Reduced NO signaling during pregnancy attenuates outward uterine artery remodeling by altering MMP expression and collagen and elastin deposition

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Hale SA, Weger L, Mandala M, Osol G. Reduced NO signaling during pregnancy attenuates outward uterine artery remodeling by altering MMP expression and collagen and elastin deposition. Am J Physiol Heart Circ Physiol 301: H1266–H1275, 2011. First published August 19, 2011; doi:10.1152/ajpheart.00519.2011.—Recent findings indicate that endothelial nitric oxide (NO) plays a key role in uterine artery outward circumferential remodeling during pregnancy. Although the underlying mechanisms are not known, they likely involve matrix metalloproteinases (MMPs). The goal of this study was to examine the linkage among NO inhibition, expansive remodeling, and MMP expression within the uterine vascular wall. Adult female rats were treated with N^3-nitro-L-arginine methyl ester [L-NAME (LPLN)] beginning on day 10 of pregnancy and until death at day 20 and compared with age-matched controls [late pregnant (LP)]. Mean arterial pressure of LPLN rats was significantly higher than controls. LPLN fetal and placental weights were significantly reduced compared with controls. Main uterine arteries (mUA) were collected to determine dimensional properties (lumen area and wall thickness), collagen and elastin content, and levels of endothelial nitric oxide synthase (eNOS) and MMP expression. Circumferential remodeling was attenuated, as evidenced by significantly smaller lumen diameters. eNOS RNA and protein were significantly (>90%) decreased in the LPLN mUA compared with LP. Collagen and elastin contents were significantly increased in LPLN rats by ~10 and 25%, respectively, compared with LP (P < 0.05). Both MMP-2 and tissue inhibitors of metalloproteinase-2 as assessed by immunofluorescence were lower in the endothelium (reduction of 60%) and adventitia (reduction of 50%) of LPLN compared with LP mUA. Membrane bound MMP-1 (MT1-MMP) as assessed by immunoblot was significantly decreased in LPLN. These data suggest a novel contribution of MMPs to gestational uterine vascular remodeling and substantiate the linkage between NO signaling and gestational remodeling of the uterine circulation via altered MMP, TIMP-2, and MT1-MMP expression and activity.

matrix metalloproteinase; extracellular matrix; hypertension; pregnancy; nitric oxide

DURING PREGNANCY, THE UTERINE vasculature undergoes significant expansive remodeling to accommodate the dramatic increase in uteroplacental blood flow that is requisite for normal pregnancy outcome. Studies from our and other laboratories (40, 41, 44, 46, 50, 61) have established that nitric oxide (NO) is a key molecule involved in vascular remodeling during pregnancy and that expression of endothelial nitric oxide synthase (eNOS) is increased during pregnancy, leading to increased synthesis and release of NO from the endothelium.

The importance of NO and NO signaling during pregnancy is underscored by the vascular and reproductive implications evident in mouse knockouts for endothelial NO synthase and in rats treated with the NO inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) during pregnancy (50, 64). Treatment of animals with L-NAME has been used repeatedly as a model of preeclampsia (3, 4, 50, 60, 65). NO-inhibited and eNOS-deficient animals have increased blood pressure, proteinuria, decreased fetal and neonatal weights, and a reduced number of viable newborn pups (50, 64, 65).

The decrease in reproductive performance in the L-NAME model is likely due to a number of factors, including decreased uteroplacental perfusion resulting from aberrant vascular remodeling and/or tone in the uterine circulation. Indeed, both small and large uterine artery outward remodeling are significantly reduced in this animal model, although the post-NO signaling mechanisms are not known (50, 63).

Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that degrade the extracellular matrix (ECM; Ref. 55). MMPs are secreted as proenzymes, must be cleaved to become active, and can be regulated by tissue inhibitors of metalloproteinases (TIMPs) by forming complexes that regulate their activity (55). For example, activation of MMP-2 can be accomplished by an enzyme complex consisting of TIMP-2 and membrane bound MMP-1 (MT1-MMP or MMP-14; Refs. 62, 68). MMPs may also be regulated by NO, although the effect of NO on MMPs is variable (6, 16, 47, 53). Interestingly, shear stress, a potent activator of NO release, has been shown to both decrease and increase MMP-2 activity, further suggesting a role for NO in the regulation of MMP activity but also underscoring the complexity of the underlying signal transduction mechanisms (16, 21, 31, 53).

