Pressure-Induced Matrix Metalloproteinase-9 Contributes to Early Hypertensive Remodeling

Stéphanie Lehoux, PhD; Catherine A. Lemarié, MSc; Bruno Esposito; H. Roger Lijnen, PhD; Alain Tedgui, PhD

Background—High blood pressure causes a change in vascular wall structure involving altered extracellular matrix composition, but how this process occurs is not fully understood.

Methods and Results—Using mouse carotid arteries maintained in organ culture for 3 days, we detected increased gelatin zymographic activity of matrix metalloproteinase (MMP)-2 (168±13%, P<0.05) in vessels kept at low intraluminal pressure (10 mm Hg) compared with vessels at 80 mm Hg (100%), whereas in vessels maintained at high pressure (150 mm Hg), both MMP-2 and MMP-9 activity was induced (182±32%, P<0.05, and 194±21%, P<0.01, respectively). MMPs were detected in endothelial and smooth muscle cells by immunohistochemistry and in situ gelatin zymography. In vessels at 150 mm Hg, MMP activation was associated with a shift in the pressure-diameter curve toward greater distensibility (P<0.01) compared with vessels at 80 mm Hg. However, distensibility was not altered in vessels at 10 mm Hg, in which only activated MMP-2 was detected. The role of MMPs in high pressure–induced vessel distensibility was confirmed by use of the MMP inhibitor FN-439, which prevented the shift in the pressure-diameter relationship. Furthermore, in carotid arteries from MMP-9–deficient mice, the pressure-dependent increase in MMP-2 and in situ gelatinolytic activity were maintained, but the upward shift in the pressure-diameter curve was abolished.

Conclusions—MMP-9 seems to play a key role in the early stages of hypertensive vascular remodeling. (Circulation. 2004;109:1041-1047.)

Key Words: arteries • hypertension • metalloproteinases • remodeling • signal transduction

Blood vessels are permanently under physiological strain because of shear stress and circumferential strain. These stresses are major determinants of vessel morphology and composition; prolonged or chronic changes in mechanical forces lead to adaptive restructuring of the vessel wall. In turn, vascular remodeling contributes to the progression of vascular disease in pathological conditions involving alterations in hemodynamic load.

Reorganization of the extracellular matrix through protein synthesis and degradation is a key characteristic of hypertensive vascular remodeling. Yet it remains unclear how mechanical forces contribute to this process at the onset of blood pressure elevation. Recent publications indicate that matrix metalloproteinases (MMPs) are activated in vascular smooth muscle cells (VSMCs) submitted to stationary stretch or cyclic stretch or in arteries exposed to longitudinal tension. However, the activation of MMPs in whole arteries under hypertensive conditions has not been studied. Furthermore, a clear causal relationship between MMP activation and functional changes in arterial vascular structure has yet to be established. We therefore evaluated the expression and gelatinase activity of MMP-2 and MMP-9 in mouse carotid arteries maintained in organ culture at different levels of intraluminal pressure and assessed the contribution of these enzymes to vessel remodeling.

Methods

Organ Culture

Male C57BL/6 mice between 8 and 10 weeks of age (n=32; Charles River, France) were killed by injection of sodium pentobarbital (50 mg/kg IP). Care and use of laboratory animals conformed to European Community standards. Left and right carotid arteries were isolated and cannulated at both extremities. Each arterial segment was connected to a closed perfusion circuit consisting of a 3-port reservoir, a peristaltic pump (Alitea), and a pressure chamber, allowing for the application of a controlled intraluminal hydrostatic pressure. Vessels were immersed in culture medium identical to that used in the intraluminal compartment, consisting of DMEM (Gibco BRL) and antibiotics (100 IU/L penicillin, 100 mg/L streptomycin, and 10 μg/L fungizone), supplemented with 5% FCS (Boehringer Mannheim). Flow within the segments was set to renew the medium at a constant rate of 0.5 cm/s. Care was taken to ensure perfusion of the segments from the proximal to the distal end. Organ culture of the carotid...
arteries was carried out under sterile conditions in a 5% CO₂ incubator at 37°C.

