Downregulation of Vascular Matrix Metalloproteinase Inducer and Activator Proteins in Hypertensive Patients

Adviye Ergul, Vera Portik-Dobos, Jimmie Hutchinson, Jennifer Franco, and Mark P. Anstadt

Background: Peripheral vasculature undergoes extensive vascular remodeling in the hypertensive state. Regulation of extracellular matrix turnover by the matrix metalloproteinase (MMP) system is an important step in the vascular remodeling process. However, the expression pattern of the vascular MMP system in human hypertension remained unknown.

Methods and Results: Internal mammary artery specimens were obtained from normotensive (n = 13) and hypertensive (n = 19) patients undergoing coronary artery bypass grafting surgery. Zymographic analysis indicated a threefold decrease in total gelatinolytic activity of MMP-2 and MMP-9 in hypertension. MMP-1 activity was also decreased by fourfold without a significant change in protein levels. Tissue levels of extracellular matrix inducer protein (EMMPRIN), MMP activator protein (MT1-MMP), MMP-1, MMP-2, and MMP-9, as well as tissue inhibitors of MMPs (TIMP-1 and TIMP-2) were assessed by immunoblotting and yielded a significant decrease in MMP-9, EMMPRIN, and MT1-MMP levels in hypertension. In addition, measurement of plasma markers of collagen synthesis (procollagen type I amino-terminal propeptide [PINP]) and collagen degradation (carboxy-terminal telopeptide of collagen type I [ICTP]) indicated no difference in PINP levels but suppressed degradation of collagen in hypertension. Evaluation of profibrotic growth factors demonstrated higher levels of fibroblast growth factor (FGF)-2 in tissue preparations from hypertensive patients but no difference in transforming growth factor-β1 levels.

Conclusions: These findings demonstrate that not only MMP-1 and MMP-9, but MMP inducer and activator proteins are also downregulated in the hypertensive state. Augmented FGF-2 levels may contribute to parallel decreases in MMP activity and MMP induction system resulting in enhanced collagen deposition in hypertension.

Key Words: Matrix metalloproteinase, vascular remodeling, extracellular matrix, growth factors.

Arterial hypertension is associated with increased risk of target organ damage resulting in stroke, left ventricular hypertrophy, and coronary artery disease. Increased extracellular matrix (ECM) content, especially collagen type I and type III, results in fibrosis and contributes to the pathogenesis of target organ damage in hypertension. Enhanced collagen deposition may be due to increased collagen synthesis or decreased degradation by matrix metalloproteinases (MMPs). The MMPs are a family of proteolytic enzymes that degrade ECM proteins such as collagen and elastin and are essential for cellular migration and tissue remodeling under physiological and pathological conditions. The members of MMP family are synthesized as latent proenzymes, which are later cleaved to active MMPs by serine proteases including trypsin and plasmin, active MMP-2, and recently described membrane type (MT)-MMPs. The MT-MMPs possess a transmembrane domain that anchors the enzyme to the membrane and can exert local MMP activation. For example, MT1-MMP contributes to the activation of MMP-2, which then can activate other secreted pro-MMPs to their active forms. The major MMP species expressed in the vasculature include MMP-1, MMP-2, MMP-9, and MT1-MMP, and endothelial and smooth muscle cells, as


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well as fibroblasts can synthesize these enzymes. The activity of MMPs is tightly regulated by tissue inhibitors of MMPs (TIMPs) and the MMP/TIMP ratio is critical for coordinating matrix production and degradation. Lavia-des and colleagues reported that plasma levels of MMP-1 and collagen type I degradation product (ICTP; carboxy-terminal telopeptide of collagen type I) were decreased in patients with essential hypertension and left ventricular hypertrophy, whereas TIMP-1 levels were increased. They also reported that treatment with lisinopril restored MMP-1 and TIMP-1 levels. These results suggested that systemic extracellular degradation of collagen type I is depressed in patients with essential hypertension and left ventricular hypertrophy, whereas TIMP-1 levels were increased. They also reported that treatment with lisinopril restored MMP-1 and TIMP-1 levels. These results suggested that systemic extracellular degradation of collagen type I is depressed in patients with essential hypertension and can be normalized with the inhibition of an angiotensin-converting enzyme. The expression and activity of the vascular MMP system in hypertensive patients remains unknown. Accordingly, we investigated the presence and activity of the components of the MMP system, changes in ECM proteins, and potential factors that may influence MMP activity in internal mammary artery specimens obtained from normotensive and hypertensive patients undergoing coronary artery bypass surgery.

