Rapid Reversal of the Diabetic Endothelial Dysfunction by Pharmacological Inhibition of Poly(ADP-Ribose) Polymerase

Francisco Garcia Soriano, Pál Pacher, Jon Mabley, Lucas Liaudet, Csaba Szabó

Abstract—Oxygen- and nitrogen-derived free radicals and oxidants play an important role in the pathogenesis of diabetic endothelial dysfunction. Recently we proposed the importance of oxidant-induced DNA strand breakage and activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) in the pathogenesis of diabetic endothelial dysfunction. In this study, we tested whether established diabetic endothelial dysfunction is reversible by PARP inhibition. The novel PARP inhibitor PJ34 (10 mg/kg per day PO) was given at various lengths (4 weeks or 3 days) for established streptozotocin-diabetic animals. In addition, we also tested whether incubation of the aortic rings with PJ34 (3 μmol/L) or a variety of other PARP inhibitors for 1 hour affects the diabetic vascular changes. Both 4-week and 3-day PARP-inhibitor treatment of streptozotocin-diabetic mice with established endothelial dysfunction fully reversed the acetylcholine-induced endothelium-dependent relaxations in vitro. Furthermore, 1-hour in vitro incubation of aortae from streptozotocin-diabetic mice with various PARP inhibitors was able to reverse the endothelial dysfunction. ATP, NAD+, and NADPH levels were markedly reduced in diabetic animals, and PARP-inhibitor treatment was able to restore these alterations. Unexpectedly, pharmacological inhibition of PARP not only prevents the development of the endothelial dysfunction but is also able to rapidly reverse it. Thus, PARP activation and the associated metabolic compromise represent an ongoing process in diabetic blood vessels. Pharmacological inhibition of this process is able to reverse diabetic endothelial dysfunction. (Circ Res. 2001;89:684-691.)

Key Words: diabetes ■ endothelium ■ nitric oxide ■ necrosis ■ apoptosis

Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme that has been implicated in the cellular response to DNA injury.1 We recently proposed a role for PARP activation in the pathogenesis of diabetic endothelial dysfunction. Endothelial cells and vascular rings from PARP+/− mice incubated in high glucose were found to exhibit metabolic suppression and loss of endothelium-dependent relaxant function, effects that did not develop in the vascular rings from PARP−/− mice and were prevented by pharmacological inhibition of PARP.2 Ex vivo experiments, examining the immunohistochemical profile and the endothelium-dependent relaxant function of diabetic aortae, demonstrated intravascular production of reactive nitrogen species, the development of DNA strand breakage, and the activation of PARP.3 Finally, in vivo treatment with PJ34, a novel potent phenanthridinone PARP inhibitor, was found to prevent the development of diabetic endothelial dysfunction in streptozotocin-diabetic mice.2

In this study we report our recent, unexpected findings that demonstrate that pharmacological PARP inhibition not only prevents the development of diabetic vascular dysfunction but also rapidly restores the function of established diabetic blood vessels.

Materials and Methods

Induction and Monitoring of Diabetes

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23 revised 1985) and was performed with approval of the local Institutional Animal Care and Use Committee.

Streptozotocin Treatment

Adult male Balb/c mice (Taconic Farms, Germantown, NY) were treated with streptozotocin (240 mg/kg IP) or vehicle (citrate buffer). Blood glucose was monitored weekly using a one-touch blood glucose meter (Lifescan). Hyperglycemia was defined as nonfasting blood glucose level >200 mg/dL.

