Regulation of vascular smooth muscle cell expression and function of matrix metalloproteinases is mediated by estrogen and progesterone exposure

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Objective: **Postmenopausal women receiving hormone replacement therapy (HRT) have been reported to have more adverse outcomes after vascular reconstructions, including increased intimal hyperplasia development and bypass graft failure. HRT may be affecting the pathway contributing to intimal hyperplasia. An important component of this pathway involves matrix metalloproteinases (MMPs), implicated in vascular remodeling due to their ability to degrade components of the extracellular matrix. We hypothesize that estrogen (Est) and progesterone (Prog) upregulate the MMP pathway in vascular smooth muscle cells (VSMCs) thereby increasing MMP activity and function.**

Methods and Results: **VSMCs were incubated with Est (5 ng/mL), Prog (50 ng/mL), Est + Prog combination (Est/Prog), and/or doxycycline (40 μg/mL; Doxy). Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis we have previously shown membrane type 1-MMP (MT1-MMP) messenger ribonucleic acid (mRNA) levels are significantly increased by Est. Here, Western blot analyses indicated MT1-MMP and MMP-2 protein levels, not tissue inhibitor of MMP-2 (TIMP-2), were increased in response to Est and Est/Prog ($P < .05$ vs control). In-gel zymography revealed that Est and Est/Prog resulted in increased MMP-2 activity (hormone groups, $P < .05$ vs control) with no significant difference among the hormone groups. VSMC migration was increased by 45 ± 14% in response to Est ($P < .05$ vs control), as measured using a modified Boyden chamber assay. Doxycycline significantly inhibited basal and Est/Prog-stimulated increases in MMP-2 activity ($P < .05$ vs control; $P < .05$ vs hormone groups), and partially blocked basal and hormonally stimulated migration ($P < .05$ vs control and Est).**

Conclusion: **Estrogen and progesterone affects the MMP pathway by increasing MMP-2 enzymatic activity, possibly via the upregulation of MT1-MMP expression without a corresponding increase in TIMP expression. This increased collagenase activity increases VSMC motility and their ability to migrate through a collagen type IV lattice. Est/Prog upregulation of MT1-MMP may contribute to the adverse effect of HRT on vascular interventions. (J Vasc Surg 2009; 49:185-91.)**

Clinical Relevance: **Postmenopausal women receiving HRT have more adverse outcomes after vascular reconstructions, including intimal hyperplasia, restenosis, and decreased graft patency. MMPs play a major role in vascular remodeling due to their degradation of components of the basement membrane separating vascular cell layers. Specifically, MMP-2 has a strong affinity for collagen type IV degradation, and MT1-MMP is a transmembrane protein known to activate MMP-2 by proteolytic cleavage. Here we provide strong evidence for MT1-MMP's role in increased MMP-2 activity and increased cellular migration in VSMCs exposed to estrogen and progesterone. Manipulations of the MMP pathway specifically targeting MT1-MMP expression at the time of vascular interventions may improve outcomes in females receiving HRT.**

Atherosclerotic disease in the coronary and peripheral arterial vasculature is a leading cause of morbidity and mortality in the United States.[1] The incidence of both cardiovascular disease and noncardiac vascular disease is greater in men compared with age-matched premenopausal women,[2,3] and there is a long-standing hypothesis that estrogen levels might contribute to this gender-based difference by providing "vascular protection". Therefore, decreased risk of coronary vascular disease has been an expected benefit of hormone replacement therapy (HRT), and early observational studies reported decreased risk in postmenopausal women taking HRT.[4,5] More recently, however, the validity of these findings have been challenged. Evidence indicates that HRT used for primary prevention in healthy postmenopausal women has an adverse effect on heart disease risk.[6] Moreover, HRT failed to slow the progression of atherosclerosis in postmenopausal women with established coronary artery disease,[7] and the use of HRT has been shown to increase the risk of coronary and thromboembolic events.[8] Postmenopausal women receiving HRT have more adverse outcomes of peripheral arterial disease and after vascular reconstruction. A previous study from our group reports postmenopausal women tak-

