Different Involvement of Extracellular Matrix Components in Small and Large Arteries During Chronic NO Synthase Inhibition

Céline Bouvet, Liz-Ann Gilbert, Daphné Girardot, Denis deBlois, Pierre Moreau

Abstract—In essential hypertension, conduit arteries present hypertrophic remodeling (increased cross-sectional area), whereas small arteries undergo eutrophic remodeling. The involvement of matrix metalloproteinases (MMPs) and de-adhesion proteins, such as tenasin-C and thrombospondin, has been relatively well characterized in large artery remodeling, but their contribution is not known in small artery remodeling. Rats received N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg per day) in their drinking water on days 1, 3, 7, 14, and 28. Arterial MMP-2 activity was measured by ELISA, whereas levels of tenasin-C and thrombospondin were assessed by Western blotting. To determine the involvement of MMPs, additional L-NAME rats received the nonselective MMP inhibitor doxycycline (30 mg/kg per day) on days 7, 14, and 28. Already, at day 1, pressure was elevated. Media/lumen ratio of mesenteric arteries and the aorta increased gradually to reach significance at 28 days. However, the cross-sectional area increased only in the aorta, confirming the heterogeneous remodeling process. In small arteries, MMP-2 activity increased after 7 and 14 days of treatment and returned to baseline at 28 days, whereas the elevation was more progressive but sustained in the aorta. The level of thrombospondin paralleled that of MMP-2 in small arteries, whereas tenasin-C levels declined rapidly and stayed below control values. Doxycycline blunted large artery remodeling but had no influence on the development of eutrophic remodeling despite elevation of MMP-2 activity in the process. Thus, in contrast to large artery hypertrophic remodeling, in which the contributions of cellular de-adhesion and matrix breakdown is manifest, the contribution of MMPs in eutrophic remodeling appears less crucial. (Hypertension. 2005;45:432-437.)

Key Words: nitric oxide synthase ▪ arteries

Vascular remodeling is considered an adaptive response to elevation of arterial pressure to normalize the wall tension. In essential hypertension, large artery remodeling is characterized by an increase in media thickness–lumen diameter (M/L) ratio and cross-sectional area (CSA). This augmentation of media mass, or hypertrophic remodeling, is explained by changes in size or number of vascular smooth muscle cells (VSMCs) and matrix collagen deposition.\textsuperscript{1} In resistance arteries (diameter <300 μm), essential hypertension is associated with a reduced lumen and increased M/L ratio but without CSA increase, producing a type of remodeling designated as inward eutrophic remodeling.\textsuperscript{2} To explain this different response between large and small arteries, it is suggested that small arteries are not submitted to an augmented wall stress because they are initially constricted.\textsuperscript{3-5} Thus, we hypothesized that inward eutrophic remodeling, which appears as a fixed form of vasoconstriction, could proceed through specific modifications of VSMC–matrix interactions.

As in essential hypertension, chronic inhibition of NO synthesis with the L-arginine analogue N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) produces hypertrophic remodeling in large arteries,\textsuperscript{6} whereas resistance arteries undergo inward eutrophic remodeling.\textsuperscript{7-9} As seen during normal development, inflammation, wound healing, and cancer, remodeling likely involves modification of cell–matrix interactions and breakdown of existing extracellular matrix (ECM). In the ECM, a group of secreted glycoproteins, the matricellular proteins, such as tenasin-C (TN-C) and thrombospondin (TSP), are believed to disrupt cell–matrix interaction by favoring de-adhesion and have been associated with remodeling. Indeed, TSP expression is augmented in hypertensive pulmonary arteries,\textsuperscript{10} and TN-C is implicated in progressive pulmonary vascular disease,\textsuperscript{11} in arterialization of human vein grafts,\textsuperscript{12} and in hypertension.\textsuperscript{13} Thus, TN-C and TSP are increased in vascular remodeling associated with cell proliferation, but their regulation in small artery eutrophic remodeling is unknown. To break down the ECM or simply cell–matrix adhesions, vascular cells produce proteases such as matrix metalloproteinases (MMPs), which are secreted in a latent form and require an enzymatic cleavage for activa-
tion. They can degrade several ECM proteins unless bound to specific tissue inhibitors of metalloproteinases (TIMPs). They have been implicated in large artery remodeling during atherosclerosis and restenosis14,15 and in hypertrophic remodeling of pulmonary arteries.16 Some evidence suggests that mechanical stretch and strain upregulate MMP-2 and MMP-9 in VSMCs.17 The same was observed when vein grafts were mechanically stretched18 or when transmural pressure was elevated ex vivo in porcine carotid arteries,19 suggesting that hypertension could trigger MMP activation in small arteries.

