Ph.D. THESIS

THE BIOLOGICAL ROLE OF REACTIVE NITROGEN INTERMEDIATE-INDUCED POLY(ADP-RIBOSE) POLYMERASE AND MATRIX METALLOPROTEINASE ACTIVATION

Péter Bai

INTRODUCTION

Nitrosative stress-induced poly(ADP-ribose) polymerase activation

Establishing the *in vivo* importance of reactive nitrogen intermediates (RNI) was one of the greatest discoveries of modern biology. Nitric oxide, the most well-known RNI, is syntesised by nitric oxide synthase enzymes (NOS). NO exists in three redox states. The one electron reduced form of NO is called nitroxyl. Nitroxyl modifies the cysteine residues of proteins and reacts with transition metal complexes. Nitroxyl was found to be cytotoxic on neurons, MCF-7 human lung carcinoma cells and V79 hamster lung fibroblast cell line.

NO is known to possess both cytoprotective and cytotoxic effects. According to our current knowledge NO itself is considered cytoprotective. NO-derived reactive species such as peroxynitrite are likely to be responsable for the cytotoxic effects. Peroxynitrite is formed in the reaction of NO with superoxide anion. Peroxinitrite is capable of inducing oxidative modification of several amino acids leading to the inactivation of several enzymes and ion channels. The targets of peroxinitrite-mediated nitration are tyrosine residues resulting in nitrotyrosine formation. Nitrotyrosine is considered as the *in vivo* marker of peroxinitrite formation. Peroxinitrite proved to be cytotoxic on several cell lines such as HaCaT keratinocytes, Raw macrophages and thymocytes.

In various conditions, increased poly(ADP-ribose) metabolism has been made responsable for the cytotoxic effects of oxidative/nitrosative stress. Poly(ADP-ribose) polymerase-1 (PARP-1) is the best known member of the PARP enzyme family consisting of 18 members. PARP-1 is activated upon DNA single- or double strand breaks. The enzyme

forms homodimers and binds to the DNA nicks. Activated PARP-1 uses NAD^+ as substrate and cleaves it. From the ADP-ribose moiety of NAD^+ PARP polymerises poly(ADP-ribose) (PAR) polymers onto glutamate side chains of different acceptor proteins.

The role of PARP has been established in the regulation of replication, gene expression, differentiation and cell death. PARP activation was linked to the cytotoxic properties of DNA damaging agents by Berger and his colleagues. Their concept was, that PARP overactivation consumes cellular NAD^+ content. The resynthesis of NAD^+ requires ATP, therefore cellular ATP content may drop. ATP depletion stalls ATP dependent processes, while NAD^+ depletion stalls the mitochondrial electron transport chain leading to the collapse of the mitochondrial membrane potential. Thereafter, the mitochondrial F1/F0 ATPase turns into an ATPase. All these processes then lead to the loss of the cellular ATP. The compromised cellular energetics may inhibit ATP dependent steps of apoptotic cell death, thus diverting cell death towards oncosis.

Matrix metalloproteinases in oxidative/nitrosative stress conditions

Matrix metalloproteinases (MMPs) are zinc endopeptidases with diverse functions. The role of the MMPs is the remodelling of the extracellular matrix under physiological and pathological conditions. MMP activation has been implicated in several diseases such as arthritis or different cardiovascular diseases.

MMPs are synthesised as inactive proenzymes. The so called autoinhibitory (or propeptide) domain on the N-terminus of the enzyme is responsible for the inhibitory functions. MMPs can be activated in two different ways. Protecolytic activation means the removal of the

autoinhibitory domain by proteolysis. Proteolysis changes the molecular mass of the MMP by removing the 8-12 kDa inhibitory domain.

The other mechanism of activation is induced by changing the structure of the zymogen. Agents like strong detergents, mercuri compounds, reactive oxigen species (ROI), or peroxynitrite. This pathway was proposed to be responsible for MMP activation in the oxidatively stressed heart. In heart failure the balance of the synthesis and degradation of the extracellular matrix is distorted. Upon the breakdown of collagen, cardiomyocyte slippage occurs leading to ventricular dilatation, contracile dysfunction and attenuation of the cardiac output.

