Inhibition of inducible nitric oxide synthase and superoxide production reduces matrix metalloproteinase-9 activity and restores coronary vasomotor function in rat cardiac allografts

Koso Egi, Nicole E. Conrad, Jennifer Kwan, Costas Schulze, Richard Schulz, Stephen M. Wildhirt

Abstract

Objective: Oxidants such as nitric oxide (NO) and superoxide are involved in coronary endothelial dysfunction, an early event in the process of allograft coronary atherogenesis, possibly by activation of matrix metalloproteinases (MMPs) and extracellular matrix proteins. We investigated the contribution of inducible nitric oxide synthase (iNOS) derived NO and superoxide on (MMP)-9 activity and to changes in coronary vasomotor function in rat cardiac allografts. Methods and Results: An allogenic (Brown Norway to Lewis rats) heterotopic cardiac transplantation model was used to study the effect of continuous treatment with a selective iNOS inhibitor, N-(3-(aminomethyl) benzyl) acetamidine (1400W), and polyethylene glycol conjugated superoxide dismutase (SOD) either alone or in combination on coronary vasomotor dysfunction. 1400W or SOD 24 h alone or their combination improved endothelium-dependent (bradykinin) and -independent (sodium nitroprusside) coronary flow reserve and inhibited enhanced MMP-9 protein and activity. In addition, histopathological study revealed that either 1400W or SOD or their combination reduced superoxide production and nitrotyrosine protein. Conclusion: The present study demonstrates for the first time that selective iNOS inhibition or SOD treatment reduces enhanced MMP-9 protein and activity associated with improvement of both, endothelium-dependent and -independent coronary vasomotor function in rat cardiac allografts. This is accompanied by reduction of nitrotyrosine and superoxide production. This suggests that the proteolytic enzyme MMP-9 is an effector molecule of oxidant-mediated coronary vasomotor dysfunction.

Keywords: Nitric oxide synthase; Superoxide; Matrix metalloproteinases; Transplantation; Endothelium

1. Introduction

Coronary endothelial dysfunction is an early and potentially reversible phenomenon in the process of native and cardiac transplant atherogenesis [1,2]. Recent evidence suggests that the oxidants nitric oxide (NO) derived by inducible nitric oxide (iNOS) and superoxide, in part by the formation of peroxynitrite, are major contributors to vasomotor dysfunction [3–7]. Two important molecules, matrix metalloproteinase (MMP)-2 and MMP-9 have been shown to play a role in vascular remodeling, plaque stability and myocardial ischemia/reperfusion [8–11]. It has been shown that oxidants such as superoxide, NO and peroxynitrite can activate MMP’s [8,12,13]. Their activity in the heart may change within seconds to minutes and contribute to acute myocardial dysfunction as shown previously in stunned myocardium following ischemia and reperfusion [14]. The relative contribution of both NO derived from iNOS and superoxide to up-regulate MMP-2 and/or -9 activities and their role in coronary vascular dysfunction remains unknown but may be of therapeutic interest and prognostic value. We hypothesized that upregulation of both NO derived from iNOS and superoxide are involved in coronary endothelial dysfunction and upregulation of extracellular matrix proteins 2 and -9 (MMP 2, -9) in rat cardiac allografts.
To test this hypothesis we studied the effects of in vivo selective iNOS inhibition and treatment with SOD after allogenic heterotopic heart transplantation on enhanced MMP-2 and -9 protein and activity. In addition, we tested whether this is associated with improvement of both, endothelium-dependent and -independent coronary vasomotor function in a rat model of isolated coronary vasomotor dysfunction.

2. Model of isolated coronary vasomotor dysfunction in rat cardiac allografts

The experimental protocol was approved by the institutional Animal Care and Use Committee (‘Guide for the Care and Use of Laboratory Animal’, NIH publication 85–23, revised 1985).

