Matrix metalloproteinase-specific inhibition of Ca^{2+} entry mechanisms of vascular contraction

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Objective: **Abdominal aortic aneurysm (AAA) is a common disease with as yet unclear cause. Increased matrix metalloproteinase (MMP) levels in the plasma and aorta are a consistent finding in AAA. Although the role of MMPs in AAA has largely been attributed to degradation of the extracellular matrix proteins, the effects of MMPs on the mechanisms of aortic contraction are unclear. The purpose of this study was to test the hypothesis that MMPs promote aortic dilation by inhibiting the Ca^{2+} mobilization mechanisms of smooth muscle contraction.**

Methods: **Isometric contraction and $^{45}Ca^{2+}$ influx were measured in aortic strips isolated from male Sprague-Dawley rats treated or not treated with MMP-2 and MMP-9.**

Results: **In normal Krebs solution (2.5 mmol/L Ca^{2+}) phenylephrine (10^{-5} mol/L) caused contraction of the aortic strips, which was significantly inhibited ($P < .05$) by MMP-2 (maximum, 48.9% ± 5.0%) and to a greater extent by MMP-9 (maximum, 69.8% ± 6.2%). The MMP-induced inhibition of phenylephrine contraction depended on concentration and time. The inhibitory effects of MMPs on phenylephrine contraction were reversible. In Ca^{2+}-free (2 mmol/L ethylene glycol bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid) Krebs solution phenylephrine caused a small contraction that was not inhibited by MMP-2 or MMP-9, which suggests that MMPs do not inhibit Ca^{2+} release from the intracellular stores. Membrane depolarization with 96 mmol/L of potassium chloride, which stimulates Ca^{2+} entry from the extracellular space, caused a time-dependent and reversible contraction, which was inhibited by MMP-2 and MMP-9. Histologic studies of MMP-treated tissues stained with hematoxylin-eosin or Verhoeff stain for elastin confirmed the absence of degradation of the extracellular matrix. MMP-2 and MMP-9 also caused significant inhibition of $^{45}Ca^{2+}$ influx induced by phenylephrine and potassium chloride.**

Conclusions: **These data suggest that MMP-2 and MMP-9 promote aortic dilation by inhibiting the Ca^{2+} entry mechanism of vascular smooth muscle contraction. (J Vasc Surg 2004;40:1001-10.)**

Clinical Relevance: **Abdominal aortic aneurysm (AAA) is a slow and progressive disease. The late stages of AAA are characterized by degenerative changes in the extracellular matrix and smooth muscle components of the aortic wall. The present study describes novel inhibitory effects of matrix metalloproteinase (MMP) on the Ca^{2+} entry mechanisms of aortic smooth muscle contraction, even in the absence of extracellular matrix degradation. The MMP-induced inhibition of aortic contraction may further explain the role of increased MMP activity particularly during the early development of AAA. Chronic exposure to MMPs may lead to protracted inhibition of aortic contraction, progressive aortic dilation, and aneurysm formation. MMP-9 is a more potent inhibitor of aortic contraction than MMP-2, consistent with a more dominant role in AAA. Restoration and preservation of smooth muscle contractile function by specific inhibitors of MMPs may represent a new strategy in preventing the progression of small AAA.**

Abdominal aortic aneurysm (AAA) is a focal dilatation of the aorta that is commonly observed in elderly persons. The prevalence of AAA has increased dramatically over the past 3 decades, in part because of the demographic distribution shifting to more elderly persons in the general population.[1,2] AAA is a progressive degenerative disease that if left uncorrected can result in aortic rupture and death. Although large AAAs can be repaired surgically, no effective treatment is currently available for small AAAs, other than careful observation until they become critically large and the risk for rupture outweighs the risks of surgery. Understanding the mechanisms of AAA formation would help identify new treatment strategies to retard the progressive growth of small AAAs.

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-containing enzymes known to degrade the connective tissue matrix.[3-5] MMPs have key roles in tissue remodeling and cellular migration, and act as regulatory molecules, both by functioning in enzyme cascades and by processing matrix proteins and adhesion molecules.[3,4,6] Increased MMP activity has been reported in various inflammatory, malignant, and degenerative disorders, including AAA, and increased MMP production or decreased expression of MMP inhibitors may have a role in the pathogenesis of AAA.[7-9]

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Several studies have suggested that the plasma level and aortic wall expression of MMPs is increased in AAA disease.[10-16] Specifically, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) seem to have an important role in aneurysm formation.[17-23] For example, targeted gene disruption of MMP-9 in mice suppresses development of experimental AAAs.[24] Also, both MMP-2 and MMP-9 are necessary to induce experimental AAA formation in mice.[22] Because of their potent elastolytic activity, the mechanism of action of MMPs in aneurysm formation has largely been attributed to their proteolytic effects on the extracellular matrix proteins and subsequent weakening of the aortic wall.[8,9] Recent studies have suggested that elastolytic enzymes, such as porcine pancreatic elastase, could have additional effects on other vascular cell types, and thereby could affect aortic contraction.[25,26] Despite the potent elastolytic activity of MMPs, little is known about the effects of MMPs on other vascular cell types, particularly on the mechanisms of aortic smooth muscle contraction.

Vascular smooth muscle contraction in response to vasoconstrictor agonists such as phenylephrine is triggered by an increase in intracellular Ca^{2+} due to Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space.[27] The purpose of this study was to test the hypothesis that MMPs promote aortic dilation by inhibiting the Ca^{2+} mobilization mechanisms of smooth muscle contraction. Experiments were designed to investigate whether MMPs inhibit phenylephrine-induced aortic smooth muscle contraction, and whether MMP-induced inhibition of aortic contraction is due to inhibition of the Ca^{2+} release mechanism from the intracellular stores or the Ca^{2+} entry mechanism from the extracellular space. The effects of MMPs on phenylephrine-induced contraction and $^{45}Ca^{2+}$ influx were measured. To further investigate the Ca^{2+} mobilization mechanism involved, the effects of MMPs on phenylephrine-induced contraction in Ca^{2+}-free Krebs solution, which is mainly due to Ca^{2+} release from the intracellular stores, were compared with their effects on high potassium chloride (KCl)–induced contraction, which is mainly due to Ca^{2+} entry from the extracellular space.

