Effect of PARP-1 inhibitors on LPS-induced inflammation in mice

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Contents.

Abbreviations.

3-AB, 3-aminobenzamide; AP-1, activator protein 1; COX-2, cyclooxygenase 2; ELAM, endothelial leukocyte cell adhesion molecule; ERK, extracellular signal-regulated kinase; HMG, high mobility group; 4-HQN, 4-hydroxyquinazoline; ICAM, intercellular adhesion molecule; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP, mitogen activated protein; MRI, magnetic resonance imaging; NF-κB, nuclear factor kappa B; p90RSK, protein of 90 kDa from the ribosomal subunit S6 kinase; PARP, poly-(ADP-ribose) polymerase; PI3, phosphatidylinositol 3; PMNs, peripheral blood mononuclear cells; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α.

Introduction.

Sepsis and septic shock

Sepsis describes a complex clinical syndrome that results from a harmful or damaging host response to infection. Many of the components of the innate immune response that are normally concerned with host defences against infection can, under some circumstances, cause cell and tissue damage and hence multiple organ failure, the clinical hallmark of sepsis. Sepsis develops when the initial, appropriate host response to an infection becomes amplified, and then dysregulated. Clinically, the onset is often insidious: features may include fever, mental confusion, transient hypotension, tachycardia, tachypnea, leukocytosis, diminished urine output or unexplained thrombocytopenia. If untreated, the patient may develop respiratory or renal failure, abnormalities of coagulation and profound and unresponsive hypotension.

During the oneset of sepsis, the inflammatory system becomes hyperactive, involving both cellular and humoral defense mechanisms. Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes, produce powerful pro-inflammatory mediators, especially tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-1 and IL-8. Simultaneously, robust production of acute-phase proteins, such as C-reactive protein, occurs and humoral defense mechanisms such as the complement system are activated, resulting in production of pro-inflammatory mediators, including C5a, the complement split product. C5a ultimately enhances cytokine and chemokine production. Furthermore, the coagulation system becomes activated through various mechanisms, often resulting in disseminated intavascular coagulopathy. The described mediators are produced early in the oneset of sepsis and reflect the overactive status of the inflammatory response. Phagocytic cells (neutrophils and macrophages) respond to many of these mediators by releasing granular enzymes and

producing reactive oxygen species (ROS) such as H_2O_2, which is a crucial product for the killing of bacteria. H_2O_2 is also capable of causing tissue damage, which ultimately leads to increased vascular permeability and organ injury. In later stages of sepsis, anti-inflammatory mediators are produced (such as IL-10, transforming growth factor-β and IL-13), leading to abatement in the production of many of the pro-inflammatory mediators. In this phase, various innate functions are supressed, especially the functions of neutrophils, leading to a hyporeactive host defense system and immunoparalysis.

The „public enemy": LPS

The commonest sites of infection are the lungs, abdominal cavity, the urinary tract and the primary infections of the blood stream and it appears often as a result of a systemic Gram-negative bacterial infection. Determining the structural components of bacteria that are responsible for initiating the septic process has been important not only in understanding the underlying mechanisms, but also in identifying potential therapeutic targets.

In Gram-negative bacteria, lipopolysaccharide (LPS; known also as endotoxin) has a dominant role (Fig. 1). The outer membrane of Gram-negative bacteria is constructed of a lipid bilayer, separeted from the inner cytoplasmic membrane by peptidoglycan.

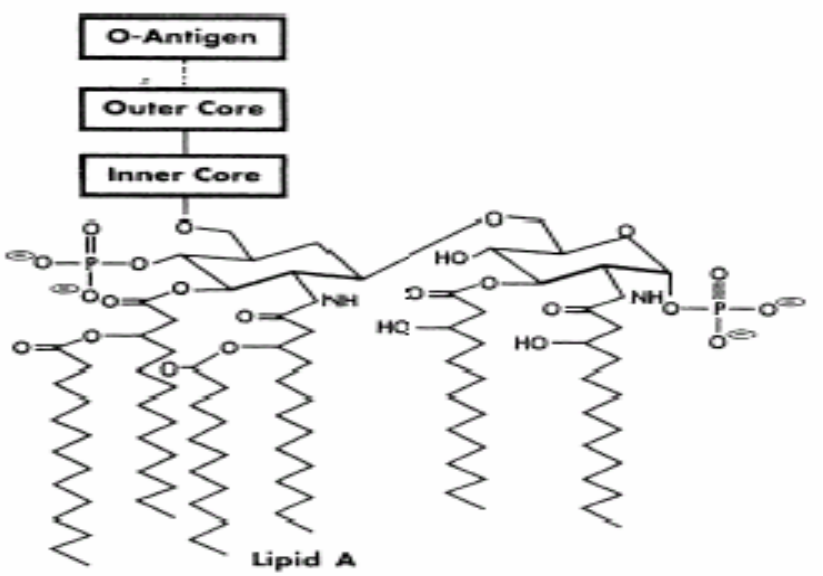

Fig. 1 **The stucture of lipopolysaccharide.**

The LPS molecule is embedded in the outer membrane and the lipid A portion of the molecule serves to anchor LPS in the bacterial cell wall. Biophysical studies on the three dimensional conformation adopted by different lipid A partial structures have revealed that, under physiological conditions, the most active forms assume the shape of a truncated cone, whereas inactive molecules prefer a lamellar structure and become progressively more cylindrical. These conformational changes seem to correlate with the ability to activate host cell membranes. There is no endotoxin in Gram-positive bacteria, but their cell walls do contain peptidoglycan and lipoteichoic acid, and several investigators have identified structural components that account their biological activity. Both peptidoglycan and lipoteichoic acid can bind to cellsurface receptors and are pro-inflammatory, although they are much less active than LPS. Their role in the pathogenesis of clinical sepsis remains uncertain because there are no convincing clinical data to show that they are present in the circulation at concentrations comparable to those used in the experimental setting.

LPS-induced septic shock

When LPSs are present in the bloodstream, as a result of a systemic Gram-negative bacterial infection, they are immediately captured by LPS-binding protein and then transferred to their cognate extracellular receptor complex composed of CD-14, MD-2 and Toll-like receptor 4 (TLR-4) (Daun JM 2000; Kaisho T 2000). Binding of LPS to the CD-14/MD-2/TLR-4 receptor complex in target cells, such as monocytes and residential macrophage populations, results via TLR-4 associated MYD88 complexes in activation of a diverse set of complex signaling cascades including the TRAF6/TAK1/IKK-α/IKK-β-IκB-NF-κB pathway (Akira S 2001). In macrophages, NF-κB might then upregulate in concert with its coactivator PARP-1 the expression of specific sets of pro-inflammatory mediators involved in the pathogenesis of septic shock: cytokines (TNF-α, MIF, IFN-γ) and interleukins. The massive production and release of cytokines by macrophages might then in turn activate in target cells, such as

monocyte/macrophage populations and epithelial and endothelial cells, the NF-κB complex in concert with other transcription factors, including STAT-1 and AP-1, which results in the repeated upregulation of the expression of inflammatory cytokines and chemokines, endothelial adhesion molecules including intercellular adhesion molecules (ICAMs), endothelial leukocyte cell adhesion molecules (ELAMs, E-selectin), platelet endothelial cell adhsion molecule-1 (P-selectin), and vascular cell adhesion molecules. The increased expression of these genes in effector cells (epithelial and endothelial cells) results in the recruitment and activation of peripheral blood mononuclear cells (PMNs) and macrophages. Secretion of pro-inflammatory cytokines by these cells leads to continued stimulation of epithelial and endothelial cells that in turn might activate the NF-κB transcription factor. Thus, a positive autoregulatory loop might be established that can amplify the inflammatory response and increase the duration of chronic inflammation resulting in a persistent activation of NF-κB transcription factor characterized by over-expression of pro-inflammatory mediators and massive recruitment of PMNs and macrophages. At the same time, TNF-α, IL-1β and IFN-γ stimulate endothelial cells, PMNs and macrophages to drastically upregulate the expression of inducible nitrogen monoxide synthase (iNOS). Cellular iNOS induction in effector and target cells results in a massive increase in the concentration of NO. NO can diffuse from PMNs and macrophages to endothelial and epithelial cells where it is converted into a cytotoxic derivative, peroxynitrite. Rapid single-strand DNA breaks are induced by the high concentration of peroxynirtite in effector cells, leading to excessive activation of PARP-1, depletion of cellular energy and necrotic cell death. Necrosis of endothelial cells then in turn might result again in a massive recruitment and activation of PMNs and macrophages as a positive feedback loop that finally leads to enhanced systemic inflammation, endothelial dysfunction and organ failure.

Poly-(ADP-ribose) polymerase (PARP-1)

Until recently, only one type of poly-(ADP-ribose) polymerase (PARP) was thought to exist: the PARP-1. However, the development of mice deficient for the PARP-1 gene has completely changed this view. To date, more than 16 new PARP family members can be found in the human genome (Shall S 2002).

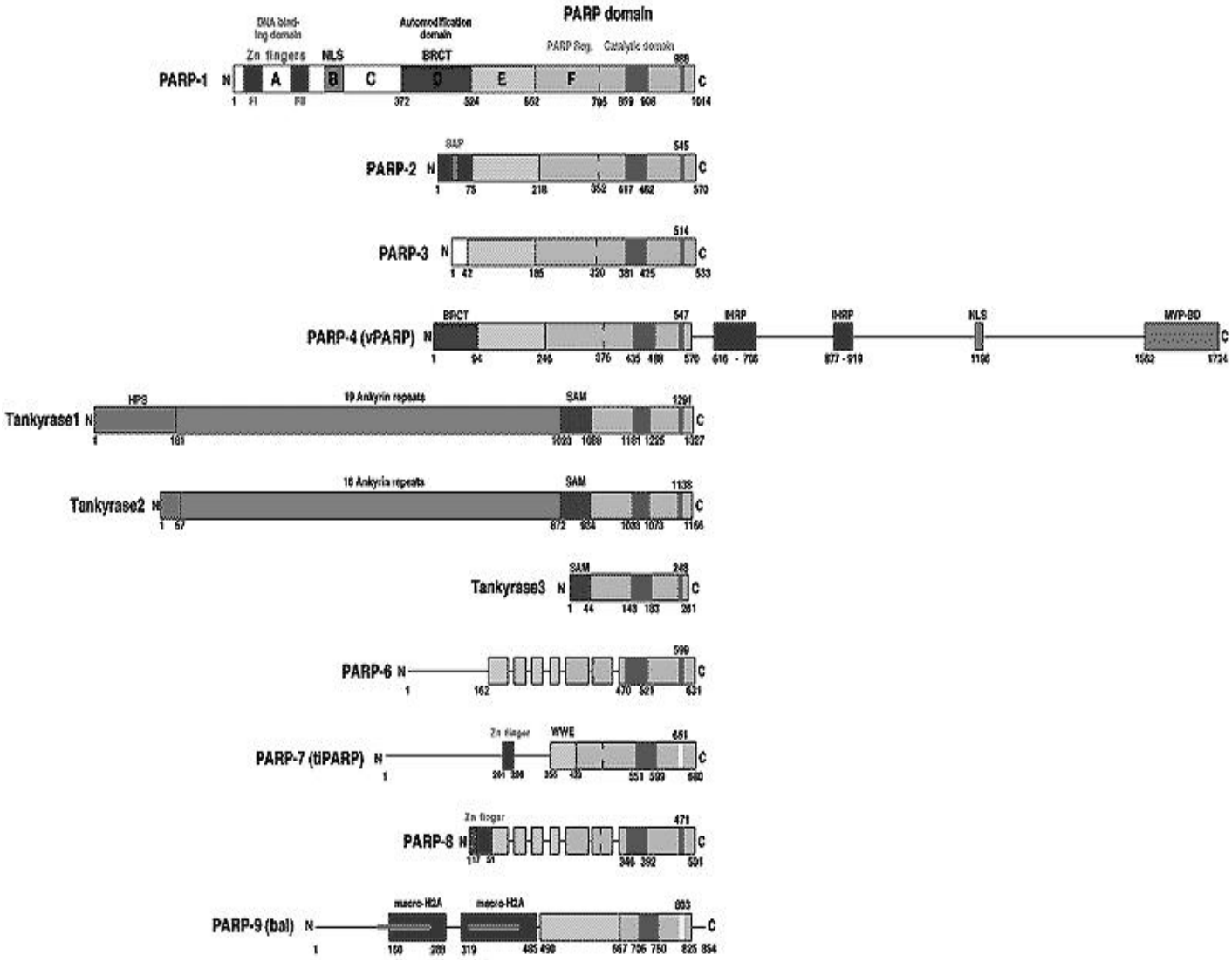

Fig. 2 **The structures of the PARP subclasses.**

These new PARPs are structurally distinct from the classical 114 kDa PARP-1 enzyme, and can be classified together with PARP-1 according to their structures and sizes into several

subgroups (Fig. 2). Only limited data are avaliable regarding the physiological roles of these nonclassical PARP family members.

Mammalian PARP-1, a 114 kDa abundant nuclear chromatin associated protein, belongs to a large family of enzymes that catalyzes the transfer of ADP-ribose units from beta-nicotinamide adenine dinucleotide (NAD^+) onto glutamic acid residues of nuclear protein acceptors. The existence of a PARP enzyme was first reported nearly 40 years ago. PARP-1 in one of the best-characterized examples of this family. The activity of PARP-1 is strongly stimulated by the presence of nicks and strand breaks in DNA (Alvarez-Gonzalez R 1994). These observations have contributed to the idea that PARP mediates stress-induced signaling and functions in an NAD^+-dependent manner in certain cellular processes (Althaus FR 1990; Althaus FR, 1992). Since then, the biological significance of PARP has been reported in many cellular processes (D'Amours D 1999; Le Rhun Y 1998; Shall S 2000). However, the physiological role of PARP-1 is still under heavy debate. Earlier studies using inhibitors of PARP enzymatic activity such as 3-AB and nicotinamide suggested that PARP-1 plays a crucial role in DNA replication, DNA base excision repair, recombination as well as regulation of telomere lenght (Shall S 2000). Other functions proposed for PARP-1 include gene expression, chromatin organization, proliferation and differentiation, cellular NAD^+ metabolism and necrosis. In addition to the PARP-related energetic depletion and suicidal cycle, PARP may have other important functions in modulating cell death. Although highly controversial, PARP (or its cleavage) may have a role in the process of apoptosis (Kaufman SH 1993; Nocholson DW 1995). Several reports demonstrated that peroxynitrite – as well as hydrogen peroxide and various other oxygen derived oxidants and free radicals – can cause apoptosis in a variety of cell types (Salgo MG 1995; Bonfoco E 1995; Lin KT 1995). It appears that sustained exposure or low levels of peroxynitrite cause apoptosis, whereas sudden exposure to high concetrations of peroxynitrite induces cell necrosis. However, the

peroxynitrite-induced apoptosis, in all cell types studied so far, cannot be attenuated by pharmacological inhibitors of PARP or PARP-/- phenotype (Virag L 1998; Leist M 1997; Wang ZQ 1997; O'Connor M 1997). PARP-1 also serves as a marker for the onset of apoptosis, after which it is cleaved by caspases into DNA-binding and catalytic fragments (Gu Y 1995; Tewari M 1995).

Structure of PARP-1

PARP-1 is found in all multicellular lower and higher eukaryotes studied so far. The structure of the type 1 PARP has been extensively characterized. PARP-1 is a highly conserved multifunctional enzyme consisting of three domains: a DNA-binding domain (DBD) containing a bipartite nuclear localization signal (NLS) which is interrupted by a caspase cleavage site, an automodification domain and a catalytic domain (Fig. 3). Tha catalytic domain is the most highly conserved region of the PARP molecule (Murcia G 1994). The N-terminal DBD of human PARP-1 spans residues 1-373 and has a molecular mass of approximately 42 kDa. This domain contains two zinc fingers (FI and FII) and two helix-turn-helix (HTH) motifs (Gradwohl G 1990; Ikejima M 1990).

