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REVIEW

Role of nitrosative stress in the pathogenesis of diabetic vascular dysfunction

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Here we overview the role of reactive nitrogen species (nitrosative stress) and associated pathways in the pathogenesis of diabetic vascular complications. Increased extracellular glucose concentration, a principal feature of diabetes mellitus, induces a dysregulation of reactive oxygen and nitrogen generating pathways. These processes lead to a loss of the vascular endothelium to produce biologically active nitric oxide (NO), which impairs vascular relaxations. Mitochondria play a crucial role in this process: endothelial cells placed in increase extracellular glucose respond with a marked increase in mitochondrial superoxide formation. Superoxide, when combining with NO generated by the endothelial cells (produced by the endothelial isoform of NO synthase), leads to the formation of peroxynitrite, a cytotoxic oxidant. Reactive oxygen and nitrogen species trigger endothelial cell dysfunction through a multitude of mechanisms including substrate depletion and uncoupling of endothelial isoform of NO synthase. Another pathomechanism involves DNA strand breakage and activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP). PARP-mediated poly(ADP-ribosyl)ation and inhibition of glyceraldehyde-3-phosphate dehydrogenase importantly contributes to the development of diabetic vascular complications: it induces activation of multiple pathways of injury including activation of nuclear factor kappa B, activation of protein kinase C and generation of intracellular advanced glycation end products. Reactive species generation and PARP play key roles in the pathogenesis of 'glucose memory' and in the development of injury in endothelial cells exposed to alternating high/low glucose concentrations.
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Abbreviations: AGE, advanced glycation end product; BH4, tetrahydrobiopterin; eNOS, endothelial isoform of nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-NAME, NG-nitro-L-arginine methyl ester; NF-κB, nuclear factor kappa B; NO, nitric oxide; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C

Introduction

Despite state-of-the-art glucose control, diabetic patients remain at a markedly increased risk of cardiovascular disease. A close correlation exists between fasting plasma glucose levels and cardiovascular morbidity and mortality (Ruderman *et al.*, 1992; Laakso, 1999; Stratton *et al.*, 2000; Reusch, 2003). The loss of endothelial function (the development of diabetic endothelial dysfunction) has been implicated both in the development of diabetic macrovascular diseases (e.g. increased incidence and severity of stroke and myocardial infarction) and in the development of microvascular diseases (neuropathy, nephropathy, retinopathy and erectile dysfunction) (Dandona *et al.*, 2004; Boyle, 2007; Cohen *et al.*, 2007; Vaughan *et al.*, 2007; Aronson, 2008). The mechanisms responsible for the development of this endothelial dysfunction have received much attention over the last three decades.

The endothelial cell produces many vasoactive substances, hormones and cytoprotective biological factors, including prostaglandins, coagulation factors and many others. Much of the recent attention in the last decade has been directed towards the loss of the ability of the diabetic vasculature to produce nitric oxide (NO: the principal 'endothelium-dependent relaxing factor', a vasodilator and anti-inflammatory hormone, a key component of the vascular homeostasis). The observation that endothelial cells in diabetes fail to produce sufficient amount of NO and fail to relax in response to the endothelium-dependent vasorelaxants (e.g. acetylcholine, bradykinin, shear stress, etc.) has been documented by multiple studies, both in animal models of the

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disease (Oyama *et al.*, 1986; Meraji *et al.*, 1987; Mayhan, 1989; Cosentino and Luscher, 1998; De Vriese *et al.*, 2000) and in human studies (Caballero *et al.*, 1999; Calles-Escandon and Cipolla, 2001; Avogaro *et al.*, 2006). The endothelial cell is especially vulnerable to the toxic effects of increased extracellular glucose, because (as opposed to most parenchymal cells) it is unable to compensate for extracellular hyperglycaemia with an increase in glucose extrusion into the extracellular space (overviewed in Brownlee, 2001). Thus, in the case of endothelial cells, increased extracellular glucose results in an increase in intracellular glucose. In the current article we overview the role of reactive oxygen and nitrogen species (oxidative and nitrosative stress respectively) and associated pathways in the pathogenesis of diabetic endothelial dysfunction/endothelial glucose toxicity.

Aldose reductase, advanced glycation end products, protein kinase C activation: the 'classical' pathways of diabetic vascular complications

Over the last three decades, a number of key pathways have been identified in the pathogenesis of diabetic vascular complications. The first pathway, originally described in the 1960s, relates to the pathological metabolism of glucose via the aldose reductase enzyme. This pathway results in a consumption of intracellular NADPH levels when glucose is reduced to sorbitol (because oxidation of NADPH to $NADP^+$), as well as a reduction of NAD^+ to NADH (when sorbitol is oxidized to fructose). One of the consequences of these changes is the depletion of key intracellular antioxidant enzymes (e.g. glutathione). The production of sorbitol from glucose increases osmotic stress, which has been implicated in the pathophysiology of diabetic complications. Pharmacological inhibition of the aldose reductase enzyme has been shown to exert significant benefit against the development of various diabetic complications in animal models (Oates and Mylari, 1999; Altan, 2003; Drel *et al.*, 2008). The second pathway (in chronological order) was discovered in the 1970s, and this involves the formation of advanced glycation end products (AGEs), which leads to intracellular and extracellular protein modifications of various types. These proteins are known to form pathological protein–protein cross links, as well as induce pathological effects through binding to membrane receptors on various cell types. Compounds that inhibit this pathway (e.g. aminoguanidine) are effective in various models of diabetes, and experimental compounds that break up the protein–protein cross links are also under intensive investigations (Altan, 2003). In the late 1980s a third pathway has been proposed, involving the activation of various protein kinase C (PKC) isoforms. Activation of these enzymes occurs through increased intracellular concentration of diacylglycerol and leads to down-regulation of endothelial NO synthesis, as well as a variety of other deleterious vascular events including coagulation abnormalities, enhanced production of vasoconstrictors (e.g. endothelin) and increase in vascular permeability and pathological alterations in angiogenetic pathways (Lee *et al.*, 1989; Sheetz and King, 2002; Coppey *et al.*, 2003). In the 1990s a fourth pathway was described. This pathway is characterized by an increased hexosamine pathway flux and over-modification of various proteins by N-acetyl-hexosamine. For more detailed overview of these pathways, the reader is referred to in-depth reviews (e.g. Brownlee, 2001; 2005).

