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INTRACELLULAR SIGNALING MECHANISMS OF SEX HORMONES IN ACUTE MYOCARDIAL INFLAMMATION AND INJURY

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1. ABSTRACT

Sex hormones are important modifiers of the acute inflammatory response to injury, an important aspect of myocardial depression and apoptosis following ischemia or endotoxemia. Hemorrhage, trauma, ischemia/reperfusion, burn and sepsis each lead to cardiac dysfunction. Gender has been shown to influence the inflammatory response as well as outcomes following acute injury. The mechanisms by which sex affects the inflammatory response and the outcome to acute injury are being actively investigated. It is now recognized that myocardial inflammation plays a crucial role in I/R-induced myocardial dysfunction. Inflammatory mediators, such as TNF-alpha are produced by cardiomyocytes and contribute to myocardial functional depression and apoptosis. Gender differences in the inflammatory response following burn injury have been demonstrated. However, gender differences in the setting of acute I/R-induced inflammation are unclear. In addition, a critical component of the signal transduction pathway leading to myocardial inflammation is the activation of p38 mitogen-activated protein kinase (MAPK). In other systems, it appears that gender differences exist in the p38 MAPK signaling pathway. The inflammatory response, including the p38 MAPK signaling cascade and expression of proinflammatory cytokines such as TNF-alpha and IL-1beta, may precipitate cardiomyocyte apoptosis following I/R injury. Apoptosis may be an essential component in the pathogenesis of heart failure, and there is evidence that myocyte apoptosis in the failing human heart is markedly lower in women than in men. The prevention of cell death attenuates I/R-induced injury on myocardial anatomy and performance. This review will: 1) examine evidence for gender differences in the outcome to acute injury; 2) explain the myocardial inflammatory response to acute injury; and 3) elucidate the various mechanisms by which gender and sex hormones affect the myocardial response to acute injury.

2. INTRODUCTION

Estrogen and testosterone have emerged as important modifiers of the acute inflammatory response to injury, which plays an important role in the development of acute myocardial injury (1-42). Cardiovascular disease is the number one cause of death among women, accounting for nearly fifty percent of female deaths (43). Statistics show that women on average develop cardiovascular disease ten to fifteen years later in life than men, and that the risk may increase after the menopause (44). This observation has led to much speculation as to what physiological change(s) associated with the menopause is responsible for the higher risk of atherosclerosis. Estrogen and its potential as a cardioprotective agent and as an immunomodulator of the inflammatory response in atherosclerosis has received the most attention from researchers. The inflammatory response to injury is a double-edged sword playing an important part in the damage produced by the injury as well as in the process of repair. In the heart, it has been shown that the inflammatory response is produced not only by resident macrophages but also by the cardiomyocytes (5, 45). The cytokines produced during an inflammatory response cause depression of cardiac function (46-48) and hence methods that block the inflammatory response may be protective (49-51). Sex differences have been noted in these responses and the potential reasons for the differences have been the subject of extensive research (1-4, 52, 53). This review will: 1) examine evidence for gender differences in the outcome to acute injury; 2) explain the myocardial inflammatory response to acute injury; and 3) elucidate the various mechanisms by which sex affects the myocardial response to acute injury.

3. CLINCAL OUTCOMES

Gender differences have been noted in outcome to acute injuries like myocardial infarction, burns, trauma

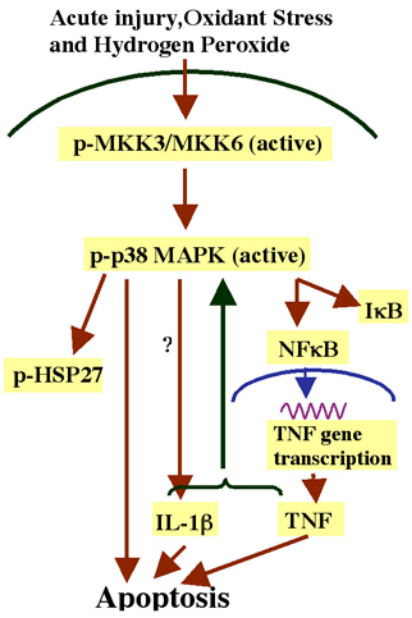

Figure 1. p38 mitogen-activated protein kinase (MAPK) signaling pathway.

and sepsis. Hospital based clinical studies have shown that females have a higher mortality rate after myocardial infarction compared to males (54-56). In general, the females in many of these studies were older, had higher risk factors (diabetes, hypertension and congestive heart failure), more complications, and lower likelihood of receiving treatment (57). Importantly, more males died from myocardial infarction before reaching the hospital and the 28 day mortality for males and females was the same (58-60). This actually suggests that females are relatively protected in the immediate aftermath of a myocardial infarction but are similar to males at the end of a month.

Sepsis and trauma are two other inflammatory conditions associated with sex dependent outcomes. For mortality in trauma, there is either no sex difference (61-63) or sex difference in blunt trauma but not in penetrating trauma (64). Studies that have found sex differences are inconsistent. Some studies showed benefit only in females > 50 years (64) while others showed benefit only in females < 50 years old (65, 66). Females however have lower incidence of pneumonia, sepsis and multi organ failure after trauma (62, 66-68). In sepsis, some studies have found a higher mortality rate in females > 80 years old (69), while others have found lower mortality rates for females (70). In a study by Schroeder and colleagues (71) involving septic patients, females demonstrated lower mortality, higher interleukin-10 (IL-10) and lower tumor necrosis factor alpha (TNF) levels. Fewer female patients in intensive care units developed sepsis, although once sepsis developed the mortality rate was the same (72). Clinical studies on sex differences in mortality after burns present inconsistent evidence. Some showed females (73) or only females aged 30-59 years (74) to have higher mortality while others showed that females have lower incidence of multi-organ dysfunction and sepsis after burns (75).

In contrast to the clinical studies, animal studies have consistently found that females do better. Protective effects of acute administration of estrogen, in an *in vivo* left anterior descending (LAD) coronary artery ischemia/reperfusion (I/R) model, have been shown in different animals (76-79). Chronic administration of estrogen provides protection from I/R injury in isolated hearts undergoing global ischemia and in hearts undergoing *in vivo* LAD obstruction (80, 81). Estrogen also protected against reperfusion induced arrhythmias after LAD I/R injury (78, 81, 82). Ovariectomized females have worse cardiac functional recovery after global I/R, in an isolated heart, than sham ovariectomized females or ovariectomized females with estradiol replacement (83, 84). After burn injury, females have lower cytokine production and better cardiac function (85). Trauma-hemorrhage leads to depressed immune function and this depression is more severe in males (86-88). The immune depression is in part caused by testosterone (89, 90) since both castration (87) and receptor blockade (91-94) attenuated this depression. Estrogen also prevented the immune depression caused by trauma-hemorrhage (95, 96).

Animal studies have consistently shown that females are protected against acute injury while clinical studies appear inconsistent. A possible reason is that in animal studies the female population is well controlled and only proestrous females are used while clinical studies have a heterogeneous population. Furthermore, the underlying condition of humans is less uniform. Indeed, the few animal studies that used diestrous females showed that diestrous females had functional recovery equivalent to males, but lower than proestrous females (85, 97). This has been borne out by a few clinical studies which showed that cardiac function fluctuates with the hormonal changes of menstrual cycle (98-100). Thus, it is important to know the hormonal status of females and future clinical studies that take this into account may produce more consistent results. The remainder of this review will focus on gender differences in the myocardial inflammatory response to acute injury.

4. MYOCARDIAL INFLAMMATION

Acute injuries lead to the production of an inflammatory cascade. The inflammatory cascade is triggered through many pathways but it may converge onto a few key regulatory proteins. Perhaps important among these are p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFkB). Ischemia reperfusion (I/R), sepsis, trauma and burn injury lead to oxidative stress (5, 101) which activates p38 MAPK (24, 102, 103) and NFkB (104, 105) (Figure 1). p38 MAPK is regulated by upstream kinases referred to as MAP kinase kinase (MAPKK) which themselves are regulated by MAP kinase kinase kinases (MAPKKK). This sequence of phosphorylation causes amplification of the signal. p38 MAPK is crucial in the cascade leading to TNF gene induction and its inhibition is protective (106-110) (Figure 1). p38 MAPK is also involved in the production of IL-1, IL-4, IL-6 and IL-8 (111-114). Wang and colleagues (108) showed that the increase in TNF, IL-1 and IL-6

production, after endotoxin infusion in an isolated heart, could be inhibited by a p38 MAPK inhibitor. TNF sequestration alone led to a decrease in IL-1 and IL-6. This suggests that TNF is upstream to IL-1 and IL-6 and that the effect of p38 MAPK on other cytokines might be mediated through TNF.

NFkB is involved in the regulation of many processes including apoptosis, cell growth, stress responses, innate and acquired immunity and sepsis. NFkB is bound to inhibitory kappa B (IkB) in the cytoplasm, this prevents its nuclear localization and DNA binding. Phosphorylation of IkB by inhibitory kappa B kinase (IKK) results in dissociation of IkB from NFkB, allowing NFkB to translocate to the nucleus. MAPK, protein kinase C (PKC) and phosphatidylinositol 3 kinase (PI3K)/Akt converge on IKK for NFkB activation (115, 116). p38 MAPK plays an important role in activation of NFkB and expression of NFkB-dependent genes (109, 115, 117, 118) (Figure 1). NFkB activation leads to production of TNF and IL-1 (36, 119, 120).

MAPK and NFkB activation also occur through other mechanisms. TNF and IL-1 activate p38 MAPK and NFkB (Figure 1). IL-1 leads to formation of a complex between IL-1 receptor-associated kinase (IRAK) and myeloid differentiation factor 88 (MyD88) (121, 122). IRAK is released and binds to TNF-receptor associated factor (TRAF). TRAF activates a MAPKKK, which activates p38 MAPK and NFkB (123). TNF also activates them through TRAF. TNF and IL-1 activate p38 MAPK and NFkB, they increase the production of TNF and IL-1, thus forming a feed-forward mechanism and amplifying the inflammatory response. Lipopolysaccharide (LPS) through its interaction with CD14 provokes rapid activation of protein tyrosine kinase which activates a pathway involving Ras/Raf-1/MEK/MAPK/NFkB (5). Recently, LPS has also been shown to use the MyD88/IRAK pathway (116, 124). These pathways have been delineated but their role in myocardial inflammatory response to sepsis is not known and should be the subject of further research.

