Transmural pressure induces matrix-degrading activity in porcine arteries ex vivo

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Transmural pressure induces matrix-degrading activity in porcine arteries ex vivo. Am. J. Physiol. 277 (Heart Circ. Physiol.: 46): H2002–H2009, 1999.—Extracellular matrix components must be degraded and resynthesized for vascular remodeling to occur. We hypothesized that the hemodynamic environment regulates activity of matrix metalloproteinases (MMPs), the primary agents for in vivo matrix degradation, during vascular remodeling in response to changes in transmural pressure and shear stress. Pathological hemodynamic conditions were reproduced in an ex vivo system in which we maintained porcine carotid arteries for 24 and 48 h. Total levels of MMP-2 and MMP-9 extracted from tissue homogenates and analyzed by SDS-PAGE zymography were stimulated by transmural pressure and were unaffected by shear stress changes. Degradation of two specific gelatinase substrates, gelatin and elastin, increased with increasing pressure, but the degradation was not affected by shear stress changes in tissue specimens analyzed using in situ zymography (gelatin) and fluorescent measurement of endogenous elastin degradation (elastin). Our results suggest that transmural pressure activates at least two members of the MMP family and that activity of these enzymes is accompanied by degradation of matrix components, effects that may be implicated in hypertensive vascular remodeling.

vascular remodeling; hemodynamics; hypertension; artery culture; matrix metalloproteinase
tion in ipsilateral or collateral arteries subject to 24 or 48 h of altered flow and pressure ex vivo. Expression and activity of gelatinases in the arterial wall were analyzed with immunohistochemistry and SDS-PAGE zymography, respectively. Matrix-degrading activity was assessed from quantification of elastin autofluorescence and by in situ zymography using a fluorescent gelatin substrate.

Traditionally, the effect of the hemodynamic environment on vascular remodeling is studied using in vivo models, which cannot separate the effect of hemodynamic forces from other systemic influences. The alternative of studying vascular cells in vitro is limited by the absence of ECM components and proper cellular architecture. The ex vivo perfusion system is a compromise modality for vascular investigation that is more physiological than cell culture and less costly and more easily controlled (hemodynamically and biochemically) than in vivo studies (6). Therefore ex vivo techniques were chosen for this investigation.

**METHODS**

Ex vivo perfusion system. Carotid arteries were harvested directly from abattoir animals (6-mo-old Landrace pigs) soon after exsanguination. They were immediately rinsed with a saline solution containing antibiotics and then were stored on ice for transport to the laboratory. After testing the vessels for leaks and tying off small side branches, the vessels were rinsed several times in sterile PBS (MediaTech, Herndon, VA) and were mounted in physiological orientation between two cannulas. These cannulas were inflow and outflow conduits for DMEM (Sigma) with 5% dextran (Sigma) added to obtain a physiological fluid viscosity in the flow loop. The vessel was then surrounded by DMEM in a sterilized chamber. Appropriate gas exchange was provided through sterile airflow filters connected to both the flow loop and chamber media (independently) to maintain physiological pH, PO2, and PCO2. Normothermia was ensured either by independent closed-loop heating and control of the internal and external media or by placing the system in a temperature-controlled incubator. This ex vivo perfusion system was described in detail elsewhere (6).

In steady (fully developed, laminar) flow, shear stress is determined by fluid flow rate, fluid viscosity, and vessel diameter according to the relationship

$$\tau = \frac{3\mu Q}{\pi D^3}$$

where $\mu$ is the fluid viscosity, $Q$ is the flow rate, and $D$ is the vessel internal diameter (21). Physiologically, arteries adapt to maintain a wall shear stress of ~15 dyn/cm$^2$ (14, 35). For a viscosity of 4 cP and a diameter of 0.5 cm, a steady flow rate of 275 ml/min gives approximately this shear stress. Decreasing the flow rate by a factor of 10 similarly decreases the shear stress.

