Proteinase Activity and Receptor Cleavage
Mechanism for Insulin Resistance in the Spontaneously Hypertensive Rat

Frank A. DeLano, Geert W. Schmid-Schönbein

Abstract—Arterial hypertension is associated with organ dysfunctions, but the mechanisms are uncertain. We hypothesized that enhanced proteolytic activity in the microcirculation of spontaneously hypertensive rats (SHRs) may be a pathophysiological mechanism causing cell membrane receptor cleavage and examine this for 2 different receptors. Immunohistochemistry of matrix-degrading metalloproteinases (matrix metalloproteinase [MMP]-9) protein shows enhanced levels in SHR microvessels, mast cells, and leukocytes compared with normotensive Wistar-Kyoto rats. In vivo microzymography shows cleavage by MMP-1 and -9 in SHRs that colocalizes with MMP-9 and is blocked by metal chelation. SHR plasma also has enhanced protease activity. We demonstrate with an antibody against the extracellular domain that the insulin receptor-α density is reduced in SHRs, in line with elevated blood glucose levels and glycohemoglobin. There is also cleavage of the binding domain of the leukocyte integrin receptor CD18 in line with previously reported reduced leukocyte adhesion. Blockade of MMPs with a broad-acting inhibitor (doxycycline, 5.4 mg/kg per day) reduces protease activity in plasma and microvessels; blocks the proteolytic cleavage of the insulin receptor, the reduced glucose transport; normalizes blood glucose levels and glycohemoglobin levels; and reduces blood pressure and enhanced microvascular oxidative stress of SHRs. The results suggest that elevated MMP activity leads to proteolytic cleavage of membrane receptors in the SHR, eg, cleavage of the insulin receptor-binding domain associated with insulin resistance. (Hypertension. 2008;52:415-423.)

Key Words: microcirculation ■ matrix metalloproteinases ■ insulin receptor ■ integrin ■ receptor cleavage ■ oxygen free radical

Arterial hypertension is associated with inflammation and organ dysfunction. Evidence from experimental models of hypertension indicates a multitude of microvascular complications that span across all segments of the microcirculation with high and low blood pressures. The origin of this organ injury is incompletely understood.

In addition to signaling activity and cause for an elevated arterial blood pressure, oxygen free radicals were proposed as a mechanism for organ injury. An interesting feature of hypertensive subjects, like the spontaneously hypertensive rat (SHR), is that, other than elevated blood pressure, a variety of complications occur, such as insulin resistance or immune suppression with impaired leukocyte-endothelial adhesion and CD18 downregulation. These diverse complications have not been conclusively linked to oxygen free radical production.

As an alternative, we studied proteolytic mechanisms. The permanently elevated leukocyte count in SHRs is accompanied by degranulation and release of proteases from lysosomal granules of neutrophils. The SHR brain, kidney, and heart have enhanced matrix metalloproteinase (MMP) activity. They may mediate cardiac, cerebral, and arterial disease processes, complications that accompany arterial hypertension. But the role of MMPs in microvascular complications and end organ damage of SHRs remains undefined.

In the current study, we measured protease and, specifically, MMP activity in SHRs. We hypothesized that SHRs may have enhanced proteolytic activity and that unique complications in this model are because of proteolytic cleavage of specific receptors, eg, the insulin receptor in insulin resistance and CD18 cleavage in leukocyte adhesion.

The objective, therefore, was to investigate the expression of MMP protein (MMP-9) and activity levels with digital microzymography in the microcirculation of SHR and Wistar-Kyoto (WKY) control together with the superoxide production and nuclear transcription factor κB (NF-κB) translocation. We demonstrated that inhibition of MMP activity by a broad-acting MMP inhibitor served to normalize in the SHR blood pressure, oxygen free radical production, and NF-κB translocation, as well as to block surface receptor cleavage and development of insulin resistance. Parts of this work was presented as abstract.

Methods

For detailed methods, please see the online supplement available at http://hyper.ahajournals.org. The study was approved by the University of California San Diego, La Jolla CA 92093-0412. E-mail gwss@bioeng.ucsd.edu

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sity of California San Diego Animal Subjects committee. Mature male SHRs and their normotensive controls, WKY rats, were studied under general anesthesia (Nembutal). Selected WKY rats and SHRs were treated with doxycycline (55 mg in drinking water, ~5.4 mg/kg per day) for 24 weeks. Total leukocyte count in femoral arterial blood, hematocrit, blood glucose, and the percentage of glycohemoglobin levels were determined.