TNF causes decreased myocardial contractile efficiency and reduced ejection fraction, hypotension, decreased systemic vascular resistance and biventricular dilation. These effects are produced through calcium dyshomeostasis, direct cytotoxicity, oxidant stress, disruption of excitation-contraction coupling, myocyte apoptosis and induction of other cardiac depressant cytokines such as IL-1 and IL-6 (5). TNF, through sphingosine, disrupts L-type channel-induced calcium release and thereby depresses calcium transients (125-127). NO appears to mediate TNF-induced desensitization of myofilaments to intracellular calcium (128, 129). Anti-TNF measures have been protective however a study using TNF receptor knockout mice showed increased infarcts in the knockout mice after LAD occlusion (130). This suggests that TNF leads to activation of both protective and damaging responses and a decrease in the excessive TNF production after acute injury may be protective but a complete absence is harmful. This might be why clinical studies of anti-TNF measures have not shown the benefit expected (131).

TNF, IL-1 and IL-6 lead to increased expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) (132-134). Neutrophil adhesion occurs leading to a respiratory burst which produces reactive oxygen species (135). The importance of neutrophils in cardiac injury is made clear by the cardioprotection obtained with neutrophil depletion (136-138). The reactive oxygen species produced during acute injury can also cause leukocyte chemotaxis (139) and adhesion (140) possibly through complement activation (141), upregulating P selectin expression (142), by inducing ICAM-1 expression (143) or by increasing the ability of ICAM–1 to bind neutrophils (144). Protective effects of antioxidant enzymes were shown in left circumflex coronary artery I/R by infusing antioxidant enzymes (145) and in isolated hearts of transgenic mice overexpressing antioxidant enzymes (146, 147). Disappointingly, clinical studies using them have found no significant benefit (148, 149).

Accumulating evidence indicates that cytokines are important mediators of cardiovascular disease. Acute injury in the form of ischemia, endotoxemia, or burn trauma results in myocardial functional suppression, in part via the local production of inflammatory mediators such as TNF, IL-1, and IL-6. Ischemia-reperfusion injury induces the local production of TNF, IL-1 and IL-6. Indeed, locally produced inflammatory mediators may be an important contributor to postischemic myocardial dysfunction and cardiomyocyte apoptosis. TNF, IL-1, or IL-6-induced depression of myocardial function in an ex vivo model has been observed. The mechanisms by which TNF causes myocardial dysfunction include calcium dyshomeostasis, direct cytotoxicity, oxidant stress, disruption of excitation-contraction coupling, and myocyte apoptosis, as well as the induction of other cardiac depressants such as IL-1 and IL-6. This study examines the mechanisms by which the heart produces these substances and uses targeted therapies to determine if blocking inflammatory cytokine production helps the heart. This information may help save heart cells during conditions of low blood flow such as heart attacks or heart surgery.

One of the signaling enzymes involved in proinflammatory cytokine production is p38 mitogen-activated protein kinase (MAPK). Ischemia-reperfusion, oxidant stress, and hydrogen peroxide directly activate p38 MAPK. Activation of p38 MAPK is required for TNF and IL-1 production in cardiomyocytes. Recently, p38 MAPK activation has been correlated with proinflammatory cytokine production in myocardium after endotoxemia and burn trauma. In addition, myocardial I/R injury in animal and human studies results in activation of myocardial p38 MAPK, and p38 MAPK inhibition results in improved myocardial function following I/R injury. Therefore, p38 MAPK may mediate post-ischemic myocardial dysfunction by upregulating the inflammatory response. However, it remains unclear how activation of p38 MAPK induces postischemic myocardial inflammatory cytokine production. Active p38 MAPK may lead to inhibitory κB (IκB) phosphorylation, which subsequently results in disruption of the NFκB-IκB complex and activates NFκB.

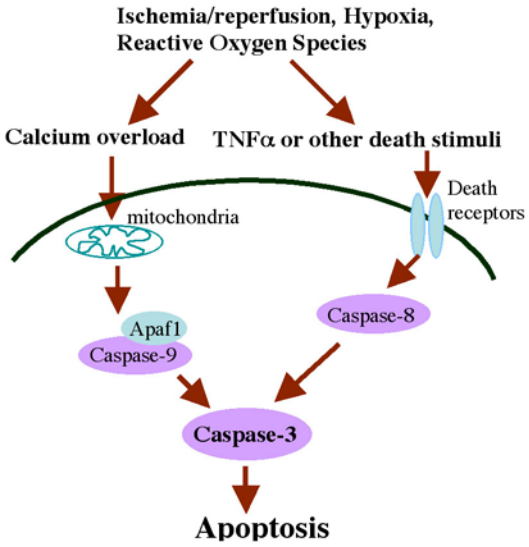

Figure 2. Myocardial apoptosis signaling pathway.

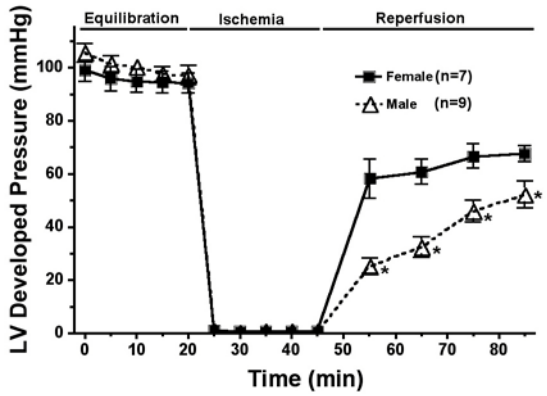

Figure 3. Changes in left ventricular developed pressure (LVDP) following ischemia and reperfusion in normal age matched male and female rat hearts perfused with modified Krebs-Henseleit solution. Results are mean +/- SEM, *p<0.01 vs. female at the corresponding time points).

Activated NFκB translocates from the cytoplasm to the nucleus, where it docks to TNF promoter and activates TNF gene transcription. Recently, caspase-1 and caspase-11 were shown to function upstream of IL-1 maturation. IL-1 is initially synthesized as an inactive precursor requiring the IL-1-converting enzyme (ICE or caspase-1) for cleavage to the mature, biologically active molecule. Caspase-11 is crucial for the activation of caspase-1. ICE is required for IL-1 activation in the postischemic heart. It has been demonstrated that caspase-11 induction by LPS and hypoxia in microglia was mediated by p38 MAPK (150, 151). However, it is still unknown whether active p38 MAPK may directly induce myocardial IL-1 after acute ischemic injury. Thus, understanding the role of the p38 MAPK signaling pathway in I/R injury may differentiate p38 MAPK, NFκB, caspase-1, or caspase-11 as putative therapeutic targets.

Apoptosis may be an essential component of myocardial dysfunction following I/R (Figure 2). Myocyte apoptosis in heart failure differs between women and men. Apoptosis can be mediated by either extrinsic death receptor signaling or intrinsic mitochondrial control pathway. The extrinsic pathway involves TNF or other death signaling binding to death receptor TNFR1, which recruits procaspase-8 and activates it. Caspase-8 then activates downstream caspase-3 and induces apoptosis. The release of cytochrome *c* from mitochondria into the cytoplasm initiates the intrinsic signaling of apoptosis. Cytochrome *c* binds to apoptotic protease-activating factor-1 (Apaf-1) and then leads to recruitment and activation of procaspase-9, which results in the activation of procaspase-3 and apoptosis. The activation of intrinsic apoptotic pathway (the release of cytochrome *c* and activation of caspase-9) during reperfusion, but not ischemia has been observed in chick cardiomyocytes and activation of caspase-8, caspase-3 exists in cardiomyocytes response to hypoxia and ischemia. However, no information exists regarding the influence of gender and sex hormones in myocardial proapoptotic signaling cascades to I/R.

5. SEX DIFFERENCES IN MYOCARDIAL INJURY

Sex differences in cytokine production after acute injury have been shown in many studies (1, 2) and appears to correlate with myocardial function (Figures 3-5). Our recent studies showed that normal female myocardium produces less TNF and IL-1 when compared to male myocardium undergoing the same insult (1). In clinical studies, Schroeder and colleagues (71) showed that females had lower TNF and higher IL-10 levels in sepsis and Oberholzer and associates (62) showed that male trauma patients had higher IL-6 levels. In animal studies, females had lower cardiomyocyte secretion and serum concentrations of TNF, IL-1, IL-6 and IL-10 after burn injury (85). Deshpande and colleagues (152) showed that estradiol attenuated the LPS-induced production of IL-1, IL-6 and TNF by macrophages and also decreased NFkB binding activity. Estradiol also attenuated the increase in IL-6 after trauma- hemorrhage (153). As stated earlier, trauma-hemorrhage leads to depressed immune function and this depression is more severe in males (86-88). The immune depression is in part caused by testosterone (89, 90) since both castration (87) and receptor blockade (91-94) attenuated this depression. Estrogen also prevented the immune depression caused by trauma-hemorrhage (95, 96). Even though gender difference in the cytokine production after acute injury has been noted, the mechanisms by which these differences are mediated are not known.

As p38 MAPK is an important regulatory protein in the inflammatory cascade, recent evidence showing sex differences in p38 MAPK point towards a possible role for it in mediating sex differences in response to acute injury. Sex differences in p38 MAPK activation have been shown after trauma-hemorrhage and I/R. Wang and colleagues (154) found that males had higher levels of phosphorylated p38 MAPK (the activated form of p38 MAPK) in isolated hearts after global I/R. They also showed that

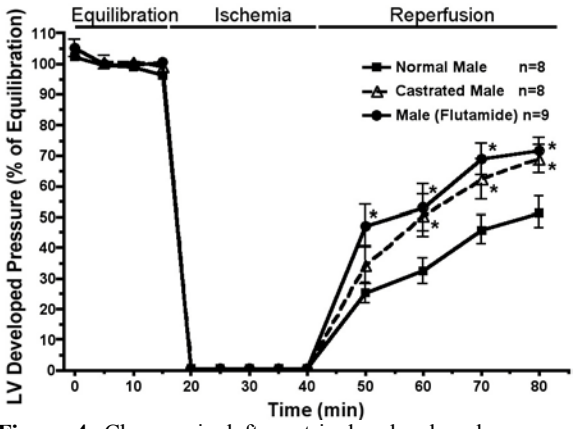

Figure 4. Changes in left ventricular developed pressure (LVDP) following ischemia and reperfusion in normal male, castrated male and male with androgen receptor blocker rat hearts. Results are mean +/- SEM, *p<0.01 vs. normal male at the corresponding time points).