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ing HRT at the time of peripheral bypass grafting have decreased primary graft patency compared to their non-HRT counterparts.[9]

Intimal hyperplasia (IH) is the general response of a vessel to injury and is considered the primary reason for late bypass graft failure. Migration of medial vascular smooth muscle cells (VSMCs) to the intima region plays a major role in IH development during vessel remodeling in arterial disorders such as atherosclerosis, hypertension, and restenosis following balloon angioplasty.[10,11] Additionally, estrogen-binding receptors have been identified on VSMCs, consistent with the theory that this vascular function may be under direct hormonal control.[12,13] An in vivo study by Miller et al reports that aged rats (12-months-old) given HRT developed more IH after balloon-induced vascular injury compared to their younger (10-week-old) female counterparts.[14]

Matrix metalloproteinases (MMPs) are implicated in vascular remodeling due to their ability to selectively degrade components of the extracellular matrix (ECM).[15,16] VSMCs in the tunica media are surrounded by ECM that must be degraded in order for cells to migrate through the vascular layers. MMP-2 and -9 are considered to play a major role in remodeling because of their ability to degrade collagen type IV, the major component of the basement membrane separating the vascular endothelial and medial cell layers. MMP expression can be induced by cytokines, growth factors, stress, or inflammation.[17,18] Additionally, we have previously shown female sex hormones affect MMP pathways through isoform-specific mechanisms.[19]

MMP proteins are synthesized in their proenzyme form and can be activated by proteolytic cleavage by other MMP family members, such as membrane type-matrix metalloproteinases (MT-MMPs).[20] Additionally, MMPs can be inhibited by tissue inhibitors of MMPs (TIMPs) binding to MMP active sites or to active MT-MMPs.[21,22] A balance in the expression and/or activity of MMPs, MT-MMPs, and TIMPs is maintained under normal physiological conditions. However, alterations within the regulatory mechanism resulting in unbalanced MMP activity have been associated with pathological remodeling.[17,23,24]

The present study was undertaken to examine estrogen- and progesterone-regulated MMP expression and activity in VSMCs, the major cell type of interest in the formation of intimal hyperplasia and restenosis. We hypothesized that estrogen and progesterone upregulate the MMP pathway in VSMCs by causing an imbalance in its regulatory mechanism, resulting in increased matrix proteolytic degradation and VSMC migration across ECM barriers. Understanding hormone-regulated modifications to the critical balance of MMP/MT-MMP/TIMP activities will enhance understanding of the mechanisms leading to development of intimal hyperplasia in women receiving HRT.

MATERIALS AND METHODS

Aortic smooth muscle cell cultures. VSMCs used in this study were human aortic smooth muscle cells (HASMC) purchased from Cascade Biologics, Inc, Portland, Ore. HASMCs obtained were cryopreserved primary cultures of female single-donor cells, and their homogeneous nature had been confirmed through positive immunocytochemical staining for α-actin and negative immunocytochemical staining for von Willebrands factor. The cultures were maintained and subcultured according to the supplier's instructions and used between passages 4 and 8. Because phenol red functions as a weak estrogen agonist,[25] cells were cultured in phenol red-free growth medium ([5% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM)] for 24 hours prior to beginning experiments. Cells were grown to 80% confluence and made quiescent by serum starvation for 24 hours.

