Ph. D. Thesis

Alterations in poly(ADP-ribose) metabolism and calcium homeostasis in oxidatively stressed HaCaT and mouse macrophage cell lines

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Introduction

Basic characteristics and enzymes of poly(ADP-ribose) metabolism

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

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

Poly(ADP-ribose) polymerase-1 (PARP-1) is one of the most abundant nuclear enzymes present in all eukaryotic cells with the exemption of yeast and terminally differentiated granulocytes. The 116 kDa enzyme consists of three 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 role of poly-ADP-ribosylation in cell death

Poly-ADP-ribosylation is a pleiotropic regulatory mechanism involved in the regulation of chromatin structure, replication, transcription, proliferation and cell death. Berger's group has implicated PARP activation in DNA damage-induced cytotoxicity. The 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 and intensity of the cytotoxic stimulus, sensitivity of the cells, cell death may occur in two main forms: apoptosis or oncosis. 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.

So far most studies aiming at revealing the role of poly(ADP-ribose) metabolism focused on the biological role of polymer synthesizing enzymes (mainly PARP-1). However, several lines of evidence points toward a key regulatory role of PARG in poly(ADP-ribose) metabolism. Swanson's group has found that PARG inhibition by gallotannin and nobotanin B protect from N-methyl-D-aspartate- or hydrogen peroxide-induced cell death. The cytoprotective effect of PARG inhibitory compounds may presumably be due to

inhibition of removal of poly(ADP-ribose) chains and to increased auto-poly-ADP-ribosylation which results in inhibition of PARP.

Poly-ADP-ribosylation and oxidative stress

Oxidative stress, the overproduction of reactive oxygen and/or nitrogen containing intermediates (ROI and RNI) accompanies various diseases. The most reactive ROI, hydroxyl radical causes DNA damage triggering a rapid activation of the DNA nick sensor enzyme PARP-1. In our experiments we have investigated the effects of another genotoxic agent: peroxynitrite which is produced in the reaction of nitric oxide (**NO·**) and superoxide (**$O_2{\cdot}^-$**). Small amount of superoxide is continously 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 **NO·**. Peroxynitrite reacts with thiols, triggers lipid peroxidation, activates or inhibits signal transduction pathways and inactivates proteins via oxidation or tyrosine/tryptophane nitration. It causes DNA breakage triggering a rapid activation of PARP.

Specific aims

Our experiments were set out to answer the following questions:

I. Development of a non-radioactive method for the detection of PARP activity using biotinylated NAD^+

1. Is the bio-NAD^+ method suitable for the detection of PARP activity in cells and tissues?

II. The role of poly(ADP-ribose) metabolism in oxidative stress-induced cytotoxicity

3. Does peroxinitrite induce elevation of intracellular calcium concentration in HaCaT cells?

4. Does PARP become activated in peroxynitrite-treated HaCaT cells?

5. Does calcium signal promote oxidative stress-induced cell death in HaCaT cells?

6. Do cell density dependent signals contribute to the regulation of sensitivity to oxidative stress in HaCaT cells?

7. Does the antioxidant and PARG inhibitory compound gallotannin modulate oxidative stress induced cytotoxicity in HaCaT cells?

Methods

Detection of PARP activation in cells and tissues

PARP activity was detected 1) by enzyme cytochemistry and enzyme histochemistry, using biotinylated NAD^+ substrate. Enzyme reaction was detected either by streptavidin-AlexaFluor-488 (Molecular Probes, Eugene, OR) or by streptavidin-conjugated peroxidase. 2) by immunodetermination of poly(ADP-ribose), the end product of the PARP-catalyzed reaction with monoclonal anti-poly(ADP-ribose) antibody. Immunreactivity was detected by biotinylated horse anti-mouse IgG and the bound antibody was visualized by using the ABC streptavidin detection system (Vector Laboratories, Burlingham, CA, USA) and 3,3'-diaminobenzidene substrate.