An appropriate length of rigid metal tubing preceded the vessel in the flow loop to ensure that the flow was fully developed and laminar. Transmural pressure was varied independently of the shear and flow parameters. Typically, the bath media was kept at atmospheric pressure, whereas the luminal pressure was varied with a pressure control valve. Pressure was measured with a pressure sensor (model 60–3002; Harvard, South Natick, MA; range 1–1,000 mmHg). A steady shear stress of 15 dyn/cm$^2$ (normal) or 1.5 dyn/cm$^2$ (low) was maintained for 24 or 48 h at either 100 or 200 mmHg (normal or high, respectively) transmural pressure. The control vessel was incubated (for either 24 or 48 h) in the external bath media with zero pressure and zero flow. At the end of the experiment, vessels were divided into sections that were either flash-frozen in liquid nitrogen for later homogenization or frozen in tissue-freezing medium (optimum cutting temperature compound; Miles, Elkhart, IN) for processing by histochemical techniques. Expression and activity of MMP-2 and MMP-9 in the homogenized tissue was analyzed by SDS-PAGE zymography. The local activity and expression of these enzymes were analyzed with in situ zymography performed on the frozen sections. Immunohistochemistry and medial elastin quantification techniques localized and quantified the presence of MMP-2 and MMP-9 and the degradation of elastin by active elastases.

SDS-PAGE zymography. Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS (Bio-Rad, Hercules, CA) in 10% discontinuous polyacrylamide gels containing 1.0 mg/ml gelatin (Bio-Rad). With this method, after electrophoretic migration, proteins with gelatinolytic activity can be detected due to their capacity to digest the gelatin substrate incorporated into the SDS-PAGE gels. Tissue samples were homogenized using 1% by volume Triton X-100, 10 mg/ml SDS, 2 mg/ml sodium azide, and 100 mg/ml deoxycholic acid in PBS stored at 4°C. Homogenized tissue samples were loaded on gels directly. Protein concentration was assayed with a modified Bio-Rad assay (Bio-Rad). Equal amounts of protein (30 µg) were loaded in each lane. The proteins in the gels were renatured by exchanging SDS with Triton X-100 (2 10-min incubations with 2.5% Triton X-100; Sigma). The gels were subsequently incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.4, containing 10 mM CaCl2 and 0.05% Brij 35. At the end of the incubation, gels were stained with Coomassie Brilliant Blue G (Sigma). Proteins having gelatinolytic activity were then visualized as areas of lytic activity on an otherwise blue gel. Migration of proteins was compared with that of prestained low-molecular-weight range markers (Bio-Rad). The Bio-Rad Gel Doc system was used to quantify the activity data (20).

Immunohistochemistry. Immunostaining for MMP-2 and MMP-9 was performed on frozen sections. After being fixed in formalin and dried, specimens were washed with PBS and preincubated with 0.3% hydrogen peroxide in PBS for reduction of endogenous peroxidase activity. Specimens were then incubated for 60 min at room temperature with primary anti-MMP-2 or anti-MMP-9 antibodies (Oncogene Science, Cambridge, MA) diluted in PBS plus 10% horse serum (Dako, Carpinteria, CA). After being washed in PBS and then in 100 mM Tris-HCl containing 150 mM NaCl and 2% horse serum, biotinylated secondary antibodies (Calbiochem, San Diego, CA) were applied, followed by avidin-biotin-peroxidase complexes. Sections were counterstained with hematoxylin-eosin (Sigma). Some specimens were also processed without the primary antibody as negative controls.

Quantification of endogenous elastin. Elastin was visualized in frozen tissue sections due to its endogenous fluorescence (4) using the blue filter on a Zeiss fluorescence microscope. Images at x10 magnification under fluorescent light were captured with a charge-coupled device (CCD) camera and the ImagePro software package. ImagePro Plus 3.0 was used to quantify the presence of elastin and the pattern of elastin autofluorescence in tissue cross sections. Specifically, elastin objects were counted within the domain between the adventitia and intima as demarcated by fluorescing collagen bundles at the edge of the outer media and the internal elastic lamina at the inner media. An elastin object was defined as three or more contiguous pixels above a certain threshold of...
luminosity. This threshold was determined independently for each image since we did not assume that lighting conditions were identical for all images captured. Fourth-order smoothing was imposed on object boundaries to reduce noise. Measured quantities included object area, luminosity, length, width, and object area as a percentage of the total irregular object area (i.e., the media). Object statistics were computed by the ImagePro software package and were saved to a spreadsheet for further analysis.

