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7-1-1997

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Chao, Juli; Stallone, John N.; Liang, Yu-Mei; Chen, Li-Mei; Wang, Dan-Zhao; and Chao, Lee, "Kallistatin Is a Potent New Vasodilator" (1997). *MUSC Faculty Journal Articles*. 107.

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## Kallistatin Is a Potent New Vasodilator

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### Abstract

Kallistatin is a serine proteinase inhibitor which binds to tissue kallikrein and inhibits its activity. The aim of this study is to evaluate if kallistatin has a direct effect on the vasculature and on blood pressure homeostasis. We found that an intravenous bolus injection of human kallistatin caused a rapid, potent, and transient reduction of mean arterial blood pressure in anesthetized rats. Infusion of purified kallistatin (0.07–1.42 nmol/kg) into cannulated rat jugular vein produced a 20–85 mmHg reduction of blood pressure in a dose-dependent manner. Hoe 140, a bradykinin B<sub>2</sub>-receptor antagonist, had no effect on the hypotensive effect of kallistatin yet it abolished the blood pressure-lowering effect of kinin and kallikrein. Relaxation of isolated aortic rings by kallistatin was observed in the presence (ED<sub>50</sub> of  $3.4 \times 10^{-9}$  M) and in the absence of endothelium (ED<sub>50</sub> of  $10^{-9}$  M). Rat kallikrein-binding protein, but not kinin or kallikrein, induced vascular relaxation of aortic rings. Neither Hoe 140 nor N<sup>ω</sup>-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, affected vasorelaxation induced by kallistatin. Kallistatin also caused dose-dependent vasodilation of the renal vasculature in the isolated, perfused rat kidney. Specific kallistatin-binding sites were identified in rat aorta by Scatchard plot analysis with a K<sub>d</sub> of  $0.25 \pm 0.07$  nM and maximal binding capacity of  $47.9 \pm 10.4$  fmol/mg protein (mean  $\pm$  SEM,  $n = 3$ ). These results indicate that kallistatin is a potent vasodilator which may function directly through a vascular smooth muscle mechanism independent of an endothelial bradykinin receptor. This study introduces the potential significance of kallistatin in directly regulating blood pressure to reduce hypertension. (*J. Clin. Invest.* 1997; 100: 11–17.) Key words: kallistatin • blood pressure • vasorelaxation • renal pressure • kallikrein

### Introduction

The tissue kallikrein-kinin system has been postulated to play an important role in blood pressure regulation (1–3). The best

characterized function of tissue kallikrein is to generate kinin peptides from precursor kininogens by limited proteolysis. The actions of kinins, such as vasodilation, blood pressure reduction, smooth muscle contraction and relaxation, pain, and inflammation, are mediated by kinin receptors (1, 4). Tissue kallikrein is synthesized as a proenzyme but is generally present in tissues and body fluids in an active form. The activity of tissue kallikrein is regulated by kallistatin, a newly identified tissue kallikrein-binding protein (5–7). Kallistatin is a serine proteinase inhibitor and is capable of inhibiting tissue kallikrein's kininogenase and amidolytic activities in vitro (6). Human tissue kallikrein, when complexed with kallistatin, has a fourfold longer half-life in the circulation of rats than that of kallikrein alone (8). These findings suggest that one of kallistatin's functions may involve the maintenance of kallikrein's bioavailability in the circulation or in tissues. Rat kallikrein-binding protein (RKBP)<sup>1</sup> has also been purified and characterized and is functionally related to human kallistatin (5, 6, 9). RKBP forms SDS-stable complexes with rat and human tissue kallikreins and inhibits their activities (9, 10). Evidence indicates that RKBP may play a role in regulating kallikrein's activity in vivo (9).

The major site of kallistatin synthesis is the liver, and to a lesser extent the pancreas, kidney, lung, heart, colon, and other tissues (7, 11, 12). Tissue distribution studies showed that immunoreactive kallistatin is present in the circulation and in tissues, blood cells, and endothelial cells involved in cardiovascular function (11). There are two lines of evidence suggesting that kallistatin may have a function other than binding and regulating the activity and bioavailability of tissue kallikrein. First, kallistatin is present in the plasma in great excess of circulating tissue kallikrein, suggesting an alternative function. Second, RKBP, the analog of human kallistatin, is significantly reduced in spontaneously hypertensive rats (5), indicating its potential role in maintaining normal blood pressure. We therefore evaluated the potential function of kallistatin by a number of biochemical and biological assays and discovered that kallistatin has potent vasodilating activity. In this study, we show that kallistatin reduces mean arterial blood pressure in anesthetized rats and renal perfusion pressure in isolated rat kidneys and induces vasorelaxation in isolated rat aortic rings. These results indicate that kallistatin has a vasodilating effect on rat vasculature and that the effect is independent of kallistatin's interaction with tissue kallikrein.

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Received for publication 7 June 1996 and accepted in revised form 2 April 1997.

*J. Clin. Invest.*

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0021-9738/97/07/0011/07 \$2.00

Volume 100, Number 1, July 1997, 11–17

1. Abbreviations used in this paper: B<sub>max</sub>, maximal binding capacity; EBDA, equilibrium binding data analysis; Endo–, endothelium denuded; Endo+, endothelium intact; KHB, Krebs-Henseleit-bicarbonate solution; L-NAME, N<sup>ω</sup>-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; RKBP, rat kallikrein-binding protein; SHR, spontaneously hypertensive rats.

