University of Alberta

Effects of Estrogen Replacement and Chronic PGHS-2 Inhibition on Vascular Function in the Aged Rat

by

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Abstract

Aging and estrogen deficiency contribute to vascular dysfunction. Young ovariectomized rats have increased prostaglandin H synthase (PGHS)-dependent vasoconstriction that can be prevented with estrogen. PGHS-2-dependent vasoconstriction predominates in aging rats. Hypothesis I) Estrogen suppresses PGHS-2-dependent constriction in aged rats, II) Chronic PGHS-2 inhibition reduces PGHS-dependent vasoconstriction in aging. Ovariectomized, aged rats given I) placebo or estrogen-treatment or II) placebo or the PGHS-2 inhibitor for one or four weeks, had their mesenteric arteries assessed for endothelium-dependent relaxation in the absence/presence of PGHS inhibitors. I) PGHS-2 inhibition increased vasorelaxation to a greater extent than PGHS-1 inhibition. Estrogen prevented PGHS-dependent constriction with an associated reduction in PGHS-2 expression. II) One week of PGHS-2 inhibition abolished a PGHS-dependent shift in relaxation while four weeks enhanced PGHS-dependent modulation of vasoconstriction. PGHS-2 expression increased with prolonged PGHS-2 inhibition, corroborating the paradoxical increase in PGHS-dependent vasoconstriction. Estrogen, but not chronic PGHS-2 inhibition may counteract aging vascular effects.

'I am a voice crying in the wilderness,
"Prepare a straight pathway for the Lord's coming."'

John 1:23

To

Corrina

You inspire me

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List of Symbols and abbreviations

α	alpha
β	beta
μ	mu/micro
E2	estrogen
HE2	high estrogen
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERE	estrogen response element
PGHS	prostaglandin H synthase
PGHS-2	inducible prostaglandin H synthase
NS-398	PGHS-2 inhibitor
iNOS	inducible nitric oxide synthase
NAD(P)H	nicotinamide adenine dinucleotide phosphate
PGH_2	prostaglandin endoperoxide
TxA_2	thromboxane A_2
PGI_2	prostacyclin
Meclo	meclofenamate
PE	phenylephrine
NO	nitric oxide
$ONOO^-$	peroxynitrite
$O_2^{\cdot-}$	superoxide
$OH^{\cdot-}$	hydroxyl radical

OVX	ovariectomy
H_2O_2	hydrogen peroxide
ROS	reactive oxygen species
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
NFκB	Nuclear factor kappa B
SOD	superoxide dismutase
CAT	catalase
BH4	tetrahydrobiopterin
EC_{50}	Effective concentration that produced 50% of the maximum
EDRF	endothelium-derived relaxing factor
EDHF	endothelium-derived hyperpolarizing factor
Ang II	angiotensin II
$[Ca^{2+}]_i$	intracellular calcium
PI3K	phosphatidylinositol 3-phosphate
cGMP	cyclic guanosine monophosphate
sGC	soluble guanylate cyclase
EUK-8	superoxide dismutase/catalase mimetic
SQ-29548	thromboxane/PGH_2 receptor blocker
U46619	thromboxane mimetic
U-51605	stable PGH_2 analogue
VS	valeryl salicylate/PGHS-1 inhibitor

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CHAPTER 1: Introduction

Age tells on us all. The phenomenon of aging encompasses a large body of scientific research to date. Aging itself is a complex process that has been investigated by a range of disciplines within the field of medicine. Specific to cardiovascular diseases, aging is a risk factor (26, 59, 61, 81). Indeed, the vasculature has been shown to have age-associated changes, both functionally and morphologically (59, 81). Large arteries develop intimal thickening (81), and become stiff and unresponsive (61). Smaller arteries, important in peripheral vascular resistance, become dysfunctional due to the effects of aging on endothelial cells and vascular smooth muscle cells (26, 59). These effects include a decline in endothelial-dependent vasorelaxation and an increase in vasoconstriction (26). Furthermore, aging alters the architecture of the blood vessel through an accumulation of extracellular matrix, an increase in collagen content and a disorganization of the elastin fibres present (7). The permeability of endothelial cells increases and the smooth muscle cells become 'activated' i.e. migrate and proliferate more readily (7). Such perturbations expose the vessel to a number of problems including hypertension (59), and atherosclerosis (7).

Aging and endothelial dysfunction are interrelated. The decreased vasorelaxation observed with age has been shown to be due to a decline in the availability of the vasodilator, nitric oxide (NO), as well as an increase in the vasoconstrictor products of the prostaglandin H synthase (PGHS) pathway (62). Both of these pathways contribute to the endothelial modulation of the vasoactive tone of the blood vessel. NO-mediated relaxation is reduced with age because it is postulated to be scavenged by the superoxide anion ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$) – a molecule that is thought to be associated with the aging process (95). Moreover, aging has been shown to reduce the PGHS product, prostacyclin, which is another important vasodilator (70). Furthermore, vasoconstrictor products of the PGHS pathway, such as thromboxane, are increased with age in the vascular endothelium (26). Indeed, our lab found that the inducible prostaglandin H synthase (PGHS-2) enzyme was upregulated with

age, thus increasing the PGHS-2-dependent vasoconstriction within the resistance arteries (89). The apparent paradox evidenced by a decrease in one type of PGHS product and an increase in another will be discussed later in the thesis.

In women, aging has often been associated with estrogen deficiency. Indeed, they are common risk factors for cardiovascular disease (9, 91). During menopause, women are subjected to a detrimental reduction in this steroid hormone due to gradual senescence of the ovaries (55). The lack of estrogen results in a shift of plasma lipoproteins away from high density lipoprotein (HDL) cholesterol and towards low density lipoprotein (LDL) cholesterol (28). Estrogen replacement restores the HDL while diminishing the LDL cholesterol (28). As well as this 'classical' benefit of the hormone, estrogen is now known to have more direct effects on blood vessel function.

In many studies estrogen has been linked to vasodilation via the potent vasodilator NO (28, 71, 96, 98). More recent work has focused on the vasoconstrictor prostaglandins as participants in the myriad of effects estrogen appears to have. Recently, our lab showed that estrogen reduces PGHS-dependent vasoconstriction in ovariectomized (to remove endogenous estrogen) young rats (25). However, the role of estrogen in an aging model remains to be determined.

There is, of course, interaction between the various pathways and vasoactive molecules discussed. One of the aims of this thesis is to try to decipher these different avenues and to see how they come together in the conditions of aging and estrogen-replacement (see Figure 1.1). For the purposes of this thesis, the NO and PGHS pathways will be focused on as they are important in the aging process as well as being relevant to the effects of estrogen replacement in postmenopausal women. The two main questions of the thesis are: 1) Does estrogen replacement suppress the vasoconstriction associated with aging by reducing the inducible prostaglandin H synthase (PGHS-2) and its products? And, 2) Is chronic PGHS-2 inhibition a suitable alternative to estrogen

replacement for improvement of vascular relaxation in the aging female by inhibiting the PGHS-2 enzyme in a similar way?

Aging and Vascular Dysfunction

Age is a major risk factor for coronary artery disease, which is the leading cause of death in men and women (9). In general, aging has been described as a gradual decline in all physiological functions (55). There are various theories as to the causes of aging. The decrease in the activity of hormones, such as estrogen, is one of these (55). Another relatively young theory of aging holds to the belief that the detrimental effects of free radical reactive oxygen species (ROS) are of vital importance in determining the life span of an organism (for a comprehensive review see (6)). Briefly, this theory outlines the balance of oxidants (such as superoxide anion and the hydroxyl radical($OH^{\cdot}$)) and antioxidants and how this balance contributes to 'oxidative stress' or the lack thereof. Aging, in this regard, appears to be a state of increased ROS production and depleted antioxidant defense. From this perspective one might think that a restoration of the balance, by addition of the appropriate antioxidant, would solve the aging problem. Indeed, much study has been undertaken which follows this thought process. The intake of vitamin E or C, for example, has garnered a lot of epidemiological evidence as to the protective and beneficial effects of antioxidants especially as pertains to the cardiovascular system (40, 80). Results are inconclusive, however, and so shed doubt on the actual benefits of taking such supplements. Indeed, ingested antioxidants may not be very effective due to compartmentalization of oxidants within the cells of the vasculature.

Oxidative stress within the vasculature, therefore, has drawn considerable attention during the last couple of decades. ROS are thought to be the instigators in the endothelial dysfunction observed in vascular disease (11, 45, 54, 63), including aging (6). Specifically, the role of endothelial cell dysfunction as a possible 'first site' of cardiovascular problems has been proposed (11, 63). ROS can be free radicals, which possess an unpaired electron ($O_2^{\cdot-}$, $OH^{\cdot}$, $NO^{\cdot}$), or oxidizing agents (H_2O_2, or peroxynitrite; $ONOO^{-}$) which are not free radicals as such (11). The conventional paradigm is that ROS are

produced by various enzymes within the endothelium and are thus of importance in regulating vascular function (11, 45, 54, 63).

Vascular Oxidative Stress

Within mammalian cells there are various enzymatic sources of ROS, including lipoxygenase, PGHS, NO synthase (NOS) and NAD(P)H oxidase (11). One mechanism for endothelial dysfunction is based on an imbalance between the production of NO from NOS and the formation of the superoxide anion from the other sources. Under normal physiological conditions, superoxide is completely reduced to water by the cytochrome oxidase complex or in steps by superoxide dismutase (SOD) (Eqn (1)), and then catalase (Eqn (2)), or peroxidase (63). The pathophysiology associated with endothelial dysfunction favors the rapid reaction between NO and superoxide (Eqn (3)) to form the cytotoxic peroxynitrite (rate = 6.7×10^9 mol/$L^{-1}.s^{-1}$), rather than the preferred dismutation of superoxide to hydrogen peroxide (Eqn (1)) by SOD (rate = 2×10^9 mol/$L^{-1}.s^{-1}$) (11, 63).

Equations:

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (1)$$

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \qquad (2)$$

$$NO + O_2^- \rightarrow ONOO^- \qquad (3)$$

Indeed, with aging, in rat aorta, NOS activity and expression was increased but the free NO levels actually dropped (95). This was attributed to an increased superoxide production that quenched the NO to form peroxynitrite. Furthermore, peroxynitrite was shown to nitrosylate the tyrosine residues of

mitochondrial manganese superoxide dismutase (MnSOD), inactivating it and thus reducing the capacity of the cell to eliminate superoxide in a less harmful manner (95).

NAD(P)H oxidase is another enzymatic source of superoxide that has attracted attention in recent years. In fact, it has been put forward as the predominant superoxide-producing enzyme within endothelial and vascular smooth muscle cells (11). Within phagocytes the oxidase is composed of a number of subunits (p21, p47, p67, gp91) most of which have counterparts in the vascular oxidase (99). Studies of angiotensin II (Ang II)-induced hypertension in rats have demonstrated an increase in superoxide generation due to a concomitant rise in NAD(P)H oxidase activity (11). Further, the p22phox subunit mRNA was elevated in response to infusion of Ang II with a corresponding increase in NAD(P)H oxidase activity (30). Moreover, in an aging female rat model, p22phox was increased in aortic rings compared to young animals and diphenyleneiodonium (DPI – an NAD(P)H oxidase inhibitor) decreased superoxide production in the aged group (35). Interestingly, recent work from our lab demonstrated that estrogen could reduce the Ang II-induced expression of the subunits of NAD(P)H oxidase, as well as peroxynitrite in cultured endothelial cells (34). Hence, estrogen may act by reducing the production of superoxide by NADPH oxidase or other enzymes which are elevated in the aged rat.

Thus, in aging, the aforementioned enhancement of NOS expression and activity appears to be a futile attempt to deal with the additional superoxide production, as the anion has been shown to inactivate NO (95). Furthermore, NOS itself has been shown to have NAD(P)H oxidase activity which is enhanced in atherogenic conditions or due to a lack of the co-factor tetrahydrobiopterin (99). PGHS activity is also a source of superoxide during synthesis of prostaglandins due to the fact that it can co-oxidize NAD(P)H (99). Therefore, in conditions of aging and oxidative stress as well as estrogen replacement, it is important to understand both the actions of and the interactions between the NOS and PGHS pathways.

Nitric Oxide

NO, as alluded to before, is potently vasoactive. Since Furchgott and Zawadzki's discovery of a necessary endothelium-derived relaxing factor (EDRF) (31) and the subsequent characterization of EDRF as NO (75), the amount of related research has increased dramatically.

NO is produced in the endothelium by the enzymatic conversion of L-arginine to L-citrulline by endothelial NOS (eNOS) (38, 66). This process involves the five-electron oxidation of the terminal guanido nitrogen of the L-arginine, with O_2 and NADPH serving as cosubstrates (66). Tetrahydrobiopterin (BH4) is an important co-factor involved as its absence causes the NOS enzyme to transfer electrons to molecular oxygen producing superoxide (79). Interestingly, the reduced availability of BH4 is involved in the endothelial dysfunction associated with atherosclerosis.(94) Further, vitamin C has been found to enhance eNOS activity through a chemical stabilization of this co-factor (3).

eNOS is only one of three known isoforms identified in mammals (66). The other two, inducible NOS (iNOS which was originally cloned from immunoactivated macrophages) and neuronal NOS (nNOS, first found in neuronal tissues) are perhaps less important in maintenance of basal vascular tone, although iNOS is expressed in vascular smooth muscle cells (66). While first thought of as constitutively expressed, eNOS and nNOS can also be induced. Similarly, iNOS being solely inducible is a misnomer in some cases (66). In fact, the main difference between the isoforms is their reliance on intracellular calcium ($[Ca^{2+}]_i$) to enable binding to the calcium regulatory protein, calmodulin (66). eNOS and nNOS appear to be regulated by $[Ca^{2+}]_i$, while iNOS is not (66).

Nitric Oxide and Aging

The relevance of NO to aging stems from its involvement in endothelial dysfunction. In male rats, eNOS and iNOS expression are increased with age (15). This effect appears to be compensatory, although eNOS activity was reduced in aging. A lack of eNOS activity could be due to the loss of essential

co-factors such as BH4 (see earlier). The iNOS activity produces more than enough NO, however, which when coupled with increased superoxide levels ends up being counterproductive as more of the potent oxidant, peroxynitrite, is formed. Therefore, with age, the counterbalance of NO is lost thus contributing to the shift towards vasoconstriction. Indeed, the Wistar-Kyoto and the spontaneous hypertensive female rat models both exhibited a reduction in NO bioavailability with age (35). Furthermore, superoxide generation was increased in both models and thought to contribute to this by NO scavenging (35). Another group went one step further by measuring 3-nitrotyrosine, the molecular footprint for peroxynitrite (95). Aged rat aorta was found to have both increased expression and activity of eNOS, as well as enhanced superoxide production (95). The increased peroxynitrite formed was shown to nitrosylate, and hence inhibit the mitochondrial manganese superoxide dismutase (MnSOD) enzyme (95). This effectively removes a 'protective' barrier and contributes to a feed-forward loop that is detrimental to the vessel. Other enzymes such as glutathione peroxidase may compensate for this, preventing apoptosis of the cells.

Human studies of forearm blood flow demonstrated the normal reduction in acetylcholine-induced relaxation by NG-monomethyl-l-arginine (L-NMMA) in young people was resistant in older subjects (92). Similarly, a functional study of isolated rat mesenteric arteries revealed a reduction in methacholine-induced vasorelaxation with NO inhibition in young animals but not in old (89). However, when there is a vascular insult, mesenteric vasculature showed N^G-nitro-L-arginine methyl ester (L-NAME, NO synthase inhibitor)-induced vasoconstriction in both young and old animals (37). The difference observed with the ischemia/reperfusion insult is probably due to an enhancement of iNOS rather than an aging effect (62). Interestingly, ovariectomy contributes to enhanced oxidative stress, which would inactivate any increased NO production, and thus, NO inhibition will have less effect in these models apart from aging (41, 43). The importance of this in estrogen replacement will be better understood after a discussion of the effects of estrogen on the NO pathway.

Nitric Oxide and Estrogen

Estrogen has been shown to upregulate the expression of eNOS, the enzyme responsible for basal production of the vasodilator (96). Moreover, physiological levels of estrogen have been shown to decrease myogenic tone (pressure-induced constriction) in rat coronary artery via an increased tonic release of NO (98). This occurs via activation of guanylyl cyclase, stimulation of cyclic GMP and subsequent activation of Ca^{2+}-activated potassium channels resulting in smooth muscle membrane hyperpolarization and vasodilation (98). In ovariectomized guinea pigs supplemented with a range of estradiol doses, coronary artery contractility to U46619 (a thromboxane mimetic) was potentiated after NO inhibition but only at the lower dosage of E2 (although physiological estrogen levels were not measured in this case) (93). Another study implicated estrogen as having a role in the scavenging of superoxide anion within bovine endothelial cells, increasing the bioavailability of NO but not altering the expression or activity of NOS itself (1). The observed decrease in superoxide production illustrates the antioxidant ability of estrogen, which is also important in the regulation of vascular function (1, 4). With aging this likely effects the production of peroxynitrite. Indeed, it has been suggested that in females, estrogen may be protective by scavenging superoxide rather than altering eNOS gene expression (4). This was evidenced in aorta of ovariectomized, estrogen-replaced female rats by a decrease in extracellular superoxide anion, and in cultured endothelial cells treated with ethinyl estradiol by a reduction in peroxynitrite generation (4). Whether or not estrogen is acting via a genomic or non-genomic mechanism is also of interest.

Estrogen's actions on the vasculature can be either genomic or non-genomic. Genomic mechanisms largely involve the nuclear estrogen receptor (ER) (28), however evidence now exists for an estrogen receptor on the cell surface (87). The ERs have been subdivided into two groups: α and β, both of which have been characterized in human and rat blood vessels (51, 68). Estrogen binds to its receptor, which, in turn, binds to a unique DNA sequence named the estrogen response element (ERE) (68). This ERE then elicits gene

transcription (68). Moreover, estrogen can elicit gene responses through transcription factors such as c-jun/c-fos, which work through the ubiquitous AP binding sites located on many genes (97). In fact, the NOS gene does not have a 'full' ERE as such, but instead contains a half-palindrome that could act with the transcription factor Sp1 (which itself is essential for eNOS transcription) to mediate estrogen's receptor effects (52). Indeed, receptor-mediated upregulation of eNOS has been characterized in pulmonary artery endothelial cells (60). Both NOS activity and protein expression were increased in response to physiological doses of estrogen, with these effects being blocked by ICI 182,780 (an ER antagonist) (60). Thus, estrogen can cause an increase in the basal production of NO by genomic modulation through its receptor.