Many MMPs are greatly increased during late pregnancy in the rat uterine artery (32). Although studies (27) implicate MMP-2 as a key component involved in pregnancy, where vascular remodeling is essential for the survival and growth of the fetus, evaluations of the effect of NO on MMP-2 in uterine arteries are lacking.

MT1-MMP is a membrane bound MMP that has an established role in vascular remodeling, both the normal and hypertensive state (reviewed by Refs. 28, 29). These effects of MT1-MMP can be separate from its role in activating MMP-2 and may be regulated by NO (17, 28). Despite current research on MT1-MMP, the role of MT1-MMP in pregnancy-induced remodeling has not been evaluated.

Aberrant expression of MMPs has been associated with a number of vascular pathologies such as hypertension, pre-eclampsia, and atherosclerosis (55). There are conflicting reports evaluating circulating levels of MMPs in hypertensive
patients. Some reports indicate that MMPs are elevated, while others show that they are decreased (9, 72). MMP-2 is elevated in the serum of preeclamptic women but decreased in umbilical cord arteries isolated from preeclamptic women (14, 15, 37, 45, 52). Dysregulation of MMPs may contribute to the inward eutropic remodeling evident in preeclamptic myometrial arteries (45, 49).

Considering the potential role of NO in the regulation of MMPs and in vascular pathologies, we hypothesized that NO synthase inhibition during pregnancy would affect MMP expression and thereby alter uterine artery structural remodeling. Here, we present data showing that NO synthase inhibition in the main uterine artery (mUA) decreases outward remodeling, increases arterial collagen and elastin content, and decreases MMP-2, TIMP-2, and MT1-MMP expression. Specifically, our data implicate MMPs in gestational uterine vascular remodeling and complement earlier observations that have documented the MMP-2/TIMP-2/MT1-MMP enzyme complex in both pathological vascular remodeling and compromised reproductive performance.

MATERIALS AND METHODS

Animals, treatments, and tissue collection. Age-matched pregnant Sprague-Dawley rats (Charles River) were housed at the University of Vermont College of Medicine Animal Facility and studied between 13 and 17 wk of age. All procedures were approved by the University of Vermont’s Animal Care and Use Committee. On day 10 of pregnancy, osmotic pumps (Alzet, Cupertino, CA) containing L-NAME were surgically implanted subcutaneously in the periscapular region. The main uterine artery (mUA) were dissected and fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 8 h and placed in 75% ethanol before embedding in paraffin blocks for subsequent immunohistochemistry. The remainder of the uterus was removed and placed in TriZOL reagent (Invitrogen, Carlsbad, CA) and homogenized as previously described (50). Tissue from each animal was used per reaction with 150 nM of the forward and reverse primers and 25 μl of cDNA. For each reaction, the cDNA was used to amplify the target genes (eNOS, MMP-2, TIMP-2, and MT1-MMP) and two housekeeping genes (Hprt1 and Ywhaz). One microliter of cDNA was used per reaction with 150 nM of the forward and reverse primers and 12.5 μl of Power Sybr Green Master Mix (Applied Biosystems, Carlsbad, CA) in a 25-μl reaction. The reactions were performed on an ABI Prism 7000 (Applied Biosystems) using an initial denaturation of 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C, followed by a melt curve analysis to ensure only the correct product was amplified. Standard curves were used to determine the relative quantities of each sample. Relative target mRNA values were normalized by dividing the target quantity by the geometric mean of the quantities of the housekeeping genes. Each sample was run in triplicate and averaged.

Use Committee.

Vessels were formalin fixed and paraffin embedded. Three serial cross-sections (6 μm) from each vessel were cut, transferred to slides, and then stained using the following protocol. Sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. Sections were placed in 10 g/ml and TIMP-2: 1:50; Abcam, Cambridge, MA) overnight, in a humidified box, at room temperature. The MMP-2 primary antibody recognizes both pro- and active forms of MMP-2. Sections were incubated with secondary antibody (MMP-2: 2 mg/ml and TIMP-2: 1:50; Abcam, Cambridge, MA) overnight, in a humidified box, at room temperature. The MMP-2 primary antibody was visualized for each slide using a Zeiss confocal microscope at 200 magnification coupled to a CCD camera and analyzed using the color threshold (for collagen and elastin analysis) and measurement capabilities (for lumen area and wall thickness) within MetaMorph (Molecular Devices, Downingtown, PA) image capture and analysis software.

