Matrix Metalloproteinase-2 Proteolysis of Calponin-1 Contributes to Vascular Hypocontractility in Endotoxemic Rats

Michele M. Castro, Jonathan Cena, Woo Jung Cho, Michael P. Walsh, Richard Schulz

Objective—Matrix metalloproteinase (MMP)-2 is activated in aorta during endotoxemia and plays a role in the hypocontractility to vasoconstrictors. Calponin-1 is a regulator of vascular smooth muscle tone with similarities to troponin, a cardiac myocyte protein that is cleaved by MMP-2 in myocardial oxidative stress injuries. We hypothesized that calponin-1 may be proteolyzed by MMP-2 in endotoxemia-induced vascular hypocontractility.

Methods and Results—Rats were given a nonlethal dose of bacterial lipopolysaccharide (LPS) or vehicle. Some rats were given the MMP inhibitors ONO-4817 or doxycycline. Six hours later, plasma nitrate+nitrite increased >15-fold in LPS-treated rats, an effect unchanged by doxycycline. Both ONO-4817 and doxycycline prevented LPS-induced aortic hypocontractility to phenylephrine. LPS activated MMP-2 in the aorta by S-glutathiolation. Calponin-1 levels decreased by 25% in endotoxemic aortae, which was prevented by doxycycline. Calponin-1 and MMP-2 communoprecipitated and both exhibited uniform cytosolic staining in medial vascular smooth muscle cells. In vitro incubation of calponin-1 with MMP-2 led to calponin-1 degradation and appearance of its cleavage product.

Conclusion—Calponin-1 is a target of MMP-2, which contributes to endotoxemia-induced vascular hypocontractility. (Arterioscler Thromb Vasc Biol. 2012;32:662-668.)

Key Words: metalloproteinases ■ calponin-1 ■ endotoxemia ■ lipopolysaccharide ■ vascular hypocontractility

Sepsis remains one of the most common causes of death worldwide.1 Its cardiovascular manifestations include myocardial dysfunction and severe arterial hypotension caused in part by vascular hyporeactivity to vasoconstrictors.2 Several mechanisms3 including lipopolysaccharide (LPS)-and interleukin-1β-mediated activation of inducible nitric oxide (NO) synthase, excess biosynthesis of NO,4,5 and peroxynitrite (ONO•−)6,7 in the vascular wall are important mediators of the vascular dysfunction in sepsis. ONO•− directly activates matrix metalloproteinases (MMPs),8,9 a group of zinc-dependent endopeptidases best known for their ability to degrade extracellular matrix proteins, causing vascular dysfunction and remodeling in many cardiovascular diseases.10-16 MMPs are synthesized as inactive zymogens in several cells, including vascular smooth muscle that express 72 kDa MMP-2 abundantly.10 It is activated by proteolytic removal of the autoinhibitory propeptide domain10 or, alternatively, by S-glutathiolation of a critical cysteine in this propeptide, on reaction with ONO•− and glutathione, resulting in 72 kDa S-glutathiolated MMP-2.9 However, whether MMP-2 activation occurs by S-glutathiolation in the vasculature is unknown.

Studies have implicated MMPs and the beneficial effects of MMP inhibition in experimental models of sepsis.17-23 MMP-2 plays an important role in LPS-induced vascular hypocontractility in rats. In vivo treatment with the MMP inhibitor doxycycline attenuated LPS-induced increase in vascular MMP-2 activity and protected against the loss of vascular contractile tone.18 Doxycycline or another MMP inhibitor GM6001 also improved LPS-induced hypocrossility to phenylephrine in isolated aorta.17 The mechanism by which MMPs contribute to this vascular hypocontractility remains unknown.

MMP-2 in particular is also an ubiquitous, intracellular protease that cleaves specific proteins inside cardiac myocytes to thereby reduce contractile function.24,25 MMP-2 activation in cardiac myocytes by oxidative stress results in the cleavage of targets to which it colocalizes including troponin I,26 myosin light chain-1,27 α-actinin,28 and titin,29 proteins essential for cardiac muscle contraction. MMP inhibitors improved contractile function in ischemic-reperfused rat hearts and prevented the proteolytic loss of these proteins.26,27,29 These intracellular effects of MMP-2 in the heart under oxidative stress suggest that MMP-2 may also have...
in intracellular targets in diseases such as sepsis associated with oxidative stress.