The role of reactive oxygen species in the pathogenesis of diabetic vascular complications

A number of early studies have suggested that reactive oxygen species play an important role in the pathogenesis of diabetic vascular dysfunction (Hattori *et al.*, 1991; Tesfamariam and Cohen, 1992; Pieper *et al.*, 1993; Diederich *et al.*, 1994; Rösen *et al.*, 1995). Most known pathways of diabetic complications have a close relationship to oxidative stress. The aldose reductase pathways is known to exert its pathological actions by enhancing the oxidative stress responses by depleting endogenous glutathione levels (Lee and Chung, 1999; Song *et al.*, 2003; Obrosova *et al.*, 2005). When endogenous antioxidants such as glutathione are depleted in endothelial cells, the cells become extremely sensitive to oxidative and nitrosative injury (Cuzzocrea *et al.*, 1998; Tagliabue *et al.*, 2005). AGEs are also known to be generated on the background of increased oxidative stress, and binding of these end products to their cellular receptors triggers pro-inflammatory responses and creates a pro-oxidative environment (Yan *et al.*, 1994; Scivittaro *et al.*, 2000; Ramasamy *et al.*, 2005; Rosca *et al.*, 2005; Goldin *et al.*, 2006). The PKC pathway has also been linked to oxidative stress because it is triggered by reactive oxygen specie, as it activates intracellular NADPH oxidases, which results in the formation of reactive oxidants (Venugopal *et al.*, 2002; Inoguchi *et al.*, 2003; Pricci *et al.*, 2003). Additional oxidative stress pathways include the uncoupling of the endothelial NO synthase or eNOS (under conditions of substrate deficiency), which, in turn, produces both superoxide and NO (which, in turn, can result in the generation of peroxynitrite, see following) (El-Remessy *et al.*, 2003; Münzel *et al.*, 2008). Activation of microsomal enzymes, xanthine oxidase, the arachidonic acid cascade and myeloperoxidase have also been implicated as sources of reactive oxygen species in diabetes.

The role of oxyradicals in the development of diabetic endothelial dysfunction is underlined by a number of functional studies demonstrating that diabetic blood vessels respond with an improved endothelium-dependent relaxant response when treated with various antioxidant agents including superoxide dismutase (Diederich *et al.*, 1994; Taylor and Poston, 1994; Tesfamariam, 1994; Voinea *et al.*, 2004). One of the mechanisms relates to the fact that the reaction of NO with superoxide anion results in its scavenging (inactivation), and therefore leads to a reduction in the biological availability of NO (Hink *et al.*, 2001; Coppey *et al.*, 2003; Bitar *et al.*, 2005; Münzel *et al.*, 2008). However, additional studies demonstrate that the mechanisms underlying diabetic endothelial dysfunction are more complex, because the same reaction can also create novel, deleterious species such as peroxynitrite (see following).

The mitochondrium represents an important source of reactive oxygen species in the diabetic endothelium. As demonstrated in 2000 by Brownlee and colleagues (Nishikawa *et al.*,

2000) endothelial cells placed in increase extracellular glucose to mimic the diabetic state[1] respond with a marked increase in reactive oxygen species formation. It was suggested by that there is a threshold value for the proton gradient in the intramitochondrial inner membrane, over which the lifetime of superoxide-generating reactive intermediates (such as ubisemiquinone) is increased. Consequently, when the increased intracellular glucose induces an overproduction of electron donors from the tricarboxylic acid cycle, the mitochondrial proton gradient increases, leading to a 'leak' of superoxide from the mitochondrial increases (Nishikawa *et al.*, 2000). Uncoupling of the mitochondria (by overexpressing uncoupling protein-1) abolishes the excess free radical formation. Antioxidant therapy exerts comparable effects (Nishikawa *et al.*, 2000; Quagliaro *et al.*, 2007). Importantly, inhibition of mitochondrial reactive species formation (by mitochondrial uncoupling or by treating the endothelial cell with superoxide dismutase or its analogues) normalizes a number of key pathways of diabetic complications, including the overactivation of PKC, the enhanced intracellular formation of AGEs and the pathological activation of the proinflammatory signal transduction pathway nuclear factor kappa B (NF-κB) (Nishikawa *et al.*, 2000; Brownlee, 2001). The mitochondrial formation of reactive oxygen species is therefore situated in an important checkpoint between increased glucose and multiple effector pathways of diabetic complications.

Role of the peroxynitrite/poly(ADP-ribose) polymerase pathway in the pathogenesis of diabetic vascular complications

In addition to scavenging NO, the formation of reactive oxidant species in the vascular endothelium can result in the generation of more deleterious oxidant species. One of these species is peroxynitrite (formed from the rapid reaction of NO and superoxide) (overviewed in Pacher *et al.*, 2007; Szabó *et al.*, 2007). There are multiple lines of evidence demonstrating the formation of peroxynitrite in the diabetic vasculature, both in experimental models (e.g. Garcia Soriano *et al.*, 2001; Pacher *et al.*, 2002) and in humans (e.g. Lyall *et al.*, 1998; Kossenjans *et al.*, 2000; Thuraisingham *et al.*, 2000; Szabo *et al.*, 2002a, 2002b; Hoeldtke *et al.*, 2003; Molnár *et al.*, 2006; Pacher and Szabo, 2008). The tissues and species where peroxynitrite has been identified in experimental animals (rodent and non-rodent species) and in humans include plasma, kidney, blood vessels (especially endothelium), retina, heart and peripheral nerves and have been overviewed recently (Pacher and Szabó, 2006). The mechanisms that underlie the peroxynitrite-induced diabetic complications and vascular alterations are multiple (Zou *et al.*, 2002; El-Remessy *et al.*, 2003; Molnar *et al.*, 2005; Pacher *et al.*, 2007; Szabó *et al.*, 2007; Ali *et al.*, 2008). Some of the deleterious biological actions of peroxynitrite, with special reference to the pathogenesis of diabetic complications, are summarized in Table 1.

One of the important pathways of peroxynitrite-mediated vascular dysfunction in diabetes involves the activation of the nuclear enzyme poly(ADP-ribose) polymerases (PARP enzymes). PARP-1 is the most abundant isoform of the PARP enzyme family (Virag and Szabo, 2002; Jagtap and Szabó, 2005). Upon binding to damaged DNA, PARP-1 forms homodimers and catalyses the cleavage of NAD^+ into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers on glutamic acid residues of a number of target proteins including histones and PARP-1 (automodification domain) itself. For many decades, PARP was mainly viewed as an enzyme primarily involved in DNA repair and maintenance of genomic stability. However, over the last decade, an additional role of PARP has been identified in the sequale of nitrosative stress. In this pathway (overviewed in Virag and Szabo, 2002), extensive oxidative and/or nitrosative stress triggers the extensive DNA breakage, overactivation of PARP and consequent depletion of the cellular stores of its substrate NAD^+, impairing glycolysis, Krebs cycle, mitochondrial electron transport, eventually resulting in ATP depletion and consequent cell dysfunction and death by necrosis. Peroxynitrite (but not its precursors superoxide or NO) has been identified as an endogenous trigger of DNA damage and PARP activation (Szabó *et al.*, 1997; Szabo *et al.*, 1998). Pharmacological inhibition of PARP or genetic deletion of the PARP-1 preserves cellular NAD^+ and ATP pools in oxidatively and/or nitrosatively stressed endothelial cells (as well as many other cell types), and thereby allowing them to function normally, or, if the apoptotic process has initiated, to utilize the apoptotic machinery and die by apoptosis instead of necrosis (Thies and Autor, 1991; Szabó *et al.*, 1997; Virág *et al.*, 1998a; Virag *et al.*, 1998b; Crocker *et al.*, 2005; Mabley *et al.*, 2005; Radovits *et al.*, 2007).