### Methods

#### Patient Enrollment and Tissue Collection

The study protocol was approved by the Human Assurance Committee at the Medical College of Georgia and written informed consent was obtained from all the participants before the surgery. Internal mammary artery specimens were obtained from patients undergoing coronary artery bypass graft surgery. Patients with diabetes were not included in this study. Patient characteristics including use of medications are summarized in Table 1. The hypertension group was designated based on the previous clinical diagnosis of hypertension and not the blood pressure (BP) readings during the hospital stay. Artery specimens were placed in cold Dulbecco’s modified Eagle medium (DMEM) and kept on ice. After surrounding fat was carefully removed, arterial specimens were rinsed in sterile saline, cut into 3-mm segments and immediately frozen.

#### Immunoblotting and Gelatin Zymography

Frozen artery specimens were homogenized as previously described. Protein levels of MMP species (MMP-1, MMP-2, MMP-9, MT1-MMP, extracellular matrix inducer protein [EMMPRIN], as well as TIMP-1 and TIMP-2; Oncogene Research Products, Cambridge, MA), transforming growth factor (TGF)-β1 (R & D Systems Inc., Minneapolis, MN), fibroblast growth factor (FGF)-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and collagen type I (Abcam Limited, Cambridge, UK) were determined by immunoblotting using antibodies specific for each species as previously described. Equal protein loading was ensured by immunoblotting the same membranes for β-actin. Basal gelatinolytic activity of MMP-2 and MMP-9 was detected using zymography as previously described. Recombinant MMP proteins were used as positive controls.

#### Collagenase Activity

Collagenase activity of vascular MMP-1 was determined using a fluorescein-conjugated collagen assay kit as recommended by the manufacturer (Molecular Probes, Eugene, OR), as described previously.

### Table 1. Patient demographics and list of medications

<table>
<thead>
<tr>
<th></th>
<th>Normotensive</th>
<th>Hypertensive</th>
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<tbody>
<tr>
<td>Age (y, mean ± SD)</td>
<td>54 ± 2.7</td>
<td>60 ± 2.5</td>
</tr>
<tr>
<td>Ethnicity (African American/white)</td>
<td>1/12</td>
<td>3/16</td>
</tr>
<tr>
<td>Sex (women/men)</td>
<td>2/11</td>
<td>6/13</td>
</tr>
<tr>
<td>Body mass index (kg/m², mean ± SD)</td>
<td>28.4 ± 1.2</td>
<td>28.5 ± 1.8</td>
</tr>
<tr>
<td>Systolic blood pressure* (mm Hg, mean ± SD)</td>
<td>124 ± 4</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg, mean ± SD)</td>
<td>66.8 ± 3.4</td>
<td>74 ± 3.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>183 (n = 8)†</td>
<td>160 (n = 6)†</td>
</tr>
<tr>
<td>Smoking history</td>
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<td>4</td>
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<tr>
<td>Medications</td>
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<td></td>
</tr>
<tr>
<td>Ca²⁺ channel blockers</td>
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<td>5</td>
</tr>
<tr>
<td>ACE inhibitors</td>
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<tr>
<td>All receptor blockers</td>
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</tr>
<tr>
<td>β-blockers</td>
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<tr>
<td>Cholesterol lowering drugs</td>
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<td>Antiaggregant therapy</td>
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<td>12</td>
</tr>
<tr>
<td>Nitrates</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme.

* Blood pressure measurements indicate readings obtained during the preoperative evaluation. Hypertension group was designated based on the previous clinical diagnosis of hypertension.

† Cholesterol information was not available from all patients.
Briefly, vascular extracts (20 μg of total protein) from normotensive and hypertensive patients (n = 5 in each group) were incubated with the substrate and increased fluorescence, which is directly proportional to the proteolytic activity of MMP-1, was measured at time 0, 2, 4, 8, and 24 h using a microplate fluorometer. The collagenase activity of samples was extrapolated from a standard curve generated by recombinant MMP-1. Other serine proteases in the tissue extracts were blocked by using 50 mmol/L phenylmethylsulfonylfluoride (PMSF).

