

Role of kallistatin in prevention of cardiac remodeling after chronic myocardial infarction

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Oxidative stress causes cardiomyocyte death and subsequent ventricular dysfunction and cardiac remodeling after myocardial infarction (MI), thus contributing to high mortality in chronic heart failure patients. We investigated the effects of kallistatin in cardiac remodeling in a chronic MI rat model and in primary cardiac cells. Human kallistatin gene was injected intramyocardially 20 min after ligation of the left coronary artery. At 4 weeks after MI, expression of human kallistatin in rat hearts was identified by reverse transcription–polymerase chain reaction, immunohistochemistry and ELISA. Kallistatin administration improved cardiac performance, increased mean arterial pressure, decreased myocardial infarct size and restored left ventricular wall thickness. Kallistatin treatment significantly attenuated cardiomyocyte size and atrial natriuretic peptide expression. Kallistatin also reduced collagen accumulation, collagen fraction volume and expression of collagen types I and III, transforming growth factor- β 1 (TGF- β 1) and plasminogen activator inhibitor-1 in the myocardium. Inhibition of cardiac hypertrophy and fibrosis by kallistatin was associated with increased cardiac nitric oxide (NO) levels and decreased superoxide formation, NADH oxidase activity and p22-phox expression. Moreover, in both primary cultured rat cardiomyocytes and myofibroblasts, recombinant kallistatin inhibited intracellular superoxide formation induced by H₂O₂, and the antioxidant effect of kallistatin was abolished by *N* ω -nitro-L-arginine methyl ester (L-NAME), indicating a NO-mediated event. Kallistatin promoted survival of cardiomyocytes subjected to H₂O₂ treatment, and inhibited H₂O₂-induced Akt and ERK phosphorylation, as well as NF- κ B activation. Furthermore, kallistatin abrogated TGF- β -induced collagen synthesis and secretion in cultured myofibroblasts. This is the first study to demonstrate that kallistatin improves cardiac performance and prevents post-MI-induced cardiac hypertrophy and fibrosis through its antioxidant action.

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Myocardial infarction (MI) leads to cardiac remodeling with complex structural alterations. After an ischemic insult, left ventricle (LV) enlargement is followed by progressive diastolic stiffness, enhanced wall stress and oxygen consumption, thus resulting in cardiomyocyte hypertrophy, interstitial fibrosis and decreased cardiac output. Increased reactive oxygen species (ROS) levels are recognized to be involved in ischemic heart failure and considered to be a major cause of cardiomyocyte death, ventricular dysfunction and heart failure progress.¹ Under pathological conditions, increased oxidative stress damages DNA and mitochondrial function, activates proapoptotic signaling kinases and leads to myocyte apoptosis or necrosis.^{2,3} Elevation of ROS activates a series of hypertrophic signaling kinases and transcription factors to stimulate cardiac hypertrophy.^{3,4} Moreover, ROS stimulate

fibroblast proliferation and activate the expression of profibrotic factors, including transforming growth factor- β 1 (TGF- β 1), that facilitate fibrosis and matrix remodeling.^{5–7} Elevation of vascular oxidative stress and ventricular remodeling contribute to high mortality in chronic heart failure (CHF) patients.⁸

Kallistatin is a member of the serine proteinase inhibitor (serpin) superfamily and was shown to have pleiotropic effects, including hypotension, anti-angiogenesis and anti-inflammation.^{9–11} Kallistatin gene delivery reduced ischemic myocardial inflammation and cardiomyocyte apoptosis at 1 day after acute ischemia/reperfusion injury.¹² Recently, we observed that increased oxidative stress after MI was associated with reduced circulating kallistatin levels, suggesting that kallistatin might be involved in the progression of

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oxidative cardiac damage. In this study, we investigated the role and mechanisms of kallistatin in cardiac remodeling in a CHF model and in primary cultured cardiomyocytes and myofibroblasts. We found that, after 4 weeks of kallistatin gene therapy, MI-induced heart failure, hypertrophy and fibrosis were greatly improved which were accompanied with inhibition of ROS though NO formation, obstruction of ROS-induced hypertrophic signaling pathway and suppression of TGF- β expression and TGF- β -induced collagen synthesis. Moreover, in cultured cardiomyocytes and myofibroblasts, recombinant kallistatin inhibited ROS formation through NO production. These combined findings indicate that kallistatin acts as an antioxidant in cardiac protection through NO formation.

MATERIALS AND METHODS

MI Surgery in Rats and Adenoviral Vector Treatment

MI was induced in male Wistar rats (250–300 g; Harlan, Indianapolis, IN, USA) by permanent ligation of the left coronary artery (LCA) as previously described.¹³ The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Adenoviral vector harboring the human kallistatin cDNA under the control of the cytomegalovirus enhancer/promoter (Ad.HKS) or adenoviral vector alone (Ad.Null) (1×10^{10} plaque-forming units) was injected intramyocardially at six different sites along the border of infarcted area and noninfarcted area at 20 min after ligation. The survival rates in the sham, MI/Ad.Null and MI/Ad.HKS groups were 100, 70 and 73%, respectively. At 4 weeks after MI surgery, survival rats ($n=6$ in each group) were killed and the myocardium from the remote noninfarcted area in LV was immediately removed for analysis.

Cardiac Hemodynamic Measurements

At 4 weeks after MI, rats were anesthetized for hemodynamic measurements as previously described.¹² A 2.5-French catheter (Millar Instrument, Houston, TX, USA) was introduced into the LV for measuring mean arterial pressure (MAP), left ventricular end diastolic pressure (LVEDP), maximum first derivative of pressure (dP/dt max.) and minimum first derivative of pressure (dP/dt min.). The data were recorded and analyzed by a model 7E polygraph (BIOPAC System Inc., Goleta, CA, USA).

Histology and Immunohistochemistry

Paraffin sections (4 μ m) were transversely obtained from the center of infarcted LV. Masson's trichrome staining was performed to distinguish areas of connective tissues and the extent of infarcted area. Cardiomyocyte size was determined by Gordon and Sweet's silver staining. A total of 40 cardiomyocytes in each noninfarcted LV were randomly chosen and traced with NIH Image software. Myocardial fibrosis was identified by sirius red staining and the percentage of collagen density in noninfarcted LV was analyzed by Adobe

PhotoShop software. Localization of recombinant human kallistatin in hearts was identified with a specific monoclonal antibody to human kallistatin as previously described.¹² For immunostaining, slides were incubated with primary antibodies against collagen I (Chemicon, Temecula, CA, USA; 1:200) and collagen III (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) at 4°C overnight.