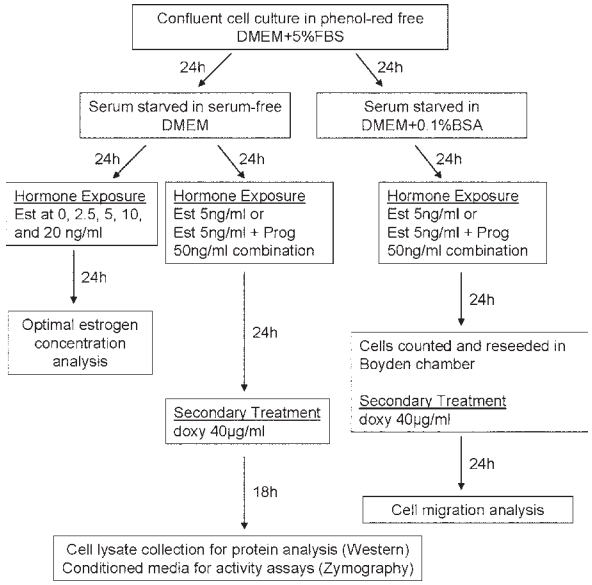

Fig 1. Schematic diagram depicting treatment timelines for each study arm. *DMEM,* Dulbecco's modified Eagle's medium; *FBS,* fetal bovine serum; *BSA,* bovine serum albumin; *Est,* estrogen; *Prog,* progesterone; *Doxy,* doxycycline.

Cell treatment. Cells were treated with β-estradiol (2.5-20 ng/mL; Est), progesterone (50 ng/mL; Prog), or a hormone combination (5 ng/mL Est + 50 ng/mL Prog; Est/Prog) for 24 hours. Randomly designated groups were maintained in their respective hormone supplemented media and subsequently incubated for 18 hours with or without MMP inhibitor doxycycline (40 μg/mL; Doxy). Cells from these treatment groups were used to measure protein levels and enzymatic activity of various members of the MMP family as well as smooth muscle cell migration. A flow chart diagramming the methods and various cell treatment arms of the study can be found in Fig 1. All other reagents were purchased from Sigma-Aldrich, St. Louis, Mo.

Western blot analyses. Total cell lysates were prepared in cell lysis buffer (Cell Signaling Technology, Danvers, Mass), and protein contents were measured using the BCA protein assay (Pierce, Rockford, Ill). Equal amounts of proteins were resolved by 10% sodium dodecyl sulfate

poly-acrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were probed with anti-MT1-MMP (Chemicon International, Temecula, Calif), anti-MMP-2, anti-MMP-9, anti-TIMP-2 (Calbiochem, La Jolla, Calif), or anti-β-actin antibodies (Sigma-Aldrich, St. Louis, Mo). The membranes were then incubated with appropriate secondary antibodies, and immune complexes were detected using chemiluminescence and autoradiography. Band intensities were quantified by densitometry and normalized to β-actin as a loading control.

In-gel zymography. MMP activities were measured in conditioned media using in-gel protease analysis by Novex (Invitrogen Co, Carlsbad, Calif). Briefly, the conditioned media were lyophilized, and the pellets were resuspended in water. The concentrated proteins were electrophoresed under non-reducing conditions into 10% SDS-PAGE gels polymerized with 1 mg/mL gelatin. Following electrophoresis, the gels were washed in renaturing buffer and incubated overnight at 37°C in developing buffer. The gels were then stained by Simply Blue SafeStain (Invitrogen Co) and destained with water. Unstained, digested regions represent areas of MMP activity. These translucent bands were quantified by densitometry.

Cell migration. VSMC migration was evaluated using 24-well Transwell migration chambers with 8.0 μm pore polycarbonate filter inserts (Corning, Inc, Lowell, Mass). The inserts were coated with 50 μg/mL collagen type IV (BD Biosciences, Bedford, Mass) in 0.05N HCl overnight at room temperature, and rehydrated with 0.1% bovine serum albumin (BSA)/DMEM). Hormone-treated VSMCs were counted and reseeded on the inserts at 10^5 cells/100 μl 0.1% BSA/DMEM in the presence or absence of Doxy. In the lower chambers, 10% FBS/DMEM served as the chemoattractant. After 8 hours at 37°C and 5% CO_2 the non-migrated cells were removed and the migrated cells on the lower side of the filters were fixed and stained with Dif-Quick stain (Dade Behring, Inc, Deerfield, Ill). Migration was quantified by counting the stained cells in three random fields per membrane at 40X magnification.

Statistical analysis. All data are reported as mean ± SEM. Statistical analyses were performed using Student's *t* test or one-way ANOVA and a post-hoc Tukey's test. Probability (*P*) values of $< .05$ were considered to be significant.