Treatment Protocols

PARP was inhibited by 10 mg/kg PJ34, administered orally via gavage once daily. In protocol A, to prevent the development of the endothelial dysfunction, the compound was given to animals between 1 to 4 and 1 to 8 weeks, followed, at 4 and 8 weeks, by killing the animals and evaluating vascular responsiveness. In protocol B, to attempt endothelial dysfunction reversion, the compound was given to two groups of animals, the first between 4 to 8 weeks after streptozotocin injection and the second for 3 days between days 26 and 28 followed by killing the animals and monitoring vascular reactivity. In protocol C, the following in vitro treatment protocols were followed: in a subset of experiments, control rings or diabetic rings obtained at 4 weeks were incubated with PJ34 (3 μmol/L) or...
vehicle for 1 hour, followed by the determination of contractile and endothelial-dependent and -independent vascular function and measurement of high-energy phosphates and pyridine nucleotides. Although PJ34 does not act as an antioxidant, many PARP inhibitors have been shown to have antioxidant or other nonspecific actions. Therefore, protocol C was subsequently repeated using a variety of structurally different PARP inhibitors: 3-aminobenzamide (3 mmol/L), 5-ido-6-amin-1,2-benzopyrene (100 μmol/L), and 1,5-dihydroxyisoquinoline (30 μmol/L). The selection of the in vivo and in vitro doses and concentrations of the PARP inhibitors was based on previous dose responses measuring the effect of the compounds on PARP activation in oxidatively challenged or high-glucose exposed endothelial cells. In an additional set of experiments, insulin-containing minipumps (Alzet Corporation, model 1004) releasing bovine insulin at a rate of 50 μg/kg per day and a volume of 0.25 μL/h were used. Diabetes was confirmed at 2 days, followed immediately by the subcutaneous implantation of the minipumps. Vascular reactivity of the insulin-treated mice was evaluated at 1 and 4 weeks.

**Measurement of Glycated Hemoglobin**

Total glycated hemoglobin content of blood samples was measured using a commercially available kit (Sigma Diagnostics).

**Vascular Studies**

**Ex Vivo Studies**

Thoracic aortae were cleared from periadventitial fat, cut into 4 pieces, and placed into chambers filled with warmed (37°C) and gas-equilibrated (95% O₂, 5% CO₂) Krebs’ solution containing (in mmol/L) CaCl₂ 1.6, MgSO₄ 1.17, EDTA 0.026, NaCl 130, NaHCO₃ 14.9, KCl 4.7, KH₂PO₄ 1.18, and glucose 5. Isometric tension was measured with isometric transducers (Kent Scientific Corporation), digitized using a MacLab A/D converter, and stored and displayed on a MacIntosh computer. The preload was 1 g, and the rings were equilibrated for 60 minutes. Dose-response curves to phenylephrine (10⁻⁶ to 3×10⁻⁴ mol/L) were first obtained. To stimulate nitric oxide (NO) release and vasorelaxation by activating the endothelial isoform of NO synthase (eNOS) in the endothelial cells, dose-response curves to the endothelium-dependent relaxant acetylcholine (10⁻⁹ to 10⁻⁴ mol/L) were obtained. The endothelium-independent relaxant sodium nitroprusside (10⁻⁶ to 3×10⁻⁴ mol/L) was tested in rings precontracted with phenylephrine (10⁻⁴ mol/L).

**Measurement of High-Energy Phosphates and Pyridine Nucleotides in Control and Diabetic Blood Vessels**

An HPLC method was used, as previously described. Aortae were homogenized in 400 μL of 0.2 mol/L potassium cyanide, 0.06 mol/L potassium hydroxide, and 1 mmol/L bathophenanthroline disulfonic acid. Samples were then extracted with chloroform and centrifuged in 4°C at 15 000 rpm for 5 minutes. A volume of 40 μL was injected on an HPLC column. A Luna, 3-μm, 150×4.60-mm, C-18(2) HPLC column (Phenomenex) was used. The detectors used were a Shimadzu RF-10AXL fluorescence detector (excitation wavelength 330, emission wavelength 460) and SPD-10AV UV detector (wavelength 254 nm). The mobile phase was 0.2 mol/L ammonium acetate and HPLC-grade methanol with pH 5.88. The flow rate was 1 mL/min, with 96% ammonium acetate and 4% MeOH initially and increasing up to 9% MeOH in 25 minutes.

**Measurements of Plasma Nitrite and Nitrate Concentration**

Serum concentrations of nitrite and nitrate, stable breakdown products of NO, were measured by the modified Griess reaction. Materials

Unless specified otherwise, all chemicals and materials listed were purchased from Sigma/Aldrich. The potent, novel, water-soluble phenanthridinone-derivative PARP inhibitor, PJ34, the hydrochloride salt of (N-(oxo-5,6-dihydro-phenanthridin-2-yl)-N, N-dimethylacetamide, was synthesized as described.
unaffected by this treatment regimen. Measurements of high-energy phosphates and pyridines nucleotides from the thoracic aortae showed a marked decrease on ATP, NAD$^+$, and NADPH levels in diabetic animals; these changes were improved after 3 days of PJ34 treatment (Table 1, Figure 4).