METHODS

Tissue preparation. Male Sprague-Dawley rats (Charles River Laboratories), 12 weeks of age and weighing 200 to 300 g, were housed in the animal facility and maintained on ad libitum standard rat chow and tap water in a 12-hour light, 12-hour dark cycle. The rats were euthanized with carbon dioxide inhalation, and complete euthanasia was judged by cessation of breathing and heartbeat. The abdominal cavity was opened, and the abdominal aorta was rapidly excised and placed in oxygenated Krebs solution.

The aorta was dissected and cleaned of adipose and connective tissue under microscopic visualization, then cut into 3-mm-wide strips. The effects of MMP on aortic contraction could be due to an effect on the endothelium or direct effects on vascular smooth muscle. To avert the contribution of the endothelium to the vascular responses to MMP and to focus mainly on the direct effects of MMP on vascular smooth muscle, the endothelium was removed by gently rubbing the vessel interior with wet filter paper. Removal of the endothelium was routinely verified by the absence of acetylcholine-induced relaxation in vascular strips pre-contracted with phenylephrine (3×10^{-7} mol/L). All procedures were performed under the guidelines of the Institutional Animal Care and Use Committee.

Isometric contraction. One end of the aortic strip was attached to a glass hook with a thread loop, and the other end was connected to a Grass force transducer (FT03). Aortic strips were stretched to L_{max} (1.5 times unloaded initial length, L). The strips were allowed to equilibrate for 1 hour in a water-jacketed, temperature-controlled tissue bath filled with 25 mL of Krebs solution continuously bubbled with 95% oxygen and 5% carbon dioxide at 37°C. The changes in isometric contraction were recorded on a Grass polygraph (Model 7D; Astro-Med).

A control contraction was elicited by applying 96 mmol/L of KCl to the tissue bath solution. Once the KCl contraction reached a plateau, the tissue was rinsed with Krebs solution 3 times for 10 minutes each. The whole procedure of KCl contraction and washing was repeated twice.

Aortic strips were stimulated with phenylephrine (10^{-5} mol/L) until the contraction reached a plateau, then treated with varying concentrations of MMP-2 or MMP-9 (0.01-1 μg/mL), and changes in phenylephrine contraction were observed. The tissues were thoroughly rinsed with Krebs solution 4 times for 15 minutes each. The tissues were then re-stimulated with phenylephrine (10^{-5} mol/L) to determine the reversibility of the inhibitory effects of MMP. To determine whether the effects of MMP are specific only to phenylephrine and α-adrenergic receptors, the effects of MMP on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contraction were also studied.

In another set of experiments the vascular strips were incubated for 2 minutes in Ca^{2+}-free (2 mmol/L ethylene glycol bis[β-aminoethylether]-N,N,N′,N′-tetra-acetic acid; EGTA) Krebs solution, then treated with phenylephrine (10^{-5} mol/L) to stimulate Ca^{2+} release from the intracellular stores and elicit a first phenylephrine contraction. The tissue was washed 4 times for 15 minutes each with normal Krebs solution to replenish the intracellular Ca^{2+} stores, and were incubated in the presence of MMP-2 or MMP-9 (1 μg/mL) for 1 hour. The bathing medium was changed to Ca^{2+}-free Krebs solution, and the tissue was stimulated with phenylephrine (10^{-5} mol/L) to elicit a second contraction. The second phenylephrine contraction was then compared with the first (control) phenylephrine contraction.

In other experiments aortic strips were stimulated with 96 mmol/L of KCl, which causes membrane depolarization and stimulates Ca^{2+} entry from the extracellular space.[27] Once the KCl response reached a plateau, the tissue strips were treated with MMP-2 or MMP-9 (1 μg/mL), and changes in KCl contraction were observed. The aortic strips were thoroughly rinsed 4 times for 15 minutes each with Krebs solution, then re-stimulated with 96

mmol/L of KCl to determine reversibility of the effects of MMP.

$^{45}Ca^{2+}$ influx. Vascular strips were incubated in Krebs solution, then stimulated with phenylephrine (10^{-5} mol/L) or 96 mmol/L of KCl for 10 minutes in the absence or presence of MMP-2 or MMP-9 (1 μg/mL). The tissues were transferred to the respective radioactive $^{45}Ca^{2+}$-labeled solution (specific activity, 5 μCi/mL; ICN) for 90 seconds. Preliminary experiments have shown that the relationship between $^{45}Ca^{2+}$ uptake versus time is linear during exposure to the $^{45}Ca^{2+}$ label for15, 30, 60, and 90 seconds. The tissues were transferred to ice-cold Ca^{2+}-free Krebs solution for 45 minutes to quench extracellular $^{45}Ca^{2+}$ label. The vascular strips were weighed, and placed in 2 mL of hypotonic (5 mmol/L) ethylenediamine tetraacetic acid for 24 hours at 4°C to disrupt the cell membranes and release the intracellular content of $^{45}Ca^{2+}$. The next day, 4 mL of Ecolite scintillation cocktail was added, and the samples were counted in a liquid scintillation counter (Packard 1500).

Histologic studies. Aortic strips were stimulated with phenylephrine (10^{-5} mol/L) for 10 minutes, then either treated or not treated with MMP-2 or MMP-9 (1 μg/mL) for 1 hour. The tissues were fixed overnight in freshly prepared neutral buffered formalin (10%), embedded in paraffin, sectioned (5-μm thick), and placed on glass slides. Tissue sections were prepared for staining with hematoxylin-eosin or Verhoeff stain for elastin to assess for integrity of the smooth muscle layer, the extracellular matrix, and elastin in the vessel wall.

