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Abstract of Dissertation

Julye Marie Adams

The Graduate School

University of Kentucky

2005

**MODULATION OF THE ADRENAL MEDULLARY RESPONSE TO
STRESS BY ESTRADIOL IN THE FEMALE RAT**

ABSTRACT OF DISSERTATION

An abstract of a dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Physiology
at the University of Kentucky

By

Julye Marie Adams

Versailles, Kentucky

Director: Dr. Brian A Jackson, Professor of Physiology

Lexington, Kentucky

2005

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Abstract of Dissertation

MODULATION OF THE ADRENAL MEDULLARY RESPONSE TO STRESS BY ESTRADIOL IN THE FEMALE RAT

The present study has established that physiological concentrations of estradiol can modulate stress-induced increases in plasma epinephrine (EPI). In anesthetized female rats, insulin-induced hypoglycemia (0.25 U/kg) increased plasma EPI concentration to a significantly greater extent in 14-day ovariectomized (OVEX) rats compared to sham-operated controls. In 17 β -estradiol (E2)-replaced OVEX rats, the hypoglycemia-induced rise in plasma EPI was significantly reduced compared to OVEX rats. This suppression was due to both decreased adrenal medullary output and increased clearance of EPI. Adrenal venous EPI concentration was significantly reduced in OVEX+E2 rats, suggesting that EPI secretion from the adrenal medulla was decreased by E2 replacement. The underlying mechanism(s) of this apparent E2-mediated reduction in secretion could not be established since 1) the expression levels of the biosynthetic enzymes tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase were not affected in OVEX+E2 rats, suggesting that EPI biosynthesis is similar in these and OVEX rats; and 2) agonist-induced increases in intracellular Ca²⁺ were identical in isolated adrenal medullary chromaffin cells exposed to E2 (10 nM) or vehicle for 48 hr, suggesting that stimulus secretion coupling is unaffected by E2 treatment. In contrast, plasma clearance of EPI was significantly increased in OVEX+E2 rats.

Although 48 hr exposure to E2 had no effect on intracellular signaling in chromaffin cells, acute (3 min) exposure to micromolar concentrations of E2 dose-dependently and reversibly inhibited agonist-induced Ca²⁺ transients. Consistent with this observation, acute (30 min) infusions of E2 also significantly reduced the insulin-induced increase in plasma EPI in OVEX rats. These data demonstrate that physiological levels of circulating E2 can modulate hypoglycemia-induced increases in plasma EPI. This effect appears to be mediated by the steroid's influence on adrenal medullary EPI output and plasma EPI clearance; however the mechanism(s) underlying these E2-mediated modulations remain undetermined. This study has also established that acute exposure to supra-physiological levels of E2 can suppress hypoglycemia-induced increases in plasma EPI, due at least in part to inhibition of stimulus-secretion coupling.

Key Words: Estradiol, Stress, Adrenal Medulla, Epinephrine, Chromaffin Cell

Julye M. Adams
September 2, 2005

**MODULATION OF THE ADRENAL MEDULLARY RESPONSE TO
STRESS BY ESTRADIOL IN THE FEMALE RAT**

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Dissertation

Julye Marie Adams

The Graduate School
University of Kentucky

2005

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STRESS BY ESTRADIOL IN THE FEMALE RAT**

DISSERTATION

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By

Julye Marie Adams

Versailles, Kentucky

Director: Dr. Brian A Jackson, Professor of Physiology

Lexington, Kentucky

2005

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This work is dedicated to my entire loving family,
especially Da and Papaw. I miss you both everyday.

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Chapter 1: Introduction

Estrogen

Estrogen and progesterone are the main steroid hormones synthesized by and secreted from the ovary. Estradiol, the most biologically active form of estrogen, is produced by the granulosa cells of antral follicles and is responsible for various actions throughout the reproductive system. For example, estradiol induces the proliferation of granulosa cells in the ovary, the epithelial and stromal layers in the uterus, and the epithelium in the vagina. The development of the lactiferous duct system in the mammary glands is also dependent upon the presence of estradiol (Kacsoh, 2000).

Typically, estradiol acts through traditional steroid receptor mechanisms. Intracellular estrogen receptors, which act as transcription factors, bind estradiol and dimerize in the cytoplasm of the target cell. The estrogen receptor dimer translocates to the nucleus and binds estrogen-responsive elements located in the promoter region of estrogen-responsive genes, thereby regulating transcription of these genes. There are at least two subtypes of estrogen receptors, the classic estrogen receptor alpha (ER α), and the more recently discovered estrogen receptor beta (ER β). Although ER α and ER β share a high degree of sequence homology, they are the product of two different genes and exhibit tissue/cell-specific expression and action (Matthews and Gustafsson, 2003 for review). In addition to acting via this traditional, relatively slow-onset genomic transcription/translation signaling pathway, several studies have demonstrated that estrogens can also initiate rapid activation of G-protein-coupled receptor signaling systems, perhaps by binding to distinct pools of estrogen receptors which locate to caveolae regions of the plasma membrane (Hall et al., 2001; Nadal et al., 2001; Levin, 2002). For example, acute exposure to estradiol stimulates a rise in intracellular Ca²⁺, and activates the protein-kinase A and protein-kinase C signaling pathways in female colon cells, while in smooth muscle cells and atrial/ventricular myocytes, short-term estradiol exposure attenuates voltage-dependent Ca²⁺ current (Levin 1999 for review; Kelly and Levin, 2001).

Extra-Reproductive Effects of Estrogen

Although primarily regarded as the female sex hormone, estradiol acts beyond the scope of the reproductive axis at various sites, including bone, liver, kidney, brain, heart, and vasculature. For instance, estradiol induces elongation of bone, the closure of the epiphyseal plates, and prevents bone resorption. This latter benefit on bone remodeling is lost at menopause, a time in normal female aging when estradiol secretion declines. This permanent decrease in circulating estradiol concentration is often accompanied by increased osteoporosis and bone fragility in postmenopausal women (Lindsay, 1996). Recent studies have shown that hormone replacement therapies, which include estrogens, can decrease the incidence and severity of osteoporosis in post-menopausal women (Lawrenson et al., 2005; Stevenson, 2005).

Several studies have shown that a number of health problems confronted by older women can be linked to the deficiency of circulating estradiol after menopause. For example, increases in the prevalence of heart disease and the incidence and severity of hypertension in post-menopausal women are reduced by hormone replacement therapy (Kannel et al., 1976; Pines et al., 1996; Prevlavic et al., 1997; Wassertheil-Smoller et al., 2000). The mechanisms underlying these benefits are quite diverse, and include estrogenic effects on lipid and lipoprotein metabolism and both vascular reactivity and growth (Prevlavic et al., 1997; Dubey et al., 2001, 2004; Mendelsohn et al., 2005). Additionally, estradiol replacement also lessens the risk and/or delays the onset of neurodegenerative conditions such as Alzheimer's disease (Henderson et al., 1996; Asthana et al., 1997; Kawas et al., 1997; Baum, 2005), and provides cognitive benefits (see Gleason et al., 2005 for review) in post-menopausal women.

Not all data are consistent with the concept that estrogen is protective against these types of illnesses. While it is widely accepted that high doses of estradiol can be harmful, increasing the risk of breast cancer, venous thrombosis, stroke, and pulmonary embolism (Gillum et al., 2000), clinicians with the Women's Health Initiative (WHI), a study which has enrolled more than 160,000 participants overall, reported in 2002 that post-menopausal women receiving lower dose hormone replacement therapy (a combination of estrogen and progesterone) also have a higher risk for heart disease,

stroke and blood clots compared to post-menopausal women receiving placebo treatment (Pradhan et al.) In 2004, it was reported that post-menopausal women receiving estrogen-only therapy are also at a greater risk for blood clots, stroke and dementia than those in the placebo control group (Anderson et al.). In light of these reports, doctors halted all hormone and estrogen-only treatment studies in the WHI. It should be noted, however, that the estrogens used in these replacement therapies were conjugated equine estrogens, which can adversely affect the liver (Turgeon et al., 2004), that the therapies were given orally, which can affect hemostasis (Scarabin et al., 1997; Luyer et al., 2001), and that the women who were enrolled in these studies were well past the onset of menopause (average age of 63 and 65 years old) when they began the therapies. Given these cohorts and the fact that the levels of estrogen administered orally in hormone and estrogen replacement therapies are typically non-physiologic, even in low-dose regimes, the effects of physiologic levels of estradiol in younger individuals have remained undefined.

Several basic science studies have investigated the effects of estradiol in more controlled paradigms. Dubal and Wise (2001) have demonstrated that both estrous and pro-estrous concentrations of estradiol replacement in ovariectomized rats protects against infarct injury during ischemic stroke in both young and middle aged rats. In these studies, estradiol protected against stroke-induced injury by inhibiting infarct-induced cell death. High doses of estradiol are also protective against ischemic brain injury, as demonstrated by Yang et al. (2000). In these studies, the injection of estradiol up to 3 hours after the initial injury attenuated infarct-induced damage by modulating both cerebral blood flow and the production of nitric oxide.

Beneficial/protective effects of estradiol have also been demonstrated in the cardiovascular system. Chronic estrogen treatment lowers blood pressure in both transgenic and spontaneously hypertensive rats (Brosnihan et al., 1997; Crofton and Share, 1997; Li et al., 1997). Pressor responses to phenylephrine are also reduced in chronically estradiol-treated ovariectomized rats, while indices of baroreflex sensitivity are greater than in vehicle treated controls (He et al., 1998). This latter conclusion is supported by a study in conscious mice, which demonstrated that estradiol facilitates baroreflex function, perhaps by modulating angiotensin II-mediated effects on the

cardiovascular system (Pamidimukkala et al., 2003). He et al. (1998) also demonstrated that an acute, intravenous injection of estradiol lowers heart rate, and renal and splanchnic nerve activity in ovariectomized rats compared to vehicle-treated controls.

Estrogen and Stress

Stress responses are affected by estradiol in a variety of manners. For example, post-menopausal women have increased behavioral responses to mental stress compared to pre-menopausal women, and estrogen therapy attenuates this increase (Lindheim et al., 1992; Owens et al., 1993). These conclusions are supported by studies in experimental animals, which have shown that physiological concentrations of estradiol reduce anxiety and depressive behavior in rats subjected to open field, elevated plus maze, and forced swim tests (Lunga and Herbert, 2004; Walf and Frye, 2005). The activity of the hypothalamic-pituitary-adrenal axis (HPA axis) is also modulated by the presence of estradiol. Clinical studies have shown that cortisol levels are significantly decreased after menopause and are restored with estrogen replacement therapy (Helgason et al., 1981). In the rat, basal plasma levels of both ACTH and corticosterone are lower in ovariectomized rats than in intact controls and estradiol replacement returns both hormones to control levels (Burgess and Handa, 1992, Seale et al., 2004, Lunga and Herbert, 2004, Serova et al., 2005). In contrast, immobilization stress-induced increases in plasma ACTH concentrations are significantly lower in estradiol-replaced ovariectomized rats than in their controls (Serova et al., 2005).

Adrenal Medullary Response to Stress

As first proposed by Cannon in 1934, it is now well established that the adrenal gland is vital in the maintenance of overall body homeostasis during times of stress. Stressors such as trauma (Hammond et al., 1956; Barton et al, 1981), hypotension (Lilly et al., 1982; Bereiter et al., 1984), cold (Gordon et al., 1966; LeBlanc et al., 1967; Kanayama et al., 1999), hypoxia (Fowler et al., 1961; Bloom et al., 1977), and hypoglycemia (Nijima, 1989) result in a marked increase in both adrenal medullary catecholamine and adrenocortical glucocorticoid secretion, resulting in adaptation to the

stressor by eliciting diverse cardiovascular, metabolic, and immunomodulatory effects (Axelrod and Reisine, 1984; Becker, 1986; Ewbank 1992). Furthermore, tolerance to such stressors as exercise (Winder et al., 1987), hemorrhage (Bond and Johnson, 1984), and hypoxia (Nahas et al., 1954) is severely reduced in adrenalectomized and/or adrenal de-medullated animals.

The principal catecholamines, epinephrine (EPI) and norepinephrine (NE) are synthesized by and released from adrenal medullary chromaffin cells, which are considered functionally similar to postsynaptic sympathetic neurons. However, these cells do not have axons, and therefore function in a more endocrine manner by secreting epinephrine and norepinephrine directly into the blood stream. Catecholamine biosynthesis occurs within the cells through a series of enzymatic reactions, with the conversion of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase as the initial and rate-limiting step. DOPA is then converted to dopamine (DA) by dopa decarboxylase (DDC) and DA is converted to NE by dopamine-beta-hydroxylase (DBH). Lastly, NE is converted to EPI by phenyl-ethanolamine-N-methyltransferase (PNMT).

It is well established that there are two separate populations of chromaffin cells (Moro et al., 1990) characterized by the presence or absence of this latter enzyme. The ratio of PNMT containing (EPI secreting) cells to non-PNMT containing (NE secreting) cells is species dependent. For example, the ratio of EPI secreting to NE secreting cells in the rat adrenal medulla is approximately 4:1 (Verhofstad et al., 1985; Tomlinson et al., 1987), while the ratio is approximately 1:1 in the pig (Verhofstad et al., 1989). In humans, approximately 80% of adrenal medullary chromaffin cells are EPI secreting while just 20% are NE (Vollmer, 1996 for review).

Catecholamines are stored in vesicles until their release, which occurs when a stress-induced increase in splanchnic nerve activity stimulates secretion from the chromaffin cell. Mechanistically, acetylcholine, released from the adrenal nerve (a branch of the splanchnic nerve), induces sodium (Na^+) and calcium (Ca^{2+}) influx via nicotinic receptor-operated ion channels (Wada et al., 1985). The resultant depolarization activates voltage gated Ca^{2+} channels and results in a transient rise in intracellular free Ca^{2+} concentration, which is the primary trigger for catecholamine

exocytosis (Kilpatrick et al, 1982). The rise in intracellular Ca^{2+} can also increase the activity of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthetic cascade and therefore enhance catecholamine biosynthesis, which allows chromaffin cells the ability to maintain catecholamine stores in cases of increased secretion and maintain the cellular catecholamine content at a relatively constant amount, a process described as stimulus-synthesis-secretion coupling (Wakade et al., 1988; Malhotra et al., 1989).

Estradiol and the Adrenal Medullary Response to Stress

While the response of the HPA axis to a stressor is usually enhanced by estradiol, clinical studies indicate that stress-induced increases in plasma catecholamines are lower in post-menopausal women receiving estrogen replacement therapy (Del Rio et al., 1993; Komesaroff et al., 1999; Ceresinsi et al., 2000; Sandoval et al., 2003). Despite these persuasive clinical observations, the mechanism(s) underlying this estrogen-dependent suppression of stress-evoked catecholamine responses, and more specifically the effects of normal circulating levels of estrogen on this response, have not been convincingly established. Conclusions from studies in the rat are contradictory and may be dependent upon several factors, including the type of stressor utilized and the age, weight, and strain of the rat. For example, the hypoglycemia-induced increase in plasma catecholamine concentration is attenuated in female rats compared to weight-matched males (Drake et al., 1998), while foot-shock evoked increases are enhanced in females compared to age-matched males (Weinstock et al, 1998).

The prospect that estradiol can affect adrenal medullary function is supported by a report which identified ER α immunoreactivity in chromaffin cells of the female but not male rat adrenal gland (Green et al., 1999). However, information regarding estrogen effects on catecholamine biosynthesis is both limited and inconclusive. For example, it has been reported that daily injections of a high dose of estradiol for 3 days increases adrenal medullary epinephrine content in ovariectomized rats (Fernandez-Ruiz et al., 1988), and that 10 days of estrogen treatment in intact female rats increases the activity of adrenal tyrosine hydroxylase (TH), the rate limiting enzyme in the biosynthetic cascade (Kohler et al., 1975). In contrast, TH activity was reported to be unaffected by

estrogen treatment in ovariectomized rats (de Miguel et al., 1989). More recently, Serova et al. (2005) have demonstrated that TH mRNA levels are increased in the adrenal medulla of non-stressed, estradiol-treated ovariectomized rats compared to vehicle-treated controls. Conversely, the treatment paradigm utilized in this study also inhibited the stress-induced increase in TH mRNA levels seen in the control group.

It is now accepted that in addition to acting via traditional steroid-receptor signaling pathways, members of the steroid receptor superfamily, can rapidly affect cell function (Losel et al., 2003 for review). Previous studies from our laboratory have identified crosstalk between the adrenal stress hormones, establishing that both acute and long-term exposure to physiologic concentrations of glucocorticoids can modulate catecholamine stimulus-secretion pathways *in vitro* in both rat and pig chromaffin cells. Notably, these effects were found to be diametrically opposite: long-term exposure to physiologic concentrations of the synthetic glucocorticoid dexamethasone potentiates voltage-gated Ca^{2+} channel current, consequently potentiating agonist-induced intracellular Ca^{2+} transients (Fuller et al., 1997a,b), while acute exposure to dexamethasone dose-dependently attenuates voltage-gated Ca^{2+} channel current and the subsequent intracellular Ca^{2+} transient (Wagner et al., 1999).

Several *in vitro* studies have demonstrated that relatively high concentrations of estrogen can also acutely and therefore, presumably non-genomically, modulate adrenal medullary function. For example, in the isolated perfused rat adrenal gland, nicotinic agonist-induced catecholamine secretion is suppressed within minutes of exposure to 17α -estradiol (Park et al., 1996). Similarly, short-term exposure to 17α -estradiol suppresses secretory responses to both nicotinic agonists and to direct depolarization with KCl in isolated perfused bovine and feline adrenal glands (Lopez et al., 1991). Mechanistically, the KCl data combined with the fact that steroid treatment also suppresses agonist-induced $^{45}\text{Ca}^{2+}$ uptake in isolated bovine chromaffin cells, suggests that the primary site of estradiol action may be the voltage-gated Ca^{2+} channel. Consistent with this concept, Kim et al., (2000) have demonstrated that short-term exposure to 17β -estradiol suppresses KCl-induced [^3H] nor-epinephrine secretion and intracellular Ca^{2+} transients in the PC-12 cell line, an established model of the medullary chromaffin cell. At least in this model, channel-selective antagonist studies

demonstrated that estrogen inhibited both L- and N-type voltage-gated Ca^{2+} channels. Evidence in favor of an acute suppressive effect of estradiol on catecholamine secretion *in vitro* is not unanimous however. Dar and Zinder (1997) have reported that, compared to the effects of progestins and androgens, estrogens in general have relatively little effect on agonist-induced catecholamine secretion in bovine chromaffin cells. While the rapid-onset effects of estradiol on catecholamine secretion described above are consistent with a non-genomic mechanism of hormone action, it is not clear whether the effects have physiological significance, since the concentrations of steroid used in all of the these studies far exceeds normal plasma levels of estrogen in the rat (Smith et al., 1975).

Based on these data that non-physiological levels of estrogen can modulate the adrenal medullary response to stress in post-menopausal women and that acute exposure to high levels of estradiol can affect stimulus-secretion coupling in the chromaffin cell, the primary goal of the studies presented in this dissertation was to specifically determine whether physiological levels of estradiol can affect stress-induced increases in plasma catecholamine concentration. The working hypothesis to be tested was that ***physiological concentrations of estradiol attenuate the stress-induced increase in plasma epinephrine concentration by directly suppressing secretion from the adrenal medullary chromaffin cell.***

Chapter 2: Materials and Methods

Animals and Experimental Treatments

All animal protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee and utilized 12-14 week-old female Sprague-Dawley rats. Animals were housed individually in a room with controlled temperature (24°C) and light (14:10-h light-dark cycle) and given free access to food and water. Bilateral ovariectomy (or sham-surgery) was performed under isoflurane anesthesia via two small flank incisions. For estradiol replacement, capsules containing either 17 β -estradiol or vehicle were implanted subcutaneously at this time. The implanted capsules were made in accordance with previously published protocols (Wise et al., 1981; Dubal et al., 2001), by injecting 0.07 mL of either 17 β -estradiol (1 mg/mL; Sigma; St. Louis, MO) or sesame oil into 30 mm lengths of SILASTIC brand tubing (0.062/0.125 in inner/outer diameter; Dow Corning; Midland, MI). Each capsule was capped at both ends with 5 mm wooden applicator sticks.

Uterine weight and the change in overall body weight 14 days after surgery are listed in Table 2.1. Plasma estradiol concentrations measured 14 days after surgery are displayed in Figure 2.1. Ovariectomized rats gained more overall body weight and had significantly lower uterine weights and plasma estradiol concentrations than the sham-operated controls. Uterine weights and plasma estradiol concentrations were significantly greater and changes in body weight were significantly reduced in the estradiol-replaced group compared to the ovariectomized group. Estradiol replacement produced plasma estradiol concentrations equivalent to those of sham rats during the proestrous phase of the estrous cycle. Uterine weights and changes in body weight also were not different between these two groups.

In Vivo Studies

The primary catecholamine secreted from the adrenal medulla is epinephrine (EPI; Vollmer, 1996). Therefore, plasma EPI concentrations in response to stress were determined in all treatment groups.

Insulin-induced hypoglycemia was used as a stressor to assess the effects of estradiol on increases in plasma EPI. This well established paradigm elicits consistent increases in plasma EPI levels in both experimental animals (Vollmer et al., 1997; Drake et al., 1998) and humans (Diamond et al., 1993; Davis et al., 2000).

A. Hypoglycemic-Stress Model

1. Measurement of Stress-Induced Plasma Epinephrine Concentrations

For these experiments, overnight-fasted rats were anesthetized with inactin (100 mg/kg, i.p.; Sigma) and following tracheotomy, the left femoral artery and femoral vein were catheterized with PE 50 tubing. Core body temperature was maintained at 36.7°C by heat pad/lamp, and blood pressure and heart rate were recorded with a Grass Model 7 polygraph (Grass Instruments Co.; Quincy, MA) for the duration of the experiment. After a 30 min equilibration period, a baseline arterial blood sample (0.6 mL) was taken and a bolus of insulin was injected via the femoral vein (Figure 2.2). Additional blood samples were taken 30 and 60 min post-insulin. Blood volume was replaced with 0.9% sterile saline. A small volume (10 μ L) from each heparinized sample was used to measure blood glucose concentrations by a OneTouch Ultra glucose monitoring system (glucometer; Lifescan-Johnson & Johnson; Milpitas, CA), and the sample was centrifuged and the plasma was stored at -80°C for later EPI analysis by enzyme-linked immunosorbent assay (ELISA). Preliminary experiments established that blood-sampling performed in the absence of insulin-induced hypoglycemia did not increase plasma epinephrine concentration above baseline (Figure 2.3).

Preliminary experiments also examined the effects of two concentrations of insulin on plasma EPI concentration. Both 0.25 U/kg and 1.0 U/kg of insulin elicited decreases in plasma glucose concentrations (Δ -67 \pm 2.1% and >-76 \pm 1.2%, respectively) and increases in plasma EPI concentrations (5 fold and 30 fold, respectively) after 30 min of hypoglycemia. (It should be noted that, in several rats, 1.0 U/kg of insulin lowered plasma glucose to below the detectable range (< 20 mg/dL) of the glucometer after 30 min of hypoglycemia.) Both the decrease in plasma glucose and the increase in plasma EPI concentrations were significantly greater in the 1.0 U/kg insulin-treated compared to the 0.25 U/kg insulin treated rats (Figure 2.4; P <0.01). After

60 min, plasma glucose and EPI concentrations returned toward baseline in 0.25 U/kg insulin-treated rats. Glucose and EPI concentrations in the 1.0 U/kg insulin-treated rats did not differ after 60 min of hypoglycemia compared to 30 min, and were significantly different than the 0.25 U/kg insulin-treated group at 60 min ($P < 0.01$). All subsequent experiments utilized the sub-maximal stress effects of the 0.25 U/kg dose of insulin to determine the effects of estradiol on hypoglycemia-induced increases in plasma EPI concentration.

2. Measurement of Adrenal Medullary Epinephrine Output

For these experiments, a method for the collection of adrenal venous blood previously published by Vollmer et al. (2000) was modified for use with the basic hypoglycemia protocol. After the femoral cannulations, a peritoneal incision was made and the abdominal wall retracted to reveal the left kidney, adrenal gland, and surrounding vasculature. Ligatures were tightened around the renal vein and artery just proximal to the hilus of the kidney. A loose (non-tightened) ligature was placed around the renal vein just distal to the vena-cava. Visible venous branches were either ligated or cauterized so that only adrenal venous flow entered the renal vein. A bolus dose of heparin (300 U/kg) was injected intravenously and a small length (9-10 mm) of capped PE 50 tubing filled with heparinized saline was placed in the renal vein distal to the adrenal vein branch. Insulin was injected after a 30 min equilibration period. After 24 min of hypoglycemia, the loose ligature at the vena cava was tightened and renal/adrenal venous flow was allowed to backflow through the uncapped renal vein catheter and was collected in a pre-weighed 1.5 mL microcentrifuge tube. The collection continued for 6 min and was weighed to quantify a flow rate which was used to determine the adrenal medullary secretion rate. No arterial blood samples were taken during these experiments.

3. Measurement of Sympathetic Nerve Activity (SNA)

The basic hypoglycemia model was modified in order to record renal nerve activity in accordance with previously published methods (Randall et al., 1994; Brown et al., 1999; Stocker et al., 2004). After the femoral cannulations, a retroperitoneal incision

was made and the left kidney was retracted allowing the isolation of a sympathetic renal nerve. After placing the nerve on a stainless steel wire electrode and covering both the nerve and wire with a silicon-based impression material (Super-Dent Light, Darby Dental Supply; Westbury, NY), the electrical signals from the nerve were obtained using a high-impedance probe and were amplified (50,000) and band-pass filtered (between 30-3,000 Hz) by a Grass P511 differential amplifier. The data were digitized at 10,000 samples/sec using a Cache 486 microprocessor and Data Translation DT2821-F analog-to-digital converter. Nerve signals were recorded for 20 min before and 60 min after an insulin bolus. The raw nerve signal was full-wave rectified and integrated (1 sec time constant) using software developed by Dr. David Brown in Visual C++ (Figure 2.5). Background noise was determined by the average value of integrated voltage over 5 min after a bolus injection of 1M KCl (3 mL/kg; i.v.) to euthanize the rat. No arterial blood samples were taken during these experiments.

Values for renal sympathetic nerve activity were measured as 1 min averages every 5 min after the insulin bolus. This activity was expressed as a percent change from an average baseline value that was calculated from 3 separate 1 min averages 10, 5, and 1 min before insulin. Background noise was subtracted from all 1 min averages prior to calculating the percent change.

B. Measurement of Epinephrine Clearance

For these experiments, methods from Azoui et al. (1997) and Marker et al. (1998) were modified (Figure 2.6) and rats were infused with 3 increasing doses of EPI for 30 min each, equaling a total infusion time of 90 min. Rats were anesthetized with inactin (100 mg/kg, i.p.) and following tracheotomy, the left femoral artery and left and right femoral veins were catheterized with PE 50 tubing. Core body temperature was maintained at 36.7°C by heat pad/lamp and blood pressure and heart rate were recorded with a Grass Model 7 polygraph for the duration of the experiment. After a 30 min equilibration period, a baseline arterial blood sample (0.6 mL) was taken and an intravenous infusion of EPI (Sigma) was started at a rate of 600 pmol/kg/min. After 30 min of infusion, an arterial blood sample (0.6 mL) was taken and the EPI infusion rate was increased to 1500 pmol/kg/min by change-out of the syringe on the infusion pump.

This ensured that the infused volume over 30 min remained consistent for all doses. An arterial blood sample was taken after 30 min and the EPI infusion rate was increased to 3000 pmol/kg/min. The last blood sample was taken 30 min later. Each heparinized blood sample was centrifuged and plasma was stored at -80°C for later catecholamine analysis by ELISA.

***In Vitro* Studies**

A. Rat Adrenal Medullary Chromaffin Cells

1. Chromaffin Cell Isolation

Chromaffin cells were isolated from the adrenal medullae of 12-14 week-old female Sprague-Dawley rats as previously described by Fuller et al. (1997a). Adrenal glands were removed from CO₂-euthanitized rats and submerged in warmed (37°C) Hank's balanced salt solution (HBSS 7.4). The adrenal medullae were dissected from the cortical tissue and cut into four pieces and washed in HBSS. The tissue was then suspended in Ca²⁺/Mg²⁺-free HBSS containing 0.3% collagenase D, 0.15% hyaluronidase, and 0.02% DNase, and transferred to a spinner flask for digestion. The medullae pieces were incubated at 37°C for approximately 30 min with gentle spinning and with trituration by a fire polished glass pipette every 10 min to break up chunks of tissue. The suspension was checked several times during the digestion for single cells, 30 min generally yielded a majority of healthy single cells. The cell suspension was then filtered through a 200 µm screen into a 15 mL centrifuge tube and the cells were washed in enzyme-free, Ca²⁺/Mg²⁺-free HBSS and centrifuged at 1500 rpm for 3 min. The cells were resuspended in DMEM containing 10% fetal bovine serum, 40 mg/L gentamicin, 100,000 U/L penicillin, 40,000 U/L nystatin, 4 g/L ascorbate, and 10 µM cytosine arabinose. Aliquots of the cell suspension were allowed to adhere to poly-L-lysine coated glass cover slips in 6-well plates for 30 min in a 37°C incubator, after which 0.5 mL of culture medium was added to the wells and the cells maintained in a humidified, 37°C environment of 5% CO₂. Culture medium was changed 24 hr after plating, and cells were used 48 hr after isolation.

2. Intracellular Calcium Measurements

Intracellular Ca^{2+} transients were analyzed using established protocols as previously described (Liu et al., 2001; Jorgensen et al., 2002). In each experiment, cytosolic Ca^{2+} was measured simultaneously in 5-12 chromaffin cells loaded with the fluorescent dye fura-2 using a dual-excitation spectrofluorometric system (Zeiss AttoFluor Ratio Vision Workstation, Atto Instruments, Inc.; Rockville, MD). Before experimental analysis, the cells were rinsed 3X with HBSS and loaded with 5 μM fura-2AM plus 0.1% Pluronic F-127 for 45 min at 37°C. After this time, the cells were rinsed again with HBSS and allowed to recover for at least 15 min. The cover slip was mounted in a closed perfusion chamber (Warner Instrument Corp.; Hamden, CT), placed on the stage of a Zeiss Axiovert inverted microscope fitted with a 40X fluorescence oil immersion objective, and constantly perfused by gravity-feed with ambient temperature HBSS at 1-3 mL/min.

For these experiments, non-stimulated (basal) fluorescence was measured for approximately 30 sec, an agonist was added by a rapid change-out of the bathing medium, and was removed after 15 sec by a second bath change-out. There was a 10 min recovery period before each re-stimulation. Fluorescence was determined using excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. Ratiometric data were collected and converted to cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) estimates based on a curve generated using a two-point calibration method and the following equation (Grynkiewicz et al., 1995):

$$[\text{Ca}^{2+}]_i = K_d [(R - R(\text{min}) / R(\text{max}) - R)] \times [\text{Den}(\text{min})/\text{Den}(\text{max})]$$

where R = measured ratio; K_d = dissociation constant for the dye; R(min) = ratio for the low standard (Ca^{2+} -free solution); R(max) = ratio for high standard (10 mM Ca^{2+} solution); Den(min) = denominator intensity for the low standard; Den(max) = denominator intensity for the high standard.

