Acta Physiologica Hungarica, Volume 90 (3), pp. 175–193 (2003)

Pathophysiological aspects of cellular pyridine nucleotide metabolism: focus on the vascular endothelium

Review

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Received: January 28, 2003
Accepted: May 21, 2003

In recent years, pyridine nucleotides NAD(H) and NADP(H) have been established as an important molecules in physiological and pathophysiological signaling and cell injury pathways. Protein modification is catalyzed by ADP-ribosyl transferases that attach the ADP-ribose moiety of NAD^+ to specific aminoacid residues of the acceptor proteins, with significant changes in the function of these acceptors. Mono(ADP-ribosyl)ation reactions have been implicated to play a role both in physiological responses and in cellular responses to bacterial toxins. Cyclic ADP-ribose formation also utilizes NAD^+ and primarily serves as physiological, signal transduction mechanisms regulating intracellular calcium homeostasis. In pathophysiological conditions associated with oxidative stress (such as various forms of inflammation and reperfusion injury), activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) occurs, with subsequent, substantial fall in cellular NAD^+ and ATP levels, which can determine the viability and function of the affected cells. In addition, NADPH oxidases can significantly affect the balance and fate of NAD^+ and NADP in oxidatively stressed cells and can facilitate the generation of various positive feedback cycles of injury. Under severe oxidant conditions, direct oxidative damage to NAD^+ has also been reported. The current review focuses on PARP and on NADPH oxidases, as pathophysiologically relevant factors in creating disturbances in the cellular pyridine nucleotide balance. A separate section describes how these mechanisms apply to the pathogenesis of endothelial cell injury in selected cardiovascular pathophysiological conditions.

Keywords: pyridine nucleotides, nitric oxide, superoxide, free radicals, oxidants, poly(ADP-ribose) synthetase, poly(ADP-ribose) polymerase, NADPH oxidase, DNA, mitochondria, endothelium, reperfusion, inflammation, shock

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0231–424X/2003/$ 20.00

Overview of the (ADP-ribosyl) action reactions

The coenzyme NAD^+, is a well-known hydrogen donor or acceptor which participates in numerous metabolic reactions. Recent work has focused on the role of this molecule in various cellular signaling pathways. It became evident that protein modifications can be catalyzed by ADP-ribosyl transferases that attach the ADP-ribose moiety of NAD^+ to specific aminoacid residues of the acceptor proteins. For many (ADP-ribosyl)ation reactions the specific transferases and their target proteins have been identified. Mono (ADP-ribosyl)ation reactions and their functions have been subject of multiple recent reviews (24, 53, 100). The eukaryotic mono ADP-ribose transferases (mADPRTs) primarily modify arginine, and, less frequently cysteine residues with (generally) an inhibition of the activity of the mono-(ADP-ribosyl)ated proteins. The target proteins of mADPRTs are multiple and their functions are diverse, as overviewed elsewhere (24, 53, 100). A large body of work focuses on mono-(ADP-ribosyl)ation reactions induced by bacterial toxins, which, when taken up into the host cells, catalyze (ADP-ribosyl)ation reactions and thus modify specific proteins. Modification of arginine residues of nucleotide-binding proteins, primarily G-proteins, followed by an inhibition of their function, appears to be a major mechanism, as established, for example, in the case of pertussis and cholera toxins. The pathophysiological alterations, that result in response to the exposure of mammalian cells to these toxins, are primarily the consequences of the activation of the adenylyl cyclase, and an increase in intracellular cyclic AMP concentration (24, 53, 100).

Cyclic ADP-ribose (cADPR), synthetized from NAD^+ by bifunctional ADP-ribosyl cyclases/cyclic ADP-ribose hydrolases represents another important physiological NAD^+-derived intracellular regulatory pathway, which has been subject of several recent overviews (22, 100). The first enzyme identified as ADP-ribosyl cyclase has subsequently been identified as the membrane protein CD38. Other ADP-ribosyl cyclases, with primarily intracellular functions also exist. Cyclic APDR increases intracellular calcium concentration, via calcium release from the endoplasmic/sarcoplasmic reticulum and acts as a specific ligand for the ryanodine receptor or an associated protein. Regulation of insulin secretion by cADPR in pancreatic β-cells has been subject of extensive investigations (27).

While the above listed two enzymes (mono ADPRT and cADPRT) both utilize cellular NAD^+, it appears that changes in their enzyme activities are not associated with drastic alterations in the overall cellular NAD^+ pools. In addition, there is no evidence which would suggest that these two enzyme systems have major roles in cardiovascular diseases or in oxidant-mediated cell injury. In pathophysiological conditions associated with oxidative stress (such as various forms of inflammation and reperfusion injury), activation of the nuclear enzyme poly(ADP-ribose) polymerase occurs, with subsequent, substantial fall in cellular NAD^+ and high energy phosphate levels, which can determine the viability and function of the affected cells. In addition, NADPH oxidases can significantly affect the balance of NAD^+ in oxidatively stressed cells. Under severe oxidant conditions, direct oxidative damage to NAD^+ has also been

reported (18, 41, 81). The subsequent chapters of the current review focus on PARP and on NADPH oxidases, as pathophysiologically relevant factors in creating disturbances in cellular NADPH balance, and consequently, cell functions, with special relevance to endothelial dysfunction and cardiovascular pathophysiology.