Arterial segments were kept for 1 hour at an intraluminal pressure of 80 mm Hg for stabilization after surgery. Thereafter, the pressure was maintained at 80 mm Hg or reset to 10 or 150 mm Hg for 72 hours. Some segments were treated with an MMP inhibitor (FN-439, 10⁻⁵ mol/L, Calbiochem). The inhibitor was added to the culture medium at the onset of the equilibration period. In another set of experiments, carotid arteries from 8- to 10-week-old male MMP-9–deficient mice and wild-type littermates (on a C57BL/6 background, n=9 and 11, respectively) were cultured at 80 or 150 mm Hg. After 72 hours, the arterial segments were removed from the organ culture bath and processed as described below.

**Tissue Extraction**

Vessel segments were ground in ice-cold lysis buffer containing (mmol/L) Tris-HCl 20 (pH 7.5), EGTA 5, NaCl 150, glycerophosphate 20, NaF 10, sodium orthovanadate 1, 1% Triton X-100,

deficient mice and wild-type littermates (on a C57BL/6 background, n=9 and 11, respectively) were cultured at 80 or 150 mm Hg. After 72 hours, the arterial segments were removed from the organ culture bath and processed as described below.

**Figure 1.** Differential MMP activation by high and low intraluminal pressure. Carotid arteries were maintained in culture at 10, 80, or 150 mm Hg for 72 hours. Top, Gelatin zymography of vessel lysates reveals greater MMP-2 activity in vessels kept at 10 or 150 mm Hg compared with 80 mm Hg. However, MMP-9 activity is enhanced only in carotid arteries maintained at high pressure. Treatment of vessels with FN-439 prevents pressure-dependent MMP activation. Bottom, Quantification of gelatinolytic activity in untreated (▫) and FN-439–treated vessels (▫) presented as mean±SEM of 6 to 8 experiments. *P<0.05 and **P<0.01.

**Figure 2.** Immunostaining for MMP-9 and MMP-2 is increased in endothelium and throughout media of vessels cultured at 150 mm Hg compared with vessels at 80 mm Hg. This coincides with an increase in total gelatinase activity, detected by enhanced fluorescence of a fluorogenic gelatin substrate, in carotid arteries at high pressure. Representative of 8 separate experiments.
CaCl$_2$ at 37°C in zymography buffer (50 mmol/L Tris-HCl, pH 7.4, and 15 mmol/L 2.5% Triton X-100 at room temperature. Gels were incubated in electrophoresis, SDS was removed from the gel by washing twice with SDS-polyacrylamide gel containing 0.1 mg/mL gelatin. After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 at room temperature. Gels were incubated in zymography buffer (50 mmol/L Tris-HCl, pH 7.4, and 15 mmol/L CaCl$_2$) at 37°C overnight and then stained with Coomassie brilliant blue. Gelatinolytic activity was visualized as clear bands of lysis against a dark background.

Gelatin Zymography

Zymography using gelatin-containing gels was performed as described previously.$^2$ Briefly, modified Laemmli buffer without mercaptoethanol was added to lysed tissue samples and loaded on an SDS-polyacrylamide gel containing 0.1 mg/mL gelatin. After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 at room temperature. Gels were incubated in zymography buffer (50 mmol/L Tris-HCl, pH 7.4, and 15 mmol/L CaCl$_2$) at 37°C overnight and then stained with Coomassie brilliant blue. Gelatinolytic activity was visualized as clear bands of lysis against a dark background.

Immunohistochemistry and In Situ Zymography

Arterial segments were embedded vertically in Tissue-tek (Sakura), and serial 15-μm sections were cut. MMP-2 and MMP-9 were detected with primary rabbit polyclonal antibodies used at 1:50 (Santa Cruz). Immunostainings were developed with avidin-biotin–horseradish peroxidase (Vectastain ABC kit, Vector Laboratories). For in situ zymography, vessel sections were incubated at 37°C for 5 hours with a fluorogenic gelatin substrate (DQ gelatin, Molecular Probes) dissolved to 25 μg/mL in zymography buffer. Proteolytic activity was detected as green fluorescence (530 nm).