In 2001 our group has tested the potential role of the PARP pathway in the development of diabetic endothelial dysfunction. We have demonstrated that generation of peroxynitrite (a reactive species formed from NO and superoxide), DNA single strand breakage and activation of the nuclear enzyme PARP occur in endothelial cells placed in increased extracellular glucose concentration, as well as in the blood vessels of diabetic rodents (Garcia Soriano *et al.*, 2001). The activation of PARP (but not the DNA single strand breakage) was prevented by pharmacological PARP inhibition or by $PARP^{-/-}$ phenotype. Subsequent studies demonstrated that the diabetes-associated loss of endothelial function is not only preventable, but also rapidly reversible with PARP inhibition. Treatment with the PARP inhibitor ameliorated vascular poly(ADP-ribose) (PAR) accumulation in the diabetic blood vessels and restored normal vascular function without altering systemic glucose levels, plasma-glycated haemoglobin levels or pancreatic insulin content (Garcia Soriano *et al.*, 2001; Soriano *et al.*, 2001). Furthermore, even *in vitro* incubation of diabetic blood vessels with PARP inhibitors significantly enhanced their endothelium-dependent relaxant responsiveness (Soriano *et al.*, 2001). The potential of PARP inhibition in reversing endothelial dysfunction has also been demonstrated in an autoimmune non-obese diabetic model

[1]Some investigators criticize this method, noting that the degree of glucose increase (25–30 $mmol \cdot L^{-1}$, when compared with 5 $mmol \cdot L^{-1}$ normal glucose) is higher than the hyperglycemia seen in most diabetics. Nevertheless, this method continues to be a useful tool to study the molecular mechanisms of diabetic complications *in vitro*.

Table 1 Peroxynitrite-mediated pathophysiological alterations in the context of diabetic vascular complications

Action of peroxynitrite	*Mechanisms involved in these actions*
Enzyme inhibition leading to cell death or endothelial dysfunction (e.g. prostacyclin synthase)	Oxidation, nitration
Membrane pump inhibition leading to cell death	Oxidation, nitration
Inhibition of eNOS dimerization and loss of endothelium-dependent relaxation	Oxidation, nitration
Antioxidant enzyme inhibition leading to sensitization of the cell to oxidative damage and cell death	Oxidation, nitration
Signal transduction pathway disturbances leading to dysregulation of multiple cellular pathways	Oxidation, nitration of various receptors (e.g. VEGF receptor, uPAR, TrKA receptor) and intracellular signal transduction pathways (including p38 mitogen-activated protein kinase)
DNA injury leading to cell death through multiple pathways including PARP activation	Oxidation, nitration, deamination, adduct formation
Antioxidant enzyme depletion leading to sensitization of the cell to oxidative damage and cell death	Glutathione, cysteine oxidation
Inhibition of BH4-dependent enzymes including eNOS leading to loss of endothelium-dependent relaxations	Direct BH4 oxidation
Inhibition of NAD^+- and NADH-dependent enzymes including eNOS leading to a loss of endothelium-dependent relaxations	NAD^+ oxidation, NAD^+ and NADPH depletion through PARP
Lipid peroxidation leading to cellular damage	Peroxidation
Oxidative chain reactions leading to and enhancing cellular damage	Lipid peroxidation, generation of reactive alpha-oxoaldehydes from glucose
Mitochondrial dysfunction leading to mitochondrial oxidant generation and further oxidant generation, thereby promoting positive feedback cycles of injury	Inhibition of cytochromes, NADH-COQ1, etc.
Up-regulation of adhesion receptors leading to leukocyte activation and vascular injury	NF-κB activation
GAPDH inhibition leading to promotion of multiple pathways of diabetic complications	Multiple, including PARP activation
Protein kinase C activation leading to vascular injury and other complications	Multiple, including GAPDH inhibition through PARP activation
Active DNA fragmentation leading to cell death	Caspase activation
Calcium dysregulation leading to cell death	Dysfunctional calcium pumps and cell energetics
Cell necrosis leading to vascular dysfunction and other disturbances	Mitochondrial injury, energetic collapse, oxidation, nitration, antioxidant depletion, calcium dysregulation
Apoptosis leading to vascular dysfunction and other disturbances	Mitochondrial injury, DNA injury, caspase activation, signal transduction disturbances, calcium dysregulation

These effects are overviewed, in more detailed, in the text of the current article and in specialized reviews (Pacher and Szabó, 2006; Pacher *et al.*, 2007; Szabó *et al.*, 2007). Specific identification of several proteins that are nitrated by peroxynitrite in diabetes has been conducted by multiple groups (Zou *et al.*, 2002; El-Remessy *et al.*, 2003; Molnar *et al.*, 2005; Ali *et al.*, 2008).
BH4, tetrahydrobiopterin; eNOS, endothelial isoform of nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor kappa B; PARP, poly(ADP-ribose) polymerase; VEGF, vascular endothelial growth factor.

of diabetes (Pacher *et al.*, 2002) and in leptin-deficient db/db mice (Szabo *et al.*, 2006a). Importantly, the hyperglycaemia-induced increase in PARP activation *in vivo* has been shown to be preventable by insulin therapy (Horváth *et al.*, 2008).

The development of the endothelial dysfunction was associated with a simultaneous loss of NAD^+ and NADPH in the vasculature, and PARP inhibition reversed these changes. Based on these observations, coupled with the known fact that eNOS is dependent on NADPH and is sensitively regulated by this co-factor, we proposed that the endothelial dysfunction in diabetes is dependent on a PARP-mediated, reversible cellular NADPH deficiency (Garcia Soriano *et al.*, 2001). However, NADPH is not the only co-factor of eNOS that is depleted in diabetes. Several studies have demonstrated that diabetic endothelial dysfunction is also associated with direct oxidation and consequent intracellular depletion of tetrahydrobiopterin, another essential co-factor of eNOS (Guzik *et al.*, 2000; 2002; Fukuda *et al.*, 2002; Pannirselvam *et al.*, 2002; Bagi and Koller, 2003; Förstermann, 2006; Satoh *et al.*, 2008) leading to increased free radical and oxidant production, oxidative damage and further exacerbation of the endothelial dysfunction (Figure 1).

