Elastase-Induced Suppression of Endothelin-Mediated Ca^{2+} Entry Mechanisms of Vascular Contraction

David K.W. Chew, Julia M. Orshal and Raouf A. Khalil

Hypertension. 2003;42:818-824; originally published online August 4, 2003; doi: 10.1161/01.HYP.0000086200.93184.8E

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/42/4/818
Elastase-Induced Suppression of Endothelin-Mediated Ca²⁺ Entry Mechanisms of Vascular Contraction

David K.W. Chew, Julia M. Orshal, Raouf A. Khalil

Abstract—Abdominal aortic aneurysm (AAA) is associated with increased endothelin (ET-1), both systemically and locally in the aorta. Also, elastase activity is increased in human AAA, and elastase perfusion of the aorta induces aneurysm formation in animal models of AAA. However, whether elastase directly affects the ET-1–induced mechanisms of aortic smooth muscle contraction is unclear. Isometric contraction and Ca²⁺ influx were measured in aortic strips isolated from male Sprague-Dawley rats and treated with elastase (5 U/mL). To avoid degradation of the extracellular matrix proteins by elastase, experiments were performed in the presence of elastin (10 mg/mL). In normal Krebs solution (2.5 mmol/L Ca²⁺), ET-1 (10⁻⁷ mol/L) caused contraction of aortic strips that was inhibited by elastase (5 U/mL). The elastase-induced inhibition of ET-1 contraction was slow in onset (4.6±0.4 minutes), time-dependent, complete in 34±3 minutes, and reversible. In Ca²⁺-free Krebs solution, caffeine (25 mmol/L) caused a small contraction that was not inhibited by elastase, suggesting that elastase does not inhibit Ca²⁺ release from the intracellular stores. Membrane depolarization by 96 mmol/L KCl, which stimulates Ca²⁺ entry from the extracellular space, caused a contraction that was inhibited by elastase in a concentration-dependent, time-dependent, and reversible fashion. The reversible inhibitory effects of elastase, particularly in the presence of elastin, suggest that they are not due to dissolution of the extracellular matrix or smooth muscle contractile proteins. Elastase also inhibited ET-1 and KCl-induced Ca²⁺ influx. Thus, elastase directly inhibits ET-1–induced Ca²⁺ entry mechanisms of vascular smooth muscle contraction, which may explain the role of elastase and ET-1 during the development of AAA. (Hypertension. 2003;42[part 2]:818-824.)

Key Words: endothelin ■ muscle, smooth ■ potassium ■ calcium ■ aorta
cellular space. Also, studies have shown that ET-1 increases intracellular Ca\(^{2+}\) and stimulates the Ca\(^{2+}\) mobilization mechanisms of vascular smooth muscle contraction. The purpose of this study was to test the hypothesis that elastase inhibits the ET-1–induced Ca\(^{2+}\) mobilization mechanisms of smooth muscle contraction. Experiments were designed to investigate (1) whether elastase inhibits ET-1–induced aortic smooth muscle contraction and (2) whether the inhibition of ET-1–induced smooth muscle contraction by elastase is due to inhibition of Ca\(^{2+}\) release from the intracellular stores or Ca\(^{2+}\) entry from the extracellular space. The effects of elastase on ET-1–induced contraction and \(^{45}\)Ca\(^{2+}\) influx were measured and compared with its effects on caffeine-induced contraction, which is mainly due to Ca\(^{2+}\) release from the intracellular stores, and its effects on high KCl-induced contraction, which is mainly due to Ca\(^{2+}\) entry from the extracellular space. To avoid the potential degradation of the extracellular matrix proteins by elastase, experiments were performed in the presence of saturating concentrations of elastin (10 mg/mL).