## Methods

**Animals.** Sprague-Dawley, Spontaneously Hypertensive, Wistar-Kyoto, Brown-Norway, Dahl salt-sensitive, or Dahl salt-resistant rats (male, 250–300 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Sprague-Dawley rats used for the isolated vasculature studies were purchased from Zivic-Miller Laboratories (Zelienople, PA).

**Materials.** Human  $\alpha$ 1-antitrypsin and  $\alpha$ 1-antichymotrypsin were purchased from Athens Research and Technology (Athens, GA). Lys-bradykinin (kallidin), bradykinin, bovine serum albumin, and  $N^G$ -nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO). D-Arg-[Hyp<sup>3</sup>, Thi<sup>5</sup>, D-Tic<sup>7</sup>, Oic<sup>8</sup>]-bradykinin (Icatibant or Hoe 140) was a gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ).

**Kallistatin and kallikrein purification.** Human kallistatin and RKBP were purified to apparent homogeneity from human and rat plasma, respectively, as previously described (5, 6). Tissue kallikreins from rat and human were purified as previously described (13, 14).

**Blood pressure measurements.** Blood pressure was determined by direct arterial cannulation in rats anesthetized with pentobarbital (50 mg/kg body weight). Two PE 10 (polyethylene) catheters filled with heparinized saline solution (20 U heparin/ml saline) were inserted via the left carotid artery and right jugular vein, respectively. The carotid catheter was connected to a pressure transducer (Guttmann-Statham Transducers, Inc., Costa Mesa, CA) coupled to a polygraph (model 7; Grass Instrument Co., Quincy, Mass). Drugs, in 0.2 ml saline, were injected and flushed with an additional 0.1 ml saline into the vena cava.

**Thoracic aortic ring preparation.** The rat thoracic aorta was used for isolated vascular ring studies. Male Sprague-Dawley rats were killed by decapitation and thoracic aortas were removed and placed in chilled (4°C) Krebs-Henseleit-bicarbonate solution (KHB, composition [mM]: 118.0 NaCl, 25.0 NaHCO<sub>3</sub>, 10.0 glucose, 4.74 KCl, 2.50 CaCl<sub>2</sub>, 1.18 MgSO<sub>4</sub>, and 1.18 KH<sub>2</sub>PO<sub>4</sub> [pH 7.4, osmolality, 292±1 mosmol/kg H<sub>2</sub>O]), which was gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The blood vessels were cleaned of all connective tissue and fat and 3-mm segments were mounted in water-jacketed tissue baths containing 15.0 ml of KHB (gassed and maintained at 37°C for measurement of isometric tension, according to Stallone (15). Passive tension was adjusted to the optimum for the male rat aorta (2.50 g) and a 2-h equilibration period was allowed before any experimental intervention (e.g., kallistatin, kallidin, tissue kallikrein, L-NAME, or Hoe 140). Extreme care was exercised during preparation of the rings to preserve the integrity of the endothelium, which was evaluated functionally in all experiments.

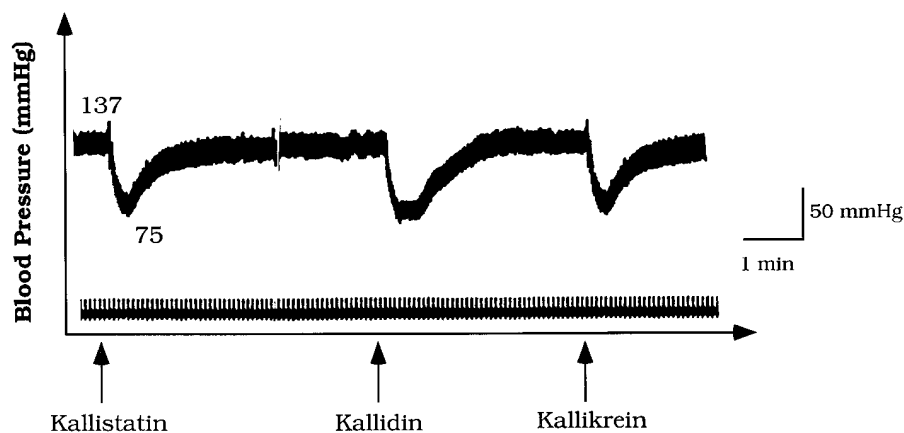
**Perfused kidney preparation.** Perfusion of the rat renal vasculature followed the methods originally described by Malik and McGiff

(16) as used recently by Cooper and Malik (17) and Burton and Stallone (18). Rats were anesthetized with ether, the left renal artery was cannulated, and the kidney was gently perfused (to avoid endothelial damage) with chilled, heparinized KHB (100 U/ml). The kidney was then isolated and transferred to a 37°C organ bath (within 60–90 s) and perfused at a constant flow rate (5.0 ml/min) with warmed, gassed KHB (37°C, 95% O<sub>2</sub>/5% CO<sub>2</sub>). The perfusate was not recirculated and was allowed to flow out the cut ends of the renal vein and ureter. Perfusion pressure was monitored continuously with a pressure transducer and recorded. Because flow rate was constant, changes in perfusion pressure reflected changes in vascular resistance. The kidney was perfused for 45–60 min before any experimental intervention. Test substances were administered as 50- $\mu$ l bolus injections or as constant infusions (0.10 ml/min) via a sidearm of the perfusate inflow cannula.