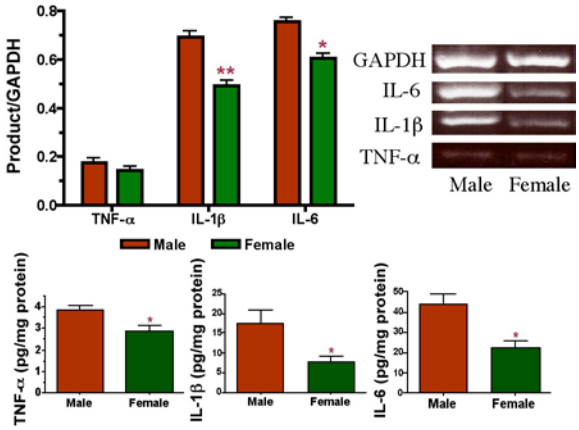

Figure 5. Cardiac TNF-α, IL-1β and IL-6 in normal age matched male and female rat hearts after I/R. Right one in the top shows relative optical density of target PCR signals, normalized to GAPDH PCR signal. Left one is representative gel photograph. Protein levels are shown in the bottom (Mean +/- SEM, n=5/group. *p<0.05, ** p<0.01 vs. male).

ovariectomized females had phosphorylated p38 MAPK equivalent to males and that treatment of males and ovariectomized females with estradiol prevented the increase in phosphorylated p38 MAPK caused by I/R. This suggests that the decreased cytokine production and cardioprotection caused by estrogen might be mediated through p38 MAPK. Indeed, our data indicates decreased p38 MAPK activation in females following ischemia (Figure 6).

Chaudry's group (155) have presented interesting findings on p38 MAPK activation after trauma-hemorrhage. They showed that sex, after trauma-hemorrhage, did not alter the expression of the nonphosphorylated p38 MAPK in macrophages but altered the expression of phosphorylated p38 MAPK. Macrophages from female shams had increased phosphorylated p38 MAPK compared with males. Trauma-hemorrhage increased phosporylated p38 MAPK in males but decreased it in females. They also showed that castration attenuated the increase in phosphorylated p38 MAPK caused by trauma-hemorrhage and that supplementation with 5alpha Dihydrotestosterone restored the ability of trauma-hemorrhage to activate p38 MAPK. This suggests that testosterone leads to p38 MAPK activation after trauma-hemorrhage. The significance of the gender difference in p38 MAPK in shams is not known and it will be interesting to see if it is valid in humans and other animals and whether it leads to differences in cytokine production. Both studies show higher p38 MAPK activity after acute injury in males suggesting that acute injuries lead to activation of similar pathways and p38 MAPK is involved in them (154, 155). Previous studies indicate that after trauma-hemorrhage there is a decreased capability of macrophages to release cytokines and that this is more severe in males. This raises the question as to whether increased p38 MAPK activation in males is the cause for the immune depression and if it is why does it lead to immune depression instead of activation. A hypothesis proposed by Chaudry's group (155) that integrates all the evidence is that the increased p38 MAPK activation leads to such an increase in the inflammatory cytokine response that it leads to an exhaustion of the capacity of the cells to respond to any further stimulus. These studies have shown that estradiol and testosterone modify p38 MAPK and that this may be responsible for gender differences in response to acute injury though further studies are needed to clarify the issue.

As oxidant stress is one of the stimuli for inflammatory cytokine production, any gender difference in antioxidants could lead to differences in the inflammatory response to acute injury. The sudden increase in production of hydroxyl radicals that occurs during reperfusion was reduced by estrogen in a canine model of cardiac ischemia and reperfusion (156). Using a similar I/R model it was found that estrogen decreased lipid peroxidation and in an in-vitro study estrogen decreased superoxide anion production from coronary artery segments undergoing hypoxia/reoxygenation (81). A possible mechanism for the antioxidant effect of estrogen is increased GSH (reduced glutathione) in the myocardium. This was supported by using a GSH synthesis antagonist, which reduced GSH and partially reversed the beneficial effects of estrogen on left ventricular diastolic pressure, systolic shortening and lipid peroxidation after LAD I/R (157). As there was only a partial reversal by using a GSH synthesis antagonist other mechanisms must also play a role in the protective effects of estrogen.

Another possible antioxidant mechanism is increased levels of superoxide dismutase in females. Barp and colleagues (158) showed decreased lipid peroxidation and increased superoxide dismutase in female hearts compared with males. Ovariectomized females had significant decrease in superoxide dismutase and an increase in lipid peroxidation. However, this study did not induce acute injury and hence the role of these changes in the protective effects seen in females after acute injury is

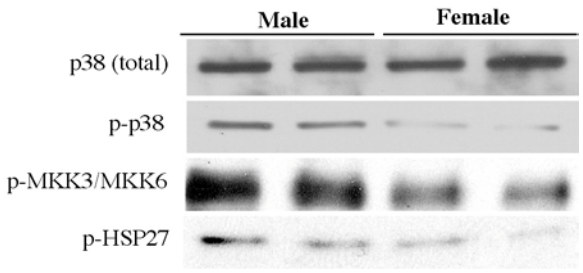

Figure 6. The expression of activated p38 MAPK signaling pathway was increased after I/R in male rat hearts compared to females. Shown are representative immunoblots. n=4/group.

unknown. Oxygen radicals have also been shown to upregulate expression of adhesion molecules. Squadrito and colleagues (159) showed that estrogen decreased serum and macrophage TNF levels and decreased ICAM-1 expression in the myocardium after left main coronary artery I/R. This led to decreased leukocyte accumulation and smaller infarcts in the estradiol treated group. Other studies have also shown that estrogen decreases neutrophil accumulation after LAD I/R (76, 77). The mechanism by which estrogen decreases the leukocyte accumulation is not well defined though there is evidence for a role of antioxidant mechanisms, nitric oxide (NO) and decreased TNF production. Many physiological and pharmacological actions have been attributed to NO and several of these are cardio-protective. The protective effects appear dependent on endothelial NO synthase (eNOS) (160) and inducible NO synthase (iNOS) (161). Estrogen increases NO production through increased expression of iNOS and eNOS in cardiomyocytes (78, 162-165). NO provides protection by reducing the expression of adhesion molecules, especially P selectin, and decreasing neutrophil accumulation (166, 167). Increased infarct size occurred when a NOS inhibitor was used (168) NO also modulates calcium channels and affects myocardial contractility.

Cardiac contraction is triggered by calcium release through L-type calcium channels and sarcoplasmic reticulum (SR). During relaxation sodium-calcium exchanger (NCX) and sarcoplasmic reticulum calcium ATPase (SRCA) act to remove intracellular free calcium. Increase in intracellular calcium occurs with myocardial ischemia and inhibition of this is protective (169, 170). NO decreases free intracellular calcium by inhibiting L-type calcium channels (171, 172) and inhibiting the calcium release from SR (173-175). The inhibition of calcium release from SR may be mediated by inactivation of ryanodine receptor calcium release channel by NO (174). NO may also modulate calcium by activating or potentiating the effects of ATP sensitive potassium (K_{ATP}) channels (176, 177). Thus, estrogen leads to increased production of NO and through it decreases neutrophil accumulation and free intracellular calcium and provides cardioprotection.

The sympathetic stimulation that occurs after acute injury acts through beta adrenoreceptors to increase cAMP and cAMP-dependent protein kinase A. This kinase phosphorylates many effector proteins, one of them being Phopholamban (PLB). PLB is a sarcoplasmic reticulum (SR) protein that when phosphorylated dissociates from sarcoplasmic reticulum calcium ATPase (SRCA) and increases the affinity of SRCA for calcium (178). This leads to increased SR calcium, increased SR calcium release and increased myocardial contractility (179-181). Golden and coworkers (182) have shown that protein kinase A also increases expression of NCX. Thus, betaadrenergic stimulation not only increases calcium release from SR but also increases NCX expression. These pathways lead to increased intracellular calcium and increased myocardial contractility but after acute injury these same pathway lead to increased damage.

Estrogen and progesterone depletion causes upregulation of $beta_1$ adrenergic receptors in the heart and this was reversed by supplementation of either estrogen or progesterone (183). Estrogen deficiency led to increased density of beta adrenoreceptors in the heart though the affinity remained the same. Isoproterenol (selective beta adrenoreceptor agonist) increases infarct size in isolated hearts undergoing I/R but estrogen protects against the injury produced by it (184). The increased damage caused by beta adrenoreceptors is probably due to increased accumulation of intracellular calcium after injury. Using Phospholamban-knockout mice, Cross and colleagues (185), found that the knockout mice had greater baseline myocardial contractility but worse myocardial recovery after I/R. This study confirmed the fact that increased calcium through SRCA leads to increased baseline myocardial contractility but it also leads to increased damage after acute injury. They also found that female knockout mice recovered better from I/R compared to male knockout mice. Use of L-NAME (a NOS inhibitor) blocked the protective effects in females while giving SNAP (a NO donor) to males provided protection equivalent to female knockouts. In another study, when isolated hearts were treated with Isoproterenol or high Ca^{2+} increased I/R injury was found in males (186). Females were protected against this injury but lost the protection when treated with L-NAME. The probable mechanism by which NO provides this protection is by inhibiting the ryanodine receptor calcium release channel and thereby inhibiting calcium release from cardiac sarcoplasmic reticulum.

Castration causes a reduction in the myocardial expression of beta adrenergic receptor, L-type calcium channel and sodium-calcium exchanger (NCX) and supplementation with androgens reversed these effects (187). This led to reduced contractility in cardiomyocytes from castrated males. Indeed, our recent studies demonstrated that endogenous testosterone contributes to the increased myocardial TNF and IL-1, and activation of p38 MAPK and caspase 3 (Figures 7-9). The impact of these changes on myocardial damage, after acute injury, has not been studied but it can be postulated that the increased calcium influx produced by them would lead to increased myocardial injury.

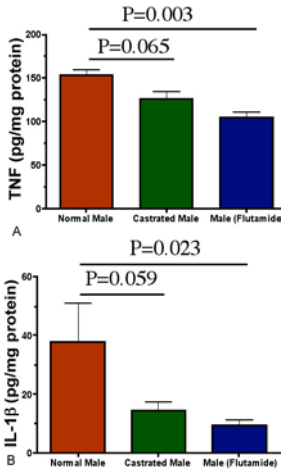

Figure 7. The expression of myocardial TNF and IL-1β following ischemia and reperfusion in normal males, castrated males and males with testosterone receptor blockade. TNF and IL-1β protein levels are lower in castrated males and males with testosterone receptor blockade than in normal males (n=5-7/group).

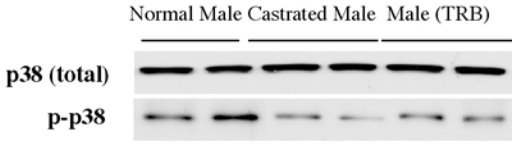

Figure 8. Myocardial p38 MAPK activation following acute ischemia and reperfusion in normal males, castrated males and males with testosterone receptor blockade. More activation of p38 MAPK exists in normal male rat hearts compare to castrated males and males with testosterone receptor blockade (n=4/group).