Nitrosative stress and the 'Grand Unifying Theory' of diabetic complications

Our group, in collaboration with the group of Dr Brownlee at the Albert Einstein University have set out to investigate the relationship between oxidative stress, nitrosative stress, DNA strand breakage, PARP activation and endothelial dysfunction. The results of these studies (Du *et al.*, 2003) revealed that: (i) inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a role in triggering the pathogenesis of multiple diabetic complications; and (ii) PARP activation is responsible for this GAPDH inhibition. These studies demonstrated that in *cells in normal glucose milieu*, antisense knockdown of GAPDH activity (to a similar degree as the one seen in endothelial cells placed into increased extracellular glucose) resulted in a similar degree of PKC activation and NF-κB activation and production of AGEs. Furthermore, GAPDH inhibition was found to be a consequence of poly(ADP-ribosyl)ation of GAPDH by activated PARP (Figure 1). Because in endothelial cells placed in increased intracellular glucose, (i) pharmacological inhibition of eNOS, (ii) neutralization of reactive oxygen species, as well

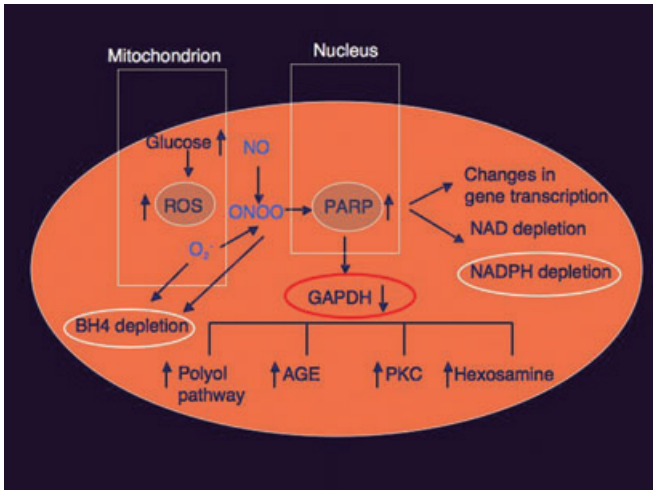

Figure 1 Selected peroxynitrite and poly(ADP-ribose) polymerase (PARP)-dependent pathways involved in the pathogenesis of diabetic complications in endothelial cells exposed to the cytotoxic effects of increased extracellular glucose. Increased glucose leads to increased mitochondrial formation of reactive oxygen species (ROS), such as superoxide, which, when reacting with nitric oxide (NO), produces peroxynitrite. Peroxynitrite induces cellular damage through depletion of the co-factor of endothelial isoform of nitric oxide synthase (eNOS), tetrahydrobiopterin (BH4), as well as multiple additional pathways not outlined in this scheme (see Table 1). One of the pathways of peroxynitrite-mediated injury involves DNA strand breakage, activation of the nuclear enzyme PARP, poly(ADP-ribosyl)ation and inhibition of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and activation of multiple pathways of diabetic complications including the polyol pathway, the advanced glycation end products (AGE) pathway, the protein kinase C (PKC) pathway and the hexosamine pathway. PARP activation can also lead to intracellular NAD^+ and NADPH depletion (the latter being an essential co-factor of eNOS), and it can also up-regulate various pro-inflammatory pathways leading to pathological modifications in adhesion molecule expression, angiogenesis and other processes.

as (iii) mitochondrial uncoupling with 2,4-dinitrophenol prevent the activation of PARP (Garcia Soriano *et al.*, 2001; Zsengeller *et al.*, 2004), it is most likely that peroxynitrite (generated when mitochondrially produced superoxide combines with constitutively generated NO in the endothelial cells) is the trigger of the DNA strand breakage, which, in turn, activates PARP (Figure 1). Both the hyperglycaemia-induced decrease in activation of GAPDH and its poly(ADP-ribosyl)ation were prevented when hyperglycaemia-induced superoxide generation was blocked by over-expression of either uncoupling protein-1 or treating the cells with manganese superoxide dismutase (Du *et al.*, 2003). Importantly, the hyperglycaemia-induced activation of PKC, the pathological changes in hexosaminase pathway flux, the activation of NF-κB, as well as the changes in AGE formation were all prevented by the pharmacological blockade or genetic inactivation of PARP-1 activity (Du *et al.*, 2003) (Figure 1).

It is important to note that many additional factors and mechanisms have also been demonstrated to contribute to the loss of endothelium-dependent relaxations in various experimental models of diabetes. These factors include the activation of the protein kinase pathway (Beckman, 2002), the post-translational modification of eNOS through the hexosamine pathway (Du *et al.*, 2001), down-regulation of the *expression* of eNOS (as opposed to inhibition of its catalytic activity) (Veves *et al.*, 1998), as well as inhibition of eNOS dimerization, inhibition of eNOS by oxidant-dependent S-nitrosylation (Wadham *et al.*, 2007), and oxidative inactivation of guanylyl cyclase, the intracellular receptor of NO within the cytosol of the vascular smooth muscle cells (Stasch *et al.*, 2006). One must keep in mind that studies in cultured endothelial cell studies are unable to mimic all aspects of the pathomechanism of diabetic endothelial dysfunction, because these mechanisms are often related to the interaction of various cell types. For instance, high glucose, as well as AGE-albumin induces a marked enhancement of the adhesion of leukocytes to the endothelial surface (Morigi *et al.*, 1998), which may contribute to the development of endothelial dysfunction, in part through the release of leukocyte-derived oxidants and free radicals. Furthermore, circulating myeloperoxidase can deposit to the vascular endothelium in diabetes, and reactive oxidant species can be produced as a result (Zhang *et al.*, 2004).

Diabetic endothelial dysfunction (in addition to the lack of ability of the blood vessel to produce NO and to respond with a normal vasorelaxant response) also includes a pathological increase in vascular permeability. This response appears to be dependent on the enhanced formation of vascular endothelial growth factor, the up-regulation of which appears to be dependent on the activation of PKC as well as the intravascular production of oxidative stress as well as PARP activation (Leung *et al.*, 1989; Kuroki *et al.*, 1996; Tilton *et al.*, 1997; Lu *et al.*, 1998; Obrosova *et al.*, 2004a).