**Kallistatin-binding assay in rat aortic membrane proteins.** Purified kallistatin was used as a radioligand for the binding assay. It was iodinated using the iodogen method, and purified through an Excullulose GF-5 column. The specific activity was 4,900 Ci/mmol. For the membrane binding assay, male Sprague-Dawley rats were anesthetized and aorta was removed and washed in ice-cold normal saline solution. Aortic tissues were then minced and homogenized with a glass homogenizer at 4°C in 50 mM Tris-HCl, pH 7.4, containing 250 mM sucrose. The homogenate was centrifuged at 500 g for 10 min at 4°C. The supernatant was collected and further centrifuged at 50,000 g for 30 min at 4°C. The pellet was resuspended in 20 mM Hepes, pH 7.6, containing 0.1% BSA, 1 mM PMSF, 50  $\mu$ g/liter soybean trypsin inhibitor, and 2 mM benzamidine. The membrane protein preparation was stored at –80°C. The concentration of membrane protein was determined by a protein assay (Bio-Rad, Hercules, CA). For saturation studies, <sup>125</sup>I-kallistatin was incubated in duplicate with 15  $\mu$ g of membrane protein at 25°C in a volume of 250  $\mu$ l for 1 h. The assay was terminated by filtration over a Fisher AcetatePlus filter membrane (0.45  $\mu$ m) (Fisher Scientific, Pittsburgh, PA), using a filter apparatus (Hofer Scientific, San Francisco, CA). The tubes and filters were rinsed three times with 4 ml of ice-cold 20 mM Hepes buffer, pH 7.6, and 0.1% BSA and the filters were counted in a 1261 Multigamma counter (Pharmacia, Turku, Finland). Saturable binding was calculated by subtracting the nonspecific binding in the presence of 1  $\mu$ M unlabeled kallistatin. The Scatchard plot was analyzed with the equilibrium binding data analysis (EBDA) program.

## Results

**Hypotensive activity of kallistatin.** An intravenous bolus injection of purified human kallistatin (1.42 nmol/kg) into the jugular vein of anesthetized rats produced a transient and



**Figure 1.** Changes in the arterial blood pressure after intravenous injection of human kallistatin (1.42 nmol/kg), kallidin (lys-bradykinin) (26 nmol/kg), or rat tissue kallikrein (0.86 nmol/kg). Sprague-Dawley rat (male, body weight 300 g) was anesthetized with pentobarbital (50 mg/kg). Blood pressure was monitored by a Grass polygraph and a Statham pressure transducer connected to the carotid artery cannula. The sample, in 0.2 ml normal saline, was injected into the right jugular vein. *Ab-scissa:* time; *ordinate:* arterial blood pressure recorded. Chart speed: 10 mm/min.

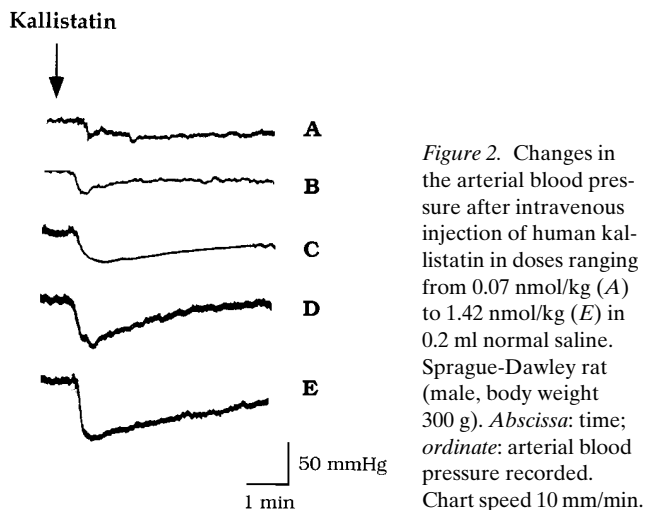


Figure 2. Changes in the arterial blood pressure after intravenous injection of human kallistatin in doses ranging from 0.07 nmol/kg (A) to 1.42 nmol/kg (E) in 0.2 ml normal saline. Sprague-Dawley rat (male, body weight 300 g). Abscissa: time; ordinate: arterial blood pressure recorded. Chart speed 10 mm/min.

rapid reduction in mean arterial blood pressure which occurred in seconds and reached maximal reduction within 1 min (Fig. 1). A reduction in mean blood pressure of similar magnitude and profile was observed when kallidin (lys-bradykinin) (26 nmol/kg) and tissue kallikrein (0.86 nmol/kg) were injected intravenously into rats (Fig. 1). Fig. 2 shows that the effect of human kallistatin was dose dependent. Injections of purified kallistatin at various doses (from 0.07 to 1.42 nmol/kg) into the rat jugular vein produced a 20–85 mmHg reduction of blood pressure in a dose-dependent manner. Repeated injections of kallistatin did not inhibit the hypotensive activity, since the magnitude of the blood pressure-lowering effect was not affected (data not shown). Transient reductions in blood pressure by bolus intravenous injections of kallistatin were observed in several strains of normotensive and hypertensive rats such as Sprague-Dawley, Wistar-Kyoto, Brown-Norway, Dahl salt-resistant, Dahl salt-sensitive, and spontaneously hypertensive rats. Other human plasma proteins such as  $\alpha$ 1-antitrypsin,  $\alpha$ 1-antichymotrypsin, and albumin at equivalent or higher doses had no effect on blood pressure of the rats (data not shown).