Male Lewis rats (250–350 g) and male Brown–Norway (BN) rats (200–300 g) were housed and cared under conventional conditions, following the National Institutes of Health guidelines. Allogenic (Brown Norway to Lewis) heterotopic cardiac transplantation was performed as described by Ono and Lindsey [15]. In the present study, an allograft model was used since it was intended to study early endothelial dysfunction, known to precede and predict allograft vasculopathy. To minimize variability between experiments, the ischemic time was standardized to 60 min and myocardial temperature maintained at 8 °C for all transplantsations.

This protocol was chosen from preliminary experiments to induce significant coronary vasomotor dysfunction in the absence of major cardiac contractile depression in rat cardiac allografts. In this model, the coronary vasomotor dysfunction observed is likely to be mediated by both ischemia-reperfusion as well as an early alloimmune response since acute rejection starts immediately after transplantation. However, examination of allografts did not show signs for acute rejection episodes including cellular infiltration, myocyte necrosis or intramyocardial bleeding within the 24 h observation period.

3. Pharmacologic protocol and continuous administration of drugs in vivo

Animals were randomly assigned to one of the four groups:

(A) Control group (saline only; n = 8),
(B) Selective iNOS inhibition by 1400W (N-(3-(Aminomethyl) benzy) acetamidine; 2.0 mg/kg/12 h, s.c.; n = 6). 1400W has been shown to be a highly selective, tight binding iNOS inhibitor in vitro and in vivo and is 1000 fold selective for iNOS than eNOS in rats [16].
(C) Superoxide scavenging by polyethylene glycol conjugated superoxide dismutase (SOD; 1000 U/kg/24 h, s.c.; n = 6),
(D) 1400W (2.0 mg/kg/12 h, s.c.) + SOD SOD; 1000 U/kg/24 h, s.c.; n = 6).

The in vivo doses of 1400W and SOD were derived from preliminary dose finding experiments showing maximal effects on vasomotor function without toxicity. Fig. 1 shows the study protocol and time sequence of drugs administered.

4. Assessment of hemodynamics and coronary flow reserve

24 h following in vivo reperfusion allografts were rapidly excised and immediately perfused on a Langendorff apparatus at constant perfusion pressure of 70 mmHg (Hugo Sachs Elektronik, Hugstetten, Germany) Left ventricular developed pressure (LVDP), heart rate (HR), peak positive dP/dt(+dP/dt) and peak negative dP/dt(−dP/dt) were continuously measured. Coronary flow (CF) and coronary flow reserve (CFR) were continuously assessed with a flow probe positioned at the ascending aorta.

Endothelium-dependent CFR was measured following infusion of bradykinin (final concentration of 1 µM, 1.5 min). To block cyclooxygenase, indomethacin (final concentration of 10 µM) was infused for 10 min before bradykinin and added to the bradykinin solution.

After drug washout and re-equilibration, sodium nitroprusside (final concentration of 200 µM, 1.5 min) was infused to assess endothelium independent vasomotor function.

Both bradykinin and SNP were given in random order to control for bias and non-specific effects which may have occurred during the perfusion phase.

CFR was expressed as the difference between baseline CF and maximum CF derived by drug administration.

Thereafter, specimens from the left ventricle were immediately frozen in liquid nitrogen and stored at −80 °C or fixed in buffered 4% formalin overnight, dehydrated and embedded in paraffin for further examination.

5. Detection of superoxide

The oxidative fluorescent dye hydroethidine was used to evaluate levels of superoxide in situ as previously described [17]. Hydroethidine is freely permeable to cells and is oxidized to fluorescent ethidium bromide by superoxide where it is trapped by intercalating with DNA. Sections (25 µm thickness) were incubated with 8 µM hydroethidine ( Molecular Probes, Mo Bi Tec GmbH, Göttingen) dissolved in DMSO for 30 min in a light-protected humidified chamber at 37 °C. To account for autofluorescence of
tissues, negative control sections were incubated with DMSO only, omitting hydroethidine. Fluorescence was detected with a 585-nm long-pass filter in a fluorescence microscope.