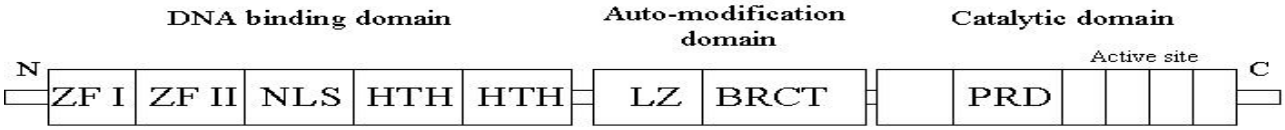

Fig. 3 **Schematic representation of the PARP-1 map.** Abbreviations: BRCT: BRCA-C-terminal domain; ZF: zinc finger; HTH: helix-turn-helix domain; LZ: leucine zipper; NLS: nuclear localization signal; PRD: PARP regulatory domain.

Studies have shown that these two zinc fingers are the main stuctures responsible for binding to double-strand breaks (DSBs) or single-strand breaks (SSBs) and for activation of PARP-1 enzyme activity. The moderate non-specific association of PARP-1 with non-damaged DNA has been proposed to depend most probably on the HTH motifs (Thibodeau J 1993). Moreover, the zinc fingers can also act as an interface with various protein partners.

The automodification domain of PARP-1 extends from residues 374 to 525 bearing a leucine zipper (LZ) motif in the N-terminal part and a BRCA1 carboxyl-terminal (BRCT) protein interaction domain in the C-terminal part (Uchida K 1993). Both the LZs and the BRCT domain are well known to be involved in protein-protein interactions (Buch SJ 1990; Callebaut I 1997). Experimental data suggested that the LZs might be responsible for homodimerization of PARP-1 (Kim JW 2000). In addition, the automodification domain contains possible auto-poly(ADP-ribosyl)ation sites implicated in the negative regulation of interactions between PARP-1 and DNA (Duriez PJ 1997; Mendoza Alvarez H 1999).

Tissue distribution, expression levels and subcellular localization of PARP-1

The tissue distribution of PARP-1 and its enzymatic activity have been examined in several rat and mouse organs (Ogura T 1999; Menegazzi M 1991). Northern blot analysis and *in situ* hybridization have revealed that PARP-1 gene is constitutively expressed in testis, spleen, brain, thymus, intestine, colon and nasal cavities. Very high levels of PARP-1 were found in lymphoid organs, especially the thymus, in the germinal centers of the spleen and in the Peyer's patches in the ileum, while only very low levels of PARP-1 expression were found in organs such as liver, kidney and heart (Dantzer F 2000). In the central nervous system (CNS), PARP-1 is highly expressed in regions with a high neuronal cell density such as hypocampal neurons of the regions CA1 and 3, granule cells of the dentate gyrus, Purkinje cells of the cerebellar cortex, as well as microglia and astrocytes in several regions (Zucconi G 1992). Interestingly, for non-neuronal cell types, a direct correlation could be observed between cell proliferation and high expression levels of PARP-1. Several studies have shown that an increase in PARP-1 mRNA levels is observed during thymocyte proliferation and upon activation of lymphocytes and peripheral blood mononuclear cells (Menegazzi M 1988; McNerney R, 1989). Moreover, the PARP-1 mRNA level reaches its peak either in the G1 or the S phases (Wesierska-Gadek J, 2000). The tissue-, cell- and cycle-specific expression

pattern of PARP-1 suggests strongly not only that PARP-1 is critical to major cellular functions but also that its expression is modulated through complex transcriptional regulation. Observation of several different tissues and cell lines using conventional fluorescence microscopy revealed that PARP-1 is exclusively localized to the nucleus (Cocha I 1989). Subsequent studies using either confocal laser scanning microscopy, electron microscopy or cell fractionation experiments showed that PARP-1 is not homogeneously distributed in the nucleus. PARP-1 was shown to be associated with nuclear matrix regions and localized to centromeres during metaphase (Kanai M 2000), while other studies indicated that PARP-1 is found preferentially in nucleoli and defined nuclear bodies (Desnoyers S 1996). Interestingly, PARP-1 was also shown to be associated with actively transcribing nucleolar regions and nuclear bodies. Treatment of cells with RNA synthesis inhibitors caused PARP-1 immunofluorescence to become evenly distributed throughout the nucleus. The association of PARP-1 with actively transcribed regions in the chromatin strongly implies a role for PARP-1 in transcription. Surprisingly, treatment with DNA synthesis inhibitors did not change the distribution of PARP-1 in the nucleus.

Poly-(ADP-ribosyl)ation responses

Activation of PARP-1 was proposed to be one of the earliest responses of mammalian cells to genotoxic stress (Lindahl T 1995). The enzymatic activity of PARP-1 is strongly stimulated *in vitro* and increased by 10- to 500-fold in the presence of nicks and double strand breaks in DNA (D'Amours D 1999). These observations have led to the idea that PARP-1 might act as a „molecular nick sensor", thereby mediating stress-induced signaling in the presence of DNA lesions in an NAD^+-dependent manner to downstream effectors involved in coordinating the cellular response to DNA damage (Althaus FR 1992). The „molecular nick sensor" signaling model proposes that PARP-1 recognizes and rapidly binds to DNA strand breaks through its zinc fingers and in turn, the catalytic domain of PARP-1 is allosterically activated and starts

to synthesize complex branched poly-(ADP-ribose) chains, resulting in automodification of PARP-1 itself and probably to extensive modification of histones at sites of DNA strand breaks. Modification of chromatin proteins and PARP-1 itself might then subsequently act as a strong signal that may rapidly recruit other DNA damage-signaling molecules.

More than 40 nuclear chromatin-associated proteins have been implicated the function as a substrate for PARP-1 and to be modified by poly-(ADP-ribose) chains *in vivo*. Target proteins include topoisomerase I and II, histones, p53 and high-mobility group proteins (Kasid UN 1989; Scovassi AI 1993; Wesierska-Gadek J 1996; Boulikas T 1991). In intact organisms, PARP-1 itself is the predominant acceptor of poly-(ADP-ribose). Except for PARP-1 itself, data about modifications of proteins by PARP-1 *in vivo* should, however, be very cautiously interpreted. Despite intense studies in the last 30 years, neither specific glutamic acid residues functioning as poly-(ADP-ribose) acceptor sites nor any specific poly-(ADP-ribosyl)ation motifs could be identified *in vitro*. Moreover, only a few of the proposed substrates of PARP-1, such as p53, topoisomerase I and histone 1 have been shown to directly interact with PARP-1 (Kumari SR 1998; Bauer PI 2001). One has also to stress that the physiological consequences of poly-(ADP-ribosyl)ation of the substrates are in most cases unknown. Lindahl and collegues (Lindahl T 1995) have even proposed that the minor degree of modification of poly-(ADP-ribose) acceptor proteins could be explained as an artificial side reaction *in vitro*.

PARP-1 $^{-/-}$ mice

In recent years, several laboratories developed mice deficient for the PARP-1 gene. The three different knockout mice were created by interruption of either exon 2, exon 4 or exon 1 of the PARP-1 gene in mice (Wang ZQ 1997; Trucco C 1998; Masutani M 1999). Surprisingly, PARP-1 $^{-/-}$ mice from all three different laboratories are viable and fertile. Furthermore, they did not show any phenotypic abnormalities such as organ failures as one would have clearly

expected from the data obtained using inhibitors of PARP enzyme activity and taking into account that knockouts of genes like XRCC1, DNA polymerase-β or APE, which play a crucial role in the BER pathway are lethal (Xanthoudakis S 1996; Tebbs RS 1999; Sugo N 2000). Indeed, carefully designed studies with PARP-1 $^{-/-}$ cells clearly demonstrated that PARP-1 is dispensable and not essential for replication, repair of DNA damage or apoptosis *in vitro* or *in vivo* (Ha HC 1999; Vodenicharov MD 2000). Interestingly, recent studies using PARP-1 $^{-/-}$ mice showed that they were protected against lipopolysaccharide (LPS)-induced septic shock, collagen-induced arthritis, streptozotocin-induced diabetes, hemorrhagic shock and neuronal damage induced by transient middle cerebral artery occlusion (MCAO) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), indicating that PARP-1 plays a crucial role in inflammatory and neurodegenerative disorders and is involved in the pathogenesis of these events (Szabo C 1997; Pieper AA 1999; Oliver FJ 1999; Mandir AS 1999; Eliasson MJ 1997; Liaudet L 2000).

Pharmacological inhibition of PARP-1 in mice

Current strategies aimed at limiting free radical-mediated and oxidant-mediated cell/organ injury include agents that catalyze superoxide or peroxynitrite, or inhibit the induction or activity of the inducible NO synthase. Less attention has been directed to strategies that interfere with intracellular cytotoxic pathways initiated by nitrogen- or oxygen-derived free radicals or their toxic derivatives. Direct and indirect experimental evidence presented in several papers supports the view that peroxynitrite-induced DNA strand breakage and PARP activation importantly contribute to the pathophysiology of various forms of inflammation.

Pharmacological inhibition of PARP, either with 3-AB (Szabo A, 1998, Shock) or with the potent, novel PARP inhibitors 5-iodo-6-amino-1,2-benzopyrone (Szabo C 1997; Bauer PI 1995) improves survival rate in mice challenged with high dose endotoxin. Also, several recent studies compared the survival times of wild-type and PARP-deficient mice in response

to high dose endotoxin, and compared the degree and nature of liver damage in the two experimental groups. In one study, all PARP-deficient animals survived high dose (20 mg/kg) LPS-mediated shock, which killed 60 % of wild-type animals (Kuhnle S 1999). Similar results were obtained by another independent group, led by DeMurcia (Oliver FJ 1999). Szabo C and his group reported that 100% mortality in the wild-type group and less than 50 % mortality in the PARP-deficient animals was observed at 48 h after intraperitoneal injection of high dose (120 mg/kg) *E. coli* endotoxin. Moreover, LPS-induced necrotic liver damage was significantly reduced in the PARP-deficient mice (Kuhnle S 1999). In contrast, when apoptotic liver damage was induced via injection of low concentrations of LPS (30 mg/kg) into D-galactosamine-sensitized mice, or via activation of hepatic cell death receptors, PARP-deficient animals were not protected. Thus, PARP activation is involved in systemic LPS toxicity, while it plays a minor role in apoptotic liver damage mediated by tumor necrosis factor or CD95.

All of the above-described experiments utilized bacterial components, such as endotoxin or hemorrhage and resusciation. It is generally believed that sepsis induced by live bacteria is more appropriate in mimicking the human septic condition. In a preliminary study, Szabo et al (in: PARP as a therapeutic target) compared the survival rates of wild-type and PARP-deficient mice to cecal ligation and puncture (CLP), a commonly used model of polymicrobial sepsis. Their found that CLP-induced death was delayed in the PARP-deficient mice when compared with wild type animals. The beneficial effects of PARP inhibition in bacterial sepsis were also confirmed in a model of sepsis induced by live *E. coli* sponge implantation in pigs. Pharmacological inhibition of PARP provides marked hemodynamic improvements and massive survival benefit (Marton A 2001).

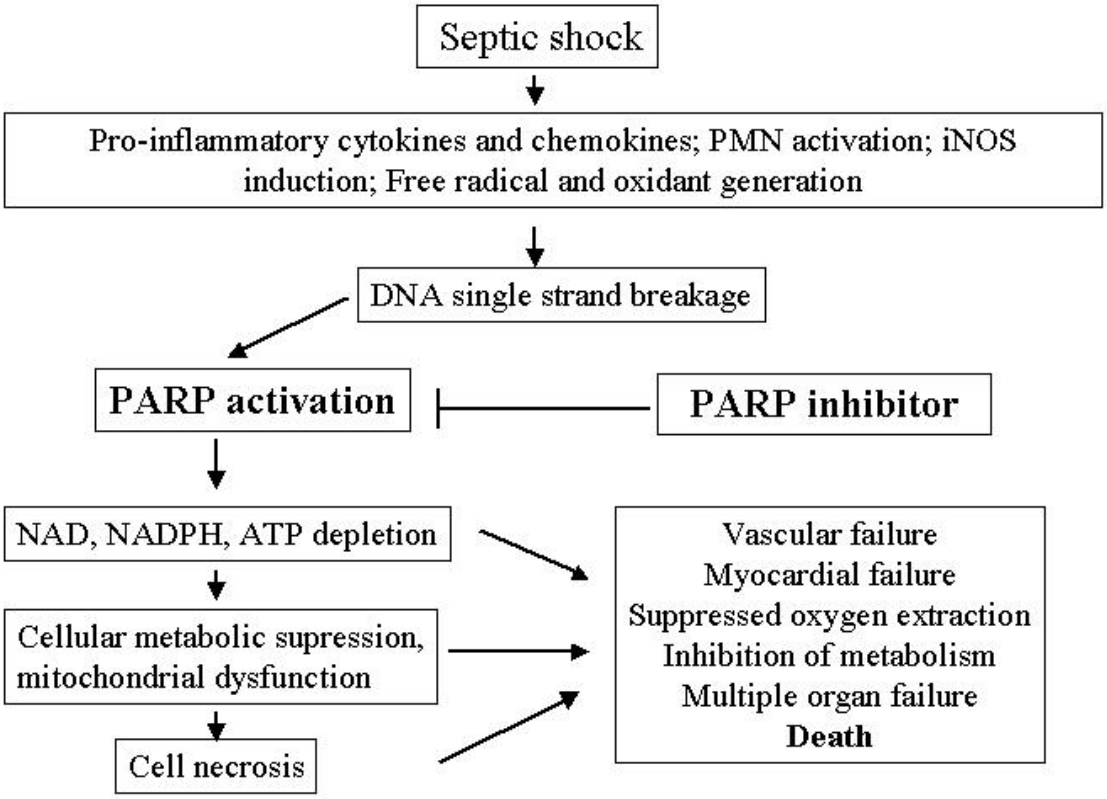

Fig. 4 **Overview of the connection between septic shock, PARP activation and inflammatory mechanisms.** In sepsis LPS induces the overproduction of pro-inflammatory cytokines and chemokines. The PMN-cell activation and the induction of iNOS leads to free radical and oxidant generation. These agents cause ssDNA breaks and activation of PARP. PARP activation leads to energy depletion, cellular metabolic supression, mitochondrial dysfunction and cell necrosis. After all these processes cause failures of several systems and subsequent death. PARP inhibitors can interfere in a relatively early stage of this process.

Based on these observations, one can conclude that, in response to pharmacological inhibition or genetic deletion of PARP, the improved hemodynamic status in shock and sepsis is due to improved vascular function and, possibly, the improved cellular energetic status in some organs. These improvements, in turn, result in an overall survival benefit in this condition (Fig. 4).

Moreover, in the past few years an increasing number of reports have appeared about the importance of the PI3-kinase/Akt and MAPK pathways in LPS-induced inflammatory mechanisms (Bozinovski S 2002; Guha M 2002; Ozes ON 1999). Recent evidence suggests

that activation of PI3-kinase, a ubiquitous lipid-modifying enzyme, may modulate positively acting signaling pathways and inhibition of LPS-induced MAPKs activation may play crucial role in the attenuation of endotoxin-induced inflammatory responses due to the modulation of transcription factors.

Objectives.

Studies utilising the traditional PARP-1 inhibitor, 3-AB, suggested that PARP-1 activity has no (Baechtold F 2001) or partial (Albertini M 2000) role in the mechanisms of septic shock. However, 3-AB is considered a poor inhibitor of PARP-1 with short cellular residence time and it has pronounced toxicity *in vivo*. A separate group in our laboratory demonstrated (Kovacs K 2002) that novel PARP-1 inhibitors protected Langendorff-perfused hearts against ischemia-reperfusion-induced damages by modulating signal transduction pathways and transcription factors.

Therefore, it seemed plausible to study whether these processes were involved in the effects of potent PARP-1 inhibitors - PJ34 and 4-HQN - on LPS-induced septic shock.