Poly(ADP-ribose) polymerase (PARP)

Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30) – also known as poly(ADP-ribose) synthetase (PARS) or poly(ADP-ribose) transferase (PADPRT) – is a nuclear enzyme present in eukaryotes. PARP-1 is a 116 kDa protein consisting of three main domains: the N-terminal DNA-binding domain containing two zinc fingers, the automodification domain, and the C-terminal catalytic domain. The primary structure of the enzyme is highly conserved in eukaryotes with the catalytic domain showing the highest degree of homology between different species. The structure and functions of PARP have been subject of several recent overviews and monographs (69, 70, 91, 99). The current review focuses on the role PARP plays in affecting cellular NAD^+ levels and cellular function in selected pathophysiological conditions.

PARP-1 catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and uses the latter to synthesize branched nucleic acid-like polymers poly(ADP-ribose) covalently attached to nuclear acceptor proteins (Fig. 1). The size of the branched polymer varies from a few to 200 ADP-ribose units. Poly(ADP-ribosyl)ated proteins can be readily identified by various methods, including using Western blotting by primary antibodies raised against poly(ADP-ribose). The covalently attached ADP-ribose polymer can affect the function of target proteins. Many signal transcription factors, including p53 and nuclear factor κB (NF-κB) have been shown to be poly(ADP-ribosyl)ated. Nevertheless, this is a relatively poorly understood area of research where intensive work needs to be performed in the future, in order to determine the exact functional role of these various poly(ADP-ribosyl)ation reactions. The most abundantly poly(ADP-ribosyl)ated protein is PARP-1 itself, and auto-poly(ADP-ribosyl)ation represents a major regulatory mechanism for PARP-1 resulting in the inhibition of enzyme activity. Histones are also considered as major acceptors of poly(ADP-ribose), and histone poly(ADP-ribosyl)ation has been implicated in chromatin remodeling, DNA repair and transcriptional regulation (69, 70, 91, 99). Poly-(ADP-ribosyl)ation is a dynamic process: two enzymes, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase, are involved in the catabolism of poly(ADP-ribose). PARG is involved in cleaving ribose–ribose bonds and the lyase serves to remove the protein proximal ADP-ribose monomer. It is noteworthy that poly(ADP-ribosyl)ation is generally viewed as a dynamic process where poly(ADP-ribose) polymer is rapidly degraded by poly(ADP-ribose) glycohydrolase and ADP ribosyl protein lyase. The half-life of the polymer is estimated to be less than 1 minute, indicating a concerted activation of poly(ADP-ribose) synthesizing and degrading enzymes. These measurements and assumptions are somewhat in contrast to cell-based

and *in vivo* observations, where a clear and time-dependent accumulation of poly(ADP-ribose) can be detected by various methods, including immunohistochemistry (as overviewed in 91). It is possible that not only the activity of PARP, but also the activity of poly(ADP-ribose) glycohydrolase can be altered in pathophysiological conditions and under conditions of oxidative stress, which may explain this apparent discrepancy.