Measurement of PINP and ICTP

The amount of collagen synthesis and degradation markers in the plasma was determined using radioimmunoassay kits, specifically designed for direct measurement of plasma procollagen type I amino-terminal propeptide (PINP) and ICTP (DiaSorin, Stillwater, MN). The sensitivity of PINP and ICTP assays are 0.5 to 25 μg/mL and 0.1 to 5 μg/mL, respectively.

Statistical Analysis

The zymograms and immunoblots were analyzed by densitometric scanning. In zymograms, lytic bands that demonstrate MMP activity at various molecular weights were analyzed by Gel Pro Image Analysis Program (Media Cybernetics, Silver Spring, MD) and expressed as optical density (pixels). In immunoblots, bands corresponding to the known molecular weight of each MMP species were analyzed in a similar manner. Blots were corrected equal protein loading using β-actin staining. The person who performed the image analysis was blinded to the images of zymograms and immunoblots. The data obtained from densitometric analyses were compared by ANOVA. The results are given as mean ± SEM. An alpha level of P < .05 was considered to be statistically significant.

Results

Vascular Matrix Metalloproteinase Activity

Total gelatinolytic activity in the internal mammary artery specimens of hypertensive patients was compared to that of patients with normal BP using substrate zymography. As shown by a representative gelatin zymogram in Fig. 1A, MMP activity detected at molecular weights corresponding to MMP-2 (~60 kDa) and MMP-9 (~85 kDa) was detected in all specimens. In addition, lytic activity corresponding to MMP-9-lipoca-
lin complex (125 kDa) was also identified mainly in tissue specimens from normotensive patients. Densitometric analysis of zymographic data demonstrated that total MMP activity (MMP-2 and MMP-9) was approximately threefold less abundant in the hypertensive tissue (P < .05) (Fig. 1B). In situ zymography experiments using tissue from limited number of patients demonstrated lytic activity in both medial and adventitial layers indicating both smooth muscle and fibroblast origin of MMP activity (data not shown). As shown in Fig. 1C, vascular homogenates from hypertensive patients displayed decreased processing of a fluorescently labeled collagen type I substrate indicating suppressed MMP-1 activity.

Matrix Metalloproteinase Protein Expression

To determine whether and to what extent MMP activity correlates with MMP protein levels, enzyme levels were determined by immunoblotting of the same extracts used in activity measurements. Bands corresponding to the molecular weight of MMP-1 (~42 kDa), active MMP-2 (~60 kDa), and MMP-9 (~85 kDa) were detected in all specimens (Fig. 2A). Densitometric comparisons of tissue from normotensive and hypertensive patients after β-actin correction demonstrated that there was no significant change in MMP-1 and MMP-2 levels, but MMP-9 levels were significantly lower in the hypertensive tissue (Fig. 2B). In an attempt to evaluate the levels of endogenous

![FIG. 2. A) A representative immunoblot demonstrating the presence of matrix metalloproteinase (MMP) in vascular tissue. Internal mammary artery specimens from normotensive (n = 8) and hypertensive (n = 8) patients were subjected to immunoblotting for MMP-1, MMP-2, and MMP-9. B) Densitometric analysis of immunoreactive bands (mean ± SEM) indicated decreased MMP-9 in the tissue from hypertensive patients.* P < .05 versus normotensive.](https://academic.oup.com/ajh/article-abstract/17/9/775/322169/)

![FIG. 3. Matrix metalloproteinase (MMP) inducer and activator proteins in vascular tissue. A) A representative immunoblot showing the abundance of 54-kDa MMP activator protein (MT1-MMP) and 58-kDa extracellular matrix inducer protein (EMMPRIN) in internal mammary artery extracts. B) Densitometric analysis of immunoreactive bands (mean ± SEM) demonstrated a decrease in MMP inducer and activator proteins in the tissue from hypertensive patients. *P < .05 versus normotensive.](https://academic.oup.com/ajh/article-abstract/17/9/775/322169/)
inhibitors of MMPs, TIMP-1, and TIMP-2 protein expression was also assessed in a small number of patients (n = 4 per group), but there was no significant difference between the groups (data not shown).

