Effect of Gender on the Release of Endothelial Factors by Acetylcholine and Shear Stress

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Abstract

A natural mechanism for lowering high blood pressure, a risk factor in cardiovascular disease, involves the synthesis and release of vasorelaxant (blood vessel-relaxing) factors such as nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). These substances are beneficial because they induce vasodilation (widening) of arteries by diffusing from the endothelium (monolayer of cells lining the inner artery) into the surrounding smooth muscle tissue, causing it to relax. Clinical studies indicate a higher risk of cardiovascular disease in men than in premenopausal women. This disparity may be partly due to a gender difference in the release of NO and EDHF. The purpose of this study was to test the hypothesis that there is a gender difference in the relative contribution of NO and EDHF to vasodilation. Using isolated tail arteries from rats, vasodilation was induced by acetylcholine (an agonist or substance that binds onto a receptor, subsequently producing some reaction) or by increasing shear stress (increased pressure by increased flow) on the endothelium. We used an NO synthesis inhibitor (L-NMMA) and elevated K+ levels to reveal the relative contributions of NO and EDHF, respectively. Data analysis showed that male arteries depend exclusively on NO for vasodilation, whereas female arteries utilize both NO and EDHF. This implies a female advantage against cardiovascular disease, consistent with clinical observations.

Faculty Mentors

The incidence and severity of cardiovascular disease is higher in men than in women. We don’t yet understand why, but many factors probably contribute to this gender difference. In his project using a rat model, Kirk Pak has identified a male-female difference in the release of blood vessel substances that cause arterial dilation. Female arteries appear to have greater capacity to maintain blood flow, which may be particularly important in cardiovascular disease. In addition to the useful results, the undergraduate research project provides a rewarding experience for both the student and the faculty mentor. It is a wonderful opportunity for the student to gain deeper understanding and practical experience related to classroom concepts. But perhaps most important is the advice and guidance provided by the mentor regarding career goals and opportunities.

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Key Terms

- Acetylcholine
- Endothelium
- Endothelium-derived Hyperpolarizing Factor
- Methoxamine
- Vasodilation
- Vasorelaxant

Author

Kirk Pak is an advocate for involvement. This athlete, editor, volunteer, and honor society member made a presentation to the American Heart Association and will be interning at the National Institutes of Health (where he will work after graduating this spring). He credits both opportunities, at least in part, to his involvement in undergraduate research. Pak hopes to continue his breadth of interests by earning both an M.D. and a Ph.D., and wants to be a “physician-scientist working within and between the academic/research and clinical worlds.”
Introduction

A number of studies have revealed a higher incidence of cardiovascular disease in men than in premenopausal women (Barrett-Connor and Stuenkel, 1999). One contributing factor may be a gender difference in endothelium-dependent modulation of arterial smooth muscle tone (McCulloch and Randall, 1998).

The endothelium controls both vascular tone and growth of the vascular smooth muscle, particularly through synthesis and release of endothelium-derived nitric oxide (NO) (Palmer et al., 1987). Another endothelium-derived relaxing factor operates by activating K⁺ channels, hyperpolarizing vascular smooth muscle. This yet unidentified substance is termed endothelium-derived hyperpolarizing factor (EDHF). Prostacyclin (PGI₂), a cyclooxygenase metabolite, is another endothelium-derived vasorelaxant factor. These three endothelial factors modulate arterial smooth muscle tone, thereby affecting arterial blood pressure.

The endothelium releases these factors via two mechanisms. In agonist-dependent vasodilatation, acetylcholine binds onto its receptor on the endothelial cell layer and initiates a G protein-coupled reaction which eventually leads to the release of NO and EDHF, relaxing the surrounding smooth muscle. In shear stress-dependent vasodilatation, an increase of flow within the artery induces NO and EDHF release. An increase of flow causes shear force or stress along the endothelium and is a mechanical stimulus for the release of these factors into the surrounding smooth muscle, leading to the relaxation and therefore dilation of the artery.