Pressure-Diameter Analysis

Carotid arteries in the organ culture setup were connected to a video-monitored perfusion system (Living Systems Instrumentation) as described previously.$^2$ The medium in the inner perfusion loop and in the organ bath was changed to a Ca$^{2+}$-free solution containing 1 mmol/L EGTA to obtain passive diameter. Pressure was controlled by a servo-perfusion system and was raised by 25-mm increments from 25 to 200 mm Hg. To account for hysteresis, 2 cycles of preconditioning were applied before data acquisition. Diameter changes were measured continuously by videomicroscopy. Distensibility (D) was calculated as $D = (A_1 - A_0)/[(A_1 + A_0)/2] \times \Delta P$, where $A_1$ and $A_0$ are vessel areas before and after pressure increment and $\Delta P$ is the change in pressure (25 mm Hg).

Data Analysis

Gelatinolytic activity was quantified by densitometric analysis using NIH Image software. Results are expressed as mean±SEM. One-way (or 2-way) ANOVA was used to compare zymographic data for different pressures (and MMP inhibition). Pressure-diameter data were analyzed by 1-way (for comparison of pressure effects) or 2-way (for analysis of effects of FN-439 treatment or of MMP-9 strain) repeated-measures ANOVA. When ANOVA analyses yielded significant results, comparisons were performed by use of Bonferroni’s test. Values of $P<0.05$ were considered statistically significant.

Results

Regulation of MMP-2 and MMP-9 Activity by Intraluminal Pressure

Gelatin zymography with extracts from carotid arteries maintained in culture for 72 hours showed lytic bands corresponding to pro-MMP-9 and pro-MMP-2 (Figure 1). In lysates from control arteries kept at an intraluminal pressure of 80 mm Hg, the gelatinolytic activity of both MMPs was assigned a value of 100%. Levels of MMP-2 increased by 68±13% ($P<0.05$) in carotid arteries exposed to an intraluminal pressure of 10 mm Hg, without a change in MMP-9. In contrast, high intraluminal pressure (150 mm Hg) was associated with an increase in both MMP-2 and MMP-9 by 82±32% ($P<0.05$) and 94±21% ($P<0.01$), respectively.

Zymographic results were confirmed by immunohistochemistry, showing increased expression of both MMP-2 and MMP-9 in vessels maintained at 150 mm Hg compared with vessels at 80 mm Hg (Figure 2). Positive staining was observed both in the endothelium and in the medial smooth muscle cells, consistent with an increase in gelatinolytic activity throughout the vessel wall, as detected by in situ zymography.

High Intraluminal Pressure Is Associated With Increased Distensibility

To assess a possible functional repercussion of maintaining vessels at different levels of pressure, carotid pressure-diameter curves were established by use of a myograph. Arteries were exposed to successive 25 mm Hg increments in intraluminal pressure, and vessel diameter was measured at each pressure level. An upward shift in the pressure-diameter...
curve ($P<0.001$), caused by greater distensibility ($P<0.01$), was observed in arteries maintained for 3 days at 150 mm Hg compared with arteries kept at 80 mm Hg, whereas the distensibility of vessels cultured at 10 mm Hg was not significantly altered (Figure 3).

**MMP Inhibition Reduces Distensibility of Vessels Kept at High Intraluminal Pressure**

To investigate the link between high pressure–induced MMP expression and increased distensibility, carotid arteries were cultured in the presence of an MMP inhibitor. Treatment with FN-439 prevented the increase in zymographic activity of both MMP-9 and MMP-2 in vessels kept at 10 or 150 mm Hg but did not affect MMP activity of vessels at 80 mm Hg (Figure 1). FN-439 also significantly reduced in situ gelatinase activity in vessels at high pressure (Figure 4). Interestingly, MMP inhibition was also associated with reduced staining for MMP-2 and a complete inhibition of staining for MMP-9 in immunohistology sections of vessels maintained at 150 mm Hg (Figure 4), indicating that chronic FN-439 treatment may actually reduce protein expression of these enzymes, rather than simply preventing their activation.