Our objectives were to determine the modulation of TN-C and TSP abundance and to examine the kinetic of MMP-2 activation during the development of hypertension-induced remodeling of large and small arteries. Moreover, we tested the impact of MMP inhibition on the progression vascular remodeling in the L-NAME model.

Methods

Treatments

Male Wistar rats (275 to 300 g) were obtained from Charles River Breeding Laboratories. To induce hypertension, 40 rats received 50 mg/kg per day of L-NAME (Sigma) in drinking water. They were treated on days 1, 3, 7, 14, and 28 (n=8 per group). In an additional 24 rats, doxycycline, a nonselective MMP inhibitor, was administered (30 mg/kg per day) in the drinking water concomitantly with L-NAME for 28 days (n=8 per group). Five age-matched control rats were studied at day 1 and 5 at day 28 to establish a baseline. Because the values of the parameters measured were not statistically different, they were pooled for further statistical analyses. Eight rats received only doxycycline during 28 days to provide additional control values. Rats were instrumented,20 and the follow-

Figure 1. MBP (A) and M/L ratio of small mesenteric arteries (B) during treatment with L-NAME alone (●), with L-NAME plus doxycycline (○) and doxycycline alone (□). Pooled control values are presented at the zero time point. C, Aortic M/L ratio and CSA, assessed from histological sections, in control rats (Ctl) and rats treated with L-NAME alone (NAME) or with doxycycline (+D) during 28 days. Results are expressed as mean±SEM; n=8 to 10 per group. *P<0.05 vs Ctl; †P<0.05 vs L-NAME alone; ANOVA with Bonferroni’s correction.

Statistical Analysis

Values are expressed as mean±SEM. Statistical analysis was done by either 1-sample t test (TN-C and TSP) or 1-way ANOVA with Bonferroni’s correction for multiple comparisons. We selected a priori the following comparisons: L-NAME and doxycycline (used alone) groups compared with the control group and L-NAME plus...
doxycycline compared with the time-matched L-NAME groups. A value of \textit{P}<0.05 was considered significant.

### Results

After 1 day of L-NAME treatment, blood pressure measure in freely moving rats was already elevated (Figure 1A). The increase was progressive over the 28 days of treatment and was not modified by concomitant doxycycline administration. Doxycycline alone had no effect on blood pressure. The increase in small artery M/L ratio became significant after 14 days of L-NAME treatment (Figure 1B). The augmented M/L ratio was not associated with a significant increase in CSA, confirming the eutrophic nature of small artery remodeling in this model (Table 1). The addition of doxycycline did not modify the evolution of the remodeling process because the M/L ratio values were not different from time-matched L-NAME–treated rats (Figure 1B). However, doxycycline seemed to reduce CSA at intermediate time points (Table 1). The mechanical properties of the arterial wall (distensibility and the geometry-independent stiffness expressed as the \(\beta\)-stiffness index) were not modified by chronic L-NAME treatment (Table 1). Moreover, the MMP inhibitor doxycycline did not modify these mechanical properties either when used alone or in combination with L-NAME.