**Effects of Hoe 140 on the blood pressure-lowering effect of kallistatin, kinin, and tissue kallikrein.** To examine whether kinin mediates the hypotensive effect of kallistatin, we evaluated the effects of Hoe 140, a specific bradykinin  $B_2$ -receptor antagonist, on kallistatin-induced blood pressure reduction. Fig. 3 A shows that the blood pressure-lowering effect of kallistatin was not affected by the administration of Hoe 140 (26 nmol/kg body wt). However, Hoe 140 at the same concentration abolished the blood pressure-lowering effect of kallidin (Fig. 3 B) and it blocked the vasodepressor effect of rat tissue kallikrein by > 80% (Fig. 3 C).

**Kallistatin induces vascular relaxation of isolated thoracic aortic rings.** Fig. 4 shows the effect of kallistatin on the relaxation of isolated rat aortic rings. Thoracic aortic rings were mounted for isometric tension recording and were precontracted with phenylephrine. Human kallistatin at concentrations in the tissue baths from  $10^{-11}$  to  $10^{-8}$  M induced dose-dependent relaxation of isolated thoracic aortas (Fig. 4 A). Kallistatin-induced vascular relaxation occurred in both endothelium-intact (Endo+) and endothelium-denuded (Endo-) preparations, although the effect was significantly greater ( $P <$

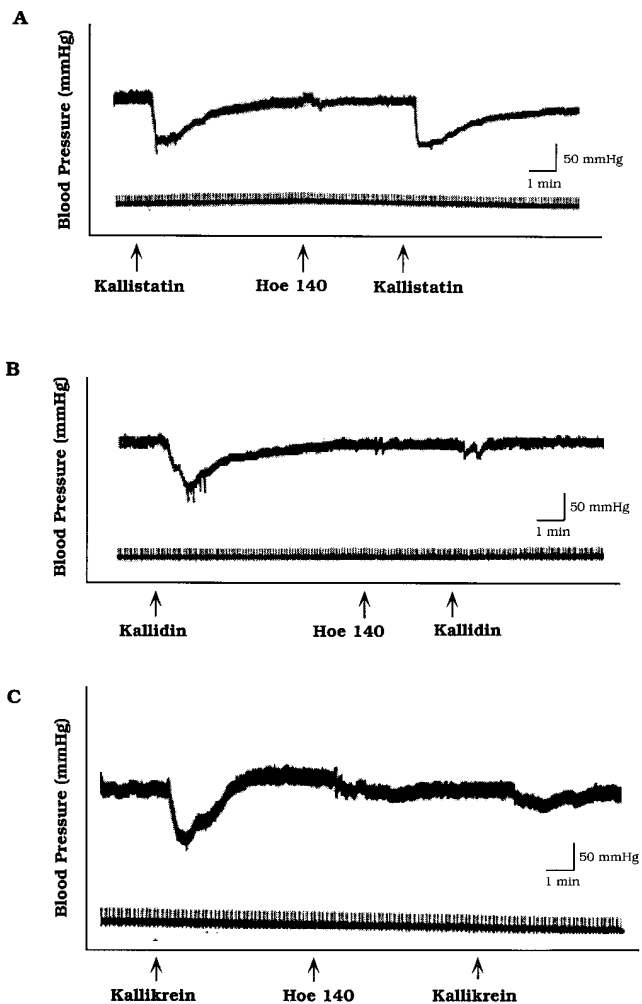


Figure 3. Effect of Hoe 140 on the blood pressure-lowering effect induced by kallistatin (A), kallidin (B), and rat tissue kallikrein (C). Abscissa: time; ordinate: arterial blood pressure recorded. Chart speed: 10 mm/min.

0.05) in the absence of the endothelium (Fig. 4 A). Kallistatin produced vascular relaxation of the isolated rat aorta with an  $ED_{50}$  of  $\sim 3.4 \times 10^{-9}$  M with Endo+ aorta and  $10^{-9}$  M with Endo- aorta. However,  $\alpha$ 1-antitrypsin at the same concentrations had no effect on vascular relaxation (data not shown). RKBP also produced dose-dependent vasorelaxation of the Endo+ and the Endo- rat aorta (Fig. 4 B). Similar to human kallistatin, RKBP exerts a greater vascular relaxation of the rat aorta in the absence of the intact endothelium. In contrast, neither kinin (Fig. 4 C) nor rat tissue kallikrein (Fig. 4 D), at concentrations ranging from  $10^{-11}$  to  $10^{-8}$  M in the tissue baths, had any significant vasorelaxing effects on the isolated rat aorta. These results suggest that kallistatin-induced vascular relaxation is endothelium independent and may act directly on vascular smooth muscle cells.

