Effects of Physical Activity on the Stress-induced Rise in C-Reactive Protein in Female Rats

A dissertation submitted to Kent State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

by

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Chapter 1 – Background

Cardiovascular Disease and Women

Cardiovascular disease is the leading cause of death throughout the world. It is also one of the heaviest financial burdens on this country’s healthcare system (Mosca et al., 2004). While overall mortality in the U.S. due to coronary heart disease has been decreasing for several decades for the population as a whole, mostly due to improved medical technology, the mortality rate for women has actually increased (Shaw et al, 2006).

One reason for this change in demographics is that recent medical advances seem to be helping men with coronary heart disease stay alive, for example, to survive a heart attack. On the other hand, a growing percentage of women experiencing cardiac arrest (52%), die before they can receive medical intervention. This percentage has surpassed that for men (47%) (Centers for Disease Control, 2004). Moreover, even women who do receive medical care following a myocardial infarct have poorer prognosis than do men, with twice the mortality rate (Daly et al., 2006).

Although women have similar early symptoms of coronary heart disease as men, these symptoms are often ignored by both the women experiencing the symptoms and their physicians. It is now also recognized that standard diagnostic tools, such as angiography, are less likely to discover artery pathology in women than in men. This is because women are less likely than men to have
blood flow-limiting stenosis, and more likely to have endothelial dysfunction that disturbs the artery’s ability to dilate (Halcox et al., 2002).

American Heart Association statistics show that more women than men have died of heart disease every year beginning in 1984, yet medical and lay people continue to associate heart disease more with men than with women. Ischemic heart disease (IHD) has traditionally been thought of as a man’s health problem. But Shu, et al. (2007) suggest it should instead be called a “woman’s affliction”. They point out that, according to the Centers for Disease Control (CDC), more women of all ages die of IHD (38% of deaths) than cancer (22%). They note that IHD results in more severe dysfunction for women than for men.

**Cardiovascular Disease as an Inflammatory Process**

It is estimated that as much as 40% of atherosclerotic events, including both coronary and cerebral, cannot be attributed to traditional risk factors. Traditional risk factors include age, smoking, obesity, hypertension, dyslipidemia, and diabetes. Verma et al. (2002) stated that, when considering only coronary vascular disease, less than 50% can be attributed to these traditional factors. The authors emphasize that recently our knowledge regarding cause and treatment of heart disease “has progressed exponentially...” (p. 1890).

This growth of knowledge began in the nineties, at the time that heart disease was first beginning to be characterized as an *inflammatory* disease. This led to the examination of a variety of “nontraditional” risk factors, the relationship
of these factors to heart disease, and the possible roles they may play in the development of heart disease. Nontraditional risk factors that have been identified and examined include: C-reactive protein, sialic acid, lipoprotein (a), and homocysteine (Mojiminiyi et al., 2002).

A number of acute phase proteins, in addition to C-reactive protein, are related to atherosclerotic risk. These include fibrinogen, plasminogen activator-1, C3 complement, and lipoprotein (a). Such nontraditional risk factors often correlate with traditional risk factors and often have even stronger predictive value.

Acute phase proteins (APPs) are synthesized primarily in the liver and increase sharply during the immune system’s inflammatory response. This APP-induced inflammatory response is a part of the acute phase response (APR) of the innate immune system and is seen most dramatically when triggered by illness or injury. However, other factors, including psychosocial stress, have been correlated with smaller and more chronic inflammatory responses.

Ludewig and Laman (2004) state that the inflammatory process is “the decisive factor that accounts for the rate of lesion formation and clinical development in patients suffering from atherosclerosis” (p. 11529). In order for atherosclerosis to occur, it is necessary for monocytes, the precursors of macrophages, to be recruited to the site of eventual atherosclerotic lesion, and then to be retained there. Monocytes are recruited to the site by cell adhesion molecules that are expressed by endothelial cells that have been stimulated in
some way, for example by the mechanical shear caused by hypertension. (Labarre & Zaloga, 2004).

Once monocytes are recruited to the atherosclerotic site, they are retained at the site by chemokines, primarily monocyte chemoattractant protein-1, which is also produced by endothelial cells. After monocytes are incorporated into the tissue of the arterial wall, they mature into macrophages. One of the functions of the mature macrophages is to phagocytose microbes and present them to T-cells.

In the arterial wall, macrophages are responsible for the uptake of LDL. These cells are capable of containing large amounts of cholesterol esters, at which point they are referred to as foam cells. This marks the beginning of the fatty streak, the first step in development of the atherosclerotic lesion. Progression from fatty streak to the more complex lesion involves migration of arterial smooth muscle cells into the intimal layer of the wall. Once smooth muscle cells are in the intimal layer, they begin producing extracellular matrix proteins which form the fibrous cap. During this time, interactions between macrophages and T cells continue to contribute to the progression of the inflammatory response.

One of the APPs that has been gaining interest in CVD research is c-reactive protein (CRP). Of the APPS, CRP has been shown to have perhaps the most extensive involvement in the inflammatory process of CVD.
C-Reactive Protein (CRP)

The CRP molecule is a pentamer consisting of five identical subunits arranged in a ring. It was first isolated and identified in patients with pneumonia and was given its name because it was observed to bind to and precipitate the C-polysaccharide of pneumococcus.

Each subunit of the CRP molecule contains a ligand-binding site on one side and an effector molecule-binding site on the other side. Szalai and McCrory (2002) have demonstrated that when CRP is produced during a normal acute phase response (APR) of the innate immune system, it functions to provide host defense against microbes. It appears to do so by reducing the growth of bacteria at the onset of infection. They show that CRP functions by activating complement, opsonization, and induction of phagocytosis.

CRP is produced primarily in the liver under the control of the inflammatory cytokine interleukin-6 (IL-6). IL-6 originates mainly from adipocytes (about 30%), but also from endothelial and other cells. IL-6 stimulates the production of CRP, and its subsequent release from the liver, in response to infection or injury to the body. CRP is usually present in very small amounts, less than 0.3 mg/dl. However, in the event of infection or injury, the acute phase response of the immune system is mobilized. Within 24 to 48 hours of the onset of the APR, levels of blood CRP rise rapidly and dramatically. In fact, CRP levels may increase to 1000 times the normal level.
The following sections include research and other information that has recently emerged regarding the various relationships between: 1) CRP and cardiovascular disease, 2) CRP and physical activity, 3) CRP and psychosocial stress, and 4) CRP, estrogen, and the stress responses.

**CRP and Cardiovascular Disease**

Acute phase proteins (APPs), the components of the acute phase response of the innate immune system, have been strongly linked to heart disease. Of the APPs, C-reactive protein (CRP) has emerged as having one of the most significant associations with heart disease. As recently as 2000, it was still not clear whether CRP was merely a biomarker of inflammation or an actual active player in the atherosclerotic process (Muscari et al., 2002).

However, during the 1990s it was becoming increasingly clear that CRP was at least correlated with vascular disease. Elevated levels of CRP were found to predict: future cardiovascular events in apparently healthy persons; reoccurrence of cardiovascular events; and even the occurrence of cardiovascular events in the absence of traditional cardiovascular risk factors. Including all traditional and nontraditional risk factors, CRP is considered by some to be the single strongest predictor of cardiovascular events (Verma et al., 2002). It is also independently associated with cardiovascular disease risk in women (Davison and Davis, 2003).
It also became apparent that CRP often correlated with other cardiovascular risk factors, both traditional and nontraditional. For this reason, CRP became a useful addition to the assessment profile for cardiovascular risk. In patients with type II diabetes mellitus, CRP was found to correlate with total homocysteine and sialic acid (Mojiminiyi, 2002). Muscari et al. (2002) found that CRP correlated with fibrinogen and C3-complement, and that CRP independently correlated with triglycerides, age, and body mass index. CRP level was also found to correlate with visceral adipose tissue and with components of insulin resistance (Saijo, 2004). Woodward et al., (2003) found that CRP correlated with most traditional risk factors except blood pressure.

The steep acute phase rise in CRP due to illness or injury provides a clear and useful means to clinically monitor levels of inflammation. In contrast to this rapid rise and fall, the levels of CRP that have been associated with atherosclerosis are not nearly as dramatic. These levels are more accurately described as low grade and chronic, that is, the levels usually are not elevated above 10 mg/L but the elevation may last for months or years.

Today, high sensitivity CRP assays can detect levels between 0.3 and 10 mg/L. Generally, levels between 1.0 and 3.0 mg/L are considered to present a moderate risk of cardiovascular disease, and levels above 3.0 mg/L are considered to present a high risk of cardiovascular disease (Labarre, 2004).

Recent research continues to elucidate the extensive involvement of CRP in the various steps of the inflammatory process. The following studies have
contributed to the body of knowledge moving CRP from a mere biomarker of inflammation, to being recognized as an active facilitator of the inflammatory process of atherosclerosis.

Torzewski et al. (2000) showed that CRP was found only in the areas of coronary arteries in which signs of atherosclerotic development were noted, and not in areas where only adaptive and diffuse thickening were found. It was demonstrated that CRP appears in the intima prior to the appearance of macrophages, and that increasing concentrations of CRP coincided with an increase in monocyte migration. It was further demonstrated that the CRP-receptor (CRP-R) is expressed by monocytes. The CRP-R was found only in areas described as early atherosclerotic lesions, primarily along the cell surface of foam cells. The authors suggest this supports the hypothesis that CRP is involved in foam cell formation by opsonizing lipids.

Pasceri et al. (2000 and 2001) further examined the inflammatory effects of CRP on vascular cells. In the first study, results indicated that human umbilical vein endothelial cells (HUVECs) incubated with CRP expressed 10 times more intercellular adhesion molecule (ICAM-1) and significantly more vascular cell adhesion molecule (VCAM-1) and E-selectin than HUVECs not incubated with CRP. When human coronary artery endothelial cells (HCACEs) were used, CRP was found to have a dose response effect on expression of ICAM-1, VCAM-1, and E-selectin.
In the second study by Pasceri et al., when HUVECs were incubated with CRP expression of monocyte chemoattractant protein (MCP-1) was significantly increased. It was also found that several anti-atherosclerosis drugs slowed down or nearly stopped the CRP effect on MCP-1.

CRP has also been shown to facilitate uptake of low-density lipoprotein (LDL) cholesterol into macrophages. Zwaka et al. (2000), utilized macrophages derived from monocytes isolated from heparinized blood to demonstrate that CRP facilitated the uptake of native LDL by opsonizing it for macrophages. The research of Yasojima et al. (2001) demonstrated that macrophages and smooth muscle-like cells of the arterial wall produce CRP and complement proteins, and that CRP and complement mRNA and proteins are upregulated in atherosclerotic plaques. Their findings indicate that CRP and complement proteins are synthesized within atherosclerotic lesions.

A relationship between CRP and monocyte chemotactic protein-1 (MCP-1) has been demonstrated and proposed to be part of the physiological pathway between inflammation and atherosclerosis. On et al. (2005) determined that both CRP and MCP-1 were also elevated in patients with microvascular angina, also referred to as cardiac syndrome X, a condition in which persons with normal cardiac angiograms experience exercise-induced angina. It is noted that cardiac syndrome X involves inflammation levels comparable to those found in other angina patients.
It is apparent that CRP contributes to the development of CVD and in fact plays an active role throughout the various steps of the inflammatory process of CVD. CRP helps to facilitate the inflammatory response in the endothelium by increasing monocyte migration to endothelial cells, increasing expression of adhesion molecules by endothelial cells, and by increasing the uptake of cholesterol into macrophages.

