Novel mechanism of angiotensin II-induced cardiac injury in hypertensive rats – critical role of ASK1 and VEGF –

Running title: Link among angiotensin II, ASK1, and VEGF

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Abstract

The present study was undertaken to elucidate a novel mechanism underlying angiotensin II-induced cardiac injury, focusing on the role of oxidative stress and myocardial capillary density. Salt-loaded Dahl salt-sensitive hypertensive rats (DS rats), a useful model of hypertensive cardiac remodeling or heart failure, were orally given irbesartan (an AT1 receptor blocker), tempol (a superoxide dismutase mimetic), or hydralazine (a vasodilator). Irbesartan significantly ameliorated left ventricular ischemia and prevented the development of cardiac hypertrophy and fibrosis in DS rats. This beneficial effect of irbesartan in DS rats was associated with the attenuation of oxidative stress, the normalization of myocardial capillary density, and the inhibition of capillary endothelial apoptosis. Moreover, DS rats with significant cardiac hypertrophy and fibrosis displayed the decreased myocardial VEGF expression and the increased cardiac ASK1 activation, and irbesartan significantly reversed them in DS rats. Tempol treatment of DS rats mimicked all the above mentioned effects of irbesartan, indicating the critical role of oxidative stress in cardiac injury. We also investigated the role of VEGF and ASK1 in oxidative stress-induced endothelial apoptosis, using cultured endothelial cells from wild type and ASK1 deficient mice. Oxidative stress-induced ASK1 activation caused endothelial apoptosis and VEGF prevented oxidative stress-induced endothelial apoptosis by inhibiting ASK1 activation. We obtained the first evidence that oxidative stress-induced cardiac VEGF downregulation and ASK1 activation, through enhancement of endothelial apoptosis, contributed to the decrease in myocardial capillary density, which was responsible for angiotensin II-induced progression of cardiac injury.

Key Words: oxidative stress; endothelial apoptosis; ASK1; VEGF; heart failure

Introduction

Accumulating evidence indicates that angiotensin II plays a key role in the transition from compensated to decompensated cardiac hypertrophy or heart failure. [1-4] Clinical evidence shows that renin-angiotensin system (RAS) blockers, such as AT1 receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors, are effective for treatment of not only cardiac hypertrophy but also decompensated cardiac hypertrophy or heart failure. [5, 6] However, the precise mechanism underlying the protective effects of RAS blockers against decompensated cardiac hypertrophy remains to be determined.

Emerging evidence shows that not only the abnormality of cardiac myocytes but also myocardial ischemia attributed to the reduction of myocardial capillary density contributes to the progression of decompensated cardiac hypertrophy. [7-10] Therefore, much attention has recently focused on the regulating mechanism of capillary density in cardiac hypertrophy. However, the role of angiotensin II in the regulation of capillary density in cardiac hypertrophy is still unclear.

In the present study, to elucidate the novel mechanism of angiotensin II-induced cardiac injury, we examined the effect of an ARB on myocardial capillary density and cardiac ischemia in Dahl salt sensitive hypertensive rats, a useful model of hypertensive decompensated cardiac hypertrophy or heart failure. We obtained the evidence that prevention of cardiac injury by angiotensin II blockade was partially attributed to the amelioration of cardiac ischemia and this beneficial effect was mediated by the suppression of oxidative stress-induced myocardial vascular endothelial growth factor (VEGF) downregulation and apoptosis signal-regulating kinase 1 (ASK1) activation.

Materials and Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. DS rats (Japan SLC, Inc, Shizuoka, Japan) were used in the present study.

Treatment of salt-loaded DS rats with irbesartan, tempol, and hydralazine

At 7 weeks of age, the diet of DS rats was switched from a 0.3 % NaCl (low-salt) to an 8 % NaCl (high-salt) diet. Previously, we [2-4]and other groups of investigators [11]have demonstrated that 12-week-old DS rats display compensated cardiac hypertrophy with normal cardiac function. Therefore, drug treatment of DS rats was initiated from 12 weeks of age. Twelve-week-old DS rats were orally given irbesartan (20 mg/kg/day), tempol (0.1 mmol/kg/day), or hydralazine (3 mg/kg/day) for 4 weeks (from 12 to 16 weeks of age). In preliminary experiments, we found that irbesartan, tempol, and hydralazine at the above mentioned dose exerted similar hypotensive effects in DS rats. Therefore, this experimental protocol allowed us to compare the effects of each drug under the same blood pressure control. Blood pressure was measured with the tail-cuff method every week. Echocardiographic assessment was performed biweekly during drug treatment. After 4 weeks of treatment, DS rats were anesthetized with ether, and then the heart was immediately excised to examine various parameters as described below.

