Sex Steroids Modulate Human Aortic Smooth Muscle Cell Matrix Protein Deposition and Matrix Metalloproteinase Expression

Alaina K. Natoli, Tanya L. Medley, Anna A. Ahimastos, Brian G. Drew, Daniel J. Thearle, Rodney J. Dilley, Bronwyn A. Kingwell

Abstract—Large artery stiffening increases cardiovascular risk and promotes isolated systolic hypertension which is more prevalent in elderly women than men. Variation in sex steroid levels between males and females and throughout life may modulate arterial stiffness. We hypothesized that sex steroids directly influence expression of important structural proteins which determine arterial biomechanical properties. Human aortic smooth muscle cells were incubated with physiological concentrations of 17β-estradiol, progesterone, 17β-estradiol and progesterone, or testosterone for 4 weeks. Collagen, elastin, and fibrillin-1 deposition was examined (histochemistry/immunohistochemistry). Gene and protein expression of 2 important matrix metalloproteinases (MMPs), MMPs 2 and 3, regulating matrix turnover was assessed. All sex steroids reduced collagen deposition relative to control (100%). However, the reduction was greater with female sex steroids than testosterone (control, 100%; 17β-estradiol plus progesterone, 20±2%; testosterone 74±12%, P<0.001). Female sex steroids increased elastin deposition compared with control (control, 100%; 17β-estradiol, 540±60%; progesterone, 290±40%; 17β-estradiol plus progesterone, 400±80%, all P<0.01). The elastin/collagen ratio was >11-fold higher in the presence of 17β-estradiol and progesterone compared with testosterone. Fibrillin-1 deposition was doubled in the presence of female sex steroids (17β-estradiol plus progesterone) compared with testosterone (P<0.01). MMP-2 gene and protein expression was unaffected by any sex steroid. Testosterone increased both gene and protein expression of MMP-3 relative to both control and female sex steroids (P<0.01). This may contribute to degradation of elastic matrix proteins. In conclusion, female sex steroids promote an elastic matrix profile, which likely contributes to variation in large artery stiffness observed between sexes and with changes in hormonal status across the lifespan. (Hypertension. 2005;46:10.1161/01.HYP.0000187016.06549.96.)

Key Words: collagen ■ elastin ■ sex ■ arterial stiffness

Stiffness of the large arteries is a key determinant of pulse pressure and independently related to cardiovascular outcome. Sex has an important influence on arterial stiffness, which may be mediated in part via the influence of sex steroids on arterial structure and function. Before menarche and after menopause, when female sex steroid secretion is low, females have stiffer large arteries and higher pulse pressure than age-matched males. Variation in sex steroid levels between males and females and throughout life may modulate arterial stiffness. We hypothesized that sex steroids directly influence expression of important structural proteins which determine arterial biomechanical properties. Human aortic smooth muscle cells were incubated with physiological concentrations of 17β-estradiol, progesterone, 17β-estradiol and progesterone, or testosterone for 4 weeks. Collagen, elastin, and fibrillin-1 deposition was examined (histochemistry/immunohistochemistry). Gene and protein expression of 2 important matrix metalloproteinases (MMPs), MMPs 2 and 3, regulating matrix turnover was assessed. All sex steroids reduced collagen deposition relative to control (100%). However, the reduction was greater with female sex steroids than testosterone (control, 100%; 17β-estradiol plus progesterone, 20±2%; testosterone 74±12%, P<0.001). Female sex steroids increased elastin deposition compared with control (control, 100%; 17β-estradiol, 540±60%; progesterone, 290±40%; 17β-estradiol plus progesterone, 400±80%, all P<0.01). The elastin/collagen ratio was >11-fold higher in the presence of 17β-estradiol and progesterone compared with testosterone. Fibrillin-1 deposition was doubled in the presence of female sex steroids (17β-estradiol plus progesterone) compared with testosterone (P<0.01). MMP-2 gene and protein expression was unaffected by any sex steroid. Testosterone increased both gene and protein expression of MMP-3 relative to both control and female sex steroids (P<0.01). This may contribute to degradation of elastic matrix proteins. In conclusion, female sex steroids promote an elastic matrix profile, which likely contributes to variation in large artery stiffness observed between sexes and with changes in hormonal status across the lifespan. (Hypertension. 2005;46:10.1161/01.HYP.0000187016.06549.96.)

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There is evidence from a variety of tissues that both male and female sex steroids regulate the extracellular matrix at a number of levels. Specifically, testosterone reduces elastin to collagen ratio in rat aorta, whereas estrogen treatment has the opposite effect. Whether such effects translate to humans or are mediated through modulation of expression of matrix proteins and their regulators, including matrix metalloproteinases (MMPs), is not known. However there is some evidence in both uterine tissue and vascular smooth muscle that sex steroids influence specific MMPs.