Reverse Transcription–Polymerase Chain Reaction and Semi-Quantitative Real-Time PCR

Reverse transcription–polymerase chain reaction (RT–PCR) for human kallistatin was performed using 5' primers (CCACAACCTGAAGTTCCTTG) and 3' primers (GCCAGA AATGGAGAAGCTTGG). Real-time PCR was carried out using the gene expression assay of Rn005616661_ml for atrial natriuretic peptide (ANP), Rn00577357_ml for p22-phox, Rn01475962_ml for TGF- β 1 and Rn00561717_ml for plasminogen activator inhibitor-1 (PAI-1) (Applied Biosystems, Foster city, CA, USA). Gene expression was normalized by the housekeeping gene GAPDH (Mm99999915_gl).

Measurements of Nitrate/Nitrite Levels, Superoxide Formation and NADH Oxidase Activity

Nitrate/nitrite (NO_x) levels, an index of NO formation, in cardiac extracts were measured by a fluorometric assay as previously described.¹⁴ At 4 weeks after MI, superoxide levels in noninfarcted myocardium were measured by a spectrophotometric assay based on rapid reduction of ferricytochrome *c* to ferrocyanochrome *c*.¹² NADH oxidase activities were measured by lucigenin-enhanced chemiluminescent detection using a luminometer (Turner Biosystems, Sunnyvale, CA, USA).¹²

Primary Culture of Cardiomyocytes and Myofibroblasts

Neonatal myofibroblasts were isolated from 1- to 3-day-old Sprague–Dawley rats by four rounds of digestion using collagenase II (Worthington, Lakewood, NJ, USA) and pancreatin (Sigma, St Louis, MO, USA).⁶ Cardiomyocytes were collected after 1 h of differential plating for myofibroblasts. Myofibroblasts were identified by positive immunostaining with α -smooth muscle actin. Cardiomyocytes at 1–2 passages or myofibroblasts at 2–3 passages were used for *in vitro* studies. Recombinant human kallistatin was purified and characterized as previously described for use in the cell culture studies.¹⁵

Intracellular Superoxide Determination and Dichlorofluorescein Fluorescence Measurement

Intracellular superoxide levels were determined by pretreating confluent cells with human kallistatin (0.5 μ mol/l) with or without *N* ω -nitro-L-arginine methyl ester (L-NAME, 200 μ mol/l) for 45 min, followed by exposure to 0 or 200 μ M H_2O_2 for 40 min.¹⁶ After removing cultured medium, cells were incubated with dihydroethidium (HE, a probe for detecting intracellular superoxide, 10 μ mol/l) for 40 min and

then photographed. For DCF fluorescence measurement, cells were washed with phenol red-free DMEM and pretreated with DCF diacetate (20 $\mu\text{mol/l}$) for 45 min.¹⁷ After removing the probe, cells were incubated with H_2O_2 (200 $\mu\text{mol/l}$) in the presence or absence of kallistatin (0.5 $\mu\text{mol/l}$) or L-NAME (200 $\mu\text{mol/l}$). DCF fluorescence was recorded (485 nm excitation, 538 nm emission) for a period of 60 min.

Western Blotting

Cytosolic fractions of noninfarcted myocardium were subjected to western blot for the detection of TGF- β 1 (Sigma; 1:1000). Cultured cardiomyocytes were serum-deprived, pretreated with or without kallistatin (0.5 $\mu\text{mol/l}$) for 45 min, and then challenged with H_2O_2 (200 $\mu\text{mol/l}$) in the presence or absence of kallistatin (0.5 $\mu\text{mol/l}$) for 15 min, using cardiomyocytes cultured in serum-free medium as control. Cultured cardiomyocyte extracts underwent western blot analysis for the detection of phospho-Akt, total Akt, phospho-ERK and total ERK (Cell Signaling Technology, Danvers, MA, USA; 1:1000). Conditioned medium from cultured myofibroblasts was collected after 24 h treatment with TGF- β 1 (10 ng/ml) with or without kallistatin (0.5 $\mu\text{mol/l}$) for the detection of collagen I by western blot (Santa Cruz; 1:400). Protein concentration was determined by Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Cell Survival Analysis

Celltiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) was used to measure viable cell levels according to manufacturer's instructions. After pretreatment with kallistatin (1.0 $\mu\text{mol/l}$) for 45 min, cells were stimulated with H_2O_2 (750 $\mu\text{mol/l}$) with or without kallistatin (1.0 $\mu\text{mol/l}$) for 24 h. Absorbance readings were measured with a photometric reader after 2 h incubation with a solution of tetrazolium compound (MTS).

Transient Transfection and NF- κ B Luciferase Activity Assay

A nucleofector kit for primary cardiomyocytes (Amaxa Biosystems, Gaithersburg, MD, USA) was used for transient transfection according to the manual bulletin. One day after transfection, cells were serum-deprived for 24 h and then were incubated with H_2O_2 (200 $\mu\text{mol/l}$) with or without kallistatin (0.5 $\mu\text{mol/l}$) for 24 h. Cells were lysed in lysis buffer, and NF- κ B firefly luciferase activity was measured with luciferase assay reagent (Promega). The data from three independent transfections were normalized with protein concentrations.

Collagenase-Sensitive [^3H]-Proline Incorporation

Collagenase-sensitive [^3H]-proline incorporation was measured for collagen synthesis in cultured myofibroblasts.¹⁸ Growth-arrested myofibroblasts were pulsed by adding 1 $\mu\text{Ci/ml}$ [^3H]-proline (PerkinElmer Life Sciences, Waltham,

MA, USA) and 10 ng/ml TGF- β 1 with or without kallistatin (0.5 $\mu\text{mol/l}$) for 48 h. Incorporated [^3H]-proline after 2 mg/ml collagenase II (Worthington) digestion was counted by a liquid scintillation counter.