B. NCI-H295R cells

1. Cell Culture

An aliquot of the adrenal cortical cell line NCI-H295R was obtained from American Type Culture Collection (ATCC; Manassas, VA) and the cells were grown

according to the product information sheet supplied. Cells were maintained in complete growth medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium + L-glutamine (2.5 mM), HEPES (15 mM), sodium pyruvate (0.5 mM), and sodium bicarbonate (1.2 g/L) , supplemented with 2.5% Nu-Serum (BD Biosciences) and the additives insulin (6.25 µg/mL), transferrin (6.25 µg/mL), selenium (6.25 µg/mL), bovine serum albumin (1.25 mg/mL), and linoleic acid (5.35 µg/mL) in a humidified, 37°C environment with 5% CO₂. Cells were subcultured weekly at a ratio of 1:4 using a solution of 0.25% Trypsin-EDTA.

2. cAMP Accumulation

Incubations were conducted on H295R cells in 24-well plates (200,000 cells/well). The well plates were placed in a 37°C water bath and the culture medium removed and replaced with warmed pre-incubation medium (0.25 mL) consisting of Eagle's Balanced Salt Solution (EBSS, pH 7.4) supplemented with the cAMP phosphodiesterase inhibitor Ro20-1724 (25 µM; Calbiochem; La Jolla, CA). After 10 min of pre-incubation, warmed incubation medium (0.25 mL) containing either vehicle or agonist in pre-incubation medium was added to the wells. The medium was removed after 10 minutes of incubation and 0.25 mL of HCl (0.01 M) was added to the wells. For protein analysis, 5% SDS (0.3 mL) was added in place of HCl to at least 4 wells from every experimental plate. The plates were stored at -80°C for later analysis by enzyme immunoassay (EIA).

Analytical Techniques

A. Plasma Epinephrine ELISA

Plasma concentration of free EPI was determined by a non-competitive, monoamine specific ELISA kit (KMI-IBL; Hamburg, Germany). EPI was extracted from kit standards, kit controls and plasma samples (0.25 mL) and was chemically converted to biotin derivatives. Basal samples were diluted 1:2, 30 min and 60 min samples were diluted 1:5 with HCl provided in the kit. Standards, controls and diluted samples (0.05 mL) were added in duplicate to the antigen-coated wells of a 96-well plate and the extracted EPI was enzymatically methylated and bound to antigens for N-

acetylmetanephrine. After a wash step, the wells were incubated in a solution of anti-biotin antibodies conjugated with alkaline phosphatase for 90 min, followed by second wash step. The sandwich complex fixed to the wells was visualized with a signal amplification system, which utilizes NADPH as the substrate for alkaline phosphatase and allows the conversion of p-iodonitrotetrazolium (colorless) to formazan (hot pink/red). The reaction was stopped after 15 min by the addition of H_2PO_4 and the developed color intensity was measured at 490 nm (reference wavelength 600-650 nm) within 1 hr using a 96-well plate reader (μ Quant, Bio-Tek Inc; Winooski, VT). All incubation steps required constant shaking (400-600 rpm) and were performed at room temperature. It was essential for assay precision that the washes utilize an auto strip washer (ELx50, Bio-Tek). Concentrations of samples were determined with the aid of KC-4 software (Bio-Tek), which interpolated the mean measured absorbancies against the standard curve (Figure 2.7) on each plate. All comparisons were intra assay and the coefficient of variation (c.v.) was $9.8 \pm 1\%$.

B. Measurement of Plasma Estradiol Concentration

1. Estradiol Extraction

Estradiol was extracted from rat plasma samples prior to assay by diethyl ether extraction. Samples (0.2 mL) and diethyl ether (3 mL) were added to 16X125 mm vortex tubes in duplicate and tightly capped. Samples were vigorously vortexed for 5 min and the aqueous layer was frozen in an ethanol/dry ice bath. The ether supernatant was decanted into 12X75 mm tubes, the ether evaporated and the tubes completely dried. This process was accelerated by heating the samples to 40°C and placing under a vacuum. The tubes were rinsed with ether and dried three additional times to ensure that the extracted estradiol accumulated at the bottom of the tube. A set of clean 12X75 mm tubes used as standard and control assay tubes were washed with ether and dried at the same time as the samples.

2. Estradiol RadiolImmunoAssay

Plasma concentration of free estradiol was determined by an estradiol-specific RIA kit (DSL; Webster, TX). After the final wash down and evaporation step of the

extraction, the samples were reconstituted in 0.2 mL of the zero standard from the kit and the appropriate volumes of kit-provided standards or controls were added to the ether washed, empty tubes. Tubes (except the total count and non-specific binding) were incubated with estradiol-antiserum (4 hr, 4°C), and I-125 labeled estradiol was added to all assay tubes and the tubes incubated again at 4°C for 20-24 hr. Tubes (except total count) were then incubated with precipitating reagent for 20 min at room temperature, centrifuged for 15 min at 1500 X g, decanted, and the iodinated estradiol contained in all tubes was measured for 1 min by gamma counter. The percent ratio of the bound fraction of labeled estradiol in each sample (B) to that in the absence of unlabelled estradiol (Bmax) was calculated and interpolated against the standard curve to determine sample concentrations (Figure 2.8). Control samples were assayed both in extracted and non-extracted form.

C. cAMP EIA

Accumulated cAMP in the H295R cells was determined by a competitive EIA kit (Cayman Chemical; Ann Arbor, MI). First, the experimental well plates were thawed and scraped to ensure total cell lysis and release of cAMP into the HCl. The samples were then transferred to 1.5 mL microcentrifuge tubes and centrifuged at 1,000 X g for 10 min. Standards, controls, and samples were acetylated in 12X75 mm tubes and 0.05 mL of each was added in duplicate to anti-rabbit IgG coated wells of a 96-well plate. cAMP acetylcholinesterase (Tracer) and cAMP antiserum were added to the appropriate wells and the plate was incubated 18-24 hrs at 4°C. The wells were washed using a plate washer (ELx50, Bio-Tek) and incubated in the dark with shaking (400-600 rpm) for 2 hrs at 25°C with the acetylcholinesterase (AChE) substrate (designated as Ellman's reagent). The intensity of the color (yellow) that developed was measured at 420 nM and was inversely proportional to the amount of free cAMP present. The percent ratio of the AChE- labeled cAMP in the sample bound to the antibody (B) to the maximum capacity of the antibody for AChE-labeled cAMP in the absence of unlabelled cAMP (Bmax) was calculated and interpolated against the standard curve (Figure 2.9) to determine sample concentrations.

D. Adrenal Medullary Gene Expression

1. RNA isolation

Adrenal medullary tissue was dissected from the adrenal glands of rats in the three treatment paradigms and was stored in RNAlater (Ambion; Houston, TX) at -20°C prior to RNA isolation. Total RNA was extracted using an RNeasy Mini Kit (Quiagen; Valencia, CA). Tissue was homogenized in a guanidine isothiocyanate-containing buffer with a Duall tissue grinder (Fisher Scientific; Pittsburg, PA). The samples were centrifuged at 12,000 X g for 3 min and the supernatant was transferred to 1.5 mL microcentrifuge tubes. One volume of ethanol (70%) was added to all samples and the solution applied to an RNeasy mini column containing a silica-gel membrane. The columns were centrifuged for 15 sec at 8,000 X g and the flow-through discarded. After an on-column DNase digestion and two high-salt washes, the total RNA was eluted from the column membrane with 0.03 mL of nanopure water and quantified spectrometrically at 260 nM.

2. Reverse Transcription

Single- stranded cDNA was synthesized from 1 µg of RNA using a Thermoscript RT kit (Invitrogen Life Technologies; Carlsbad, CA) and a protocol previously described (Carrithers et al., 2000). The samples were incubated for 5 min at 65°C with random hexamers (final concentration of 5 µM) and dNTPs (final concentration of 1 mM) and immediately placed on ice. RT components were added as a master mix containing (in final concentrations): 1X cDNA synthesis buffer (25 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8mM magnesium acetate); 0.75 U Thermoscript reverse transcriptase; 5 mM DTT; 2U RNase OUT. Samples were incubated at 25°C for 10 min, 55°C for 60 min, and 85°C for 5 min. RNase H (1 µL) was added to each reaction and incubated at 37°C for 20 min to degrade existing RNA molecules. The cDNA synthesis reactions were stored at -20°C or used immediately for polymerase chain reaction.

3. Polymerase chain reaction (PCR)

An initial PCR reaction determined the amount of β-actin generated by 1 µL of each RT reaction at 26 cycles. Subsequent PCR reactions were prepared with volumes

of RT reaction which were estimated (by CDD camera) to generate equal amounts of β -actin. The 50 μ L PCR reaction mixture consisted of (in final concentrations): cDNA template (~ 5% of RT reaction); 20 mM Tris-HCl (pH 8.0); 50 mM KCl; 0.2 mM dNTPs; 0.4 μ M primers; 2 mM $MgCl_2$ and 2.5 U of Platinum Taq Polymerase (Invitrogen Life Technologies). PCR reactions utilized a Perkin Elmer GeneAmp PCR system 2400 (Applied Biosystems; Foster City, CA). Control reactions were performed in the absence of cDNA. Samples (20% of reaction volume) were size fractionated by 3% agarose gel electrophoresis. Ethidium bromide stained DNA bands were visualized by a UV Foto/analyst image analysis system equipped with a convertible dual transilluminator (Fotodyne; Hartland, WI). The signal intensity of each PCR product was determined by an electronic imaging and analysis system with a CCD video camera (Fotodyne) and analyzed by UN-SCAN-IT software (Silk Scientific Inc; Orem, UT). The primer sequences and cycle protocols for tyrosine hydroxylase (TH; Murru et al., 1997), phenylethanolamine N-methyltransferase (PNMT; Morita et al., 2001), estrogen receptor α (ER α ; Kuiper et al., 1997), estrogen receptor β (ER β ; Byers et al., 1997), and β -actin (Carrithers et al., 2000) have been previously published and are listed in Table 2.2.

E. Adrenal Medullary Protein Expression

1. Protein Isolation

Adrenal medullary tissue was dissected from the adrenal glands of ovariectomized, vehicle and estradiol-treated rats and stored at -80°C prior to protein isolation. Samples were homogenized in a lysis buffer (pH 6.4) consisting of 25mM MES, 0.15mM NaCl, 1% TritonX-100, and 60mM n-octyl- β -D-glucopyranoside, stored on ice for 15 min, centrifuged (14,000g) at 4°C for 10 min. The supernate was collected and stored at -20°C . Prior to use, 5X protein sample buffer (pH 6.8) consisting of 0.31M Tris, 2.5% (w/v) SDS, 50% glycerol and 0.125% bromophenol blue was added to a final concentration of 1X. All samples were heated to 95°C immediately prior to loading.

2. Immunoblot

Proteins were separated on a 12.5% polyacrylamide gel at 50mA (constant current) and subsequently transferred to PVDF membrane (0.45 μ M; Sigma) at 50V (constant voltage) for 2 hours. Membranes were blocked with blotting buffer consisting of TBS plus 0.5% Tween 20 and 5% dry milk for 1 hr at 22 °C. Primary antibodies for ER α (Upstate Biotechnology; Lake Placid, NY), TH (BD Transduction Labs; San Jose, CA), and PNMT (Dia Sorin Inc; Stillwater, MN) were diluted in blotting buffer and incubated with blocked membranes for 1-24 hrs at 22°C. Membranes were washed four times for 5 min in a buffer containing TBS plus 0.5% Tween 20 and 0.2% dry milk. Horseradish peroxidase conjugated IgGs directed against the appropriate host IgG were diluted and incubated with membranes for up to 1 hr. Membranes were washed four times and visualized using chemiluminescence. The relative signal intensities were determined densitometrically. Equality of protein loading was determined qualitatively by Ponceau S staining of membranes.

F. Protein Concentration Analysis

Protein concentrations of H295R cells and adrenal medullary tissue samples were determined by the colorimetric DC Protein Assay (Bio Rad; Hercules, CA). This technique is similar to Lowry protein analysis. Samples were thawed, triturated and 0.05 mL was added in duplicate to 12 X 75 mm tubes as were bovine serum albumin standards (in 5% SDS, [0, 2.5, 5, 10, 25, and 50 mg/mL]). A working solution of 0.02 mL Reagent S per 1 mL Reagent A (alkaline copper tartrate solution) was made and 0.2 mL added to all tubes along with 1.5 mL of Reagent B (dilute Folin solution). All tubes were vortexed vigorously and the color (blue) developed for 15 min at 25°C. Absorbance was measured at 550 nM.

G. Statistical Analysis

Results are expressed as Mean \pm SEM. Statistical significance was determined using a t-test, paired t-test or ANOVA as appropriate. Post-hoc analysis to compare multiple treatment groups utilized the Student-Newman-Keuls test. Significance level was set at P<0.05.

| | Uterine Weight (mg/mm) 2 Weeks After Surgery | Δ Body Weight (g) 2 Weeks After Surgery |
|----------|---|---|
| SHAM | 123 \pm 9.5 | -1.5 \pm 5.6 |
| OVEX | 50 \pm 8.6* | +39 \pm 1.5* |
| OVEX +E2 | 110 \pm 7.4** | -8 \pm 0.9** |

Table 2.1

Effect of Ovariectomy and Estradiol-Replacement in Ovariectomized Rats on Uterine Weight and Total Body Weight

Uterine weight and changes in body weight 14 days after sham-surgery (SHAM), ovariectomy (OVEX), or ovariectomy + E2 replacement (OVEX +E2). Values are Mean \pm SEM of data from 5 separate experiments. * P< 0.01 compared to the SHAM group. ** P< 0.05 compared to the OVEX group.

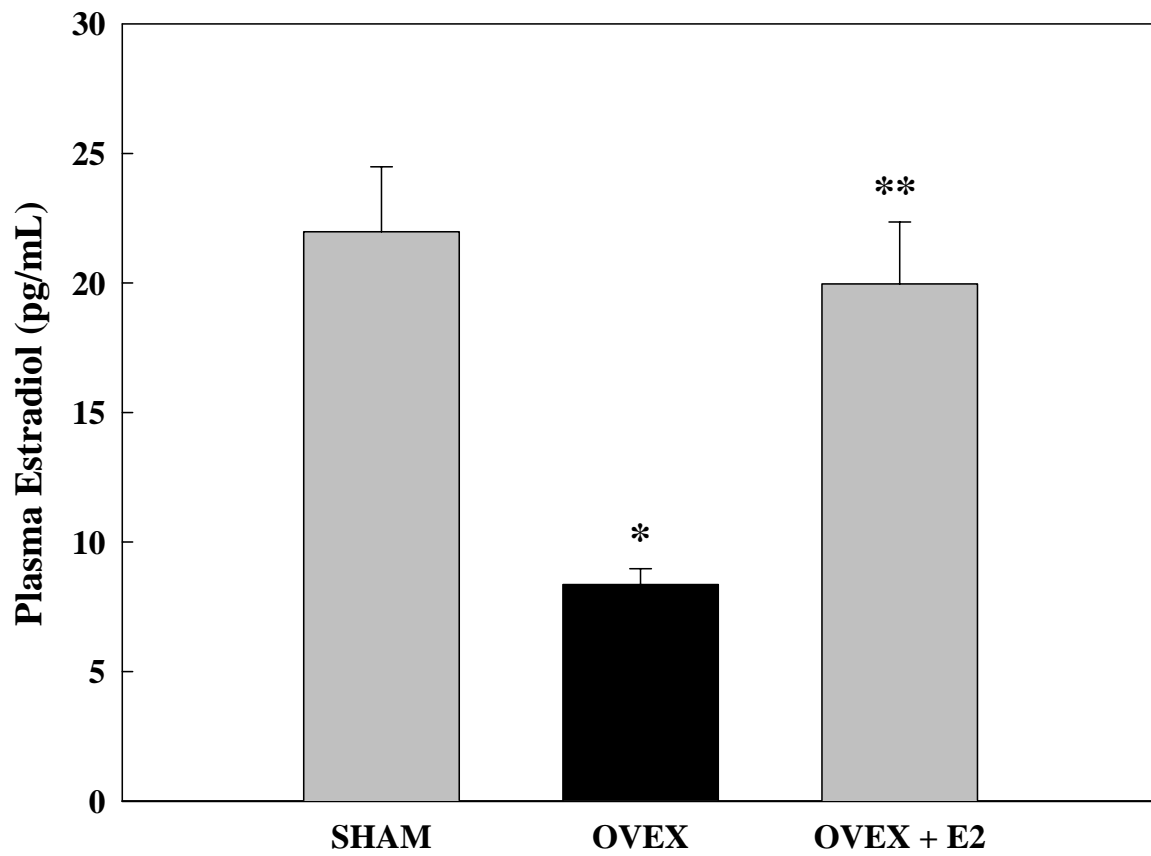


Figure 2.1

Plasma Estradiol Concentrations

Plasma estradiol concentrations were measured 14 days after ovariectomy (OVEX), or ovariectomy + E2 replacement (OVEX +E2). Plasma estradiol concentration was measured 12-16 days after surgery in the sham-operated controls (SHAM) on the pro-estrous day of the estrous cycle. Columns represent Mean \pm SEM of data from 5 separate experiments. * P< 0.01 compared to the SHAM group. ** P< 0.01 compared to the OVEX group.

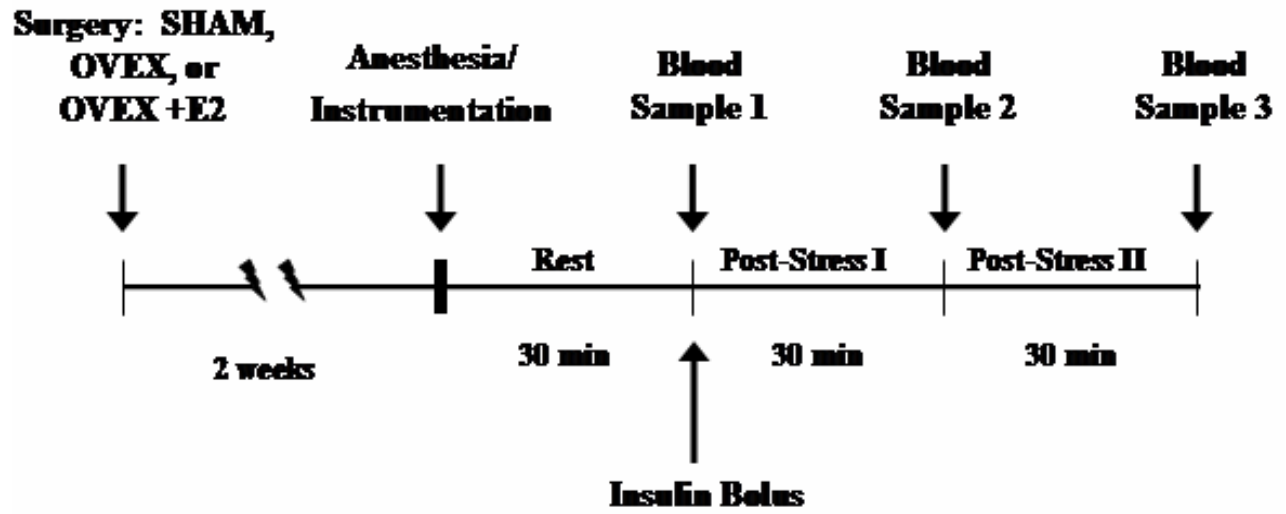


Figure 2.2

Summary of the Insulin-Induced Hypoglycemia Paradigm

OVEX: ovariectomy; E2: 17 β -estradiol

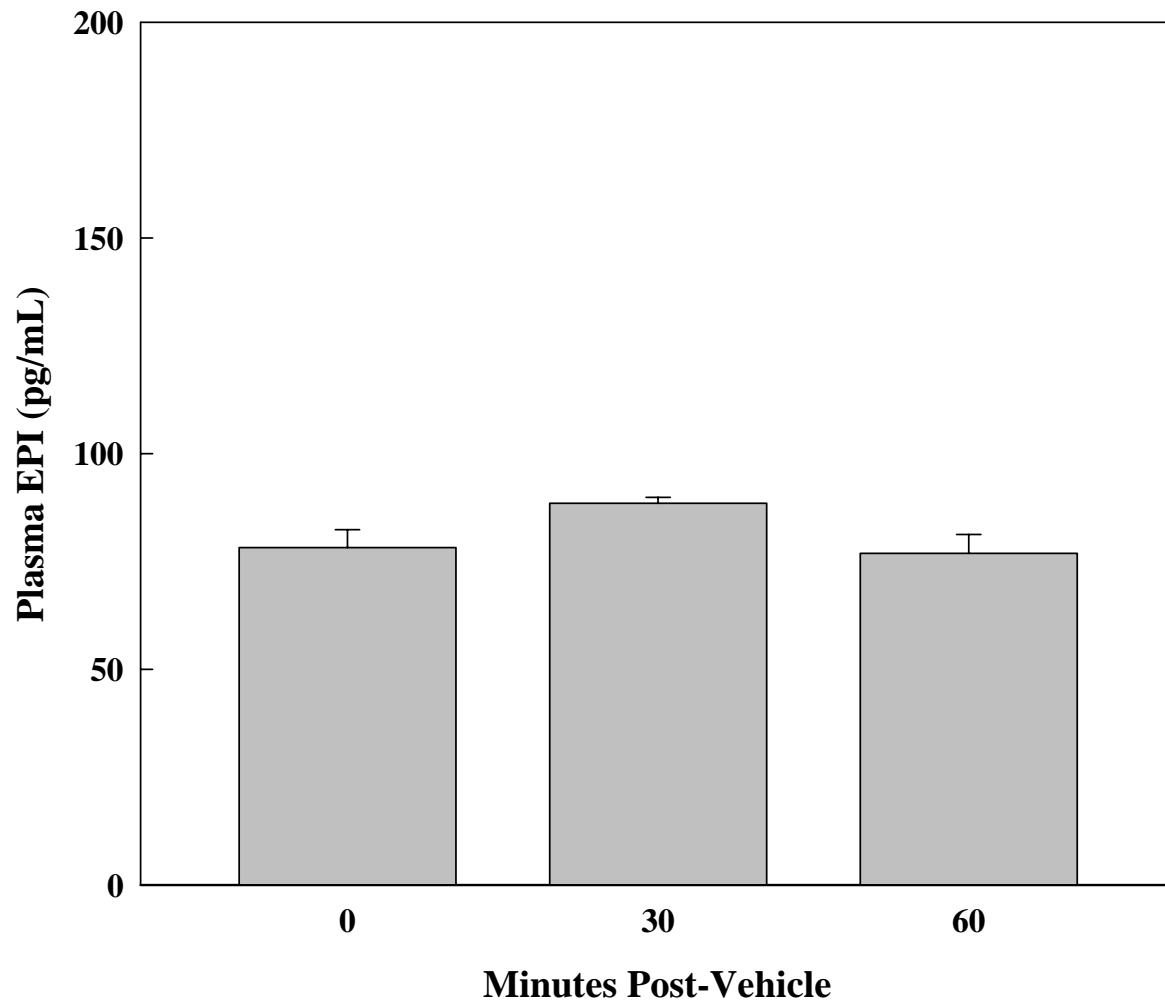
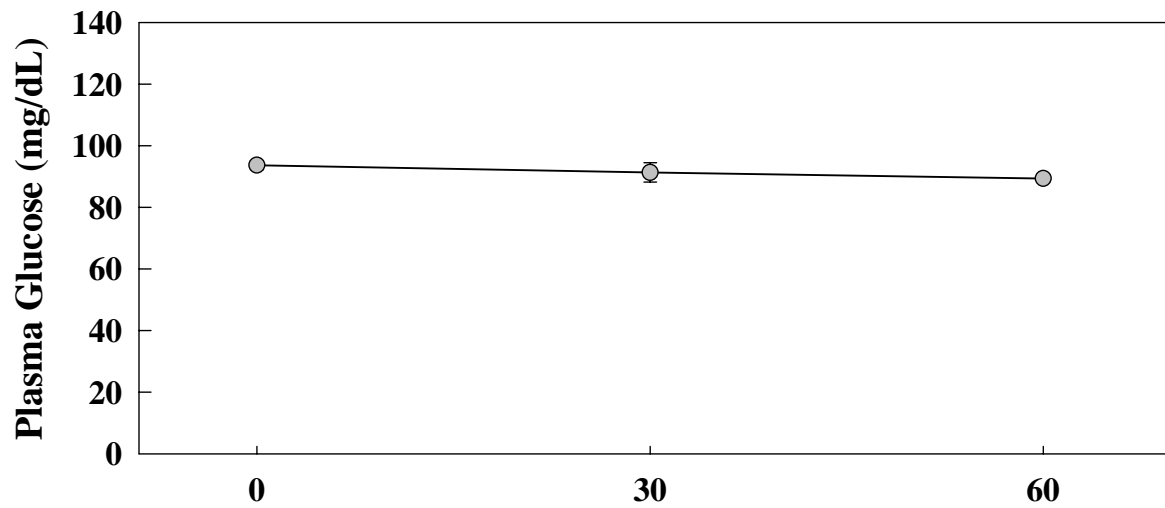


Figure 2.3

Effects of the Blood-Sampling Protocol on Epinephrine Concentrations

Plasma glucose and epinephrine (EPI) concentrations in sham-operated rats during the hypoglycemic-stress paradigm blood sampling protocol in the absence of insulin. Vehicle (saline; 100 μ L) was administered intravenously as a bolus at time 0. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the vehicle. Upper Panel: Plasma glucose concentrations. Lower Panel: Plasma EPI concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 4-5 separate experiments.

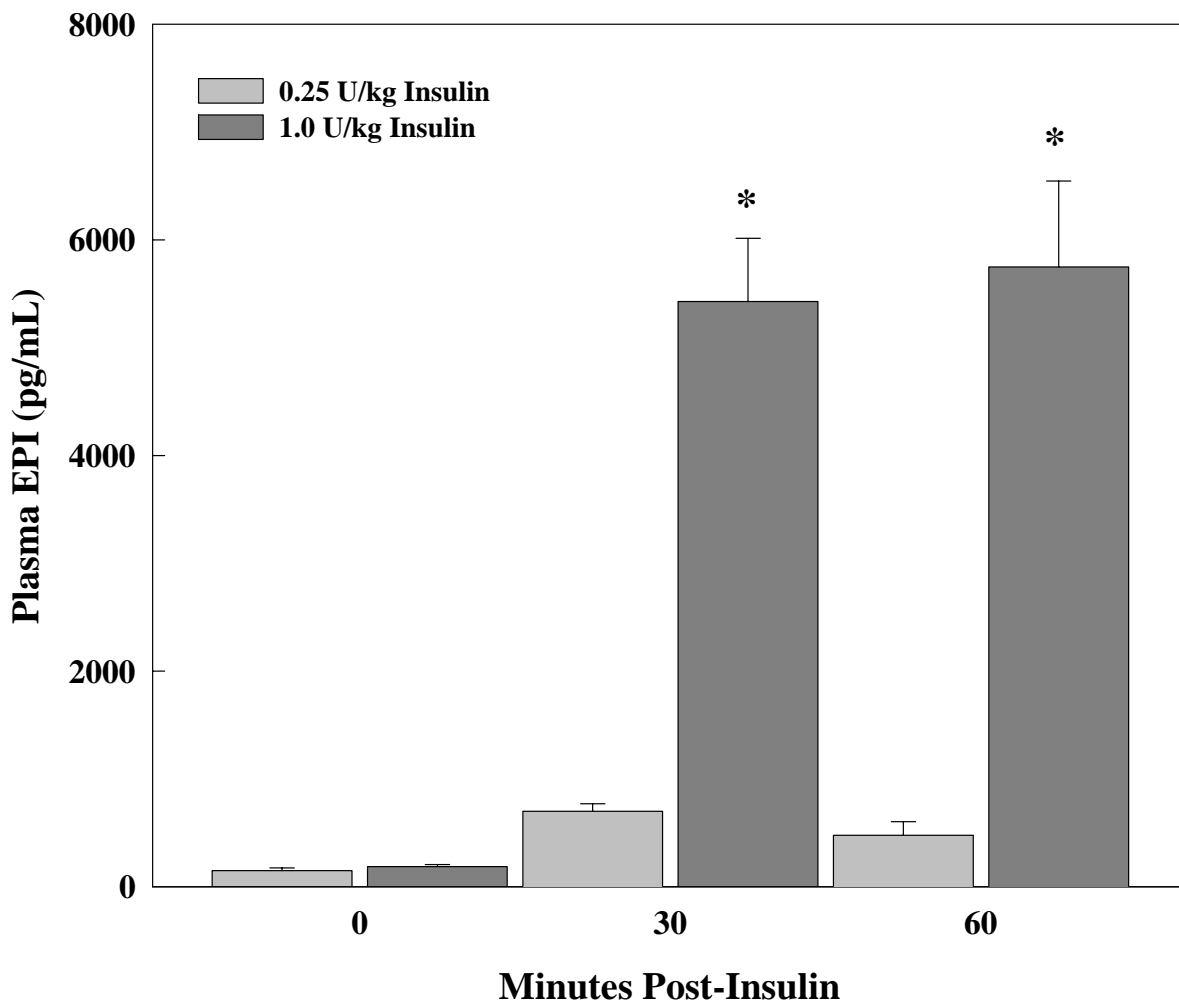
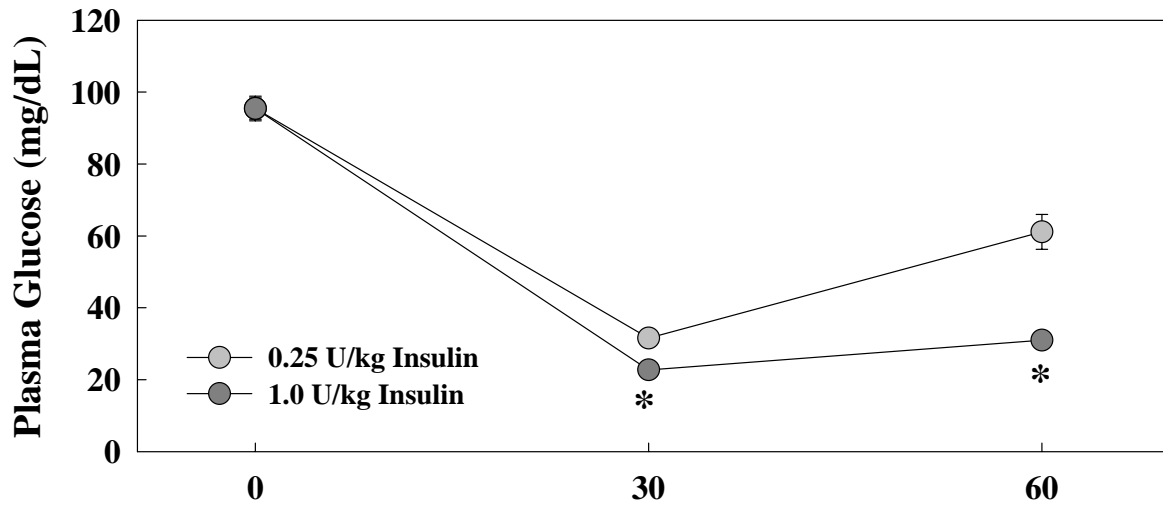


Figure 2.4

Effect of 0.25 U/kg and 1.0 U/kg Insulin Bolus on Plasma Glucose and Epinephrine Concentrations

Experiments were conducted 14 days after sham-surgery. Insulin (0.25 U/kg or 1.0 U/kg) was administered intravenously as a bolus at time 0. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the insulin. Upper Panel: Plasma glucose concentrations. Lower Panel: Plasma epinephrine (EPI) concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 6-8 separate experiments. * $P < 0.01$ compared to the 0.25 U/kg treated group.