With a view to understanding sex differences in large artery biomechanical properties, this study investigated the effects of 17β-estradiol, progesterone and testosterone on expression of specific extracellular matrix components and MMPs. The study was conducted using human aortic smooth muscle cell culture and examined the direct actions of sex steroids independently of their hemodynamic actions present
in whole animal models. The focus was on major matrix proteins including collagen, elastin, and fibrillin-1. In addition, we studied sex steroid regulation of MMP-2 and MMP-3, which both play an important role in vascular remodeling of healthy tissue. These MMPs are constitutively expressed and have a broad substrate spectrum. MMP-2 degrades elastin, fibronectin, and type IV collagen, and discordant regulation contributes to vascular aging.\(^{17}\) MMP-3 is a potent member of the stromelysin group of MMPs and degrades fibronectin, proteoglycans, elastin, fibrillin-1, and nonhelical collagens.\(^{18}\) In addition, MMP-3 is also an activator of proMMPs.\(^{19,20}\) We hypothesize that female sex steroids stimulate higher elastin/collagen ratios compared with testosterone and that such effects may be mediated in part through altered MMP expression.

**Methods**

**Study Design**

Primary human aortic smooth muscle cells were grown for 4 weeks with physiological concentrations of: (1) 17\(\beta\)-estradiol; (2) progesterone; (3) 17\(\beta\)-estradiol plus progesterone; and (4) testosterone. At completion of the 4-week period, deposition of collagen and elastin were assessed using histochemistry and fibrillin-1 by immunohistochemistry. Fibrillin-1, MMP-2, and MMP-3 gene expression was quantified by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and MMP-2 and MMP-3 protein expression via Western blot.

A human aortic smooth muscle cell line derived from an 11-month-old white female was used for all studies (CRL-1999; American Type Culture Collection). Cells were cultured in Dulbecco’s Modified Eagle Medium/Hams F12K 50:50 (DMEM/F12), phenol red-free (Invitrogen) containing 10% fetal calf serum, 90 mmol/L NaHCO\(_3\), 4 mmol/L L-glutamine, 1 U/mL penicillin, and 1 \(\mu\)g/mL streptomycin at 37°C and 5% CO\(_2\). Culture medium was changed every 2 to 3 days. Passage 5 cells were thawed, grown until confluent, and split. At passage 6, confluent cells were cultured with physiological concentrations of sex steroids (17\(\beta\)-estradiol, 700 pmol/L; progesterone, 50 nmol/L; 17\(\beta\)-estradiol, 700 pmol/L; and progesterone, 50 nmol/L or testosterone, 150 pmol/L; Sigma). Testosterone concentrations were based on the average level of circulating free testosterone in an adult male, and female hormone levels were the average circulating levels in the mid-luteal phase of the menstrual cycle. Sex steroids did not affect cell viability and total protein content did not vary between treatments. Six separate experiments (replicates) including a control were performed for each hormonal treatment and each analysis (n=6).

**Matrix Deposition (Collagen, Elastin, Fibrillin-1)**

Deposition of collagen was assessed using picrosirius red histochemistry, which stains collagen types I, II, and III.\(^{21}\) Elastin was assessed by orcein histochemistry.\(^{22}\) Deposition of fibrillin-1 was examined using immunohistochemistry with normal horse serum (CSL Biosciences) used to block nonspecific binding. A mouse anti-human fibrillin-1 monoclonal antibody (Clone 11C1.3; Neomarkers) was used in conjunction with a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories). Staining was visualized with diaminobenzidine (DAB; Sigma). Light microscope images were captured at a magnification of \(\times850\) with a color video camera (JVC) and image analysis software (Optimas, Media Cybernetics). Using a color threshold detection system, standardized for each protein, the proportion of stained area within each field was calculated as a percentage of the total area. Ten fields per slide were analyzed and averaged for each of 6 treatment replicates and data expressed relative to control of 100%.

**Real Time RT-PCR (Fibrillin-1, MMP-2, MMP-3)**

MMP-2, MMP-3, and fibrillin-1 mRNA levels were determined using real-time RT-PCR sequence detection (ABI Prism 7700; Applied Biosystems) in duplicate on 2 separate occasions (ie, 4 times). mRNA levels were normalized to 18S rRNA (Applied Biosystems) and fold expression was determined as previously described.\(^{23}\)

**Western Blotting (MMP-2 and MMP-3)**

Total protein expression of MMP-2 and MMP-3 was determined by Western blotting using relevant antibodies (primary: anti-MMP-2 and anti-MMP-3, Sigma, Saint Louis, Mo; secondary: anti-rabbit polyclonal MMP-2 and anti-mouse monoclonal MMP-3, Amersham Pharmacia Biotech), and proteins were visualized using the ECL technique. Bands/protein levels were then quantified on digitized films as the product of band density and area using Optimas 6.1 Software (Media Cybernetics, L.P., Silverspring, Md) and expressed relative to a control level of 1.

