

Ph. D. Thesis (short form)

Oxidative stress-induced poly(ADP-ribose) polymerase activation
in the skin and in other organs

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Introduction

Poly(ADP-ribose) metabolism

Poly(ADP-ribose) metabolism is based on the synthesis and degradation of (ADP-ribose)_n polymer. Enzymes capable of synthesizing the polymer are called poly(ADP-ribose) polymerases (PARP). During this reaction the enzymes cleave NAD⁺ into ADP-ribose and nicotinamide and polymerize ADP-ribose onto appropriate adapter proteins. The length of the branched (ADP-ribose)_n polymer ranges from a few to about 200 monomers. The polymer confers negative charge to the acceptor proteins and thereby modifies their physicochemical properties and function.

Poly-ADP-ribosylation is a dynamic process as the polymer is rapidly catabolyzed by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase. The half-life of the polymer is estimated to be less than a minute indicating the concerted action of poly(ADP-ribose) synthesizing and catabolyzing enzymes.

Structure and function of poly(ADP-ribose) polymerase 1 (PARP-1)

Poly(ADP-ribose) polymerase-1 (PARP-1) is one of the most abundant nuclear enzyme present in all eukaryotic cells with the exemption of yeast and terminally differentiated granulocytes. The 116 kDa enzyme consists of three main domains: The N-terminal DNA binding domain contains two Zn-finger motives whereas the catalytic domain is located on the C terminus. The automodification site can be found between these two domains. Intermolecular auto-ADP-ribosylation downregulates the enzyme's activity. PARP's structure is highly conserved among eukaryotes with human and murine PARP showing 92% homology at the amino acid level. The catalytic domain is the most conserved and contains a 50 amino acid PARP signature sequence displaying 100% homology among vertebrates.

PARP-1 becomes activated in response to single and double stranded DNA breaks. Upon binding to the site of DNA damage mainly through the second zinc finger, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ and synthesis of the polymer. In addition to PARP-1, the most important acceptors for poly(ADP-ribose) are histones which gain negative charge from the polymer leading to electrostatic repulsion between DNA and histones and as a consequence, to loosening of chromatin structure. Several other nuclear proteins such as transcription factors, replication factors as well as repair proteins (effectors and adapters) may serve as acceptors for poly(ADP-ribose).

The biological role of poly-ADP-ribosylation with special regard to cell death

The first identified role of poly-ADP-ribosylation was its contribution to DNA repair. Intensive research of the last decade revealed that poly-ADP-ribosylation is a pleiotropic regulatory mechanism involved in the regulation of chromatin structure, replication, transcription, proliferation and cell death. For our current work, PARP's role in cell death and transcription was of special importance.

Berger's group has implicated PARP activation in DNA damage induced cytotoxicity. Berger's suicide theory proposed that overactivation of PARP consumes cellular NAD^+ and ATP leading to energetic catastrophe and cell demise. In latest years it has become a highly debated issue whether the PARP-mediated suicide occurs in the form of apoptosis or oncosis. Depending on the nature, intensity of the cytotoxic stimulus, sensitivity of the cells, cell death may occur in two main forms: apoptosis or oncosis. Apoptotic cells are characterized by compact morphology, chromatin condensation, preservation of plasma membrane integrity and rapid elimination of the apoptotic cells by macrophages. As PARP-1 was among the first identified substrates of caspases (cysteine/aspartate-specific proteases), the main executors of apoptosis, PARP's role in apoptotic death was considered first. The role of PARP cleavage is likely to prevent activation of the enzyme by oligonucleosomal DNA fragmentation, a late

event of apoptosis. Thus PARP can not compromise cellular energetics and the energy-dependent steps of the apoptotic process can be carried out. Whereas the role of PARP-1 in apoptosis is quite controversial, the PARP-mediated suicidal death is becoming more and more accepted to occur in the form of oncosis. The most striking difference between these two forms of cell death is that in oncosis, plasma membrane integrity is compromised and oncotic cells swell as opposed to the compact morphology of apoptotic cells. Leakage of cell content of oncotic cells may trigger inflammation whilst apoptotic cells are safely cleared from the tissues. The beneficial effect of PARP inhibitors as observed in various models of inflammation and reperfusion injury can be explained, at least in part, by prevention of oncosis. The other important mechanism is based on the transcriptional regulatory role of PARP-1.

