Different mechanisms in the regulation of NF-κB and kinase cascades in inflammation

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Contents

List of publications

This work is based on the following articles:

1. ***Radnai B***, Tucsek Z, Bognar Z, Antus C, Mark L, Berente Z, Gallyas F Jr, Sumegi B, Veres B.: Ferulaldehyde, a water-soluble degradation product of polyphenols, inhibits the lipopolysaccharide-induced inflammatory response in mice.
J Nutr. 2009 Feb;139(2):291-7.
Impact factor: **3.647** *(2008)*
Citation: **0**

2. 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 lipopolysaccharide-induced inflammation in mice.
J Pharmacol Exp Ther. 2004 Jul;310(1):247-55.
Impact factor: **4.335** *(2004)*
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Further publications:

3. Bognar Z, Kalai T, Palfi A, Hanto K, Bognar B, Mark L, Szabo Z, Tapodi A, ***Radnai B***, Sarszegi Z, Szanto A, Gallyas F Jr, Hideg K, Sumegi B, Varbiro G.: A novel SOD-mimetic permeability transition inhibitor agent protects ischemic heart by inhibiting both apoptotic and necrotic cell death.
Free Radic Biol Med. 2006 Sep 1;41(5):835-48.
Impact factor: **5.440** *(2006)*
Citations: **11** *(2010.I.7.)*

Total impact factor: **13.422**
Total citations: **50** *(2010.I.7.)*

Abbreviations

3-AB, 3-aminobenzamide

AP1, activator protein 1

COX, cyclooxygenase

ERK1/2, extracellular signal-regulated kinase ½

FA, ferulaldehyde

FCA, ferulic acid

HMG, high-mobility group

4HQN, 4-hidroxyquinazoline

IFNγ, interferon γ

IL, interleukin

iNOS, inducible NO-synthase

JNK, c-Jun N-terminal kinase

LPS, lipopolysaccharide

MIP-2, macrophage inflammatory protein-2

MAPK, mitogen activated protein kinase

MD-2, myeloid differentiation-2

MKK, MAPK kinase

MLK, mixed-lineage kinase

MRI, magnetic resonance imaging

NF-κB, nuclear factor-kappa B

PAMP, pathogen-associated molecular pattern

PARP, poly-(ADP-ribose) polymerase

PI3, phosphatidylinositol 3

p90RSK, protein of 90 kDa from the ribosomal subunit S6 kinase

PRR, pathogen recognition receptor

PSS, physiological saline solution

RNS, reactive nitrogen species

ROS, reactive oxygen species

TLR, toll-like receptor

TNFα, tumor necrosis factor α

TRAF-6, TNF-receptor associated factor-6

Abbreviations of treatment groups

CTRL, vehicle treated group

LPS, treated with LPS alone

FA, treated with FA alone

4HQN, treated with 4HQN alone

LPS + FA, treated with LPS and FA

LPS + 4HQN, treated with LPS and 4HQN

Introduction

4.1 Sepsis

The word sepsis is derived from the Greek term for rotten or "to make putrid". Sepsis is a systemic host response against microorganisms invading in previously sterile tissues. It is a syndrome related to severe infections and is characterized by end-organ dysfunctions away from the primary site of the infection (*Silva et al., 2008*). Despite decades of efforts and significant advance in extensive research, sepsis remains the most common cause of death with a current estimate of at least 750,000 cases per year and 215,000 deaths annually, in the intensive care units, just in the USA (*Leon et al., 2008*). Sepsis is the third leading cause of death in developed societies equaling the cases of fatal acute myocardial infarction. Although there are many effective antibiotics available, sepsis still remains a major cause of death, in part because antibiotics are able to eliminate bacteria but cannot control systemic inflammation (*Ulloa et al., 2005)*. The development of a standard therapy is rendered more difficult, because of the altered symptoms and different stages of sepsis in the affected patients. According to these, the definition of sepsis was found to be too broad and common to heterogeneous groups of patients who do not necessarily had the same disorder (*Abraham et al., 2000; Matot et al., 2001*).

4.1.1 Definition of sepsis, septic shock and severe sepsis*:* Sepsis is defined by the clinical signs of systemic immune response to infection. The diagnosis of sepsis requires the presence of a bacterial infection and at least two of these clinical signs:

- Alterations in body temperature *(hypothermia or hyperthermia).*
- Abnormalities in heart rate *(tachycardia).*
- Altered respiratory rate *(tachypnea).*
- Anomalies in the amount of white blood cells *(leukocytopenia or leukocytosis)* (*Abraham et al., 2000; Matot et al., 2001*).

But there are at least two different clinical syndromes which are traditionally associated with sepsis and are distinguished upon their clinical signs and cytokine profiles (*Ulloa et al., 2005)*. The first, called septic shock, is a cardiovascular shock. It is highly lethal

and kills within 24-48h after onset. The lethal effect of septic shock is due to a cardiovascular collapse and ischemic necrosis as well. The most patients suffering from sepsis produce a second clinical syndrome, the severe sepsis. Severe sepsis is defined as sepsis associated with multiple organ dysfunctions, such as hypoxemia, lactic acidosis, oliguria, elevated liver enzymes or altered cerebral function. This less acute syndrome kills more slowly. Depending on the severity of underlying organ failure and on the amount of affected organs, the time-range of its lethal effect progresses over 7–14 days, with a mortality rate of 30–70%. Although these two clinical syndromes seem to be very different, they are not completely independent from each other. In some cases, these syndromes represent two different stages of the progression of sepsis. Patients, who survived the state of highly lethal septic shock, usually develop severe sepsis that is characterized by a progressive damage in the affected organs and tissues. Despite of these facts, not all patients with septic shock will necessarily develop severe sepsis, and in several clinical scenarios patients will develop a protracted form of severe sepsis without ever developing septic shock (*Ulloa et al., 2005*). Because sepsis and sepsis related syndromes are due to a systemic immune response against pathogens, it is of primary importance to know the mechanism by which the immunsystem recognizes the infection and develops a response, which can be lethal for the invaders and for the organism itself.

4.2 The endogen toxin: Lipopolysaccharide

The recognition of pathogens by their multicellular hosts is initiated by the activation of pathogen recognition receptors (PRR). These receptors recognize specific pathogen-associated molecular patterns (PAMP) (*Zhong et al., 2007*). Typical PAMPs are lipopolysaccharide (LPS) of Gram-negative bacteria, peptidoglycan with its muramyl dipeptide components of Gram-positive bacteria, fungal beta-glucans and double-stranded RNAs. Toll-like receptor 4 (TLR4) is an important part of the PRR complex that is involved in the activation of the immune system via the specific binding of LPS (*Iliev et al., 2005*). This critical event is essential for the immune response to Gram-negative bacteria, because lipopolysaccharide is the most biologically active component of these microorganisms.

LPS contains a heteropolysaccharide and a lipid component, termed lipid A (*Raetz et al., 1991).* The presence of the hydrophilic polysaccharide and the covalently bound lipid A gives a common structural principle *(Fig.1.).* The heteropolysaccharide region can be further

subdivided into the O-specific chain and the core oligosaccharide, in the case of Enterobacteriaceae. (*Rietschel 1994; Lodowska et al., 2007*).