Gender differences in agonist-induced release of endothelial substances have been reported. Acetylcholine-stimulated release of nitrite, and therefore possibly NO, is greater in female rat and rabbit aortae (Sanchez et al., 1996). In agonist-stimulated release of vasodilators in mesenteric arteries from female rats, NO appears to contribute differently than EDHF to endothelium-dependent relaxation; however, this does not seem to be true in mesenteric arteries from male rats (McCulloch and Randall, 1998). To determine whether this occurs in tail arteries, we observed the effects of blocking NO before and after blocking EDHF. Interactive relationships between endothelium-derived substances, NO, PGI₂, and EDHF have been well-documented (Doni et al., 1998). Whether a gender difference exists in this interactive relationship requires further investigation. In addition to agonist-induced release of vasodilators, shear stress is an important physiological stimulus for the release of endothelial factors.

Little is known about the influence of gender on shear stress-induced release of endothelial vasodilators (Macedo and Lautt, 1996).

To explore gender differences in the release of endothelial factors, the rat tail artery was chosen because agonist- and shear stress-induced release of NO and EDHF are well-documented in this preparation (Rembold and Chen, 1998). The purpose of this study is to determine whether there is a male-female difference in the relative contribution of endothelial factors (NO and EDHF) to vasodilator responses, either agonist- or shear stress-dependent. Since clinical studies imply a female advantage against cardiovascular disease, the hypothesis in this study is that arteries from females will have a greater contribution of NO and EDHF to either agonist- or shear stress-dependent vasodilation than arteries from males. This study seeks to elucidate mechanisms that may underlie the gender differences seen in clinical studies and provide further information that could lead to important treatments for cardiovascular disease such as atherosclerosis.

Materials and Methods

Animals

Approval for animal procedures was granted by the Animal Care and Use Committee of the University of California, Irvine (IUCAC Protocol Number 1999-2048). Male and female 3-4 month old Fisher 344 rats weighing 304 + 5 g for males and 163 + 5 g for females were used.

Tissue isolation and preparation

Rats were decapitated and tail arteries removed and submerged in cold, oxygenated physiological salt solution (PSS) containing (in mM): CaCl₂, 1.6; KH₂PO₄, 1.2; NaCl, 118; KCl, 4.8; NaHCO₃, 25; MgSO₄, 1.2; ascorbic acid, 0.3; and glucose, 11.5. Arterial segments of 3-4 cm were cannulated at both ends and perfused and superfused (2.0 ml/min) with PSS saturated with 95% O₂-5% CO₂ at 37 °C. Smooth muscle constriction was measured as changes in perfusion pressure (mmHg), monitored with a Statham P23 Ac transducer and a MacLab analog to digital converter.

Shear stress release of endothelial factors

Vasoconstrictor responses to methoxamine, an α₁-adrenergic agonist (which produces an adrenalin-like response), were obtained before and during exposure to the NOS inhibitor, N⁶-monomethyl-L-arginine acetate (L-NMMA) or to moderately elevated K⁺ (27 mM) to block effects of EDHF (Adeagbo and Triggle, 1993). Indomethacin (10⁻⁵ M) was present throughout the experiments to inhibit cyclooxygenase.
Vasoconstrictor responses were expressed as either developed pressure (mmHg) or as percent potentiation from the control response.

**Acetylcholine-induced release of endothelial factors**

Arteries were precontracted with methoxamine (0.3-1.0 µM). Subsequent vasodilatory responses to acetylcholine (10⁻⁵ M) were then quantified as percentages of pressure developed during the preconstriction. L-NMMA (10⁻⁴ M) was used to inhibit NO synthase and moderately elevated K⁺ (27 mM) to inhibit the effects of EDHF. Indomethacin was present throughout the experiments to inhibit cyclooxygenase.

