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CONGRESS PAPER

Contractile dysfunction in experimental cardiac allograft rejection: role of the poly (ADP-ribose) polymerase pathway*

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Summary

Recent studies suggested that the peroxynitrite-poly (ADP-ribose) polymerase (PARP) pathway is activated during acute allograft rejection. We investigated whether PARP inhibition improves transplant function during cardiac rejection. Isogeneic Lewis-to-Lewis and allogeneic Dark Agouti-to-Lewis rat cardiac transplants were studied under treatment with placebo or with the PARP-inhibitor INO-1001 (1 mk/kg/day), Functional, biochemical and histological analysis were performed 3 and 5 days after transplantation. After 3 days, baseline left ventricular pressure–volume relationships did not differ between the groups. However, coronary blood flow (4.3 ± 0.5 vs. 2.2 ± 0.2 vs. 4.1 ± 0.3 ml/min/g, $P < 0.05$) and contractile response to dobutamine (Δ+dP/dt: 98 ± 11 vs. 57 ± 7 vs. 88 ± 8%, $P < 0.05$) decreased significantly in the placebo group, which was abolished by INO-1001. Vasodilatory response to acethylcholine was reduced in the placebo group (78 ± 6 vs. 36 ± 9 vs. 72 ± 7%, $P < 0.05$). After 5 days, baseline systolic and diastolic pressure–volume relationships were impaired ($P < 0.05$) in the placebo group and the response to dobutamine and to acethylcholine deteriorated further which was abolished by INO-1001. Histology confirmed mild to moderate rejection after 3 days and severe acute rejection after 5 days in the allogeneic groups. Thus, contractile and vasomotor dysfunction occur in a typical time dependent manner during cardiac rejection, which can be reduced by PARP-inhibition.

Introduction

Cardiac transplantation is still the most successful treatment option for end-stage heart disease. Despite the improvement of mid- and long-term mortality, after cardiac transplantation by the introduction of cyclosporin A and other novel immunosuppressants, acute rejection remains one of the most serious complications of the early postoperative course [1]. Recently, we showed [2] that acute cardiac rejection leads to a typical sequence of cardiac dysfunction: mild to moderate rejection results in an early impairment of myocardial blood flow with preserved basal left ventricular contractility and compliance and reduced inotropic reserve. During severe rejection complex systolic and diastolic dysfunction occur. Impaired cardiac function can be detected also in the clinical arena. In clinical studies, decreased diastolic function indexed by isovolumic relaxation times, diastolic filling period and pressure half times was proposed to be associated with early rejection, while systolic performance was relatively preserved [2–4]. In other studies, changes in myocardial blood flow or flow reserve were suggested

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to be a marker for the onset and resolution of acute rejection [5,6]. At least at the later stages of cardiac rejection cardiac dysfunction becomes clinically symptomatic. As it is very difficult to reverse severe rejection by immunosuppressive therapy, once it has occurred, and irreversible myocyte necrosis may lead to cardiac insufficiency even if the immunologic reaction is reversed, it would be favorable to have therapeutic options, which reduce myocyte death and cardiac dysfunction associated with acute rejection.

There is an increasing evidence that inducible nitric oxide synthase (iNOS) expression is up-regulated during acute cardiac-allograft rejection [7,8] which in turn leads to increased nitrosative stress and, subsequently, cardiac muscle cell death and apoptosis. Oxidative and nitrosative stress triggers the activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which contributes to the pathogenesis of various cardiovascular diseases, including arteriosclerosis, myocardial infarction and diabetic endothelial dysfunction [see overview in 9]. PARP functions primarily as DNA damage sensor in the nucleus and mediates the cellular response to DNA strand breaks [9]. Recent studies suggest that, in addition to, DNA damage repair, PARP has several other important effects: induction of an energy-consuming, futile repair cycle that eventually leads to cell dysfunction and cell death via the necrotic route [9]. In addition, PARP participates in inflammatory response by regulating inflammatory cell infiltration [9]. Furthermore, the blockade of the nitric oxide–peroxynitrite–PARP pathway at different levels may reduce the incidence and severity of acute rejection and thereby improve transplant outcome [10,11]. Therefore, in the present study, we investigated how far PARP inhibition influences functional cardiac changes during ongoing rejection.

