THE INTERACTION BETWEEN INCREASED ESTROGEN AND INCREASED CORTISOL IS NECESSARY FOR NORMAL BLOOD PRESSURE CONTROL IN LATE GESTATION

By

FENG LI

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2003
This dissertation is dedicated to my parents and husband.
ACKNOWLEDGMENTS

I would like to thank my mentors (Dr. Maureen Keller-Wood and Dr. Charles E. Wood) for making the happiest and most productive years of my scientific career, for introducing me to the world of physiology, for immeasurable guidance and dedication to my education, and for the intellectual and scientific training I received in their laboratories (which provides me with a solid base of scientific skills). Memories of these years will remain with me for a lifetime.

I would also like to thank Dr. Michael Katovich, Dr. Michael Meldrum and Dr. Williams Millard, for serving as members of my committee, and for their helpful advice and academic support throughout this project and other doctoral activities.

I would like to give special thanks to Dr. Elaine M. Sumners, for teaching me everything patiently and for being an elegant role model. I thank Dr. Joanna Peris for believing in me when even I did not believe in myself. My deep appreciation goes to Dr. Daying Zhang and Mrs. Xiaoying Fang. They have been dear friends whom I could always count on.

I would like to thank my fellow graduate students (Amanda, Caren, Christine, Ellen, Everlyn, Jason, Jian, Justin, Kelly, Melanie, Scott, Sharon Toni, Xiaofei, and Yi) for their support and help. I would like to thank Anastasia, Donna, Paulette for their friendship and kindness.

I thank Dr. Dawson for his support during my first half year in the Ph.D program, although he could not see this dissertation.
Finally, heartful thanks go to my parents, sister and brother and husband, I could not have finished this dissertation without their love.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>General Background and Significance</td>
<td>1</td>
</tr>
<tr>
<td>Addison’s Disease</td>
<td>1</td>
</tr>
<tr>
<td>Estrogen Effects on Blood Pressure via Interaction with Nitric Oxide (NO)</td>
<td>2</td>
</tr>
<tr>
<td>Effects of Corticosteroids on Blood Pressure and NO</td>
<td>4</td>
</tr>
<tr>
<td>Significance</td>
<td>6</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>7</td>
</tr>
<tr>
<td>Aim I</td>
<td>7</td>
</tr>
<tr>
<td>Aim II</td>
<td>7</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>8</td>
</tr>
<tr>
<td>Steroid Hormones</td>
<td>8</td>
</tr>
<tr>
<td>Estrogens and Effects</td>
<td>8</td>
</tr>
<tr>
<td>Estrogen Receptors</td>
<td>9</td>
</tr>
<tr>
<td>Effects of Estrogen</td>
<td>9</td>
</tr>
<tr>
<td>The Effects of Estrogen on Cardiovascular System</td>
<td>10</td>
</tr>
<tr>
<td>Glucocorticoid Receptor (GR) and Mineralocorticoid Receptor (MR)</td>
<td>18</td>
</tr>
<tr>
<td>Mineralocorticoids and Effects</td>
<td>20</td>
</tr>
<tr>
<td>Effects of Aldosterone</td>
<td>21</td>
</tr>
<tr>
<td>Renal Effects of Aldosterone</td>
<td>21</td>
</tr>
<tr>
<td>Vascular Effects of Mineralocorticoids</td>
<td>23</td>
</tr>
<tr>
<td>Glucocorticoids and Effects</td>
<td>24</td>
</tr>
<tr>
<td>Renal Effects of Glucocorticoids</td>
<td>24</td>
</tr>
<tr>
<td>Other Effects of Glucocorticoids</td>
<td>27</td>
</tr>
</tbody>
</table>
EFFECTS OF ESTROGEN ON EXPRESSION OF Nitric oxide synthase isoforms IN KIDNEY AND VASCULATURE IN HYPOADRENocorticism

Introduction
Materials and Methods
Results
  Expression of eNOS mRNA and Protein by Endothelial Cells
  Expression of iNOS and nNOS mRNA and Protein in Whole Blood Vessels
Discussion

SUMMARY AND CONCLUSIONS
LIST OF REFERENCES
BIOGRAPHICAL SKETCH
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1</td>
<td>Plasma cortisol, aldosterone, estradiol, electrolyte, protein concentration and packed cell volume at 18-20 hours after withdrawal of corticosteroids</td>
</tr>
<tr>
<td>5-1</td>
<td>Plasma cortisol, aldosterone, estradiol, protein concentration and packed cell volume 28 hours after withdrawal of corticosteroids</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1</td>
<td>The experimental design.</td>
</tr>
<tr>
<td>4-2</td>
<td>MAP at 18-20 hours after corticosteroid withdrawal.</td>
</tr>
<tr>
<td>4-3</td>
<td>The change in MAP after corticosteroid withdrawal.</td>
</tr>
<tr>
<td>4-4</td>
<td>MAP before and after L-NAME.</td>
</tr>
<tr>
<td>4-5</td>
<td>The change in MAP after L-NAME.</td>
</tr>
<tr>
<td>4-6</td>
<td>Pressor responsiveness.</td>
</tr>
<tr>
<td>4-7</td>
<td>Plasma NOx level.</td>
</tr>
<tr>
<td>5-2</td>
<td>MAP after corticosteroids withdrawal.</td>
</tr>
<tr>
<td>5-3</td>
<td>Mean arterial blood pressure over 3 minutes in each ewe.</td>
</tr>
<tr>
<td>5-4</td>
<td>The change in plasma potassium concentration after corticosteroids withdrawal.</td>
</tr>
<tr>
<td>5-5</td>
<td>The change in plasma sodium concentration after corticosteroids withdrawal.</td>
</tr>
<tr>
<td>5-6</td>
<td>Change in MAP after L-NAME.</td>
</tr>
<tr>
<td>5-7</td>
<td>Plasma NOx level.</td>
</tr>
<tr>
<td>5-8</td>
<td>Pressor responsiveness.</td>
</tr>
<tr>
<td>6-1</td>
<td>Study I Results: eNOS mRNA in endothelial cells from aorta (left) and uterine artery (UA, right).</td>
</tr>
<tr>
<td>6-2</td>
<td>Study I Results: eNOS protein in endothelial cells of aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA).</td>
</tr>
<tr>
<td>6-3</td>
<td>Study II Results: eNOS mRNA in aorta, uterine artery (UA) and mesenteric artery (MA).</td>
</tr>
<tr>
<td>6-4</td>
<td>Study II Results: eNOS protein in endothelial cells of aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA).</td>
</tr>
</tbody>
</table>
6-5 Study I Results: iNOS mRNA in aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA).

6-6 Study I Results: nNOS mRNA in aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA).

6-7 Study I Results: eNOS iNOS and nNOS mRNA in renal cortex.

6-8 Study I Results: eNOS iNOS and nNOS mRNA in renal medulla.

6-9 Study I Results: nNOS protein in renal cortex (RC) and renal medulla (RM).

6-10 Study II Results: eNOS mRNA in renal cortex (RC) and renal medulla (RM).

6-11 Study II Results: eNOS protein in renal cortex (RC) and renal medulla (RM).

6-12 Study I Results: nNOS protein in renal cortex (RC) and renal medulla (RM).
LIST OF ABBREVIATION

ADI:  Adrenal Gland Intact
ADX:  Adrenalectomized
ACTH: Adrenocorticotropic Hormone
ASDN: Aldosterone-Sensitive Distal Nephron
AVP:  Arginine Vasopressin
CIC:  Calcium Dependent Chloride Channel
CRH:  Corticotropin-Releasing Hormone
DOC:  Deoxycorticosterone
E2:   17β- estradiol
ENaC: Epithelial Sodium Channel
ER:   Estrogen Receptor
GFR:  Glomerular Filtration Rate
GR:   Glucocorticoid Receptor
11β-HSD: 11β- Hydroxysteroid Dehydrogenase
HR:   Heart Rate
L-NAME: L-nitro-N-Arginine Methyl Ester
MAP:  Mean Arterial Pressure
MP:   Methlprednisolone
MR:   Mineralocorticoid Receptor
NO:   Nitric Oxide
NOx:  Nitrate/Nitrite
eNOS: Endothelial Nitric Oxide Synthase
iNOS: Inducible Nitric Oxide Synthase
nNOS Neuronal Nitric Oxide Synthase
PE:   Phenylephrine
PKC:  Protin Kinase C
RBF:  Renal Blood Flow
SGK:  Serun- and Glucocorticoid- regulated Kinase (SGK)
The interaction between increased estrogen and increased cortisol is necessary for normal blood pressure control in late gestation

By

Feng Li

December 2003

Chair: Maureen Keller-Wood
Major Department: Pharmacodynamics

During pregnancy, plasma concentrations of cortisol and estrogen usually increase. In previous studies, we observed that hypotension resulting from hypoadrenocorticism occurs more quickly in late-gestation pregnant ewes, and is frequently fatal to both mother and fetus. This dissertation examines the effect of increased estrogen on the hypoadrenal state (Addison’s disease).

Increased estrogen concentration did not decrease mean arterial pressure (MAP) in ovariectomized ewes who were either adrenalectomized or adrenal gland intact. However, the adrenalectomized ewes showed cyclic change in MAP after 20 hours withdrawal of corticosteroids; this pattern did not occur in adrenalectomized ewes treated with estrogen. Estrogen treatment did not alter the vascular responsensness to phenylephrine either before or after infusion of the nitric oxide inhibitor L-NAME (L-nitro-N-arginine methyl ester) in adrenal intact or hypoadrenal ewes.
The abundance of mRNA and protein of nitric oxide synthase (NOS) was also examined by PCR and Western blot techniques. The mRNA of endothelial nitric oxide synthase (eNOS) was upregulated by estrogen and downregulated by adrenalectomy in endothelial cells of the aorta and uterine artery. The estrogen treatment increased eNOS protein levels in endothelial cells of aorta, renal, uterine, and mesenteric arteries; adrenalectomy had the opposite effect on eNOS protein. However, there was no significant difference in circulating NOx (nitrate/nitrite) among the groups of ewes after stopping infusion of corticosteroids for 18-20 hours. On the other hand, the estrogen-treated ewes had higher circulating NOx than adrenalectomized ewes after stopping infusion of corticosteroids for 28 hours in Study II.

The data from these studies refute the hypothesis that estradiol treatment results in a more precipitous decline in blood pressure and vascular reactivity. However, estradiol did protect the adrenalectomized ewes from the dramatic cyclic blood pressure changes occurring 24-28 hours after steroid withdrawal. In the estrogen-treated ewes, plasma potassium and sodium changes were also less dramatic, suggesting the mechanism underlying this effect of estrogen may be the effect of estrogen on renal ion channels.
CHAPTER 1
INTRODUCTION

General Background and Significance

Addison’s Disease

Primary adrenal insufficiency is termed Addison’s disease. Addison’s disease is a relatively rare disease and it is more common in women. Addison’s disease is most commonly caused by autoimmune disease resulting in antibodies being directed against the $P_{450}$ enzymes of the adrenal gland. When the antibodies are directed against $P_{450} \text{c17}$, 17-hydroxylase, gonadal steroid production is also reduced, generally resulting in infertility. However, many women with Addison’s disease can become pregnant if the disease is appropriately treated. The patients require long-term adrenal-gland hormone replacement therapy. This disease can be successfully managed during pregnancy, which appears to require increased glucocorticoid doses, particularly during labor and delivery [Albert and Dalaker, 1989].

On the other hand, untreated Addison’s disease causes orthostatic hypotension, hypovolemia, and hyperkalemia. The hyponatremia, hyperkalemia, and hypovolemia result from a lack of mineralocorticoid action at the kidney, allowing loss of sodium through urination, and a lack of glucocorticoid stimulation of cellular sodium/potassium ATPase activity, allowing a shift of potassium from the intracellular to the extracellular fluid compartments. Additionally, severe hypoadrenocorticism reduces or eliminates the responsiveness of vascular smooth muscle to the action of vasoconstrictor agents such as epinephrine or phenylephrine [Loriaux, 1990]. So, if Addison’s disease is present in late
gestation, the outcome can be disastrous. In two case reports (human), hypoadrenal hypotensive crisis in late gestation or in labor [Seaward et al., 1989; Simcock, 1996] resulted in maternal and/or fetal demise.

Therefore, although hypoadrenocortism in the pregnant subject is rare, the effect may be devastating. In our laboratory, we have also found that when the cortisol replacement dose is equivalent to that for nonpregnant ewes, the incidence of abortion and fetal death is greater in pregnant adrenalectomized ewes. Ewes appear to be particularly vulnerable at about 128-130 days gestation. Some ewes that aborted, or delivered at term, had abrupt symptoms of Addisonian’s crisis: three of these ewes died suddenly during delivery or immediately thereafter [Keller-Wood et al., 1998; Kell-Wood 1998].

**Estrogen Effects on Blood Pressure via Interaction with Nitric Oxide (NO)**

Blood pressure is known to be regulated at a lower set point during pregnancy [Humphreys and Joels, 1974; Kassab et al., 1998] and this may be a factor in increasing the vulnerability of pregnant sheep and humans to Addisonian crisis. Estradiol is thought to be the primary mediator of decreased mean arterial pressure and peripheral resistance during pregnancy [Longo 1983]. Since both the pregnant ewes and women appear to be the most vulnerable to Addisonian crisis very late in gestation (when the estrogen to progesterone ratio is greatest), the vascular effects of estrogen may be critical in producing the hypotensive crisis.