We have investigated the role of RNIs in doxorubicin (DOX) cardiomyopathy. Doxorubicin is a broad-spectrum antracycline-based antitumor antibiotic used in the treatment of solid tumors, lymphomas and leukemias. The biggest drawback of the clinical application of DOX is it`s severe cardiotoxicity. The mechanism underlying the cardiotoxic effect is free radical production resulting from the kinon-semikinon cycling of DOX in the mitochondria of cardiac cells. The reduced semikinon form of DOX is instable, and readily donates electrons creating reactive intermediates. DOX treatment distorts the energetic state of cardiac cells, damages cellular DNA and may also lead to NFκB activation.

AIMS

Mechanism of nitroxyl-induced cytotoxicity

- Does nitroxyl activate the DNA strand break – PARP activation pathway?
- Does nitroxyl-induced cytotoxicity have the same characteristics as cytotoxicity evoked by other oxidants (hydrogen peroxide, peroxinitrite)?
- Is there peroxynitirite formation upon nitroxyl treatment?

Possible role of PARP activation and free radicals in doxorubicin-induced cardiomyopathy

- Is there PARP activation in doxorubicin-induced cariomyopathy?
- Is there MMP activation in the heart in doxorubicin-induced cardiomyopathy?

MATERIALS AND METHODS

Thymocyte preparation and treatment

The thymus of C57BL6 mice (6-8 weeks old) was used. The cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum ($37^{o}C$, 5% CO_2). For the production of nitroxyl Angeli`s salt (AS) was used as a donor compound, for the inhibition of PARP 3-amino-benzamide (3AB) was used.

Doxorubicin treatment

Male Balb/c mice (25-30 g) were treated with a single dose of DOX (25 mg/kg i.p.). Mice were used for the measurements detailed bellow after two days. Treatment with the PARP inhibitor PJ34 was started 1 hour prior to DOX treatment (20 mg/kg i.p.) and was continued at a daily dose of 3x10 mg/kg/day. FP15 treatment started two hours prior to DOX treatment (1 mg/kg/day p.o., mixed into the drinking water).

Detection of DNA single strand breaks

DNA single strand breaks were detected using the Trevingen comet assay kit.

Radioactive PARP activity measurement

Thymocytes were pretreated with PARP inhibitor for 30 minutes, then AS was added to the system. Cell culture media was replaced with 0,5 ml activity measurement buffer containing 0,5 μCi/ml ^{3}H-NAD$^+$. Thirty minutes later cellular proteins were precipitated with 50% ice cold TCA. The precipitate was solubilised in 2% SDS/0.1 N NaOH solution and radioactivity was determined by liquid scintillation.

Immunodotblot detection of poly(ADP-ribose)

Thymocytes were treated as above. The cells were lysed in RIPA buffer supplemented with NaOH and EDTA. From each sample 20 μg protein was dotted onto Hybond N+ membrane. The blot was fixed in 0,4 M NaOH, then blocked. PAR was detected using a specific antibody.

Determination of cytotoxicity

AS evoked cytotoxicity was determined by propidium iodide (PI) uptake. Cells were treated with PARP inhibitor for 30 minutes followed by a 4 hour long AS treatment. Thymocytes were stained with PI (2,5 μg/ml, 15 minutes) and the samples were analysed by flow cytometry.

Annexin-FITC - propidium idodie double staining

Three hours after AS treatment 10^5 cells were stained with 5 μl Annexin V-FITC and 5 μg/ml PI. The samples were analysed by flow cytometry.

Detection of mitochondrial membrane depolarisation and superoxide production

Changes of the mitocondrial membrane potential and secondary superoxide production were detected by 3,3'dihexiloxakarbocianine iodide and hydroetidium double staining, respectively. Fluorescence was analysed by flow cytometry.

Measurement of Caspase-3 activity

Six hours after the AS treatment, thymocytes were lysed. Lysates were combined with reaction buffer containing the caspase-3 specific DEVD-pNA substrate (300 μM). After one hour of incubation liberated pNA was determined spectrophotometrically.

Determination of DNA fragmentation (DNA laddering)

Intranucleosomal DNA fragmentation was detected by agarose gel electrophoresis 6 hours after AS treatment. Cells were loaded onto agarose gel. In the course of the electrophoresis, SDS and proteinase K was ran through them. DNA was visualised by ethidium bromide staining.

Western blot detection of nitrotyrosine

Cells were treated with AS (30 min) and were lysed in RIPA buffer. From each sample (15 µg) was protein separated by SDS-PAGE and was blotted onto nitrocellulose membrane. Nitrotyrosine was detected with a specific antibody.