**Statistics**

All data are presented as mean±SEM. A 1-way ANOVA was used to compare the 4 hormone treatments with control. When the ANOVA was significant (\(P<0.05\)), a least significant difference post-hoc test was used to compare individual means. All analyses were conducted using SPSS (Version 12.0; SPSS Inc, Chicago, Ill). Statistical significance was deemed to have been achieved when \(P<0.05\).

**Results**

**Matrix Deposition (Collagen, Elastin, Fibrillin-1)**

Collagen deposition was reduced by 17\(\beta\)-estradiol, progestrone, and the combination of 17\(\beta\) estradiol and progesterone (control, 100%; 17\(\beta\)-estradiol, 74±10%; \(P=0.03\); progesterone, 30±5%; \(P=0.001\); 17\(\beta\)-estradiol plus progesterone 20±2%; \(P=0.001\); Figure 1, upper panel). The combination of progesterone and 17\(\beta\)-estradiol reduced collagen deposition significantly more than either treatment alone (\(P<0.05\)). Collagen deposition was also reduced by testosterone (control, 100%; testosterone, 74±12%; \(P=0.03\); Figure 1, upper panel). The reduction in collagen deposition with the combination of female sex steroids was 2.5-fold greater than for testosterone alone (\(P<0.001\)).

Elastin deposition was increased by all female sex steroids (control, 100%; 17\(\beta\)-estradiol, 540±60%; \(P<0.001\); progestrone, 290±40%; \(P=0.003\); 17\(\beta\)-estradiol plus progesterone 400±80%; \(P<0.001\); Figure 1, lower panel). 17\(\beta\)-estradiol had the greatest effect, whereas progesterone had a smaller effect which was similar whether delivered alone or in combination with 17\(\beta\)-estradiol. Testosterone did not significantly influence elastin deposition.

In the presence of both female sex steroids, the elastin/collagen ratio was double that of either 17\(\beta\)-estradiol or progesterone alone and >11-fold higher compared with testosterone.

Testosterone significantly reduced fibrillin-1 deposition relative to control. Although female sex steroids alone or combination did not change fibrillin-1 deposition from control, the combination of 17\(\beta\)-estradiol plus progesterone resulted in approximately double the fibrillin-1 deposition of testosterone (\(P=0.003\); Figure 2, upper panel). mRNA levels were not different between treatments indicating that this was not mediated by an effect on gene transcription (Figure 2, lower panel).
Matrix Metalloproteinases (MMP-2, MMP-3)

MMP-2 gene and protein expression was unaffected by any sex steroid tested (Figure 3, left panels). Testosterone increased MMP-3 protein 2-fold relative to both control (P=0.002) and the combination of progesterone and 17β-estradiol (P=0.001). This was largely caused by an increase in transcription (Figure 3, lower, right panel). Progesterone also increased MMP-3 mRNA levels but this did not translate to a significant effect at the protein level.

Discussion

The major finding of this study is that sex steroids influence expression of important matrix proteins including collagen, elastin, and fibrillin-1 and their regulators (MMPs) in a relevant human model. Such effects may, in part, explain differences in large artery stiffness between males and females and across the life span.

Compared with testosterone, the elastin/collagen ratio was >7-fold higher with either female sex steroid alone and >11-fold higher with the combination. It is interesting to note that whereas female sex steroids acted in an additive fashion to decrease collagen deposition, progesterone attenuated the increase in elastin deposition observed with 17 β-estradiol alone. Female sex steroids, when administered together, increased fibrillin-1 deposition relative to testosterone. The lower elastin and fibrillin-1 deposition in the presence of testosterone relative to 17β-estradiol and progesterone may relate to elevated expression of MMP-3. These findings provide a structural basis for the observations that: (1) females have more compliant large arteries during the reproductive years compared with either before puberty or after menopause and (2) males experience an increase in arterial stiffness during the pubertal transition. Because large artery stiffness is an important determinant of systolic/pulse pressure and ultimately cardiovascular risk, these findings are of relevance to sex differences in cardiovascular risk. Specifically, low levels of female sex steroids after menopause may explain the known increase in arterial stiffness relative to both the reproductive years and age-matched males, and may contribute to the greater prevalence of isolated systolic hypertension in elderly females. Animal models will be required to further substantiate this contention.