During ovine pregnancy, uterine vascular responses to ephedrine are decreased [Li et al., 1996] and uterine arterial production of NO and levels of cGMP are increased [Magness et al., 1993]. Infusion of estradiol decreases mean arterial pressure in nonpregnant ewes; lower doses do not decrease mean arterial pressure, but cause uterine
vasodilation [Magness et al., 1989]. In vivo, estradiol treatment in ewes enhances the in vitro vasodilator response to bradykinin and increases citrulline production in the uterine artery, but not the renal artery [Veille et al., 1996]. Estradiol treatment increases cGMP secretion into the uterine vasculature; and this effect is blocked by infusion of nitric oxide synthase (NOS) inhibitor L-nitro-N-arginine methyl ester (L-NAME) [Rosenfeld et al., 1996]. Estradiol also increases endothelial nitric oxide synthase abundance in the uterine artery of nonpregnant ewes, suggesting that the reduced blood pressure and decreased response to vasoconstrictors during pregnancy is caused by estrogen stimulation of NO generation in the uterine endothelium [Vagnoni et al., 1998]. In the rat, NO production in the aorta and in the kidney also appears to be induced during pregnancy or estradiol treatment. The infusion of L-NAME resulted in a great increase in mean arterial pressure in pregnant as compared to nonpregnant rats [Kasab et al., 1998; Natan et al., 1995]. During pregnancy in the rat, there is an increase in endothelial Nitric Oxide (eNOS) in the renal medulla; and an increase in renal excretion of cGMP and nitrates/nitrites [Conrad et al., 1993]. The eNOS is also increased in the rat aorta during pregnancy [Goetz et al., 1994]. Aortas from pregnant rats exhibit decreased constrictor responses to vasoconstrictors and increased vasodilator responses to acetylcholine; the relaxation is inhibited by inhibition of NOS by L-NAME [Honda et al., 1996]. The eNOS in the aorta and renal medulla is also increased during estradiol treatment of nonpregnant rats [Goetz et al., 1994; Neugarten et al., 1997]. In isolated aortic rings from estrogen-treated rats, the maximum contraction in response to several vasoconstrictors is reduced, and tone-dependent NO release is increased [Andersen et al., 1999].
These results suggest that during pregnancy in the rat, the decrease in blood pressure and response to vasoconstrictors is caused by estradiol stimulation of eNOS in the aorta and renal vasculature, as well as uterine vasculature. However in the ewe, estradiol may also act on other vascular endothelia. Estradiol also increases eNOS in omental and mammary arteries, although the time course of the effect is markedly slower than in uterine artery, requiring 10 days of estradiol infusion [Vagnoni et al., 1998].

**Effects of Corticosteroids on Blood Pressure and NO**

In humans, there is some evidence that cortisol may increase blood pressure by an inhibitory effect on NO generation. During infusion of 80 mg/day of cortisol (approximately twice the normal daily production rate) over 5 days, plasma nitrate/nitrite levels are reduced [Kelly et al., 1998]. The effect of adrenal corticosteroids on the production of nitric oxide has been studied primarily the context of the anti-inflammatory actions of cortisol. Glucocorticoids reduce inducible nitric oxide synthase (iNOS) production by macrophages [Di Rosa et al., 1990]. Glucocorticoids also inhibit endotoxin-stimulated, calcium-independent iNOS activity in the liver, lung, and aorta [Knowles et al., 1990; Rees et al., 1990]. It has been proposed that glucocorticoids inhibit iNOS, but not eNOS production in vascular endothelial cells [Radomski et al., 1990]. However, this is based on measures of calcium-dependent eNOS activity in cultured endothelial cells grown in 20% fetal calf serum, which contains corticosteroids. This design may artificially de-emphasize any effect of added corticosteroids on basal or constitutive activity. The glucocorticoid effect to inhibit iNOS production in vasculature in response to endotoxin stimulation is thought to mediate the recovery from endotoxin shock. In adrenalectomized rats, endotoxin produces a more severe hypotension than in adrenalectomized rats treated with the synthetic glucocorticoid, dexamethasone. The
iNOS activity in the lungs, although not altered by adrenalectomy per se, was increased in adrenalectomized rats after endotoxin as compared to endotoxin-treated control or adrenalectomized and dexamethasone-treated rats [Szabo et al., 1993]. Glucocorticoids have also been shown to inhibit constitutive, calcium-dependent neuronal nitric oxide synthase (nNOS) in hippocampal neurons [Lopez-Figueroa et al., 1998], and in neuroblastoma cells [Schwarz et al., 1995].

Adrenalectomy increases mRNA for nitric oxide synthase (nNOS), and dexamethasone treatment decreases nNOS mRNA. This form of NOS is also found in skeletal muscle and macular densa. Sex steroids also appear to influence nNOS in some tissues, including caruncular tissue in estrogen-treated nonpregnant ewes [Zhang et al., 1999]. The corticosteroid effect on NOS transcription appears to be mediated by response elements other than the glucocorticoid response element (GRE). In the case of glucocorticoid effects on iNOS, the effect appears to be via inhibition of NF-κB binding to DNA [Katsuyama et al., 1999]. The mechanisms of the effects on eNOS and nNOS have not been investigated. Estrogen effects on eNOS also do not appear to be mediated by ERE (estrogen response element), since no full ERE sites have been found in the 5′flanking region of the eNOS gene. However, consensus sequences for AP-1, S1, and NF-kB (all of which have been shown to be mediators of estrogen effects in other tissues) have been found in the 5′flanking region of the nNOS gene [Forstermann et al., 1998]. The NF-κB, activator protein-1 (AP-1), and Sp-1 sites have also been found in the 5′flanking region of the nNOS gene. Activated glucocorticoid receptors have been found to inhibit AP-1-dependent gene transcription and to decrease the activity of NF-κB [Funder 1997; Gottlicher et al., 1998; McKay and Cidlowski 1998]. Given the presence
of glucocorticoid receptors in vascular smooth muscle, endothelia, skeletal muscle, and some cells in the kidney, it is possible that glucocorticoid receptors might alter eNOS and nNOS expression, and this action may underlie some of the “permissive” effects of adrenal corticosteroids that are important for normal physiology.

There is evidence of interaction between cortisol and estrogen in several systems studied, including estrogen interaction with glucocorticoid feedback control of ACTH secretion, effects of steroids on osteoclasts, and effects on hippocampal neuron growth and survival. Glucocorticoid receptors (GR) appear to antagonize ER effects at the AP-1 response element [Uht et al., 1997]. This suggests that an antagonism between GR and ER mediated effects on NO may normally exist in some target tissues during late pregnancy. The withdrawal of GR “tone” may be exacerbated in a condition of high ER activity, such as in the peripartal period.

Significance

As a model of Addison’s disease, the ewe is preferable to rodent species because, sheep (like humans and dogs) have marked hypotension and loss of vascular reactivity after adrenalectomy unless steroids are replaced. Unlike the situation in the rat, treatment with saline alone will not maintain blood pressure in the ewe.

This research is designed to determine the effect of adrenal steroid and estrogen on the control of baseline NO production, and the possible role of NO in pregnant subjects and its effects on hypotension of Addison’s disease. This may provide guidelines for clinical therapy to pregnant women with Addison’s disease.
Specific Aims

The overall goal of this research is to test the hypothesis that an interaction between increased cortisol and increased estrogen is necessary for normal blood pressure control in late gestation.

Aim I

To evaluate the effects of estrogens on blood pressure, and vascular reactivity, and plasma potassium, I tested the hypothesis that estradiol treatment results in a more precipitous decline in blood pressure and vascular reactivity, and a more precipitous rise in plasma potassium in hypoadrenal animals.

Blood pressure, and vascular reactivity to phenylephrine and plasma potassium were evaluated in adrenalectomized sheep with estradiol treatment, and in adrenalectomized sheep without estradiol treatment.

Aim II

To evaluate the effect of estrogen on the synthesis of nitric oxide, I tested the hypothesis that estradiol treatment exacerbates the increase in synthesis of nitric oxide in endothelium and/or vascular smooth muscle and in renal tubules occurring with decreased adrenal corticosteroids. Three isoforms of NOS were evaluated in various tissues in response to both estrogen treatment and corticosteroid withdrawal, and before and after infusion of L-NAME. Nitrate/nitrite level was evaluated. Blood pressure and vascular responsiveness were also evaluated before and after infusion of L-NAME.
CHAPTER 2
LITERATURE REVIEW

Steroid Hormones

The family of steroid hormones is a big family, including estrogen, progesterone, testosterone, corticosteroids, and mineralocorticoids. The structure of all of the steroids is based on the steroid nucleus, which is similar to that of cholesterol. Different steroid hormones are secreted by different tissues: estrogen and progesterone from the ovaries (or from the placenta during gestation), testosterone from the testes, and glucocorticoids and mineralocorticoids from the adrenal cortex. Steroid hormones mediate their activity via interaction and activation of their respective receptors, and they play important roles in regulating the normal physiological function of the body. Recently, investigators have discovered novel effects of steroid hormones besides their classical functions. For example, estrogen was thought mainly to affect the female reproductive system and development of the fetus; now some investigators also think it is to be a natural protective agent of the cardiovascular system based on more recent studies [Tolbert et al., 2001]. This review focuses on the direct and/or indirect effects of estrogen and corticosteroids on the cardiovascular physiology.

Estrogens and Effects

There are three major estrogens in humans: estrone, estradiol, and estriol. The most potent of the estrogens in terms of physiological function is estradiol. Most estrogen is synthesized in the ovary and is regulated by LH and FSH. During pregnancy, the placenta produces a tremendous amount of estrogen. The plasma concentration of estrogen
dramatically increases toward the end of pregnancy in humans, and increases about 3- to 4 fold during late gestation in ewes [Robertson and Smeaton 1973].

**Estrogen Receptors**

Estrogen acts by binding and activation of its receptor, the estrogen receptor (ER). So far, two isoforms of estrogen receptor have been identified: estrogen receptor $\alpha$ (ER\(\alpha\)) and estrogen receptor $\beta$ (ER\(\beta\)) [Green et al., 1986; Jensen and Jacobson 1960; Kuiper et al., 1996]. The receptor of estrogens, glucocorticoids and mineralocorticoids are all membranes of the nuclear receptor family [Evans 1988].

In humans, ER\(\alpha\) gene is located at the long arm of chromosome 6, and ER\(\beta\) gene is located at band q22-24 of chromosome 14. The distributions of ER\(\alpha\) and ER\(\beta\) are different. In the rat, ER\(\alpha\) shows highest expression in the uterus, testis, pituitary, ovary, kidney, epididymis and adrenals, whereas ER\(\beta\) is most expressed in the brain, bone marrow, endothelial cell, prostate, ovary, lung parenchyma, bladder, and epididymis [Enmark et al., 1997]. The Kd of estrogen binding to ER\(\beta\) is the same as that of ER\(\alpha\) in humans. (0.6nM) [Kuiper et al., 1998].

**Effects of Estrogen**

The classical function of the estrogens is to cause cellular proliferation and growth of the tissues of the sex organs and other tissues related to reproduction. During recent decades, investigators have discovered that estrogens also play important roles in a number of non-reproductive tissues, such as bone tissue, the cardiovascular system, and the central nervous system (CNS).
The Effects of Estrogen on Cardiovascular System

Estrogens are thought to have protective effects on the vascular system. Epidemiological studies show that premenopausal women had significantly lower incidence of cardiovascular diseases compared to age-matched men and postmenopausal women.

Estrogen has many systemic effects, including alteration of serum lipid concentrations, coagulation and fibrinolytic systems, platelet aggregation, adhesion molecules, and growth factors. Recent studies also suggest that estrogen has direct actions on blood vessels and this effect contributes significantly to the cardiovascular protective effects of estrogen. The vasculature, like the reproductive tissues, is thought to be an important target of estrogen action [Tolbert et al., 2001].

**Systemic cardiovascular effects of estrogen** Estrogen was reported to reduce total cholesterol and LDL-C, and to increase HDL-C and triglycerides [Shlipak et al., 2000]. Shlipak and colleagues also discovered a significant reduction in apolipoprotein levels in women taking hormone replacement therapy (HRT) compared to placebo. Furthermore, they found that relative hazards for cardiovascular events were lower for women using HRT who had baseline apolipoprotein levels in the highest three quartiles compared to women taking placebo.

The mechanism of estrogen’s effect on blood lipid profile is not clear. The antioxidant properties of estrogen have been investigated as a possible mechanism for its lipid-lowering effects, and the results have been inconsistent. Wen [Wen et al., 1999] examined the effect of combined estrogen and progestin on LDL-X oxidation in healthy postmenopausal women and found no significant antioxidant effect. In vitro studies, however, have shown that physiologic concentrations of 17-β-estradiol inhibit oxidative
modification of LDL-C [Shwaery et al., 1997]. Other investigations have revealed that estrogen enhances postprandial clearance of chylomicrons and chylomicron remnants and that HRT increases the ratio of arachidonic acid to lineolic acid, thus increasing the precursor for eicosanoids with important cardiovascular functions [Weintraub et al., 1999; Lewis-Barned et al., 2000].

The action of estrogen on hemostasis is also related to its cardiovascular system protective effect. Bar [Bar et al., 2000] found that 17β-estradiol significantly inhibited ADP-mediated platelet aggregation in platelet rich plasma. Furthermore, this effect was reversed by the addition of ICI 182780 and 14-hydroxytamoxifen. Selles [Selles et al., 2001] reported that rat aortic strips significantly increase its capacity to inhibit ADP-mediated platelet aggregation after administration of physiological concentrations of 17β-estradiol. These effects were significantly reduced after addition of L-NAME. This suggests that hormone treatment may stimulate the tissue NO synthase activity.

The effects of estrogen on adhesion molecules and cytokines were studied by Scarabin [Scarabin et al., 1999]. They reported that the adhesion molecule ICAM-1, which is involved in the early stages of atherosclerosis, was expressed at lower levels in postmenopausal women taking oral estrogen therapy.

**Direct effects of estrogen on the blood vessel wall** As mentioned before, both ERα and ERβ are detected in blood vessels. Both isoforms of ER are expressed in endothelium whereas ERβ is thought to be the predominant subtype in human smooth-muscle cells, particularly in women [Hodges et al., 2000]. As discussed before, estrogen mediates its activity via binding and activation of estrogen receptors. Furthermore, this estrogen-receptor complex affects many different signal transduction pathways either
with or without transcriptional effect(s) [Mendelsohn and Karas 1999]. Several studies have shown that one of the most important targets of estrogen in the vessel walls is the nitric oxide (NO) pathway. Hayashi [Hayashi et al., 1992] reported that basal level of NO production was significantly higher in female rabbit aorta. The work from the Puglisi laboratory [Bolego et al., 1997] showed that administering estrogen to ovariectomized rats restored the impaired *ex vivo* basal release of NO, but this was not the case in the aorta of estrogen-implanted intact or castrated male rats [Cignarella et al., 2000].