6. Immunostaining for nitrotyrosine and MMP-9 protein

Nitrotyrosine formation has been shown to be an estimate for peroxynitrite mediated nitrosilation of proteins by binding to the phenolic ring of tyrosine. We therefore studied the effects of 1400W and SOD on nitrotyrosine immunoreactivity as a measure of peroxynitrite formation [18]. In brief, paraffin-embedded sections 5 μm in thickness were used for immunohistochemistry. Following deparaffinization, the peroxidase technique (EnVision™, Dako) was used for immunohistochemical detection of nitrotyrosine (polyclonal anti-nitrotyrosine-antibody, Upstate/Biozol, dilution 1:100, for 45 min and MMP-9 (polyclonal anti-MMP-9-antibody, 10 μg/ml for 3 h). Color development was carried out with the peroxidase substrates streptavidine/AEC (Vector laboratories). Nitrotyrosine and MMP-9 detection were semiquantified by three independent observers using four separate transmural sections of left ventricular from three animals in each group.

7. Measurement of MMP activities by zymography

Zymography was performed as described previously [14]. Briefly, equal amounts of protein were loaded onto 8% polyacrylamide gels containing 1 mg/ml gelatin (Sigma, St. Louis, MO) and electrophoresis was performed. After washing with 2.5% (v:v) Triton X-100 in water (BDH Inc., Toronto, Canada) and then incubation buffer (50 mM Tris–HCl, 0.15 M NaCl, 5 mM CaCl₂) to remove SDS, gels were incubated at 37 °C in incubation. Subsequent staining with 0.05%. For densitometric analysis zymogram intensities were analyzed using Sigma Gel measurement software (Jandel Co, San Rafael, CA).

8. Statistical analysis

Hemodynamic data and dose response profiles are expressed as mean ± SEM Box plots were used to present data for all other results. The Bartlett test was employed to
test for homogeneity of variances. One-way analysis of variance (ANOVA), followed by Fisher’s PLSD test was used to assess data on hemodynamics, dose response profiles, CFR and MMP activities. The Kruskal-Wallis test, followed by Scheffe test was used to analyze data on superoxide, nitrotyrosine and MMP-9 staining. A value of $P < 0.05$ was considered statistically significant.

9. Results

9.1. Cardiac hemodynamics and CFR

Hemodynamic parameters are shown in Table 1. According to our protocol, no differences were observed with regard to left ventricular developed pressure (LVDP), heart rate (HR), max $\pm dP/dt$ and baseline coronary flow between groups.

Dose response studies showed that administration of either drug in vivo resulted in a dose dependent improvement of endothelium-dependent CFR in response to bradykinin (Fig. 1A and B). All doses used for both drugs were well tolerated and no drug related morbidity or mortality was noted during the course of the study.

At doses of 2.0 mg/kg/day s.c. for 1400W and 1000 U/kg/day s.c. for SOD either given alone or in combination resulted in a significant improvement in CFR in response to endothelium dependent and -independent stimulation; combined treatment at these doses did not result in any further protective effects on CFR (Fig. 2A and B).

Effects of 1400W and SOD on nitrotyrosine and MMP-9 immunoreactivity and superoxide generation

Specific nitrotyrosine immunoreactivity was confined to myocardial cells and the perivascular region of intramyocardial vessels in non-treated control animals (Fig. 3).

In contrast, treatment with 1400W, SOD, or combined treatment significantly reduced specific nitrotyrosine immunoreactivity within myocardial cells (Fig. 3). Semiquantification revealed significant reduction of nitrotyrosine immunoreactivity in all treatment groups: control: $3.6 \pm 0.5$, 1400W: $1.8 \pm 0.3\^*$, SOD: $1.6 \pm 0.5\^*$, 1400W + SOD: $1.5 \pm 0.4\^*$; $^*P < 0.05$ vs control.

No immunoreactivity was observed for negative controls when omitting the primary antibody (data not shown).

Specific fluorescence for superoxide was detected in non-treated hearts which was significantly reduced by continuous treatment with either of the drugs (Fig. 4).