Materials and methods

Heterotopic transplantation and drug treatment

Male Lewis and Dark Agouti rats weighing 250–300 g were used in these experiments. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1996).

Isogeneic (Lewis-to-Lewis, $n = 12$) and allogeneic ($n = 24$ Dark Agouti-to-Lewis) heterotopic abdominal cardiac transplantation were performed by one surgeon using the technique of Ono and Lindsay, as previously described [12,13]. Allogeneic animals were treated either vehicle or with the ultrapotent selective isoindolinone-based PARP inhibitor INO-1001 1 mg/kg/day. Three or 5 days ($n = 6$/each subgroup) after transplantation the abdomen was reopened and functional measurements were performed in situ as described below. Then, the grafts were explanted and prepared for further histological and biochemical analysis.

Functional measurements

LV pressure–volume relations [12,13]

A latex balloon was introduced into the left ventricle (LV) of the graft via the apex and was connected to a precision calibrated syringe for administration and withdrawal of fluid and to a Millar Micro-Tip catheter (Millar Instruments, Inc., Houston, TX, USA) to determine peak systolic pressure (LVSP) and end-diastolic pressure (LVEDP) at different LV volumes. Data for a complete pressure volume curve were obtained through incremental increases in ventricular volume by 0.03 ml until a ventricular volume of 0.14 ml was reached. After baseline measurements, the infusion of dobutamine was started at a rate of 5 μg/kg/min and the functional measurements were repeated.

Coronary blood flow

Total coronary blood flow (CBF) was measured by a perivascular ultrasonic flow probe on the donor aorta. After baseline measurements, the endothelium-dependent vasodilator acetylcholine (ACH, 1 nM, 0.2 ml) and bradykinin (BK 0.1 nM, 0.2 ml) as well as the endothelium-independent vasodilator sodium–nitroprusside (SNP, 10 nM, 0.2 ml) were administered directly into the coronary arteries of the graft via the donor aorta. Between the infusions, CBF was allowed to return to baseline levels. Vasodilator response was expressed as maximum percent change of CBF from baseline.

Histology

After functional measurements were completed, the hearts were excised and cut in two pieces. One piece was fixed in formalin for histologic analysis, the other piece was immediately immersed in fluid nitrogen (−196 °C) and stored frozen at −80 °C for later biochemical analysis.

Myocardial tissue sections were stained with hematoxylin–eosin and examined by light microscopy to detect and identify any structural changes or development of necrosis. Twelve sections per heart were examined by two independent investigators to limit the influence of observer basis. Rejection grade was scored by the International Society of Heart and Lung Transplantation (ISHLT) grading system.

Immunhistology was performed to detect poly (ADP-ribose), the product of PARP, as described previously [13]. Formalin fixed sections were stained by pri-

mary mouse monoclonal anti-poly (ADP-ribose) antibody (Alexis, San Diego, CA, USA). Sections were then counterstained with nuclear fast red. Immunohistologic stainings were evaluated by the COLIM software package (Pictron Ltd., Budapest, Hungary) as described previously [13]. Briefly, on the base of the measured intensity in 20 adjacent field (400× power magnification), the color classes were coupled with score values as follows: 0, no positive staining, 1–3 increasing degrees of intermediate staining and 4, extensive staining. The program automatically measured the area of the objects in each class in each field, assigned an area score (1, up to 10% positive cells, 2, 11–50% positive cells, 3, 51–80% positive cells, 4, >80% positive cells), and calculated an average score for the whole picture (intensity score multiplied by area score). Finally, each specimen was characterized with the average of the 20 adjacent fields.