Immunofluorescence. Vessels were placed in modified RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, and 10% glycerol) supplemented with Halt protease inhibitor cocktail (Pierce, Rockford, IL) and homogenized with Garnet Matrix A (MP Bio) using three 10-s pulses on a Biospec Bead Beater and rested on ice for 10 s in between each pulse. Protein quantity was determined by Bradford assay (Bio-Rad), and 25 μg from each sample were separated using a 6% polyacrylamide gel. Proteins were transferred to nitrocellulose and analyzed by immunoblot as described previously (38), using mouse monoclonal TIMP-2 (1:100; Abcam, Cambridge, MA), rabbit monoclonal anti-MT1-MMP (MMP-14: 1:200; Epitomics, Burlingame, CA) or rabbit polyclonal anti-NOS3 (eNOS: 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-β-tubulin (1:250; Cell Signaling, Danvers, MA), and rabbit polyclonal anti-GAPDH (1:1000, Cell Signaling). Immunoblots were analyzed by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

RNA extraction, PCR array, and quantitative RT-PCR. Tissue from mUA was homogenized in Trizol (Invitrogen) and Garnet Matrix A (MP Bio, Solon, OH) on a Biospec Bead Beater (Bartlesville, OK). This solution was then purified using a RNeasy Micro spin column (Qiagen, Valencia, CA) following manufacturer’s instructions. Residual DNA was removed using Ambion Turbo Dnase (Ambion, Austin, TX). RNA concentrations were determined by a Nanodrop spectrometer (Nanodrop, Wilmington, DE). Before quantitative (q)PCR, RNA integrity was analyzed on an Agilent Bioanalyzer (Agilent, Santa Clara, CA).

RT-qPCR studies were performed in a two-step process. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA. For each sample, the cDNA was used to amplify the target genes (eNOS, MMP-2, TIMP-2, and MT1-MMP) and two housekeeping genes (Hprt1 and Ywhaz) using 10 mg/kg and TIMP-2: 1:50. Sections were incubated with primary antibody (MMP-2: 2 mg/ml and TIMP-2: 1:50; Abcam, Cambridge, MA) overnight, in a humidified box, at room temperature. The MMP-2 primary antibody was visualized for each slide using a Zeiss confocal microscope at 200 magnification coupled to a CCD camera and analyzed using the color threshold (for collagen and elastin analysis) and measurement capabilities (for lumen area and wall thickness) within MetaMorph (Molecular Devices, Downingtown, PA) image capture and analysis software.

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Both fetal and placental weights were significantly lower in the LP group compared with LP controls at both time points of day 5 (LP: 101 ± 2 mmHg and LPLN: 132 ± 4 mmHg; P < 0.001; n = 8 for both groups) and day 10 of treatment (LP: 92 ± 3 mmHg and LPLN: 128 ± 4 mmHg; P < 0.001; n = 8 for both groups).

Litter size and resorption rate were not affected by L-NAME treatment (P = 0.24 and P = 0.44, respectively). However, both fetal and placental weights were significantly lower in the L-NAME-treated group (fetal weights: LP: 2.4 ± 0.06 g and LPLN: 2.2 ± 0.07 g; P = 0.01; n = 8 for both groups; placental weights: LP: 0.46 ± 0.008 g and LPLN: 0.43 ± 0.01 g; P = 0.02; n = 8 for both groups).

Maternal mUA characteristics. Relative to LP controls, LPLN-treated mUA had significantly smaller lumen diameters, unchanged wall thickness, and an increased wall-to-lumen ratio (Table 1).

Expression of eNOS RNA and protein expression. Expression of eNOS RNA in the mUA was assessed using RT-qPCR; L-NAME treatment significantly reduced eNOS RNA expression (Fig. 1A). eNOS and GADPH protein levels were assessed by immunoblot and quantified using densitometry (Fig. 1B, top and bottom, respectively). GADPH was used as a loading control.

