Estrogen replacement reduces PGHS-2-dependent vasoconstriction in the aged rat

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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 (9, 29) has demonstrated the importance of estrogen in the prostaglandin H synthase (PGHS) pathway. Our data (9) indicated that estrogen suppresses PGHS-dependent vasoconstriction in an ovariectomized rat model. However, this study as well as previous studies regarding estrogen replacement in ovariectomized animals has largely been conducted with young adult animal models (5, 14, 16, 33). Importantly, the physiological processes due to aging (which are important relative to effects on postmenopausal 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 inducible PGHS-2 through activation of the nuclear transcription factor nuclear factor (NF)-κB in the rat kidney (18) and in aging brain cells (21). More recently, we showed (30) that PGHS-2 protein expression is upregulated with aging in rat mesenteric arteries and that vessel tone is increased via this eicosanoid pathway. 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 constriction.

METHODS

Animal model. Aged (24 mo; n = 10) and young (3 mo; n = 6) female Fisher rats were obtained from the National Institute for Aging. Aged animals were ovariectomized and given a placebo (Innovative Research of America) or 17β-estradiol pellet (0.5-mg pellet; 60-day release; Innovative Research of America).
America) subcutaneously. Ovariectomy was performed to control for the large variability in estrogen levels that is characteristic of animals approaching reproductive senescence (constant estrus). 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 whether estrogen replacement in aged animals was restorative of vascular function. Four weeks after ovariectomy, rats were killed while under light anesthesia (50 mg/kg body wt ip sodium brietal). Plasma samples were obtained by heart puncture and subsequent centrifugation. Samples were frozen at −80°C for later measurement of 17b-estradiol levels with a double-antibody radioimmunoassay kit (Diagnostic Products). 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 HEPES-buffered physiological saline solution (HEPES-PSS). The HEPES-PSS contained (in mmol/l) 142 NaCl, 4.7 KCl, 1.17 MgSO4, 1.56 CaCl2, 1.18 KH2PO4, 10 HEPES, and 5.5 glucose. Resistance-sized mesenteric arteries (∼250 μm in diameter) were dissected from surrounding adipose tissue, cut into 2-mm lengths, and threaded with 20-μm-thick wires. The wires were fastened to polycrylamide blocks connected to the isometric myograph system (Kent Scientific). 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 0.8L100, 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).

**Experimental design.** Phenytoin was administered in the initial dose-response curves to determine the concentration needed for 50% maximal constriction (EC50) of each segment. This EC50 was added in subsequent curves to obtain a preconstriction baseline from which vasorelaxation curves could be measured. Cumulative doses of methacholine (1 nmol/l to 1 μmol/l) were added to assess endothelium-dependent relaxation. Inhibitors of NOS [Nω-monomethyl-L-arginine (L-NMMA); 100 μmol/l] and PGHS [1 μmol/l meclofenamate, 3 mmol/l valeryl salicylate (PGHS-1), and 10 μmol/l NS-398 (PGHS-2); Cayman Chemical, Ann Arbor, MI] were incubated in different baths for 15 min before the curves. The effects of repeating curves and time controls were incorporated into the experimental protocol. Between curves, a washout period of 30 min was maintained, with fresh HEPES-PSS buffer being added every 10 min.

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

**Data analysis.** Data from each dose-response curve were fitted to the Hill equation, and a straight line was generated by linear least-squares regression analysis. EC50 was determined from this line, and means ± SE were calculated from the curves. ANOVA was used for statistical analysis. Post hoc analysis was performed with 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 with 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 with the aged placebo-treated rats (231.8 ± 1.8 vs. 285.5 ± 8.3 g; P < 0.05).

Phenytoin 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, whereas the aged estrogen-replaced group restored relaxation to the level seen in the young animals, as evidenced by their respective EC50 values: Aged (placebo) 0.28 ± 0.07 μmol/l; Aged + Estrogen (E2): 0.05 ± 0.009 μmol/l; and Young: 0.05 ± 0.01 μmol/l (P < 0.05; Fig. 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 of 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 and P = 0.667, respectively; Fig. 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. 2B), whereas little change was observed in arteries from the Aged + E2 rats (Fig. 2B), suggesting that estrogen replacement prevented PGHS-dependent constrictor modulation of vascular function that was observed in the ovariectomized controls.