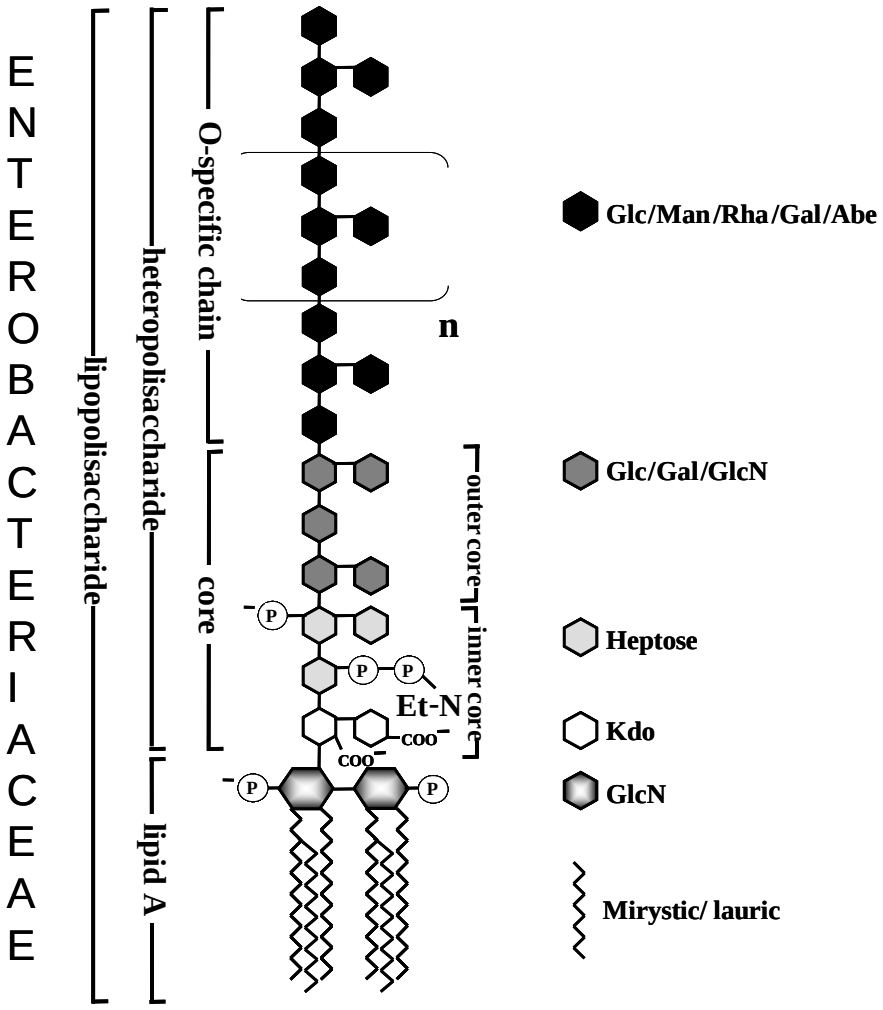


Fig.1. Molecular structure of lipopolysaccharide.

4.2.1 O-specific chain: The O-specific chain is an oligosaccharide polymer with repeating units from one to eight glycosyl residues, like glucose (Glc), rhamnose (Rha), mannose (Man), galactose (Gal) among others *(Fig.1.)*. Within a serotype, the structure of the units differs in different strains, which means distinct ring form, sequence, substitution and type of linkage between monosaccharides residues (*Rietschel 1994*). This produces an enormous structural variability, which determines the serological specificity of LPS and

bacteria and thereby LPS functions as an important surface antigen. Because of this property, LPS is often called, O-antigen (*Rietschel 1994*).

4.2.2 Core: Another part of enterobacterial LPS, called core region is a heterooligosaccharide that can formally be subdivided into an outer and an inner core *(Fig.1.)*. In contrast to the O-specific chain, the core is structurally more uniform. A structural diversity was primarily found in the outer core region. In the *Salmonella* and *E. coli* serotypes the outer core contains the common hexoses: D-glucose, D-galactose, and N-acetyl-D-glucosamine (GlcN) (*Rietschel 1994*). The inner core contains the characteristic and LPS specific components, like heptose (Hep), mainly in the L-glycero-D-manno configuration, and 3-deoxy-D-manno-octulosonic (2-keto-3-deoxyoctonic) acid (Kdo). The Hep and Kdo molecules, which are commonly substituted by charged groups like phosphate, pyrophosphate, 2-aminoethylphosphate, and 2-aminoethylpyrophosphate (Ethanolamine (Et-N)). These charged groups are able to form an agglomeration of negatively charged residues in the inner core, leading to the accumulation of bivalent cations such as Ca^{2+} and Mg^{2+} in the close environment of the cell surface. This phenomenon provides a physiological significance to the negatively charged residues, because in this area cations are required for the structural and functional integrity of the outer membrane (*Rietschel 1994*).

Interestingly, a structurally different LPS type is found in some pathogens such as *Neisseria, Haemophilus,* and *Bordetella*. In these cases, LPS molecule does not possess the complete structure because it lacks an O-chain and it is called rough LPS (rLPS). The inner core seems to be well conserved in the Gram-negative taxa. Kdo forms the linkage to lipid A also in these rLPS molecules. The smallest LPS found in pathogenic bacteria derives from the genus *Chlamydia*, where the core region consists of only a Kdo-trisaccharide with the following sequence: αKdo(2→8)αKdo(2→4)αKdo (*Rietschel 1994*).

4.2.3 Lipid A: As a structural component of the LPS molecule, lipid A is responsible for the pathophysiological effects associated with Gram-negative bacterial infections. The lipid A saccharide backbone consists of two glucosamine units in β(1→6) glycosidic linkage, in the majority of Gram-negative bacteria. Amino groups (at positions 2 and 2') and hydroxy groups (at positions 3 and 3') of glucosamines are in general substituted by 3-hydroxyfatty acids, most often by 3-hydroxytetradecanoic acid *(Fig.1.)*. Other, usually saturated and unbranched fatty acids are ester-linked by their hydroxy group to hydroxyacids. Lipid A of different microorganisms appears to possess fatty acids of high diversity, like mirystic

(tetradecanoic, 14:0) and lauric (dodecanoic, 12:0) acids and their hydroxylated derivatives and such unique structures as cis-11-octadecenoic acid (*Rhodospirillum salinarum*), 3-hydroxy-5-dodecenoic acid (*Phenylobacterium immobile*), and iso-2,3-dihydroxytetradecanoic acid (*Legionella pneumophila*). Even in this conservative component of endotoxin, in lipid A, structural differences between species and strains may occur. That can be different type of amino-sugars, variant degree of substitution in the disaccharide core by fatty acids, phosphate and/or ethanolamine, and also the type, quantity and distribution of fatty acids. The saccharide core of some bacterial lipid A may consist of sugars different from glucosamine, like 2,3-diamino-2,3-dideoxy-D-glucose. Other substituents in this region of LPS molecule, besides phosphate groups and ethanolamine, are β-mannopyranose, 4-aminoarabinose, galacturonic acid, and glycine. This conservative component of endotoxin, the lipid A part is responsible for the biological activity of LPS molecule and thereby for the LPS-induced inflammatory processes (*Rietschel 1994, Lodowska et al., 2007*).

4.3 LPS-induced inflammatory processes

4.3.1 LPS receptors, TLR4 signaling: The innate immune response is the first line of defense against infectious agents and is devoted to recognize highly conserved pathogen motifs the PAMPs (*Zhong et al., 2007*). Macrophage activation by LPS has been extensively studied in an attempt to define the mechanisms that underlie innate immunity against bacterial pathogens. The biological actions of LPS are mediated, at least in part by both LPS-binding proteins (LBP) and LPS receptors (TLR4, CD14, the macrophage scavenger receptor and the b2 integrins) (*Fenton et al., 1998*). Toll-like receptor 4 is the central signaling receptor for LPS in mammals (*Leon et al., 2008; Beutler et al., 2003; Rhee et al., 2000*), that activates a family of adaptor proteins, which recruit downstream protein kinases to activate transcription factors such as nuclear factor-kB (NF-κB) (*Bannerman et al., 2003; Medzhitov et al., 1997; Rhee et al., 2000*) and members of the interferon (IFN)-regulatory factor (IRF) family (*O'Neill et al., 2007*) *(Fig.2.)*. LPS signaling involves the binding of the LPS-binding protein to LPS. After the binding of the ligand TLR4 builds up a complex with CD14. In addition a small molecule, myeloid differentiation 2 receptor (MD-2) - which is sensitive to the acylation pattern of the lipid A moiety of the LPS monomer (*Shimazu et al., 1999*) - participates in this complex by associating with the TLR4 extracellular domain. Association of the MD-2/LPS complex to the ectodomain of the TLR4 finally transduces the signal via association to intracellular Toll/Interleukin-1 receptor (TIR) domain recruiting the adapter proteins that

triggers the signaling cascade. TLR4 uses the myeloid differentiation primary-response gene 88 adapter like protein (MAL) as a bridging adaptor to recruit the myeloid differentiation primary-response gene 88 (MyD88) that activates the NF-κB, mitogen activated protein kinases (MAPKs) via TNF-receptor associated factor 6 (TRAF6) (*Leon et al., 2008*, *Doyle et al., 2006*) and PI3K/Akt pathway. Another pathway activated by TLR4 involves TIR-domain-containing adapter-inducing interferon-β (TRIF)-related adaptor *(Fig.2.)*.