Measurement of cardiac ischemia

Cardiac hypoxia was estimated with Hypoxyprobe^{TM}-1 (Hypoxyprobe, Burlington) according to the manufacture's instructions. In brief, Hypoxyprobe^{TM}-1 (60 mg/kg body weight) was intraperitoneally injected to DS rats. At 15 minutes after the injection, DS rats were anesthetized with ether, and then the heart was immediately perfused with phosphate-buffered saline and removed. Pimonidazole, that is an element of Hypoxyprobe^{TM}-1 and reductively activated in hypoxic cells, was stained with DAB (DAKO,

Glostrop, Denmark). Pimonidazole positive area was quantified using Lumina Vision version 2.2, analysis software, in 10-sections taken at x200 magnification.

Measurement of capillary density

For assessment of cardiac capillary density, the cross-sectional heart samples were immunostained with anti-CD31 antibody (Santa Cruz) (working dilution 1:200). The number of cardiac CD31-positive cells was counted in 10-sections, taken at x200 magnification, in individual rats; the average of CD31-positive cell number was obtained in individual rats. Capillary density was expressed as the average number of capillaries per cardiomyocyte.

Measurement of capillary apoptosis

Cardiac capillary apoptosis of DS rats was detected with the TdT-mediated dUTP nick-end labeling (TUNEL) by utilizing in situ Apoptosis Detection Kit (Takara, Shiga Japan) in combination with immunostainings for anti-CD31 antibody (Santa Cruz). The number of cardiac TUNEL and CD31 double-positive cells and only CD31-positive cells was counted in 10-sections, taken at x200 magnification, in individual rats; the average ratio of the number of TUNEL and CD31 double-positive cells to that of only CD31-positive cells was obtained in individual rats.

Measurement of cardiac superoxide

The cardiac tissue, removed from DS rats, was immediately frozen in Tissue-Tek O.C.T embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate the levels of cardiac and vascular superoxide in situ, as described in detail. [12]

Western blot analysis

Our detailed method has been described previously. [13] Antibodies used were as follows: anti-VEGF (x2000, Santa Cruz), anti-phospho apoptosis signal-regulating kinase 1 (ASK1) (x1000). [14] The intensity of the bands was quantified using NIH Image analysis software

v1.61. In individual samples, each value was corrected for that of α-tubulin.

In vitro experiments on cultured endothelial cells

Aortic endothelial cells were isolated by EasySep Mouse PE Selection kit (STEMCELL, Tokyo Japan) from male ASK1-/- mice[15] and wild type mice (C57BL/6J). Endothelial cells were grown in EGM-2 BulletKit (Lonza, Basel Switherland) and used between passages 4 and 6.

To examine the role of ASK1 and VEGF in H_2O_2-induced endothelial apoptosis, subconfluent endothelial cells, starved over night in EBM-2 (Lonza, Basel Switherland) containing 1.2% serum in the absence or presence of 2 ng/ml $VEGF_{164}$ (R&D), were incubated for 24 hr in starvation medium supplemented with H_2O_2 (200 μM). For assessment of apoptosis, endothelial cells were immunostained with anti-active caspase 3 antibody (R&D) (working dilution 1:200). The number of active caspase 3-positive cells was counted in 10-sections taken at x400 magnification, containing 300-400 endothelial cells.

To examine the role of VEGF in H_2O_2-induced endothelial ASK1 activation, subconfluent endothelial cells, starved over night in EBM-2 containing 1.2% serum in the absence or presence of 2 ng/ml $VEGF_{164,}$ were incubated for 5 or 30 minute in starvation medium supplemented with H_2O_2 (200 μM). For assessment of ASK1 activation in endothelial cells, western blot analysis was performed as described above.