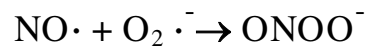
The role of PARP-1 in the regulation of transcription

Several lines of evidence points toward a possible role of poly-ADP ribosylation in the regulation of transcription. By conferring negative charge to histones, poly-ADP-ribosylation loosens up chromatin structure and thus makes genes more accessible to the transcriptional apparatus. Furthermore, PARP-1 has been shown to participate in the formation of preinitiation complex and to serve as co-activator for various transcription factors. Of special importance is NF- κ B, a key transcription factor involved in the expression of inflammatory mediators (e.g. iNOS), cytokines and adhesion molecules.

Peroxynitrite-induced poly-ADP-ribosylation in pathophysiological conditions

In the last twenty years, free radical biology and medicine revealed the important role of reactive oxygen- and nitrogen centered radicals/intermediates (ROI és RNI) in numerous conditions affecting major populations. As peroxynitrite has been in the focus of our investigation, here I briefly summarize the formation and biological effects of this reactive oxidant species.

Peroxynitrite ONOO^- is produced in the reaction of nitric oxide ($\text{NO}\cdot$) and superoxide ($\text{O}_2\cdot^-$). Small amount of superoxide is continuously leaking from the mitochondrial electron transport chain and is also produced by NADPH oxidase and xanthine oxidase. The only known molecule capable of competing for superoxide with superoxide dismutase is $\text{NO}\cdot$. Therefore Joseph Beckman proposed that in conditions where $\text{NO}\cdot$ production is associated with tissue injury, $\text{NO}\cdot$ combines with superoxide to form peroxynitrite:



The rate of the reaction approaches the diffusion limit ($6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) indicating that almost all collision between $\text{NO}\cdot$ and superoxide results in peroxynitrite formation. Although peroxynitrite is not a free radical (it has no unpaired electrons), it is more reactive than its parent molecules. Peroxynitrite reacts with thiols, triggers lipid peroxidation, DNA breakage, activates or inhibits signal transduction pathways and inactivates proteins via oxidation or tyrosine/tryptophane nitration.

Peroxynitrite and PARP activation in the pathology of the skin and other organs

Nitrotyrosin (the „footprint of peroxynitrite”) has been detected by Western blot or ELISA in skin homogenates derived from various diseases (burns, chronic UVB radiation, murine leishmaniasis, systemic sclerosis). Intracutaneous activation of PARP has only been proposed in sunburn erythema and sulfur mustard-induced vesication.

PARP activation occurs in ischaemia-reperfusion injury (myocardial infarction, stroke), inflammation, diabetes, trauma and shock. Although the PARP activating stimulus is difficult to identify in these conditions, in vitro data point toward a possible role of ROI/RNI-induced DNA breakage. Pharmacological inhibition of poly-ADP-ribosylation markedly improved the

most important clinical and laboratory parameters such as infarct size, survival, organ function, cell death, migration of inflammatory cells, cytokine production. In line with pharmacological results, PARP-1 knock out mice show remarkable protection in models of ischaemia-reperfusion and inflammation.

Specific aims

We set out to address the following questions centered around two themes:

Peroxynitrite and PARP activation in the skin

1. Is peroxynitrite produced in the skin during CHS?
2. Is DNA breakage and PARP activation detectable in the skin during CHS?
3. What is the mechanism of peroxynitrite-induced cytotoxicity in HaCaT keratinocytes?
4. Does PARP become activated in peroxynitrite-treated HaCaT cells?
5. Is PARP involved in the regulation of cytokine-induced IL-8 production and ICAM-1 expression in HaCaT cells?

Peroxynitrite and PARP activation in pathophysiological conditions characterized by oxidative stress

6. Is peroxynitrite produced in hemorrhagic shock (HS)?
7. Is PARP activation detectable in HS and what may be its role?
8. Is the peroxynitrite→ DNA breakage→ PARP activation pathway detectable in diabetic vasculopathy?
9. Does PARP inhibition affect extravasation of inflammatory cells in experimental uveitis?