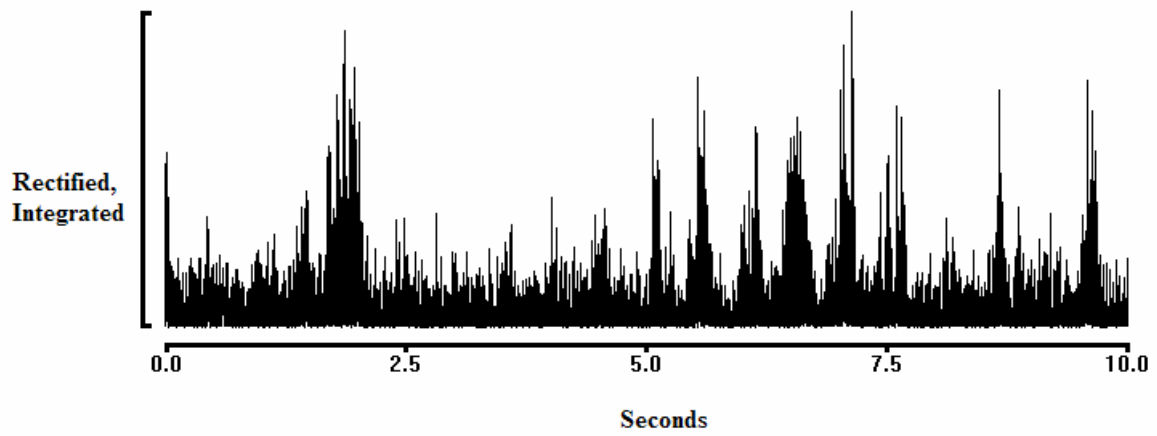
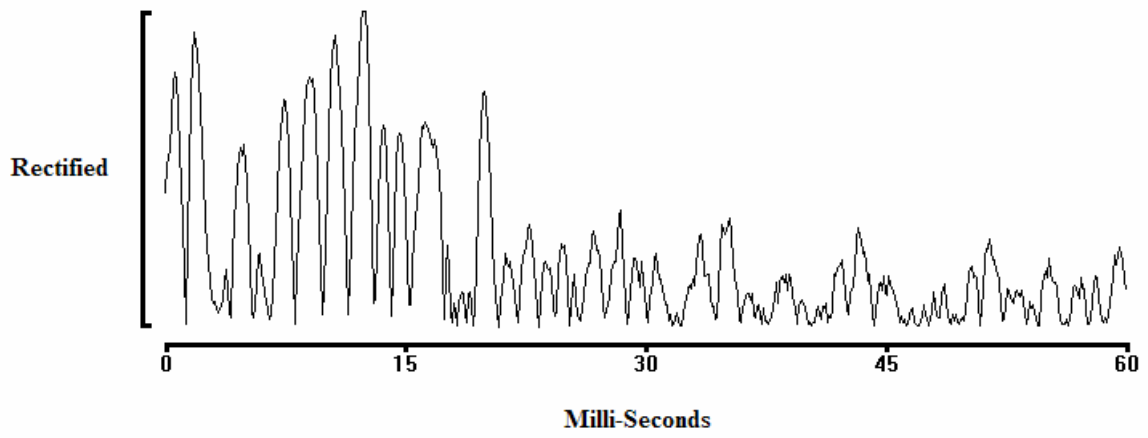
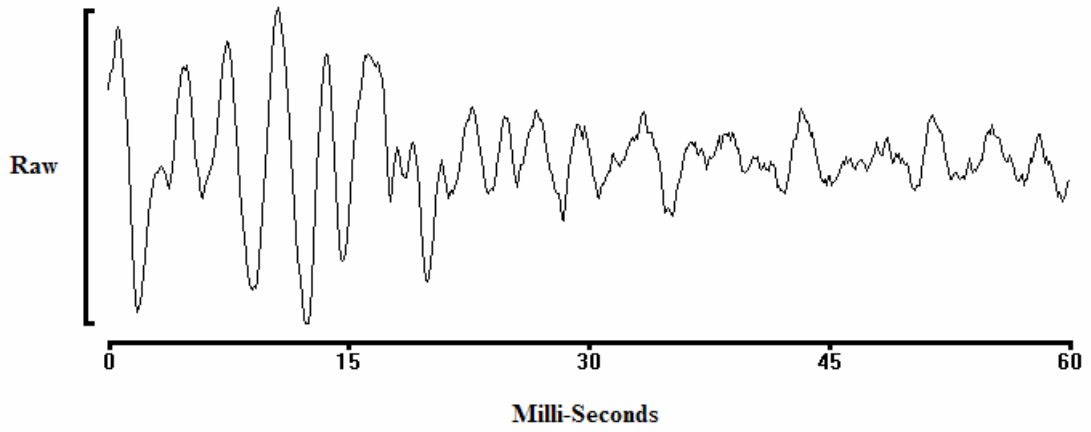


Figure 2.5

Typical Renal Sympathetic Nerve Recording

Upper Panel: Raw nerve activity over 60 milliseconds. Middle Panel: Rectified nerve activity over 60 milliseconds. Lower Panel: Rectified nerve activity integrated over 0.01 second integrals for 10 seconds.

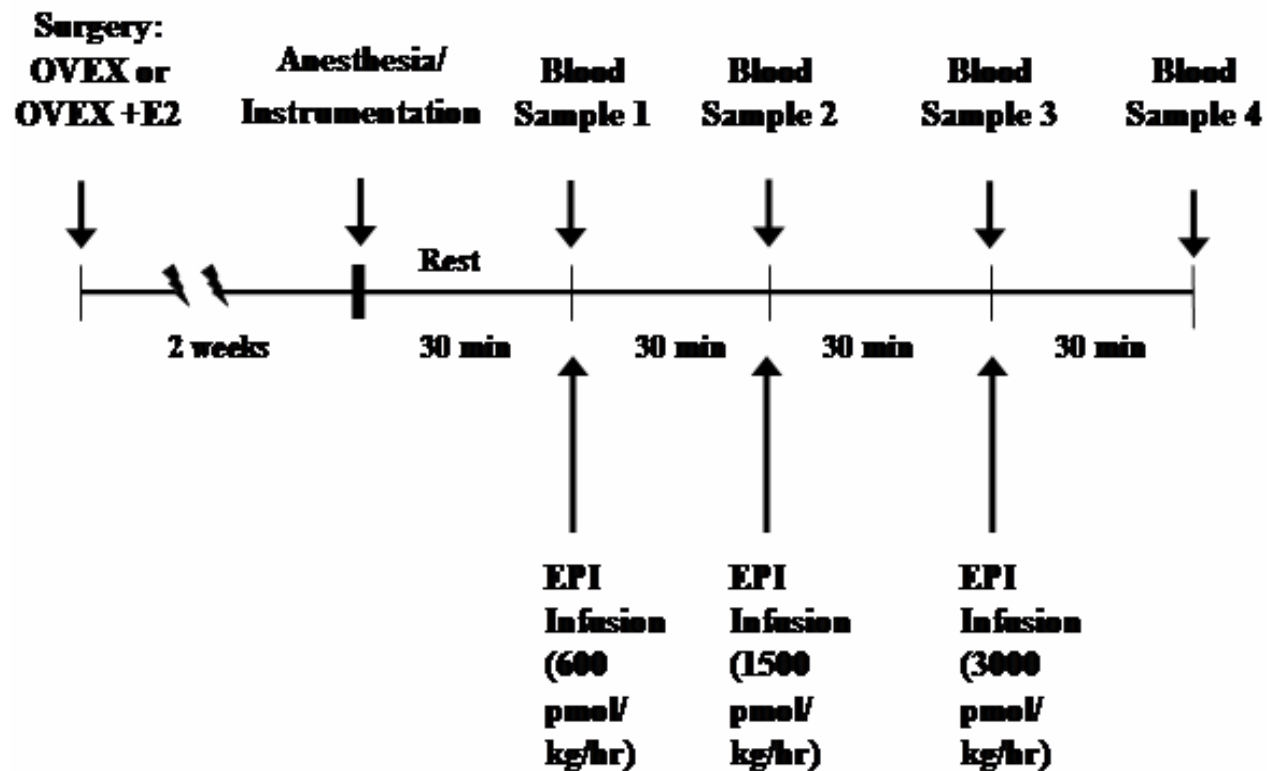


Figure 2.6

Protocol for Measurement of Plasma Epinephrine Clearance

OVEX: ovariectomy; E2: 17 β -estradiol; EPI: epinephrine

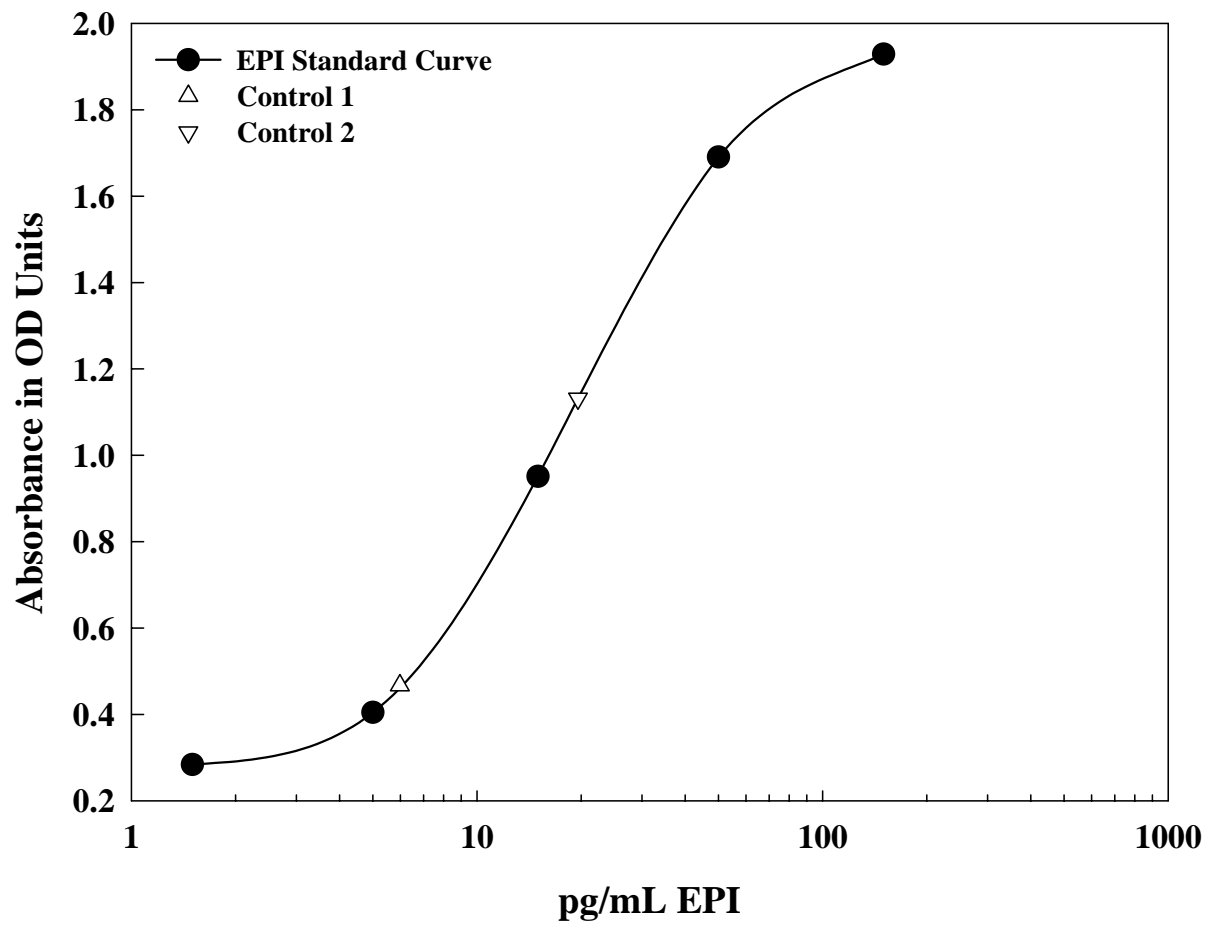


Figure 2.7

Representative Standard Curve Used for the Determination of Epinephrine Concentration by ELISA

Standard curve used for the determination of epinephrine (EPI) concentrations in rat plasma. Up-pointing triangle represents the actual measured concentration (5.6 pg/mL) of a kit-provided low-concentration control (Control 1) with a range of 3.1-6.5 pg/mL. Down-pointing triangle represents the actual measured concentration (19.6 pg/mL) of a kit-provided high-concentration control (Control 2) with a range of 13.4-26.1 pg/mL.

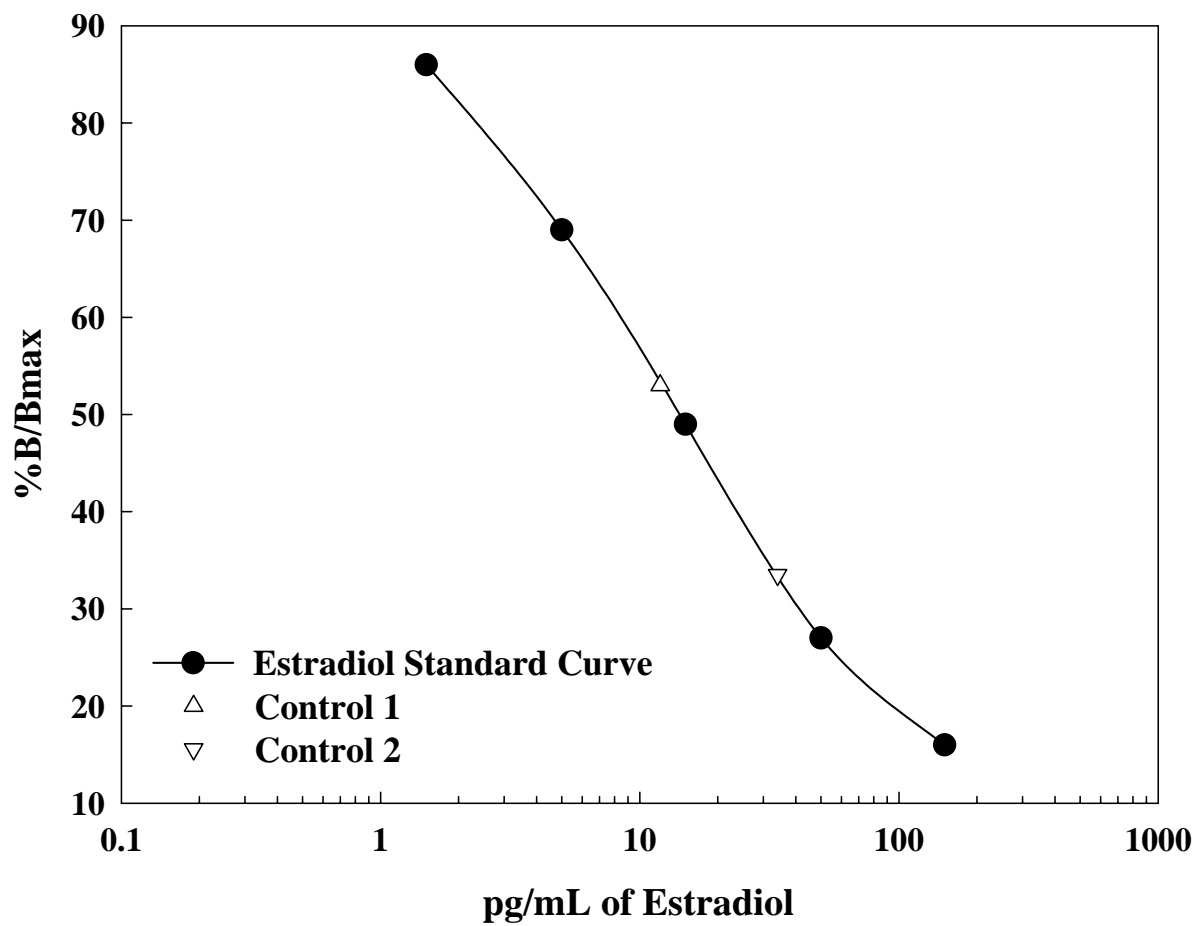


Figure 2.8

Representative Standard Curve Used for Determination of Estradiol Concentrations by
RIA

Standard curve used for the determination of estradiol concentrations in rat plasma. Up-pointing triangle represents the actual measured concentration (12 pg/mL) of an extracted, kit-provided low-concentration control (Control 1) with a range of 10 ± 3 pg/mL. Down-pointing triangle represents the actual measured, concentration (34 pg/mL) of an extracted, kit-provided high-concentration control (Control 2) with a range of 30 ± 9 pg/mL.

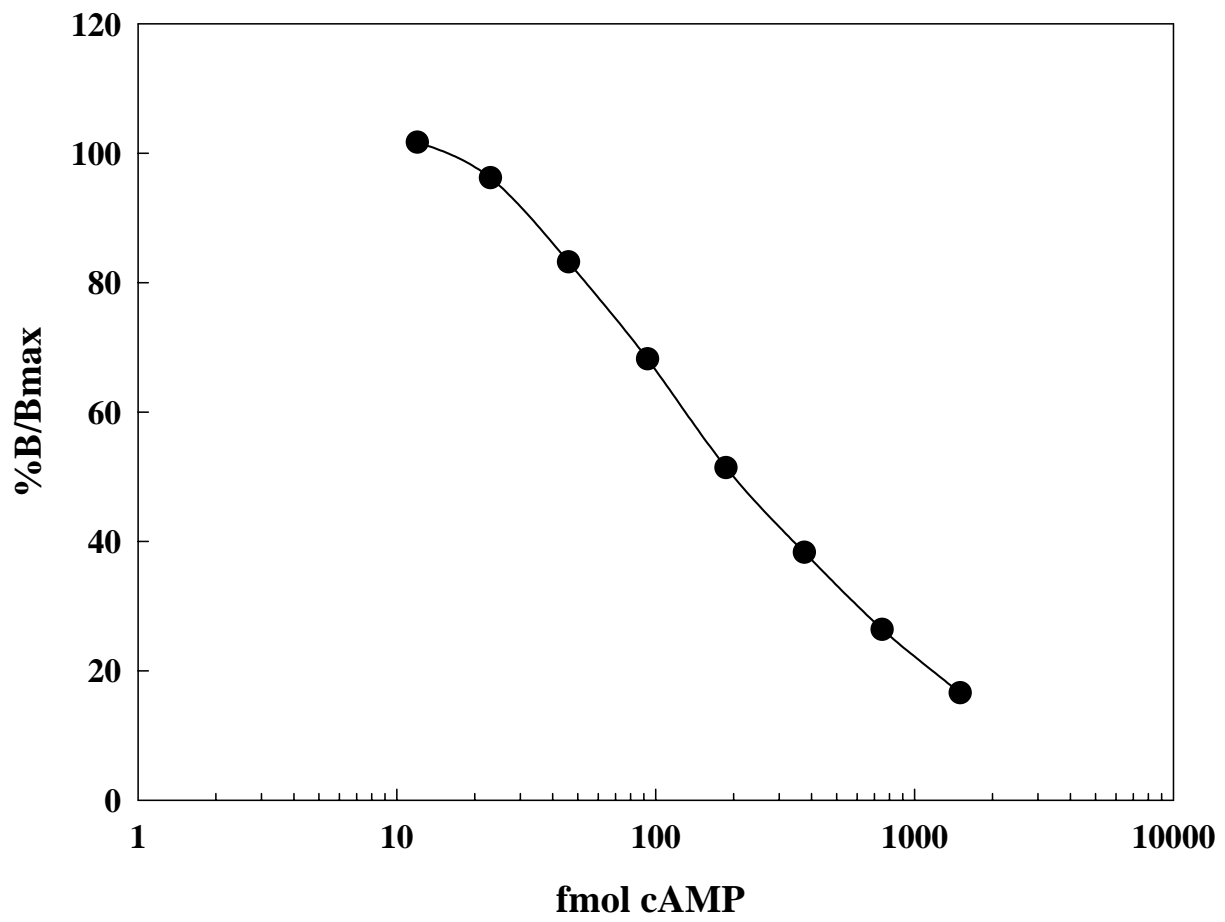


Figure 2.9

Representative Standard Curve Used for the
Determination of cAMP Levels by EIA

| Gene | Primer Sequence (5' to 3') | base pairs | Cycle # | Cycle Protocol | |
|----------------|---|------------|---------|----------------|---------|
| | | | | Temp | Time |
| TH | (+) ACATTTGAACTTAAAATTCAC (-) CGGGTGGTCCAGGTCCAGAT | 263 | 29 | 94°C | 0.5 min |
| | | | | 55°C | 0.5 min |
| | | | | 72°C | 1.0 min |
| PNMT | (+) CAGACTTCTTGGAGGTCAACCG (-) TTATTAGGTGCCACTTCGGGTG | 610 | 30 | 94°C | 0.5 min |
| | | | | 55°C | 0.5 min |
| | | | | 72°C | 1.0 min |
| ER α | (+) AATTCTGACAATCGACGCCAG (-) GTGCTTCAACATTCTCCCTCCTC | 344 | 36 | 95°C | 1.0 min |
| | | | | 55°C | 1.0 min |
| | | | | 72°C | 2.0 min |
| ER β | (+) AAAGCCAAGAGAAACGGTGGGC (-) GCCAATCATGTGCACCAGTTCC | 203 | 38 | 94°C | 0.5 min |
| | | | | 62°C | 1.0 min |
| | | | | 72°C | 1.5 min |
| β -actin | (+) TGGTGGGAATGGGTGTCAGAAGGACTC (-) CATGGCTGGGGTGTGAAGGTCTCA | 265 | 26 | 94°C | 1.0 min |
| | | | | 63°C | 1.0 min |
| | | | | 72°C | 1.0 min |

Table 2.2

PCR Primer Sequences and Protocols

TH: tyrosine hydroxylase; PNMT: phenylethanolamine N-methyltransferase; ER α :
estrogen receptor α ; ER β : estrogen receptor β

Chapter 3: Results

A. Estrogen Receptor Expression

In order for estradiol to affect adrenal medullary function via conventional steroid pathways, receptors for the steroid must be present in the tissue.

RT-PCR analysis identified mRNA for both estrogen receptor α (ER α) and β (ER β) in the adrenal medullary tissue of sham-operated controls (Figure 3.1; upper panel). ER β expression was more evident than ER α expression in the sham group. The expression of both receptors in medulla was markedly lower than in uterine (ER α) and the ovarian (ER β) tissue. While the expression of ER α appeared to be increased by ovariectomy, densometric comparisons (Figure 3.1; lower panel) were not significant and estradiol replacement did not affect ER α expression in ovariectomized (OVEX) rats. ER β mRNA levels did not differ between ovariectomized and sham groups or estradiol-replaced and vehicle-treated OVEX groups. A limited number of immunoblot samples confirmed that ER α did translate to receptor protein in the medulla, but at a reduced level compared to expression in the uterus (Figure 3.2; upper panel). Expression levels did not differ between vehicle-treated and estradiol-replaced OVEX groups (Figure 3.2; lower panel). Attempts to measure ER β protein expression proved unsuccessful.

B. Effects of Physiological Concentrations of Estrogen

1. Effects of the Loss of Ovarian Steroids

Experiment 1 compared the effects of insulin-induced hypoglycemia on plasma epinephrine concentration in 14-day OVEX and sham-operated rats. Pre-insulin plasma glucose concentration did not differ between the groups, and the glucose lowering effects of 0.25 U/kg insulin were identical in both groups at 30 ($\Delta -67 \pm 2.3\%$) and 60 ($\Delta -36 \pm 2.3\%$) minutes post-treatment (Figure 3.3; upper panel). Pre-insulin plasma epinephrine concentrations also did not differ between groups (150 ± 25 vs. 150 ± 26 pg/mL). However, 30 min of hypoglycemia increased plasma epinephrine concentration to a significantly greater extent ($\Delta +112\%$; $P < 0.01$) in the OVEX group compared to the sham-operated controls (Figure 3.3; lower panel). At 60 min post-insulin, plasma epinephrine concentrations were lower than at 30 min and did not differ between the

groups. Heart rate did not differ between the groups at any time point (Figure 3.4; upper panel). Mean arterial blood pressure (MAP) was significantly decreased ($P < 0.001$) to exactly the same extent after 30 min of hypoglycemia in the sham and OVEX groups ($\Delta -20 \pm 4.0\%$ and $-21 \pm 2.3\%$ respectively) and remained depressed after 60 min (Figure 3.4; lower panel).

2. Effects of Estradiol Replacement

In order to assess the effects of estradiol *per se* in this response, experiment 2 compared the effects of insulin-induced hypoglycemia on plasma epinephrine in 14-day OVEX and estradiol-replaced OVEX rats. For these experiments, one SILASTIC capsule containing either 17β -estradiol (E2) or sesame oil (vehicle) was implanted at the time of ovariectomy. As described in the materials and methods section, this replacement paradigm produced plasma E2 concentrations that were almost identical to those seen in intact, late proestrous phase, female rats (20.0 ± 2.4 and 22.0 ± 2.5 pg/ml respectively). Plasma E2 concentration in the OVEX animals (8.5 ± 0.6 pg/mL) was significantly lower than in both the intact and the E2-replaced OVEX rats ($P < 0.01$).

Pre-insulin plasma glucose concentrations did not differ between vehicle-treated and E2-treated OVEX rats (95 ± 3.4 and 103 ± 3.5 , respectively) and were decreased to the same extent 30 min after insulin ($\Delta -64 \pm 2.6\%$; Figure 3.5; upper panel). Pre-insulin plasma epinephrine concentrations also did not differ between groups (133 ± 29 vs. 152 ± 30 pg/mL). However, at 30 min post-insulin, the hypoglycemia-induced increase in plasma epinephrine concentration was significantly lower ($\Delta -56\%$; $P < 0.001$) in the OVEX +E2 group compared to the vehicle-treated OVEX (Figure 3.5; lower panel). Comparing across experiments, the increase in plasma epinephrine in the E2-replaced group was, in fact similar to that elicited in the SHAM group of experiment 1 ($\Delta 416 \pm 75$ vs. 550 ± 73 pg/mL). At 60 min post-insulin, plasma epinephrine concentrations again did not differ between groups despite the fact that plasma glucose was significantly lower in the OVEX compared to the OVEX +E2 group at this time point (61 ± 4.8 vs. 88 ± 9.0 mg/dL respectively; $P < 0.05$). Heart rate and MAP during hypoglycemia were similar to those seen in experiment 1. Heart rate remained constant in both groups and was not different between groups at any time point (Figure 3.6; upper panel). MAP

significantly decreased after 30 min of hypoglycemia in both groups ($\Delta -21 \pm 2.5\%$ and $-17 \pm 2.4\%$, OVEX and OVEX +E2 respectively; $P < 0.001$) and remained depressed after 60 min (Figure 3.6; lower panel), but was not different between groups at any time point.

To this point, experiments demonstrate that physiological levels of E2 can suppress stress (hypoglycemia)-induced increases in plasma epinephrine. Theoretically, this could result from a steroid-mediated decrease in epinephrine secretion from the adrenal medulla and/or increase in the plasma clearance of epinephrine. Measurement of epinephrine levels in blood collected directly from the adrenal vein was utilized as an index of epinephrine secretion. Given the highly invasive nature of these studies, blood sampling was restricted to one 6 min period beginning 24 min post insulin, during a time of lowest plasma glucose. Consistent with the plasma data, epinephrine secretion during this time period was significantly lower in the OVEX +E2 group compared to the OVEX ($\Delta -44\%$; $P < 0.005$; Figure 3.7). This E2-mediated decrease in secretion could be due to several factors, including decreased epinephrine synthesis in the adrenal medulla, suppressed intracellular signaling for secretion in the chromaffin cells of the medulla, and/or decreased neural input to the medulla.

Our data do not support the concept that E2 affects epinephrine biosynthesis. Using semi-quantitative RT-PCR, mRNA expression levels for tyrosine hydroxylase (TH), the rate limiting enzyme in the catecholamine biosynthetic cascade, and for phenylethanolamine *N*-methyltransferase (PNMT), the enzyme necessary for the conversion of nor-epinephrine to epinephrine, did not differ between OVEX and sham or E2-replaced and vehicle-treated OVEX groups (Figure 3.8). Similarly, protein expression of these enzymes measured by immunoblot also was not different in OVEX +E2 compared to OVEX rats (Figure 3.9).

Isolated adrenal medullary chromaffin cells maintained in primary culture were used to assess the effects of E2 on agonist-induced increases in intracellular Ca^{2+} , which is the primary stimulus for epinephrine secretion. Exposure to 10 nM E2 for 48 hr had no significant effect on Ca^{2+} transients elicited by either 50 μM nicotine (NIC) or by 60 mM KCl when compared to vehicle-treated control cells (Figure 3.10). In contrast,

short-term treatment (3 min) with relatively high concentrations of E2 dose-dependently suppressed agonist-induced Ca^{2+} transients in chromaffin cells. At 5 μM E2, NIC-induced Ca^{2+} transients were significantly suppressed by $33 \pm 2.0\%$ ($P < 0.001$; Figure 3.11; upper panel); transients were almost completely abolished with exposure to 50 μM E2 ($\Delta -88 \pm 4.2\%$; $P < 0.001$). The suppressive effects of E2 were rapidly reversible. For example, exposure to 10 μM E2 inhibited NIC-induced responses by $48 \pm 4.6\%$ ($P < 0.001$). Following removal of E2, the amplitude of the Ca^{2+} transient returned to $92 \pm 4.6\%$ of control within 10 min (Figure 3.11; lower panel). Increasing the time of exposure to E2 to 10 and 20 minutes had no additional inhibitory effect (Figure 3.12; upper panel). Ca^{2+} transients stimulated by KCl were inhibited 50% ($P < 0.001$) by 3 min treatment with 50 μM E2 (Figure 3.12; lower panel).

The effects of these two *in vitro* treatment paradigms were diametrically opposite in adrenal cortical cells compared to chromaffin cells. Pituitary adenylate cyclase-activating polypeptide-27 (PACAP-27; 100 nM)- induced increases in intracellular cAMP accumulation were significantly decreased in cells from a human adrenal cortical cell line (H295R) exposed to 10 nM E2 for 48 hr compared to vehicle-treated controls ($\Delta -33\%$; $P < 0.05$; Figure 3.13). However, acute exposure to a supra-physiological concentration of E2 (50 μM) did not affect agonist-induced cAMP accumulation in this cell line (Figure 3.14).

Subsequent experiments assessed the effects of E2 on renal sympathetic nerve activity (RSNA) as an index of neural input to the adrenal medulla. As in previous experiments, insulin reduced plasma glucose concentrations to the same extent in both groups. Hypoglycemia elicited a maximal increase in RSNA above baseline approximately 25 min after insulin in both groups and was significantly greater in the OVEX +E2 compared to the OVEX group ($49 \pm 6.0\%$ vs $16 \pm 1.8\%$; $P < 0.01$). Activity returned toward baseline after peak, but remained significantly augmented above baseline for the duration of the experiment in the OVEX +E2 group. However, in the OVEX group, RSNA had returned to baseline after 45 min of hypoglycemia, and was thereafter significantly decreased below baseline levels (Figure 3.15).

To evaluate the effects of estradiol on epinephrine clearance, plasma epinephrine concentrations were compared in OVEX and OVEX +E2 rats before and

during infusion of three successively increasing doses of exogenous epinephrine. Pre-infusion plasma epinephrine concentrations did not differ between the groups (108 ± 19 vs. 92 ± 9 pg/mL). As anticipated, plasma epinephrine concentrations increased as the epinephrine infusion rate was increased in both groups. These increases in plasma epinephrine levels were significantly lower in the E2-replaced OVEX animals (Δ -39% at 30 min, Δ -34% at 60 min, and Δ -42% at 90 min; $P < 0.05$; Figure 3.16), suggesting that the rate of epinephrine clearance is greater in this group compared to the OVEX group.