Treatment with FN-439 did not affect the distensibility of vessels maintained at 10 mm Hg (data not shown) or at 80 mm Hg, whereas it completely abolished the increase in distensibility normally observed in arteries kept at 150 mm Hg (Figure 5). Hence, the intraluminal pressure-induced enhancement in carotid distensibility requires gelatinase activity.

**Loss of Pressure-Induced Distensibility in MMP-9–Deficient Mice**

Figure 6 confirms that MMP-9 expression was completely absent from MMP-9–deficient mice compared with wild-type littermates, whereas MMP-2 activity was equivalent in both genotypes. Also, MMP-2 levels in vessels at high pressure were not significantly altered in MMP-9–deficient mice compared with wild-type littermates (Figure 6). However, the MMP inhibitor did not alter distensibility of vessels cultured at 80 mm Hg. Data are mean±SEM of 4 to 7 experiments. **$P<0.01$ and ***$P<0.001$ for 150 vs 80 mm Hg.

**Figure 4.** Treatment with FN-439 prevents MMP-9 induction and reduces MMP-2 levels in carotid arteries maintained in culture at 150 mm Hg. In situ gelatinase activity is also decreased by MMP inhibitor. Representative of 4 separate experiments.

**Figure 5.** FN-439 prevents shift in pressure-diameter curve (left) and blocks increase in distensibility (right) in carotid arteries maintained at 150 mm Hg for 3 days. However, the MMP inhibitor does not alter distensibility of vessels cultured at 80 mm Hg. Data are mean±SEM of 4 to 7 experiments. **$P<0.01$ and ***$P<0.001$ for 150 vs 80 mm Hg.
pressure was comparable in wild-type and knockout animals. Immunohistology corroborated enhanced MMP-2 and MMP-9 staining in vessels from wild-type animals show pressure-dependent MMP-9 activity, whereas no MMP-9 is detected in arteries from knockout mice, as expected. However, MMP-2 induction is enhanced in all vessels cultured at 150 mm Hg compared with 80 mm Hg, irrespective of genotype. Representative of 4 experiments.

Figure 6. Gelatin zymographic activity of carotid arteries from MMP-9–deficient mice or wild-type littermates maintained in culture for 3 days at 80 or 150 mm Hg. Vessels from wild-type animals show pressure-dependent MMP-9 activity, whereas no MMP-9 is detected in arteries from knockout mice, as expected. However, MMP-2 induction is enhanced in all vessels cultured at 150 mm Hg compared with 80 mm Hg, irrespective of genotype. Representative of 4 experiments.

Discussion

This study demonstrates the fundamental role of MMP-9 in pressure-induced blood vessel remodeling. MMP-9 expression and activity is enhanced in arteries maintained at 150 mm Hg for 3 days compared with vessels kept at 10 or 80 mm Hg and is associated with an increase in vascular distensibility. In vessels treated with a MMP inhibitor or derived from MMP-9–deficient mice, the increase in vascular distensibility at high intraluminal pressure is no longer observed. Hence, MMP-9 is likely to contribute to the hypertensive remodeling process.

Previous studies have shown evidence of MMP activation by mechanical strain. In cultured VSMCs, cyclic strain stimulates MMP-2 production and release,1,3,7–9 and VSMCs under stationary strain are characterized by increased MMP-2 and MMP-9 levels.1 However, data on whole vessels are less consistent. In saphenous veins, arterial conditions (pulsatile pressure and flow) lower MMP-2 and MMP-9 synthesis and activation,10 whereas cyclic 50% longitudinal stretch increases their secretion and activity.2 In comparison, MMP-2 and MMP-9 are activated in porcine arteries cultured at 100 and 200 mm Hg, respectively, relative to controls at zero pressure.11 Hence, thus far the link between intraluminal pressure state and vessel MMP production and activity was not clearly established, and the role of MMPs in hypertensive vascular remodeling remained speculative.

