Vascular endothelial dysfunction and superoxide anion production in heart failure are p38 MAP kinase-dependent

Julian Widder, Thomas Behr, Daniela Fraccarollo, Kai Hu, Paolo Galuppo, Piet Tas, Christiane E. Angermann, Georg Ertl, Johann Bauersachs

Medizinische Klinik, Julius-Maximilians-Universität, Würzburg, Germany

Medizinische Poliklinik, Julius-Maximilians-Universität, Würzburg, Germany

Klinik für Anaesthesiologie, Julius-Maximilians-Universität, Würzburg, Germany

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Abstract

Objective: The mitogen-activated protein (MAP) kinase system, especially the p38 MAP kinase, is activated in chronic heart failure (CHF). However, the role of vascular p38 MAP kinase in CHF has not been analyzed yet. Methods and results: In aortic rings from rats with CHF 10 weeks after myocardial infarction, acetylcholine-induced relaxation was attenuated (maximum relaxation, \( R_{\text{max}}: 54 \pm 5\% \)) compared to sham-operated animals (\( R_{\text{max}}: 77 \pm 5\%, p < 0.01 \)), while endothelium-independent relaxation elicited by sodium nitroprusside was not significantly changed. Aortic levels of phosphorylated p38 MAP kinase protein were significantly elevated in rats with CHF. In addition, phosphorylation of MAP kinase-activated protein kinase-2 (MAPKAPK-2), an index of p38 MAP kinase activity, was increased. Aortic superoxide anion generation was significantly enhanced in rats with CHF accompanied by elevation of the NAD(P)H oxidase subunit p47phox protein expression. Inhibition of p38 MAP kinase by treatment with the p38 MAP kinase inhibitor SB239063 (800 ppm in standard rat chow) reduced MAPKAPK-2 phosphorylation, preserved acetylcholine-induced relaxation (\( R_{\text{max}}: 80 \pm 4\% \), \( p < 0.01 \)), and reduced vascular superoxide formation. SB239063 treatment did not affect blood pressure and left ventricular enddiastolic pressure. In aortic tissue from CHF animals treated with the angiotensin-converting enzyme (ACE) inhibitor trandolapril, p38 MAP kinase phosphorylation was significantly reduced. Conclusions: Vascular p38 MAP kinase is markedly activated in rats with CHF. Chronic p38 MAP kinase inhibition with SB239063 prevented endothelial vasomotor dysfunction through reduction of superoxide anion production.

Keywords: Endothelial function; MAP kinase; Myocardial infarction; Oxygen radicals; Rats

1. Introduction

Impaired endothelium-dependent vasodilation of coronary, large-conductance and peripheral arteries is a characteristic feature of ischemic as well as non-ischemic chronic heart failure (CHF) in patients [1,2] and experimental models of cardiac dysfunction [3,4]. The endothelium is an important therapeutic target as the normalization of endothelial function reduces vascular resistance and enhances arterial compliance, tissue perfusion, and exercise capacity [5,6]. The mechanisms underlying endothelial dysfunction are not completely clear. Nevertheless, evidence suggests that activation of different endogenous neurohumoral systems, such as the renin–angiotensin and endothelin system, increases oxidative stress in the vessel wall. Indeed, endothelial dysfunction in experimental CHF is associated with enhanced generation of reactive oxygen species (ROS) leading to reduced NO bioavailability [7]. Neurohormonal effectors involved in left ventricular remodeling and heart failure progression as well as endothelial dysfunction such as angiotensin II or endothelin-1 have been shown to activate p38 mitogen-activated protein (MAP) kinase [8,9]. The p38 MAP kinase belongs to the...
MAP kinase superfamily which transduces signals from the cell membrane to the nucleus in response to various stimuli. Three major subgroups of MAP kinases have been identified, the extracellular signal-regulated kinase, c-jun-NH2-terminal kinase and p38 MAP kinase [10]. Indeed, p38 MAP kinase is activated in heart failure [11]. Furthermore, generation of ROS in response to various external stimuli is known to activate MAP kinases, e.g. angiotensin II stimulates p38 MAP kinase by a redox sensitive mechanism in vascular smooth muscle cells [8,12]. Recently, in spontaneously hypertensive-stroke prone rats on salt/fat diet, Ju et al. [13] have demonstrated that hypertension-induced endothelial dysfunction depends on p38 MAP kinase activation.

In the present study, we investigated the role of vascular p38 MAP kinase in rats with heart failure after myocardial infarction and the effects of chronic p38 MAP kinase inhibition on endothelial vasomotor function and vascular superoxide anion production.