Solutions, drugs and chemicals. Normal Krebs solution contained NaCl, 120 mmol/L; KCl, 5.9 mmol/L; $NaHCO_3$, 25 mmol/L; NaH_2PO_4, 1.2 mmol/L; dextrose, 11.5 mmol/L; $MgCl_2$, 1.2 mmol/L; and $CaCl_2$, 2.5 mmol/L, at pH 7.4. For Ca^{2+}-free Krebs solution, $CaCl_2$ was omitted and EGTA (2 mmol/L) was added. Solution of 96 mmol/L KCl was prepared as for Krebs solution, with substitution of KCl for NaCl. Phenylephrine and $PGF_{2\alpha}$ were obtained from Sigma. MMP-2 and MMP-9 (Biomol) were not the whole molecules, but the active catalytic subunit of the respective enzyme cloned from human complementary DNA. The specific activities of MMPs were confirmed with the colorimetric thiopeptolide Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-Oet as substrate. All other chemicals were of reagent grade or better.

Statistical analysis. Data were analyzed and expressed as mean ± SEM. Data were compared with the Student *t* test for unpaired and paired data. Percentage changes in data were analyzed with the Mann-Whitney *U* test and EPISTAT statistical program. Differences were considered statistically significant at $P < .05$.

RESULTS

Effect of MMP-2 and MMP-9 on Phe contraction in Ca^{2+}-containing solution. In normal Krebs solution (2.5 mmol/L Ca^{2+}), phenylephrine (10^{-5} mol/L) caused a significant contraction that reached a steady state in approximately 10 minutes. The phenylephrine contraction was maintained with no significant decline for at least 30 minutes (Fig 1, *A*). The phenylephrine contraction was reproducible. After washing the tissue with normal Krebs solution for 1 hour a second phenylephrine contraction was not significantly different from the first (control) phenylephrine contraction (Fig 1, *A*). Application of MMP-2 (1 μg/mL) caused significant inhibition of the phenylephrine-induced contraction (Fig 1, B; and Fig 2). In a similar fashion, application of MMP-9 (1 μg/mL) also caused significant inhibition of the phenylephrine-induced contraction (Fig 1, C; and Fig 2). MMP-9 showed a greater inhibitory effect on phenylephrine-induced aortic contraction when compared with MMP-2 (Figs 1 and 2). In control experiments in which phenylephrine and the MMPs were placed together before adding to the strips the initial phenylephrine contraction was not significantly affected; however, the maintained phenylephrine contraction

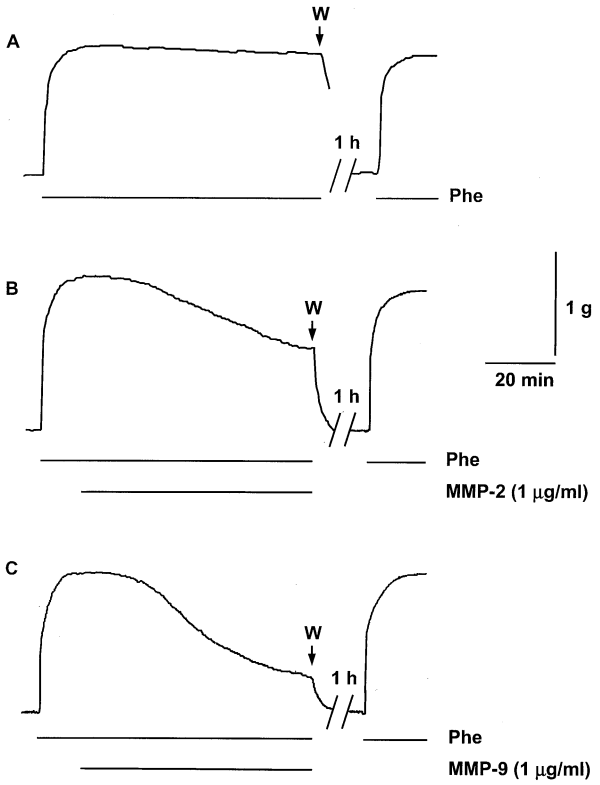

Fig 1. Inhibition of phenylephrine *(Phe)* contraction by matrix metalloproteinases *MMP-2* and *MMP-9*. Aortic strips were incubated in normal Krebs solution (2.5 mmol/L Ca^{2+}), then stimulated with phenylephrine (10^{-5} mol/L). When the phenylephrine contraction reached a plateau, tissue was either not treated **(A)** or treated with 1 μg/mL of MMP-2 **(B)** or MMP-9 **(C)**, and inhibition of phenylephrine contraction was observed. Tissues were washed *(W)* with Krebs solution 4 times for 15 minutes each, and a second phenylephrine contraction was elicited to determine reversibility of the effects of MMPs. Tracings represent results from 5 nontreated 4 MMP-2-treated and 4 MMP-9- treated vascular strips from different rats.