RESULTS

Estrogen affects MMP-2 production in a concentration-dependent manner. Cells were treated with estrogen concentrations ranging from 2.5-20 ng/mL for 24 hours. An increase in MMP-2 protein levels was evident at 5 ng/mL (Fig 2). Typical estrogen: progesterone levels for premenopausal women prior to ovulation are at a ratio of 1:10.[26] HRT is commonly prescribed at this ratio to postmenopausal women to reduce side effects and risks accompanying menopause. Given our objective to investigate hormonally-stimulated effects on MMPs at a translational level, we chose hormone combination conditions (Est 5 ng/mL + Prog 50 ng/mL) that maintained this relevant estrogen/progesterone ratio.

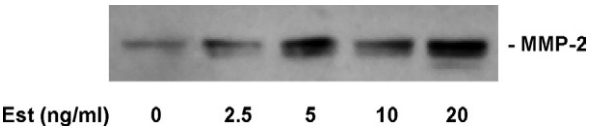

Fig 2. Determination of optimal hormone concentration. Vascular smooth muscle cells (VSMCs) were treated with the indicated concentrations of estrogen (Est) for 24 hours. Total cell lysates were analyzed by Western blot analysis using anti-matrix metalloproteinase (MMP)-2 antibodies in order to determine the minimum concentration resulting in upregulation of MMPs.

Hormone stimulation increases MT1-MMP and MMP-2 protein levels with no effect on TIMP-2. We have recently reported that estrogen and progesterone differentially regulate VSMCs MMP gene expression via isoform-specific mechanisms.[19] RT-PCR analyses of total ribonucleic acid (RNA) indicated Est increases MT1-MMP mRNA levels independent of inflammatory cascade signaling or the presence of inflammatory cytokines. Here, to more closely examine hormonal effects on MMP family protein production, VSMCs were treated with individual and combination hormones as previously described. Western blot analyses of highly homogenized cell lysates confirmed an increase in basal MT1-MMP expression at the protein level in response to estrogen (percent increase vs control; Est, 31 ± 11* [*$P < .05$ vs control; n = 3; Fig 3, *A*]). Furthermore, Western blot analyses of total cell lysates indicated an increase in basal MMP-2 protein levels in response to both estrogen and estrogen/progesterone combination (percent increase vs control; Est, 47 ± 5%*; Est/Prog, 31 ± 10%* [*$P < .05$ vs control; n = 4; Fig 3, *A*]). As expected, doxycycline had no significant inhibitory effect on MT1-MMP or MMP-2 protein levels (P = NS vs control; n = 3-4; Fig 3, *A*). There was no significant difference among the hormone groups. Furthermore, Western blot analyses indicated no significant change in TIMP-2 protein levels in any group (Fig 3, *B*). No VSMC-produced MMP-9 protein was detectable by Western blot analyses, while an MMP-9 positive control source produced a readily visible signal (data not shown).

Hormone stimulation increases MMP-2 activity. In-gel zymographic analysis was used to examine hormonally regulated enzymatic activity. Estrogen and combination hormone treatment resulted in increases in basal MMP-2 activity (percent increase vs control; Est, 27 ± 5%*; Est/Prog, 46 ± 6%* [*$P < .05$ vs control; n = 4; Fig 4]). There was no significant difference among the hormone groups. As expected, doxycycline significantly inhibited MMP-2 activity (percent decrease vs control; Doxy, 47 ± 10%* [*$P < 0.05$ vs control; n = 4; Fig 4]). We were not able to detect basal or hormonally stimulated MMP-9 enzymatic activity. Hormone treatment did not affect the presence of MMP-2/TIMP-2 complex in reverse zymogram (data not shown), further confirming hormones do not have counter-regulatory effect on TIMP-2.