C. Effects of Supra-Physiological Concentrations of Estradiol

Consistent with other reports, our work has demonstrated that acute exposure to high concentrations of estradiol can suppress agonist-induced Ca^{2+} transients in the chromaffin cell. Based on previous work from our laboratory (Wagner et al., 1999; Jorgensen et al., 2002), we would anticipate that this reduction in Ca^{2+} signaling would result in decreased epinephrine secretion. Subsequently, experiments were designed to determine whether acute increases in circulating E2 can affect hypoglycemia-induced increases in plasma epinephrine. The following modifications were applied to the basic hypoglycemia protocol for these experiments: only ovariectomized rats were used and a bolus dose of E2 (150 $\mu\text{g}/\text{kg}$) or vehicle (10% EtOH) was intravenously infused over 2 min 30 min prior to the insulin bolus. To maintain a high plasma concentration of free E2, rats were infused with either E2 or vehicle at a rate of 75 $\mu\text{g}/\text{kg}/\text{hr}$ for the remainder of the experiment.

Plasma glucose concentrations measured immediately prior to and 30 min after E2 or vehicle administration did not differ and the glucose lowering effects of insulin were identical in both groups at 30 and 60 min post-insulin (Δ -66 \pm 1.6% and Δ -35 \pm 3.1%; Figure 3.17; upper panel). Plasma epinephrine concentrations also did not differ prior to (82 ± 29.9 vs. 105 ± 16.5 pg/mL) or 30 min after (154 ± 23.0 vs. 203 ± 32.3 pg/mL) E2 or vehicle administration. E2 treatment significantly suppressed the rise in plasma epinephrine concentration after 30 min of hypoglycemia (Δ -34%; $P < 0.05$), and tended to suppress the rise at 60 min post-insulin (Figure 3.17; lower panel). In contrast to experiment 1 and 2, heart rate did increase after 30 min in the E2-infused group, and after 60 min in the vehicle-infused group, but these increases did not reach significance

($P=0.06$ and $P=0.07$ respectively; Figure 3.18; upper panel). Also in contrast to experiments 1 and 2, MAP remained stable for the duration of the 60 min hypoglycemic test in both groups. Neither heart rate nor MAP was different between the groups at any time point (Figure 3.18; lower panel).

D. Effects of Gender

Since the data to this point have established that both physiological and supra-physiological levels of E2 can modulate stress-induced epinephrine responses, a final series of experiments compared hypoglycemia-induced increases in plasma epinephrine concentration in age-matched (14 week-old) male and female rats. Plasma glucose concentration did not differ between the groups prior to insulin treatment (92 ± 1.2 and 98 ± 3.3 mg/dL; male and female respectively). The glucose lowering effects of insulin were identical in both groups ($\Delta -62 \pm 2.2\%$) after 30 min of hypoglycemia. Plasma glucose levels were returned toward baseline after 60 min in both groups, but had returned to a greater degree in the female rats (73 ± 3.7 vs. 56 ± 3.7 mg/dL; $P<0.01$ p) (Figure 3.19; upper panel). Pre-insulin plasma epinephrine concentrations were significantly higher in the female group compared to the male group (113 ± 6 vs. 79 ± 11 pg/mL; $P<0.05$). Hypoglycemia-induced increases in plasma epinephrine concentration were greater in the female group after 30 min ($\Delta + 308\%$; $P<0.05$) and did not differ after 60 min (Figure 3.19; lower panel), but heart rate was significantly increased above baseline after 30 min of hypoglycemia in the female group ($\Delta +10\%$; $P<0.05$). Heart rate did not differ between the groups at any time point (Figure 3.20; upper panel). MAP significantly decreased over time in both groups, but also did not differ between groups (Figure 3.20; lower panel).

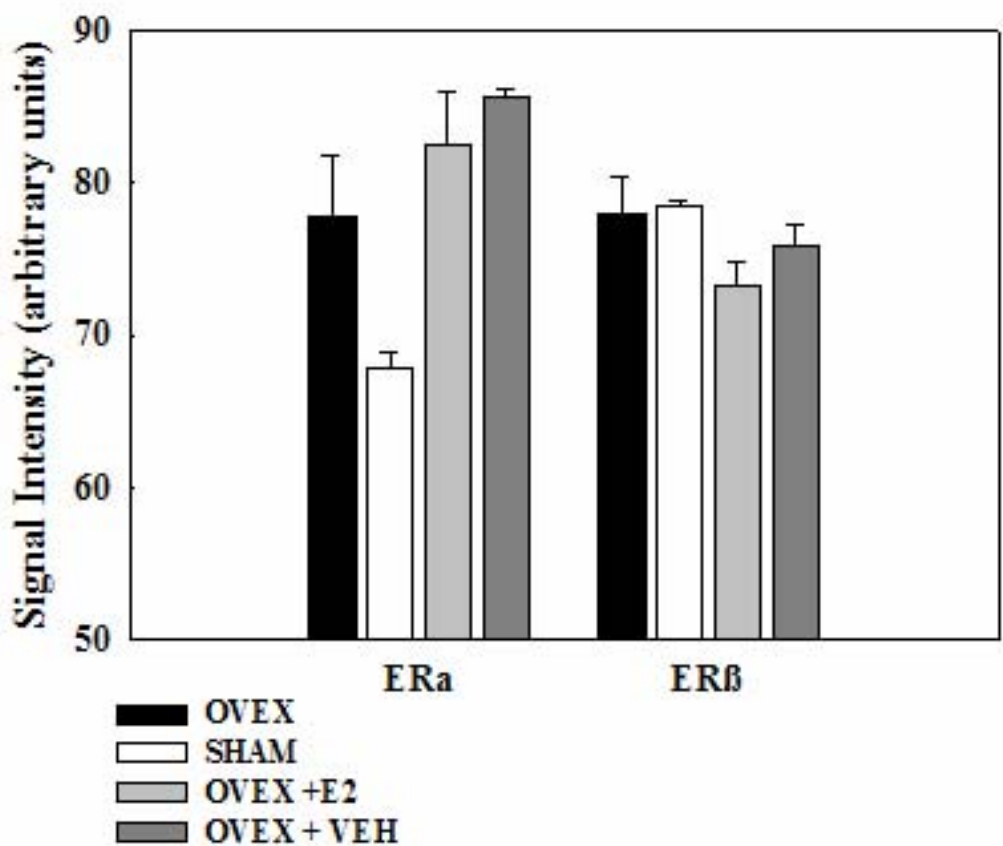
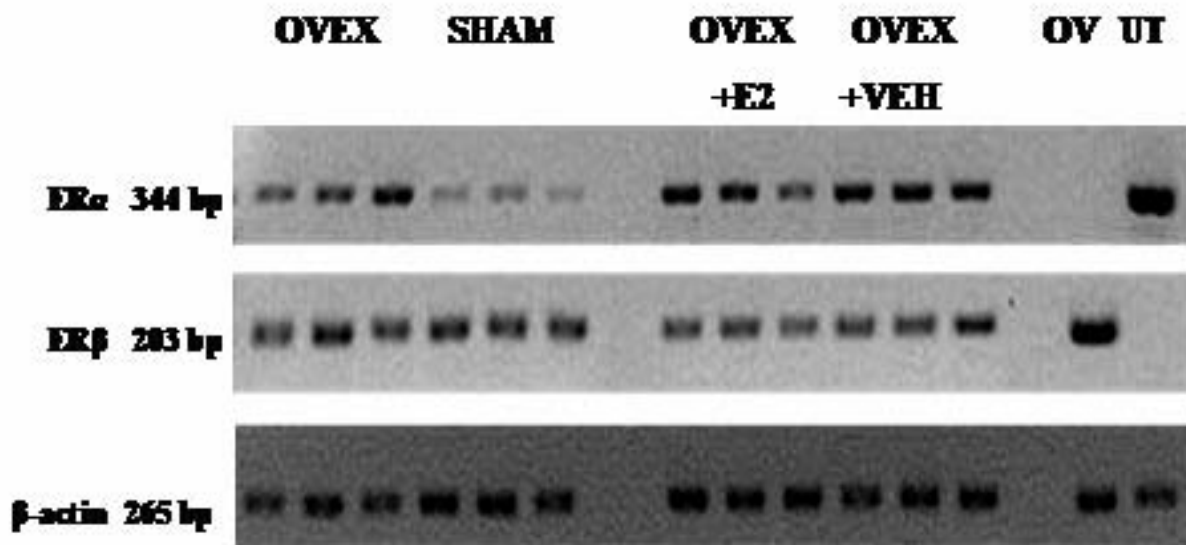


Figure 3.1

Estrogen Receptor Gene Expression in the Adrenal Medulla

Semi-quantitative RT-PCR analysis of adrenal medullary mRNA levels for estrogen receptor α (ER α) and estrogen receptor β (ER β). Tissue was collected 14 days after ovariectomy (OVEX), SHAM- surgery, ovariectomy + estradiol replacement (OVEX + E2) or + vehicle treatment (OVEX + VEH). Upper Panel: This ethidium bromide-stained agarose gel illustrates each PCR product (n = 3 per group). The size of each product is listed in the left column. Uterine (UT) tissue was used as a control for ER α expression, ovarian (OV) tissue was used as a control for ER β expression; β -actin was used as an internal control for each reaction. Lower Panel: Densitometric analysis of the relative signal intensities. Columns (lower panel) represent Mean \pm SEM of data from the 3 individual experiments in the upper panel.

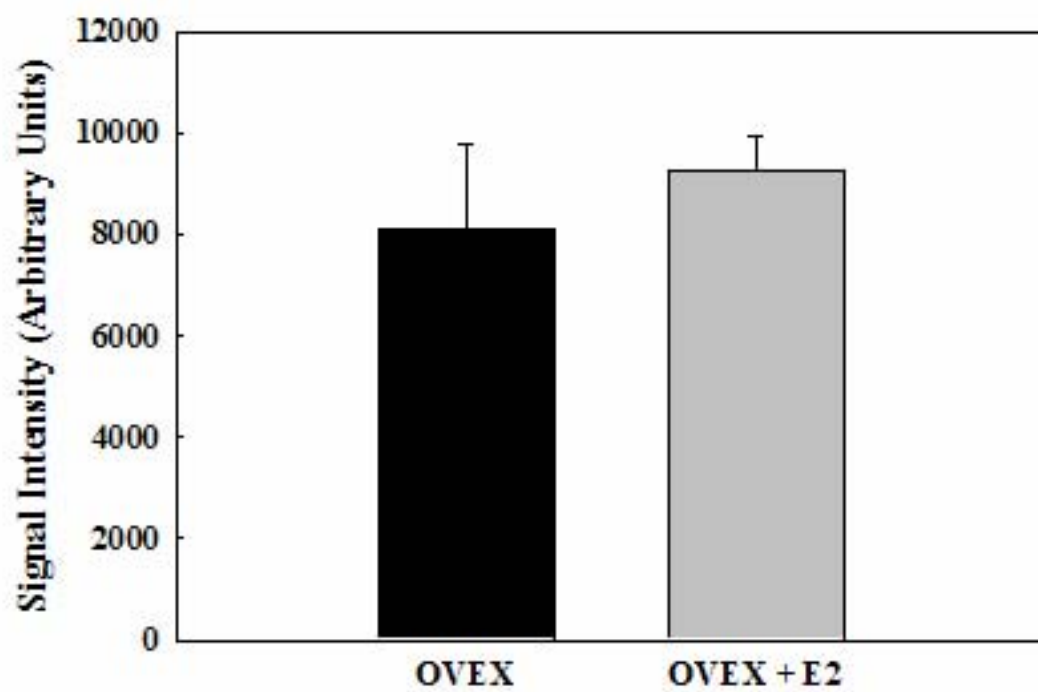


Figure 3.2

Estrogen Receptor α Protein Expression in the Adrenal Medulla

Immunoblot analysis of adrenal medullary protein levels for estrogen receptor α (ER α). Tissue was collected 14 days after ovariectomy + vehicle (OVEX) or estradiol replacement (OVEX + E2). Upper Panel: 100 μ g of adrenal medullary and 20 μ g of uterine (UT) protein were separated by SDS-PAGE (n = 3 per group) and subjected to immunoblot analysis with rabbit anti ER α in a dilution of 1:2000 overnight. The size of the product is listed in the left column. Lower Panel: Densitometric analysis of the relative signal intensities. Columns (lower panel) represent Mean \pm SEM of data from the 3 individual experiments in the upper panel.

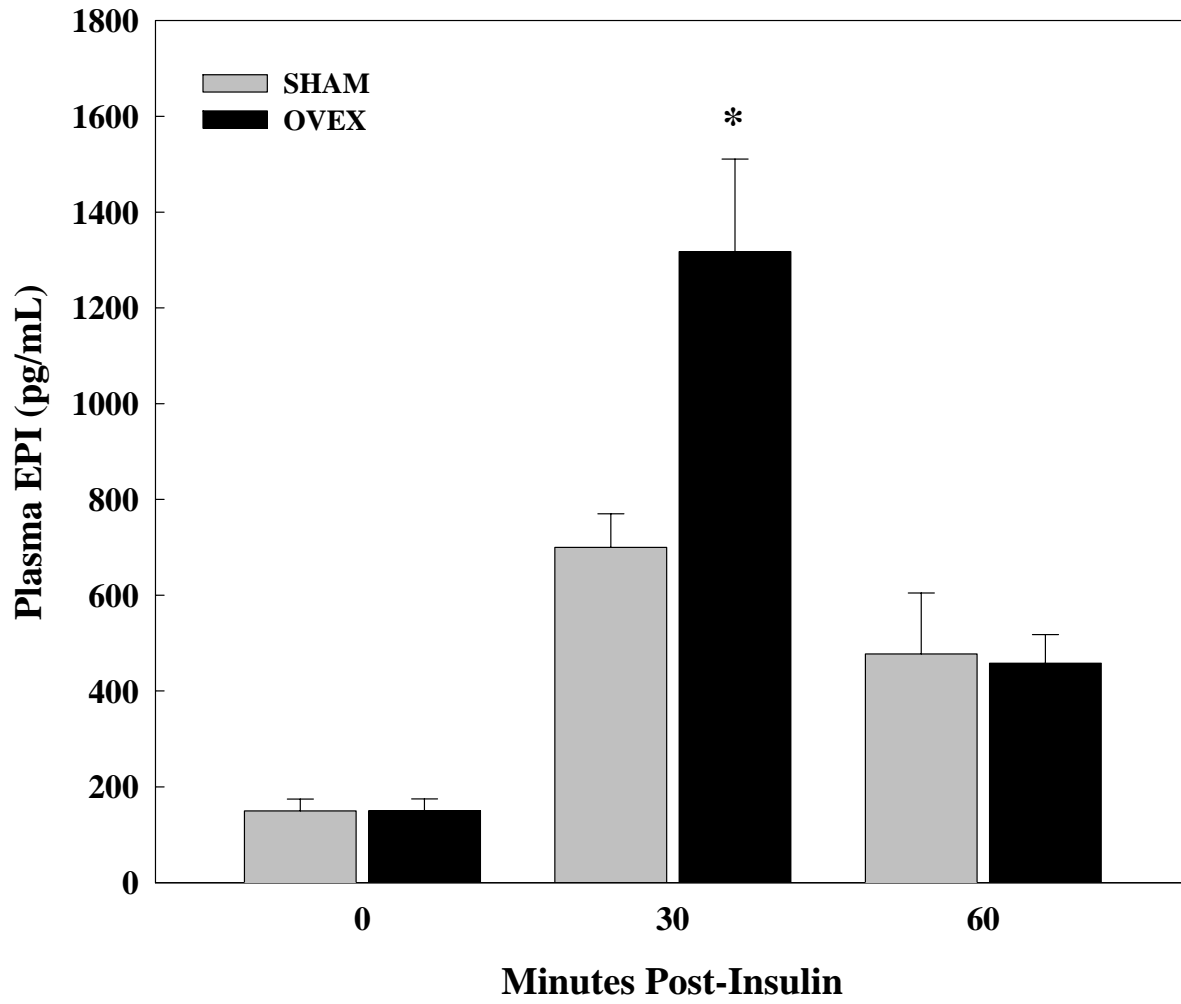
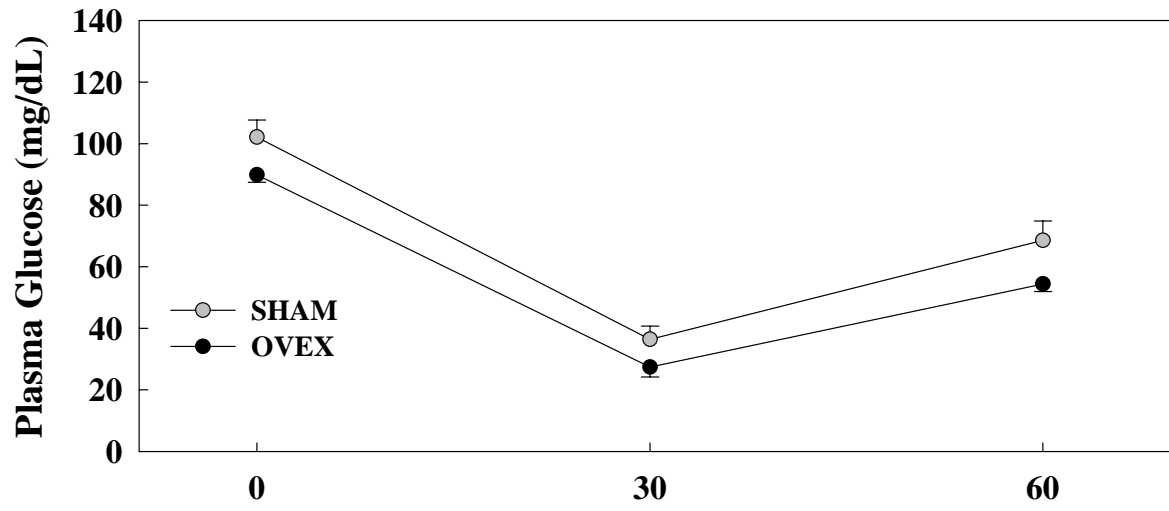


Figure 3.3

Effect of Ovariectomy on Hypoglycemia-Induced Increases in Plasma Epinephrine Concentration

Experiments were conducted 14 days after ovariectomy (OVEX) or sham-surgery (SHAM). Insulin (0.25 U/kg) was administered intravenously as a bolus at time 0. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the insulin. Upper Panel: Effect of insulin on plasma glucose concentrations. Lower Panel: Effect of hypoglycemia on plasma epinephrine (EPI) concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 7-8 separate experiments. * $P < 0.01$ compared to SHAM group.

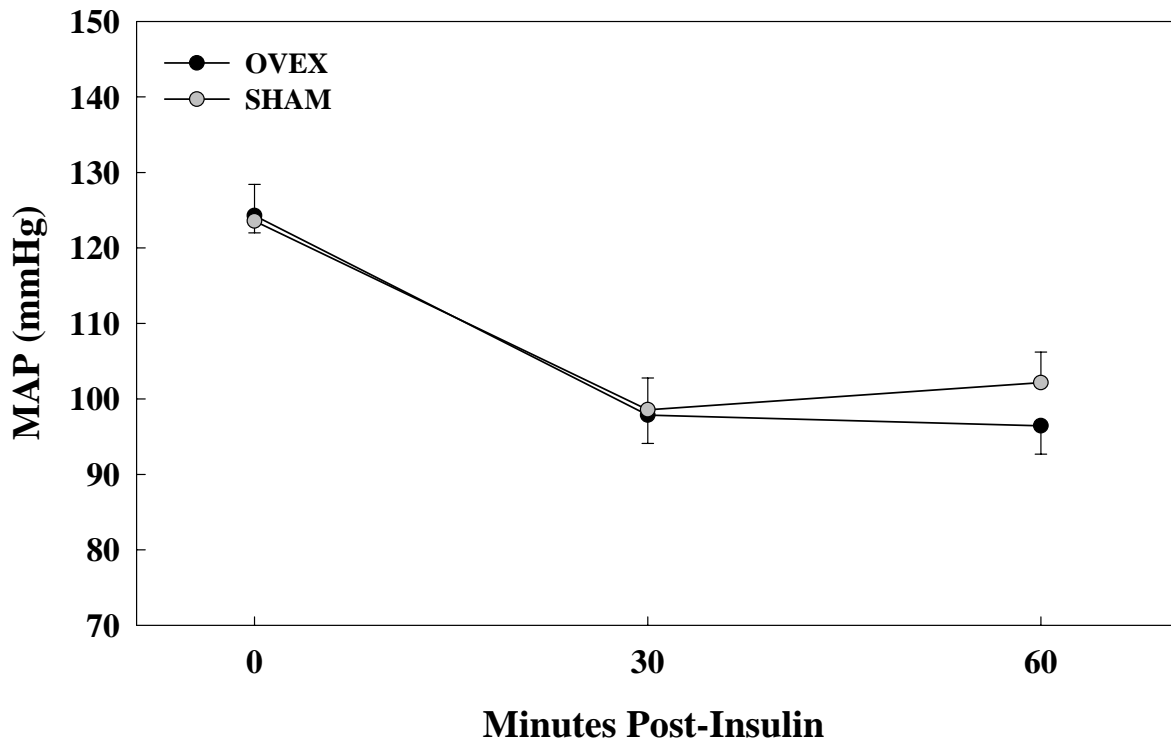
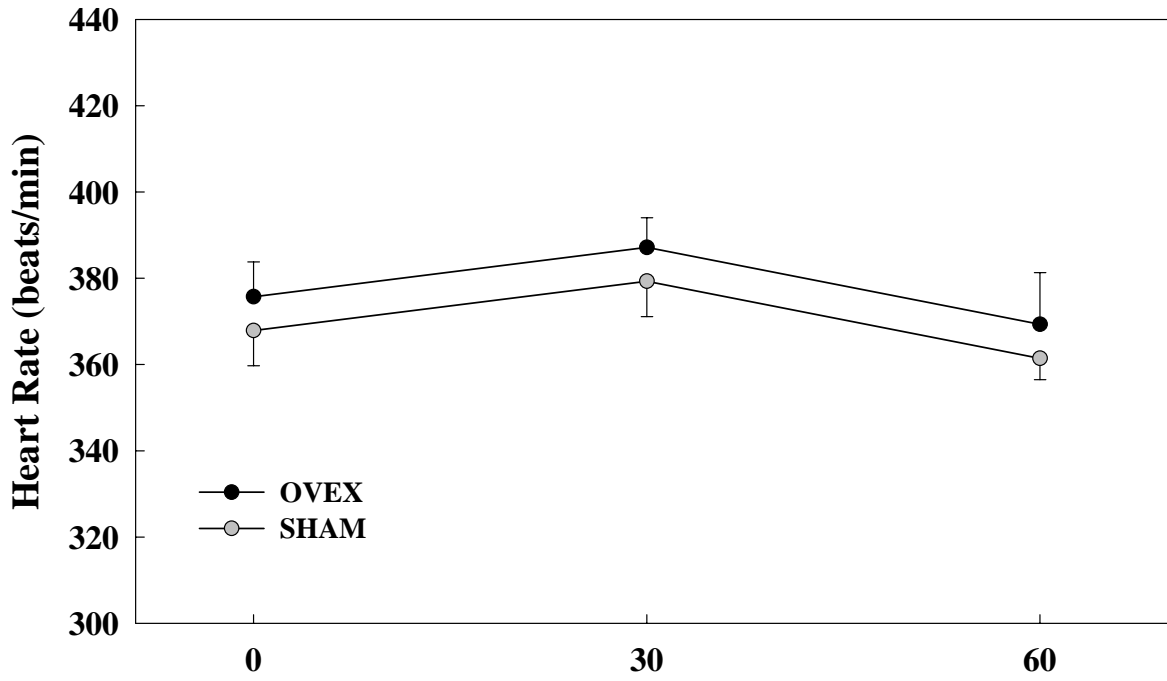


Figure 3.4

Effect of Ovariectomy on Hypoglycemia-Induced Changes in Heart Rate and Mean Arterial Blood Pressure

Upper Panel: Effect of hypoglycemia on heart rate. Lower Panel: Effect of hypoglycemia on mean arterial blood pressure (MAP). Experiments were conducted 14 days after ovariectomy (OVEX) or sham-surgery (SHAM). Insulin was administered intravenously as a bolus at time 0. Circles represent Mean \pm SEM of data from 7-8 separate experiments.

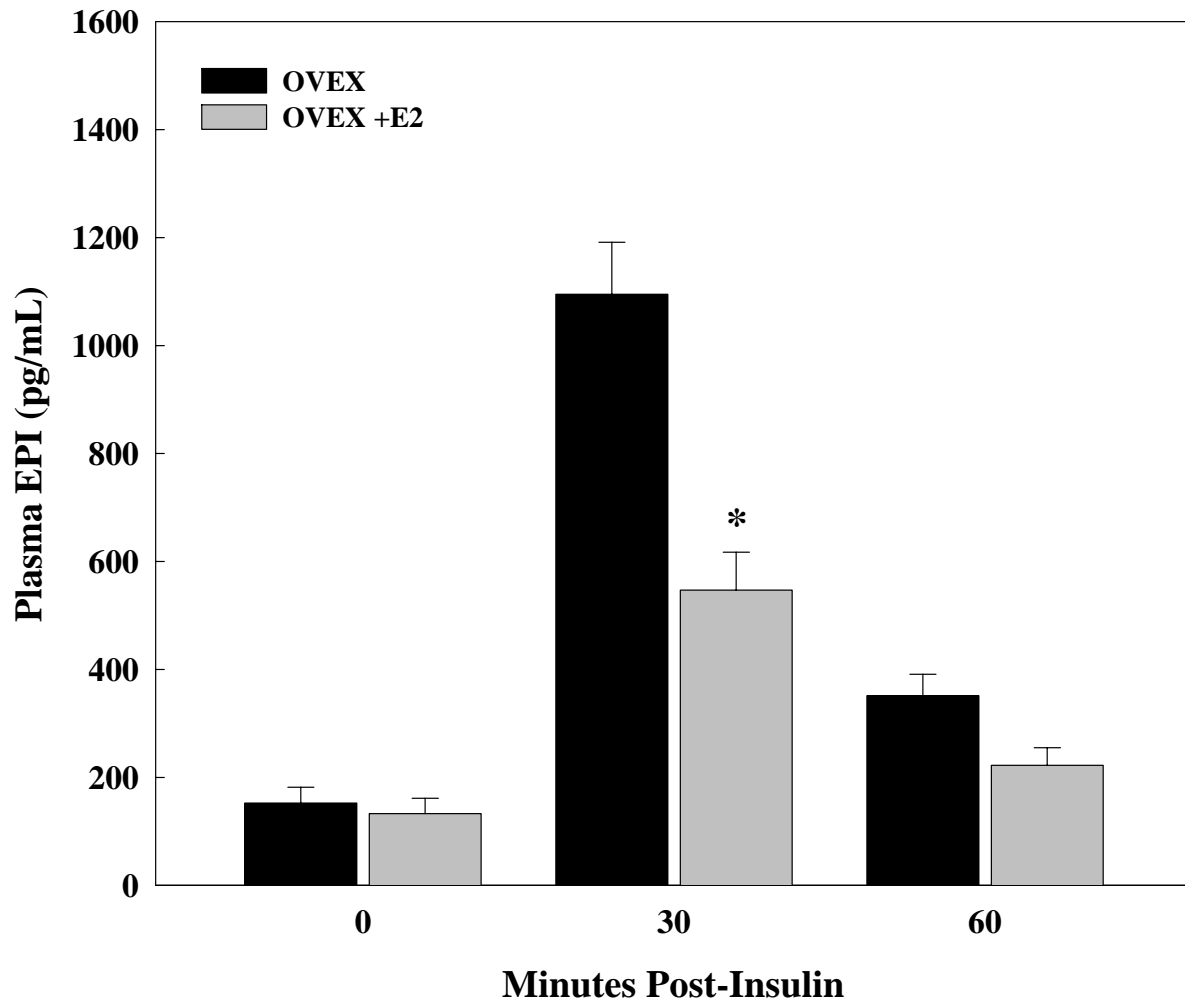
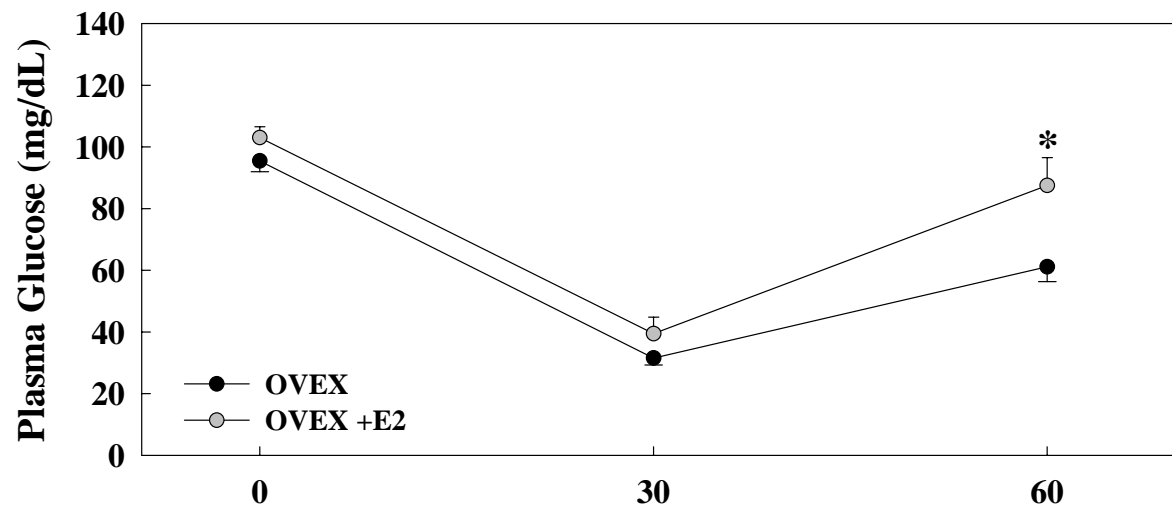


Figure 3.5

Effect of Estradiol-Replacement in Ovariectomized Rats on Hypoglycemia-Induced Increases in Plasma Epinephrine Concentration

Experiments were conducted 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the insulin. Upper Panel: Effect of insulin on plasma glucose concentrations. Lower Panel: Effect of hypoglycemia on plasma EPI concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 7-8 separate experiments. * $P < 0.001$ compared to OVEX group.