High energy phosphates

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) contents were assessed with standard photometry using an enzyme-kinetic assay [13].

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Repeated measures of analysis of variance (ANOVA) followed by the Scheffe's post-hoc test were used to compare individual means. A value of $P < 0.05$ was considered statistically significant.

Results

Histology

After 3 days mild to moderate, after 5 days severe acute rejection could be observed in the vehicle-treated allogeneic group. Application of INO-1001 reduced the ISHLT rejection score significantly both at 3 and 5 days (Table 1).

Poly (ADP-ribose) immunochemistry showed significant PARP activation in the vehicle-treated allogeneic group at 3 days in comparison with the isogeneic group, which further increased 5 days after transplantation. INO-1001 completely prevented rejection associated PARP activation, PARP histology scores did not differ significantly between the INO-treated allogeneic and the isogeneic group (Table 1).

Baseline hemodynamics

Heart rate (HR), CBF, LVSP and LVEDP showed no significant differences within the isogeneic subgroups 3 and 5 days after transplantation (Fig. 1). In the allogeneic group, a significant ($P < 0.05$) decrease in CBF was observed 3 days after transplantation, which was prevented by INO-1001 treatment, while the other hemodynamic parameters did not significantly differ from the time-matched isogeneic group. With the onset of severe rejection (5 days after transplantation) a rapid deterioration of systolic and diastolic function occurred. LVSP decreased significantly ($P < 0.05$) indicating reduced contractility. The increase of LVEDP reflects to a deterioration of diastolic compliance. PARP inhibition significantly improved both systolic and diastolic function. The complete pressure–volume relationships 3 and 5 days after transplantation are shown in Fig. 2. The significant rightward shift of the systolic pressure–volume relationship and the significant leftward shift of the diastolic pressure–volume relationship indicate a reduced systolic and diastolic performance after 5 days respectively.

Table 1. Histologic scoring.

	Isorafts	Allografts control	Allografts INO-1001
ISHLT rejection grade			
POD 3	–	2.1 ± 0.1	0.5 ± 0.1‡
POD 5	–	3.4 ± 0.2*	1.9 ± 0.3*‡
Poly (ADP-Ribose) staining			
POD 3	0.3 ± 0.1†	3.5 ± 0.3†	0.5 ± 0.2‡
POD 5	0.2 ± 0.1†	5.7 ± 0.4*†	0.9 ± 0.4‡

POD, postoperative day. All values are given as mean ± SEM.
*$P < 0.05$ POD 5 versus POD 3.
†$P < 0.05$ allografts versus isografts.
‡$P < 0.05$ allograft INO-1001 versus vehicle.

Effects of dobutamine stress

The response of HR, and LVSP to administration of dobutamine is given as percent change to baseline in Fig. 3. The chronotropic response was similar in both iso- and allografts 3 days after transplantation. After 5 days, HR showed a significantly ($P < 0.05$) higher increase in the isogeneic than in the allogeneic group. The response of maximum LVSP and CBF to dobutamine was significantly ($P < 0.05$) reduced after 3 days in the allogeneic group and deteriorated further after 5 days. INO-1001 treatment completely prevented the reduced responses to dobutamine 3 days after transplantation. After 5 days, the response to dobutamine was significantly ($P < 0.05$) more pronounced in the INO-1001 group in comparison with the vehicle-treated allogeneic group, however, it was reduced in comparison with the isogeneic group.