In situ zymography. In situ zymography was performed on tissue sections as described previously (12). Briefly, tissue specimens were incubated on slides coated with FITC-labeled gelatin in a humidified chamber for 1–4 days with maximum contrast visible at 3 days. Lysis of the fluorescent gelatin substrate demonstrates the presence of active enzymes and localizes the sites of enzymatic activity in the tissue.

RESULTS

Viability of the artery for 7 days in this apparatus was confirmed by the following two assays: one in situ and one in vitro (data not shown). Arterial vasoconstriction in response to norepinephrine and vasodilation in response to a subsequent dose of ACh were measured with a CCD video camera. Because this pathway requires an intact endothelium and the response requires contractile smooth muscle cells (24, 27), the viability of the entire artery was tested. In vitro testing demonstrated that endothelial cells harvested from the artery after 7 days in organ culture proliferated in sterile cell culture conditions. Morphological assessment of the arterial endothelium after perfusion by scanning electron microscopy studies also revealed a healthy-appearing, intact endothelium.

Levels of MMP-2 and MMP-9 expression. Quantitative measurements of total levels (zymogen and activated forms) of MMP-2 and MMP-9 in arterial tissue homogenates by SDS-PAGE zymography showed increasing expression with transmural pressure at both 24 (data not shown) and 48 h (Fig. 1). Tissue extracts of vessels subjected to 100 mmHg transmural pressure for 24 h under normal flow conditions (15 dyn/cm² shear stress) contained significantly more MMP-2 than control vessels maintained at zero pressure with zero flow (P < 0.05). Vessels perfused at 200 mmHg contained more MMP-2 than control conditions but less than vessels perfused at 100 mmHg. However, the difference

Fig. 1. Effects of transmural pressure and flow on total levels of matrix metalloproteinase (MMP)-2 and -9 in homogenates of arteries maintained ex vivo for up to 48 h, as quantified from SDS-PAGE zymography. A: MMP-2 levels at 48 h were quantified from densitometric analysis of gelatin-containing SDS-PAGE gels. Under either normal or low-flow conditions, vessels subjected to 100 mmHg transmural pressure show significantly more activity (*P < 0.02) than the static, 0 pressure control or than the vessels subjected to 200 mmHg transmural pressure. B: MMP-9 levels at 48 h as quantified from SDS-PAGE zymography. Under either normal or low-flow conditions, arteries subjected to 200 mmHg transmural pressure show more MMP-9 than arteries maintained at 100 mmHg and significantly more MMP-9 (*P < 0.05) than the arteries maintained under static, 0 pressure control. Values obtained in independent experiments (n = 3–4 vessels for each condition) were measured from gels by scanning densitometry and were normalized to respective control levels. Bars represent averages ± SD. C: raw SDS-PAGE data for homogenates at normal flow conditions for 48 h in the 72-kDa range showing the zymogen (pro-MMP-2) and activated (MMP-2) forms. D: raw SDS-PAGE data for homogenates at normal flow conditions for 48 h in the 92-kDa range.
between the 200 mmHg condition and 100 mmHg was not statistically significant at 24 h. After 48 h, this trend obtained statistical significance (Fig. 1A). Vessels subjected to 100 mmHg transmural pressure showed fivefold more MMP-2-associated activity than control vessels ($P < 0.02$) and roughly threefold more activity than vessels perfused at 200 mmHg ($P < 0.02$). Total levels of MMP-2 at 200 mmHg were not statistically different from control values, and flow did not affect MMP-2.

Transmural pressure also induced MMP-9 activity in porcine carotid arteries. After 48 h in perfused organ culture (Fig. 1B), MMP-9 levels at 100 mmHg transmural pressure were higher than control levels, although not statistically significant. Levels of MMP-9 extracted from arteries perfused at 200 mmHg, quantified by SDS-PAGE zymography, were roughly 30-fold higher than control levels ($P < 0.05$) in the normal shear stress conditions and 20 times higher than control levels in the low shear conditions.