PJ34: Phenanthridinone was described as a fairly potent inhibitor of PARP-1. However, the compound is not water soluble, and it has not been tested in models of shock and reperfusion injury *in vivo*. PJ34 (N-(6-Oxo-5,6-dihydrophenanthridin-2-yl)-(N,N-dimethylamino)acetamide hydrochloride, $C_{17}H_{18}ClN_3O_2 \cdot 0.5H_2O$) (Fig. 5) is a selected water soluble member of the PJ series of phenanthridinone-based PARP-1 inhibitors (Jagtap P 2002).

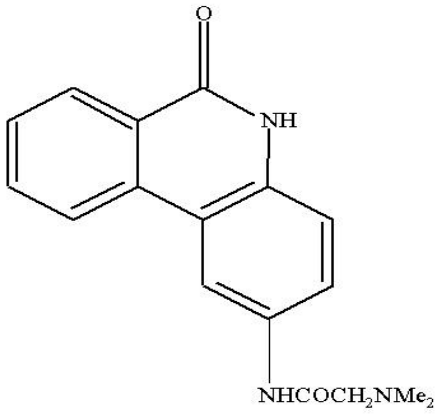

Fig. 5 **Basic structure of PJ34.**

4-HQN: Several different quinazoline-derivatives were reported as potent PARP-1 inhibitors (Banasik M 1992) and 4-HQN (Fig. 6) is one of them. It is structurally unrelated to PJ34 and it was reported to protect hearts from ischemia-reperfusion-induced damages (Halmosi R 2001).

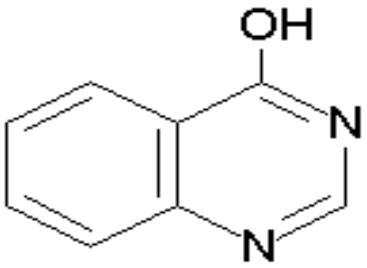

Fig. 6 **Basic structure of 4-hydroxyquinazoline.**

Materials and Methods.

Animals. BALB/c mice were purchased from Charles River Hungary Breeding LTD. The animals were kept under standardized conditions; tap water and mouse chow were provided *ad libitum* during the whole experimental procedure. Animals were treated in compliance with approved institutional animal care guidelines.

Materials. LPS from *Escherichia coli* 0127:B8 was purchased from Sigma/Aldrich; N-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide HCl (PJ34) was synthesized as previously described and it was a kind gift of Szabo C., 4-hydroxyquinazoline (4-HQN) was purchased from Sigma/Aldrich. Primary antibodies, anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-Akt (Ser473, Thr308), anti-phospho-GSK-3β (Ser9), anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-phospho-p90RSK (Thr359/Ser363) were from Cell Signaling Technology; anti-phospho-p38 (Thr180/Tyr182) was from Sigma; anti-HMG-1 was from Becton Dickinson and anti-COX-2 was from Santa Cruz Biotechnology.

Sepsis model. To induce murine endotoxic shock, BALB/c mice were injected i.p. with LPS at a dose of 20 mg/kg body weight in a volume of 250 µl. PJ34 (10 mg/kg) was administered i.p. in a volume of 250 µl three times on the day preceding the LPS challenge (pre-treatment), or as a single dose 1 or 6 hours after the LPS injection. With 4-HQN (100 mg/kg) treatment, we used the same protocol. Control mice received the same volume of sterile saline solution instead of the PARP-1 inhibitors. The mice were monitored for clinical signs of endotoxemia and lethality every hour for 48 hrs, after which time they were monitored three times a day for 1 week. No late deaths were observed in any of the experimental groups. Each experimental group consisted of 10 mice. Data represent mean of 3 independent experiments.

4-HQN was reported as a potential PARP-1 inhibitor (Banasik M 1992) with an IC_{50} 9,5 microM. We have measured the inhibitory effect of 4-HQN on PARP-1, and our results were in agreement with the above mentioned data (IC_{50} 8-12 microM). In accordance with the notion that an animal model requires higher dose than an in vitro system we selected 100 mg/kg dose, which had a significant protective effect in the survival studies. In a previous study (Halmosi R 2001), we have established that this dose of 4-HQN effectively inhibited PARP-1 activity in Langerdorff-perfused hearts, because it prevented the ischemia-induced ADP-ribosylation of proteins. Since this substance has a high potency on PARP-1 and no effects of it on enzymes other than PARP have been documented, it seemed likely that our observations could assigned to its PARP inhibitory effect.

Western blot analysis. For Western blot analysis, groups of 4 BALB/c mice were pre-treated or not with 10 mg/kg PJ34 or 100 mg/kg 4-HQN three times a day one day prior to the LPS-challenge (20 mg/kg). Liver, heart, lung, spleen and small intestine were removed from the animals 16 hours after the LPS treatment, were frozen in liquid N_2 and were processed exactly as described previously (Szabados E 2000). Protein load was 35 μg/lane. We applied the primary antibodies: anti-COX-2, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-GSK-3β (Ser9), anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-phospho-p90RSK (Thr359/Ser363), anti-phospho-p38 (Thr180/Tyr182) and anti-HMG-1 protein at 4°C overnight at a dilution of 1:500 (anti COX-2) and 1:1000 (all the rest). The secondary antibodies were horseradish peroxidase-conjugated rabbit IgG. Peroxidase labeling was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Quantification of band intensities (E_{540}) of the blots was performed by a Beckman DU-62 spectrophotometer equipped with a densitometry attachment (Beckman Instruments)

and ImageJ software. Data representing 3 independent experiments are expressed as % of the untreated control, mean ± S.E.M.

TNF-α determination. Mice were treated exactly as for Western blot analysis. Serum TNF-α concentrations were determined with the Quantikine M TNF-α immunoassay kit (R&D Systems). Blood samples were taken 1.5 hours after LPS administration and were allowed to clot for 0.5 hour at room temperature before centrifuging for 20 minutes at 2000 × g. The serum was removed and assayed immediately. To calculate the results, we created a standard curve by reducing the data using computer software to generate a four parameter logistic (4-PL) curve-fit. The mouse TNF-α standard dilution series was prepared in accordance with the protocol of the manufacturer. Data represent mean ± S.E.M. of 12 independent values (three independent experiments with 4 mice in each group.).

Determination of NF-κB and AP-1/c-Fos activation. Mice were treated exactly as for Western blot analysis. For nucleus isolation, liver, spleen and lung were removed 1.5 hours after the LPS-treatment and were homogenised immediately according to the procedure described previously (Edmead C 1999). Protein concentrations in nuclear extracts were determined using a bicinchoninic acid assay with bovine serum albumin (BSA) as standard (Sigma). To monitor NF-κB and AP-1 c-Fos activation in tissues, we used Trans-AM^{TM} Transcription Factor Assay Kits (Active Motif). The kits consists of 96-well plates into which oligonucleotides containing the NF-κB and AP-1 c-Fos consensus sites (5'-GGGACTTTCC-3'; 5'-TGAGTCA-3' respectively) are bound. The active forms of the above mentioned transcription factors in the nuclear extract specifically bind to these consensus sites and are recognised by primary antibodies. A horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. Results were expressed as percentages

of the positive controls (TNF-α stimulated HeLa whole cell extract and WI-38 nuclear cell extract respectively) provided by the manufacturer and represent mean ± S.E.M. of 12 independent values (Three independent experiments with 4 mice in each group.).

Histopathological analysis. Mice were treated exactly as for Western blot analysis. For histopathological analysis, liver, kidney, heart, spleen and small intestine were removed from the animals 16 hrs after LPS injection, fixed in 4% formaldehyde, blocked, cut into sections of 10 μm in thickness, and stained with hematoxylin and eosin. Scoring of the LPS-induced histopathological changes was performed by an expert pathologist who was blind to the experiment.

MRI analysis. Mice were treated exactly as for Western blot analysis. Six hours after LPS treatment, the animals were anaesthetised with urethane (1.7 g/kg administered i.p.) and were placed into an epoxy resin animal holder tube.

MRI measurements were performed on a Varian ^{UNITY}INOVA 400 spectrometer (Varian, Inc.) with a 89 mm vertical bore magnet of 9.4 T (Oxford Instruments Ltd.) using a 35 mm inner diameter hollow micro-imaging probe with a built-in self-shielded gradient system up to 400 mT/m (Doty Scientific, Inc.). After tuning, shimming (^{1}H linewidth ≈ 150 Hz) and RF calibration, the slice of interest was selected using a T_1-weighted multislice spin-echo sequence (4.0 ms sinc pulses, TR = 1000 ms, TE = 12 ms, slice thickness = 1 mm, FOV = 30 mm × 30 mm, acquisition matrix 128 × 128). T_2-weighted images were recorded using a multislice spin-echo sequence (parameters were like at T_1-weighting, except TR = 3000 ms and TE = 50 ms). One average was taken and images were reconstructed as 256 × 256 matrices. The intensities of the images were standardised to the signal of a 1 mm inner diameter tube filled with water : glycerol = 9 : 1, which was placed near the animal during the measurements. Mean signal intensities were measured and expressed as a percentage of the

signal intensity of the internal standard in characteristic regions delineated as freehand areas by experts who were blind to the experiment. Due to individual differences among the animals, with special respect to uncertainity of the their posture and position (i.e. orientation of their internal organs) within the magnet of the NMR instrument, this scoring should be considered as semiquantitative. Experiments were repeated 3 times.

Inflammation affected regions appear in T2-weighted MRI with increased signal intensity, due to cellular invasion and edema, as reported recently for paraspinal or epidural inflammation and soft tissue inflammation (Ledermann HP 2002). Agreement between MRI data and histopathology was found to be satisfactory for acute local infection in mouse muscle (Ruiz-Cabello J 2002).

Statistical analysis. When pertinent, data were presented as means ± S.E.M. For multiple comparisons of groups, ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's t-test, with Bonferroni's correction.

Results.

Survival studies

Mice treated with a single dose of LPS (20 mg/kg) died within 65 hours. When the mice were pre-treated three times a day, one day before the LPS challenge by PJ34 (10 mg/kg) or by 4-HQN (100 mg/kg), 100 % or 80 % of the animals in the group survived, respectively. Even when groups of mice received only a single shot of PJ34 (10 mg/kg) 1 or 6 hours after the LPS challenge, the PARP-1 inhibitor significantly protected the animals against LPS-induced death, with 86 and 43 % surviving mice, respectively (Fig. 7).

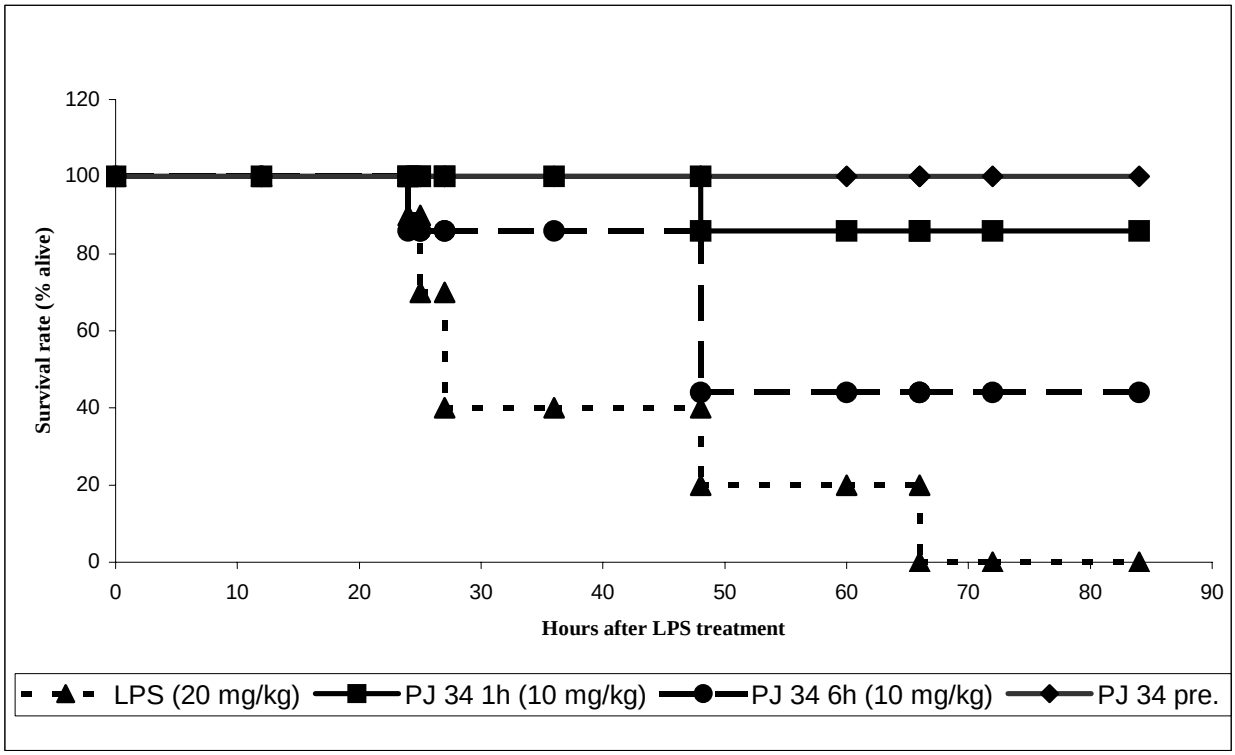

Fig. 7 **Effect of PJ34 on the LPS-induced mortality in mice.** PJ34 (10 mg/kg, i.p.) was injected three times a day one day prior the LPS (20 mg/kg, i.p.) treatment (pre-treatment with PARP-inhibitor) or was injected in a single dose (10 mg/kg, i.p.) 1 or 6 hours after LPS (post-treatment with PARP-inhibitor).

A single shot of 4-HQN (100 mg/kg) 1 hour after the LPS challenge, with a less efficiency in compared with PJ34, but significantly protected the animals against LPS-induced death, with 30 % surviving mice. In contrast to PJ34, when 4-HQN were administered 6 hours after LPS challenge in a single shot (100 mg/kg) it had no protective effect (Fig. 8). PJ34 or 4-HQN treatment itself did not induce death or any obvious damage (data not shown).

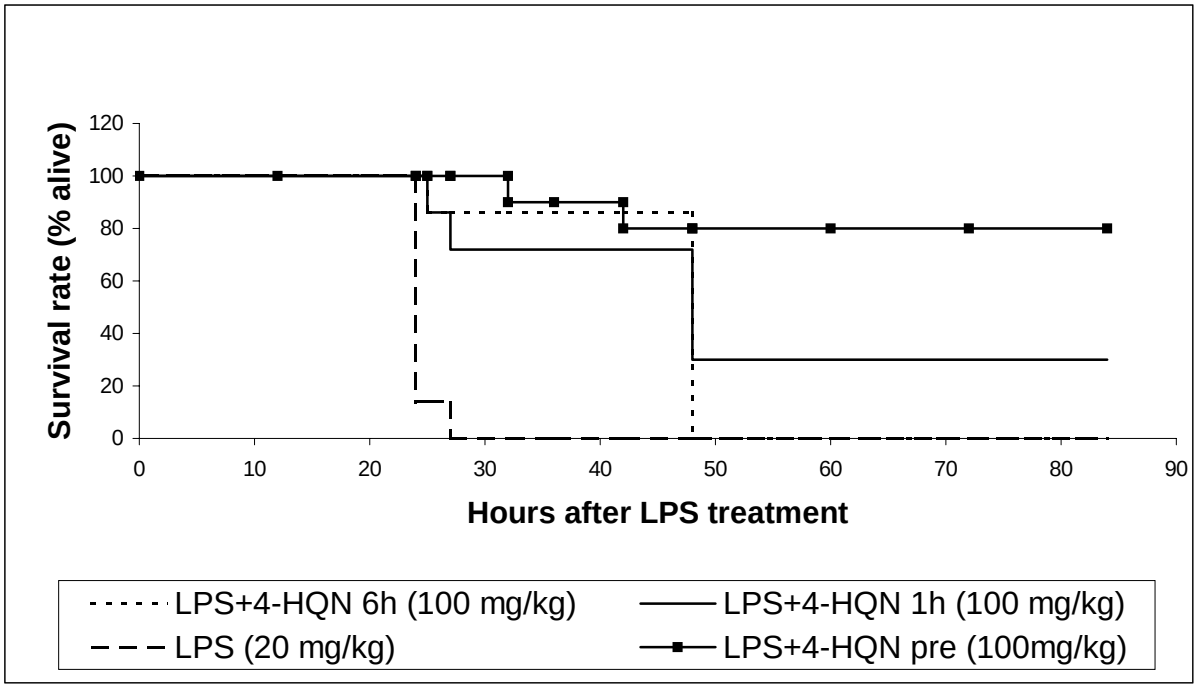

Fig. 8 **Effect of 4-HQN on the LPS-induced mortality in mice.** 4-HQN (100 mg/kg, i.p.) was injected three times a day one day prior the LPS (20 mg/kg, i.p.) treatment (pre-treatment with PARP-1 inhibitor) or was injected in a single dose (100 mg/kg, i.p.) 1 or 6 hours after LPS (post-treatment with PARP-1 inhibitor).

