Activation of Poly(ADP-Ribose) Polymerase Contributes to Development of Doxorubicin-Induced Heart Failure

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Received October 7, 2001; accepted November 27, 2001 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) by oxidant-mediated DNA damage is an important pathway of cell dysfunction and tissue injury in conditions associated with oxidative stress. Increased oxidative stress is a major factor implicated in the cardiotoxicity of doxorubicin (DOX), a widely used antitumor anthracycline antibiotic. Thus, we hypothesized that the activation of PARP may contribute to the DOX-induced cardiotoxicity. Using a dual approach of PARP-1 suppression, by genetic deletion or pharmacological inhibition with the phenanthridinone PARP inhibitor PJ34, we now demonstrate the role of PARP in the development of cardiac dysfunction induced by DOX. PARP−1+/+ and PARP−1−/− mice received a single injection of DOX (25 mg/kg i.p.). Five days after DOX administration, left ventricular performance was significantly depressed in PARP−1+/+ mice, but only to a smaller extent in PARP−1−/− ones. Similar experiments were conducted in BALB/c mice treated with PJ34 or vehicle. Treatment with a PJ34 significantly improved cardiac dysfunction and increased the survival of the animals. In addition PJ34 significantly reduced the DOX-induced increase in the serum lactate dehydrogenase and creatine kinase activities but not metalloproteinase activation in the heart. Thus, PARP activation contributes to the cardiotoxicity of DOX. PARP inhibitors may exert protective effects against the development of severe cardiac complications associated with the DOX treatment.

Poly(ADP-ribose) polymerase (PARP), also known as poly(ADP ribose) synthetase (PARS), is an abundant nuclear enzyme of eukaryotic cells. When activated by DNA single-strand breaks, PARP initiates an energy-consuming cycle by transferring ADP ribose units from NAD⁺ to nuclear proteins. This process results in rapid depletion of the intracellular NAD⁺ and ATP pools, slowing the rate of glycolysis and mitochondrial respiration and eventually leading to cellular dysfunction and death (Eliasson et al., 1997; Szabó et al., 1997; Zingarelli et al., 1998; Burkart et al., 1999; Szabó, 2000). Overactivation of PARP represents an important mechanism of tissue damage in various pathological conditions associated with oxidant stress, including myocardial reperfusion injury (Zingarelli et al., 1998), stroke (Eliasson et al., 1997), circulatory shock (Szabó et al., 1997; Oliver et al., 1999; Liaudet et al., 2000), and autoimmune β-cell destruction (Burkart et al., 1999; Pieper et al., 1999). Activation of PARP also contributes to the development of cardiovascular dysfunction in diabetes (Soriano et al., 2001a,b; Pacher et al., 2002).

Doxorubicin (DOX; Adriamycin; Pharmacia & Upjohn, Peapack, NJ) is a broad-spectrum antitumor anthracycline antibiotic that is commonly used to treat a variety of cancers, including severe leukemias, lymphomas, and solid tumors (Blum and Carter, 1974; Young et al., 1981; Singal et al., 1987; Hortobagyi, 1997; Singal and Iliskovic, 1998). However, the clinical use of DOX is limited because of its serious cardiotoxicity, which leads to irreversible degenerative cardiomyopathy and heart failure (Singal et al., 1987; Singal and Iliskovic, 1998).

The cardiotoxicity of DOX may involve increased oxidative stress in cardiomyocytes, alteration of cardiac energetics, and direct effect on the DNA. However the exact mechanisms implicated have not been established, and optimal therapeutic approaches for cardioprotection are not fully defined (Meyers et al., 1977; Olson et al., 1981; Doroshow and Davies, 1986; Liu, 1989; Siveski-Iliskovic et al., 1994; Li and Singal, 2000; Weinstein et al., 2000).

Herein, we tested whether the impairment of cardiac function in doxorubicin-induced acute heart failure is dependent upon the activation of the PARP pathway within the heart.

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; PARS, poly(ADP-ribose) synthetase; DOX, doxorubicin; +dp/dt, maximal slope of systolic pressure increment; −dp/dt, maximal slope of diastolic pressure decrement; LDH, lactate dehydrogenase; CK, creatine kinase; MMP, metalloproteinase.
Materials and Methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication 85-23, revised 1985) and was performed with the approval of the local Institutional Animal Care and Use Committee.