Collagen and elastin content. To evaluate the effect NO inhibition on ECM composition, we evaluated changes in collagen and elastin within the vessel cross-section as described above. L-NAME treatment significantly increased collagen (Fig. 2A, blue staining) content in the mUA artery cross-section compared with controls (Fig. 2, A and B). Elastin content in the mUA (Fig. 2, A and C, dark purple/black staining) was also significantly increased.

### RESULTS

Maternal blood pressure and fetal characteristics. Maternal mean arterial pressure was significantly higher in the LPLN rats compared with LP controls at both time points of day 5 (LP: 101 ± 2 mmHg and LPLN: 132 ± 4 mmHg; P < 0.001; n = 8 for both groups) and day 10 of treatment (LP: 92 ± 3 mmHg and LPLN: 128 ± 4 mmHg; P < 0.001; n = 8 for both groups).

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### Statistical analysis

Statistical differences between treatment groups were determined from individual vessel data. Statistical significance for all comparisons was assessed using Student’s t-test within the Sigma Plot statistical package (Systat Software, San Jose, CA). Data are presented as means ± SE, with n values representing the number of animals. P < 0.05 was considered to be statistically significant.

MMP-2, TIMP-2, and MT1-MMP RNA levels. To determine the effect of NO inhibition on MMP levels, in particular the complex known to mediate MMP-2 activity, we first evaluated MMP-2, TIMP-2, and MT1-MMP RNA expression. Although mean values were reduced, L-NAME treatment did not produce a statistically significant effect on MMP-2 RNA expression (Fig. 3A; P = 0.17). However, TIMP-2 RNA expression was decreased by 50% in LPLN mUA (Fig. 3B; P = 0.05). While...
L-NAME treatment did not significantly decrease MT1-MMP RNA; RNA expression tended to be 50% lower in LPLN mUA (Fig. 3C; \( P = 0.06 \)).

MMP-2 protein expression. Because MMP-2 can be post-transcriptionally regulated (5, 22), we evaluated protein levels of MMP-2 using immunofluorescence (Fig. 4A). We also quantified MMP-2 levels separately in the three main areas of the mUA wall: endothelium, vascular smooth muscle cell (VSMC) and adventitia (Fig. 4B; \( n = 6 \) LP and \( n = 7 \) LPLN; \( P = 0.06 \)).

![Fig. 2. Lumen area is decreased and extracellular matrix components are increased in LPLN-treated mUA. A: LP and LPLN arteries were dissected, arterial lumens flushed of blood, fixed, and stained using elastic Van Gieson and Masson’s trichrome stain as described in MATERIALS AND METHODS. Collagen is stained blue, and elastin is stained dark purple. B: collagen content was assessed using the color threshold capability within MetaMorph set to distinguish between collagen and elastin staining. Collagen staining is significantly increased in LPLN mUA compared with LP (\( n = 7 \), both groups). C: elastin staining was determined as for collagen; however, the color threshold was set to only include dark purple stained structures within the section. Elastin staining is significantly increased in LPLN mUA (\( n = 5 \), both groups).

* \( P < 0.05 \) by Student’s \( t \)-test.

![Fig. 3. L-NAME treatment of LP rats decreases mRNA expression of tissue inhibitors of metalloproteinase-2 (TIMP-2) and membrane bound matrix metalloproteinase-1 (MT1-MMP) but not matrix metalloproteinase-2 (MMP-2) within the mUA. LP and LPLN mUA were dissected, placed immediately in Trizol reagent for subsequent RNA extraction and qPCR analysis as described in MATERIALS AND METHODS. Hprt1 and Ywhaz were used as endogenous controls for the qPCR. A: although somewhat reduced, LPLN MMP-2 mRNA is not significantly different from LP (\( P = 0.17 \)). B: L-NAME significantly decreased TIMP-2 mRNA expression in mUA, compared with LP (\( P = 0.05 \)). C: MT1-MMP trended be lower in LPLN mUA, although this difference did not achieve statistical significance (\( P = 0.06 \)). * \( P < 0.05 \) by Student’s \( t \)-test.
and found that both endothelial and adventitial levels were significantly reduced while VSMC MMP-2 was unchanged.