Methods

Contact hypersensitivity model (CHS)

Mice were sensitized by applying 100 µl 2% oxazolone onto the belly (CHS group). Animals in the control group were treated the same way but with 100 µl solvent (acetone : olive oil = 4:1). After seven days, ears were treated with

20 µl 0,5%-os oxazolone. Skin samples were taken after 24 hours and were stored either in formalin or in cryoembedding medium.

Experimental uveitis

Ten Lewis rats were treated with lipopolysaccharide (serotype 0127:B8 from E.coli, 150 µg/rat, subcutaneously into the rear right paw). Control animals received the same amount of physiological saline. Degree of inflammation was assessed after 18 hours by means of counting CD11⁺ cells in the iris.

Histology

In vivo peroxynitrite production, PARP activation and inflammatory cell migration have been detected by immunohistochemical stainings for nitrotyrosine, poly(ADP-ribose) and CD11, respectively. PARP activity was in some cases detected by enzyme histochemistry using biotinylated NAD⁺ as PARP substrate and streptavidin-peroxidase for detection. DNA breakage was detected by a commercially available TUNEL (TdT-mediated dUTP nick end labelling) kit.

Measurement of cell proliferation, cytotoxicity and PARP activity

Proliferation of HaCaT cells was determined with a commercially available ELISA (Amersham, Pharmacia Biotechnology, Piscataway, NJ) based on the measurement of BrDU incorporation. Cytotoxicity was quantified in a colorimetric MTT assay. PARP activity was determined with a standard ³H-NAD⁺ incorporation assay.

Caspase activity and DNA fragmentation

A fluorogenic tetrapeptide-amino-4-methylcumarin conjugate (DEVD-AMC) has been used for the measurement of caspase activity which is proportional to the liberation of AMC by the cell lysates. DNA fragmentation was quantified in a commercially available ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) measuring cytoplasmic histone-associated DNA fragments.

ICAM-1 expression and interleukin-8 (IL-8) production

HaCaT cells were stimulated for 24h with 30 ng/ml (300 U/ml) recombinant human interferon-gamma (R&D Systems, Minneapolis, MN). Cells were then stained with FITC conjugated monoclonal anti-human ICAM-1 antibody (Pharmingen, San Diego, CA), and then fixed in 1%-os paraformaldehyde. Cell fluorescence was measured with a FACSCalibur flow cytometer (Becton Dickinson, San Diego, Ca). For IL-8 production, cells were stimulated for 24h with 20 ng/ml human recombinant IL-1 β (R&D Systems, Minneapolis, MN). The IL-8 secreted into the media was quantified in a sandwich ELISA system (R&D Systems, Minneapolis, MN).

Results and Discussion

Sulfur mustard-induced tyrosine nitration and PARP activation in the skin

After applying sulfur mustard on the mouse skin, we have looked for signs of peroxynitrite formation (anti-nitrotyrosine immunohistochemistry) and PARP activation [anti-poly(ADP-ribose) immunohistochemistry]. Nitrotyrosine could be detected in all layers of the epidermis, whereas PARP activation was only detectable in the basal layer of the epidermis. We propose that intracutaneous peroxynitrite may be responsible for PARP activation in this model.

Contact hypersensitivity

As peroxynitrite can nitrate tyrosyl residues of proteins, detection of nitrotyrosine in tissues indicates in vivo peroxynitrite production. We could detect nitrotyrosine in CHS but not in the skin of non-sensitized animals. Tyrosine nitration was most intense in keratinocytes surrounding inflammatory foci. As peroxynitrite is known to trigger PARP activation via DNA breakage, we have investigated the activity of this pathway in CHS. Whilst control skin contained only few scattered TUNEL positive cells indicative of DNA damage, skin obtained from CHS animals was densely packed with TUNEL positive cells

(keratinocytes and inflammatory cells). PARP activity, as detected by PARP enzyme histochemistry also showed strong positivity in keratinocytes and in some dermal cells in CHS but not in control skin.