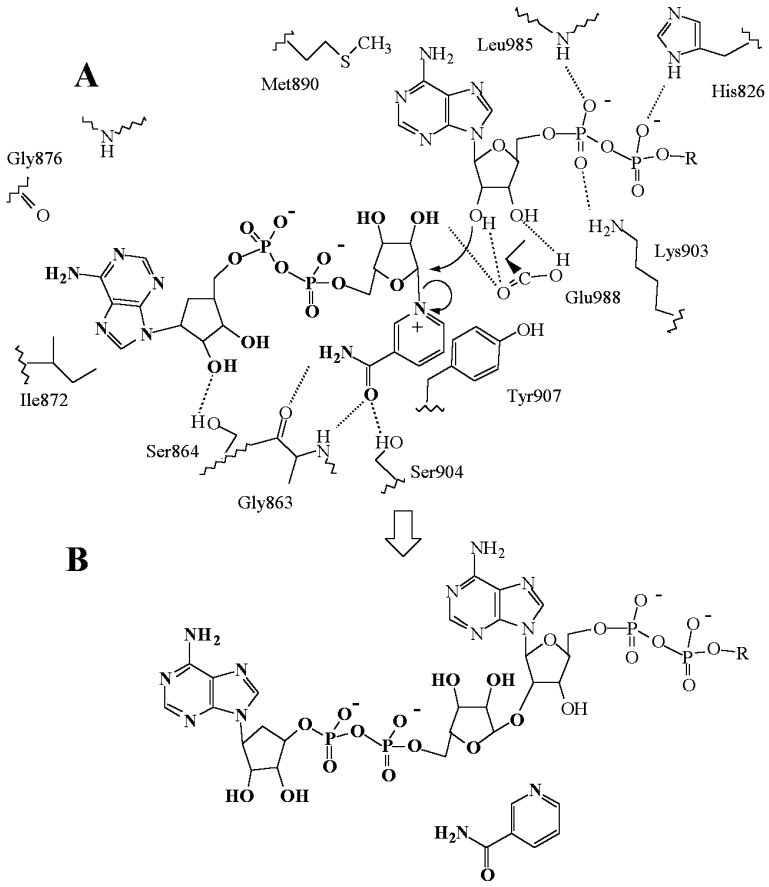

Fig. 1. A representation of the binding of NAD^+ to PARP and the catalysis of the polymerization elongation reaction. **(A):** NAD^+ (bold atoms) is bound in a cleft in the PARP protein. Some key amino acid residues involved in the interaction with NAD^+ are indicated. Dotted lines represent hydrogen bonding interactions. The curved arrows represent the movement of electrons during nucleophilic attack by the 2' oxygen of the adenosine ribose of the acceptor polymer on the NAD^+ molecule. **(B):** Nicotinamide is displaced from the NAD^+, the rest of which (ADP-ribose) becomes bonded to the acceptor polymer resulting in elongation of the polymer chain

PARP-1 can be viewed as a DNA damage sensor and signaling molecule. Its catalytic activity is triggered by binding to broken (primarily single-stranded) DNA through zinc finger domains. As there are breaks in the DNA under normal (physiological) conditions, it is not surprising that a low, but detectable activity of PARP can be seen in cells under baseline conditions (e.g. 1, 6, 26, 28, 33, 36, 37, 61). Traditionally PARP-1 is viewed as a housekeeping enzyme involved in DNA repair and maintenance of genomic integrity – even though PARP-1 deficient animals and cells do not show signs of major DNA defects and are not prone to the spontaneous development of malignancies or other disturbances one would normally expect from an essential DNA repair enzyme (69, 70, 91, 99).

PARP becomes greatly activated when multiple, simultaneous breaks in the DNA develop. These breaks can develop as consequence of severe oxidant or free radical generation. In specific, hydroxyl radical, peroxynitrite and nitroxyl anion can directly trigger the single strand breakage in DNA, whereas superoxide and nitric oxide do not directly induce DNA breaks. Nevertheless, nitric oxide donor compounds, at high concentrations, can induce DNA breakage, which is most likely related to their transition into peroxynitrite in the cellular environment (as overviewed in 70, 91). As overviewed elsewhere (91) the concept that under pathophysiological conditions, genotoxic stress can occur, is a relatively new one. Nevertheless, several studies now indicate the development of DNA breaks and base modifications in various inflammatory and reperfusion conditions. These breaks in DNA activate an intracellular cascade widely known as the "PARP activation – cell suicide cascade". The initial studies on the role of PARP and cell death were performed using pharmacological inhibitors of PARP (most frequently, 3-aminobenzamide and nicotinamide) and have been previously reviewed (69, 70). These agents can exert additional actions, for example as free radical scavengers. Studies, using cells from PARP knockout animals confirmed the role of the PARP pathway in oxidant-mediated cell injury. In the first such study, pancreatic islets of Langerhans of the $PARP^{-/-}$ mice were found to be resistant to injury elicited by a nitric oxide donor agent, when compared to the response in islets of the wild-type mice (26). Similarly, we observed that pulmonary fibroblasts from the $PARP^{-/-}$ mice are protected from peroxynitrite-induced cell injury when compared to the fibroblasts of the corresponding wild-type animals (72). Furthermore, Eliasson and colleagues demonstrated protection by PARP negative phenotype in brain slices exposed to N-methyl-D-aspartate, a neurotoxin which releases nitric oxide, superoxide and peroxynitrite, which mediates much of its neurotoxic effects (15). The cell death that occurs in response to PARP activation is primarily of the necrotic type, and is characterized by depletion of cellular NAD^+ and ATP levels, mitochondrial swelling and dysfunction, breakdown of membrane potential, release of mitochondrial suicide factors including cytochrome c and apoptosis inducing factor and leakage of cellular content to the extracellular space (6, 74, 87–90, 97). Protection by PARP inhibition can be achieved in many but not all cell types against this type of necrotic cell death, but, importantly, not against apoptotic cell death. It is still a widely held view that necrosis is a futile process which cannot be influenced by pharmacological means

(while apoptosis is viewed as a sophisticated process which is under the control of a complex cellular machinery and is amenable to pharmacological intervention). The above evidence suggests that the necrotic process is, indeed, amenable to pharmacological interventions. In fact, the marked protection seen in many experimental models of inflammation and reperfusion injury in the absence of functional PARP indicates that necrosis, and not apoptosis is the likely predominant form of cell death and organ dysfunction in many diseases (as overviewed in 91).