Measurement of Intracellular Reactive Oxygen Species in vitro

The determination of intracellular oxidative formation was based on the oxidation of 2',7'-dichlorofluorescin diacetate (H2DCFDA, Sigma) to yield an intracellular-trapped fluorescent compound. After 24 hours of starvation with or without $VEGF_{164}$ (2 ng/ml), endothelial cells were pre-loaded with H2DCFDA at a final concentration of 10 μM for 15 min. H_2O_2 was added at a final concentration of 200 μM for 5 min, and then the medium

was removed and replaced with PBS. The fluorescent images were captured with fluorescence microscope, and the fluorescence intensity was quantitated with image-analysis software (Lumina Vision).

Echocardiographic assessment

Transthoracic echocardiographic studies were performed with an echocardiographic system equipped with 12-MHz echocardiographic probe (PHILIPS SONOS-4500) as previously described in detail. [2, 4] In brief, DS rats were lightly anesthetized with intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and were held in the half left-lateral position. M-mode tracings were recorded through left ventricular (LV) anterior and posterior walls (AW and PW, respectively) at the papillary muscle level to measure left ventricular end-diastolic dimension, left ventricular end-systolic dimension, fractional shortening, left ventricular ejection fraction, left ventricular anterior wall thickness at end diastole, and posterior wall thickness at end diastole.

RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) from left ventricular tissue. Complementary DNAs were synthesized by standard techniques, using QuantiTect® Reverse Transcription Kit (QIAGEN Inc., Hilden, Germany). Real time PCR reactions were performed, recorded, and analyzed using Thermal Cycler Dice® Real Time System (TaKaRa Bio Inc., Shiga, Japan) with SYBR Green I detection as described previously. [13] cDNA was amplified using SYBR® Premix Ex TaqTM (Perfect Real Time) PCR kit (TaKaRa Bio Inc.) with specific BNP primers: primer forward: 5'-GTCTCCAGAACAATCCACGATG-3': primer reverse: 5'-AAGGCGCTGTCTTGAGACCTAA-3'

Histological analysis and immunohistochemistry

The hearts were fixed with 4% formalin overnight, embedded in paraffin, cut into 4 μm thick coronal sections, and stained with Sirius Red F3BA (0.5% in saturated aqueous picric

acid, Aldrich Chemical Company) for assessment of cardiac interstitial fibrosis. The area of fibrosis was assessed by using Lumina Vision version 2.2 analysis software. [16]

For assessment of cardiac macrophage infiltration, the sections of heart were immunostained with anti-ED-1 antibody (BMA Biomedicals AG) (working dilution 1:500) for identification of monocytes/macrophages, as described by us. [16] The number of cardiac ED-1 positive cells was counted in 10- sections in individual rats; the average of ED-1 positive cell number was obtained in individual rats.

Statistical analysis

All data are presented as mean±SEM. The data on time course experiments were analyzed by two-way ANOVA, followed by Fisher's PLSD test, using StatView for Windows (SAS Institute, Inc. Cary, U.S.A.). In all other data, statistical significance was determined with one-way ANOVA, followed by Fisher's PLSD test. In all tests, differences were considered statistically significant at a value of $P<0.05$.

Results

Effects on blood pressure and left ventricular weight

As shown in Fig. 1, irbesartan, tempol, or hydralazine significantly and similarly reduced blood pressure of DS rats throughout the treatment, and there was no significant difference in blood pressure among all drug treatment. As shown in Fig. 2 and Table1, despite comparable hypotensive effect, irbesartan or tempol reduced left ventricular weight, and left ventricular BNP expression, macrophage infiltration, and interstitial fibrosis in DS rats, to a greater extent than hydralazine. Irbesartan and tempol significantly reduced the increase in lung weight in DS rats, while hydralazine failed to reduce the increase in lung weight (Table 1).

As shown in Table 2, there was no significant difference in left ventricular end-diastolic dimension, end-systolic dimension, ejection fraction, and fractional shortening between high-salt and low-salt fed DS rats, indicating that there was no apparent cardiac dysfunction in salt-loaded DS rats in this study. Thus, the change of lung weight in salt loaded DS rats described above seems to be attributed to the increase in body fluid retention well recognized in DS rats, but not to cardiac dysfunction.

Effects on left ventricular oxidative stress and myocardial ischemia

As shown in Fig. 3 (A), irbesartan or tempol, but not hydralazine, significantly prevented the increase in left ventricular oxidative stress of DS rats. Interestingly, the significant reduction of left ventricular superoxide by irbesartan or tempol was closely associated with a significant attenuation of left ventricular ischemia, as shown by the decrease in left ventricular pimonidazole positive area (Fig. 3 (B)). Hydralazine failed to reduce cardiac oxidative stress and pimonidazole positive area of DS rats.