The next three sections will focus on the various factors that are known to influence CRP levels. These include psychosocial stress, physical activity/fitness, and estrogen

**CRP and Psychosocial Stress**

Several studies showed a link between psychosocial stress and elevated CRP. As a result, suggestions have been made regarding the nature of this relationship and the probable contributions of the stress responses (SNS and HPA) in the initiation of the acute phase response (APR), including CRP, of the innate immune system.

Paul Black (2002) has provided one of the most extensive and persuasive explanations of the relationship between psychosocial stress and the immune system, most notably inflammation and the disease states that can result. Black points out that the inflammatory aspects of the acute phase response elicited by an invading microorganism or tissue injury are very similar to those elicited by “psychogenic stimuli”. He suggests that this makes a great deal of sense: “The
coupling has obvious survival advantage for an animal engaged in, or recuperating from, combat since an inflammatory response would help deal with any infectious organisms introduced during combat”. He notes that both somatic and autonomic nerves are “intimately associated with inflammatory cells”, and he proposes that the stress response evolved from the inflammatory response.

He further explains the dynamic relationship between the SNS and the HPA. For example, corticotropin releasing factor (CRF) leads to the production of ACTH by the anterior pituitary, which subsequently stimulates the adrenal cortex to release corticosteroids. CRF also stimulates the locus coeruleus to release norepinephrine (NE) from sympathetic nerves, thereby stimulating those organs involved in the fight-or-flight response, including the adrenal medulla, which releases additional NE as well as epinephrine.

A study conducted by Palmas, et al. (2007) also demonstrates a link between sympathetic stimulation and elevation of CRP. In this study of 2340 individuals taking medications for hypertension, those taking a beta blocker had significantly lower CRP levels than those not taking a beta blocker, including those taking another type of antihypertensive. Zhang et al. (2003) point out the mutually reinforcing effects of sympathetic nerves and proinflammatory cytokines. Others have noted that sympathetic nervous stimulation causes an increase in the proinflammatory cytokine interleukin-6 (IL-6). In a study by De Jongh et al. (2003), IL-6 was seen to lead to activation of the HPA axis during psychosocial stress in rats.
McDade et al. (2006) hypothesized that inflammation may be a mechanism through which stress facilitates progression of CV disease, and therefore sought to determine the contribution of psychosocial stress to CRP levels. Subjects were a representative sample of middle-aged and older adults. In addition to anthropometric data, behavioral (including smoking, sleep patterns, alcohol consumption) and psychosocial data (including perceived stress, perceived social support, depressive symptoms) were obtained. The following factors were independently associated with higher levels of CRP: waist circumference, smoking, latency to sleep, and perceived stress.

Using data from the Multi-Ethnic Study of Atherosclerosis, Ranjit et al. (2007) looked at the association between psychosocial factors and the inflammatory indicators IL-6, CRP, and fibrinogen. Results indicated a positive correlation between chronic stress and higher levels of IL-6 and CRP.

These studies demonstrate the impact of psychosocial stress on the inflammatory process in general and more specifically on CRP. Psychosocial stress is seen to positively correlate with the inflammatory markers IL-6 and CRP, and CRP in turn positively correlates with incidents of ischemia triggered by mental stress. One mechanism of action that has been proposed to explain the psychosocial stress/CRP connection incorporates the autonomic nervous system. That is, increased psychosocial stress increases sympathetic activity, which promotes the release of proinflammatory cytokines (Black 2002), resulting in an increase in CRP. Likewise, psychosocial stress decreases parasympathetic
activity, thus further promoting the release of proinflammatory cytokines (Shah et al., 2006).

**CRP and Physical Activity**

A number of studies have indicated that higher physical activity is associated with lower CRP in both men and women.

Metabolic syndrome is a set of metabolic risk factors that together predict risk of developing coronary heart disease, stroke, and type II diabetes. The metabolic risk factors include abdominal obesity, elevated triglycerides, low HDL cholesterol, high LDL cholesterol, elevated blood pressure, and elevated fasting plasma glucose (AHA 2009). Lamonte et al. (2005) found that women with elevated CRP (here defined as higher than 2.0 mg/L) were more likely to have metabolic syndrome than women with “low” CRP. Aronson et al. (2004) looked at CRP levels in subjects with metabolic syndrome and found that those with a higher fitness level had a lower CRP level than those with a lower fitness level.

In a study that utilized individuals determined to be at risk for developing ischemic heart disease (one of the criteria was elevated CRP), Smith et al. (1999) found that long-term exercise suppressed the atherogenic activity of blood mononuclear cells and caused a 35% decrease in serum levels of CRP.

Some lifestyle change studies have found that CRP levels were lowered by regular exercise even when weight loss or adipose loss did not occur. Milani et al. (2004) compared CHD patients who completed phase II of a cardiac
rehabilitation and exercise training program with CHD patients who did not participate in cardiac rehabilitation. Although they note that weight reduction and statin therapy have been shown to reduce CRP, the authors sought to determine the independent effects of a cardiac rehabilitation program on CRP level. It was found that CRP levels were significantly decreased for all subjects completing the cardiac rehabilitation program, whether or not they lost weight or were taking a statin drug.

In a study conducted by Giannopoulou et al. (2005), the effects of diet and exercise on inflammatory cytokines were compared in postmenopausal women with type II diabetes. Three groups were compared: diet only, exercise only, and diet plus exercise. The diet only group and the diet plus exercise group both lost more weight than did the exercise only group. However, each of the three groups decreased their CRP levels about 15%.

Utilizing self-reports of physical activity and estimates of aerobic fitness among 3803 adults aged 25 to 74, Borodulin et al. (2006) found physical activity to be inversely correlated with CRP in women. Similar results for men bordered on significance. It was concluded that “physical activity and fitness may have an anti-inflammatory effect, which is independent of obesity”.

In addition to this apparent inverse relationship between physical activity and CRP, physical exercise has been shown to have a general anti-inflammatory effect in humans. CRP was decreased and VO₂max was increased in healthy but sedentary workers who participated in a 12-week aerobic exercise program.
consisting of four sessions per week at 65% of VO$_2$max (Hewitt et al., 2008). Kullo et al. (2007) also found that markers of inflammation, including IL-6, CRP, and fibrinogen, correlated negatively with VO$_2$max in men without symptoms of heart disease.

In a study utilizing patients with Type 2 diabetes mellitus without CV disease (Kadoglou et al., 2007) subjects who participated in an aerobic exercise program (four times per week, 45-60 minutes per week, for six months) decreased high sensitivity CRP significantly. Subjects also improved lipid profiles, glucose control, and VO$_2$max. The authors conclude that the exercise training produced anti-inflammatory results without significant weight loss (de Lemos, 2007).

Exercise training also has an anti-inflammatory effect in rats with Type 2 diabetes. The physical activity of swimming one hour a day, three days per week for 12 weeks resulted in a significant increase in adiponectin and a significant decrease in CRP in ZDF (type 2) diabetic rats. This result was achieved without change in body weight. The metabolic abnormalities typical for these animals were partially or totally prevented in those animals in the swimming protocol.

In the studies presented above, the physical activity/fitness variable was determined in several ways, some more subjective than others. These include self-report, observed completion of a specific exercise program, and VO$_2$max.

In summary, physical activity and fitness appear to have general anti-inflammatory effects, that is, an inverse relationship with several inflammatory
markers including CRP. This effect was seen in healthy humans as well as in humans and rats with Type II diabetes. The anti-inflammatory effect was also seen both in individuals at risk for developing CVD and in those recovering from CVD.

Moreover, some studies have indicated that increasing physical activity/fitness leads to lower CRP levels even without significant weight loss. Gallistl (2001), noting that weight loss due to physical training results in decreased blood pressure and heart rate, suggested that this decrease in sympathetic stimulation indicates a more favorable balance between the sympathetic and parasympathetic systems. It seems likely that this decrease in cardiovascular reactivity would be facilitated by a more efficient cardiovascular system, one that can rise to a physical task with less effort.

If exercise facilitates a more favorable sympathetic/parasympathetic balance, as Gallistl suggests, and sympathetic stimulation contributes to activation of the inflammatory response, as pointed out in the previous section, it follows that regular exercise would have an anti-inflammatory effect. It also follows that regular physical activity would have beneficial effects on inflammatory diseases such as heart disease and type 2 diabetes mellitus.

**CRP and Estrogen**

Several studies have indicated a relationship between estrogen, stress responses, and CRP; however, the associations here are much more complex
than the associations previously mentioned between CRP and exercise, stress, or heart disease. The following description will attempt to dissect the variables into: type of steroid, dosage and route of administration, and physical activity.

Background

It is well established that CRP levels increase for women as they transition through menopause, during which time estrogen levels fall. Several studies have shown a negative correlation between endogenous estrogen level and CRP. For example, Blum et al., 2005, reported that soluble IL-6 receptor concentrations, as well as CRP levels, were highest in women during the early follicular phase, when estrogen and progesterone levels are low.

However, it is not clear whether postmenopausal increases in CRP can be attributed to a direct decrease in estrogen. In contrast to observational studies that indicated hormone replacement therapy (HRT) reduced the risk of cardiovascular events (Henderson et al. 1991, Ettinger et al. 1996), randomized trials have demonstrated a positive correlation between hormone replacement therapy and CV disease (Hully et al. 1998, Rossouw et al., 2002), and HRT and CRP (Koh et al., 2003, Cooper et al., 2007). This initially came as a surprise due to the well-documented ability of endogenous estrogen to protect the cardiovascular system, especially in animal studies. Although the relationship between estrogen and CRP levels is complex, further and ongoing studies have resulted in some clarification of this relationship.
Steroid Type

Replacement of progesterone, in conjunction with replacement of estrogen, may help to truncate the CRP elevation seen with estrogen replacement alone, although this combination does not return CRP to premenopausal levels (Yildirir et al. 2002).

The Yildirir et al (2002) study helped to reveal the potential difference between ERT (estrogen alone) and HRT (estrogen plus progesterone). In this study, estrogen replacement alone resulted in significantly increased serum CRP in postmenopausal women, while estrogen along with medroxyprogesterone acetate (MPA) did not increase CRP.

In contrast to the Yildirir findings, Koh (2003) found that the use of either conjugated equine estrogen (CEE, an oral form of ERT) or MPA or CEE with micronized progesterone (MP) in postmenopausal women “tended to increase CRP levels from baseline” (p. 371). Also, Cooper (2007) found that the use of conjugated estrogens along with MPA in postmenopausal women increased CRP 121% compared to 32% for placebo. However, as previously mentioned, a number of studies have found that oral, but not transdermal, ERT raises CRP. Therefore, the finding in the latter two studies that natural or synthetic progesterones did not attenuate the CRP-elevating of ERT may be due to the oral route of administration of the estrogen.

Animal studies have demonstrated similar findings regarding replacement estrogen and CRP, as those findings mentioned above pertaining to women.
Yang et al. (2005), demonstrated that estradiol replacement in ovariectomized rats resulted in significant elevation of CRP.