The ileocecal portion of the mesentery was observed by digital intravital microscopy. The cellular distribution of MMP proteins (MMP-9 and gelatinase-B) in the whole mount mesentery was delineated by biotin-avidin immunolabeling. Tissue specimen without primary antibody served as a control. No counterstain was applied to facilitate quantitative label intensity measurements, because microvascular structures can be readily identified on the labeled mesentery.

MMP-1 and -9 activity were determined by in vivo digital microangiography during superfusion of the mesentery with fluorescent dye (D2293, Sigma-Aldrich) in the suffusate that is cleaved by MMP-1 (collagenase-1) and MMP-9 (gelatinase B). The mesentery was loaded with substrate 10 minutes before image collection and suffused with substrate throughout the experiment. The associated fluorescence intensity was recorded under standardized microscope settings in digital units after subtraction of background in the absence of tissue. Reproducibility of measurements was within 3%.

Fresh plasma samples were tested for overall protease activity with a fluorescent protease assay kit (EnzChek, Molecular Probes). The substrate was cleaved by multiple proteases (metallo-, serine, acid, and sulfhydryl proteases). Protease activity was determined from fluorescent intensity after cleavage fluorescent units). The substrate was cleaved by multiple proteases (metallo-, serine, acid, and sulfhydryl proteases). Protease activity was determined from fluorescent intensity after cleavage fluorescent units).

Results

Blood Pressures, Heart Rates, and Blood Cell Counts

The elevated blood pressure of SHRs was reduced by chronic doxycycline treatment (Table 1). After doxycycline treatment, the blood pressure of the SHRs reached values comparable to WKY rats before treatment, whereas the arterial pressure of WKY rats was reduced to values below the blood pressure of Wistar rats (≈105 mm Hg) from which the WKY and SHR strains were bred. Heart rates were not significantly affected by the MMP inhibition. Acute topical doxycycline administration under general anesthesia at the same concentration (5.4 mg/kg) had no significant effect on WKY or SHR blood pressure (n=3 rats per strain) over a 6-hour monitoring period.

The hematocrit levels of WKY rats and SHRs were the same and slightly decreased after doxycycline treatment (Table 1). In contrast, the elevated leukocyte counts of both SHRs and WKY rats fell during the treatment; the SHR fell to values <50% of its usually high count.

Blood Glucose and Glycated Hemoglobin

The SHR has a significantly elevated blood glucose level compared with WKY rats, which is reduced by doxycycline treatment (Table 2). The elevated glycohemoglobin values (percentage of glycohemoglobin) in the SHRs are also reduced to WKY values, although the WKY rats also had lower percentages of glycohemoglobin values after MMP inhibition.

MMP-9 Protein Levels in Neutrophils

The anti–MMP-9 antibody label was located predominantly in the cytoplasm (Figure 1A). Undetectable levels of substrate labeling were observed in the absence of the primary antibody (data not shown). The MMP-9 distribution on individual neutrophils, as determined by optical density measurements, showed, on average, significantly higher values in the SHR.
(Figure 1B). After doxycycline treatment, the MMP-9 levels fell to average values without a significant difference among strains (Figure 1).

**MMP-9 Levels in Mesentery Microcirculation**

Undetectable levels of immunolabel substrate were present in the mesentery without primary antibody or in specimens treated with a nonbinding antibody (data not shown). MMP-9 protein was detectable in all of the major structures of the mesentery, including the endothelium and interstitial cells. The general pattern of the MMP-9 protein labeling in arterioles, capillaries, and venules was similar for both WKY and SHR mesentery (Figure 2A). Endothelial cells have nonuniform protein labeling, such that cells with an enhanced label are located side by side with cells that have low or even undetectable substrate levels. Venules for both WKY rats and SHRs revealed an increased MMP-9 receptor density compared with comparable arterioles.

The average antibody label density as measured by light absorption over randomly selected arterioles and venules in mesentery was significantly higher in the SHRs (Figure 2B). No difference in the level of MMP-9 protein levels of capillaries was detected between WKY rats and SHRs. Avascular tissue areas of the SHR mesentery had a significantly enhanced level of MMP-9.

After doxycycline treatment, the MMP-9 levels decreased significantly in both WKY rats and SHRs (Figure 2B). The SHRs reached control levels that were not significantly different from the values in WKY rats.