Calponin-1 is a 34 kDa actin-binding protein with regions of sequence homology to cardiac troponin I and T.\textsuperscript{30–32} It is located in the cytoskeleton and contractile apparatus of differentiated smooth muscle cells.\textsuperscript{33} There are 3 isoforms of calponin: calponin-1 (h1 or basic), calponin-2 (h2 or neutral), and calponin-3 (h3 or acidic). Calponin-1 is implicated in the regulation of smooth muscle contraction by mediating intracellular signaling responses to some vasoconstrictors by acting as a contractile scaffold protein.\textsuperscript{32,34} This mechanism is based on results in aortic smooth muscle cells stimulated with phenylephrine, which showed calponin-1 connecting protein kinase C and ERK1/2 pathways to promote contractility. Although calponin-1 also inhibits the actin-activated myosin MgATPase, this action is abrogated by protein kinase C-catalyzed phosphorylation of calponin-1\textsuperscript{35,36} which may occur in sepsis. Calponin-1 knockout mice exhibit both reduced vasoconstriction and a diminished rise in mean arterial pressure in response to \( \alpha \)-adrenergic agonists.\textsuperscript{37} Thus, in theory, cleavage of calponin-1 would result in a decreased vascular contractile response. Although lower calponin-1 levels have been reported in sepsis-induced vascular hypocontractility. Here we hypothesized that calponin-1 may be colocalized with and degraded by MMP-2 in the vasculature to contribute to endotoxia-induced vascular hypocontractility.

### Methods

#### Rat Endotoxemia

Male Sprague Dawley rats (250–300 g) were injected with either a nonlethal dose of LPS (Salmonella typhosa, 4 mg/kg IP; Sigma) or pyrogen-free water vehicle (control; Sigma). Some rats were given the MMP inhibitors ONO-4817 (100 mg/kg by gavage, diluted in 0.5% carboxymethylcellulose) 2 hours prior to LPS or doxycycline (4 mg/kg IP, Sigma) 30 minutes after LPS.\textsuperscript{18} Doxycycline preferentially inhibits MMP-2, -9, and -8 activities and is a much weaker inhibitor of MMP-1.\textsuperscript{39,40} ONO-4817 is a more selective MMP inhibitor with a \( K_d \) in the nanomolar range for MMP-2 and MMP-9 and almost no inhibitory activity up to 100 \( \mu \)mol/L against several other proteases.\textsuperscript{41} Six hours after LPS rats were euthanized using an overdose of sodium pentobarbital (100 mg/kg, IP). See details in online-only Data Supplement. This study was approved and performed according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

#### Determination of Nitrate and Nitrite (\( \text{NO}_x^- \)) Concentrations in Plasma

Plasma samples were analyzed for total \( \text{NO}_x^- \) levels using the Nitrate and Nitrite Colorimetric Assay kit (Cayman Chemical). For details, see online-only Data Supplement.

#### Assessment of Vascular Hypocontractility

Aortic rings 5 mm in length were mounted in organ baths for isometric tension recording. Rings were exposed to increasing concentrations of phenylephrine (10\textsuperscript{-9} to 10\textsuperscript{-5} mol/L) as previously described.\textsuperscript{18}

### Table. Plasma \( \text{NO}_x^- \) Concentration 6 Hours Following LPS Administration

<table>
<thead>
<tr>
<th>Groups</th>
<th>( \text{NO}_x^- ) Concentration, ( \mu )mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.1±1.4</td>
</tr>
<tr>
<td>DOXY</td>
<td>8.2±3.1</td>
</tr>
<tr>
<td>LPS</td>
<td>164.6±43.4*</td>
</tr>
<tr>
<td>LPS+DOXY</td>
<td>141.1±63.0*</td>
</tr>
</tbody>
</table>

\*P<0.001 vs control and doxy groups.

**N=12–15 rats per group.**

LPS, lipopolysaccharide. DOXY, doxycycline.

#### Western Blot Analysis, Coimmunoprecipitation Studies, and MMP-2 Activation

Calponin-1 protein levels were examined in aortic homogenates by Western blot.\textsuperscript{18} To investigate whether LPS induces MMP-2 activation by S-glutathiolation, aortic homogenates were incubated without or with dithiothreitol (0.1, 0.3 or 1.0 mmol/L) and then communo-precipitation analysis was performed with specific antibodies against glutathione and MMP-2 (online-only Data Supplement). Coimmunoprecipitation studies of MMP-2 with calponin-1 in aorta were also performed. For details, see online-only Data Supplement.

#### Immunohistochemistry and Confocal Microscopy

Aortae were fixed with 4% paraformaldehyde for 4 hours at room temperature and then cryoprotected in 30% sucrose in 0.1 mol/L sodium phosphate buffer overnight at 4°C. After that, cryoprotected aortae were vertically embedded in Tissue-Tek OCT compound and then frozen and serially cryosectioned (5-\( \mu \)m sections). Double-immunofluorescence with primary antibodies from different host species was performed.\textsuperscript{42} For details, see online-only Data Supplement. Calponin-1 levels in aortic cryosections were obtained by quantification of fluorescence intensity/area using ImageJ software.