Antiproliferative and cytotoxic effects of peroxynitrite in HaCaT keratinocytes: the role of PARP activation

Peroxynitrite caused a concentration dependent inhibition in proliferation and was cytotoxic at high concentrations. The antiproliferative effects of peroxynitrite is unlikely to be related to PARP activation as it was not inhibitable by 3-aminobenzamide (3-AB). Nonetheless, the strong antiproliferative effect of peroxynitrite as observed at high concentrations is at least in part due to PARP activation indicated by the significant inhibitory effect of 3-AB. The cytotoxic effect of peroxynitrite could be significantly reduced by 3-AB. Cytoprotection provided by 3-AB was not accompanied by changes in apoptotic parameters (caspase activation, DNA fragmentation) indicating that PARP-mediated cytotoxicity is not apoptotic in HaCaT cells.

The role of PARP in keratinocyte activation

Human recombinant interferon- γ (30 ng/ml) induced a ninefold elevation in cell surface ICAM-1 expression. The PARP inhibitor 3-AB suppressed interferon- γ induced ICAM-1 expression in a concentration-dependent manner. Furthermore, HaCaT cells are known to produce IL-8 in serum-containing media. We found that both spontaneous and az IL-1 β -stimulated IL-8 production of HaCaT was inhibited by 3-AB indicating a possible role of PARP in the regulation of IL-8 expression.

The peroxynitrite $\rightarrow$ DNA damage $\rightarrow$ PARP activation pathway in the skin

Detection of nitrotyrosine in the two models of skin inflammation indicates in vivo peroxynitrite formation. Keratinocytes showed the most intense nitrotyrosine staining. This finding identifies keratinocytes as prime targets of peroxynitrite attack in the skin. Tyrosine nitration is likely to result from cytokine-induced iNOS expression and overproduction of superoxide.

Superoxide combines with iNOS-derived NO to form peroxynitrite that nitrates tyrosine. Oxidative/nitrosative stress is also indicated by the high ratio of cells showing signs of DNA damage. In light of the extensive DNA damage, it is not surprising that PARP activation could also be detected by enzyme histochemical method. The activation of the enzyme was strongest in the basal layer of epidermis, whereas superficial, differentiated keratinocytes showed no activity. We propose that basal proliferative keratinocytes may either have a more active metabolic rate, a compromised antioxidant status or alteration in calcium homeostasis which renders them more susceptible to oxidative stress.

Based on our current data with HaCaT cells and on preliminary pharmacological experiments, PARP inhibition is likely to beneficially influence CHS or sulfur mustard-induced vesication. Such effect could be due to improved viability or suppressed activation of keratinocytes.

Haemorrhagic shock (HS)-induced tyrosine nitration and PARP activation in the gut

Oxidative stress plays a major role in the rather complex pathomechanism of haemorrhagic shock. The effectiveness of antioxidant treatment strategies also supports the role of ROI in haemorrhagic shock. A role for nitric oxide and NO-derived RNI in HS is also supported by experimental data. Of special importance may be the inducible NO synthase (iNOS) as indicated by the potent anti-shock effects of iNOS specific inhibitors such as L-NIL or MEG. Based on the overproduction of both ROI and RNI species it was plausible to propose that peroxynitrite may also be formed and PARP may become activated in HS.

We have prepared paraffin-embedded sections from the small intestines of wild type (PARP^{+/+}) and PARP knock out (PARP^{-/-}) mice that underwent haemorrhagic shock (HS) or sham treatment. In the HS group we were able to detect poly(ADP-ribose) (PAR) by immunohistochemistry. The most intense PAR staining was seen at the tip of the villi where staining localized to the nuclei of the epithelial cells as well as a diffuse stromal staining could be

observed. In the samples of PARP^{-/-} mice no PAR could be detected neither in the HS nor in the control group. Moreover, we have observed massive tyrosine nitration in the gut of PARP^{+/+} mice that underwent HS. The strongest staining was found at the tip of the villi. Some nitrotyrosine staining was also seen in the PARP^{-/-} group but the morphology of the gut architecture was not significantly damaged. Results of immunohistochemical stainings were in line with the protection of PARP^{-/-} mice against HS-induced mortality and gut hyperpermeability. (results obtained by Dr. Lucas Liaudet). In summary, these data indicate that PARP activation does play a role in the pathomechanism of HS.