Statistical Analysis

Data were expressed as mean \pm s.e.m. and were compared among experimental groups with the use of one-way ANOVA followed by Newman-Keuls multiple comparison test. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Recombinant Human Kallistatin in Rat Hearts

Four weeks after local injection of adenovirus containing the human kallistatin gene in rat hearts, expression and localization of recombinant human kallistatin protein and mRNA were identified in both infarcted and noninfarcted areas by immunohistochemistry (Figure 1a) and by RT-PCR (Figure 1b). Moreover, using a human kallistatin specific ELISA,¹² immunoreactive human kallistatin levels in noninfarcted left ventricular extracts were 0.54 ± 0.23 ng/mg protein ($n = 4$) and 0.10 ± 0.01 ng/mg protein ($n = 4$) at 1 and 4 weeks, respectively, after kallistatin gene delivery. Neither human kallistatin mRNA nor protein was detectable in the corresponding tissues of rats injected with control virus. Human kallistatin was undetectable in the circulation after local gene delivery, and serum protein levels of rat kallistatin remained unchanged in all groups, as determined by specific human and rat kallistatins (data not shown).

Kallistatin Gene Delivery Improves Hemodynamic Parameters

The effects of kallistatin on cardiac function at 4 weeks after MI are shown in Table 1. Heart weight (HW) to body weight (BW) ratio was increased by 12.3% in MI/Ad.Null group, and increased by only 6.3% after Ad.HKS treatment, compared with the sham group. However, there was no statistical difference in HW/BW among the groups. MAP was decreased in the MI control group compared with the sham group, and kallistatin treatment significantly restored MAP levels. Rats injected with Ad.Null gene showed decreased cardiac contractile and relaxing function, as indicated by increased LVEDP and decreased dP/dt (dP/dt max and dP/dt min). Kallistatin gene transfer significantly improved cardiac performance.

Kallistatin Gene Delivery Reduces Infarct Size and Restores Left Ventricular Wall Thickness

Representative Masson's trichrome staining showed that kallistatin treatment reduced infarcted size after MI (Figure 1c). Quantitative analysis indicated that kallistatin gene transfer significantly reduced the ratio of infarcted area to area at risk from 37.4 ± 2.6 to $28.6 \pm 3.0\%$ at 4 weeks after MI ($P < 0.05$, $n = 6$) (Figure 1d). Moreover, LV wall thickness was

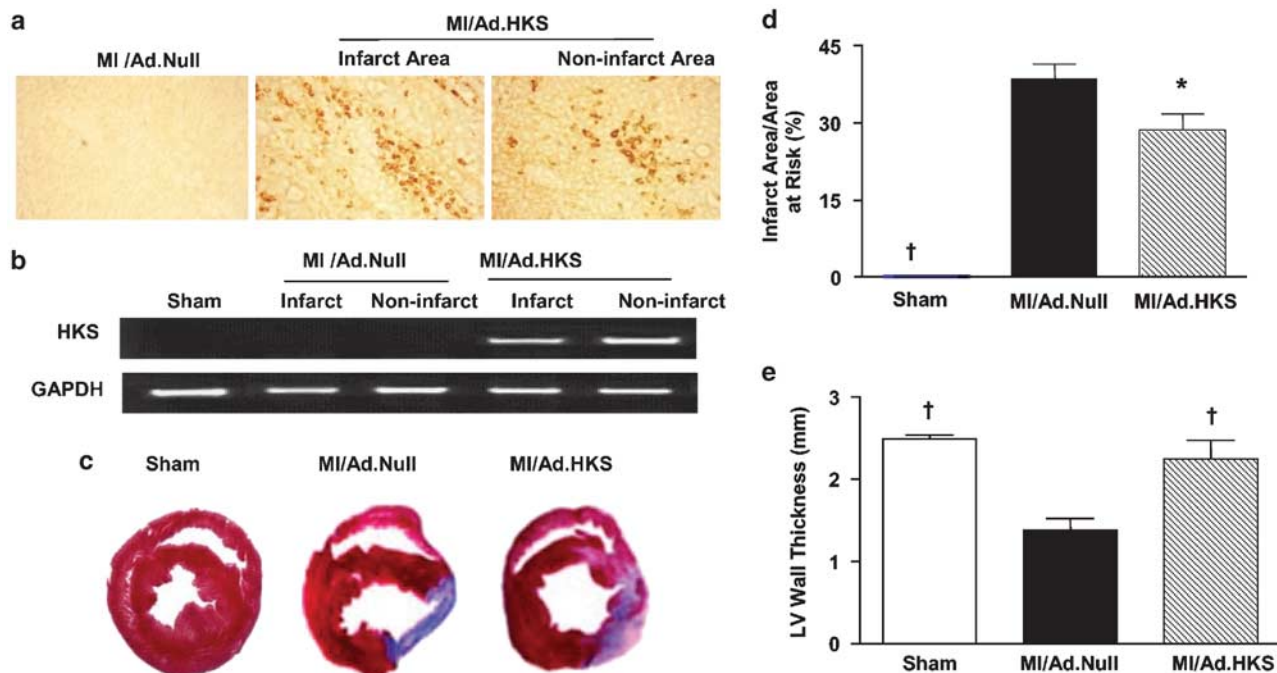


Figure 1 Expression and localization of kallistatin and effects of kallistatin on infarct size and LV wall thickness at 4 weeks after MI. (a) Identification of human kallistatin protein levels immunohistochemical staining (original magnification is $\times 200$) in both infarct area and noninfarct area myocardium. (b) mRNA expression of human kallistatin by RT-PCR. (c) Representative photographs of Masson's trichrome staining to depict infarct size (original magnification is $\times 50$). (d) Analysis of infarcted area is expressed as a ratio of infarcted area to area at risk in LV. (e) LV wall thickness quantification results; * $P < 0.05$, † $P < 0.01$ vs MI/Ad.Null. Data are expressed as mean \pm s.e.m., $n = 6$.

Table 1 Effects of kallistatin gene delivery on cardiac function at 4 weeks after MI

	Sham	MI/Ad.Null	MI/Ad.HKS
HW/BW (g/kg)	3.50 \pm 0.15	3.93 \pm 0.20	3.72 \pm 0.05
MAP (mm Hg)	109.50 \pm 3.12†	58.25 \pm 3.12	70.00 \pm 3.30*
LVEDP (mm Hg)	1.54 \pm 0.32†	15.19 \pm 1.34	9.47 \pm 1.21†
dP/dt max. (mm Hg/s)	3994.17 \pm 87.47†	2032.13 \pm 96.75	2560.33 \pm 104.63†
DP/dt min. (mm Hg/s)	3530.33 \pm 90.40†	1465.50 \pm 105.74	1926.33 \pm 139.40†

BW, body weight; dP/dt max., maximum first derivative of pressure; dP/dt min., minimum first derivative of pressure; HW, heart weight; LVEDP, left ventricular end diastolic pressure; MAP, mean arterial pressure.