Animals. Male BALB/c, PARS+/+, and PARS−/− mice weighing 25 to 35 g were administered a single dose of DOX HCl (Sigma/Aldrich, St. Louis, MO) at 25 mg/kg i.p., and used for functional measurements 5 days later. This time point was chosen as more than five final half-lives of elimination of DOX from both plasma and cardiac tissue in mice (van der Vlugh et al., 1990). Treatment with the PARP inhibitor PJ34 (20 mg/kg i.p.) started 1 h before the DOX injection and continued (3 × 10 mg/kg i.p./day) until the hemodynamic measurements were made. A similar dosing regimen with PJ34 has previously been shown to be sufficient to block vascular PARP activation in rats and mice (Soriano et al., 2001a,b).

Hemodynamic Measurements in Mice. Five days after DOX administration analysis of left ventricular performance was measured in mice anesthetized with i.p. injections of ketamine (80 mg/kg) and xylazine (10 mg/kg). The animals were placed on controlled heating pads, and core temperature measured via a rectal probe was maintained at 36–38°C.

A microtip catheter transducer (SPR-671; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle under pressure control. After stabilization for 15 to 20 min, the pressure signal was continuously recorded using a MacLab A/D converter (AD Instruments, Mountain View, CA), and stored and displayed on an Apple Macintosh personal computer. The heart rate and left ventricular systolic and end-diastolic pressures were measured and the maximal slope of systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt), and indexes of contractility and relaxation were calculated. After these measurements, the catheter was pulled back into the aorta for the measurement of arterial blood pressure. After the hemodynamic measurements were made, animals were sacrificed.

Serum Lactate Dehydrogenase (LDH) and Creatine Kinase (CK) Measurement. Forty-eight hours after DOX treatment, mice were sacrificed, and blood was drawn form the vena cava inferior. Samples were allowed to clot and serum was used for activity measurement. LDH and CK activities were determined by endpoint activity assay kits (Sigma Diagnostics Canada, Mississauga, ON, Canada) according to the manufacturer’s instructions. LDH and CK activities were expressed as units per liter.

Metalloproteinase Zymography. Forty-eight hours after DOX treatment mice were sacrificed, and hearts were perfused with physiological saline and excised. Samples were homogenized in TNC buffer (50 mM Tris, 0.15 mM NaCl, 10 mM CaCl2, 0.05% Brij 35, 0.02% NaN3, pH 7.4) (Koyama et al., 2000), and cellular debris was removed by centrifugation. Protein content was assayed by the method of Bradford (1976) and samples were mixed with equal volume of 2× SDS sample buffer (Invitrogen, Carlsbad, CA). Samples were incubated at room temperature for 15 min and were applied to gelatin or casein zymography gels. After electrophoresis (125 V, 90 min) proteins were renatured in zymography renaturing buffer (Invitrogen) for 30 min at room temperature under continuous shaking and were then placed to 37°C for overnight developing in developing buffer (Invitrogen). Undigested substrate was visualized by Coomassie brilliant blue staining (0.1% Coomassie brilliant blue, 45.5% methanol, 9% acetic acid). To confirm that digested bands are due to Ca2+-dependent proteases, replicate gels were developed in Ca2+-free buffer containing 20 mM EDTA.

Survival Experiments. Animals (142) exposed to DOX (25 mg/kg i.p.) received either PJ34 (3 × 10 mg/kg i.p.; n = 55) or vehicle (isotonic saline, 0.2 ml i.p.; n = 87), starting from 1 h before DOX injection. Mortality was monitored and recorded over a 4-week period.

Statistical Analysis. Results are reported as mean ± S.E.M. Statistical significance between two measurements was determined by the two-tailed unpaired Student’s t test, and among groups it was determined by analysis of variance with Bonferroni’s correction. In the survival experiments the survival curves of the different groups were compared using log-rank test. Probability values of P < 0.05 were considered significant.