MRI-analysis

Untreated, PARP-inhibitor pre-treated, LPS-treated and LPS + PARP-inhibitor pre-treated mice underwent MRI-analysis. T_2-weighted transversal spin-echo images were taken from the thoracic and lower abdominal regions. Signal intensities of T_2 images were proportional to the inflammatory response.

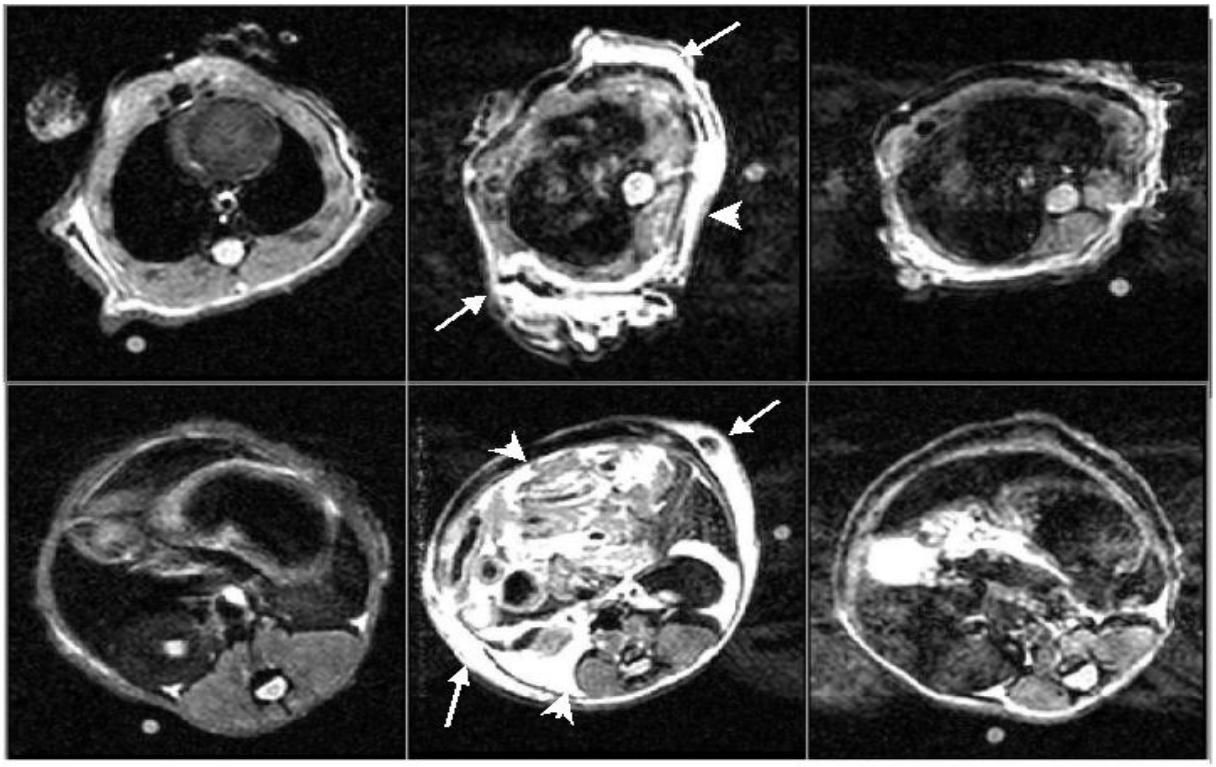

Fig. 9

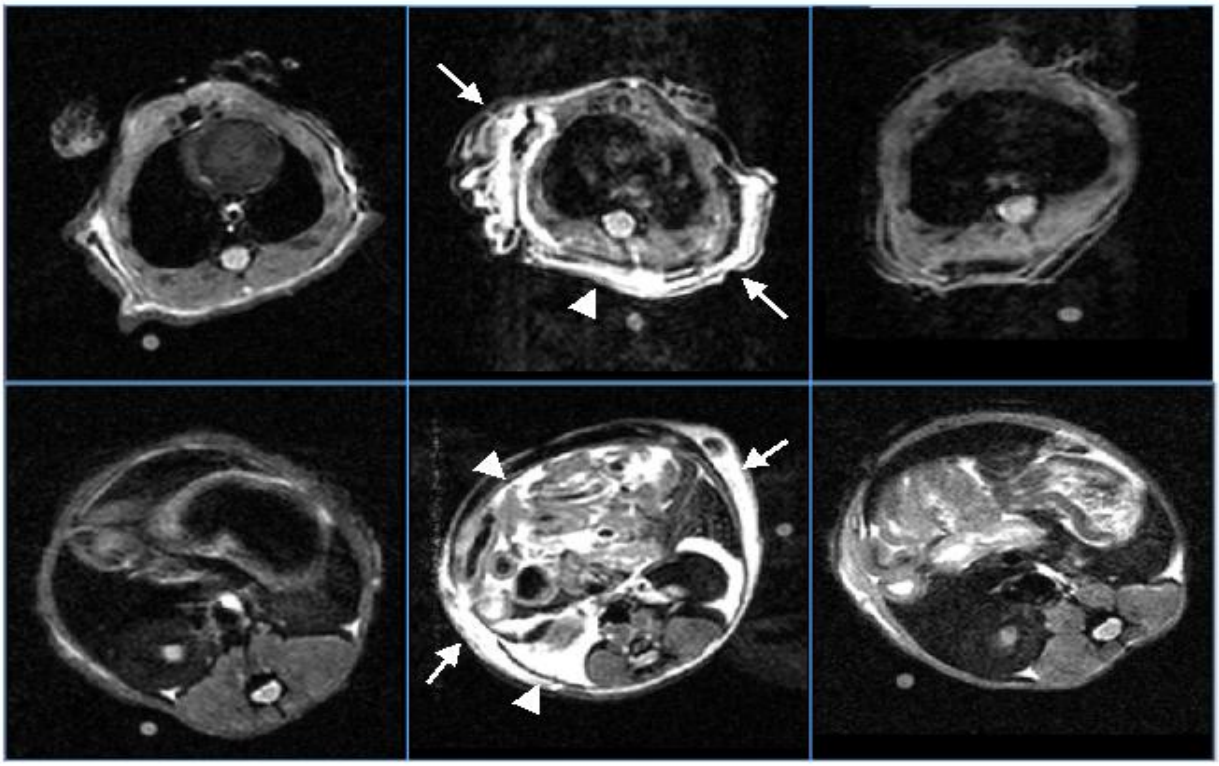

Fig. 10

Fig. 9-10 **Effect of PARP inhibitor treatment on T_2-weighted MR images of the thoracic (upper row) and the lower abdominal (lower row) region in mice**. Typical T_2-weighted images of untreated (left column), LPS (20 mg/kg, i.p.) treated (middle column), LPS + PJ34 (20 mg/kg, i.p. LPS and 3 × 10 mg/kg PJ34 i.p.) treated (right column) (Fig. 7) and untreated (left column), LPS (20 mg/kg, i.p.) treated (middle column) and LPS + 4-HQN (20 mg/kg, i.p. LPS and 3 × 100 mg/kg 4-HQN i.p.) treated (right column) mice (n = 4 in each group) are shown. The thoracic region of LPS treated mice showed a marked increase of intensity (inflammation) especially in the peripheral (arrows) and in the dorsal subcutaneous (arrowhead) regions. The lower abdominal region of LPS treated mice showed marked increase of intensity (inflammation) especially in the lateral subcutaneous regions (arrows) and the inter-intestinal cavities as well as around the kidneys (arrowheads). In animals treated with LPS and PJ34 or LPS and 4-HQN, the T_2-weighted intensities were markedly lower, so the inflammatory response was significantly smaller. T_2-weighted images of animals treated with PJ34 or 4-HQN alone were basically identical to images of untreated mice.

Table 1. Normalized pixel intensities of MRI T_2-weighted images

	Untreated	LPS-treated	LPS+4HQN treated	LPS+PJ34 treated
thorax whole slice	54.4 ± 2.4	95.1 ± 5.7*	68.2 ± 2.6 #	62.7 ± 4.4#
thorax muscles and subcutaneous region	87.3 ± 2.4	124.7 ± 5.5*	106.2 ± 4.6 #	97 ± 8.1#
abdomen whole slice	42.8 ± 1.3	145.5 ± 5.0*	78.5 ± 2.6 #	80.1 ± 3.4#
abdomen gastro-intestinal tract	34.7 ± 1.6	162.0 ± 6.4*	75.6 ± 5.3 #	84.2 ± 6.9#

Mean pixel intensities (± SEM) of T_2-weighted images were expressed as the percentage of the mean intensity of the internal standard (water : glycerol = 9 : 1).

∗ $P < 0.01$ vs. untreated; # $P < 0.05$ vs. LPS-treated.

Representative images selected from 12 images for each group are presented in Fig. 9-10 and normalized mean pixel intensities for all the images were summarized in Table I.

T_2-weighted images of the thoracic regions showed considerably increased intensities in the dorsal subcutaneous region, moderately increased intensities in the intramuscular regions and no observable difference in and inside the pleura of the LPS-treated mice. In the abdominal regions, characteristic increases were observed around the kidneys, in the inter-intestinal cavities and in the gastro-intestinal tract. On the other hand, no signal increase could be observed inside the kidneys and in skeletal muscle, neither in the paravertebral nor in the femoral muscles. All increases in signal intensities were significantly attenuated in mice pre-treated with PJ34 or 4-HQN, indicating that the PARP-1 inhibitors reversed the LPS-induced morphological changes. Mice pre-treated with PJ34 or 4-HQN alone were identical to the untreated control (data not shown).

Effect of PARP inhibitors on LPS-induced pathological changes.

Within 2 hours after the LPS-injection, we observed hypomotility, hypothermia, tremor, diarrhea and a characteristic crouching gait among the treated mice. Despite the serious symptoms and the rapid death, we found surprisingly few pathological changes 16 hours after the LPS-treatment. There were no notable pathological changes in the heart, spleen and kidney. In the small intestine, LPS induced a considerable atrophy of villi (Fig. 11b) that was prevented by PJ34 pre-treatment (Fig. 11c) but not by 4-HQN (data not shown). In the liver, we observed focal necrotic and necrobiotic damages (Fig. 11e), sporadic basophilic apoptotic nuclei (Fig. 11f) and blood vessels obstructed with cell-rich material either attaching to the wall of the blood vessels or obstructing the whole lumen (Fig. 11g). PJ34 pre-treatment significantly protected the tissue against the necrotic and apoptotic damage, as well as against

the obstruction of blood vessels caused by LPS (Fig. 11h). 4-HQN had no significantly protective effect on the above mentioned pathological changes (data not shown). Mice treated with PJ34 or 4-HQN alone did not show any pathological changes.

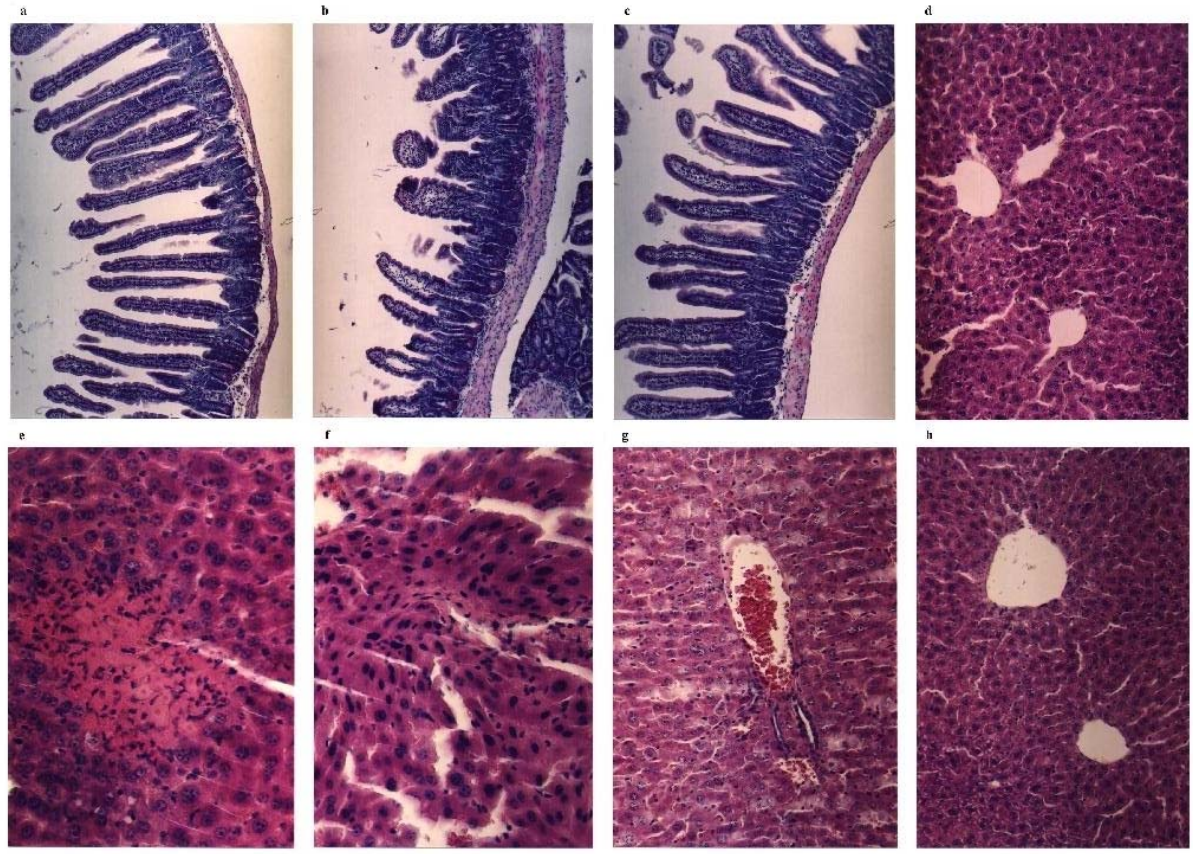

Fig. 11 **Histology of the liver and the small intestine.** Mice were treated with LPS (20 mg/kg, i.p.) either with or without of PJ34 (3 × 10 mg/kg, i.p.) pre-treatment. Tissues were collected 16 hours after LPS injection. Sections were stained with hematoxylin-eosin (HE). a: tissue of normal control gut; b: LPS-induced atrophy of villi; c: atrophy of villi prevented by PJ34 pre-treatment; d: tissue of normal control liver; LPS-induced necrotic (e), apoptotic damage (f) and obstructed blood vessels (g); h: PJ34 pre-treatment largely prevented the effects of LPS in the liver. Original magnification: 100 × (a, b, c); 250 × (d, g, h); 300 × (e, f). Tissues from animals treated with PJ34 alone were identical to control.

Effect of PARP inhibitors on LPS-induced TNF-α production.