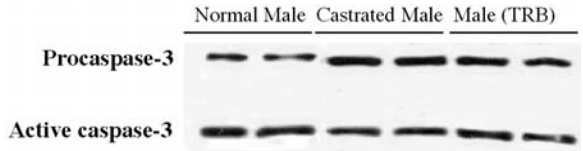

Figure 9. Myocardial caspase-3 after acute ischemia and reperfusion in normal males, castrated males and males with testosterone receptor blockade. More active caspase-3 and less procaspase-3 in normal male rat hearts was observed compared to castrated males and males with testosterone receptor blockade (n=4/group).

Horton and colleagues (85) found that the cardiomyocyte secretion of inflammatory cytokines after burns by diestrous rats was similar to males but the sodium/calcium (Na^+/Ca^{2+}) accumulation was significantly less and diestrous rats had better cardiac function. This suggested that differences in Na^+/Ca^{2+} accumulation are instrumental in mediating sex differences in cardiac function after burns. Sodium – calcium exchanger (NCX) is present on the sarcolemmal membrane and countertransports three Na^+ ions for one Ca^{2+} ion. NCX can function in either direction depending on the transmembrane gradients of the ions and the membrane potential. A role for this protein in myocardial injury was suspected because manipulations of intracellular sodium led to corresponding changes in intracellular calcium and that affected the myocardial injury produced (188, 189). NCX normally works in the calcium removal mode but in ischemia there is an increase in intracellular Na^+ and change in the membrane potential, which leads to reversing of the NCX (190). Isolated hearts of transgenic male mice overexpressing NCX had greater I/R injury than wild type mice (191). Female transgenic mice were protected from the increased I/R injury compared to male transgenics and this protection was lost partially when female transgenic mice were ovariectomized. The mechanism of the protective effect in these transgenic females is unknown as male and female transgenic hearts did not have a difference in the overexpression of NCX. The possible mechanisms are that females are better able to withstand the higher intracellular calcium produced by NCX overexpression or that even with similar NCX overexpression females have lower intracellular calcium due to lower intracellular sodium. Evidence in support of the latter has been provided by Sugishita and colleagues (192). They studied myocytes isolated from NCX overexpressing mice and found lower intracellular calcium after metabolic inhibition in females. They also found lower intracellular sodium in females suggesting that the decreased calcium in females might be due to this. Due to the stoichiometry of NCX, exchanges 3 sodium ions for 1 calcium ion, even small differences in intracellular sodium would lead to larger differences in calcium. This suggests that females may be protected due to lower intracellular sodium after acute injury though the reason for the lower intracellular sodium in females remains unanswered.

ATP sensitive potassium (K_{ATP}) channels have been shown to be present in cardiac mitochondria and to mediate protection against ischemic injury. These channels are activated by many stimuli including adenosine (193), NO (177) and free radicals and these then activate Protein Kinase C (PKC) which links to mitochondrial K_{ATP} channels (194, 195). Mitochondrial calcium overload plays an important part in I/R injury (196, 197). Opening of mitochondrial K_{ATP} channels results in potassium influx, which decreases the driving force for calcium uptake (198). Use of K_{ATP} channel agonists has shown the decreased mitochondrial calcium concentration caused by opening of K_{ATP} channels (199). Estrogen has been shown to decrease infarct size in canine LAD I/R through mitochondrial K_{ATP} channels (200) and decrease reperfusion-induced arrhythmias through sarcolemmal K_{ATP} channels (201). The mechanism by which estrogen activates these channels is unknown. The evidence that estrogen increases NO production and that NO can activate these channels indicates that this might be a possible mediator. Estrogen has been used in coronary angioplasty patients and has been shown to reduce myocardial ischemia caused by balloon inflation, possibly through K_{ATP} channels (202).

Apoptosis is a genetically controlled process by which cells undergo nonnecrotic cellular death. Testosterone has been shown to promote apoptosis in vascular endothelial cells (203) and renal tubular cells (204) while anabolic-androgenic steroids do so in

cardiomyocytes (205). Verzola and colleagues (204) showed a dose dependent effect of testosterone on apoptosis in renal tubular cells. They also showed that testosterone upregulated Fas, Fas ligand and Fas associated death domain. The use of caspase-3 inhibitor, caspase-8 inhibitor or caspase-9 inhibitor reduced the apoptosis produced by testosterone. Testosterone was shown to decrease Bcl-2 expression (203). These studies indicate the possible role of testosterone in promoting apoptosis though further research is needed to delineate the mechanisms.

Estrogen has been shown to decrease apoptosis of cardiac myocytes induced by staurosporine and this was associated with decreased caspase 3 activity and decreased NFkB (206). Wang and colleagues (154) showed that females had lower active caspase 3, and active caspase 8 but higher Bcl-2 after global I/R, in isolated hearts. They also showed that ovariectomized females had increased active caspase-3 while estradiol administration to males and ovariectomized females reduced the level of active caspase-3. Camper-Kirby and colleagues (207) found that females have higher nuclear localization of phospho-Akt in myocardium. Akt kinase activity in female nuclei was higher and they had higher phospho-forkhead levels (which is a downstream target of Akt). Administration of estradiol increased nuclear localization of phopho-Akt. Akt has previously been shown to inhibit apoptosis in cardiomyocytes in I/R (208, 209). It has also been shown to mediate the anti-apoptotic effects of insulin growth factor-1 (IGF-1) (210). The possible mechanisms of the antiapoptotic effects of Akt are phosphorylation of BAD, (211) phosphorylation of caspase 9 (212) and phosphorylation of FKHRL1 which leads to blocking of Fas ligand expression (213). This suggests that Akt might be a mediator for the anti-apoptotic effect of estrogen after acute injury.

TNF is also known to produce apoptosis. TNF induces apoptosis by binding to either TNF type 1 receptor or Fas (5, 124). Both are associated with death domains, TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD). These death domains interact with receptor-interaction protein and activate endonucleases. The endonucleases destroy the nuclear DNA leading to apoptosis. As stated earlier, estrogen decreases TNF production after acute injury and hence may decrease the apoptosis produced by TNF.

6. ESTROGEN AND TESTOSTERONE

In recent studies (1-3) we have provided evidence that inherent gender differences affect the otherwise normal myocardial response to acute ischemic injury. These results suggest that differences between the female and male response to I/R are related to the correlation between myocardial function and inflammatory signaling. Understanding the mechanisms that lead to these differences may allow low side effect therapeutics. The main findings of those studies were that after I/R, compared to normal males, castrated males and males treated with testosterone receptor blockade (TRB=flutamide): 1) exhibited cardiac functional protection; 2) had decreased proinflammatory cytokine production (TNF-α, IL-1β and IL-6), and active p38 MAPK and caspase-1; 3) had decreased expression of apoptotic-related proteins caspase-3 and caspase-11; and 4) had increased expression of anti-apoptotic protein Bcl-2.

Sex differences exist in the response of myocardium to acute injury (85, 227, 228). Most studies have focused on the role of estrogen in cardiac protection, whereas little data exists regarding the effect of testosterone on cardiovascular disease. The role of testosterone on cardiac injury may be important because the heart can accumulate testosterone at higher concentrations than other androgen target organs (229), and functional androgen receptors are present in isolated cardiac myocytes (230). Furthermore, testosterone can modulate nuclear transcription by membrane receptor second messenger cascades (231); therefore, testosterone may be involved in mediating L-type calcium channel activity, coronary vasomotion by NO-dependent (232) and NO-independent mechanisms (233), and apoptotic cell death of cardiomyocytes (205, 234). In this study, rat hearts from castrated males and flutamide-treated males exhibited better functional recovery after acute I/R compared to normal males. This observation is consistent with other reports of protected cardiac performance in animals with testosterone depletion or testosterone receptor blockade after trauma-hemorrhage (93, 235), and suggests that endogenous testosterone may have a negative effect on the heart subjected to acute I/R.

Accumulating evidence indicates that cytokines are important mediators of injury-induced cardiovascular dysfunction. Acute injury in the form of ischemia, endotoxemia, or burn trauma results in myocardial functional suppression, in part via the local production of inflammatory mediators such as TNF and IL-1. Ischemia-reperfusion injury induces the local production of TNF, IL-1, and IL-6 (5, 214, 215). Indeed, locally produced proinflammatory mediators may be important contributors to postischemic myocardial dysfunction and cardiomyocyte apoptosis. p38 MAPK has been shown to be an important mediator of TNF production. In our study, castrated males and TRB-treated males, both of which had improved post-ischemic functional recovery compared to normal males, also had less myocardial TNF, IL-1 and Il-6 production, and decreased p38 MAPK activation.

It remains unclear how I/R induces myocardial inflammatory cytokine production. One possible mechanism may involve myocardial p38 MAPK signaling. Stimuli such as ischemia-reperfusion, oxidant stress, and hydrogen peroxide directly activate p38 MAPK (5). Activation of p38 MAPK is required for TNF and IL-1 production in cardiomyocytes (220, 225, 226). TNF production following I/R is dependent on NFκB translocation, which may occur via p38 MAPK activation, and regulation of this process occurs pretranscriptionally (5). On the other hand, caspase-1 and caspase-11 are shown to function upstream of IL-1 maturation (92). Caspase-1 is recognized as the IL-1-converting enzyme

(ICE) for cleavage of IL-1 precursor to the mature form (236, 237). Precursor caspase-1 results in the generation of mature p10/p20 and p10/p20 form ICE (238). ICE is required for IL-1 activation in the postischemic heart (239). Furthermore, the activation of caspase-1 is dependent on caspase-11 (240). Caspase-11 is thought to activate downstream signals caspase-1 and caspase-3, and thus, it may be important in both inflammation and apoptosis (241). It has been demonstrated that p38 MAPK mediates caspase-11 induction in microglia subjected to hypoxia and endotoxin exposure (150, 151). In our study, we observed increased procaspase-1, but decreased caspase-1 p20 and caspase-11 in castrated males and TRB-treated males compared to untreated males.

Myocardial apoptosis is responsible for the loss of cardiomyocytes and depression of myocardial function following I/R. Myocyte apoptosis in heart failure is reduced in women compared to men (242). Apoptosis may be mediated by either the extrinsic death receptor signaling pathway or the intrinsic mitochondrial control pathway (243). Caspases play a crucial role in each of these pathways. The activation of the intrinsic apoptotic pathway (the release of cytochrome *c* and the activation of caspase-9) during reperfusion, but not ischemia, has been observed in chick cardiomyocytes (244), and activation of caspase-8 and caspase-3 occurs in response to hypoxia or ischemia (245). However, no information exists regarding the influence of testosterone on myocardial proapoptotic signaling cascades following acute I/R. In our study, we observed decreased activation of pro-apoptotic signaling cascade (caspase-3, caspase-11) in castrated males and TRB-treated males compared to untreated male hearts following I/R, whereas anti-apoptotic Bcl-2 was increased in the latter group. Clinically, anabolic androgenic steroid abuse has been associated with myocardial ischemia and sudden cardiac death (246). Some studies have demonstrated that anabolic androgenic steroids induce injury and apoptosis in myocardial cells (205, 234) and skeletal muscle cells in culture (247). Our observations of decreased apoptotic signaling with testosterone depletion or testosterone receptor blockade are consistent with these studies.

