From specific aim 1. *Identification of the role of p38 MAP kinase, JNK and MAP kinase phosphatase-1 (MKP-1) in oxidative stress induced necrotic cell death.*

Studies with synthetic PARP inhibitors

1. Determining the effect of the PARP-1 on the activation of MAP kinase systems including ERK1/2, p38 MAP kinase and JNK, and on the cell death process. Our data show that inhibition of PARP-1 decrease the cell death in oxidative stress both necrotic and apoptotic processed as determined from propidium iodid and fluorescein labeled-Annexin V labeling. PARP inhibition **activates ERK1/2** determined by phosphorylation specific antibodies as described before, and **decreases the activation JNK and p38** MAP kinase.

2. In oxidative stress, we provided evidence that PARP inhibition increase the oxidative stress dependent expression of MKP-1 which is the main phosphatase responsible for the inactivation of JNK and p38 MAP kinase, and so it can play a role in the PARP inhibition mediated mitochondrial protection.

Studies with the transdominant expression of PARP-1 DNA-binding domain –*which prevents PARP-1 activation***- and by suppression of PARP-1 by siRNA.**

These techniques exclude the possibility that a synthetic molecule, beside PARP inhibition, might effects other signaling pathways.

1. We provided evidence for the PARP inhibition/suppression induced suppression of JNK and p38 MAP kinases.
2. We identified the overexpression of MKP-1/Dusp1 on the effect of PARP inhibition/suppression in oxidative stress.
3. Providing evidence by suppression MKP-1/Dusp1 (with siRNA) on its critical role in the PARP inhibition regulatory effect on MAP kinases.

In vivo studies.

We provided evidence in several pathological systems, that the regulatory effects of PARP inhibition on kinases cascades are extremely important in cytoprotection.

1. Identifying the importance of the PARP inhibition induced suppression of JNK and p38 MAP kinases in the protection against hypertensive cardiopathy.
2. Providing evidence for the role of MAP kinase and PI-3-kinase-Akt pathways in the protection against neurodegenerative diseases.

From specific aim 2. *Identification and proteomic analysis of the mitochondrial membrane proteins which are phosphorylated by Akt, and which can mediate the protective effect of Akt on mitochondria in oxidative stress.*

Using control mouse liver and liver from LPS induced secptic shock models we isolated mitochondria, and isolated proteins by anti-Akt substrates antibodies. That way we can isolates mitochondrial targets of Akt kinase, and comparing the data we can see which proteins are phosphorylated in septic shock. Since septic shock model animals mitochondrial permeability transition is taking place while in normal liver it is not, therefore these data may show which protein phosphorylation can contribute to mitochondrial permeability transition.

Phosphoprotein analysis.

We performed the analysis of Akt phosphorylated proteins by using antibody which recognizes the phosphorylated Akt sites. . Using anti-Akt substrates antibodies we identified proteins by immunoprecitation followed by proteolysis and MS analysis. MS analysis were performed by nano-HPLC-Maxis (Brucker) system we identified GSK-3-beta phosphorylated on Ser-9 and phosphorylated cyclophilin D (site is unknown) as possible mitochondrial Akt targets. We found several other phosphoproteins with MS analysis but they did not were related to mitochondrial permeability transition. In addition we identified them by Western blot with anti-p-GSK-3beta and anti-cyclophilin antibodies. The two independent techniques provided reliable data that Akt can regulate two important mitochondrial proteins which can play significant role in the regulation of mitochondrial permeability transition.

Data from the literature indicated that cyclophilin D can undergo covalent modifications, which can be catalyzed by either extracellular signal regulated kinase (ERK) or glycogen synthase kinase (GSK-3beta) (*A. Rasola, M. Sciacovelli, F. Chiara, B. Pantic, W.S. Brusilow, P. Bernardi, Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition, Proc. Natl Acad. Sci. USA 107 (2010) 726–731*.). In addition it is suggested that recombinant GSK3 could phosphorylate cyclophilin D and an in silico analysis identified possible GSK3 target residues on [*Rasola et al.*]. It is known that Akt phosphorylates GSK-3beta at Ser-9, which inactivates the enzyme, and so protect against mitochondrial permeability transition. However, it is the first data showing that Akt can phosphorylate cyclophilin D and thereby can prevent cyclophilin D dependent mitochondrial permeability transition.