The non-genomic actions of estrogen have come to the forefront in recent years. One study measured vasorelaxation to 17 β-estradiol (for structure see Figure 2) added exogenously to depolarized rat aortic rings (29). It was found that relaxation was not prevented by cycloheximide (inhibitor of protein de novo synthesis) or actinomycin D (gene transcription inhibitor). These data suggest a nongenomic role for 17 β-estradiol in relaxation observed within the rodent aorta (29). Another study focused on the nongenomic activation of eNOS via the ERα in an ovine endothelial cell culture model (16). 17 β-estradiol elicited acute activation of eNOS, which was not blocked by actinomycin D but was inhibited by simultaneous treatment with specific ER antagonists. Further, eNOS activation by 17 β-estradiol was inhibited by blocking the mitogen-activated protein (MAP) kinase pathway, suggesting that non-genomic activation of ERα is mediated through this pathway (16). Moreover, 17 β-estradiol was shown to stimulate NO release in a nongenomic manner through ERα located in caveolae ("little caves" or invaginations) at the plasma membrane (50). This is thought to occur by the activation and internalization of eNOS associated with caveolae and is a rapid receptor-mediated effect (65). Finally, this rapid effect of estradiol was found in HUVECs to be independent of the cytosolic Ca^{2+} release usually associated with eNOS activity (14). By contrast, estrogen elicited acute effects in airway

epithelial and endothelial cells (83) and human peripheral monocytes (87) in a calcium-dependent manner, but similarly through ERα (83, 87) and MAP kinase mechanisms (83).

Recent studies in human endothelial cells have implicated the phosphatidylinositol-3-OH (PI3)-kinase-Akt pathway as important in the rapid ER activation of eNOS (39, 86). The increased NO production induced by 17 β-estradiol was blocked by the PI3 kinase inhibitor, LY294002, as well as the ER antagonist ICI 182,780 (39). Estrogen was shown to stimulate Akt phosphorylation which, in turn, phosphorylated and activated eNOS (39). Moreover, the plasma membrane impermeable estrogen-BSA conjugate, known to bind to endothelial cell membrane sites, also induced rapid Akt phosphorylation and subsequent eNOS activation suggesting the involvement of a membrane ER (39). Another group discovered a direct interaction of ERα with the p85 regulatory subunit of PI3-kinase as well as inhibition of eNOS activation by the PI3 kinase inhibitor, wortmannin (64, 86). Interestingly, ERβ did not interact directly with p85 (86). Yet, whole cell studies indicate that ERβ may also elicit rapid eNOS activation (64). These observations indicate differential eNOS activation pathways between the two known estrogen receptors.

Therefore, attention has turned to the second estrogen receptor, ERβ, first cloned from rat prostate (53). In studies of carotid arterial response to injury, estrogen replacement suppressed the effects in both female wild type and ERα knockout mice to a similar degree (44). A similar expression level of ERβ mRNA in both types of mice was thought to explain estrogen's maintained protection (44). Interestingly, findings of ERβ induction in male carotid balloon vascular injury (56) suggested that ERβ could compensate for ERα, demonstrating a redundancy between the two types.

Thus, estrogen can affect the vasculature through rapid, nongenomic actions or via more long-term genomic alterations. Whether or not the more recently discovered rapid actions of estrogen play any physiological role remains to be determined. Furthermore, due to methylation and inactivation of

the ERα gene with age, receptor-mediated genomic changes with estrogen are probably less relevant in the aging vascular system (78). Moreover, the decline in estrogen with aging would further contribute to this effect since the hormone can upregulate its own receptor. It is for these reasons that the remainder of this thesis focuses on the downstream physiological effects of estrogen replacement and aging rather than the molecular aspects of estrogen receptor activation and signaling. To understand estrogen's actions from a physiological perspective, therefore, it is important to understand what is occurring within the vascular endothelium during aging and estrogen replacement. 17 β-estradiol and the more stable 17 α-ethinyl estradiol have both been shown to enhance eNOS mRNA and protein expression in human endothelial EA.hy 926 cells (52), which could be blocked by the ER antagonist RU58668. Indeed, estrogen's beneficial cardiovascular effects have been attributed to NO- and cGMP-dependent relaxation in porcine coronary arteries (21). As discussed, however, this dependence on NO does not appear to predominate in conditions of aging (62) or ovariectomy (43) (relevant to the postmenopausal woman). Therefore, it is necessary to investigate alternative pathways to determine how estrogen replacement is of benefit to the vasculature of aging females. The synthesis of prostaglandins has been shown important in this regard.

PGHS pathway

Prostaglandins have a more extended history than NO, and are involved in vascular reactivity (72), and the inflammatory response (67). Prostaglandins are bioactive lipid molecules that are produced from arachidonic acid by the enzyme prostaglandin endoperoxide synthase (PGHS) (33). After arachidonic acid is liberated from the phospholipid membrane of the endothelial cell by phospholipase A_2, PGHS catalyses the incorporation of two molecules of oxygen to produce the hydroperoxy endoperoxide, PGG_2 (33). This is the cyclooxygenase activity of the enzyme (33). PGG_2 is then reduced by the peroxidase activity of PGHS to form the prostaglandin intermediate, PGH_2 (33). Next, PGH_2 can be converted into various eicosanoids by different downstream enzymes (33). Two that are particularly important in vascular response are

thromboxane (TxA_2) and prostacyclin (PGI_2). These are produced by their respective synthases. Thromboxane stimulates platelet aggregation and is a potent vasoconstrictor (10). Prostacyclin, on the other hand, is a potent vasorelaxant that inhibits platelet adherence and aggregation (10). The balance achieved by these two eicosanoids is therefore important in the regulation of platelet aggregation and smooth muscle tone.

PGHS has two isoforms: PGHS-1 and PGHS-2. PGHS-1 is the constitutive enzyme that can also be regulated, while PGHS-2 is primarily the inducible form that is constitutively present in some cells (33). PGHS-2 is highly expressed at sites of inflammation (69). Both isoforms have been localized within endothelial and smooth muscle cells of the vasculature (42).

PGHS and Aging

With aging, impaired vascular function has been associated with an increase in PGHS-dependent vasoconstrictors due to a concomitant rise in lipid peroxidation (24). This can be evidenced as an increase in sensitivity to phenylephrine and/or a decrease in endothelial-dependent relaxation to methacholine in aged female rat arteries (24). Furthermore, in a rat model of oxidative stress via vitamin E deprivation, meclofenamate (a PGHS inhibitor), and SQ-29548 (a thromboxane A_2/PGH_2 receptor blocker) potentiated the endothelial-dependent relaxation (23). Recently, an induction of the PGHS-2 isoform in arteries from aging rats was discovered (89). This will be discussed in more detail in the following sections.

One might suppose that an increase of the products of PGHS would also lead to elevated levels of the vasodilator, prostacyclin. This is not likely the case with aging. As mentioned previously, the oxidative stress indicative of the aging process leads to the formation of the cellular toxin, peroxynitrite (95). Peroxynitrite has been shown to nitrate and inactivate prostacyclin synthase (PGI_2 synthase) in atherosclerotic bovine coronary arteries (100). In addition, peroxynitrite decreased PGI_2 synthase protein levels in endothelial cells (19). Theoretically, then, a shunt is created which results in accumulation of the vasoconstrictive PGH_2, and production of various other vasoconstrictor

eicosanoids, including thromboxane (see Figure 1.1). Another possibility arose from a recent hypothesis that stated that colocalization of PGI_2 synthase and PGHS is vital for prostacyclin synthesis (57). It was found that PGI_2 synthase and PGHS-1 were colocalized to the nuclear envelope and endoplasmic reticulum in bovine aortic endothelial cells (57). However, in cells stimulated with phorbol ester to induce PGHS-2 there was no colocalization with PGI_2 synthase, accompanied with a lack of prostacyclin production (57). Hence, one might speculate that higher levels of PGHS-2 induced with aging might not colocalize with PGI_2 synthase and therefore only produce vasoconstrictor prostanoids. Overall, PGHS contributes to the changes in vascular function associated with aging by producing vasoconstrictor eicosanoids, whilst the vasodilator prostacyclin is diminished.

PGHS and Estrogen

Estrogen, too, affects the PGHS pathway. Bovine microvascular endothelial cells exposed to 17 β-estradiol for 24 hours were found to have reduced TxB_2 and 6-keto $PGF_{1\alpha}$ production (stable metabolites of thromboxane and prostacyclin respectively) (90). This suppression of PGHS-dependent products by estrogen was shown to be receptor dependent (90). Furthermore, estrogen has been shown to effect vessel function by reducing the production of these prostanoids (20, 25). It was discovered that vasodilation to methacholine was blunted in arteries from ovariectomized rats compared to estrogen-replaced rats (25). Inhibitors of the PGHS pathway enhanced vasorelaxation in the ovariectomized rats making their response similar to that seen with estrogen-replacement. Thus, it was demonstrated in young female rats that removal of estrogen increases endothelium-dependent, PGHS-dependent vasoconstriction (25). This is additional to estrogen's effects on NO. Another study determined the effects of estrogen replacement on endothelium-dependent vasodilation in ovariectomized female Sprague-Dawley rats using a different agent (13). Arterial relaxation to the histamine H(1) agonist, 2-thiazolylethylamine was inhibited by N-omega-nitro-L-arginine (LNA; NOS inhibitor) in ovary-intact and ovariectomized + estrogen rats but not in ovariectomized alone rats (13).

Furthermore, addition of indomethacin (a non-selective PGHS inhibitor) decreased arterial sensitivity to the H(1) agonist slightly in ovary-intact rats, significantly in ovariectomized rats and not at all in estrogen-treated rats (13). It was concluded that in these young (3 months) animals estrogen increases the NO modulation and decreases the PGHS modulation of endothelium-dependent vasodilation (13). Hence, estrogen appears to modulate some 'cross talk' between NO and the PGHS pathway. The significance of this interaction remains to be determined in aging female vasculature. However, recent cellular studies have investigated the interactions between these two pathways (33, 73).

Nitric Oxide and PGHS

NO is known to both stimulate and suppress prostaglandin production. In endothelial cells the calcium ionophore A23187 was shown to stimulate NO synthesis as well as thromboxane and prostacyclin production (22). Furthermore, inhibiting or inactivating NO (with hemoglobin) decreased prostacyclin production. PGHS-1 and –2 expression levels remained unchanged, however it was concluded that NO increased prostaglandin production through activation of the PGHS enzyme (22). Within another cell type, NO was shown to suppress the induced PGHS-2 expression due to lipopolysaccharide challenge, thus inhibiting the activity of the enzyme (73). However, in another study NO was demonstrated to enhance PGHS-2 activity without altering protein levels (33). One explanation for the contrary findings could be that under certain conditions peroxynitrite is being formed and this molecule has been shown to induce the PGHS-2 enzyme (27), although other work from our lab showed that peroxynitrite was unable to increase the protein mass of PGHS-2 perhaps due to the lack of essential cofactors in the media (19).

Clearly, the discrepancies and controversy evidenced by such studies must be investigated further. Recent work in macrophages revealed that NO enhanced PGE_2 (another PGHS-derived vasoactive product) release that was inhibitable by indomethacin but not by NS-398 (a specific PGHS-2 inhibitor) (18). Using PGHS-1 or PGHS-2 knockout mice they further showed that NO stimulated PGE_2 release in PGHS-2 deficient cells without changing PGHS-1

mRNA or protein levels. By contrast, NO blocked PGHS-2-mediated PGE_2 production in PGHS-1 deficient cells that was associated with decreased expression and nitration of PGHS-2 (18). Moreover, in vascular endothelial cells, the production of 6-keto $PGF_{1\alpha}$ was attenuated in cells expressing PGHS-2 but unaffected in cells expressing PGHS-1 after coincubation of arachidonic acid and a NO donor (74). To confirm these results aorta were isolated from normal and LPS-treated rats. The 'normal' aortae, where PGHS-1 expression was dominant, showed no response in PGHS activity with the NO donor, while the aortae taken from those treated with LPS, where PGHS-2 expression was dominant, exhibited a significant reduction in PGHS activity with NO (74). Thus, in vascular endothelium it appears that NO suppresses PGHS-2 activity alone.

Such an interaction could help to explain the increase in PGHS-2 activity associated with aging (refer to next section). If, as previously discussed, oxidative stress causes NO to be scavenged by superoxide to form peroxynitrite, then less of the dilator is available to interact with PGHS-2, thus maintaining its activity. Indeed, the expression of PGHS-2 and iNOS were colocalized in atherosclerotic lesions from human coronary arteries (2). Moreover, nitrotyrosine (peroxynitrite indicator) was detectable in the same distribution (2). Since PGHS-2 is itself capable of producing moderate amounts of superoxide (99) it is conceivable that it can protect itself from suppression by NO. Therefore, it can be concluded that PGHS-2 is a crucial component in the enhanced vasoconstriction observed with aging. Inhibition of this enzyme could afford protection to the vasculature by altering the activity of both the NO and PGHS pathways.

PGHS Inhibition

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) that has long been used to reduce pain and fever. Interestingly, it acts by binding to PGHS and acetylating the serine 530 residue in the active site. Indeed, commercials now report aspirin as 'anti-hypertensive' based on the research previously discussed. The drug is now sold 'coated', however, due to the fact that PGHS-1

inhibition has adverse effects within the gastrointestinal tract (such as ulcer formation, bleeding and anemia). This is the main reason for the development of more specific PGHS-2 inhibitors in the last decade.

NS-398 or N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide is one such drug. In humans, NS-398 is about 40 times more selective for PGHS-2 than for PGHS-1 (5). In fact, one study showed that NS-398 selectively inhibited monocyte PGHS-2 from human plasma (76). Therefore, this drug has often been employed in studies that require specific inhibition of PGHS-2.

PGHS-2 and Aging

Selective PGHS-2 inhibition targets the isoform that has been implicated in inflammation (47), atherosclerosis (88), and aging (89). Indeed, in human atherosclerotic carotid arteries (88), and in blood vessels adjacent to human rheumatoid arthritis (47), PGHS-2 expression was detectable. Our lab demonstrated an upregulation of PGHS-2 protein in aged female rats using Western immunoblot analysis (89). It was further shown that the blunted methacholine relaxation observed in arteries from the aged animals could be enhanced with acute PGHS-2 inhibition (NS-398) (89). This effectively restored vessel function to that observed in young rats. Conversely, NO modulation of arterial function was decreased in the aged rat, while specific PGHS-1 inhibition (valeryl salicylate) had little effect. Thus, it was concluded that PGHS-2-dependent vasoconstriction was of primary importance in the aged female rat model (89).

Oxidative stress is also thought to play a role in PGHS-2 expression. The redox-sensitive transcription factor, NFκB, was found to have enhanced activity that coincided with the increased ROS associated with aging (49), and the neurodegenerative disorder, Alzheimer's disease (58). This was accompanied by an increase in PGHS-2 mRNA and protein levels (49). Furthermore, in human vascular endothelium, hypoxia was shown to transactivate NFκB via the high-mobility-group protein I(Y), and hence induce PGHS-2 promoter activity (46). A rat vascular function study of oxidative stress, induced by *tert*-butyl hydroperoxide, demonstrated an increase in

vasoconstriction of the aorta mediated by PGHS-2 metabolites (32). Interestingly, another rat study showed that dietary restriction attenuated the upregulation of PGHS-2 with age (17), demonstrating that anti-oxidative action can reduce the activity of NFκB. Thus, estrogen's antioxidant effects could potentially reduce NFκB activity and decrease the expression of PGHS-2.

PGHS-2 and Estrogen

Less is known, however, about estrogen's effects on PGHS-2 expression and activity. In mouse bone marrow supernatants from ovariectomized or estrogen-replaced mice, ovariectomy alone increased PGHS-2 levels twofold and estrogen was found to diminish PGHS-2 as evidenced by decreased PGHS-2 mRNA and PGE_2 levels (48). Similarly, in rabbit uterine cervical fibroblasts treated with interleukin-1α, PGE_2 levels were suppressed by progesterone and 17 β-estradiol at physiological concentrations as well as by indomethacin and NS-398 (specific PGHS-2 inhibitor) (82). These data are suggestive of a direct effect of estrogen, perhaps through modulation of gene transcription, to decrease the expression and activity of PGHS-2. Conversely, the rapid vasodilatory effects to acetylcholine were enhanced with acute estrogen administration following aspirin, diclofenac or placebo. This could be abolished by celecoxib (specific PGHS-2 inhibitor, see below)(12), suggesting an induction of PGHS-2 dependent vasodilators as a nongenomic mechanism observed with estrogen treatment. Another interesting study looked at a 'combination' of estrogen's nongenomic and genomic effects on PGHS-2. This involved what was termed Membrane-Initiated Steroid Signalling or MISS. In this paradigm the estrogen binds to a receptor on the membrane and instead of eliciting nongenomic actions it leads to gene transcription by activating several kinase cascades. Estrogen was found to stimulate gene and protein expression of PGHS-2 within 60 minutes as well as increasing PGI_2 and PGE_2 production in cultured endothelial cells (77). This could be a result of MISS which perhaps explains the reason for the fast-acting genomic effects observed (77). This process was inhibited using the phosphatidylinositol 3-phosphate (PI3K) blocker LY49002. Furthermore, functional endothelial cell migration induced by estrogen could be blocked with

NS-398, a specific PGHS-2 inhibitor. In this instance, then, estrogen was serving to induce the PGHS-2 pathway. However, since our studies involved the long-term administration of the steroid hormone, one would expect the genomic action of reducing PGHS-2 expression and activity to occur. Also, developmental age is important since estrogen can induce PGI_2 production in ovine fetal pulmonary endothelium (84), but PGI_2 synthase is inactive in conditions of oxidative stress such as atherosclerosis (100). Thus, in our model, estrogen may reduce the PGHS-2-dependent vasoconstriction associated with aging and oxidative stress (see Hypotheses).