LPS-treatment resulted in a rapid increase in serum TNF-α concentration which reached 2960 ± 112 pg/ml after 90 minutes (Fig. 12). In the PJ34 and 4-HQN treated mice, LPS challenge resulted in a significantly lower TNF-α concentrations (1150 ± 122 pg/ml; 1500 ± 135 pg/ml

respectively). PJ34 and 4-HQN alone did not exert any significant effect on serum TNF-α levels (Fig. 12).

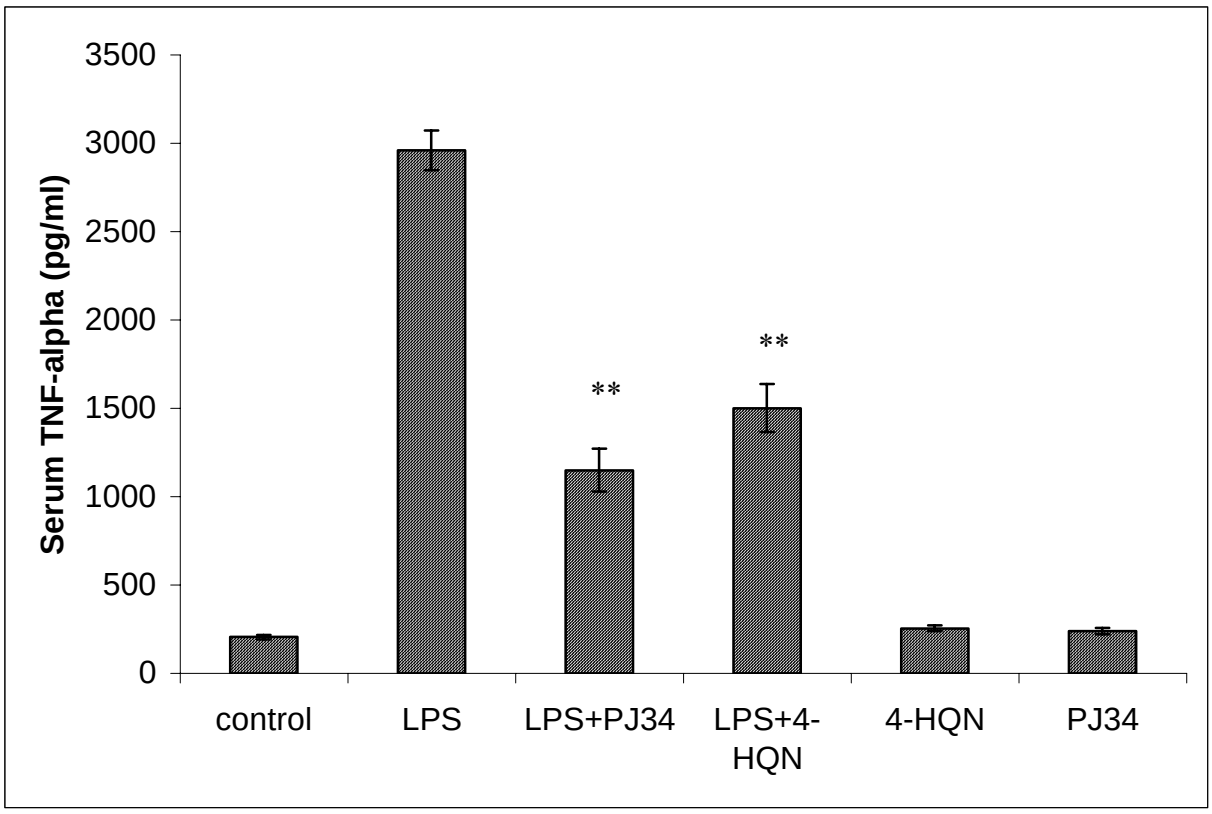

Fig. 12 **Effect of PJ34 and 4-HQN on the serum TNF-α concentrations of LPS-treated mice.** Serum TNF-α concentrations were measured 1.5 hours after LPS injection. In control animals (without LPS), TNF-α concentration was near to the detection limit. Control: injected with physiological saline; 4-HQN: injected with 4-HQN (3 × 100 mg/kg, i.p.); PJ34: injected with PJ34 (3 × 10 mg/kg, i.p.); LPS: injected with LPS (20 mg/kg, i.p.); LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.); LPS+PJ34: pre-treated with PJ34 (3 × 10 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.). Data is expressed as mean ± SEM of 12 independent values. ** $P < 0.01$ vs. LPS.

Effect of PARP inhibitors on LPS-induced phosphorylation of various kinases in liver, lung, heart, small intestine and spleen.

Phospho ERK ½, phospho p38, phospho Akt, phospho GSK-3β, phospho p90RSK and phospho JNK expression were determined by Western blotting from lung, liver and spleen of untreated mice, mice treated with LPS or 4-HQN, and from 4-HQN + LPS-treated mice.

Utilising phosphorylation-specific antibody against phospho-Akt (Ser^{473}) region, we were able to demonstrate activation of Akt under our experimental conditions. We did not find Akt activation in tissues of untreated animals or in animals treated with LPS alone. In every tissue we studied there was a marked increase in the phosphorylation and thereby the activation of Akt of 4-HQN and 4-HQN + LPS treated mice (Fig. 13). This activation reached 2880 ± 144 % in spleen, 835 ± 54 % in liver and 957 ± 103 % in lung in animals treated with 4-HQN +LPS and 416 ± 57 %, 554 ± 19 % and 1158 ± 97 % in animals treated with 4-HQN alone, respectively. Using an antibody against phospho Akt Thr^{308} region we did not find Akt activation neither with LPS nor with 4-HQN treatment (data not shown). Under our experimental condition we could not detect phospho-GSK-3β (Ser^{9}) synthesis even though our assay system was able to detect GSK-3β phosphorylation in other systems (data not shown).

Extracellular signal-regulated kinase phosphorylation and activation was determined by Western blotting using an anti-phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) antibody. LPS-treatment resulted a marked increase in activation of ERK ½ in the spleen (4360 ± 270%), liver (5025 ± 345%) and in the lung (4575 ± 298 %). Pre-treatment with 4-HQN significantly attenuated this activation in all three tissues (2022 ± 125 %, 1358 ± 97 % and 2174 ± 203 % respectively). 4-HQN treatment itself did not have any effect on the activation of phospho-ERK ½ in the tissues we studied (Fig. 14).

p90RSK is a downstream target of p44/42 MAP kinase in the ERK pathway. Phosphorylation of p90RSK was determined using an anti-phospho p90RSK antibody. In the lung of LPS-treated mice we found a marked activation of p90RSK (5964 ± 420 %), which was attenuated by 4-HQN pre-treatment (4550 ± 121 %) (Fig. 15). However, in spleen and in liver LPS did not induce the activation of p90RSK and 4-HQN had no additional effect (data not shown).

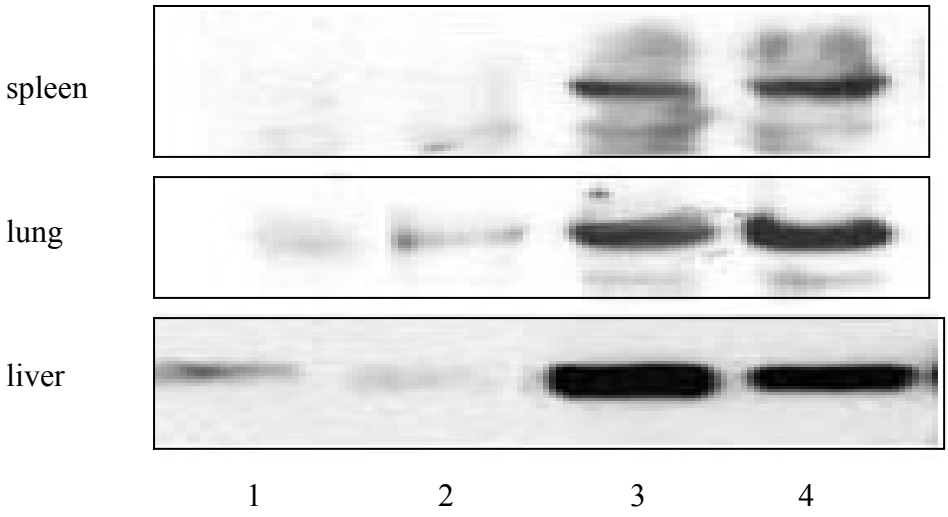

Fig. 13 **Activation of Akt by 4-HQN pre-treatment.** The effect of 4-HQN pre-treatment on Akt activation was evaluated by Western blot analysis in the extracts of various tissues. Lane1: control (injected with physiological saline); Lane 2: LPS (20 mg/kg, i.p.); Lane 3: LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.); Lane 4: 4-HQN (injected with 100 mg/kg 4-HQN alone).

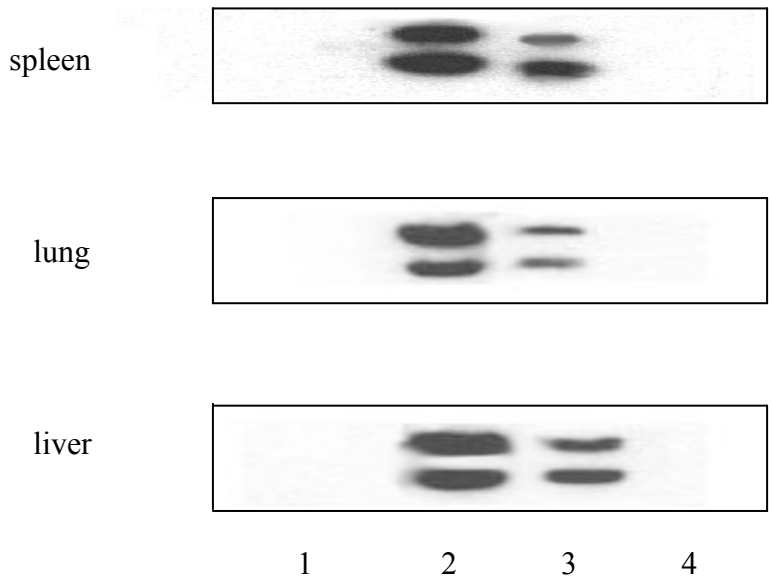

Fig. 14 **Inhibition of ERK 1/2 MAPK by 4-HQN pre-treatment.** The effect of 4-HQN pre-treatment on ERK 1/2 inhibition was evaluated by Western blot analysis in the extracts of various tissues. Lane1: control (injected with physiological saline); Lane 2: LPS (20 mg/kg, i.p.); Lane 3: LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.); Lane 4: 4-HQN (injected with 100
mg/kg 4-HQN alone).

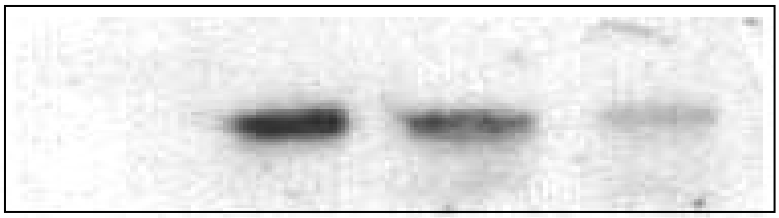

Fig. 15 **Effect of 4-HQN on phosphorylation of p90RSK in lung.** Lane1: control (injected with physiological saline); Lane 2: LPS (20 mg/kg, i.p.); Lane 3: LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.); Lane 4: 4-HQN (injected with 100 mg/kg 4-HQN alone).

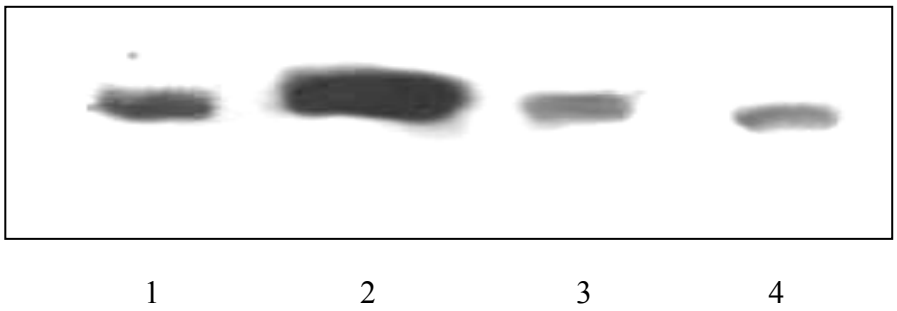

Fig. 16 **Inhibition of p38 MAPK by 4-HQN pre-treatment in lung.** Lane1: control (injected with physiological saline); Lane 2: LPS (20 mg/kg, i.p.); Lane 3: LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.); Lane 4: 4-HQN (injected with 100 mg/kg 4-HQN alone).

Phosphorylation of MAPK p38 was determined using an anti-phospho p38 antibody. As shown in Fig. 16, LPS-stimulated p38 activation in the lung (368 ± 42 %) was completely abolished by 4-HQN pre-treatment. However, in spleen and in liver LPS did not induce the activation of p38 and 4-HQN had no additional effect (data not shown).

Under our experimental condition we could not detect phospho-JNK (Thr^{183}/Tyr^{185}) synthesis even though our assay system was able to detect JNK phosphorylation in other systems (data not shown).

COX-2 and HMG-1 expression was determined by Western blotting from lung, heart, liver and small intestine of untreated mice, mice treated with LPS or PJ34, and from

PJ34+LPS-treated mice. Regardless of the treatment, we did not find significant changes in COX-2 and HMG-1 expression in these tissues (Fig. 17).

Utilising phosphorylation-specific antibodies against phospho-Akt (Ser^{473} and Thr^{308}) regions, we were able to demonstrate activation of Akt under our experimental conditions. There was an activation of the Akt-system close to the detection limit in all tissues of untreated animals (Ser^{473} liver: 4040 ± 354; small intestine: 4601 ± 478; Thr^{308} liver: 2126 ± 155; small intestine: 1945 ± 177) (Fig. 17) or in animals treated with PJ34 alone (data not shown).

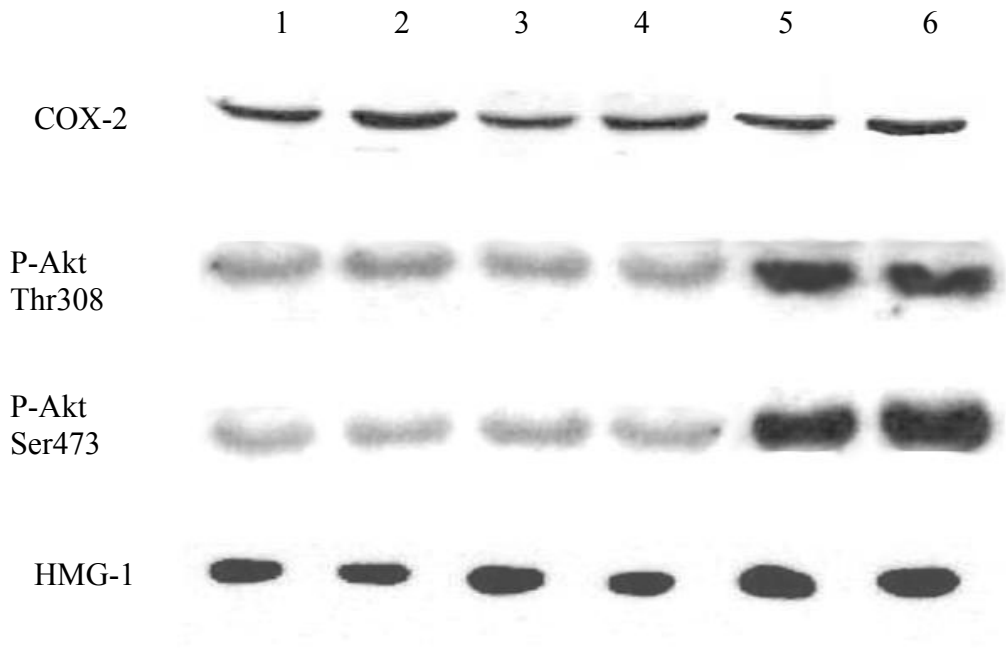

Fig. 17 **Western blot analysis** of COX-2, phospho-Akt Thr308, phospho-Akt Ser473 and HMG-1 expression in the liver and in the small intestine. Lanes; 1: control liver; 2: control small intestine; 3: liver of LPS treated mice; 4: small intestine of LPS treated mice; 5: liver of LPS + PJ34 treated mice; 6: small intestine of LPS + PJ34 treated mice. Control: injected with physiological saline; PJ34: injected with PJ34 (3 × 10 mg/kg, i.p.); LPS: injected with LPS (20 mg/kg, i.p.); LPS+PJ34: pre-treated with PJ34 (3 × 10 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.).