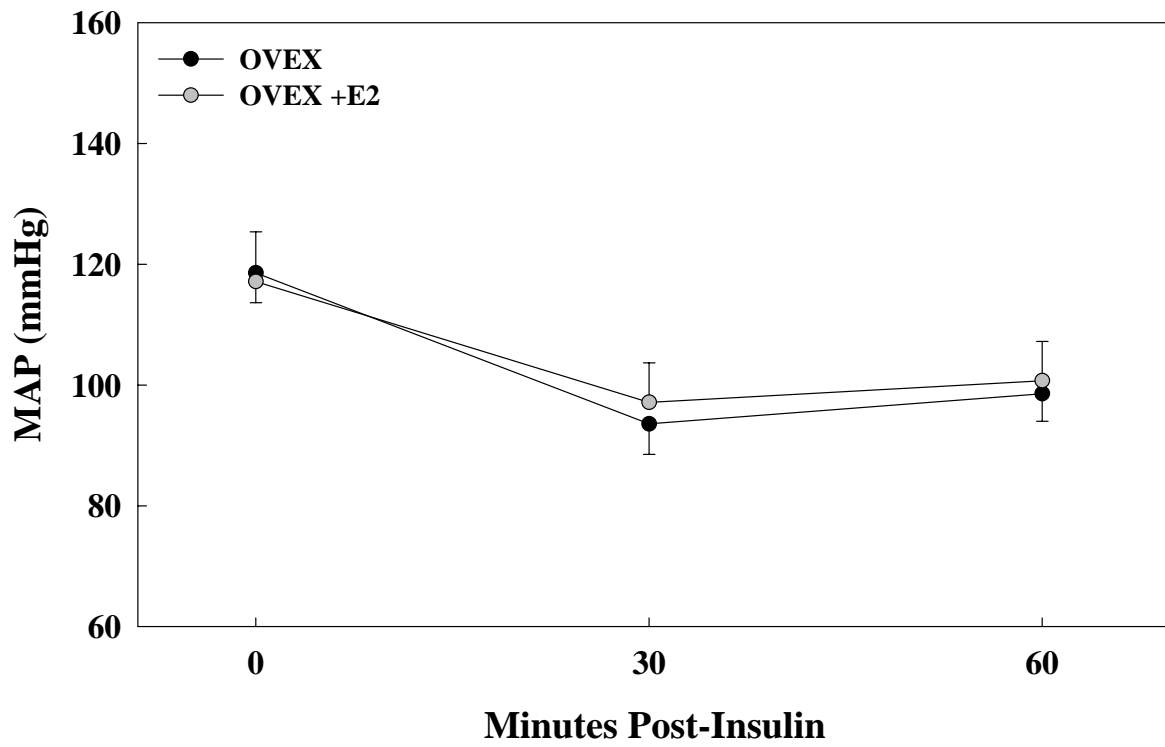
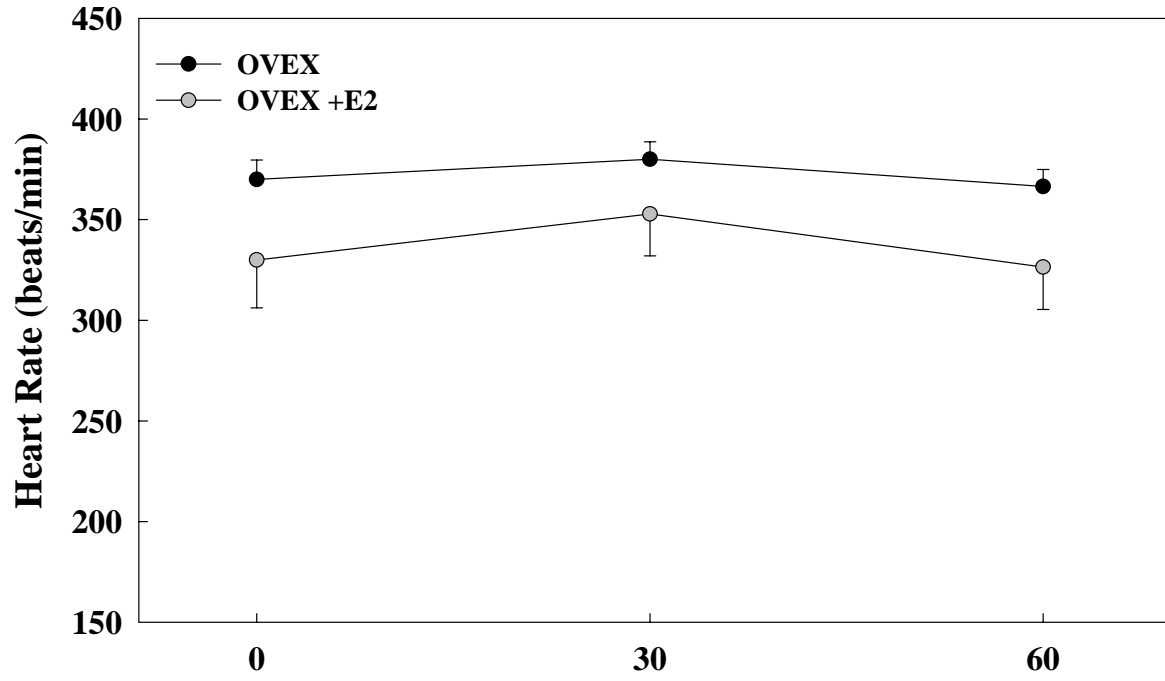


Figure 3.6

Effect of Estradiol-Replacement in Ovariectomized Rats on Hypoglycemia-Induced Changes in Heart Rate and Mean Arterial Blood Pressure

Upper Panel: Effect of hypoglycemia on heart rate. Lower Panel: Effect of hypoglycemia on mean arterial blood pressure (MAP). Experiments were conducted 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. Circles represent Mean \pm SEM of data from 7-8 separate experiments.

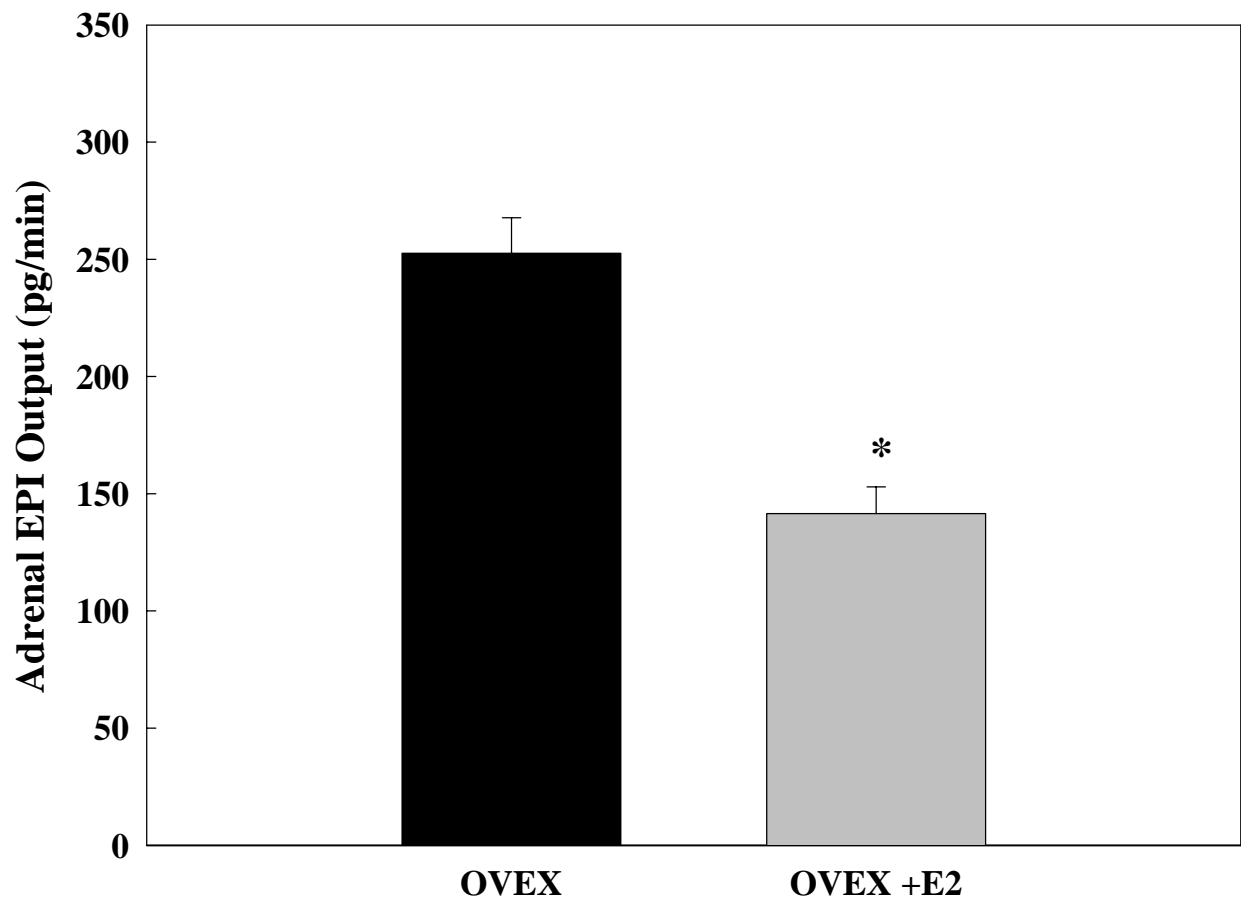


Figure 3.7

Effect of Estradiol-Replacement in Ovariectomized Rats on Hypoglycemia-Induced Increases in Adrenal Epinephrine Output

Experiments were conducted 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. The left renal vein was isolated so that blood flow from the adrenal vein branch could be sampled. Epinephrine (EPI) concentrations were measured in blood samples collected during one 6 min period starting 24 min after the insulin. Columns represent Mean \pm SEM of data from 4-5 separate experiments. * $P < 0.005$ compared to OVEX group.

Figure 3.8

Effects of Ovariectomy and Estradiol-Replacement in Ovariectomized Rats on Adrenal Medullary Gene Expression of Catecholamine Biosynthetic Enzymes

Adrenal medullary tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT) mRNA expression analysis by semi-quantitative RT-PCR. Tissue was collected 14 days after ovariectomy (OVEX), SHAM- surgery, ovariectomy + estradiol replacement (OVEX +E2) or + vehicle treatment (OVEX +VEH). Upper Panel: Ethidium bromide-stained agarose gel illustrating each PCR product (n = 3 per group). The size of each product is listed in the left column. Lower Panel: Densitometric analysis of the relative signal intensities. Columns (lower panel) represent Mean \pm SEM of data from the 3 individual experiments in the upper panel.

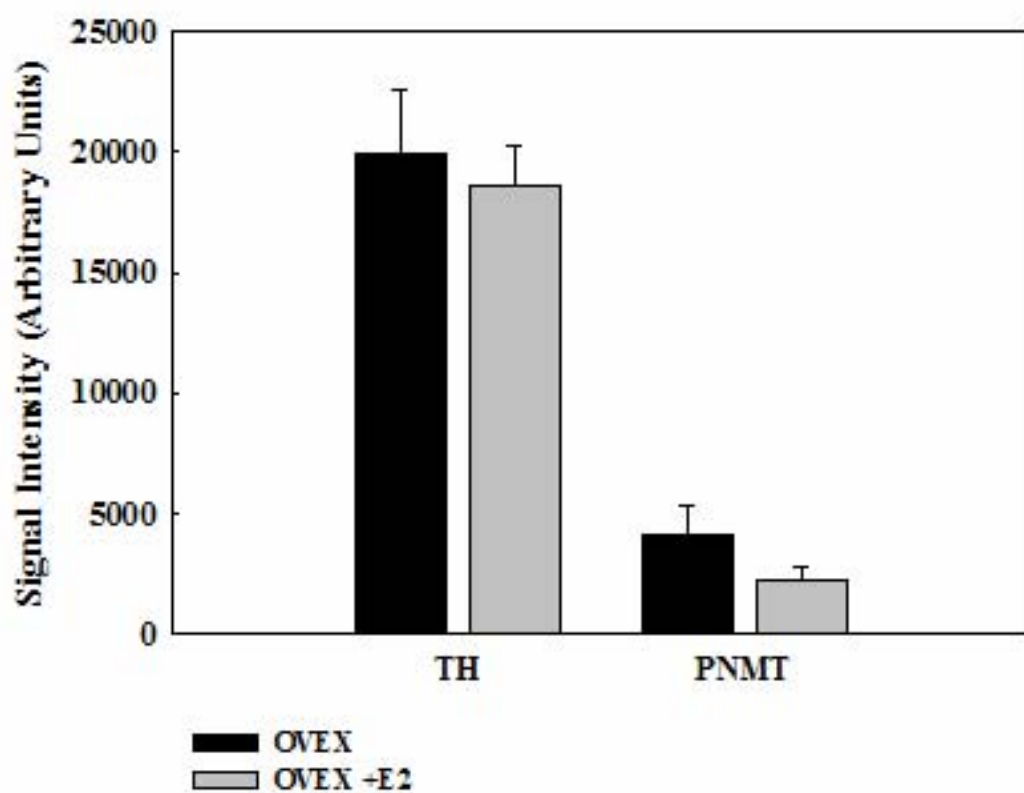
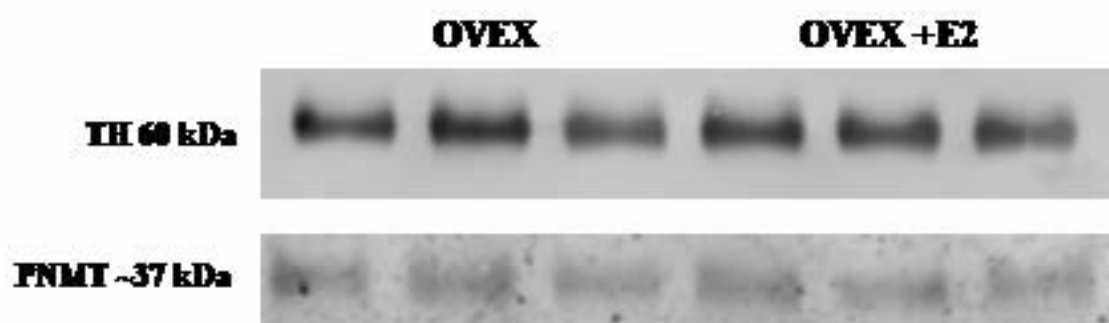


Figure 3.9

Effect of Estradiol-Replacement in Ovariectomized Rats on Adrenal Medullary Protein Expression of Catecholamine Biosynthetic Enzymes

Immunoblot analysis of adrenal medullary protein levels for tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT). Tissue was collected 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. Upper Panel: 20 μ g of adrenal medullary protein samples were separated by SDS-PAGE ($n = 3$ per group) and subjected to immunoblot analysis with rabbit anti TH or rabbit anti PNMT in a dilution of 1:1000 for 1 hr. The size of the product is listed in the left column. Lower Panel: Densitometric analysis of the relative signal intensities. Columns (lower panel) represent Mean \pm SEM of data from the 3 individual experiments in the upper panel.

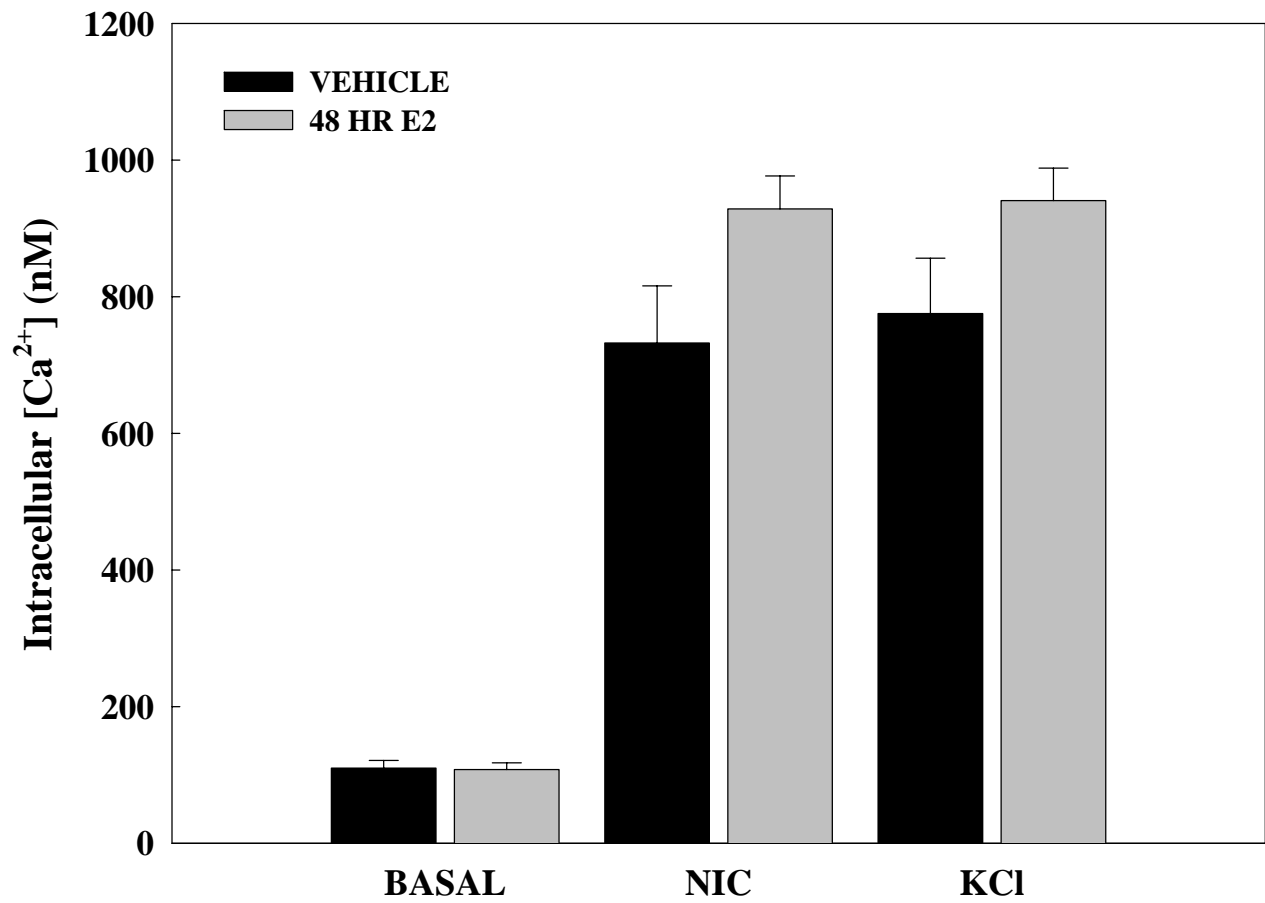


Figure 3.10

Effect of 48 hr Estradiol Treatment on Basal and Agonist-Induced Ca^{2+} Transients in Rat Chromaffin Cells

Cells were exposed to either vehicle or 10 nM estradiol (E2) for 48 hr prior to analysis. Data are expressed as peak intracellular Ca^{2+} concentration achieved in response to either 50 μM nicotine (NIC) or to 60 mM KCl. Columns represent Mean \pm SEM of data from 3 separate isolations.

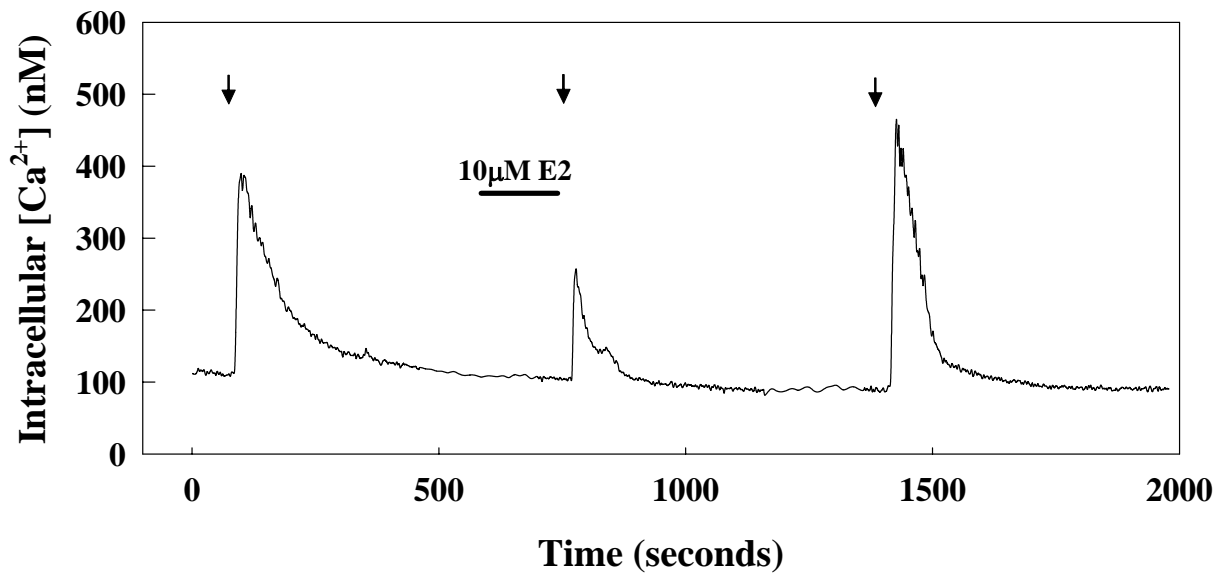
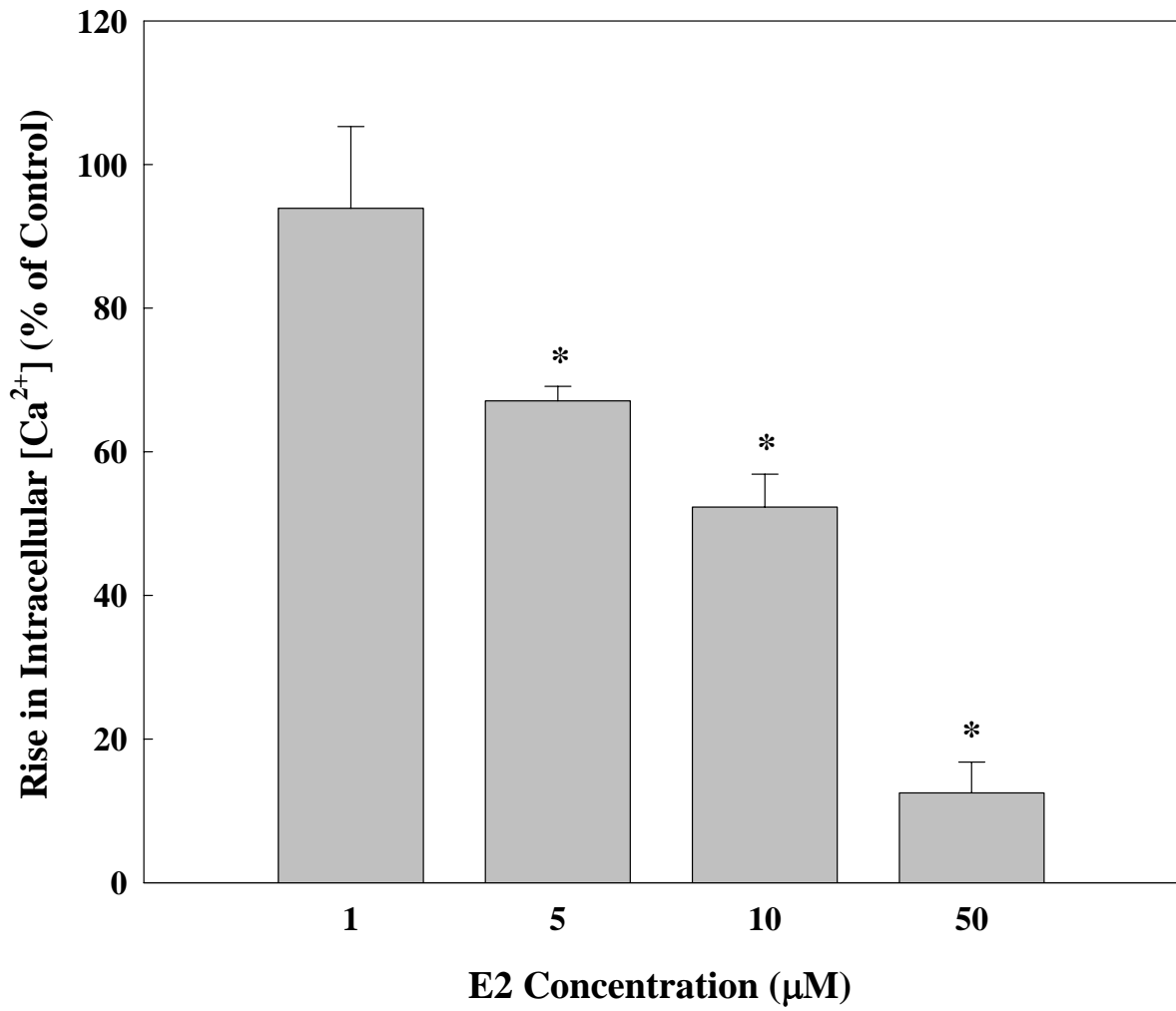


Figure 3.11

Effect of Acute Estradiol Treatment on Agonist-Induced Ca²⁺ Transients in Rat Chromaffin Cells: Dose Dependent Effects and Representative Trace

Upper Panel: Dose-dependent effects of acute estradiol (E2) exposure on nicotine (NIC)-induced intracellular Ca²⁺ transients in rat chromaffin cells. Cells were exposed to various concentrations of E2 for 3 min prior to stimulation with a 15-sec pulse of 50 μ M NIC. Data are expressed as a percent of a control NIC-induced Ca²⁺ transient measured prior to E2 treatment. Lower Panel: Representative trace of intracellular Ca²⁺ responses to 15-sec pulses of 50 μ M NIC (arrows) prior to E2 exposure, after 3 min exposure to 10 μ M E2, and after 10 min washout of E2. Columns (upper panel) represent Mean \pm SEM of data from 3-4 separate isolations. * P<0.001 compared to control transients

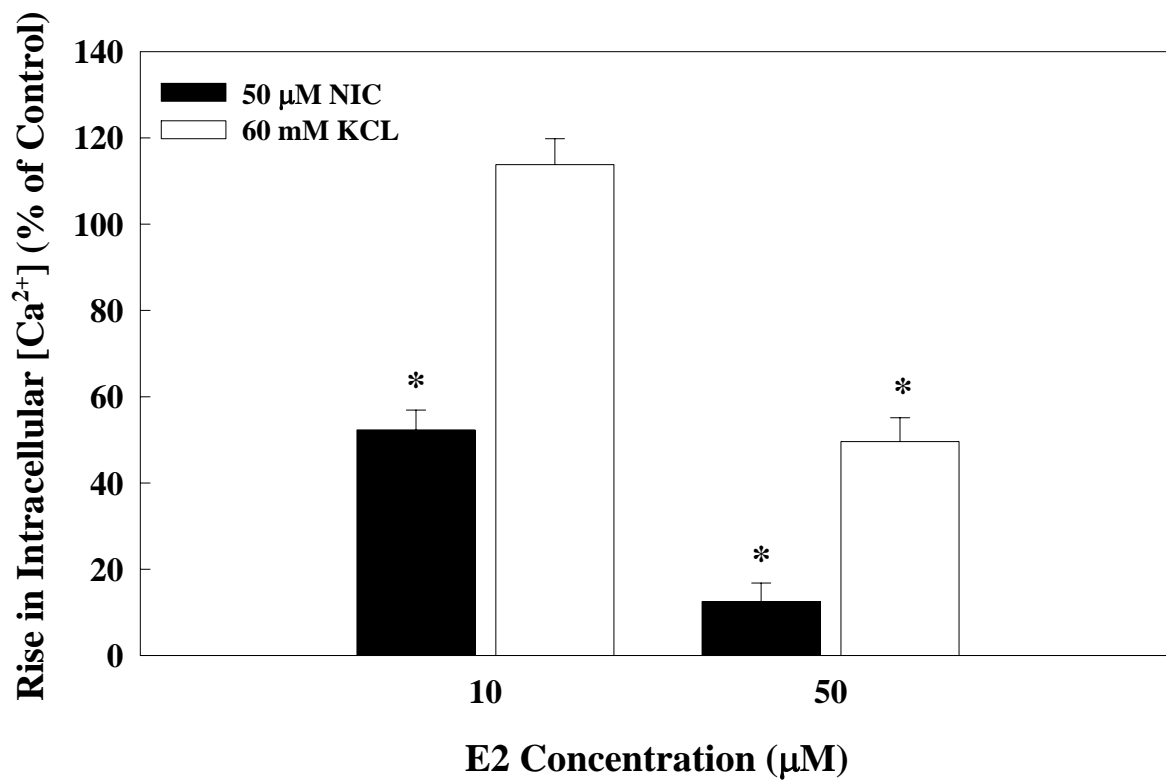
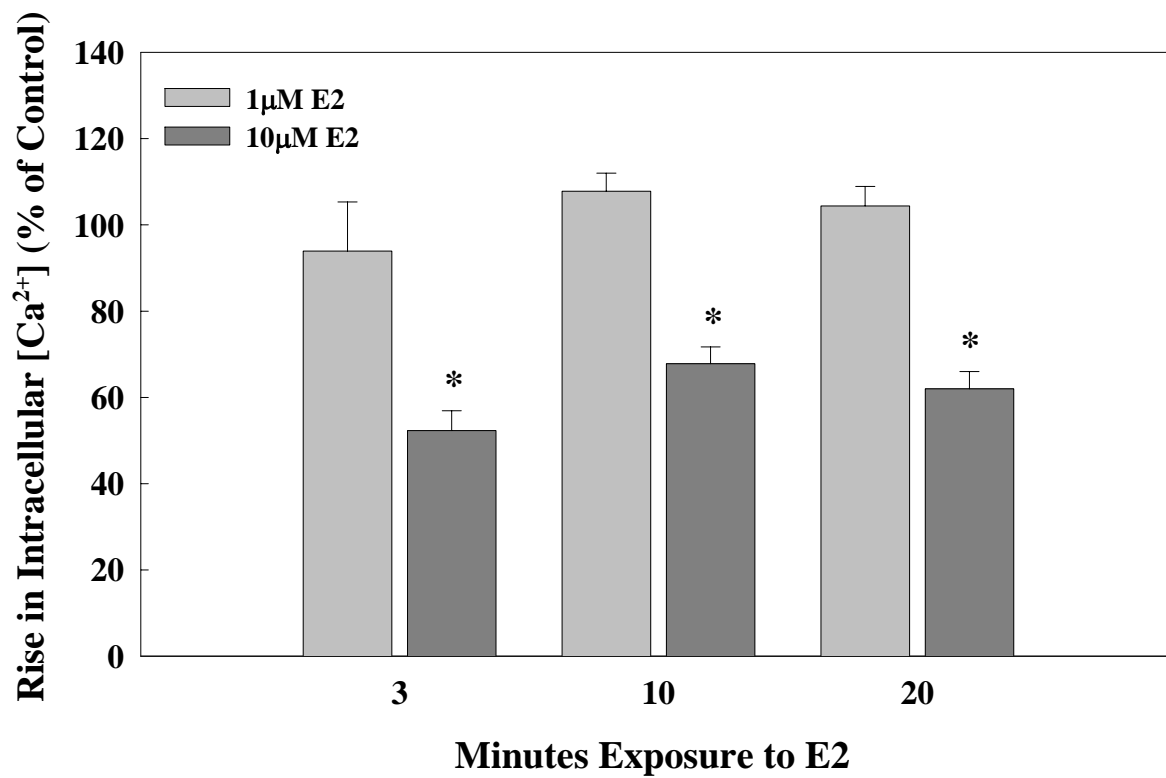


Figure 3.12

Effect of Acute Estradiol Treatment on Agonist-Induced Ca²⁺ Transients in Rat Chromaffin Cells: Time Dependent Effects and Nicotine vs KCl-Induced Transients

Upper Panel: Time dependent effects of acute estradiol (E2) exposure on nicotine (NIC)-induced intracellular Ca²⁺ transients in rat chromaffin cells. Cells were exposed to either 1 or 10 μ M concentrations of E2 for 3, 10, and 20 min prior to stimulation with a 15-sec pulse of 50 μ M NIC. Data are expressed as a percent of a control NIC-induced Ca²⁺ transient measured prior to E2 treatment. Lower Panel: Effect of acute E2 exposure on NIC- and KCl-induced intracellular Ca²⁺ transients in rat chromaffin cells. Cells were exposed to either 10 or 50 μ M concentrations of E2 for 3 min prior to stimulation with a 15-sec pulse of 50 μ M NIC or 60 mM KCl. Columns represent Mean \pm SEM of data from 3-4 separate isolations. * P<0.001 compared to control transients.