Measurement of cytotoxicity, cellular PARP activity and caspase activity

Cytotoxicity was determined 1) by the colorimetric MTT assay, 2) by lactate dehydrogenase (LDH) release using the commercially available LDH activity assay kit (Roche, Basel, Switzerland), and 3) by fluorescent microscopy after propidium iodide staining. Quantification of cellular PARP activity was carried out by cellular ELISA method using biotinylated NAD^+ substrate. Caspase activity was measured with the "Caspase-3 Intracellular Activity Assay kit" purchased from Calbiochem (San Diego, CA) following manufacturer`s instructions. The assay is based on the use of the fluorogenic peptide substrate PhiPhiLux^{TM}.

Measurement of cellular NAD^+ content

Following peroxinitrite or hydrogen-peroxide treatment, cells were extracted and centrifuged. Supernatants were mixed with a reaction medium containing 0.1 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 0.9 mM phenazine methosulfate, 13 units/ml alcohol dehydrogenase, 100 mM nicotinamide, and 5.7% ethanol in 61 mM Gly-Gly buffer (pH 7.4). The $A_{560\ nm}$ was determined immediately and after 10 min.

Statistical analysis

Data of cytotoxicity, PARP activity, caspase activity are reported as mean ± SD of quadruplicate samples. Experiments were repeated at least n=3-4 times. Student's t-test was used to compare mean values. Statistical differences were declared significant for $p<0.05$. Results of immunocytological experiments represent typical results obtained in at least 3 independent experiments.

Results and discussion

Detection of PARP activation using biotinylated NAD^+ substrate

For a long time the only assay in which PARP inhibitors could be tested was a radioactive one using either ^{32}P or ^{3}H-labeled NAD^+. We have developed three applications such as enzyme cytochemistry, enzyme histochemistry and cellular ELISA to detect the activation of poly(ADP-ribose) polymerase in oxidatively stressed cells and tissues. The methods utilize a novel commercially available substrate 6-biotin-17-nicotinamide-adenine-dinucleotide (bio-NAD^+) from which PARP incorporates biotinylated (ADP-ribose) into nuclear acceptor proteins. To prove the identity of the product synthesized by the cells from bio-NAD^+, we used wild-type ($PARP^{+/+}$) and PARP-deficient ($PARP^{-/-}$) macrophages. Exposure of cells to 200 μM hydrogen peroxide induced bio-NAD^+ incorporation into the nuclei of wild-type but not of PARP-deficient macrophages. Bio-NAD^+ incorporation could be inhibited with PJ-34 or 3-aminobenzamide, inhibitors of PARP. These results indicate that PARP-1 is responsible for bio-NAD^+ incorporation into hydrogen peroxide-treated macrophages.

The ability of the bio-NAD^+ method to detect PARP activation in tissues was demonstrated by enzyme histochemistry. Frozen sections were cut from untreated and hydrogen peroxide-treated mouse skin. In control (vehicle-treated) skin, no detectable ADP ribosylation was found. Hydrogen peroxide treatment activated PARP in the skin, as indicated by the appearance of darkly stained

cells. Staining was nuclear and was most intense in keratinocytes. The presence of the PARP inhibitor 3-aminobenzamide (5 mM) abolished peroxynitrite-induced bio-ADP-ribose incorporation, demonstrating that PARP activation was responsible for the staining.

The cellular ELISA method allows the quantification of PARP activity. J774 macrophages seeded in 96-well plates were exposed to hydrogen peroxide in the presence or absence of PJ-34. Hydrogen peroxide induced a dose-dependent PARP activation in J774 cells, and pretreatment with the PARP inhibitor PJ-34 suppressed hydrogen peroxide-induced PARP activation. As a reference method, [^{3}H]-NAD incorporation was also used to measure PARP activity and the two assays showed good correlation (r^2 = 0.92) with the bio-NAD^+ method giving higher induction results.