**Drugs and solutions**

The following drugs were used: indomethacin, acetylcholine chloride, and methoxamine HCl (Sigma Chemical Company); and N⁶-monomethyl-L-arginine acetate (L-NMMA) (RBI Company). Indomethacin was initially diluted in 0.1 M Na₂CO₃ to produce a stock solution of 10 mM.

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using analysis of variance (ANOVA) with Tukey’s test. Levels of p<0.05 indicated statistical significance.

**Results**

**Effects of inhibiting NOS and EDHF**

In order to observe possible interactions between NO and EDHF as well as their contributions to vasoconstriction and vasodilation, indomethacin (10⁻⁵ M) was added to eliminate any possible effects of cyclooxygenase products throughout the experiment. Methoxamine (3 x 10⁻⁷ M - 1 x 10⁻⁶ M), a selective α₁-adrenergic agonist, was used to constrict the artery and increase shear stress. The effects of NOS inhibition by L-NMMA (10⁻⁴ M) were observed before and after inhibiting EDHF with 27 mM K⁺ (Figure 1).

Inhibition of NOS with L-NMMA increased methoxamine vasoconstriction approximately three-fold in arteries from males, with a smaller effect in arteries from females (p<0.001, Figure 2). Subsequent addition of K⁺ to inhibit actions of EDHF did not have any significant additional effect in arteries from males. In contrast, in arteries from females, addition of K⁺ resulted in significantly greater potentiation of methoxamine-induced contraction. Similar trends were observed when EDHF was inhibited before inhibition of NOS, but these did not reach statistical significance.

On the other hand, inhibition of EDHF failed to yield a mean perfusion pressure that was significantly different from
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The effects of inhibition of NOS and EDHF on vasodilator responses to acetylcholine (0.3-1.0 µM) in tail arteries from male and female rats. Responses are calculated as a percent of control methoxamine response. The first three pairs of columns from left to right correspond to the protocol illustrated in the upper tracing of Figure 1. In this case, control acetylcholine-induced vasodilation was expressed as a percent of methoxamine-induced preconstriction. Subsequent percent vasodilations were divided by the control percent vasodilation and multiplied by 100. Control relaxation was thereby calibrated to 100%. The first three pairs of columns from left to right correspond to the protocol shown in the lower tracing of Figure 1 where NOS was inhibited prior to inhibiting the effects of EDHF. Control responses to acetylcholine (% of methoxamine-induced preconstriction) were 58 ± 7% (n=6) in arteries from males and 43 ± 6% (n=7) in arteries from females. *Significantly different from control, P<0.01. †Significantly different from L-NMMA + K+, P<0.05.

In arteries from females, inhibition of NOS with L-NMMA reduced acetylcholine-induced vasodilation to approximately 33% of control (p<0.01). Additional inhibition of EDHF with K+ alone significantly decreased the vasodilation to acetylcholine (p<0.01). In the presence of 27 mM K+, L-NMMA caused a significant further decline in the vasodilatory response to acetylcholine (p<0.01).

Figure 3 illustrates the percent contribution of inhibiting either NOS or EDHF, for either shear stress or acetylcholine-induced dilation. Arteries from males were proportionately more sensitive to L-NMMA in both cases than arteries from females. Correspondingly, male arteries were proportionately less sensitive to inhibition of EDHF in enhancing methoxamine contraction or inhibiting dilation to acetylcholine.
The major finding of this study is that EDHF contributes more to vasodilation in arteries from females compared to those of males. Female arteries displayed more sensitivity to K+ than male arteries, suggesting that in female arteries EDHF contributes more to responses to shear stress. Male arteries, on the other hand, appeared to be highly insensitive to K+ and relatively more sensitive to L-NMMA. Male arteries, therefore, appear to depend primarily on NO for vasodilation whereas female arteries utilize both NO and EDHF.