**Effects of Hoe 140 and L-NAME on kallistatin-induced vascular relaxation of isolated thoracic aortic rings.** To determine the role of the bradykinin receptor on the vascular action of kallistatin, we examined the effects of bradykinin receptor blockade on kallistatin-induced vasorelaxation. Pretreatment

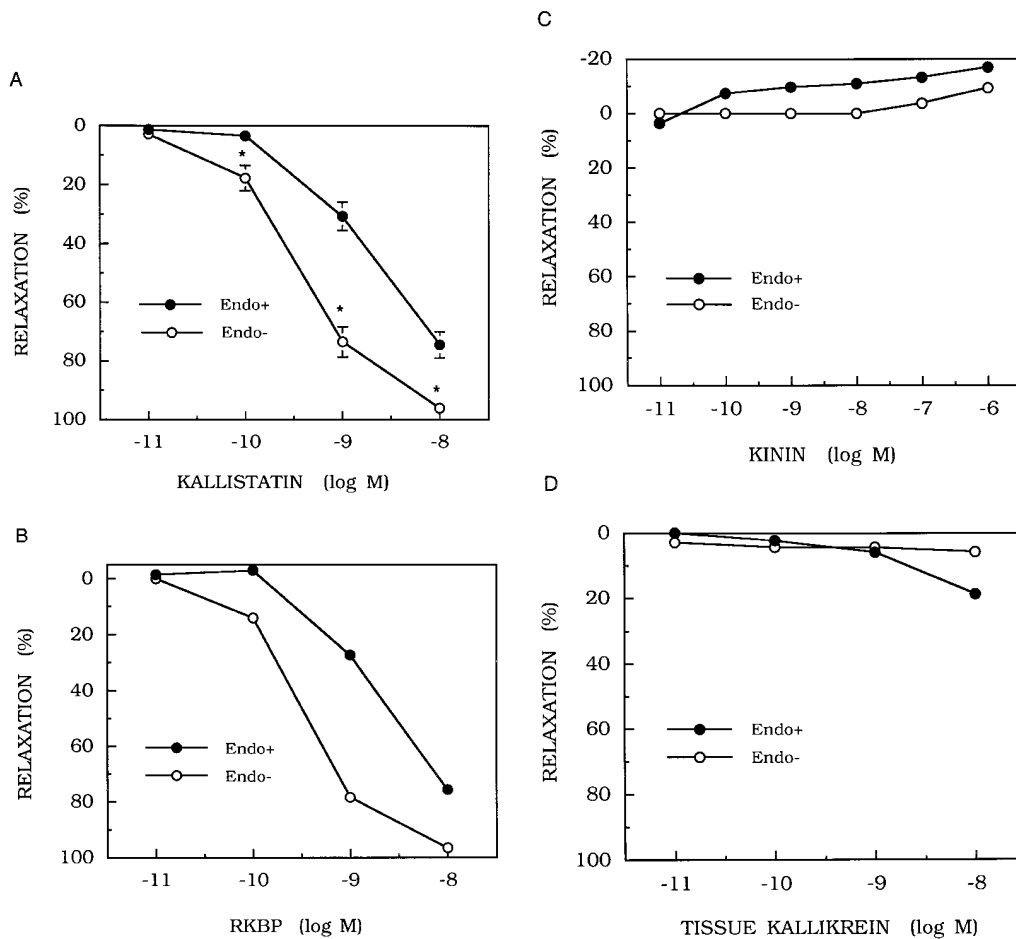


Figure 4. Dose-response effects of kallistatin and RKBP on relaxation of isolated rat aortic rings. Thoracic aortic rings were prepared from male Sprague-Dawley rats and mounted for isometric tension recording (in KHB, 37°C, 2.5 g passive tension). Paired aortic rings were studied with their Endo+ or Endo-. Aortas were precontracted with phenylephrine to an active baseline tension of 2–3 g. Data points are means  $\pm$  standard error. (A) Effect of human kallistatin in paired Endo+ and Endo- aortas ( $n = 4$  animals) (\* $P < 0.05$ ). (B) Effect of RKBP in paired Endo+ and Endo- aortas (representative data). (C) Effect of kinin in paired Endo+ and Endo- aortas (representative data). (D) Effect of rat tissue kallikrein in paired Endo+ and Endo- aortas (representative data).

of Endo+ aortic rings with the bradykinin  $B_2$ -receptor blocker, Hoe 140, ( $10^{-6}$  M) had no significant effect ( $P > 0.05$ ) on kallistatin-induced vascular relaxation (Fig. 5 A). To determine the role of nitric oxide in the vascular action of kallistatin, we examined the effects of nitric oxide synthase inhibition with L-NAME on kallistatin-induced vasorelaxation. Pretreatment of Endo+ aortic rings with L-NAME ( $2.5 \times 10^{-4}$  M) had no significant effect ( $P > 0.05$ ) on kallistatin-induced vascular relaxation (Fig. 5 B).