Chronic PGHS-2 Inhibition

Chronic PGHS-2 inhibition has become an area of clinical interest in the past few years. The development of specific PGHS-2 inhibitors has crossed from the bench to the bedside. Celecoxib was the first drug to be clinically approved and launched. The drug is currently used as an anti-inflammatory agent for patients suffering from conditions such as rheumatoid arthritis. Celecoxib was soon to be followed by rofecoxib. These new drugs have been marketed as 'revolutionary', but, in truth, they have sparked some controversy in the medical and scientific communities. A recent randomized controlled trial tested the gastrointestinal toxicity with celecoxib vs. other NSAIDs (85). This 'Celecoxib Long-term Arthritis Safety Study' ('CLASS') demonstrated that patients treated with celecoxib had a lower incidence of symptomatic ulcers, although this was at higher doses than those given clinically (85). The CLASS trial further revealed no significant change in cardiovascular events. Conversely, the 'Vioxx Gastrointestinal Outcomes Research Study' ('VIGOR'), which compared the gastrointestinal toxicity with rofecoxib or naproxen (nonselective PGHS inhibitor), showed a significant increase in the risk of cardiovascular events such as myocardial infarction with this selective PGHS-2 inhibitor (8). Indeed, a number of basic science studies have called into question the efficacy and other effects of these drugs. One study showed that an increased supply of arachidonic acid alongside elevated PGHS-2 expression actually decreased the efficacy of the selective PGHS-2 inhibitors, celecoxib and

NS-398 (36). Furthermore, a pharmacological study revealed that celecoxib (dose = 10mg kg^{-1} daily for 3 weeks) elevates blood pressure and increases leukocyte adherence in young, normal and hypertensive rats (69). This was thought due to an inhibition of PGI_2 synthesis, however, serum $PGF_{1\alpha}$ levels (the stable metabolite of PGI_2) were unaltered by the drug.

Controversy aside, various studies indicate that chronic, selective PGHS-2 inhibition by drugs such as celecoxib and NS-398 is potentially useful as an alternative to aspirin in the treatment of a number of pathological conditions, especially involving aging and oxidative stress. Whether or not chronic PGHS-2 inhibition is an effective alternative to estrogen replacement in reducing hypertension in the aged patient remains to be determined. Indeed, the rationale behind employing drugs as an alternative to hormone replacement has been of interest for many years because

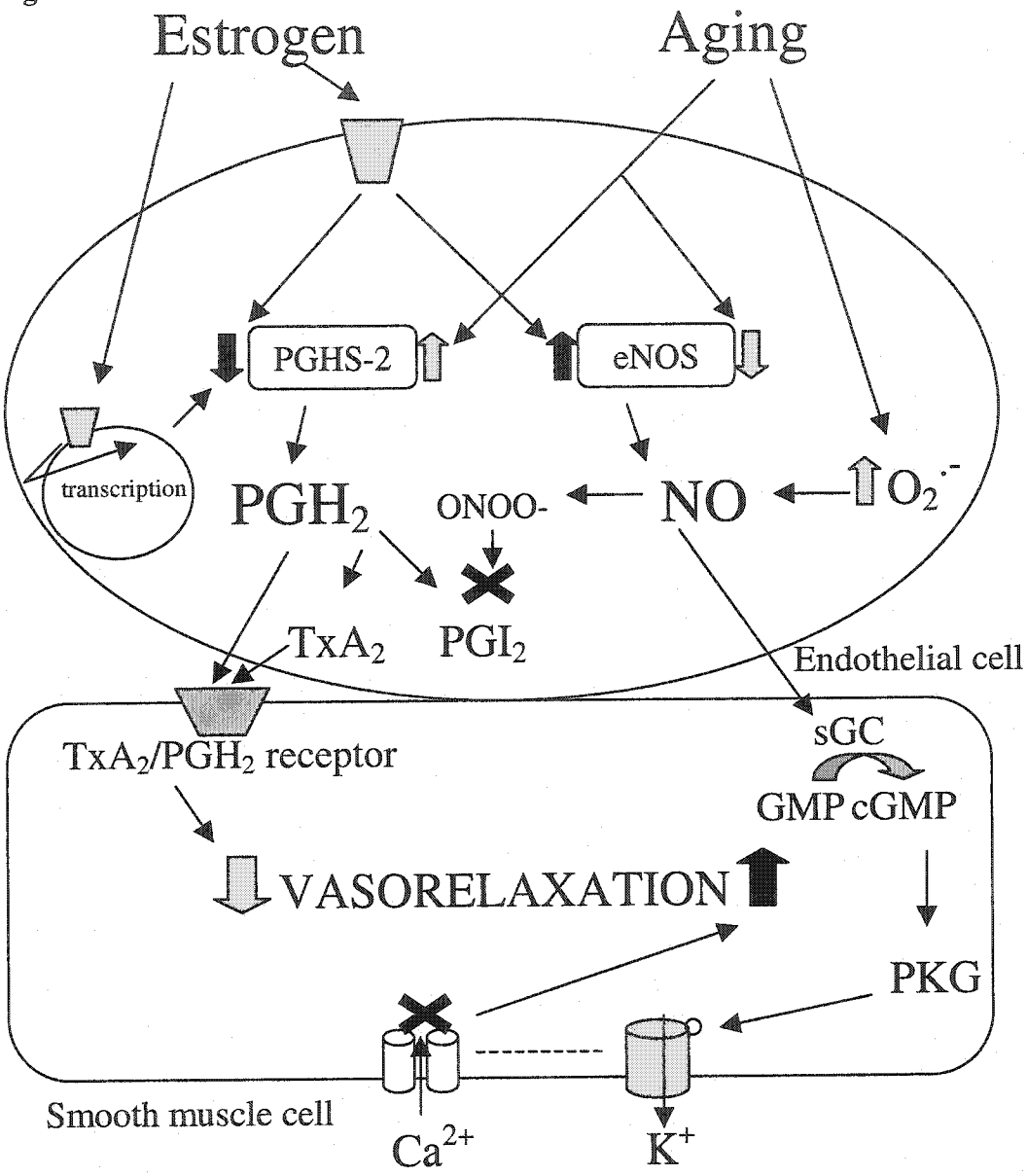
> "compounds might be found that eliminate the undesirable action of such hormones and yet retain its beneficial effects on arteries"
>
> Samuel A. Levine, 1958 (101). This idea is central to this thesis.

In conclusion, we can hypothesize the protection of estrogen replacement on aging female rat vasculature to be three-fold: 1) estrogen can activate eNOS to produce more of the vasodilator, NO, 2) estrogen can scavenge the superoxide associated with aging and oxidative stress, preventing the formation of detrimental factors, such as peroxynitrite, and 3) estrogen may act to reduce the activity and/or expression of PGHS-2 (associated with aging) indirectly through the interaction of NO with the enzyme or directly via gene modulation. The effects of NO are less important to vasorelaxation in aging and therefore, the effects of estrogen are probably dependent on how it interacts with superoxide and the PGHS-2 enzyme itself.

Hypotheses

My first hypothesis is that *estrogen replacement in aged female rats will restore vasorelaxation in mesenteric arteries via a suppression of the PGHS-2 enzyme and its vasoconstrictor products* (see Figure 1.1 for more details). Furthermore, chronic PGHS-2 inhibition might be one possible alternative to estrogen replacement that could benefit the vasculature of aging females. The induction of this enzyme with aging makes it a primary target for therapeutic intervention in postmenopausal women and in men. Those who decide against hormone replacement therapy for whatever reason may want to take a specific PGHS-2 inhibitor to protect themselves from hypertension and/or other cardiovascular problems. Therefore, we proposed a study using the aging, ovariectomized female rat model to investigate the effects of chronic PGHS-2 inhibition with NS-398 on the function of the mesenteric arteries. Hence, our second hypothesis is that *chronic PGHS-2 inhibition, via NS-398 injection, will restore the impaired function of the mesenteric arteries in the aged, ovariectomized female rat to that observed in the estrogen replacement study.*

Figure 1.1



Hypothesis

In aging, PGHS-2 enzyme is induced while eNOS production/activity is suppressed in vascular endothelial cells. Superoxide levels are elevated, and thus NO is scavenged to form peroxynitrite. $ONOO^-$ can nitrate and inactivate PGI_2 synthase reducing the production of this dilator. In addition, PGH_2 metabolism is shunted towards TxA_2 (a vasoconstrictor) production leading to enhanced PGHS-dependent constriction. Estrogen is known to enhance eNOS and may reduce PGHS-2 expression, thus reversing the vascular aging effects.

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CHAPTER 2: Estrogen Replacement Reduces PGHS-2-dependent Vasoconstriction in the Aged Rat

Introduction

After menopause, women become more susceptible to cardiovascular dysfunction, largely as a result of the estrogen deficit that is incurred (22, 28). This lack of estrogen has been found to affect vascular function, not only by loss of protection of favorable blood lipid levels (19), but also through direct mechanisms. Numerous studies have focused on the effect of estrogen on endothelial modulation of vascular tone by nitric oxide (NO). For instance, estrogen replacement is thought to improve relaxation by elevating the expression of endothelial NO synthase (eNOS) and subsequently producing more NO (13, 25, 36). In addition, estrogen enhances the bioavailability of NO by inhibiting superoxide anion production (2). However, not all of the vascular effects of estrogen can be attributed to the NO pathway.

Our laboratory has demonstrated the importance of estrogen on the prostaglandin H synthase (PGHS) pathway (9, 30). Our data indicated that estrogen suppresses PGHS-dependent vasoconstriction in an ovariectomized rat model (9). However, in this study as well as in previous studies regarding estrogen replacement in ovariectomized animals, studies have largely been conducted using young adult animal models (5, 14, 16, 33). Importantly, the physiological processes due to aging (which is important relative to effects on post-menopausal women) are not taken into account with this model. For instance, the aging process contributes to enhanced oxidative stress on the vasculature, which could lead to further scavenging of NO (reduced bioavailability) as well as resulting in enhanced peroxynitrite (3). Peroxynitrite reduces the expression and activity of prostacyclin synthase (6, 38), the enzyme that produces the vasorelaxant prostacyclin. Also, reactive oxygen species, such as superoxide, have been shown to enhance the formation of the inducible PGHS-2 through activation of the nuclear transcription factor NFκB in rat kidney (18) and in aging brain cells (21). More recently we have shown that

PGHS-2 protein expression is upregulated with aging in rat mesenteric arteries, and that vessel tone is increased via this eicosanoid pathway (29). However, the effect of estrogen-replacement on the PGHS pathway in an aged animal remains to be determined. Thus, we hypothesized that estradiol would enhance vasorelaxation in aged rats by reducing PGHS-2-dependent vasoconstriction.

Methods

Animal Model

Aged (n=10, 24 months) and young (n=6, 3 months) female Fisher rats were obtained from the National Institute for Aging (NIA). Aged animals were ovariectomized and given a placebo (Innovative Research of America) or 17 β-estradiol pellet (0.5mg/pellet/60 day release, Innovative Research of America) subcutaneously one month before experimentation. Ovariectomy was performed in order to control for the large variability in estrogen levels that is characteristic of animals approaching reproductive senescence (constant estrous). Ultimately our study was designed to assess the effect of exogenous estrogen in the aging vasculature. Intact young animals were used as a reference point to determine if estrogen replacement in aged animals was restorative of vascular function. Four weeks following ovariectomy, rats were sacrificed while under light anesthesia (sodium brietal, i.p. injection, 50mg/kg-body wt.). Plasma samples were obtained by heart puncture and subsequent centrifugation. Samples were frozen at –80°C for later measurement of 17 β-estradiol levels using a double antibody radioimmunoassay kit (Diagnostic Products Corp.). Animal protocols were approved by the University of Alberta Animal Welfare Committee, following guidelines outlined by the Canada Council on Animal Care.

Vessel Preparation

A portion of mesentery was rapidly excised and immersed in ice-cold *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]-buffered physiological saline solution (HEPES-PSS). The HEPES-PSS contained (in mmol/L): NaCl 142, KCl 4.7, $MgSO_4$ 1.17, Ca_2Cl 1.56, KH_2PO_4 1.18, HEPES 10, and glucose

5.5. Resistance-sized mesenteric arteries (~ 250 μm in diameter) were dissected from surrounding adipose tissue, cut into 2mm lengths, and threaded with 20μm thick wires. The wires were fastened to polyacrylamide blocks connected to the isometric myograph system (Kent Scientific Corp.). Arteries were mounted in four glass-jacketed organ baths and maintained at 37°C in HEPES-PSS. Each vessel was set at a passive tension of $0.8L_{100}$, the point on the curve that provides maximum active force with minimum passive tension. Force production was recorded on a data acquisition system (Workbench, Strawberry Tree Inc.).

Experimental Design

Phenylephrine was administered in the initial dose-response curves to determine the concentration needed for 50% maximal constriction (EC_{50}) of each segment. This EC_{50} was added in subsequent curves to obtain a preconstriction base line from which vasorelaxation curves could be measured. Cumulative doses of methacholine were added (1 nmol/L to 1 μmol/L) to assess endothelium-dependent relaxation. Inhibitors of NOS (L-NMMA, 100 μmol/L) and PGHS (Meclofenamate, 1 μmol/L; PGHS-1: Valeryl Salicylate, 3 mmol/L; and PGHS-2: NS-398, 10 μmol/L; Cayman Chemical Co., Ann Arbor, MI, USA) were incubated in different baths for 15 minutes before the curves. The effect of repeating curves and time controls were incorporated into the experimental protocol. Between curves, a washout period of 30 minutes was maintained, with fresh HEPES-PSS buffer being added every 10 minutes.

Western Immunoblot

Rat aortas were harvested and homogenized. Protein concentrations were measured using the Bradford protein assay (4). Western immunoblots were performed as described before using antibodies for eNOS, PGHS-1, PGHS-2 and α-actin (rabbit polyclonal anti-eNOS, Santa Cruz Biotechnology, Inc.; mouse polyclonal anti-PGHS-1 and anti-PGHS-2, Cayman Chemical Co.; monoclonal anti-α-actin, Boehringer Mannheim.) (8).

Data Analysis

Data from each dose-response curve was fitted to the Hill equation, and a straight line generated by linear least-squares regression analysis. EC_{50} was determined from this line and the mean ± SE calculated from the curves. ANOVA was used for statistical analysis. Post hoc analysis was performed using Tukey's test. Western immunoblots of protein expression were analyzed with a Student's *t* test. Tests with values of $P<0.05$ were considered significant.

Results

Plasma estradiol levels were significantly higher in the estrogen-replaced rats compared to ovariectomized controls (99.7 ± 27.9 vs. 3.80 ± 2.02 pg/ml, $P<0.05$). Body weights were reduced in aged, estrogen-replaced rats compared to the aged, placebo-treated rats (231.8 ± 1.8 vs. 285.5 ± 8.3 g, $P<0.05$).

Phenylephrine elicited a similar dose response in both aged groups as well as in the young reference group ($P=0.660$). Methacholine-induced relaxation of preconstricted mesenteric arteries was blunted in the aged placebo group whilst the aged estrogen-replaced group restored relaxation to the level seen in the young animals, as evidenced by their respective EC_{50} values: Aged (placebo): 0.28 ± 0.07 μmol/L, Aged +Estrogen (E2): 0.05 ± 0.009 μmol/L, Young: 0.05 ± 0.01 μmol/L ($P<0.05$; Fig. 2.1).

To assess potential mechanism(s) for the effect of estrogen on the aging vasculature, methacholine relaxation curves were repeated in the presence of inhibitors to NOS and PGHS activity. Preincubation with the pharmacological inhibitors did not alter the resting baseline tension of the arteries. Surprisingly, NOS inhibition with L-NMMA significantly enhanced the relaxation to methacholine in the Aged group but did not alter relaxation in the Aged+E2 group ($P<0.05$ & $P=0.667$; respectively. Fig. 2.2A). Therefore, estrogen did not enhance NO-dependent relaxation. However, PGHS inhibition with meclofenamate restored relaxation in the arteries of the Aged animals ($P<0.05$; Fig. 2.2B), while little change was observed in arteries from the Aged+E2 rats (Fig. 2.2B), suggesting that estrogen replacement prevented PGHS-dependent

constrictor modulation of vascular function that was observed in the ovariectomized controls.

The role of each PGHS isoform was investigated to determine if one had a more predominant effect. Both PGHS-2 inhibition with NS-398 and PGHS-1 inhibition with valeryl salicylate significantly enhanced vasorelaxation in the Aged, placebo group ($P<0.05$; Fig. 2.3A & 2.3B respectively), while estrogen prevented the PGHS-dependent constrictor modulation of vascular function (Fig. 2.3A & 2.3B). To compare the relative effects of PGHS-1 and PGHS-2 in the Aged group, we assessed the delta change between the EC_{50} for methacholine relaxation alone and with the specific inhibitors. PGHS-2 inhibition evidenced a 91 ± 3% reduction in the methacholine EC_{50}, while PGHS-1 inhibition was found to reduce the EC_{50} by 76 ± 7% ($P<0.05$).

We have previously reported a specific increase in PGHS-2 expression in mesenteric arteries of aged, Sprague Dawley rats (29). In the present study, age-induced expression of PGHS-2 was significantly reduced in aortas from the Aged+E2 group compared to the Aged group ($P<0.05$; Fig. 2.4A). There was no significant change in PGHS-1 expression (Fig. 2.4B) or eNOS expression (Fig. 2.5) between both aged groups.

Discussion

Previous studies using ovariectomized young (3-6 months old) rats to assess the effect of exogenous estrogen on the vasculature have revealed a role for NO (14, 16, 33) as well as the PGHS pathway (5, 9). However, in aging the relative importance of these pathways may be altered. Our model of ovariectomized, aged rats indicates that estrogen replacement suppressed PGHS-dependent vasoconstriction but did not enhance NO-mediated relaxation. Although specific inhibition of either PGHS-1 or PGHS-2 enhanced relaxation in the aged, placebo-treated animals, there was a greater effect with PGHS-2 inhibition. Moreover, only PGHS-2 protein expression was reduced with estrogen replacement.