The EMMPRIN and MT1-MMP protein levels were analyzed to determine whether proteins involved in the induction and activation of MMPs are modulated in the hypertensive state. As shown in Fig. 3A, both EMMPRIN and MT1-MMP were readily detectable in internal mammary artery specimens and both were decreased in tissue specimens from hypertensive patients (Fig. 3B).

**Extracellular Matrix Deposition**

To investigate whether decreased MMP activity is associated with enhanced ECM accumulation, collagen type I and fibronectin levels in the same tissue extracts were determined by immunoblotting. Bands corresponding to both the α1 (120 kDa) and α2 (200 kDa) chains of collagen type I were detected (Fig. 4A) and densitometric analysis demonstrated a fourfold increase in collagen type I in the tissue from hypertensive subjects (P < .05 vs control). In addition, analysis of the circulating markers of collagen synthesis (PINP) and degradation (ICTP) demonstrated similar plasma PINP levels in both groups, whereas ICTP levels were elevated providing further evidence that collagen type I metabolism is altered in hypertensive patients.

**Profibrotic Growth Factors**

To identify possible mechanisms contributing to decreased MMP activity and increased ECM deposition, tissue TGF-β1 and FGF-2 levels were evaluated in the same preparations (Fig. 5). There was no significant difference in TGF-β1 (data not shown). However, a ~18-kDa band corresponding FGF-2 was detected in all specimens and densitometric analysis of immunoblots demonstrated threefold higher levels of FGF-2 in the arterial tissue from hypertensive patients as compared to normotensive individuals.

**Discussion**

This study demonstrated that MMP inducer (EMMPRIN) and activator (MT1-MMP) proteins as well as MMP-1 and MMP-9-mediated proteolytic activity are downregulated in the peripheral arterial vasculature of hypertensive patients. Furthermore, this decrease in MMP induction and activation system is associated with parallel increases in ECM deposition and FGF-2 expression. Pathologic changes in the vessel wall structure contributes to the
development of target organ damage in hypertension and findings of the present study provide important information regarding the molecular and cellular basis of structural changes in the peripheral vasculature in hypertension and contribute to our understanding of the potential mechanisms responsible for hypertensive complications.

Pathologic vascular remodeling of resistance arteries in hypertension involves growth, ECM expansion, and fibrosis. The ECM is a dynamic structure that requires constant synthesis and degradation by MMPs. This degradation process is tightly controlled by tissue inhibitors of MMPs known as TIMPs. Several species are commonly expressed in the vasculature, including MMP-1, MMP-2, and MMP-9, by endothelial and smooth muscle cells as well as fibroblasts. All these enzymes are secreted in zymogen forms, which are later activated by other tissue and plasma proteinases such as trypsin and plasmin as well as active MMP-2 and MT1-MMP. Recent studies have demonstrated that EMMPRIN, another membrane-bound protein, can induce MMP-1, MMP-2, and MMP-3 expression in fibroblasts. As demonstrated by these past reports, regulation of MMPs is a complex process that involves inducer, activator, and inhibitor proteins. A comprehensive analysis of the vascular MMP system in hypertension remained unknown. Thus, this study focused on the regulation of ECM proteins by the MMP family of proteases. Although we did not perform morphometric studies, our results provided evidence that both inducer and activator proteins of the arterial MMP system are reduced in hypertension leading to enhanced ECM deposition.

Several groups of investigators reported changes in the soluble markers of vascular remodeling in hypertension. They demonstrated that at baseline MMP-1 was decreased and TIMP-1 was increased in hypertensives with no difference in ICTP levels. In hypertensive patients with baseline left ventricular hypertrophy, both MMP-1 and ICTP were decreased compared with normotensive subjects. Treatment with lisinopril restored MMP-1 and TIMP-1 levels. Based on these results it was speculated that depressed degradation of collagen type I may facilitate organ fibrosis in hypertension. Recently, Lindsay and colleagues investigated the correlation between indices of left ventricular function and ICTP, TIMP-1, and propeptide of collagen type I (PINP), which is a marker for collagen synthesis. The TIMP-1 levels were significantly elevated in patients with diastolic function. Zervoudaki et al reported that plasma levels of MMP-2 and MMP-9 proteins are decreased in hypertensive patients. In the current study, MMP-1 activity was decreased without a significant change in protein levels determined by immunoblotting. This finding suggests that post-translational modifications of MMP-1 might play an important role in suppressed enzyme activity in hypertensive patients. On the other hand, we found parallel decreases in MMP-9 protein expression levels and activity. Our findings on decreased MMP expression and activity in internal mammary artery specimens obtained from hypertensive patients support these past studies and provide evidence of decreased MMP abundance/activity and ECM degradation at the tissue level.