It has also been shown that estrogen stimulates nitric oxide synthase activity in endothelial cells, reducing its calcium-dependence [Caulin-Glaser et al., 1997]. This effect had a rapid onset and did not involve changes in gene expression. It was also found that the selective estrogen-receptor modulator raloxifene can trigger nongenomic ER signaling and lead to rapid activation of nitric oxide synthesis in human endothelial cells [Simoncini et al., 2002]. Conversely, 17β-estradiol prevented activation of the inducible isoform of nitric oxide synthase stimulated by inflammatory mediators in most vascular cells [Kauser et al., 1998; Zancan et al., 1999]. This in turn may represent a protective mechanism against uncontrolled progression of inflammatory processes and oxidative damage such as those occurring in atherosclerosis, although other reports were not consistent with this report. Interestingly enough, Zhu [Zhu et al 2002] showed that the treating endothelium–denuded rings with estradiol led to increased accumulation of the iNOS protein which was fully inhibited by simultaneous treatment of the rings with the ER antagonist ICI-182, 780. It seems that endothelium is the key for this effect. Recent studies showed that the stimulatory effect of estrogen on endothelial nitric oxide synthase was ERα-dependent and required interaction of ERα with heat shock protein 90 [Chen et
As for smooth muscle cells, estrogen has been shown to inhibit their proliferation and migration and has been shown to increase relaxation [Kolodgie et al., 1996]. Estrogen has shown protective effects also on animal models of atherosclerosis such as that of carotid injury resulting in neointimal proliferation. Such intimal thickening was greater in males than in females in two different rat models of vascular injury [Akishita et al., 1997; Oparil et al., 1997]. In both studies, treatment with 17β-estradiol effectively prevented the ovariectomy-induced increase in neointimal formation in females. Recently, 17β-estradiol was reported to retard progression of atherosclerosis in ovariectomized rabbits after cholesterol feeding and aortic balloon injury owing to NO-related improved endothelial function [Hayashi et al., 2000]. Most of these effects on vascular-smooth muscle function are receptor-mediated. For instance, the inhibitory effects on 17β-estradiol and other estrogenic compounds on human aortic smooth muscle cell biology were reversed by the non-selective ER antagonist ICI 182,780 [Dubey et al., 2000]. Estrogen protects against vascular trauma seen in the rat carotid injury model with ER-dependent mechanisms, as shown again by treatment with ICI 182,780 [Bakir et al., 2000]. Ying [Ying et al., 2000] studied the methylation status of ERα in human aortic smooth muscle cells, either in situ (normal aortic tissue, contractile phenotype), or the same cells explanted from the aorta and cultured in vitro (de-differentiated phenotype). They found the ERα promoter became methylated in proliferating aortic smooth muscle and this did not show in situ (normal aorta). This result suggests that methylation of the ERα could contribute to the switch in phenotype observed in these cells. An interesting study also showed that ER antagonism by ICI 182,780 increased ischemic injury after middle cerebral artery
occlusion in female mice, indicating that ER-mediated mechanisms were important to estrogen’s protective properties in the brain [Sawada et al., 2000].

In this regard, it has been shown that 17β-estradiol stimulated NO release from granulocytes within seconds through activation of a cell membrane ER, triggering an increase in intracellular Ca^{2+}. Interestingly, the process was not affected by ICI 182,780 but by tamoxifen, a synthetic ER ligand with mixed agonist/antagonist activity. In human endothelial cells, membrane-impermeant forms of 17β-estradiol stimulated cGMP production and NO release within minutes, and this effect was blocked by ICI 182,780. In the same cells, an anti-ERα antibody detected two proteins, which were associated with the genomic and non-genomic responses mediated by ERα, respectively [Russell et al., 2000; Stefano et al., 2000]. Invaluable help in understanding the vascular role of ER has been provided from studies carried out on ER knock-out (KO) mice [Couse et al., 1999]. In ERα-KO mice, the basal level of NO was significantly lower in the aortas of males than females and this was regarded as a reflection of the significantly greater amount of ERα expressed in the aorta of male wild-type mice [Rubanyi et al., 1997]. There was no difference though in the response to vasodilators such as acetylcholine, which releases endogenous NO, or nitroglycerine, an exogenous NO donor. This study therefore suggested that the level of ERα may be critical for the control of endothelial basal NO formation. In another study, the model of carotid arterial injury was used in wild-type and ERα-KO mice [Iafrati et al., 1997]. Vascular injury led to similar increases in the medial area and smooth-muscle cell proliferation in both groups. Treatment with 17β-estradiol provided vascular protection significantly reducing these measures of injury in wild-type as well as ERα-KO mice, indicating the existence of a functionally
distinct system from ERα involved in vascular protection. Once identified, ERβ became an obvious candidate to explain the vasoprotective effect of 17β-estradiol observed in the above study. This hypothesis gained further credit when two reports came out showing that expression of ERβ mRNA, but not of ERα mRNA, increased in the arteries of male and female rats after vascular injury [Lindner et al., 1998; Makela et al., 1999]. In both cases, ERβ expression remained elevated in vascular cells even 28 days after injury. In this context, it is also worth mentioning that ERβ appears to be endowed with a general antiproliferative activity as shown in other tissues such as the prostate or the uterus [Zhang et al., 2000; Lau et al., 2000]. Surprisingly, when the vascular-injury model was tested in ERβ-KO mice, a similar degree of protection was provided by 17β-estradiol in both wild-type and ERβ-KO mice, in full agreement with the results seen in ERα-KO mice [Karas et al., 1999]. Therefore, it has been proposed that ERα and ERβ redundancy takes place in mediating vascular protection; alternatively, a third ER or some non-receptor-mediated signaling mechanisms may be involved. Ongoing studies for the double ERα and ERβ KO mouse will probably help unravel this tangled web. Certainly, the observation that the double ERα- and ERβ- KO mouse survives to adulthood points to the possible relevance of some as yet unidentified mechanisms underlying the biological effects of estrogen in vivo [Couse and Koraach 1999].

**Estrogen and nitric oxide** So far, three isoforms of nitric oxide synthase are identified. They are neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). nNOS was originally purified from brain [Bredt et al. 1990]. It is primarily a soluble enzyme with a molecular weight of 160 kDa, and is also present in human skeletal muscle, and renal macular densa cells. eNOS
was identified in endothelial cells. It is mostly particulate in nature and has a molecular weight of 135 kDa [Forstermann et al. 1994]. Myristylation at the N-terminal glycine is likely to be responsible for the particulate nature of the enzyme [Sessa et al. 1992]. nNOS and eNOS are regulated by calcium and CaM; eNOS is also regulated by pulsatile flow and shear stress. iNOS has been shown to be induced by bacterial endotoxin and/or cytokines. (Hevel, 91) in macrophages, endothelial cells, and other cells. iNOS is a predominantly soluble enzyme with molecular weight of 125-130 kDa, and is not regulated by calcium.

Estrogen can increase the bioavailability of endothelially derived nitric oxide in a variety of models [Mendelsohn and Karas 1999]. When administered directly into a coronary artery of a nonhuman primate or a human, estradiol causes rapid vasodilation by activating endothelial nitric oxide production. The molecular mechanism(s) by which estrogen activates eNOS is still unknown. More and more attention focuses on the estrogen-ER-eNOS rapid activation pathway [Mendelsohn 2002]. In 1997, Lantin-Hermoso [Lantin-Hermoso et al., 1997] and Caulin-Glaser [Caulin-Glaser et al., 1997] reported that relatively specific antagonists of the ERs could inhibit nitric oxide release from cultured endothelial cells exposed to physiologic concentration of estrogen. Lantin-Hermoso demonstrated that eNOS activity increased in a dose-dependent manner within 5 minutes after ovine pulmonary artery endothelial cells were exposed to 17β-estradiol. In their studies, removal of ionic calcium completely inhibited 17β-estradiol-stimulated eNOS activity, supporting the hypothesis that 17β-estradiol acutely stimulates eNOS by increasing Ca2+ influx. The ER antagonist ICI-182, 780 fully inhibited 17β-estradiol-stimulated eNOS activity in these studies. Caulin-Glaser demonstrated a rapid increase in
nitric oxide release from endothelial cells from the female human umbilical vein in response to 17β-estradiol. They similarly found that the ER antagonist ICI-164,384 inhibited this increase in nitric oxide release. These surprising observation suggested for the first time that the ligand-activated transcription factors might have a second, entirely novel function: activation of signal transduction events that could culminate in the rapid activation of eNOS.

In 1999, Chen [Chen et al., 1999] showed that ERα can mediate the short-term effects of estrogen on eNOS activity. In intact endothelial cells, 17β-estradiol caused activation of eNOS within 5 minutes. This activation was specific to 17β-estradiol, required the ERα ligand binding domain, and was blocked by ER antagonists. The acute response of eNOS to 17β-estradiol could be reconstituted in COS cells cotransfected with ERα and eNOS, but not in those transfected with eNOS alone. 17β-estradiol caused the rapid, ER-dependent activation of the mitogen-activated protein (MAP) kinase, and the MAP kinase inhibitor PD-98059 blocked the ability of 17β-estradiol to activate eNOS in intact endothelial cells. These finding demonstrate that the rapid activation of eNOS is mediated by ERα functioning in a novel, nongenomic manner. The pharmacologic data from this study also suggest that the MAP kinase pathway might be recruited by activation of ER and be involved in eNOS activation.

In 2000, two laboratories provided substantial evidence for the involvement of a second signal transduction pathway in eNOS activation: phosphatidylinositol (PI) 3-kinase-Akt pathway [Haynes et al., 2000; Simoncini et al., 2000]. Haynes et al [Haynes et al., 2000] demonstrated that PI 3-kinase is capable of activating Akt (protein kinase B, a serine/threonine kinase downstream of PI 3-kinase), which, it had been shown by several
groups, could in turn directly phosphorylate and activate eNOS. Simoncini et al. showned that human recombinant ERα (but not ERβ) binds in a ligand-dependent manner to the p85α regulatory subunit of PI 3-kinase. Stimulation with estrogen increased ERα-associated PI 3-kinase activity, leading to activation of Akt and eNOS. Interesting results from Arnal’s group showed that estradiol altered nitric oxide production in the mouse aorta through the α-, but not β-, estrogen receptor. They reported that basal NO production was increased and the sensitivity to acetylcholine decreased in ERβ knockout mice in response to estradiol, whereas this effect was abolished in ERα knockout mice [Darnal et al., 2002]. Chambliss [Chambliss et al., 2000] also reported in 2000 that the ER-eNOS signaling system is localized to endothelial cell caveolae, the plasma membrane subdomains known to be enriched in many signaling molecules, including eNOS. The relative importance of the MAP kinase and PI 3-kinase signaling pathways in ER-dependent activation of eNOS is not yet understood. Furthermore, the relative importance of this rapid activation pathway in the physiology of estrogen action in the vasculature remains uncertain.

**Glucocorticoid Receptor (GR) and Mineralocorticoid Receptor (MR)**

Both GR and MR are the members of the nuclear receptor and bind corticosteroids secreted by the adrenal cortex. Like other members of nuclear receptor family, the GR and MR can be divided into several regions from N-terminal to C-terminal: A/B (NH2-terminal domain), C (DNA-binding domain), D, E and F (ligand binding domain). The N-terminal A/B domain is highly variable in sequence and length (15% homology) DNA-binding domain is highly conserved (94% homology) (DBD) (C), which contains two zinc fingers involving in specific DNA binding and receptor dimerization, and a less well
conserved ligand-binding domain (57% homology) (LBD) (E) harboring regions important for ligand binding, receptor dimerization, nuclear localization and interactions with transcriptional coactivators and corepressors. The hinge (D) domain links the DBD and LBD. The function of the C-terminal F domain is not well known.

**Distribution**  GR broadly exists in whole body whereas the distribution of MR is limited. The classical tissues expressing MR include the distal nephron, distal colon, salivary and sweat gland. Recently, MR is also found in neurons, cardiac myocytes, endothelial cells and smooth muscle cells [Krozowski and Funder 1983].

**Selectivity**  The endogenous ligand to MR, GR is mineralocorticoids (aldosterone) and glucocorticoids (cortisol). The affinity of glucocorticoids binding to MR is same as mineralocorticoids (Kd=0.5-2 nM) in vitro [Farman and Bocchi 2000]. During normal physiological conditions, MR is protected from the much higher concentration of the endogenous cortisol. Although there are several candidate factors may contribute the selectivity of MR, the 11β- HSD-2 play a critical role to convert glucocorticoids into their 11-keto receptor inactive congeners [Funder et al., 1988].

MR and 11β- HSD-2 coexist in epithelial cells, and aldosterone plays a dominant role in these tissues (as discussed before). In other tissues the relationship between GR and MR agonists in other tissues is not clearly elucidated. Nonepithelial cells usually express MR without 11β- HSD-2 except vascular smooth muscle cells and amygdala [Roboson et al., 1998]. Therefore in most nonepithelial tissues, cortisol is not inactivated by 11β- HSD-2 and therefore has appreciable binding at MR.

In vascular smooth muscle cells MR and 11β- HSD-2 are coexpressed. Alzamora et al reported that cortisol alone had no effect on Na+/H+ exchanger even its concentration
was much higher than normal plasma level. When 11β-HSD-2 was inhibited by carbenoxolone, cortisol activation of the Na+/H+ exchanger was much close to that of aldosterone [Alzamora et al., 2000]. Glucocorticoids appear to have an opposing action on MR activity in brain and heart Gomez-Sanchez et al reported that intracerebroventricular infusion of aldosterone alone increased blood pressure in rat. Interestingly, corticosterone alone did not increase blood pressure whereas RU26988 (a GR agonist) blocked the increase of blood pressure to aldosterone [Gomez-Sanchez et al., 1990]. Young discovered that uninephrectomized rats developed cardiac hypertrophy, perivascular and interstitial fibrosis after treatment with aldosterone (0.75 microgram/h) for 8 weeks. Both hypertrophy and fibrosis were significantly improved by infusion of corticosterone at same time [Young et al., 1995]. In these non-epithelial tissues, glucocorticoids have opposite effects compared to aldosterone. However, the mechanism underlying the different effects of glucocorticoids on different tissues via MR is unclear.

**Mineralocorticoids and Effects**

Aldosterone is the principal mineralocorticoid. The other major endogenous mineralocorticoid is 11-Deoxycorticosterone (DOC), however its potency as a mineralocorticoid only 2% that of aldosterone. Aldosterone is secreted by the zona glomerulosa, the outermost and very thin layer of cells on the surface of adrenal cortex. The synthesis of aldosterone is regulated by Angiotensin II. The availability of Angiotensin II is mainly controlled by body sodium status, and extracellular volume. An increase in extracellular potassium concentration is a potent, renin-independent stimulus to aldosterone secretion. The potassium concentration sets the slope of the relationship between angiotensin II and aldosterone [Connell et al., 2001]. Importantly, the
responsiveness of aldosterone secretion rate to all agonists depends on whether the subject is sodium-loaded (low sensitivity) or sodium-depleted (high sensitivity) [Oelkers et al., 1974; Hollenberg et al., 1974].