**TIMP-2 protein expression.** Given the role of TIMPs in regulation of MMP-2 (11, 34, 66), we evaluated the expression level of TIMP-2 in mUA from LPLN vs. LP animals. Interestingly, TIMP-2 expression in LPLN arteries paralleled that of MMP-2, with reductions in endothelial and adventitial levels and no change in VSMC (Fig. 5, A and B). These findings were confirmed with immunoblotting, which showed a 60% decrease in TIMP-2 protein levels in mUA from LPLN vs. LP animals (Fig. 5, C and D).

**MT1-MMP protein expression.** MT1-MMP and TIMP-2 are known to form an activation complex with MMP-2 (13, 48, 62). As we found both MMP-2 and TIMP-2 protein levels to be decreased with L-NAME treatment, we also evaluated MT1-
Fig. 5. Endothelial and adventitial TIMP-2 protein expression is decreased in late pregnant mUAs isolated from LPLN- rats. A: mUAs were dissected from LP and LPLN-treated animals, fixed, paraffin-embedded, cross-sectioned, and analyzed using immunofluorescence to detect TIMP-2 (green). DAPI was used to stain nuclei (blue). Images were taken at ×25 and ×100 as described in MATERIALS AND METHODS. Bar = 50 μm. DAPI images represent the secondary antibody control images where there was no incubation with primary antibody. B: quantification of TIMP-2 protein immunofluorescence was measured using the integrated intensity function on MetaMorph software and normalizing for area measured. Relative integrated intensity is expressed as a ratio to LP control per area (μm²). TIMP-2 expression was significantly attenuated in the endothelial and adventitial layers of LPLN mUA. MUA TIMP-2 was unchanged by L-NAME in the vascular smooth muscle. *n = 7 LP; n = 7 LPLN, except n = 6 LP adventitia (Adv). C: LP and LPLN mUA were dissected and placed in modified RIPA buffer for protein analysis as described in MATERIALS AND METHODS. Twenty-five micrograms of protein were loaded on the gel. Shown is a representative immunoblot. D: densitometry, as described in MATERIALS AND METHODS, was used to quantify the immunoblots probed for TIMP-2 and β-tubulin. TIMP-2 is expressed as a ratio to β-tubulin. TIMP-2 is significantly decreased in late pregnant L-NAME-treated mUA. n = 6 LP; n = 5 LPLN. *P < 0.05 by Student’s t-test.
MMP levels. In L-NAME-treated animals, mUA MT1-MMP protein levels were reduced by ~80% (Fig. 6, left, immunoblot, and right, quantification).

DISCUSSION

To our knowledge, this study is the first to evaluate extracellular matrix components in uterine arteries from pregnant animals coupled with a potential mechanism via MMP inactivation. Likewise, the current study is the first to provide a feasible mechanism to substantiate the increased deposition of ECM components observed in myometrial arteries from hypertensive preeclamptic women (49). Further, the work presented here extends previous findings (50) showing the abrogation of uterine artery expansion during pregnancy by a reduction in NO signaling secondary to pharmacologic inhibition and provides a potential mechanism for uterine artery outward remodeling during pregnancy.

Previous research describing blood pressure responses in NO-inhibited animals show hypertension developing 2–3 days following the initiation of L-NAME administration, after which time blood pressures stabilize but then decrease in the last 3–4 days of pregnancy (3, 10, 23, 39, 50). We used a higher dose of L-NAME than in previous studies and administered L-NAME via a subcutaneous osmotic pump; mean arterial pressure (MAP) was significantly elevated on day 5 and 10 (days 15 and 20 of pregnancy, respectively) after initiation of L-NAME treatment. The consistent delivery system (5 μL/h) and increased L-NAME dosage could account for the MAP remaining high on day 10, whereas other studies have found a decrease in MAP at this time point.

Normal vascular remodeling during pregnancy is outward hypertrophic, as both lumen diameter and cross-sectional area are increased (51). Consistent with our finding of decreased mUA lumen area, L-NAME treatment has been associated with inward eutrophic remodeling characteristic of hypertension (8, 50) in both pregnant and nonpregnant animals. However, earlier studies from our laboratory (50) show that amelioration of hypertension with hydralazine cotreatment did not reverse the inward eutrophic remodeling due to L-NAME treatment.

This finding points to NO inhibition, rather than hypertension, as the signaling mechanism responsible for the inward eutrophic remodeling of the mUA in L-NAME treated animals. Aberrant NO signaling has been suggested as a contributor to preeclampsia, and our data support this mechanism (18, 19, 42).