During the development of eutrophic remodeling, a transient elevation of MMP-2 activity was observed (Figure 2A). Total MMP-2 abundance, assessed after activation of the proform, was not modified in small arteries throughout the experimental protocol (Figure 2B). Gel zymograms of mesenteric arteries confirmed that MMP-2 activity was enhanced (Figure 2C). Interestingly, TIMP-2 was not reduced, but its abundance increased during L-NAME treatment (Table 1). Doxycycline inhibited MMP-2 activity (Figure 2A and 2C), had no effect on total MMP (Figure 2B), and increased TIMP-2 levels even further (Table 1). In small arteries, TN-C levels decreased gradually to reach 35% of control values at

### Table 1. Morphological Characteristic, Mechanical Properties, and Protein Expression in Small Mesenteric Arteries

<table>
<thead>
<tr>
<th>Variables</th>
<th>Treatment</th>
<th>Control</th>
<th>1 Day</th>
<th>3 Days</th>
<th>7 Days</th>
<th>14 Days</th>
<th>28 Days</th>
<th>Doxy 28 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID, (\mu m)</td>
<td>L-NAME</td>
<td>245±7</td>
<td>264±7</td>
<td>248±9</td>
<td>258±10</td>
<td>226±9</td>
<td>241±6</td>
<td>257±10</td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>211±10</td>
<td>227±9</td>
<td>222±7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Media, (\mu m)</td>
<td>L-NAME</td>
<td>17.0±0.7</td>
<td>18.5±1.3</td>
<td>16.5±0.9</td>
<td>18.1±1.0</td>
<td>19.0±0.9</td>
<td>20.0±1.1</td>
<td>16.8±1.2</td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>17.4±0.6</td>
<td>16.9±0.8</td>
<td>19.4±1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CSA, (\times 10^5) (\mu m^2)</td>
<td>L-NAME</td>
<td>15.5±0.6</td>
<td>17.5±1.3</td>
<td>15.4±0.95</td>
<td>17.4±0.85</td>
<td>16.2±0.9</td>
<td>17.2±0.8</td>
<td>14.8±0.9</td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>14.2±0.8†</td>
<td>14.2±1.1</td>
<td>17.1±0.6</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Distensibility, (\mu m\cdot mm Hg^{-1})</td>
<td>L-NAME</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
<td>1.3±0.1</td>
<td>1.3±0.2</td>
<td>1.0±0.03</td>
<td>0.9±0.04</td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>1.2±0.1</td>
<td>1.3±0.2</td>
<td>1.1±0.1</td>
<td></td>
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<tr>
<td>(\beta)-stiffness index</td>
<td>L-NAME</td>
<td>2.77±0.14</td>
<td>2.84±0.12</td>
<td>2.66±0.17</td>
<td>2.75±0.16</td>
<td>2.56±0.23</td>
<td>3.07±0.10</td>
<td>3.03±0.09</td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>2.67±0.24</td>
<td>2.64±0.17</td>
<td>3.00±0.20</td>
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<tr>
<td>TIMP-2</td>
<td>L-NAME</td>
<td>78.5±22.5</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>168.2±15.6*</td>
<td>265.7±88.5*</td>
<td>291.3±37.5*</td>
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<tr>
<td>TSP</td>
<td>L-NAME</td>
<td>100</td>
<td>112.4±11.7</td>
<td>114.8±14.6</td>
<td>111.2±6.4</td>
<td>175.6±11.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Doxy</td>
<td></td>
<td>351.7±70.9†</td>
<td>415.5±60.5†</td>
<td>303.3±109.8</td>
<td></td>
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</tr>
<tr>
<td>TN-C</td>
<td>L-NAME</td>
<td>100</td>
<td>67.6±14.3</td>
<td>79.6±11.6</td>
<td>50.4±15.9*</td>
<td>34.4±4.6*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Doxy** indicates doxycycline; ID, internal diameter; Media, media thickness. Media/lumen ratio is presented in Figure 1. For TSP and TN-C, \(n=4\); \(*P<0.05\) vs control; †\(P<0.05\) vs L-NAME alone.
day 14 (Table 1). In contrast, TSP levels did not initially change but increased at day 14.