Aldosterone acts by binding to mineralocorticoid receptor (MR). MR has been cloned and characterized in both epithelia (e.g. kidney) and non-epithelial (e.g. hippocampus) tissues [Arriza et al., 1987]. In both tissues in vitro, MR has an equivalent high affinity for corticosterone, cortisol and aldosterone [Krozowski and Funder 1983]. In renal tissues, MR coexists with the protective enzyme 11β hydroxysteroid dehydrogenase-2 (11βHSD-2), which converts cortisol and corticosterone (but not aldosterone) to the 11-keto receptor-inactive metabolite. 11βHSD-1 functions as both dehydrogenase and oxoreductase. [Funder et al., 1988]. Non-epithelial tissues usually do not coexpress 11βHSD-2, with two exceptions (amygdala and smooth muscle cells).

Effects of Aldosterone

The major targets for aldosterone are those epithelial tissues that accomplish unidirectional sodium transport, particularly the distal nephron, the large intestine and the salivary and sweat glands. However, recent evidence now strongly suggests that aldosterone also affects the electrolyte metabolism of leukocyte and aspects of cardiac, vascular and brain function [Connell et al., 2001].

Renal Effects of Aldosterone

Aldosterone controls extracellular volume and blood pressure mainly through its effects on kidney. In the aldosterone-sensitive distal nephron (ASDN), mineralocorticoid receptor occupancy results in sodium reabsorption across epithelial cells. The increased entry of sodium into the tubular cell from the lumen via the amiloride-sensitive
sodium/hydrogen exchanger is mediated by a coordinate action on the luminal sodium channel and the basolateral Na,K-ATPase. The precise mechanism of this action is not fully understood.

The physiological responses to aldosterone in transporting epithelial can be divided into short-term and long-term effects. The short-term aldosterone action (early effect) on sodium reabsorption and potassium secretion can be observed as rapidly as 30 minutes after the beginning of an aldosterone treatment [Horisberger and Diezi 1983]. In the long-term (late effect), aldosterone induces a more durable increase in the transport capacity of the target cells [Verrey et al., 2000; Verrey et al., 2003].

The amiloride-sensitive epithelial sodium channel (ENaC) expressed along the aldosterone – sensitive distal nephron (ASDN) is a main target of aldosterone. Loffing et al reported kidneys and isolated tubules of adrenalectomized rats that received a single dose of aldosterone showed that the expression of ENaC α subunit was regulated in the short term, at the mRNA and protein levels, whereas the β and γ subunits were expressed constitutionally [Loffing et al., 2001]. Studies in cell cultures systems has also shown that the half-life of ENaC subunits was short [De La Rosa et al., 2002]. Thus, the rapid up- and down-regulation of the total cellular amount of αβγENaC can play an important role in the short-term regulation of transepithelial sodium transport. David et al showed the serum- and glucocorticoid-regulated kinase (SGK1) is an aldosterone – regulated protein that strongly stimulates ENaC cell-surface expression and function when expressed in Xenopus oocyte. In a later study, Loffing et al reported SGK1 was indeed rapidly induced at the mRNA and protein levels in the segment-specific cells of the entire ASDN [Loffing et al., 2001]. Verrey and colleague observed the increase of Na,K-ATPase mRNA (α
subunit), protein, and activity in the animals treated with aldosterone for long term [Verrey et al., 1996]. The same group also found that aldosterone acted in the short-term on the cell-surface expression of the basolateral Na,K-ATPase in a manner similar to its action onto apical ENaC and SGK1 also played an important role on the pathway that led from the transcriptional regulation to the functional response [Summa et al., 2001]. Further studies will be required to explain how the short-term and long-term effects of aldosterone cooperate with each other, and provide the mechanism of aldosterone’s effects on kidney.

**Vascular Effects of Mineralocorticoids**

Mineralocorticoid receptor is also identified in the endothelial and smooth muscle cells of blood vessels [Krozowski and Funder 1983; Lombes et al., 1992]. Unlike other nonepithelial cells, vascular smooth muscle cells also express 11-β HSD2 [Alzamora et al., 2000]. This suggests that aldosterone may have physiological and pathophysiological effects on the blood vessels. Christ et al reported that aldosterone significantly stimulated the activity of sodium transport in primary cultured vascular smooth muscle cells of rat thoracic aorta [Christ and Douwes 1995]. Alzamora and coworker also discovered this effect of aldosterone in strips of human uterine and chorionic arteries [Alzamora et al., 2000]. They suggest that the rapid nongenomic pathway involved in this effect of aldosterone. These results indicate that aldosterone mediates its effects on blood pressure regulation not only through their renal effects, but also vascular effects.

Additional studies will be needed to integrate the renal and vascular effects of aldosterone in order to elucidate the mechanism of the effects of aldosterone on blood pressure regulation.
Glucocorticoids and Effects

Cortisol is the major glucocorticoids in human, whereas corticosterone is the major glucocorticoid in rodents. Glucocorticoids are synthesized in zona fasciculata and zona reticularis, which are the middle and deep layers of adrenal cortex. The secretion of glucocorticoids is regulated by ACTH (adrenocorticotropic hormone) from the anterior pituitary. The availability of ACTH is further controlled by CRH (corticotropin-releasing hormone) and AVP (arginine vasopressin) from the hypothalamus. The negative feedback effect exists in every step of the hypothalamus-pituitary-adrenal gland axis. During pregnancy, the plasma concentration of cortisol increases in human and sheep [Keller-Wood et al., 1998]

Renal Effects of Glucocorticoids

Glomerular filtration and renal blood flow The effects of glucocorticoids on kidney are more complicated compared to mineralocorticoids. Several studies have shown that glucocorticoids increase the renal blood flow (RBF) and glomerular filtration rate (GFR) in a variety of animal models. De Bermudez et al first reported that methylprednisolone (MP: a synthetic glucocorticoid) treatment significantly increased the renal blood flow in the rat [De Bermudez et al., 1972]. Then, Baylis and her colleagues also discovered that the plasma flow rate increased approximately 25% in the rat treated with MP [Baylis and Brenner 1978]. The study by Cardoso and co-workers provided even more evidence for physiological effects of glucocorticoids. They found single-nephron glomerular filtration rate in the superficial nephrons decreased 20% and 57% in normal and chronic renal failure rats after administration of the glucocorticoid inhibitor RU-486. Connel and colleague also found cortisol significantly increased GFR in human subjects [Connel et al., 1987].
Although these results suggest RBF and GFR are stimulated by glucocorticoids, the mechanism underlying this effect is still unknown. De Matteo et al suggest that nitric oxide (NO) may be involved in cortisol’s effect on renal function. They indicated that GFR significantly increased in sheep after administration of cortisol intravenously or intrarenally. Additionally, this effect of cortisol was abolished by infusion of L-NAME (nitric oxide synthase inhibitor); the GFR still maintained the increased level after infusion of vasoconstrictor angiotensin II intrarenally [De Matteo and May 1997]. However, in this study, they did not directly measure nitric oxide synthase activity or the concentrations of NO of nitric oxide metabolites (such as citrulline or nitrate/nitrite). Further studies will be required to provide more convincing evidence to support this hypothesis.

**Proximal tubules** The effects of glucocorticoids on the different segments of the renal tubule vary. Under normal physiological conditions in humans, glucocorticoids have almost little effect on the distal nephrons because 11β-HSD-2 converts cortisol into the inactive hormone cortisone. The expression of 11β-HSD is different in different species; therefore, the effects of glucocorticoids on different animal models are different.

Lee et al [Lee et al., 1995] reported that the mRNA level of Na⁺, K⁺ ATPase significantly increased in proximal tubule cells of rats after incubation with dexamethasone for 5 hours; protein level and activity of the enzyme also increased about 50% and 58% respectively. All of these effects were inhibited by addition of cycloheximide, an inhibitor of translation.

Data from different labs have shown that glucocorticoids also affect the ion channels in the apical membrane of proximal tubules. Baum et al discovered that NHE-3
mRNA (isoform of Na+/H+ antiporter) level in renal brush-border membrane significantly increased after chronic administration of dexamethasone. Incubation of proximal tubules for 3 h with dexamethasone increased proximal tubule Na\(^+\)-H\(^+\) antiporter activity significantly without change of its mRNA level [Baum et al., 1994]. Yun found that chronic incubation with dexamethasone activated NHE3 independent of gene induction in PS120 and opossum kidney cells [Yun et al., 2002]. These results suggest that the acute effect of glucocorticoids on NHE3 may be through nongenomic pathway. Park et al discovered that the acute treatment with cortisol decreased phosphate (Pi) uptake in primary rabbit renal proximal tubule cells in a time- and concentration-dependent manner, and aldosterone had no such effect. This inhibitory effect of cortisol was abolished by neomycin or U73122 (phospholipase C inhibitors), and two protein kinase C (PKC) inhibitors (staurosporine or bisindolylmaleimide I) but not by cycloheximide. They suggest that cortisol may inhibit renal Pi uptake via a nongenomic mechanism, which involves the PLC/PKC pathway [Park et al., 2001].

Taken together, glucocorticoids may have different effects on different ion channels of either apical or basolateral membrane in renal proximal tubules through classical genomic and/or nongenomic pathways.

**Distal tubules** In humans, a lack of 11-\(\beta\) HSD-2 expression results in hypertension, even with undetectable circulating aldosterone concentration. This disease was named apparent mineralocorticoid excess (AME), and results from a deficiency of 11-\(\beta\) HSD-2 due to mutation in exon 3, 4 or 5 of this gene. In animal models if 11-\(\beta\) HSD-2 is inhibited by carbenoxolone, glucocorticoids mediate their effects on distal nephron through interaction and activation of GR (glucocorticoid receptor) and MR
(mineralocorticoid receptor), also resulting in hypertension despite low circulating aldosterone levels.

Rayson et al reported dexamethasone elicited a 27% increase in tubular Na⁺, K⁺ ATPase activity in 6 h and a 32% increase in 24 h in rat kidney tubules derived from the distal nephron [Rayson and Edelman 1982; Kornel et al., 1993]. In MR knockout mice, Schulz-Baldes [Schulz-Baldes et al., 2001] and colleagues indicated glucocorticoids induced an amiloride-sensitive sodium absorption in renal cortical collecting duct of the MR-/− mice of about 25% compared to wild-type. ENaC α subunit mRNA was also increased. The regulation of ENaC expression was the same in both genotypes. They suggest that MR is not prerequisite for the activation of ENaC transcription and activity, and that the respective mechanisms can be stimulated via GR.

As discussed in the section on aldosterone, SGK increases the activity of ion channels and pumps in distal nephron. Alvarez et al discovered the constitutive expression of SGK under basal conditions was high and this was maintained by glucocorticoids [Alvarez et al., 2003]. These data suggest that glucocorticoids may indirectly affect distal nephron through SGK (or other mediators), and resulting in transepithelial sodium transport, although glucocorticoids directly bind to MR and/or GR in distal tubules in pathological condition. Further studies will be needed to elucidate the mechanisms of their effects.

**Other Effects of Glucocorticoids**

**Blood vessels** Glucocorticoids increase the vascular reactivity to vasoconstrictor such as angiotensin II. They also have effects on the ion channels of smooth muscle cells.
Kornel et al [Kornel et al., 1993] reported that Sodium and calcium ion influx were increased in vascular smooth muscle cells after treated with glucocorticoids for 24 hours.

Development and maturation  Glucocorticoids are necessary for development and maturation of fetus. The GR/- mice die shortly after birth [Cole et al., 1995].

Central nervous system  Glucocorticoids have broadly effects on CNS. In human, cortisol affects numerous cognitive domains including attention, perception, memory, and emotional processing [Erickson et al., 2003]

Others  Glucocorticoids play an important role in inhibition of inflammation and the metabolism of carbohydrate, protein and fat. Anti-inflammatory effects of glucocorticoids result from their reducing almost all steps of the inflammatory process [Barnes 1998].
CHAPTER 3
GENERAL METHODOLOGY

Animal Care

The ewes used in these experiments were non-pregnant adult weighting 45-62kg. The animals were housed in pens in the health center animal resources department under controlled lighting and temperature (12-hour light/dark cycle and constant 19-20 °C temperature). The husbandry staff cleaned pens daily and ewes had free access to food and water. The University of Florida’s Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

General Surgical Procedures

All surgical procedures were performed using aseptic technique. Ewes were fasted 24 hours prior to surgery. During surgery, anesthesia was maintained with 1% -2.5% v/v halothane in oxygen. Following intubation ewes were connected to a ventilator. Ventilatory CO2 and O2 respirations were all monitored during the surgery. After surgery, ewes were given 750 mg of ampicillin (Polyflex, Aveco, Fort Dodge, IA) twice a day for 5 days.

All ewes were subjected to chronic vascular catheterization. With ewes positioned in dorsal recumbency, catheters were inserted into both femoral arteries and veins, 6 and 10 inches respectively. Bilateral incisions were made at the femoral triangles to expose the femoral arteries and veins. The distal end of the vessel was tied off using 0 silk (Ethicon, Webster, Inc., Staring, MA) and a polyvinyl chloride catheter (0.050 i.d., 0.090 o.d.) was inserted into the vessel. The catheters were advanced approximately 6 and 10
inches to the iliac artery and the inferior vena cava, respectively. The catheters were then secured in place with ligature using 0 silk, and flushed with 0.9% physiological saline (Baxter Healthcare Corporation, Deerfield, IL) and heparin (1000 U/mL) (Elkins-Sinn, Cherry Hill, NJ). Catheters were then routed subcutaneously to the flank plugged with sterile nails, and stored in pocket attached to the flank of the ewe with an expandable bandage.

Bilateral ovariectomy was performed using a single midline abdominal incision. The peritoneum was incised along the linear albae and each ovary was located. The vasculature to the ovary was ligated using 0 silk, and the ovary was excised using electrocautery. The peritoneum was sutured using Braunamid, (Braun Melsugen AG, Melsugen, Germany) suture and the skin was also closed using Braunamid.

Adrenalectomy was performed with the ewes in sternal recumbency using bilateral flank incisions. Incisions were made just caudal to the diaphragm and lateral to the transverse processes of the lumbar vertebra. The adrenal glands were isolated, and the arterial and venous vasculature to and from the adrenal glands was ligated, using 2-0 silk ligature, and/or stainless steel clips (Hemo-clip, Roxame Laboratories Inc, Columbus, OH), and were cauterized. The wounds were closed in layers with 2-0 chromic gut for the internal layers and with Braunamid multifilament polyamide for the skin.

Hormone Replacement

Study I There were four groups in this study: ovariectomy (OVX), ovariectomy + 17β- estradiol treatment (OVX+E2), ovariectomy + adrenalectomy (OVX+ADX), and ovariectomy + adrenalectomy + 17β- estradiol treatment (OVX+ADX+E2).