2. Methods

2.1. Study protocol, myocardial infarction, hemodynamic measurements

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Left coronary artery ligations were performed in adult male Wistar rats (250–300 g, obtained from Charles River, Sulzfeld, Germany) as previously described [14]. Briefly, the thorax was opened under isoflurane anesthesia, the heart exteriorized and a ligature placed around the proximal left coronary artery. Subsequently, the heart was returned to its normal position and the thorax closed. Rats were randomly allocated to treatment with placebo or the p38 MAP kinase inhibitor SB239063 (800 ppm in chow) with free access to standard rat chow and water. SB239063 displays specific and high-affinity binding to p38 MAP kinase, resulting in potent inhibition of its catalytic activity, with an IC50 of 44 nM [15,16]. Hemodynamic studies were performed 10 weeks after coronary artery ligation under isoflurane anesthesia and controlled respiration.

2.2. Vascular reactivity studies

Only animals suffering from heart failure (left ventricular end-diastolic pressure >15 mm Hg) were included in the study. The descending thoracic aorta was dissected following removal of the heart and cleaned of connective tissue. One section (10 mm) was immediately frozen in liquid nitrogen for Western blot analysis. Another section (10 mm) was used for measurement of superoxide anion production, while the remainder was cut into rings (3 mm in length) which were mounted in an organ bath (Förh Medical Instruments, Seeheim, Germany) for isometric force measurements [17]. The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O2, 5% CO2) Krebs-Henseleit solution (NaCl 118 mmol/l, KCl 4.7 mmol/l, MgSO4 1.2 mmol/l, CaCl2 1.6 mmol/l, KH2PO4 1.2 mmol/l, NaHCO3 25 mmol/l, glucose 12 mmol/l; pH 7.4, 37 °C) containing diclofenac (1 μmol/l) [17]. Rings were repeatedly contracted by KCl (with a maximum of 100 mmol/l) until reproducible responses were obtained. Thereafter, the rings were preconstricted with phenylephrine (0.3–1 μmol/l) to comparable constriction levels (2.81 ± 0.16 g in placebo-treated sham, 2.76 ± 0.14 g in CHF and 2.40 ± 0.12 g in CHF treated with SB239063) and the relaxant responses to cumulative doses of acetylcholine and to sodium nitroprusside were assessed.

2.3. Measurement of superoxide anion formation

Vascular superoxide anion formation was measured using lucigenin-enhanced chemiluminescence. The light reaction between superoxide anions and lucigenin (5 μmol/l) was detected in a luminometer (Wallac, Freiburg, Germany) during incubation of rings in a HEPES-modified Krebs buffer (pH 7.40) without and with addition of 100 μmol/l NADH. Signals were integrated over 30 s and averages of the plateau phase used for further calculation. The specific chemiluminescence-signal was expressed as counts per min per mg dry weight of tissue (cpm/mg). The oxidative fluorescent dye hydroethidine was used to evaluate in situ production of superoxide anion. Unfixed frozen ring segments were cut into 10-μm-thick sections and placed on a glass slide. Hydroethidine (2 μmol/l) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37 °C for 30 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Aortic rings from CHF animals and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from CHF and control specimens. Fluorescence was detected with a 585-nm long-pass filter [17].

2.4. Western blot analysis

Aortic samples were homogenized in ice-cold RIPA buffer (150 mmol/l NaCl, 50 mmol/l Tris–HCl, 5 mmol/l EDTA, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 100 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Proteins were determined by Bradford assay. Aortic extracts were mixed with sample loading buffer and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electrotransferred onto PVDF membrane (Immun-Blot® 0.2 μm, Bio-Rad) for 60 min at 100 V using Criterion Blotter (Bio-Rad). After transfer, the membranes were blocked in blotting solution.
(20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.05% Tween 20, pH 7.5) with 5% blocking agent (Amersham) overnight at 4 °C, followed by incubation with primary antibody in blotting solution with 0.5% blocking agent overnight at 4 °C. Primary antibodies used recognize: p47phox (sc-7660, Santa Cruz Biotechnology); MAP kinase-activated protein kinase-2 (MAPKAPK-2, 3042, Cell Signaling Technology); MAPKAPK-2 phosphorylated at Thr222 (3044, Cell Signaling Technology); and β-actin (6276, Abcam). The blots were washed five times in blotting solution and incubated with matching secondary antibody for 1 hour at room temperature. After extensive washing, the bands were detected using chemiluminescence assay (ECL + Plus, Amersham). Anti-β-actin was used to normalize for loading variations.