Hormone stimulation induces migration of VSMCs through a collagen type IV lattice. To examine the functional effects of Est and Prog on VSMC migration, we used a modified Boyden chamber assay. Est significantly increased VSMC migration across the membrane by 45 ± 14%* (*$P <$ .05 vs control; n = 3; Fig 5). Interestingly, combination hormone treatment (Est + Prog) resulted in no significant difference. Doxycycline inhibited both basal and Est-induced cell migration (percent decrease vs control; Doxy, 60 ± 16%#; Est + Doxy, 42 ± 17%‡; #$P < 0.05$ vs control; ‡$P < 0.05$ vs Est; n = 3; Fig 5).

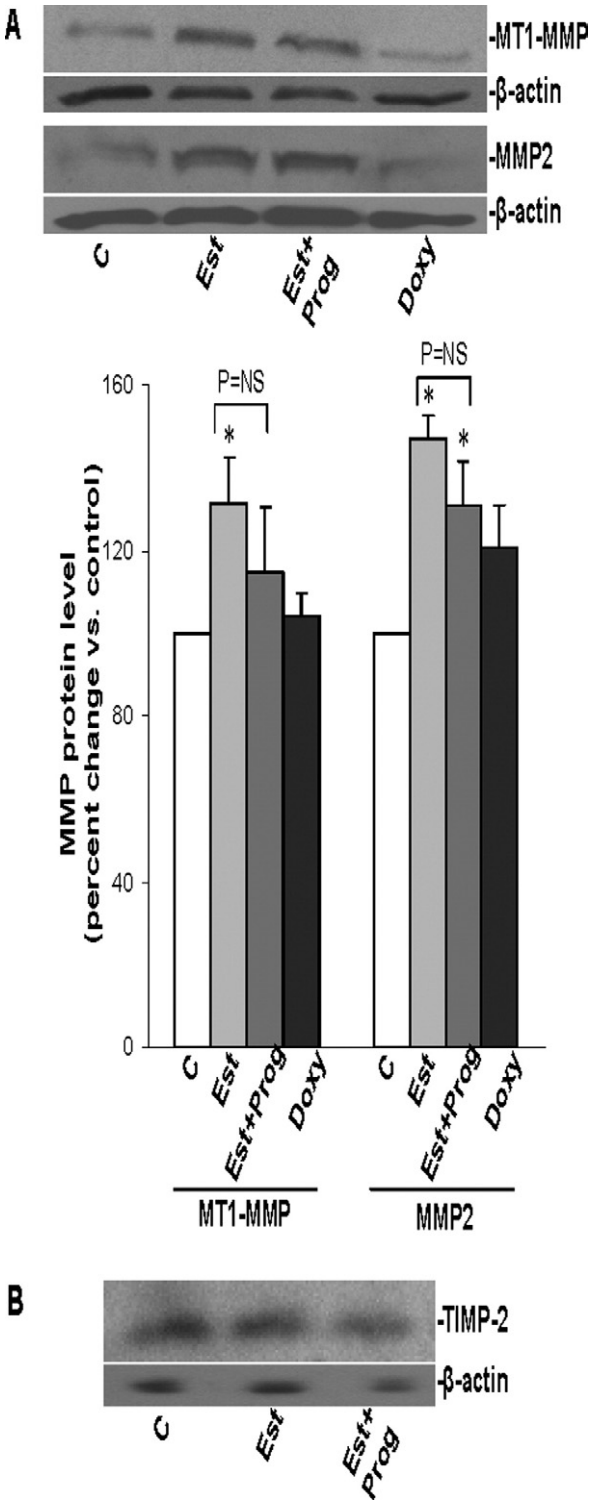

Fig 3. Hormone stimulation increases membrane type 1-MMP (MT1-MMP) and MMP-2 protein levels with no corresponding increase in tissue inhibitors of metalloproteinase (TIMP)-2. Vascular smooth muscle cells (VSMCs) were pretreated with estrogen (5 ng/mL; Est) with (Est/Prog) and without progesterone (50 ng/mL; Prog) for 24 hours followed by doxycycline (40 μg/mL; Doxy) for 18 hours. Total cell lysates were analyzed by Western blot using anti-MT1-MMP, anti-MMP-2, and anti-TIMP-2 antibodies. **A and B** demonstrate representative images from 3 or 4 independent experiments. The graph exhibits the mean data ± SEM for MT1-MMP and MMP-2 protein levels. All values have been normalized with β-actin *$P <$ .05 vs control (C); n = 4.