**Methods**

**Tissue Preparation**

Male Sprague-Dawley rats (12 weeks of age, 200 to 300 g in weight) were purchased from Charles River Laboratory, housed in the animal facility, and maintained on ad libitum standard rat chow and tap water in a 12 hour/12 hour light/dark cycle. The rats were euthanized by CO\(_2\) inhalation, and complete euthanasia was judged by cessation of breathing and heart beat. The chest and abdominal cavities were opened, and both the thoracic and abdominal aorta were rapidly perfused of the connective tissue matrix of the aorta by elastase, the tissue was incubated in the presence of increasing concentrations of elastin (1, 3, 6, and 10 mg/mL). The inhibitory effects of elastase on KCl contraction were completely reversible in the presence of elastin (10 mg/mL); therefore all experiments were performed in the presence of this concentration of elastin unless indicated otherwise.

Experiments were also designed to test the effects of elastase on ET-1 contraction. Previous studies have shown that the plasma ET-1 levels are elevated to \(\approx 3 \times 10^{-11}\) mol/L in patients with AAA. However, since ET-1 is a locally acting paracrine or autocrine protein rather than a circulating hormone, the plasma levels may not reflect a potentially much higher local ET-1 concentration at the interface between the endothelium and vascular smooth muscle cells. Preliminary experiments have shown that ET-1 causes concentration-dependent contraction of rat aortic strips. At concentrations \(<10^{-7}\) mol/L, the ET-1 contraction was slow in onset, small in magnitude, and reached steady state after a long period of time and thus made it difficult to measure the inhibitory effects of elastase. On the other hand, at \(10^{-7}\) mol/L, ET-1 caused a relatively rapid and maximal contraction that reached steady state in \(\approx 30\) minutes and thus made it feasible to analyze quantitatively the inhibitory effects of elastase. Therefore, aortic strips were stimulated with ET-1 \(10^{-7}\) mol/L until the contraction reached a plateau, then were treated with elastase (5 U/mL), and the changes in contraction were observed. Reversibility of the inhibitory effects of elastase was tested by treating the aortic strips with elastase (5 U/mL) in the absence or presence of elastin (10 mg/mL) for 30 minutes, then thoroughly rinsing the strips with Krebs solution, followed by stimulation with ET-1 \(10^{-7}\) mol/L.

In another set of experiments, the vascular strips were incubated for 45 minutes in Ca\(^{2+}\)-free (2 mmol/L ethylene glycol bis(\(\beta\) aminoethyl ether)-\(N,N,N',N'\)-tetra-acetic acid; EGTA) Krebs, then treated with 25 mmol/L caffeine, which stimulates Ca\(^{2+}\) release from the intracellular stores, to elicit a first contraction. The tissue was washed 6 times with normal Krebs for a total of 1 hour to replenish the intracellular Ca\(^{2+}\) stores and then incubated in the presence of elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes. The bathing medium was changed to Ca\(^{2+}\)-free Krebs and the tissue was treated with caffeine to elicit a second contraction. The second caffeine contraction was then presented as a percentage of the first caffeine contraction.

In other experiments, the vascular strips were stimulated with 96 mmol/L KCl, which causes membrane depolarization and Ca\(^{2+}\) entry from the extracellular space. Once the KCl response reached a plateau, the tissue was treated with elastase (5 U/mL) in the absence or presence of elastin (10 mg/mL), and the changes in KCl contraction were observed. The strips were thoroughly rinsed with Krebs solution and then restimulated with 96 mmol/L KCl to determine the reversibility of the effects of elastase.