Determination of serum lactate dehydrogenase and creatin kinase activity

Two days after the DOX treatment blood was drained from the vena cava inferior. Blood was left to clot and both lactate dehydrogenase and creatin kinase activities were determined using colorimetric kits. Activity was expressed in U/L.

Metalloproteinase zymography

Two days after DOX treatment, the hearts of the mice were perfused with physiological saline and were excised. Tissue samples were homogenised in calcium containing buffer. The homogenates were mixed with 2X SDS sample buffer and were incubated at room temperature for 15 min. Out of these samples 7,5 µg protein was loaded onto zymography gels. Gels were renatured for 30 min and were developed overnight at 37°C. The undigested substrate was stained with Brilliant Blue.

RESULTS

Nitroxyl- induced thymocyte cell death

AS treatment (200μM) caused DNA single strand breaks in the genomic DNA of thymocytes. In permeabilised thymocytes after AS treatment the amount of TCA-precipitable tritiated proteins was elevated, indicating an increase in PARP activity. The PARP specificity of the activity was verified by 3AB pretreatment. Measuring PARP activity with the dot blot technique showed similar results.

AS treatment lead to a concentration-dependent oncosis of thymocytes. Pretreatment with the free radical scavenger $K_3Fe(CN)_6$ and TEMPOL protected thymocytes against the AS-induced cell death suggesting nitroxyl as the inducer of the cytotoxicity. Chelating intracellular calcium showed significant protection similarly to the PARP inhibitor 3AB.

To characterise AS-induced cytotoxicity, Annexin-FITC – PI double staining was performed. The thymocytes form three populations. The FITC-PI double negative cells represent the normal population, the FITC-PI double positive cells represent the oncotic, while the FITC single positive cells the apoptotic population. Treating cells with AS resulted in an increase in the ratio of both the apoptotic and oncotic cells compared to the control sample. 3AB pretreatment slightly lowered the ratio of the oncotic cells and at the same time elevated the level of the normal and apoptotic cells.

AS treatment (150 μM) of the thymocytes resulted in the loss of the mitochondrial membrane potential. Secondary superoxide production also

increased. PARP inhibition significantly augmented these alterations, but did not fully prevent them.

In order to further characterise AS-induced cell death, caspase-3 activity and DNA laddering were investigated. Caspase-3 activation showed a mild, elongated increase followed by decline. In the presence of a PARP inhibitor, the characteristics of caspase-3 activation were similar, but began to increase at lower AS concentrations and reached higher levels compared to the control activation curve. DNA laddering showed the same characteristics.

There was no significant protein nitration in the AS treated thymocytes, while the positive control – thymocytes treated with authentic peroxinitrite – gave strong signal.

Role of PARP and free radicals in doxorubicin cardiomyopathy

DOX treatment resulted in cardiomyocyte death, marked by increased serum levels of CK and LDH. Both FP15 and PJ34 pretreatment inhibited CK and LDH release. Therefore, DOX-induced cell death involves the action of free radicals and PARP activation.

MMP activation was detected by zymography. Control hearts showed no MMP activity. DOX treatment resulted in the activation of a 34 kDa gelatinase. MMP activity was approximated by the densitometry of the bands and it showed a four time increase in the DOX treated samples compared to the control ones. FP15 treatment inhibited MMP activation in this model, while PJ34 treatment did not. The calcium dependecy of the MMP activity was verified with the development of replica gels in calcium free buffer.

DISCUSSION

Nitroxyl-induced cytotoxicity

Nitroxyl showed concentration dependent cytotoxicity on thymocytes. Nitroxyl evoked DNA single strand breaks, as published in the literature. DNA strand breakage caused PARP activation as showed by dot-blot and ^{3}H-NAD$^+$ incorporation techniques. PARP inhibition provided significant, but not full protection against cytotoxicity. Nitroxyl cytotoxicity showed correlation with the elevation of the intracellular calcium concentration. Our results suggest, that the DNA breakage – PARP activation – oncosis pathway is not the only mechanism initiated by nitroxyl.

It is questionable, wether nitroxyl itself or other nitroxyl-derived reactive species are responsible for the AS-induced cytotoxicity. To exclude the possibility of peroxinitrite production, we were looking for the presence of nitrotyrosine in the proteins of AS-treated thymocytes. There was no nitrotyrosine formation after AS exposure, so there was no peroxynitrite production in our experimental system. However, other nitroxyl-induced modifications such as cysteine nitrosylation and oxidative modification may have occured.