Effect of gallotannin on oxidative stress–induced cytotoxicity in HaCaT keratinocytes

Treatment of HaCaT keratinocytes with hydrogen peroxide or peroxynitrite caused a concentration- dependent cytotoxicity as detected with the lactate dehydrogenase (LDH) release assay or the propidium iodide uptake assay. Both assays detect the breakdown of plasma membrane integrity resulting in leakage of the cell content (e.g. LDH) or uptake of cell-impermeable dyes such as propidium iodide. Degree of lactate-dehydrogenase release detected after exposure of cells to oxidative stress could be significantly diminished by gallotannin pretreatment, in case of both hydrogen peroxide- and peroxynitrite-induced oxidative stress. Similary, propidium iodide-uptake of hydrogen peroxide- and peroxynitrite-treated cells could be abolished when cells were pretreated with gallotannin

Effect of gallotannin on cellular poly(ADP-ribose) content

In our previous work we have investigated the mechanism of oxidative stress-induced cytotoxicity in HaCaT keratinocytes. We found that hydrogen peroxide and peroxynitrite activated poly(ADP-ribose) polymerase. After the

demonstration of cytoprotective effect of gallotannin in oxidative stress induced cytotoxicity we set out to investigate the effect of this compound on cellular poly(ADP-ribose) metabolism. Treatment of cells with both hydrogen peroxide and peroxynitrite resulted in increased polymer content most likely due to activation of poly(ADP-ribose) polymerase. Poly(ADP-ribose) accumulation was blocked in cells pretreated with the PARP inhibitor 3-aminobenzamide. In the contrary, gallotannin treatment did not prevent (or slightly enhanced) poly(ADP-ribose) accumulation, as indicated by the more intense poly(ADP-ribose) staining in gallotannin+oxidant treated cells as compared to control (untreated cells) or to oxidant treated cells. Moreover, HaCaT cells are not likely to have a high basal poly(ADP-ribose) synthetic activity as the polymer did not accumulate in gallotannin treated cells when oxidants were not applied.

Effect of gallotannin on the NAD^+ content

Changes in cellular NAD^+ content also can be considered as another characteristic factor of poly(ADP-ribose) metabolism. Hydrogen peroxide or peroxynitrite depleted cellular NAD^+ content. Pretreatment of cells with gallotannin provided significant protection against NAD^+ loss in oxidatively stressed cells. These findings support the presumption that PARG inhibition by gallotannin indirectly breaks the cycle of accelerated poly(ADP-ribose) metabolism by leaving PARP in the hyper-ADP-ribosylated inactive form.

Effect of gallotannin on oxidative-stress induced PARP-activity

A significant increase in PARP activity can be observed in both hydrogen peroxide- and peroxynitrite-treated cells. Oxidative stress-induced PARP activity was fully inhibited by 3-aminobenzamide or gallotannin pretreatment. The question arises, however, what is the mechanism underlying the cytoprotective effect of gallotannin in oxidatively injured cells. One possible mechanism is the inhibition of poly(ADP-ribose) turnover by gallotannin. It has been known for years that overactivation of poly(ADP-ribose) polymerase-1 depletes NAD^+, the substrate of PARP and leads to ATP depletion. This cellular

energetic failure also referred to as the "cellular suicide pathway" results in necrotic type cell death. The primary acceptor of poly(ADP-ribose) is PARP-1 itself and auto-poly-ADP-ribosylation downregulates the enzyme activity. PARG is likely to "reactivate" PARP-1 by removing the polymer from its automodification domain. This hypothesis is also supported by our finding that inhibition of PARG by gallotannin results in polymer accumulation and cellular PARP inhibition. Cellular PARP activity was almost fully blocked by 50 μM gallotannin. Additional factors which need to be considered are the the known antioxidant and direct PARP inhibitory effects of gallotannin. Here we have shown that gallotannin has a direct inhibitory effect on PARP-1. At cytoprotective concentrations (20-50 μM) gallotannin caused a 50% inhibition of PARP activity. Whether this effect is a real pharmacological inhibition or is related to the known protein precipitating effect of gallotannin (a defining characteristic of tannins), is not known. Nonetheless, we propose that the antioxidant and PARP inhibitory effects can not be made fully responsible for the cytoprotective effects of gallotannin. Our data showing poly(ADP-ribose) accumulation in gallotannin+peroxynitrite/hydrogen peroxide treated cells indicate that a substantial amount of radicals were not scavenged by gallotannin and these radicals must have reached the cells to cause PARP activation. Accumulation of the polymer also indicates that initially PARP could be activated in the presence of gallotannin.