**MMP Activity in Mesentery Microcirculation**

MMP-1 and -9 enzymatic activity, as detected by fluorogenic substrate cleavage, was present in all of the major structures of the mesentery, including the endothelium and interstitial cells (Figure 3A). The microvascular distribution of the MMP-1 and -9 activity closely resembles the MMP-9 immunolabeling pattern (Figure 2A). Endothelial cells have a nonuniform pattern of substrate cleavage such that cells with enhanced enzyme activity may be located side by side to cells with low or even undetectable levels of substrate label. Venules for both WKY rats and SHRs revealed an increased MMP-9 receptor density when compared with arterioles of similar diameter. The majority of mast cells exhibited enhanced levels of MMP-9 enzyme activity compared with fibroblast or mesothelial cells in the mesentery. The average peptide substrate density, as determined by digital light intensity measurements over randomly selected mesenteric microvessels (arterioles, capillaries, and venules), was higher in the SHRs for the MMP-9 enzyme (Figure 3B). Avascular tissue areas of the mesentery sheet in SHRs showed enhanced MMP (MMP-1 and -9) enzyme activity.

Chronic doxycycline treatment led to a significant reduction of the MMP activity in endothelial cells and fibroblasts (Figure 3) in both rat strains. The intensities in SHRs reached levels that were the same as in control WKY rats.

Application of the metal chelators (EDTA, 5 mmol/L; hydroxamic acid, GM6009, Sigma, 5 mmol/L) reduced the fluorescent intensity generated by the fluorogenic substrate to

<table>
<thead>
<tr>
<th>Blood Glucose Indices</th>
<th>Control Groups</th>
<th>Doxycycline Groups</th>
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<tbody>
<tr>
<td></td>
<td>WKY (n=5)</td>
<td>SHR (n=5)</td>
</tr>
<tr>
<td>Glucose level, mg/mL</td>
<td>91.5±12.8</td>
<td>117.8±0.4*</td>
</tr>
<tr>
<td>Glycated hemoglobin,</td>
<td>4.1±0.3</td>
<td>5.7±0.4*</td>
</tr>
<tr>
<td>%</td>
<td>87±5.1</td>
<td>77.4±14.2†</td>
</tr>
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*P<0.05 WKY control vs SHR control and WKY doxycycline vs SHR doxycycline.
†P<0.05 WKY control vs WKY doxycycline and SHR control vs SHR doxycycline.

**Figure 1.** A, MMP-9 levels as detected by labeling with primary antibody and Vector NovaRED substrate in selected circulating leukocytes of SHRs and WKY rats before (left) and after treatment (right) with doxycycline. B, Light absorption (see Methods section) because of MMP 9 labeling over individual leukocytes in control (C) and doxycycline-treated rats (D). Mean±SD for each group is derived from the means of 30 cells per rat and 3 rats per group. *P<0.05 vs WKY rat; †P<0.05 vs WKY rat without doxycycline treatment (control); ††P<0.05 vs SHR without doxycycline.
values that were undetectable with the current imaging system (results not shown). This observation confirms that MMPs are predominantly responsible for the substrate cleavage.

**Plasma Protease Activity**

The SHR had a significantly elevated plasma protease activity (Figure 4), as detected by the fluorogenic substrate (casein substrate), that was dramatically reduced by doxycycline treatment. The plasma protease activity in the WKY rats was also reduced after chronic MMP inhibition. The addition of EDTA to the plasma sample significantly reduced the plasma protease activity on average by ~29%, indicating that, in contrast to the mesentery microvessels, the plasma protease activity was only in part attributable to MMP activity.

**Membrane Receptor Cleavage in the SHR**

Immunolabeling of the extracellular domain of the insulin receptor-α on the membrane of fresh leukocytes and mesenteric interstitial cells showed reduced levels in the SHR (Figure 5A and 5C). There is also considerable cleavage of the receptor in the WKY rats. The average density of the
insulin receptor label on leukocytes was significantly enhanced after doxycycline treatment in both WKY rats and SHRs (Figure 5C).

Furthermore, a 30-minute exposure of naive Wistar leukocytes to SHR and, to a lesser degree, also WKY rat plasma leads to a significant reduction of the density of extracellular binding sites of insulin receptor-α (Figure 6A). Doxycycline treatment reversed this trend (Figure 6B) hand in hand with a normalization of the glucose and glycohemoglobin levels (Table 1).

Cleavage of the insulin receptor also caused reduced glucose transport as detected by intracellular accumulation of a fluorescent glucose analog. The intracellular fluorescence intensity in naive Wistar leukocytes was reduced from a control value in fresh Wistar plasma (13.1±9.5 digital units) to ≈39% after 30 minutes of incubation in fresh SHR plasma (5.2±3.2 digital units; P<0.0001) and to ≈87% in WKY plasma (11.4±7.2 digital units; P<0.001; Figure S1).