Values are presented as mean \pm s.e.m.

* $P < 0.05$, † $P < 0.01$ vs MI/Ad.Null ($n = 6$).

significantly reduced by permanent MI compared with sham, but was restored by kallistatin gene delivery (Figure 1e).

Kallistatin Attenuates Cardiac Hypertrophy

Representative Gordon and Sweet's silver staining showed that kallistatin reduced enlarged cardiomyocyte size in non-infarcted area at 4 weeks after MI (Figure 2a). Quantitative analysis showed that kallistatin gene delivery significantly reduced cardiomyocyte size compared with the MI/Ad.Null group (Figure 2b). The inhibitory effect of kallistatin

on hypertrophy was associated with a significant reduction of ANP mRNA levels, a marker of cardiac hypertrophy (Figure 2c).

Kallistatin Attenuates Collagen Deposition and Expression of Profibrotic Factors

Examination and quantitative analysis of heart sections by sirius red staining indicated that kallistatin reduced MI-induced collagen deposition and collagen fraction volume (Figure 3a and b). Similarly, immunostaining showed that kallistatin reduced collagen types I and III levels (Figure 3a). Reduction of collagens I and III accumulation by kallistatin was further confirmed by measurements of their mRNA levels (Figure 3c and d). Western blot and quantitative analysis showed that kallistatin treatment prevented elevation of TGF- β 1 levels induced by MI (Figure 3e). Kallistatin also reduced TGF- β 1 and PAI-1 mRNA levels in noninfarcted myocardium (Figure 3f and g).

Kallistatin Increases NO Formation and Reduces Oxidative Stress after MI

At 4 weeks after MI, kallistatin gene delivery significantly increased cardiac NO_x content compared with sham and MI groups (Figure 4a). Kallistatin gene delivery significantly inhibited superoxide formation and NADH oxidase activity compared with the MI/Ad.Null group in the noninfarcted myocardium (Figure 4b and c). Furthermore, inhibition of

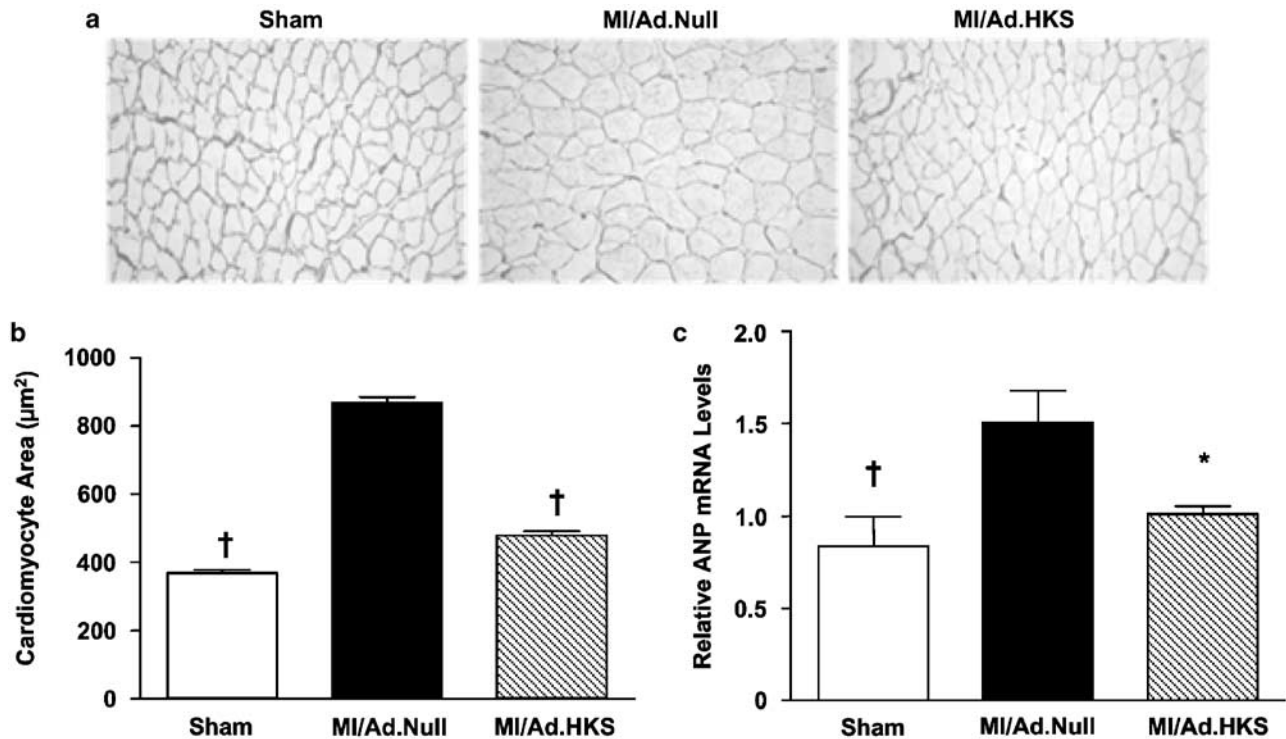


Figure 2 Kallistatin prevents cardiac hypertrophy at 4 weeks after MI. (a) Cardiomyocyte size was visualized by silver staining in noninfarcted myocardium (original magnification is $\times 400$). (b) Quantification analysis of cardiomyocyte size from silver staining. (c) ANP mRNA expression in noninfarcted myocardium by real-time PCR; * $P < 0.05$, † $P < 0.01$ vs MI/Ad.Null. Data are expressed as mean \pm s.e.m., $n = 6$.

NADH oxidase activity by kallistatin was accompanied by a reduction in p22-phox mRNA levels (Figure 4d).

Effects of Recombinant Kallistatin on ROS Formation and ROS-Induced Prohypertrophic Signaling Pathway in Cultured Cardiomyocytes

In primary cardiomyocytes, recombinant kallistatin inhibited H_2O_2 -induced intracellular superoxide formation as determined by HE fluorescence intensity, and the inhibitory effect of kallistatin on *in situ* ROS formation was abrogated by L-NAME, indicating a NO-mediated event (Figure 5a). H_2O_2 -induced DCF fluorescence in a time-dependent manner and kallistatin inhibited the increases (Figure 5b). Again, kallistatin's effect on H_2O_2 -induced ROS formation was blocked by L-NAME. Kallistatin significantly promoted cell survival (Figure 5c) and inhibited H_2O_2 -stimulated Akt and ERK phosphorylation (Figure 5d) and NF- κ B activation (Figure 5e) in cultured cardiomyocytes.