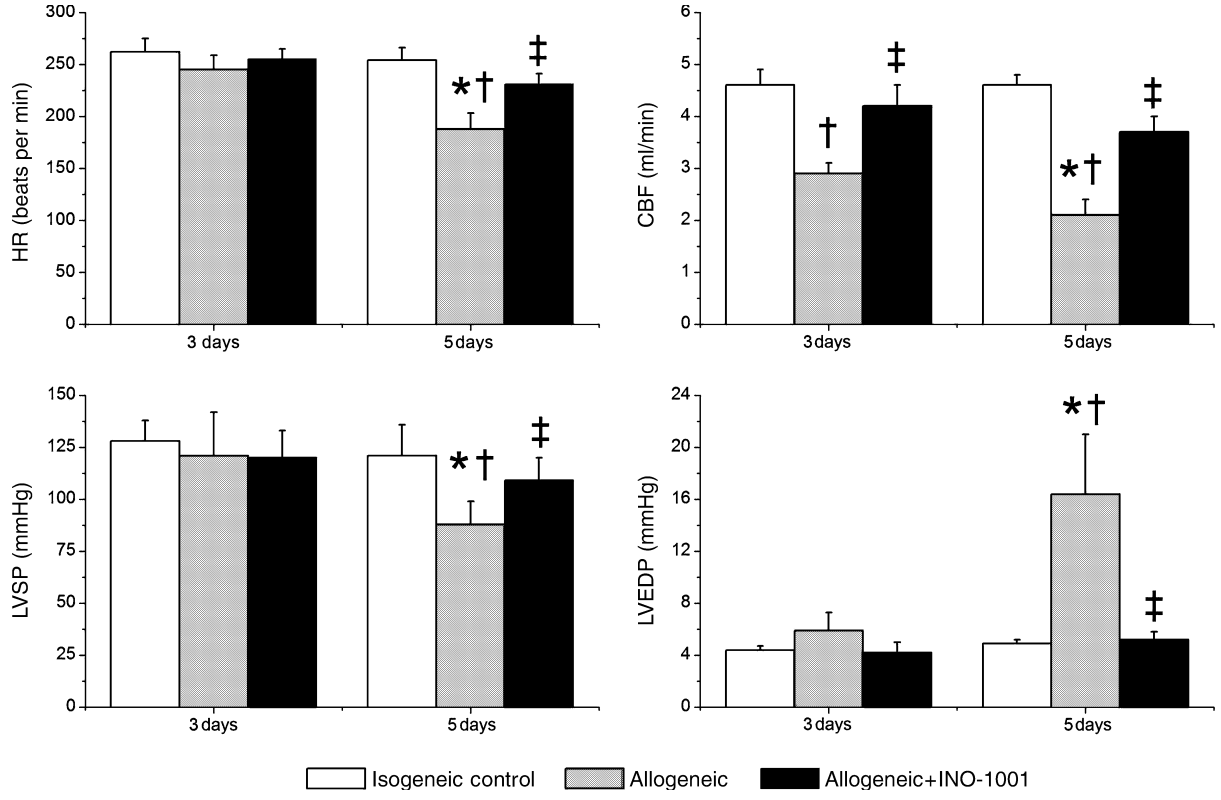

Figure 1 Hemodynamic data at 3 and 5 days after transplantation. HR, heart rate; CBF, coronary blood flow; LVSP, left ventricular systolic pressure; LVEDP, left ventricular enddiastolic pressure. All values are given as mean ± SEM; *$P < 0.05$ POD 5 versus POD 3, †$P < 0.05$ allografts versus isografts, ‡$P < 0.05$ allograft INO-1001 versus vehicle.