\(^{45}\)Ca\(^{2+}\) Influx

Vascular strips were incubated in Krebs solution and then stimulated with ET-1 \(10^{-7}\) mol/L or 96 mmol/L KCl for 10 minutes in the absence or presence of elastase (5 U/mL) plus elastin (10 mg/mL). The tissues were transferred to the respective radioactive \(^{45}\)Ca\(^{2+}\)-labeled solution (specific activity, 5 \(\mu\)Ci/mL, ICN) for 90 seconds. Preliminary experiments have shown that the relationship between \(^{45}\)Ca\(^{2+}\) uptake versus time is linear during 15, 30, 60, and 90 seconds of exposure to the \(^{45}\)Ca\(^{2+}\) label. The tissues were then transferred to ice-cold Ca\(^{2+}\)-free Krebs for 45 minutes to quench extracellular \(^{45}\)Ca\(^{2+}\) label. The vascular strips were weighed and placed in 2 mL hypotonic (5 mmol/L) ethylenediamine-tetracetic acid (EDTA) 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 scintillation counter (Beckman LS 6500).
Histological Studies
Aortic strips were incubated for 30 minutes in Krebs solution in the absence or presence of elastase (5 U/mL) and elastin (10 mg/mL). Tissue sections were prepared for histological studies with the use of hematoxylin and eosin and Verhoeff elastin stain to assess for the integrity of the extracellular matrix and elastin in the vessel wall.

Solutions, Drugs, and Chemicals
Normal Krebs solution contained (in mmol/L): NaCl, 120; KCl, 5.9; NaHCO3, 25; NaH2PO4, 1.2; dextrose, 11.5; MgCl2, 1.2; CaCl2, 2.5; at pH 7.4. For Ca2⁺-free Krebs solution, CaCl2 was omitted and EGTA 2 mmol/L was added. KCl solution (96 mmol/L) was prepared as Krebs solution, with substitution of NaCl with KCl. ET-1, caffeine, and elastin were from Sigma. Porcine elastase grade II was from Boehringer Mannheim. All other chemicals were of reagent grade or better.

Statistical Analysis
Data were analyzed and expressed as mean±SEM. Data were compared by using the Student t test for unpaired and paired data. Differences were considered statistically significant at a level of P<0.05.

Results
In normal Krebs solution (2.5 mmol/L Ca2⁺), 10⁻⁷ mol/L ET-1 caused a significant contraction that reached a maximum in 26±4 minutes (Figure 1). Elastase (5 U/mL) caused inhibition of ET-1 contraction that was slow in onset (mean delay, 4.6±0.4 minutes) and complete in 34±3 minutes (Figure 1). In tissues treated with elastase (5 U/mL) for 30 minutes followed by rinsing with Krebs for 1 hour, the inhibitory effects of elastase on ET-1–induced contraction were partially reversible (Figure 2). Elastin (10 mg/mL) alone did not have any significant effect on the basal or ET-1–induced contraction. In tissues treated with elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes, the inhibitory effects of elastase on ET-1–induced contraction were completely reversible (Figure 2).

To determine if treatment with elastase in the absence or presence of elastin causes degradation of the medial smooth muscle or elastin, tissue sections were prepared for histological examination with the use of hematoxylin and eosin staining and Verhoeff stain for elastin. Tissue sections of the control aorta showed an intact tunica media and preserved elastin layer (Figure 3). In tissues treated with elastase (5 U/mL) for 30 minutes, marked elastin degradation in the tunica media was observed (Figure 3). In contrast, in tissues treated with elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes, the tunica media appeared to be preserved, and no apparent elastin degradation was detected when compared with control tissue (Figure 3).
In Ca\textsuperscript{2+}-free (2 mmol/L EGTA) Krebs solution, 25 mmol/L caffeine, which stimulates Ca\textsuperscript{2+} release from the intracellular stores,\textsuperscript{12} caused a small contraction (Figure 4A). The aortic strips were rinsed with normal Krebs for 1 hour to replenish the intracellular Ca\textsuperscript{2+} stores, and a second caffeine contraction was elicited in Ca\textsuperscript{2+}-free Krebs. In these control experiments, the second caffeine contraction was not significantly different from the first caffeine contraction (Figures 4A and 4C). In tissues treated with elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes, the second caffeine contraction was not significantly different from the first caffeine contraction (Figures 4B and 4C), suggesting that the intracellular Ca\textsuperscript{2+} release mechanisms are preserved during treatment with elastase.