Study II There were two groups in this study: OVX+ADX and OVX+ADX+E2.
All of the adrenalectomized ewes were treated with a maintenance dose of aldosterone and cortisol to produce a mean plasma cortisol concentration of 2-3 ng/ml, and mean aldosterone concentration of about 25 pg/ml by iv infusion of cortisol: 0.3 mg/kg/day, aldosterone: 3 µg/kg/day. Half of the ewes also were treated with 17β-estradiol. Estradiol was delivered to ewes as continuous intravenous infusion of 17β-estradiol (4µg/kg/day) following an intravenous bolus of 17β-estradiol of 5µg/kg. Estradiol was infused for all 10 days of study.

**Blood Pressure Measurement**

Blood pressure and heart rate were measured using Cobe pressure transducers (Gould, Oxnard, CA), which were connected directly to a National Instruments analog-to-digital conversion board (LabView, National Instruments, Austin, TX) and Pentium-class personal computer. Heart rate and blood pressure were calculated in real-time. Both raw and calculated data were stored on the computer. Calculated resulted were then imported to Excel, SigmaStat (Academic Superstore, Austin,TX), or SigmaPlot for data analysis and graphing.

**Blood Collection and Handling**

To avoid direct contact of the animals with investigators during the experiments, femoral arterial and venous catheters of 4 meters or 3 meters long were used. Catheters were removed from the pocket and threaded up and out through a vinyl duct (Ace Hardware Corp., Oak Brook, IL) so that the catheters were accessible to investigators without disturbing the sheep by entering the pens. In addition, the rectal body temperature of each ewe was measured before each experiment to monitor the health of the animal (normal temperature of sheep is about 39 °C).
All blood samples for hormone analysis were collected in centrifuge tubes containing 0.05 ml of 0.3 M Na4EDTA (Sigma Chemical Co., St. Louis, MO) per 1 ml of blood. The volume of dead space (about 10 ml) was taken out, and blood sample was collected (about 10 mL). Samples for determination of plasma Na+ and K+ concentration were taken in heparinized 3 ml syringes. Then, the 10 mL of blood of dead space was given back to the animal. The total volume of blood sampled was approximately 50 ml for the experimental day, which should not have effect on circulating hormones and/or arterial blood pressures. All of tubes were kept on ice until the end of the experiment. The blood samples were then centrifuged at 3000g/minute for 20 minutes at 4°C (Sorvall RT 6000B, Dupont, Newtown, CT).

Plasma Na+ and K+ concentration were determined by using a bedside ABL 77 Blood Gas analyzer (Radiometer, Copenhagen, Denmark). Plasma Na+ and K+ concentration were detected by ion-specific electrodes.

Plasma protein concentration and packed cell volume (PCV) were measured during every experiment with a refractometer (Fisher Scientific, Inc) and micro-capillary reader (Damon/IEC Division, MA) respectively, and utilized as an index for changes in blood volume. Plasma protein concentration was read to nearest tenth of an mg/100ml of plasma while PCV was read to nearest of 0.5%.

**Experimental Procedures**

**Study I**

**Experiment** On day 9 post surgery, the adrenalectomized ewes were withdrawn from cortisol and aldosterone in order to produce the hypoadrenal (or Addisonian) state. Prior to the end and of the infusion, blood pressure was measured as basal level and blood samples were collected. Beginning 8 hours after stopping corticosteroids infusion,
plasma glucose, sodium, potassium, and protein concentration were measured at two-hour intervals, and blood samples also were collected for further hormone assays. Vascular reactivity in response to phenylephrine was tested (3 µg/kg) 18-20 hours after stopping infusion of corticosteroids in vivo. Following the return of blood pressure to normal (about 20 minutes) a bolus of L-NAME (Sigma Chemical Co., St. Louis, MO) (10 mg/kg i.v.) was administered, and after 5 minutes the same dose of phenylephrine was repeated. Blood pressure and heart rate were measured throughout the test period. Additional plasma samples were collected after 5 minute of L-NAME infusion in order to test for reduction in plasma nitrate/nitrite concentration by L-NAME blockade of NOS activity.

**Necropsy** At the end of the experiment, all animals were anesthetized with sodium pentothal. After achieving surgical depth anesthesia, the uterine artery, a section of mesenteric artery, both renal arteries and the aorta were sequentially collected as soon as possible. RNA was extracted by using Trizol (Life Technologies, Inc., Frederic, MA) immediately. Animals were euthanized by an overdose of pentobarbital and other tissues were collected and rapidly frozen in liquid nitrogen, then stored in -80°C for further protein and mRNA determinations.

Endothelial cells were isolated from the whole blood vessels. Briefly, the vessels were dissected and collected into beaker containing Krebs solution (sigma chemical Co., St. Louis, MO). The blood was washed off, the adventitia was removed, the vessels were opened with scissors and spread out flat on the paper. The blood vessels were handled gently and kept on ice at all the time. Endothelial cells were scraped using round end
spatula and collected in the lysis buffer and Trizol for protein and mRNA determinations respectively.

**Study II**

**Experiment** On day 9 post surgery, all animals were withdrawn from cortisol and aldosterone in order to produce hypotension. Prior to stopping the steroid infusion, basal blood pressure was measured and blood samples were collected. Blood pressure and plasma electrolytes were also measured 12 hours after stopping infusion corticosteroids to assure animals were not hypotensive. Beginning 20 hours after stopping corticosteroids infusion, plasma glucose, sodium, potassium, and protein concentration were measured at two-hour intervals, and blood samples also were collected for further hormone assays. Vascular reactivity *in vivo* in response to phenylephrine was also tested as described in Study I.

**Necropsy** As described in Study I.

**Nitrate and Nitrite Assay**

Total plasma nitrate/nitrite levels were measured as described in the Cayman Nitrate/Nitrite Assay Kit (Cat# 780001). Initially, plasma designated for nitrate/nitrite determination was collected in EDTA glass tube and kept frozen (at -20°C) until assayed. Prior to the nitrate/nitrite analysis, plasma was ultrafiltered through a 30 kDa molecular weight cut-off filter (Amicon, Inc., Beverly, MA) using microfuge ultrafiltration of plasma. For nitrite/nitrate measurement, 40 µl plasma was mixed with equal amount of assay buffer (Cayman Chemical Co., Ann Arbor, MI) to a final volume of 80 µl and pipetted into a microtiter plate. Then, 10 µl of enzyme cofactor and nitrate reductase were added to each of the wells (standard and unknown) followed by incubation at room
temperature for 3 hours. After the required incubation time, 50µl of Griess Reagent #1 (Sulfanamide; Cayman Chemical, MI), and Griess Reagent #2 (Ethylenediamine; Cayman Chemical, MI) were added to each of the wells (standard and unknown). Color was allowed to develop for 10 minutes at room temperature and the absorbance was measured at 540 nm (Autoreader, Cayman Chemical, MI). Total nitrate/nitrite concentrations were determined by extrapolation from the prepared nitrate standard curve.

**Hormone Assay**

**Cortisol**

Plasma cortisol concentration were measured as previously described (Wood, Cudd, Kane, & Engelke, 1993) using antibody raised in rabbits and tritiated cortisol purchased from Amersham (# TRK-407) and cortisol standard from Sigma (Sigma Chemical Co., St. Louis, MO). Cortisol was extracted from 50 µl plasma with 1ml ethanol, and standards were prepared in ethanol. Standards and samples were dried under vacuum and immediately reconstituted with 500 µl assay buffer for assay [0.05 M phosphate buffer pH 7.0, with 0.15 M NaCl, 0.1% w/v gelatin, and 0.1 w/v sodium azide]. The lower limit of detection for this assay was 0.2 ng/ml.

**Estradiol**

Plasma estradiol concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit from Oxford Biochemical Inc. (Cat# EA70). Estradiol was extracted from 0.5mL plasma by 3-5 fold volume of ether. Samples were dried down under air. Extracted samples were reconstituted overnight in 120-150µL extraction buffer; 50µl of the reconstituted extract was assayed for estradiol in duplicate.
Western Blot

Total tissue eNOS, iNOS and nNOS were estimated using a semi-quantitative Western blot procedure. Based on the results of the protein assay, samples were diluted so the same amount protein could be loaded into each lane in a precast 7.5% polyacrylamide, Tris-HCL gel (Bio Rad, San Rafael, CA) and electrophoresed at 200 V for 1 hour. Besides unknown samples, each gel also had two lanes for the molecular weight marker and positive control (human endothelial cell, mouse macrophage cell, and rat cerebella cell for e,i,n NOS respectively, respectively. In preliminary experiments, vascular smooth muscle cells were tested as negative control for eNOS. Gels were transferred at 100V for 1 hour onto a nitrocellulose membrane. After the membrane was washed by PBST, it was blocked overnight at room temperature and incubated with primary antibody (1 hour for eNOS at room temperature, overnight for iNOS and nNOS at 4°C, 1:2500 dilution of the three primary antibody) (e,i,nNOS, Transduction Laboratories, Lexington, KY) followed by incubation with secondary antibody (1 hour at room temperature, 1:10000 dilution). The protein was visualized using the ECL chemiluminescence (Amersham, Piscataway, NJ). The density of bands was quantified by Quantity One densitometric analysis software (Bio Rad, San Rafael, CA). The results of the densitometry were expressed as relative optical density (OD) units and normalized by β-actin.

Two-Step Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

All the tissues that were collected and snap frozen in liquid nitrogen were extracted in Trizol according to manufacture’s instructions (Life Technologies, St. Paul, MN). Messenger RNA expression for eNOS, iNOS, nNOS were determined by a real time RT-
PCR method. This system allows for a very small amount of high quality of mRNA to be analyzed and quantified relatively quickly and accurate. For this method, we used a two-step method of RT-PCR. First, 2 μg total mRNA was converted into cDNA, then 10 ng of cDNA was used in the PCR reaction to quantitate the mRNA of the gene of interest. All reagents and primer/probes used in these reactions were obtained from Applied Biosystems. (Biosystems, Foster City, CA).

The plate was set up to include sample unknowns in triplicate from all animal groups for a single gene, and controls with no template added for each primer/probe set. For all genes, the 18s ribosomal was used as the internal control for all comparisons.

For real-time PCR, primers and Taqman probes were designed using Primer Express 1.0 (Applied Biosystems). The primer probe sequences from all genes investigated are presented. The Taqman primer and probes for the 18s ribosomal control, were supplied by Applied Biosystems in a control reagents kit. PCR reactions were carried out in a 25 μl volumes. The reaction times and temperatures were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Primer and probe sequences used in real-time RT-PCR as follows [Giroux02]:

- eNOS: TTTCAGGCACAGTCCTCTCA (forward sequence 5’-3’)
  AGGCGCACCAGGATGTT (reverse sequence 5’-3’)
  TGGAAAAACCTGCAAAGCAGCAAGTCC (probe)

- iNOS: TGATGCAGAAAGGCATGTCA (forward sequence 5’-3’)
  TCTCCCTGTCTCTGTTGCAAAG (reverse sequence 5’-3’)
  CCAGGGTCAGAGGCAGCAAGTC (probe)

- nNOS: AAAACCTTCAAGTCCAAAATCCA (forward sequence 5’-3’)
AGCTCCTGATTCCCGTTGGT (reverse sequence 5’-3’)

TGGAAAAACCTGCCAAAGCAGCAAGTCC (probe)

Calculations for real-time PCR: In each assay, the cycle threshold (C_T) value for each sample for each gene was determined in triplicate. The mean value for these was used to determine a ΔC_T by subtracting the mean values from triplicates of the 18s ribosomal RNA values from each sample (18s ribosomal RNA was used as a housekeeping or control gene for normalization of RNA/sample). The mean value of ΔΔC_T was also determined (ΔΔC_T = ΔC_T of experimental animals - ΔC_T of control animals). The fold change was determined by equation: \(2^{-\Delta\Delta C_T}\). In the study I, the ADI group served as control. In study II, ADX served as control. Statistical analysis for mRNA data was done by using ΔC_T, as this variable should be linearly distributed.

**Statistical Analysis**

Methods of statistical analysis were chosen based on experimental design and the character of the obtained data set. In each chapter, the statistical methods are detailed for each experiment. For all statistical tests, the null hypothesis was rejected when \(P<0.05\).
CHAPTER 4
EFFECTS OF INCREASED ESTROGEN ON VASCULAR RESPONSIVENESS AND MEAN ARTERIAL PRESSURE DURING WITHDRAWAL FROM CORTICOSTEROIDS

Introduction

During pregnancy, both estrogen and cortisol concentration normally increase. The physiologic significance of this increase of cortisol is still not clear. However, there is some evidence that cortisol may increase blood pressure by an inhibitory effect on NO generation. At same time, estradiol is thought to be the primary mediator of the decrease in mean arterial pressure and peripheral resistance via the increase in production of NO. (see Chapter 1,2 for detailed discussion about the effects of estrogen and cortisol on NO) The effects of increased cortisol may balance the effects of increased estrogen, this counterbalance may be important for normal blood pressure and volume control in pregnancy. Insufficient secretion of adrenal steroids results in a hypoadrenocorticoid state (Addisonian crisis), which is marked by critical hypotension, hypovolaemia, hyponatraemia and hyperkalaemia. Perhaps even more important, hypoadrenocorticoid humans experience a markedly decreased vasoconstrictor response to exogenous administration of vasoconstrictors, such as norepinephrine [Loriaux et al., 1990]. Orbach et al discovered the same results in sheep model [Orbach et al., 2001].

In our previous project, we observed that hypotension resulting from hypoadrenocorticism occurs more quickly in late gestation pregnant ewes, and it frequently was fatal.
In this study, we tested the hypothesis that increased estrogen levels may exacerbate the decreased vascular reactivity and hypotension of hypoadrenocorticism.

**Materials and Methods**

**Experimental Procedures**

For this study, twenty-two adult ewes of mixed Western breeds were studied. The ewes were assigned to one of four groups at the time of surgery: ADI (n=6), ADI+E₂ (n=4), ADX (n=6), and ADX+ E₂ (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; E₂: treated with 17β-estradiol.)

Surgery was performed as described in Chapter 3. After surgery, animals in the ADX groups were continuously infused with cortisol and aldosterone for 9 days (cortisol: 0.3 mg/kg/day, aldosterone: 3µg/kg/day). Animals in the E₂ groups were continuously infused with 17β-estradiol (4µg/kg/day) for all 10 days of study.