What are, then, the biochemical steps leading to cell dysfunction and cell death in response to PARP-mediated rapid cellular NAD^+ depletion? First of all, there seems to be a discrepancy between the known auto-inhibition of PARP by auto-ADP-ribosylation and the observed prolonged nature of PARP activation and NAD^+ depletion in oxidatively stressed cells. In response to rapidly decomposing oxidants (such as peroxynitrite), one can observe a rapid depletion of NAD^+ (to about 10–15% of basal NAD^+ levels within 5–10 minutes), followed by a partial recovery over the subsequent several hours (61, 74). This pattern may be consistent with gradual partial auto-inhibition of PARP, as the alternative explanation (a rapid repair of the oxidative DNA breaks, followed by inactivation of PARP) is unlikely. The mechanisms whereby the decreases in cytosolic NAD^+ levels lead to a shut-down of glycolysis, oxidative phosphorylation, mitochondrial and cell membrane potential and various cellular functions (culminating in necrosis) are incompletely understood, although they appear to involve a futile effort of the cell to attempt to re-synthesize NAD^+. In this process, first the conversion of nicotinamide to nicotinamide mononucleotide (NMN) by phosphoribosyl transferase occurs, which uses phosphoribosyl pyrophosphate, obtained from ATP. ATP is also required for the metabolism of NMN into NAD^+ by an enzyme termed mononucleotide adenylyl transferase. The re-synthesis of each molecule of NAD^+ requires four molecules of ATP. Eventually, the process leads to the depletion of the main NAD^+ dependent metabolic pathways such as anaerobic glycolysis and oxidative phosphorylation (17, 48, 100). Recent work demonstrates that substrates that bypass glycolysis can rescue cells after PARP activation. In a murine cortical astrocyte and astrocyte-neuron co-cultures, PARP was activated with the DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Studies using the 2-deoxyglucose method confirmed that glycolytic flux was reduced by more than 90% in MNNG-treated cultures. The addition of alpha-ketoglutarate, pyruvate, or other mitochondrial substrates to the cultures after MNNG treatment markedly reduced cell death (96). The above-described model of NAD^+ depletion and secondary ATP depletion assumes that cytosolic and mitochondrial NAD^+ pools communicate with each other, an issue which is debated by many investigators (100). We must point out, nevertheless, that PARP activation, in oxidatively stressed cells, and in pathophysiological conditions, does not occur in isolation: a variety of oxidative or nitrosative cellular modifications simultaneously affect the energy-generating processes of the cell at multiple levels and checkpoints (via affecting key enzymes of glycolysis and mitochondrial oxidative phosphorylation). Under these conditions, it is likely that many of the biochemical equations and assumptions (which have been established in intact, physiologically

functioning cells) lose some of their validity. For instance, the permeability of the mitochondrial membranes may increase, thereby allowing the efflux of mitochondrial NAD^+ into the cytosol. There is some recent evidence, which shows that mitochondria also contain poly(ADP-ribosyl)ating activities (13). The potential existence of an additional, intramitochondrial, PARP-related NAD^+ metabolizing pathway (possibly triggered by injury to the mitochondrial DNA?) is an intriguing possibility, which should be further explored in the future.

While initially the formation of poly(ADP-ribose) was viewed a one-way process (essentially forming an NAD^+ sink), recent work has demonstrated that poly(ADP-ribose) polymer can serve as an alternative source of energy used by the base excision machinery to synthesize ATP (14). However, it appears that in oxidative stress, this re-synthesis of ATP is not sufficient to maintain cellular energetics and cellular function, presumably due to the rapid shutdown of cellular energy-generating pathways in cells with low cytosolic NAD^+ levels (see above).

It is noteworthy that – in addition to the process of NAD^+ depletion and the induction of cellular dysfunction – part of the PARP-overactivation induced cell dysfunction and necrosis is related to intracellular acidification. Part of this process is related to inhibition of sodium/hydrogen exchange in energy-depleted cells (29). Another part of this process is due to a direct acidification: when PARP catabolizes NAD^+, in addition to ADP-ribose and nicotinamide a "byproduct" of the reaction is H^+, which directly induces intracellular acidification, with direct consequences for cell viability (1).

It is interesting that under oxidative stress conditions, an up-regulation of certain NAD^+ catabolizing pathways can occur, perhaps in order to substitute for the NAD^+ that is being over-utilized by PARP. One such pathway involves the up-regulation of oxidative tryptophan catabolism via the kynurenine pathway (20).

As mentioned above, the end-result of PARP overactivation mediated depletion of cellular energy leads to cell necrosis. In many pathophysiological conditions full-fledged parenchimal cell necrosis can, indeed, occur. Typical examples are myocardial infarction, stroke, or various forms of liver disease where intracellular cellular content from the dying cells is being released into the circulation. (In fact, these released enzymes frequently serve as diagnostic markers in routine internal medicine practice – for instance the myocardial specific creatinine phosphokinase to detect myocardial infarction, or the so-called "liver enzymes" to help diagnose various hepatic diseases associated with parenchymal necrosis.) In these conditions, pharmacological inhibition or genetic deficiency of PARP, indeed, salvages significant portions of the ischemic and reperfused heart and brain tissue that would otherwise undergo necrosis (91). On the other hand, in many pathophysiological conditions, one must distinguish between full-fledged necrotic cells and severely disturbed, but living cells (i.e. cells which show evidence of DNA breaks, PARP activation and depletion of cellular energetics). This latter cell population may be called "pre-necrotic". Examples of such a cell population are the oxidatively stressed intestinal epithelial cells in inflammatory bowel disease, or cultured epithelial cells exposed to pro-inflammatory cytokines (a reductionist *in vitro*