2.5. Materials

All biochemicals were obtained in the highest purity available from Sigma (Deisenhofen, Germany). SB239063 was kindly provided by Robert N. Willette; Glaxo Smith Kline (King of Prussia, Philadelphia, USA).

2.6. Statistics

Relaxant responses were given as percentage relaxation relative to the preconstriction level. Values are expressed as mean ± S.E.M. of n experiments with segments from different arteries. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparison followed by a post hoc Bonferroni test. P values < 0.05 were considered statistically significant.

3. Results

Left ventricular end-diastolic pressure was significantly elevated in rats with CHF after myocardial infarction (27.6 ± 5.5 mm Hg) as compared to sham-operated animals (6.4 ± 0.5 mm Hg), and left ventricular systolic pressure was diminished (126 ± 7 resp. 146 ± 4 mm Hg). Both hemodynamic parameters were not influenced by treatment with SB239063 (26.6 ± 3.1 resp. 120 ± 4 mm Hg). Systolic and diastolic blood pressures were slightly reduced in heart failure (128 ± 8 resp. 96 ± 5 mm Hg) compared to sham-operated animals (138 ± 8 resp. 104 ± 5 mm Hg). This was not influenced by treatment with the p38 MAP kinase inhibitor (125 ± 4 resp. 97 ± 4 mm Hg).

Acetylcholine-induced relaxation of aortic rings preconstricted with phenylephrine was blunted in aortae from rats with CHF (Fig. 1), and restored by chronic treatment with the p38 MAP kinase inhibitor SB239063. Maximum endothelium-independent relaxation induced by sodium nitroprusside did not differ among the groups, however, the concentration response curve was slightly shifted to the right in CHF placebo (pD2 = 7.54 ± 0.08 resp. −7.28 ± 0.09 mol/l, p n.s.), and shifted to the left by treatment with SB239063 (−7.81 ± 0.09 mol/l, Fig. 1).

Superoxide anion formation was significantly increased in aortae from rats with CHF versus sham-operated rats. Vascular superoxide anion formation in animals with CHF treated with SB239063 was significantly lower compared to placebo-treated animals. NADH-stimulated superoxide anion formation was significantly higher in aortae from placebo-treated rats with CHF compared to sham-operated rats, and was significantly reduced in aortae from SB239063 treated animals (Figs. 2 and 3). NAD(P)H oxidase subunit p47phox protein levels were elevated in aortae from rats with CHF versus sham-operated animals and reduced by treat-
ment with SB239063 (Fig. 3). Expression of the NAD(P)H oxidase subunit nox1 did not differ between CHF and sham-operated animals.

Phosphorylation of p38 MAP kinase was significantly increased in the aorta from rats after myocardial infarction compared to sham-operated animals (Fig. 4). The phosphorylation of MAPKAPK-2, a direct downstream target, was measured as an index of p38 MAP kinase activity. Phospho-MAPKAPK-2/MAPKAPK-2 ratio was significantly elevated in aortae from placebo-treated rats with CHF compared to sham-operated animals and normalized in aortae from animals treated with SB239063 (Fig. 4).

In additional experiments using aortic tissue obtained in a study performed earlier, the effect of chronic treatment with the angiotensin-converting enzyme (ACE) inhibitor trandolapril (0.3 mg/kg body weight per day) on vascular p38 MAP kinase phosphorylation in CHF rats was investigated. Treatment with trandolapril improved endothelium-depen-
dent relaxation, reduced left ventricular pressure and diminished aortic superoxide production in CHF rats in that study [18]. As shown in Fig. 5, ACE inhibition significantly reduced p38 MAP kinase phosphorylation in the aortae from CHF animals.

4. Discussion

p38 MAP kinase phosphorylates specific serine and threonine residues of target protein substrates thus regulating a variety of cellular activities. p38 MAP kinase activation is involved in cardiomyocyte hypertrophy, vascular remodeling or apoptosis. In response to external stress such as hypoxia, pressure overload or ischemia/reperfusion injury, p38 MAP kinase is activated in the heart as well as in the vessel wall [10]. During heart failure progression, activation of cardiac p38 MAP kinase has been shown [11]. p38 MAP kinase inhibition with SB239063 enhanced survival and improved vasoreactivity in a rat model of hypertensive cardiac damage [15].