**Kallistatin induces vasodilation of isolated perfused rat kidney.** Fig. 6 shows the dose-response effects of kallistatin on the perfusion pressure in the isolated rat kidney. Under constant flow conditions, the renal vasculature was precontracted with phenylephrine to a perfusion pressure of  $\sim 125$  mmHg and constant infusions of kallistatin or rat tissue kallikrein ( $0.1$  ml/min for 10 min;  $10^{-9}$ – $10^{-5}$  M) were administered into the perfusion circuit, producing final concentrations from  $2 \times 10^{-11}$  to  $2 \times 10^{-7}$  M. Kallistatin produced dose-dependent reductions in renal vascular perfusion pressure that averaged  $21 \pm 4$  mmHg at the highest dose tested ( $2 \times 10^{-7}$  M). In contrast, rat tissue kallikrein failed to alter renal vascular perfusion pressure (data not shown). For comparison, we evaluated the dose-response effects of bradykinin on renal vascular perfusion pressure. Under constant flow conditions with the renal vasculature precontracted with phenylephrine, 50- $\mu$ l bolus injections of bradykinin ( $10^{-9}$ – $10^{-4}$  M) produced dose-dependent biphasic changes in renal perfusion pressure that con-

sisted of an initial, rapid vasodilation and a secondary, prolonged vasoconstriction. At the highest dose ( $10^{-4}$  M), bradykinin produced an initial vasodilation of 18 mmHg and a secondary vasoconstriction of 26 mmHg (data not shown).

**Kallistatin-binding sites in aorta.** The binding of  $^{125}$ I-kallistatin at 25°C to rat aortic membrane proteins was a time-dependent process. An apparent binding equilibrium was reached after 1 h; the specific binding was  $\sim 35$ –40% of the total binding. The subsequent binding activity study was performed at 25°C for 1 h. Saturation binding of  $^{125}$ I-kallistatin to rat aortic membrane proteins is shown in Fig. 7. Scatchard plot analysis of the binding data revealed the presence of a single class of binding sites for kallistatin with the apparent  $K_d$  of  $0.25 \pm 0.07$  nM and the maximal binding capacity ( $B_{max}$ ) of  $47.9 \pm 10.4$  fmol/mg protein (mean  $\pm$  SEM,  $n = 3$ ). These results indicate that specific kallistatin-binding sites exist on aortic membrane proteins.

## Discussion

This study shows that kallistatin has potent vasodilating activities through a mechanism independent of its interaction with tissue kallikrein. It produces a rapid and transient reduction in mean arterial blood pressure in anesthetized rats, causes vasorelaxation in rat thoracic aortic rings, and vasodilation of the isolated, perfused rat kidney in a dose-dependent manner. On a molar basis, the potencies of kallistatin and tissue kallikrein

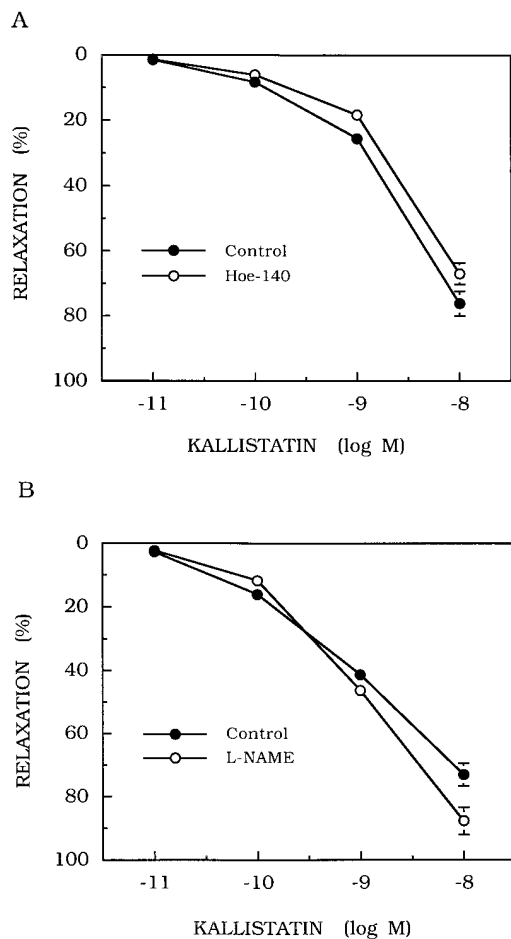


Figure 5. Effects of Hoe 140 and L-NAME on kallistatin-induced relaxation of isolated rat thoracic aortic rings. (A) Effect of Hoe 140 ( $10^{-6}$  M) on kallistatin-induced vasorelaxation ( $n = 4$  animals). (B) Effect of L-NAME ( $2.5 \times 10^{-4}$  M) on kallistatin-induced vasorelaxation ( $n = 4$  animals).

for lowering blood pressure in rats are similar. In contrast, both bradykinin and kallidin are less potent in vasodilation, possibly due to their effective degradation after a single pass through the pulmonary vascular bed. The blood pressure-lowering effect and vasorelaxation induced by kallistatin are not affected by Hoe 140 (19). Hoe 140 abolishes the transient blood pressure reduction induced by kinin or kallikrein, indicating that kallikrein's activity as a vasodilator is mediated by kinin activation of the  $B_2$ -receptor. In contrast, the vasodepressor effect of kallistatin was not affected by the administration of Hoe 140. These observations support the notion that kallistatin has a direct vasodilating action independent of kinin or tissue kallikrein and participates in regulating vascular tone independent of the endothelial bradykinin receptor. The relaxation of vascular smooth muscle, induced by both kallistatin and RKBP, is further enhanced by the absence of endothelium. These observations suggest that a constrictor may be released by the endothelium in response to kallistatin, thus blunting the vasorelaxant effect on the muscle cells. Alternatively, kallistatin may bind directly to vascular smooth muscle cell receptors to mediate biological actions. It is also possible that kallistatin receptors are located in the muscle layer and