A strong fluorescence signal was observed in the non-treated control group. This was observed predominantly in the vascular endothelial and smooth muscle cells. In contrast, potent reduction in the specific fluorescence signal was observed in all treatment groups (Fig. 4). Semiquantification revealed significant reduction of fluorescence for superoxide in all treatment groups: control: $3.6 \pm 0.5$, 1400W: $2.5 \pm 0.4\^*$, SOD: $2.3 \pm 0.5\^*$, 1400W + SOD: $2.2 \pm 0.4\^*$; $^*P < 0.05$ vs control.

9.2. Effects of 1400W and SOD on MMP-2 and -9 activities

Representative zymogram reveals the presence of 92 kDa (MMP-9) and 72-kDa (MMP-2) gelatinolytic activities in left ventricles from transplanted rat hearts (6, A). Treatment with SOD nearly abolished 92 kDa activity whereas treatment with 1400W or combined treatment with SOD and 1400W resulted in a significant reduction in 92 kDa activity. On the other hand, there was no significant difference of 72 kDa activity between the groups (Fig. 6B and C).

10. Discussion

The present study investigated, whether selective iNOS inhibition by 1400W or continuous administration of a cell permeable superoxide dismutase (SOD) protects coronary vascular function in rat cardiac allografts. Moreover, we
tested if continuous antioxidant treatment with 1400W and SOD would have protective effects on coronary vasomotor function via inhibition of extracellular matrix proteins, MMP-2 and MMP-9, potential effector molecules of reactive oxygen species.

Here we demonstrate for the first time that continuous treatment with either a highly selective iNOS inhibitor 1400W or SOD reduces MMP-9 protein and activity associated with improvement of both endothelium-dependent and -independent coronary vasomotor function in rat cardiac allografts. Combined treatment with 1400W and SOD does not result in further effects on coronary vascular protection (Fig. 2). In addition, nitrotyrosine and superoxide staining as observed in non-treated control group is nearly abolished in all treatment groups (Figs. 3B and 5).

The question occurs, why iNOS inhibition or superoxide scavenging by SOD results in comparable protective effects. One explanation may be the possibility that under pathological conditions iNOS up-regulation results in the production of both endothelium-dependent and -independent coronary vasomotor function in rat cardiac allografts. Combined treatment with 1400W and SOD does not result in further effects on coronary vascular protection (Fig. 2). In addition, nitrotyrosine and superoxide staining as observed in non-treated control group is nearly abolished in all treatment groups (Figs. 3B and 5).

An interesting finding in the present study is the detection of nitrotyrosine protein in cardiomyocytes and the perivascular region of intramyocardial vessels in non-treated cardiac allografts. The observation that hemodynamics were comparable despite highly significant reduction in coronary vascular function in the present study remain uncertain. However, nitrotyrosine formation correlates with tissue peroxynitrite in the presence of enhanced superoxide production and increased NO production by iNOS formation in dysfunctional hearts of endotoxemic rats [18]. This has also been showed in various settings including experimental myocarditis and in lungs taken from endotoxin treated rats [20,21].

Fig. 2. The increase in flow (ml/min) from baseline coronary flow is shown. The effects of 1400W or SOD on (A) endothelium-dependent increase in coronary flow (CF) as assessed by infusion of bradykinin (1 μM, 1.5 min) and (B) endothelium-independent increase in CF as assessed by infusion of sodium nitroprusside (SNP, 200 μM, 1.5 min) are plotted. Both, the selective iNOS inhibition by N-(3-(Aminomethyl) benzyl) acetamidine (1400W), and polyethylene glycol conjugated superoxide dismutase (SOD) significantly improved the increase in CF in response to both endothelium dependent and -independent stimulation in all treatment groups. (A) control (n = 8), (B) 1400W (n = 6), (C) SOD (n = 6), D) 1400W + SOD (n = 6). *P < 0.05 vs. control. Doses used: 1400W: 2.0 mg/kg/day s.c.; SOD: 1000 U/kg/day s.c.
Fig. 3. Representative immunostainings of nitrotyrosine protein expression. Nitrotyrosine protein expression was detected in the control group. However, nearly no nitrotyrosine expression was observed in the 1400W group, SOD group and 1400W + SOD group. Doses used: 1400W: 2.0 mg/kg/day; SOD: 1000 U/kg/day.