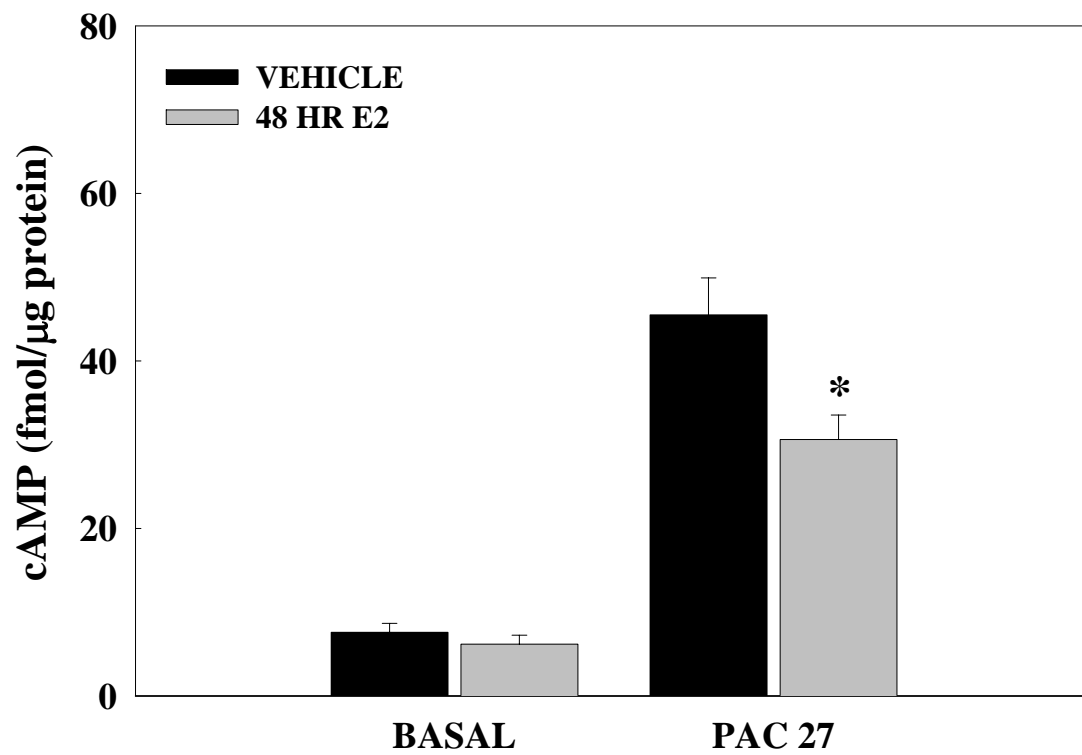


Figure 3.13

Effect of 48 hr Estradiol Treatment on Basal and Agonist-Induced cAMP Accumulation
in H295R Cells

Cells were exposed to either vehicle or 10 nM estradiol (E2) for 48 hr prior to incubation (10 min) with PACAP-27 (PAC 27; 10^{-7} M). All incubations included the phosphodiesterase inhibitor Ro20-1724 (25 μ M). Columns represent Mean \pm SEM of duplicate determinations from 3 experiments. * $P < 0.05$ compared to vehicle-treated cells.

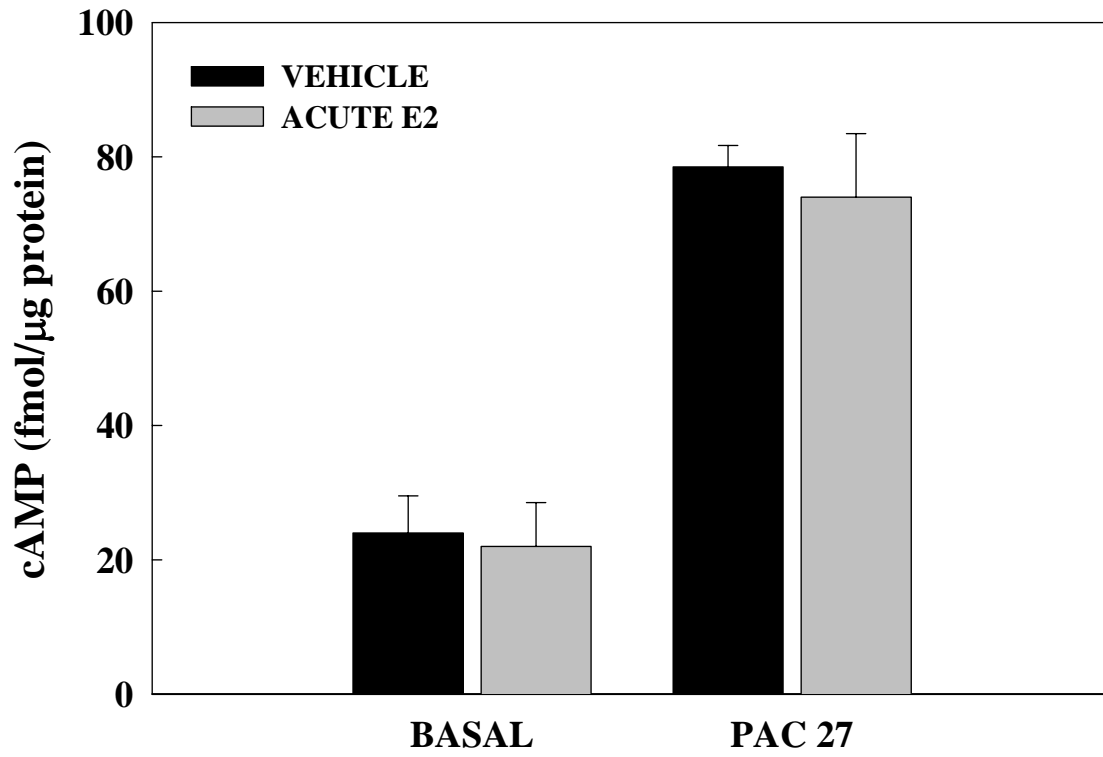


Figure 3.14

Effect of Acute Estradiol Treatment on Basal and Agonist-Induced cAMP Accumulation in H295R Cells

Cells were exposed to either vehicle or 10 nM estradiol (E2) for 10 min prior to incubation (10 min) with PACAP-27 (PAC 27; 10^{-7} M). All incubations included the phosphodiesterase inhibitor Ro20-1724 (25 μ M). Columns represent Mean \pm SEM of duplicate determinations from 3 experiments.

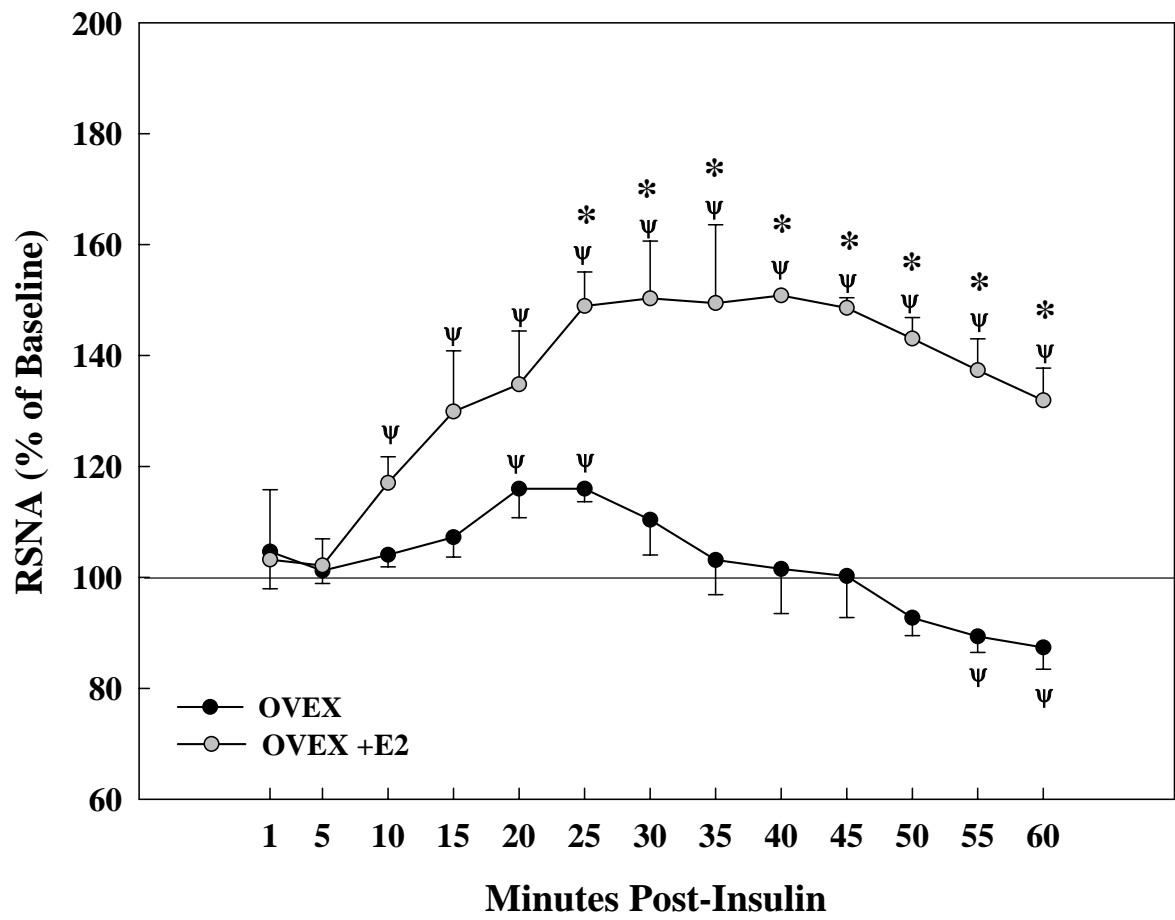


Figure 3.15

Effect of Estradiol-Replacement in Ovariectomized Rats on Hypoglycemia-Induced Changes in Renal Sympathetic Nerve Activity

Experiments were conducted 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. A renal sympathetic nerve fiber was isolated and placed on a stainless steel electrode and stabilized with a silicon-based impression material. Renal sympathetic nerve activity (RSNA) was recorded for 20 min prior to insulin to calculate baseline activity. Data are expressed as a percent of the baseline shown as the solid line at 100%. Circles represent Mean \pm SEM of data from 3 separate experiments. * $P < 0.05$ compared to OVEX group. Ψ $P < 0.05$ compared to baseline.

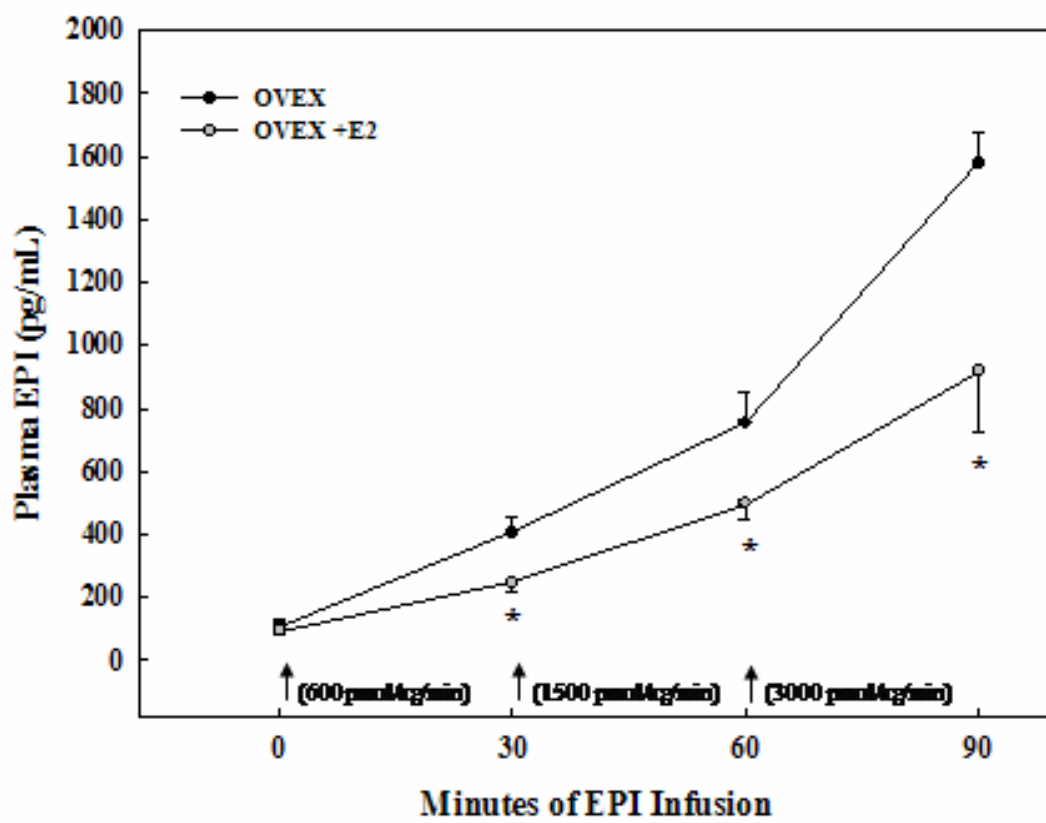


Figure 3.16

Effect of Estradiol-Replacement in Ovariectomized Rats on Plasma Epinephrine Clearance Rates

Plasma epinephrine (EPI) concentrations before and during EPI infusions at a rate of 600, 1500, and 3000 pmol/kg/min (arrows). An arterial blood sample (0.6 mL) was taken prior to the start of the infusion and after 30 min of infusion with each dose. Experiments were conducted 14 days after ovariectomy (OVEX); estradiol (E2)- or vehicle (sesame oil)-containing SILASTIC capsules were implanted at the time of surgery. Circles represent Mean \pm SEM of data from 4-5 separate experiments. *P<0.05 compared to OVEX group.

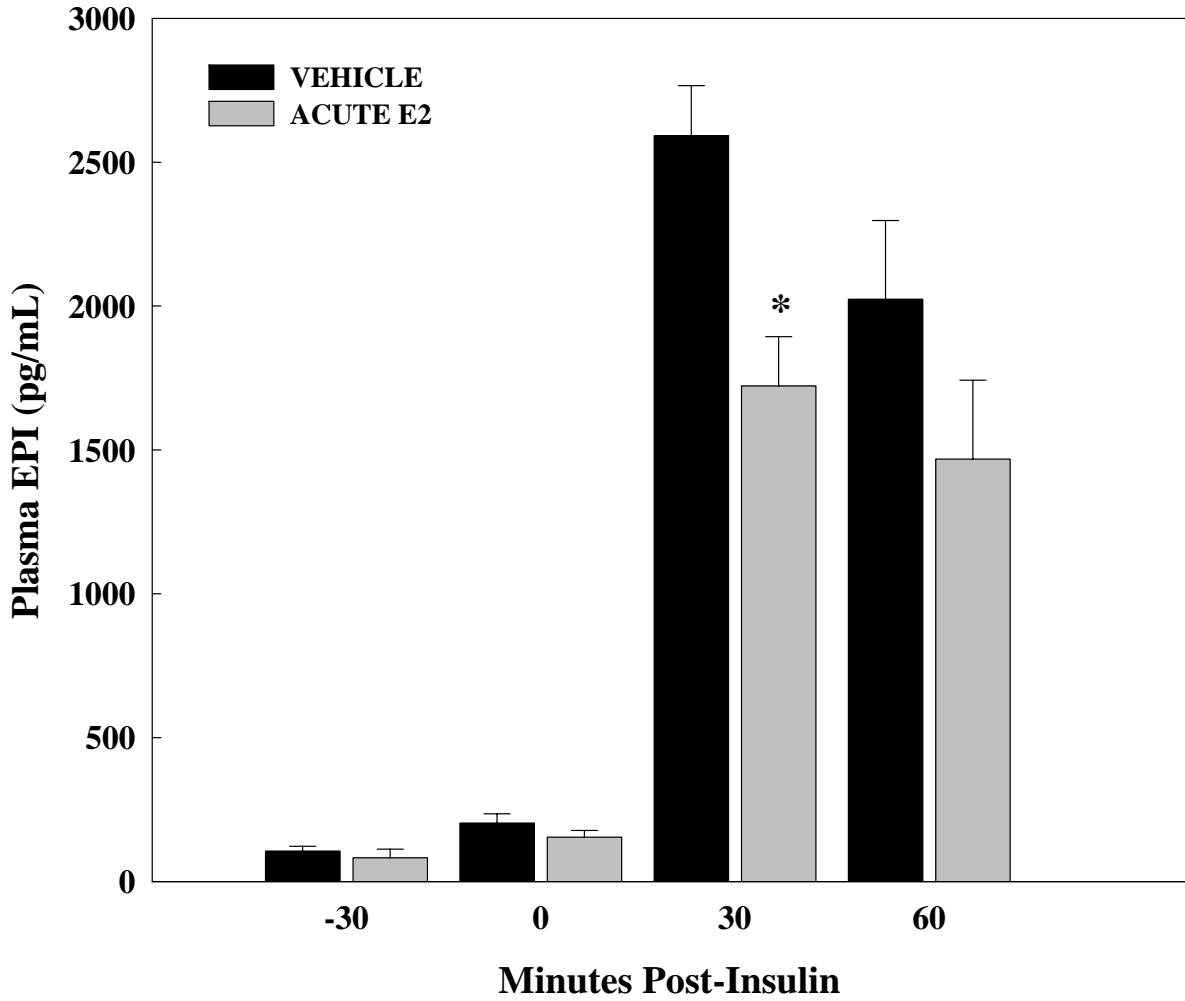
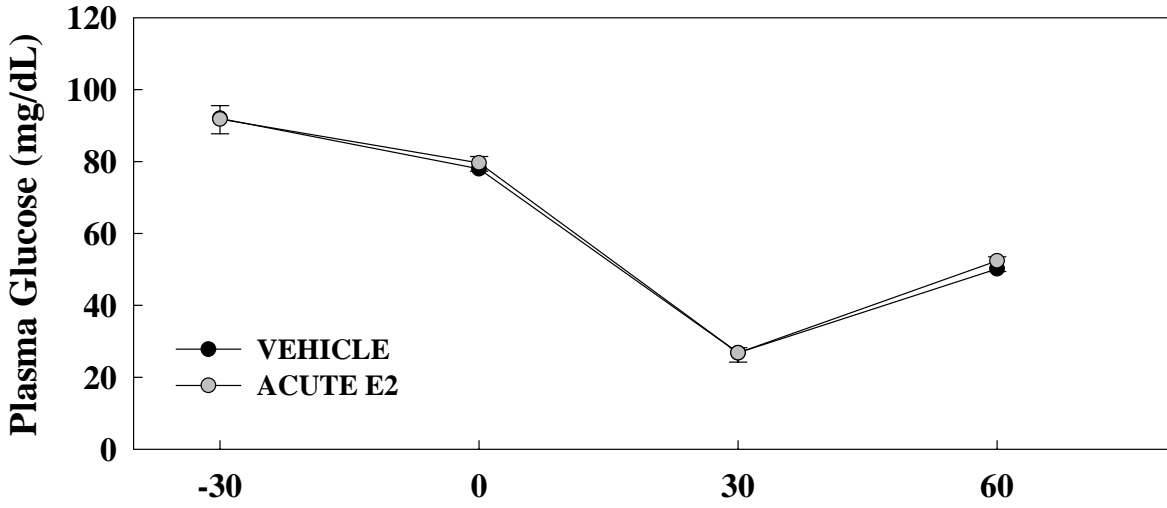


Figure 3.17

Effect of Acute Estradiol Treatment in Ovariectomized Rats on Hypoglycemia-Induced Increases Plasma Epinephrine Concentration

Experiments were conducted 14 days after ovariectomy. 17β -Estradiol (E2; 150 $\mu\text{g}/\text{kg}$) was administered IV over a 2 min period 30 min prior to insulin, followed by a constant infusion (75 $\mu\text{g}/\text{kg}/\text{hr}$) for the duration of the experiment. Insulin (0.25 U/kg) was administered intravenously as a bolus at time 0. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the insulin. Upper Panel: Effect of insulin on plasma glucose concentrations. Lower Panel: Effect of hypoglycemia on plasma epinephrine (EPI) concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 6-7 separate experiments. * $P < 0.01$ compared to vehicle-treated group.

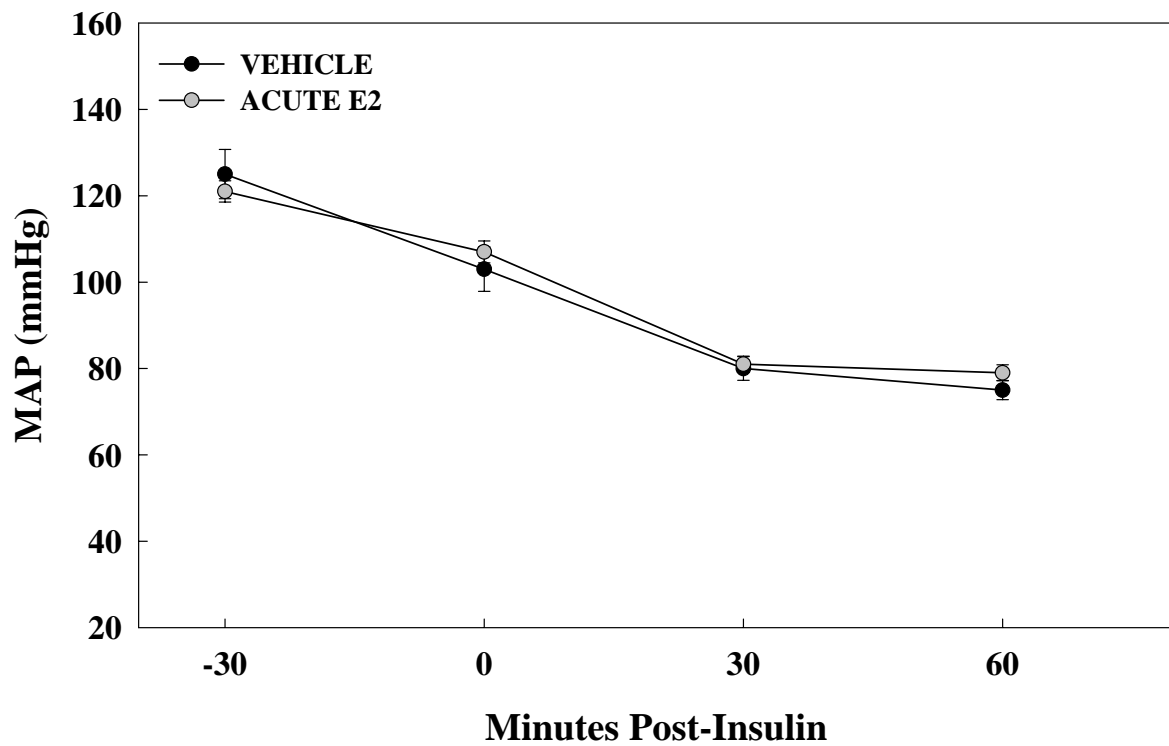
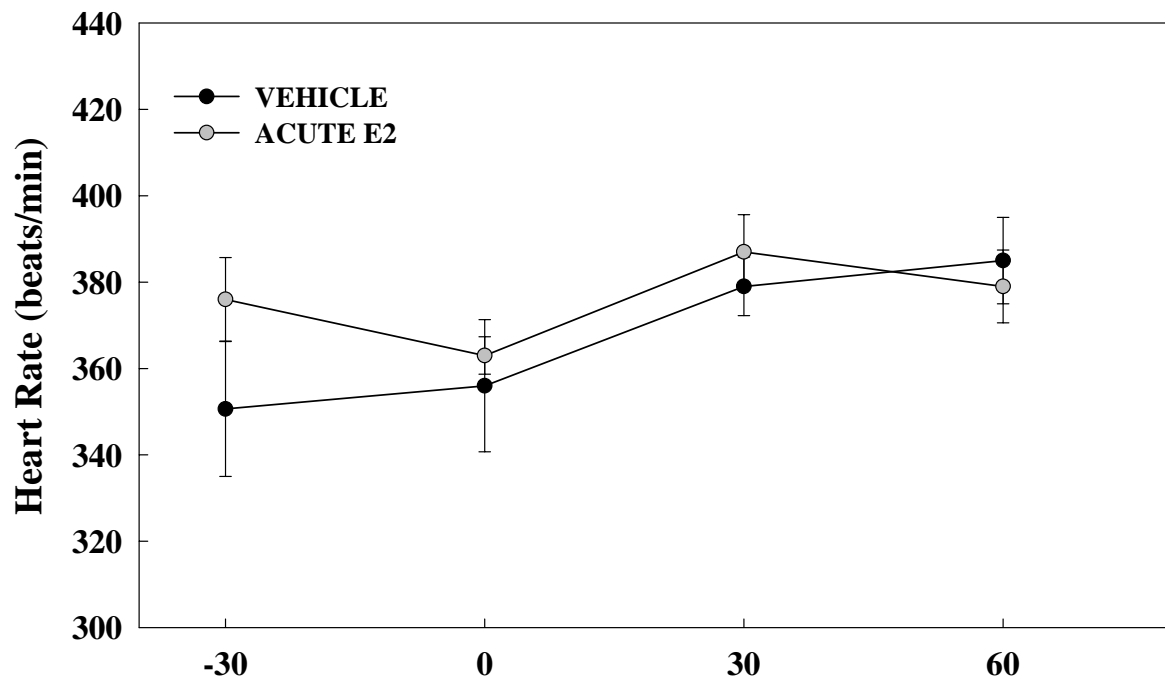


Figure 3.18

Effect of Acute Estradiol Treatment in Ovariectomized Rats on Hypoglycemia-Induced Changes in Heart Rate and Mean Arterial Blood Pressure

Upper Panel: Effect of hypoglycemia on heart rate. Lower Panel: Effect of hypoglycemia on mean arterial blood pressure (MAP). Experiments were conducted 14 days after ovariectomy. 17β -Estradiol (E2) or vehicle was administered IV over a 2 min period 30 min prior to insulin, followed by a constant infusion of the steroid for the duration of the experiment. Insulin was administered intravenously as a bolus at time 0. Circles represent Mean \pm SEM of data from 6-7 separate experiments.

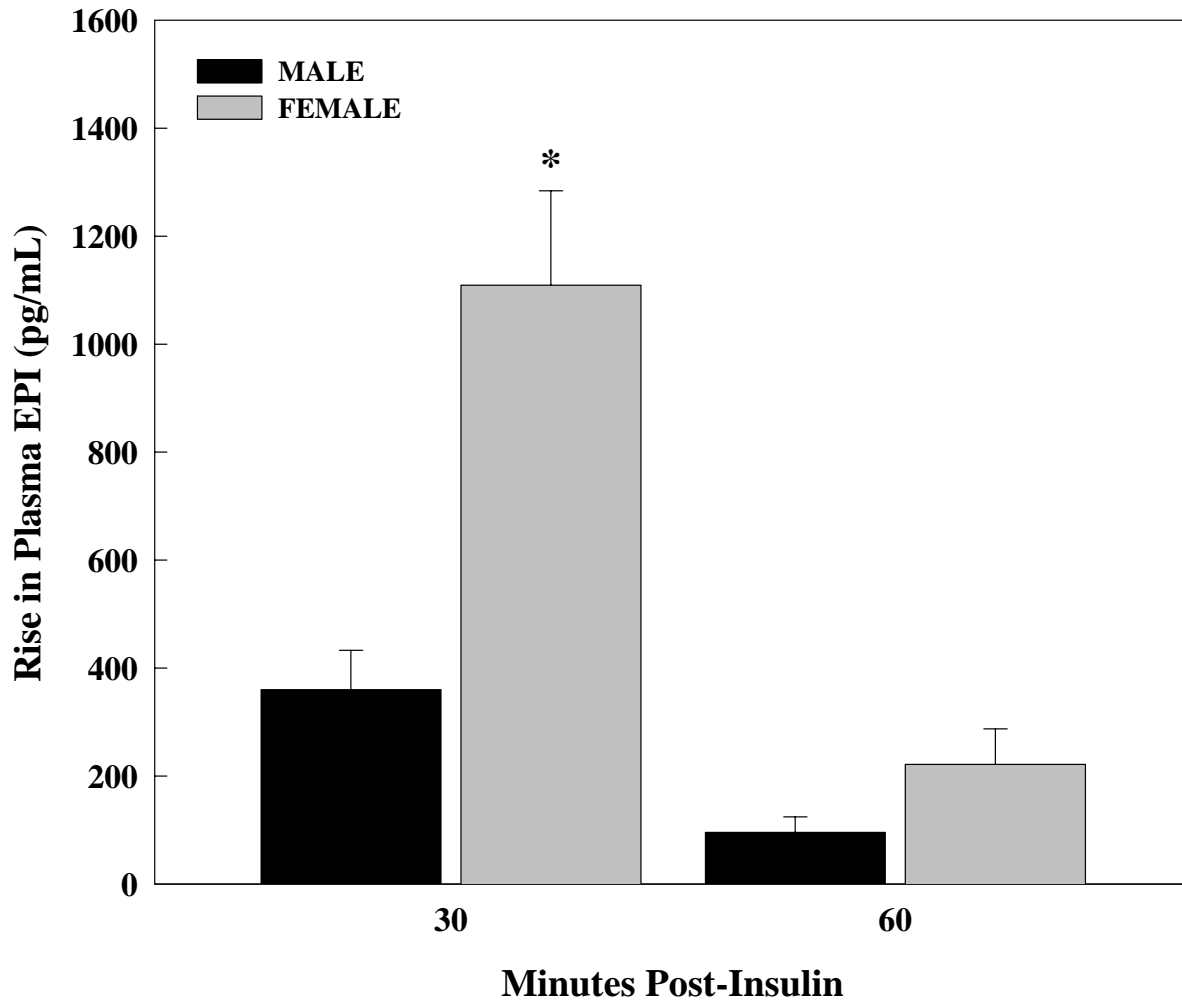
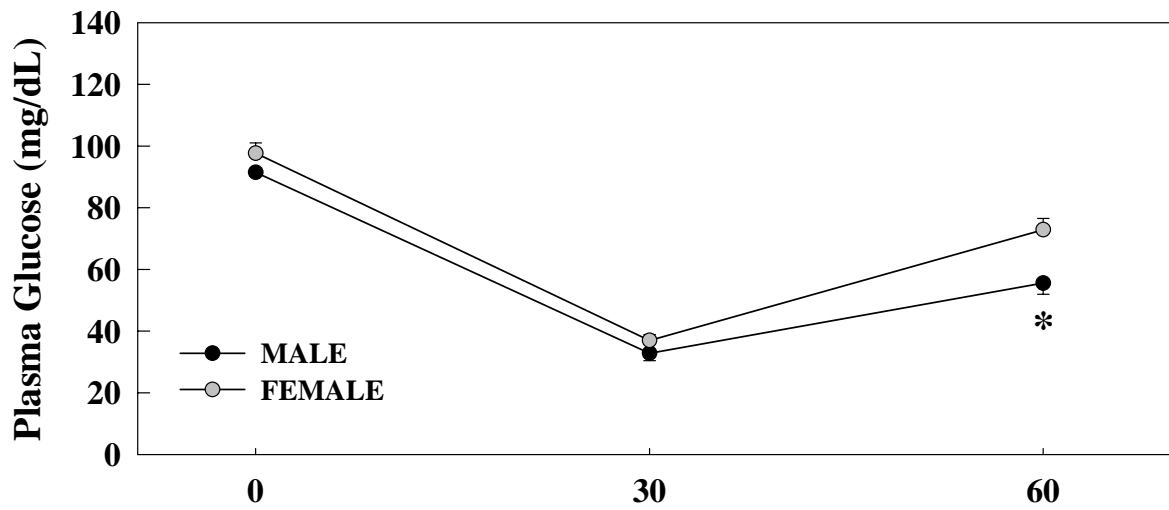


Figure 3.19

Effect of Gender on Hypoglycemia-Induced Increases in Plasma Epinephrine Concentration

Experiments were conducted in 14 week-old male and female rats. Insulin (0.25 U/kg) was administered intravenously as a bolus at time 0. Arterial blood samples (0.6 mL) were taken immediately prior to and 30 and 60 minutes after the insulin. Upper Panel: Effect of insulin on plasma glucose concentrations. Lower Panel: Effect of hypoglycemia on plasma epinephrine (EPI) concentrations. Circles (upper panel) and columns (lower panel) represent Mean \pm SEM of data from 6-7 separate experiments. * $P < 0.01$ compared to male group.