In the present study, we demonstrate for the first time a marked activation of vascular p38 MAP kinase and its downstream target kinase, MAPKAPK-2, in rats with heart failure. Specific inhibition of the p38 MAP kinase with SB239063 normalized vascular p38 MAP kinase activity and prevented endothelial dysfunction in this model. As confirmed in the present study, endothelial dysfunction in heart failure is related to elevated vascular superoxide anion production with subsequent reduction of NO bioavailability [7]. Recently, a correlation between superoxide anion formation and the degree of impairment of endothelium-dependent relaxation was demonstrated in the rat model of heart failure after myocardial infarction [19]. Increased superoxide formation in the media of the artery as supported by superoxide detection using hydroethidine may also be responsible for the slight rightward shift of the concentration response curve to sodium nitroprusside.

Treatment with SB239063 reduced aortic superoxide anion production. The attenuation of endothelial dysfunction and vascular superoxide anion formation by SB239063...
suggests that p38 MAP kinase activation is involved in vascular ROS production in heart failure. This observation fits to the role of p38 MAP kinase in oxidative stress signaling in other cell types. In bovine leukocytes and human neutrophils, p38 MAP kinase has been described to regulate the activation of the NAD(P)H-oxidase [20,21]. Our results indicate that vascular p38 MAP kinase activation in heart failure is involved both in induction and activation of the vascular NAD(P)H-oxidase. The mechanism of NAD(P)H oxidase activation is complex and not yet completely understood. The regulatory components p47phox, p67phox, and the small GTP-binding protein rac1 translocate from the cytosol to the cell membrane. There, they associate with the membrane-bound flavocytochrome b555, which is a heterodimer of gp91phox and p22phox. gp91phox (nox2) is one member of five homologous proteins termed nox1–5. In vascular smooth muscle cells, nox1 substitutes for gp91 phox [22]. Furthermore, in the vessel wall, the components of the NAD(P)H-oxidase are constitutively expressed and at least in part preassembled in the membrane. p47phox facilitates the activation of the NAD(P)H-oxidase and has been shown to play an important role for vascular NAD(P)H-oxidase-mediated superoxide formation [23]. The up-regulation of p47phox expression together with the augmented activity of NAD(P)H oxidase in aortae from CHF rats suggest that both the induction of NADPH oxidase as well as facilitated activation of the enzyme may contribute to increased vascular superoxide formation in CHF.

Besides NAD(P)H-oxidase, potential dysregulation of other enzymes may increase superoxide in the vessel wall, e.g. reduced extracellular superoxide dismutase activity has been demonstrated in patients with heart failure [24]. However, as the rat vessels contain a very low amount of extracellular superoxide dismutase compared to other mammals [25], a major influence in the rat model is unlikely. Furthermore, uncoupling of the endothelial nitric oxide synthase (eNOS) in pathophysiological conditions such as diabetes mellitus is known to result in superoxide anion production [26]. However, eNOS uncoupling has not yet been demonstrated in the vessel wall in heart failure. In the CHF model after coronary ligation in the rat, removal of the endothelium did not reduce vascular superoxide anion production [7]. In addition, the NOS inhibitor N-nitro-L-arginine methyl ester did not diminish aortic superoxide generation (J.B., unpublished data) suggesting that uncoupling of eNOS is unlikely to contribute to the increased vascular superoxide anion production in this model of heart failure.

As treatment with SB239063 did not significantly affect left ventricular pressure and function in the present study, it is unlikely that improvement of the heart failure status is responsible for the changes in vascular function. Indeed, the p38 MAP kinase inhibitor appears to exert specific beneficial effects on the vascular wall. This is further supported by the fact that p38 MAP kinase expression was much higher in the vascular wall than in the myocardium (data not shown). Improvement of endothelium-dependent relaxation is generally believed to be quite positive, and the effects of several established treatments for heart failure have been attributed at least in part to reversal of endothelial dysfunction [5]. However, firm answers about the value of p38 MAP kinase inhibition for heart failure are not possible from the present study.

p38 MAP kinase activation by angiotensin II has been repeatedly demonstrated [8,12,27], and angiotensin receptor blockade prevented angiotensin II-induced p38 MAP kinase phosphorylation in vascular smooth muscle cells [27]. In the present study, angiotensin-converting enzyme inhibition in rats with heart failure by treatment with trandolapril reduced vascular p38 MAP kinase phosphorylation. These data suggest that neurohumoral activation with increased angiotensin II levels in heart failure contributes to p38 MAP kinase activation in the aorta.

In conclusion, we found a marked activation of vascular p38 MAP kinase in rats with CHF. Chronic p38 MAP kinase inhibition with SB239063 prevented endothelial vasomotor dysfunction through reduction of superoxide anion production.

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