Regardless of the treatment, this activation did not increase in lung and heart (data not shown). LPS-treatment resulted in a slight, but not significant Akt activation (Fig. 17) in liver

(Ser^{473} 4865 ± 455; Thr^{308} 2479 ± 255) and small intestine (Ser^{473} 4996 ± 604; Thr^{308} 2416 ± 306). However, there was a marked increase in the phosphorylation and thereby the activation of Akt in the liver (Ser^{473} 14104 ± 587; Thr^{308} 10942 ± 455) and small intestine (Ser^{473} 14186 ± 688; Thr^{308} 13035 ± 870) of PJ34 + LPS treated mice.

Effect of PARP inhibitors on LPS-induced transcription factor activation.

Ninety minutes after LPS-treatment, NF-κB activation was assessed in lung and liver.

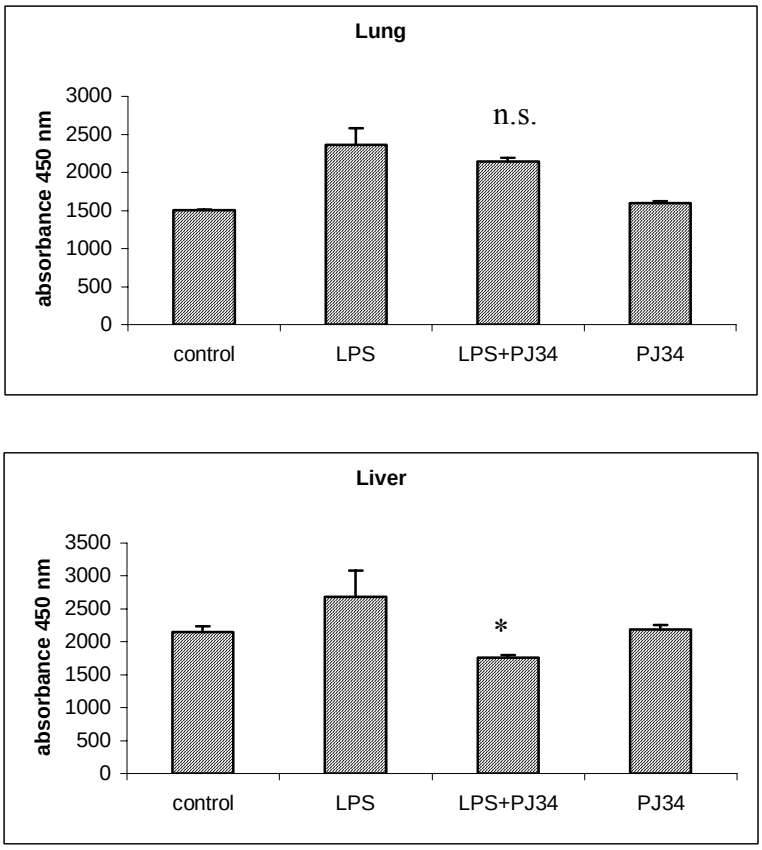

Fig. 18 **Effect of PJ34 on NF-κB activation in the lung and in the liver in mice** at 1.5 hours after LPS injection, with and without PJ34 pre-treatment. Results are expressed as percentages of the positive control provided by the manufacturer. Control: injected with physiological saline; PJ34: injected with PJ34 (3 × 10 mg/kg, i.p.); LPS: injected with LPS (20 mg/kg, i.p.); LPS+PJ34: pre-treated with PJ34 (3 × 10 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.). Data is expressed as mean ± SEM of 12 independent values. n.s. = non significant, ∗ $P < 0.01$ vs. LPS.

LPS-treatment caused a significant increase in NF-κB activation in both tissues. NF-κB activation in the lung was attenuated slightly but statistically not significantly by PJ34 pre-treatment. In contrast to the lung, NF-κB activation in the liver was prevented by PJ34 pre-treatment. PJ34 treatment itself did not have any effect on NF-κB activation in the tissues we studied (Fig. 18).

However, 4-HQN pre-treatment decreased the LPS-induced NF-κB activation in the lung and in the liver also. 4-HQN pre-treatment itself did not have any effect on NF-κB activation in the these tissues (Fig. 19).

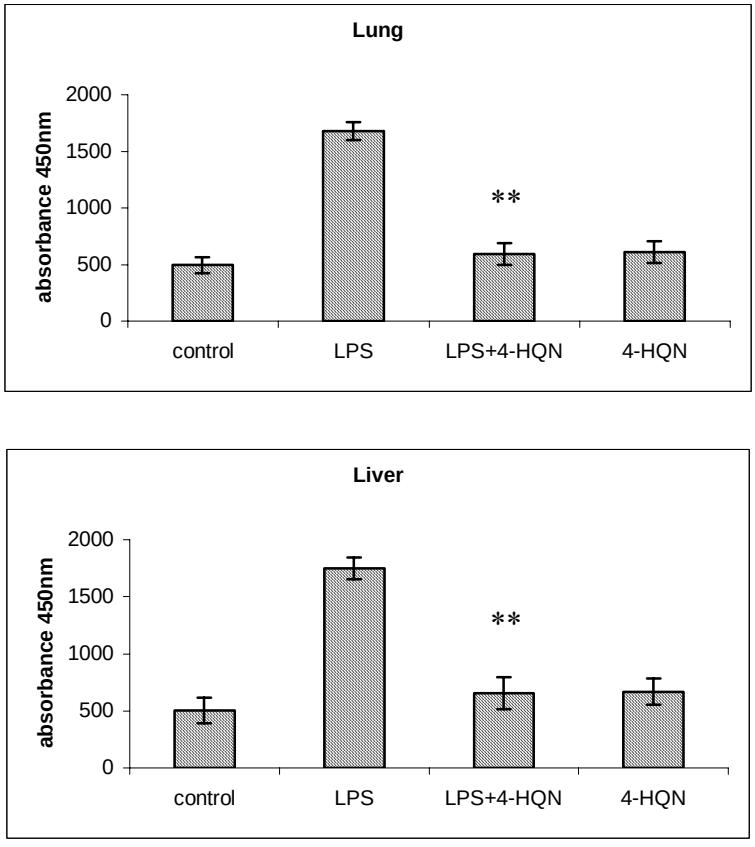

Fig. 19 **Effect of 4-HQN on NF-κB activation in the lung and liver in mice** at 1.5 hours after LPS injection, with and without 4-HQN pre-treatment. Results are expressed as percentages of the positive control provided by the manufacturer. Control: injected with physiological saline; 4-HQN: injected with 4-HQN (3 × 100 mg/kg, i.p.); LPS: injected with LPS (20 mg/kg, i.p.); LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.). Data is expressed as mean ± SEM of 12 independent values. n.s. = non significant, ** $P < 0.01$ vs. LPS.

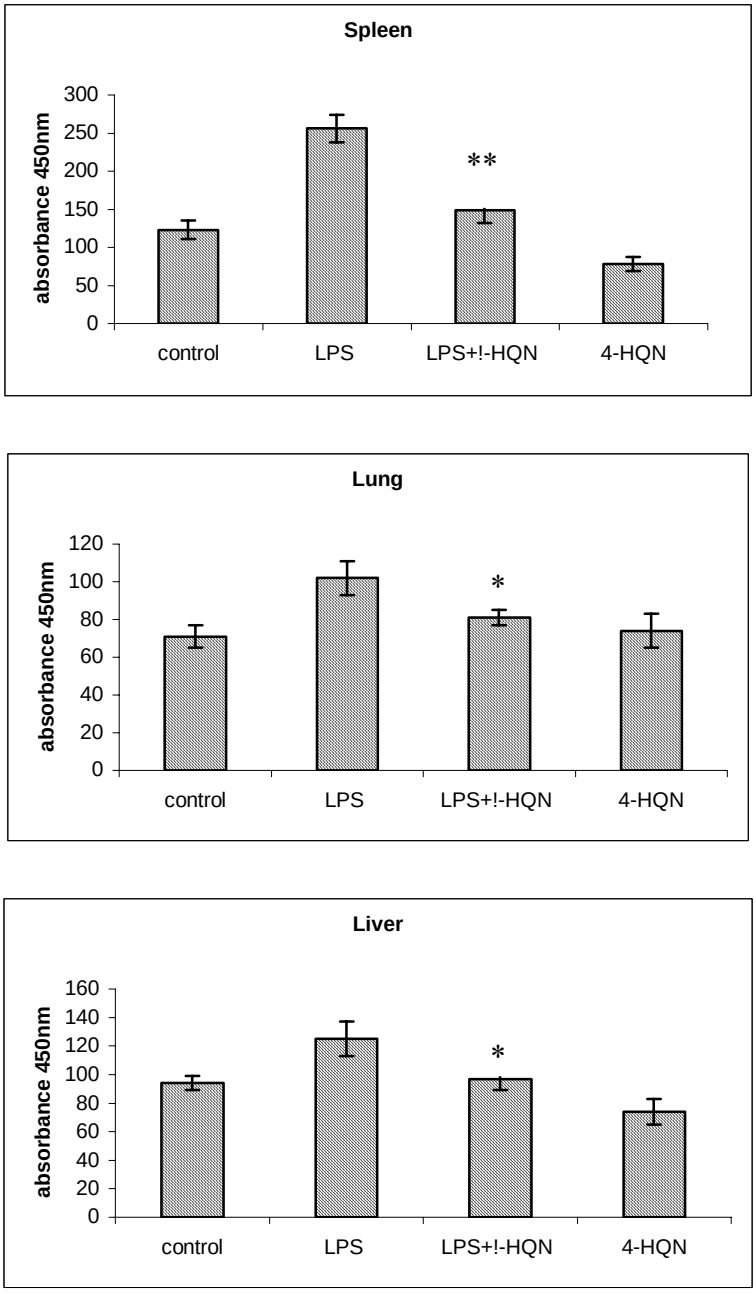

Fig. 20 **Effect of 4-HQN on AP-1 (c-Fos) activation in the spleen, lung and liver in mice** at 1.5 hours after LPS injection, with and without 4-HQN pre-treatment. Results are expressed as percentages of the positive control provided by the manufacturer. Control: injected with physiological saline; 4-HQN: injected with 4-HQN (3 × 100 mg/kg, i.p.); LPS: injected with LPS (20 mg/kg, i.p.); LPS + 4-HQN: pre-treated with 4-HQN (3 × 100 mg/kg, i.p.) and injected with LPS (20 mg/kg, i.p.). Data is expressed as mean ± SEM of 12 independent values. ∗ $P < 0.05$; ∗∗ $P < 0{,}01$ vs. LPS.

Ninety minutes after LPS-treatment, phosphorylation of AP-1 family member c-Fos and thereby the activation of this transcription factor and the effect of 4-HQN pre-treatment on this transcription factor were assessed in spleen, liver and lung. LPS-treatment caused a

significant increase in c-Fos activation in all three tissues. 4-HQN pre-treatment significantly inhibited this activation in the tissues we studied (Fig. 20). 4-HQN treatment itself did not have any effect on c-Fos activation in the studied tissues.

Discussion.

Despite the importance of LPS as a trigger of innate host defenses and inflammation, very little is known of the pathomechanism and actual transduction pathways activated by this endotoxin. Our study demonstrates that PJ34 and 4-hydroxyquinazoline, potent PARP-1 inhibitors, had protective effect in LPS-induced murine septic shock model, in part by activating the PI3 kinase/Akt cytoprotective pathway and inhibiting the ERK 1/2 and p38 MAPK pathways.

Within 2 hours after LPS-injection, the mice exhibited signs of hypomotility, hypothermia, tremor, diarrhea and a characteristic crouching gait. Despite the serious symptoms and the rapid death (within 65 hours), we found surprisingly few pathological changes 16 hours after the LPS treatment. We detected LPS-induced pathological changes only in the liver and small intestine among the tissues studied. In a recent paper, Pacher et al. (2002) describe severe depression of the systolic and diastolic contractile function, tachycardia, and a reduction in mean arterial blood pressure in both rats and mice as a consequence of LPS challenge. They used a much higher dose of LPS than us (55 vs. 20 mg/kg, same serotype), but did not perform a pathological study, so we are unable to determine if the absence of pathological changes in hearts in our study was due to the lower LPS dose. On the other hand, obstructed blood vessels in the liver as well as the hypothermia of the LPS-treated mice reflected disturbed cardiac functions under our experimental conditions too. Necrotic and necrobiotic damage and the appearance of apoptotic cells

observed by us in the liver of LPS treated mice indicated disturbed microcirculation and the resulting tissue hypoxia (Pacher P 2002). It is in accordance with the previous observation that LPS treatment decreases liver oxygen uptake from blood vessels (Collins AS 1990).

The other tissue with observable LPS-induced pathological changes was the small intestine. Atrophy of villi may reflect the diarrhea observed in the LPS-treated animals and is in agreement with the results of Abreu et al. (2001) who found a Fas-mediated apoptosis in intestinal epithelial cells that was sensitized by inhibitors of phosphatidylinositol 3-kinase and opposed by expressing constitutively active Akt.

The *in vivo* response to endotoxin-induced septic shock was detected by MRI technics. Six hours after LPS administration, we observed markedly increased intensities of T_2-weighted MRI images of the thoracic and lower abdominal region of the animals. This method provides a real time insight into inflammatory processes in living animals even in cases where no histologic alteration can be detected. Among all the observed LPS-induced inflammatory responses, we found the most characteristic and most pronounced increases in the gastro-intestinal and in the thoracic tract (Fig. 2) similar to that found in a porcine endotoxic shock model (Oldner A 1999). The importance of these tracts in the mediation of sepsis and multiple-system organ failure is well documented (Boulares AH 2003; Standiford TJ 1995). Pre-treatment of the animals with potent PARP-1 inhibitors, PJ34 and 4-HQN, diminished the thoracic and abdominal inflammatory responses as revealed by T_2 imaging (Fig. 2) and abolished the above mentioned pathological changes.

These data together with the finding that PARP-1 knock out mice were resistant to septic shock (Oliver FJ 1999; Soriano FG 2002) indicate that PARP activity is involved in the mechanism of septic shock. This view was previously challenged (Baechtold F 2001; Albertini M 2000). However, these studies utilised 3-AB, an inhibitor with low potency and considerable toxicity *in vivo*. One of these studies has utilised a cecal ligature and puncture

(CLP) model that may represent a more clinically relevant animal model of sepsis than the LPS challenge used by us. However, they have found higher 24-hour mortality (5/15) in their CLP + 3AB group than in their CLP only group (1/15). This difference, although CLP + 3AB not statistically significant, may reflect the in vivo toxicity of 3-AB. It is also noteworthy that since in the vehicle treated CLP group only a very low mortality was detected, the design of this above referenced particular study (Baechtold F 2001) would not have permitted a demonstration of significant improvements in survival rate by PARP inhibitors or by any other pharmacological agent.

In order to determine whether PARP-1 inhibition interferes with early or late mediators of LPS-induced septic shock, we performed survival studies in which we administered the PARP-1 inhibitors 1 or 6 hours after the LPS challenge. With a decreasing efficacy (compare mortality of 4/30 for +1 hour and 17/30 for +6 hours), PJ34 protected the mice against LPS-induced death even when added after the induction of the septic shock. These findings suggest that PJ34 may interfere with both the early and late mediators of the effect of LPS. We have found that 4-HQN had a protective effect (30% survivor) when it was added 1 but not when it was added 6 hours after LPS injection suggesting that 4-HQN may interfere preferably with the early mediators of the effect of LPS (Fig. 1). To support these observations we studied some important late mediators of septic shock by Western blotting and found that LPS treatment did not affect the HMG-1 and COX-2 expression in lung, heart, small intestine, liver or spleen and PJ34 and 4-HQN did not have any additional effect on these factors (data not shown). It suggests that the beneficial effect of the post-treatment of PJ34 was evolving due a different mechanism.