As described in Chapter 3, in ADX ewes, the hypoadrenal (or Addisonian) state was produced by stopping the cortisol and aldosterone infusion on post-operative day 9. The animals were monitored until the ewes became hypoadrenocorticoid. For the purposes of the present study, ewes were considered hypocorticoïd when plasma potassium exceeded 6.0 mEq/L. These levels were achieved 18-20h after the end of the infusion of cortisol and aldosterone.

Basal MAP and heart rate (HR) were recorded, and blood samples were collected for plasma hormone, electrolyte, protein and PCV determinations at 0 h and at 12-20 h, as described in Chapter 3. Vascular responsiveness in response to phenylephrine was tested (3 µg/kg) 18-20 hours after stopping infusion of corticosteroids in vivo. Following the return of blood pressure to normal (about 20 minutes) a bolus of L-NAME (sigma
chemical Co., St. Louis, MO) (10 mg/kg i.v.) was administered, and after 5 minutes the same dose of phenylephrine was repeated. Vascular responsiveness was calculated as the mean arterial blood pressure in the first minute after infusion of phenylephrine minus mean arterial blood pressure for the 30 seconds before infusion of phenylephrine. Blood pressure and heart rate were measured throughout the test period. The experimental design is shown in Figure 4-1.

Statistics

All data except MAP before and after L-NAME (Figure 4-5) were analyzed using two-way ANOVA. For MAP before and after L-NAME (Figure 4-5), a paired-test was used. Differences between individual means were analyzed using Duncan’s post hoc test. All values are reported as the mean ± SD.

Results

Plasma Cortisol, Aldosterone and 17-β estradiol Concentration

As shown in Table 4-1, the plasma cortisol and aldosterone concentrations of adrenalectomized groups were significantly lower than those of adrenal intact groups. Although plasma estrogen level tended to increase in ADX+E2 group, there were no significant different among ewes.

Plasma Electrolyte, Protein, and Hematocrit

Plasma K⁺ concentration and protein concentration, of adrenalectomized ewes were significantly higher than those in the adrenal intact groups (Table 4-1). Plasma Na⁺ concentrations in the adrenalectomized groups were significantly lower than in the adrenal intact groups. There were no significant differences in any of these variables between ADI and ADI+ E₂ groups, or between ADX and ADX+ E₂ groups (Table 4-1).
Packed cell volume (PCV) of adrenalectomized ewes treated with E2 was significantly higher than those in the adrenal intact groups (Table 4-1).

**Effect of 17β-estradiol on MAP**

After withdrawal of corticosteroid for 18-20 hours, the MAP values among the four groups of ewes were not significantly different (Figure 4-2). However, the changes in MAP over time from Day 9 to Day 10 was significantly greater in the ADX groups after withdrawal from corticosteroids than in ADI groups (ADX: -10.8± 3.2, ADX+ E2: -15.4± 5.2 respectively)(Figure 4-3)

**Effect of L-NAME Administration on MAP**

After L-NAME treatment, the MAP was significantly increased in all groups except for the ADI+ E2 group. In the groups treated with E2, the increase in MAP in response to L-NAME was significantly reduced relative to the groups without E2 treatment (Figure 4-4, 4-5).

**Vascular Responsiveness**

Injection of PE increased the MAP in all four groups of animals. However, there was no significant differences in the MAP after PE among the four groups of ewes, either before or after infusion of L-NAME (Figure 4-6).

**Effect of 17β-estradiol on Circulating NOx Level**

All ewes had relatively low plasma NOx levels; these levels did not significantly differ among groups. After withdrawal of corticosteroid for 18-20 hours, there was no significant changes in plasma NOx. However, after infusion of L-NAME, the NOx levels in all animals were significantly decreased (Figure 4-7).
Discussion

In this study we used an adrenalectomized sheep model to study the interaction between cortisol and estradiol on blood pressure and vascular reactivity. We had hypothesized that estradiol treatment would result in a more precipitous decline in blood pressure and vascular reactivity in hypoadrenal animals. However, the adrenalectomized ewes with E2 administration did not have more severe hypotension and vascular reactivity compared to non- E2 treatment ewes. We also found that chronic E2 administration did not reduce the MAP and vascular reactivity in adrenal intact ewes. Recently, Salhab et al also observed nonpregnant ovariectomized ewes receiving intermittent daily intravenous 17-β E2 (1µg/kg) had no change in basal MAP and heart rate [Salhab et al., 2000]. These results differ from studies by the Magness lab. They reported MAP fell about 9% and heart rate increased about 20% in nonpregnant ovariectomized ewes treated with 17-β E2 at a dose of 5µg/kg iv followed by 220µg /day for 14 days. The difference between their studies and ours may be due to duration of estrogen administration (compared to 10 days in our experiment) [Magness et al., 1993], or differences in duration of blood pressure measurement.

Although plasma NOx concentrations were not significantly different among the four groups before infusion of L-NAME, plasma NOx concentrations in the ewes treated with E2 tended to be lower. Recently, Khorram et al observed replacement of estrogen in OVX monkeys resulted in reduced circulating NOx compared to OVX monkeys. Circulating NOx levels in these animals correlated negatively with E2 levels [Khorram et al., 2002]. This result differs from several studies showing a positive correlation between E2 and NO levels [Rosselli et al., 1995]. These suggest the relationship between E2 and
circulating NO is not clear. It is possible estrogen may influence NOS activity or expression differently in different tissues. (see discussion in Chapter 6). This result may explain why the increase in MAP after infusion of NOS inhibitor L-NAME was smaller in E2-treated animals and there was no change in vascular reactivity. Because the E2 treated ewes had low NO production, there was a smaller increase in MAP after L-NAME than in the ewes not treated with E2. Taken together, the data refute the hypothesis that estrogen results in a generalized increase in nitric oxide generation and therefore increases the decrement in blood pressure with steroid withdrawal.

Glucocorticoids are thought to increase the vasoconstrictive effects of catecholamines and angiotensin II [Brem 2001]. However, our data showed there were no significant differences in blood pressure or vascular response to phenylephrine among the four groups of ewes, which suggest that there might be unknown mechanism underlying the vascular effects of glucocorticoids.

Although our estradiol assay data did not detect significant differences between estrogen-treated animals and nonestrogen-treated animals, which may be due to the method of extraction and reconstitution which minimized changes at the low end of the standard curve. However, the estrogen-treated animals exhibited the effects of estrogen such as the enlargement of mammary gland, vaginal hyperaemia, and uterine artery enlargement.
Table 4-1  Plasma cortisol, aldosterone, estradiol, electrolyte, protein concentration and packed cell volume at 18-20 hours after withdrawal of corticosteroids. (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17 β-estradiol.)

<table>
<thead>
<tr>
<th>Variable</th>
<th>ADI</th>
<th>ADI+E2</th>
<th>ADX</th>
<th>ADX+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (ng/mL)</td>
<td>6.4 ±3.6</td>
<td>7.5 ±4.4</td>
<td>1.6 ±1.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.3 ±0.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aldosterone (pg/mL)</td>
<td>68.5 ±28.5</td>
<td>49.8 ±27.8</td>
<td>12.0 ±0.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>13.6 ±2.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>40 ±8</td>
<td>40 ±6</td>
<td>49 ±12</td>
<td>65 ±20</td>
</tr>
<tr>
<td>K⁺ (mEq/L)</td>
<td>4.3 ±0.2</td>
<td>4.2 ±0.3</td>
<td>7.8 ±1.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.0 ±1.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Na⁺ (mEq/L)</td>
<td>144.2 ±1.3</td>
<td>144.8 ±1.9</td>
<td>135.2 ±3.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>138.6 ±2.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>7.7 ±0.4</td>
<td>7.6 ±0.4</td>
<td>9.4 ±0.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>9.1 ±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCV</td>
<td>28 ±3</td>
<td>28 ±6</td>
<td>37 ±9</td>
<td>41 ±7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are the mean±SEM. a p< 0.05 compared with ADI b p<0.05 compared with ADI+E2 K⁺: plasma [potassium]; Na⁺: Plasma [sodium]; PCV: packed cell volume.

Figure 4-1 The experimental design: Cort: cortisol, Aldo: aldosterone, PE: phenelephrine
Figure 4-2 MAP at 18-20 hours after corticosteroid withdrawal. ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17 β-estradiol.)

Figure 4-3 The change in MAP after corticosteroid withdrawal. a, p<0.05, compared with ADI, b, p<0.05, compared with ADI+E2. (two way ANOVA). ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17 β-estradiol.)
**Figure 4-4** MAP before and after L-NAME. *p<0.05, compared with before L-NAME (paired t-test) ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol).

**Figure 4-5** The change in MAP after L-NAME. *p<0.05 compared with groups without estrogen (two way ANOVA). ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol).
Figure 4-6 Pressor responsiveness (difference in MAP in response to 3 µg/kg phenylephrine). PE1: before administration of L-NAME; PE2: after L-NAME. ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6) (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol).

Figure 4-7 Plasma NOx level. * p<0.05, compared with basal level. ADI (n=6), ADI+E2 (n=4), ADX (n=6), ADX+E2 (n=6). (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17β-estradiol).
CHAPTER 5
EFFECTS OF INCREASED ESTROGEN ON VASCULAR REACTIVITY AND MEAN ARTERIAL PRESSURE DURING EXTENDED LONGER TIME COURSE OF CORTICOSTEROID WITHDRAWAL

Introduction

In the previous study, we found that 18-22 hours after withdrawal of corticosteroids the adrenalectomized ewes showed some symptoms of Addison’s disease such as reduced plasma Na⁺ concentration, increased plasma protein and K⁺ concentration, and elevated PCV. However, the ewes were not hypotensive. Therefore, although the ewes exhibited some symptoms of hypoadrenal patients, they did not exhibit the profound hypotension or hypovolemia of Addisonian crisis. Therefore, another series of experiments was performed in which the period of steroid withdrawal was extended so that the animals would be clinically hypotensive.

Materials and Methods

Experimental Procedure

In this study twelve adult ewes of mixed Western breeds were studied. The ewes were assigned to one of two groups at the time of surgery: ADX or ADX+E₂.

The surgery was performed as described in Chapter 3. As in the preceding study, after surgery all ewes were infused with cortisol (0.33 mg/kg/d) and aldosterone (3μg/kg/d) until day 9. ADX+E₂ ewes were also infused with 17β-estradiol (6μg/kg/d) throughout the study.

As described in Chapter 3, in both the ADX and ADX+ E₂ groups, the hypoadrenocorticoic state was produced by stopping the cortisol and aldosterone...
infusion. Blood pressure was measured and blood samples for plasma electrolytes, plasma hormones, plasma protein concentrations and PCV determination were collected at 20-28 hours after the aldosterone and cortisol infusion was stopped (Figure 5-1).

Statistics

MAP, vascular reactivity and other parameters were analyzed using ANOVA and \( t \)-test. The occurrence of cyclic change in MAP was analyzed using chi square test. All values are reported as the mean\( \pm \) SD. The Cochran and Cox approximation was used for unequal variances.

Results

As in the preceding study, plasma cortisol and aldosterone levels were low on day 10 after stopping the infusion of cortisol and aldosterone in ADX and ADX+E2 ewes (Table 5-1). Plasma estradiol concentrations were significantly higher in the ADX+ E\(_2\) ewes as compared with the ADX ewes (Table 5-1).

There were no significant differences in PCV and plasma protein between the ADX and ADX+E2 groups (Table 5-1).

MAP was significantly decreased in both groups of adrenalectomized animals after stopping the infusion of corticosteroids. The mean change in MAP over time did not differ between the groups, as shown in Figure 5-2. However, there were marked differences in the pattern of change in MAP in the two groups of ewes. In the ADX+ E\(_2\) ewes, MAP dropped to 70-80 mmHg by 20 h after stopping the corticosteroid treatment. This level of MAP was sustained until sacrifice at 28 hour. In the ADX group without E\(_2\) treatment, the MAP varied greatly between animals and the experiment was terminated in 2 animals before 28 hours due to profound hypotension in the ADX group. In ADX ewes, three of six ewes showed cyclic changes in MAP prior to L-NAME injection; this change
in pattern usually happened 22-26 hours after withdrawal of corticosteroids (Figure 5-3). The adrenalectomized ewes treated with estrogen never shown these cyclic changes. The occurrence of the cyclic change in MAP was significant higher in ADX ewes than ADX + E2 ewes (Chi square test).

Plasma sodium and potassium and sodium concentrations were not significantly different at 0 hour between ADX and ADX+ E2 ewes. The withdrawal of corticosteroids significantly increased the plasma potassium concentration and decreased the plasma sodium concentration. Overall the estrogen treated ewes had significant lower plasma potassium concentration and significant higher plasma sodium concentration, although there were no significant difference in either plasma sodium or potassium concentration at any time point between two groups of the ewes. In the ADX ewes plasma sodium concentrations were significantly reduced and plasma potassium concentrations were significantly increased by 12 h after stopping the infusion of corticoids. In the ADX+E2 ewes, these changes only reached significance after 26 h (Figure 5-4, 5-5). There were no statistically significant differences in plasma sodium and potassium concentration between ewes with cyclic pattern of MAP as compared to those without a cyclic pattern in MAP.