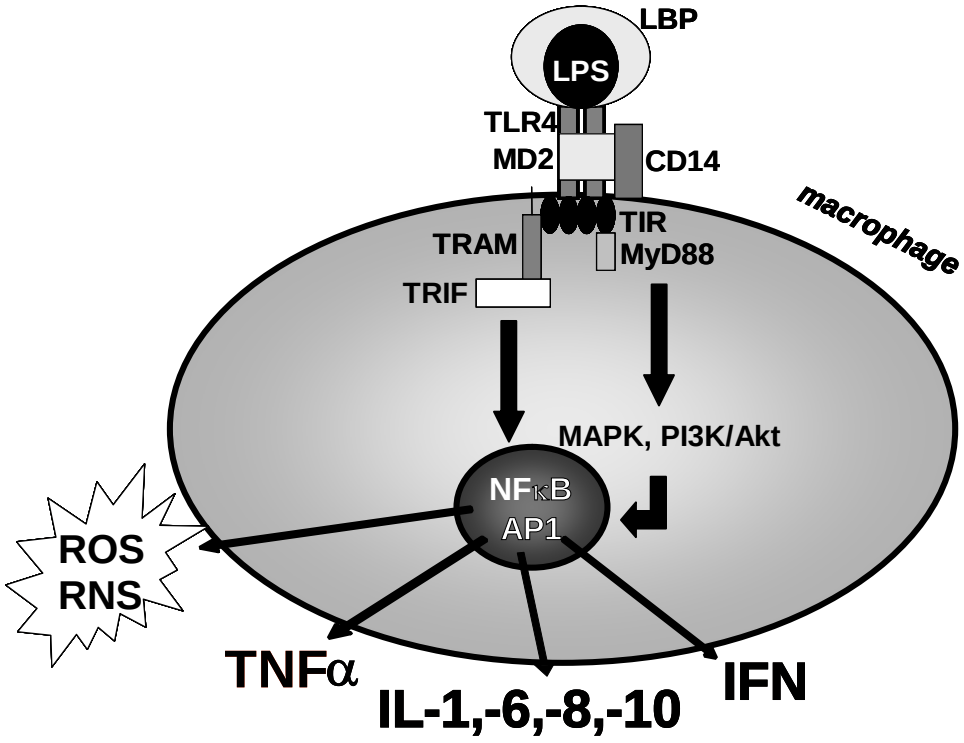


Fig.2. LPS signaling: from the receptor to the inflammatory mediators.

4.3.2 Signaling pathways, MAPK and Akt: Binding of LPS to the CD14 and TLR4/MD2 complex induces the aforementioned mitogen activated protein kinase pathways (*Fang et al., 2007*), including extracellular signal-regulated kinase (p42/44 MAPK or ERK1/2), p38 kinase and c-Jun N-terminal kinase (JNK) as well as the phosphatidylinositol 3 kinase (PI3K)/Akt pathway (*Murakami A et al., 2005*; *Dumitru et al., 2000*) *(Fig.2.)*. MAPKs and Akt are capable of modulating functional responses through phosphorylation of transcription factors and activation of other kinases.

The activated p42/44 MAPK will, in turn, phosphorylate an array of cellular substrates, including downstream Ser/Thr effector kinases such as p90RSK (protein of 90 kDa from the ribosomal subunit S6 kinase) (*Sturgill et al., 1988*). LPS and H_2O_2 can increase the PI3K activity, PTEN inactivation, NF-κB-inducing kinase (NIK) activation and Akt phosphorylation (*Brazil et al., 2001; Monick et al., 2001; Ozes et al., 1999; Luyendyk et al., 2008*) independently from MAPK-pathways. This phenomenon shows these important pathways (MAPK and Akt) to be partly independent and demonstrates the deep involvement of reactive oxygen species in the pathomechanism of sepsis *(Fig.3.)*.

4.3.3 Transcription factors, NF-κB: MAPKs and Akt can mediate the activation of nuclear transcription factors, such as activator protein 1 (AP-1) or the activation and nuclear translocation of cytoplasmic transcription factors such as NF-κB, which activate the expression of numerous genes (*Bone et al., 1997; Bozinovski et al., 2002*) *(Fig.2.)*.

AP-1 an obligatory transcription factor of innate immunity in inflammation exists either as a homodimeric c-Jun complex or as a c-Jun/c-Fos heterodimer that is regulated by transcription and direct phosphorylation. Phosphorylation of c-Fos stabilizes the transcription factor and enhances the trans-activation and DNA binding of AP-1 (*Shaywitz et al., 1999*).

Another important inflammation related transcription factor is NF-κB that is responsible for the gene expression of cytokines, chemokines, growth factors, cell adhesion molecules and some acute phase proteins, which are strongly involved in the LPS-induced inflammatory processes. NF-κB is ubiquitous in almost all animal cell types and mediates cellular responses against stimuli such as stress, cytokines, free radicals (*Cadenas et al., 2002; Victor et al., 2005*), ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. Depending on its actual role, NF-κB has been linked to cancer, inflammatory and autoimmune diseases, viral infection and to improper immune development. Since NF-κB is a primary transcription factor, it is present in the cells in an inactive state and do not require synthesis of new proteins for its activation. Binding of specific molecules on cell-surface receptors, such as TNFR or TLR-4 activates NF-κB and leads to rapid changes in gene expression. Some subunits of NF-κB, which play important role in modulating the specificity of NF-κB function, like p50 and p52 do not contain transactivation domains in their C terminal halves in contrast to RelA, RelB and c-Rel. Despite of the general repressor activity of homodimers in κB site transcription, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB or c-Rel. In addition, the aforementioned homodimers of p50 and p52 can also bind to the nuclear protein Bcl-3.

Complexes like this can function as transcriptional activators (*Karin et al., 2000, Senftleben et al., 2001*).

NF-κB, as a central transcription factor in many ROS related syndromes (*Li et al., 2002)*, possesses both antiapoptotic and apoptotic properties (*Bannerman et al., 2003)*. The fact, that many different types of human tumors have constitutively active NF-κB (*Okamoto et al., 2007*), shows the antiapoptotic nature of this transcription factor. In these cells, permanent activation of NF-κB induces numerous genes, which keep the cells proliferating and protect them from apoptosis. This nonstop activity is due to mutations in genes encoding the NF-κB themselves or in genes that control NF-κB activity, like IκB genes. Furthermore, tumor cells are able to secrete certain factors, which activate this important transcription factor. In these cases, inhibition of NF-κB can order tumor cells to stop proliferating or to die. At least, blocking NF-κB makes the cells more sensitive against anti-tumor agents.

Otherwise, NF-κB controls many genes involved in inflammation *(Brasier et al., 2006)*, which are chronically active in many inflammatory diseases, such as inflammatory bowel disease, arthritis and asthma among others. Many natural products including anti-oxidants with anti-cancer and anti-inflammatory property have also been shown to inhibit NF-κB (*Kim et al., 2008*). It seems there is a connection between inflammation and cancer and this possible link can be NF-κB. These findings emphasize the value of drugs and natural products including polyphenols that regulate the activity of NF-κB (*Gilmore et al., 2006*).