Effect of Recombinant Kallistatin on ROS Formation, and TGF- β -Induced Collagen Synthesis in Cultured Myofibroblasts

In myofibroblasts, kallistatin inhibited H_2O_2 -induced intracellular oxidant production and the effect was blocked by L-NAME as determined by both HE and DCF fluorescence measurements (Figure 6a and b). Kallistatin significantly inhibited TGF- β 1-induced [3H]-proline incorporation

(Figure 6c) in cultured myofibroblasts. To confirm that the observed effect was specific to the synthesis of collagen I, immunoblotting was used to analyze non-cross-linked collagen secreted into the cultured medium. Kallistatin inhibited TGF- β 1-induced collagen I secretion in the medium of cultured myofibroblasts (Figure 6d).

DISCUSSION

This study identified a novel role of kallistatin in preventing cardiac injury and remodeling by inhibiting oxidative stress and ROS-related hypertrophy and fibrosis in an animal model of postinfarcted and CHF. Our lab has previously shown that kallistatin plays a protective role in heart during acute ischemia by preventing apoptosis and inflammation in infarcted myocardium.¹² This study adds to the previous work by showing the longer-term benefits of kallistatin in MI. We demonstrate here that administration of kallistatin prevents cardiac fibrosis and hypertrophy, leading to a reduction in cardiac remodeling. This study shows that ROS levels are diminished after kallistatin gene delivery suggesting kallistatin may act as an antioxidant to prevent cardiac remodeling and oxidative cardiac damage. Moreover, kallistatin was found to reduce ROS formation and inhibit ROS-related prohypertrophic and profibrotic signaling pathway. Our previous work focused on the acute effects of kallistatin with myocardial ischemia and reperfusion, whereas this study investigated chronic effects of MI and the ability of kallistatin

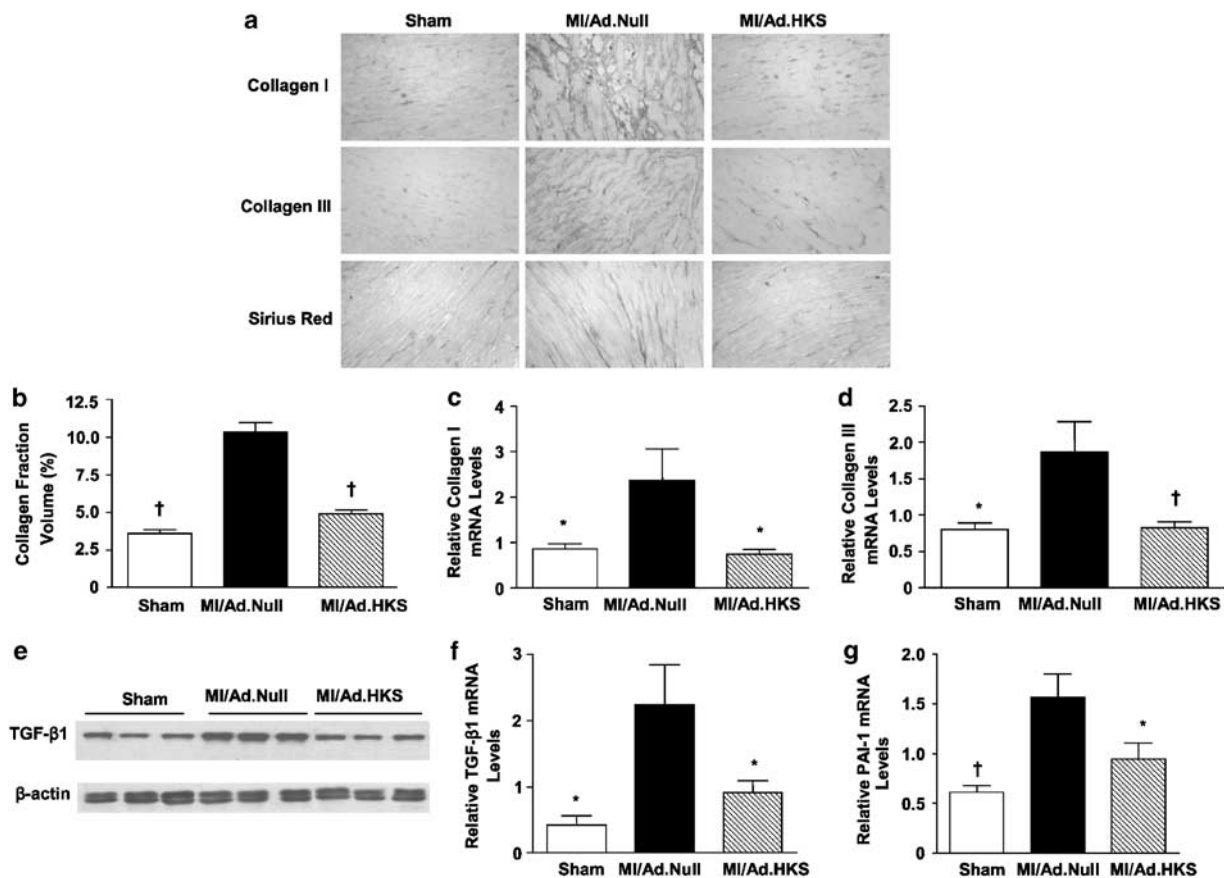


Figure 3 Kallistatin reduces collagen deposition and TGF-β1 and PAI-1 levels in noninfarcted myocardium. (a) Representative sections of sirius red staining and immunohistochemistry of collagens I and III (original magnification is × 400). (b) Collagen fraction volume resulting from quantification of sirius red staining. (c) Collagen I and (d) collagen III mRNA expression by real-time PCR in noninfarcted myocardium. (e) Representative TGF-β1 protein levels in noninfarcted myocardium by western blot. (f) TGF-β1 and (g) PAI-1 mRNA levels determined by real-time PCR in noninfarcted myocardium; **P* < 0.05, [†]*P* < 0.01 vs MI/Ad.Null. Data are expressed as mean ± s.e.m., *n* = 6.