#### In Silico Analysis, In Vitro Degradation Assay, and Mass Spectrometry

Putative cleavage site motifs for MMP-2 (PVS, LRS, PEA, LRG, PEG, LRV, PAV, MTS and LAA, ITA, as shown by the arrows) were identified within the primary sequence of human calponin-1 using the SIM Alignment tool for protein sequences. To test whether calponin-1 is susceptible to proteolysis by MMP-2 in vitro, purified human recombinant calponin-1 was incubated with human recombinant MMP-2 at 37°C in 50 mmol/L Tris-HCl buffer. To verify that the resulting degradation products were in fact derived from calponin-1, they were digested with trypsin and analyzed by LC-MS/MS using a Thermo LTQ Orbitrap XL mass spectrometer and the MASCOT database to identify the protein (www.matrixscience.com). For details, see online-only Data Supplement.

#### Statistical Analysis

Results are expressed as means ± SEM. Between-groups comparisons were assessed using either Student's t-test, 2 or one-way ANOVA followed by Tukey or Dunnett's posthoc tests, as appropriate. Pearson correlations and linear regression were calculated in all groups comparing aortic calponin-1 levels with plasma \( \text{NO}_x^- \) (GraphPad Prism 5.01 software). \( P<0.05 \) was considered significant.

#### Results

**Signs of Endotoxemia and Plasma \( \text{NO}_x^- \) levels**

Six hours after LPS administration, rats developed marked symptoms of endotoxemia including lethargic behavior, piloerection, diarrhea, and porphyrin secretion from the snout and eyes. Treatment with either ONO-4817 or doxycycline did not ameliorate these symptoms. To further test whether rats were endotoxemic, plasma \( \text{NO}_x^- \) was measured. Table
LPS-induced loss of calponin-1 (0.05, Figure 3A). The activity (0.01, Figure 1) whereas doxycycline did not reduce the LPS-induced increase in NOx versus control, Figure 2). Although doxycycline did not significantly prevent LPS-induced hypocontractility to vasoconstrictors, the contractility of isolated rat aortic rings to phenylephrine was assessed. Aortae from LPS rats contracted less well to phenylephrine (P<0.01 versus control) whereas either ONO-4817 or doxycycline significantly prevented LPS-induced hypocontractility (P<0.01, Figure 1).

**MMP Inhibition Prevented LPS-Induced Aortic Hypocontractility to Phenylephrine**

To test whether MMP inhibition protects against LPS-induced vasocostriction, the contractility of isolated rat aortic rings to phenylephrine was assessed. Aortae from LPS rats contracted less well to phenylephrine (P<0.01 versus control) whereas either ONO-4817 or doxycycline significantly prevented LPS-induced hypocontractility (P<0.01, Figure 1).

**LPS Enhanced MMP-2 S-Glutathiolation in Aorta**

As a means to determine the mechanism of activation of MMP-2 in aorta, immunoblot analysis for MMP-2 of antiguacthionine, but not control IgG1 immunoprecipitates, showed 30% higher levels of S-glutathiolated 72 kDa MMP-2 in LPS aorta compared to control (P<0.05; Figure 2). As expected for S-glutathiolation reactions of proteins, MMP-2 S-glutathiolation was reversed by dithiothreitol in a concentration-dependent manner. These results indicate that LPS enhances MMP-2 S-glutathiolation in aorta.

**MMP Inhibition Prevented LPS-Induced Loss of Calponin-1 in Aorta**

We determined whether MMP-mediated aortic hypocontractility in endotoxemia is related to calponin-1 proteolysis. Examination of LPS-treated rat aortae by Western blot revealed a 25% loss of 34 kDa calponin-1 protein (P<0.05 versus control, Figure 3A). Although doxycycline did not alter the level of calponin-1 in control aortae, it prevented LPS-induced loss of calponin-1 (P<0.05, Figure 3A). The extent of calponin-1 loss in aortae among the LPS-treated rats was variable; however the aortic level of calponin-1 correlated inversely with plasma NOx in this group (Figure 3B), whereas no correlation existed between calponin-1 and NOx levels in control, doxy or LPS+doxy groups (Figure 3B).

We also assessed calponin-1 levels in aortae by immunofluorescence. Six hours after LPS, calponin-1 staining was significantly less throughout the aortic medial smooth muscle when compared with controls (P<0.05, Figure 3C). Doxycycline partially prevented LPS-induced loss of aortic calponin-1, which was associated with its better preservation in luminal smooth muscle.