After infusion of nitric oxide synthase inhibitor L-NAME, there was no significant difference in the change of the MAP between these two groups (Figure 5-6). The plasma NOx concentration was significant higher in ADX + E2 group ewes than ADX group ewes at 28 hours after stopping infusion of corticosteroids (Figure 5-7). There was no significant difference in the response to phenylephrine between the two groups either before or after administration of L-NAME (Figure5-8).
Discussion

As we expected, the MAP of both groups decreased dramatically with the longer time interval after corticosteroids withdrawal. The mechanisms by which cortisol and aldosterone maintain the blood pressure and blood volume are not completely understood. Aldosterone increases blood pressure by causing an exchange in the transport of sodium and potassium, which leads to sodium and water retention [Connell et al., 2001]. The mechanisms in which cortisol increases blood pressure are more complicated. Despite the widespread assumption that cortisol raises blood pressure as a consequence of renal sodium retention, there are few data consistent with that notion. Although cortisol has a plethora of actions on the brain, heart, blood vessels, kidney, and body fluid compartments, precisely how it elevates blood pressure is unclear. Rayson et al [Rayson and Edelman 1982; Kornel et al., 1993] reported dexamethasone elicited a 27% increase in tubular Na⁺, K⁺ ATPase activity in 6 h and a 32% increase in 24 h in rat kidney tubules derived from the distal nephron Alvarez [Alvarez et al. 2003] discovered the constitutive expression of serum- and glucocorticoid-regulated kinase (SGK, an aldosterone – regulated protein that strongly stimulates epithelial sodium channel) under basal conditions was high and this was maintained by glucocorticoids. These data suggest that glucocorticoids may indirectly affect distal nephron through SGK (or other mediators), and resulting in transepithelial sodium transport. Kornel et al [Kornel et al., 1993] reported that Sodium and calcium ion influx were increased in vascular smooth muscle cells after treated with glucocorticoids for 24 hours. Other possible mechanisms currently being examined include inhibition of the vasodilator nitric oxide system and increases in vasoconstrictor erythropoietin concentration [Judith et al., 2000].
The most interesting and important result we obtained from this experiment is that E\textsubscript{2} treatment appeared to have a protective effect after the steroid withdrawal. Although the changes in MAP did not differ between the groups, in the ADX+ E\textsubscript{2} group, the MAP dropped to 70-80 mmHg and was sustained at this level over many hours. In contrast to this, in the ADX ewes, the MAP varied greatly between animals and the experiment was terminated in 2 animals before 28 hours due to profound hypotension. The blood pressure recordings from them (prior to L-NAME injection) showed cyclic changes in MAP; cyclic change in MAP were not observed in the ADX + E\textsubscript{2} ewes. The reason for the cyclic changes in MAP is still not clear. One explanation might be that the high plasma K+ and low plasma Na+ concentrations disturb the ion gradient between extra- and cellular concentration in myocytes, thus leading to arrhythmia. On the other hand, adrenalectomized ewes with E\textsubscript{2} have relatively stable plasma Na+ and K+ concentrations, which protect them from arrhythmia. Saleh observed that the baroreflex sensitivity in ovariectomized female rats can be improved following acute intravenous administration of 17\beta-estradiol. El-Mas and Abdel-Rahman observed similar effects but only following chronic subcutaneous administration of 17\beta-estradiol [Saleh et al., 2000]. The increased baroreflex sensitivity might help the adrenalectomized sheep treated with E\textsubscript{2} from cyclic changes in the MAP. However, the mechanism underlying this function remains to be explained.

In term of electrolytes, ADX ewes had significant lower sodium and higher potassium concentration from 12 hours after withdrawal of corticosteroids to the end of the experiment. In the ADX+E2 ewes, these changes only reached significance after 26h. E\textsubscript{2} appears to act on the kidney to reduce Na\textsuperscript{+} loss and K\textsuperscript{+} retention after steroid
withdrawal. The mechanism underlying the estrogen protective effect might be that both estrogen and aldosterone activate the same signal transduction pathway. Nascimento D.S. [Nascimento et al., 2003] reported that estrogen and aldosterone control the calcium-dependent chloride channel (CIC-2) chloride channel expression in rat kidney. The decreased renal expression of CIC-2 mRNA and protein observed in ovariectomized rats resorted to control levels after treatment with low doses of E2. Higher doses of estradiol lead to an even greater increase in CIC-2 mRNA and protein expression. Recent evidence points to protein kinase C isoforms as highly specific signal transduction pathway for both aldosterone and estradiol in epithelia. The end targets of this kinase activation are Na⁺/H⁺ exchange and K⁺ and Ca²⁺ channels [Harvey et al., 2001]. The effect of E₂ on ion channels may explain why adrenalectomized ewes with E₂ can keep plasma Na⁺ and K⁺ levels relatively stable.

Unlike the NOx data of Chapter 4, estrogen-treated ewes had significant higher level of NOx, as we expected. This may explain the fact that MAP in estrogen-treated ewes tended to be increased a greater degree after infusion of L-NAME. After infusion of L-NAME, NO production was decreased, which resulted in increased blood pressure. After infusion of L-NAME, the circulation NOx level tended to decrease, but there was no significant difference. This might be due to mistakes I made during the experiment.

In ADX group, there was a small response to phenylephrine, but estrogen-treated ewes showed better response to phenylephrine (p=0.054). This suggests that estrogen may maintain the vascular reactivity to vasoconstrictors. As discussed in Study I, the response to PE either before or after L-NAME did not differ between two groups.
The protective effects of estrogen are not likely to be due to estrogen effects on NOS or vascular responsiveness. The protective effect of estrogen is probably through kidney ion channel function or CNS effects on blood pressure control systems.

Table 5-1  Plasma cortisol, aldosterone, estradiol, protein concentration and packed cell volume 28 hours after withdrawal of corticosteroids. (ADX: adrenalectomized; E2: treated with 17β-estradiol.)

<table>
<thead>
<tr>
<th>Variable</th>
<th>ADX</th>
<th>ADX+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol (ng/mL)</td>
<td>0.3±0.1</td>
<td>0.5 ±0.4</td>
</tr>
<tr>
<td>Aldosterone(pg/mL)</td>
<td>12±0</td>
<td>12±0</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
<td>23±6</td>
<td>56±24*</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>4.9±0.7</td>
<td>6.8±0.4</td>
</tr>
<tr>
<td>PCV</td>
<td>38±9</td>
<td>36±9</td>
</tr>
</tbody>
</table>

Data are the mean±SD. *p< 0.05 compared with ADX.
Figure 5-1 Experimental design.

Figure 5-2 MAP after corticosteroids withdrawal. ADX(n=6), ADX+E2(n=6). (ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol.)
Figure 5-3 Mean arterial blood pressure over 3 minutes in each ewe (chi square test).

ADX (n=6), ADX+E2 (n=6). ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol. (ADX: adrenalectomized; ADX+E2: treated with 17 β-estradiol.)
Figure 5-4 The change in plasma potassium concentration after corticosteroids withdrawal. *P<0.05 compared with 0 hour (two way ANOVA). ADX(n=6), ADX+E2(n=6). (ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol.)

Figure 5-5 The change in plasma sodium concentration after corticosteroids withdrawal. *P<0.05, compared with 0 hour (two way ANOVA). ADX(n=6), ADX+E2(n=6). (ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol.)
Figure 5-6 Change in MAP after L-NAME. ADX(n=6), ADX+E2(n=6). (ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol.)

Figure 5-7 Plasma NOx level. * P<0.05, compare with ADX. ADX(n=6), ADX+E2(n=6). (ADX: adrenalectomized; ADX+E2: treated with 17β-estradiol.)
Figure 5-8 Pressor responsiveness (Vascular responsiveness was calculated as the mean arterial blood pressure in the first minute after infusion of phenylephrine minus mean arterial blood pressure for the 30 seconds before infusion of phenylephrine). PE₁: before administration of L-NAME; (p=0.054), PE₂: after L-NAME. ADX(n=6), ADX+E₂(n=6). (ADX: adrenalectomized; ADX+E₂: treated with 17 β-estradiol.)
CHAPTER 6
EFFECTS OF ESTROGEN ON EXPRESSION OF NITRIC OXIDE SYNTHASE ISOFORMS IN KIDNEY AND VASCULATURE IN HYPOADRENOCORTICISM

Introduction

Three isoforms of nitric oxide synthase have been identified: neural, inducible and endothelial nitric oxide synthase. It is proposed that estrogen can increase the activity and/or expression of endothelial nitric oxide synthase (eNOS) in a variety of models [Mendelsohn and Karas 1999; Moncada et al., 1993] The molecular mechanism(s) by which estrogen activates eNOS is/ are still unknown. (Chapters 1 and 2 for detailed discussion). The effect of adrenal corticosteroids on the production of nitric oxide has been primarily studied within the context of the anti-inflammatory actions of cortisol. Glucocorticoids reduce inducible nitric oxide synthase production by macrophages [Di Rosa et al., 1990]. It has been proposed that glucocorticoids inhibit iNOS, but not eNOS production in vascular endothelial cells [Radomshi et al 1990]. However, other studies have shown that adrenalectomy increases mRNA for nNOS and dexamethasone treatment decreases nNOS mRNA. Since there are no identified GRE (glucocorticoid response element) in the promoters for eNOS, iNOS or nNOS, the corticosteroid effect on NOS transcription appears to be mediated by response elements other than the GRE. In the case of glucocorticoid effects on iNOS, the effect appears to be via inhibition of NF-κB binding to DNA [Katsuyana et al., 1999]. This effect involves dimerization of GR (the activated glucocorticoid receptor) preventing (by a protein/protein interaction) the binding of transcription factor NF-κB to the NOS II promoter, thus, inhibiting the
induction of NOS II transcription [Kleinert et al., 1996]. The mechanism of glucocorticoid effects on eNOS and nNOS has not been investigated. Estrogen effects on eNOS also do not appear to be mediated by the ERE (estrogen response element). Estradiol effects may be mediated via AP-1, Sp1, and NF-κB [Forstermann et al., 1998]. Given the presence of glucocorticoid receptors in vascular smooth muscle, endothelia, skeletal muscle and some cells in the kidney, it is possible that glucocorticoid receptors might alter eNOS and nNOS expression, and this action may underlie some of the “permissive” effects of adrenal corticosteroids that are important for normal physiology (see Chapters 1 and 2 for detailed discussion).

There is evidence of interaction between cortisol and estrogen in several systems studied, including estrogen interaction with glucocorticoid feedback control of ACTH secretion, effects on the steroids on osteoclasts, and effects on hippocampal neuron growth and survival. Glucocorticoid receptors (GR) appear to antagonize ER effects at the AP-1 response element [Uht et al., 1997]. This suggests that an antagonism between GR and ER mediated effects on NO may normally exist in some target tissues during late pregnancy. The withdrawal of GR “tone” may be exacerbated, therefore, in a condition of high ER activity, such as in the peripartal period.

We therefore tested the possibility that estradiol and cortisol interact to control expression of NOS in vasculature and/or kidney, by examining the NOS expression after cortisol withdrawal in estradiol-treated ewes.

**Materials and Methods**

The present experiments were performed using tissues collected from all four groups of adult sheep of Study I, and in both groups of animals in Study II, as previously
described (Chapter 3). In study I: ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5). In uterine artery, ADX + E2 (n=4). (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17 β-estradiol.). In study II: ADX (n=6), ADX+ E2 (n=6). Briefly, all tissues were collected as quickly as possible from deeply anesthetized ewes. Endothelial cells and whole blood vessels were extracted for RNA immediately, renal cortex and renal medulla were rapidly frozen in liquid nitrogen and stored at -80°C until further analysis by Western blot and PCR as described in Chapter 3.

Statistical analyses were performed using ANOVA for data of Study I and t-tests for data of Study II as described for other values in Chapters 4 and 5.

Results

Expression of eNOS mRNA and Protein by Endothelial Cells

The results of Study I (Figure 6-1) showed that estrogen treatment upregulated the expression of eNOS mRNA by endothelial cells from uterine artery (p<0.05), whereas adrenalectomy downregulated its expression (p<0.05). In aortic endothelial cells. E2 did not significantly increase the eNOS mRNA expression in ADI ewes, but adrenalectomy downregulated its expression (p<0.05). The endothelial cells from both aorta and uterine artery showed higher eNOS mRNA level in ADX+E2 ewes compared to ADX ewes (P<0.05).

Expression of eNOS protein level showed the same pattern of decrease in ADX and increase with E2 treatment in the four types of endothelial cells as in eNOS mRNA. In endothelial cells of uterine artery, estrogen treatment significantly increased eNOS protein expression (p<0.05) and ADX significantly decreased eNOS expression (p<0.05). The overall effect of estrogen on eNOS protein expression was increased and overall
effect of ADX on eNOS expression was decreased in endothelial cells of aorta, although there were no significant differences in levels of eNOS protein between any two groups (Figure 6-2).

The results of Study II similarly show an increase in eNOS expression in the E2-treated vessels; in this study it was not possible to obtain mRNA for eNOS from endothelial cells but whole vessel eNOS mRNA shows a similar pattern: Expression of eNOS mRNA was significantly increased in mesenteric and uterine artery. However, the increase in expression of eNOS protein was only significantly increased in uterine artery (Figure 6-3,6-4).

Expression of iNOS and nNOS mRNA and Protein in Whole Blood Vessels

In aorta, estrogen treatment significantly increased iNOS and nNOS mRNA expression (p<0.05), and ADX significantly decreased iNOS and nNOS expression (p<0.05) (Figure 6-5,6-6). In renal artery, both E2 and ADX significantly decreased the iNOS and nNOS mRNA expression (p<0.05); the iNOS mRNA level in ADX+E2 ewes was significantly lower compared to ADX ewes (p<0.05) (Figure 6-5,6-6). In uterine artery, the ewes in the ADI+E2 group had significant lower iNOS and nNOS level (P<0.05); however, the ewes in the ADX group had high iNOS and nNOS level (p<0.05). The ewes in the ADX+E2 group had lower level of iNOS and nNOS compared to the ADX ewes (p<0.05) (Figure 6-5,6-6). In mesenteric artery, E2 upregulated the iNOS mRNA and ADX downregulated it (p<0.05) (Figure 6-5,6-6). The ewes in ADX+E2 group had lower iNOS and nNOS mRNA level compared to the ADI group, and also had lower nNOS level compared to the ADX ewes (p<0.05).

No iNOS or nNOS protein was detectable by Western blot in any of the blood vessels tested.
Expression of NOS mRNA and Protein in Renal Cortex and Renal Medulla

The expression of eNOS mRNA in renal cortex was significantly increased by adrenalectomy, however there was no effect of E2-treatment, either in ADI or ADX ewes (Figure 6-7). On the other hand, eNOS expression in the renal medulla was significantly increased by E2 treatment, but was not altered by ADX alone (Figure 6-8).

There were no significant differences in eNOS protein level in renal cortex and medulla, although eNOS protein in renal cortex tended to be higher in the E2-treated ewes (Figure 6-9).

In Study II eNOS mRNA expression in renal cortex and medulla was significantly increased by E2 treatment (Figure 6-10). However, there were no differences in expression of eNOS protein detected in either region of kidney (Figure 6-11).

There were no differences in expression of iNOS or nNOS mRNA in the renal cortex with steroid withdrawal or E2 treatment. The ewes in the ADX+E2 group had lower iNOS mRNA levels compared to ADI ewes, and nNOS expression was higher than in ADI and ADX ewes (Figure 6.7). In the adrenal medulla, there were no significant differences in iNOS mRNA expression among four groups, however nNOS levels were higher in the ADX and ADX+E2 ewes as compared to ADI ewes (Figure 6.8) (P<0.05).

There were no significant differences in nNOS protein expression in the renal cortex and renal medulla (Figure 6.12). iNOS protein was undetectable.

