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

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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.

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.

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). 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. 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.

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 or with the potent, novel PARP inhibitors 5-iodo-6-amino-1,2-benzopyrone 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. Similar results were obtained by another independent group, led by DeMurcia. 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. 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.

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. 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 or partial 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 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, $C_{17}H_{18}ClN_3O_2 \cdot 0.5H_2O$) is a selected water soluble member of the PJ series of phenanthridinone-based PARP-1 inhibitors.

4-HQN: Several different quinazoline-derivatives were reported as potent PARP-1 inhibitors and 4-HQN is one of them. It is structurally unrelated to PJ34 and it was reported to protect hearts from ischemia-reperfusion-induced damages.

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 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, 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. 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).

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™ 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. Agreement between MRI data and histopathology was found to be satisfactory for acute local infection in mouse muscle.

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.

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. PJ34 or 4-HQN treatment itself did not induce death or any obvious damage.

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.

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.

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 that was prevented by PJ34 pre-treatment but not by 4-HQN. In the liver, we observed focal necrotic and necrobiotic damages, sporadic basophilic apoptotic nuclei and blood vessels obstructed with cell-rich material either attaching to the wall of the blood vessels or obstructing the whole lumen. 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. 4-HQN had no significantly protective effect on the above mentioned pathological changes. Mice treated with PJ34 or 4-HQN alone did not show any pathological changes.

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. 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.

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. 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. 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.

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.

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 %). However, in spleen and in liver LPS did not induce the activation of p90RSK and 4-HQN had no additional effect.

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.

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.

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.

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) or in animals treated with PJ34 alone.

Regardless of the treatment, this activation did not increase in lung and heart. LPS-treatment resulted in a slight, but not significant Akt activation 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. 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.

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.

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. 4-HQN treatment itself did not have any effect on c-Fos activation in the studied tissues.

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.

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