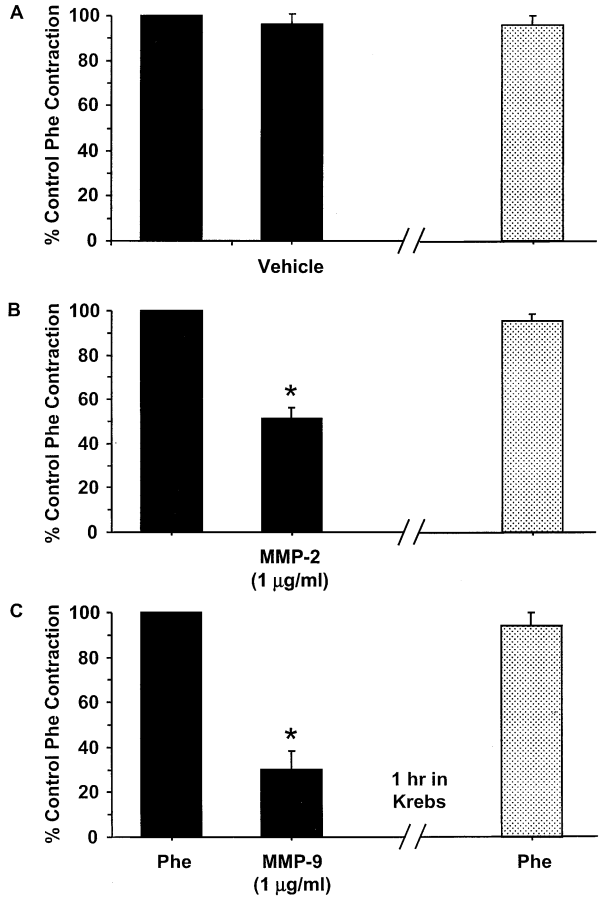

Fig 2. Reversibility of matrix metalloproteinase *(MMP)*–induced inhibition of phenylephrine contraction. Aortic strips were incubated in normal Krebs solution, then stimulated with phenylephrine (10^{-5} mol/L). The tissues were either not treated **(A)** or treated for 1 hour with 1 μg/mL of MMP-2 **(B)** or MMP-9 **(C)**. Tissues were washed with Krebs solution 4 times for 15 minutes each, and a second phenylephrine contraction was elicited and presented as percentage of control phenylephrine contraction. Data bars represent mean ± SEM of measurements in 5 nontreated 4 MMP-2-treated and 4 MMP-9-treated vascular strips from different rats. *Significantly different ($P < .05$) from control phenylephrine contraction, or phenylephrine contraction in tissues stimulated in parallel with phenylephrine for the same period of time but not treated with MMP **(A)**.

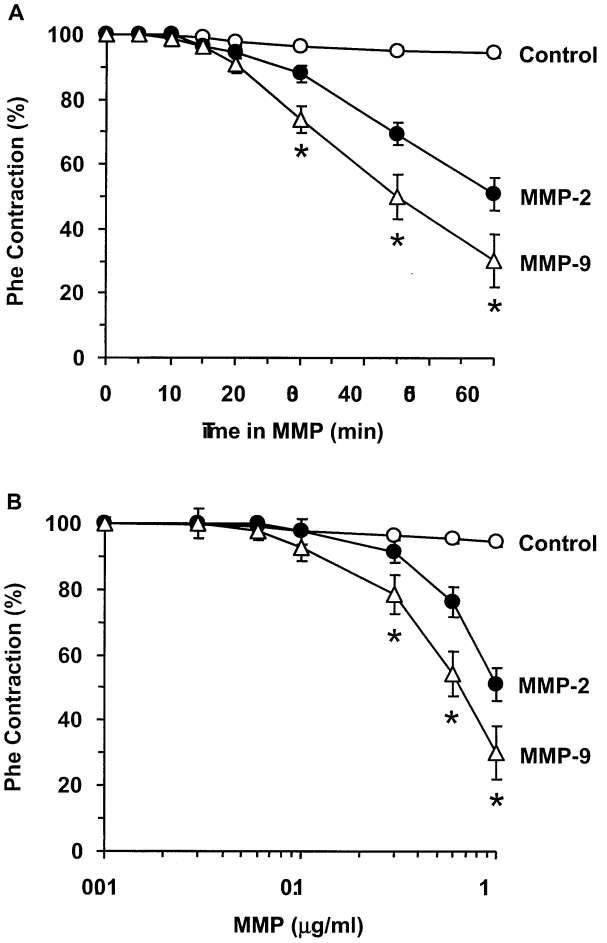

Fig 3. Matrix metalloproteinase *(MMP)*–induced inhibition of phenylephrine contraction is time-dependent and concentration-dependent. Aortic strips were stimulated with phenylephrine (10^{-5} mol/L), then treated with MMP-2 or MMP-9 (0.01-1 μg/mL). **A,** To illustrate that the effects of MMP were time-dependent, inhibition of phenylephrine contraction in response to MMP-2 or MMP-9 (1 μg/mL) was measured at different times. **B,** To illustrate that the effects of MMP were concentration-dependent, inhibition of Phe contraction after 1 hour was compared for different concentrations of MMP-2 and MMP-9. For each concentration of MMP, 4 vascular strips from different rats were used. Data are presented as mean ± SEM. *Significantly different ($P < .05$) from control phenylephrine contraction.

showed slow decline, similar in time course and magnitude to that observed with topical application of MMP on phenylephrine contraction. These control experiments suggest that the observed inhibitory effects of MMPs are not due to direct phenylephrine and MMP interaction.

The reversibility of the inhibitory effects of MMP-2 and MMP-9 on phenylephrine-induced aortic contraction was tested. In tissues treated with MMP-2 and MMP-9 (1 μg/mL) for 1 hour, followed by rinsing with Krebs solution for 1 hour, the inhibitory effects of MMP-2 and MMP-9 on phenylephrine-induced contraction were reversible (Figs 1 and 2). In these tissues the second phenylephrine contraction was not significantly different compared with the first (control) phenylephrine contraction.

Inhibition of phenylephrine contraction by MMP-2 and MMP-9 was dependent on time and concentration (Fig 3). At 1 μg/mL of MMP-2, inhibition of phenylephrine contraction was slow in onset (18.8 ± 3.2 minutes; n = 4), and maximum inhibition was 48.9% ± 5.0%. At 1 μg/mL of MMP-9, inhibition of phenylephrine contraction was also slow in onset (20.0 ± 3.5 minutes; n = 4), but maximum inhibition was significantly greater (69.8% ± 6.2%) than that induced by MMP-2 ($P = .039$).