Discussion

We hypothesized that the expression of nitric oxide synthase would be increased after withdrawal of corticosteroids. Interestingly enough, our data showed the opposite results. The adrenalectomy significantly decreased the mRNA level of eNOS in the endothelial cells from the uterine artery and aorta. At the protein level, the results from
endothelial cells of four different blood vessels also showed the same decreasing trend in the ADX ewes. There are several candidate explanations for this. First, cortisol might not decrease the eNOS production. In 1990, Radomski, et al reported that hydrocortisone and dexamethasone inhibited the expression of the inducible but not the constitutive, nitric oxide synthase in vascular endothelial cells [Radomski et al., 1990]. Since then, there were no more papers supporting this results until 2002; Liao’s lab published that high-dose corticosteroids exert cardiovascular protection through a novel mechanism involving the rapid, non-transcriptional activation of endothelial nitric oxide synthase (eNOS) [Hafezi-Moghadam et al., 2002]. Binding of corticosteroids to the glucocorticoid receptor (GR) stimulated phosphatidylinositol 3-kinase and protein kinase Akt, leading to eNOS activation and nitric oxide dependent vasorelaxation [Hafezi-Moghadam et al., 2002]. This suggests that glucocorticoids increase the expression and/or activity of eNOS under a certain circumstances. However, additional studies are needed to provide evidence to support this hypothesis.

Second, aldosterone might have some unknown effect(s) on eNOS. So far, although several studies have done on the relationship between aldosterone and NOS, the effect of aldosterone on eNOS is still not clear [Salemi et al., 2001; Turban et al., 2003].

Third, the major regulator of eNOS gene expression in vivo is shear-stress which is exerted on the luminal side of the endothelium [Li et al., 2002]. Schafer and coworkers reported that in severe CHF, impaired left-ventricular function leads to reduce blood flow in conductance and peripheral arteries and therefore results in less shear-stress. This resulted in vascular eNOS expression is significantly attenuated [Schafer et al., 2003]. Our results are in concordance with this hypothesis. About 20 hours after withdrawal of
corticosteroids, the MAP of adrenalectomized ewes decreased about 20mmHg, which led to the reduced blood flow to the aorta, renal, uterine and mesenteric arteries. So there were less shear-stress on these endothelial cells, then dramatically reduced the expression of eNOS. In fact, in the vessels collected at 28h after steroid withdrawal (Study II) it was difficult to obtain sufficient mRNA for eNOS mRNA analysis; we believe this is because the extreme hypotension and very low shear stress after anesthesia and at the time of tissue collection.

Taking all of these theories together, although the withdrawal of corticosteroids might increase the expression of eNOS, the effect of shear-stress on eNOS might be dominant, so that the net effect of withdrawal of corticosteroids on eNOS is a decrease in expression, which may lead to low circulating NOx level due to low production of NO.

As we expected, E₂ treatment increased eNOS protein expression significantly in uterine artery and E₂ also tended to increase eNOS expression by endothelial cells in other vessels. Magness et al observed the same result in uterine artery [Magness et al., 1997]. Although, estrogen treatment increased eNOS production in uterine artery and other vessels showed the same trend, the MAP in estrogen-treated ewes did not significantly increase. This suggests that there might be other mechanism involve in blood pressure regulation besides NO. Stachenfeld et al. reported that estrogen administration increased AVP release by hypothalamus [Stachenfeld et al. 1998]. Derkx et al reported that estrogen increased angiotensinogen release by liver [Derkx et al. 1986]. Taken together, the effects of estrogen on blood pressure are more complicated and involve in multiple mechanism.
eNOS protein in renal medulla and renal cortex was not significantly increased by E2. eNOS mRNA was upregulated by estrogen treatment in renal medulla and estrogen treatment increased eNOS mRNA in the more severely hypoadrenal ewes in both renal cortex and medulla in study II. These data suggest that estrogen treatment and ADX may have different effect(s) on transcription and post-transcriptional modification. This difference between renal cortex and medulla may also due to the structure of endothelial cells is different between them [Mauller 1958]. This indicates a different influence of E2 on eNOS activity or expression in different tissues. This might be the reason that MAP had no significant difference among ewes in study I and II.

In addition, E2 and ADX alter the different NOS isoforms differently. ADX downregulated iNOS and nNOS mRNA expression in aorta, renal and mesenteric arteries. This suggests that shear-stress may have same effect(s) on iNOS and nNOS as its effect on eNOS. So far, a few papers of the effect of shear-stress on iNOS and nNOS have been published, but they are not consistent [Gosgnach et al. 2003; Schoen and Lautt 2001; Da Silva-Azevedo et al. 2002]. On the other hand, ADX upregulated iNOS and nNOS mRNA expression as we hypothesized that NOS expression was increase without inhibitory effect of glucocorticoid after withdrawal of corticosteroids. In the case of glucocorticoid effects on iNOS, the effect appears to be via inhibition of NF-κB binding to DNA [Katsuyama et al., 1999]. This effect involves dimerization of GR (the activated glucocorticoid receptor) preventing (by a protein/protein interaction) the binding of transcription factor NF-κB to the NOS II promoter, thus, inhibiting the induction of NOS II transcription [Kleinert et al., 1996]. The mechanism of glucocorticoid effects on eNOS and nNOS has not been investigated.
Estrogen treatment upregulated iNOS and nNOS mRNA in aorta, and downregulated iNOS and nNOS mRNA in renal artery and uterine artery. Therefore, the tendency for a decrease in circulating nitrate/nitrite concentrations, measured in the animals in Study I, reflects the combination of stimulatory effects of E2 on eNOS and inhibition of iNOS and nNOS, which are both tissue-dependent.

The results of second series of experiments also showed that estrogen treatment increased eNOS mRNA expression in uterine and mesenteric artery; it also had the same effect on renal cortex and medulla. eNOS protein level was also upregulated by estrogen treatment. The abundance of mRNA and protein of eNOS seemed much lower in the ewes of study II. These provided evidence to support the shear-stress hypothesis. These changes also might explain the higher plasma NOx levels in estrogen-treated ewes. Taken together the NOx results from Chapter 4 and 5, this suggests that the relationship between estrogen and plasma NO is still unknown.

![Figure 6-1 Study I Results: eNOS mRNA in endothelial cells from aorta (left) and uterine artery (UA, right). a, p<0.05, compared with ADI, b, p<0.05, compared with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+E2 (n=4). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17 β-estradiol).](image-url)
Figure 6-2 Study I Results: eNOS protein in endothelial cells of aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA). a, p<0.05, compared with ADI, b, p<0.05, compared with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5, n=4 in UA). ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17 β-estradiol).

Figure 6-3 Study II Results: eNOS mRNA in aorta, uterine artery (UA) and mesenteric artery (MA). *p<0.05 compare with ADX (t-test). (ADX: adrenalectomized; +E2: treated with 17 β-estradiol).
Figure 6-4 Study II Results: eNOS protein in endothelial cells of aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA). *p<0.05 compare with ADX (t-test). (ADX: adrenalectomized; +E2: treated with 17 β-estradiol).

Figure 6-5 Study I Results: iNOS mRNA in aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA). a, p<0.05, compared with ADI, b, p<0.05, compared with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17 β-estradiol.)
Figure 6-6 Study I Results: nNOS mRNA in aorta, renal artery (RA), uterine artery (UA), and mesenteric artery (MA). a, p<0.05, compared with ADI, b, p<0.05, compared with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol.)

Figure 6-7 Study I Results: eNOS iNOS and nNOS mRNA in renal cortex. a, p<0.05, compare with ADI; b, p<0.05 compare with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol.)
Figure 6-8 Study I Results: eNOS iNOS and nNOS mRNA in renal medulla. a, p<0.05, compare with ADI; b, p<0.05 compare with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol.)

Figure 6-9 Study I Results: nNOS protein in renal cortex (RC) and renal medulla (RM). a, p<0.05, compare with ADI; b, p<0.05 compare with ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+ E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17β-estradiol.)
Figure 6-10 Study II Results: eNOS mRNA in renal cortex (RC) and renal medulla (RM)  
*p<0.05 compare with ADX (t-test). ADX (n=6), ADX+E2 (n=6). (ADX: adrenalectomized; +E2: treated with 17 β-estradiol).

Figure 6-11. Study II Results: eNOS protein in renal cortex (RC) and renal medulla (RM)  
ADX (n=6), ADX+E2 (n=6). (ADX: adrenalectomized; +E2: treated with 17 β-estradiol).
Figure 6-12. Study I Results: nNOS protein in renal cortex (RC) and renal medulla (RM) ADX (two way ANOVA). ADI (n=4), ADI+E2 (n=4), ADX (n=4), and ADX+E2 (n=5). (ADI: adrenal gland intact; ADX: adrenalectomized; +E2: treated with 17 β-estradiol).
CHAPTER 7
SUMMARY AND CONCLUSIONS

The purpose of the studies described in this dissertation was to test the interaction between increased estrogen and increased cortisol on blood pressure regulation, and to determine whether the interaction between estrogen and cortisol is via nitric oxide synthase and/or plasma electrolytes. These studies investigated the effect of estrogen on hypoadrenal ewes.

The results of the studies presented in Chapter 4 suggest estrogen treatment did not decrease MAP and vascular reactivity in either eucorticoid or hypocorticoid ewes; estrogen treatment also did not increase the circulating NOx level either. Surprisingly, estrogen treatment reduced the increase in MAP after inhibition of NO production by L-NAME. Adrenalectomy decreased MAP and plasma sodium concentration but increased plasma potassium concentration, which suggests that the adrenalectomized animal model was successful.

The studies presented in Chapter 5 were designed to determine the effect of estrogen on animals in a severe hypocorticoid state (Addisionian crisis). These studies did not reveal that estrogen treatment exaggerate the decrease in MAP as we hypothesized. However, estrogen-treated ewes did not show cyclic patterns of MAP whereas half of non-estrogen treated ewes showed this change. Estrogen-treated ewes had less dramatic changes in plasma electrolyte concentration. The mechanism underlying the protective effect(s) of estrogen is still unknown. The candidate reasons might be 1. estrogen may increase the sensitivity of baroreflex [Saleh et al., 2000]. The increased
baroreflex sensitivity might help the adrenalectomized sheep treated with estrogen from cyclic changes in the MAP. 2. Estrogen may have effect(s) on ion channels in kidney, which may increase sodium reabsorption and potassium excretion [Nascimento et al., 2003]. 3. The protective effect of estrogen may be through central nervous system mechanisms controlling heart rate. Further studies will be required to provide evidence to support these hypotheses.

Although estrogen treatment had no effect on L-NAME responses in intact or non-hypotensive adrenalectomized ewes, estrogen treatment ewes increased MAP after inhibition of NO production by L-NAME in the more severely hypotensive adrenalectomized ewes, and estrogen treatment also increased the circulating NOx level as we expected. Taken together the NOx results from Chapter 4 and 5, this suggests that the relationship between estrogen and plasma NO levels is complex, and is influenced by changes in multiple isoforms of NOS in multiple tissues.

The results of the studies presented in Chapter 6 suggest estrogen treatment did increase eNOS mRNA and protein level in endothelial cells of different blood vessels whereas adrenalectomy decreased it. In renal medulla, estrogen treatment also increased eNOS mRNA. However, eNOS mRNA was increased by adrenalectomy in renal cortex and estrogen treatment resulted in a further increase in eNOS in both renal cortex and medulla in hypoadrenal ewes. The different results between renal cortex and renal medulla suggest estrogen and adrenalectomy may play different roles in different parts of kidney. The different pattern of eNOS mRNA in different parts of kidney may also due to the different structure of endothelial cells between renal cortex and medulla.
Estrogen treatment had different effects on iNOS and nNOS mRNA on different tissues. Estrogen treatment increased iNOS and nNOS only in aorta, and decreased iNOS and nNOS mRNA in renal artery and uterine artery. Like estrogen, adrenalectomy also had different effects on iNOS and nNOS mRNA on different tissues. Adrenalectomy decreased iNOS and nNOS mRNA in renal, mesenteric artery and aorta, but adrenalectomy increased iNOS and nNOS mRNA in uterine artery. The mechanism of this tissue-dependent effect of estrogen treatment and adrenalectomy on iNOS and nNOS mRNA is still unknown.

Although estrogen did increase eNOS expression in several vessels, it did not statistically alter MAP or the response to the inhibition of NOS. Although adrenalectomy caused a decrease in MAP, the expression of eNOS by endothelial cells from aorta, renal artery, uterine artery, and mesenteric artery was decreased rather than increased. This suggests that factors other than arterial endothelial NOS expression cause the decrease in blood pressure with corticosteroid withdrawal, and the protective effect of estrogen.

Together these studies, we conclude that estradiol treatment does not exacerbate the hypotension after corticosteroid withdrawal. Conversely, estradiol appears to buffer the effects of hypoadrenocorticism. Estradiol appears to act on the kidney to reduce sodium loss and potassium retention after steroid withdrawal.

Further experiments are needed to test: 1. the mechanism underlying the effect(s) of adrenalectomy on eNOS expression, 2. the mechanism of effects of estrogen on kidney ion channels and the interaction between estrogen and aldosterone on kidney ion channels, 3. the interaction between estrogen and aldosterone on vascular ion channels, 4. the mechanism of effects of estrogen on central nervous system, especially the vasomotor
center, 5. the mechanism underlying the cyclic change in MAP in severe hypoadrenal state, and 6. the mechanism of effects of estrogen on NO production.
LIST OF REFERENCES


Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 1999; 20; 358-417.


Krozowski ZS, Funder JW. Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc Natl Acad Sci USA* 1983; 80: 6056-6060.


Mendelsohn ME, Genomic and nongenomic effects of estrogen in the vasculature. Am J Cardiol 2002; 90(suppl): 3F-6F.


Schoen JM, Lautt WW. iNOS is not involved in shear stress-induced nitric oxide release, which triggers the liver regeneration cascade. _Proc West Pharmacol Soc._ 2001; 44:181-182.


Shlipak MG, et al. Estrogen and progestin, lipoprotein (a), and the risk of recurrent coronary heart disease events after menopause. JAMA 2000; 283: 1845-52.


BIOGRAPHICAL SKETCH

Feng Li was born and raised in China. She received her Bachelor of Medicine degree at the Shanghai Medical University in 1993. She received her Master of Pharmacology degree at the Shanghai Medical University in 1996. She came to Gainesville, FL, in August 1998 and joined Dr. Keller-Wood’s laboratory in June 1999.

In December 2003, she received the Doctor of Philosophy degree from the Department of Pharmacodynamics, College of Pharmacy, with a concentration in animal physiology.

Feng Li then began postdoctoral studies at the University of North Carolina at Chapel Hill.