model of intestinal inflammation) (35, 37, 38). Another example of such cell population is the vascular endothelium during hyperglycemia, or exposure of cultured endothelial cells to elevated extracellular glucose concentration (a reductionist *in vitro* model of diabetic vascular complications) (65). In these cell populations, cellular NAD^+ and ATP levels are suppressed. Cellular functions can be rapidly restored by PARP inhibition, or by replenishing the cellular NAD^+ levels by the administration of liposomal NAD^+ preparation (35, 37, 38, 65). This reversible nature of the cell dysfunction is consistent with direct regulation by NAD^+ of various cellular energetic and functional processes. For example, it appears that in endothelial cells exposed to high extracellular glucose concentrations, cellular NAD^+ levels secondarily influence cellular NADPH levels. Changes in cellular levels of NADPH, being a co-factor of the endothelial nitric oxide synthase, directly and reversibly influence the ability of the endothelial cell to produce NO in response to hormones that induce endothelium-dependent vascular relaxations (see Fig. 2 and, in detail, below).

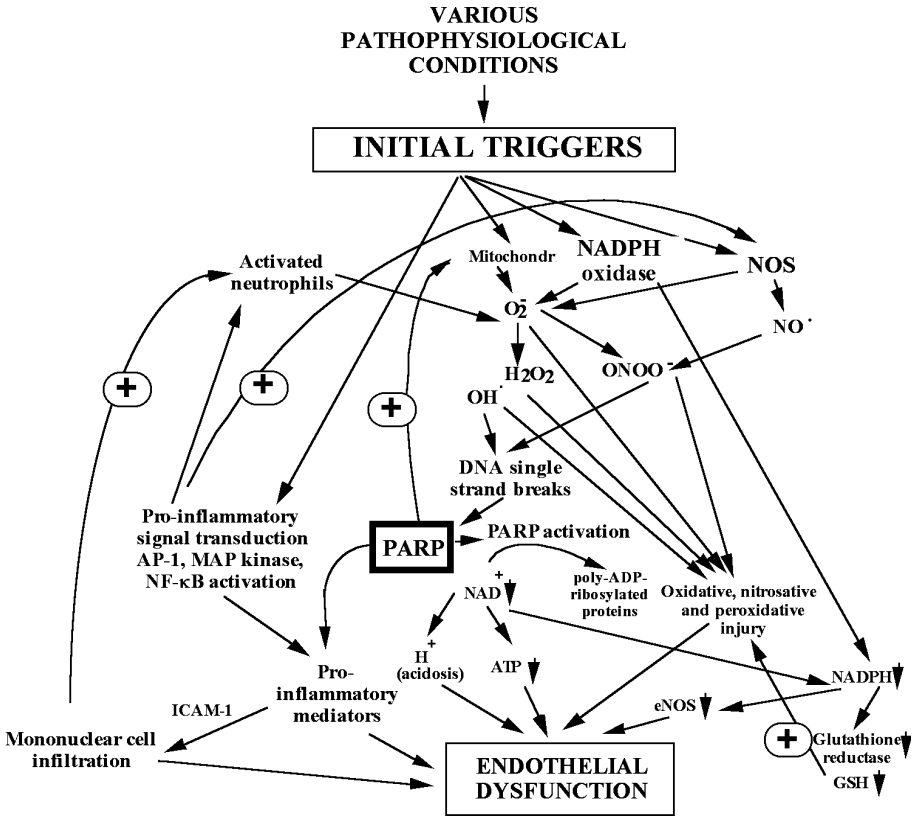

Fig. 2

It is important to note that until recently, PARP activity was thought to result from the function of a single enzyme. Following the observation that PARP-1 deficient cells have some residual PARP activity (62), intensive research began to identify enzymes responsible for this activity. In recent years numerous additional enzymes possessing poly(ADP-ribosyl)ation activity have been described (overviewed in 91). The functions of these minor (i.e. low-expression, low-level) PARP isoforms are largely unknown and