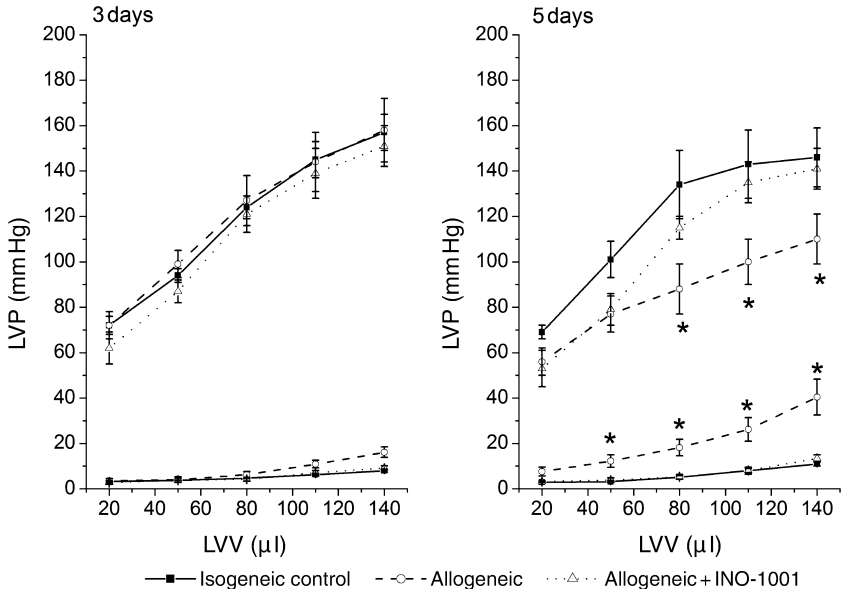

Figure 2 Systolic and enddiastolic pressure–volume relationships. ISO, isografts; ALLO, allografts; LVSP, left ventricular systolic pressure; LVEDP, left ventricular enddiastolic pressure; LVV, left ventricular volume; POD, postoperative day. All values are given as mean ± SEM; *$P < 0.05$ versus other groups.

Coronary vascular function

Vasodilatory responses to acetylcholine and bradykinin were significantly reduced in the vehicle-treated allogeneic group after 3 days, which was completely abolished by INO-treatment (Fig. 4). After 5 days, the responses to acetylcholine and bradykinin deteriorated further in the vehicle-treated allogeneic group, which was partly reversed

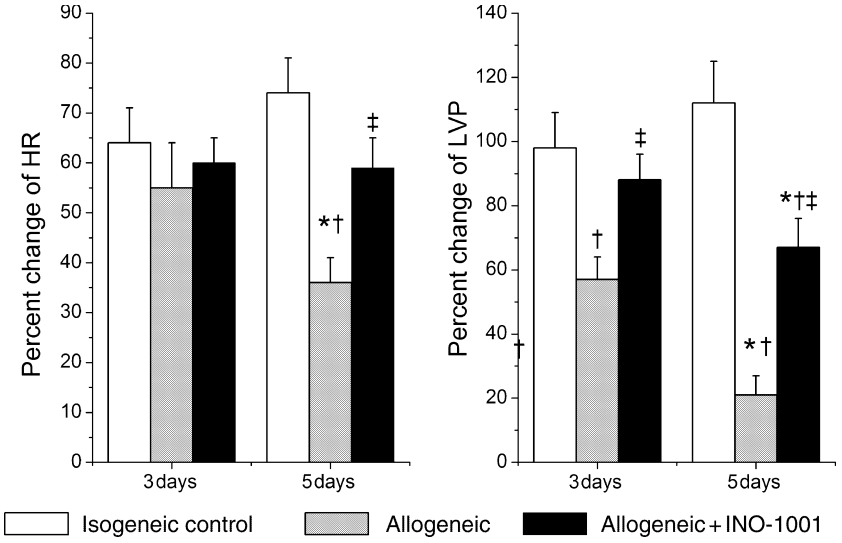

Figure 3 Percent changes of heart rate (HR), and left ventricular systolic pressure (LVSP) after administration of dobutamine. ISO, isografts; ALLO, allografts; POD, postoperative day. All values are given as mean ± SEM; **P* < 0.05 POD 5 versus POD 3, †*P* < 0.05 allografts versus isografts, ‡*P* < 0.05 allograft INO-1001 versus vehicle.