Furthermore, exposure of naive leukocytes from normotensive Wistar rats to plasma from WKY rats or SHRs caused a significant reduction of the extracellular domain of CD18 by ≈25% to 35% (Figure 7). This evidence indicated that plasma from the SHR and also from the WKY rat has the ability to cleave the extracellular domain of membrane receptors to a degree that exceeds the activity in plasma from their normotensive Wistar controls.

Oxygen Free Radical Formation in Rat Mesentery Microcirculation

The elevated free radical production in the mesentery microcirculation of the SHR, as detected by NBT labeling (Figure 8A), was reduced by blockade of MMPs. This reduction was observed not only in arterioles but was even more notable in low-pressure venules and in interstitial cells. Doxycycline treatment also served to reduce the zymogen deposits in the normotensive WKY rats, a feature that is confirmed by quantitative light absorption measurements (Figure 8B).

NF-κB Levels

The transcription factor NF-κB was distributed throughout the cytoplasm and nucleus of SHR leukocytes (Figure 9A). Its average level was elevated in control SHRs, an effect that was significantly reduced by chronic MMP inhibition (Figure 9B).

Discussion

These results indicate that SHRs have elevated protease activities in plasma (Figure 4) and in microvascular endothelium (MMP-1 and -9; Figures 1 and 3) that are associated with...
with reduced density of extracellular binding domains of the insulin receptor and CD18 membrane adhesion molecule (Figures 5 and 8). Chronic blockade of the protease activity in SHRs with a broad-acting MMP inhibitor reduces blood pressure, normalizes the elevated blood cell counts (Table 1) and plasma protease activity (Figure 4), reduces MMP protein and activity levels (Figures 1 to 3), and reduces receptor cleavage caused by SHR plasma on naive cells (Figures 6 and 7). Furthermore, the MMP inhibition reduces the enhanced oxidative stress levels in SHR microvessels (Figure 8) and the level of NF-κB translocation (Figure 9). Protease activity may constitute a pathogenic mechanism in SHRs that is associated with its insulin resistance4,19 and suppression of leukocyte adhesion to the endothelium.6,20

**Microvascular MMP Activity**

The study demonstrates the use of fluorogenic substrates for detection of matrix metalloproteinase (eg, MMP-1 and -9) activity with intravital microscopy as an important aspect of the inflammation in the SHR microcirculation (Figure 3). The fact that the metal chelation suppresses the emitted light after cleavage of the fluorescent substrate confirms the central role of MMPs in the proteolytic activity of the SHR. The substrate does not distinguish between MMP-1 and MMP-9 activity and, therefore, was supplemented with immunolabeling to confirm enhanced levels of MMP protein in a microvascular pattern that coincides with the protease activity (Figures 2A and 3A).

There is a remarkable colocalization between MMP protein and activity levels and NBT reduction by oxygen free radicals (Figure 8A). Both are dominant in microvascular endothelium on the arteriolar and venular side of the SHR. Other than endothelium and leukocytes, we also saw in tissue fibroblast MMP-9 protease (Figure 2A) that is colocalized with protease activity (Figure 3A). The colocalization is in line with the observation that either inhibition of MMP activity in this study or superoxide scavenging2 alleviates many symptoms in SHRs. Enzyme activity is also observed to a lower degree in the low blood pressure WKY rat, a strain that has elevated arterial pressure compared with the Wistar strain from which it originated.

**Pathophysiological Aspects of MMP Activity in SHRs**

MMPs exert diverse pathophysiological effects, eg, proteolytic remodeling of the extracellular matrix or breakdown of connective tissue. MMP activity has also been observed in complications that accompany the hypertensive state. For example, in the left ventricle with age there is an increase in MMP-1 mRNA, whereas at the same time there is a decrease
in the WKY rat. Activation of MMPs leads to decreased cardiac tissue tensile strength. Left ventricular volume and MMP-2 activity in obese male spontaneously hypertensive heart failure rats are enhanced. After cerebral ischemia, SHRs, compared with control brain, have an acute increase in type IV collagenase (MMP-9) that is maintained over several days, whereas gelatinase A (MMP-2) is elevated only after several days. In situ zymography of the stroke-prone SHR brain shows an increase in plasminogen activator/plasmin activity that colocalizes with cerebral damage. Concomitant MMP-2 activation is observed in damaged brain areas. In human hypertensive subjects, the plasma MMP activity has been correlated with enhanced systolic pressure.