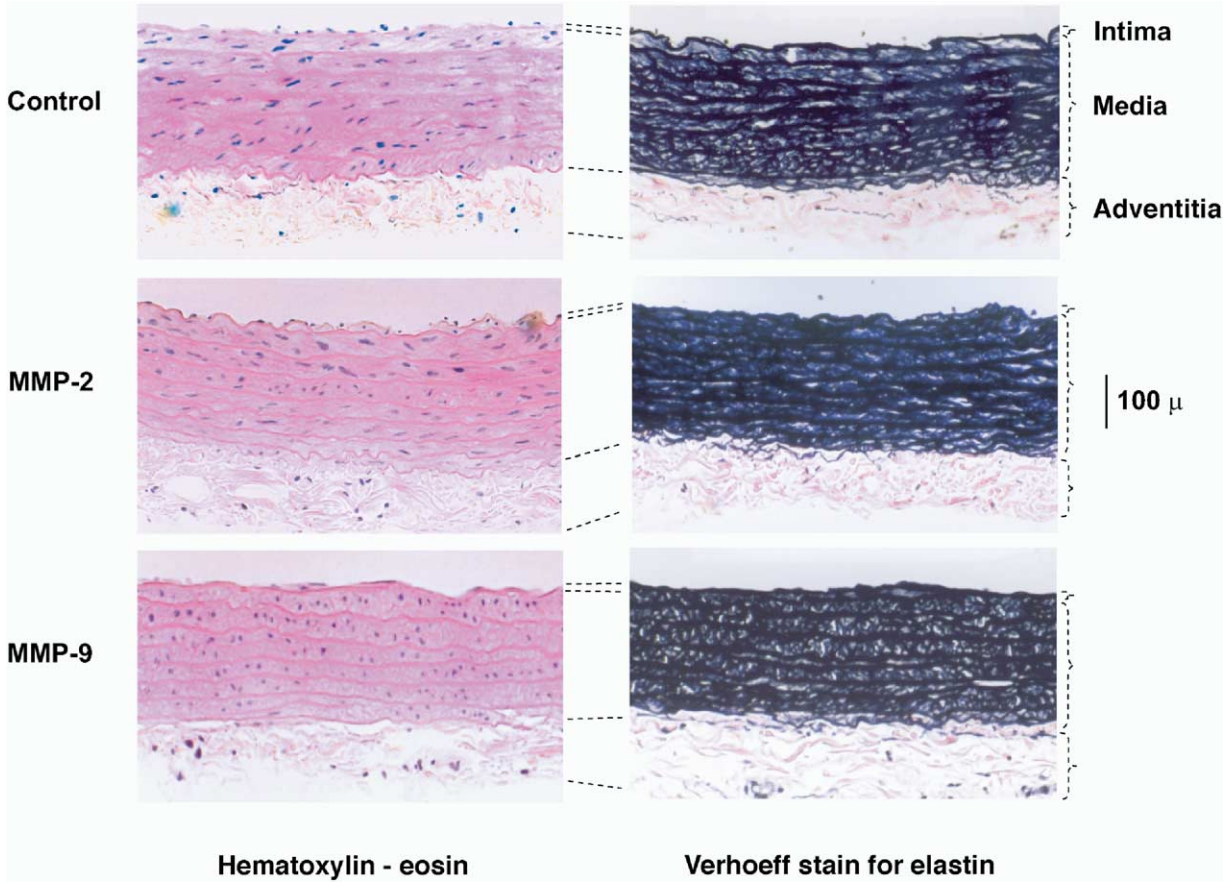

Fig 4. Effect of matrix metalloproteinase *(MMP)* on structure of rat aorta. Rat aortic strips were either nontreated or treated for 1 hour with 1 μg/mL of MMP-2 or MMP-9. Tissue sections were prepared and stained with hematoxylin-eosin *(left panels)* or Verhoeff stain for elastin *(right panels)*. *Dashed lines* indicate different layers of vessel. No structural differences were found at subjective examination of MMP-treated and MMP-nontreated vessels.

To investigate whether the effects of MMPs were specific only to phenylephrine and α-adrenergic receptors, we tested the effect of MMP-9 on $PGF_{2\alpha}$ (10^{-5} mol/L)–induced aortic contraction. MMP-9 (1 μg/mL) caused 68.5% inhibition of $PGF_{2\alpha}$-induced contraction.

Effect of MMPs on aortic wall structure. To determine whether treatment with MMP-2 or MMP-9 caused disruption of the structural integrity of the vessel wall or degradation of elastin, tissue sections were prepared for histologic examination with hematoxylin-eosin stain and Verhoeff stain for elastin. Tissue sections of the control aorta showed an intact tunica media and preserved elastin layer. In tissues treated with either MMP-2 or MMP-9 (1 μg/mL) for 1 hour the tunica media remained intact and no significant elastin degradation was seen, compared with control tissue (Fig 4). The aortic media–total wall thickness was 57.9% ± 0.7% in nontreated vessels, which was not significantly different ($P > .06$) from that in vessels treated with MMP-2 (60.1% ± 1.7%; n = 5) or MMP-9 (60.9% ± 1.9%; n = 5).

Effect of MMPs on aortic contraction in Ca^{2+}-free solution. Agonist-induced contraction in Ca^{2+}-free solution represents the Ca^{2+} entry-independent mechanisms of vascular contraction, and is often used as a measure of Ca^{2+} release from the intracellular Ca^{2+} stores in the smooth muscle sarcoplasmic reticulum.[27] In Ca^{2+}-free (2 mmol/L of EGTA) Krebs solution phenylephrine (10^{-5} mol/L) caused a small contraction. The aortic strips were rinsed with normal Krebs solution for 1 hour to replenish the intracellular Ca^{2+} stores, and then a second phenylephrine contraction was elicited in Ca^{2+}-free Krebs solution. In control experiments the second phenylephrine contraction did not appear to be different from the first phenylephrine contraction (Fig 5, *A*). In tissues treated with MMP-2 or MMP-9 (1 μg/mL) for 1 hour the second phenylephrine contraction was not different from the first phenylephrine contraction, which suggests preservation of the intracellular Ca^{2+} release mechanism (Fig 5, *B* and *C*).