In young animals, estrogen enhances expression of NOS (32, 33) and NO-dependent relaxation (32, 33) in a variety of vascular beds. However, in conditions of aging and oxidative stress, the effect of estrogen through the NO pathway may be reduced due to the scavenging of NO by free radicals (17). Indeed, aging has been shown to affect human vasculature by decreasing the inhibitory effect of L-NMMA (NO blocker) in acetylcholine-induced forearm dilatation (31). In our study assessing mesenteric arteries from aged rats, there was actually enhanced relaxation with NO inhibition. We speculate that in the absence of NO, superoxide production in the aged vasculature is being converted to H_2O_2, a vasorelaxant. Ultimately, estrogen replacement did not enhance NO-mediated relaxation in the small mesenteric arteries of the aged animal and eNOS expression in the aorta remained the same between both aged groups. Therefore, in aging, the actions of estrogen on the PGHS pathway may become more predominant. Our previous findings in young rats demonstrated that chronic estrogen replacement inhibited PGHS-dependent constriction that occurred in ovariectomized Sprague-Dawley rats (9). In agreement with our data, ovariectomized spontaneous hypertensive rats similarly restored altered endothelium-dependent responses with estradiol as well as with indomethacin and sodium diclofenac (non-selective PGHS inhibitors) (7). Together these data indicate a role for estrogen to inhibit PGHS-dependent vasoconstriction, however, differences in rat strains may limit the ability to compare data among the studies.

With aging and oxidative stress it has previously been shown that the inducible PGHS-2 is upregulated (18, 21, 29). Moreover, our lab reported that PGHS-2-dependent vasoconstriction is increased with age that was associated with increased PGHS-2 expression (29). Furthermore, oxidative stress in the form of reactive oxygen intermediates (11) can induce PGHS-2 via the redox-sensitive factor, NFκB (18). However, little is known about the effect of estrogen on PGHS-2 expression and activity within arteries.

Estrogen has been found to decrease PGHS-2 expression in a number of cell types, including bovine endometrial cells (35) and bovine chondrocytes

(26). Our data also indicate that *in vivo*, estrogen decreases arterial PGHS-2 expression. In contrast, estrogen has also been found to increase PGHS-2 expression in sheep (34) and rat myometrium (10) as well as in human umbilical vein endothelial cells (1). These discrepancies indicate a difference in the effects of estrogen depending on its serum concentration and could be attributed to the reproductive condition of the animal and/or the vascular bed being studied.

The level of PGHS expression, however, does not necessarily translate to product formation and, ultimately, effects on function. Estrogen has been shown to decrease PGHS-dependent products in bovine microvascular endothelial cells (30). A recent study using rabbit uterine cervical fibroblasts showed that 17 β-estradiol suppressed the levels of PGE_2 that were previously augmented by interleukin-1α (27). Furthermore, indomethacin and NS-398 similarly suppressed the PGE_2 levels (27). Our results are in agreement with these data since estrogen-replacement suppressed PGHS-dependent vasoconstrictor products. Both meclofenamate and NS-398 incubation showed no significant change in arterial relaxation of estrogen-replaced rats but elicited a marked change in vasorelaxation of placebo-treated aged rats, restoring it to the level observed with estrogen replacement. A significant increase in relaxation responses was also observed with specific PGHS-1 inhibition in the placebo-treated group. This is in agreement with a recent study that implicated both PGHS-1 and PGHS-2 in age-associated endothelial dysfunction of male rat aortic rings (15). However, the enhanced relaxation with PGHS-1 inhibition in our study did not reach the level seen in the estrogen-replaced group. Moreover, the delta change determined between methacholine-induced relaxation in the presence of PGHS-2 inhibition was significantly greater than the change found with PGHS-1 inhibition. Furthermore, there was no difference in PGHS-1 protein expression between groups. Thus, the defining difference between the vasoreactivity of the two aged groups of animals is likely due to the constrictor eicosanoids produced by PGHS-2.

Our data indicate that PGHS was the predominant pathway for the effect of estrogen on vascular function in mesenteric arteries in the aged rat model. These actions of estrogen could be due to an increase sensitivity of muscarinic receptors with estrogen replacement. In addition, it is interesting to note that methacholine-mediated relaxation in rat mesenteric arteries is not greatly modulated by NO (24, 37), therefore other pathways other than NO may predominate. We observed that estrogen reduced PGHS-dependent vasoconstriction, however estrogen may affect other vasoactive pathways such as endothelial-derived hyperpolarizing factor (EDHF). Very little work has been done to assess the combined effects of estrogen and aging on arterial relaxation mediated by EDHF. One study of young female Wistar rats investigated the effects of estrogen deficiency on EDHF-mediated relaxation in mesenteric arteries (20). The reduction in endothelial-dependent relaxation was attributed to a diminished EDHF response in the estrogen-deficient rats (20). Furthermore, in aging, the EDHF-dependent portion of endothelial-dependent relaxation has been shown to be reduced (12). Indeed, this may be explained by the reduction in the number of voltage and Ca^{2+}-activated K^+ channels as has been observed in coronary arteries from old male F344 rats (23). As such, both aging and ovariectomy will reduce the EDHF-mediated response. Whether estrogen replacement is restorative of this response in aging will need to be determined.

In conclusion, our results demonstrate that estrogen can reduce the vasoconstriction associated with aging by suppressing PGHS-dependent vasoconstriction. Moreover, PGHS-2 is the predominant isoform affected by estrogen. Consequently, PGHS-2 inhibition is one possible alternative to estrogen replacement that could afford a direct vascular benefit in the aged population.

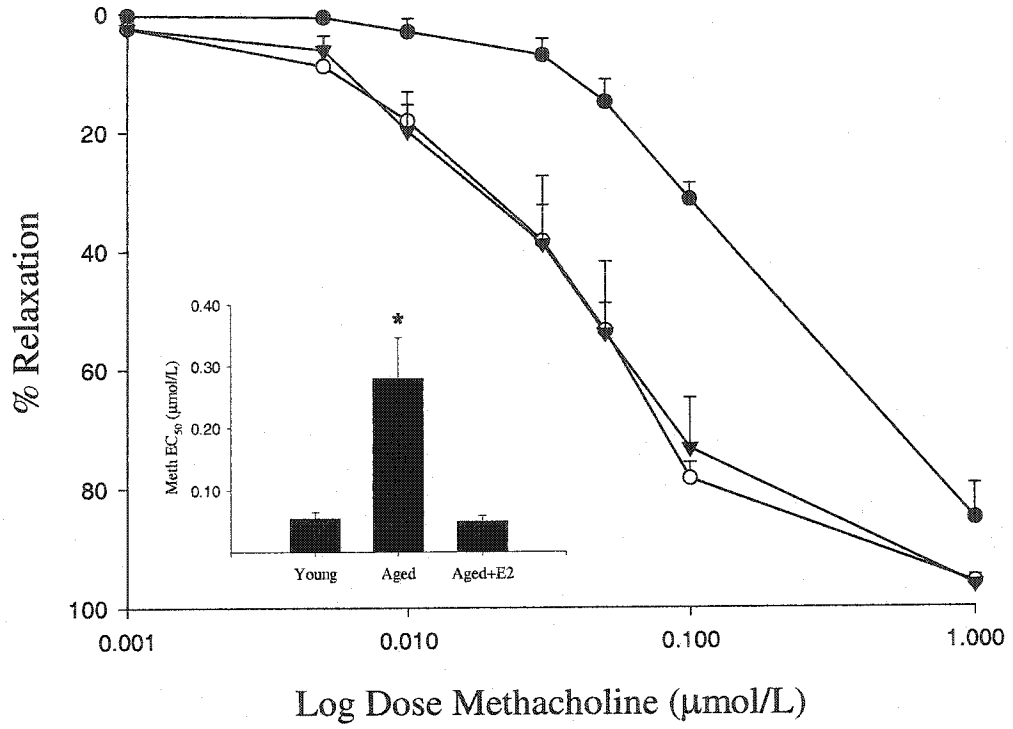

Figure 2.1 Concentration-response curves to methacholine in mesenteric arteries of ovariectomized, aged placebo-treated rats (n=6, filled circles), aged estrogen-replaced rats (n=4, triangles), and intact young rats (n=6, open circles). *Inset*: EC_{50} values of arteries from Young, Aged (placebo-treated rats), Aged + E2 (estrogen-replaced, aged rats). Bars represent mean ± SE. * $P < 0.05$ vs. Young and Aged + E2.

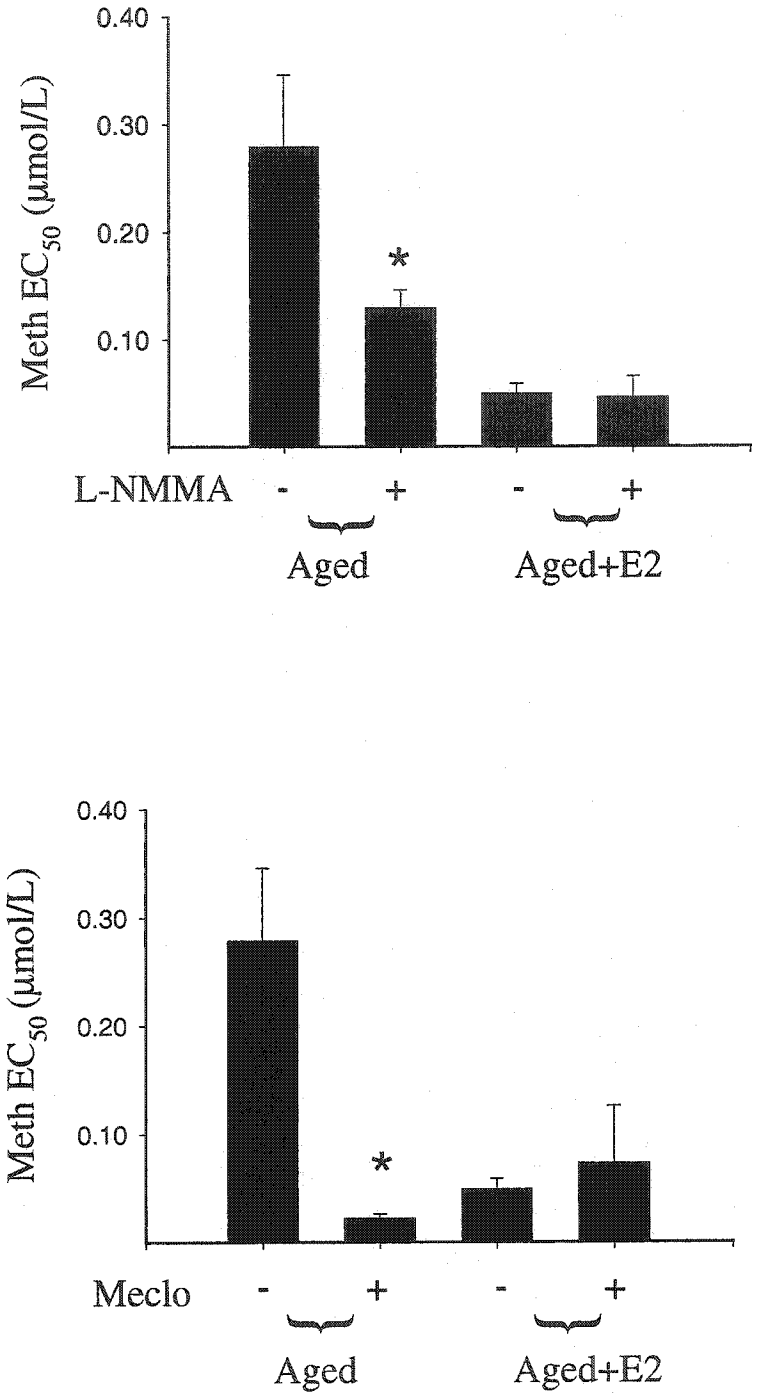

Figure 2.2 Effective concentration that produced 50% of the maximum response (EC_{50}) for methacholine in mesenteric arteries of Aged (n=6), and Aged + E2 (n=3-4) rats in the absence (-) and presence (+) of Top Panel - the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 100 µmol/L) or Bottom Panel - the non-selective PGHS inhibitor meclofenamate (Meclo, 1 µmol/L). Bars represent mean ± SE. * $P < 0.05$.

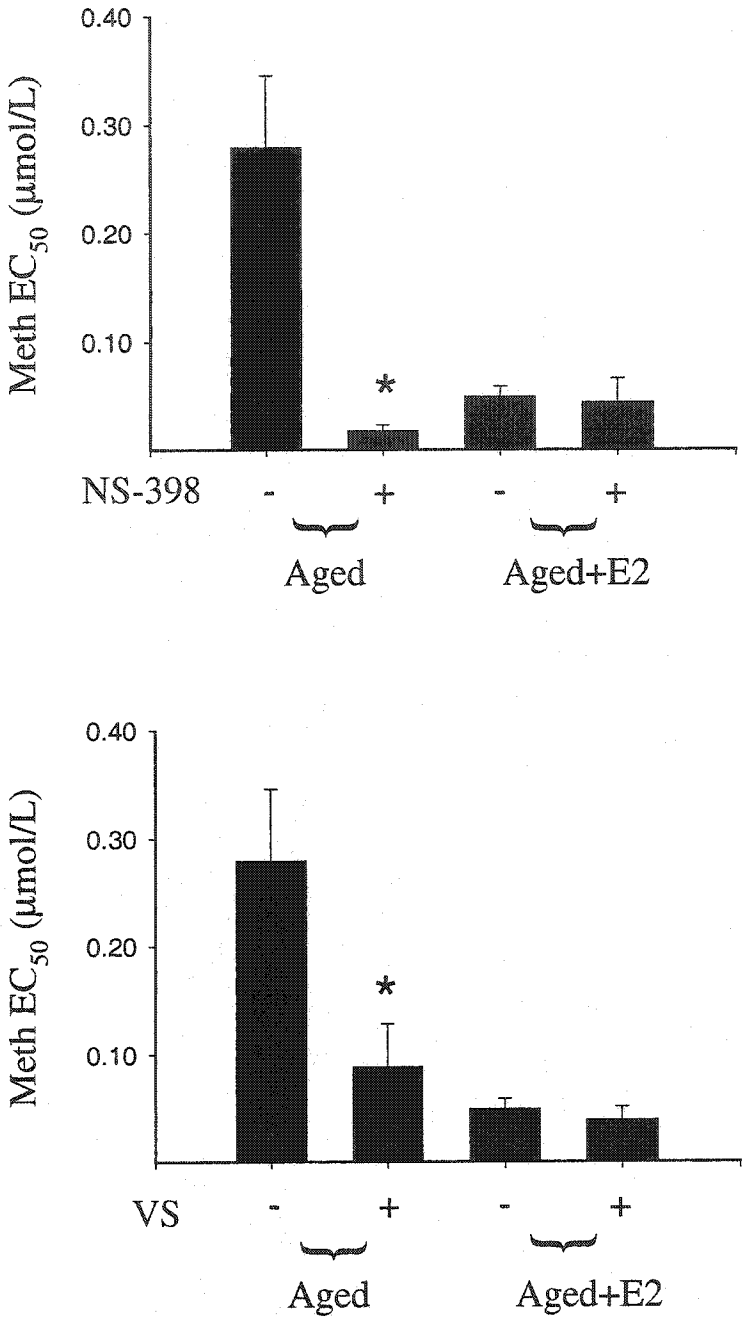

Figure 2.3 Methacholine EC_{50} values from mesenteric arteries of Aged (n=6), and Aged + E2 (n=3-4) rats. Top Panel is in the absence (-) and presence (+) of the PGHS-2 inhibitor NS-398 (10 µmol/L). * $P < 0.05$. Bottom Panel is in the absence (-) and presence (+) of the PGHS-1 inhibitor valeryl salicylate (VS, 3 mmol/L). Bars represent mean ± SE. * $P < 0.05$

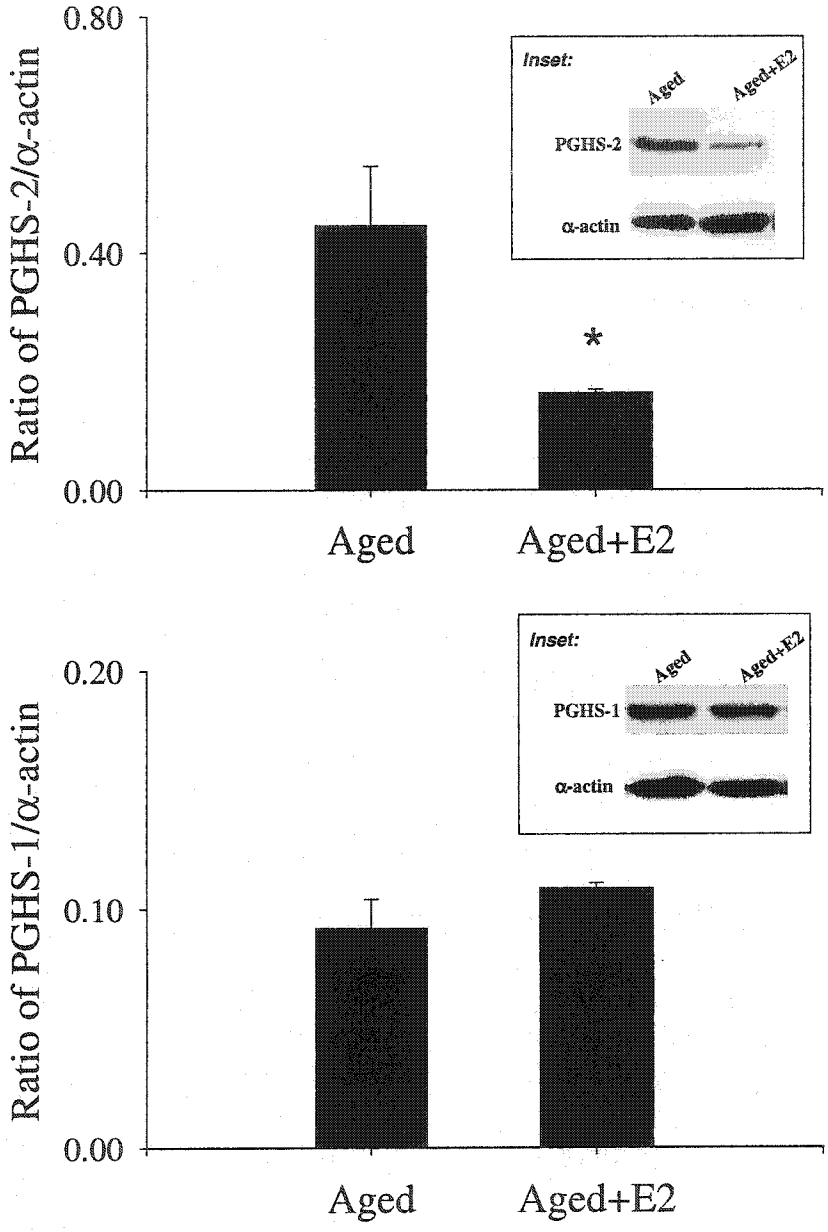

Figure 2.4 Top Panel - Western immunoblot summary graph for PGHS-2 in aorta from Aged (n=6) and Aged + E2 (n=4) rats. *Inset:* representative PGHS-2 blot. Bottom Panel - Western immunoblot summary graph for PGHS-1 in aorta from Aged and Aged + E2 rats. *Inset:* representative PGHS-1 blot. Bands were normalized to α-actin. Bars represent mean ± SE. * $P < 0.05$.