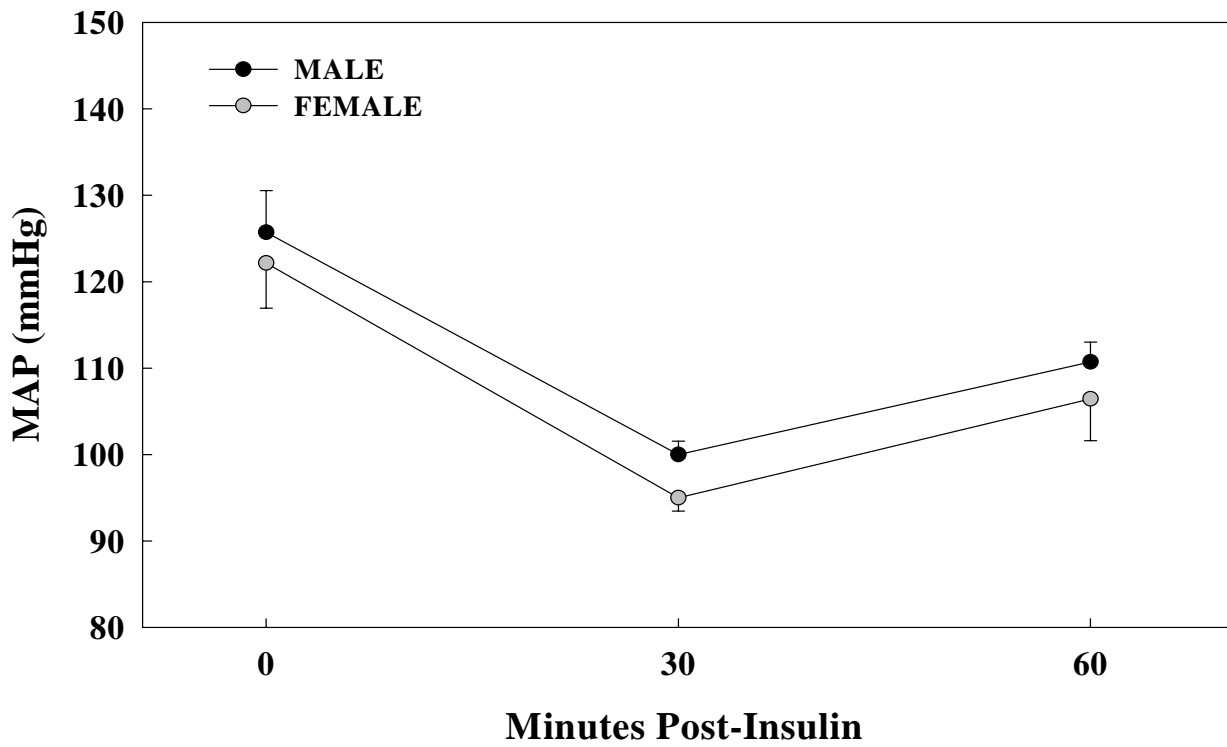
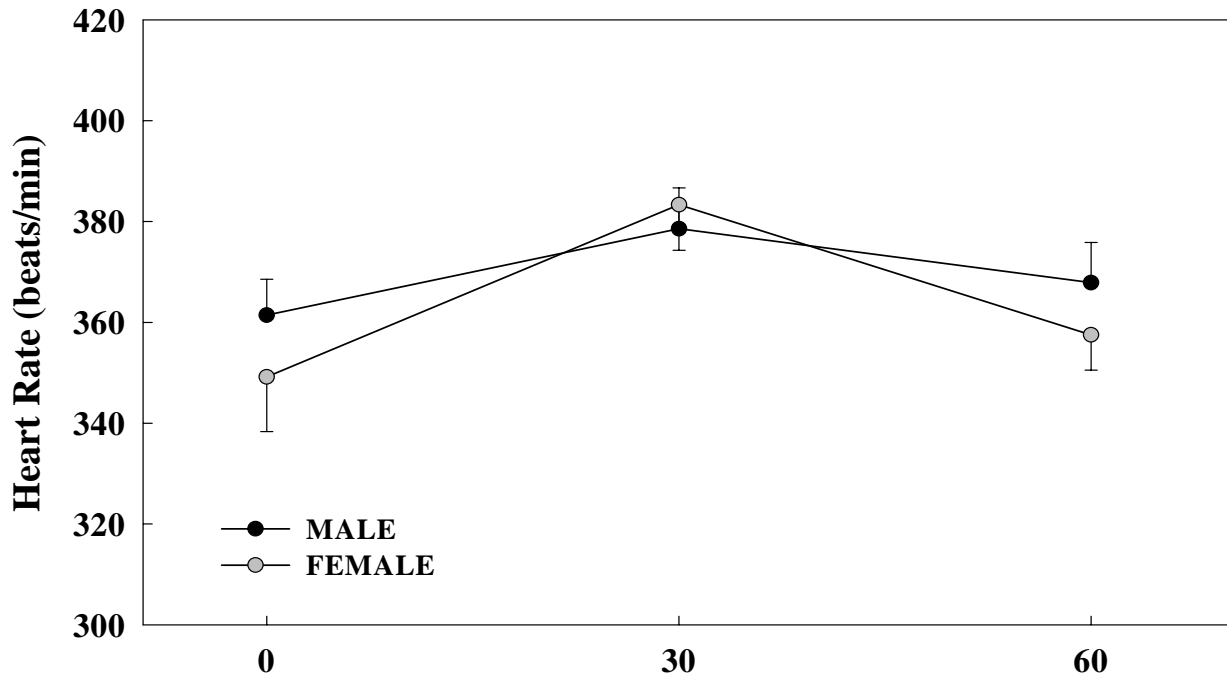


Figure 3.20

Effect of Gender on Hypoglycemia-Induced Changes in Heart Rate and Mean Arterial Blood Pressure

Upper Panel: Effect of hypoglycemia on heart rate. Lower Panel: Effect of hypoglycemia on mean arterial blood pressure (MAP). Experiments were conducted in 14 week-old male and female rats. Insulin was administered intravenously as a bolus at time 0. Circles represent Mean \pm SEM of data from 6-7 separate experiments.

Chapter 4: Discussion

Effects of Estradiol

A number of clinical studies have reported that stress-induced increases in plasma epinephrine levels are decreased in post-menopausal subjects receiving estrogen-replacement therapies (Del Rio et al., 1993; Komesaroff et al., 1999; Ceresinsi et al., 2000; Sandoval et al., 2003). Estrogen supplementation even attenuates mental stress-induced increases in plasma epinephrine in hypogonadal, elderly men (Komesaroff et al., 2002). While these data illustrate that administration of exogenous estradiol suppresses the adrenal medullary response to stress, it remains to be established whether normal circulating levels of estradiol can modulate this response, due to the fact that the levels and types of estrogen administered in these replacement therapies were typically non-physiologic and were almost always administered orally. Also, these reports were limited to a cohort of aging subjects, and most often investigate the effect of replacement therapies in women whose circulating estradiol concentrations have been severely depleted for months or even years. In this context, the timing of estradiol replacement after the loss of the steroid (by ovariectomy) can affect final outcomes. For example, the ovariectomy-induced rise in circulating luteinizing hormone (LH) level in rats is abolished by estradiol replacement at the time of ovariectomy, while estradiol replacement three weeks after ovariectomy reduces the serum LH level by only 50% (King et al., 1987). The same study also reported that luteinizing hormone-releasing hormone quantities in the medial basal hypothalamus are higher in ovariectomized rats administered estradiol replacement at the time of surgery, compared to rats that were estradiol replaced three weeks after ovariectomy. In order to avoid these confounds, the primary goal of the present study was to assess the adrenal medullary response to stress in young, but sexually mature rats in which estradiol levels were controlled by ovariectomy and immediate estradiol replacement.

Our studies have established that physiological circulating levels of 17β -estradiol suppress stress-induced increases in plasma epinephrine in the young adult, female rat. This conclusion is based on two fundamental observations. First, insulin-induced hypoglycemia increases plasma epinephrine concentration to a significantly greater

extent in 14-day ovariectomized rats than in sham-operated controls. Second, and perhaps more specifically, the rise in plasma epinephrine is significantly lower in ovariectomized, estradiol-replaced rats than in vehicle-treated ovariectomized rats. This replacement regime elicited plasma estradiol concentrations virtually identical to those of sham-operated rats during the pro-estrous phase of the estrus cycle. Comparing across groups, the rise in plasma epinephrine attained with 30 min of hypoglycemia in the estradiol-replaced rats was, in fact, quite comparable to that of the sham-operated controls, suggesting indirectly that the modulation of plasma catecholamine levels is due to an effect of physiological levels of circulating estradiol alone and not in combination with other ovarian steroids.

Insulin-induced hypoglycemia is a standard method used for assessing adrenal stress responses, despite the fact that the mechanism by which insulin elicits an increase in plasma catecholamines is still not completely understood. While there is evidence that insulin can directly stimulate epinephrine release from the adrenal medulla *in vitro* (Macho et al., 1996), the preponderance of evidence suggests that the *in vivo* response requires a centrally-mediated neuronal activation. For example, it has been shown that adrenal denervation inhibits the hypoglycemia-induced increases in both plasma epinephrine concentration (Khalil et al., 1986) and adrenal medullary tyrosine hydroxylase gene expression (Vietor et al., 1996). Increases in plasma epinephrine are also attenuated in brain/neuron specific insulin receptor knockout mice (Fisher et al., 2005).

Despite this uncertainty surrounding the mechanism, there are several advantages in utilizing an insulin-induced hypoglycemic model of stress. Firstly, it is well established that hypoglycemia consistently increases plasma epinephrine levels in both experimental animals (Fujino and Fujii, 1995; Vollmer et al., 1997; Drake et al., 1998) and humans (Diamond et al., 1993; Davis et al., 2000; Sandoval et al., 2003; Heise et al., 2004). Secondly, in contrast to other stress paradigms such as footshock (Weinstock et al., 1998), hypoxia (Bloom et al., 1977), and cold (Kanayama et al., 1999), the magnitude of the epinephrine increase in response to hypoglycemia is insulin dose-dependent. A third advantage of this model is that it can be utilized reliably in both conscious (Goldstein et al., 1993; Vollmer et al., 1997; Batista et al., 2005; Fisher et al.,

2005) and anesthetized (Mokuda and Sakamoto, 1994; Lamarche et al., 1995; Drake et al., 1998) experimental animals. We selected the anesthetized model for use in the present studies for a number of reasons. For example, anesthesia decreases many of the environmental confounds that can affect the stress response. Second, this model eliminates the need for multiple survival surgeries. Finally, from a productivity perspective, the turnover rate of chronically instrumented, conscious animal studies is much slower than that of anesthetized studies.

The utilization of an anesthetized model in these studies was, however, also precipitated by our early work which focused on the development of a classical conditioning paradigm so that stress responses in the conscious female rat could be assessed. Using a paradigm modified from Randall et al. (1994), rats were first habituated to handling and restraint in a cloth sock for 1-2 days, after which each rat was exposed for 1 week to 5 trials per day of a 15-second pulsed tone followed by a 0.5 second, 0.4 mA tail shock. On the day of the experiment, the rats were placed in the sock and exposed to 1 tone + shock. This paradigm has been shown to evoke immediate, short-duration increases in sympathetic nerve activity (Randall et al., 1994; Brown et al., 1999) and in principle, should have resulted in epinephrine secretion from the adrenal medulla. However, this paradigm failed to elicit even marginal increases in plasma epinephrine levels, suggesting that the adrenal medulla does not secrete epinephrine in response to this model of stress.

The estrogen-dependent suppression of hypoglycemia-induced increases in plasma epinephrine could, in theory, be due to either decreased adrenal medullary secretion and/or increased peripheral clearance of epinephrine. We do have data to support the concept that estradiol replacement suppresses adrenal medullary secretion in ovariectomized rats. This conclusion is based on measurements of epinephrine concentration from the adrenal vein during hypoglycemia. Our approach in these experiments was a modification of a standard method used to assess adrenal catecholamine output (Engeland and Gann, 1989; Gaumann et al., 1990; Vollmer et al., 2000; Lamouche and Yamaguchi, 2001), which allows blood to flow from the vein for the duration of the experiment while donor blood is infused through a peripheral vein. In order to minimize confounds associated with continuous bleeding and donor

replacement, blood was collected over just one 6 min period after 24 min of hypoglycemia in the present study. The epinephrine measured during this time should represent peak adrenal output because both the hypoglycemia-induced decrease in blood glucose concentration and rise in sympathetic nerve activity are maximal at this time. Ideally, to confirm this assumption these experiments should be repeated to allow sampling at other post-insulin time points as well as to establish pre-insulin epinephrine secretory levels. Recognizing the inherent limitations of this approach, considerable time was initially invested on attempting to establish a method for the continuous measurement of adrenal epinephrine output using *in vivo* chronoamperometry. This technique successfully detects and quantifies catecholamines in the brain (Freund et al., 2003; Moxon et al., 2004; Burmeister et al., 2005; Glaser et al., 2005). With assistance from Dr. Greg Gerhardt and members of his laboratory, both a Nafion-coated carbon fiber electrode and a ceramic-based multisite electrode were successfully introduced into the adrenal vein and the adrenal medulla. However, both electrodes failed to detect epinephrine at either site, due, at least in part, to the electrodes' inability to oxidize catecholamines in blood or highly vascularized tissue.

Subsequent experiments addressed the question of whether estradiol-dependent suppression of the adrenal output of epinephrine was due to decreased synthesis and/or secretion of epinephrine by the chromaffin cell. Green et al. (1999), initially reported estrogen receptor- α (ER α) immunoreactivity in chromaffin cells of the female but not male rat adrenal gland. The RT-PCR and immunoblot data in the present study confirms and expands on this report, since ER- α message and protein expression and estrogen receptor- β (ER β) message can be detected in the female rat adrenal medulla, albeit at considerably lower levels of expression than in the uterus and ovary respectively.

Despite the probable existence of functional estradiol receptors in the medulla, the present studies do not support the hypothesis that this steroid directly affects either epinephrine biosynthesis in or secretion from the adrenal medulla. At the gene level, semi-quantitative RT-PCR analysis revealed that mRNA levels for both tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT) are not different in adrenal medullae taken from ovariectomized versus sham-operated animals or from

estradiol- versus vehicle-replaced ovariectomized rats. At the protein level, neither TH nor PNMT expression was modulated by estradiol treatment in ovariectomized rats.

There are, in fact, already a number of contradictory reports on the effects of estradiol on catecholamine biosynthesis in the adrenal medulla. Recently, Serova et al. (2005) have reported that TH mRNA levels are increased in ovariectomized rats treated with estradiol benzoate for 16 days compared to vehicle-treated ovariectomized rats. And while Kohler et al. (1975) have reported that 10 days of estrogen administration to intact female rats increases TH activity, de Miguel et al. (1989) have reported that TH activity is unaffected by estrogen treatment in ovariectomized rats. Additionally, it has also been reported that TH activity is not different in the adrenal medulla of aged (22 months old) female rats compared to young (2 months old), but adrenal medullary epinephrine content is greater in the aged rats (Fernandez-Ruiz et al., 1989).

With regard to secretion, acetylcholine released by the adrenal nerve stimulates Na^+ influx via nicotinic acetylcholine receptor-operated ion channels on the chromaffin cell (Wada et al., 1985). The resultant depolarization activates voltage-gated Ca^{2+} channels and results in a transient rise in intracellular free Ca^{2+} concentration, which is the primary trigger for catecholamine exocytosis (Kilpatrick et al., 1982). Previous studies from our laboratory established that 48 hr exposure to the synthetic glucocorticoid dexamethasone significantly potentiated agonist-induced intracellular Ca^{2+} transients in isolated adrenal medullary chromaffin cells (Fuller et al., 1997a, b). In the present study, 48 hr exposure to a relatively physiologic concentration of estradiol (10 nM) had no significant effect on nicotine-induced intracellular Ca^{2+} transients, suggesting, albeit indirectly, that the effects of estradiol *in vivo* are not due to a suppression of agonist-induced Ca^{2+} signaling in, and as a consequence epinephrine secretion from, the chromaffin cell.

While nicotinic acetylcholine receptor mediated Ca^{2+} signaling is the primary stimulatory pathway in these cells, it is well-established that chromaffin cells also express muscarinic acetylcholine receptors (Inoue and Kuriyama, 1991; Inoue and Imanaga, 1995), which, upon activation, can also directly stimulate catecholamine secretion from rat the chromaffin cell (Finnegan et al., 1996). There is evidence that the number of muscarinic acetylcholine receptors is increased in the hippocampus of ovariectomized

rats, and that this increase is abolished by estradiol treatment (Cardoso et al.; 2004), therefore future studies should also investigate the effects of estradiol on muscarinic-receptor mediated contributions to secretion.

Although adrenal output and intracellular Ca^{2+} signaling are valid means by which to assess stress-induced chromaffin cell secretion, cellular processes 'downstream' of Ca^{2+} signaling were not directly examined in these studies. Previous studies from our laboratory have determined nicotinic-induced catecholamine secretion in single cells by carbon-fiber amperometry (Liu et al., 2001). One of the limitations of this approach is that measurements can reliably only be compared within the same cell, making this technique more appropriate for acute drug exposure studies. The effects of longer-term (ie. 48 hr) exposure to a steroid (dexamethasone) on agonist-induced epinephrine secretion have been assessed in populations of cells by HPLC (Fuller et al., 1997b), but this method requires a large number of cells for each experiment and cell yields from rat adrenal glands are typically too low for this type of analysis. However, while chronoamperometry has not proven effective for measuring plasma concentrations of epinephrine, it may be possible to utilize this approach to assess the effects of estradiol on secretion *in vitro*. Our *in vivo* attempts at chronoamperometry gave us experience in calibrating carbon-fiber electrodes for epinephrine in solution and it may be possible to use these types of electrodes in order to measure secretion from a small number of cells.

A more global approach to examine the effects of estradiol on stimulus-secretion coupling pathways in the adrenal medulla would be to utilize gene array technology. However, our experience in this area was not very encouraging. In experiments to assess the effects of age on gene expression in the adrenal medulla, the expression levels of 62 known genes were significantly increased and the expression levels of 67 known genes were significantly decreased by a minimum of 1.5 fold in the adrenal medulla of aged compared to young rats. However, none of the genes that were affected by age were known to be particularly important in secretory signaling or biosynthetic pathways, even though it is well established that both stress-induced plasma catecholamine levels (McCarty 1986; Mabry et al., 1995) and expression of tyrosine hydroxylase (Voogt et al., 1990; Tumer et al., 1992; 1997; Tumer and Larohelle, 1995) are significantly increased with age.

In contrast to the apparent lack of effect of circulating levels of estradiol on chromaffin cell function discussed earlier, short-term exposure to high, presumably non-physiologic levels of estradiol can suppress agonist-induced Ca^{2+} signaling in the chromaffin cell. These data are fundamentally consistent with several previous reports in which acute exposure to micromolar concentrations of estradiol significantly depressed nicotinic receptor-triggered increases in intracellular Ca^{2+} concentration in bovine chromaffin cells (Liu et al., 2002; Machado et al., 2002) and KCl-induced transients in PC-12 cells (Kim et al., 2000). The present study demonstrated that 3 min of exposure to estradiol is sufficient to dose-dependently suppress nicotine and KCl-induced Ca^{2+} transients in rat chromaffin cells. The fact that the effect was both rapid and reversible suggests that estradiol is acting non-genomically. Although catecholamine secretion was not measured in this study, previous work from our laboratory has shown that a suppression of nicotine-induced intracellular Ca^{2+} transients of this magnitude reduces nicotine-induced catecholamine secretion from porcine chromaffin cells (Wagner et al., 1999). However, it has been reported that short-term exposure to estrogens, including 17β -estradiol, has no effect on acetylcholine-induced secretion from bovine chromaffin cells, while androgens inhibit agonist-induced secretion (Dar and Zinder, 1997).

Due to the high concentrations of estradiol used, it is plausible that the acute effects of estradiol on chromaffin cell signaling are non-cell specific. The present study has demonstrated that acute exposure to a high concentration of estradiol does not affect function in every adrenal cell type. The NCI-H295R cell line was derived from human adrenocortical cells. These cells retain the ability to secrete mineralocorticoids, glucocorticoids, and adrenal androgens (Rainey et al., 1994), and like primary adrenal cortical cells, express pituitary adenylate cyclase-activating polypeptide (PACAP) receptors that, when activated, stimulate cAMP synthesis and the subsequent release of aldosterone and/or cortisol (Bodart et al., 1997; Haidan et al., 1998). Acute exposure to a high concentration of estradiol did not affect PACAP-mediated cAMP accumulation in these cells. In contrast, 48 hr exposure to a physiologic concentration of estradiol significantly attenuated PACAP-induced increases in cAMP accumulation in this cell line. While it is difficult to correlate data between primary cultures of cells and a

cell line, as well as between species, these data do suggest that both the acute and long-term effects of estradiol on intracellular signaling processes are cell-type specific.

Given that acute treatment with estradiol attenuated agonist-induced Ca^{2+} transients in the chromaffin cell, epinephrine secretion should also be suppressed, which would result in decreased stress-induced rises in plasma epinephrine levels *in vivo*. Using the hypoglycemic stress model, we tested this hypothesis and have now shown that an acute infusion of supra-physiological levels of estradiol does suppress the insulin-induced rise in plasma epinephrine in ovariectomized rats. It should be noted however, that the overall effect on plasma epinephrine levels is relatively small given the large increase in plasma estradiol levels that occurred with this infusion paradigm. Therefore, it is unlikely that a physiological increase in estradiol could occur that would be of the magnitude needed to elicit an acute effect on circulating epinephrine levels.

The studies to this point do not support the concept that the estradiol-dependent suppression of stress-induced adrenal epinephrine output is due to modulation of either epinephrine biosynthesis or stimulus-secretion coupling in the chromaffin cell. Therefore, subsequent experiments addressed the possibility that estradiol can modulate stress-induced increases in sympathetic nerve activity, which is the initial trigger for catecholamine secretion from the adrenal medulla. Although previous reports have demonstrated that intravenous administration of supra-physiologic concentrations of estrogen can rapidly suppress both renal and splanchnic nerve activity in ovariectomized rats (He et al., 1998), and that chronic estrogen treatment can affect sympathetically-mediated baroreflex sensitivity (He et al., 1999; Pamidimukkala et al., 2003), in the present study, hypoglycemia-induced increases in nerve activity were actually greater in the estradiol-replaced ovariectomized rats compared to vehicle-treated ovariectomized rats. Clearly these data are not consistent with the concept that estradiol suppresses epinephrine secretion by modulating neural input, since this increased nerve activity should elicit a greater epinephrine response to stress.

One limitation of these data is that recordings were made from the more easily accessible renal nerve, while secretion is predominately regulated by the activity of the splanchnic/adrenal nerve. Therefore, the activity of these nerves during hypoglycemia should also be examined in order to fully assess the effect of estradiol on neural input to

the adrenal medulla. This requirement is further supported by the fact that a given stimulus can induce a diverse pattern of sympathetic responses. For example, Scislo et al. (2001) demonstrated that stimulation of A2A receptors increased adrenal nerve activity, decreased renal nerve activity, and did not affect lumbar nerve activity. This same group also reported that A1 receptor stimulation increased the activity of the adrenal, renal, and lumbar nerves, but the magnitude of the increase was greatest in the adrenal nerve and lowest in the lumbar nerve. In addition, it has been reported that estrogen potentiates presynaptic function in cultured hippocampal neurons (Yokomaku et al., 2003) and enhances glutamate receptor-mediated excitatory postsynaptic potential (Foy et al., 1999); but there is little published research investigating the effects of physiological concentrations of estradiol on nerve properties such as conductivity, pre- and post-synaptic function and synapse plasticity. The information from such studies would certainly offer insight into how estrogen may modulate the neural input to the adrenal medulla.

In review of the results thus far, the reduced adrenal output of epinephrine in estradiol-replaced, ovariectomized rats is consistent with the observed depressive effects of physiological levels of estradiol on the hypoglycemia-induced increases in plasma epinephrine concentration. The mechanism(s) underlying the suppressive effects of estradiol on adrenal medullary secretion remain to be defined. A final series of experiments assessed whether the difference in plasma epinephrine concentration during hypoglycemia may also be attributed to differential rates of plasma catecholamine clearance. To assess the effects of estradiol on plasma epinephrine handling, 30 min sequential intravenous infusions of epinephrine (600, 1500, and 3000 pmol/kg/min) were administered in vehicle and estradiol-treated ovariectomized rats. At all epinephrine infusion rates, plasma epinephrine concentrations were lower in the estradiol-replaced ovariectomized group compared to the vehicle-treated ovariectomized rats after each infusion, suggesting that the clearance of epinephrine from the circulation was higher in these animals.

However, these data are not consistent with the majority of available information which suggests that estrogen negatively regulates both catecholamine metabolism and cellular reuptake. For example, the activity of catechol-O-methyltransferase (COMT),

the main enzyme responsible for the metabolism of catecholamines, is at its lowest level in the female rat brain and adrenal gland during the pro-estrous phase of the estrous cycle (Parvez et al., 1978), the period of highest circulating estradiol concentration (Smith et al., 1975). Also, both the gene expression and the activity of COMT are down-regulated by physiologic concentrations of estradiol in MCF-7 cells (Xie et al., 1999; Jiang et al., 2003). It has also been reported that estradiol can affect nor-epinephrine transporter function in bovine chromaffin cells, suppressing cellular reuptake (Toyohira et al., 2003). Consequently, these effects on metabolism and reuptake should elevate plasma epinephrine levels.

Effects of Gender

As noted earlier in the discussion, estradiol supplementation can also reduce the epinephrine response to stress in men (Komesaroff et al., 2002). These data are consistent with both clinical (Diamond et al., 1993; Davis et al., 2000; Galassetti et al., 2001) and basic science (Drake et al., 1998) studies which have demonstrated that stress-induced increases in plasma catecholamines are lower in females than in males. In contrast, however, the data from our experiments on gender are diametrically opposed to these reported observations, since hypoglycemia-induced increases in plasma epinephrine levels are greater in female rats compared to age-matched males. Our data are, however, consistent with at least one published report, which demonstrated that the extent and duration of both the corticosterone and epinephrine response to foot-shock is also higher in females compared to age-matched males (Weinstock et al, 1998).

Summary

The studies contained in this dissertation have clearly demonstrated that *physiological* circulating levels of estradiol suppress stress-induced increases in plasma epinephrine concentration. This suppression may be due to both a reduction in adrenal medullary epinephrine secretion and an increase in plasma clearance of the catecholamine. However, the specific mechanism(s) underlying these effects have yet to be established. For example, while the decreased adrenal vein epinephrine

concentration is consistent with a reduction in epinephrine secretion, our data suggest that this effect could not be attributed to decreased biosynthesis, attenuation of intracellular Ca^{2+} signaling, or decreased sympathetic nerve activity. Additionally, while epinephrine clearance is markedly increased in estradiol-replaced rats, available data in the literature do not support that this increase could be attributed to the increased activity of catecholamine metabolizing enzymes such as COMT. We have also confirmed previous *in vitro* reports that short-term exposure to high concentrations of estradiol can attenuate agonist-induced Ca^{2+} signaling in the chromaffin cell and shown for the first time that acute exposure to high, presumably non-physiological, levels of estradiol can suppress stress-induced increases in plasma epinephrine *in vivo*.

References

- Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, Bonds D, Brunner R, Brzyski R, Caan B, Chlebowski R, Curb D, Gass M, Hays J, Heiss G, Hendrix S, Howard BV, Hsia J, Hubbell A, Jackson R, Johnson KC, Judd H, Kotchen JM, Kuller L, LaCroix AZ, Lane D, Langer RD, Lasser N, Lewis CE, Manson J, Margolis K, Ockene J, O'Sullivan MJ, Phillips L, Prentice RL, Ritenbaugh C, Robbins J, Rossouw JE, Sarto G, Stefanick ML, Van Horn L, Wactawski-Wende J, Wallace R, Wassertheil-Smoller S; Women's Health Initiative Steering Committee. 2004. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA* 291(14):1701-1712.
- Asthana M, Hoshimaru M, Uemura Y, Tokime T, Kojima M, Ohtsuka T, Matsuura N, Aoki T, Shibahara K, Kikuchi H. 1999. Expression of interleukin-1 β converting enzyme gene family and bcl-2 gene family in the rat brain following permanent occlusion of the middle cerebral artery. *J Cereb Blood Flow Metab* 17:11-18.
- Axelrod J and Reisine TD. 1984. Stress hormones: Their interaction and regulation. *Science* 224:452-459.
- Azoui R, Schneider J, Dong W, Dabire H, Safar M, Cuche J. 1997. Red blood cells participate in the metabolic clearance of catecholamines in the rat. *Life Sci* 60:357-367.
- Barton RN and Passingham BJ. 1981. Effect of binding to plasma proteins on the integration of plasma cortisol concentrations after accidental injury. *Clin Sci* 61:399-405.
- Batista MR, Smith MS, Snead WL, Connolly CC, Lacy DB, Moore MC. 2005. Chronic estradiol and progesterone treatment in the conscious dog: effects on insulin sensitivity and the response to hypoglycemia. *Am J Physiol* [In press; Epub ahead of print].
- Baum LW. 2005. Sex, hormones, and Alzheimer's disease. *J Gerontol A Biol Sci Med Sci* 60:736-743.
- Becker BA. 1986. The phenomenon of stress: concepts and mechanisms associated with stress-induced responses of the neuroendocrine system. *Vet Res Commun* 11:443-456.
- Bereiter HA, Zaid AM, Gann DS. 1984. Adrenocorticotropin response to graded blood loss in the cat. *Am J Physiol* 247:E398-E404.
- Bloom SR, Edwards AV, Hardy RN. 1977. Adrenal and pancreatic endocrine responses to hypoxia and hypercapnia in the calf. *J Physiol* 269(1):131-154.

- Bodart V, Babinski K, Ong H, De Lean A. 1997. Comparative effect of pituitary adenylate cyclase-activating polypeptide on aldosterone secretion in normal bovine and human tumorous adrenal cells. *Endocrinology* 138(2):566-573.
- Bond RF and Johnson G. 1984. Cardiovascular adrenoceptor function during compensatory and decompensatory hemorrhagic shock. *Circ Shock* 12:9-24.
- Brosnihan KB, Li P, Ganten D, Ferrario C. 1997. Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *Am J Physiol* 273:R1908-R1915.
- Brown DR, Li SG, Lawler JE, Randall DC. 1999. Sympathetic control of BP and BP variability in borderline hypertensive rats on high-vs. low-salt diet. *Am J Physiol* 46:R650-657.
- Burgess LH and Handa RJ. 1992. Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* 131:1261-1269.
- Burmeister JJ, Palmer M, Gerhardt GA. 2005. L-lactate measures in brain tissue with ceramic-based multi-site microelectrodes. *Biosens Bioelectron* 20(9):1772-1779.
- Byers M, Kuiper GG, Gustafsson JA, Park-Sarge OK. 1997. Estrogen receptor-beta mRNA expression in rat ovary: down-regulation by gonadotropins. *Mol Endocrinology* 11:172-182.
- Cannon WB. 1934. *Bodily changes in pain, hunger, fear, rage*. New York: Appleton.
- Cardoso CC, Pereira RT, Koyama CA, Porto CS, Abdalla FM. 2004. Effects of estrogen on muscarinic acetylcholine receptors in the rat hippocampus. *Neuroendocrinology* 80(6):379-386.
- Carrithers S, Taylor B, Cai W, Johnson B, Ott C, Greenberg R, Jackson B. 2000. Guanylyl cyclase-C receptor mRNA distribution along the rat nephron. *Reg Pep* 95:65-74.
- Ceresini G, Freddi M, Morganti S, Rebecchi I, Modena AB, Rinaldi M, Manca C, Amaducci A, Del Rio G, Valenti G. 2000. The effects of transdermal estradiol on the response to mental stress in postmenopausal women: A randomized trial. *Am J Med* 109:463-468.
- Crofton JT and Share L. 1997. Gonadal hormones modulate deoxycorticosterone-salt hypertension in male and female rats. *Hypertension* 29(1 Pt 2):494-499.
- Dar DE, and Zinder O. 1997. Short term effect of steroids on catecholamine secretion from bovine adrenal medulla chromaffin cells. *Neuropharmacol* 36:1783-1788.