The potential role of the angiotensin II/nitrosative stress/PARP pathway in the pathogenesis of diabetic complications

Another factor to be considered in the process of diabetic endothelial dysfunction is angiotensin II, which is known to play a role in the pathogenesis of diabetic nephropathy, cardiomyopathy and retinopathy. The protective effects of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists go beyond the blood pressure-lowering effects of these agents (Bell, 2003; Bui *et al.*, 2003; Siragy, 2008). In this context it is noteworthy that angiotensin II can induce direct, pro-oxidative effects on the vascular endothelium, leading to an impairment of vascular relaxations. These effects are mediated, at least in part, by intraendothelial reactive species formation through a new family of NADPH oxidase subunits, known as the non-phagocytic NADPH oxidase proteins. Reactive oxidant species produced following angiotensin II-mediated stimulation of NADPH oxidases can exert direct oxidative effects, can inhibit the dimerization of but can also signal through pathways such as MAP kinases, tyrosine kinases and transcription factors, and can lead to inflammation, hypertrophy, remodelling and angiogenesis (Cai and Harrison, 2000; Cai *et al.*, 2003). Angiotensin II can also induce intraendothelial peroxynitrite formation (Wattanapitayakul *et al.*, 2000; Mihm *et al.*, 2003), as well as PARP activation (Szabo *et al.*, 2004). Angiotensin-induced intracellular oxidants can also lead to uncoupling of eNOS and reduction of tetrahydrobiopterin availability (Satoh *et al.*, 2008). Administration of

angiotensin II triggers the activation of PARP in cultured endothelial cells *in vitro*, which is inhibited by apocynin, indicating the involvement of NADPH oxidase-generated superoxide anions (Szabo *et al.*, 2004). Angiotensin II-induced PARP activation is also inhibited by the eNOS inhibitor L-NAME (NG-nitro-L-arginine methyl ester), as well as the NADPH oxidase inhibitor diphenyleneiodonium (Szabo *et al.*, 2004). Thus, angiotensin II triggers the endothelial generation of reactive oxygen species from NADPH oxidase, which react with constitutively produced NO, to produce peroxynitrite and other reactive nitrogen species, which induce DNA breakage and activate PARP in the vascular endothelium, leading to the development of endothelial dysfunction. The angiotensin II/PARP pathway also appears to be operative *in vivo*, in animal models, as shown in a rat model of essential hypertension (Szabo *et al.*, 2004) and in human diabetes, as evidenced by the reduction of PARP activation by the angiotensin II receptor blocker valsartan in microvascular endothelial cells obtained in skin biopsies (Shrikhande *et al.*, 2006).

Protective effect of peroxynitrite neutralization and PARP inhibition in animal models of diabetic complications

Over the last decade, a number of peroxynitrite decomposition catalysts (e.g. the metalloporphyrinic compounds FP-15, FeTPPS and FeTMPS) and pharmacological inhibitors of PARP (3-aminobenzamide, nicotinamide, PJ-34, INO-1001, 1,25 isoquinolinediol) have been tested in a variety of experimental models of diabetic complications in various animals. The results of these studies (overviewed in Table 2) demonstrated that both peroxynitrite neutralization (Coppey *et al.*, 2001; Szabo *et al.*, 2002b; DeRubertis *et al.*, 2004; Nangle *et al.*, 2004; Obrosova *et al.*, 2004a, 2004b; Sugawara *et al.*, 2004; Drel *et al.*, 2007a,b; Obrosova *et al.*, 2007; Vareniuk *et al.*, 2007; Ali *et al.*, 2008) and PARP inhibition (Wahlberg *et al.*, 1985; Garcia Soriano *et al.*, 2001; Soriano *et al.*, 2001; Pacher *et al.*, 2002; Li *et al.*, 2004; Mabley *et al.*, 2004; Obrosova *et al.*, 2004b; 2005; 2008; Sugawara *et al.*, 2004; Zheng *et al.*, 2004; Gibson *et al.*,

Table 2 Effect of neutralization of peroxynitrite or pharmacological inhibition of poly(ADP-ribose) polymerase (PARP) on the development of diabetic complications in various experimental models of diabetes mellitus

	Peroxynitrite neutralization	*PARP inhibition*
Diabetic endothelial dysfunction	FP-15 improved endothelium-dependent relaxations in streptozotocin-diabetic rats (Szabo *et al.*, 2002b). FP-15 improved endothelium-dependent relaxations of coronary and mesenteric arteries from streptozotocin-diabetic rats (Drel *et al.*, 2007a).	PJ-34 or INO-1001 improved endothelium-dependent relaxations in streptozotocin-induced diabetes models in mice and rats, in non-obese diabetic mice and in db/db mice. PARP-deficient mice are also resistant against the development of diabetic endothelial dysfunction (Garcia Soriano *et al.*, 2001; Soriano *et al.*, 2001; Pacher *et al.*, 2002; Szabo *et al.*, 2006a). PJ-34 improved cerebrovascular endothelium-dependent relaxations in diabetic rats (Arrick *et al.*, 2007).
Diabetic cardiomyopathy	FP-15 improved myocardial contractile function in streptozotocin-diabetic rats (Szabo *et al.*, 2002a).	PJ-34 improved myocardial contractile function in streptozotocin-induced diabetes models in mice and rats and in non-obese diabetic mice (Pacher *et al.*, 2002). 3-aminobenzamide attenuates the up-regulation of extracellular matrix proteins in the heart and reduces myocardial hypertrophy (Chiu *et al.*, 2008b).
Diabetic neuropathy	FP-15 improved motor and sensory nerve conduction velocity and improves sensory dysfunction in streptozotocin-diabetic mice and rats and in non-obese diabetic mice (Drel *et al.*, 2007a, 2007b; Obrosova *et al.*, 2007). FeTMPS improves motor and sensory neuronal conduction velocity and improves sensory nerve fibre dysfunction and degeneration in diabetic mice (Drel *et al.*, 2007a). FeTMPS and FP-15 both improve motor and sensory nerve conduction velocities in diabetic leptin-deficient ob/ob mice (Vareniuk *et al.*, 2007).	Pharmacological inhibition of PARP inhibitors of various classes (including 3-aminobenzamide, nicotinamide, PJ-34, 4-amino-1,8-naphthalimide, GPI-15427 and 1,5-isoquinolinediol) improves motor and sensory nerve conduction velocity and improves sensory dysfunction in streptozotocin-diabetic rats (Li *et al.*, 2004; Obrosova *et al.*, 2004a,b; 2005; 2008; Ilnytska *et al.*, 2006; Stevens *et al.*, 2007; Sharma *et al.*, 2008). PARP-deficient mice are also resistant against the development of diabetic neuropathy (Obrosova *et al.*, 2004a, 2004b).
Diabetic nephropathy	No published studies using specific peroxynitrite decomposition catalysts. Reduced kidney injury in diabetic db/db mice over-expressing superoxide dismutase suggests an involvement of peroxynitrite (DeRubertis *et al.*, 2004).	Nicotinamide reduced glomerular depositions of IgG in streptozotocin-diabetic rats (Wahlberg *et al.*, 1985). INO-1001 improved renal function and morphology in the db/db mouse model of type 2 diabetes (Szabo *et al.*, 2006a).
Diabetic retinopathy	FP-15 reduced the entrapment of leukocytes in the diabetic retina in streptozotocin-diabetic rats (Sugawara *et al.*, 2004). FeTPPS inhibits the neurodegenerative responses in the retina of diabetic rats (Ali *et al.*, 2008).	PJ-34 treatment improved retinal morphology (pericyte loss, formation of acellular capillaries) in streptozotocin-induced diabetes in rats and mice and reduced leukocyte entrapment in the retina of diabetic rats (Mabley *et al.*, 2004; Sugawara *et al.*, 2004; Zheng *et al.*, 2004).
Diabetic autonomic dysfunction	FeTMPyP treatment improves the function of corpus cavernosum from diabetic mice *in vitro* (Nangle *et al.*, 2004).	3-aminobenzamide improves non-adrenergic non-cholinergic relaxations of gastric fundus rings obtained from diabetic rats (Gibson *et al.*, 2006).