Effect of peroxinitrite on intracellular Ca^{2+} levels in HaCaT cells

First we tested if the application of peroxinitrite would alter $[Ca^{2+}]_i$, and tried to assess the source of calcium ions entering the cytoplasm. Based on the results obtained by Dr. Mónika Gönczi and Dr. László Csernoch (Dept. Physiol. Univ. Debrecen) it can be stated that peroxynitrite caused a dramatic increase in $[Ca^{2+}]_i$. Although the source of this intracellular calcium "signal" was mainly

the extracellular compartment, the release of calcium from intracellular sources was also significant.

Buffering of intracellular calcium by BAPTA-AM protects HaCaT cells from peroxynitrite-induced cytotoxicity

Our previous work has shown that peroxynitrite is cytotoxic to HaCaT cells. In order to investigate whether or not peroxynitrite-induced calcium mobilization contributes to the cytotoxic effects of the oxidant, HaCaT cells were pre-treated with the cell-permeable calcium chelator BAPTA-AM (2,5 μM) for 30 min and then exposed to peroxynitrite. BAPTA-AM provided significant protection from peroxynitrite-induced cytotoxicity, supporting the importance of intracellular calcium levels during this process. In order to establish whether this phenomenon is peroxynitrite-specific or is a general feature of oxidative stress, we used hydrogen peroxide and superoxide as triggers of oxidative stress in HaCaT cells. While BAPTA-AM exerted significant cytoprotective effects in hydrogen peroxide treated cells, it provided no significant protection against superoxide. The observation that calcium chelation did not protect HaCaT cells from the cytotoxic effect of superoxide indicates that distinct cytotoxic pathways are triggered by different reactive species.

Role of cell density-dependent signals in the regulation of peroxynitrite sensitivity of HaCaT cells

Upon reaching confluence, HaCaT cells undergo differentiation; a process similar to that occuring in the skin during passage of keratinocytes from the basal toward superficial layers of the epidermis. We studied the effect of this cell contact-induced differentiation process on the peroxynitrite sensitivity of HaCaT cells. Our data show that subconfluent cultures (20-80%) of HaCaT cells displayed equal sensitivity to oxidative stress. In contrary, confluent cultures

showed significant resistance toward both peroxynitrite, hydrogen peroxide and superoxide. We hypothesize that different metabolic (e.g. mitochondrial) activity may be responsible for the differences in oxidative stress sensitivity of subconfluent (proliferating) and confluent (differentiating) keratinocytes.

BAPTA-AM and high density signaling inhibits oxidant-induced poly(ADP-ribose) polymerase activation in HaCaT cells

Our previous work has identified poly(ADP-ribose) polymerase (PARP) activation as a mechanism responsible for peroxynitrite-induced HaCaT cell death. There have been multiple pathways identified in the peroxynitrite-induced cytotoxicity in HaCaT cells. At lower concentrations of the oxidant, caspase-mediated apoptotic cell death predominates. However, at more intense oxidative stress, the apoptotic machinery is disabled and HaCaT cells die by a PARP-mediated necrotic mechanism. The "apoptosis to necrosis switch role" of PARP likely involves depletion of intracellular stores of NAD^+ and ATP and thus interfering with energy-dependent steps of the apoptotic machinery. We set out to investigate whether protective effects of intracellular calcium buffering and high cell density are related to PARP activation. We have found that chelation of intracellular calcium by BAPTA-AM significantly inhibited peroxynitrite-induced PARP activation. As the enzyme does not require calcium for activity, calcium signal is likely to be a proximal step of a signal transduction pathway leading to PARP activation. Similarly, high-density HaCaT cultures were resistant to peroxynitrite-induced PARP activation, providing further evidence supporting the importance of calcium signaling and cell density in the regulation of processes induced by oxidative stress.