Although it is apparent that the effects of exogenous HRT are not entirely comparable to those of endogenous estrogen, some studies suggest that, in addition to its apparently harmful effects, exogenous estrogen replacement may retain some of the cardioprotective effects of endogenous estrogen.

Koh et al. (2004) suggest that HRT affects a wide variety of mechanisms impacting CV disease, resulting in both harmful and protective CV effects. Yildirir et al. (2002) examined the effects of estrogen replacement therapy (ERT) versus hormone (both estrogen and progesterone) replacement therapy (HRT) and found, among other things, that both therapies resulted in the cardioprotective effect of significantly reducing plasma levels of the artery-damaging amino acid homocysteine. Wakatsuki (2004) found that both standard and low doses of CEE (without progesterone) significantly increased flow-mediated vasodilation in the brachial artery.

Using a rat model, Miller et al. (2004) showed that estradiol (E2) significantly slowed the influx of inflammatory cells into the vessel wall of injured arteries. In a related study (Wang et al., 2005), human CRP transgenic mice showed an increased response to vascular injury, i.e., increased neointimal formation, compared to their nontransgenic counterparts due to the presence of human CRP. Intact transgenic and nontransgenic mice and ovariectomized (OVX) (treated with E2 or vehicle) transgenic and nontransgenic mice were used.
In both intact and OVX transgenic mice, neointimal formation was exaggerated. However, this effect was largely eliminated in the OVX mice with the addition of E$_2$.

Dosage and Route of Administration Variables

At least two studies found that using a lower dose of oral estrogen than that found in standard ERT (0.625 mg) eliminated the CRP-elevating effect usually seen with the standard dose. Koh et al. (2004) compared standard dose ERT (0.625 mg CEE) plus MP with a lower dose ERT (0.3 mg CEE) plus MP in postmenopausal women. Standard dose ERT significantly raised CRP while the lower dose ERT did not raise CRP.

In a similar study, Wakatsuki (2004) compared standard dose ERT (0.625 mg CEE) with a lower dose ERT (0.3125 mg CEE). No progesterone was used in this study. The standard dose ERT resulted in significant increases in CRP as well as two other inflammatory markers, serum amyloid A and IL-6. The lower dose ERT did not raise any of these inflammatory markers.

Although CRP has been shown to increase in response to oral conjugated estrogen, in response to transdermal administration of estrogen, CRP does not increase, or increases only slightly (Bukowska et al. 2004, Miller et al. 2003, Vongpatanasin et al. 2003, Decensi et al. 2002, Ho et al. 2006).

Silvestri (2003) concluded that a rise in CRP resulting from HRT may not be due to an actual rise in inflammation. In this study the use of oral HRT
resulted in a rise in CRP but a decrease in several other markers of inflammation, that is, intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin.

Similarly, Sumino et al. (2005) found that when transdermal HRT (estradiol and medroxyprogesterone) was administered to postmenopausal women, CRP levels remained unchanged, but other inflammatory markers were decreased (ICAM-1, VCAM-1, E-selectin, and monocyte chemoattractant protein-1 (MCP-1). Wakatsuki (2004) found that standard dose, but not low dose CEE (without progesterone) raised CRP. However, both standard and low doses of CEE (without progesterone) significantly lowered E-selectin concentration and did not change ICAM-1 or VCAM-1.

In summary, ERT does not automatically restore the exact same benefits provided by endogenous estrogen. Sometimes ERT has been found to be detrimental to cardiovascular health. Some of the problems encountered with estrogen replacement may have been caused by replacing estrogen alone, without also replacing progesterone, and by overly high replacement doses. It also appears that estrogen replacement benefits cardiovascular health when it is started prior to endothelial damage, but is detrimental to cardiovascular health when started after endothelial damage has begun. Finally, it appears that the route of administration of ERT can determine whether replacement estrogen raises CRP.
Physical Activity Variables

Another variable complicating the estrogen-CRP relationship is physical activity level. Stauffer et al. (2004) compared four groups of postmenopausal women: sedentary women using HRT, sedentary women not using HRT, physically active women using HRT, and physically active women not using HRT. “Physically active” was defined as endurance trained. Sedentary women using HRT had a 75% higher level of CRP than sedentary women not using HRT. Physically active women using HRT did not have a significantly higher level of CRP than physically active women not using HRT. Overall, the physically active women had a 65% lower level of CRP than sedentary women.

Potential Mechanism

If estrogen does, in fact, suppress CRP levels prior to menopause, a likely mechanism for this action would be estrogen’s modulating effect on the stress response involving the SNS. In the following study, estrogen was shown to decrease the SNS response to a stressor. Eikelis and Van Den Buuse (2000) examined the relationship between sex hormones and the SNS by exposing rats to open field novelty (colony) stress within the context of several experiments, two of which will be mentioned here. Blood pressure and heart rate were measured as an indicator of the SNS activity resulting from the open field stress. In each of the experiments, blood pressure and heart rate were measured twenty minutes prior to, and during the psychosocial stressor. One of the experiments
utilized male and female rats, all of which were exposed to the open field stress. Cardiovascular responses were compared between: male rats, female rats in diestrous stage of their estrous cycle (having the lowest levels of estrogen and progesterone), and female rats not in diestrous (having higher levels of estrogen and progesterone). Results indicated that female rats in diestrous had significantly greater blood pressure and heart rate than did females not in diestrous or male rats.

A second experiment utilized ovariectomized rats, half of which received estrogen replacement. All animals were exposed to the open field stressor. The rats receiving estrogen replacement then began receiving progesterone replacement as well, followed by another open field stress. It was found that estrogen replacement, along with progesterone replacement, significantly decreased heart rate and blood pressure responses to the open field stress compared to no hormone replacement. This decrease in heart rate and blood pressure indicated a decrease in SNS response to the stressor. However, estrogen replacement alone did not change blood pressure and heart rate measures compared to no hormone replacement.

In contrast to estrogen’s apparent restraining effect on the SNS, estrogen has been seen to correlate with glucocorticoid elevation in response to stress. Rats that were exposed to tailshock or swim stress manifested increased levels of estradiol above basal levels. Simultaneously, glucocorticoid levels were elevated for all female rats. In fact, glucocorticoid levels were higher for female
rats in both basal and stress conditions than for male rats in basal and stress conditions (Shors et al., 1999). The authors point out that estrogen is known to increase corticosterone production by the adrenals.

In conclusion, the relationship between estrogen and CRP remains complex. Although endogenous estrogen is associated with lower CRP levels, the same is not always so for exogenous estrogen. However, it is clear that in premenopausal women, endogenous estrogen is found to be strongly cardioprotective. One of the factors contributing to this protection may be the suppressive effect of estrogen on CRP. Given that sympathetic activation has been linked to the inflammatory response, and estrogen has been shown to decrease sympathetic reactivity, a reasonable assumption is that estrogen suppresses levels of CRP by decreasing sympathetic reactivity. The following research will address this question.
Chapter 2 – Exercise, Stress, and the Stress Response Variables

Introduction

Objectives

1. To determine if acute psychosocial stress produces a rise in CRP.
2. To determine if increased voluntary physical activity decreases the rise in CRP caused by an acute psychosocial stressor.
3. To determine if plasma norepinephrine, corticosterone, and interleukin 6 (IL-6) are physiological mediators underlying the acute psychosocial stress-induced rise in CRP.

Hypotheses

1. Psychosocial stress will cause a rise in CRP for high activity (HA) and low activity (LA) subjects.
2. The stress-induced rise in CRP will be smaller for HA subjects than for LA subjects.
3. Norepinephrine, via the sympathetic nervous system, and corticosterone, via the hypothalamic-pituitary-adrenal axis, cause a rise in IL-6 and CRP.
Methods

Animals
Female WKY/UA age 3.5 to 4 months and weighing between 170 and 200 g at the onset of each experiment were used. Animals were placed individually in polyethylene cages (45x25x20) with stainless steel tops and heat treated wood chip bedding (P.J. Murphy Forest Products, Montville, NJ). Rat chow (Tekland Rodent diet, Madison, WI) and water were given ad libitum. All animals were kept in the same room, which was maintained on a 12-hour light/12-hour dark schedule, with lights on at 7:00 am, and temperature between 24 and 26 degrees C, and relative humidity 50%. Animals were given two weeks to acclimate to individual cages prior to beginning treatment conditions. Cages were cleaned weekly. Animals were weighed weekly. All procedures were approved by the University of Akron Institutional Animal Care and Use Committee.

Conditions
Animals were divided and placed in one of three conditions:
1) Control – animals remained singly housed in standard cages and did not receive any treatment.
2) Low activity (LA) – animals remained singly housed in standard cages and received colony stress and pharmacological antagonist treatments as described below.
3) High activity (HA) – animals were placed in cages with running wheels and allowed to exercise at will. Distance each animal ran daily was determined from
number of wheel revolutions recorded daily by means of mechanical counter or
distance run in km recorded by cyclometer (Schwinn, Coral Gables, Fla). Animals
were considered to be conditioned after running at least 3 km per day for at least
5 weeks. Animals received colony stress and pharmacological antagonist
treatments as described below.

The animals were kept in these conditions for the remainder of the
experiment.
TABLE 2.1. Exercise, Stress, and Pharmacological Antagonist Treatments

<table>
<thead>
<tr>
<th>Condition</th>
<th>No tx</th>
<th>S</th>
<th>Ex</th>
<th>S</th>
<th>Ex</th>
<th>S+AA</th>
<th>Ex</th>
<th>S+BA</th>
<th>Ex</th>
<th>S+CA</th>
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<tr>
<td>LA (n=12)</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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<td>2 wks</td>
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<td>2 wks</td>
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</tr>
</tbody>
</table>

No tx = No treatment
Control = No treatment.
LA = Low Activity Condition
HA = High Activity Condition
S = One-hour Colony Stress
Ex = Unlimited 24/7 access to exercise wheel
AA = Alpha-adrenergic antagonist (Prazosin)
BA = Beta-adrenergic antagonist (Metoprolol)
CA = Corticosterone (GR and MR) antagonists (Mifepristone + Spironolactone)

Following two-week acclimation period, animals in the LA and HA conditions received one-hour colony stress treatment followed by blood acquisition 6 hours later. After 8 weeks, animals in the LA and HA conditions
again received one-hour colony stress treatment followed by blood acquisition 6 hours later.

After additional 2 weeks, animals in the LA and HA conditions were injected with alpha-adrenergic antagonist Prazosin (0.5 mg/kg BW) followed immediately by one-hour colony stress treatment. Blood acquisition was 6 hours later.

After additional 2 weeks, animals in the LA and HA conditions were injected with beta-adrenergic antagonist Metoprolol (50 mg/kg BW) followed immediately by one-hour colony stress treatment. Blood acquisition was 6 hours later.

After additional 2 weeks, animals in the LA and HA conditions were injected with corticosterone antagonists Mifepristone (50 mg/kg BW) and Spironolactone (20 mg/kg BW) followed immediately by one-hour colony stress treatment. Blood acquisition was 6 hours later.