← Fig. 2. Selected schemes of oxidative stress and PARP and NADPH oxidase dependent cytotoxic pathways in the pathogenesis of endothelial dysfunction. Superoxide and other reactive oxidants can be generated from extracellular sources, as well as from various intracellular sources including the mitochondrial electron transport chain, xanthine oxidase, NADPH oxidase and other sources. Endothelial NO synthase (eNOS), and in certain conditions, the inducible NO synthase (iNOS) produce nitric oxide (NO). NO, in turn, combines with superoxide to yield peroxynitrite. Hydroxyl radical (produced from superoxide via the iron-catalyzed Haber-Weiss reaction) and peroxynitrite or peroxynitrous acid induce the development of DNA single strand breakage, with consequent activation of PARP. Depletion of the cellular NAD^+ leads to inhibition of cellular ATP-generating pathways. Depletion of NAD results in secondary depletion of NADPH. Because NADPH is a co-factor of NOS, its depletion directly impairs the ability of the endothelial cell to produce NO (and thus, leads in diminished endothelium-dependent relaxant ability of blood vessels). NADPH is also a co-factor fo enzymes involved in the regeneration of cellular glutathione, thereby indirectly protecting cells from oxidative damage. Under conditions of low cellular NADPH, this pathway is compromised, thereby making cells more susceptible to oxidative damage, which exacerbates positive feedback cycles of oxidant generation. Under oxidative stress (conditions of low cellular L-arginine, or oxidant-mediated depletion of tetrahydrobiopterin), NOS may produce superoxide, rather than NO, which then can combine to form peroxynitrite, thereby further exacerbating oxidative stress (top of the scheme, right part). In later stages of oxidative stress (bottom of scheme), the enzymatic activity of NOS is compromised due to NADPH depletion. Not discussed in the current review are the mechanisms whereby oxidative stress (in part, via mechanisms involving PARP activation), can promote the activation of nuclear factor kappa B (NF-κB), AP-1, MAP kinases, and the expression of pro-inflammatory mediators, adhesion molecules (such as intercellular adhesion molecule-1 [ICAM-1]), and of iNOS, thereby activating positive feedback cycles of inflammation and oxidative cell injury (overviewed in 91). In order to simplify the scheme, the pathways leading to the direct oxidative damage (and subsequent depletion) of NADH, NADPH and tetrahydrobiopterin (BH4) are not depicted. PARP activation and mitochondrial oxidant production form another positive feedback cycle, with subsequent release of mitochondrial death effectors such as cytochrome c and AIF (not depicted in the scheme). Please note that all of the above changes occur on a background of a damaged cell (with oxidatively or nitrosatively damaged DNA, lipids and proteins: the latter includes compromised energy-generating mitochondrial enzymes and membrane pumps). Positive feedback circles are marked with a "+" symbol in a circle. The current scheme represents a summary of many pathways, the importance of some of which has only been proven in cell culture systems, and not *in vivo*. The relative importance or participation of the depicted pathways is likely to depend on the cell type involved, the severity of the oxidative stress, the degree cell injury and the mode of cell death. The present scheme depicts interacting, interrelated possibilities: it is likely that all of the pathophysiological pathways depicted in the scheme do not present themselves simultaneously. For example, the pro-inflammatory pathways depicted on the left side of the scheme (characterized by mononuclear cell infiltration, adhesion molecule expression, chemokine and cytokine production) are prominently associated with the endothelial dysfunction associated with shock and certain forms of reperfusion, but much less so with the endothelial dysfunction associated with hypertension. Hyperglycemia (diabetic cardiovascular complications) is a special example, where many additional oxidant-generating pathways (e.g. glucose auto-oxidation, aldose reductase, etc.) are present, and some (but not all) of the pro-inflammatory pathways depicted here are also activated

are subject to intensive research. Because of their low level of cellular expression, and because most of them do not contain DNA-damage recognizing motifs, it is unlikely that activation of the minor PARP isoforms would significantly alter overall cellular NAD^+ levels. In fact, islet cells, fibroblasts and neurons exposed to severe oxidative stress maintain most of their cellular NAD^+ levels after genetic deletion of PARP-1 (15, 26, 72), indicating that PARP-1 is the major NAD^+ utilizing PARP isoform. The protection of the PARP-1 deficient mice against cell and organ injury in various forms of inflammation and reperfusion injury and the similarity of the outcome of experiments in PARP-1 knockout mice and in wild-type mice treated with PARP inhibitors (overviewed in 67, 91) also indicates that PARP-1 is the major, pathophysiologically relevant isoform. Thus, PARP-1 (rather than the minor PARP isoforms) is likely to become the main target for pharmacological intervention and drug development for the experimental therapy of shock, inflammation, reperfusion injury and vascular diseases.

NADPH oxidase

$NADP^+$ is essential for several biosynthetic pathways, energy homeostasis and signal transduction. Its synthesis is catalyzed by NAD^+ kinase, a tetrameric enzyme that is highly selective for its substrates, NAD^+ and ATP (44, 48). Cellular ATP level appears to be a primary determinant in controlling the levels of intracellular pyridine cofactors, including that of $NADP^+$ (60). NADPH levels, similar to NAD^+ levels, decrease rapidly in response to oxidative cell injury, and this drop can be prevented or restored by pharmacological inhibition of PARP (28, 34, 65). These data are consistent with the notion that cellular NAD^+ consumption by PARP can secondarily affect NADPH levels. NADPH is an essential co-factor of nitric oxide synthases (NOS) as well (85). The relevance of this pathway for the development of endothelial dysfunction is discussed in a separate section (see below). It is well known that NADPH acts as a hydride (hydrogen anion) donor in a variety of enzymatic processes. One example is the re-reduction of the oxidized form of glutathione (GSSG) to the reduced form (GSH), catalyzed by glutathione reductase. Because of this reaction, NADPH has been suggested to also act as an indirectly operating antioxidant, thus maintaining the antioxidative capacity of glutathione. Under conditions of cellular NADPH depletion, the recycling of glutathione may diminish, and the antioxidant defenses of the cells may be compromised (Fig. 2).