In situ gelatinase activity and MMP-2 and MMP-9 expression. Use of homogenized tissue allows quantification of total levels of MMPs but obscures any local effects on MMP expression or activity. Thus we investigated local distribution of MMP activity and matrix degradation in histological specimens. Immunocytochemical detection of MMP-2 protein showed that transmural pressure affected the distribution of MMP-2 expression across the arterial wall (Fig. 2). In the control arteries (zero pressure, zero flow condition), MMP-2 was weak and homogeneously distributed throughout the wall. In contrast, overall staining in the vessels perfused under pressure was increased, and MMP-2 expression was highest in the outer media. More importantly, the gelatinolytic activity detected using in situ zymography, a method that allows identification of active gelatinases, was absent in the control vessels, whereas it was present in vessels perfused at 200 mmHg. In vessels maintained at 100 mmHg, both degradation (by in situ zymography) and expression (by immunohistochemistry) were visible throughout the vessel wall, distributed homogeneously (data not shown). Furthermore, at 200 mmHg, gelatin degradation colocalized with areas of intense MMP-2 staining in the outer tunica media, suggesting that transmural pressure increased not only MMP-2 expression but also the enzymatic activity.

Fig. 2. Distribution of MMP-2 expression and gelatinolytic activity by immunocytochemistry (A and B) and by in situ zymography (C and D) of vessels maintained for 24 h in artery culture. A and C: representative specimens of control vessels (maintained at 0 mmHg). Control vessels present low levels of homogeneously distributed immunopositive MMP-2 and no gelatinolytic activity. B and D: arteries maintained at 200 mmHg. In the first specimen, MMP-2 staining was strongest in the outer media (arrows); the intense staining colocalized with gelatin degradation (dark areas) in the second specimen. Specimens for in situ zymography (C and D) were incubated on the FITC-labeled gelatin substrate for 3 days. White dotted lines mark the luminal side of the artery. Gelatin degradation is evident in the 200 mmHg vessel in the outer media as loss of fluorescence, whereas little loss of fluorescence is seen in the static control vessel. Negative controls not shown. All images were taken at $\times 10$ original magnification and were scaled identically.
Immunohistochemistry staining for MMP-9 in vessels maintained at 0, 100, and 200 mmHg for 24 h (Fig. 3, A, B, and C, respectively) showed that MMP-9 expression also increased with increasing transmural pressure. In contrast to the static condition (A), at pressures of 100 mmHg, intracellular staining of smooth muscle cells was evident (B). In arteries maintained at 200 mmHg, more intense MMP-9 staining was seen throughout the vessel, and endothelial cells also stained positive (C). Furthermore, increasing pressure visibly altered the pattern of endogenous elastin, a substrate for gelatinases. Representative fluorescent images of elastin content in cross sections from vessels cultured for 48 h under normal flow conditions are shown in Fig. 3, D–F.

Because both MMP-2 and MMP-9 are effective elastases and collagenases (26, 29), activated forms of either enzyme can degrade elastin effectively. Quantification of the elastin content supported the visual impression of increased MMP expression and matrix degradation found by examination of MMP immunostaining, in situ zymography, and elastin autofluorescence (Fig. 4). Image analysis of endogenous elastin showed that control vessels contained large amounts of elastin, predominantly in long, contiguous elastic laminae in cross section. The total percentage area of elastin occupied in sections of arteries cultured at the different pressure and flow conditions is shown in Fig. 4A. All differences between pressure conditions (within each flow group) were statistically significant, with \( P < 0.05 \). Differences between flow conditions at the same pressure were not statistically different, suggesting that decreasing the shear stress had no effect on the elastin content. The mean percentage area of each elastin structure occupied within the cross section of the arterial wall at different pressure and flow conditions is shown in Fig. 4B. In this case, the average area of each elastin segment was plotted instead of the summation of all areas. Arteries subject to transmural pressure for 48 h showed less elastin content overall, and the elastin