Large Artery Structure and Arterial Stiffness

The biomechanical properties of the large arteries are conveyed largely by the extracellular matrix and its interaction with smooth muscle cells within the lamellar unit. Collagen types I, III, IV, V, and VI are all found in human aorta and play an important structural and load bearing role. The
Elastic lamellae are composed primarily of elastin and are structurally important in conveying elasticity to the aorta. Elastin, fibrillin-1, and fibronectin are all key components of distensible fibrils connecting smooth muscle cells with the elastic lamellae. Although the extracellular matrix in the human aorta is highly complex, it is clear that the collagens primarily convey strength, whereas elastin and related proteins including fibrillin-1 convey distensibility. Increases or decreases in elastin/collagen ratio have been shown to reflect increased or decreased distensibility, respectively.

The effect of sex steroids on vascular extracellular matrix regulation in human cells has not previously been examined. In a series of studies, however, Fischer et al have explored this relationship in animal models. Rats receiving estradiol had a higher elastin/collagen ratio and lower systolic blood pressure than those receiving testosterone. Furthermore, in an atherosclerotic rabbit model, 3 months of treatment with the contraceptive steroid mestranol-norethynodrel inhibited collagen synthesis in the aorta. Conversely, ovariectomized atherosclerotic rabbits had elevated collagen deposition, which was ameliorated by estradiol treatment but not by progesterone. In the same model, female rabbits had elevated collagen deposition when treated with testosterone or progesterone. The current study extends this animal work to a human model and shows that sex steroids exert their influence independently of the hemodynamic effects operating in in vivo models.

Mechanisms
Receptors for estrogen, progesterone, and testosterone are variably expressed in both the endothelium and vascular smooth muscle cells of multiple vessels. It is well known that sex steroids modulate vascular tone through receptor-mediated effects, which are both genomic and nongenomic. Sex steroids thus likely influence arterial stiffness by passive mechanisms related to both effects on mean arterial pressure and changes in the relative loading of collagen and elastin within the arterial wall. Such mechanisms likely mediate changes in arterial biomechanical properties throughout the menstrual cycle. More controversial is whether sex steroids mediate structural effects in the large arteries that contribute to sex differences in large artery properties. The current data indicate that such effects are likely to have an important influence on arterial biomechanical properties.

Matrix protein deposition is the net result of matrix synthesis and degradation. Previous studies report that 17β-estradiol reduces collagen biosynthesis in bovine aortic smooth muscle cells and mesangial cells. Testosterone, however, had no effect on collagen synthesis in mesangial cells. Little is known regarding the effect of progesterone on matrix biosynthesis or the effect of any sex steroid on the biosynthesis of elastic proteins, including elastin and fibrillin-1.

In the current study it is not possible to definitively determine whether sex steroids exerted their effects via matrix protein synthesis or degradation. However, the elevation in MMP-3 gene and protein expression with testosterone likely contributed to degradation and thus lower deposition of elastin and fibrillin-1. Conversely, the higher levels of elastic matrix proteins with the combination of progesterone and 17β-estradiol may be caused by lower levels of MMP-3.

Conclusion
Sex steroid modulation of arterial properties may contribute to variation in large artery stiffness and pulse pressure between sexes and across the lifespan. The mechanisms underlying postmenopausal loss of cardiovascular protection are unclear but systolic and pulse pressure elevation may contribute. Postmenopausal normotensive and untreated hypertensive women exhibit a greater increase in pulse pressure (PP) compared with men in a similar age range, caused mainly by a greater increase in systolic blood pressure (SBP). Thus, there is a greater incidence of isolated systolic hypertension in elderly women compared with men. The current data imply that modulation of arterial biomechanical properties may be important among the mech-
anisms by which sex steroids modulate vascular properties, blood pressure and thus cardiovascular risk. Our data indicate that the combination of 17β-estradiol and progesterone increase elastin/collagen ratio in an additive manner. This was not caused by effects on elastin, in which the 2 hormones had counteractive effects, but was rather related to the additive effects of female sex steroids on inhibition of collagen deposition. Progesterone is frequently described as opposing the effects of estradiol; however, there is increasing evidence, supported by our collagen data, that this may be an oversimplification. Thus, both low levels of female sex steroids and testosterone may contribute to the elevated risk of isolated systolic hypertension.

Perspectives
The combination of 17β-estradiol and progesterone resulted in an elastin/collagen ratio 11-times higher than testosterone. The lower elastin deposition associated with testosterone may be secondary to greater MMP-3 activity and increased degradation of elastic matrix components. Sex steroids likely be related to the additive effects of female sex steroids on the greater prevalence of systolic hypertension in elderly women. Our data indicate that the combination of 17β-estradiol and progesterone resulted in an elastin/collagen ratio 11-times higher than testosterone. The lower elastin deposition associated with testosterone may be secondary to greater MMP-3 activity and increased degradation of elastic matrix components. Sex steroids likely play an important role in mediating sex differences in large artery stiffness through regulation of the extracellular matrix.

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References


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