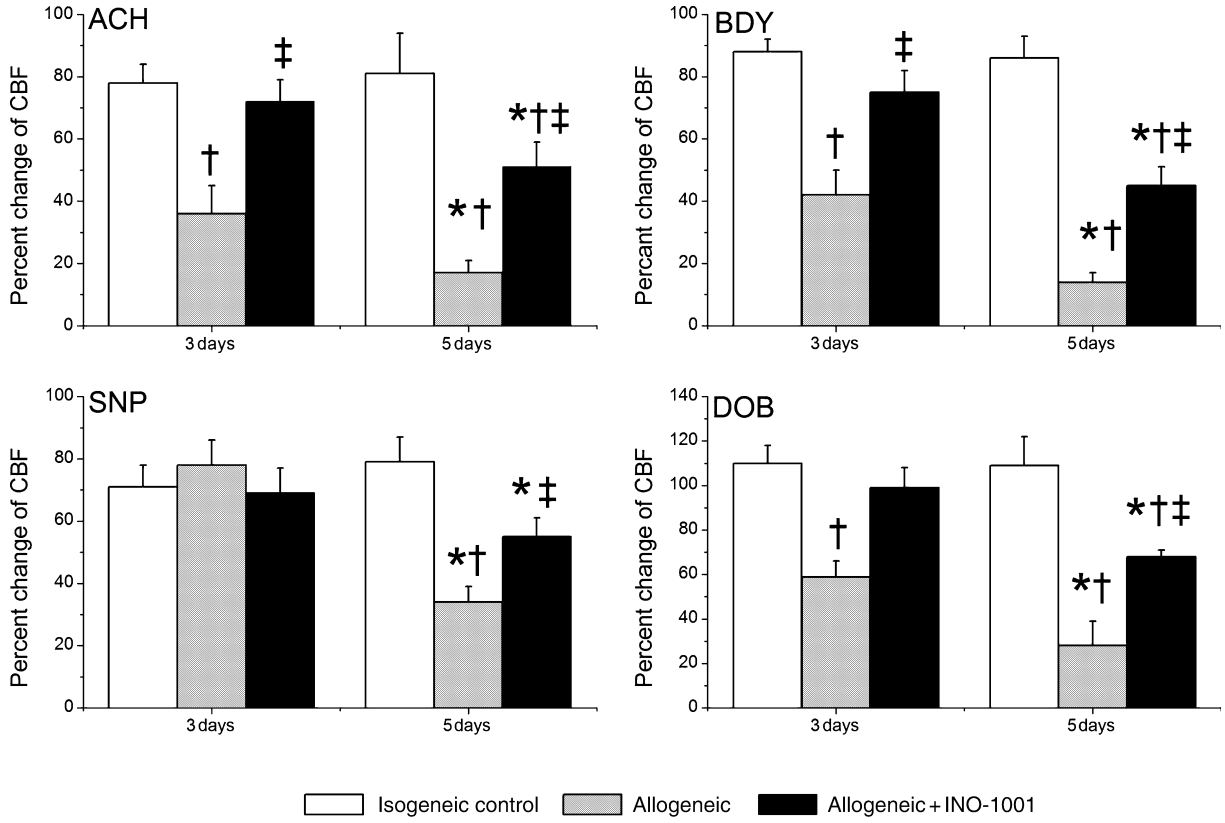

Figure 4 Vasodilatory response to acetylcholine (ACH), bradykinin (BDY) and sodium–nitroprusside (SNP) as well as dobutamine (DOB) is expressed as percent change of baseline coronary blood flow. **P* < 0.05 POD 5 versus POD 3, †*P* < 0.05 allografts versus isografts, ‡*P* < 0.05 allograft INO-1001 versus vehicle.

in the INO group. Response to SNP did not differ between the groups after 3 days, but was reduced after 5 days in the vehicle-treated allogeneic group. The response of CBF to dobutamine deteriorated progressively at 3 and 5 days after transplantation in the vehicle treated allogeneic group, which was abolished by INO-1001 (Fig. 4).