Reversal of the Diabetic Endothelial Dysfunction by PARP Inhibition In Vitro

In an additional set of experiments, we attempted to reverse the endothelial dysfunction by in vitro inhibition of PARP activation in diabetic vascular rings. Vascular rings obtained from 4-week-diabetic animals exhibited impaired endothelium-dependent relaxation. The relaxation was fully normalized by in vitro incubation of the vessels with the PARP inhibitor PJ34 (3 μmol/L, 1 hour incubation) (Figure 5). Relaxations to sodium nitroprusside were not different between any of the experimental groups (not shown). Effective concentrations of PARP inhibitor compounds of different structural classes (using a benzamide, an isoquinoline, and a benzopyrene PARP inhibitor) provided a comparable degree of restoration of the endothelium-dependent relaxant capacity of the diabetic blood vessels (Figure 6). In vitro incubation of the diabetic rings with all four PARP inhibitors significantly improved vascular NAD$^+$ and NADPH levels in the diabetic blood vessels (Table 2).

Discussion

Time Course of Vascular Dysfunction in Mice: Rapid Reversibility of the Vascular Alterations by PARP Inhibition

To establish the time course of endothelial dysfunction in the murine model, we have studied the vascular reactivity of aorta at 1, 2, 4, and 8 weeks after streptozotocin injection. The results showed that endothelial dysfunction is fully established at 4 weeks of diabetes and still persists at 8 weeks. The vascular contractility showed a decrease at 8 weeks but not at earlier time points. Our studies demonstrate that the vascular dysfunction in our model is specifically related to elevated blood glucose, because vascular function was preserved in animals treated with insulin. After establishing the time course of the endothelial dysfunction, we subsequently investigated the reversibility of the endothelial dysfunction by PARP inhibition in the time frame between 4 and 8 weeks.
Both 4-week and 3-day PARP inhibitor treatment of streptozotocin-diabetic mice with established endothelial dysfunction fully reversed the acetylcholine-induced endothelium-dependent relaxations in vitro. Furthermore, 1-hour in vitro incubation of aortae from streptozotocin-diabetic mice with four structurally different PARP inhibitors was also able to reverse vascular metabolic alterations as well as the associated endothelial dysfunction.

**Role of PARP in Diabetes and Diabetic Vascular Alterations**

Our recent studies identified PARP activation as a novel pathway of diabetic vasculopathy. In vitro and in vivo endothelial cell dysfunction in response to high-glucose concentration was found to be associated with increased poly(ADP-ribose)ylation. Furthermore, the deterioration of endothelial function was prevented in endothelial cells and in vascular rings of animals in which PARP was genetically inactivated or PARP was pharmacologically inhibited.

Two main pathways were proposed to explain the role of PARP in diabetic vascular alterations. The first pathway, the proinflammatory mediator production pathway, is related to data implicating a role for PARP in modulating the activation of nuclear factor-κB (NF-κB) and, subsequently, proinflammatory gene expression. The genes, activation of which are suppressed in the absence of PARP, include the gene for intercellular adhesion molecule-1 (ICAM-1) and the gene for iNOS. Adhesion molecules such as ICAM-1, iNOS expression, and NF-κB activation have been implicated in the pathogenesis of the endothelial dysfunction associated with diabetes. Therefore, it is conceivable that suppression of these proinflammatory mechanisms is a potential mode of protection by the absence of functional PARP against diabetic endothelial dysfunction in vivo. This hypothesis is additionally supported by our present data demonstrating that PJ34 treatment for 4 weeks normalizes circulating nitrite and nitrate levels as well as by recent data demonstrating that PARP deficiency suppresses NF-κB activation in endothelial cells placed in high glucose.