2006; Ilnytska *et al.*, 2006; Szabo *et al.*, 2006a; Arrick *et al.*, 2007; Stevens *et al.*, 2007; Chiu *et al.*, 2008b; Sharma *et al.*, 2008) exert significant beneficial effects and reduce, among others, the development of diabetic endothelial dysfunction, cardiomyopathy, retinopathy, nephropathy and neuropathy. PARP overactivation also plays a role in diabetic neuropathy associated brain dysfunction (Kuchmerovska *et al.*, 2004) and in the exaggerated myocyte injury in diabetic rats in response to coronary occlusion and reperfusion myocardial infarction (Xiao *et al.*, 2004).

The therapeutic potential of both peroxynitrite neutralization and PARP inhibition goes well beyond the experimental therapy of diabetic complications is covered in separate overviews (e.g. Virag and Szabo, 2002; Jagtap and Szabó, 2005; Pacher and Szabó, 2007).

Nitrosative stress, PARP activation and the vascular complications of diabetes mellitus in human subjects

A number of studies have demonstrated the formation of nitrotyrosine (a marker of peroxynitrite generation) in tissues from diabetic subjects (e.g. Ceriello *et al.*, 2001; 2002a,b). In 2002, in collaboration with Dr Aristidis Veves at Harvard University, we set out to investigate the relationship between PARP activation, nitrosative stress and endothelial dysfunction in human diabetic subjects. We have immunohistochemically analysed forearm skin biopsy samples from healthy individuals with parental history of type 2 diabetes, subjects with impaired glucose tolerance and a group of type 2 diabetic patients. The results of this study (Szabo *et al.*, 2002a) demonstrated that the percentage of PARP-positive endothelial nuclei was higher in the group of parental history of type 2 diabetes and diabetic patients when compared with the controls. In addition, significant correlations were observed between the percentage of PARP-positive endothelial nuclei and fasting blood glucose, resting skin blood flow, maximal skin vasodilatory response to the iontophoresis of acetylcholine (which indicates endothelium-dependent vasodilation) and nitrotyrosine immunostaining intensity. Nitrotyrosine immunoreactivity was higher in the diabetic patients when compared with all other groups. Significant correlations were observed between nitrotyrosine immunostaining intensity and fasting blood glucose, HbA1c, intracellular adhesion molecule and vascular cellular adhesion molecule. No differences in the expression of eNOS and receptor for AGE were found among all four groups. The polymorphism of the eNOS gene was also studied and was not found to influence eNOS expression or microvascular functional measurements. Thus, in humans, PARP activation is present in healthy subjects at risk of developing diabetes, as well as in established type 2 diabetic patients, and it correlates with impairments in the vascular reactivity in the skin microcirculation (Szabo *et al.*, 2002a). Because interventional studies with PARP inhibitors in humans with diabetic endothelial dysfunction have not yet been conducted, it remains to be seen whether PARP activation in diabetic or prediabetic humans can be seen as a predictor or early marker for the development of diabetic vascular complications.

It is also important to note, in the context of PARP activation and diabetic complications an increasing body of clinical literature demonstrating increased DNA damage in peripheral blood leukocytes from diabetic patients, a phenomenon closely associated with increased oxidative/nitrosative stress (Anderson *et al.*, 1998; Astley *et al.*, 1999; Dincer *et al.*, 2003; Adaikalakoteswari *et al.*, 2007). In peripheral blood leukocytes, the DNA damage has also been demonstrated to show a close correlation with the degree of PARP activation (Adaikalakoteswari *et al.*, 2007).

The role of reactive nitrogen species and PARP in the phenomenon of 'glucose memory'

The phenomenon of 'glucose memory' is one of the long-standing enigmas in the field of diabetes. Two decades ago it was demonstrated that there is a persistence or 'memory' of the induced expression of basement membrane mRNAs (fibronectin, collagen IV) long after high glucose levels were normalized in endothelial cells in culture and in diabetic rats (Cagliero *et al.*, 1988; Roy *et al.*, 1990), suggesting the possibility for a long-lasting deleterious effects that persists beyond the period of hyperglycaemia. These data were consistent with findings from studies in dogs that retinopathy progression can persist even after the normalization of glycaemic control (Engerman and Kern, 1987).

In a minimalist *in vitro* model of diabetic glucose memory (in cultured endothelial and retinal cells), Ihnat and colleagues have recently demonstrated the persistence of high glucose stress markers after glucose normalization (Ihnat *et al.*, 2007). In addition to PAR and nitrotyrosine, additional markers of high glucose stress included the basement membrane protein fibronectin, the signalling kinase PKC-β, the mitochondrial pro-apoptotic protein Bcl-2 family member Bax and p47phox, an inducible subunit of the enzyme NADPH oxidase. After 1 week of increased glucose environment, reactive oxygen species production continued for a subsequent week during which the cells were returned into normal glucose. In addition, all of the proteins studied remained increased even 1 week after normalization of extracellular glucose. When the antioxidant alpha-lipoic acid was added during the last week of normal glucose levels, significant decreases in the induction of all the markers of high glucose stress were seen. As expected (based on the crucial role of mitochondria in the generation of reactive species in high-glucose conditions), over-expression of the mitochondrial respiratory chain uncoupling protein-2 during the normalization period reduced the induction of all the stress proteins. PARP inhibition had similar protective effects (Ihnat *et al.*, 2007).

As with the isolated endothelial cells, levels of nitrotyrosine and PAR, as well as fibronectin, activated PKC-β, p47phox, nitrotyrosine and PAR remained increased in the retina of the rats that were hyperglycaemic for 2 weeks followed by normoglycaemic for 1 week, but were normalized when the animals were treated with the mitochondrial antioxidant nutritional supplement, alpha-lipoic acid (Ihnat *et al.*, 2007). The findings with alpha-lipoic acid are clinically important, because this compound has previously been shown to inter-

rupt target organ damage when given chronically to diabetic animals (Kowluru and Odenbach, 2004; Lin *et al.*, 2006) and has also produced encouraging clinical effects in human diabetes trials (Ziegler *et al.*, 2006; Foster, 2007; Du *et al.*, 2008). However, because this compound is a 'general antioxidant' (Scott *et al.*, 1994; Savitha *et al.*, 2005) we cannot imply the role of specific reactive species (e.g. superoxide, peroxynitrite, etc.) in the process. From the above findings it appears that antioxidant therapy may be of potential utility to interrupt the ongoing deleterious pro-oxidant cycles and continuing activation of diabetic complication pathways in diabetes. Indeed, in a small-scale human study in type 1 diabetes it has been demonstrated that neither normalization of glucose with insulin alone, nor the antioxidant ascorbic acid alone restores the normal endothelium-dependent relaxations, but the combination of the two approaches is effective (Ceriello *et al.*, 2007).