Membrane depolarization by 96 mmol/L KCl, which is known to stimulate Ca\textsuperscript{2+} entry from the extracellular space,\textsuperscript{12} caused a significant contraction that reached a plateau in 9.5±0.4 minutes. Elastase caused inhibition of the KCl-induced contraction that was rapid in onset and concentration- and time-dependent (Figure 5). In tissues treated with elastase (5 U/mL) for 30 minutes, the inhibitory effects of elastase on KCl-induced contraction were partially reversible (Figures 6A and 6C). Elastin (10 mg/mL) alone did not have any significant effect on KCl-induced contraction. In tissues treated with elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes, the inhibitory effects of elastase on KCl-induced contraction were completely reversible (Figures 6B and 6D).

ET-1 (10\textsuperscript{-7} mol/L) and 96 mmol/L KCl caused significant increases in \textsuperscript{45}Ca\textsuperscript{2+} influx (Figure 7). In tissues treated with elastase (5 U/mL) plus elastin (10 mg/mL) for 30 minutes, the inhibitory effects of elastase on KCl-induced contraction were completely reversible (Figures 6B and 6D).

ET-1 (10\textsuperscript{-7} mol/L) and 96 mmol/L KCl caused significant

**Discussion**

The main findings of the present study are (1) elastase inhibits ET-1-induced aortic contraction, (2) elastase does not affect caffeine-induced contraction, which is a measure of Ca\textsuperscript{2+} release from the intracellular stores, and (3) elastase inhibits KCl-induced aortic contraction and \textsuperscript{45}Ca\textsuperscript{2+} influx.

The present study showed that elastase caused significant inhibition of ET-1–induced aortic contraction. The inhibition of ET-1–induced contraction by elastase could be due to an
effect on one of the components of the vessel wall, namely the endothelium, the smooth muscle, or the extracellular matrix. It is unlikely that the observed inhibition of vascular contraction is a result of an effect of elastase on endothelium-derived relaxing factors because the present experiments were performed in endothelium-denuded vascular strips. Another possibility is that elastase may cause elastin degradation and disruption of the extracellular matrix, which could then decrease the ability of the blood vessel to maintain contraction. This is supported by reports that elastase causes degradation of the extracellular matrix proteins in animal models of AAA.\textsuperscript{15,16} This is also supported by the present observations that the elastase-induced inhibition of ET-1 and KCl contraction were only partially reversible in tissues treated with elastase alone. Additionally, the present histological studies have shown marked elastin degradation in tissues treated with elastase alone. To minimize the potential proteolytic effects of elastase on the vessel wall, we tested the effects of elastase in the presence of saturating concentrations of elastin. The effectiveness of this strategy was confirmed by the histological finding of elastin preservation in the tunica media of tissues treated with elastase plus elastin. Also, the complete reversibility of the effects of elastase plus elastin on ET-1–induced and KCl-induced contraction and the lack of effect of elastase plus elastin on the caffeine-induced contraction suggest that the observed inhibitory effects of elastase are not due to irreversible degradation of the extracellular matrix. Conversely, the observed inhibitory effects of elastase on ET-1 contraction may involve direct effects on the mechanisms of vascular smooth muscle contraction.

Figure 5. Elastase-induced inhibition of KCl contraction is concentration- and time-dependent. Aortic strips were stimulated with 96 mmol/L KCl, then treated with increasing concentrations of elastase (1 to 30 U/mL), and the inhibition of KCl contraction was observed. Data points represent mean ± SEM of measurements in 6 vascular strips from 6 rats.

Figure 6. Reversibility of elastase-induced inhibition of KCl contraction. Aortic strips were stimulated with 96 mmol/L KCl, then treated with elastase (5 U/mL in the absence (A) or presence of elastin (10 mg/mL) (B), and the inhibition of KCl contraction was observed. Tissues were washed with Krebs solution 3 times for a total of 1 hour, and a second KCl contraction was elicited to determine the reversibility of elastase-induced inhibition. The second KCl contraction was then presented as percentage of the control 96 mmol/L KCl contraction (C and D). Data bars represent mean ± SEM of measurements in 6 vascular strips from 6 rats.