For the last several decades, the best known NADPH oxidase was that of phagocytes-neutrophils and monocytes. In these cells, this enzyme produces large quantities of superoxide and other reactive oxidants that serve to kill invading microorganisms. Recent studies, however, have suggested that a number of other tissues, including the vascular endothelium, contain NADPH oxidases. The rates of oxidant production by these other cell types are quite low, when compared to

phagocytic cells. Nevertheless, the oxidant production by these additional cell types is certainly sufficient to serve both signaling functions and to underlie a variety of pathophysiological processes (5, 83).

The most important NADPH oxidase for the purpose of the current review is the NADPH oxidase expressed in endothelial cells. This enzyme exhibits baseline activity and generates a low, basal level of intracellular reactive oxygen species (5, 83). Recent work revealed co-localization of the major oxidase subunits, i.e. gp91(phox), p22(phox), p47(phox), and p67(phox), in a mainly perinuclear distribution. In contrast to the neutrophil enzyme, a substantial proportion of the NADPH oxidase in unstimulated endothelial cells exists as a preassembled intracellular complex associated with the cytoskeleton, and a significant amount of the NADPH oxidase is located in the vicinity of the nucleus (46).

The endothelial NADPH oxidase – similarly to the neutrophil enzyme – primarily utilizes NADPH, rather than NADH (45). The activation of the endothelial cell NADPH oxidase is associated with the upregulation of various enzyme subunits and an increase in vascular superoxide formation. These processes, in significant part, occur via protein kinase C activation (52). The NADPH oxidase derived superoxide production has been demonstrated to contribute to a variety of pathophysiological signal transduction and cytotoxic processes (as overviewed in 51, 78, 83, 98). For instance, NADPH oxidase-mediated superoxide generation has been shown to contribute to the induction of DNA single strand breaks, and subsequently to PARP activation, as demonstrated in cultured neurons (30, 39). A simultaneous activation of PARP and NADPH oxidase could result in an accelerated depletion of cellular pyridine nucleotides under pathophysiological conditions.

Pyridine nucleotide metabolism and endothelial dysfunction

In many pathophysiological conditions the vascular endothelium develops a suppressed ability for mediator production (including an attenuated production of the main physiological, endothelium-dependent relaxant factor, nitric oxide [NO]). These conditions include various forms of shock, inflammation, reperfusion injury, as well as diabetes mellitus. It is now clear that the development of endothelial dysfunction (which can be an early trigger, or even the predictor of many cardiovascular diseases), is not necessarily and not usually associated with structural changes of the endothelial cells, but rather, represents a dysfunctional state of the cells, which, under certain conditions, can be reversible. The endothelium is often viewed as a distinct endocrine and homeostatic organ (4, 84), and its dysfunction is sometimes termed “Endothelial Dysfunction Syndrome”. It is conceivable that this syndrome, in the future, will be considered a separate disease entity, which, as most diseases, can develop on basis of a variety of underlying conditions, may involve a variety of pathomechanisms, but nevertheless presents itself as a well characterizable and diagnosable entity (31, 43, 50, 82).

The contribution of the PARP pathway to the development of endothelial dysfunction has been proposed by us in 1997. We have utilized an endotoxic shock model in the rat, which is known to induce severe oxidative and nitrosative stress in the vicinity of the vascular endothelium, due to the upregulation of the inducible nitric oxide synthase (iNOS), as well as the activation of various superoxide-generating sources including NADPH oxidase. In vascular rings taken from rats subjected to endotoxic shock, we observed a loss of the endothelium-dependent relaxations, and these alterations were prevented by pharmacological inhibition of PARP with 3-aminobenzamide (68). In *in vitro* studies, vascular rings exposed to peroxynitrite also exhibited reduced endothelium-dependent relaxations in response to acetylcholine, and the development of this endothelial dysfunction was ameliorated by 3-aminobenzamide (68). These findings were consistent with previous *in vitro* data from our laboratory and other groups demonstrating that PARP inhibition protects against the metabolic suppression and death of oxidatively (3, 36, 40, 42, 79) or nitrosatively (68) injured endothelial cells. These findings were also consistent with studies where endothelial cells were incubated *in vitro* with various pathophysiologically relevant factors that induce oxidative stress, including homocysteine (a model of a variety of cardiovascular diseases) (8) or elevated glucose concentrations (a model of diabetic vascular complications) (65).