After additional 2 weeks, animals in the LA and HA conditions were injected with the GR antagonist Mifepristone (50 mg/kg BW) and the MR antagonist Spironolactone (20 mg/kg BW), as described above, one hour prior to the colony stress treatment. The animals were injected with both Prazosin (0.5 mg/kg BW) and Metoprolol (50 mg/kg BW), as described above, immediately prior to the one-hour colony stress treatment. Blood acquisition was 6 hours later. Blood was also obtained from the animals in the Control condition at this time.
While sedated, hearts, adrenal glands, and thymus glands were removed and weighed.

Treatments and Procedures

*Colony stress*

For each one-hour colony stress treatment, animals from the LA condition and the HA condition were placed together in a colony box. The colony box consisted of Plexiglas floor and sides, with floor covered with standard bedding (type), and screen covering. The box measured 1.2m X 1.2m X 0.3m. During each colony stress treatment, an opaque cloth was placed over the colony box in order to minimize light and to not inhibit activity. Following each colony stress treatment, each animal was returned to its individual standard or wheel cage, and the colony box was cleaned with 50% vinegar solution to remove odors.

*Running Wheels*

Animals in the HA condition were placed in cages with running wheels. These animals had access to the wheel at all times and were able to run at will. For wheels with mechanical counters, circumference of wheels was measured in meters. Number of wheel turns was recorded daily, converted to kilometers and charted. For wheels with electronic cyclometers, distance run per day was recorded in kilometers and charted.
Pharmacological antagonist treatments

1. For the alpha-adrenergic receptor blockade - LA and HA animals were injected with 0.5 mg/kg BW Prazosin IP immediately prior to the colony stress treatment (S+AA in Table 1 above).

2. For the beta-adrenergic receptor blockade – LA and HA animals were injected with 50 mg/kg BW Metoprolol IP immediately prior to the colony stress treatment (S+BA in Table 1 above.)

3. For the corticosterone receptor blockade –LA and HA animals were injected with 50 mg/kg BW Mifepristone and 20 mg/kg BW Spironolactone IP one hour prior to the colony stress treatment (S+CA in Table 1 above).

4. For the final drug treatment, HA and LA animals were injected with the GR and MR antagonists Mifepristone and Spironolactone, as described above, one hour prior to the colony stress treatment. The HA and LA animals were then injected with both Prazosin and Metoprolol, as described above, immediately prior to the colony stress treatment.

Blood sampling procedure

Blood samples were taken 6 hours post-treatment. Prior to blood acquisition, animals were sedated with 50 mg/kg Brevital IP injection using a 26-guage needle. As some of the plasma biochemical measurements have circadian rhythm effects, Colony Stress and receptor antagonist treatments were timed such that blood acquisition was always at the same time of day. Blood was obtained retro-orbitally using a capillary tube to initiate blood flow. Two ml blood
was obtained from each animal and placed in EDTA tube (size) and immediately placed on ice. Blood was centrifuged (SORVALL RT7) at 2800 rpm with chamber temperature 5 degrees C for 15 minutes. Each plasma sample was aliquoted into three separate bullet tubes and placed in a –80 degree C. freezer.

Plasma corticosterone, catecholamine, IL-6, and CRP assays

Norepinephrine was measured using high pressure liquid chromatography with electrochemical detection (Waters 2465, Millford, MA). The mobile phase for catecholamines consisted of citric acid (35 mM), sodium acetate (90 mM), octyl sodium sulfate (690 uM), EDTA (130 uM) and 10% methanol, pH adjusted to 4.7. The HPLC pump (Waters 1515) was set at 1.4 ml/min for catecholamines. 50 ul of extracted supernatant was injected onto the column (Waters Symmetry C18, 5 um 4.6 X 150 mm column) preceded by a guard column (LC-18, Supelco, Bellfonte, PA). The samples were maintained at 10 degrees C. throughout injections using the 717 plus autosampler (Waters, Millford, MA). The calculations for NE were achieved by using the Waters Breeze system software (Waters, Millford, MA). The minimum sensitivity of the assay is 30 pg/ml for NE.

IL-6 was measured using solid phase enzyme linked immunosorbent assay (ALPCO Diagnostics, Salem, NH) specific for rats. The intra-assay coefficient of variation was 3.7 to 4.7. The inter-assay coefficient of variation was 3.5 to 7.2 percent. The recovery of rat IL-6 added to serum averaged 108.4 percent. No cross-reactivity was found for a panel of substances.
CRP was measured using a highly sensitive two-site (double antibody sandwich) enzyme linked immunoassay (ALPCO Diagnostics, Salem, NH) specific for rats. Intra-assay coefficient of variation was <10%. Inter-assay coefficient of variation was <10%. Control serum recovery was >85%.

The corticosterone assay utilized a double antibody $^{125}$I radioimmunoassay (MP Biomedicals, LLC, Diagnostics division, Orangeburg, NY) technique for rats and mice. Intra-assay coefficient of variation was 4.4 to 10.3 percent. Inter-assay coefficient of variation was 6.5 to 7.2 percent. Specificity was 100 percent for corticosterone, and the highest cross-reactivity for potential interfering steroids was 0.34 percent for deoxycorticosterone and 0.10 percent for testosterone. The recovery of rat corticosterone added to steroid diluent averaged 100.1 percent.

Termination procedure

During the final blood acquisition, animals were terminated by an overdose of Brevital (50 mg/kg IP). Heart and adrenal glands were removed from each animal and weighed.

Statistics

One-way ANOVA, Two-Way ANOVA, and Two-Way ANOVA with Repeated Measures statistical analyses, with post-hoc Pairwise Multiple Comparison Procedures (Holm-Sidak Method) were used as appropriate Student’s t-test and Mann-Whitney Rank Sum Test were used to compare individual groups. SigmaStat and SigmaPlot software (Jandel Scientific, San
Rafael, CA) were used for statistical analyses, with significance assumed at p<0.05.

**Results**

**Running**

Animals in the HA group had access to turning wheels 24/7 and were able to run at will. The 12 HA ran at least 3 km per day, meeting the conditioning requirement. HA were running 3 or more km per day within 3 weeks (Figure 2.1a).

**Body Weight Gains**

Weight gains for the duration of the study were compared for Control, LA, and HA. There was no significant difference between the amount of weight gained for Control, LA, and HA (Figure 2.1b)

**Heart and Adrenal Relative Weights**

At termination, hearts and adrenal glands were removed and weighed. Relative heart weight was compared for Control, LA, and HA. There was not a significant difference between Control and LA relative heart weight (0.328 and 0.321 mg/gms BW). Relative heart weight for HA (0.377 mg/gms BW) was significantly higher than relative heart weight for Control (0.328 mg/gms BW) (p<0.001). Relative heart weight for HA (.0377 mg/gms BW) was significantly higher than relative heart weight for LA (0.321 mg/gms BW)(p<0.001) (Figure 2.2).
No significant differences were found for relative adrenal weight between Control (.0286 mg/gms BW), LA (.0333 mg/gms BW), and HA (.0344 mg/gms BW). However, difference between relative adrenal weights for Control and HA was close to significant (p<0.064) (Figure 2.3).
Figure 2.1a High activity animals were running more than the minimal 3 km/day by 4 weeks. After 8 weeks, average running distance remained between 5 and 8 km per day.
Average Body Weight Gains for Control, Low Activity, and High Activity

Figure 2.1b Weight gains for the duration of the study were compared for Control (Ctl), Low Activity (LA), and High Activity (HA). There was no significant difference between amount of weight gained for Ctl, LA, and HA. Values expressed as mean, +/- SE, n=12 per group.
Figure 2.2 There was not a significant difference between Control (Ctl) and Low Activity (LA) relative heart weight. Relative heart weight was significantly higher for High Activity (HA) than for Ctl. Relative heart weight was significantly higher for HA than for LA (***p<0.001 compared to Ctl and LA). Values expressed as mean, +/- SE, n=12 for each group.
Relative Adrenal Weight for Control, Low Activity and High Activity WKY Females

Figure 2.3 No significant differences were found for relative adrenal weights between Control (Ctl), Low Activity (LA), and High Activity (HA). The difference between relative adrenal weight for Ctl and HA was close to significant (p<0.064). Values expressed as mean, +/- SE, n=12 for each group.
Norepinephrine (NE)

Two Way Repeated ANOVA statistical analysis for NE showed a significant treatment effect ($F=7.038$) ($P<.001$). Group effect and Group x Treatment interaction effect were not significant. Pairwise Multiple Comparison Procedures showed the following significant differences: PRS+P vs. Baseline; PRS+P vs. Stress Baseline; PRS+P vs. PRS+CA; PRS+P vs. PRS+M; PRS+P vs. PRS+ALL; PRS+P vs. PRS.

The following results were obtained using Student's t-test and Mann Whitney Rank Sum Test. Plasma NE was significantly higher for LA during PRS+P, PRS+M, and PRS+All than during Stress Baseline ($p<0.05$) (Figure 2.4). Plasma NE was significantly higher for HA during PRS+P than at Stress Baseline ($p<0.05$) (Figure 2.5).

Plasma NE was significantly higher for LA than for HA during PRS+ALL ($p<0.01$) (Figure 2.6). Plasma NE was also significantly higher for LA than for Control during PRS+ALL ($p<0.01$) (Figure 2.6). Plasma NE was significantly higher for LA than for HA during PRS with Metoprolol (PRS+M) ($p<0.05$) (Figure 2.7). Plasma NE was significantly higher for LA during PRS+M than during Stress Baseline ($p<0.05$) (Figure 2.8). However, plasma NE was not significantly different between HA at PRS+M and Stress Baseline (Figure 2.8). Plasma NE was significantly higher for LA during PRS+All than during Stress Baseline ($p<0.05$) (Figure 2.9). However, plasma NE was not significantly different
between HA at PRS+ALL and HA at Stress Baseline (Figure 2.9). No other significant differences were found.

Interleukin-6 (IL-6)

Two Way Repeated Measures ANOVA statistical analysis for IL-6 showed a significant effect for Group (F= 3.269) (p=0.049) and for Treatment (F=6.938) (p<0.001). Group x Treatment interaction effect was not significant. Pairwise Multiple Comparison Procedures (Holm-Sidak Method) showed the following significant differences: PRS+ALL vs. Baseline; PRS+CA vs. Baseline; PRS+ALL vs. Stress Baseline; PRS+CA vs. Stress Baseline; PRS+ALL vs. PRS; PRS+CA vs. PRS; PRS+ALL vs. PRS+P; PRS+CA vs. PRS+P; PRS+ALL vs. PRS+M.