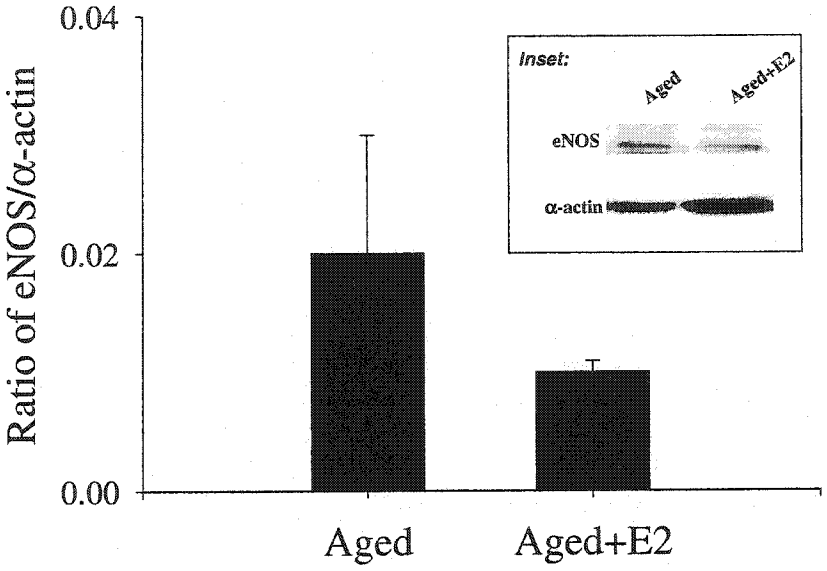

Figure 2.5 Western immunoblot summary graph for eNOS in aorta from Aged (n=6) and Aged + E2 (n=4) rats. *Inset:* representative eNOS blot. Bands were normalized to α-actin. Bars represent mean ± SE.

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CHAPTER 3: Effects of Chronic PGHS-2 Inhibition on PGHS-dependent Vasoconstriction in the Aged Rat

Introduction

Aging and estrogen deficiency are both risk factors for cardiovascular disease, the leading cause of death among postmenopausal women (28). Aging is associated with an increase in vasoconstriction due, in part, to alterations in the vascular endothelium (11). The enzyme prostaglandin H synthase (PGHS) has been shown to be important in this process. Two isoforms, PGHS-1 and PGHS-2, metabolize arachidonic acid to produce the intermediate PGH_2. Subsequently, eicosanoids such as prostacyclin and thromboxane are formed. Normally, prostacyclin, which elicits vasodilation, would balance or supercede the constriction promoted by thromboxane (5). In aging, however, the production of prostacyclin is reduced(4) and thromboxane is preferentially generated (23).

Prior to menopause, women are protected from age-related vascular dysfunction by the actions of estrogen (13). One effect of estrogen is to inhibit PGHS-dependent vasoconstriction in young, ovariectomized rats (9). Recently we demonstrated that PGHS-2 protein expression is upregulated with aging in female rat mesenteric arteries, and that vessel tone is enhanced through this pathway (27). Moreover, estrogen replacement in an ovariectomized, aged rat suppressed vasoconstriction dependent upon the PGHS-2 pathway (1). This was associated with a reduction in PGHS-2 protein expression in the vasculature from estrogen replaced animals (1). Estrogen has also been shown to reduce PGHS-2 mRNA expression in bovine chondrocytes (21). Since estrogen replacement is contraindicated for some women, specific PGHS-2 inhibition may be an alternative therapy to provide vascular benefits.

Selective PGHS-2 inhibitors have been developed to treat chronic inflammatory conditions while attempting to reduce the gastrointestinal toxicity observed with traditional NSAIDs (2, 24). The rationale behind selective PGHS-2 inhibition is to target those eicosanoids involved in the inflammatory response and bypass the prostaglandins that protect against gastric ulceration

(15). These drugs have been used successfully in conditions such as acute lung injury,(7) arthritis(26) and colon cancer (32). However, the chronic effects of PGHS-2 inhibitors on vascular responses in an aging model are not known.

These data led to the hypothesis that chronic PGHS-2 inhibition should facilitate enhanced vasorelaxation by reducing PGHS-dependent vasoconstriction in the aged, ovariectomized rat.

Methods

Animal Model

Female Sprague-Dawley rats were obtained from Charles River and were housed in our facilities until 11-12 months of age. Rats were ovariectomized (OVX) and given a placebo pellet (Innovative Research of America; n=7), or a daily subcutaneous injection of the PGHS-2 inhibitor, NS-398 (3 mg/kg), for one week (1 wk, n=7) or four weeks (4 wks, n=6) prior to experimentation. Ovariectomy was performed in order to control for the potential variability in estrogen levels that is characteristic of animals approaching reproductive senescence (constant estrous). The NS-398 dose was chosen based on effective PGHS-2 inhibition from previous studies in the rat (19). Rats were killed by exsanguination while under anesthesia (sodium pentobarbitol, ~60 mg/kg-body wt.). A blood sample was taken and serum obtained by centrifugation. To confirm a successful ovariectomy, samples were snap-frozen (–80°C) for subsequent measurement of 17 β-estradiol levels. Animal protocols were approved by the University of Alberta Animal Welfare Committee.

Vessel Preparation

A portion of the mesentery was excised and immersed in ice-cold *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]-buffered physiological saline solution (HEPES-PSS) which contained the following (in mmol/L): NaCl 142, KCl 4.7, $MgSO_4$ 1.17, Ca_2Cl 1.56, KH_2PO_4 1.18, HEPES 10, and glucose 5.5. Resistance-sized arteries (diameter ~ 250 μm) were dissected and connected to the myograph system (Kent Scientific Corp.) as previously described (1). Force production was recorded on a data acquisition system (Workbench, Strawberry Tree Inc.).

Experimental Design

Phenylephrine was administered in the initial curves to determine the concentration needed for 50% maximal constriction (EC_{50}) of each segment. This provided a preconstriction base line from which vasorelaxation curves could be measured. Sodium nitroprusside (1nmol/L to 1μmol/L) was used to assess endothelium-independent relaxation. Methacholine was added (1nmol/L to 1μmol/L) to assess endothelium-dependent relaxation. The PGHS inhibitor, meclofenamate (1μmol/L; Cayman Chemical Co., Ann Arbor, MI), and the stable PGH_2 analogue, U-51605 (50μmol/L; Cayman Chemical Co., Ann Arbor, MI), which is capable of inhibiting PGH_2- and thromboxane-dependent constriction, were incubated in different baths, 15 minutes before various curves, to study the functional significance of eicosanoids in these arteries. This design enabled us to determine whether or not chronic PGHS-2 inhibition was successful in the animals and/or if it altered PGHS-dependent vascular effects. We hypothesized that the rats that were administered chronic NS-398 treatment would have little or no change in vascular function with addition of the PGHS inhibitor to the bath.

Western Immunoblot

Mesenteric arteries were dissected and homogenized in eppendorf tubes (containing a protease inhibitor cocktail to inhibit serine, cysteine, and aspartic proteases to prevent degradation; Sigma, Saint Louis, Missouri) using a small tissue homogenizer. The Bradford assay was employed to measure protein concentration (3). Western immunoblots were performed as previously described (8). Polyclonal antibodies for PGHS-2 and monoclonal antibodies for α-actin (Cayman Chemical Co. and Boehringer Mannheim, respectively) were used. Bands were quantified by densitometric analysis and normalized to α-actin.

Data Analysis

Data from each dose-response curve was fitted to the Hill equation, and a straight line generated by linear least-squares regression analysis. EC_{50} was determined from this line and the mean ± SE calculated from the curves.

ANOVA was used for statistical analysis among groups as well as for Western immunoblot bands. Post hoc analysis was performed using Tukey's or Student-Newman-Keuls tests. A Student's *t* test was used to compare EC_{50} between two groups. Tests were considered significant at $P<0.05$.

Results

Body weights were not significantly different among the groups. The plasma estradiol levels were below <5pg/ml (detectable limit of assay) in all animal groups.

Contrary to our hypothesis, methacholine-induced relaxation was not significantly altered among all the groups. However, the influence of PGHS in modifying vascular responses varied between the groups. Meclofenamate (PGHS inhibitor) enhanced methacholine-induced relaxation in the placebo, aged group (Fig. 3.1A, $P<0.05$) but did not alter relaxation in the one week treatment group (Fig. 3.1B, $P=0.733$), suggesting the presence of PGHS-dependent vasoconstriction in the aged rat which could be prevented by chronic PGHS-2 inhibition for one week. Interestingly, in the four week treatment group, meclofenamate enhanced relaxation (Fig. 3.1C, $P<0.05$), suggesting the reoccurence of PGHS-dependent vasoconstriction. Inhibition of downstream eicosanoids with the stable PGH_2 analogue, U-51605, had similar effects to meclofenamate (Fig. 3.2), suggesting that PGH_2/thromboxane is involved in the PGHS-dependent constriction.

Phenylephrine elicited a similar dose response in all groups. In the presence of the PGHS inhibitor there was little effect in the placebo group, although there was a trend towards an increase in the dose required to elicit 50% constriction with the drug (Fig. 3.3A, +Meclo $P=0.126$). The one week treated group exhibited a significant increase in EC_{50} with meclofenamate in the bath (Fig. 3.3B, +Meclo, $P<0.05$), indicating a small role for PGHS-dependent constriction with one week of chronic PGHS-2 inhibition. Four weeks of NS-398 treatment also revealed a significant increase in EC_{50} for phenylephrine after PGHS inhibition in the bath (Fig. 3.3C, +Meclo, $P<0.05$). The increase in

PGHS-dependent constriction in the four week group was the most marked, however, and observed in both the phenylephrine and methacholine curves further supporting an enhancement of the PGHS vasoconstrictor pathway over time with chronic PGHS-2 inhibition.

In support of this, Western immunoblots revealed a time-dependent increase in PGHS-2 expression between the one and four week treatment with the PGHS-2 inhibitor (Fig. 3.4, $P<0.05$).

Discussion

Our data indicate that although the PGHS-2 pathway impairs vascular function in the aging rat model, long term use of specific PGHS-2 inhibitors may not have vascular benefits. Chronic inhibition of PGHS-2 with NS-398 paradoxically caused an increase in PGHS-2 expression and PGHS-dependent vasoconstriction in arteries from the aged, ovariectomized rat.

A similar feedback effect has been observed in studies using aspirin. Aspirin administered orally to rats induced a significant increase in PGHS-2 mRNA and protein expression in the superficial mucosa of the stomach.(10) Furthermore, aspirin enhanced PGHS-2 expression between 30 minutes and 5 hours when administered every 30 minutes in placental cytotrophoblasts.(17) In contrast, aspirin given immediately prior to lipopolysaccharide challenge suppressed PGHS-2 mRNA expression in murine peritoneal macrophages.(31) These studies indicate a time-dependent response for the actions of aspirin on PGHS-2 levels. Our study also demonstrated a time-dependent feedback response to induce the PGHS-2 enzyme in animals given a specific PGHS-2 inhibitor.

Contrary to our hypothesis, chronic PGHS-2 inhibition did not enhance endothelium-dependent relaxation nor did it blunt adrenergic vasoconstriction in the aged rat. However, the role of PGHS-dependence within the vascular responses was shifted among the treatment groups. As previously reported, the PGHS-dependent portion of the age-related constriction was substantial in the ovariectomized, placebo-treated animals.(1) Moreover, one week of PGHS-2 inhibition abolished this PGHS-sensitive component of methacholine relaxation,

albeit with a small role still detectable in phenylephrine constriction. Interestingly, however, an extended four week period of chronic treatment markedly enhanced the PGHS-dependent portion of the constriction, shifting the balance back towards the placebo controls.

PGHS-2 is capable of producing both vasodilator and vasoconstrictor prostanoids. The ability of the enzyme to elicit a downstream effect is largely dependent upon the activity of terminal synthases, such as prostacyclin or thromboxane synthase. Some reports have concluded that PGHS-2 inhibition may be mediating its vascular effects by blocking prostacyclin production.(4, 16, 25) However, in the female aging rat model, PGHS-dependent constriction predominates. Indeed, our data revealed an enhanced relaxation to methacholine and a blunted constriction to phenylephrine after non-selective PGHS blockade with meclofenamate. Furthermore, inhibition of PGH_2/thromboxane with U-51605 demonstrated a similar increase in methacholine-induced relaxation, which was suggestive of a role for these constrictor eicosanoids in the placebo and four-week treated animals. Prostacyclin exhibited a minimal role in this modulation. This could be due to the nitration and inactivation of prostacyclin synthase by peroxynitrite (the cellular toxin produced by the reaction between nitric oxide and superoxide).(33) Indeed, vascular aging has been shown to be associated with enhanced peroxynitrite formation(29) and our lab demonstrated that peroxynitrite decreased the protein mass of prostacyclin synthase in bovine microvascular endothelial cells.(6) Interestingly, peroxynitrite has been shown to induce PGHS-2 in human endothelial cells.(12) Ultimately, a feed forward loop could occur whereby there is an increased production of PGHS-2 (which itself can be a source of superoxide anion) leading to further peroxynitrite production, inactivation of prostacyclin synthase and hence PGHS-2-dependent constriction.

Interestingly, although there was difference in modulation by PGHS-dependent constrictors among the groups, relaxation to methacholine was not different. We speculate that the enhanced PGHS-dependent constriction may be compensated for by a concomitant increase in an undetermined vasodilator

pathway. Indeed, there could be an enhancement of vasodilation due to other downstream effects on arachidonate metabolism. In rat liver cells, the selective PGHS-2 inhibitor, celecoxib, and the nonselective PGHS inhibitor indomethacin stimulated the release of arachidonic acid at micromolar concentrations.(18) This was after 6 hours of incubation of the drugs and it is quite possible that extended time periods of incubation with other NSAIDs may mimic the response. As such, our chronic treatment with the PGHS-2 inhibitor, NS-398, may have predisposed the vasculature to release arachidonic acid. Since our in vitro protocol involved inhibiting PGHS activity, the available arachidonic acid could be shunted down an alternative pathway, such as lipoxygenase or cytochrome P450. Indeed, recent work in rat mesenteric and bovine coronary arteries revealed arachidonic acid-induced vasodilation dependent upon downstream lipoxygenase metabolites (eg.12-HETE),(20) or epoxygenase products (eg.14,15-EET)(14) respectively. This would need to be verified in our aging model. Whether or not these mechanisms occur in humans, particularly in aging, remains to be determined.

Recently, two randomized trials were performed on large populations (>8000) to assess the efficacy of the PGHS-2 inhibitors, celecoxib and rofecoxib (Vioxx), on gastrointestinal and other outcomes. These were the Celecoxib Long-term Arthritis Safety Study (CLASS) (24) and the Vioxx Gastrointestinal Outcomes Research (VIGOR) Study.(2) For cardiovascular outcomes, the CLASS study showed no significant change in events such as myocardial infarction between celecoxib and the traditional NSAIDs.(22) In contrast, the VIGOR study demonstrated a significant increase in the relative risk of cardiovascular events between rofecoxib and the non-selective PGHS inhibitor, naproxen.(22) Furthermore, a meta-analysis between these studies and a placebo group revealed an increase in myocardial infarction rates with both PGHS-2 inhibitors.(22) Interestingly, more patients in the VIGOR study developed hypertension with rofecoxib treatment compared with naproxen treatment.(22) By contrast, a study of forearm blood flow response to acetylcholine in young, healthy humans given either rofecoxib or naproxen for

one week revealed no significant changes in endothelial-dependent vasodilation.(30) Indeed, our data indicated no change in vasodilation to methacholine between the aged, PGHS-2 inhibited (1 week or 4 week) and aged control animals as well as little PGHS-dependent vasoconstriction in the one week group. However, our data indicates a paradoxical increase in PGHS-dependent vasoconstriction with prolonged PGHS-2 inhibition.

Perspectives

Our study revealed direct vascular effects after chronic inhibition of PGHS-2 with a selective inhibitor. Importantly, these findings provide a possible mechanism for the adverse vascular function. Moreover, the increased expression of PGHS-2 in the resistance vasculature may be a mechanism for the hypertension observed in the VIGOR study.(2) That is, chronic PGHS-2 inhibition appears to have a detrimental feedback effect on vascular function leading to greater PGHS-dependent constriction. Further studies to understand the feedback effects of aspirin and PGHS-2 inhibitors will be important to understand the long-term effects of these widely used drugs. We speculate that the aging population will be contraindicated for selective PGHS-2 inhibition. Moreover, since chronic PGHS-2 inhibition is currently used in cancer therapy and for treatment of inflammatory conditions, caution needs to be observed regarding the vascular effects. Hence, PGHS-2 inhibitors may not be the panacea for reducing vascular complications in conditions where PGHS-dependent constriction predominates.