In an effort to better understand the potential mechanisms of decreased MMP activity in arterial tissue from hypertensive patients, the presence of inflammation as well as expression of profibrotic growth factors were evaluated. Inflammation contributes to complications associated with hypertension and inflammatory cells like neutrophils and macrophages express the components of the MMP system. Thus, the current study also assessed neutrophil-derived MMP activity. The MMP-9 forms a complex with neutrophil gelatinase-associated lipocalin protein that can be detected as a high molecular weight band on zymograms. Surprisingly, the MMP-9/lipocalin complex was lower in the hypertensive patients. Although increased infiltration of inflammatory cells could contribute to enhanced MMP levels/activity, a decrease in MMP activity could not be solely explained by lack of inflammation. Thus, future studies are needed to evaluate the role of inflammation in long-term regulation of MMP activity in hypertension. Laviades et al reported that increased plasma TGF-β1 levels are associated with decreased collagen degradation in hypertensive patients, suggesting an inhibitory role for this profibrotic growth factor in MMP regulation. A recent study reported that in stroke-prone spontaneously hypertensive rats fed a high salt diet, renal MMP-2 activity was augmented by fivefold. At the same time, procollagen I expression as well as tissue levels of potent fibrogenic growth factors TGF-β1 and FGF-2 were increased. In the current study, we also observed higher levels of FGF-2, which might contribute to collagen deposition by suppressing MMP activity. Ammarguellat and colleagues studied potential mechanisms of cardiac fibrosis in DOCA-salt hypertensive rats and demonstrated that ET-1 promotes fibrosis through activation of MMP-2 and MMP-9 activity in this model. We have recently shown that in a borderline hypertension model, behavioral stress stimulates MMP activity before the development of hypertension. These recent reports suggest that MMPs are activated early in the disease process. In contrast to early MMP activation observed in these experimental models of hypertension, our results demonstrated a decrease in vascular MMP activity and expression in hypertensive patients. Possible explanations for this difference are twofold. First, the current study investigated MMP expression and activity in hypertensive patients with a long-standing history of hypertension rather than in experimental models. Second, the changes in MMP synthesis and activity might be time dependent. We speculate that in the early phase of hypertension the MMP system is activated to allow the smooth muscle cells to migrate and restructure the vessel wall. However, with the progression of disease, the MMP system is suppressed causing ECM deposition and fibrosis.
It is difficult to assess the impact of coronary artery disease on the MMP system in our model. Second, the internal mammary artery is not a resistance vessel and indices of vascular remodeling (ie, wall thickness and vascular smooth muscle cell growth) might differ from remodeling observed in small arteries. Third, all the patients were receiving therapy for coronary artery disease or hypertension. A majority of the patients were on combination therapy. Although medication use appears to be similar in both groups, the number of subjects in each group is limited to study complex drug interactions on MMP expression/activity. It should also be noted that BP of the patients in the hypertension group at the time of surgery was not significantly different than that of the normotensive group. However, the hypertension group designation was based on the previous clinical diagnosis of hypertension and similar BP readings in both groups indicate that BP control was achieved at the time of surgery. Due to the heterogeneity between the study groups, we cannot specifically attribute the changes in MMP expression/activity to hypertension but speculate that there is an association between hypertension and MMP levels.

In summary, this study demonstrated that both MMP inducer/activator proteins as well as MMP-1 and MMP-9 are decreased in hypertensive subjects suggesting that the vascular MMP induction/activation system may be a potential target for ECM regulation by cytokines and growth factors in hypertension.

**References**