From specific aim 3. *Providing evidence for the oxidative stress induced cytoplasmic (released from the nucleus) poly-ADP-ribose (PAR) and for PAR induced signal-transducting effects.*

The main question of this proposal was mechanism by which PARP activation/inhibition can regulate kinase cascades. First we thought that PAR polymer may get out to cytoplasm and can regulate directly mitochondria and kinases. Previously, it was suggested that the release of nuclearly formed ADP-ribose polymers or ADP-ribosylated proteins could be responsible for the cytosolic and mitochondrial effects of poly(ADP-ribose) polymerase (PARP)-1 activation in oxidative stress. Dawsons group showed previously, that nuclear poly-ADP-ribose can get out to cytoplasm and can destabilize mitochondrial membrane system (*Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death. Yu SW, Andrabi SA, Wang H, Kim NS, Poirier GG, Dawson TM, Dawson VL. Proc Natl Acad Sci U S A. 2006 Nov 28;103(48):18314-9.).* In our systems we can not get these data, and the suppressing of PARG which mediates increases in poly-ADP-ribose content has complex role in cell survival (*Dual role of poly(ADP-ribose) glycohydrolase in the regulation of cell death in oxidatively stressed A549 cells. Erdélyi K, Bai P, Kovács I, Szabó E, Mocsár G, Kakuk A, Szabó C, Gergely P, Virág L. FASEB J. 2009 Oct;23(10):3553-63.*). These and other data indicate that that Yu et al. data can be valid only their special cell systems. In other experimental systems several laboratories showed that inhibiting certain signaling pathways can prevent PARP activation induced cell death (*Trends in Biochemical Sciences. Volume 31, Issue 6, June 2006, Pages 309–311Players in the PARP-1 cell-death pathway: JNK1 joins the cast. Conrad C. Alano, Raymond A. Swanson*). Our previous data also showed that PARP activation mediated mitochondrial destabilization is a complex signaling processes (*Pivotal role of Akt activation in mitochondrial protection and cell survival by poly(ADP-ribose)polymerase-1 inhibition in oxidative stress. Tapodi A, Debreceni B, Hanto K, Bognar Z, Wittmann I, Gallyas F Jr, Varbiro G, Sumegi B. J Biol Chem. 2005 Oct 21;280(42):35767-75.).*

Therefore the main question of this proposal was to discover the mechanism by which PARP activation/inhibition can regulate mitochondrial integrity and the activity of kinase cascades. Our studies *(Racz et al. Free Radic Biol Med. 2010 Dec 15;49(12):1978-88*), we provide a novel alternative mechanism. We found that reactive oxygen species-activated PARP-1 regulated the activation of JNK and p38 mitogen-activated protein kinases (MAPKs) because inhibition of PARP-1 by pharmacons, small interfering RNA silencing of PARP-1 expression, or the transdominant expression of enzymatically inactive PARP-1 resulted in the inactivation of these MAPKs. This regulation was achieved by increased expression and enlarged cytoplasmic localization of MAPK phosphatase-1 (MKP-1) upon PARP-1 inhibition in oxidative stress because changes in MKP-1 expression were reflected in the phosphorylation states of JNK and p38. Furthermore, we found that in MKP-1-silenced cells, PARP inhibition was unable to exert its protective effect, indicating the pivotal roles of JNK and p38 in mediating the oxidative-stress-induced cell death as well as that of increased MKP-1 expression in mediating the protective effect of PARP inhibition. We suggest that regulation of a protein that can directly influence cytoplasmic signaling cascades at the expression level represents a novel mechanism for the cytoplasmic action of PARP-1 inhibition.

Furthermore, we identified the mechanism by which PARP-1 directly modulated (poly-ADP-ribosylates) nuclear transcription factors involved in the regulation of MKP-1, 2 & 3 expression.