Reagents. All reagents were obtained from Sigma/Aldrich, unless indicated otherwise. The potent, novel, water-soluble phenanthridine-oxo-5,6-dihydro-phenanthridin-2-yl-N,N-dimethylacetamide, was synthesized as described (Soriano et al., 2001b). In cell-free PARP assay, with NAD + and purified PARP-1 enzyme, PJ34 inhibited PARP activity in a dose-dependent manner, with an EC50 value of 20 nM. The EC50 value of the prototypical PARP inhibitor 3-aminobenzamide was 200 μM. Peroxynitrite- and hydrogen peroxide-induced oxidation of dihydrodimerodamine-123 was unaffected by PJ34, in the concentration range of 1 μM to 10 mM, indicating that the compound does not act as an antioxidant. The details of the synthesis and pharmacological characterization of PJ34 were published previously (Soriano et al., 2001b).

Results

Cardiac Function

Ventricular Function in PARP-1+/+ and PARP-1−/− Mice. In PARP-1+/+ mice treated with DOX, heart rate, mean blood pressure, left ventricular systolic pressure, +dP/dt, and −dP/dt were significantly decreased, whereas left ventricular end-diastolic pressure increased (Fig. 1). In contrast PARP-1−/− mice treated with DOX showed significantly improved left ventricular performance (Fig. 1). There was no significant difference in the left ventricular function between PARP-1+/+ and PARP-1−/− mice in the absence of DOX treatment (Fig. 1).

Effects of PJ34 on Doxorubicin-Induced Cardiac Dysfunction in BALB/c Mice. DOX induced a significant increase in left ventricular end-diastolic pressure and decrease in heart rate, mean blood pressure, left ventricular systolic pressure, +dP/dt, and −dP/dt in BALB/c mice (Fig. 2). Treatment with PJ34 significantly attenuated the DOX-induced changes in left ventricular systolic pressure, mean blood pressure, systolic +dP/dt, diastolic −dP/dt, and left ventricular end-diastolic pressure (Fig. 2). The PARP inhibitor exerted no significant effects on hemodynamic parameters in control mice (Fig. 2).

Serum LDH and CK Measurement

Serum LDH and CK activities were significantly elevated 48 h after DOX injection compared with the activities measured in the control mice (Fig. 3, A and B). Treatment with PJ34 significantly attenuated the DOX-induced elevations in serum LDH and CK activities.

Metalloproteinase Zymography

Heart extracts were subjected to metalloproteinase zymography. Extracts were assayed after 48 h of DOX or DOX + PJ34 treatment. Hearts of untreated mice were used as control.

On the gelatin zymography gels only one band was detected with an apparent molecular mass of 34 kDa. Densitometric analysis of these bands showed increases up to 412% (P < 0.05) of metalloproteinase (MMP) activity in hearts from DOX-treated mice compared with control. PJ34 treatment of
animals resulted in a moderate, not significant, reduction in MMP activity (315% of control) (Fig. 4). No gelatinolytic activity could be detected using Ca²⁺/H₁₁₀₀₁-free developing buffer (data not shown). No caseinolytic activity was detected in the heart extracts.

Survival Experiments

The results of the survival experiments are shown in Fig. 5. Treatment with PJ34 significantly decreased the DOX-induced mortality. The overall mortality of mice treated with DOX was 76% (66/87) and 77% (67/87) at 20 (Fig. 5) and 28 (data not shown) days of observation period. In DOX + PJ34-treated group mortality was 36% (20/55) (Fig. 5) and 40% (22/55) (data not shown), respectively.

Discussion

DOX continues to be a commonly used broad-spectrum chemotherapeutic agent. However, the clinical use is limited because of its serious dose-dependent cardiotoxicity, which leads to irreversible degenerative cardiomyopathy and heart failure (Blum and Carter, 1974; Young et al., 1981; Singal et al., 1987; Hortobagyi, 1997; Singal and Iliskovic, 1998). Several mechanisms have been implicated in the etiology of DOX-induced cardiotoxicity, including increased oxidative stress in cardiomyocytes, alteration of cardiac energetics, and direct effect on the DNA, the putative mechanism by which injury occurs remains poorly understood (Myers et al., 1977; Olson et al., 1981; Doroshow and Davies, 1986; Liu, 1989; Siveski-Iliskovic et al., 1994; Li and Singal, 2000; Weinstein et al., 2000).