**Fig. 1.** Concentration-response curves to methacholine (Meth) in mesenteric arteries of ovariectomized aged placebo-treated rats (●), aged estrogen (E2)-replaced rats (●), and intact young rats (○). Inset: effective concentration that produced 50% of the maximum response (EC50) values of arteries from Young, Aged (placebo-treated rats), and Aged + E2 (estrogen-replaced, aged rats) groups. Bars represent means ± SE. *P < 0.05 vs. Young and Aged + E2.
The role of each PGHS isoform was investigated to determine whether 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 \((P < 0.05; \text{Fig. 3, A and B, respectively})\), whereas estrogen prevented the PGHS-dependent constrictor modulation of vascular function (Fig. 3). 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\% reduction in the methacholine EC\(_{50}\), whereas PGHS-1 inhibition was found to reduce the EC\(_{50}\) by 76\% \((P < 0.05)\).

We previously reported (30) a specific increase in PGHS-2 expression in mesenteric arteries of aged Sprague-Dawley rats. In the present study, age-induced expression of PGHS-2 was significantly reduced in aortas from the Aged + E\(_2\) group compared with the Aged group \((P < 0.05; \text{Fig. 4A})\). There was no significant change in PGHS-1 expression (Fig. 4B) or eNOS expression (Fig. 5) between the aged groups.

**DISCUSSION**

Previous studies with ovariectomized young (3–6 mo old) rats to assess the effect of exogenous estrogen on the vasculature 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, under conditions of aging and oxidative stress, the effect of estrogen through the NO pathway may be reduced because of the scavenging of NO by free radicals (17). Indeed, aging has been shown to affect human vasculature by decreasing the inhibitory effect of \(\text{L-NMMA} (\text{NO blocker})\) 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 \(\text{H}_2\text{O}_2\), a vasorelaxant. Ultimately, estrogen replacement did not enhance NO-mediated relax-

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**Fig. 2.** EC\(_{50}\) for Meth in mesenteric arteries of Aged and Aged + E\(_2\) rats in the absence (−) and presence (+) of the nitric oxide synthase (NOS) inhibitor \(\text{N}^0\)-monomethyl-L-arginine (L-NMMA, 100 μmol/l; A) and the absence and presence of the nonselective prostaglandin H synthase (PGHS) inhibitor meclofenamate (Meclo, 1 μmol/l; B). Bars represent means ± SE. *\(P < 0.05\).

**Fig. 3.** Meth EC\(_{50}\) values from mesenteric arteries of Aged and Aged + E\(_2\) rats in the absence and presence of the PGHS-2 inhibitor NS-398 (10 μmol/l; A) and the absence and presence of the PGHS-1 inhibitor valeryl salicylate (VS, 3 mmol/l; B). Bars represent means ± SE. *\(P < 0.05\).
ation in the small mesenteric arteries of the aged animals and eNOS expression in the aorta remained the same in both aged groups. Therefore, in aging, the actions of estrogen on the PGHS pathway may become more predominant. Our previous findings (9) in young rats demonstrated that chronic estrogen replacement inhibited PGHS-dependent constriction that occurred in ovariectomized Sprague-Dawley rats. In agreement with our data, ovariectomized spontaneously hypertensive rats similarly restored altered endothelium-dependent responses with estradiol as well as with indomethacin and sodium diclofenac (nonselective PGHS inhibitors) (7). Together, these data indicate a role for estrogen to inhibit PGHS-dependent vasoconstriction; however, differences in rat strains may limit our ability to compare data among the studies.

It was shown previously that inducible PGHS-2 is upregulated with aging and oxidative stress (18, 21, 30). Moreover, our lab reported (30) that PGHS-2-dependent vasoconstriction is increased with age, which was associated with increased PGHS-2 expression. Furthermore, oxidative stress in the form of reactive oxygen intermediates (11) can induce PGHS-2 via the redox-sensitive factor NF-κB (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 (10) myometrium 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 (29). A recent study with rabbit uterine cervical fibroblasts showed that 17β-estradiol suppressed the levels of PGE₂ that were previously augmented by interleukin-1α (27). Furthermore, indomethacin and NS-398 similarly suppressed PGE₂ levels (27). Our results are in agreement with these data because estrogen replacement suppressed PGHS-dependent vasoconstrictor products. 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 with 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 increased 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, pathways other than NO may predominate. We observed that estrogen reduced PGHS-dependent vasoconstriction; however, estrogen may affect other vasoactive pathways such as endothelium-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 endothelium-dependent relaxation was attributed to a diminished EDHF response in the estrogen-deficient rats (20). Furthermore, in aging, the EDHF-dependent portion of endothelium-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 aged male F344 rats (23). Therefore, 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.

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