Enhanced reactive species generation in response to intermittent high/low/high/low extracellular glucose concentration

Several series of studies demonstrated that intermittent low- and high-glucose conditions are more deleterious to the function of endothelial cells than even a steady, constant increase of glucose (Risso *et al.*, 2001; Quagliaro *et al.*, 2003; Piconi *et al.*, 2004). These conditions also induce a more pro-inflammatory state of the endothelial cells, associated with the up-regulation of various adhesion molecules and pro-inflammatory cytokines (Piconi *et al.*, 2004). Some of the pathways implicated in these exacerbated cellular responses involve activation of PKC and NADPH oxidases, as well as mitochondrial oxidants (Quagliaro *et al.*, 2003; 2005). In addition, the fact that rosuvastatin treatment of the endothelial cells reduced their oxidant production and protected against cell death may implicate the 3-hydroxy-3-methylglutaryl coenzyme A reductase in the process (Piconi *et al.*, 2008). In a rat model of streptozotocin-induced diabetes, induction of rapid glycaemic swings with short-acting insulin was more deleterious to the function of the endothelium (as assessed by endothelium-dependent relaxant function of thoracic aortic rings) than even the steady hyperglycaemia associated with diabetes (Horvath *et al.*, 2009). Likewise, clinical data demonstrate that rapid glycaemic swings are associated with an exacerbated degree of oxidant production in human diabetes (Monnier *et al.*, 2006) and are very deleterious to the endothelial function of patients with type 2 diabetes (Ceriello *et al.*, 2008). Overall, these data outline the importance of steady glucose control and the potential involvement of oxidative and nitrosative stress in the pathogenesis of complications due to poorly controlled diabetes.

Unanswered questions and future directions

A number of important questions remain unanswered in relation with the pathogenesis of increased extracellular glucose-mediated endothelial toxicity and diabetic complications. In the current section, we briefly overview some of these.

1. The molecular mechanism of glucose memory

As mentioned previously, the mechanism of increased superoxide generation from the mitochondria is related to exceeding a threshold value for the proton gradient in the intramitochondrial inner membrane due an overproduction of electron donors from the tricarboxylic acid cycle. However, if this mechanism would be solely responsible for the observed effects, one would expect that reactive oxygen generation and nitrosative stress and PARP activation would return to normal after 1 week of normalization of extracellular glucose. As aforementioned (Ihnat *et al.*, 2007), this is not the case. It is possible that irreversible cellular alterations, or possibly changes in the expression of mitochondrial proteins occur over the days/weeks in increased glucose. It was suggested that damage to the mitochondrial DNA results in the expression of 'faulty' mitochondrial proteins, which, in turn, result in permanent defects in the mitochondrial electron transport chain (Brownlee, 2001). It is also possible that prolonged activation of the various pathways of diabetes induces a secondary generation of reactive oxidant species (e.g. through NADPH oxidases or other pathways), which 'take over' or participate in a positive feedback cycle of oxidant generation. In this context it is interesting to note that in the Ihnat glucose memory study, blocking NADPH oxidase using apocynin or xanthine oxidoreductase with oxypurinol during the glucose normalization period in endothelial cells also interrupted the induction of some of the high glucose stress markers (Ihnat *et al.*, 2007). The molecular basis of these findings needs to be investigated in future studies.

2. Why is alternating high/low glucose more deleterious than constant increase of glucose?

Like in the case of glucose memory, a simple 'overdrive' of the mitochondria does not seem to be sufficient to explain these findings. Is it possible that some trigger in the mitochondrial electron transport chain is dysregulated? Alternatively, is possible that steady increases of glucose induce certain compensatory protective mechanisms, which are not switched on in the intermittent high/low-glucose mode? A related, and clinically relevant question is whether, in diabetic patients, the overall average of blood glucose concentration (as estimated by detection of glycated haemoglobin levels) is the best indicator for a risk of diabetic complications, or is it possible that this measure should be combined with other measures aimed at detecting the frequency and magnitude of 'rapid glycaemic swings'?

3. The intracellular downstream effectors of PARP activation

Multiple series of recent studies have demonstrated that the PARP-mediated cell injury pathways can involve a variety of pathways in addition to the depletion of intracellular NAD^+ levels. For instance, PARP activation can regulate the mitochondrial-to-nuclear translocation of apoptosis-inducing factor (Yu *et al.*, 2006; Dawson and Dawson, 2004). AIF is a

67 kDa mitochondrial death-promoting protein, which induces DNA fragmentation by initiating the activation of a yet unidentified nuclease, and which is now viewed as a key terminal effector of cell death (Lorenzo and Susin, 2007). The importance of this pathway in the context of diabetic complications has not yet been investigated. Likewise, the potential cytotoxic effect of free PAR polymer (Andrabi *et al.*, 2006) has not yet been investigated in the context of hyperglycaemic endothelial cell injury, even though it is conceivable that poly(ADP-ribosyl)ation of GAPDH (a primarily cytosolic protein) by PARP (a primarily nuclear protein) may occur through the nuclear-to-cytosolic translocation of free PAR polymer, followed by its binding to GAPDH.

4. The exact role of pyridine nucleotides in the pathogenesis of diabetic complications

As described in the current overview, activation of the aldose reductase pathway results in the depletion of intracellular NADPH levels (through oxidation of NADPH to $NADP^+$), as well as a reduction of NAD^+ to NADH. These processes result in a pathological increase in the cytosolic NAD/NADH ratio. Additionally, the activation of PARP results in the direct depletion of cytosolic NAD^+ and NADPH. In effect, one can view this response as if cytosolic NAD^+ pools would get transferred to the nucleus (in the form of PAR polymers). Incidentally, these PAR pools can be later used as a source of energy for DNA repair enzymes (Oei and Ziegler, 2000). These alterations, which have been demonstrated both *in vitro* (in cultured endothelial cells) (Garcia Soriano *et al.*, 2001) and *in vivo* (for instance in diabetic neurons) (Obrosova *et al.*, 2005) may have significant effects on vascular function through several mechanisms (in addition to the previously discussed inhibition of GAPDH enzyme). For instance, NADPH depletion can result in an inhibition of eNOS activity through lack of co-factor availability. A comprehensive characterization of pyridine nucleotide metabolism remains to be performed in endothelial cells placed in increased glucose, with special reference to the recently identified distinct roles of cytosolic and mitochondrial NAD^+ pools in PARP-dependent and independent cellular responses. In this respect it is noteworthy that increase of tissue pyruvate levels (which drives oxidation of NADH to NAD^+) can substitute for the missing cytosolic NAD^+ levels and rescues cells from PARP-dependent death (Ying *et al.*, 2002; Zeng *et al.*, 2007). Some of the vascular alterations induced by increased glucose levels have been shown to be prevented by pyruvate treatment (Williamson *et al.*, 1993; Kashiwagi *et al.*, 1997).