**Association of MMP-2 with Calponin-1 in Aorta**

To test the possible association between MMP-2 and calponin-1 in aorta, we first performed coimmunoprecipitation studies using calponin-1 antibody followed by Western blot for MMP-2. Immunoblot analysis for MMP-2 of anticalponin-1, but not IgG immunoprecipitates, showed the presence of 72 kDa MMP-2, suggesting a strong association between these proteins (Figure 4A). Double-immunofluorescence confocal microscopy was also performed to examine this. Figure 4B shows that both proteins exhibited uniform cytosolic staining in vascular smooth muscle cells in the aortic wall and colocalized to each other in this compartment, strengthening the hypothesis that MMP-2 may proteolyse calponin-1 in aortic smooth muscle.

**MMP-2 Cleaves Calponin-1 In Vitro**

In silico mapping of putative MMP-2 cleavage sites within the amino acid sequence of human calponin-1 revealed sites in the troponin I-like/actin-binding region (Pro 154–Glu 159) and in the second calponin repeat within the C-terminal domain (Gln 216–Ala 221). This search was restricted to
calponin-1 sites with at least 50% identity to 6 amino acids of different putative MMP-2 cleavage motifs (Figure 5A).

We also tested using either Coomassie Blue stained gels or Western blotting whether calponin-1 is susceptible to proteolysis by MMP-2 in vitro. Incubation of calponin-1 with human recombinant MMP-2 (100:1 molar ratio, 37°C) led to the complete loss of the 34 kDa calponin-1 band and the concomitant appearance of a 15 kDa degradation product at 2 hours. Inhibition of MMP activity with GM6001 significantly prevented calponin-1 degradation (Figure 5B). Troponin I was used as a positive control because it is effectively degraded by MMP-2 in vitro.26 We found similar results when Western blot for calponin-1 was performed after its incubation with MMP-2 at 100:1 molar ratio for 2 hours (data not shown). Densitometric analysis showed the disappearance of the 34 kDa calponin-1 band and the simultaneous appearance of a 15 kDa degradation product at both 300:1 and 100:1 molar ratios (Figure 5C). Mass spectrometric analysis of the excised and trypsin-digested band of 15 kDa identified it as a mixture of 2 calponin-1 fragments because 17 peptides with coverage of 67% of the entire protein were identified with a MASCOT score of 2857 (see Table, in the online-only Data Supplement).

**Discussion**

MMP-2 is activated in the vascular wall during oxidative stress induced by endotoxemia and plays an important role in the resultant hypotcontractility to vasoconstrictors.17,18 We evaluated here a potential mechanism by which MMP-2 contributes to this. We found that LPS treatment activates aortic MMP-2 by S-glutathiolation and that MMP-2 colocalizes with and degrades calponin-1 to contribute to endotoxemia-induced vascular hypocontractility, as this was reduced in rats given 2 chemically distinct MMP inhibitors, ONO-4817 or doxycycline. To our knowledge this is the first study showing an intracellular action of MMP-2 in vascular smooth muscle.

MMP-2 is recognized for its ability to degrade extra- and intracellular proteins and this effect contributes to cardiovascular...
In silico analysis

Human calponin-1
Putative MMP-2 cleavage sites

Human calponin-1
Putative MMP-2 cleavage sites

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A

**Figure 5. A.** The amino acid sequence of human calponin-1 was compared with a variety of 6 amino acid matrix metalloproteinase (MMP)-2 cleavage motifs. The sequence 154 to 159, which lies within the troponin I-like/actin-binding region of calponin-1, exhibits 50% identity to 3 different MMP-2 recognition sequences. The 216 to 221 sequence within the second calponin repeat domain in the C-terminal region of calponin-1 shows 50% identity to 2 different MMP-2 cleavage motifs. Underlined letters indicate identical amino acids. B. Representative gel stained with Coomassie Blue showing troponin I and calponin-1 degradation by MMP-2. Purified human calponin-1 was incubated for 2 hours with human recombinant MMP-2 at 100:1 molar ratio at 37°C in the absence or presence of GM6001. The ~15 kDa band was identified by mass spectrometry to be a calponin-1 fragment. Troponin I was used as a positive control for MMP-2 degradation. C. Densitometric analysis showing the disappearance of the 34 kDa calponin-1 band and appearance of a 15 kDa degradation product at both 300:1 and 100:1 molar ratios. n=3 per group.*P<0.01 versus control.