The aorta of rats having received L-NAME during 28 days had a significantly larger internal diameter and an increased media thickness compared with that of control rats, resulting in an increased M/L ratio (Figure 1; Table 2). However, in contrast to small arteries, the CSA was elevated after 28 days of treatment. Remodeling did not seem to be consistent at earlier time points because these parameters were variable until 14 days of treatment. Using the 3D dissector method, we observed that the number of cells per length was not different between rats treated with L-NAME for 28 days (1607 ± 128) and control rats (1481 ± 218), suggesting cellular hypertrophy rather than hyperplasia to explain the increased CSA. Coadministration of doxycycline during 28 days prevented hypertrophic remodeling of the aorta; it significantly reduced lumen diameter, CSA, and M/L ratio (Figure 1; Table 2). The major novel finding is the transient increase of MMP-2 activity after 14 days of hypertension that parallels the establishment of stable and significant remodeling of small mesenteric arteries. Indeed, to our knowledge, this is the first study to report the evolution of MMP-2 activity in VSMCs during hypertension-induced eutrophic remodeling. The total amount of the enzyme was not increased, as suggested by the activity assay after APMA treatment. Moreover, the augmentation of MMP-2 activity cannot be explained by a decrease of its inhibitor, TIMP-2. Thus, our results suggest that MMP-2 could be implicated in eutrophic remodeling, as it is in several forms of cardiovascular remodeling.

Continuous L-NAME treatment induced a progressive elevation of MMP-2 activity in the aorta, which became significant after 28 days (Figure 2A). It was paralleled by an increase of total MMP-2 levels (Figure 2B). MMP-2 activity was reduced by doxycycline, mainly during the last week of treatment (Figure 2A). Total MMP-2 levels were also reduced but not to the same extent.

### Discussion

Administration of L-NAME increased MBP, as expected from several studies using this NO synthase inhibitor. The M/L ratio gradually increased in the aorta and mesenteric arteries, suggesting that it took several days of hypertension to modify the matrix more permanently. However, CSA increased only in the aorta, confirming that remodeling is heterogeneous along the vascular tree. Thus, the aorta adapted to sustained pressure elevation by hypertrophic remodeling, whereas mesenteric resistance arteries underwent eutrophic remodeling. Interestingly, this heterogeneity also occurs in essential hypertension, making the L-NAME model particularly suitable to study hypertension-induced vascular remodeling.

Although there have been reports on heterogeneous responses in rats in terms of activation of the renin-angiotensin system (RAS) and the development of left ventricular hypertrophy and renal alterations, we never observed RAS activation in our experimental conditions.

There is ample evidence that MMPs in general, and MMP-2 more specifically, are involved in hypertrophic cardiovascular remodeling (see introduction). We chose MMP-2 as our primary candidate because MMP-2 is expressed at lower transmural pressures in coronary arteries studied ex vivo. Furthermore, a predominance of MMP-2 activity in VSMCs was observed under mechanical stretch and strain. Consistent with previous findings, the present study demonstrates a progressive augmentation of total MMP-2 level and activity in hypertrophic remodeling of large arteries. The major novel finding is the transient increase of MMP-2 activity after 14 days of hypertension that parallels the establishment of stable and significant remodeling of small mesenteric arteries. Indeed, to our knowledge, this is the first study to report the evolution of MMP-2 activity in hypertensive-induced eutrophic remodeling. The total amount of the enzyme was not increased, as suggested by the activity assay after APMA treatment. Moreover, the augmentation of MMP-2 activity cannot be explained by a decrease of its inhibitor, TIMP-2. Thus, our results suggest that MMP-2 could be implicated in eutrophic remodeling, as it is in several forms of cardiovascular remodeling.

To examine the direct involvement of MMP-2 activation in eutrophic remodeling, rats were treated with doxycycline, a tetracycline known to inhibit MMPs nonselectively. The mechanism of this inhibition is not completely understood, but doxycycline binds directly the Zn$^{2+}$ or Ca$^{2+}$ ion associated with MMPs, thus either blocking their active site or modifying their activation. Inhibition of MMPs could also be related to inhibition of the transcription of MMP mRNAs. In our study, doxycycline significantly reduced aortic MMP-2 upregulation and MMP-2 activity in the 2 types of arteries. This suggests that it could act on MMP-2 expression, activation,