Effect of MMPs on KCl contraction. Membrane depolarization with 96 mmol/L of KCl, which stimulates

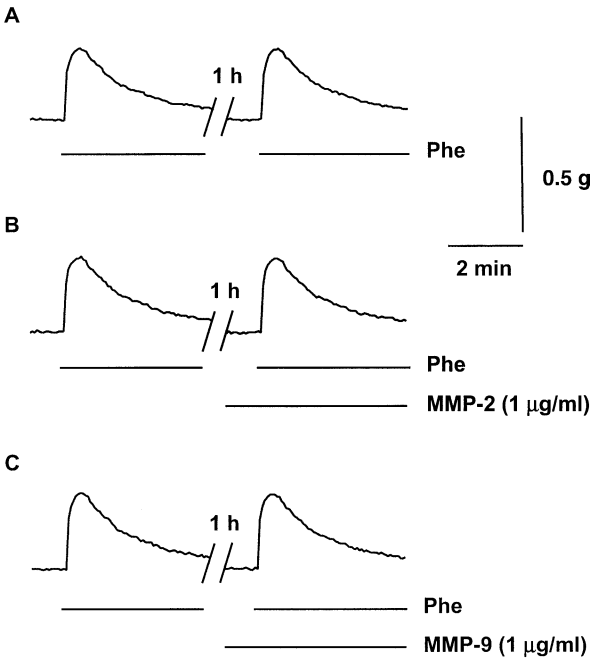

Fig 5. Effect of matrix metalloproteinase *(MMP)* on phenylephrine-induced contraction in Ca^{2+}-free Krebs solution. Aortic strips were incubated for 2 minutes in Ca^{2+}-free (2 mmol/L ethylene glycol bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid) Krebs solution, then treated with phenylephrine (10^{-5} mol/L) to stimulate Ca^{2+} release from the intracellular stores and elicit a first contraction. Tissue was washed with normal Krebs solution for 1 hour to replenish intracellular Ca^{2+} stores, then incubated in the absence **(A)** or presence of 1 μg/mL of MMP-2 **(B)** or MMP-9 **(C)**. Bathing medium was changed to Ca^{2+}-free Krebs solution, and a second phenylephrine contraction was elicited and compared with the first phenylephrine contraction.

Ca^{2+} entry from the extracellular space,[27] caused a significant contraction, which reached a plateau in approximately 10 minutes. The KCl contraction was completely inhibited by the Ca^{2+} channel blocker verapamil (10^{-5} mol/L), confirming that the KCl contraction is mainly due to Ca^{2+} entry from the extracellular space. MMP-2 and MMP-9 caused inhibition of the KCl-induced aortic contraction that was slow in onset (13.3 ± 6.0 minutes, n = 3; and 12.9 ± 1.5 minutes, n = 7) and time-dependent. The inhibitory effects of MMPs on KCl-induced contraction were completely reversible, and a second KCl contraction was not significantly different from the first (control) KCl contraction (Fig 6).

Effect of MMPs on $^{45}Ca^{2+}$ influx. Phenylephrine (10^{-5} mol/L) and 96 mmol/L of KCl caused significant increase in $^{45}Ca^{2+}$ influx in control aortic strips. In tissues treated with MMP-2 or MMP-9 (1 μg/mL), phenylephrine-induced and KCl-induced $^{45}Ca^{2+}$ influx were significantly reduced compared with control tissues (Fig 7).

DISCUSSION

The main findings of the present study are that MMP-2 and MMP-9 reversibly inhibit phenylephrine-induced aortic contraction; MMP-2 and MMP-9 treatment does not inhibit phenylephrine-induced contraction in Ca^{2+}-free Krebs solution, which is a measure of Ca^{2+} release from the intracellular stores; MMP-2 and MMP-9 treatment inhibit KCl-induced contraction, and phenylephrine-induced and KCl-induced $^{45}Ca^{2+}$ influx; and MMP-9 is a more potent inhibitor of aortic contraction than MMP-2 is.

To our knowledge, no previous studies have examined the effects of MMP on vascular smooth muscle contraction or relaxation. The inhibitory effects of MMPs on phenylephrine-induced aortic contraction could be due to an effect on the endothelium, the connective tissue matrix, or the smooth muscle. Because in the in vivo conditions the endothelium is present on the aortic wall, at least in the initial stages of aneurysm development, the effects of MMP on aortic contraction could be due to an effect on the endothelium. For example, the vascular endothelium releases endothelium-dependent relaxing factors, such as nitric oxide, and thereby cause smooth muscle relaxation. However, it is unlikely that the observed inhibition of vascular contraction is due to an effect of MMPs on endothelium-derived relaxing factors, because the present experiments were performed on endothelium-denuded vascular strips. Another possibility is that MMPs may cause elastin degradation and disruption of the connective tissue matrix, which could decrease the ability of the blood vessel to maintain contraction. However, the present histologic studies showed no evidence of extracellular matrix destruction in tissues treated with MMP-2 or MMP-9 at the concentrations and relatively short duration of treatment tested. Also, the complete reversibility of the effects of MMPs on phenylephrine-induced and KCl-induced contraction and the lack of effect of MMPs on phenylephrine-induced contraction in Ca^{2+}-free Krebs solution suggest that the inhibitory effects of MMPs are not solely due to irreversible degradation of the extracellular matrix. It also appears unlikely that the inhibitory effects of MMPs are due to an effect on phenylephrine or α-adrenergic receptors, because MMPs did not inhibit phenylephrine contraction in Ca^{2+}-free solution. Furthermore, MMPs inhibited $PGF_{2\alpha}$-induced contraction, which suggests that the inhibitory effects of MMPs are not specific to a particular agonist or receptor, but more likely involve direct effects of MMPs on a common signaling mechanism of vascular smooth muscle contraction downstream from receptor activation.

Vascular smooth muscle contraction is triggered by an increase in intracellular Ca^{2+} due to Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space.[27] The observation that MMPs did not inhibit phenylephrine-induced contraction in Ca^{2+}-free Krebs solution suggests that the MMPs may not work by inhibiting the Ca^{2+} release mechanism from the intracellular stores. On the other hand, the observations that MMPs inhibit KCl-induced contraction and the phenylephrine-induced and KCl-induced $^{45}Ca^{2+}$ influx suggest that they may function by inhibiting Ca^{2+} entry from the extracellular space.