One of the first large-scale studies of the cardioprotective role of estrogen investigated the ability of hormone replacement therapy to prevent secondary coronary events. The Heart and Estrogen/progestin Replacement Study (248) (HERS) was a randomized trial involving nearly 3,000 postmenopausal women under eighty years of age (mean of 66.7 years) who had a history of coronary artery disease. The women were treated with either placebo or combination hormone therapy (0.625 mg conjugated estrogen and 2.5 mg medroxyprogesterone daily). Interestingly, no significant differences in primary outcome (CVD death or nonfatal myocardial infarction) or secondary outcome (stroke, peripheral arterial disease, congestive heart failure, resuscitated arrest, unstable angina, or resuscitated arrest) were noted between the hormone treated groups and those treated with placebo after a four-year follow-up, despite a favorable effect of the hormone therapy on the lipid profile. It was found, however, that more women in the hormone-treated group suffered coronary events during the first year of treatment than those taking the placebo. Women in the hormone treated group also experienced more thrombosis events such as pulmonary embolism. During years four and five of the study, however, hormone-treated women had fewer cardiovascular events. DM Herrington, *et al* (249) also investigated the ability of HRT (combination as well as unopposed estrogen) to provide secondary protection against cardiovascular (CV) events by administering unopposed, conjugated estrogen, estrogen and medroxyprogesterone, or placebo to postmenopausal women with coronary disease. It was concluded that HRT administration did not prevent the progression of atherosclerotic plaques, despite, once again, a favorable effect on the lipid profile. The Estrogen in the Prevention of Atherosclerosis Trial (250) was organized to assess estrogen's ability to attenuate the progression of subclinical atherosclerotic plaques, and the protocol included post-menopausal women with no history of CVD receiving unopposed doses of beta-estradiol to ensure no interference from progesterone. This trial concluded that unopposed estradiol treatment can slow the progression of subclinical atherosclerosis to much the same extent as traditional lipid-lowering therapy (250).

Other studies, along with HERS, investigating estrogen's preventative effects have also written of a seemingly higher "early-harm" risk of cardiovascular events during the first year of treatment in post-menopausal women taking hormone replacement therapy (44). These studies included not only secondary prevention studies, but primary prevention studies as well. One such primary prevention study is the Women's Health Initiative (224), which administered combination hormone therapy to healthy postmenopausal women. It was found (and the trial ceased) at the five-year follow-up due to a higher occurrence of stroke, cardiac events, thromboembolic events, and invasive breast cancer among those on a hormone regimen. It was determined by this trial that HRT should not be used as a primary preventative measure for cardiovascular disease. The Nurses Health Study (251), as well as observational studies that show evidence of early-harm for women who have prior CVD and are beginning hormone regimens (252, 253), supported the findings of HERS (254). Studies that were administering estrogen to men with coronary artery disease also were stopped early for safety consideration because those receiving hormone therapy were suffering more cardiac events than the control group (255). It is expected that this high-risk period for HRT use (the first year of therapy) is so pronounced because it is during this time that the events occur in those with pre-existing risk factors, such as those with a tendency to develop thromboemboli (252, 254). Several studies have documented that the net effect of estrogen on the coagulation system is procoagulant (249, 256). Another possible explanation for this finding could be that progestin interferes with some of the acute beneficial effects of estrogen on vasoreactivity (257-259). Cannon, *et al* (260), found that estrogen administration tends to increase levels of matrix metalloproteinase-9, which is critical to T-cell migration and tends to promote lesion rupture. This could

imply that although the long-term increase in MMP-9 may be able to prevent the aggregation of matrix proteins, this acute effect of estrogen may put women with vulnerable plaques at risk during early use (260).

All that can be derived from these studies at this point is that although estrogen therapy may possibly be able to play a role in the prevention of the formation of the plaques associated with atherosclerosis or to slow progression during very early stages of the disease, HRT cannot repair damage that is already done and is not as effective at slowing progression at later stages of the disease. Therefore, the timing of the initiation of hormone treatment relative to age and menopause may be a crucial point to consider (261) There are a couple of possible explanations for this observation (261). First, early-stage atherosclerotic lesions are composed of less dense fibrous tissue than the later-stage lesions (262),and aging can decrease the expression of the estrogen α-receptor, making the estrogen less able to exert its effects (263). Overall, more clinical studies are needed to adequately assess both the primary and secondary protective capabilities of estrogen. Special attention should be given to the nature and health status of the study participants, which form of HRT (combination or estrogen) and dosage that is given, and to the mode of administration (oral or transdermal), as these are often linked to differences in the beneficial or detrimental effects of estrogen therapy (254).

It is well-established that estrogen has favorable effects on some of the major risk factors for the development of atherosclerosis and therefore CVD. Although there is some concern as to estrogen's tendency to significantly increase plasma triglyceride levels (264), estrogen therapy has also been shown to reduce levels of low-density lipoprotein (LDL) and increase levels of high-density lipoprotein (HDL) in postmenopausal women. This restores the lipid profile back to one that resembles the profile of premenopausal women, who are at lower risk for CVD (265). Estrogen administration has also been shown to decrease levels of collagen production in rabbit aortas, which has implications in the formation of advanced atherosclerotic plaques (266). Additionally, Godsland and coworkers (267) demonstrated the estrogen may have a favorable effect on insulin resistance. Estrogen has also been targeted as having a role in the change in fat distribution that many women experience after menopause. Menopause usually results in an abdominal concentration of body fat (as opposed to peripheral), which is a risk factor for cardiovascular disease. HRT can prevent increases in abdominal fat and distribute it elsewhere, therefore having a favorable effect on cardiovascular risk (268). It is, however, estimated that the above-mentioned effects account for only about 30 to 50 percent of estrogen's cardioprotective effects (269). There has been intense investigation into the other possibilities for estrogen's effects. Postmenopausal women experience changes in the levels of many factors involved with hemostasis that put them at higher risk for thrombosis. Therefore much investigation has been directed at dertermining estrogen's effects on coagulation factors (232). Endothelial function and how estrogen may alter vasoreactivity have also been investigated (232), as have the hormone's effects on inflammatory markers such as the cytokines, C-reactive protein, and the adhesion molecules (270).

Several studies are also investigating the effects of tamoxifen, an estrogen receptor modulator used to treat breast cancer (271), and raloxifene, an estrogen receptor modulator used in the treatment of osteroporosis (272). Tamoxifen, although it acts as an estrogen antagonist in the breast (273), often mimics that actions of estrogen in tissues other than the breast, and has also been shown to have favorable effects on lipid profiles (274). Raloxifene, although it also demonstrates an ability to lower low-density lipoprotein levels (275), does not demonstrate estrogen-like effects in the uterus or breast (272, 276).

Much research attention has been given to the effects of estrogen and hormone replacement therapy on blood coagulation and the formation of emboli, as these are major risk factors for cardiovascular disease and myocardial infarction (277), and it has been demonstrated that levels of several coagulation factors such as factors VII and VIII as well as fibrinogen increase after menopause (278-282). Much of the attention given to hormone therapy's impact on coagulation factors originated from the aftermath of the HERS study, during which the women in the hormone-treated group suffered more thromboembolic events (248, 283). Additionally, tamoxifen treatments have been linked to higher rates of thromboembolic events in women (271). Studies have produced conflicting conclusions concerning what effect estrogen has on coagulation and thrombosis, as hormone therapy effects the various fibrinolytic components differently (284) Because pro-inflammatory effects of estrogen have been found with D-dimer, metalloproteinase 9, and Factor VII, whereas anti-inflammatory effects have been found with fibrinogen, endothelial adhesion molecules, and plasminogen activator inhibitor-1, it is difficult to determine which effect will prevail (260, 285, 286). It is therefore challenging to assess whether estrogen's effects on the coagulation pathway have the power to explain some of estrogen's proposed cardioprotective effects. Much of the concern that estrogens and hormone replacement therapy may heighten risk for thromboembolism stems from the available data on the use of oral contraceptives and their tendency to increase such risk (287). Indeed, several studies have concluded that the use of oral contraceptives with high doses of estrogen is associated with a higher rate of thromboemboli formation (288). However, estrogens in hormone replacement therapy (HRT) have different effects on the separate components of the coagulation system, so many studies have assessed the effects of HRT on factors such as the level of fibrinolysis, the activation of coagulation, levels of plasminogen activator, and levels of plasminogen-activator inhibitor type 1 (287).

It has been shown that estrogen may exert anti-coagulant effects by interfering with platelet aggregation (287). After a blood vessel wall is injured, collagen fibers allow for platelets to adhere to the injury site, and the platelets simultaneously produce several compounds

involved with coagulation including a prostaglandin, thromboxane A2, which allows platelets to adhere to one another and acts as a potent vessel constrictor. Thromboxane A2 is broken down to thromboxane B2, which serves as an indicator of platelet activity (287). Although it has been shown that pre-menopausal women produce significantly less thromboxane B2 than age-matched men, women show a linear increase in the level of the prostaglandin during the post menopausal years whereas such an increase has not been demonstrated in men (287). This could partially explain the increased incidence of atherosclerosis and associated cardiovascular disease in post-menopausal women in comparison to pre-menopausal women. It was demonstrated that lipopolysaccharide-induced thromboxane B2 levels in post-menopausal women were nearly restored to the levels seen in pre-menopausal women (levels reduced by 35%) after one year of treatment with HRT for both orally and transdermally administered estrogen, suggesting that HRT could possibly decrease platelet reactivity (289). The same study cited significantly reduced levels of tissue factor (TF) expression and decreased tumor necrosis factor release in response to HRT. Reduced levels of tissue factor lessen the ability of factor VIIa to bind to its receptor, a key step in the initiation of coagulation (289).

Nozaki, *et al* (277) also conducted a study on the effects of HRT on fibrinolytic components by administering unopposed conjugated equine estrogen (CEE) to women who had undergone hysterectomy and oophorectomy, combination hormone therapy (CEE and MPA) to a group of postmenopausal women, and a placebo to a control group. Levels of various fibrinolytic components (Factor VII, protein C, fibrinogen level, antithrombin III, plasminogen activator inhibitor-1 (PAI-1), and tissue type plasminogen) were measured at 1, 3, and 6 months of treatment—during the period of time when women are thought to be at the greatest risk for a thromboembolic event after starting hormone therapy. Whereas antithrombin III and tissue-type plasminogen levels remained stable throughout treatment, protein C activity increased roughly 10 percent in each treatment group, fibrinogen levels dropped nearly seven percent, and the levels of PAI-1 decreased by nearly sixteen percent. In both the cases of decreased levels, the unopposed CEE treatment group saw a slightly greater result than the combination therapy group, whereas the combination group saw the greater increase in protein C activity. The activity of factor VII increased by ten percent in the CEE group only. They conclude that with the exception of the raise in the factor VII levels among the CEE treated patients, HRT has no adverse effects on coagulation and fibrinolysis (277).