Figure 7. Basal, ET-1–induced (10\textsuperscript{–7} mol/L), and KCl-induced (96 mmol/L) ⁴⁰Ca\textsuperscript{2+} influx in aortic strips not treated or treated with 5 U/mL elastase. Data bars represent mean ± SEM of measurements in 25 to 30 vascular strips from 5 to 6 rats.
Vascular smooth muscle contraction is triggered by an increase in intracellular Ca\(^{2+}\) caused by Ca\(^{2+}\) release from the intracellular stores and Ca\(^{2+}\) entry from the extracellular space.\(^2\) The observation that elastase did not significantly inhibit caffeine-induced contraction suggests that elastase may not act by inhibiting the Ca\(^{2+}\) release mechanisms from the intracellular stores. On the other hand, the observation that elastase significantly inhibited KCl-induced contraction suggests that it may function through the inhibition of Ca\(^{2+}\) entry from the extracellular space. This is supported by the observation that elastase inhibited both ET-1-induced and KCl-induced \(^{45}\)Ca\(^{2+}\) influx.

The specific mechanism by which elastase inhibits Ca\(^{2+}\) entry is unclear at the present time. The elastase-induced inhibition of Ca\(^{2+}\) entry does not appear to be due to membrane hyperpolarization and activation of potassium channels because elastase inhibited KCl-induced contraction and Ca\(^{2+}\) influx despite the fact that the high extracellular KCl gradient does not favor the diffusion of potassium ions from the intracellular to the extracellular space. Other possible mechanisms may include (1) direct interaction of elastase with the Ca\(^{2+}\) channels, (2) an effect mediated by integrin receptors that are coupled to the Ca\(^{2+}\) channels, or (3) an effect mediated by elastase-sensitive receptors, which activate signaling pathways that block the Ca\(^{2+}\) channels. It has been suggested that the discharge of elastase from neutrophils can degrade denatured collagen and release Arg-Gly-Asp (RGD)-containing peptides, which may bind to \(\alpha\)\(_{v}\)\(\beta\)\(_{3}\) integrin receptors and thereby inhibit Ca\(^{2+}\) entry by inhibiting integrin-mediated tyrosine kinase-dependent phosphorylation and activation of the Ca\(^{2+}\) channels.\(^17\)-\(^20\) Also, elastase is a member of a family of serine proteases that may stimulate protease-activated receptors (PARs) in a multitude of cell types. PARs mediate a myriad of cellular responses to various extracellular proteases through G-protein–coupled intracellular signaling pathways.\(^21\) Recent studies have identified PARs in smooth muscle cells, which may play a role in smooth muscle proliferation and migration.\(^22\) Other studies have shown that activation of PARs causes vasodilation through a nitric oxide–dependent mechanism.\(^23\) Further studies are needed to define the role of integrins and PARs as possible molecular mechanisms by which elastase could inhibit Ca\(^{2+}\) entry into the smooth muscle.