High energy phosphates

Table 2 shows the values of high energy phosphates. After 3 days, there were no significant differences between the groups. Five days after transplantation myocardial ATP contents were significantly ($P < 0.05$) reduced in the allogeneic group without treatment in comparison with the other two groups.

Discussion

Although allograft survival during acute cardiac allograft rejection could be significantly increased by immunosuppressive drugs, there have been recent additional efforts to enhance the salvage of cardiac muscle cells during acute rejection and to thereby preserve ventricular function [14]. In correspondence with our previous studies [12,15], we observed a typical sequence of cardiac dysfunction during the development of acute cardiac rejection: preserved baseline systolic and diastolic function with reduced contractile reserve at the stages of mild to moderate rejection followed by progressive systolic and diastolic dysfunction during severe rejection. Reduced responsiveness to β-adrenergic stimulation at preserved baseline contractility has been also described at early stages of cardiac rejection in ventricular myocytes [16], isolated papillary muscles [17], *ex vivo* Langendorff preparation [18] and rejecting canine orthografts [19].

We also observed characteristic changes of coronary blood flow and endothelial function during the development of cardiac rejection: baseline coronary blood flow decreases even at the early stages of cardiac rejection with concomitant decrease of endothelium dependent vasodilatory function (in response to acetylcholine and bradykinin) while endothelium-independent vasodilatory function remained intact. During severe rejection a progressive deterioration of coronary blood flow and both coronary endothelial and smooth muscle function could be documented. Reduction of myocardial or coronary blood flow has been reported in heterotopic and orthotopic models of heart transplantation during early stages of acute rejection [12,20]. Bergsland *et al.* [20] also found a clear evidence of histologically confirmed rejection and concomitant severe decrease in blood flow of cardiac rat allografts, although the hearts appeared to function normally. Nitenberg *et al.* [5] noted a marked decrease in coronary flow, as well as flow reserve during cardiac rejection in humans. In accordance with previous *in vitro* studies [21], we demonstrated *in vivo* that allograft rejection leads to an early endothelial dysfunction followed by deterioration of endothelium-independent vasorelaxation. The pathomechanisms of endothelial dysfunction include both G-protein dependent and -independent pathways [21].

Table 2. High energy phosphates.

	Isografts	Allografts control	Allografts INO-1001
ATP (μmol/g drw)			
POD 3	4.2 ± 0.6	3.6 ± 0.7	4.4 ± 0.4
POD 5	3.9 ± 1.0	2.1 ± 0.2*†	4.0 ± 10‡
ADP (μmol/g drw)			
POD 3	2.5 ± 0.4	2.8 ± 0.5	2.2 ± 0.4
POD 5	2.6 ± 0.2	2.1 ± 0.3	2.3 ± 0.2
AMP (μmol/g drw)			
POD 3	0.9 ± 0.2	1.4 ± 0.6	1.5 ± 0.3
POD 5	1.2 ± 0.5	1.6 ± 0.2	1.0 ± 0.5

POD, postoperative day; drw, dry wait. All values are given as mean ± SEM.
*$P < 0.05$ POD 5 versus POD 3.
†$P < 0.05$ allografts versus isografts.
‡$P < 0.05$ allograft INO-1001 versus vehicle.

A major finding of the present study is that inhibition of PARP markedly improves allograft function during acute rejection. Previous studies showed that protein nitration and subsequent peroxynitrite generation play an important role in cardiac rejection. It has been demonstrated that inhibitors of iNOS-dimerization or genetic deletion of iNOS reduce cardiac rejection and apoptosis [14,22]. A recent study showed, that WW85, a peroxynitrite decomposition catalyst, also reduces acute cardiac rejection [10]. Peroxynitrite is a potent activator of DNA strand breaks and PARP [9]. PARP activation has been shown after reperfusion injury in rat cardiac transplants [13] or following alloimmune activation and rejection in rat tracheal [23] and cardiac allografts [11]. Piper *et al.* [10] found that the peroxynitrite decomposition catalyst WW85 given alone decreased poly (ADP-ribose) suggesting that it acted, in part, by decreasing PARP activation proving that PARP activation in acute cardiac rejection may be related, at least in part, to peroxynitrite formation.