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and activity. However, because the processing of tissues required for activity measurements (gelatin zymography and ELISA) is likely to remove doxycycline bound to the enzyme, we may have measured mainly in vivo activation inhibition rather than blockade of its activity. Nonetheless, as a result of MMP inhibition, hypertrophic arterial remodeling was prevented. This is in line with studies showing prevention of blood flow–induced artery enlargement and inhibition of several phases of intimal thickening after arterial injury. In contrast to results obtained in large arteries, doxycycline treatment did not modify vascular remodeling in mesenteric arteries, suggesting that despite the increase of MMP-2 activity, this family of enzymes does not appear to be necessary for the eutrophic remodeling process to occur. Interestingly, doxycycline appeared to influence small artery “hypertrophy,” although it is not a main feature in this model. To investigate whether MMPs could influence other parameters than the overall structure during the remodeling process, we studied the distensibility of small arteries during L-NAME and doxycycline treatment. According to our observations, and in accordance with several studies in experimental hypertension, eutrophic remodeling was not associated with modification of the mechanical properties of the arteries. Doxycycline did not modify these properties, suggesting that the elevation of MMP-2 observed during L-NAME treatment is not involved in the control of small artery stiffness.

In large pulmonary arteries, hypertrophic remodeling appears to involve reduced adhesion of cells to the ECM, because several matricellular molecules with reduced adhesion properties, such as TN-C and TSP, are overexpressed. This could be necessary to allow cells to grow, proliferate, and migrate within the matrix. In the present study, only TN-C was elevated in the aorta and could serve such a function. The expression of TSP was not modified during the development of aortic hypertrophy, suggesting that its involvement may be less than in pulmonary arteries. In small arteries facing a chronic elevation of arterial pressure, cell adhesion may have to be weakened to allow VSMCs to move within the contracted matrix. Indeed, eutrophic remodeling is considered to be a rearrangement of cells around a smaller lumen, and after an initial shortening, cells appear to regain initial elongation despite a sustained contracted vessel structure. In line with this, TSP was upregulated at the same time (14 days) remodeling became significant. Alternatively, enhanced adhesion could be important to keep the cells attached to the contracted matrix, to allow them to pursue their vasomotion function. To support this alternative hypothesis, TN-C was reduced throughout the treatment period, with a clear reduction at 14 days. The results concerning these matricellular proteins could also be interpreted in light of MMP-2 activity. Indeed, TSP-1 has been shown to stimulate MMP-2 activity, and both proteins show concurrent kinetics in our study. In large elastic arteries, TN-C abundance appears to parallel that of MMP activity, as it is the case in the present study. It has been suggested that TN-C induces MMP expression. Our results do not support such a relationship in small arteries but are in line with a recent report demonstrating that TN-C could be a substrate of MMP-2. Thus, the decline of TN-C abundance that was observed could result from enhanced MMP-2 activity. Our results support, but do not prove, the interplay between MMP-2 and matricellular proteins during the remodeling process, and more work is needed to identify their exact role.

**Perspectives**

We postulated that to stabilize vasoconstriction into eutrophic remodeling, VSMCs had to modify their matrix interactions. Although such alterations could be demonstrated during the development of this process, we were unable to demonstrate a causative role of MMPs, in contrast to what can be observed in large arteries. Thus, the development of eutrophic remodeling of small arteries may involve a complex interplay of ECM enzymes and de-adhesive proteins that is not decisive for the remodeling to proceed. However, future studies may involve the evaluation of plasmin, which is known to degrade glycoprotein components of the ECM. Plasmin is formed by enzymatic cleavage of plasminogen by tissue plasminogen activator and urokinase plasminogen activator (u-PA). Indeed, a very recent study (abstract form) has shown that during reverse vascular remodeling induced by antihypertensive therapy, u-PA activity was more important than MMP-2 activity. Because doxycycline does not inhibit u-PA, our study did not evaluate this possibility.

In conclusion, the development of eutropic remodeling of small arteries is associated, in a timely fashion, with varying expression of several proteins involved in cell–matrix interactions. In contrast to large artery hypertrophic remodeling, eutrophic remodeling does not appear to depend on enhanced MMP activity to evolve in its final form. Thus, we provide evidence that the remodeling process in arteries with different functions also involves alternative mechanisms.

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