We also measured cardiac eNOS in each group of rats and found no significant difference in cardiac phospho-eNOS and total eNOS among each group of rats.

Effects on left ventricular capillary density and capillary endothelial apoptosis

As shown in Fig.4 (A), cross-sectional area of cardiomyocytes in salt loaded DS rats, which was significantly larger than that of control DS rats, was significantly attenuated by irbesartan or tempol, but not hydralazine. As shown in Fig 4. (B), left ventricular capillary density in DS rats was significantly smaller than that of control DS rats (low salt fed DS rats), as shown by the number of left ventricular CD31-positive cells per cardiomyocyte. Irbesartan or tempol treatment significantly and similarly prevented the decrease in left ventricular capillary density of DS rats, while hydralazine did not significantly prevent it.

As shown in Fig. 4 (C), DS rats displayed the increase in left ventricular capillary endothelial apoptosis compared with control DS rats, as shown by double staining of TUNEL and CD31. Interestingly, irbesartan or tempol, but not hydralazine, significantly prevented the increase in capillary endothelial apoptosis in DS rats.

On the other hand, 12-week-old DS rats with compensated cardiac hypertrophy had no decrease in left ventricular capillary density and no increase in capillary endothelial apoptosis (data not shown).

Effects on left ventricular VEGF and ASK1

As shown in Fig. 5 (A), left ventricular VEGF protein levels in DS rats were significantly smaller than those in control DS rats. Irbesartan or tempol treatment significantly attenuated the decrease in left ventricular VEGF protein levels in DS rats, while hydralazine did not increase VEGF levels in DS rats.

As shown in Fig. 5 (B), left ventricular ASK1 phosphorylation in DS rats was significantly higher than that in control DS rats. Irbesartan or tempol significantly reduced left ventricular ASK1 phosphorylation in DS rats to a greater extent than hydralazine treatment.

On the other hand, 12-week-old DS rats with compensated cardiac hypertrophy had no decrease in VEGF and no increase in left ventricular ASK1 activation (data not shown).

Role of ASK1 and VEGF in endothelial apoptosis

As shown in Fig. 6 (A), H_2O_2 significantly increased vascular endothelial apoptosis as shown by the increase in active caspase 3-positive cell number. On the other hand, endothelial apoptosis induced by H_2O_2 was less from ASK1 deficient mice than that from wild type mice. As shown in Fig. 6 (B), pretreatment with VEGF significantly suppressed H_2O_2-induced vascular endothelial cell apoptosis. As shown in Fig. 6 (C), H_2O_2 significantly activated endothelial ASK1 from wild type mice. However, pretreatment with VEGF significantly prevented H_2O_2-induced activation of endothelial ASK1.

As shown in Fig. 6 (D), VEGF treatment significantly attenuated H_2O_2-derived oxidative stress in endothelial cells ($P<0.01$).

Discussion

The major findings of this study were that the prevention of cardiac hypertrophy by angiotensin II blockade was accompanied by the amelioration of left ventricular ischemia due to the suppression of reduction of myocardial capillary density, and these beneficial effects were mediated by the amelioration of oxidative stress-induced endothelial apoptosis through the normalization of VEGF and ASK1. Thus, our present work provided a novel insight into the molecular mechanism underlying angiotensin II-induced cardiac injury.

Compensated cardiac hypertrophy is associated with a normal or increased number of myocardial capillaries. [7-10] On the other hand, prolonged cardiac hypertrophy leads to cardiac ischemia due to the reduction of myocardial capillary density, which is regarded as one of the main mechanisms responsible for the transition from compensated to decompensated cardiac hypertrophy. [7-10] Importantly, the decrease in myocardial capillary density by blockade of VEGF in pressure overload-induced cardiac hypertrophy model results in the transition from compensated to decompensated cardiac hypertrophy, leading to the development of heart failure. [10] Moreover, pressure overload-induced cardiac hypertrophy in VEGF-deficient mice leads to a reduction in myocardial capillary density and accelerates the transition from compensated myocardial hypertrophy to heart failure. [17] Thus, myocardial VEGF plays a protective role against the development of decompensated cardiac hypertrophy. However, the potential contribution of angiotensin II to the regulation of capillary density in cardiac hypertrophy remains to be defined. In the present study, by comparing the effects of irbesartan and hydralazine on DS rats, we obtained the evidence that DS rats at the stage of marked cardiac hypertrophy and fibrosis had significant left ventricular ischemia due to the decreased capillary density, which was mediated by angiotensin II, independently of blood pressure. Of note, angiotensin II-induced cardiac ischemia in DS rats was associated with the increased endothelial apoptosis, the reduced left ventricular

VEGF expression, and the increased left ventricular ASK1 activation.