The specific mechanism by which the MMPs inhibit Ca^{2+} entry is unclear, but could be related to possible

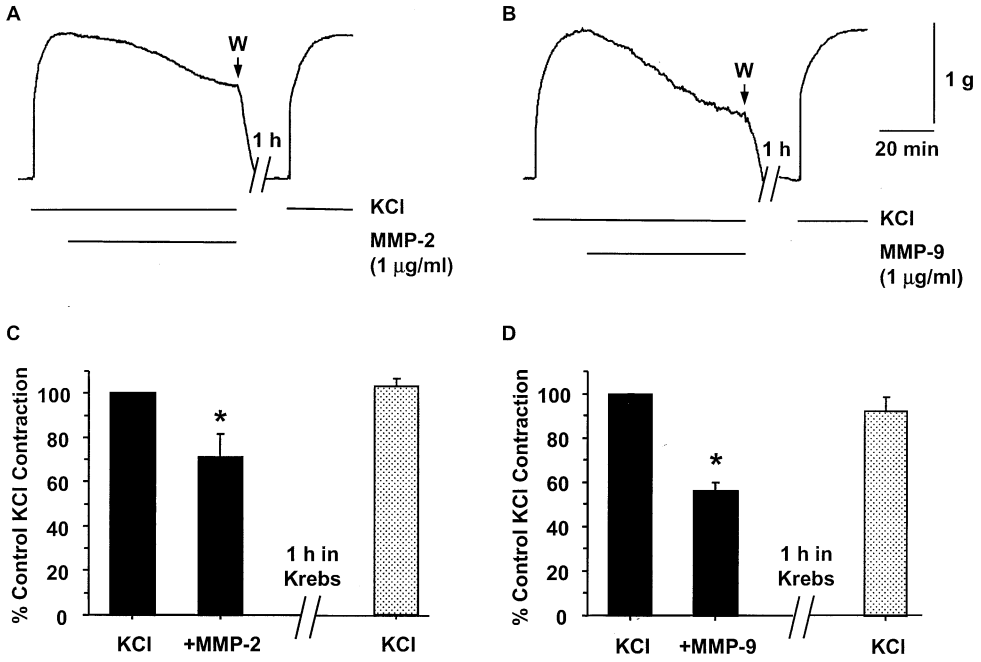

Fig 6. Matrix metalloproteinase *(MMP)*-induced inhibition of potassium chloride *(KCl)* contraction. Aortic strips were stimulated with 96 mmol/L of KCl, then treated with 1 μg/mL of MMP-2 (**A** and **C**) or MMP-9 (**B** and **D**), and inhibition of KCl contraction was observed. Tissues were washed with Krebs solution 4 times for 15 minutes each, and a second KCl contraction was elicited to determine the reversibility of MMP-induced inhibition. **C** and **D**, Second KCl contraction was then presented as percent of control 96 mmol/L of KCl contraction. Data bars represent mean ± SEM of measurements in 5 vascular strips treated with MMP-2 and 7 vascular strips treated with MMP-9. *Significantly different ($P < .05$) from control 96 mmol/L of KCl contraction.

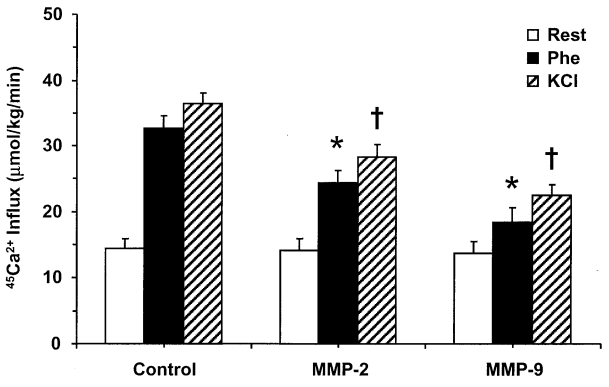

Fig 7. Basal, phenylephrine (10^{-5} mol/L)–induced, and potassium chloride (*KCl*; 96 mmol/L)–induced $^{45}Ca^{2+}$ influx in aortic strips nontreated or treated with 1 μg/mL of matrix metalloproteinase *(MMP)* MMP-2 or MMP-9. Data bars represent mean ± SEM of measurements in 5 vascular strips. *Phenylephrine-induced Ca^{2+} influx is significantly less ($P < .05$) in MMP-treated compared with nontreated strips. †KCl-induced Ca^{2+} influx is significantly less ($P < .05$) in MMP-treated compared with nontreated strips.

direct effects of MMPs on the Ca^{2+} channels. MMPs may also cause degradation of collagen and produce Arg-Gly-Asp–containing peptides, which bind to $\alpha_v\beta_3$ integrin receptors and inhibit Ca^{2+} entry in vascular smooth muscle.[28-31] MMPs may also interact with specific cell surface proteins, such as intercellular adhesion molecule–1, or stimulate protease-activated receptors and activate signaling pathways, which could lead to blockade of smooth muscle Ca^{2+} channels.[32-34] This is supported by reports that proteases such as thrombin could activate protease-activated receptors and cause vascular smooth muscle relaxation by inhibiting Ca^{2+} influx.[35] The greater inhibitory effects of MMP-9 compared with MMP-2 could be due to greater affinity to the Ca^{2+} channels or to other modulating molecules such as integrins and protease-activated receptors. Further studies are needed to define the role of integrins and protease-activated receptors as possible molecular mechanisms by which MMPs could inhibit Ca^{2+} entry into vascular smooth muscle.

We have observed that MMPs cause greater inhibition of phenylephrine-induced contraction than KCl-induced contraction. Inasmuch as KCL contraction is mainly due to Ca^{2+} entry,[27] the greater inhibition of phenylephrine by MMPs could be due to inhibition of other signaling mechanisms of vascular contraction, such as protein kinase C, Rho-kinase, or mitogen-activated protein kinase, and should represent important areas for future investigation.