Nitroxyl-induced cell death showed similar characteristics to cell death induced by other oxidants: the mitochondrial membrane potential decreased, and the secondary superoxide production increased. Nitroxyl has similar characteristics to NO, therefore it may be able to modify the protein-metal complexes of the mitochondria and the iron-sulphur complexes. This may also lead to the decrease of the mitochondrial membrane potential and to secondary superoxide production.

AS treatment resulted in apoptosis (DNA fragmentation, caspase-3 activation, annexin-PI staining), which turned into oncosis in a concentration-dependent manner. The apoptosis-oncosis switch functioned similarly as in other forms of cell death induced by other oxidants but the switch was less dominant. The switch between the two forms of cell death could be PARP activation leading to the decrease of ATP-NAD$^+$ levels. On the other hand, AS treatment may have altered the activity of proapoptotic proteins either by nitrosylation or by oxidative modification.

Doxorubicin-induced cardiomyopathy

Our results have shown that nitrotyrosine was present in the hearts of DOX treated mice, suggesting peroxynitrite production. A possible source of NO necessary for peroxinitrite production is the inducible NOS (iNOS). iNOS expression is regulated by the transcription factor NFκB, which is inducible by DOX.

The most important reactive intermediate produced during the kinone-semikinone cycling of DOX is superoxide anion. The superoxide anion may form peroxynitrite with NO. The peroxynitrite decomposition catalyst FP15 *in vivo* effectively catalysed the decomposition of peroxynitrite, thus preventing nitrotyrosine formation.

According to the results of Pál Pacher, the left ventricular heart functions of DOX treated mice decreased significantly to the fifth day after DOX treatment. Both PJ34 and FP15 reduced DOX-induced heart dysfunction. However, while FP15 provided full protection, the protection provided by PJ34 was not complete. In terms of cytoprotection, both compounds had the same efficiency in keeping CK and LDH levels at the control level. These results suggest the involvment

of the free radical production - DNA strand breakage – PARP activation – ATP/NAD$^+$ depletion pathway in the cell death of the DOX treated cardiomyocytes. Probably, as in other cell models, PARP inhibition partially diverted these cells from oncosis towards apoptosis. Cardiomyocytes dieing on the apoptotic pathway may cause less stress for the organism than necrotic cells.

Our results suggest a novel mechanism, the activation of MMPs in the cardiotoxicity of DOX. We have shown the activation of a 34 kDa MMP in the heart of DOX treated mice. We propose the role of free radicals in the activation of the 34 kDa MMP, but PARP probably does not play a major role in this process. It is plausible to hypothesize that activated MMPs degrade the extracellular matrix of the heart, therefore the contractions of the cardiomyocytes will be less efficient due to the slippage of the myocytes. Through this mechanism, MMPs may contribute to the DOX-evoked contractile dysfunction.

For the better understanding of the pathophysiological role of MMP activation further investigations are necessary. If the connection between the contractile dysfunction and MMP activation can be confirmed it may identify MMPs as new pharmacological target in the prevention of DOX cardiotoxicity. The application of MMP inhibitors in the DOX therapy of solid tumors would not only lower the cardiotoxicity, but also may prevent the metastasis forming capability of these tumors.

CONCLUSION

1. Nitroxyl causes PARP activation through the initiation of DNA single strand breaks in thymocytes.

2. Nitroxyl triggers the collapse of the mitochondrial membrane potential and production of superoxide.

3. There is no nitrotyrosine formation after Angeli`s salt treatment suggesting the lack of peroxynitrite production.

4. Our results suggest that the DNA breakage – PARP activation - NAD^+/ATP depletion pathway plays a significant but not a major role in the Angeli`s salt-induced cytotoxicity.

5. In doxorubicin-induced cardiomyopathy, peroxintirite production and PARP activation contributes to the oncotic cell death of cardiomyocytes.

6. A 34 kDa MMP becomes activated in doxorubicin-induced cardiomyopathy likely by free radicals.

PUBLICATION LIST

Publications the Ph.D. thesis is based on

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

IF.: 5.082

Pacher P, Liaudet L, **Bai P**, Virag L, Mabley JG, Hasko G, Szabo C (2002) The activation of poly(ADP-ribose) polymerase contributes to the development of doxorubicin-induced heart failure. *J. Pharmacol. Exp. Ther.* **300**:862-867.