4.3.4 Inflammatory cytokines: NF-κB plays a major role in inducing inflammatory processes during infection caused by Gram-negative bacteria. Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes produce powerful pro-inflammatory mediators via activation of the aforementioned transcription factors, especially tumor necrosis factor α (TNFα), interleukin (IL)-6, IL-1 and IL-8, E-selectin, inter-cellular adhesion molecule-1 (ICAM-1 also known as CD54), vascular cell adhesion molecule-1 (VCAM-1 also known as CD106), tissue factor (TF) and high-mobility group box-1 (HMGB-1) among many other inflammatory molecules playing important roles in the induction of sepsis (*Stone, 1994*). These cytokines trigger a beneficial inflammatory response that promotes local coagulation to confine tissue damage. However, the excessive production of these proinflammatory cytokines can be even more dangerous than the original stimulus, overcoming the normal regulation of the immune response and producing pathological inflammatory disorders. This is especially notable in severe sepsis, in which the excessive production of proinflammatory cytokines causes capillary leakage, tissue injury leading to lethal organ failure. A number of

different approaches have been investigated to try to medicate and/or prevent the septic shock associated with infections caused by Gram-negative bacteria, including inhibition of one or more of the cytokines induced by LPS *(Leon et al., 2008)*. Experimental strategies neutralizing these cytokines (monoclonal antibodies against TNF, IL-1-receptor antagonists and TNF-receptor fusion proteins) are successful therapeutic approaches against several inflammatory disorders, including rheumatoid arthritis and Crohn's disease, but could not solve the overall problem of sepsis and sepsis related diseases.

4.3.5 Inflammation and oxidative stress: Beside the inflammatory cytokines, the pathomechanism of septic shock involves oxidative stress *(Cadenas et al., 2002)* via expression of the most important free radical producing enzymes such as cyclooxygenase-2 (COX-2) (*Woo et al., 2007 Chiu et al., 2008*) that produces superoxide ($O_2^{\bullet -}$), a reactive oxygen species (ROS) or inducible NO synthase (iNOS) that generate nitric-oxide, a reactive nitrogen species (RNS) *(Fig.3.)*. (*Soriano et al., 2002; Jagtap et al., 2002; Goldfarb et al., 2002; Szabo, 2000; Szabo, 2002*).

The possible sources of ROS in inflammation:

- the mitochondrial respiratory chain,
- the metabolic cascade of arachidonic acid (COX, LOX),
- the protease-mediated enzyme xanthine oxidase,
- granulocytes and other phagocytes activated by complement, bacteria, endotoxin, lysosomal enzymes, etc.,
- other oxidases, mainly NADPH oxidase (*Victor et al., 2005*).

Oxidative stress has been implicated in playing a crucial role in the pathogenesis of a number of diseases including neurodegenerative disorders such as Alzheimer's disease, sepsis and septic shock *(Cadenas et al., 2002)*. Oxygen or nitrogen containing radicals are highly reactive and may cause cellular dysfunctions by modifying and inactivating proteins, lipids, DNA and RNA. To prevent free radical-induced cellular damage, the organism has developed the antioxidative system as a defense mechanism. This system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), and glutathione reductase (GSSGR), as well as non-catalytic antioxidants such as glutathion and

plasma proteins (*Victor et al., 2005*). Glutathion plays a key role in maintaining the physiological balance between pro-oxidants and antioxidants. But if this balance is pushed toward the production of pro-oxidants the cell suffers from oxidative stress. Reactive oxygen species have been implicated in the regulation of the most important inflammation related transcription factor the NF-κB *(Fig.3.)* (*Kim et al., 2008*), which plays an important role in cell death or survival as aforesaid in *4.3.3*.

Many antioxidants are shown to downregulate the NF-κB dependent inflammatory genes, like iNOS, TNF-α, IL-1β, and COX-2 by scavenging superoxide and peroxide in the LPS-stimulated macrophages. Furthermore, ROS and RNS can induce DNA-breaks in the affected cells and thereby activate the nuclear enzyme poly-(ADP-ribose) polymerase (PARP), which can dramatically contribute to the tissue damaging processes of sepsis.

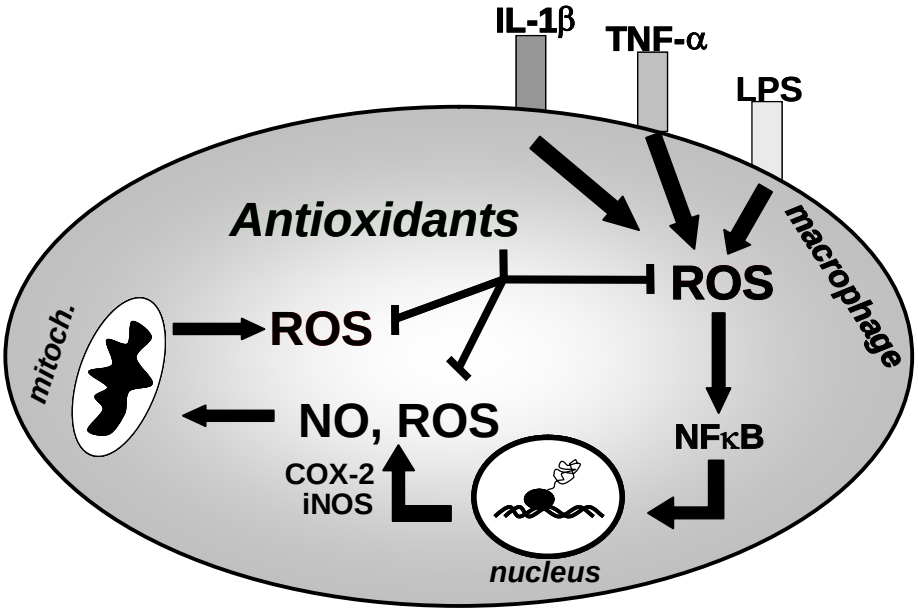


Fig.3. Induction of ROS and RNS production, possible role of antioxidants.

4.4 Poly-(ADP-ribose)-polymerase

4.4.1 PARP-1 "knock out" mice: Mammalian poly-(ADP-ribose) polymerase-1 is an abundant nuclear enzyme, which catalyzes the transfer of ADP-ribose units from its substrate beta-nicotinamide adenine dinucleotide (NAD^+) to itself and other nuclear proteins. The activation of PARP-1 is part of the DNA-repair mechanism but overactivation of this enzyme can lead to the collapse of the cellular ATP and NAD^+ pool causing rather necrotic than apoptotic cell death (*Boulares et al., 2003).* According to these facts, PARP-1 knockout mice were extensively tested and found to be protected against myocardial infarction,

streptozotocin-induced diabetes, zymosan-induced multiple organ failure and lipopolysaccharide-induced septic shock, indicating that PARP-1 is strongly involved in the pathogenesis of these disorders (*Soriano et al., 2002).* PARP-1 and the previously delineated NF-κB have been both suggested to play a crucial role in inflammatory disorders such as sepsis and septic shock. Many reports demonstrate that there is a connection between PARP-1 and NF-κB activation (*Olivier et al., 1999),* because PARP-1 can act as a coactivator of NF-κB. In these experiments PARP-1-deficient cells were found to be defective in NF-κB-dependent transcription activation, but not in its nuclear translocation in response to TNFα. Challenging mice with LPS induced a rapid NF-κB activation in macrophages from PARP-$1^{+/+}$ but not from PARP-$1^{-/-}$ mice and PARP-$1^{-/-}$ animals were highly resistant to LPS-induced endotoxic shock (*Hassa et al., 2003*). This dramatic resistance was due to an almost complete abrogation of NF-κB-dependent TNFα production and to the down-regulation of iNOS, leading to decreased NO synthesis, which is one of the main sources of free radicals in inflammation. Because of this functional association between PARP-1 and NF-κB, PARP-1 was thought to be an important molecular target to regulate systemic inflammatory processes *(Andreone et al., 2003; Albertini et al., 2000).*

4.4.2 Pharmacological inhibition of PARP: After publishing the first results, which proved the crucial role of PARP-1 in many disorders (*Chiarugi et al., 2002; Goldfarb et al., 2001; Halmosi et al., 2001; Jagtap et al., 2002*, *Banasik et al., 1992; Zingarelli et al., 2003*), the needs to develop and to test potent PARP-1 inhibitors were strongly increased. Studies with the traditional PARP inhibitor 3-aminobenzamid (3-AB), suggested that PARP activity has no (*Baechtold et al., 2001*) or partial (*Albertini et al., 2000*) role in the mechanisms of septic shock. However, 3-AB is considered to be a poor inhibitor of PARP and has pronounced toxicity *in vivo*. On the other hand novel, potent PARP inhibitors were found to protect against LPS-induced tissue damage (*Liaudet et al., 2002; Ivanyi et al., 2003; Veres et al., 2003*).