The present study demonstrates severe depression of left ventricular function involving both systolic pressure development and relaxation in a well established murine model of DOX cardiotoxicity (Figs. 1 and 2). These results are in agreement with earlier reports showing depressed cardiac performance in different mouse and rat models of DOX-induced heart failure and are consistent with clinical observations (Siveski-Iliskovic et al., 1994; Singal and Iliskovic, 1998; Weinstein et al., 2000).

Importantly, the results presented herein document for the first time that in murine model of DOX-induced heart failure the activation of PARP in the myocardium may contribute to the impaired cardiac function, because PARP-1−/− mice
were more resistant to the cardiotoxic effects of DOX than PARP-1+/+ ones (Fig. 1), and pharmacological inhibition of PARP with PJ34 attenuated the DOX-induced cardiac dysfunction (Fig. 2) and the DOX-induced elevations in serum LDH and CK levels (Fig. 3), indirect indexes of cardiac myocyte necrosis. This finding is consistent with PARP inhibition’s molecular mode of action [i.e., the prevention of cell necrosis triggered by energetic failure (see below)]. Furthermore, the PJ34 treatment significantly increased the survival of the animals treated with DOX (Fig. 5).

In addition, we demonstrate that DOX induces metalloproteinase activation in the heart (Fig. 4), which is considered to be an important contributory factor to the development of various pathological conditions, including dilated cardiomyopathy, congestive heart failure, and reperfusion injury (Mann and Spinale, 1998; Thomas et al., 1998; Cheung et al., 2000; Creemers et al., 2001). The metalloproteinase activation was not prevented by PJ34 treatment. Because metalloproteinase activation is dependent on oxidative stress, our finding is consistent with our proposed scheme, where PARP activation lays downstream from the generation of oxidants.

Clinical and experimental investigations suggested that increased oxidative stress associated with an impaired antioxidant defense status may play a critical role in subcellular remodeling, calcium-handling abnormalities, alteration of cardiac energetics, and subsequent cardiomyopathy and heart failure associated with DOX treatment (Myers et al., 1977; Olson et al., 1981; Doroshow and Davies, 1986; Siveski-Iliiskovic et al., 1994; Li and Singal, 2000; Weinstein et al., 2000). Consistent with this concept, increased nitric oxide synthase II induction and massive nitrotyrosine formation have been shown in cardiomyocytes of mice 5 days after a single dose of DOX (Weinstein et al., 2000).

Superoxide anion interacts with nitric oxide, forming the oxidant peroxynitrite (ONOO⁻), which attacks various biomolecules, leading to, among others, the production of a modified amino acid (nitrotyrosine) (Beckman and Koppenol, 1996; Szabó, 1996). Although nitrotyrosine was initially considered a specific marker of peroxynitrite generation, other pathways can also induce tyrosine nitration (Eiserich et al., 1998). Thus, nitrotyrosine is now generally considered a collective index of reactive nitrogen species, rather than a spe-
cific indicator of peroxynitrite formation (Halliwell, 1997; Eiserich et al., 1998). Nevertheless, the increase in nitrotyrosine in myocytes of DOX-treated mice suggested that a causative link exist between oxidative stress and cardiotoxicity of DOX. Furthermore, the extent of protein nitration observed in the hearts of DOX-treated mice highly correlates to left ventricular dysfunction measured by Doppler echocardiography (Weinstein et al., 2000).

Oxidative stress induced by DOX in myocytes is accompanied by increased formation of hydrogen peroxide and peroxynitrite, which are endogenous inducers of DNA single-strand breakage (Doroshow and Davies, 1986; Weinstein et al., 2000; Xu et al., 2001). DNA single-strand breakage is the obligatory trigger of PARP activation (Szabo et al., 1997, 1998; Szabo, 2000), which in turn may result in rapid depletion of the intracellular NAD\(^+\) and ATP pools, slowing the rate of glycolysis and mitochondrial respiration and eventually leading to cellular dysfunction and necrosis. The importance of the PARP pathway is well documented in various models of myocardial ischemia-reperfusion injury and diabetic cardiomyopathy (another conditions where oxidative stress plays a key pathogenetic role) (Thiemermann et al., 1997; Zingarelli et al., 1997, 1998; Grupp et al., 1999; Pieper et al., 2000; Yang et al., 2000; Pacher et al., 2002). Based on the results of the current study, we conclude that the reactive oxygen/nitrogen species PARP pathway also plays a pathogenetic role in the development of DOX-induced cardiomyopathy.