The following results were obtained from Student’s t-test and Mann-Whitney Rank Sum test. Plasma IL-6 showed a significant Group effect (F= 3.269) (p=0.049) and Treatment effect (F=6.938) (p<0.001). Plasma IL-6 was significantly higher for LA during PRS+CA than during Stress Baseline (p<0.05) (Figure 2.10).
Figure 2.4 Plasma NE for LA at PRS+P, PRS+M, and PRS+All were significantly higher than plasma NE at Stress Baseline (*p<0.05 compared to Stress Baseline). Values expressed as mean, +/- SE, n=12 for each treatment.
Plasma NE for High Activity (HA)
During Various Treatment Conditions

Figure 2.5 Plasma NE was significantly higher at PRS+P than at Stress Baseline. Plasma NE was not significantly higher at any of the other treatment conditions compared to Stress Baseline (*p<0.05 compared to all other treatment conditions). Values expressed as mean, +/- SE, n=12 for each treatment.
Plasma NE for Control (Ctl), Low Activity (LA), and High Activity (HA) During Post-running Stress+All Blockers

Figure 2.6 Plasma NE was significantly higher for LA than for HA or Ctl during PRS+All (**p<0.01 compared to HA and Ctl). Plasma NE for HA was not significantly higher than for control during this condition. Values expressed as mean, +/- SE, n=12 for each group.
Plasma NE for Low Activity (LA) and High Activity (HA) During Post-running Stress+Metoprolol (PRS+M)

**Figure 2.7** Plasma NE was significantly higher for LA than for HA during PRS+M (*p<0.05 compared to HA). Values expressed as mean, +/- SE, n=12 for each group.
Plasma NE for Low Activity (LA) and High Activity (HA) During Post-running Stress+Metoprolol (PRS+M) and Stress Baseline

**Figure 2.8** Plasma NE was significantly higher for LA during Stress Baseline (*p<0.05 compared to LA at Stress Baseline). Plasma NE for was not significantly different between PRS+M and Stress Baseline. Values expressed as mean, +/- SE, n=12 for each group and condition.
Plasma NE for Low Activity (LA) and High Activity (HA) During Post-running Stress+All Blockers (PRS+All) and Stress Baseline

Figure 2.9 Plasma NE was significantly higher for LA during PRS+All than during Stress Baseline (*p<0.05 compared to LA at Stress Baseline). Plasma NE for HA was not significantly different between PRS+All and Stress Baseline. Values expressed as mean, +/- SE, n=12 for each group and treatment.
Figure 2.10 Plasma IL-6 was significantly higher for LA at PRS+CA and at PRS+All than at Stress Baseline. Plasma IL-6 for LA at PRS, PRS+P, and PRS+M were not significantly higher than at Stress Baseline (*p<0.05 compared to Stress Baseline, **p<0.01 compared to Stress Baseline). Values expressed as mean, +/- SE, n=12 for each treatment.
Plasma IL-6 was also significantly higher for LA during PRS+All than during Stress Baseline (p<0.01) (Figure 2.10). Plasma IL-6 was significantly higher for HA during PRS+CA than during Stress Baseline (p<0.01) and for HA during PRS+All than during Stress Baseline (p<0.05) (Figure 2.11). Plasma IL-6 was significantly higher for HA than for Control during PRS+M and during PRS+CA (p<0.05) (Figure 2.12). No significant differences were found between LA and HA at any of the treatment conditions. No other significant differences were found.

C-Reactive Protein (CRP)

Two Way Repeated Measures ANOVA statistical analysis for CRP did not show any significant differences for Group, Treatment, or GroupXTreatment, although the effect for Treatment showed a strong trend toward significance (F=1.811) (p=0.100). The following results were obtained using Student’s t-test and Mann-Whitney Rank Sum test. Plasma CRP was significantly elevated for both LA and HA during Stress Baseline compared to Baseline (p<0.05) (Figure 2.13). No other significant differences were found between treatment conditions for Ctl, LA, or HA. No significant differences were found between Control, LA, or HA for any of the treatment conditions.

Corticosterone

Plasma corticosterone showed significant effects for Treatment (F=8.638) (p<0.001) and for Group x Treatment interaction effect (F=2.143) (p=0.017). The effect for Group showed a strong trend toward significance (F=2.472) (p=0.099)
(Two Way Repeated Measures ANOVA). Pairwise Multiple Comparison Procedures (Holm-Sidak Method) showed the following significant differences:

PRS+ALL vs. Baseline; PRS+M vs. Baseline; PRS+ALL vs. Stress Baseline;
PRS+M vs. Stress Baseline; PRS+P vs. Baseline; PRS+CA vs. Baseline;
PRS+ALL vs. PRS. The following results were obtained using Student’s test and Mann-Whitney Rank Sum test: Plasma corticosterone was significantly higher for LA at PRS+CA and at PRS+All than at Stress Baseline (p<0.05), PRS (p<0.05), PRS+P (p<0.01), and PRS+M (p<0.05) (Figure 2.14). Plasma corticosterone was significantly higher for HA during PRS+All than during Stress
Figure 2.11 Plasma IL-6 was significantly higher for HA during PRS+CA and during PRS+All than at Stress Baseline (*p<0.05 compared to Stress Baseline, **p<0.01 compared to Stress Baseline). Values expressed as mean, +/- SE, n=12 for each treatment.
Plasma IL-6 for High Activity (HA) and Control (Ctl) During Post-running Stress+Metoprolol (PRS+M) and Post-running Stress+Corticosterone Antagonist (PRS+CA)

Figure 2.12 Plasma IL-6 was significantly higher for HA than for Ctl at PRS+M and at PRS+CA (*p<0.05 compared to Ctl at PRS+M, t-p<0.05). No significant differences were found between Ctl, LA, and HA at Stress Baseline, PRS+P, or PRS+All. Values expressed as mean, +/- SE, n=12 for each treatment group and condition.
Plasma CRP for Control (Ctl), Low Activity (LA), and High Activity (HA) During Various Treatment Conditions

Figure 2.13 Plasma CRP was significantly higher for both LA and HA after Stress Baseline compared to Baseline (*p<0.05 compared to Baseline). No other significant differences were found between treatment conditions for Ctl, LA, or HA. No significant differences were found between Control, LA, or HA for any of the treatment conditions. Values expressed as mean +/- SE. (n=12 for each treatment group and condition).
Plasma Corticosterone for Low Activity (LA) During Various Treatment Conditions

Figure 2.14 Plasma corticosterone was significantly higher for LA at PRS+CA and PRS+All than during Stress Baseline, PRS, PRS+P, and PRS+M (*p<0.05 compared to Stress Baseline, PRS, and PRS+M; **p<0.01 compared to PRS+P, t=p<0.05 compared to Stress Baseline, PRS, and PRS+M; ttt=p<0.01 compared to PRS+P).

Values are expressed as mean, +/- SE, n=12 for each treatment.
Baseline (p<0.01) and PRS (p<0.05) (Figure 2.15). No other significant differences were found between treatment conditions for Control, LA, or HA.

Plasma corticosterone was significantly higher for LA than Control at PRS+All (p<0.05). There was not a significant difference between HA and Control plasma corticosterone at PRS+All (Figure 2.16). Plasma corticosterone was significantly higher for LA than Control at PRS+CA (p<0.01). There was not a significant difference between HA and Control plasma corticosterone at PRS+CA (Figure 2.17).

**Discussion**

**Effect of High Physical Activity on the Stress Variables**

The overriding purpose of the present study was to provide some answers to the questions, “Does exercise decrease the negative effects of stress?” and “If exercise decreases the negative effects of stress, how does it do so?” In fact, animals in the high activity (HA) group showed protective effects against stress in two major areas: decreased corticosterone response to psychosocial stress and decreased norepinephrine (NE) response to psychosocial stress.

The protective effects of exercise against plasma corticosterone and NE stress responses were seen to increase over time. For example, the attenuation of stress-induced NE in HA animals did not become evident until the third post-running stress treatment, in which beta receptors were blocked. The attenuation of stress-induced corticosterone in HA animals did not become evident until the
Plasma Corticosterone for High Activity (HA)
During Various Treatment Conditions

![Graph showing plasma corticosterone levels for different conditions](image)

**Figure 2.15** Plasma corticosterone was significantly higher for HA at PRS+All than at Stress Baseline (**p<0.01 compared to Stress Baseline). Values expressed as mean, +/- SE, n=12 for each treatment.
Plasma Corticosterone for Control (Ctl), Low Activity (LA), and High Activity (HA) During Post-running Stress + All Blockers (PRS+ALL)

Figure 2.16 Plasma corticosterone was significantly higher for LA than for Ctl during PRS+All (*p<0.05 compared to Ctl). There was no significant difference between HA and Ctl during PRS+All. Values expressed as mean, +/- SE, n=12 for each group.
Plasma Corticosterone for Control (Ctl), Low Activity (LA), and High Activity (HA) During Post-running Stress+
Corticosterone Antagonist (PRS+CA)

Figure 2.17 Plasma corticosterone was significantly higher for LA than for Ctl at PRS+CA (**p<0.01 compared to Ctl). There was no significant difference in plasma corticosterone between HA and Ctl during PRS+CA. Values expressed as mean, +/- SE, n=12 for each group.
fourth post-running treatment condition, in which corticosterone receptors were blocked. At the time in which these stress variable-lowering effects became evident, the animals had been running nine to eleven weeks. This makes sense since increased cardiovascular fitness in the HA animals would have been acquired over time.

It is important in a relatively long-term study such as this to be able to eliminate the time element as a possible variable impacting results. Although NE, IL-6, and corticosterone levels increased throughout the course of the experiment for LA animals (NE, IL-6 and corticosterone) and for HA animals (IL-6), no such elevations over time occurred for the Control animals. This indicates that the differences in the stress variables occurred due to the Colony Stress, receptor antagonist, and running treatments.

Therefore, the acquired protection against the effects of stress no doubt reflects the HA animals’ increasing level of fitness and the stress benefits such fitness affords. Exercise provides protection against the harmful effects of the stress responses in a number of ways. In the present study, blocking corticosterone receptors prior to Colony Stress resulted in a significant increase in corticosterone for both LA and HA. However, the corticosterone level remained significantly lower for HA than for LA. The high physical activity of HA appears to have truncated the stress-and corticosterone antagonist-induced rise in corticosterone.
The ability of exercise to attenuate the stress-induced rise in corticosterone has been demonstrated in previous studies. When rats were exposed to either predictable, controllable shock or to unpredictable, uncontrollable shock, plasma corticosterone was lower when the rats were allowed to run for three hours following this stressor, than when they were not allowed to run (Starzec et al., 1983). Although a stress-induced rise in corticosterone has been shown to habituate and decrease when exposed repeatedly to the same stressor (Jeferi and Bhatnagar, 2007), exercise has also been shown to speed up this habituation. Also, exercising rats showed lower corticosterone stress responses when exposed to a novel environment than did non-exercising controls (Droste et al., 2007).

When exercising and non-exercising rats were repeatedly exposed to a 98 dB noise stressor, exercising rats' corticosterone stress response habituated significantly sooner than did that of the controls. While the exercising rats had significantly less plasma corticosterone in response to stress than did the controls, they did not have significantly less adrenocorticotropic hormone (ACTH). The authors suggest that the decrease in stress-induced corticosterone resulting from exercise may involve a decrease in adrenal gland sensitivity to ACTH (Sasse et al., 2008).

In the present study, exercise also attenuated the stress-induced rise in plasma NE. Stress-induced NE was significantly lower for HA than for LA when beta-adrenergic receptors were blocked. This effect was seen again in the final
treatment condition in which alpha- and beta-adrenergic receptors and corticosterone receptors were blocked. Increased cardiovascular fitness has previously been shown to decrease cardiovascular reactivity, i.e., heart and blood pressure increases in response to a mental or physical challenge.