4.5 Role of antioxidants in inflammation

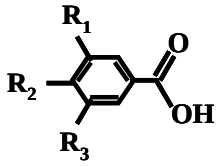
4.5.1 Naturally occurring antioxidants, polyphenols: Polyphenols are the most common antioxidants in our diet and are present in fruits, vegetables, cereals, olive, dry legumes and beverages such as chocolate, tea, coffee, beer or wine (*D'Archivio et al., 2007).* Their biological role in plants, as a product of their secondary metabolism is to eliminate bacteria, fungi and viruses attacking the plant tissues. Despite their wide distribution and their

use with medical herbs in the traditional medicine, nutritionists and researchers have paid attention to their healthy effects, only in the last decades (*Perron et al., 2009*). These compounds are potent antioxidants and they may protect cells against oxidative damage. With this way of action they limit the risk of various degenerative diseases associated to oxidative stress. Many studies have shown an important role in the prevention of cardiovascular disease, cancer, osteoporosis, diabetes mellitus and neurodegenerative disease (*Singh et al., 2008*). Furthermore, polyphenol consumption limits the development of atheromatous lesions, inhibited the oxidation of low density lipoprotein (*Giovannini et al., 2008*), which is considered a key mechanism in the endothelial lesions occurring in atherosclerosis. However, emerging findings suggest that the protective effects of polyphenols are due just in part to their conventional antioxidant activities. Depending on the experimental conditions they can act as antioxidant and pro-oxidant compounds (*Perron et al., 2009*). As antioxidants they improve cell survival, as pro-oxidants they may induce apoptosis and block cell proliferation. Polyphenols possesses the potential to inhibit or reduce the amount of different enzymes such as telomerase, cycloxygenase, lipoxygenase. They are able to interact with signal transduction pathways and cell receptors, can affect caspase-dependent pathways, cell cycle regulation and platelet functions (*Rahman et al., 2006; Gonzales et al., 2008, Hatcher et al., 2008*).

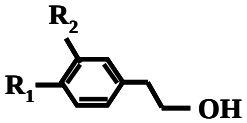
Polyphenols comprise a wide variety of molecules with polyphenol structure. That means the binding several hydroxyl groups on aromatic rings, but also on molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of polyphenols are: flavonoids, phenolic acids, phenolic alcohols, lignans and stilbenes *(Fig.4.)* (*D'Archivio et al., 2007*).

4.5.2 Resveratrol: One of the most investigated and potent polyphenolic compounds regulating inflammation is a stilbene, called resveratrol *(Fig.4.)*. It is produced by plants in response to infections or to a variety of stress conditions. Resveratrol was found in seeds and fruits, like berries, peanuts and grapes, therefore in red wine (*Chen et al., 2008, Guerrero et al., 2009*). The carrier of this phytoalexine is rising up from the 80's as it was thought to play an important role in the "French paradox" as a result of a moderate wine consumption. The French paradox is an observation that the French people suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats (*Simini et al., 2000; de Lorgeril et al., 2002; de Lange et al., 2007*). However, some health researchers

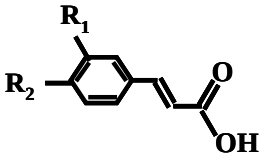
question the validity of this paradox; in 2008 it was found that high doses of resveratrol mimicked some of the benefits of caloric restriction, including reduced effects of aging in a mice study (*Pearson et al., 2008*), which confirmed some earlier works showing a longer lifespan in yeast and flies by activating sirtuin2 (Sir2) a histone deacetylase (*Howitz et al. 2003*). Controversially, some papers suggest the reexamination of the mechanism accounting for putative life-longevity effects of resveratrol since they queries its activating effect on Sir2 (*Kaeberlein et al., 2005*). Despite of these contradictory data, many papers indicate a beneficial role on neurological, hepatic and cardiovascular systems and inflammatory processes (*Zou et al., 2009*) and emphasize its chemopreventive potential in inhibiting cancer development by blocking the complex process of carcinogenesis at various stages of tumor initiation, promotion and progression.



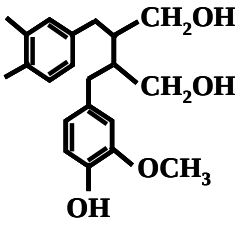
Hydroxybenzoic acids



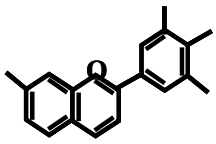
Phenolic alcohols



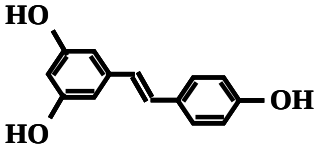
Hydroxycinnamic acids



Lignans
(Secoisolariciresinol)



Flavonoids



Stilbenes
(Resveratrol)

Fig.4. Classification of polyphenols

A possible mechanism for its biological activity in inflammation involves downregulation of the inflammatory response via inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of activated immune cells or inhibiting such free-radical generating processes as iNOS and COX-2 by inhibiting NF-κB or AP-1 (*Das et al., 2007; Fröjdö et al., 2007; de la Lastra et al., 2007; de la Lastra et al., 2005*).

Unfortunately, bioavailability of an *in vitro* investigated compound can dramatically determinate the biological effect *in vivo*. Resveratrol was shown to have a low bioavailability and rapid clearance from the plasma (*de la Lastra et al., 2005)*. Therefore, the aim of many researchers can be to find compounds with the positive biological effects of resveratrol, but with higher bioavailability. For this role, some phenolic acids and aldehydes may be potent candidates.

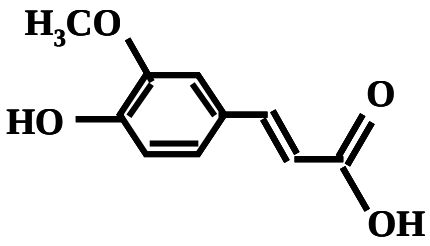
4.5.3 Phenolic acids / aldehydes: These compounds can be divided in two classes:

- derivatives of benzoic acid (*gallic acid, protocatechuic acid*)
- derivatives of cinnamic acid (*coumaric, caffeic and ferulic acid).*

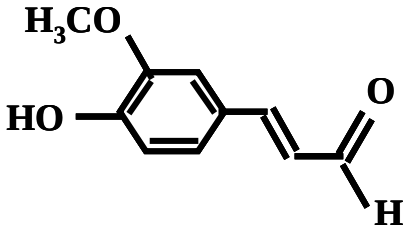
The first group, the benzoic acid derivatives (hydroxybenzoic acids), such as gallic acid and protocatechuic acid are present just in very few plants playing important role in human diet. Some red fruits, like blackberries contain benzoic acid derivative in 270 mg/kg fresh weight, but their concentration in other plants is generally very low. Therewith, tea is an important source of gallic acid, which may contain up to 4.5 g/kg fresh weight of this polyphenol. Raspberry and olive oil contains up to 100 mg/kg and 0.22 mg/kg fresh weight of protocatechuic acid, respectively. Interestingly, this hydroxybenzoic acid was reported to be the major human metabolite of anthocyanins, such as cyanidin-3-glucoside, as it was detected in human serum and feces after consumption of cyanidin-rich food (*D'Archivio et al., 2007*).