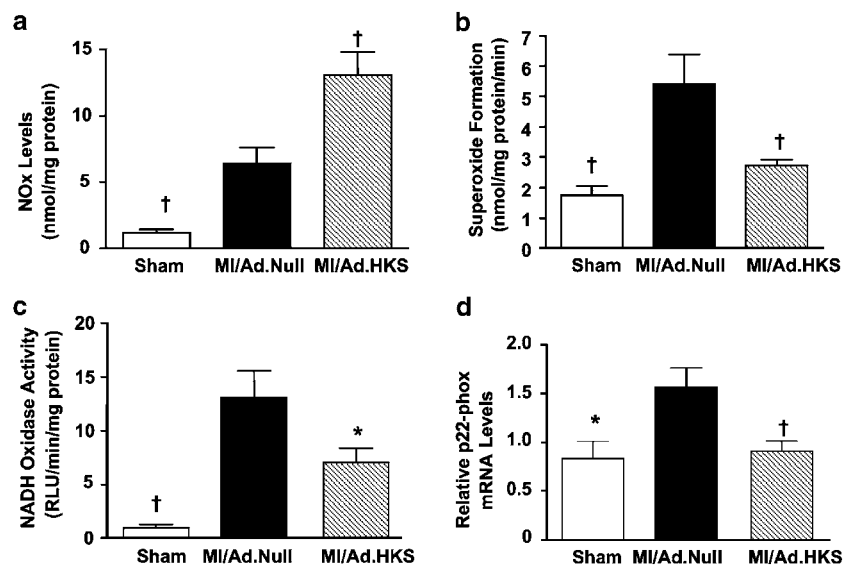


Figure 4 Kallistatin increases NO_x levels, and inhibits ROS formation and NADH oxidase activity. (a) NO levels, (b) superoxide anion formation, (c) LV NADH oxidase activity and (d) p22-phox mRNA expression in noninfarcted myocardium at 4 weeks after MI; **P* < 0.05, [†]*P* < 0.01 vs MI/Ad.Null. Data are expressed as mean ± s.e.m., *n* = 6.

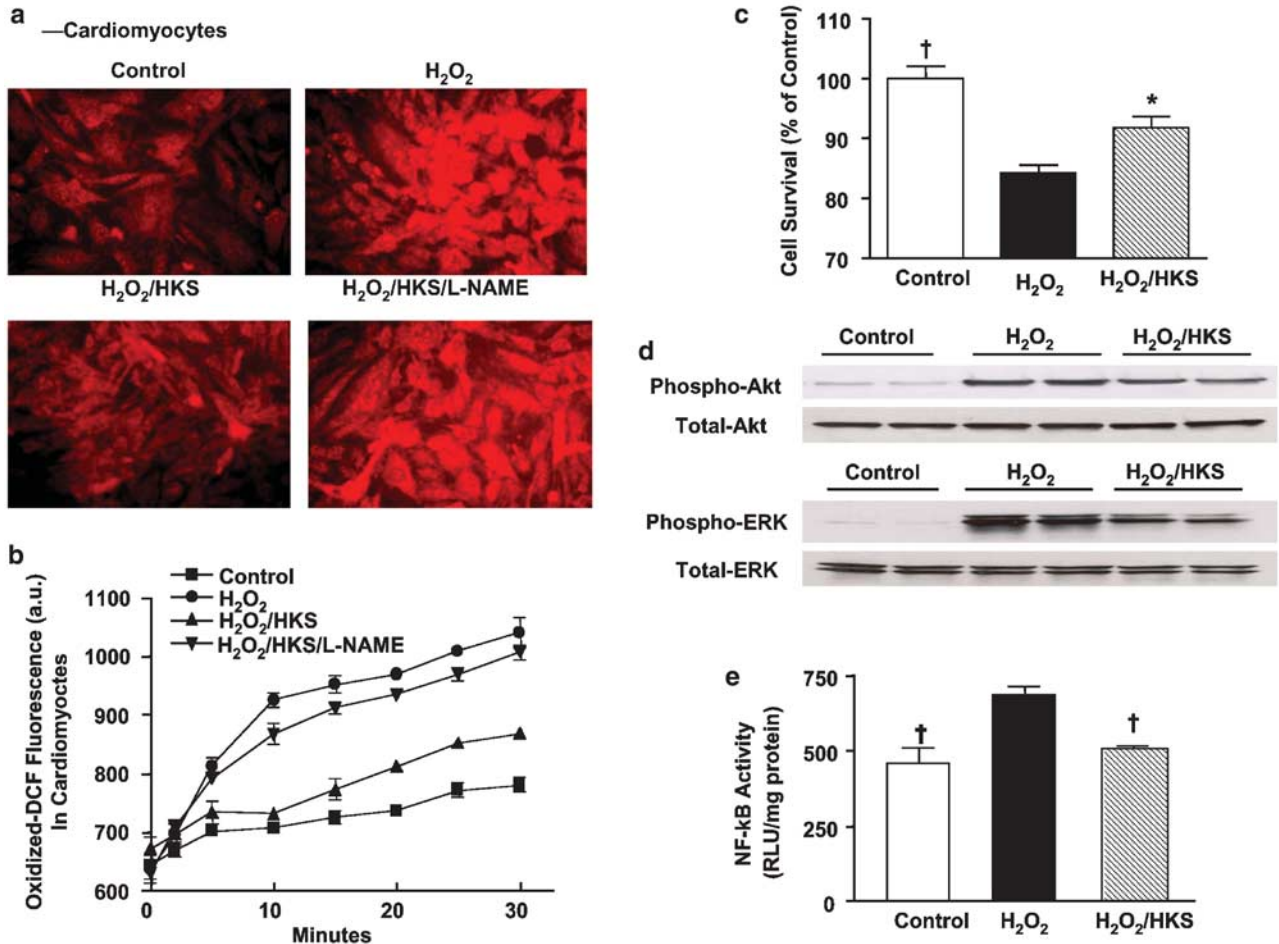


Figure 5 Kallistatin inhibits ROS formation and ROS-induced hypertrophic signaling in cardiomyocytes. Detection of intracellular ROS by (a) *in situ* HE fluorescence staining and (b) oxidized DCF fluorescence measurement (a.u., arbitrary units). (c) Cell survival determined by MTS assay. (d) Western blots of phosphorylated and total Akt and ERK. (e) NF- κ B activation in cultured cardiomyocytes (RLU, relative light units); * $P < 0.05$, † $P < 0.01$ vs H₂O₂. Data from independent triplicate experiments are expressed as mean \pm s.e.m., $n = 3$.

to modulate them. We believe that kallistatin affords cardiac protection both at the early and later stages of cardiac remodeling induced by myocardial ischemia.