5. A potential role of PARP in regulation of gene expression in diabetes

As already noted previously, PARP, through modulation of GAPDH, and, subsequently, through modulation of the PKC and NF-κB pathways, may influence the expression of various genes. However, PARP can regulate the expression of a variety of additional genes and gene products through other mechanisms as well. One level of gene regulation is related to the regulation of poly(ADP-ribosyl)ation of histones, and by unwinding the strands of the DNA and making it more accessible for mRNA transcription mechanisms (Wacker *et al.*, 2007; Krishnakumar *et al.*, 2008). It has been demonstrated that PARP regulates the expression of various proteins, for example, inducible NO synthase, tumour necrosis factor alpha, intercellular adhesion molecule-1, the inducible isoform of cyxlooxygenase and major histocompatibility complex class II. The absence of functional PARP-1 (either genetic or pharmacological) decreased the expression of a host of pro-inflammatory mediators, including cytokines, chemokines, adhesion molecules and enzymes, and it also reduced tissue infiltration with activated phagocytes in various experimental models of inflammation and cardiovascular disease (reviewed in Jagtap and Szabó, 2005). It has been shown that PARP inhibition suppresses the diabetes-induced up-regulation of endothelin-1 and its receptor in the kidney (Minchenko *et al.*, 2003) and of the diabetes-induced up-regulation of the pro-angiogenic factor vascular endothelial growth factor in the diabetic retina (Obrosova *et al.*, 2004a), while the anti-angiogenetic factor PEDF (pigment epithelium derived factor) may be down-regulated (Chen *et al.*, 2008). In the retina, kidney and heart of diabetic rats PARP was implicated in the up-regulation of endothelin-1, as well as fibronectin (Chiu *et al.*, 2008a). A comprehensive survey investigating the role of PARP in the expression of various genes in diabetes remains to be investigated.

6. A potential relationship between PARP and sirtuins

Another area worthy of additional exploration is the relationship of PARP with sirtuin activation. The sirtuin pathway has recently been implicated in the context of diabetic complications (Porcu and Chiarugi, 2005; Tikoo *et al.*, 2007). In this context it is important to remember that sirtuins are NAD^+-dependent deacetylases and it is likely that changes in NAD^+ metabolism due to PARP activation or inhibition could impact sirtuin function (Frye, 1999; Chong *et al.*, 2005; Kruszewski and Szumiel, 2005). Activation of sirtuins has been shown to be protective in a number of aging-related disorders, and these pathways likely interact (overviewed in Hassa *et al.*, 2006). Furthermore, the PARP-1-dependent cardiac myocyte cell death during heart failure may also be mediated by NAD^+ depletion and reduced Sir2alpha deacetylase activity (Pillai *et al.*, 2005). The sirtuin/PARP relationship may be worthy for additional investigations in the context of diabetic complications.

7. Endogenous regulators of oxidative stress, nitrosative stress and PARP in diabetes

There are many endogenous factors (antioxidants, vitamins, including vitamin C and vitamin E) that can serve as endogenous modulators of the production and action of endogenous reactive species. Although many clinical trials with such antioxidant vitamins investigating the progression of diabetic complications conclude as negative or inconclusive (e.g. Giannini *et al.*, 2007; Evans, 2008; Milman *et al.*, 2008), one may wish to see additional studies, perhaps ones involving combination of these vitamins, or mega-dose therapies. It is interesting to note that benfothiamine, a transketolase activator, has been shown to rectify some of the defective path-

ways of diabetes, by diverting them to an alternative pathway (Hammes *et al.*, 2003). This compound has been shown to beneficially affect the development of various diabetic complications in humans (Stirban *et al.*, 2006). It is noteworthy that recent studies demonstrated that the regulation of PARP is more dynamic than previously thought, and endogenous purines, caffeine, the active form of vitamin D (1,25-dihydroxycholecalciferol), gender (presumably the female sex hormone oestrogen) and many other factors can act as negative regulators of PARP activation *in vitro* and *in vivo* (Mabley *et al.*, 2005; 2007; overviewed in Szabó *et al.*, 2006b). It is also conceivable that clinically used drugs – including the angiotensin receptor blocker valsartan (Shrikhande *et al.*, 2006), rosiglitazone (Cuzzocrea *et al.*, 2004), thiazolidinediones (Da Ros *et al.*, 2004) and angiotensin convertin enzyme inhibitors or angiotensin receptor blockers (Szabo *et al.*, 2004) may indirectly (through reduction of intracellular and extracellular reactive species generation) inhibit the activation of PARP in diabetes. It remains to be seen whether the appropriate use of such agents (alone, or in combination) may be of benefit against the development and progression of diabetic vascular complications.

8. The concept of combination therapies

As in many other diseases, it may be too simplistic to expect that pharmacological modulation or any single pathway can be completely effective for the therapy of diabetic complications. Accordingly, an appropriate mixture of drugs targeting various interrelated but independent pathways of injury may be a potential future approach for the experimental therapy of diabetes. As mentioned above, glucose control and antioxidant therapy has been shown to demonstrate synergistic clinical benefit for preserving endothelial function (Ceriello *et al.*, 2007). The combination of alpha-lipoic acid and benfothiamine has also been demonstrated to exert marked protective effects in a small-scale human trial (Du *et al.*, 2008). Perhaps a combination of a statin (the pharmacological action of which includes antioxidant effects, up-regulation of the endothelial NO synthesis, indirect inhibition of PARP and other effects), with other antioxidants (perhaps of the catalytic type), PARP inhibitors and angiotensin pathway blockers may be worth considering. Interestingly, in a recent study, the combination of the PARP inhibitor 1,25 isoquinolinediol, in combination with the angiotensin-converting enzyme inhibitor lisinopril, as well as the combination of the PARP inhibitor with the β_2 receptor agonist salbutamol was found more effective than each individual agent in improving nerve conduction velocity in diabetes, and in fully restored the responses to the levels seen in normal non-diabetic animals (Obrosova *et al.*, 2005).

Although many questions remain unanswered, it is clear that significant progress has been made with respect to the oxidative and nitrosative stress-related mechanisms of endothelial cell injury in diabetes over the last decades. Studies in this field have identified a number of approaches and therapies that indirectly or directly reduce the activation of various pathways of oxidative and nitrosative stress, and thereby hold the potential of improving the function of the endothelial cells to provide therapeutic benefit for patients with diabetes.

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