TNF-α is a substantial early mediator of endotoxemia since the production of this cytokine returned to a normal level 4 hours after LPS treatment (James PE 2002; Zanetti G 1992). When administered before LPS treatment, both PARP-1 inhibitor attenuated the LPS-

induced elevation in the serum TNF-α concentrations by approximately 50 % (Fig. 3), consistent with the notion that PJ34 and 4-HQN partially inhibits the expression of TNF-α gene.

Due to the importance of the functional state of different organs during septic shock, we investigated in lung, liver and spleen various protein kinases that lead to transcription factor activation. Previous works had shown that LPS did induce activation of MAPK pathways in different cell lines, and it played key role in the transduction of the LPS signal between the extracellular receptor and the cytoplasmic and nuclear response resulting in activation of gene expression (Bozinovski et al., 2002; Dumitru et al., 2000). Furthermore, several papers showed that there is a cross-talk between ERK and other MAP kinases in different cell lines (Guha M 2002; Dumitru CD 2000; Xiao YQ 2002). In agreement with these results, we found that ERK and p38 MAPK had indeed a similar LPS-induced activation in lung but not in spleen and liver. In monocytes, macrophages and Tpl2 knockout mice, a functional association between ERK and JNK was reported that was not detectable in our BALB/c mice sepsis model. However, we have found that p90RSK, a downstream target of ERK, showed a similar activation pattern in lung as did ERK and p38 in response to the LPS treatment. The significance of this finding can be understood by considering that phosphorylated (i.e. activated) p90RSK can activate nuclear transcription factors such as c-Fos and NF-κB.

Since ERK is a key element of the signal transduction pathway that regulates the LPS-mediated transcription factor activity, inhibition of this MAP kinase can contribute to reducing the endotoxin-induced inflammatory responses. Under our experimental conditions, ERK was activated by LPS in all the tissues studied (liver, lung and spleen) but p38 MAP kinase had detectable activation only in lung indicating that the signalling from cell surface CD14-TLR4/MD2 LPS receptor to the activation of the different branches of MAP kinases

has tissue specific components. Furthermore, we did not find detectable activation of JNK pathway that suggests that there is no functional association between either JNK pathway and cell surface LPS receptor or ERK and JNK pathways in these tissues in our experimental system. Interestingly, the phosphorylation of the ERK substrate p90RSK also showed tissue specificity (Fig. 6) indicating that ERK activation does not necessarily trigger the activation of p90RSK and the activation of p90RSK-dependent transcription factors. LPS induced the activation of ERK, p90RSK and p38 MAP kinase but not JNK in lung, and there was a significant increase in the activation of NF-κB and c-Fos transcription factors showing that gene expressions in lung are regulated by ERK or p38 MAP kinase but not by JNK pathway. In liver and spleen, out of the MAP kinases, only the ERK pathway was activated by LPS, and it is likely that this pathway activated the c-Fos and NF-κB transcription factors. In the absence of PARP-1 inhibitors, LPS did not affected PI-3-kinase/Akt pathway, therefore, MAP kinase pathways had to play the major role in activation of Ap-1 and NF-κB.

When comparing the pattern of the activation and inhibition of the kinase cascades in the different organs of LPS or LPS + PARP-1 inhibitors treated mice, there was sometimes a poor correlation with the MRI data. One reason for this is that the hyperintensity in MRI signals due to inflammation and oedema originates from the intercellular space and not the organs themselves. Furthermore, by masking contrast, this hyperintensity makes the outlining of certain anatomical features within the field difficult or sometimes impossible. Thus, we found both the feasibility and relevance of such a comparison to be questionable. On the other hand, both the inflammatory response visualized by the MRI technique and the changes in the activities of the kinase cascades were clearly the result of the LPS treatment, and were attenuated by PARP-1 inhibitors. A more profound understanding of the events and mechanisms underlying LPS-induced septic shock could help to resolve this problem.

The most clear effect of PARP-1 inhibitors on the kinase cascades was the activation of Akt in the absence and in the presence of LPS in all studied tissues (Fig. 4). In monocytes, it was found that inhibition of PI-3-kinase/Akt pathway can activate MAP kinases (Guha M 2002). Therefore, it is likely that activated Akt in the presence of PARP-1 inhibitor mediates the inhibition of MAP kinases and so the inhibition of NF-κB and AP-1 transcription factors resulting in inhibition of inflammatory gene expression. In conclusion, the most important protective effect of PARP-1 inhibitors in different organs can be the Akt activation and MAP kinase inhibition, and the inhibition of related transcription factors (Fig. 10).

Since Akt activation can be induced by structurally different PARP-1 inhibitors, such as quinazoline derivatives, phenantridine derivatives and carboxamino benzimidazol derivatives (unpublished data), it is likely that this effect is related to the PARP-inhibitory property of these molecules, although, it is not clear how the inhibition of the nuclear PARP can activate cytoplasmic Akt. Furthermore, Luo et al. have recently showed in an other experimental model that PARP inhibitors prevent deactivation of Akt in N-methyl-D-aspartate-induced neurotoxicity (Luo HR 2003). In spite of the lack of the precise molecular mechanism, the finding that PARP-1 inhibitors reverse the inflammatory processes and organ damages (Fig 2) probably via the activation one of the most important protective kinase cascade, the PI-3-kinase/Akt pathway, provide a novel possibility to prevent multiorgan failure in septic shock. While the Akt activation seems to occur in all studied tissues (Fig 4), the regulation of transcription factor activation appears to have some tissue specificity indicating that there are some tissue specific components between the MAP kinase pathway and the transcription factors regulated by this pathway. It can explain that Akt activation by PJ34 could supress NF-κB activation only in liver (Fig) and by 4-HQN in lung and in liver (Fig. 8), while c-Fos activation was suppressed by 4-HQN in all studied tissues (lung, liver, spleen) (Fig. 9).

Isoquinasolines such as 4-HQN and phenantridinons such as PJ34 inhibit PARP-1 activity by competitive binding to the NAD binding site (Banasik M 1992; Jagtap P 2002). Thus, it is likely that all PARP isoforms are inhibited by PJ34 and 4-HQN since the catalytic site is highly conserved among the various PARP isoforms. In fact, no effects of these drugs on enzymes other than PARP have been reported. The high potency of the compound on PARP together with the fact that our findings on PJ34 and 4-HQN treated animals were very similar to those that were reported in PARP-1 deficient mice makes it likely that the principal action of the drugs were mediated via PARP inhibition. The various classes of recently emerging potent, non-toxic PARP inhibitors will help to further clarify this question.

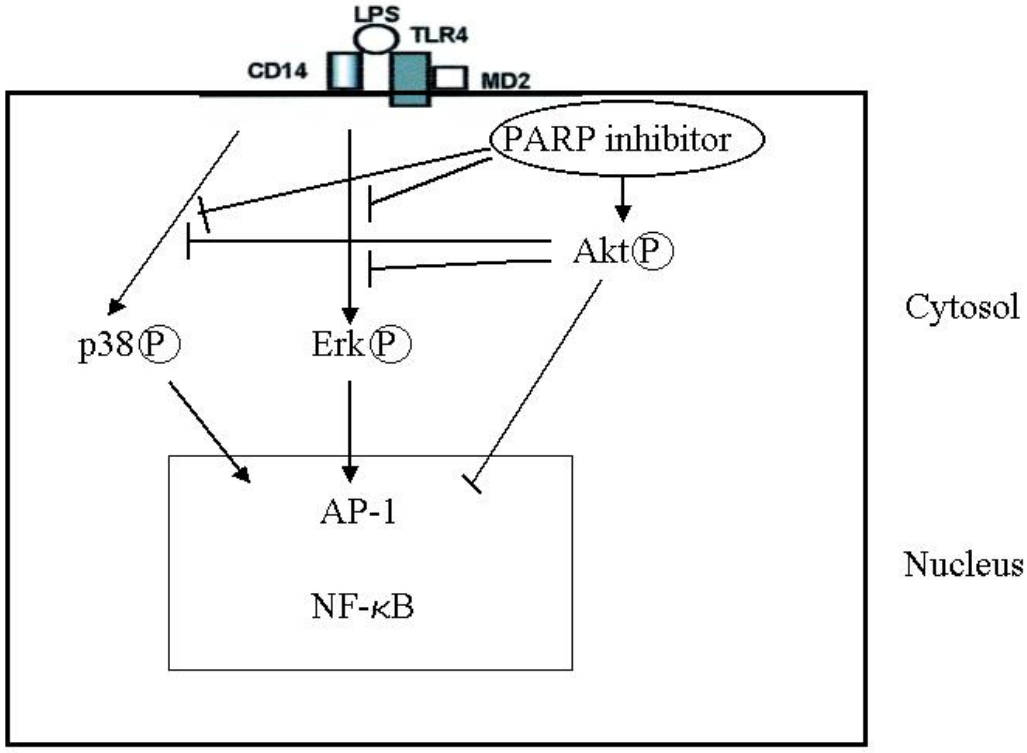

Fig. **Schematic representation of the effect of 4-hydroxyquinazoline on Akt and MAPK activation, and NF-kappaB and Ap-1 activation**. Binding of LPS to the CD14 and TLR4/MD2 complex activates the MAPK pathways. PARP-1 inhibitor directly or indirectly activates the PI3 kinase/Akt pathway and inactivates the ERK ½ and p38 MAPKs. Activation of Akt via PARP-1 inhibition can also inactivate these kinases. Inactivation of nuclear transcription factors (NF-κB, AP-1) is the consequence, at least partially, of the forementioned processes.

Taken together, our data show that LPS induces a different extent of MAP kinase activation in different organs, which can activate Ap-1 and NF-κB transcription factors in a tissue specific manner. Activation of these transcription factors induces activation of pro-inflammatory genes that are most likely responsible for the tissue damages during septic shock. PARP-1 inhibitors beside their well-known effect of inhibiting NAD^+ and ATP depletion, influence LPS-induced transcription factor activation and gene expression. These effects of PARP-1 inhibitors are mediated by the activation of PI-3-kinase/Akt pathway which can inhibit MAP kinase activation and can attenuate transcription factor activation and inflammatory tissue damage (Fig. 10) in a tissue specific manner.

List of Publications.

Publications supporting the dissertation:

Veres B., Radnai B., Gallyas F. Jr, Varbiro G., Berente Z., Osz E., Sumegi B.: Regulation of kinase cascades and transcription factors by a poly-(ADP-ribose) polymerase-1 inhibitor – 4-hydroxyquinazoline – in LPS-induced inflammation in mice. **J. Pharm. Exp. Ther.** (2004) In press

Veres B., Gallyas F., Varbiro G., Berente Z., Osz E., Szekeres G., Szabo C., Sumegi B.: Decrease of the inflammatory response and induction of the Akt/protein kinase B pathway by poly-(ADP-ribose) polymerase 1 inhibitor in endotoxin-induced septic shock. **Biochem. Pharmacol.** (2003) 65(8):1373-82.

Other publications:

Varbiro G., Toth A., Tapodi A., Bognar Z., *Veres B.*, Sumegi B. and Gallyas F.: Protective effect of amiodarone but not N-desethyl-amiodarone on postischemic hearts through the inhibition of mitochondrial permeability transition. **J. Pharm. Exp. Ther.** (2003) 307(2): 615-625.

Varbiro G., Toth A., Tapodi A., *Veres B.*, Gallyas F., Sumegi B.: The concentration dependent mitochondrial effect of amiodarone. **Biochem. Pharmacol.** (2003) 65(7):1115-28.

Varbiro G., *Veres B.*, Gallyas F., Sumegi B.: Direct effect of taxol on free radical formation and mitochondrial permeability transition. **Free Rad. Biol. Med.** (2001) 31(4):548-58.

Abstracts, posters and presentations supporting the dissertation:

Veres B., Gallyas F. Jr., Varbiro G., Berente Z., Osz E., Szekeres G., Szabo C., Sumegi B.: Decrease of the inflammatory response and induction of the Akt/protein kinase B pathway by poly-(ADP-ribose) polymerase 1 inhibitor in endotoxin-induced septic shock. (Abstract) **Experimental Clinical Cardiology** 2003, 8 (1), pp. 52.

Veres B., Gallyas F. Jr., Berente Z., Osz E., Szekeres Gy., Sumegi B., Szabo C.: Pharmacological inhibition of poly(ADP-ribose) polymerase (PARP) protects mice against LPS-induced septic shock by the decreasing the inflammatory response and by enhancing the cytoprotective Akt/protein kinase B pathway. (Abstract) **FASEB Journal** (2003) 17:A242

Veres B., Radnai B., Gallyas F. Jr, Várbíró G., Berente Z., Ősz E., Sümegi B.: Effect of poly-(ADP-ribose) polymerase-1 inhibitor – 4-hydroxyquinazoline – on LPS-induced inflammation: modulation of kinase cascades and activation of transcription factors. Pecs, IV. International Symposium on Myocardial Cytoprotection, 2003. szeptember 25-27.

Veres B., Radnai B., Gallyas F. Jr, Várbíró G., Berente Z., Ősz E., Sümegi B.: Effect of poly-(ADP-ribose) polymerase-1 inhibitor – 4-hydroxyquinazoline – on LPS-induced inflammation: modulation of kinase cascades and activation of transcription factors. Debrecen, Hungary, FEBS Advanced Course: Poly-ADP-ribosylation in health and disease. 2003. August 27-30.

Veres B., Tapodi A., Radnai B., Várbíró G., Sümegi B.: A 4-hidroxikinazolin hatása az LPS-indukálta szeptikus sokkra. Sümeg XXXIII. Membrán-Transzport Konferencia, 2003. május 20-23.

Veres B., Gallyas F. Jr., Berente Z., Osz E., Szekeres Gy., Szabo C., Sumegi B.: Pharmacological inhibition of poly(ADP-ribose) polymerase (PARP) protects mice against LPS-induced septic shock by the decreasing the inflammatory response and by enhancing the cytoprotective Akt/protein kinase B pathway. San Diego, CA., Experimental Biology 2003. Translating the Genome, 2003. Április 11-15.

Veres B., Tapodi A., Varbiro G., ifj. Gallyas F., Sumegi B.: PARP-gátlók hatása az LPS indukálta szepszisre. Sümeg, XXXII. Membrán-Transzport Konferencia, 2002. Május 21-24.

Veres B., Varbiro G., Tapodi A., ifj. Gallyas F., Sumegi B.: PARP-gátlók hatása a szeptikus sokkra. Siófok, X. Sejt- és Fejlődésbiológiai Napok, 2002. Március 27-29.

Other abstracts, posters and presentations:

Kovacs K., Toth A., Deres P., Osz E., *Veres B.*, Radnai B., Sumegi B.: Impact of poly(ADP-ribose) polymerase inhibitors on the activation of PI3-kinase-Akt and mitogen-activated protein kinase pathways in postischemic myocardium. (Abstract) **Free Radical Research** 2003, 37, pp.101.

Bognar Z., Varbiro G., *Veres B.*, Kovacs K., Osz E., Sumegi B.: The direct induction of mitochondrial free radical formation by taxol. (Abstract) **Free Radical Research** (2003) 37, pp.110.

Varbiro G., Tapodi A., *Veres B.*, Gallyas F. Jr., Sumegi B.: The induction of COX-2 expression through an NFkB dependent pathway in liver cells by amiodarone. (Abstract) **Z. Gastroenterologie** (2002)

Varbiro G., *Veres B.*, ifj. Gallyas F., Sumegi B.: The effect of Taxol on liver mitochondrial free radical formation and mitochondrial permeability transition. (Abstract) **Z. Gastroenterologie** (2001) 5:428

Varbiro G., Bognar Z., *Veres B.*, Tapodi A., Radnai B., Sumegi B.: The effect of phosphatidic acid on the permeability transition and membrane potential in isolated rat liver mitochondria. Madrid, Spain, CNIO Cancer Conference: Apoptosis and Cancer. 2003. December 1-3.