Hydroxycinnamic acids are commonly present in all part of the most fruits, but the highest concentrations of these compounds were found in the outer part of ripe fruit. They consist primarily of coumaric, caffeic and ferulic acid (FCA) *(Fig.5.)*. Rather the glycosylated derivatives or esters of quinic, shikimic or tartaric acid occur, than the free form. For example, blueberries contain 2 g/kg fresh weight of hydroxycinnamic acids and kiwis contain up to 1 g/kg fresh weight of caffeic acid, which is the most abundant hydroxycinnamic acid

represents between 75% and 100% of the total contents in most fruits. FCA is the most abundant phenolic acid found in cereal grains: its content of wheat grain is about 0.8-2 g/kg dry weight, which may represent up to 90% of total polyphenols. The high amounts of different phenolic acids show the high potential of polyphenols in human diet and suppose a special role of FCA - as the most abundant phenolic acid in cereal grains - in the human nutrition, which may provide a positive effect for the human health *(D'Archivio et al., 2007)*.



Ferulic acid



Ferulaldehyde

Fig.5. Molecular structure of ferulic acid and ferulaldehyde

4.5.4 Ferulic acid and ferulaldehyde *(Fig.5.)**:*** Ferulic acid is produced by the Shikimate pathway in plants and is a product of the phenylalanine and tyrosine metabolism. It is a major constituent of fruits like orange, some vegetables such as tomato, carrot and sweet corn *(Srinivasan et al., 2007).* Both of the free and covalently bound forms - in lignin and in other biopolymers - exist in seeds, leaves and in wheat, ester linked to cell wall carbohydrates. Because of its molecular structure, ferulic acid (4-hydroxy-3-methoxy cinnamic acid) belongs to the group of hydroxycinnamic acids, which means two functional groups on a benzene ring; a 3-methoxy group and a 4-hydroxyl group. The carboxylic acid group binds with an

adjacent unsaturated bond to the ring, which allows for cis-trans isomerisation. The trans-isomer predominates and accounts for 90% of the total phenolic acids in common flour. Ferulic acid, similarly to other hydroxycinnamic acids, is reported to be a potent antioxidant.

The antioxidant potential of FCA can usually be attributed to its structural characteristics *(Fig.5.)*. In its structure, there are three motifs that are responsible for the radical scavenging effect, or can at least contribute to (*Graf et al., 1992)*:

- Methoxy group (H_3CO-)
- Hydroxyl group (OH-)
- C-C double bond (-HC=CH-)

The methoxy and hydroxyl groups terminates the free radical chain reactions as electron donating groups, and the C-C double bond can provide attack sites for free radicals. FCA with its aforementioned molecular structure is able to form a resonance stabilized phenoxy radical, which significantly accounts for its potent antioxidant activity. Highly reactive radicals colliding with ferulic acid easily abstract a hydrogen atom to form phenoxy radical. The produced phenoxy radical is highly resonance stabilized by the delocalization of the unpaired electron across the entire molecule and by the extended conjugation in the unsaturated side chain. FCA in its stable phenoxy radical form is unable to initiate or propagate a radical chain reaction. *(Srinivasan et al., 2007)* Furthermore, the collision and condensation of two ferulate radical probably leads to the formation of its dimer, curcumin. The presence of a second phenolic hydroxyl group substantially enhances the radical scavenging activity due to additional resonance stabilization and o-quinone formation (*Graf et al., 1992; Chang et al.; 2007*).

In part, because of its strong antioxidant activity, FCA exhibits a wide range of therapeutic effects against various diseases such as cancer, diabetes, cardiovascular and neurodegenerative disorders (*Srinivasan et al., 2007)*. Furthermore, it has been reported that ferulic acid or related ester derivatives decrease the levels of some inflammatory mediators such as prostaglandin E2 (*Xu et al., 1990*), TNFα (*Han et al., 2007*) and iNOS expression and function in LPS stimulated cells (*Huang et al., 2009*). Hydrophobic ester derivatives of FCA were shown to have enhanced inhibitory activity on iNOS protein expression in LPS+IFNγ activated RAW 264.7 macrophages. It was reported that feruloyl-myoinositols, the derivatives of ferulic acid, suppressed cyclooxygenase-2 promoter activity in human colon cancer DLD-1 cells (*Hosoda et al., 2002*). It was also reported that FCA dose dependently inhibited the

production of murine macrophages inflammatory protein-2 (MIP-2), a member of chemokine superfamily, in LPS-stimulated RAW 264.7 cells (*Murakami et al., 2005; Sakai et al., 1997*). These evidences strongly suggest that ferulic acid, as a potent antioxidant, has anti-inflammatory effect.

Its reduced form, ferulaldehyde (FA) *(Fig.5.)* possesses the same structural characteristic and main molecular motifs as ferulic acid up to the aldehyde group. Because of the structural similarity, FA is thought to have very similar or maybe better biological activity as ferulic acid, because of the reactive aldehyde group, which can potentially be oxidized to carboxylic group. In the literature there are numbers of reports explaining the effect of ferulic acid under numerous experimental conditions, but the effect of ferulaldehyde remains still unclear. In a few papers, published about FA, it was reported to inhibit LPS-induced iNOS expression and NO synthesis in murine macrophage-like RAW 264.7 cells (*Kim et al., 1999*) and to have a good antioxidant activity in about the same degree as ferulic acid (*Nenadis et al., 2003*).

Recently, a number of natural products or ingredients of traditional medicines and healthy foods such as resveratrol, curcumin and proanthocyanidins were extensively investigated, even subjected to clinical trials as anti-inflammatory agents (*Rahman et al., 2006; Hatcher et al., 2008*). Since solubility of these compounds is limited, it is questionable whether their bioavailability could account for their pharmacological effect. Furthermore, recent publications show that polyphenols in healthy foods or drinks such as chocolate, red wine or beer are readily metabolized to phenolic acids and aldehydes by the microflora of the intestines, raising the possibility that these metabolites, rather than the original natural products or food ingredients, are responsible for their anti-inflammatory properties (*Rios et al., 2003; Gonthier 2003*). Ferulic acid and ferulaldehyde are potential end-products of dietary polyphenol degradation since they were found at a high concentration in human urine after red wine and chocolate consumption (*Rios et al., 2003; Gonthier 2003*). Furthermore, FCA was reported to stay in the blood longer than other antioxidants such as vitamin C (*Adam et al., 2002*), and have higher bioavailability than that of other dietary flavonoids and monophenolics studied so far (*Beecher et al., 1998*).

Aims of study

1. Although previously we provided evidence for the anti-inflammatory effect of PARP inhibition in a mouse model of septic shock and demonstrated significant involvement of the PI3K/Akt pathway in this effect, the exact signaling mechanisms still remained elusive. Therefore, our first aim was to identify other pathways implicated in the LPS-induced signaling mechanisms such as MAPK pathways, which can potentially be affected in the anti-inflammatory effect of PARP inhibition.

2. Based on *in vitro* evidences, some recent publications raised the possibility that microbial degradation products of polyphenols rather than the polyphenols themselves are responsible for the anti-inflammatory and antioxidant effects of polyphenol containing healthy food, drink or traditional medicines. To provide *in vivo* experimental basis for this theory, our second aim was to investigate the effect of FA, an antioxidant and microbial metabolite of several polyphenols, in a mouse model of septic shock as well as in primary hepatocytes activated by LPS and IFNγ. Also, we intended to identify signaling mechanisms, transcription factors and inflammatory cytokines involved in the anti-inflammatory effect of FA.

3. PARP activation and oxidative stress can be both responsible for the activation and nuclear translocation of the most important inflammatory transcription factor the NF-κB. Therefore, we compared the effect of 4HQN, a PARP inhibitor with negligible antioxidant properties, and FA, a potent antioxidant with no effect on PARP, on regulation of LPS-induced inflammatory processes focusing mainly on the signaling mechanisms leading to NF-κB activation.

4. In addition many reports provide evidence about the altered sensitivity of differential mouse strains in response to many harmful stimuli, like LPS, which can be widely confirmed by our findings. But the molecular basis of this phenomenon has not been properly clarified. Accordingly, our last aim was to find molecular mechanisms potentially causing the observed different immunresponse, by the comparison of the signaling mechanisms induced by LPS, in our experimental animals derived from different strains.

