubing inserted into the vein near its junction with the left atrium

APPENDIX II

Effect of 7-day estradiol treatment on the systemic pressor response of ovariectomized rats to infused ANG II

Objectives:

1. To determine whether chronic estrogen treatment affects the pressor response to infused ANG II.
2. To correlate these findings with the absence of an effect of chronic estradiol treatment on mesenteric artery ANG II receptor density.

Experimental protocol:

Eight ovariectomized (OVX) rats and 8 OVX-estradiol-treated rats were treated and used as described in chapter 3 for the *in vivo* aldosterone release study. On day-7 control and treated rats were paired, anesthetized sequentially with ether and cannulated one group at a time (4 groups of 2 controls and 2 treated). The right jugular vein and carotid artery were cannulated distally, rinsed with 5% dextrose-containing 100 U/ml of heparin and the two plastic PE-50 tubes were passed under the skin and brought out at the scruff of the neck. Rats were allowed 2 hours to recover from anesthesia before starting the experiment. Using one group at a time, the carotid artery of each rat was connected to a pressure transducer (Bell and Howell) and recorded on a Devices Polygraph. The jugular veins of one control-OVX rat and one control OVX-estradiol treated rat were connected to syringes containing 5% dextrose. The jugular veins of the remaining OVX-rat and OVX-estradiol treated rats were connected to syringes

containing ANG II diluted in 5% dextrose. After 10 minutes of arterial pressure stabilization, infusions of ANG II (40 pmol/10 µl/ minute/rat) and 5% dextrose (10 µl/minute/rat) with a Sage pump (model 220-1, Sage Instruments, White Plains, N.Y.) were started and carotid artery blood pressure readings were recorded for 30 minutes. Data were analyzed by two-tailed paired and unpaired Student's t test.

Results

Basal arterial pressure readings were not significantly different between OVX-rats (89 ± 2 mmHg) and OVX-estradiol treated rats (92 ± 2 mmHg). Following ANG II infusion, pressure readings in both OVX rats and OVX-estradiol treated rats began to rise after approximately 4 minutes from the beginning of the infusion and reached a plateau after approximately 6 minutes. When compared to pre-infusion basal values, ANG II infusion significantly increased blood pressure in both OVX-rats and OVX-estradiol treated rats ($p < 0.01$ paired t-test). Blood pressure was not affected by infusion of 5% dextrose vehicle into OVX-rats or OVX-estradiol rats (Figure 1). Pressure increments (pressure reading at 6 minutes minus basal) were not significantly different between OVX rats (Δ 31 mmHg, n=4) and OVX-estradiol treated rats (Δ 35 mmHg, n=4) ($p > 0.05$ unpaired Student's t-test).

Discussion

Interpretations of this study are limited by the small number of experimental groups (n=4). Chronic estradiol treatment of OVX-rats for 7 days does not appear to induce refractoriness to the pressor effects of a physiological infusion dose of ANG II. In fact, it appeared as if estrogen treatment might enhance the pressor response to ANG II (Δ 35 mmHg in treated animals versus Δ 31 mmHg in controls). However, this

difference was not found to be statistically significant. These findings tend to agree with results described in chapter 3 where estrogen treatment was shown not to affect ANG II receptor density in the mesenteric artery.

A. OVX + Vehicle

B. OVX + ANG II

C. OVX + Estradiol + Vehicle

D. OVX + Estradiol + ANG II

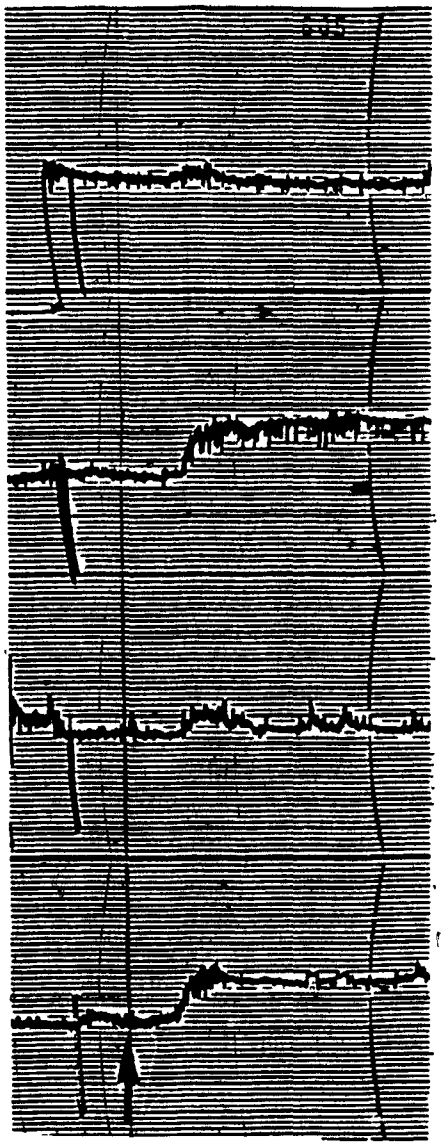


Figure 1

Carotid artery blood pressure recordings following the intravenous infusion of 5% dextrose vehicle or ANG II (40 pmol/10 µl/minute/rat). Vehicle infusion into ovariectomized (OVX)-only (A) or OVX-estradiol-treated rats (C) did not affect blood pressure recordings. Angiotensin II infusion produced a similar rapid sustained increase in blood pressure in the OVX-only rat (B) (from 85 to 125 mmHg) as well as in the OVX-estradiol treated rat (D) (from 80 to 120 mmHg). (On the abscissa one square represents 2 minutes and on the ordinate one square represents 25 mmHg). Arrow indicates the start of ANG II infusion.