The role of PARP activation in diabetic endothelial dysfunction

Hyperglycaemia has long been known to play a key role in diabetic vasculopathy. Moreover, this has been linked to endothelial dysfunction caused by hyperglycaemia. Hyperglycaemia triggers three virtually unrelated pathways culminating in deterioration of endothelial function: a) formation of AGEs (advanced glycation endproducts), b) glucose-induced protein kinase C activation and c) aldose reductase pathway. Brownlee's group identified ROI as the „smallest common denominator” in hyperglycaemia-related endotheliopathy. They have proved that hyperglycaemia stimulates ROI production in endothelial cells and blocking ROI production (e.g. by inhibiting mitochondrial electron transfer or oxidative phosphorylation or by overexpression of superoxide dismutase) prevented activation of all three pathological pathways.

Our group has established PARP activation as the „fourth” ROI-stimulated pathway contributing to endothelial dysfunction. In aortas obtained from diabetic mice we have observed intense nitrotyrosine staining indicating peroxynitrite production in these dysfunctional blood vessels. Nitrotyrosine staining could also be observed in diabetic mice treated with the PARP inhibitor PJ-34. Furthermore, TUNEL staining revealed a high number of cells with damaged DNA both in aortas of diabetic and of PJ-34-treated diabetic animals.

Moreover, PARP activation was visualized in the aortas of diabetic mice by means of enzyme histochemistry. PARP inhibitor treatment abolished diabetes-induced PARP activation in the vessels.

Data obtained by my colleague, Dr. Francisco Garcia Soriano prove that hyperglycaemia causes endothelial dysfunction in part by increased poly-ADP-ribosylation. (PJ-34 markedly improved vascular relaxation). The most likely scenario taking place in this model is that hyperglycaemia stimulates iNOS expression in the vessel wall leading to overproduction of NO. In parallel, mitochondrial overproduction of superoxide provides ideal conditions for peroxynitrite formation. Peroxynitrite activates the DNA breakage-PARP activation-NAD⁺/ATP depletion pathway leading to endothelial dysfunction.

Experimental uveitis

Experimental autoimmune uveitis is triggered by immunisation with endogenous ocular proteins. A similar inflammation can be induced by intraplantar injection of bacterial lipopolysaccharide (LPS). Using this latter approach we have observed a marked increase in the number of CD11⁺ inflammatory cells in the iris of Lewis rats. Inflammatory cell migration could be significantly reduced by both the iNOS inhibitor MEG and the PARP inhibitor PJ-34. These results support the idea that the NO → ONOO⁻ → PARP activation pathway is active in experimental uveitis. It is known from the literature that iNOS expression and tyrosine nitration occurs in experimental uveitis. Our data indicate that PARP becomes activated and that activation of the enzyme is most prominent in the projection of the vasculature (either in the endothelium or in circulating blood cells).

Conclusion

1. Tyrosine nitration, DNA breakage and PARP activation occurs in the keratinocytes during CHS.
2. Peroxynitrite induces PARP activation in the skin.
3. Peroxynitrite induces apoptosis in HaCaT keratinocytes, as characterized by caspase activation and DNA fragmentation.
4. Higher concentrations of peroxynitrite induces oncosis in HaCaT keratinocytes.
5. Peroxynitrite activates PARP in HaCaT cells.
6. PARP regulates cytokine-induced IL-8 production and ICAM-1 expression.
7. Tyrosine nitration and PARP activation occurs in the intestinal epithelial and stromal cells during HS and likely contributes to increased gut permeability in HS.
8. Tyrosine nitration, DNA breakage and PARP activation occurs in the blood vessels of diabetic mice.
9. Inhibition of poly-ADP-ribosylation by PJ-34 suppresses migration of CD11⁺ cells during experimental uveitis.
10. Peroxynitrite-induced poly-ADP-ribosylation may represent a lucrative target for therapeutic intervention in inflammatory conditions and ischemia-reperfusion injury.

Publications

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