The smooth muscle cells in the tunica media serve important functions for mechanical support as well as maintenance of vascular tone. Atrophy of the tunica media and depletion of smooth muscle cells are consistent histological findings in end-stage large AAA in humans.\(^24\) Ultrastructural evidence of smooth muscle cell injury has also been described in the elastase-treated animal models of AAA.\(^25\) Nevertheless, the possible role and significance of smooth muscle dysfunction in the pathogenesis of AAA is unclear. In the present study, we have shown that one of the initial effects of elastase on the aortic wall is inhibition of ET-1-induced smooth muscle contraction. This inhibitory effect of elastase on smooth muscle contraction may explain the initial dilatation of the aorta that is commonly observed immediately after the administration of elastase in animal models of AAA and that has been solely attributed to loss of elastin.\(^26\) The present study suggests that in addition to causing elastin degradation in the vessel wall, elastase also inhibits smooth muscle contraction by suppression of the Ca\(^{2+}\) entry mechanisms, which may represent some of the early events during aneurysm formation. We should note that the acute in vitro effects of elastase appear to be reversible at the dose and duration of elastase treatment tested. However, the effects of elastase may be different in vivo, where an initial elastase insult could initiate a cascade of events, leading to progressive smooth muscle dysfunction and cell death. Also, the effects of a single exposure to elastase may be different from repeated and chronic exposure to elastase. The degradative enzymes matrix metalloproteinases (MMPs) have significant elastolytic activities.\(^27\) MMP 2, MMP 3, and MMP 9 have been found in abundance in aneurysm tissues and have therefore been implicated in the pathogenesis of AAA.\(^28\) MMP 2 has the greatest elastase activity and is produced mainly by mesenchymal cells such as smooth muscle cells and fibroblasts.\(^29\) On the other hand, MMP 9 is produced mainly by the aneurysm-infiltrating macrophages, which are part of the inflammatory cellular infiltrate in AAA disease.\(^30\) It seems reasonable to postulate that chronic exposure to elastase and other related elastolytic enzymes could lead to protracted inhibition of the Ca\(^{2+}\)-dependent mechanisms of aortic smooth muscle contraction. This prolonged inhibition of vascular contraction may diminish the ability of the aortic wall to withstand the hemodynamic forces generated with each cardiac cycle, leading to progressive dilation and aneurysm formation over time. The effects of chronic exposure to elastase on smooth muscle function, both in vitro and in vivo, should therefore represent important areas for future investigations.

**Perspectives**

An increase in elastase activity is a consistent finding in human AAA, and elastase perfusion of the aorta induces the formation of aneurysm in animal models of AAA.\(^1\)-\(^7\) Also, the levels of ET-1 are elevated systemically in the plasma of patients with AAA\(^11\) and locally in sites of elastic tissue defects in the aorta of rats prone to develop aneurysm.\(^31\) The increased ET-1 and elastase activities in patients with AAA and in animal models of AAA have suggested possible modulating effects of elastase on the vasoconstrictive actions of ET-1 during AAA formation. The present study has shown that elastase inhibits ET-1–induced Ca\(^{2+}\) entry mechanisms of aortic smooth muscle contraction. Elastase also inhibited the contraction and Ca\(^{2+}\) influx in response to high KCl depolarizing solution, suggesting that it inhibits Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels. Further electrophysiological and molecular studies are needed to determine the mechanisms through which elastase could inhibit the Ca\(^{2+}\) entry pathways and whether it involves direct interaction with the Ca\(^{2+}\) channel or possible interactions with integrins or elastase-sensitive receptors. These studies should help explain the relation between the increased ET-1 levels and elastase activity during the development of AAA. However, vasoconstrictor agonists other than ET-1 may also play a role in AAA. Angiotensin II has been implicated in aneurysm formation in atherosclerosis-prone apolipoprotein E–deficient (apoE/–/–)
mice, and ACE inhibitors suppress aneurysm formation in the elastase-perfused rat aorta. Investigation of the effects of elastase on the Ca²⁺ mobilization mechanisms of arterial contraction in response to angiotensin II and other vasoconstrictor agonists such as norepinephrine or prostaglandin F₂α would determine whether the effects of elastase are specific to a particular agonist/receptor or involve effects on post-receptor signaling mechanisms.

Acknowledgments
This work was supported by grants from the National Heart, Lung, and Blood Institute (HL-52696 and HL-65998). Dr Khalil is an Established Investigator of the American Heart Association. We gratefully acknowledge the assistance and expertise of Sandra Zapata-Taylor in the tissue sectioning and staining for the histological studies.

References