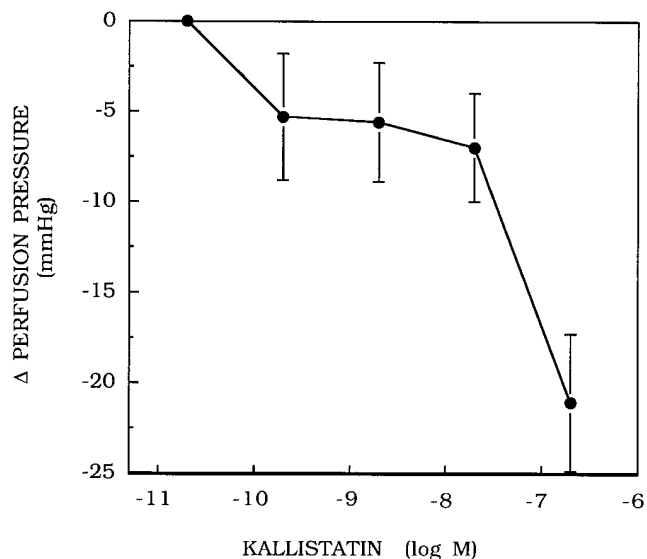


Figure 6. Concentration-response effects of kallistatin on the perfusion pressure of the isolated perfused rat kidney. Under constant flow conditions (perfused with KHB solution,  $37^\circ\text{C}$ , 95%  $\text{O}_2$ /5%  $\text{CO}_2$ ), the renal vasculature was precontracted with phenylephrine to a perfusion pressure of 125 mmHg and constant infusions of kallistatin ( $0.10$  ml/min for 10 min) were administered into the perfusion circuit, producing final concentrations of  $2 \times 10^{-11}$  to  $2 \times 10^{-7}$  M ( $n = 4$  animals).

that delivery of kallistatin from the blood through the endothelium leads to degradation of the kallistatin or its translocation to other sites of action.

In the combined studies of the isolated, perfused rat kidney and the isolated vascular rings from rat aorta, we presented

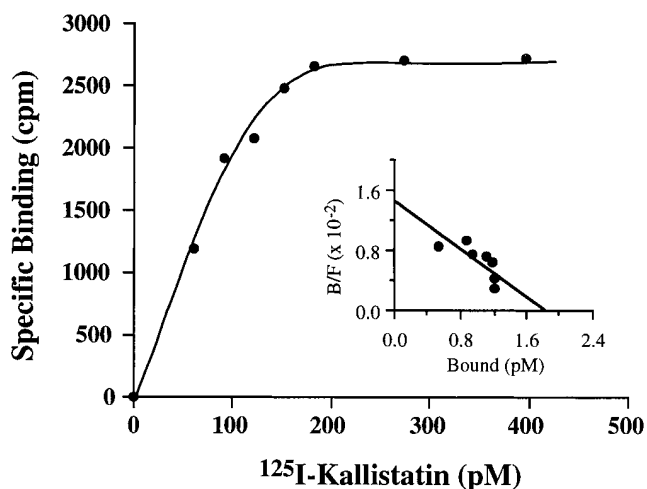


Figure 7. Representative saturation binding curve and Scatchard plot analysis of kallistatin binding in rat aorta. The specific binding of kallistatin (y axis) to the crude membrane proteins from rat aorta is shown with increasing concentrations of  $^{125}\text{I}$ -kallistatin (x axis). Scatchard plot analysis revealed that the membrane of the rat aorta contained specific kallistatin binding sites with a  $K_d$  of  $0.25 \pm 0.07$  nM and  $B_{\text{max}}$  of  $47.9 \pm 10.4$  fmol/mg protein (mean  $\pm$  SEM,  $n = 3$ ) as determined by analyzing saturation data from three independent experiments in duplicate with the EBDA program.

data in support of the hypothesis that kallistatin exerts a direct vasodilatory effect on the vasculature, which is responsible, at least in part, for its hypotensive effect on systemic blood pressure. As with virtually all directly vasoactive compounds, it is likely that the vasodilatory effect of kallistatin is subject to modulation by local vasodilators and/or vasoconstricting substances produced in endothelium and/or vascular smooth muscle. Our experiments with Endo- preparations suggest that a constrictor factor from the endothelium, perhaps endothelin (or a constrictor prostanoid), attenuates the vasodilatory effects of kallistatin. The most important finding of these experiments is that kallistatin-induced vasodilation involves an endothelium-independent action on the blood vessel (probably vascular smooth muscle) to produce vasorelaxation. We intend to examine the mechanisms of kallistatin action in vascular smooth muscle and its ability to produce vasorelaxation in our future studies.