Effect of BAPTA-AM and cell density signaling on peroxynitrite-induced caspase activation in HaCaT cells

Peroxynitrite-induced cytotoxicity is characterized by both apoptotic and necrotic features. We and others have previously shown that in various cell lines the switch from the default apoptotic toward necrotic death is mediated in part by PARP activation. We have investigated the effect of calcium mobilization and cell density on peroxynitrite-induced caspase activation, a characteristic feature of apoptosis. Peroxynitrite induced caspase-3 like activity in HaCaT cells could be significantly inhibited by pretreatment of the cells with BAPTA-AM. Similarly, in confluent cultures, caspase activation was less intense compared to activities measured in subconfluent cells. As caspase activity does not directly depend on calcium, elevated intracellular calcium levels may be involved in signaling peroxynitrite-induced apoptosis at a step proximal to caspase activation.

Conclusions

1. The bio-NAD^+ assays represent sensitive, specific, and non-radioactive alternatives for the detection of oxidative stress-induced PARP activation in cells and tissues.
2. Gallotannin provides protection from peroxynitrite- and hydrogen peroxide-induced necrotic cell death.
3. Inhibition of PARG by gallotannin results in poly(ADP-ribose) accumulation and PARP inhibition.
4. PARG inhibition may represent a viable strategy in many of the disease models where PARP inhibition previously proved useful.
5. Application of peroxynitrite resulted in a dramatic increase in $[Ca^{2+}]_i$ in HaCaT cells.
6. Increase in $[Ca^{2+}]_i$ plays a crucial role in peroxynitrite-induced cytotoxicity.
7. Cell density-dependent signals are also of great importance concerning the regulation of peroxynitrite sensitivity of HaCaT cells

Publications

The Ph.D. thesis is based on the following publications

1. **Bakondi E**, Bai P, Szabó É, Hunyadi J, Gergely P, Szabó C, Virág L: Detection of poly(ADP-ribose) polymerase activation in oxidatively stressed cells and tissues by using biotinylated NAD substrate. *J. Histochem. Cytochem.* 50(1):91-98, 2002

IF.: 2.718

2. **Bakondi E**, Gönczi M, Szabó É, Bai P, Gergely P, Kovács L, Hunyadi J, Szabó C, Csernoch L, Virág L: Intracellular calcium and cell density-dependent signalling regulate oxidative stress sensitivity of HaCaT keratinocytes. *J. Invest. Dermatol. (in press)*

IF.: 4.645

3. **Bakondi E,** Bai P, Erdélyi K, Szabó É, Gergely P, Hunyadi J, Szabó C, Virág L: Cytoprotective effect of gallotannin in oxidatively stressed HaCaT keratinocytes: the role of poly(ADP-ribose) metabolism. *Experimental Dermatology (in press)*

IF.: 2.234

Other publications

4. Virág L, Szabó É, **Bakondi E**, Bai P, Gergely P, Hunyadi J, Szabó C: The nitric oxide – peroxynitrite - poly(ADP-ribose) polymerase pathway in the skin. *Experimental Dermatology* 11(3):189-202, 2002

IF.: 2.234

5. Bai P, **Bakondi E**, Szabó É, Gergely P, Szabó C, Virág L: Partial protection by poly(ADP-ribose)polymerase inhibitors from nitroxyl-induced cytotoxicity in thymocytes. *Free Rad. Biol. Med*. 15;31(12):1616-1623, 2001

IF.: 5.082

6. Szabó E, Virág L, **Bakondi E**, Gyüre L, Haskó G, Bai P, Hunyadi J, Gergely P, Szabó C: Peroxynitrite-induced poly(ADP-ribose) polymerase activation in keratinocytes: implications for contact hypersensitivity. *J. Invest. Dermatol.* 117(1):74-80, 2001

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