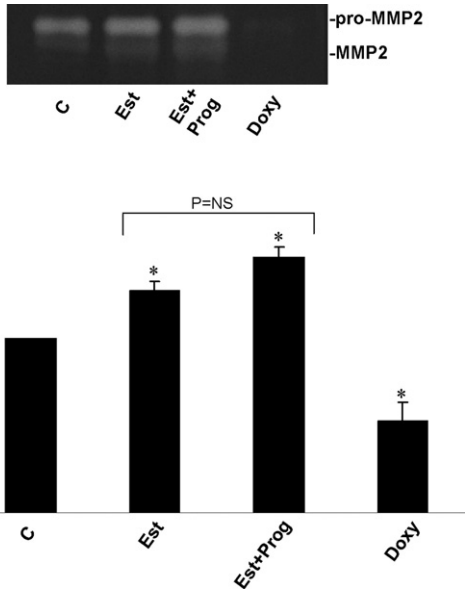

Fig 4. Hormone stimulation increases matrix metalloproteinase (MMP)-2 activity. Vascular smooth muscle cells (VSMCs) were pretreated with estrogen (5 ng/mL; Est) with (Est/Prog) and without progesterone (50 ng/mL; Prog) for 24 hours followed by doxycycline (40 μg/mL; Doxy) for 18 hours. Concentrated conditioned media were used to measure matrix metalloproteinase (MMP)-2 activity using gelatin in-gel zymography. The lower panel exhibits the mean data ± SEM. *$P <$ .05 vs control (C); n = 3-4.

DISCUSSION

The incidence of both cardiovascular disease and noncardiac vascular disease is greater in men compared with age-matched premenopausal women.[2,3] Women develop both pathologies later in life. This difference has been attributed to the protective effect of female estrogen hor-