As with most pharmacological inhibitors, we cannot fully exclude the possibility that PJ34 may also act at pharmacological sites other than inhibiting PARP in the heart. Thus, the contribution of such effects to the observed benefit of the compound in DOX-induced acute heart failure model in mice cannot be excluded until further studies with other new specific PARP inhibitors strengthen these observations. Nevertheless, based on the protection seen with PARP-1-deficient animals (in addition to PJ34), we believe that the most likely possibility is that PJ34 indeed works via inhibition of PARP activity. As mentioned above, PJ34 is one of the most potent and effective bioavailable PARP inhibitors published to date (Soriano et al., 2001b). We have analyzed the antioxidant potential of PJ34 and found that it does not act as an antioxidant (Soriano et al.,

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**Fig. 3.** Pharmacological inhibition of PARP decreases the DOX-induced increase in serum LDH (A) and CK (B) activities, indirect indexes of myocardial tissue damage. CO, control; DOX, doxorubicin-treated (a single dose of 25 mg/kg). DOX + PJ34, doxorubicin (a single dose of 25 mg/kg i.p.) and PJ34 (3 × 10 mg/kg i.p. for 5 days) treated. LDH and CK activities were measured 48 h after DOX administration. Results are mean ± S.E.M. of five experiments. *, P < 0.05 versus CO; #, P < 0.05 versus DOX.

**Fig. 4.** DOX induces MMP activation in the hearts; lack of effect of PARP inhibition. On the gelatin zymography gels, only one band was detected with an apparent molecule mass of 34 kDa. Densitometric analysis of these bands showed significant increases up to 412% of MMP activity in hearts from doxorubicin-treated mice compared with control. PJ34 treatment of animals resulted in a moderate, not significant reduction in MMP activity (315% of control). Mice hearts were assayed after 48 h of DOX + PJ34 treatment. Hearts of untreated mice were used as control.

**Fig. 5.** Pharmacological inhibition of PARP with PJ34 improves survival of mice treated with DOX. Treatment with PJ34 (3 × 10 mg/kg) significantly decreased the DOX (a single dose of 25 mg/kg)-induced mortality. The overall mortality of mice treated with DOX reached 76% (66/87) at day 20 of the observation period, whereas in DOX + PJ34-treated group it was only 36% (20/55). *, P < 0.05, log-rank test.
Other pharmacological inhibitors of PARP (e.g., nicotinamide and 3-aminobenzamide) have been shown to act as free radical scavengers complicating the evaluation of the relative contribution of PARP inhibitory effect and free radical scavenging properties of these compounds in a model associated with increased production of reactive oxygen species in the heart.

Further strengthening our point that PJ34 lacks antioxidant effects was the finding that chronic oral treatment with PJ34 inhibited PARP activation in diabetic bellies ex vivo, but did not affect the degree of tyrosine nitration, an indicative of vascular nitrosative stress (Soriano et al., 2001b). In addition, the DOX-induced metalloproteinase activation in the heart, which is also dependent on oxidative stress, was not prevented by PJ34 treatment (Fig. 4).

We have previously shown that the dose regimen of PJ34 used in the current study effectively inhibit PARP activation in different tissues (Jagtap et al., 2001; Mabley et al., 2001; Soriano et al., 2001a,b; Liaudet et al., 2002), including heart (Goldfarb et al., 2002; Pacher et al., 2002) in various pathophysiological conditions. In addition to the beneficial effects of pharmacological inhibition of PARP with PJ34 in mouse model of DOX-induced acute heart failure we also provided evidence that the genetic deletion of PARP-1 is associated with protection against DOX-induced cardiotoxicity. Thus, we believe that our data are sufficient to support the proposal that PARP activation is likely to contribute to the cardiotoxicity of DOX. Further work is required to clarify whether PARP inhibition may exert beneficial effects against cardiotoxicity of DOX in humans.

References


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