Kovacs K., Toth A., Deres P., Osz E., *Veres B.*, Radnai B., Sumegi B.: Impact of poly(ADP-ribose) polymerase inhibitors on the activation of PI3-kinase-Akt and mitogen-activated protein kinase pathways in postischemic myocardium. Ioannina, Greece, SFRR-Europe Meeting 2003, Free Radicals and Oxidative Stress: Chemistry, Biochemistry and Pathophysiological Implications, 2003. June 26-29.

Bognar Z., Varbiro G., *Veres B.*, Kovacs K., Osz E., Sumegi B.: The direct induction of mitochondrial free radical formation by taxol. Ioannina, Greece, SFRR-Europe Meeting 2003,

Free Radicals and Oxidative Stress: Chemistry, Biochemistry and Pathophysiological Implications, 2003. June 26-29.

Tapodi A., *Veres B.*, Varbiro G., ifj. Gallyas F., Sumegi B.: PARP-gátlók hatása oxidatív stresszben. Sümeg, XXXII. Membrán-Transzport Konferencia, 2002. Május 21-24.

Varbiro G., *Veres B.*, ifj. Gallyas F., Sumegi B.: The effect of Taxol on liver mitochondrial free radical formation and mitochondrial permeability transition. Balatonaliga, 43rd Annual Meeting of The Hungarian Society of Gastroenterology, 2001. Jun 5-9.

Tapodi A., *Veres B.*, Varbiro G., ifj. Gallyas F., Sumegi B.: Az Amiodarone mitokondriális hatásai. Sümeg, XXXI. Membrán-transzport konferencia, 2001. Május 22-25.

Varbiro G., *Veres B.*, ifj. Gallyas F., Tapodi A., Sumegi B.: A Taxol közvetlen hatása a mitokondriális permeability transitionra és a szabadgyökképződésre. Sümeg, XXXI. Membrán-transzport konferencia, 2001. Május 22-25.

References.

Akira S. et al. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2:675-680.

Albertini M. et al. (2000) Role of poly-(ADP-ribose) synthetase in lipopolysaccharide-induced vascular failure and acute lung injury in pigs. J. Crit. Care 15:73-83.

Althaus F. R. (1992) Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. J. Cell Sci. 102:663-670.

Althaus F. R. et al. (1990) The poly-ADP-ribosylation system of higher eukaryotes: a protein shuttle mechanism in chromatin? Acta. Biol. Hung. 41.9-18.

Alvarez-Gonzalez R. et al. (1994) Enzymology of ADP-ribose polymer synthesis. Mol. Cell. Biochem. 138:33-37.

Baechtold F. et al. (2001) Does the activation of poly (ADP-ribose) synthetase mediate tissue injury in the sepsis induced by cecal ligation and puncture? Shock 16:137-142.

Banasik M. et al. (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J. Biol. Chem. 267:1569-1575.

Bauer P. I. et al. (1995) Reversion of malignant phenotype by 5-iodo-6-amino-1,2-benzopyrone a non-covalently binding of poly(ADP-ribose) polymerase. Biochimie 77:374-7.

Bauer P. I. et al. (2001) Molecular interactions between poly(ADP-ribose) polymerase (PARP1) and topoisomerase I (Topo I): identification of topology of binding. FEBS Lett. 506:239-242.

Bonfoco E. et al. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with NMDA or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. U.S.A. 92:7162-6.

Boulares A. H. et al (2003) Gene knockout or pharmacological inhibition of poly(ADP-ribose) polymerase-1 prevents lung inflammation in a murine model of asthma. Am. J. Respir. Cell Mol. Biol. 28:322-9.

Boulikas T. (1991) Relation between carcinogenesis, chromatin structure and poly(ADP-ribosylation). Anticancer Res. 11:489-527.

Bozinovski et al. (2002) Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NF-kappaB and AP-1 in vivo. J. Biol. Chem. 277:42808-42814.

Buch S. J. and Sassone-Corsi P. (1990) Dimers, leucine zippers and DNA-binding domain. Trends Genet. 6:36-40.

Callebaut I. and Mornon J. P. (1997) From BRCA1 to RAP1: a widespread BRCT modul closely associated with DNA repair. FEBS Lett. 400:25-30.

Cocha I. et al. (1989) Intracellular distribution of poly(ADP-ribose) synthetase in rat spermatogenic cells. Exp. Cell Res. 180:353-366.

Collins A. S. (1990) Gastrointestinal complications in shock. Crit. Care Nurs. Clin. North Am. 2:269-277.

D'Amours D. et al. (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. 342:249-268.

Dantzer F. et al. (2000) Base excision repair is impaired in mammalian cells lacking poly(ADP-ribose) polymerase-1. Biochemistry 39:7559-7569.

Daum J. M. and Fenton. M. J. (2000) Interleukin-1/Toll receptor family members: receptor structure and signal transduction pathways. J. Interferon Cytokine Res. 20:843-855.

De Murcia G. et al. (1994) Struture and function of poly(ADP-ribose) polymerase. Mol. Cell Biochem. 138:15-24.

Desnoyers S. et al. (1996) Association of poly(ADP-ribose) polymerase with nuclear subfractions catalyzed with sodium tetrathionate and hydrogene peroxide crosslinks. Mol. Cell Biochem. 159:155-161.

Dumitru C. D. et al. (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103:1071-1083.

Duriez P. J. et al. (1997) Characterization of anti-peptide antibodies directed towards the automodification domain and apoptotic fragment of poly(ADP-ribose) polymerase. Biochim. Biophys. Acta 1334:65-72.

Edmead C. et al. (1999) Thrombin activates transcription factors sp1, NF-kappaB, and CREB: importance of the use of phosphatase inhibitors during nuclear protein extraction for the assessment of transcription factor DNA-binding activities. Anal. Biochem. 275:180-6.

Eliasson M. J. et al. (1997) Poly(ADP-ribose) polymerase gene desruption renders mice resistant to serebral ischemia. Nat. Med. 3:1089-1095.

Gradwohl G. et al. (1990) The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA. Proc. Natl. Acad. Sci. U.S.A. 87:2990-4.

Gu Y. et al. (1995) Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. J. Biol. Chem. 270:18715-8.

Guha M. and Mackman N. (2002) The phosphatidylinositol 3-kinase-Akt pathway limits LPS activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J. Biol. Chem. 277:32124-32132.

Ha H. C. and Snyder S. H. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc. Natl. Acad. Sci. U.S.A. 96:13978-13982.

Halmosi R. et al. (2001) Effect of poly(ADP-ribose) polymerase inhibitors on the ischemia-reperfusion-induced oxidative cell damage and mitochondrial metabolism in Langendorff heart perfusion system. Mol. Pharmacol. 59:1497-1505.

Ikejima M. et al. (1990) The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks and the consequent enzyme activation: other stuctures recognize intact DNA. J. Biol. Chem. 265:21907-21913.

Jagtap P. et al. (2002) Novel phenanthridinone inhibitors of poly (adenosine 5'-diphosphate-ribose) synthetase: potent cytoprotective and antishock agents. Crit. Care. Med. 30:1071-1082.

James P. E. et al. (2002) Endotoxin-induced liver hypoxia: defective oxygen delivery versus oxygen consumption. Nitric Oxide 6**:**18-28.

Kaisho T. and Akira S. (2000) Critical roles of Toll-like receptors in host defense. Crit. Rev. Immunol. 20:393-405.

Kanai M. et al. (2000) Poly(ADP-ribose) polymerase localizes to the centrosomes and chromosomes. Biochem. Biophys. Res. Commun. 278:385-9.

Kasid U. N. et al. (1989) Poly(ADP-ribose)-mediated post-translational modification of chromatin-associated human topoisomerase I: inhibitory effects on catalytic activity. J. Biol. Chem. 264:18687-18692.

Kaufman S. H. et al. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer. Res. 53:3976-9.

Kim J. W. et al. (2000) Inhibition of homodimerization of poly(ADP-ribose) polymerase by its C-terminal cleavage products produced during apoptosis. J. Biol. Chem. 275:8121-5.

Kovacs K. et al. (2002) Effect of poly(ADP–ribose) polymerase inhibitors on the activation of ischemia-reperfusion induced inflammatory processes in Langendorff perfused hearts. In: Boros M editor. Proceedings of the 37th Congress of the European Society for Surgical Research 2002. pp. 63-68. Monduzzi Editione, Italy

Kuhnle S. et al. (1999) Prevention of endotoxin-induced lethality, but not of liver apoptosis in poly(ADP-ribose) polymerase-deficient mice. Biochem. Biophys. Res. Commun. 263:433-8.

Kumari S. R. et al. (1998) Functional interactions of p53 with poly(ADP-ribose) polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and noncovalent binding of p53 to the M® 85000 proteolytic fragment. Cancer Res. 58:5075-8.

Le Rhun Y. et al. (1998) Cellular responses to DNA damage in the absence of poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Commun. 245:1-10.

Leist M. et al. (1997) Apoptosis in the absence of poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Commun. 233:518-522.

Liaudet L. et al. (2000) Protection against hemorrhagic shock in mice genetically deficient in poly(ADP-ribose) polymerase. Proc. Natl. Acad. Sci. U.S.A. 97:10203-8.

Lin K. T. et al. (1995) Peroxynitrite-induced apoptosis in HL-60 cells. J. Biol. Chem. 270:487-90.

Lindahl T. et al. (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem. Sci. 20:405-411.

Luo H. R. et al. (2003) Akt as a mediator of cell death. Proc Natl Acad Sci USA 100**:**11712-7.

Mandir A. S. (1999) Poly(ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism. Proc. Natl. Acad. Sci. U.S.A. 96:5774-9.

Marton A. et al. (2001) Protective effects of a novel PARP inhibitor in a porcine model of sepsis. FASEB J. 15:A939.

Masutani M. et al. (1999) Function of poly(ADP-ribose) polymerase in response to DNA damage: gene-desruption study in mice. Mol. Cell Biochem. 193:149-152.

McNerney R. et al. (1989) Changes in mRNA levels of poly(ADP-ribose) polymerase during activation of human lymphocytes. Biochim. Biophys. Acta 1009:185-7.

Mendoza-Alvarez H. and Alvarez-Gonzalez R. (1999) Biochemical characterization of mono(ADP-rybosil)ated poly(ADP-ribose) polymerase. Biochemistry 38:3948-3953.

Menegazzi M. et al. (1988) Induction of poly(ADP-ribose) polymerase gene expression in lectin-stimulated human T lymphocytes is dependent on protein synthesis. Biochem. Biophys. Res. Commun. 156:995-9.

Menegazzi M. et al. (1991) Differential expression of poly(ADP-ribose) polymerase and DNA polymerase beta in rat tissues. Exp. Cell Res. 197:66-74.

Nicholson D. W. et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37-43.

O'Connor M. et al. (1997) Role of peroxynitrite in the protein oxidation and apoptotic DNA fragmentation in immunostimulated vascular smooth muscle cells. Shock 8:439-443.

Ogura T. et al. (1990) Striking similarity of the distribution patterns of the poly(ADP-ribose) polymerase and DNA polymerase beta among various mouse organs. Biochem. Biophys. Res. Commun. 172:377-384.

Oldner A. et al. (1999) Tissue hypoxanthine reflects gut vulnerability in porcine endotoxin shock. Crit Care Med 27:790-7.

Oliver F. J. (1999) Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly(ADP-ribose) polymerase-1 deficient mice. EMBO J. 18:4446-4454.

Ozes et al. (1999) NF-kappaB activation by tumor necrosis factor requires the Akt serine-threonine kinase. Nature 401**:**82-5.

Pacher P. et al (2002) Role of poly(ADP-ribose) polymerase activation in endotoxin-induced cardiac collapse in rodents. Biochem. Pharmacol. 64:1785-1791.

Pieper A. A. (1999) Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes. Proc. Natl. Acad. Sci. U.S.A. 96:3059-3064.

Salgo M. G. et al. (1995) Peroyxynitrite causes apoptosis in rat thymocytes. Biochem. Biophys. Res. Commun. 215:1111-8.

Scovassi A. I. et al. (1993) ADP-ribosylation of nonhistone proteins in HeLa cells: modification of DNA topoisomerase II. Exp. Cell Res. 206:177-181.

Shall S. (2002) Poly(ADP-ribosylation) - a common control process? Bioessays 24:197-201.

Shall S. and de Murcia G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mutat. Res. 460:1-15.

Standiford T. J. et al. (1995) Macrophage inflammatory protein-1 alpha mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. J Immunol. 155:1515-1524.

Sugo N. et al. (2000) Neonatal lethality with abnormal neurogenesis in mice deficient in DNA polymerase beta. EMBO J. 19:1397-1404.

Szabados E. et al. (2000) BGP-15, a nicotinic amidoxime derivate protecting heart from ischemia reperfusion injury through modulation of poly(ADP-ribose) polymerase. Biochem. Pharmacol. 59:937-945.

Szabo A. et al. (1998) 3-Aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase, improves hemodynamics and prolongs survival in a porcine model of hemorrhagic shock. Shock 10:347-353.

Szabo C. et al. (1997) Inhibition of poly(ADP-ribose) synthetase attenuates neutrophil recruitment and exerts anti-inflammatory effects. J. Exp. Med. 186:1041-9.

Szabo C. et al. (1997) Regulation of components of the inflammatory response by 5-iodo-6-amino-1,2-benzopyrone, and inhibitor of poly(ADP-ribose) synthetase and pleiotropic modifier of cellular signal pathways. Int. J. Oncol. 10:1093-8.

Tebbs R. S. et al. (1999) Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Dev. Biol. 208:513-529.

Tewari M. et al. (1995) Yama/CPP32 beta, a mammalian homolog of CED-2, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81:801-9.

Thibodeau J. et al. (1993) Expression in Escherichia coli of the 36 kDa domain of poly(ADP-ribose) polymerase and investigation of its DNA binding properties. Biochim. Biophys. Acta 1163:49-53.

Trucco C. et al. (1998) DNA repair defect in poly(ADP-ribose) polymerase deficient cell lines. Nucleic Acids Res. 26:2644-9.

Uchida K. et al. (1993) Cloning of cDNA encoding Drosophila poly(ADP-ribose) polymerase: leucine zipper in the automodification domain. Proc. Natl. Acad. Sci. U.S.A. 90:3481-5.

Virag L. et al. (1998) Poly(ADP-ribose) synthetase activation mediates mitochondrial injury during oxidant-induced cell death. J. Immunol. 161:3753-9.

Vodenicharov M. D. (2000) Base excision repair is efficient in cells lacking poly(ADP-ribose) polymerase 1. Nucleic Acids Res. 28:3887-3896.

Wang Z. Q. et al. (1997) PARP is important for genomic stability but dispensable in apoptosis. Genes Dev. 11:2347-2358.

Wesierska-Gadek J. and Schmid G. (2000) Overexpressed poly(ADP-ribose) polymerase delays the release of rat cells from p53-mediated checkpoint. J. Cell Biochem. 80:85-103.

Wesierska-Gadek J. et al. (1996) ADP-ribosylation of p53 tumor supressor protein: mutant but not wild-type p53 is modified. J. Cell Biochem. 62:92-101.

Xanthoudakis S. et al. (1996) The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc. Natl. Acad. Sci. U.S.A. 93:8919-8923.

Xiao Y. Q. et al. (2002) Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. J. Biol. Chem. 277:14884-14893.

Zanetti G. et al. (1992) Cytokine production after intravenous or peritoneal gram-negative bacterial challenge in mice. Comparative protective efficacy of antibodies to tumor necrosis factor-alpha and to lipopolysaccharide. J Immunol 148**:**1890-7.

Zucconi G. et al. (1992) DNA repair enzymes in the brain: DNA polymerase beta and poly(ADP-ribose) polymerase. Ann. NY Acad. Sci. 663:432-5.

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