The activity of kallistatin can be easily distinguished from that of tissue kallikrein and kinin when assayed with isolated aortic rings. Neither tissue kallikrein nor kinin exerts any vasorelaxing effect on isolated aortic rings when tested over a wide range of concentrations (Fig. 4 and reference 20). In contrast, kallistatin showed a strong vasorelaxation effect in this assay. The physiological concentration of kallistatin in the plasma is  $3 \times 10^{-7}$  M (20–25  $\mu\text{g/ml}$  plasma) (11). The concentrations of kallistatin introduced into the rat jugular vein and to which isolated aortic rings were exposed were  $10^{-8}$  to  $10^{-11}$  M which were within the normal physiologic concentration of this plasma protein. In another assay, kallistatin has been shown to reduce perfusion pressure in the renal vasculature in a dose-dependent manner. The response is clearly different from that of bradykinin which expresses a biphasic effect with initial vasodilation and subsequent vasoconstriction. This difference in renal responses strongly suggests that different mechanisms may be involved in mediating the actions of kallistatin and bradykinin.

Whether kallistatin-induced vasorelaxation is mediated via a receptor-binding mechanism was further evaluated by a kallistatin-ligand binding assay. Specific kallistatin-binding sites in the aortic membrane proteins were identified by analyzing saturation data with the EBDA program. Scatchard plot analysis revealed that rat aorta membrane proteins contained specific kallistatin-binding sites with a  $K_d$  of  $0.25 \pm 0.07$  nM and  $B_{\text{max}}$  of  $47.9 \pm 10.4$  fmol/mg protein (mean  $\pm$  SEM,  $n = 3$ ), which are comparable with those of bradykinin and its  $B_2$ -receptor binding in rat cultured arterial smooth muscle cells (21). These results support the notion of a receptor binding mechanism for kallistatin's effects on aortic smooth muscle cells. The signal transduction pathways after receptor binding and the mechanism for kallistatin-induced smooth muscle cell relaxation remain to be investigated.

Although it is known that bradykinin activates the G-protein-coupled endothelial  $B_2$ -receptor to stimulate phospholipases  $A_2$  and C with a concomitant increase in cytosolic calcium and the formation of the potent vasodilators, prostacyclin and nitric oxide (NO) (22), our studies indicate that the second messenger for kallistatin-induced vasorelaxation does not involve the generation of NO. The concentration of L-NAME used in our aortic ring experiments (250  $\mu\text{M}$ ) is more than sufficient to fully inhibit NO synthase activity. This was verified in the studies by testing the response to acetylcholine, which was completely inhibited in the presence of L-NAME. In our pre-

vious studies (15, 23), another inhibitor of NO synthase was used,  $N^G$ -methyl-L-arginine, which fully blocked NO synthase function at 250  $\mu\text{M}$ , even though it is fivefold less potent than L-NAME (24). Since we demonstrated that kallistatin-induced vascular relaxation of the rat aorta is endothelium independent, it seems quite unlikely that it is mediated by NO. Another possibility is that NO is derived from vascular smooth muscle, which seems unlikely under normal conditions. Nonetheless, we tested the effects of L-NAME and confirmed our findings from the Endo- rat aorta that kallistatin is both endothelium and NO independent. The potential for prostacyclin to mediate kallistatin-induced vasodilation is also unlikely since our experiments demonstrated that the effects of kallistatin are independent of endothelium which is the major source of this prostanoid in blood vessels. Whether the overall effects of kallistatin are mediated via calcium ions, potassium or chloride channels, phospholipase activation, or other second messengers remains to be investigated.

This report shows that human kallistatin and RKBP are potent vasodilators. However, the physiological functions of kallistatin are complex due to kallistatin's ability to form a specific and tight complex with tissue kallikrein. Our previous studies suggest a role for kallistatin in regulating tissue kallikrein's activity and/or bioavailability (8). In light of the current findings, it seems likely that kallistatin may have other functions independent of its interaction with tissue kallikrein. There are several lines of evidence indicating that kallistatin might play an important role in blood pressure homeostasis. The expression of RKBP, a functional analog of human kallistatin, is significantly lower in spontaneously hypertensive rats (SHR) as compared to that of the normotensive Wistar-Kyoto rats (5, 25). A number of restriction fragment length polymorphisms in the RKBP gene locus have been identified in the SHR, indicating that mutations exist either within or linked to the RKBP gene in this hypertensive animal model (5). A strong association has been established between an RKBP gene polymorphism and the blood pressure phenotype in a stroke-prone SHR after salt loading (26). Moreover, excessive urinary kallistatin excretion has been observed in patients with pregnancy-induced hypertension (27). In our recent study, we showed that transgenic mice overexpressing RKBP, an analog of human kallistatin, are hypotensive (28). Also, intravenous injection of purified RKBP into mice via a catheter produced a dose-dependent reduction in the mean arterial blood pressure. These findings suggest that RKBP may function as a vasodilator *in vivo*, independent of regulating the activity of tissue kallikrein. Furthermore, we showed that adenovirus-mediated delivery of the human kallistatin gene results in a reduction of blood pressure in SHR, again suggesting that kallistatin may function as a vasodilator *in vivo* (29). These two new findings lend strong support to the conclusion of this study that kallistatin acts as a vasodilator. Collectively, these findings suggest that kallistatin may play an important and direct role in regulating systemic blood pressure and raise the possibility for the development of new pharmacological treatments for hypertension.

## Acknowledgments

We thank Drs. Paolo Madeddu and Gary Richards for critical reading of the manuscript.



The work was supported by National Institutes of Health grants HL-44083 and HL-29397 and grant HL-47432 to J.N. Stallone.

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