For example, when female SHR rats voluntarily ran for eight weeks, they showed significantly lower spontaneous changes in arterial pressure and reflex heart rate responses (Collins et al., 2000). The authors concluded that these changes resulted from a decrease in sympathetic control of the baroreflex and an increase in parasympathetic control. Other studies have demonstrated that exercise decreases cardiovascular reactivity by decreasing sympathetic activity (Chen et al., 1995; DiCarlo, 2001; Kulics et al., 1999). When rats ran daily for four weeks, they showed significant decreases in coronary vasoconstrictor tone. It was shown that these cardiovascular changes resulted from a decrease in alpha-adrenergic receptor-mediated vasoconstrictor tone, which allowed an increase in beta2 adrenergic receptor control and subsequent vasodilation (DiCarlo et al., 1988).

A relationship between exercise, adipose tissue, and NE has been proposed. Lambert and Jonsdottir (1998) showed that exercising rats had significantly decreased amounts of both abdominal fat and hypothalamic NE. Arterial NE positively correlated with hypothalamic NE. The authors suggest an afferent link from adipose tissue to hypothalamic noradrenergic neurons, which subsequently impacts sympathetic nervous system activity. When abdominal fat
is decreased, as with exercise, afferent signals to the hypothalamus also decrease, resulting in a reduction in sympathetic activity.

In the present study, the significantly reduced NE stress response seen in HA compared to LA during later treatment conditions suggests that cardiovascular fitness and a resulting decrease in cardiovascular reactivity had occurred.

Evidence of High Physical Activity and Cardiovascular Fitness for the High Activity (HA) Group

One of the surprising results of this study was the high amount of running voluntarily undertaken by the HA animals. In the present study, peak average daily running for the female rats was as high as 13.5 km. Many previous studies have used male rats and achieved running levels adequate for the study’s purpose. Typical daily running amounts in these studies were 8.3 (Griffin, 1990), 6.7 (Hoffman et al., 1987), and 4-7 km (Droste et al., 2007). However, at least one previous study, in which rats ran about 10.5 km a day, used female rats because they are known to run more than do male rats (Stones et al., 2008).

It has been suggested that female running behavior is consistent with a generally higher level of exploring behavior in female rats compared to males (Campbell, 2003). In addition to a possible desire to run more, female rats are also able to run more than males. Cortright et al. (1997) showed that when male and female rats engaged in voluntary wheel running, females experienced less
energy deficit than did males. The males had greater increases in fat mass and protein mass than did the females, even though the males ran less than the females. Tate and Holtz (1998) suggest that female rats were able to run more than males due to an ability to more efficiently use fat stores for energy, thereby using less muscle glycogen. The authors point out that although the precise physiological mechanism for this difference is not clear, it appears to be related to circulating estrogen, as male rats receiving estrogen showed similar activity and energy usage as the females.

The designated minimum to qualify as high voluntary running activity for this protocol was to have reached a daily running level of four km per day by the end of three weeks. These parameters were based on a review of the literature of studies utilizing rats and voluntary wheel running, in which some measure of improved cardiovascular functioning was achieved, e.g., increased relative heart weight, decreased blood pressure. In those studies surveyed, typical daily running ranged from three to over ten km per day by two or three weeks of access to running wheels. O'Dell et al. (2007) found that male rats had increased their daily running to six km per day by two weeks. Male rats were running four or eight km per day by the end of three weeks access to running wheels (Bakos et al., 2007). In other studies: male rats ran four to six km per day by three weeks (Howells et al., 2005); female rats ran 10.5 km per day by four weeks (Stones et al., 2008).
As the majority of HA animals in our study ran between 4.5 and 15 km per day after four weeks access to running wheels, these findings support the conclusion that HA had higher levels of cardiovascular fitness than LA.

In the present study, cardiac hypertrophy was also used as an indicator of increased cardiovascular fitness in the HA group relative to the non-runners in the LA and Control groups, and is consistent with the following studies that examined the impact of physical activity on rat cardiac function. Different exercise protocols elicit varying degrees of heart remodeling. Endurance training in male rats (swimming 90 minutes/day for 5 days a week for 60 days) increased left ventricular capillary density by 45% and left ventricular cardiomyocyte cross-sectional area by 40% (Garciaarena et al., 2009). Swimming endurance training resulted in a 13% increase in left ventricular weight and a 21% increase in myocyte dimension in male rats (Medeiros et al., 2004). Involuntary treadmill training resulted in a 22% increase in left ventricular wall thickness and a 25% increase in left ventricular cavity (eccentric hypertrophy) in male rats (Diaz-Herrera et al., 2001). The authors consider this cardiac hypertrophy to be an indicator of training efficiency. Relative heart weight was significantly higher (12%) for trained (running wheel) rats compared to sedentary rats (Stones et al., 2008).

Eccentric cardiac hypertrophy occurs when specific genes are triggered. The primary triggers for hypertrophy are hypoxic responsive elements in genes. For instance, increased physical activity has been shown to cause eccentric
cardiac hypertrophy by increasing gene expression involved in efficient oxidative capacity and resistance to apoptosis (Watson et al., 2007). They showed that exercise-induced increases in left ventricular respiratory capacity correlated with left ventricular mass for both rats (involuntary treadmill) and mice (voluntary wheel running). Also, exercise correlated with increases in the transcription factor cyclic-nucleotide regulatory element binding protein (CREB), and that increase in CREB resulted in increased anti-apoptotic proteins, increased mitochondrial respiratory capacity and rates, and increased mitochondrial protein content.

Effect of Acute Colony Stress on the Physiological Variables for Low Activity (LA) and High Activity (HA) Groups

In the present study, CRP level increased from Baseline to Stress Baseline for LA and HA. This stress-induced increase in CRP is not surprising based on previous research, and supports our hypothesis that psychosocial stress will increase CRP.

The particular stressor used in this study was Colony Stress, frequently referred to in the literature as “open-field” stress. Colony stress is one type of “novel environment” stress in which the animals must adapt to the environment and to each other. It is therefore considered a psychosocial stressor and has been shown to be effective in inducing a physiological stress response. Open-field stress resulted in increased blood pressure and heart rate in rats (Eikelis and van den Busse, 2000, van den Buuse et al., 2001).
CRP is one of the major components of the inflammatory response initiated by IL-6 (Labarre, 2004). In several studies, exposure to open-field stress has been shown to cause elevations in IL-6, a known mediator of the acute phase response including CRP (LeMay et al., 1990; Zhou et al., 1993). Zhou et al. (1993) state that the release of IL-6 into the plasma is likely the result of both neural and endocrine responses to stress. The HPA axis also is involved in increasing the production of IL-6. When male Lewis strain rats were adrenalectomized, increases in IL-6 were largely attenuated (Zhou et al., 1993).

When stress activates the sympathetic nervous system, production of IL-6 is increased, initiating an inflammatory response that potentially has adaptive benefits for the stressed organism.

CRP has been shown to correlate with activation of the sympathetic nervous system stress response. Involvement of the SNS in CRP elevations was indicated when individuals taking a beta blocker for hypertension had significantly lower CRP than those taking another type of hypertension medication (Shibao et al., 2005). In the present study, NE did not increase significantly above Baseline during Colony Stress, except when adrenergic receptors were blocked. However, it is likely that NE did increase in response to Colony Stress, but had returned to Baseline level at 6 hours post-stress when blood was drawn.

A CRP elevation in humans has also been shown to correlate with psychosocial stress (McDade et al., 2006). Ranjit et al. (2007) found a positive correlation between chronic stress and higher levels of IL-6 and CRP.
Effect of blocking corticosterone receptors on plasma corticosterone

When corticosterone receptors (MR and GR) were blocked (PRS+CA and PRS+ALL), the LA and HA animals’ corticosterone levels became elevated above all previous treatment levels in which Colony Stress occurred. These were the only treatment conditions in which corticosterone was significantly elevated above Colony Stress Baseline, indicating that blocking the corticosterone receptors prior to the Colony Stress resulted in an increase in plasma corticosterone 6 hours following the stress.

In addition, corticosterone was higher for LA than for Control when corticosterone receptors were blocked, while HA corticosterone was not higher than Control during the same condition. These results indicate that, although blocking the corticosterone receptors resulted in an increase in plasma corticosterone, the voluntary running significantly truncated this rise for the HA animals compared to the non-running LA animals. Several previous studies relating to HPA habituation, HPA negative feedback control, and the effect of exercise on corticosterone, provide insight into potential mechanisms for these findings.

For example, the HPA axis has been shown to habituate to repeated stress. Jaferi and Bhatnagar (2007) showed that the HPA axis habituates and gradually decreases its activity when repeatedly exposed to the same stressor. This habituation appears to be enhanced by voluntary wheel running (Sasse et al., 2008). In this study the authors found that initial corticosterone responses to
audiogenic stress were similar for both trained (voluntary wheel running) and sedentary animals. While both groups of animals habituated to the stressor over 15 weeks, the trained animals were seen to habituate significantly sooner than the sedentary animals.

The increase in corticosterone that was seen in the present study when corticosterone receptors were blocked apparently resulted from the HPA axis negative feedback system. Glucocorticoid synthesis and release is regulated through negative feedback loops. Glucocorticoid occupation of the two primary glucocorticoid receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) results in “long-loop” negative feedback to the hypothalamus and the pituitary to decrease the stimulus and release of CRH, ACTH, and glucocorticoid (primarily corticosterone in the rat). Blocking corticosterone receptors has been shown to disrupt the glucocorticoid negative feedback mechanism and result in increased levels of corticosterone. Blocking glucocorticoid receptors also causes ACTH secretion to be stimulated (Berne and Levy, 1998).

MR receptors have been thought to be mostly occupied during basal, non-stress conditions, and that GR receptors are available and responsive to stress-induced increased levels of glucocorticoids, thereby acting as the negative feedback regulators of corticosterone. When the MR antagonist RU28318 was used to determine the degree to which MR receptors also were available and participated in the negative feedback during a stressor, it was found that
treatment with this MR antagonist significantly increased the corticosterone response to a mild stressor (novel environment) but did not increase the corticosterone response to a moderate stressor (restraint stress). Restraint stress caused a significantly higher corticosterone response than did novel environment stress (Pace and Spencer, 2005). In the present study, the stressor utilized was Colony Stress, a form of novel environment stress, and therefore could be categorized as a mild stressor. Consistent with the above results found by Pace and Spencer, treatment with corticosterone antagonists significantly increased the plasma corticosterone response to Colony Stress.

Blocking MR receptors has been shown to override the habituation typically seen in the corticosterone stress response. When rats were repeatedly exposed to restraint stress, the corticosterone stress response decreased with each exposure. However, when MR receptors were blocked with the selective MR antagonist RU28318, the corticosterone response did not show habituation to a repeated stressor. The authors concluded that the corticosterone stress response habituation depends on MR-mediated corticosteroid negative feedback during acute stress in order to minimize exposure of target tissues to corticosteroids during repeated stress.

Effect of blocking corticosterone receptors on interleukin-6 (IL-6)

IL-6 is a major cytokine of the immune system that is produced by macrophages and lymphocytes as a part of the inflammatory process. (Salome et
al., 2008). This inflammatory process occurs in humans and animals during most physical challenges but also during psychological stress (Black, 2002). In the present study, when corticosterone receptors were blocked, stress-induced levels of IL-6 rose above those levels found with any of the other treatment conditions. These results are similar to those we found for corticosterone when corticosterone receptors were blocked, and are consistent with known interactions between the HPA axis and inflammatory cytokines such as IL-6.