![Fig. 3. Effect of ex vivo transmural pressure on localization of MMP-9 expression and endogenous elastin fluorescence in porcine arteries. Immunohistochemistry staining for MMP-9 in vessels maintained at 0 mmHg (A and D), at 100 mmHg (B and E), and at 200 mmHg (C and F) for 48 h. A: In unpressurized arteries, no positive staining was visible. B: Positive perinuclear staining for MMP-9 is present in approximately one-half of the smooth muscle cells in the section, whereas no staining is detectable in the endothelial cells. C: Increased positive staining of smooth muscle cells and endothelial cells is detectable in arteries maintained at higher transmural pressure. All images were taken at \( \times 40 \) original magnification and were scaled identically. D–F: Representative images of elastin autofluorescence in vessels perfused for 48 h at 0 (D), 100 (E), and 200 (F) mmHg pressure. Internal elastic lamina on the luminal side of the vessel (top) and collagen bundles at the adventitial side (which fluoresce when found in the 3-part braided structure, bottom) are visible in all three images. D: In control vessels maintained at 0 mmHg, elastin lamellae are intact. E: Increased pressure led to progressive fragmentation of elastin lamellae. Elastin lamellae in arteries maintained at 100 mmHg would be quantified as several small pieces of elastin. F: A few discontinuous lamellae near the internal elastic lamina can still be seen in arteries maintained at 200 mmHg. Images were taken at \( \times 10 \) original magnification and were scaled identically.](image-url)
present was made up of shorter segments than those in the control vessels, suggesting elastin degradation or restructuring. Vessels cultured at 200 mmHg transmural pressure had the smallest elastin content and the most fragmented elastin. In both flow groups, the pressurized vessels were statistically different from the zero pressure control \( (P < 0.05 \text{ and } P < 0.002) \) but were not different from one another. The average luminosity of elastin in each specimen cross section was not different for any of the conditions, indicating that the threshold for elastin identification did not change with either pressure or flow.

**DISCUSSION**

In vivo structural reorganization of arterial vessels has been previously studied in response to various hemodynamic perturbations. Changes in blood flow rate and shear stress over the course of six or more weeks were found to induce revisions in the internal radius until the wall shear stress was restored to control levels (5, 15, 18, 35). In adult rabbits, diameter changes to reduced flow were initially vasomotor in origin and became chronic within 2 wk without net changes in vessel wall elastin, collagen, or DNA (23). A combination of increased flow and changing pressure in a rat aortocaval fistula led to increased fistula diameter and increased wall elastin and collagen content (8). The enzymatic factors involved in low-flow remodeling were investigated by Bassiouny et al. (2), who found that low flow upregulated injury-induced MMP-2 mRNA and MMP-2 activity after 7 days. Doubling and halving of flow rates in the carotid arteries of immature rabbits led to significant changes in elastin structure within 5 wk, as measured by confocal laser microscopy in an en face view of the internal elastic laminae (34).

According to our analyses of homogenized tissue and histology, no changes in MMP activity or matrix-degrading activity were measured in vessels subject to low flow for 48 h or less compared with normal flow. The enzymatic upregulation due to low flow reported in vivo may require times of exposure longer than 48 h. Cell culture experiments that show endothelial cell morphological, biochemical, and structural changes to flow within minutes cannot take into account the effect of the underlying smooth muscle cells and ECM. We did not specifically investigate expression of MMP inhibitors (TIMPs) in the remodeling process. However, we used in situ zymography, which reveals the balance between degrading activity by active MMPs and inhibitory action of TIMPs.

In response to experimentally induced hypertension, new matrix synthesis, endothelial cell proliferation, and changes in smooth muscle cell morphology have been reported to occur (1, 8, 25, 28, 30, 32, 33). Typically, the vascular wall thickness increases monotonically with time after an increase in transmural pressure (8, 25). In rat aortas, increased fibronectin synthesis was shown to correlate with hypertension (28). Fibronectin expression in response to hemodynamic stimuli was studied in an ex vivo system similar to ours (1). After 3–5 five days in culture, expression of fibronectin was significantly higher at high transmural pressure (150 mmHg) compared with low (80 mmHg) or zero pressure and was potentiated by the addition of 20% FCS to the perfusate. In vitro, pressure-induced cyclic stretch was shown to stimulate endothelial cell
proliferation (33) and to affect smooth muscle cell orientation (32) and morphology (30). Ambient pressure stimulated immortalized human aortic endothelial cells to increase MMP-1 (interstitial collagenase) production in another in vitro study (19).