The present study involving shear stress-stimulated endothelium-dependent dilation parallels a study conducted by McCulloch and Randall (1998) involving agonist-induced endothelium-dependent relaxation. Similar to the present study, NO seemed to be dominant in the mesenteric arterial bed from males, and EDHF seemed to be more important in mesenteric arteries from females. It is possible that the NOS inhibitor, L-NMMA, may have also acted on inducible and/or neuronal nitric oxide synthase instead of endothelial NOS. However, it is not believed that L-NMMA acts on non-endothelial forms of NOS in the tail artery, because in an endothelium-denuded preparation, L-NMMA had no effect (unpublished results).

In the present investigation, there was a greater EDHF effect in shear stress- than in agonist-induced release of endothelial factors. Both male and female arteries seemed to be more sensitive to elevated levels of K+ in methoxamine-induced shear, than in acetylcholine-stimulated dilation. During agonist-induced endothelium-dependent relaxation, acetylcholine binds to its receptor on endothelial cells, eventually leading to synthesis of NO and EDHF. Shear stress provides a mechanical stimulus to endothelial cytoskeletal membrane proteins, increasing intracellular Ca++ or sensitivity to intracellular Ca++, resulting in release of endothelium-derived factors (Davies, 1995). The data in this study suggest that tail arteries are more sensitive to K+ via a shear stress-induced mechanism compared to an agonist-induced mechanism.

Much evidence suggests that gonadal steroids modulate the release of endothelial factors, which may contribute to gender differences in vascular regulation. Estrogen plays an important role in the prevention of cardiovascular disease (Barrett-Conner and Stuenkel, 1999), which can only partially be explained by beneficial changes in plasma lipids (Barrett-Conner and Bush, 1991). Although the responsible mechanisms are not completely understood, one potential target for estrogen is the vascular endothelium (Kauser and Rubanyi, 1994). Estrogen treatment has been shown to result in increased levels of endothelial NO synthase protein (McNeill et al., 1999), and to influence either the synthesis or effect of EDHF (McCulloch and Randall, 1998). Estrogen also appears to potentiate shear stress-dependent NO release (Kauser and Rubanyi, 1994). In humans, elevated estrogen during either the follicular or luteal stages of the menstrual cycle is associated with greater shear stress-dependent dilation (Hashimoto et al., 1995). These observations support the hypothesis that there may be greater shear stress-induced release of NO, and perhaps other endothelial factors as well, in arteries from females than males.

Effects of testosterone are not as well understood. In 1974, Greenberg et al. suggested that male gonadal steroids enhance the sensitivity of canine arteries to catecholamines. Testosterone increases coronary vascular resistance in the rat heart via a rapid, possibly nongenomic mechanism (Ceballos et al., 1998). Although testosterone has been shown to inhibit va-
The hypothesis that gonadal steroids affect the release of multiple endogenous endothelium-derived factors has important clinical implications. The vasodilatory properties of NO, PGI\(_2\), and EDHF reduce vascular resistance, vascular contractility, and blood pressure while increasing vascular distensibility; combined, this may delay the onset or severity of hypertension (Kahonen et al., 1998). In addition, both PGI\(_2\) and NO reduce proliferation and migration of vascular smooth muscle tissue, inhibit blood coagulation (Chen et al., 1996), and interfere with the oxidation of deposited low-density lipoprotein cholesterol particles (Hayashi et al., 1995). Therefore, beneficial effects of female gonadal steroids may be a consequence, in part, of the combined effects of endothelium-derived factors.

In conclusion, this study has shown a gender difference in the relative contribution of endothelial factors, NO and EDHF, to agonist- and shear stress-dependent vasodilation in tail arteries from rats. For vasodilation, male arteries seem to be restricted to NO, whereas female arteries seem to be able to use both NO and EDHF. This supports and may partially explain why males have a higher incidence of cardiovascular disease than females. This study has contributed to a fundamental understanding of the physiological dynamics of blood pressure and, therefore, has provided some basis by which to navigate the search for potential treatments against cardiovascular disease.

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**Works Cited**