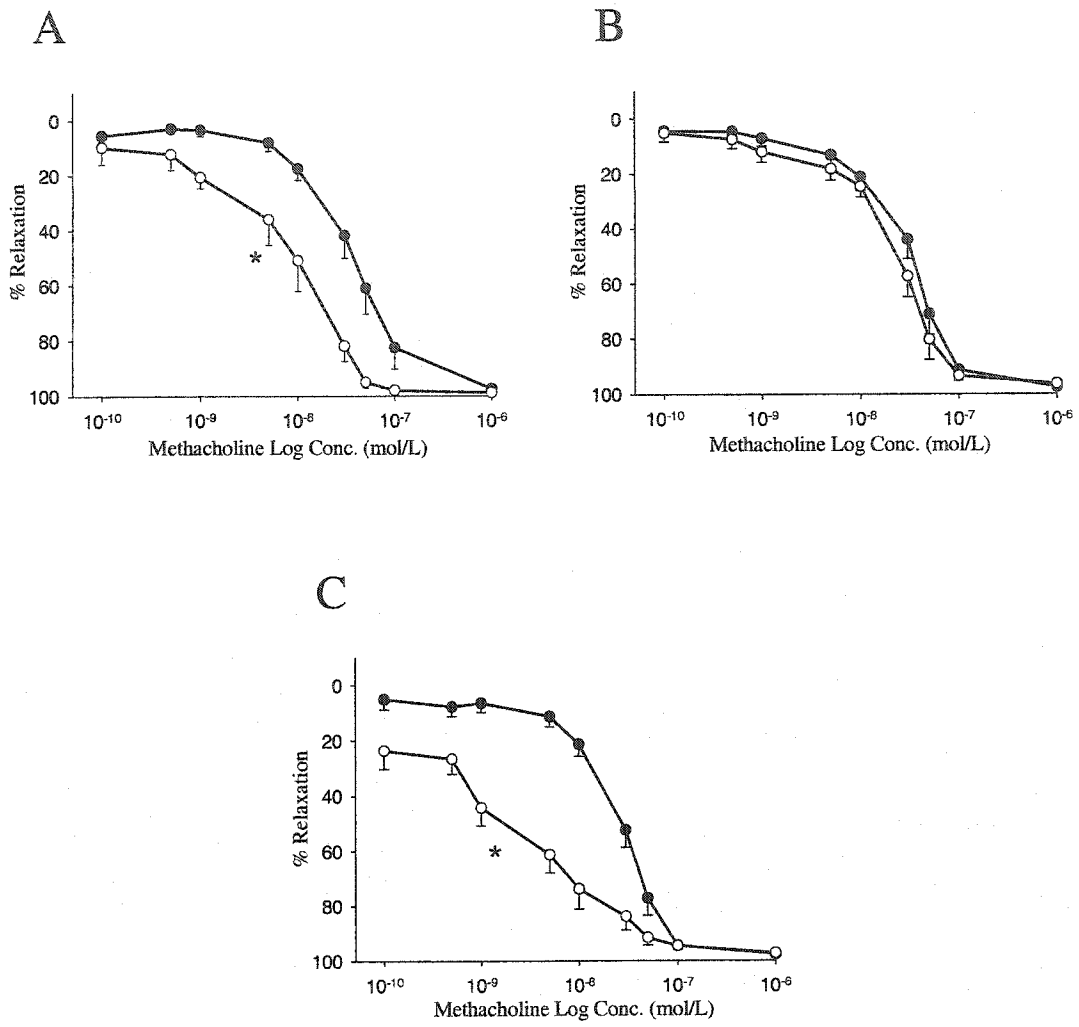

Figure 3.1 Methacholine concentration response curves from mesenteric arteries of aged, ovariectomized rats. Panel A- Placebo (n=7), Panel B- *in vivo* PGHS-2 inhibition (NS-398, 3 mg/kg) for 1 week (n=7), Panel C- *in vivo* PGHS-2 inhibition for 4 weeks (n=6) in the absence (•) and presence (o) of 1 μmol/L Meclofenamate (a non-selective PGHS inhibitor) in the bath. *EC_{50} $P<0.05$ vs. methacholine alone

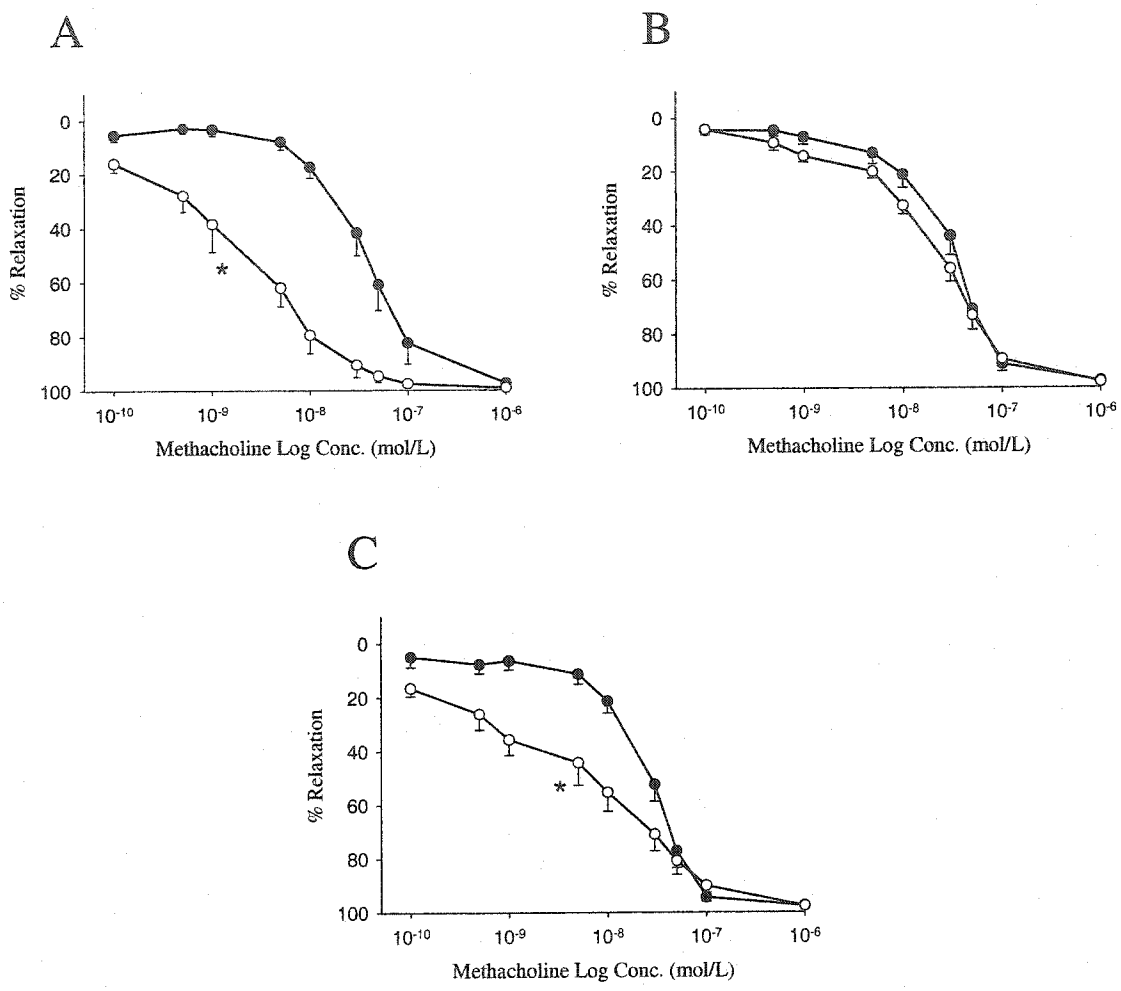

Figure 3.2 Methacholine concentration response curves from mesenteric arteries of aged, ovariectomized rats. Panel A- Placebo (n=7), Panel B- *in vivo* PGHS-2 inhibition (NS-398, 3 mg/kg) for 1 week (n=7), Panel C- *in vivo* PGHS-2 inhibition for 4 weeks (n=6) in the absence (•) and presence (o) of 50 μmol/L U-51605 (a stable PGH_2 analogue/antagonist) in the bath. *EC_{50} $P<0.05$ vs. methacholine alone

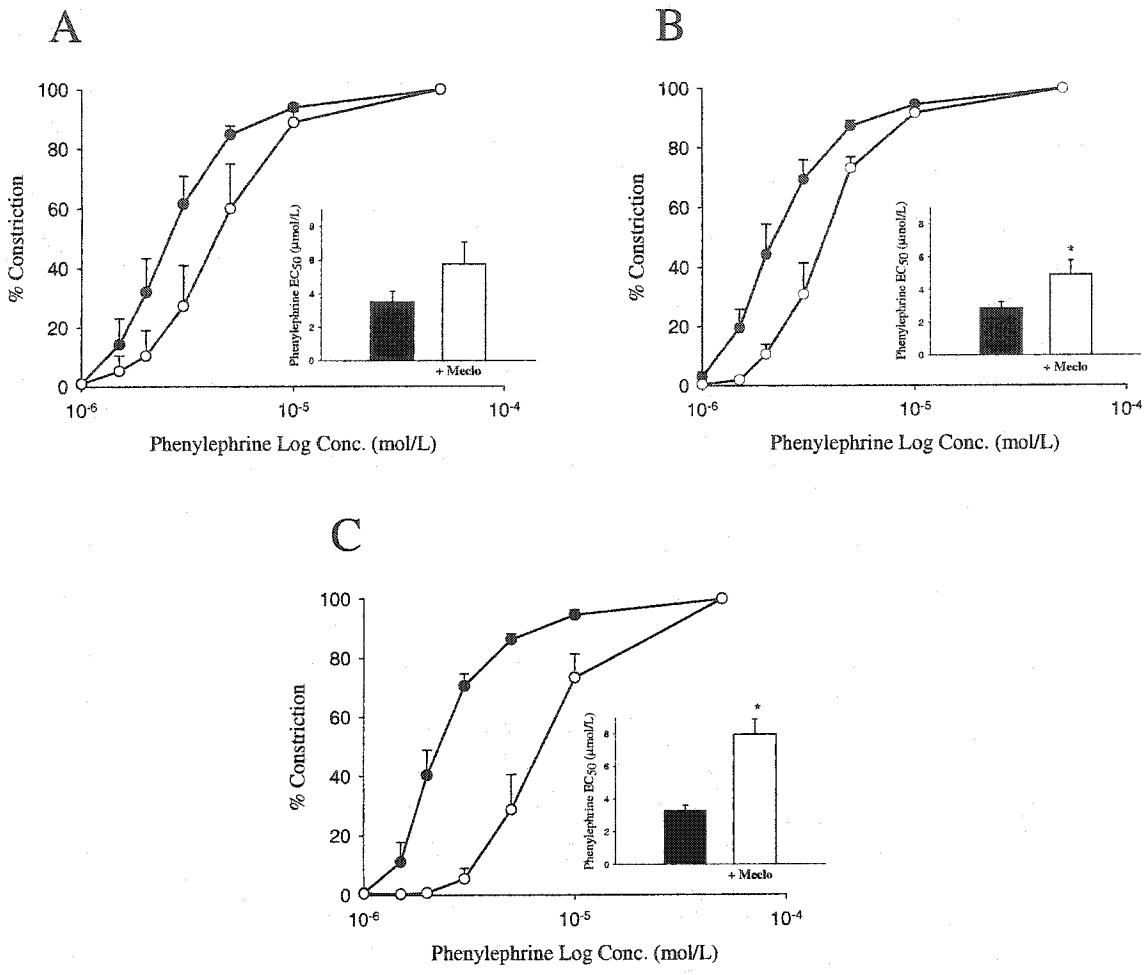

Figure 3.3 Phenylephrine concentration response curves and EC_{50} bar graphs from mesenteric arteries of aged, ovariectomized rats. Panel A - Placebo (n=5-7), Panel B - *in vivo* PGHS-2 inhibition for 1 week (n=5-7), Panel C - *in vivo* PGHS-2 inhibition for 4 weeks (n=6), in the absence (•) and presence (o) of meclofenamate (Meclo, 1 μmol/L). * EC_{50} $P<0.05$

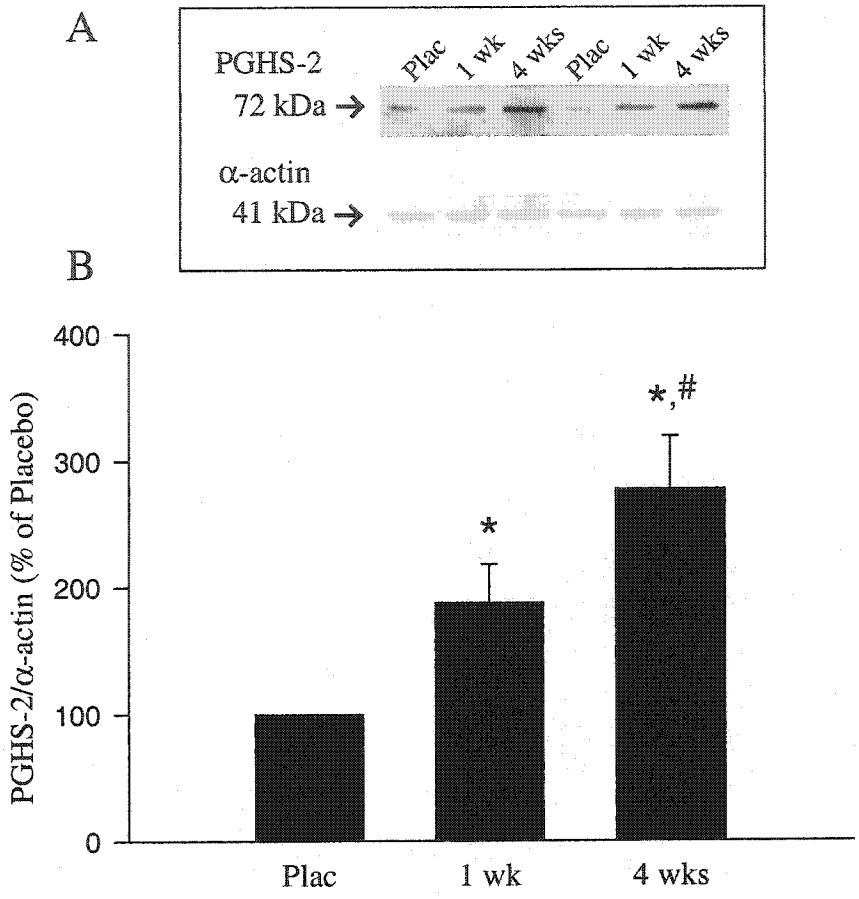

Figure 3.4 Panel A- Representative Western blot for PGHS-2 expression in mesenteric arteries from aged, ovariectomized placebo-treated rats (n=7), and aged, ovariectomized rats given the PGHS-2 inhibitor for 1 (n=7) or 4 weeks (n=6). Panel B- Ratio of the densitometric analysis of PGHS-2 bands normalized to α-actin. Bar and error represents the mean + SEM of samples. *P<0.05 vs. placebo, #P<0.05 vs. 1 wk

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CHAPTER 4: General Discussion

Estrogen's role as a cardiovascular protective agent, particularly in aging, is still unresolved. Whether or not this hormone is of benefit to the aging cardiovascular system might be dependent on the dose and conditions by which it is administered. If this is the case, the detrimental effects observed in the most recent epidemiological studies could be attributed to the amount of estrogen as well as the physiologic state of each individual. As such, the effects of estrogen on the aged rat vasculature provides specific information on this important subject. The aim of this thesis was to assess the actions of estrogen on the PGHS pathway in mesenteric arteries from aged rats. As well, the role of chronic PGHS-2 inhibition as an alternative to estrogen replacement was determined in an aging model.

Aging, in the cardiovascular system, is partly evidenced as altered vascular endothelial cell function. Within blood vessels, oxidative stress contributes to aging through the modification/induction of proteins (20), such as PGHS-2. Indeed, aging has been shown to increase vasoconstriction by enhancing the production of vasoconstrictor products from the PGHS-2 pathway.

Previous work in our lab determined the role of PGHS-2-dependent vasoconstriction in the aging female rat (21). Moreover, it was demonstrated that estrogen could reduce vasoconstriction dependent upon PGHS in mesenteric arteries from 3 month old ovariectomized rats (8) and another study revealed that estrogen decreased PGHS products in bovine endothelial cells (22). These findings led us to our first hypothesis: estrogen replacement decreases PGHS-2-dependent vasoconstriction in the aged rat.

Paper 1- Estrogen Reduces Vascular PGHS-2

Our results indicated a beneficial response to estrogen replacement within the arteries from the aged rat, as evidenced by a vasodilation similar to that observed in three month old animals. Blunted relaxation was restored by the PGHS-2 inhibitor, NS-398, in isolated mesenteric arteries from placebo-

treated aged animals. The enhanced relaxation observed with estrogen replacement was not increased further by PGHS-2 inhibition in the bath. Moreover, PGHS-2 protein levels were reduced with estrogen treatment affirming the functional data. In this study, estrogen replacement was found to reduce PGHS-2-dependent vasoconstriction in aged rats (2).

The effects of estrogen on PGHS have been characterized in different experimental models. Confirming our data, estrogen reduced PGHS-2 expression in bovine chondrocyte (16) and endometrial cells (27), however it has been shown to induce the enzyme in rat myometrial (9) and human umbilical vein endothelial cells (1). These conflicting results may be dependent on varying doses and/or different cell types as well as estrogen's capacity to act as an antioxidant or oxidant molecule.

The fact that estrogen has a phenolic moiety (see Fig. 4.1-A) helps to explain this discrepancy between antioxidant and prooxidant activity (15). The basic chemistry behind this is such that the steroid can undergo further hydroxylation by a hydroxylase enzyme to form a catechol estrogen. The actions of catechol estrogen derivatives are prooxidant (refer to Appendix) while estrogen itself is antioxidant in nature. Conflict between these opposing metabolic pathways could account for differential effects of the hormone under varying conditions.