There is evidence that activation of PARP occurs in a variety of cell types in response to oxidant damage, ischemia/reperfusion, hemorrhagic shock, and local and systemic inflammation [9] as well as various forms of chronic heart failure [24,25]. In all these settings, DNA damage and single-strand DNA breaks (mediated in part by NO or peroxynitrite) are thought to trigger the activation of PARP and subsequent deleterious effects. The mechanisms of INO-1001's protective action are multiple. In various types of ischemia/reperfusion or inflammatory injury, the prevention of PARP-activation results in a better preservation of the high-energy phosphate content resulting in an improved energy status [9,13,26], which is consistent with the findings of the present study. Indeed,

Benvenuti *et al.* [27] found a significant decrease in ATP content in human cardiac transplants when moderate or severe rejection with focal or diffuse aggressive infiltrates were present. In a subpopulation of this cohort, sequential analysis showed a significant increase in ATP content after rejection therapy concomittant with histologic improvement. In accordance with this study, ATP content was significantly decreased in the placebo-treated allogeneic group, which was completely prevented by PARP inhibition. Beside its direct effects on myocardial metabolism, PARP-activation contributes to the expression of adhesion molecules such as P-selectin and ICAM-1 [9,13,26] and consequently to the recruitment of neutrophils into the jeopardized tissue. It is likely that both an inhibition of the energetic component of PARP-mediated cell dysfunction and the suppression of proinflammatory pathways contribute to cardioprotective effects [9,26]. On the base of the cumulative data, we propose a positive feedback cycle: early hydroxyl radical and peroxynitrite production → PARP-related endothelial injury → neutrophil infiltration → more hydroxyl and peroxynitrite production. Inhibition of PARP would interrupt this cycle at the level of endothelial injury.

It remains unclear how far direct immunosuppressive effects of PARP-inhibition contribute to prolonged survival and improved cardiac function during cardiac rejection. The fact that histologic rejection grade was reduced in the INO-treated group implies that apart of its anti-inflammatory effects PARP inhibition may have a direct immunmodulatory effect. As described previously, PARP inhibitors reduce basal and cytokine-induced expression of pro-inflammatory surface antigens [9] on several types of antigen-presenting cells. In settings of inflammation, ischemia/reperfusion or graft rejection such depressed expression of cell adhesion molecules or immune costimulator ligands may mediate anti-inflammatory and immunomodulatory effects as seen in our animal model.

One further possible explanation of PARP-mediated immunosuppression is the direct effect of PARP on NAD and ADP-ribose metabolism. It has been recently shown [28] that NAD and ADP-ribose inhibit T-lymphocyte proliferation. Activated PARP scavange NAD and ADP-ribose and thereby may induce a vicious cycle during graft rejection: reduced NAD and ADP-ribose concentrations → PARP related increase of T-lymphocyte proliferation → more cytokine production and neutrophil infiltration → more PARP activation. Inhibition of PARP would interrupt this cycle at the level of NAD metabolism and would stimulate intrinsic immunosuppressive modulation. As the molecular mechanism of PARP mediated immunomodulation was not the primary scope of this study, unfortunately, we do not have any evidence at these points.

In summary, we showed that PARP is activated and contributes to cardiac dysfunction during acute cardiac rejection. The inhibition of PARP attenuates the development of acute cardiac rejection and improves allograft function. Furthermore, the data suggest, that beneficial effects of PARP inhibition are might not be restricted to the reduction of free radical mediated injury, but involve direct immunomodulatory effects on cellular immunity. Here, further studies are warranted to elucidate how PARP modulate immune function.

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