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dysfunction and remodeling in many diseases. A significant involvement of MMPs and the beneficial effects of their inhibition have been shown in experimental models of sepsis although proteolytic targets have not yet been identified. MMP-2 plays an important role in LPS-induced aortic hypocontractility in vivo and in vitro as its effects were completely prevented with either doxycycline or GM6001. Because doxycycline and GM6001 inhibit several MMPs, we here also tested ONO-4817 (Ki in the nanomolar range for MMP-2 and -9 and almost no inhibitory activity up to 100 μmol/L against several other proteases) as a further pharmacological control. ONO-4817 was also able to prevent LPS-induced aortic hypocontractility similar to the effect of doxycycline. Studies suggested that high concentrations of tetracyclines may decrease inducible NO synthase protein expression via destabilization of its mRNA resulting in a decrease of NO production. Here and consistent with previous work, inductible NO synthase is upregulated in LPS-treated rats as we observed increased plasma NOX levels. However, doxycycline did not reduce this elevation of NOX. This indicates that the protective effect of MMP inhibition by doxycycline in our model of endotoxemia is independent of action on inducible NO synthase.

MMP-2 is localized within cardiac myocytes to specific subcellular organelles including the sarcomere, where it colocalizes with proteins of the contractile machinery including troponin I. MMP-2 degrades these proteins in cardiac muscle undergoing enhanced oxidative stress to cause impaired contractile function. MMP inhibitors improved mechanical function of hearts subjected to ischemia and reperfusion injury and prevented the proteolytic loss of these MMP-2 target proteins. Based on these earlier studies in cardiac muscle, and because endotoxemia is also associated with enhanced oxidative stress and aortic hypocontractility, we investigated whether a troponin-like protein, calponin-1, is an intracellular target of MMP-2 in vascular smooth muscle during endotoxemia.

Calponin-1 is an actin-binding protein located in the cytoskeleton and contractile apparatus of differentiated smooth muscle cells. A strong association between MMP-2 and calponin-1 in vascular smooth muscle was demonstrated by both coimmunoprecipitation and confocal microscopy studies (Figure 4A and 4B). We showed using either Western blot or immunofluorescence that 6 hours of LPS treatment reduced calponin-1 levels in aorta, an effect that was abrogated by doxycycline (Figure 3A and 3C). The 3 known calponin isoforms contain a highly conserved N-terminal portion (which includes the calponin homology domain), a troponin I-like/actin-binding domain and 3 C-terminal repeats that differ among the 3 isoforms. Calponin-1 regulates smooth muscle contraction either by mediating intracellular signaling of vasoconstrictors by acting as a contractile scaffold protein, or by inhibiting actin-myosin cross-bridge cycling, which is alleviated by protein kinase C-catalyzed phosphorylation of calponin-1. The scaffold mechanism is based on results in aortic smooth muscle cells stimulated with phenylephrine, which showed calponin-1 as an adaptor protein connecting protein kinase C and ERK1/2 path-
ways to promote contractility.\textsuperscript{32,34} Mice containing a truncated form of the C-terminal of calponin-1, lacking the troponin I-like domain and the 3 C-terminal repeats, presented with both reduced vasoconstriction and a reduced vasopressor response to \(\alpha\)-adrenergic agonists.\textsuperscript{32,37}

MMP-2 activation is essential for its proteolytic effect both inside and outside the cell. MMP-2 activity is regulated at multiple levels including gene transcription, posttranslational modifications, and by interaction with its endogenous tissue inhibitors.\textsuperscript{24} In addition to its activation by proteolysis, 72 kDa MMP-2 cleaves susceptible contractile proteins to which it localizes such as calponin-1 to contribute to LPS-induced aortic hypocontractility. The red disc represents the catalytic site of MMP-2 containing Zn\textsuperscript{2+}.}

pressure, future studies should also be carried out in resistance arteries.

We showed that MMP-2 cleaves calponin-1 in vitro resulting in a 15 kDa cleavage product which was prevented by an MMP inhibitor. By mass spectrometry we showed that this 15 kDa band contains 2 fragments of MMP-2–degraded calponin-1 (see Table in the online-only Data Supplement). \textit{In silico} analysis of putative MMP-2 cleavage sites in human calponin-1 showed at least 2 sites with 50\% identity in its troponin I-like/actin-binding (Pro 154–Glu 159) and C-terminal (Gln 216–Ala 221) domains. Cleavage at Lys 156 would generate 2 fragments of similar size: an N-terminal fragment of 17,704 Da and a C-terminal fragment of 15,484 Da, consistent with the mass spectrometric data. The C-terminal portion of calponin-1 seems to be important for its action on vascular smooth muscle contraction. We used the monoclonal rabbit calponin-1 (C-terminus) antibody to detect calponin-1 and observed, whether by Western blotting (Figure 3A) or by confocal microscopy (Figure 3C), less of it in aortae of LPS-treated rats which was prevented by doxycycline. Similar results were obtained if either mouse monoclonal calponin-1 (full-length) or rabbit polyclonal calponin-1 (N-terminus) antibodies were used in the Western blot experiments (data not shown).