It is well known that oxidative stress causes cardiomyocyte death, ventricular dysfunction and remodeling after MI, thus contributing to high mortality in CHF patients. Our results showed that kallistatin increased NO_x levels and inhibited NADH oxidase activity and superoxide formation through downregulation of p22-phox expression in failing hearts at 4 weeks after MI. These results indicate a clue that, through NO formation, kallistatin prevented hearts from undergoing cardiac remodeling by inhibiting oxidative stress and ROS-induced cardiomyocyte apoptosis, cardiac hypertrophy and fibrosis.

Ischemia-generated ROS-induced cardiomyocyte apoptosis and necrotic cell death.¹⁹ Our current study showed that kallistatin improved the survival of cultured cardiomyocytes subjected to high levels of ROS. At 4 weeks after MI, a mature scar had already formed in the area of infarction, being

mainly consisting of acellular and fibrillar collagen. Thus, at this point, apoptotic cells and signals were undetectable in the infarcted heart. Reduction of infarcted size and restoration of LV wall thickness also provide direct evidence for increased cardiomyocyte survival after chronic MI. This finding is consistent with our previous study showing that kallistatin gene transfer inhibited cardiomyocyte apoptosis and inflammation in infarcted myocardium after acute ischemia/reperfusion injury.¹² Thus, considering that reversible cardiomyocyte injury during the acute phase is important for the subsequent ventricular remodeling in postinfarcted heart failure,²⁰ the protective effects of kallistatin observed in the chronic phase may be partially attributed to its ability to improve cardiomyocyte survival under oxidative stress damage or attenuate cardiomyocyte apoptosis and inflammation in the early phase after MI.

It is well known that the development of hypertrophy is tightly coupled with ROS, which trigger redox-related hypertrophic signaling pathways. Among them, the PI3-kinase/

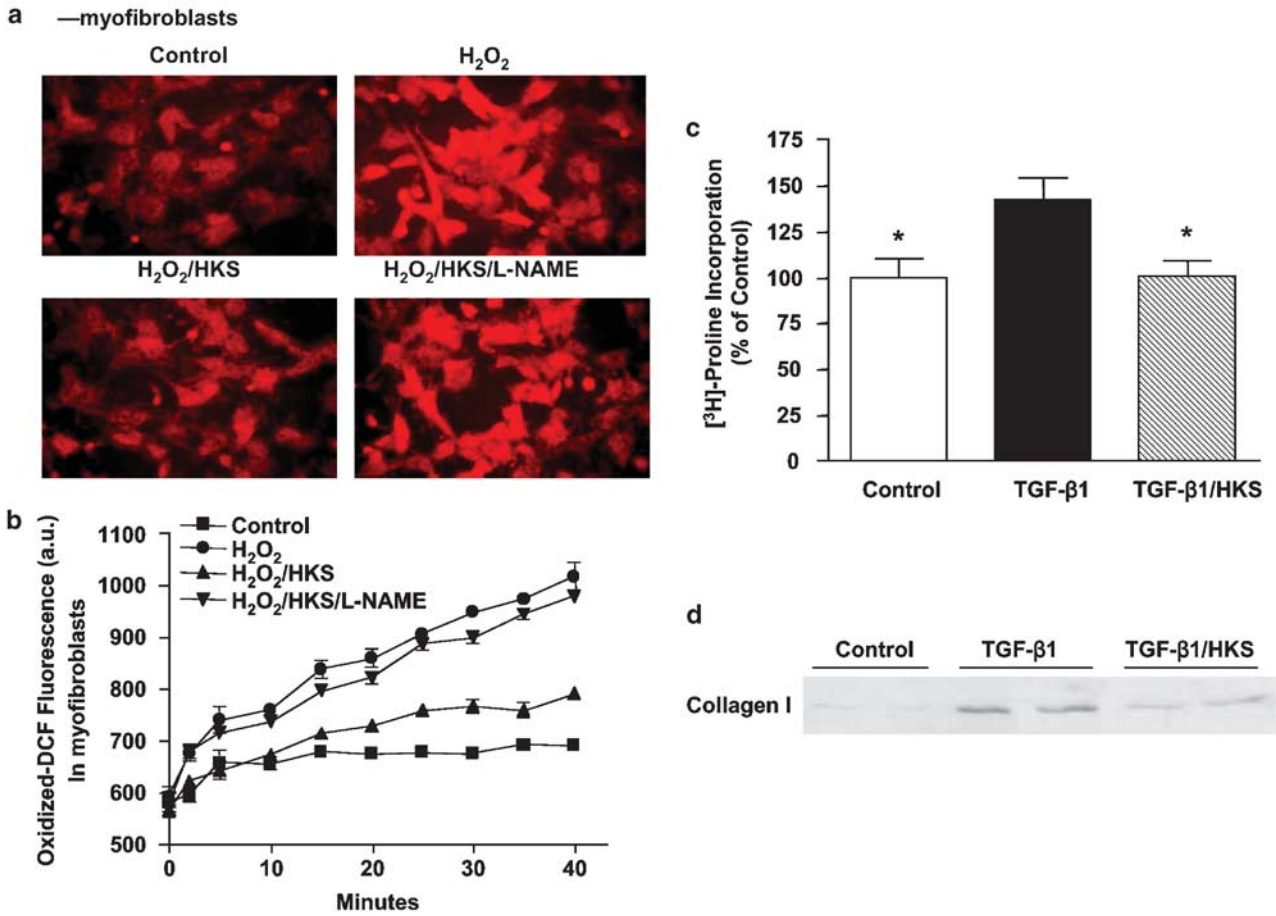


Figure 6 Kallistatin abrogates ROS levels and inhibits TGF-β-induced collagen synthesis and secretion in cultured myofibroblasts. Determination of intracellular ROS by (a) *in situ* HE fluorescence staining and (b) oxidized DCF fluorescence measurement. (c) TGF-β1-induced collagen synthesis determined by collagenase-sensitive [³H]-proline incorporation. (d) TGF-β1-induced collagen secretion in cultured medium from myofibroblasts; *P<0.05, †P<0.01 vs TGF-β1. Data from independent triplicate experiments are expressed as mean ± s.e.m., n = 3.