Therefore, the beneficial effects of estrogen on arterial function appear to be obtained by an 'optimal' dose of this steroid hormone. We achieved this physiological dosage in our first study using Fisher rats but were not able to duplicate the response in aged Sprague-Dawley rats (see Limitations). As such, what 'works' for one group of animals does not necessarily translate to another. This is indicative of the age-old problem of drug efficacy in some patients but not in others. Our therapeutic goal, then, should be to find the optimal amount of estrogen for a particular subset of animals/people (refer to Appendix – Fig. A.6). When this dosage is too high or too low as to be detrimental then we must turn to alternatives as foreseen by Samuel Levine's statement in 1958:

"compounds might be found that eliminate the undesirable action of such hormones and yet retain its beneficial effects on arteries" (28).

Given the results of the first paper, the alternative focussed on in this thesis was the PGHS-2 inhibitor, NS-398 (Fig. 4.1B). Interestingly, recent work investigated the chemical structure of PGHS-2 inhibitors tracing them back to the structure of estradiol (14). The search for non-steroid estrogens had led to the development of the drugs clomiphene and tamoxifen while cyclized analogues of these structures eventually yielded the selective estrogen receptor modulator raloxifene (14). Indole analogues showed good anti-inflammatory activity but were somewhat problematic. Thiazole substitution and a few other modifications created the drug known as celecoxib. The fact that the structure of this well known PGHS-2 inhibitor can be linked through a number of transformations to estrogen (see Figure 4.1) makes such drugs prime candidates as therapeutic alternatives.

Paper 2- PGHS-2 Inhibition Enhances Vascular PGHS-2

Within arteries, increased PGHS-2 expression has been associated with both an increase in vasoconstriction or vasodilation depending on the conditions. Our lab discovered enhanced PGHS-2-dependent vasoconstriction in the aged animal (21). Further, the first paper of this thesis revealed an increase in the PGHS-2 vasoconstrictor pathway in aged, ovariectomized (to remove any endogenous estrogen) rats that could be prevented with estrogen replacement (2). These and other data led to the hypothesis that chronic inhibition of the PGHS-2 pathway should mimick the benefit of estrogen by reducing PGHS-2-dependent constriction in the aged, ovariectomized rat.

Indeed, one might surmise that a PGHS-2 inhibitor that has its chemical origins in estrogen itself should elicit some similar effects (14). However, this was not the case in our second study. Indeed, contrary to our hypothesis, chronic PGHS-2 inhibition did not improve vasorelaxation in this model. In fact, there was an increase in PGHS-dependent vasoconstriction after four weeks of chronic inhibition. By contrast, one week of inhibition did not enhance PGHS-dependent constriction implying that the PGHS pathway was effectively

blocked. As we had previously shown in the first paper, placebo treated rats exhibited enhanced methacholine relaxation after PGHS inhibition. The four week inhibited animals had a similar profile to this, indicating a return to PGHS-dependent vasoconstriction. Furthermore, these rats revealed increases in PGHS-2 expression compared to control suggesting a feedback response to the PGHS-2 inhibitor. In contrast, four weeks of estrogen replacement (previous study) reduced PGHS-dependent vasoconstriction with a concomitant suppression of the PGHS-2 protein. Thus, chronic PGHS-2 inhibition, under these conditions, was not found to be a therapeutic alternative to estrogen replacement for improved vascular function in the aged, ovariectomized rat.

We speculate that the inhibition of PGHS-2 *activity* with the drug NS-398 prevented the suicide inactivation of the PGHS-2 enzyme. Normally, the enzyme goes through approximately 1300 mol arachidonate/mol enzyme before it is inactivated (7). Chronic PGHS-2 inhibition would prevent the protein from running through these cycles and hence the enzyme could persist. This suggests a preservation of existing enzyme on top of the normal production of PGHS-2. Alternatively, the inhibition of PGHS-2 could elicit a feedback response whereby transcription of the enzyme is induced. Moreover, the PGHS-2 protein may have been induced due to enhanced ROS levels produced as a result of the oxidative stress characteristic of aging, or potentially from an inflammatory response after the multiple injections that the animals received.

Caloric intake has also been shown important in altering the PGHS pathway. A study of dietary restriction revealed that *ad libitum* fed rats had increased PGHS-2 and ROS levels in the aging kidney that could be suppressed in animals fed a controlled diet (5). Furthermore, a recent study of endothelial cells treated with a high glucose media revealed an induction of PGHS-2 that was dependent upon the stimulation of ROS production (6). The link with aging can occur at numerous levels, the most obvious being elevated ROS levels. Another important interaction is the combination of macromolecular proteins such as albumin with glucose to form advanced glycation end-products or 'AGE's. These in turn bind to their receptor, termed 'RAGE' (11), which

further enhances ROS production and so plays a part in the continuing cycle of oxidative stress. AGE-RAGE interactions have been revealed in diabetes (24), as well as in aging (19). This is a site of potential therapeutic intervention by either preventing AGE formation or via binding the receptor (see Future Directions).

Since aging and ovariectomy (13) have both been found to enhance PGHS-2, our aged, ovariectomized rats provided a good model to study the implications of this enzyme and its products on vasoconstriction.

Limitations

One caveat between the two studies is that we used two different rat strains (Fisher - Paper 1 vs. Sprague-Dawley - Paper 2). At first glance this appears to limit the comparison between the studies. However, an earlier study from our lab revealed similar effects of estrogen reducing PGHS-dependent vasoconstriction in young Sprague-Dawley animals (8). By contrast, another aging study in our lab investigated Sprague-Dawley rats given the same estrogen dose (0.5 mg/pellet) that was implanted in the aged Fisher rats. Surprisingly, this work revealed no beneficial arterial effects along with limited estrogen levels detectable in the plasma (data not published). Hence, the effect of rat strain differences may or may not be negligible. Furthermore, given the smaller size of the Fisher rats, we were unable to obtain enough protein to do Westerns on mesenteric arteries in the first study so we had to look at a more systemic effect on their aortas. This was in contrast to the 'pooled' mesenteric arteries that were used for protein in the second study. In this instance, pooling the arteries did not account for any expression differences across different orders of blood vessels.

Another issue that arose in the second study was that of treatments and their appropriate controls. Initially, we had planned to give estrogen to the Sprague Dawley rats to confirm our findings in the Fisher animals. However, the possibility that the optimal doses did not overlap between strains could explain the failure to obtain a 'physiological' dose in the second study. We attempted to extrapolate a new estrogen dosage based on the increased size and

weight of the Sprague-Dawley animals. Previous work had shown an estrogen dose of 2.5 mg/pellet (60 day release) to result in a physiological dose of 70-80 pg/ml in the plasma of 250-300g rats (data not published). Based on these findings as well as the fact that our rats were at least twice as large we calculated a 7.5 mg/pellet to be within the required dose range. However, this size of estrogen pellet actually gave us a pharmacologic dose approximately ten times what we had aimed for. Evidently we did not account for the appetite suppressant effects of this estrogen dose since the animals from that particular group were significantly lower in body weight than the other groups.

Despite these problems the overall comparison of the two studies is well founded since the animals underwent the same experimental procedures. Furthermore, the increase in vasoconstriction due to the PGHS-2 pathway in the aged, ovariectomized animals was confirmed in both paper 1 and paper 2. Since the response of the drug was unable to mimic the benefit of the first study we were prompted to investigate other therapeutic alternatives.

Future Directions

Peroxynitrite and superoxide are two possible candidate factors that could be responsible for the vascular dysfunction observed with aging (10, 23) and estrogen deficiency (4). Peroxynitrite can have deleterious actions within the cell, while it may also have beneficial effects by forming NO-donor species with thiols (26). Since aging is a state of depleted antioxidant defence the actions of this molecule are likely more detrimental. Hence, inhibitors and scavengers of these molecules could mimic the effects of estrogen therapy in the aging vasculature. We have already begun studies investigating the effects of a superoxide dismutase-catalase mimetic (named EUK-8) on vascular function in the aged, ovariectomized rat. EUK-8 is a salen-manganese complex that has been effective in preventing ischemia-reperfusion injury in the iron overloaded heart (18) as well as in other models of oxidative stress (3). Our initial findings revealed no benefit of this drug on vessel function. Previous work in aortic rings showed that EUK-8 was capable of reducing the levels of ROS indicating a benefit of the drug (25). We had concerns about the intraperitoneal injection

of the EUK-8 since the site of delivery was just adjacent to the rat's mesentery. Furthermore, the drug itself was not ineffective since our concurrent heart studies showed a beneficial effect on recovery after ischemia (data not published). We have decided to continue the study of EUK-8, this time with an osmotic mini-pump mode of delivery.

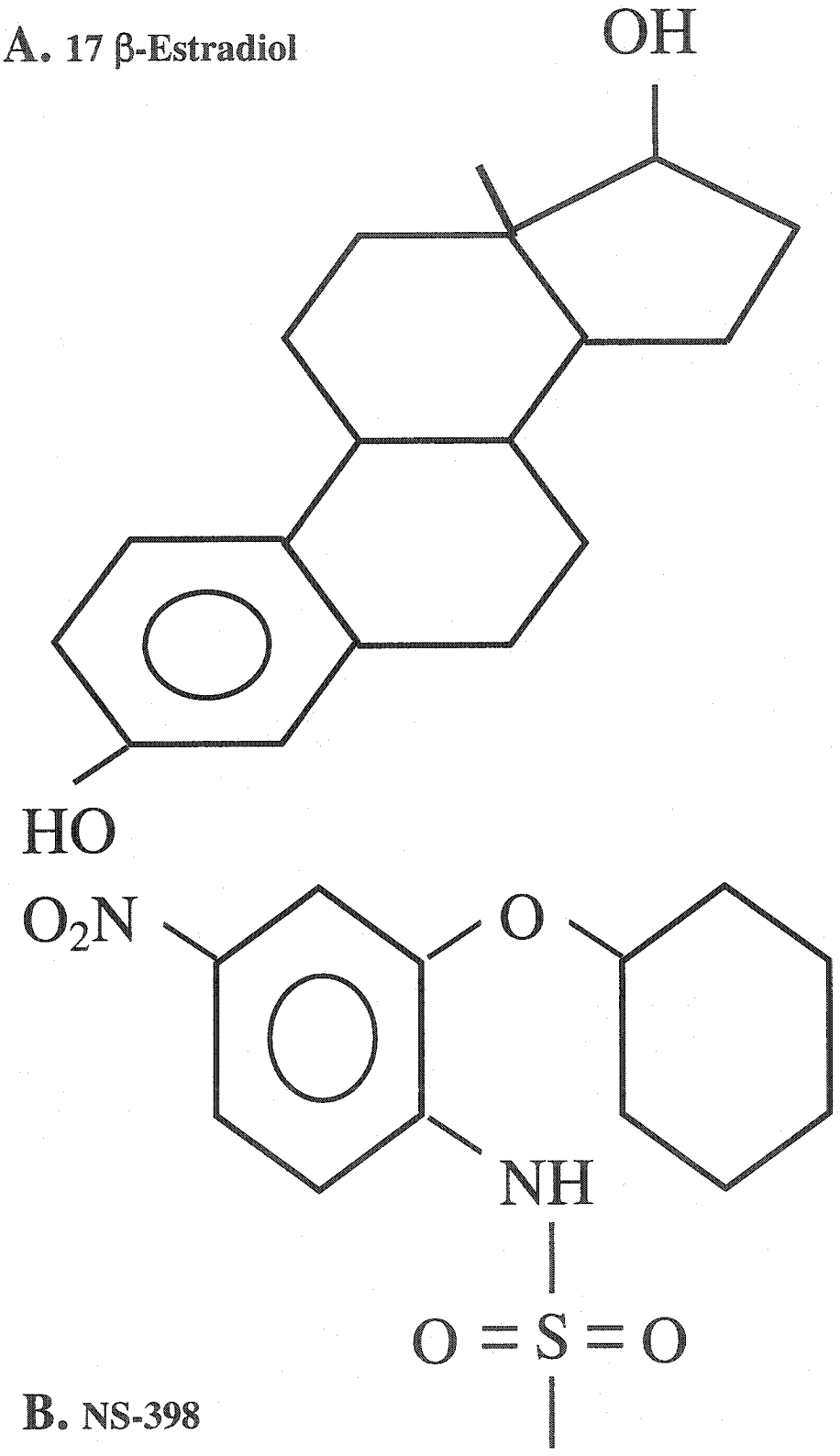
Another possible 'estrogen alternative' is the antioxidant vitamin alpha(α)-lipoic acid. This drug is thought to act as a free radical scavenger and is put forth as an anti-aging remedy in many countries. Recent studies have implicated alpha-lipoic acid as a peroxynitrite scavenger (17), and an antioxidant in endothelial cells (12). Both its oxidised and reduced forms (dihydroalpha-lipoic acid) have been shown effective in this regard. Hence, this antioxidant has therapeutic potential in our aging rat model.

These future projects would not only investigate vascular function but (as mentioned) cardiac work as well. As such, a well-rounded view of the effects of estrogen and other alternatives on the cardiovascular system of these aged female rats would be obtained.

Conclusion

The name estrogen comes from the same root word as Easter or *Estre* who was the goddess of spring, fertility and the rising sun. As such, the name itself implies a beneficial effect of this steroid hormone whether it be in aiding the formation of a new life during a pregnancy or restoring life to an aging human. Unfortunately the effects of estrogen have not been shown to be universally good. Indeed, an excess of the hormone appears to be just as detrimental as a lack thereof as pertains to the aging vasculature of the rat. Moreover, the use of chronic PGHS-2 inhibition as an alternative to estrogen was not of any benefit in this model. Again, there was the uncertainty of an 'appropriate' dose and administration of the drug. Perhaps in future we will be able to discover a drug or series of drugs that are more universal in their action to reverse the effects of aging. In the end, it may depend upon the tell-tale signs of aging versus the rejuvenating effects of estrogen (or an alternative to it) and the balance that is found between them.

Figure 4.1



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Appendix- High Estrogen Enhances PGHS-2-Dependent Vasoconstriction in the Aged Rat

Introduction

Recent controversy in the field of estrogen replacement and cardiovascular disease has prompted investigation as to the actual therapeutic value of this hormone on the heart and blood vessels. This may be dependent on the dose of the hormone since the latest trials are based on a higher dose estrogen-progestin regimen (3). Indeed, the antioxidant versus prooxidant effects of estrogen could be dose-dependent. It has been shown that increased levels of estrogen facilitates the formation of catechol estrogens. These, in turn, can form semiquinones and subsequently quinones, which are reactive species capable of free radical production (5). As such, estrogen could potentially induce or reduce oxidant sensitive proteins, depending on its metabolism. We found that a pharmacological dose of estrogen (~1000pg/ml or ≈ 10X *in vivo* dose in rats) resulted in a paradoxical induction of PGHS-2-dependent vasoconstriction in the aged rat as evidenced by enhanced methacholine relaxation and blunted phenylephrine constriction in the presence of NS-398. Furthermore, PGHS-2 expression was increased in the high estrogen group, contrary to our previous work with physiological estrogen replacement (see Chapter 2).

Methods

Sprague-Dawley rats were aged to 11 months and ovariectomized. At the time of surgery, animals were given an estrogen pellet (7.5mg/ pellet, 60 day release) or a placebo subcutaneously. After one month of treatment rats were euthanized and their mesenteric arteries dissected out for use on the wire myograph. Intact aged (12 months old) rats were used as a control.

Arteries were subjected to concentration-response curves for the alpha-adrenergic agonist phenylephrine and the endothelium-dependent muscarinic relaxant methacholine. Arteries were preconstricted to 50% of their maximal constriction (EC50) prior to the addition of methacholine. Curves were done in

the absence or presence of the PGHS-2 inhibitor, NS-398 (10μM), which was incubated in the baths 12 minutes before they were begun.

Mesenteric arteries were dissected and homogenized in eppendorf tubes using a small tissue homogenizer. The Bradford assay was employed to measure protein concentration. Western immunoblots were performed as previously described (2). Polyclonal antibodies for PGHS-2 and monoclonal antibodies for α-actin (Cayman Chemical Co. and Boehringer Mannheim, respectively) were used. Bands were quantified by densitometric analysis and normalized to α-actin.

Results

The estrogen-replaced (HE2) animals from the second study (see Chapter 3) were of a significantly lower body weight than their controls ($P<0.05$). Furthermore, the uterine weights were significantly increased ($P<0.05$). Problems with the radioimmunoassay for 17 β-Estradiol enabled only a qualitative assessment of the levels. The HE2 group was approximately 10-20 times as much as the other groups indicating a pharmacological level of the hormone.

Methacholine-induced relaxation was not significantly enhanced in the HE2 group indicating that any beneficial effect of estrogen is lost (Fig. A.1). Relaxation to methacholine was significantly increased in the HE2 animals in the presence of the PGHS-2 inhibitor (Fig. A.2). Vasoconstriction to phenylephrine was significantly elevated in the HE2 group indicating a greater adrenergic sensitivity (Fig. A.3). There was a trend towards blunted phenylephrine constriction after PGHS-2 inhibition in the HE2 rats (Fig. A.4). Furthermore, PGHS-2 protein expression was increased in the HE2 group compared to the controls (Fig. A.5).

Discussion

Higher levels of estrogen appear to be detrimental to the aged rat vasculature. This is in contrast to our previous findings in animals given a physiological dose of estrogen replacement (Chapter 2). 17 β-estradiol can

undergo a number of chemical transformations including the formation of what are known as 'catechol' estrogens. These can be further reduced to form semiquinones and quinones. These species, in turn, are able to produce free radicals thus contributing to the oxidative stress of the estrogen-replaced animal. Since the PGHS-2 enzyme is oxidant inducible it is possible that this process is occuring in the HE2 rats.

The effects of estrogen, then, whether beneficial or detrimental, can be construed to fall under a 'bell shaped curve' whereby the optimal dose and benefit is found in the center (Fig. A.6). As such, too high or too low a dose of the hormone would lend towards more harmful effects in the vasculature. Indeed, we observed an increase in PGHS-2-dependent vasoconstriction and PGHS-2 protein expression at both high (HE2) and low or absent (Plac) levels of estrogen.