We performed control experiments to test whether the inhibitory effects of MMPs on the mechanisms of vascular contraction could be reversed by MMP inhibitors, such as MMP-2 or MMP-9 inhibitor IV and α2-macroglobulin. These MMP inhibitors exerted an inhibitory effect on vascular smooth muscle contraction (data not shown). These MMP inhibitors have a Zn^{2+} binding site, and therefore could bind Zn^{2+} ion or other divalent cations such as Ca^{2+}. Therefore it is likely that these MMP inhibitors bind extracellular Ca^{2+}, and thereby would confound the present results. Future studies should determine whether the inhibitory effects of MMPs on vascular contraction are reversed by other MMP inhibitors, such as tissue inhibitor of metalloproteinases (TIMP), TIMP-1 or TIMP-2.

We have considered performing control experiments with an MMP that has not been shown to be present in aortic aneurysm. However, the list of MMPs that could have a role in AAA formation is growing, and includes, in addition to MMP-2 and MMP-9, MMP-1, MMP-3, MMP-7, MMP-8, and MMP-12.[16,36-38] The focus of the present study was to understand the mechanism of MMP-induced inhibition of aortic smooth muscle contraction. Comparison of the effects of MMP-2 and MMP-9 with several other MMPs less described in AAA could be the subject of future comparative studies. An excellent control experiment would be with an MMP that is least likely to have a role in AAA, such as the tooth enamel MMP-20 (enamelysin), and as it becomes commercially available it will be a useful tool for control experiments in future studies.

The observed effects of MMPs on the mechanisms of aortic smooth muscle contraction could be of importance in the pathogenesis of AAAs. Increased expression of MMPs is often found in tissue samples from the aneurysm wall.[10-12,16,17] The median concentration of MMP-2 in AAA wall is 0.04 μg/mL,[39] and that of MMP-9 is 0.5 μg/g of tissue weight.[40] Also, the levels of MMP-2 and MMP-9 isolated from fibroblasts and smooth muscle cells cultured from AAA wall are between 0.01 and 1.2 μg/mL.[19] Furthermore, patients with AAA have elevated plasma levels of MMP-2 and MMP-9, in the range of 0.06 to 0.6 μg/mL.[13,14,41] MMP-2 has the greatest elastolytic activity, and is produced mainly by smooth muscle and fibroblasts.[42,43] MMP-9 is the most abundantly expressed MMP in AAA tissue, and is produced mainly by aneurysm-infiltrating macrophages.[11,18,36] Although degradation of the extracellular matrix by MMPs could have a significant role in the pathogenesis of AAA, the present data show an additional novel effect of MMP-2 and MMP-9 on the Ca^{2+}-entry mechanisms of vascular smooth muscle contraction that may also contribute to AAA formation. For example, the inhibitory effects of the MMPs on aortic smooth muscle contraction were observed at concentrations similar to those observed in plasma and tissue samples from patients with AAA. Also, the observed MMP-induced inhibition of aortic smooth muscle contraction occurred even in the absence of degradation of the connective tissue matrix of the vessel wall. Furthermore, the present data show that MMP-9 is a more potent inhibitor of aortic contraction than MMP-2 is, consistent with the observations that MMP-9 is the dominant MMP in AAA wall.[11,18,23,36,44]

The extracellular matrix proteins elastin and collagen provide significant structural support of the aorta. On the other hand, the contribution of vascular smooth muscle contraction to the overall tensile strength of the aortic wall is less clear. There is evidence, however, to suggest that the integrity of aortic smooth muscle contractile function may contribute to the structural integrity of the aortic wall. First, the tunica media composes a major portion of the aortic wall, and is formed mainly of circumferentially arranged alternating lamellae of smooth muscle cells and elastin fibers. An active and tonic contraction of the smooth muscle is predicted to limit the tendency of the aorta to dilate in response to pulsatile forces generated with each cardiac cycle. Second, atrophy of the tunica media and depletion or apoptosis of smooth muscle cells are consistent histologic findings in AAA.[45,46] Third, disruption of the structural integrity of the tunica media, for example, in chronic aortic dissection, often leads to late aneurysm formation. Thus the MMP-induced inhibition of vascular smooth muscle contraction could function synergistically with their degradation of the extracellular matrix, and thereby contribute to further weakening of the aortic wall and aneurysm formation.

In the present study we tested the acute in vitro effects of MMPs on vascular function. The acute effects of MMPs on aortic contraction were reversible at the concentrations and duration of treatment tested. It remains to be examined whether the acute inhibitory effects of MMPs on smooth muscle contraction represent an early event in AAA formation. However, the effects of MMPs may be different in vivo, where an initial MMP insult could initiate a cascade of events leading to progressive smooth muscle dysfunction and cell death. Also, because AAA is a slow and progressive disease, prolonged exposure to MMPs may lead to protracted inhibition of the Ca^{2+}-entry mechanisms of aortic smooth muscle contraction. This prolonged inhibition of vascular contraction may contribute to progressive aortic dilation and aneurysm formation over time. Therefore the effects of prolonged exposure to MMPs on smooth muscle function, both in vitro and in vivo, should be examined in future studies. We should mention that the present study tested the effects of MMPs on aortic smooth muscle in normotensive rats. Future studies should examine the effects of MMPs on vascular smooth muscle of animal models of hypertension, because the relation between vessel tonicity and diastolic hypertension could be a factor in aneurysm formation in human beings.

In conclusion, the present data suggest that MMP-2 and MMP-9 inhibit the Ca^{2+} entry mechanisms of aortic smooth muscle contraction, and may further explain the role of increased MMP activity during early development of AAA. MMP-9 is a more potent inhibitor of the mechanisms of vascular contraction than MMP-2 is, consistent with

experimental evidence in mice of its dominant role in AAA formation.

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