In conclusion, MMP-2 and calponin-1 are associated together in aortic vascular smooth muscle cells. MMP-2 is activated in vascular smooth muscle during endotoxemia by S-glutathiolation, which then proteolyses calponin-1 to contribute to LPS-induced hypocontractility, an effect abolished by MMP inhibitors. The intracellular actions of MMP-2 in vascular smooth muscle in health and disease, its other potential proteolytic targets, and the possibility of inhibitors specifically targeting MMP-2 as therapeutic agents for septic shock will be important future investigations.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

DETAILED METHODS

This study was approved and performed according to the Canadian Council on Animal Care *Guide to the Care and Use of Experimental Animals*. Male Sprague Dawley rats (250 - 300 g) were used in all experiments.

**Rat endotoxemia**

Male Sprague-Dawley rats (250 – 300 g) were given either an intraperitoneal injection of a non-lethal dose of lipopolysaccharide (LPS, *Salmonella typhosa*, 4 mg/kg i.p.; Sigma; n = 15) or pyrogen-free water vehicle (Control; Sigma; n = 15). Two hours before and 30 min after LPS administration, some rats were given the MMP inhibitors ONO-4817 (100 mg/kg by gavage, diluted in 0.5% carboxymethylcellulose; n = 4)\(^1\).\(^2\) or doxycycline (4 mg/kg i.p., Sigma; n = 12)\(^3\). Doxycycline preferentially inhibits MMP-2, -9 and -8 activities and is a much weaker inhibitor of MMP-1\(^4\),\(^5\). ONO-4817 is a more selective MMP inhibitor with a \(K_i\) in the nanomolar range for MMP-2 and MMP-9 and almost no inhibitory activity up to 100 µmol/L against several other proteases\(^6\). Six hours after LPS administration, rats were euthanized using an overdose of sodium pentobarbital (100 mg/kg, i.p.). We and others have shown that at this time point NO production is increased and blood pressure, cardiac function and vascular reactivity to phenylephrine are significantly depressed\(^3\),\(^7\)-\(^9\). The aortae were collected, cleaned from fat and connective tissue, divided into three portions and then consistently distributed for each experiment. One-third was used for functional studies. Another third was fixed in 4% paraformaldehyde in sodium phosphate buffer for immunohistochemistry studies and the remaining third was flash frozen in liquid nitrogen and then stored at -80°C for biochemical analyses. Blood was also collected at this time from the vena cava using sodium citrate as the anticoagulant. Plasma was obtained following centrifugation (6,500 g for 5 min, 4°C) to measure nitrate and nitrite (\(\text{NO}_x\)) levels (a marker of NO biosynthesis).
Determination of NO$_x$ concentrations in plasma

Plasma samples were filtered through a 10 kDa molecular weight cut-off filter (Amicon Ultra-0.5 centrifugal filter unit with ultracel-10 membrane, Millipore) and then ultrafiltrates (diluted 1:5 in distilled water) were analyzed for total NO$_x$ levels using the Nitrate and Nitrite Colorimetric Assay kit (Cayman Chemical) according to the manufacturer's instructions.

Assessment of vascular hypocontractility

Aortic rings 5 mm in length were cut and mounted for isometric tension recording in organ baths. The rings were placed in bath chambers filled with Krebs-Henseleit buffer (20 mL) with the following composition (mmol/L): NaCl 111.4, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ · 7H$_2$O 1.2, CaCl$_2$ · 2H$_2$O 2.5, D-glucose 11.1 and NaHCO$_3$ 25. This solution was maintained at 37°C, pH 7.4, and bubbled with 95% O$_2$ and 5% CO$_2$. Isometric tension was measured using force transducers (Grass FT03) and recorded using AcqKnowledge 3.1 software. The rings were subjected to a tension of 1 g for a 60 min equilibration period with the Krebs-Henseleit buffer being replaced at 20 min intervals. To assess aortic contractility, rings were exposed to increasing concentrations of phenylephrine (10$^{-9}$ to 10$^{-5}$ mol/L) and the resulting changes in tension were recorded.