For example, during stress, the innate immune system and the HPA axis become activated simultaneously in an integrated manner. IL-6 has been shown to activate the HPA axis (Dunn, 2009; Berczi et al., 2009). IL-6, via its receptors on the hypothalamus, stimulates CRH and therefore ACTH secretion, resulting in increased synthesis and secretion of corticosterone. This makes sense for the stressed organism, whose survival may depend on the mobilization of energy substrate facilitated by corticosterone.

However, as IL-6 continues to rise, it can lead to destructive levels of inflammation and potential tissue damage if left unchecked. As IL-6 rises, the corresponding rise in corticosterone acts to inhibit further IL-6 release from macrophages and lymphocytes. For example, Yang et al. (2008) report that glucocorticoids downregulate cytokine synthesis and suppress the immune response. Also, Hager et al. (2009) found that administering corticosterone to rats prior to surgery reduced post-operative plasma IL-6, thereby suppressing the inflammatory response. When an increase in serum IL-6 was induced by ETOH
in mice, it was found that corticosterone suppressed the ETOH-induced rise in IL-6 (Glover and Pruett, 2006).

In the present study, when corticosterone receptors were blocked prior to the Colony Stress, the corticosterone suppressive effects on IL-6 production described above did not happen. In fact, when corticosterone receptors were blocked, corticosterone was not able to bind to the corticosterone receptors on leukocytes. Therefore, the inhibition of IL-6 production by leukocytes did not occur, and IL-6 levels rose unchecked.

A similar result, i.e., high plasma IL-6, was found in a study using humans and relaxin, a GR agonist. Relaxin was seen to bind to the GR receptor on leukocytes and stopped leukocyte production of IL-6 in the same way that glucocorticoids such as cortisol and corticosterone do. When GR receptors were blocked with RU-486, thereby preventing relaxin from binding to GR receptors, IL-6 production was unchecked (Dschiætzig, 2005).

Effect of blocking alpha-adrenergic receptors and beta-adrenergic receptors on the stress variables

When animals or humans experience a physical threat, or the perception of a threat, the organism’s sympathetic nervous system becomes activated resulting in the fight-or-flight response. This is facilitated by the rapid neuronal release of NE, which activates organs necessary in the fight-or-flight process. Men subjected to an intense interview about upsetting subjects they were facing
had significant elevations in systolic blood pressure (16 mm) and diastolic blood pressure (13mm). The blood pressure increases correlated with an 83% increase in plasma NE (Dimsdale et al., 1987). When young and old, male and female (diestrous and estus) rats were subjected to restraint stress, all had elevated plasma catecholamines, including NE (DeTurck and Vogel, 1980). Restraint stress increased transcription of the genes encoding enzymes involved in norepinephrine synthesis in rats (Serova et al., 2009).

One of the target organs of the sympathetic nervous system is the adrenal medulla, which responds to sympathetic stimulation by rapidly increasing production of epinephrine and NE, which are then released into the blood and become systemic.

In the present study blood was taken 6 hours post-stress as this length of time was previously found in a pilot study in our lab to be necessary to capture a stress-induced rise in CRP. However, it was not expected that plasma catecholamines would be elevated at 6 hours post-stress because NE and Epi do not stay in the circulation very long. In humans and rats, the half-life of NE and Epi is only one to three minutes (Berne and Levy, 1998). This short duration is important as the effects of these catecholamines is dramatic and prolonged effects could be detrimental to the organism.

Plasma levels of NE were still elevated compared to Stress Baseline six hours following Colony Stress only when alpha receptors were blocked with Prazosin or beta receptors were blocked with Metoprolol. This effect occurred for
LA animals but not for HA animals; as previously mentioned, NE was elevated only for LA and not for HA in the latter treatment conditions, apparently due to the acquired protective effect of running. One possibility for this prolonged elevation of NE is that alpha receptor blockade prevented NE from binding to alpha receptors, thus interrupting NE negative feedback. Therefore, elevated levels of NE continued several hours following the Colony Stress. These results are consistent with previous studies in which alpha blockade was used. For example, plasma NE response to exercise for women was 48% higher when alpha receptors were blocked compared to controls (Mazzeo et al., 2003).

As in the present study, previous studies have also shown an elevation in plasma NE when beta receptors were blocked. For example, it has been shown that for myocardial infarct patients, plasma NE did not increase in response to the beta blocker Metoprolol during rest, but did increase plasma NE during physical exercise compared to exercising controls that did not receive Metoprolol (Delius et al., 1979). Similarly, plasma NE was unchanged during basal conditions for male patients with moderate hypertension taking Metoprolol compared to when they were taking a placebo. However, plasma NE significantly elevated during submaximal exercise when these patients were taking Metoprolol compared to when they were taking a placebo (Hansson et al., 1977).

Also in support of our findings, it has been noted that anti-hypertensive medications can trigger sympathetic activity when a fall in blood pressure causes baroreceptor unloading. Grassi et al. (2000) suggested this as a mechanism of
action causing the increase in plasma NE following administration of the alpha-adrenergic antagonist Prazosin, the alpha block used in the present study.
Chapter 3 – Exercise, Colony Stress, and Estrogen

Introduction

Objective

To determine if blocking the estrogen receptor with tamoxifen increases the rise in CRP caused by an acute psychosocial stressor.

Hypothesis

The stress-induced rise in CRP will be higher for those subjects with tamoxifen (LA and HA, after tamoxifen implant) than for those subjects without tamoxifen (Control). The stress-induced rise in CRP will be higher for LA subjects than for HA subjects, both before and after receiving tamoxifen.

Methods

Animals

Female WKY/UA age 3.5 to 4 months and weighing between 170 and 200 g at the onset of each experiment were used. Animals were placed individually in polyethylene cages (45x25x20 cm) with stainless steel tops and heat treated wood chip bedding (P.J. Murphy Forest Products, Montville, NJ). Rat chow (Tekland Rodent diet, Madison, WI) and water were given ad libitum. All animals were kept in the same room, which was maintained on a 12-hour light/12-hour dark schedule, with lights on at 7:00 am, and temperature between 24 and 26
degrees C, and relative humidity 50%. Animals were given two weeks to acclimate to individual cages prior to beginning treatment conditions. Cages were cleaned weekly. Animals were weighed weekly. All procedures were approved by the University of Akron Institutional Animal Care and Use Committee.

Conditions

Animals were divided and placed in one of three conditions:

1) Control – animals remained singly housed in standard cages and did not receive tamoxifen implants. They did receive Colony Stress treatment.

2) Low activity + Tamoxifen – animals remained singly housed in standard cages and received Colony Stress and tamoxifen treatments.

3) High activity + Tamoxifen – animals were placed in wheel cages and allowed to exercise at will. Distance each animal ran daily was determined from number of wheel turns recorded daily by means of mechanical counter or distance run in km recorded by Schwinn cyclometer (Pacific Cycle, Madison, WI).

The animals were kept in these conditions for the remainder of the experiment.
Experimental Design

TABLE 3.1 Experiment 2: Exercise, Stress, and Tamoxifen

<table>
<thead>
<tr>
<th>Condition</th>
<th>No tx</th>
<th>S</th>
<th>Ex</th>
<th>TAM Implant</th>
<th>Ex</th>
<th>S</th>
</tr>
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<tbody>
<tr>
<td>Control(n=8)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LA+TAM(n=8)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HA+TAM(n=8)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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<td>1 hr</td>
<td>5 wks</td>
<td>2 wks</td>
<td>1 hr</td>
<td></td>
</tr>
</tbody>
</table>

No tx = No treatment

Control = No treatment

LA + TAM = Low Activity + Tamoxifen Condition

HA + TAM = High Activity + Tamoxifen Condition

S = One-hour Colony Stress

Ex = Unlimited 24/7 access to exercise wheel

Following two-week acclimation period, animals in the Control, LA+TAM and HA+TAM groups received one-hour colony stress followed by blood acquisition 6 hours later.

After animals in the HA condition had been running for 5 weeks, LA and HA animals were implanted with Tamoxifen capsules. Control animals were given a sham implant.

After an additional two weeks, animals in Control, LA, and HA groups received one-hour colony stress followed by blood acquisition 6 hours later.
While sedated, hearts, adrenal glands, and thymus glands were removed and weighed.

Treatments and Procedures

Tamoxifen Treatment

Tamoxifen (Sigma Chemical Co., St. Louis, MO) was administered via Silastic laboratory tubing (0.058 inch ID X 0.077 inch OD) cut into 3 cm lengths. The resulting capsules were implanted subcutaneously into the dorsal neck region of each rat. Each rat was implanted with two 3-cm capsules containing a total of 28 mg of tamoxifen citrate (Mannino et al., 2007). Control animals were implanted with empty Silastic tubes.

Running Wheels

Animals in the HA condition were placed in wheel cages as described in Experiment I.

Blood sampling procedure

Blood was obtained, centrifuged, and stored as described in Experiment I.

Plasma corticosterone, catecholamine, IL-6, and CRP assays

Plasma samples were assayed as described in Experiment I.

Termination procedure

Animals were terminated as described in Experiment I.

Statistics

One-way ANOVA, Two-Way ANOVA, and Two-Way ANOVA with Repeated Measures statistical analyses were used as appropriate. Student’s t-
test and Mann-Whitney Rank Sum Test were used to compare individual groups. SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) were used for statistical analyses, with significance assumed at p<0.5.

Results

Tamoxifen Administration

At termination, the content of tamoxifen in the silastic tubes was checked for each animal. For each animal, both tubes were present and had some tamoxifen left in the tube, indicating that tamoxifen had continued to be released throughout the experiment. The tubes had between 40 and 70% of tamoxifen left.

Running

Animals in the HA group had access to turning wheels 24/7 and were able to run at will. The HA animals were running three or more km per day within 3 weeks, meeting the conditioning requirement. (Figure 3.1a).

Body Weight Gains

There were no significant differences in weight gain between Control, LA, and HA (Figure 3.1b). However, weight gain for OVX rats used in a previous pilot study in our lab (also WKY-UA and similar in age and beginning weight to those in the present study) was significantly higher than for those animals receiving tamoxifen in the present study (LA and HA, p<0.001) (Figure 3.2).
Average Running Distance for High Activity WKY Females
Before and After Tamoxifen Implants

Figure 3.1a The high activity animals were running more than the minimal 3 km/day by 2 weeks. Running distance was not impacted by the tamoxifen implants, which were surgically implanted between weeks 8 and 9. The arrow indicates the time of the tamoxifen implants.
Figure 3.1b There were no significant differences in weight gain between Control (Ctl), Low Activity (LA), and High Activity (HA). Values expressed as mean +/- SE, n=8 for each group.
Figure 3.2 Average weight gain for Ovariectmized (OVX) animals was significantly higher than average weight gain for Intact and average weight gain for animals receiving tamoxifen (TAM) (**p<0.01 compared to Intact, ***p<0.001 compared to TAM).

Values expressed as mean +/- SE, n=36 Intact, n=12 OVX, n=16 TAM.
Relative Heart and Adrenal Weights

Relative heart weight was significantly higher for HA (0.398 mg/100 kg body weight) compared to LA (0.342 mg/100 kg body weight, p<0.01) and compared to Control (0.351 mg/100 kg body weight, p<0.05). There was no significant difference between Control and LA relative heart weight (Figure 3.3).