Materials and methods

6.1 Animals

BALB/c and C57BL/6 mice were purchased from Charles River Hungary Breeding LTD. Animals were kept under standardized conditions; tap water and mouse chow were (CRLT/N, Szindbad Kft, Hungary) provided *ad libitum* during the whole experimental procedure. Animals received human care according to the Guide for the Care and Use of Laboratory Animals published by the US NIH, and the experiment was approved by the Animal Research Review Committee of the University of Pecs, Medical School. Animals were treated in compliance with the approved institutional animal care guidelines.

6.2 Materials

LPS from *Escherichia coli* 0127:B8 and 4-hydroxyquinazoline were purchased from Sigma/Aldrich Corporation (Saint Louis, MO or Budapest, Hungary); 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 Signalling Technology (Waltham, MA), anti-phospho-p38-MAPK (Thr180/Tyr182) was from Sigma, anti-COX-2 was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-HMG-1 was from Becton Dickinson (San Diego, CA). Ferulaldehyde was a generous gift of Prof. Kalman Hideg (Department of Organic and Pharmacological Chemistry, Faculty of Medicine, University of Pecs, Hungary).

6.3 Cell culture

Primary hepatocytes of C57BL/6 mice of 21-24 g body mass were isolated according to Le Cam A. (*Le Cam, 1993*) with slight modifications. Briefly, livers were perfused *in situ* with 50 mL of physiological saline solution (PSS) containing 6000 U/L heparin and 0.66 mmol/L EGTA followed by 50 mL of PSS then 35 mL of PSS containing 0.7 g/L collagenase H (Roche) and 10 mmol/L $CaCl_2$ at 37°C. Hepatocytes were seeded to 24- or 96-well plates coated with rat tail collagen type I (Sigma-Aldrich Ltd.) in DMEM containing 1% MEM non-essential amino acid solution, 0.05% insulin, 0.1% penicillin-streptomycin, 10% fetal calf serum and 0.1% dexamethasone.

6.4 Sepsis model

To induce murine endotoxic shock, BALB/c mice were injected intraperitoneally (i.p.) with LPS at a dose of 20 mg/kg in a volume of 250 µl. 4HQN (100 mg/kg) was administered i.p. in a volume of 250 µl three times a day proceeding the day of LPS-treatment, or as a single dose 1 or 6 hours following the LPS injection. Control mice received the same volume of sterile saline solution instead of the PARP-1 inhibitor.

To induce murine endotoxic shock in the C57BL/6 mice, animals were injected i.p. with a single dose of LPS (20 mg/kg assigned as low or 40 mg/kg assigned as high dose). FA (6 mg/kg) was administered i.p. in every 12 h, the first injection was given one hour before the LPS treatment. Mice treated with FA alone received 6 mg/kg FA, and control mice received the same volume of PSS instead of FA. The mice were monitored for clinical signs of endotoxemia and lethality every hour for 84 h, after which time they were monitored three times a day for 1 wk. No late deaths were observed in any of the experimental groups. Experiments were repeated three times with 10 mice in each experimental group. Data represent mean of 3 independent experiments.

6.5 Western blot analysis

For Western blot analysis, groups of 4 BALB/c mice were pre-treated or not with 100 mg/kg 4HQN three times a day preceding the day of LPS-challenge (20 mg/kg). Liver, lung and spleen were removed from the animals 6 hours after the LPS treatment, were frozen in liquid N_2, and were processed exactly as described previously (*Veres et al., 2003*). Protein load was 35 µg/lane.

C57BL/6 mice were pre-treated with 6 mg/kg FA one h prior to LPS challenge (20 or 40 mg/kg). Livers were removed from the mice 1.5 h after the LPS treatment, were frozen in liquid N_2, and were processed exactly as described above. We applied the primary antibodies at $4^{\circ}C$ overnight at a dilution of 1:1000. The secondary antibodies were horseradish peroxidase-conjugated rabbit IgG. Peroxidase labeling was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

6.6 TNFα, IL-1β, IL-6, IL-10 determination

Mice were treated exactly as for Western blot analysis. Blood samples were taken 1.5 and 3 h after LPS administration, and were allowed to clot for 0.5 hour at room temperature before centrifuging for 20 minutes at 2000 x g. The serum was removed and assayed immediately. Selection of these time points was based on the published activation kinetics of the given cytokine. In these experiments, we used FA in 6 mg/kg dose injected 1 h before the LPS, which was applied in 20 mg/kg (low) and 40 mg/kg (high) doses. Serum TNFα, IL-1β, IL-6 and IL-10 concentrations were determined with the Quantikine M TNFα immunoassay kit (R&D Systems) and with IL-1β, IL-6, IL-10 ready-set-go kits (eBioscience). The ELISA-kits were used in accordance with the protocol of the manufacturer. Three independent experiments with three mice in each experimental group were performed. Data were pooled (n = 9) and values are means ± SEM.

6.7 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 homogenized immediately according to the procedure described previously (*Veres et al, 2003*). 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, Rixensart, Belgium). The kit 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 recognized by primary antibodies. A horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. The ELISA-kit was used in accordance with the protocol of the manufacturer. Three independent experiments with three mice in each experimental group were performed. Results were expressed as mean ± S.E.M. of 12 independent values (Three independent experiments with 4 mice in each group.).

6.8 MRI analysis

Mice were treated exactly as for Western blot analysis. Six hours after LPS treatment, the animals were anaesthetized 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., Paolo Alto, CA) with a 89 mm vertical bore magnet of 9.4 T (Oxford Instruments Ltd., Abingdon, UK) 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., Columbia, SC). After tuning, shimming (^{1}H linewidth ≈ 150 Hz) and RF calibration, the slice of interest was selected using a T_1-weighted multi-slice 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 multi-slice 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 standardized 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. Despite the internal standard, we were not able to quantify accurately the T_2-weighted images due to individual differences among the animals and differences in their positioning inside the probe. Instead, we used qualitative scoring of the standardized T_2-weighted images performed by experts who were blind to the experiment. Experiments were repeated 3 times.

6.9 Determination of ROS production

Culturing medium was replaced with a fresh one without dexamethasone, and the primary hepatocytes were incubated in the presence of 5 mg/L LPS + 50 μg/L interferon-γ (IFNγ) alone, or together with FA (1-100 μmol/L) for 24 h. Then 2,4-dichlorodihydrofluorescein-diacetate (C400, Invitrogen) at a final concentration of 2 mg/L was added to the medium for an additional two hours. Fluorescence was measured at 485 nm excitation and 555 nm emission wavelengths by using a Fluostar Optima (BMG Labtechnologies, Heidelberg, Germany) fluorescent microplate reader. All experiments were run in six parallels and repeated three times (n = 18).

6.10 Measurement of nitrite concentration

Culturing medium was replaced with a fresh one without dexamethasone, and the primary hepatocytes were incubated in the presence of 5 mg/L LPS + 50 mg/L IFNγ alone, or together with FA (1-100 μmol/L) for 24 h. Then NO_2^- production was measured by adding to 50 μL culture supernatant equal volume of Griess-reagent (1% sulphanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) and measuring light absorption at 550 nm by means of an Anthos 2010 (Rosys, Wiena, Austria) microplate reader. All experiments were run in six parallels and repeated three times (n = 18).

6.11 Measurement of free radical scavenging activity

Oxidation of the redox dye dihydrorhodamine123 was induced by 10 μmol/L H_2O_2 and 60 μmol/L EDTA Fe^{2+} salt in the presence and absence of 5 to 100 μmol/L of FA or resveratrol. Fluorescent intensity of the oxidized dye was measured at 494 nm excitation and 517 nm emission wavelengths by using a LS50B spectrofluorimeter (Perkin-Elmer Ltd, Budapest, Hungary). Experiments were repeated three times (n = 3). Values are means ± SEM.