Further studies involving plasminogen activator inhibitor-1 have shown that postmenopausal women tend to have a higher level of PAI-1, a potent inhibitor of fibrinolysis, than premenopausal women, possibly putting them at higher risk for thromboemboli or cardiovascular events (290). Koh, *et al* (286), studied the effects of combination and unopposed estradiol therapy (orally and transdermally administered) on the levels of PAI-1 and on the levels of D-dimer (a by-product of fibrinolysis). D-dimer levels increased proportionately with the decreasing PAI-I levels. Orally-administered estradiol and estradiol combined with progestin led to a reduction in PAI-1 levels by approximately 50% as opposed to the transdemally-administered therapy, which showed no significant effect. This agrees with the findings of Kroon (291) and Gilbert (292) showing that passage of the hormone through the liver has an impact on estrogen's effect on PAI-1. In fact, it has been suggested that the liver, along with the endothelium, is a main source of PAI-1 (293). Van Kesteren *et al* (294) found that the adminstration of ethinylestradiol (100 μg/day) in combination with an anti-androgen compound to male→female transsexuals decreased both tissue plasminogen and PAI-1 levels. Sobel, *et al* (295) demonstrated that 17beta-estradiol affected plasminogen activator (tPA), which is necessary for fibrinolysis, in a biphasic dose-dependent manner using bovine aortic endothelial cells. Increased secretion of tPA from the endothelial cells was seen at lower, physiological concentrations of the hormone, whereas decreased levels of tPA were seen at higher concentrations of the hormones. The most pronounced stimulatory effect was seen at 10^{-12} mol/L estradiol. A similar, reciprocal pattern was seen with estrogen's effect on the presence of PAI-1 antigen. Inhibition was seen at 10^{-12} mol/L estrogen, whereas stimulation was seen at 10^{-7} mol/L.Similar results were seen with progesterone. Because fibrinolysis is a mechanism balanced by the interaction of tPA and PAI-1, and because studies have shown that physiological levels of estradiol tend to decrease endothelial PAI-1 levels and increase tPA levels (290), these results support that restoring estrogen to normal physiological levels after menopause may be beneficial in the potentiation of fibrinolysis.

Contrary to what was laid out above about estrogen's potential effects on fibrinolysis, many studies have demonstrated that estrogen activates the coagulation system (278). Aside from the data that state oral contraceptives can increase the risk for thromboembolic events (279, 288) and the knowledge that HERS participants treated with hormones showed a higher incidence of thrombosis events, Caine *et al* (296) administered 0.625 mg or 1.25 mg CEE to healthy postmenopausal women and noted a dose-dependent increase in thrombin generation and fibrinopeptide A. Levels of antithrombin and protein S, inhibitors of thrombin activation and formation, respectively, were also shown to decrease. Scarabin *et al* (297) found that a regimen of 2mg oral estrogen with cyclic progesterone increased levels of prothrombin fragments 1 and 2 and decreased antithrombin activity in healthy postmenopausal women, whereas no result was seen with the transdermal regimen. Current evidence indicates that while estrogen therapy seems to have a favorable effect on fibrinolysis, it has a simultaneous unfavorable effect on the activation of coagulation. This led Koh *et al* (278) to wonder if the pro-fibrinolytic effects of estrogen were a secondary response to the coagulation activation induced by estrogen. 0.625mg of CEE was administered to nine postmenopausal women. After one month, eight women had lower PAI-1 activity

and seven had marginally greater t-PA activity. The ratio of t-PA to PAI-1 activity, as a measure of fibrinolytic potential (298), increased six to 650 percent in all nine women, whereas no significant change for the group was seen in coagulation activation markers prothrombin fragments 1 and 2 levels or in levels of thrombin-antithrombin. This study, although small, suggests that coagulation activation by estrogen is not necessary for the increased potentiation of fibrinolysis (278).

Once the monocytes have migrated into the vessel wall and converted to tissue macrophages, they, along with the platelets, smooth muscle cells, endothelial cells, and lymphocytes, are capable of producing a wide variety of molecules such as cytokines and growth factors that aid in the progression of atherosclerotic lesions as they act in a paracrine, juxtacrine, and autocrine manner and disrupt endothelial function (299). These growth factors and the cytokines play central roles in the development of atherosclerosis, as they are involved in nearly every aspect of the inflammatory process; they are often the stimulators of cell proliferation, migration, adhesion, and regulators of vasomotor tone (300). The interactions that estrogen has with these molecules therefore may provide insight into estrogen's cardioprotective effects. Although there is no definitive statement as to what effect estrogen has on the cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), estrogen receptors have been found on human monocytes, suggesting that estrogen may modulate cytokine release (301).

A link between estrogen levels and IL-1 levels was discovered by Cannon and Dinarello (302) who found that IL-1 activity was increased in women immediately post-ovulation. However, it was then suggested that conjugated estrogens had no effect on IL-1 release in bone marrow aspirates (303). Patients who had undergone a complete hysterectomy showed higher levels of IL-1 than other postmenopausal women who were on a hormone replacement regimen (304). This decrease in IL-1 expression is apparently mediated by estrogen at the transcriptional level, as lower levels of IL-1 mRNA were found in human monocytes and peritoneal macrophages (305). IL-6 protein and its transcripts have been identified in atherosclerotic lesions as well, and it has been shown that IL-6 can induce monocyte chemotactic protein-1 in mononuclear cells (306). It was demonstrated that administration of 17beta- estradiol to ex-vivo aortic tissue of male apolipoprotein-E knockout mice reduced IL-6 secretion by 50 percent (307). Although possible, it is unlikely that the lower levels of testosterone were responsible for the decrease in IL-6 secretion. This suggests that the suppression of IL-6 secretion from the macrophages may serve to protect by inhibiting autocrine stimulation of chemokines involved in cell recruitment (307).

Upon investigating the effects of estriol on the transmigratory behavior and cytokine profile of autoreactive T-cells, YC Zang and colleagues (308) found that estradiol can inhibit T cell transmigration at an estradiol level mimicking pregnancy. Estriol also upregulated the T cell secretion of IL-10 and decreased secretion of TNF in a non antigen-dependent manner. It was also shown that estriol can inhibit nuclear transcription factor kappa B (NF-kappaB) (308), which is typically activated by injury and the associated cytokines (300). Because NF-kappa B regulates a number of genes involved in the immune response, it may be through NF-kappa B that estrogen exerts some of its effects on the markers of inflammation. For example, Selzman, *et al* (309) demonstrated that the mitogenic effect of TNF on human vascular smooth muscle cells is dependent on NF kappaB activation, as the administration of an NF kappaB inhibitor led to a decrease in vascular cell proliferation and a decrease in the levels of IL-6 as well. Zang, *et al* (308) additionally noted a decreased level of MMP expression in T-cells treated with estriol; however, several other studies have found HRT to be associated with higher MMP-9 levels. Hormone replacement therapy was found to be associated with higher levels of MMP-9, which digests the matrix and makes lesions more prone to rupture (310), in a study by Cannon, *et al* (311). Zanger, *et al* (260) also reported that women on hormone replacement therapy tended to have higher levels of serum MMP-9 than those women taking a placebo.

Aune and coworkers (289) found that after twelve months of HRT, the levels of TNF produced by lipopolysaccharide-stimulated macrophages had decreased significantly in both patients receiving estrogen orally and those receiving treatment transdermally. Pacifici, *et al* (304) determined that the spontaneous macrophage secretion of TNF and IL-1was decreased in women taking hormone therapy in comparison to postmenopausal women not on a regimen. Other studies, such as that conducted by Stock, *et al* (281), found that estrogen had little or no effect on spontaneous macrophage IL-1 secretion. Zhang and coworkers (312) additionally found that pre-incubating murine bone marrow-derived macrophages in17beta estradiol significantly inhibited lipopolysaccharide-induced TNF release. Kamada *et al* (313) studied the levels of fifteen cytokines in postmenopausal women on HRT and those who were not on a regimen. Although higher levels of TNF and lower levels of transforming growth factor beta1 (TGF) were found in the late postmenopausal women, no significant differences were found in cytokine levels between the group on HRT and the group not receiving hormones. The women on the hormone therapy did experience a significant increase of colony-stimulating factor, which is known to decrease serum cholesterol (313).

Insulin-like growth factor, basic fibroblast growth factor, PDGF, and epidermal growth factor are the main growth factors involved in the proliferation of smooth muscle cells, endothelial cells, and matrix proteins (299), and therefore have been pinpointed at possibly playing an important role in the development of atherosclerotic lesions. It has been shown that one of the earliest nuclear events associated with the action of growth factors is the induction of the proto-oncogene *c-fos*. Breast cancer research has shown that estrogen may upregulate this oncogene and cause cell proliferation synergistically with IGF (314). Although most studies investigating the linkage

between estrogen and growth factor signaling pathways have been conducted using tissue other than vascular tissue (299), it has been shown that postmenopausal women on HRT show lower levels of serum IGF-1 when compared to those not on a hormone regimen (315). In a study on the effects of estrogen on transplant atherosclerosis, Saito *et al* (316) determined that estrogen administration to rats undergoing cardiovascular transplantation inhibited the typical increase in insulin-like growth factor-1 that occurs during the onset of posttransplantation atherosclerosis. The estrogen regimen (20 micrograms/kg daily for 2 days prior to surgery and until euthanasia) also decreased expression of the major histocompatability complex class II antigen (316). Recent findings have shown that TNF can downregulate insulin-like growth factor-1 and upregulate insulin-like growth factor binding protein-3 in vascular smooth muscle cells (224). Selzman, *et al* (299) found that estrogen replacement could decrease the aortic accumulation of TGF in sheep, indicating that estrogen may modulate the growth factor's impact on VSMC proliferation. The *in vitro* incubation of human aortic smooth muscle cells with estrogen showed increased levels of TGF secretion and increased potentiation of TGF's inhibition of VSMC proliferation.