Using normal pressure (80 mm Hg) as a reference, the present study demonstrates that both low and high intraluminal pressures induce MMPs. However, whereas MMP-2 is activated in conditions of both high and low pressure, MMP-9 is activated only in vessels at 150 mm Hg, coincident with

Figure 7. MMP expression and in situ gelatinase activity in carotid arteries from MMP-9–deficient mice maintained 3 days at 80 or 150 mm Hg. No staining for MMP-9 is detected in vessels, irrespective of pressure setting. Nonetheless, pressure-dependent induction and gelatinase activity of MMP-2 is sustained. Representative of 3 to 5 separate experiments.
increased vessel distensibility. These data indicate that MMP-9 and MMP-2 are likely to play different roles in vascular remodeling. In a previous study, cuffing rabbit carotid arteries such that circumferential wall tension was off-loaded resulted in artery remodeling, including apoptosis, wall atrophy, and increased activity of MMP-2 and MMP-9.12 MMPs were hypothesized to promote cell apoptosis by depriving cells of antiapoptotic matrix–integrin interactions. Similarly, we previously reported a loss of smooth muscle marker proteins in rabbit aortas in organ culture at 10 mm Hg compared with 80 mm Hg.13 Because in our model, MMP-9 is not activated by low pressure, we propose that MMP-2 may be preferentially involved in the processes of apoptosis and dedifferentiation in understretched vessels. A recent report showing that MMP-9 but not MMP-2 modulates collagen organization by VSMCs further exemplifies the disparity of roles taken on by these 2 enzymes.14

Our data concur to some extent with recent in vivo observations in a model of arterial longitudinal stretch. Jackson et al4 found that axial strain in rabbit carotid arteries caused an increase in the area of fenestrae in the internal elastic lamina, leading to vessel stretch, concurrent with a transient rise in MMP-2 and MMP-9 activity. However, the authors did not investigate the function of MMPs in the remodeling process directly using inhibitors. These authors also found that axial strain was associated with increased VSMC apoptosis, which we have not observed in vessels cultured at high pressure.15 Hence, the mechanisms of remodeling induced by axial strain and circumferential stretch seem to be different, but both suggest an important role for MMPs in strain-induced remodeling. Our data actually demonstrate this role of MMPs, and more specifically MMP-9, in pressure-induced vascular distensibility. Indeed, MMP inhibitor treatment prevented the pressure-induced increase in vessel distensibility, and the pressure-diameter relationship was likewise restored in vessels from MMP-9–knockout mice placed at high intraluminal pressure despite MMP-2 activation.

In hypertensive patients, circulating MMP-9 levels are generally low compared with normotensive control subjects and remain low even after antihypertensive treatment, although MMP-2 levels can be restored by such treatment.16,17 Also, hypertensives tend to have relatively rigid arteries, unlike the more distensible vessels we found associated with high intraluminal pressure. However, we believe that increased MMP activation and enhanced distensibility are hallmarks of early hypertensive remodeling, allowing the vessel to expand to accommodate the new pressure setting. Because extracellular matrix synthesis is also stimulated in vessels at high intraluminal pressure5 and cultured VSMCs exposed to cyclic stretch,6,18 these proteins may very well contribute to later rigidification of the vessel wall.

In summary, we have shown that high intraluminal pressure–induced MMPs are directly involved in increased distensibility in the carotid artery. Although the contribution of other MMPs may not be excluded, MMP-9 seems to be critical for vessel expansion under pressure, playing a role in the early stages of hypertensive vascular remodeling.

References


Pressure-Induced Matrix Metalloproteinase-9 Contributes to Early Hypertensive Remodeling
Stéphanie Lehoux, Catherine A. Lemarié, Bruno Esposito, H. Roger Lijnen and Alain Tedgui

Circulation. 2004;109:1041-1047; originally published online February 16, 2004;
doi: 10.1161/01.CIR.0000115521.95662.7A
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/8/1041

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/