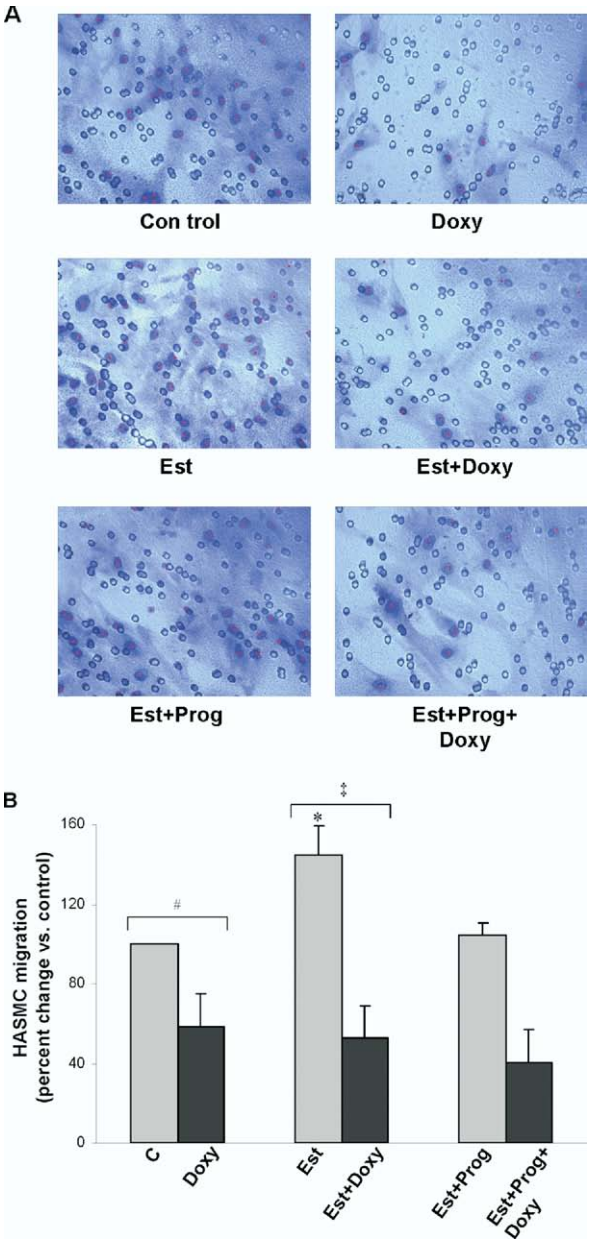

Fig 5. Hormone stimulation induces migration of vascular smooth muscle cells (VSMCs). The ability of cells to migrate through a collagen type IV lattice was assessed using a modified Boyden chamber assay. VSMCs were pretreated with estrogen with and without progesterone (Est, 5 ng/mL; Prog, 50 ng/mL) for 24 hours. Cells were seeded on porous membranes with or without doxycycline (40 μg/mL; Doxy) for an additional 8 hours. **A,** demonstrates representative images from three independent experiments. Cell migration was quantified by counting the number of migrated cells present on the lower surface in three random fields per membrane at 40X magnification. **B,** represents the mean data ± SEM. $^{*}P < .05$ vs control (C); $^{\#}P < .05$ vs control (C); $^{\ddagger}P < .05$ vs Est; n = 3. *HASMC,* human aortic smooth muscle cells.

mones lost at menopause. However, the long-standing hypothesis that estrogen levels might contribute to this gender-based difference by providing "vascular protection" has recently been challenged. Postmenopausal women receiving HRT have more adverse outcomes of peripheral arterial disease and after vascular reconstruction. It has been reported that HRT is an independent predictor of decreased primary graft patency.[9] The effect of HRT on vascular remodeling may be playing a central role in the gender-related differences seen with atherosclerotic progression and outcomes.

Dysfunctional remodeling underlies the pathogenesis of major vascular diseases, such as atherosclerosis and restenosis.[24] The progression of these diseases may be halted by restoring balance to the processes involved in remodeling of the ECM. A key group of enzymes involved in these processes are MMPs, implicated in vascular remodeling due to their ability to selectively degrade components of the ECM.[15,16] Additionally, it has been reported that increased expression of active MMPs within plaques may contribute to plaque rupture, hemorrhage, and aneurysm formation.[23,27] Rigorous regulation of MMP production and activity is a crucial part of ECM homeostasis.

Here we studied the effect of estrogen and progesterone exposure on MMPs synthesized by VSMCs. In this study, we confirm that estrogen enhances the release of MMP-2 from vascular smooth muscle cells.[27] Natoli et al have shown that female sex steroids reduced collagen deposition in human aortic smooth muscle cells, but were unable to demonstrate an effect on MMP-2 gene or protein expression with any sex steroid.[28] The effect of hormone exposure on the MMP pathway can be further explained based on these and previous results. Hormone exposure increases MT1-MMP at the transcriptional level, without a counter-regulatory effect on TIMP-2 expression,[19] ultimately leading to unbalanced MMP regulation. Liao et al have found similar results in other tissues, demonstrating estrogen-induced upregulation of MT1-MMP expression in human osteoblasts.[29] Here, hormone exposure resulted in increased MMP-2 enzymatic activity and increased cell migration through a collagen IV barrier. Based on our results and previous reports that MT1-MMP initiates the activation of MMP-2,[20] we propose that this hormonally stimulated upregulation of MMP-2 enzymatic activity is regulated by increased MT1-MMP expression. To examine if hormonally-stimulated increased cell migration could be reversed by inhibiting increased activity of MMP-2, VSMCs were exposed to doxycycline, a known inhibitor of MMP activity. Doxycycline significantly decreased VSMC migration, as well as MMP-2 activity independent of RNA or protein levels. Collectively these data support that hormonally stimulated increases in VSMC migration occur via increased collagenase activity, primarily MMP-2.