The inward eutrophic remodeling may be a symptom of decreased plasticity of the outer vessel wall due to changes in ECM composition and vessel wall content. Collagen and elastin are two ECM components that have received much attention, especially with regard to hypertensive changes in arteries. Collagen is a very stiff protein that does not contribute to the elasticity of the vessel at lower pressures but is a mediator of the passive pressure/diameter relationship of arteries at higher pressures (1). Arteries isolated from women with preeclampsia show increased collagen content in the umbilical cord artery (2). The L-NAME mUA evaluated in our study echo the literature’s description of preclamptic maternal arteries as our vessels also had increased collagen and elastin content and increased wall-to-lumen ratios (2, 49).

There is inconsistency in the literature regarding the effect of L-NAME on eNOS levels. Some researchers (59) have found that eNOS levels are not changed by L-NAME treatment but that the phosphorylation of eNOS is decreased by L-NAME. Other studies (7) have shown that both eNOS protein levels and phosphorylation levels are decreased with L-NAME treatment. We found a remarkable reduction in eNOS RNA and protein (~90%) with L-NAME treatment. In light of the previously published reports, it was surprising to find such a dramatic decrease in eNOS protein in response to L-NAME treatment. While we did not evaluate neuronal or inducible NO synthase, it is possible that inhibition of neuronal NO synthase or inducible NO synthase is contributing to the attenuation of outward remodeling induced by L-NAME.

Because lack of NO signaling rather than hypertension seems to be the primary mechanism for the lack of circumferential remodeling in our L-NAME model, and considering the increased deposition of ECM components, we hypothesized that decreased NO signaling would have a negative effect on MMP expression and thus contribute to the increased deposition of ECM. Therefore, we sought to expand on the role of MMPs in pregnancy and delineate the effect of NO inhibition on the expression of MMPs in the late pregnant mUA. MMPs are key proteases involved in remodeling and have been linked to NO signaling (17, 33, 54). Others (32, 45) have established that many MMPs and TIMPs, including MT1-MMP, MMP-2, and TIMP-2, are upregulated during rat pregnancy, especially during the day before parturition. We found a significant decrease in TIMP-2 RNA with L-NAME treatment. Differences in MMP-2 and MT1-MMP RNA secondary to L-NAME treatment were evident in terms of mean values (which differed by 30 and 50%, respectively); however, these did not reach statistical significance (P = 0.17 and 0.06, respectively). Unfortunately, our statistical power was limited due to small sample size in the RNA experiments, and we recognize this to be an unfortunate limitation of this study. Nevertheless, we found significant decreases in MMP-2, MT1-MMP, and TIMP-2 protein expression in vessels isolated from the NO synthase-inhibited animals. Based on the consistency in the pattern of change in RNA and protein levels in control vs. NO synthase-inhibited animals, we suspect that NO regulation of

![Fig. 6. Treatment of rats with L-NAME decreases MT1-MMP protein expression in LP mUA. Left: LP and LPLN mUA were dissected and placed in modified RIPA buffer for protein analysis as described in MATERIALS AND METHODS. Twenty-five micrograms of protein were loaded on a denaturing gel. Shown is a representative immunoblot. Right: densitometry, as described in MATERIALS AND METHODS, was used to quantify the immunoblots probed for MT1-MMP and β-tubulin. MT1-MMP is significantly decreased in mUA of LPLN animals compared with LP. *P < 0.05 by Student’s t-test.](http://ajpheart.physiology.org/)

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MMPs and TIMPs occurs primarily at the transcriptional level, with consequent changes in protein expression, although this issue cannot yet be conclusively resolved and translational or posttranslational regulation of MMPs and TIMPs by NO cannot be discounted.

Interestingly, NO inhibition only affected MMP-2 and TIMP-2 protein levels within the endothelium and adventitia. This is of particular importance since much of vascular NO signaling is initiated by the endothelium. Further, the decreased expression within the adventitia suggests a direct effect of MMPs on ECM turnover. Indeed, decreased MMP activity/ expression has been associated with NO inhibition and increased collagen deposition (25, 26, 36, 59). The literature describing MMP expression in pregnancy-induced hypertension is conflicting; some studies (32, 67) suggest that MMPs are increased in preeclampsia while others found that ECM degradation is compromised in preeclampsia due to decreased MMP expression. These disparate results could be due to sampling location, since studies that evaluate serum or plasma levels of MMPs in preeclampsia generally report elevated levels of MMPs while those that evaluate MMP and TIMP expression within the umbilical cord artery or the placenta report decreased expression of MMPs and TIMPs (14, 15, 37, 45, 52).