Measurement of calponin-1 levels in aortae by western blot analysis

Aortic sample preparation

Frozen aortae were crushed at liquid nitrogen temperature, placed in homogenization buffer [50 mmol/L Tris-HCl, 3.1 mmol/L sucrose, 1 mmol/L dithiothreitol (DTT), 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, protease inhibitor cocktail (1:1000 v/v; Sigma) and 0.1% Triton X-100, pH 7.4] at a ratio of 1:4 wt/v and quickly homogenized on ice using a pellet pestle hand homogenizer (Kontes®). Aortic homogenates were centrifuged at 10,000 g for 5 min at 4°C and the supernatants were collected for analysis.
Western blot analysis was carried out as previously described\(^3\). Briefly, aortic homogenates, normalized for protein concentration (20 µg protein/sample), were heated for 3 min at 95°C and loaded onto 10% polyacrylamide gels. After electrophoresis, gels were electroblotted onto nitrocellulose membranes in Towbin buffer (20% methanol, 25 mmol/L Tris-base, 192 mmol/L glycine and 0.05% w/v sodium dodecyl sulphate) to determine the levels of calponin-1. Positive standard as HT1080 (human fibrosarcoma cells) and/or molecular weight standards (Precision Plus protein dual color standards, BioRad) were also loaded onto gels to assist in the identification of proteins of interest. Membranes were blocked with 5% w/v skim milk powder in TTBS (0.001% v/v Tween-20, 2 mol/L Tris pH 7.6, 0.1 mol/L NaCl) for 1 hour at room temperature and then probed overnight with rabbit monoclonal calponin-1 (C-terminus) antibody (1:20,000 dilution; Chemicon Cat. # 04-589). In some experiments, mouse monoclonal calponin-1 (1:200 dilution; Santa Cruz Cat. # 58707) and rabbit polyclonal calponin-1 (N-terminus) (1:200 dilution; Santa Cruz Cat. # 16604-R) antibodies were used. Blots were then probed with either mouse or rabbit secondary horseradish peroxidase-conjugated antibodies (Transduction Laboratories, Cat. # CLCC30007 and # CLCC42007, as appropriate), for 1 hour at room temperature and visualized using a chemiluminescence reaction kit (Amersham Pharmacia Biotech). Band intensity was quantified using ImageJ software (National Institutes of Health, USA). Rabbit hypoxanthine phosphoribosyltransferase 1 (HPRT) polyclonal antibody (1:1000 dilution; Abcam Cat. # ab10479) was used as a loading control to normalize protein levels on each membrane.

**Co-immunoprecipitation studies**

To investigate whether LPS induces MMP-2 activation by its S-glutathiolation, aortic homogenates were first normalized for protein concentration (100 - 300 µg protein/sample) and then incubated without or with dithiothreitol (DTT, 0.1, 0.3 or 1.0 mmol/L) in Tris buffer (50 mmol/L Tris-HCl and 3.1 mmol/L sucrose, pH 8.0) for 10 min at 30°C. DTT was used as a control as S-glutathiolation is reversible with this reducing agent\(^10\). Homogenates were pre-cleared by incubation with a Protein-A Sepharose bead suspension (BioVision, USA) for 60 min at 4°C. After centrifugation, supernatants were incubated with
either mouse monoclonal IgG₁ (negative control, 1:100 dilution) or mouse monoclonal glutathione [D8 (clone number)] antibody (GSH, 1:250 dilution; Abcam Cat. # ab19534) at 4°C overnight. Protein A-Sepharose bead suspension (1:10 v/v) was added and further incubated under agitation at 4°C overnight. After centrifugation (12,000 x g for 30 s), supernatants were discarded and pellets were washed three times in Tris buffer at 4°C. The final pellets were suspended in 30 µl of sample buffer and then heated to 95°C for 5 min and centrifuged. Supernatants were used for SDS-PAGE followed by western blotting for MMP-2. Briefly, gels were electroblotted onto polyvinylidene difluoride membranes in Towbin buffer (20% methanol, 25 mmol/L Tris-base, 192 mmol/L glycine and 0.05% w/v sodium dodecyl sulphate) to determine the levels of MMP-2. Molecular weight standards (Precision Plus protein dual color standards, BioRad) were also loaded onto gels to assist in the identification of proteins of interest. Membranes were blocked with 5% w/v skim milk powder in TTBS (0.001% v/v Tween-20, 2 mol/L Tris pH 7.6, 0.1 mol/L NaCl) for 1 hour at room temperature and then probed overnight with a mouse anti-human MMP-2 antibody (1:1000 dilution; Chemicon Cat. # MAB3308). Blots were then probed with mouse secondary horseradish peroxidase-conjugated antibodies (Transduction Laboratories, Cat. # CLCC30007) for 1 hour at room temperature and visualized using a chemiluminescence reaction kit (Amersham Pharmacia Biotech). Band intensity was quantified using ImageJ software (National Institutes of Health, USA).

Similar analysis was performed to examine whether MMP-2 co-immunoprecipitates with calponin-1 in aorta. The only modification here was that the homogenates were incubated with either control IgG (1:100) or the calponin-1 (C-terminus) antibody (1:250 dilution; Chemicon) at 4°C overnight.

**Immunohistochemistry and confocal microscopy**

Aortae were fixed with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2 – 7.4) for 4 hours at room temperature. Fixed aortae were rinsed with 0.1 mol/L sodium phosphate buffer and then cryoprotected in 30% v/v sucrose in 0.1 mol/L sodium phosphate buffer overnight at 4°C. After that,
cryoprotected aortae were vertically embedded with Tissue-Tek® O.C.T. compound, frozen and serially
cyrosectioned (5 µm sections).