Over the last 5 years, the list of pathophysiological conditions where the endothelial dysfunction has been demonstrated to be dependent on PARP activation has increased. The list, in addition to various forms of shock (12, 33, 47, 68) now includes complement-mediated endothelial injury (11), myocardial infarction and various forms of myocardial reperfusion injury and heart transplantation (76, 101), as well as the endothelial dysfunction associated with chronic heart failure (54), aging (56), hypertension (57) and diabetes mellitus (55, 64, 65, 73). The latter subject has been covered in recent reviews (66, 75). Relevant for the purpose of the current review are the data related to the cellular and molecular mechanism of diabetes mellitus induced vascular dysfunction. In a rat model of Type I diabetes, we observed that the diabetes-associated loss of endothelial function is not only preventable, but also rapidly reversible with PARP inhibition (64). Furthermore, the endothelial dysfunction is associated with a simultaneous loss of NAD^+ and NADPH in the vasculature, and PARP inhibition reverses both of these changes. Based on these observations, and the known fact that ecNOS, the NOS isoform present in the vascular endothelial cells – is dependent on NADPH and is sensitively regulated by this co-factor, we hypothesized that the endothelial dysfunction in diabetes is dependent on a PARP-mediated cellular NADPH deficiency (64). In fact, previous *in vitro* work demonstrated that the NADPH depletion in oxidatively stressed cells is dependent on PARP activation (10, 28, 43). It is interesting to note that other groups have demonstrated that diabetic endothelial dysfunction is also associated with direct oxidation and consequent cellular depletion of other co-factors of ecNOS, such as tetrahydrobiopterin (BH4) (16, 23, 58, 93). Since in the absence of tetrahydrobiopterin a functional uncoupling of ecNOS occurs and the enzyme produces superoxide and peroxynitrite, rather than NO (85), the consequences

of these processes are increased free radical and oxidant production, oxidative damage and further exacerbation of the Endothelial Dysfunction Syndrome. It is also worth mentioning here that global cellular NADPH depletion is not the only mechanism whereby ecNOS NADPH availability can be reduced in pathophysiological conditions: Venturini and colleagues have recently reported that amyloid peptide fragments can directly interact with NADPH, thereby reducing NADPH availability for NOS and subsequently reducing NO formation by the enzyme (86).

As briefly mentioned in the preceding section, the upregulation of NADPH oxidases can also significantly contribute to the development of endothelial dysfunction in various pathophysiological conditions (recently overviewed in 5, 78, 83, 94). The list of cardiovascular diseases where endothelial NADPH oxidase activation plays a pathogenic role is long and rapidly expanding and includes atherosclerosis, hypertension (reviewed in 5, 7, 19, 21, 63, 78, 80, 83, 94), diabetes mellitus (23), endothelial dysfunction associated with hyperglycemia (9, 27, 32) or hyperlipidemia (32, 49), aging (25), hyperoxic vascular injury (59) and hypoxia-reoxygenation, and possibly ischemia-reperfusion (1, 102, 103). Important for the pathogenesis of a variety of cardiovascular diseases is that angiotensin II (ANG II) – a hormone that has multiple effects on cardiovascular and renal cells, including vasoconstriction, cell growth, induction of proinflammatory cytokines, and profibrogenic actions – can stimulate the intracellular formation of reactive oxygen species via activation of membrane-bound NADPH-oxidases. Current evidence suggests that ANG II, through AT1-receptor activation, upregulates several subunits of this multienzyme complex, resulting in an increase in intracellular superoxide concentration. ANG II-induced reactive oxidant formation plays a pivotal role in several pathophysiological situations of vascular and renal cells such as hypertension, nitrate tolerance, atherosclerosis, and cellular remodeling (5, 7, 19, 78, 80, 83, 94). It is interesting to note that low dose ANG II infusions (i.e. doses that do not induce changes in blood pressure) lead to a significant degree of tyrosine nitration (a marker of peroxynitrite generation), and associated loss of endothelium-dependent vascular relaxant function (92). We have recently observed that the same low doses of ANG II infusion lead to PARP activation, and the endothelial dysfunction is PARP-dependent: it is preventable and restorable by potent pharmacological inhibitors of PARP (71).

Conclusions and future prospects

Based on the evidence reviewed herein, one can conclude that pyridine nucleotides play very important regulatory roles in the pathogenesis of vascular endothelial dysfunction in pathophysiological conditions associated with oxidative stress. Although the cellular and molecular mechanisms of these alterations require further characterization, it is clear that pathological pyridine nucleotide utilization underlies the pathogenesis of a wide range of cardiovascular diseases. Potential approaches to interfere with these alterations include antioxidant therapy (such as potent, catalytic superoxide dismutase

mimics and peroxynitrite decomposition catalysts), pharmacological inhibitors of PARP, inhibitors of NADPH oxidase and various approaches to neutralize the conversion and cellular action of ANG II. Based on the experimental findings described in the present review, it is clear that all of these approaches hold great therapeutic promise (separately, as well as possibly as combination approaches). Most of these approaches (except certain approaches to neutralize ANG II) are still in various stages of preclinical testing. The comparative therapeutic utility of these approaches, and the applicability of these approaches for the treatment of human diseases must be explored by additional preclinical and subsequent clinical investigations.

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