Shanker, *et al* (317). determined that the addition of estrogen to cells from the monocyte/macrophage THP-1 line treated with 12-O-tetradecanoylphorbol 13 acetate (TPA) increased the level of PDGF-A transcript by 61 and 190 percent after 48 and 96 hours, respectively. Results for PDGF-B were not conclusive. The addition of estrogen to cells stimulated with TPA as well as with lipopolysaccharide caused no significant change in the amount of the PDGF-A transcript. It has been shown that the differentiation of THP-1 line cells as induced by TPA is similar to the transformation of monocytes to macrophages (138, 318), which is crucial during initiation and progression of atherosclerosis. Due to the fact that the actual role of PDGF in atherogenesis is still not clear, it is difficult to assess whether this effect of estrogen is cardioprotective or not. It has been suggested that PDGF-A may play an important role in early-stage atherosclerotic lesions, and that PDGF-A can inhibit the migratory activity of cultured smooth muscle cells (319). Estrogen's upregulation of PDGF-A (317) in the context of this study may be a mechanism by which it decreases the level of cell migration early in the lesion formation process. This is difficult to confirm, as most studies investigating the proliferative effects of PDGF involve late-stage lesions (317). It has been previously shown that estrogen can inhibit the migration of vascular smooth muscle cells *in vitro* (320). Kikuchi (321) *et al*, however, concluded that the addition of either estriol or estrone sulfate to cultured VSMC decreased transcript levels for PDGF-A, IL-1, and IL-6. These results were similar to those of Okubo, *et al* (321) Dai-Do, *et al* (272), who found that PDGF-BB stimulated DNA synthesis in VSMCs cultured from both postmenopausal females and age-matched males, and that the administration of estradiol inhibited the ability of both bFGF and PDGF to upregulate DNA synthesis in both males and females. Saito, *et al* (316) additionally found that the adminstration of 20 μg/kg per day to male rats undergong aortic allograft transplantation diminished the post-transplant increases in PDGF, IGF-1, and bFGF that were seen with the placebo group.

Another study conducted by Somjen *et al* (322) showed that 17beta estradiol administration had differential effects on DNA synthesis in vascular smooth muscle cells and in endothelial cells. Low doses (0.3 nmol/L) of estradiol stimulated DNA synthesis whereas higher doses (30 nmol/L) inhibited DNA synthesis as measured by [^{3}H] thymidine incorportion in VSMCs. In contrast, estradiol increased DNA synthesis in a dose-dependent manner in endothelial cells (E304 cells). High doses of estradiol inhibited PDGF and IGF-1 induced DNA synthesis in the VSMCs, whereas estradiol increased the growth factor-induced DNA synthesis in endothelial cells. It is suggested that estrogen's inhibition of VSMC proliferation and stimulation of DNA synthesis in the endothelium may characterize its role in vascular remodeling (322). When cells were incubated with tamoxifen and raloxifene, the modulators had the same effect on DNA synthesis as estrogen in both cell types. When cells were incubated with a combination of 0.3 nmol/L estradiol and either tamoxifen or raloxifene, the estradiol-induced increase in DNA synthesis was inhibited in endothelial cells but not in VSMCs (322).

Another marker of inflammation, C-reactive protein (CRP), has been shown to be an independent risk factor for CVD in both sexes (270, 323). High serum levels of this marker was the strongest overall predictor for cardiovascular disease in women overall (270). Vicenzino Pasceri and coworkers (324) investigated the effect of C-reactive protein on several other factors in the inflammatory response, including vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), and E-selectin. Incubation of human coronary artery endothelium in human serum with CRP led to an increase in the expression of ICAM-1 and VCAM-1 from 5μg/ml to 50 μg/ml and also increased the expression of E-selectin. CRP also induces tissue factor (325). It is unclear at this point whether CRP itself plays a direct role in atherosclerosis, or whether it simply serves as a marker of inflammation. (324) A few studies have concluded that HRT administered to postmenopausal women actually raises the level of CRP. Ridker and colleagues (326) conducted a study of 493 healthy postmenopausal women and found that those on HRT generally had higher levels of CRP than those not taking supplemental hormones. During the PEPI trial, (285) levels of CRP rose over the three-year period in women taking each of the four different hormone regimens (progesterone included). The serum concentration of CRP correlates to that of TNF-α (270) and IL-6, which is the main inducer of hepatic CRP production (270, 327). It was found by Walsh, *et al* (328) the increases in CRP levels in response to HRT administration were indeed higher for those women with high IL-6 levels, and that raloxifene, an estrogen receptor modulator, is not associated with higher CRP levels in postmenopausal women. However, this increased level of CRP was also seen in women after their IL-6 levels had decreased, suggesting that estrogen may exert its effects on CRP production by acting on the liver directly (328).

Estrogen has also been shown to affect various adhesion molecules and chemotactic proteins that are involved in the pathogenesis of atherosclerosis. Caulin-Glaser, *et al* (329)incubated human umbilical vein endothelial cells in estradiol and found that it strongly inhibited IL-1 mediated membrane E-selectin and vascular cell adhesion molecule-1 induction. Estradiol treatment also inhibited intercellular adhesion molecule-1 hyperinduction. This could possibly be a mode by which estrogen exerts is cardioprotective effects, as lower levels of these molecules lessen the ability of leukocytes and other inflammatory molecules to bind to endothelial cell injury sites. Caulin-Glaser (330) also found during a human study that men with CAD, as well as postmenopausal women with CAD not on a hormone regimen, had higher levels of circulating cellular adhesion molecules in comparison to the women with CAD taking estrogen. Those postmenopausal women with CAD taking estrogen, however, had increased levels of VCAM-1 than those not taking estrogen, as did the premenopausal women with CAD. In a study involving healthy individuals, Van Baal et al (331) found that the administration of unopposed estrogen or the combination of estrogen/trimegestone for twelve weeks to postmenopausal women without CAD led to decreases in ICAM-1, VCAM-1, and thrombomodulin. A decrease in endothelin levels was seen only in the group given combination therapy. There is also evidence that the effect that HRT or estrogen treatment may have on the expression of various adhesion molecules may be a function of its mode of adminstration. Seljeflot *et al* (332) reported that after twelve months of HRT, lower levels of E-selectin, ICAM-1, and VCAM-1 were seen in postmenopausal women with CAD who were receiving the treatment transdermally rather than orally. Oger (333) found that the lowering effect of HRT on ICAM-1 was dependent on treatment duration for those on a transdermal regimen, whereas this was not seen among those taking HRT orally.

Additionally, it was discovered in a study using human aortic endothelial cells and THP-1 monocytes that 48 hours of incubation with 17beta estradiol decreased monocyte adhesion by 28 percent, as well as decreased cytokine (TNF, IL-1 or both)-induced adhesion by 30-35 percent after a four hour addition of cytokines. Of importance, however, was that the estradiol could not inhibit endothelial cell-monocyte adhesion if the cells were incubated with the cytokines for 24 hours (334). Conversely, Zhang, *et al* (221) found that both estradiol and testosterone increase the expression of E-selectin and VCAM-1 in a receptor-mediated manner in human umbilical vein endothelial cells incubated simultaneously with TNF and the steroid hormone. The conclusion that estradiol upregulates expression of adhesion molecules as induced by TNF is supported by the work of Cid *et al* (54). It was suggested, however, that estradiol could decrease expression of monocyte-chemotactic protein-1 (MCP-1) in human artery endothelial cells. Estradiol decreased MCP-1 mRNA expression by 30 percent, although this effect was seen only in the arterial cells and not in umbilical vein cells (335). This suggests that estrogen may function to attenuate macrophage recruitment.

It has long been suspected that at least some of estrogen's proposed ability to protect from the progression of atherosclerosis and cardiovascular disease comes from its effect on endothelial function, as the endothelium and it's metabolically active cells are crucial in the development of atherosclerosis (336). The endothelium serves as a physical barrier of protection for the underlying components of the blood vessel, as well as a "docking point" for monocytes, leukocytes, and neutrophils (300). It also functions to prevent clotting and promote smooth blood flow through the vessel (300). The endothelium is also largely responsible for regulating vascular tone, as it is a source of vessel constrictors such as thromboxane, free radicals, endothelins, and cytokines as well as of vessel relaxants such as prostacyclin, and nitric oxide. (300) It was also recently documented that estradiol may lessen the extent of TNF-induced apoptosis among endothelial cells (337). As mentioned previously, Aune *et al* (289) found that after one year of hormone replacement therapy, there was a significant decrease in the production of the lipopolysaccharide-induced prostaglandin thromboxane B_2 , a potent vasoconstrictor.

Similarly, it has been suggested that estrogen can regulate the secretion of the prostaglandin prostacyclin, which acts as a vasodilator and inhibitor of platelet aggregation (299), although the results have been contradictory. Higher levels of 6-keto-prostaglandin were noted in the uterine arteries of premenopausal, but not postmenopausal women (338). Findings supporting this data have been obtained by tissue culture (339). O'Sullivan and colleagues (340) placed ovariectomized female monkeys on an atherogenic diet and treated a select group with Premarin and found that there was an 165 percent increase in arachidonate-induced prostacyclin production by vessels treated with estrogen, although there was no difference in the basal prostacyclin release. Additionally, prostacyclin production was inversely related to plaque size (340). Ospina *et al* (341) found that estrogen treatment increases cerebrovascular prostacyclin production in the rat by increasing levels of cyclooxygenase-1 as well as prostacyclin synthase. It was also found that estrogen can stimulate prostacyclin synthesis in ovine pulmonary artery endothelium (342). Conversely, Redmond and coworkers (343) found that estradiol had an inhibitory effect on flow- and acute hypoxia-induced prostacyclin release from endothelial cells. This was supported by a more recent study by Christodoulakos *et al* (335) which concluded that 17beta estradiol and raloxifene had a lowering (unfavorable) effect on prostacyclin levels.

van Kesteren *et al* (294) conducted a study using a subset of male→ female and female→ male transsexuals in order to assess the effects of transgender hormone therapy on coagulation factors as well as on markers of endothelial functioning such as big endothelin-1 (ET-1). Baseline levels were similar for the vasoconstrictor ET-1 between groups. The administration of estrogens to the M→ F transsexuals led to significant decreases in the level of big ET-1, whereas the F→M transsexuals receiving testosterone treatment saw an increase in ET-1. Other human studes have also shown that estradiol adminstration can attenuate ET-1 induced vessel constriction (335, 344,

345). Lee and colleagues (345) concluded that intracoronary treatment with estrogen decreased levels of ET-1 in post-coronary angioplasty patients. Saitta, et al (344) found that estrogen therapy with noresthisterone acetate as well as raloxifene therapy lowered ET-1 levels in healthy postmenopausal women after a sixth-month trial. It was noted by Jhund, *et al* (346), however, that estrogen treatment administered to women with coronary artery disease lost its ability to modulate ET-1-induced vasoconstriction after three months. In addition to its action as a vasoconstrictor, ET-1 has also been shown to promote endothelial cell adhesion molecule expression (347).