Double-immunofluorescence with primary antibodies from different host species was performed as
previously described11. Briefly, cryosections were dried for 20 min at room temperature followed by two
washes with 0.3% Triton-X 100 in sterile phosphate-buffered saline (PBS, pH 7.2 - 7.4) and one wash
with PBS alone. To reduce the binding of non-specific proteins, cryosections were first incubated with
10% normal donkey serum (Jackson ImmunoResearch Laboratories, Cat. # 017-000-121) for 1 hour.
Mouse anti-human monoclonal MMP-2 antibody (1:200 dilution, Chemicon Cat. # MAB3308) and rabbit
monoclonal calponin-1 (C-terminus) antibody (1:250 dilution, Chemicon Cat. # 04-589) were mixed
together, applied to cryosections and then incubated for 18 hours at room temperature in humidified
chambers. On the following day, cryosections were washed twice with 0.3% Triton-X 100 in sterile PBS
followed by one wash with PBS alone. Two different secondary antibodies conjugated to either donkey
anti-mouse fluorescent cyanine (Cy3, Jackson ImmunoResearch Laboratories, Cat. # 715-165-151) or
donkey anti-rabbit Alexa® 488 (Invitrogen, Cat. # A21206) were mixed together, applied to cryosections
and then incubated for 2 hours at room temperature in dark humidified chambers. During this incubation,
2% v/v normal donkey serum was added to the cryosections for stabilization of the antibodies and
reduction of any background staining. To determine the specificity of the immunolabeling, either the
primary or secondary antibodies were omitted from samples. The immunolabeled cryosections were
observed by confocal laser scanning microscopy (LSM 510, Carl Zeiss Co., Germany). Cy3 (red) was
scanned with a helium/neon green laser (543 nm) with a long pass 590 filter. Alexa 488 (green) was
captured by an argon laser (488 nm) with a band pass 500-530 filter. Line profile analysis using Image-
Pro Plus software (Media Cybernetics, Inc.) was performed to study the potential co-localization of MMP-
2 and calponin-1 in aortic tissue.

The levels of calponin-1 (green) in aortic cryosections were obtained by quantification of their
fluorescence intensity/area using ImageJ software. An arbitrary area was equally selected around the
vessel image (at 40x magnification) in all groups to assess the fluorescence intensity. This area corresponds to approximately 20-30% of the total aortic area being studied.

**In silico analysis**

Putative cleavage site motifs for MMP-2 (PVS↓LRS, PEA↓LRG, PEG↓LRV, PAV↓MTS and LAAD↓ITA as indicated by the arrows) were identified within the primary sequence of human calponin-1 using the SIM Alignment tool for protein sequences. These consensus sequences showed a significant selectivity for MMP-2 over other MMPs, including MMP-9\(^1\).\(^2\).\(^3\). These sequences were aligned especially against the troponin I-like/actin-binding and the C-terminal domains of calponin-1 and the results were restricted to the calponin-1 sites having at least 50% identity to six amino acids of the cleavage motif.

**In vitro degradation assay and mass spectrometry**

To test whether calponin-1 is susceptible to proteolysis by MMP-2 *in vitro*, purified human recombinant calponin-1, prepared as described by Winder and Walsh\(^4\), was incubated with human recombinant 64 kDa MMP-2 (Calbiochem) at 300:1 and 100:1 molar ratios at 37°C in 50 mmol/L Tris-HCl pH 7.6, 5 mmol/L CaCl\(_2\), 150 mmol/L NaCl for 2 h. In additional experiments, MMP-2 was pre-incubated with the MMP inhibitor GM6001 (100 nmol/L) for 15 min at 37°C before addition to calponin-1. The reaction mixtures were analyzed by either Coomassie Blue stained gels or western blot using anti-rabbit calponin-1 raised against the full-length protein). The intensities of both the intact calponin-1 band and its degradation product were quantified using ImageJ software. The potential degradation products of calponin-1, which were detected by the Coomassie blue stained gel, were excised for digestion with trypsin and the tryptic peptides were analyzed by LC-MS/MS using a Thermo LTQ Orbitrap XL mass spectrometer. To verify that these degradation products were in fact derived from calponin-1, we used the MASCOT program which classifies the proteins from primary sequence databases (www.matrixscience.com).
SUPPLEMENTAL REFERENCES


**SUPPLEMENTAL TABLE.** Analysis of the 15 kDa calponin-1 degradation product by LC-MS/MS using a Thermo LTQ Orbitrap XL mass spectrometer and the MASCOT database.

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