Our data demonstrate localized, specific activation of matrix-degrading enzymes and degradation of elastin in response to increased transmural pressure. Control vessels incubated at zero pressure showed low levels of MMP expression and activity and maintained intact elastin. MMP-2 was expressed in these arteries, as detected in the homogenized tissue and by immunocytochemistry, but the expression was not associated with gelatin-degrading activity, as indicated by in situ zymography. We conclude that static conditions do not stimulate MMP activity and, in the absence of enzyme activity, medial elastin lamellae remain intact. MMP-2 extracted from whole tissue homogenates of vessels perfused at 100 mmHg, measured by SDS-PAGE zymography, was increased by about sixfold over static control vessels. Levels from homogenates of vessels perfused at 200 mmHg were not statistically different from control. However, immunohistochemistry and in situ detection of enzymatic activity showed intense staining and FITC-labeled gelatin degradation in the outer media in vessels maintained at 200 mmHg. Thus both MMP-2 expression and matrix degradation are locally enhanced at the higher transmural pressure (200 mmHg), although the increase was not detectable when the whole tissue content was analyzed. These observations suggest that variations in the local distribution of MMP-2 expression and activity may be more relevant to vascular remodeling than changes in the overall levels. Localized data were not sought using SDS-PAGE zymography but may warrant further study.

MMP-9 expression was typically undetectable or very low in control arteries in this study but increased monotonically with increasing pressure and was not significantly affected by decreasing flow rates below normal levels. The SDS-PAGE zymography data showed a robust increase over control in the higher-pressure vessels confirmed by histology. Elastin degradation also increased monotonically with pressure. The intermediate level of elastin degradation detected at the physiological mean in vivo pressure of 100 mmHg may be due to the sudden increase in pressure over static storage conditions (always <6 h) or the steady, nonphysiological nature of the pressure stimulus. On the other hand, continual turnover of elastin in vivo likely occurs at pressures in the normal range. Analysis of correlation between elastin degradation and levels of MMP-9 in homogenates, measured by SDS-PAGE zymography, yielded a weakly positive correlation coefficient of 0.60. Through these linked pathways for enzyme activation and matrix degradation, high pressure may enable vascular remodeling by breaking down structural impediments in the vessel wall. At 200 mmHg pressure, both MMP-2 and MMP-9 are expressed and may act in a combined fashion to degrade ECM components. Presumably, other mechanical or biochemical stimuli then induce matrix component synthesis in smooth muscle cells during a later stage of remodeling. Increased transmural pressure increased expression of MMP-9, inducible in smooth muscle cells, and led to degradation of elastin, a matrix component. Increased pressure affected the distribution of MMP-2, which is constitutively secreted by smooth muscle cells. Thus localized activation of MMP-2 and the associated localized degradation of gelatin (collagen substitute) may aid remodeling secondary to hypertension by removing specific structural components in the outer vessel wall. The mechanical advantage for prioritized degradation of the outer media versus the inner or entire media is unclear.

Our data demonstrate that enzymes that degrade ECM components are involved in the early structural changes that occur in response to increased transmural pressure and thus may be clinically relevant to early vascular remodeling in hypertensive vascular disease.

Transmural pressure induces MMP activity within 48 h ex vivo and results in elastin degradation. MMP-9 activity increases with increasing pressure up to 200 mmHg; total MMP-2 expression is significantly higher at 100 mmHg than at either 0 or 200 mmHg pressure and shows very localized distribution at the higher pressure. This localization is coincident with enzymatic gelatinase activity measured by in situ zymography. Vascular wall shear stress has no significant effect on MMP-2 or MMP-9 expression, elastin degradation, or gelatinolytic activity at early time points. Our data identify two agents that likely participate in the remodeling process secondary to hypertension and detail their stimuli in an ex vivo perfusion system. The relationship between hemodynamics and MMP activity is an important component of physiological and pathological vascular remodeling.

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