The second pathway is related to the so-called metabolic hypothesis of PARP activation. According to this pathway, glucose triggers the production by endothelial cells of oxygen- and nitrogen-derived free radical and oxidant species, which, in turn, induce DNA single-strand breakage,
which then activates PARP, leading to a cellular energetic crisis, which is prevented by PARP inhibition. By this fashion, PARP activation in the endothelial cells leads to a cellular energetic impairment, which subsequently impairs the ability of the endothelial cells to generate NO when stimulated by an endothelium-dependent relaxant agonist, such as acetylcholine. The metabolic hypothesis is supported by the following previous findings. First, exposure of endothelial cells to oxidants, such as hydrogen peroxide, leads to cellular NAD$^+$ depletion, which is prevented by PARP inhibition. Second, when vascular rings are exposed to oxidants, the resulting impairment of the endothelial function (loss of endothelium-dependent relaxant responses) can be prevented by PARP inhibition. Third, when vascular rings from wild-type and PARP-deficient mice were placed into high-glucose solution, the PARP-deficient rings are resistant against the development of endothelial dysfunction. Fourth, compromising the energetic status of the endothelial cells by various PARP-independent means can lead to selective endothelial dysfunction. For example, inhibition of oxidative metabolism with either amytal or rotenone in vascular rings induces a markedly impaired relaxation to acetylcholine but does not affect smooth muscle relaxations to exogenously administered NO donors. Similar effects were reported in rings incubated with 2-deoxylgucose. Fifth, high glucose induces significant, PARP-dependent changes in endothelial ATP content as well as pyridine nucleotide levels (including NADPH). Sixth, our present findings (Tables 1 and 2) are also in line with the importance of the metabolic hypothesis. Because eNOS is an NADPH-dependent enzyme, it is conceivable that a PARP-dependent depletion of NADPH in

### TABLE 1. Changes in High-Energy Phosphates and Pyridine Nucleotide Levels in Diabetic Thoracic Aorta Homogenates: Effect of PARP Inhibition

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic</th>
<th>Diabetic</th>
<th>Diabetic + PJ34</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>31.33±1.29</td>
<td>18.04±5.35</td>
<td>28.46±0.35†</td>
</tr>
<tr>
<td>ADP</td>
<td>2.83±0.84</td>
<td>3.17±0.66</td>
<td>2.38±0.50</td>
</tr>
<tr>
<td>AMP</td>
<td>2.49±0.46</td>
<td>2.90±0.11</td>
<td>2.04±0.29</td>
</tr>
<tr>
<td>NAD</td>
<td>19.18±2.17</td>
<td>5.84±0.41*</td>
<td>11.39±1.24†</td>
</tr>
<tr>
<td>NADH</td>
<td>1.58±0.04</td>
<td>0.94±0.04*</td>
<td>1.46±0.40</td>
</tr>
<tr>
<td>NADP</td>
<td>6.48±0.64</td>
<td>2.76±1.10*</td>
<td>6.12±0.47†</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.81±0.04</td>
<td>0.37±0.05*</td>
<td>0.65±0.06†</td>
</tr>
</tbody>
</table>

Values represent control (nondiabetic) male Balb/c mice, mice 4 weeks after streptozotocin-induced diabetes, and mice with diabetes in the presence of PARP-inhibitor treatment (PJ34, 10 mg/kg oral gavage once a day) starting 3 days before the termination of the experiments at 4 weeks. NAD$^+$, NADH, NADP$, NADPH$, ATP, ADP, and AMP values are expressed in nmol/mg of wet tissue. Data shown are mean±SEM of n=4 to 5 experiments.

*P<0.05 indicates significant difference between the values in control and diabetic groups.

†P<0.05 indicates significant difference between the values in the diabetic group and in the PJ34-treated group.

![Figure 3. Vascular responsiveness. Endothelium-dependent relaxations induced by acetylcholine, contractions induced by phenylephrine, and endothelium-independent relaxations induced by sodium nitroprusside (SNP) in control (nondiabetic) male Balb/c mice and 4 weeks after streptozotocin-induced diabetes. Vehicle or PARP inhibitor (PJ34, 10 mg/kg oral gavage once a day) treatment started 3 days before the termination of the experiments at 4 weeks. There was a marked and selective impairment of the endothelium-dependent relaxant ability of the vascular rings in diabetes at 4 weeks. Treatment with the PARP inhibitor for the last 3 days of the experiment improved the endothelium-dependent relaxant ability of the diabetic vessels despite the persistence of hyperglycemia. ${}^2 P<0.05$ for differences between experimental groups, as indicated. n=8 per group.