Accumulating evidence indicates that oxidative stress plays a key role in the pathophysiology of heart failure. [18-20] Moreover, angiotensin II is well known to induce cardiovascular oxidative stress, which plays a pivotal role in the mechanism of angiotensin II-induced cardiac injury. [18-20] Therefore, in this study, we also investigated the effect of tempol, a SOD mimetic, in DS rats to elucidate the contribution of oxidative stress to cardiac injury. We found that the attenuation of left ventricular oxidative stress by tempol prevented cardiac hypertrophy and fibrosis, and the abnormality of cardiac capillary density, endothelial apoptosis, VEGF and ASK1 in DS rats, to a similar extent to irbesartan. These results indicate the critical role of oxidative stress in cardiac injury. Taken together with the findings that irbesartan attenuated cardiac oxidative stress in DS rats to a comparable degree to tempol, our present findings demonstrated that the above mentioned protective effects of irbesartan against cardiac injury were mainly mediated by the attenuation of oxidative stress.

As described above, the present observations showed that the increased left ventricular endothelial apoptosis in DS rats with significant cardiac hypertrophy was attributed to oxidative stress. ASK1 is known to be one of the key molecules involved in oxidative stress-induced cellular apoptosis. [14, 21] These findings encouraged us to determine the role of ASK1 and VEGF in oxidative stress-induced endothelial apoptosis. Using cultured endothelial cells from wild type and ASK1 deficient mice, we found that ASK1 deficiency significantly attenuated H_2O_2-induced endothelial apoptosis, demonstrating the important role of ASK1 in oxidative stress-induced endothelial apoptosis. Interestingly, VEGF treatment mimicked the effect of ASK1 deficiency, as shown by the suppression of endothelial apoptosis by VEGF. Furthermore, this effect of VEGF was associated with the significant inhibition of oxidative stress-induced endothelial ASK1 activation. This suppression of ASK1 activation by VEGF can be at least in part explained by the attenuation of endothelial

oxidative stress by VEGF. Our observations provided the first evidence that VEGF prevented oxidative stress-induced endothelial apoptosis, through the suppression of ASK1.

Study limitations

Several study limitations should be considered in the present study. First, in this study, we found no significant difference in cardiac phospho-eNOS and total eNOS among all groups of DS rats, thereby providing no evidence for the involvement of eNOS in cardiac injury. However, the potential role of eNOS in cardiac injury cannot be completely ruled out, because eNOS is one of the major source producing oxidative stress and VEGF can phosphorylate eNOS. Second, in the present study, we did not examine the effect of each drug treatment on hemodynamics in DS rats. Therefore, our present work did not allow us to determine whether or not the abnormal changes in cardiac VEGF and ASK1 observed in this study were associated with the transition from compensated to decompensated cardiac hypertrophy. Third, it should be noted that blood pressure measurement was performed by not direct method but tail-cuff method, because measurement of a large number of rats was needed in this study. Fourth, the detailed mechanism underlying the suppression of ASK1 activation by VEGF remains to be defined. Finally, in the present study, it also remains to be determined whether ASK1 is directly involved in the regulation of VEGF expression. Further study is necessary to address these key issues.

In conclusion, in DS rats with significant cardiac hypertrophy and fibrosis, angiotensin II, through oxidative stress, decreased myocardial VEGF and activated ASK1. These detrimental effects of angiotensin II induced endothelial apoptosis, thereby causing the reduction of capillary density and subsequent myocardial ischemia. Thus, our present work provided a novel mechanism responsible for angiotensin II-induced cardiac injury. However, further study is needed to elucidate whether or not our present findings indeed contribute to the transition from compensated to decompensated cardiac hypertrophy in hypertensive rats.

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Conflicts of interest

There are no conflicts of interest.

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