Other MMPs may be upregulated in the SHR, together with downregulation of tissue inhibitors of MMPs. These need to be explored in the future.

What Mechanism may Induce MMP Activity in the SHR?

It may be argued that an elevated blood pressure may induce MMP expression. Indeed, application of elevated blood pressure and stretch of endothelial cells can lead to MMP expression. Mechanical stress on vascular smooth muscle cells enhances the message for MMP-2 and proenzyme release via an reduced nicotinamide-adenine dinucleotide phosphate oxidase pathway, an enzyme that is also enhanced in the wall of the same mesentery microvessels in which we see the MMP activity in the current study. However, the high expression of MMPs in SHRs is prominent not only in arterioles but also in venules (Figure 2), with blood pressures that are indistinguishable between WKY rats and SHRs and even in circulating leukocytes that pass both high- and low-pressure regions of the circulation. The lack of a unique arteriolar localization of indicators for organ injury is observed also with markers for apoptosis and oxidative enzymes. This collective evidence suggests that the MMPs in SHRs may be induced through a mechanism other than only the elevated blood pressure, eg, involving oxygen free radicals and NF-κB translocation.

The colocalization between the MMP protein levels and the NBT reduction (Figures 3 and 8) supports the possibility that the MMP expression may be associated with the oxidative stress in the same endothelium and NF-κB translocation (Figure 9). The elevated oxidative stress in the SHR versus WKY endothelium is also present in SHR endothelial cultures (unpublished results) under identical fluid pressures and shear stresses, supporting the hypothesis that it is of genetic origin. Enhanced levels of superoxide, hydrogen peroxide, and peroxynitrite formation modulate and activate MMP-2 and -9 activity and inactivate tissue inhibitors of MMPs.

MMP Inhibition

Other than its antimicrobial activity, doxycycline inhibits a variety of MMPs, including MMP-1, -2, -8, and -9, and blocks serpinolytic activity (degradation of α-1-antitrypsin) and plasmin. It has high affinity for a bivalent ion. It inhibits in endothelial cells the induction of MMP-8 and -9 and or in epithelial cell induction of MMP-9 after transforming growth factor stimulation. Although it is evident that doxycycline blocked MMP-1 and -9 (Figures 1 to 3), other actions, like tissue inhibitor of MMP regulation, need to be explored.

Receptor Cleavage by Protease Activity

The current results provide evidence for proteolytic activity in SHR plasma and on endothelial cells (Figures 3 and 4). Although the specific protease(s) remain to be determined, the enzymatic activity may be associated with receptor cleavage. Membrane CD18 expression levels, which are reduced on SHR neutrophils, can be cleaved by its plasma
The SHR has reduced P-selectin expression levels on postcapillary venules, a situation that leads to a reduced rolling interaction between leukocytes and the endothelium on these postcapillary venules. The presence of elevated soluble P-selectin levels in hypertensives is also in line with receptor cleavage.

Furthermore, in SHRs we see cleavage of insulin receptor that is attenuated by chronic blockade of the MMP activity. Insulin receptor cleavage can be caused by enzymatic activity in the SHR plasma. The loss of receptor sites is accompanied by reduced glucose transport and, thus, may be a cause of insulin resistance in the SHR, including the increase in blood glucose levels and glycohemoglobin levels. MMP inhibition blocks the insulin receptor cleavage, normalizes blood glucose, and reduces glycohemoglobin levels. The receptor cleavage hypothesis is also in line with the observation that glycation of proteins in diabetic rats is reduced by doxycycline and by nonantimicrobial chemically modified tetracycline derivatives. Inhibition of MMP reduces tumor necrosis factor- levels, which are associated with insulin resistance. Tumor necrosis factor- is synthesized as a membrane-bound precursor that is proteolytically processed by an MMP-like enzyme to its active form.

Thus, protease activity in hypertension may not only affect suspended proteins but also lead to cleavage of membrane receptors. Unchecked enzymatic activity may affect also other membrane receptors, thus forming one of the reasons for the diversity of microvascular dysfunctions encountered in the SHR.

**Perspectives**

The SHR has enhanced MMP-9 protein levels and MMP-1 and -9 activity in the microcirculation and enhanced plasma protease activity, as well as cleavage of the extracellular domain of CD18 and the insulin receptor. The results point the first time to a mechanism that may explain the multiple and diverse cell dysfunctions encountered in SHRs, such as insulin resistance and leukocyte adhesion deficiency.

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**Disclosures**

None.

**References**

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