Relative adrenal weight was significantly higher for HA (0.0374 mg/100 kg body weight) compared to LA (0.0303 mg/100 kg body weight, p<0.05) and compared to Control (0.0307 mg/100 kg body weight, p<0.01) (Figure 3.4). There was no significant difference between Control and LA relative heart weight.

Norepinephrine (NE)

Plasma NE showed a significant Treatment effect (Two Way Repeated ANOVA) (F=3.238) (p=0.051). Group effect and GroupXTreatment effect were not significant. The following results were obtained using Student’s t-test and Mann-Whitney Rank Sum test: NE for both Control and LA were significantly higher at PRS Stress+TAM than at Baseline (p<0.05), while HA NE was not significantly higher at PRS+TAM than at Baseline (Figure 3.5). There were no other significant differences between Control, LA, and HA or between treatments.

C-Reactive Protein (CRP)

Plasma CRP showed a significant Group effect (F=4.335) (p=0.026) and Treatment effect (Two Way Repeated ANOVA) (F=10.906) (p<0.001). GroupXTreatment interaction was not significant. Pairwise Multiple Comparison Procedures (Holm-Sidak Method) showed the following significant differences:
Ctl vs. HA, Baseline vs. Stress Baseline, Stress Baseline vs. PRS+TAM. The following results were obtained using Student’s t-test and Mann-Whitney Rank Sum test: Plasma CRP for Control, LA, and HA were significantly higher at Colony Stress than at Baseline for Control, LA, and HA (p<0.05) (Figure 3.6). Plasma CRP was significantly higher for Control at PRS+TAM than at Baseline (p<0.01), while plasma CRP was not significantly higher for LA or HA at PRS+TAM than at Baseline (Figure 3.7).
Relative Heart Weight for Ctl, LA, and HA

Figure 3.3 Relative heart weight was significantly higher for High Activity (HA) than for Control (Ctl). Relative heart weight was significantly higher for HA than for Low Activity (LA) (*p<0.05 compared to Ctl, **p<0.01 compared to LA). Values expressed as mean, +/- SE, n=8 for each group.
Relative Adrenal Weight for Ctl, LA, and HA

Figure 3.4 Relative adrenal weight was significantly higher for High Activity (HA) than for Control (Ctl). Relative adrenal weight was significantly higher for HA than for Low Activity (LA) (*p<0.05 compared to LA, **p<0.01 compared to Ctl). Values expressed as mean, +/- SE, n=8 for each group.
Figure 3.5 Plasma NE for both Control (Ctl) and Low Activity (LA) were significantly higher during PRS+TAM than during Baseline, while plasma NE for High Activity (HA) was not significantly higher during PRS+TAM than at Baseline (*p<0.05 compared to Ctl Baseline; t=p<0.05 compared to LA Baseline).

Values expressed as mean, +/- SE, n=8 for each group and treatment.
Figure 3.6 Plasma CRP was significantly higher at Colony Stress than at Baseline for Control (Ctl), Low Activity (LA), and High Activity (HA) (*p<0.05 compared to Baseline). Values expressed as mean, +/- SE, n=8 for each group for each treatment.
Plasma CRP for Control (Ctl), Low Activity (LA), and High Activity (HA) During Baseline and Post-running Stress+TAM (PRS+TAM)

![Bar chart showing Plasma CRP levels for Ctl, LA, and HA during Baseline and PRS+TAM](image)

**Figure 3.7** Plasma CRP was significantly higher for Control (Ctl) during PRS+TAM than at Baseline (**p<0.01** compared to Ctl during Baseline), while there was no significant difference between Low Activity (LA) or High Activity (HA) at PRS+TAM than at Baseline. Values expressed as mean, +/- SE, n=8 for each group and treatment.
Plasma CRP was significantly higher for Control than for LA (p=0.05) at PRS+TAM. Plasma CRP was higher for Control than for HA at PRS+TAM, and showed a strong trend toward significance (p=0.075) (Figure 3.8). There were no significant differences between Control, LA, and HA at Baseline or Colony Stress.

**Corticosterone**

There were no significant differences between treatments or groups for plasma corticosterone.

**Discussion**

**Effect of Blocking Estrogen Receptors with Tamoxifen**

In the present study, it was hypothesized that blocking estrogen receptors would increase the CRP physiological stress effect, but the opposite happened. Tamoxifen decreased the CRP response to stress for HA and LA compared to Control. In general, the results for tamoxifen were much more similar to intact animals than to OVX, indicating that although the estrogen receptor was blocked, this did not eliminate all estrogen-like effects. The similarities of tamoxifen animals to intact animals in the present study included weight gain, running behavior, and CRP response to Colony Stress.

Several studies provide insight into the possible mechanism underlying these results. Although tamoxifen has been shown to compete with estrogen for the estrogen estrogen receptor (Li et al., 2002), tamoxifen occupation of the ER
Plasma CRP for Control (Ctl), Low Activity (LA), and High Activity (HA) During Post-running Stress+Tamoxifen (PRS+TAM)

**Figure 3.8** Plasma CRP was significantly higher for Control (Ctl) than for Low Activity (LA) during PRS+TAM (*p<0.05 compared to LA). Plasma CRP was higher for Ctl than for High Activity (HA) during PRS+TAM, and showed a strong trend toward significance (p=0.075). Values expressed as mean, +/- SE, n=8 for each group.
receptor mimics some estrogen effects, especially those on behavior and response to stress. For example, rats became physically inactive after ovariectomy (OVX) compared to controls. When the OVX rats were given either tamoxifen or estrogen, running behavior and weight returned to their pre-OVX norm (Gorzek et al., 2007).

Wade and Heller (1993) concluded that tamoxifen mimics the effects of several estrogen activities including the physiological and behavioral regulation of energy balance. They demonstrated that tamoxifen decreases white adipose tissue wet weight and lipoprotein lipase activity in OVX rats in a manner similar to estrogen. Also similar to estrogen, tamoxifen caused a significant decrease in food intake and weight.

In the present study, the CRP response to stress for tamoxifen was significantly lower than for Control animals not receiving tamoxifen. In previous studies, Specific Estrogen Receptor Modulators (SERMs) have been shown to decrease vasopressin-induced mean blood pressure and increase acetylcholine-induced vasorelaxation in OVX rats (Ikeno et al., 2009), to restore endothelial function by enhancing nitric oxide synthesis (Leitzbach et al., 2005; Wassmann, 2002), and to inhibit NE responsiveness (Morales et al., 2007). As NE, cardiovascular reactivity, and endothelial dysfunction are all associated with increases in CRP, the above studies provide insight into the effect of tamoxifen on CRP in the present study.
Similar to the present study, tamoxifen has been shown to lower circulating CRP. For example, tamoxifen significantly lowered CRP in healthy hysterectomized women even when 10 mg doses were given rather than the standard 20 mg dose (Bonanni et al., 2003). Tamoxifen lowered several inflammatory markers in healthy women, including a 26% reduction in CRP (Cushman et al., 2001).

In the present study, CRP stress response for tamoxifen animals was not merely similar to intact animals, it was significantly lower than for intact controls. This suggests that for certain behavioral and physiological effects, tamoxifen not only mimics estrogen, but may actually intensify those effects.

Evidence of High Physical Activity and Cardiovascular Fitness for the High Activity (HA Group)

As in Experiment I, average daily running exceeded the minimum of 3 km per day. Average daily running was between 10 and 12 km on many days. HA also had significantly higher relative heart weights than did LA or Control. These two facts were considered indicators that HA had achieved higher cardiovascular fitness than the non-running Control and LA. Previous studies have found significant increases in cardiovascular functioning and hypertrophy using running protocols in which rats ran similar or smaller amounts than in the present study (O’Dell et al., 2007; Bakos et al., 2007; Droste, 2007; Stones et al., 2008).
Effect of High Physical Activity on Adrenal Weight

Relative adrenal weight was significantly higher for HA than for LA and Control, suggesting that the running activity of the HA contributed to adrenal enlargement. This is consistent with previous research that has shown exercise-induced increases in the adrenal gland. For example, when rats were trained at near capacity for 10 weeks on motorized wheels, adrenal gland size increased by 40%. This increase in size was shown to result from both hyperplasia and hypertrophy of the adrenal gland cells (Song et al., 1973). In another study, when young and old rats underwent 10 weeks of treadmill training for 60 minutes per day, adrenal medullary volume increased by 48% in young and 18% in old rats. Medullary epinephrine content increased by 36% in young and by 24% in old rats. However, adrenal epinephrine and norepinephrine concentration did not increase, indicating that the increase in epinephrine content resulted from an increase in the size of the medulla (Schmidt et al., 1992).

Effect of High Physical Activity on the Stress Variables

As in Experiment I, high physical activity appears to have had a protective effect against stress. During the final Colony Stress, HA animals NE was not significantly higher than during Baseline, while Control and LA had significant NE stress effect compared to Baseline. This is consistent with previous research indicating that increased cardiovascular fitness results in a decrease in cardiovascular reactivity, i.e., heart rate and blood pressure increases in
response to a mental or physical challenge (Collins et al., 2000; Chen et al., 1995; DiCarlo., 2001; Kuliks et al., 1999; DiCarlo et al., 1988).

Effect of Acute Colony Stress on the Physiological Variables

As in Experiment I, the novel colony situation (Colony Stress) was shown to elicit a stress response. Colony Stress significantly raised CRP for Control, LA, and HA compared to Baseline, and Colony Stress significantly raised NE for LA and Control during PRS+TAM. This is consistent with previous research indicating that Colony Stress is a viable means of inducing a measurable social stress response in rats. For example, open-field stress resulted in increased blood pressure and heart rate in rats (Eikelis et al., 2000, van den Buuse et al., 2001). In several studies, exposure to open-field stress has been shown to cause elevations in IL-6, a known mediator of the acute phase response including CRP (LeMay et al., 1990, Zhou et al., 1993).
Chapter 4 – Conclusion

In this study increasing physical activity was seen to minimize the potentially harmful NE and corticosterone responses to stress. Recently, stress has become increasingly recognized as a major contributor to most health conditions. In humans, NE and cortisol (the equivalent to corticosterone in rats) are largely responsible for the health consequences of stress. Although the high activity animals in this study received the same stressors (Colony Stress) as did the low activity rats, this stress did not cause the same rise in NE and corticosterone for the high activity animals. This indicates that the higher physical activity had helped to “stress proof” the runners.

In addition, blocking the estrogen receptor with Tamoxifen helped to prevent the CRP response to stress, thereby minimizing the harmful effects of the inflammatory response. Although Tamoxifen has primarily been used as an estrogen antagonist in the treatment of breast cancer, additional actions and benefits are continually being explored. Many of Tamoxifen’s future clinical uses are likely to stem from its estrogen agonist effects. In other words, Tamoxifen is likely to be used to mimic some of the positive effects of estrogen, thereby alleviating some of the health consequences of menopause.

In future studies, it will be beneficial to examine the variables in this study, i.e., the impact of physical activity on the stress responses, within the context of
aging. In addition, exploring the potential of high physical activity to reduce the impact of stress on brain structures such as the hippocampus and amygdala would shed light on the potential of exercise to reduce stress-induced cognitive changes such as memory difficulties and clinical depression.
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