Our findings that MMP-2, TIMP-2, and MT1-MMP are decreased in the mUA are consistent with studies evaluating MMP expression in the umbilical cord artery. Given the labile nature of enzymes, systemic regulation may not be reflective of local, vascular levels of MMPs. We suggest that MMP protein expression within the vascular wall is likely quite different than systemic levels of MMP.

Studies evaluating the effect of NO signaling on TIMP-2 are lacking. The few published reports are contradictory, showing both increased and decreased and no change in expression of TIMP-2 RNA and protein with NO inhibition (56, 70, 71, 73). The results of this study support the regulation of TIMP-2 by NO on two levels: both transcriptionally and posttranscriptionally.

Apart from its role in activating MMP-2, MT1-MMP has an established role in both physiologic and pathophysiologic vascular remodeling (12, 29). MT1-MMP null mice are infertile and have inadequate collagen turnover and decreased lifespan (24). Considering this, MT1-MMP may have an influence on pregnancy-induced vascular remodeling that is independent of MMP-2 and TIMP-2. Accordingly, we found a significant decrease in MT1-MMP with NO inhibition. In line with this finding, MT1-MMP has been implicated in outward remodeling of carotid arteries that is lost in mice that are heterozygous for MT1-MMP (12). MT1-MMP has also been shown to be regulated by NO in endothelial cells; in fact, NO increases MT1-MMP expression and, likewise, NO inhibition decreases MT1-MMP and inhibits endothelial migration and tube formation (17).

Mechanisms by which vascular NO signaling may be altered include changes in mechanical forces (e.g., strain/tensile deformation) secondary to increased intravascular pressure or by hormonal signaling (43, 69). Pregnancy-associated changes in lumen diameter secondary to uterine arterial remodeling and the biomechanical properties of the vascular wall (such as the stress-strain relationship) would each have distinct effects on wall stress and, most likely, circumferential pulsatile strain.

This could directly induce MMP activation, as has been shown in isolated fibroblasts (58). Endocrine changes, particularly the elevations in relaxin and estrogen characteristic of mammalian pregnancy, may exert a synergistic effect, as both hormones have been implicated in MMP regulation and activation in the pregnant state (30, 35). Further, MMP activation is essential in blood-flow-mediated vascular enlargement, potentially through an estrogen-mediated mechanism where increased estrogen increases MT1-MMP and MMP-2 expression (20, 35, 63).

In conclusion, hypertension and/or NO inhibition leads to inward eutrophic remodeling of the uterine circulation that features decreased MMPs, in particular, MMP-2, TIMP-2, and MT1-MMP. In addition to facilitating vasodilation to normalize increased shear stress produced by increased uterine blood flow during pregnancy, our data suggest that NO is also involved in regulation of the deposition of extracellular matrix through the modulation of MMP-2, TIMP-2, and MT1-MMP protein expression. We propose that during normal pregnancy, enhanced NO signaling due to a confluence of increased estrogen levels, augmented shear stress and/or cyclic circumferential strain leads to increases in MMP-2, TIMP-2, and MT1-MMP. Conversely, in the absence of NO signaling, these MMPs and TIMP-2 decrease, contributing to increased collagen and elastin deposition and inward eutrophic remodeling that opposes the outward hypotrophic remodeling characteristic of pregnancy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

16. H1274 NO DECREASES MMP EXPRESSION
24. 17. Intengan HD, Schiffrin EL.
18. 26. Intengan HD, Deng LY, Li JS, Schiffrin EL.
20. Grandas OH, Mountain DH, Kirkpatrick SS, Cassada DC, Stevens JL.
23. Helmbrecht GD, Farhat MY, LaMarca BB, Sedeek M, Murphy SR, Granger JP.
27. 29. Hadler-Olsen E, Fadnes E, Sylte I, Uhlin-Hansen L, Winberg JO.