IF.: 3.555

Pacher P, Liaudet L, **Bai P**, Mabley J, Kaminsky PM, Virag L, Deb A, Szabo E, Ungvari Z, Wolin MS, Szabo C (2003) A potent peroxynitrite decomposition catalyst, FP15, protects against the development of doxorubicin-induced cardiac dysfunction. *Circulation* **107**: 896-904.

IF.: 10.517

Other publications

Bai P, Mabley JG, Liaudet L, Virág L, Szabó C, Pacher P: Matrix metalloproteinase activation is an early event in doxorubicin-induced cardiotoxicity. *Oncol. Rep.* (in press)

IF.: 1.171

Bakondi E, **Bai P**, Erdélyi K, Szabo E, Gergely P, Hunyadi J, Szabo C, Virag L: Cytoprotective effect of gallotannin in oxidatívely stressed HaCaT keratinocytes: the role of poly(ADP-ribose) metabolism. *Exp. Derm.* (in press)

IF.: 2.234

Bakondi E, Gönczi M, Szabó É, **Bai P**, Pacher P, Gergely P, Kovács L, Hunyadi J, Szabó C, Csernoch L, Virág L (2003) Role of intracellular calcium mobilization and cell density-dependent signaling in oxidatíve stress-induced cytotoxicity in HaCaT keratinocytes. *J. Invest. Dermatol.* **121(1)**:88-95.

IF.: 4.645

Virag L, Szabó E, Bakondi E, **Bai P**, Gergely P, Hunyadi J, Szabo C (2002): The nitric oxide – peroxynitrite - poly(ADP-ribose) polymerase pathway in the skin. *Exp. Derm.* **11(3)**:189-202.

IF.: 2.234

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

IF.: 2.718

Szabó E, Virag L, Bakondi E, Gyüre L, Haskó G, **Bai P**, Hunyadi J, Gergely P, Szabo C (2001) Peroxynitrite-induced poly(ADP-ribose)

polymerase activation in keratinocytes: implications for contact hypersensitivity. *J. Invest. Dermatol*. **117(1)**:74-80.
IF.: 4.645

Bai, P., Erdélyi, K., Bakondi, E., Hegedus, C., Gergely, P., Szabo, C., Virag, L. Potential role of peroxynitrite in the DNA damage, poly(ADP-ribose) polymerase activation and cytotoxicity caused by the alkylating agent N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in thymocytes. (közlésre benyújtva)

Mabley, J.G., Pacher, P., **Bai, P.**, Wallace, R., Goonesekera, S., Virag, L., Southan, G.J., Szabo, C. Suppression of intestinal polyposis in *Apc*^min/+ mice by inhibition of inducible nitric oxide synthase, inhibition of poly (ADP-ribose) synthetase or neutralization of peroxynitrite. (közlésre benyújtva)

Abstracts

Bai, P., Bakondi, E., Gergely, P., Szabo, C., Virág, L. (2002) Mechanism of nitroxyl (NO$^-$/HNO)-induced cytotoxicity in thymocytes. *FASEB J.* **16(5)**, A1166-A1167.

Bakondi, E., **Bai, P.**, Bak, I., Szabó, E., Gergely, P., Szabó, C., Virág, L. (2002) Role of poly(ADP-ribose) polymerase in contact hypersensitivity. FASEB J. **16(4)**, A674-A675.

Virág, L., **Bai, P.**, Bak, I., Bakondi, E., Szabo, E., Liaudet, L., Gergely, P., Szabo, C. (2002) Inhibition of poly(ADP-ribose) polymerase

attenuates leukocyte migration in an ovalbumin-induced asthma model in mice. *FASEB J.* **16(4)**, A674.

Pacher, P., Liaudet, L., **Bai, P.**, Virag, L., Mabley, J.G., Szabo, C. (2002) Activation of poly(ADP-ribose)-polymerase contributes to the development of doxorubicine-induced heart failure. *FASEB J.* **16(4)**, A570.

Pacher, P., Liaudet, L., **Bai, P.**, Virag, L., Mabley, J.G., Szabo, C. (2002) A potent peroxynitrite decomposition catalyst, FP15, protects against the doxorubicine-induced heart failure. *FASEB J.* **16(4)**, A177.

Szabo E, Bakondi E, **Bai P**, Hunyadi J, Gergely P, Szabo C, Virag L (2001) Regulation of oxidatíve stress-induced cytotoxicity in HaCaT keratinocytes. *J. Eur. Acad. Dermatol. Venerol.* **15(Suppl 2)**:200.