There were some unanticipated outcomes of the study. First, there was no significant difference in the increase in MMP-2 activity between estrogen alone or in combination with progesterone, indicating MMP upregulation may be a general phenomenon of estrogen availability. Progesterone

has been previously reported to play a protective role in other cell types and pathologies.[30,31] Further investigation is needed to understand the effects of progesterone in combination with estrogen and their individual and combined influence in vascular pathology. Secondly, our study was unable to demonstrate an upregulation of MMP-9, most likely due to the negligible amounts of MMP-9 found in normal aortic tissue. MMP-2 is constitutively expressed in VSMC, whereas MMP-9 is sequestered in neutrophils, macrophages, and other inflammatory cells. In vivo the presence of MMP-9 in aortic tissue is primarily due to macrophage infiltration during inflammation,[23] making MMP-9 evaluation in isolated aortic smooth muscle cells difficult at best.

Our working hypothesis formulated from the current study is that inhibition of MT1-MMP expression will result in inhibition of hormonally-stimulated MMP-2 activation and downstream effectors such as migration. In vitro studies of targeted silencing in this proposed MT1-MMP/MMP-2 pathway are currently ongoing in our laboratory. Blockade of estrogen receptors using specific receptor antagonists alone and in combination with hormones are being used to verify changes in MT1-MMP expression and MMP-2 activity are a direct result of receptor signaling prior to silencing. In addition, in vivo studies of vascular injury in subjects exposed to HRT are needed to assay the benefits of this approach as well. Alternatively, the urokinase pathway is a known mechanism of MMP activation and has been shown to play an important role in vascular remodeling.[32] Urokinase plasminogen activator (uPA) – uPA receptor (uPAR) signaling converts plasminogen to plasmin, which can in turn activate proMMP to active MMP. An alternative hypothesis for future investigation is that estrogen stimulated increases in MMP-2 activity occur via this uPA-uPAR signaling mechanism.

In conclusion, in the present study we have provided strong supportive evidence for a regulatory role of MT1-MMP in increased migration of VSMC exposed to estrogen and progesterone, mediated by an increase in MMP-2 activity. We previously demonstrated that hormone stimulation increases MT1-MMP mRNA expression, with no effect on MMP-2 or TIMP-2. Here hormone stimulation increases MT1-MMP and MMP-2 protein levels with no effect on TIMP-2. This further confirms estrogen and progesterone are not involved in TIMP-regulated MMP inhibition, leaving hormonally stimulated upregulation of MMP unbalanced. Collectively, the downstream effect of hormone stimulation results in increasing MMP-2 enzymatic activity. This may play a major role in the development of intimal hyperplasia because of VSMCs ability to degrade collagen type IV, allowing for migration and intimal thickening.

The clinical implication of our findings rests in the fact that broad, non-specific inhibition of MMPs may not result in a decrease of intimal hyperplasia in females receiving HRT. Overarching inhibition of the MMP pathway (ie, TIMPs, MT-MMPs, and MMPs) likely results in unbalanced MMP activity. Understanding the hormone-regulated modifications to the balance of MMP/MT-MMP/TIMP activity could lead to the development of more specific therapeutic options for postmenopausal patients with vascular disease undergoing interventions. Specifically targeting MT1-MMP expression at the time of vascular interventions in females receiving HRT may result in better outcomes. It has been postulated that timing of exposure to HRT in regard to the development of atherosclerotic lesions and subsequent interventions may be responsible for the differential response to local injury in previously diseased vessels. However, this variable cannot be controlled in most cases, as atherosclerotic disease is a continuous process and interventions may be necessary while the patient is receiving HRT. Therefore, alternative approaches, such as tissue targeted gene therapy need to be examined.

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AUTHOR CONTRIBUTIONS

Conception and design: OG, DM, MF
Analysis and interpretation: OG, DM, SK, MF, MG
Data collection: DM, SK
Writing the article: OG, DM
Critical revision of the article: OG, DM, SK, DC, SS, MF, MG
Final approval of the article: OG, DM, SK, DC, SS, MF, MG
Statistical analysis: DM
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Overall responsibility: OG, MF

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