From specific aim 4*. Proteomic analysis of the cytoplasmic poly-ADP-ribose binding proteins.*

We isolated cytoplasmic protein in from hydrogene peroxide treated WRL68 cells and LPS treated mouse liver and subjected to either poly-ADP-ribose (PAR) affinity chromatography, or PAR immuno-precipitation by anti-PAR antibodies. By these methods we precipitated the cytoplasmic poly-ADP-ribose-binding proteins and cytoplasmic PARylated proteins. Proteins were separated by electrophoresis, or 2D electrophoresis, and identified by Brucker MALDI and MAXIS mass spectrometers.

After several disappointing efforts we identified the following proteins binding to poly-ADP-ribose, or PARylated proteins (see below).

Q8BQD8 mouse **Nucleolin**,
AAH09004, **Protein phosphatase 1G,** Mg-dep.
Q8C1W9 nucleosome assembly protein 1-like 4, isoform.
YBOX1 Mouse (**Nuclease sensitive element-binding protein**)
BAA34736 likely Nucleosome assembly protein,
and 4 other proteins with unknown function.

After several unsuccessful experiment with these proteins we identified Creb2 as a PARylated protein in completely independent studies which regulated the expression of MKP-1(plus MKP-2, MKP-3), MAK kinase activation and cell death, therefore our interest turned to Creb2.

From specific aim 5. *Identification of those PAR-binding proteins which can regulate MAP kinases, or PI-3-kinase-Akt systems, and can play a role in necrotic cell death.*

Analysis of cytoplasmic effect of PARP inhibition take a new turn under our studies, as we discovered that the cytoplasmic effect of PARP inhibition on mitochondrial dependent cell death and MAP kinase regulation takes place by the activation of the expression and partially cytoplasmically localized MKP-1(and some other member of MKP family (*manuscript under preparation*)). Therefore, we focus on this new mechanism (Racz B, Hanto K, Tapodi A, Solti I, Kalman N, Jakus P, Kovacs K, Debreceni B, Gallyas F Jr, Sumegi B. Regulation of MKP-1 expression and MAPK activation by PARP-1 in oxidative stress: a new mechanism for the cytoplasmic effect of PARP-1 activation. Free Radic Biol Med. 2010 Dec 15;49(12):1978-88), and we try to identify the PARP regulated transcription factor(s) which can be responsible for the regulation of certain members of MKP family.
After two years of hard work we identified Creb2 as the transcription factor being responsible for PARP-1 mediated MKP expression in oxidative stress. We provided evidence that in oxidative stress PARP-1 poly-ADP-ribosylates and inactivates Creb2 including the DNA binding of Creb2 (*Racz et al. J. Biol. Chem submitted for publication*).
Therefore, PARP-1 activation in oxidative stress, by poly-ADP-ribosylation and inactivation of Creb2, decrease MKP-1 expression, and so activates JNK1 and p38MAPK which initiates mitochondrial permeability pore formation and cell death. That is, poly-ADP-ribosylation is an important step in the regulation of cytoplasmic pathways, our data show that this retrograde communication is taking place by the regulation of MAP kinase phosphatases the main regulators of MAP kinases.

We identified another PARylated protein which can be shuttled between nucleus and cytoplasm, and which can be responsible for PARP inhibition induced Akt activation. This protein was ATM kinase which is regulated and PARylated by PARP-1, and through PARylation PARP-1 can influence of its localization in cells (*manuscript under preparation*). It was showed before that ATM cytoplasmic export plays a key role in Akt1 activation (*ATM protein kinase mediates full activation of Akt and regulates glucose transporter 4 translocation by insulin in muscle cells. Halaby MJ, Hibma JC, He J, Yang DQ. Cell Signal. 2008 Aug;20(8):1555-63*.).

That is, our efforts to identify poly-ADP-ribosylated proteins which can be involved in cell death, MAP kinase regulation and PI-3-K-Akt pathway regulation has been successfully accomplished after a lot of efforts. Our data showed that in these two retrograde pathways either ATM nuclear export, or the regulation of MKP-1 mRNA synthesis and export plays critical role, and nuclear PARylation are involved, and not the cytoplasmic export of PAR.

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