Akt pathway is required for transition from compensated to decompensated hypertrophy, as transgenic expression of Akt in the heart was shown to lead to an increase in cardiomyocyte size.^{4,21} In addition, ANP, a cardiac hypertrophy marker, is activated in an Akt-dependent manner.²² However, one study reported that short-term activation of Akt-induced cardiac hypertrophy without ANP gene expression.²³ Moreover, ERK is considered to be activated by mechanical stress, which induces a G-protein-coupled hypertrophic stimulation in heart failure.²⁴ The transcription factor NF-κB has also been observed to be activated by ROS and is responsible for the induction of hypertrophy in cardiomyocytes.²⁵ In this study, kallistatin therapy minimized postinfarcted LV hypertrophy evidenced by diminished HW/BW, cardiomyocyte size, ANP expression, as well as inhibition of Akt/ERK/NF-κB activation induced by H₂O₂. Our results indicate that, under oxidative stress circumstances induced by MI, the antihypertrophic effect of kallistatin is, at least partially, mediated by the inhibition of ROS-related prohypertrophic signaling pathway in cardiomyocytes (Figure 7).

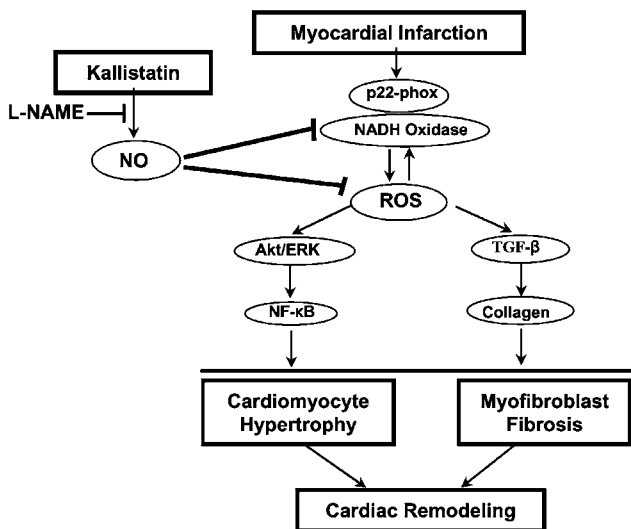


Figure 7 Schematic diagram of kallistatin through inhibition of oxidative stress and protection against cardiac remodeling after ischemic infarction.

Fibrosis is one of the most refractory clinical problems at the late stage of cardiac remodeling.²⁶ At this stage, TGF- β 1 activates fibrogenic pathways by inducing extracellular matrix deposition and contributes to the pathogenesis of left ventricular remodeling by promoting fibrosis of the non-infarcted myocardium.^{18,27} TGF- β can also prevent the degradation of collagen via increased PAI-1 expression.^{17,28} It has been reported that ROS modulate fibroblast proliferation and their transition into matrix-producing myofibroblasts in the heart.^{5,29} Furthermore, ROS induce expression of TGF- β 1 and type I collagen, and lead to activation of TGF- β from its latent form.^{6,7,30} Treatment with the antioxidant taurine was shown to attenuate hepatic fibrosis by inhibiting oxidative stress and TGF- β gene expression.³¹ In this study, kallistatin decreased the levels of the profibrotic molecules such as TGF- β 1 and PAI-1 in the ischemic myocardium, and directly inhibited TGF- β 1-induced collagen I synthesis and secretion in cultured myofibroblasts, thus preventing the occurrence and expansion of cardiac fibrosis (Figure 7).

After ischemic injury, endogenous ROS formation increases due to an imbalance of oxidant-generating and -catalyzing systems. Superoxide anion, the primary precursor in ROS metabolic pathways, decreases NO bioavailability and attenuates vasodilatation, thus increasing cardiac loading in CHF.³² NO is known to abolish mitochondrial oxidant damage and calcium overload, and reduce superoxide anion production via an efficient suppression on the activity of NAD(P)H oxidase and the assembly of NAD(P)H oxidase subunits.^{1,33} Maintenance of NO levels is critical for recovery of heart failure after ischemic injury. As part of a self-protective process, we found that CHF causes a modest increase in interstitial NO levels (consistent with a previous publication³⁴) and may be derived from activated NOS isoforms and/or a NOS-independent pathway.^{35,36} We have previously reported that kallistatin treatment increased NO levels through activating endothelial nitric oxide synthase (eNOS) in infarcted myocardium at 1 day after acute ischemia/reperfusion injury.¹² The present study showed that at 4 weeks after permanent LCA ligation, kallistatin gene transfer also significantly increased NO levels and inhibited ROS formation in noninfarcted myocardium, compared with Ad.Null group. However, we found that kallistatin treatment increased cardiac NO levels in conjunction with elevation of inducible NOS (iNOS) expression in noninfarcted myocardium. Similarly, kallistatin also stimulated iNOS expression in cultured cardiomyocytes and myofibroblasts (data not shown). Moreover, our study found that inhibition of NOS by L-NAME abolished the antioxidative effect of kallistatin on ROS formation in both cardiomyocytes and myofibroblasts. As long-term treatment with L-NAME could cause unwanted side effects such as hypertension *in vivo*, L-NAME was used only *in vitro* to determine the effects of NO. As reported, iNOS was initially perceived to generate NO in order to protect cells from apoptosis under chronic conditions, especially in proin-

flammatory situations.^{37,38} Moreover, in iNOS knockout mice, the antioxidant resveratrol had no protective effects on attenuating cardiac injury, though it protected against cardiac damage in wild-type mice, supporting a protective role of iNOS-related NO as an antioxidant.³⁹ NO formation through increased NOS activity should be an important protective mechanism during kallistatin treatment after MI injury.

Taken together, these findings indicate that kallistatin inhibits ROS injury through NO formation and thus protects against cardiomyocyte apoptosis, necrosis, hypertrophy and fibrosis by suppressing ROS-induced Akt/ERK and NF- κ B activation and ANP, TGF- β 1, PAI-1 and collagen expression (Figure 7). Kallistatin should be recognized as an important regulator in protection of post-MI injury and cardiac remodeling.

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