Fig. 4. In situ detection of superoxide in cardiac allografts. Representative fluorescent photomicrographs of non-treated and treated hearts were incubated with hydroethidine. Specimens from non-treated cardiac allografts show highly specific fluorescence in vasculature and myocardial cells. In contrast, treatment with 1400W, SOD, or combined treatment reduced specific fluorescence within myocardial cells. Original magnification ×200. Doses used: 1400W: 2.0 mg/kg/day; SOD: 1000 U/kg/day. (Magnification ×200).
myeloperoxidase and other metalloproteinases, or substrates supporting peroxidase-dependent tyrosine nitration such as NO, NO$_2$ and hydroperoxides [17,23].

Involvement of MMPs in coronary vasomotor dysfunction

A novel finding in the present study is that enhancement of MMP-9 expression and activation is significantly reduced by either 1400W or SOD. This is associated with significant improvement of coronary vascular function. The combined treatment with both 1400W and SOD resulted in comparable effects on inhibition of MMP-9 activity and vascular protection (Fig. 6).

Under inflammatory conditions oxidant species including superoxide and peroxynitrite are potent activators of gelatinases in inflammatory and vascular smooth muscle cells as shown previously [8,11,13,24]. In particular MMP-1, MMP-2 and MMP-9 were upregulated in myocardial and vascular dysfunction [11,14,24]. In this regard, Cheung et al. demonstrated an immediate upregulation of MMP-2 activity following ischemia and reperfusion in isolated rat hearts. Inhibition of MMPs by doxycycline or phenanthroline improved cardiac function, suggesting that MMP-2 may play a direct role in affecting myocardial contractile function [14].

The exact mechanism by which MMP activation alters vascular function remains unclear. Moreover, the present data do not provide proof whether or not vascular dysfunction and enhanced MMP-9 activity are dependent phenomena. It may as well be solely parallel effects and to clarify this issue, a selective MMP-9 inhibitor for in vivo treatment would be helpful. However, MMP-9 protein was localized predominantly in intramyocardial vessels in the present study. In this regard, Gurjar and colleagues demonstrated enhanced activity of MMP-9 in vascular smooth muscle cells following increased superoxide production [25]. It has been shown that MMPs may cause degradation of the extracellular matrix, enhanced extravasation of inflammatory cells, platelet aggregation and increased smooth muscle cell migration [11]. All of these are potential mediators of endothelial and vascular dysfunction. These findings are in line with the present data suggesting, that MMP-9 plays an important role in the development of oxidant mediated coronary vascular dysfunction.

11. Conclusion

In conclusion, the present study demonstrates for the first time that continuous treatment with either a highly selective iNOS inhibitor 1400W or SOD reduces MMP-9 activation associated with improvement of both endothelium-dependent and -independent coronary vasomotor function in rat cardiac allografts. This is related to attenuation of enhanced endogenous superoxide production and a decrease in nitrotyrosine expression 24 h after transplantation. This is of therapeutic and prognostic value and further studies are required to link early vascular dysfunction associated with induction of iNOS, superoxide production and enhanced MMP-9 activity to morphologic changes observed in cardiac allograft vasculopathy or classic arteriosclerosis.
Acknowledgements

Koso Egi is a research fellow in the Department of Cardiac Surgery, University of Munich and supported by the Dr Karl Wamsler Foundation, Munich, Germany. The study was in part supported by the German Israel Foundation and the Deutsche Forschungsgemeinschaft and the Canadian Institutes of Health Research (MT-11563). The authors would like to thank Andrea Bedynek; Holger Schlaszu; Joachim F. Schneider and Haluk Akdemir for their excellent technical assistance. We acknowledge the collaboration with the Department of Cardio Thoracic Surgery (Director: Prof. Makoto Sunamori), Tokyo Medical and Dental University, Tokyo Japan.

References