It has also been suggested that estrogen may have an effect on the production of nitric oxide, a vasodilator that is also capable of preventing platelet aggregation, leukocyte chemotaxis, and T-cell proliferation (348) within the endothelium. Wagner and colleagues (349) investigated the hypothesis that estrogen exhibited an anti-atherosclerotic effect by shifting the balance between nitric oxide (NO) and the free radical superoxide in the vessel wall. They showed the 17beta estradiol caused a concentration-dependent decrease in the expression of NADPH oxidase, an enzyme required for the generation of superoxide. 17beta estradiol also increased the production of nitric oxide in the vessel wall by a factor of two over the same concentration range. Incubation of the human vein umbilical endothelial cells with the estrogen also decreased the ability of the cells to produce superoxide upon phorbol ester stimulation. It appears estradiol may act as an antioxidant which can improve the NO/superoxide ratio and therefore normalize the response to injury. Saito *et al* (348) concluded that while estrogen therapy can suppress cytokine-inducible nitric oxide synthase (iNOS) (possibly via estrogen's effects on NF kappa-B) in macrophages and smooth muscle cells, it can also upregulate endothelial nitric oxide synthase (eNOS). In this case, estrogen is indirectly blocking the cytokines' ability to activate the inflammatory pathway. The study by Christodoulakos, *et al* (335) found that both the administration of estrogen plus noresthisterone acetate and estrogen and norethisterone plus raloxifene decreased ET-1 levels and inceased the nitric oxide/ET-1 ratio to produce a possibly cardioprotective effect. The study by Saitta (344) also concluded that estrogen treatments and raloxifene treatments can increase NO production.

Another recent study focused in on the questions as to whether estrogen's cardioprotective effect is a function of its effect on nitric oxide production (350). After treating human aortic endothelial cells with 17beta estradiol and measuring eNOS expression after 24 hour and 7 day regimens using intro-arterial infusion of interbrachial N^G-monomethyl-1-arginine, it was determined that there was no change in eNOS expression after acute or chronic estradiol treatment. A study by the same group (350) in which the vasoconstrictor response to a substrate inhibitor of nitric oxide synthase was measured in 10 healthy postmenopausal women receiving 80μg transdermal estrogen daily for four weeks also showed no difference in vasoconstrictor responses as mediated by nitric oxide.

It has also been shown that estrogen administration may effect the vasodilator response to acetylcholine (350). There is evidence that oestrogen modulates the vasodilator response to acetylcholine in rabbit aortas, (351) and that continuous estrogen administration can reduce the acetylcholine-induced vasoconstriction in atherosclerotic ovariectomized monkeys (352).

Collins, *et al* (353) demonstrated that estrogen could decrease or abolish the amount of vasoconstriction in response to acetylcholine (Ach) in post-menopausal women with coronary artery disease, and that this was a phenomena seen in females, but not in males. This attenuation of Ach-induced vasoconstriction in women by estrogen was also supported by the work of Reis, *et al* (354).

Estrogen has also been shown to have some protective capabilities against ischemia-reperfusion injury in various organs. Shi *et al* (207) used ovariectomized rats to assess estrogen's ability to protect from cortical ischemia-reperfusion injury by inducing one hour of middle cerebral artery occlusion and using sequential diffusion weighted MRI to obtain resultant lesion size. It was shown that while both the groups of ovariectomized rats (one receiving 100μg/kg estrogen 2 hours prior to the procedure, the other one not receiving estrogen) developed similar lesions in response to ischemia, the cortical-area lesions of the rats receiving estrogen decreased in size by 50-60 percent during reperfusion, whereas such a decrease was not seen in the rats not on an estrogen regimen. This and other studies (355-357) suggest that estrogen may act as a neuroprotective agent and selectively protect cortical tissue from ischemic damage during occlusion and therefore may be useful in the treatment of stroke. Additionally, Nonaka *et al* (358) discovered that the administration of estradiol reduced leukocyte accumulation by 35.7 percent 24 hours post-retinal ischemia reperfusion. There was also evidence that the administration of the estradiol lessened the extent of retinal damage 168 hours post-reperfusion.

Various animal studies have also studied the effects of estrogen on ischemia reperfusion injury in cardiac muscle. Squadrito, *et al* (359) exposed rats treated with 17beta estradiol and untreated rats to one hour of left coronary artery occlusion followed by one hour of reperfusion, using various markers of injury such as levels of necrosis, creatinine phosphokinase, TNF, and ICAM-1 to indicate the level of damage. Administration of estradiol to rats 5 minutes after the induction of injury decreased levels of necrosis, decreased levels of macrophage and serum TNF as well as creatinine phosphokinase activity, and prevented an increase in ICAM-1 staining. Zhai, *et al* (360) found that hearts from female, ovariectomized rats given estrogen supplements and from sham-operated female rats had higher coronary flow rates and left ventricular pressures as well as higher nitrate production post-ischemia/reperfusion than ovariectomized rats not undergoing hormone treatment. However, nitrate production and coronary flow rate were not consistently different throughout reperfusion. The same investigators

also found the dietary phytoestrogens may lessen the extent of inschemia-reperfusion injury in female rats (361). McNulty, *et al* (362) found, however, no difference in infarct size or cardiac function between estrogen treated rats and untreated rats after ischemia-reperfusion injury irrespective of gender. It has also been shown through experiments with canine hearts that estrogen treatment may limit the size of infarction as mediated by activation of mitochondrial ATP -sensitive potassium channels in myocardial tissue in both male and female dogs (97).

Yet another recent path of study has focused on estrogen's ability to modulate the cardiac heat shock protein 70, whose expression can be stimulated by exercise (363). Hsp 70, which encodes intracellular proteins that can prevent protein abnormalities and serve in cytoprotection (363), has been found to have the ability to protect from cardiac injury and even improve ATP synthesis (364, 365). A close temporal relationship has also been documented between the accumulation of Hsp 70 and recovery from ischemia-reperfusion injury (366). Paroo, *et al* (367) found that male rats exercised on treadmills experienced a two-fold increase in the expression of Hsp 70, whereas this increase was not seen in exercised female rats. Additionally, ovariectomized female rats also had a significant increase in Hsp 70 levels, and the administration of supplemental estrogen to these rats reversed that effect and was accompanied by lower levels of exercise-induced Hsp 70. It was also concluded that although cardiac cells contain both estrogen receptors alpha and beta, that this is not the mechanism by which estrogen mediates the Hsp 70 expression as treatment with tamoxifen (and estrogen antagonist) did not change the response in comparison to controls. Additional findings that 17alpha estradiol (which does not activate receptors) also showed the ability to inhibit exercise induced expression of Hsp70 further supports that estrogen does not mediate Hsp 70 via its receptors. (367) The authors speculate that estrogen may mediate the response through a membrane-stabilizing mechanism, and that although females do show less of a stress response to exercise, this can be disadvantageous as it is an indication that certain protective forces against ischemia-reperfusion injury are not initiated (366). This could partially explain why premenopausal women who do suffer cardiac events have a higher mortality rate than men who suffer similar events (368).

Due to the apparent rise in cardiac events in postmenopausal women as compared to premenopausal women, the vast majority of research involving the steroid sex hormones and influences on cardiac functioning have focused on estrogen and its possible cardioprotective role. However, several of the studies investigating the effects of estrogens on factors involved in the development of atherosclerosis and cardiovascular disease also conducted trials using testosterone or its derivatives, as testosterone's effects are not exclusively immunosuppressive (43). For example, Somjen *et al* (322) concluded that dihydroxytestosterone (DHT) had very similar effects to those of estrogen on DNA synthesis in endothelial cells and vascular smooth muscle cells. DHT dose-dependently increased DNA synthesis in endothelial cells as measured by [^{3} H] thymidine incorporation. In VSMCs, testosterone enhanced DNA synthesis at low concentrations (3 nmol/L) and inhibited synthesis at higher concentrations (300 nmol/L).

The androgen antagonist flutamide inhibited DHT's actions on both endothelial and VSM cells. Van Kesteren *et al* (294) studied female→ male transsexuals taking testosterone hormone therapy and found significant increases in endotelin-1 levels. Levels of plasminogen activator and PAI-1 did not change. Additionally, Sobel and colleagues (295) found that incubation of bovine aortic endothelial cells with testosterone tended to increase tissue plasminogen activator at low concentrations of hormone (10^{-12} M) and decrease its secretion at higher concentrations (10^{-7} M). Testosterone at a concentration of 10^{-7} mol/L also increased the secretion of PAI-1. Webb, *et al* (369) found that intracoronary infusion of testosterone improves coronary blood flow possibly by inducing vessel dilation in men with coronary artery disease, although dilation was shown to be reduced in women taking high doses of androgens (370). Studies have also found that higher levels of testosterone in men are associated with higher levels of high density lipoprotein, contrary to popular belief.

Zhang, *et al* (221) reported that testosterone increases the TNF-α induced expression of VCAM-1 and E-selectin in endothelial cells by a receptor-mediated mechanism. This report agrees with the findings of McCrohon, *et al* (371), who found that dihydroxytestosterone increases the expression of endothelial VCAM-1. A conflicting report by Mukherjee, *et al* (372) proposed that testosterone inhibited TNF induced VCAM-1 expression in human umbilical vein endothelial cells by conversion to estradiol via the enzyme aromatase. They found that dihyroxytestosterone, an unaromatizable testosterone, had no effect on VCAM-1 mRNA expression, whereas testosterone adminstration (30nM-1μM) decreased VCAM-1 expression in a dose-dependent manner. Because the addition of anastrozole (an aromatase inhibior) lessened testosterone's inhibitory effect on VCAM-1 expression, it was concluded that testosterone exerts its effect on the expression of VCAM-1 by converting to estradiol via aromatase present in endothelial cells (372). Dihydroxytestosteone has also been linked to increases in platelet accumulation (373).

7. SUMMARY AND PERSPECTIVE

The known mechanisms of myocardial injury are becoming increasingly complex. Not long ago, mechanisms of necrosis and ion dyshomeostasis were appreciated as the primary culprits in myocardial injury. Our progressive understanding of the mechanisms of apoptosis, stunning, hybernation, and preconditioning has served to bring us closer to enhanced myocardial preservation but, at the same time, has made understanding injury more complex and difficult. Although estrogen and testosterone have long been respected for their potent effects, our recent understanding of acute inflammation following ischemia has lit the spotlight on another theater in which these players are active. Testosterone appears to

be a potent promoter of proinflammatory and proapoptotic signaling processes in the heart and estrogen appears to have the counter effect. The results of our studies, and those of many of our colleagues around the country, most notably Dr. Chaudry, have revealed that there is much work to do in further understanding the role that sex hormones may have in altering the course of inflammation during ischemia, sepsis, and trauma.

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