Davis SN, Shavers C, Costa F. 2000. Gender-related differences in counterregulatory responses to antecedent hypoglycemia in normal humans. *J Clin Endocrinol Metab* 85:2148-2157.

de Miguel R, Fernandez-Ruiz JJ, Hernandez ML, Ramos JA. 1989. Role of ovarian steroids on the catecholamine synthesis and release in female rat adrenal: In vivo and in vitro studies. *Life Sci* 44:1979-1986.

Del Rio G, Velardo A, Zizzo G, Marrama P, Della Casa L. 1993. Sex differences in catecholamine response to clonidine. *Int J Obes* 17:465-469.

Diamond M P, Jones T, Caprio S, Hallarman L, Diamond MC, Addabbo M, Tamborlane WV, Sherwin RS. 1993. Gender influences counterregulatory hormone responses to hypoglycemia. *Metabolism* 42: 1568-1572.

Drake K, Gateva E, Deutsch J, Cohen WR. 1998. Sex differences in the adrenal catecholamine response to hypoglycemia in rats. *Metabolism* 47:121-124.

Dubal DB, and Wise PM. 2001. Neuroprotective effects of estradiol in middle-aged female rats. *Endocrinology* 142:43-48.

Dubey RK, Jackson EK, Keller PJ, Imthurn B, Rosselli M. 2001. Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism. *Hypertension* 37:640-644.

Dubey RK, Tofovic SP, Jackson EK. 2004. Cardiovascular pharmacology of estradiol metabolites. *J Pharmacol Exp Ther* 308:403-409.

Engeland WC and Gann DS. 1989. Splanchnic nerve stimulation modulates steroid secretion in hypophysectomized dogs. *Neuroendocrinology* 50(2):124-131.

Ewbank R. 1992. Stress: a general overview. In: *Farm animals and the environment*. pps 255-261, Eds: C Phillips and D Piggins, CAB Int.

Fernandez-Ruiz JJ, Bukhari AR, Martinez-Arrieta R, Tresguerres JA, and Ramos JA. 1988. Effects of estrogens and progesterone on the catecholaminergic activity of the adrenal medulla in female rats. *Life Sci* 42:1019-1028.

Fernandez-Ruiz JJ, Bukhari AR, Hernandez ML, Alemany J, and Ramos JA. 1989. Sex and age-related changes in catecholamine metabolism and release of rat adrenal gland. *Neurobiol of Aging* 10:331-335.

Finnegan JM, Borges R, Wightman RM. 1996. Comparison of cytosolic Ca²⁺ and exocytosis responses from single rat and bovine chromaffin cells. *Neuroscience* 71(3):833-843.

Fisher SJ, Bruning JC, Lannon S, Kahn CR. 2005. Insulin signaling in the central nervous system is critical for the normal sympathoadrenal response to hypoglycemia. *Diabetes* 54:1447-1451.

Fowler NO, Shabetai R, Holmes JC. 1961. Adrenal medullary secretion during hypoxia bleeding and rapid intravenous infusion. *Cir Res* 9:427-435.

Foy MR, Xu J, Xie X, Brinton RD, Thompson RF, Berger TW. 1999. 17β estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. *J Neurophysiol* 81:925-929.

Freund RK, Gerhardt GA, Marshall KE, Palmer MR. 2003. Differences in norepinephrine clearance in cerebellar slices from low-alcohol-sensitive and high-alcohol-sensitive rats. *Alcohol* 30(1):9-18.

Fujino Y and Fujii T. 1995. Insulin-induced hypoglycemia stimulates both adrenaline and noradrenaline release from adrenal medulla in 21-day-old rats. *Jpn J Pharmacol* 69(4):413-420.

Fuller L, Lu C, McMahon D, Alaudin E, Jorgensen M, Rau S, Sicken J, Jackson B. 1997a. Effect of dexamethasone on voltage-gated Ca^{2+} channels and cytosolic Ca^{2+} in rat chromaffin cells. *Neuroreport* 8:1169-1172.

Fuller L, Lu C, McMahon D, Lindemann M, Jorgensen M, Rau S, Sicken J, Jackson B. 1997b. Stimulus-secretion coupling in porcine adrenal chromaffin cells: effect of dexamethasone. *J Neurosci Res* 49:416-424.

Galassetti P, Neill AR, Tate D, Ertl AC, Wasserman DH, Davis SN. 2001. Sexual dimorphism in counterregulatory responses to hypoglycemia after antecedent exercise. *J Clin Endocrinol Metab* 86(8):3516-24.

Gaumann DM, Yaksh TL, Tyce GM. 1990. Effects of sufentanil and naltrexone on adrenal vein catecholamines and neuropeptides during splanchnic nerve stimulation in cats. *Synapse* 6(4):392-394.

Gillum LA, Mamidipudi SK, Johnston SC. 2000. Ischemic stroke risk with oral contraceptives. *JAMA* 284:72-78.

Glaser PE, Thomas TC, Joyce BM, Castellanos FX, Gerhardt GA. 2005. Differential effects of amphetamine isomers on dopamine release in the rat striatum and nucleus accumbens core. *Psychopharmacology* 178(2-3):250-258.

Gleason CE, Cholerton B, Carlsson CM, Johnson SC, and Asthana S. 2005. Neuroprotective effects of female sex steroids in humans: current controversies and future directions. *Cell Mol Life Sci* 62:299-312.

Green PG, Dahlqvist SR, Isenberg WM, Strausbaugh HJ, Miao FJ, Levine JD. 1999. Sex steroid regulation of the inflammatory response: sympathoadrenal dependence in the female rat. *J Neurosci* 19:4082-4089.

Goldstein DS, Garty M, Bagdy G, Szemeredi K, Sternberg EM, Listwak S, Pacak K, Deka-Starosta A, Hoffman A, and Chang PC. 1993. Role of CRH in glucopenia-induced adrenomedullary activation in rats. *J Neuroendocrinol* 5:475-86.

Gordon R, Spector S, Sjoerdsma A, Udenfriend S. 1966. Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold. *J Pharmacol Exp Ther* 153(3):440-447.

Grynkiewicz G, Poenie M, Tsien RY. 1995. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440-3450.

Haidan A, Hilbers U, Bornstein SR, Ehrhart-Bornstein M. 1998. Human adrenocortical NCI-H295 cells express VIP receptors. Steroidogenic effect of vasoactive intestinal peptide (VIP). *Peptides* 19(9):1511-1517.

Hall JM, Couse JF, Korach KS. 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* 276(40):36869-36872.

Hammond WG, Aronow L, Moore FD. 1956. Studies in surgical endocrinology III. Plasma concentrations of epinephrine and norepinephrine in anesthesia, trauma and surgery, as measured by a modification of the method of Weil-Malherbe and bone. *Ann Surg* 715-731.

He X-R, Wang W, Crofton JT, Share L. 1998. Effects of 17β -estradiol on sympathetic activity and pressor response to phenylephrine in ovariectomized rats. *Am J Physiol* 275:R1202-R1208.

He X-R, Wang W, Crofton JT, Share L. 1999. Effects of 17β -estradiol on the baroreflex control of sympathetic activity in conscious ovariectomized rats. *Am J Physiol* 277:R493-R498.

Heise T, Heinemann L, Heller S, Weyer C, Wang Y, Strobel S, Kolterman O, Maggs D. 2004. Effect of pramlintide on symptom, catecholamine, and glucagon responses to hypoglycemia in healthy subjects. *Metabolism* 53:1227-1232.

Helgason S, Carlstrom K, Damber MG, Damber JE, Selstam G, von Schoultz B. 1981. Effects of various oestrogens on circulating androgens and cortisol during replacement therapy in post-menopausal women. *Maturitas* 3:301-308.

Henderson VW, Watt L, Buckwalter JG. 1996. Cognitive skills associated with estrogen replacement in women with Alzheimer's disease. *Psychoneuro-endocrinology* 21:421-430.

Inoue M and Kuriyama H. 1991. Muscarinic receptor is coupled with a cation channel through a GTP-binding protein in guinea-pig chromaffin cells. *J Physiol* 436:511-529.

Inoue M and Imanaga I. 1995. Mechanism of activation of non selective cation channels by putative M₄ muscarinic receptor in guinea-pig chromaffin cells. *Br J Pharmacol* 114:419-427.

Jiang H, Xie T, Ramsden D, Ho S. 2003. Human catechol-o-methyltransferase down-regulation by estradiol. *Neuropharm* 45:1011-1018.

Kacsoh, B. 2000. In: *Endocrine Physiology*. McGraw-Hill Publishers, New York pp. 502-597.

Jorgensen M, Liu J, Adams J, Titlow W, Jackson B. 2002. Inhibition of voltage-gated Ca²⁺ current by PACAP in rat adrenal chromaffin cells. *Reg Pep* 103:59-65.

Kanayama N, Khatun S, Belayet H, She L, Terao T. 1999. Chronic local cold stress to the soles induces hypertension in rats. *Am J Hypertens* 12:1124-1129.

Kannel WB, Hjortland MC, McNamara PM, Gordon T. 1976. Menopause and the risk of cardiovascular disease; the Framingham study. *Ann Intern Med* 85:447-452.

Kawas C, Resnick S, Morrison A, Brookmeyer R, Corrada M, Zonderman A, Bacal C, Lingle D, Metter E. 1997. A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. *Neurology* 48:1517-1521.

Kelly MJ and Levin ER. 2001. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12(4):152-156.

Khalil Z, Marley PD, Livett B. 1986. Elevation in plasma catecholamines in response to insulin stress is under both neuronal and nonneuronal control. *Endocrinology* 119:159-167.

Kilpatrick DL, Slepatis RJ, Corcoran JJ, Kirschner N. 1982. Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J Neurochem* 38:427-435.

Kim Y-J, Hur E-M, Park T-J, Kim K-T. 2000. Nongenomic inhibition of catecholamine secretion by 17 β -estradiol in PC12 cells. *J Neurochem* 74:2490-2496.

King JC, Anthony EL, Damassa DA, Elkind-Hirsch KE. 1987. Morphological evidence that luteinizing hormone-releasing hormone neurons participate in the suppression by estradiol of pituitary luteinizing hormone secretion in ovariectomized rats. *Neuroendocrinology* 45:1-13

Kohler C, Berkowitz BA, Spector S. 1975. Sex hormones and tyrosine hydroxylase activity in vascular and adrenal tissue. *Endocrinology* 97:1316-1320.

Komesaroff PA, Esler MD, Sudhir K. 1999. Estrogen supplementation attenuates glucocorticoid and catecholamine responses to mental stress in perimenopausal women. *J Clin Endocrinol Metab* 84:606-610.

Komesaroff PA, Fullerton M, Esler MD, Jennings G, Sudhir K. 2002. Oestrogen supplementation attenuates responses to psychological stress in elderly men rendered hypogonadal after treatment for prostate cancer. *Clin Endocrinology* 56(6):745-753.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138:863-870.

Lamouche S and Yamaguchi N. 2001. Role of PAC(1) receptor in adrenal catecholamine secretion induced by PACAP and VIP in vivo. *Am J Physiol* 280(2):R510-R518.

Lawrenson R, Nicholls P, Rivers-Latham R, Brown T, Barnardo J, Gray R. 2005. PIXI bone density screening for osteoporosis in postmenopausal women. *Maturitas* [In press; Epub ahead of print].

LeBlanc J, Robinson D, Sharman DF, Tousignant P. 1967. Catecholamines and short-term adaptation to cold in mice. *Am J Physiol* 213(6):1419-1422.

Levin BE. 2002. Metabolic sensors: viewing glucosensing neurons from a broader perspective. *Physiol Behav* 76:397-401.

Levin E. 1993. Cellular functions of the plasma membrane estrogen receptor. *TEM* 10(9): 374-377.

Li P, Ferrario CM, Ganten D, Brosnihan KB. 1997. Chronic estrogen treatment in female transgenic (mRen2)27 hypertensive rats augments endothelium-derived nitric oxide release. *Am J Hypertens* 10(6):662-670.

Lilly MP, Engleland WC, Gann DS. 1982. Adrenal medullary responses to repeated hemorrhage in the anesthetized dog. *Endocrinology* 111:1917-1924.

- Lindheim SR, Legro RS, Bernstein L, Stanczyk FZ, Vijod MA, Presser SC, Lobo RA. 1992. Behavioral stress responses in premenopausal and postmenopausal women and the effects of estrogen. *Am J Obstet Gynecol* 167(6):1831-1836.
- Lindsay R. 1996. The menopause and osteoporosis. *Obstet Gynecol* 87:16S-19S.
- Liu J, Jorgensen M, Adams J, Titlow W, Nikolova-Karakashian M, Jackson B. 2001. Ceramide modulates nicotinic receptor-dependent Ca^{2+} signaling in rat chromaffin cells. *J Neurosci Res* 66:559-564.
- Liu PS and Lin C. 2002. Phthalates suppress the calcium signaling of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. *Tox Applied Pharmacol* 183:92-98.
- Lopez MG, Abad F, Sancho C, de Pascual R, Borges R, Maroto R, Dixon W, Garcia AG. 1991. Membrane-mediated effects of the steroid 17α -estradiol on adrenal catecholamine release. *J Pharmacol Exp Ther* 259:279-285.
- Losel R, Falkenstein E, Feuring M, Schultz A, Tillmann H, Rossol-Haseroth K, Wehling M. 2003. Nongenomic steroid action: controversies, questions, and answers. *Physiol Rev* 82:965-1016.
- Lunga P and Herbert J. 2004. 17β -Oestradiol modulates glucocorticoid, neural and behavioural adaptations to repeated restraint stress in female rats. *J of Neuroendocrinol* 16: 776-785.
- Luyer MDP, Khosla S, Owen WG, Miller VM. 2001. Prospective randomized study of effects of unopposed estrogen replacement therapy on markers of coagulation and inflammation in postmenopausal women. *J Clin Endocrinol Metab* 86:3629-3634.
- Mabry TR, Gold PE, McCarty R. 1995. Stress, aging, and memory. Involvement of peripheral catecholamines. *Ann N Y Acad Sci*. 771:512-522.
- Machado J, Alonso C, Morales A, Gomez J, Borges R. 2002. Nongenomic regulation of the kinetics of exocytosis by estrogens. *J of Pharmacol Exp Ther* 301:631-637.
- Macho L, Zorad S, Blazicek P, Pickova M, Kvetnansky R. 1996. Direct effects of insulin and corticosterone on catecholamine release from rat adrenal medulla. In: McCarty R, Aguilera G, Sabban E, Kvetnansky R (Eds.), *Stress: Molecular genetic and neurobiological advances*. Gordon and Breach Science Publishers, New York, pp. 333-342.
- Malhotra RK, Wakade TD, Wakade AR. 1989. Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol triphosphate, protein kinase C, and calcium in rat adrenal medulla. *J Neurosci* 9:4150-4157.

- Marker JC, Clutter WE, Cryer PE. 1998. Reduced epinephrine clearance and glycemic sensitivity to epinephrine in older individuals. *Endocrinol Metab* 38:E770-E776.
- Matthews J and Gustafsson J. 2003. Estrogen signaling: A subtle balance between E_{α} and E_{β} . *Molecular Interventions* 3(5):281-292.
- McCarty R. 1986. Age-related alterations in sympathetic-adrenal medullary responses to stress. *Gerontology* 32(3):172-183.
- Mendelsohn ME and Karas RH. 2005. Molecular and cellular basis of cardiovascular gender differences. *Science* 308:1583-1587.
- Mokuda O and Sakamoto Y. 2004. Insulin-induced hypoglycemia stimulates gluconeogenesis from ^{14}C -lactate independently of glucagon and adrenaline releases in rats. *Exp Clin Endocrinol* 102(4):351-354.
- Morita K, Kuwada A, Sano A, Sakata M, Sei H, Morita Y. 2001. Semi-quantitative RT-PCR analysis of environmental influence on P450 $_{scc}$ and PNMT mRNA expression in rat adrenal glands. *Life Sci* 70:73-80.
- Moro MA, Lopez M, Gandia L, Michelena P, Garcia A. 1990. Separation and culture of living adrenalin-and noradrenaline-containing cells from bovine adrenal medullae. *Anal Biochem* 185:243-248.
- Moxon KA, Leiser SC, Gerhardt GA, Barbee KA, Chapin JK. 2004. Ceramic-based multisite electrode arrays for chronic single-neuron recording. *IEEE Trans Biomed Eng* 51(4):647-656.
- Murru S, Pani L, Poddie D, Gessa G, Cao A, Pirastu M. 1997. Rat tyrosine hydroxylase gene polymorphisms. *Brain Res Mol Brain Res* 45:345-348.
- Nadal A, Diaz M, Valverde MA. 2001. The estrogen trinity: membrane, cytosolic, and nuclear effects. *News Physiol Sci* 16:251-255.
- Nahas GG, Mather GW, Wargo JDM, Adams WL. 1954. Influence of acute hypoxia on sympathectomized and adrenalectomized dogs. *Am J Physiol* 177:13-15.
- Nijima A. 1989. Neural mechanisms in the control of blood glucose concentration. *J Nutr* 119(6):833-840.
- Owens JF, Stoney CM, Matthews KA. 1993. Menopausal status influences ambulatory blood pressure levels and blood pressure changes during mental stress. *Circulation* 88(6):2794-2802.

Pamidimukkala J, Taylor J, Welshon W, Lubahn D, Hay M. 2003. Estrogen modulation of baroreflex function in conscious mice. *Am J Physiol* 284:R983-R989.

Park Y-H, Cho G-S, Cho E-T, Park Y-K, Lee M-J, Chung J-Y, Hong S-P, Lee J-J, Jang Y, Yoo H-J, Choi C-H, Lim D-Y. 1996. Influence of 17α -estradiol on catecholamine secretion from the perfused rat adrenal gland. *Korean J Intern Med* 11:25-39.

Parvez S, Ismahan G, Raza-Bukhari A, Youdim M. 1978. Activity of catechol-o-methyltransferase in brain regions and adrenal gland during the oestrus cycle. *J Neural Transm* 42:305-312.

Pines A, Fisman EZ, Shapira I, Drory Y, Weiss A, Eckstein N, Levo Y, Averbuch M, Motro M, Rotmensch HH, Ayalon D. 1996. Exercise echocardiography in postmenopausal hormone users with mild systemic hypertension. *Am J Cardiol* 78(12):1385-1389.

Pradhan AD, Manson JE, Rossouw JE, Siscovick DS, Mouton CP, Rifai N, Wallace RB, Jackson RD, Pettinger MB, Ridker PM. 2002. Inflammatory biomarkers, hormone replacement therapy, and incident coronary heart disease: prospective analysis from the Women's Health Initiative observational study. *JAMA* 2002 288(8):980-987.

Prelevic GM, and Jacobs HS. 1997. New developments in post-menopausal hormone replacement therapy. *Curr Opin Obstet Gynecol* 9:207-212.

Rainey WE, Bird IM, and Mason JI. 1994. The NCI-H295R cell line: a pluripotent model for human adrenocortical studies. *Mol and Cell Endocrinol* 100:45-50.

Randall DC, Brown DR, Brown LV, Kilgore JM. 1994. Sympathetic nervous activity and arterial blood pressure control in conscious rat during rest and behavioral stress. *Am J Physiol* 267:R1241-1249.

Sandoval D, Ertl A, Richardson A, Tate D, Davis S. 2003. Estrogen blunts neuroendocrine and metabolic responses to hypoglycemia. *Diabetes* 52:1749-2204.

Scarabin PY, Alhenc-Gelas M, Plu-Bureau G, Taisne P, Agher R, Aiach M. 1997. Effects of oral and transdermal estrogen/progesterone regimens in blood coagulation and fibrinolysis in postmenopausal women: a randomized clinical trial. *Arterioscler Thromb Vasc Biol* 17:3071-3078.

Scislo TJ, Kitchen AM, Augustyniak RA, O'Leary DS. 2001. Differential patterns of sympathetic responses to selective stimulation of nucleus tractus solitarius purinergic receptor subtypes. *Clin Exp Pharmacol Physiol* 28(1-2):120-124.

Seale JV, Wood SA, Atkinson HC, Bate E, Lightman SL, Ingram CD, Jessop DS, Harbuz MS. 2004. Gonadectomy reverses the sexually diergic patterns of circadian and stress-induced hypothalamic-pituitary-adrenal axis activity in male and female rats. *J Neuroendocrinol* 16(6):516-524.

Serova LI, Maharjan S, Sabban EL. 2005. Estrogen modifies stress response of catecholamine biosynthetic enzyme genes and cardiovascular system in ovariectomized female rats. *Neuroscience* 132:249-259.

Smith M, Freeman M, Neill J. 1975. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* 96:219-226.

Stevenson JC. 2005. Justification for the use of HRT in the long-term prevention of osteoporosis. *Maturitas* 51:113-126.

Stocker S, Keith K, Toney G. 2004. Acute inhibition of the hypothalamic paraventricular nucleus decreases renal sympathetic nerve activity and arterial blood pressure in water-deprived rats. *Am J Physiol* 286:R719-R725.

Tomlinson A, Durbin J, Coupland RE. 1987. A quantitative analysis of rat adrenal chromaffin tissue: morphometric analysis at tissue and cellular level correlated with catecholamine content. *Neurosci* 20:895-905.

Toyohira Y, Utsunomiya K, Ueno S, Minami K, Uezono Y, Yoshimura R, Tsutsui M, Izumi F, Yanagihara N. 2003. Inhibition of the norepinephrine transporter function in cultured bovine adrenal medullary cells by bisphenol A. *Biochem Pharm* 65:2049-2054.

Tumer N, Hale C, Lawler J, Strong R. 1992. Modulation of tyrosine hydroxylase gene expression in the rat adrenal gland by exercise: effects of age. *Brain Res Mol Brain Res* 14(1-2):51-6.

Tumer N and Larochele JS. 1995. Tyrosine hydroxylase expression in rat adrenal medulla: influence of age and cold. *Pharmacol Biochem Behav* 51(4):775-80, 1995.

Tumer N, Bowman CJ, LaRochelee JS, Kelley A, Scarpace PJ. 1997. Induction of tyrosine hydroxylase by forskolin: modulation with age. *Eur J Pharmacol* 324: 57-62.

Turgeon JL, McDonnell DP, Martin KA, Wise PM. 2004. Hormone Therapy: Physiological complexity belies therapeutic simplicity. *Science* 304 (5675):1269-1273.

Verhofstad AAJ, Coupland RE, Parker TR, Goldstein M. 1985. Immunohistochemical and biochemical study on the development of the noradrenaline- and adrenalin-storing cells of the adrenal medulla of the rat. *Cell Tissue Res* 242:233-243.

- Verhofsatd AAJ, Coupland RE, Colendbrander B. 1989. Immunohistochemical and biochemical study on the development of the noradrenaline- and adrenalin-storing cells of the adrenal medulla of the rat and pig. *Arch Histol Cytol* 52:351-360.
- Vietor I, Rusnak M, Viskupic E, Blazicek P, Sabban EL, Kvetnansky R. 1996. Glucoprivation by insulin leads to trans-synaptic increase in rat adrenal tyrosine hydroxylase mRNA levels. *Eur J Pharmacol* 313:119-127.
- Vollmer RR. 1996. Selective neural regulation of epinephrine and norepinephrine cells in the adrenal medulla--cardiovascular implications. *Clin Exp Hypertens* 18:731-751.
- Vollmer RR, Balcita JJ, Sved AF, Edwards DJ. 1997. Adrenal epinephrine and norepinephrine release to hypoglycemia measured by microdialysis in conscious rats. *Am J Physiol*. 273:R1758-R1763.
- Vollmer RR, Balcita-Pedicino JJ, Debnam AJ, Edwards DJ. 2000. Adrenal medullary catecholamine secretion patterns in rats evoked by reflex and direct neural stimulation. *Clin and Exper hypertension* 22:705-715.
- Voogt JL, Arbogast LA, Quadri SK, Andrews G. 1990. Tyrosine hydroxylase messenger RNA in the hypothalamus, substantia nigra and adrenal medulla of old female rats. *Brain Res Mol Brain Res* 8(1):55-62.
- Wada A, Takara H, Izumi F, Kobayashi H, Yanagihara N. 1985. Influx of $^{22}\text{Na}^+$ through acetylcholine receptor-associated Na^+ channels: Relationship between $^{22}\text{Na}^+$ influx, $^{45}\text{Ca}^{2+}$ influx and secretion of catecholamines in cultured bovine adrenal medulla cells. *Neurosci* 15:283-292.
- Wagner P, Jorgensen M, Arden W, Jackson B. 1999. Stimulus-secretion coupling in porcine adrenal chromaffin cells: acute effects of glucocorticoids. *J Neurosci Res* 57:643-650.
- Wakade AR, Wakade TK, Malhotra RK. 1988. Restoration of catecholamine content of previously depleted adrenal medulla in vitro: importance of synthesis in maintaining the catecholamine stores. *J Neurochem* 51:820-829.
- Walf A and Frye C. 2005. Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity. *Neuropsychopharmacology* 30:1288-1301.
- Wassertheil-Smoller S, Anderson G, Psaty B, Kotchen T, Langer R, Lasser N. 2000. Hypertension and its treatment in postmenopausal women: baseline data from women's health initiative. *Hyperten* 36:780-789.

Weinstock M, Razin M, Schorer-Apelbau D, Men D, McCarty R. 1998. Gender differences in sympathoadrenal activity in rats at rest and in response to footshock stress. *Int J Devl Neurosci.* 16:289-295.

Winder WW, Yang HT, Jaussi AW, Hopkins CR. 1987. Epinephrine, glucose, and lactate infusion in exercising adrenodemedullated rats. *J Appl Physiol* 62:1442-1447.

Wise P, Rance N, Barraclough C. 1981. Effects of estradiol and progesterone on catecholamine turnover rates in discrete hypothalamic regions in ovariectomized rats. *Endocrinol* 108:2186-2193.

Xie T, Ho S, Ramsden D. 1999. Characterization and implications of estrogenic down-regulation of human catechol-o-methyltransferase gene transcription. *Mol Pharm* 56:31-38.

Yang SH, Shi J, Day AL, Simpkins JW. 2000. Estradiol exerts neuroprotective effects when administered after ischemic insult. *Stroke* 31:745-750.

Yokomaku D, Numakawa T, Numakawa Y, Suzuki S, Matsumoto T, Adachi N, Nishio C, Taguchi T, Hatanaka H. 2003. Estrogen enhances depolarization-induced glutamate release through activation of phosphatidylinositol 3-kinase and mitogen activated protein kinase in cultured hippocampal neurons. *Mol Endocrinol* 17:831-844.

Society Memberships:

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|-------------------------|--------------------------------|
| 2004-Present | The Endocrine Society |
| 2000-Present | Society for Neuroscience |
| 1998-1999; 2004-Present | American Physiological Society |

Honors:

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|---------------------------------|---|
| 08/2002 | Graduate Student Representative, University of Kentucky Department of Physiology Chair Search Committee |
| 07/2001-06/2003 | Pre-Doctoral Fellowship American Heart Association, Ohio Valley Affiliate |
| Lindsey Wilson College: 1997 | Magna Cum Laude Graduate |
| 1997 | Biology Award |
| 1996 | Journalism Award |
| 1995-1997 | Member, Alpha Chi National Honor Society |
| 1995-1996 | Class President |
| 1992-1997 | Dean's List |
| 1992-1997 | Athletic and Academic Scholarship |

Teaching Experience:

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|-------------|--|
| Summer 2004 | University of KY Outreach Center STARS Program, Lexington KY |
| Fall 2003 | Renal Physiology block, undergraduate level Systems Physiology Course: University of KY, Lexington, KY |
| Summer 2003 | University of KY Outreach Center STARS Program, Lexington KY |
| Spring 2003 | Renal Physiology block, undergraduate level Systems Physiology Course: Asbury College, Wilmore, KY |

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| Fall 2002 | Renal Physiology block, undergraduate level Systems Physiology Course: University of KY, Lexington, KY |
| Spring 2002 | Undergraduate level Systems Physiology Companion Course: University of KY, Lexington, KY |
| Spring 2002 | Renal Physiology block, Undergraduate Systems Physiology Course: Asbury College, Wilmore, KY |
| Spring 1997 | Biology, 10th grade level: Woodford County High School, Versailles, KY (Practicum in Teaching for completion of Secondary Education degree, Lindsey Wilson College; Supervisor: Melissa S. Arnold) |

Relevant Course Work:

Ethics in Scientific Research

Seminars in Teaching Medical Science
(Medical Science Teaching I)

Practicum in Teaching Medical Science
(Medical Science Teaching II)

Bibliography:

Peer-Reviewed Manuscripts:

Adams J, Legan S, Ott C, Jackson B. Modulation of Hypoglycemia-Induced Increases in Plasma Epinephrine by Estrogen in the Female Rat. *J of Neurosci Res* 79:360-367, 2005.

Jorgensen M, Liu J, **Adams J**, Titlow W, Jackson B. Inhibition of Voltage- Gated Ca²⁺ Current by PACAP in Rat Adrenal Chromaffin Cells. *Reg Peptides* 103:59-65, 2002.

Liu J, Jorgensen M, **Adams J**, Titlow W, Nikolova-Karakashian M, Jackson B. Ceramide Modulates Nicotinic Receptor-Dependent Ca²⁺ Signaling in Rat Chromaffin Cells. *J of Neurosci Res* 66:559-564, 2001.

Abstracts:

Adams J, Legan S, Ott C, Jackson B, 2004. Modulation of Stress-Induced Increases in Plasma Epinephrine by Estrogen in the Female Rat. (*Gill Heart Institute Research Day, Lexington, KY October 2004.*)

Adams J, Legan S, Ott C, Jackson B, 2004. 17 β -Estradiol Suppresses Hypoglycemia-Induced Increases in Plasma Epinephrine in the Female Rat. (*The Society for Neuroscience Annual Meeting, San Diego October 2004.*)

Adams J, Titlow W, Ott C, Legan S, Jackson B, 2003. Effects of 17 β -Estradiol on Hypoglycemia-Induced Increases in Plasma Catecholamines in the Rat. (*The Society for Neuroscience Annual Meeting, New Orleans November 2003.*)

Adams J, Jackson B, 2002. Effect of Aging on the Gene Expression Profile of the Rat Adrenal Medulla. (*The Society for Neuroscience Annual Meeting, Orlando, FL November 2002.*)

Liu J, **Adams J**, Jorgensen M, Titlow W, Nikolova-Karakashian M, Jackson B, 2000. Modulation of Nicotinic Receptor-Dependent Calcium Signaling by Ceramide in Rat Adrenal Chromaffin Cells. (*The Society for Neuroscience Annual Meeting, New Orleans, LA November 2000.*)

Liu J, **Adams J**, Jorgensen M, Titlow W, Nikolova-Karakashian M, Jackson B, 2000. Modulation of Nicotinic Receptor-Dependent Calcium Signaling by Ceramide in Rat Adrenal Chromaffin Cells. (*Gill Heart Institute Research Day, Lexington, KY October 2000.*)

Adams J, Kilpatrick E, Taylor B, Carrithers S, Ott C, Jackson B, 1999. Modulation of Cytochrome P450 4A Gene Expression in the Rat Kidney. (*Experimental Biology, Washington, DC April 1999.*)