![Figure 4. Typical traces of HPLC with fluorescent detector from aortic tissues. The corresponding peak for each pyridine is labeled. A, Sample from healthy mouse. B, Sample from diabetic mouse at 4 weeks of diabetes. C, Sample from diabetic mouse at 4 weeks of diabetes when treated for the final 3 days with PJ34.](http://circres.ahajournals.org/)

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endothelial cells exposed to high glucose is directly responsible for the suppression of eNOS activity and the reduction in the endothelium-dependent relaxant ability of the diabetic vessels.

On the basis of the present findings demonstrating the rapid reversal of the endothelium-dependent relaxations by PARP inhibition in vivo or in vitro, we conclude that from the two above-listed mechanisms, the metabolic theory is the more feasible one to explain the mechanism of diabetic endothelial dysfunction in our experimental model. We thus propose that PARP activation represents a continuing, ongoing intravascular process, most likely related to a continuous intravascular production of oxygen- and nitrogen-derived free radicals and oxidants within the diabetic vascular endothelium. There is abundant evidence supporting the importance of these reactive species in inducing and maintaining diabetic endothelial dysfunction.22–27 We now propose that these oxidants represent a continuous trigger of DNA single-strand breakage that, in turn, keeps PARP in an activated state, which continuously uses and depletes endothelial NAD$^+$ and NADPH, thereby impairing the endothelium-dependent relaxant responses. Under this continuing oxidative and nitrosative stress, the endothelial cells are likely to exist in a state of metabolic suppression and associated dysfunction, which cannot be classified as a normal, necrotic, or apoptotic state, although the cells do exhibit certain patterns that are characteristic for either of the latter two states, such as increased DNA strand breakage2 or mitochondrial dysfunction.28 Although to a lesser extent than the endothelial dysfunction, diabetic blood vessels also showed an impairment of the contractility, which may be a consequence of a combination of oxidative and nitrosative stress and energy starvation.

The rapid reversibility of the diabetic endothelial dysfunction may have important future therapeutic implications. One can expect that a relatively short treatment course with a potent PARP inhibitor may be sufficient to improve vascular status in diabetic subjects. Future work must investigate whether the improvement by PARP inhibition of the early functional alterations in diabetes (such as the endothelial dysfunction) can also translate to improvements in the more delayed severe vascular and extravascular changes, including accelerated atherosclerosis, hypertension, and foot ulceration.29–31

Although several series of novel, potent PARP inhibitors are in various stages of preclinical development for a variety of therapeutic indications,32–34 there are presently no potent, selective PARP inhibitors available for human trials. Nicotinamide is a weak PARP inhibitor,35 which, nevertheless, has an excellent safety profile in humans.36 One can envision clinical trials designed to reverse diabetic endothelial dysfunction by pharmacological inhibition of PARP with nicotinamide. The design could follow the design of recent
The role of PARP activation in diabetes is not limited to the vascular dysfunction. A vast body of evidence supports the role of PARP activation in the primary process of diabetes, i.e., the process of islet cell destruction. Pretreatment of the animals with PARP inhibitors is known to protect against streptozotocin-induced β-cell necrosis and hyperglycemia. Similarly, PARP-deficient mice are resistant against streptozotocin-induced diabetes. However, to effectively prevent the primary process of islet cell death, PARP inhibitors must be applied in a pretreatment regimen. PARP inhibitors rapidly lose their effectiveness in protecting the primary process of diabetic islet cell death, when their administration is delayed. In fact, this phenomenon has been exploited to our advantage in the present experimental design: the start of the PARP inhibitor administration was delayed to the time when the primary islet cell destruction was complete to minimize interference with the diabetic hyperglycemia. However, the streptozotocin models of diabetes produce a rapidly progressing disease phenotype, where functional islet cells are rapidly eliminated. In contrast, human diabetes can feature ongoing autoimmune islet cell destruction. Under such circumstances, one can expect that pharmacological PARP inhibition may have some additional beneficial effects on the primary process as well.

On the basis of the data presented in this study, we propose that PARP inhibition may be a suitable strategy to reconstitute normal vascular function in diabetes mellitus. Preclinical and clinical studies with suitably selected PARP inhibitors are needed to additionally address this question.

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References
9. Zingarelli B, Salzman AL, Szabo C. Genetic disruption of poly (ADP-ribose) synthetase inhibits the expression of P-selectin and intercellular...
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