A recent study provides an interesting comparison to this. In human aortic endothelial cells exposed to normal and high levels of glucose, the high glucose caused PGHS-2 mRNA and protein to be induced (1). Moreover, the high glucose-treated cells had increased thromboxane A_2 (TxA2) and reduced PGI_2 production. Peroxynitrite was implicated in this as evidenced by nitrotyrosine and PGI_2 synthase colocalization (1). This may also be occurring in our aging model. Since PGI_2 synthase is nitrated and inactivated the available PGH2 can be shunted to produce TxA_2 as well as producing a vasoconstrictor effect itself. The high glucose study also revealed that these effects could be prevented by inhibition of reactive oxygen species formation through blockade of NAD(P)H oxidase (1). A couple of studies, including one from our own lab have shown that estrogen can reduce expression of the various NAD(P)H oxidase subunits (4, 6). Whether or not high estrogen levels would produce an opposite effect, similar to that seen with high glucose, is a potential direction for future study in this field.

Speculation

We speculate that high estrogen levels constitute an oxidative insult on the aged rat vasculature which, in turn, causes the induction of the oxidant-sensitive PGHS-2 enzyme.

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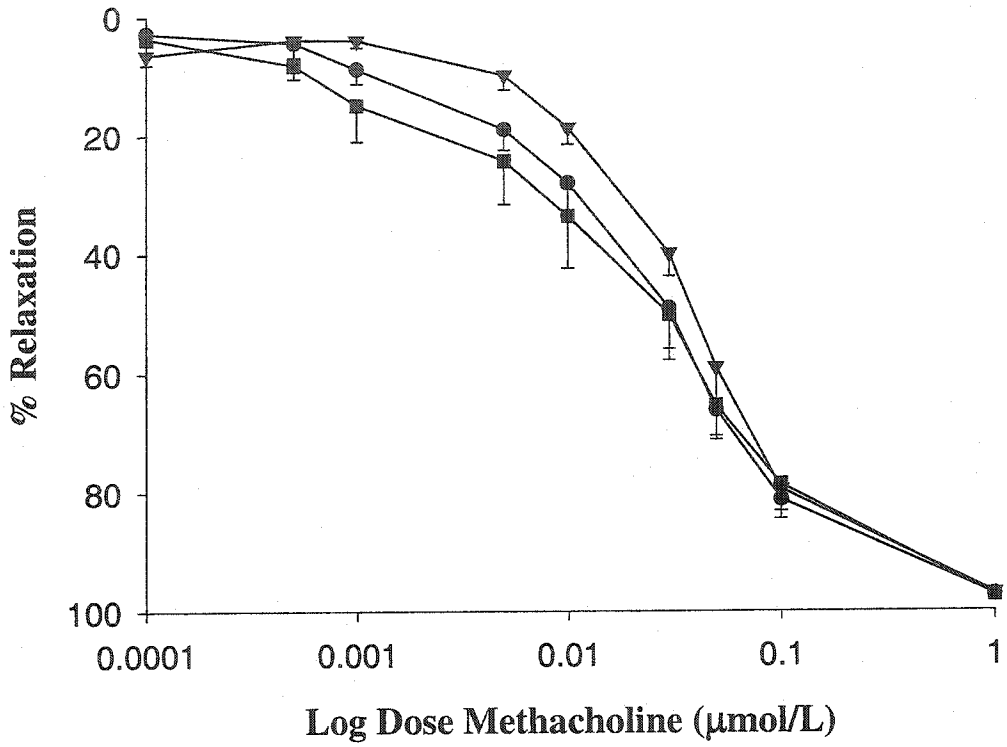

Figure A.1 Methacholine-induced vasorelaxation was not different between aged intact (●), and ovariectomized rats given a high estrogen dose (■, 7.5 mg/pellet, 60 day release) or a placebo (▼).

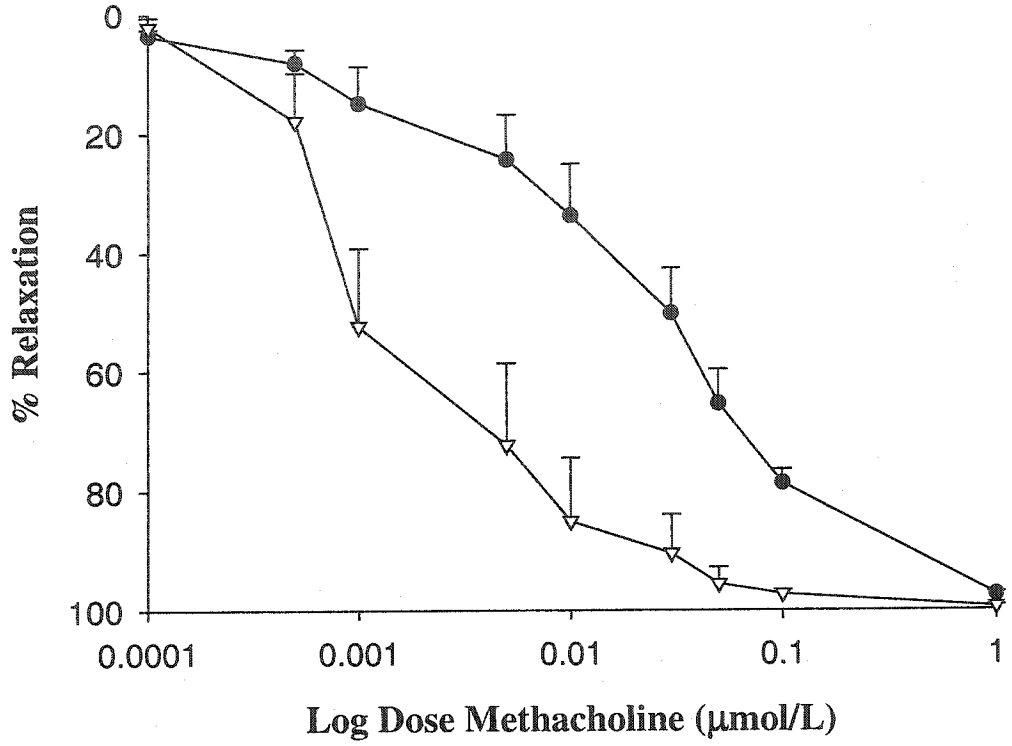

Figure A.2 Methacholine-induced vasorelaxation in the HE2 group (●) was enhanced in the presence of the PGHS-2 inhibitor (NS-398, 10μM) (▽). * $P<0.05$

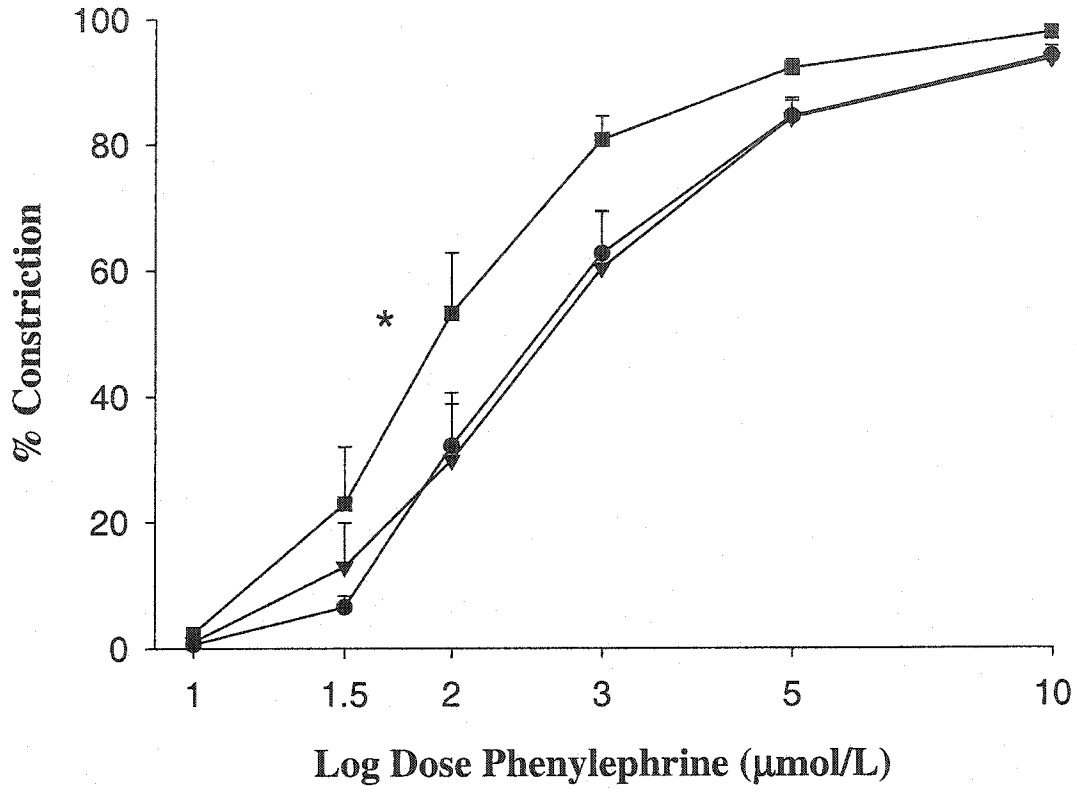

Figure A.3 Phenylephrine-induced vasoconstriction was enhanced in the HE2 group (■) compared to the intact (●) and placebo (▼) controls. * $P<0.05$

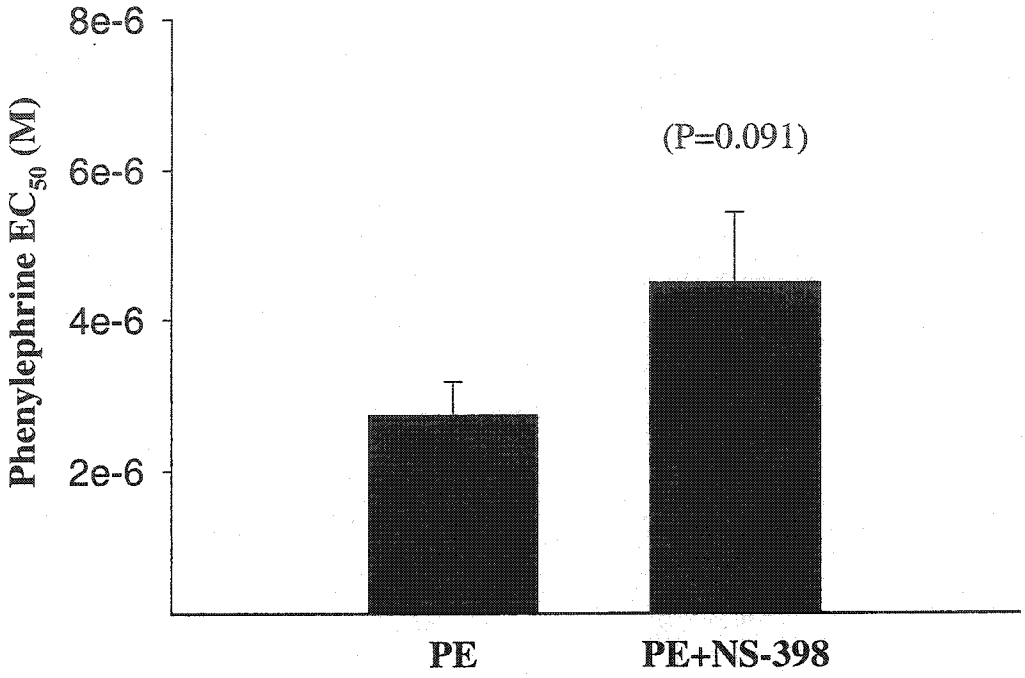

Figure A.4 Phenylephrine-induced vasoconstriction was blunted in the HE2 group after PGHS-2 inhibition (NS-398, 10μM) as shown by a trend for an increase in the EC_{50}. P=0.091 vs. PE alone

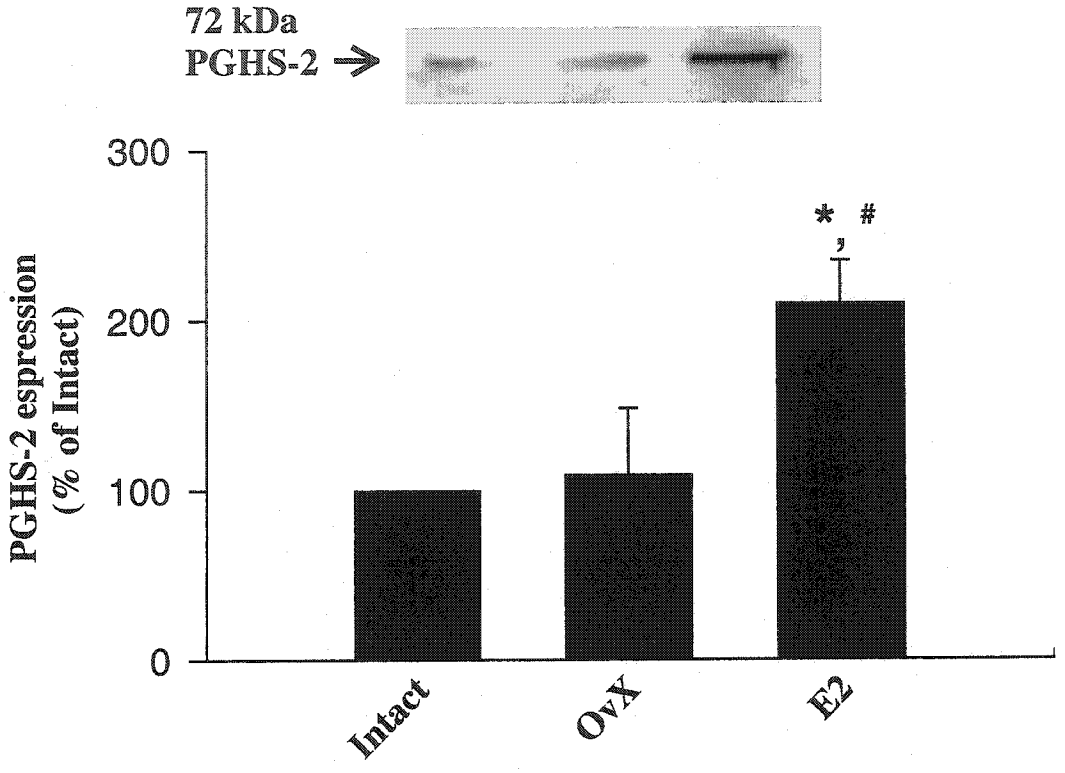

Figure A.5 PGHS-2 protein expression was increased in the HE2 group compared to placebo and intact controls. *,# P<0.05 vs. Intact and OVX

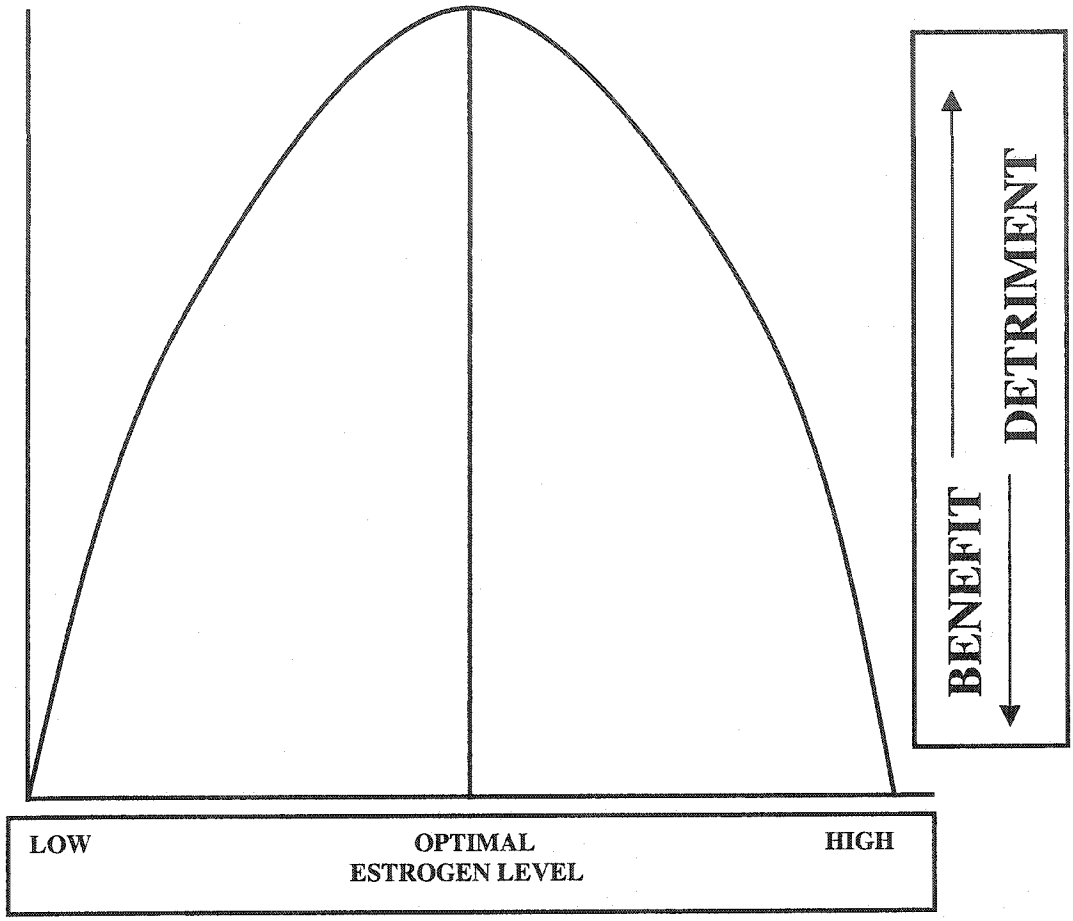

Figure A.6 Proposed Biphasic Dose Response Curve illustrating the effects of estrogen. At lower estrogen levels the vasculature would experience detriment. The optimal dose of estrogen would be of most benefit to the blood vessel. Higher estrogen levels would also be detrimental to the vessel. Interestingly, the profile for chronic PGHS-2 inhibition fits this curve as well with short and long term inhibition exhibiting the most PGHS-dependent constriction and the intermediate time (1 week) revealing a less marked constriction due to the PGHS pathway.