6.12 Statistical analysis

When pertinent, data were presented as means ± S.E.M. For multiple comparisons of groups, ANOVA was used. In some experimental models, because of the different doses (20 mg/kg and 40 mg/kg) of LPS, data were analyzed using one-way or two-way ANOVA followed by Bonferroni's correction. When F-test indicated unequal variances, Kruskal-Wallis test was used. For survival experiments, Mantel-Cox's logrank test was used. Differences were considered statistically significant at $P<0.05$.

Results

7.1 Effect of 4HQN on LPS-induced inflammatory processes *in vivo*

***7.1.1 Effect of 4HQN on survival of LPS-treated mice*:** Mice treated with a single dose of LPS (20 mg/kg) died within 50 hours. When the mice were pre-treated three times a day, one day before the LPS challenge by 4HQN (100 mg/kg), 80 % of the animals in the group survived. Even when groups of mice received only a single shot of 4HQN (100 mg/kg) 1 hour after the LPS challenge, the PARP-1 inhibitor significantly protected the animals against LPS-induced death demonstrated by a survival rate of 30 % (*Fig.6.*). When 4HQN were administered 6 hours after LPS challenge in a single shot (100 mg/kg) it did not have protective effect (*Fig.6.*). 4HQN treatment itself did not induce death or any obvious damage (*data not shown*).

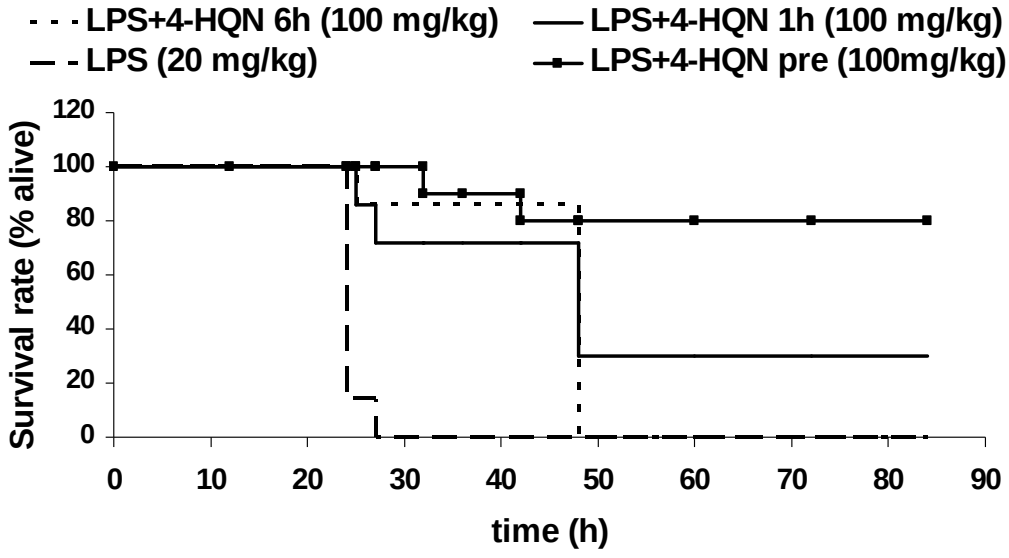


***Fig. 6.* Effect of 4HQN on the LPS-induced mortality in mice.** 4HQN (100 mg/kg i.p.) was injected three times a day one day prior the LPS (20 mg/kg i.p.) treatment (pre-treatment with PARP-1 inhibitor) or was injected in a single dose (100 mg/kg i.p.) 1 or 6 hours after LPS (post-treatment with PARP-1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

7.1.2 MRI-analysis: Untreated, LPS-treated and LPS + 4HQN-treated mice underwent MRI-analysis. T_2-weighted transversal spin-echo images were taken from the thoracic and

lower abdominal regions (*Fig.7.*). Signal intensities of T_2 images were proportional to the inflammatory response. Representative images selected from 12 images for each group are presented in *Fig.7.*.

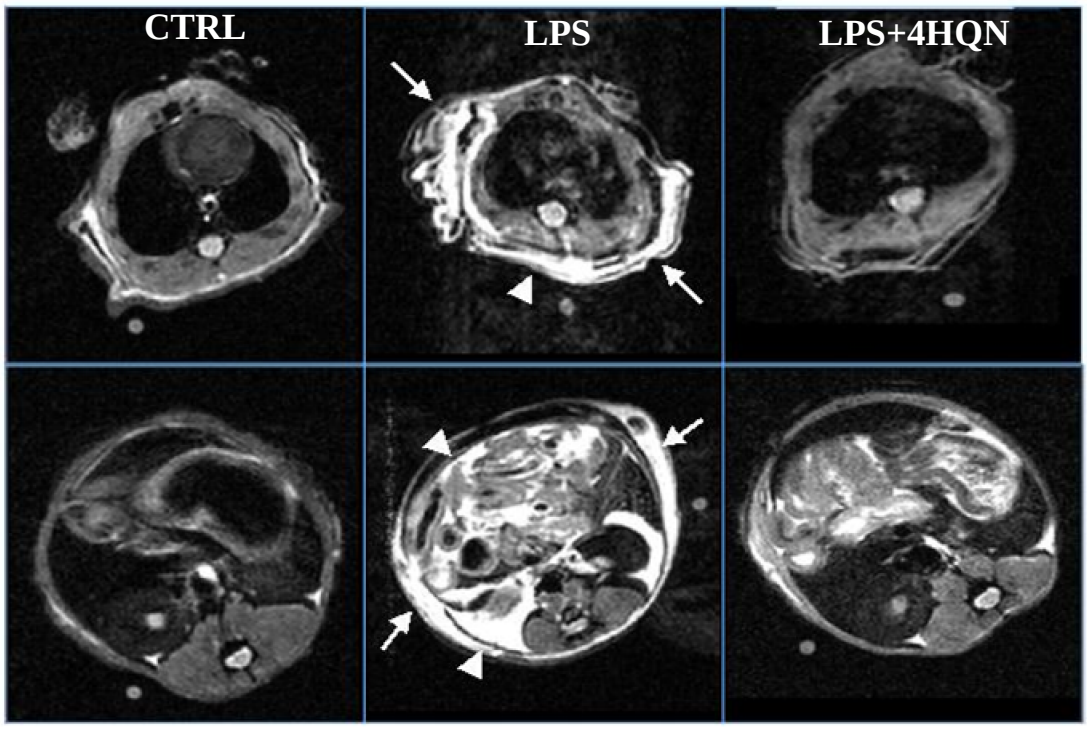


***Fig. 7.* Effect of 4HQN treatment on T_2-weighted MR images of the thoracic (upper row) and the lower abdominal (lower row) region in mice**. Typical T_2-weighted images of untreated (left column), LPS (20 mg/kg i.p.) treated (middle column) and LPS + 4HQN (20 mg/kg i.p. LPS and 3 × 100 mg/kg 4HQN 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 4HQN, the T_2-weighted intensities were markedly lower, so the inflammatory response was significantly smaller. T_2-weighted images of animals treated with 4HQN alone were basically identical to images of untreated mice.

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 and in the inter-intestinal cavities (*Fig.7.*). Among all the observed LPS-induced inflammatory responses, we found the most characteristic and most pronounced increases in the gastro-intestinal tract (*Fig.7.*). On the other hand, no increase in the signal could be observed inside the kidneys and in skeletal muscle, neither in the paravertebral nor in the femoral muscles (*data not shown*). All increases in signal intensities were significantly attenuated in mice treated with 4HQN, indicating that the PARP-1 inhibitor reversed the LPS-induced morphological changes (*Fig.7.*). Mice treated with 4HQN only were identical to the untreated control (*data not shown*).

7.1.3 Effect of 4HQN on LPS-induced TNFα production: LPS treatment resulted in a rapid increase in serum TNFα concentration which reached 2960 ± 112 pg/mL after 90 minutes (*Fig.8.*). In the 4HQN treated mice, LPS challenge resulted in significantly lower TNFα concentrations (1500 ± 135 pg/mL). 4HQN alone did not exert any significant effect on serum TNF-α levels (*Fig.8.*).

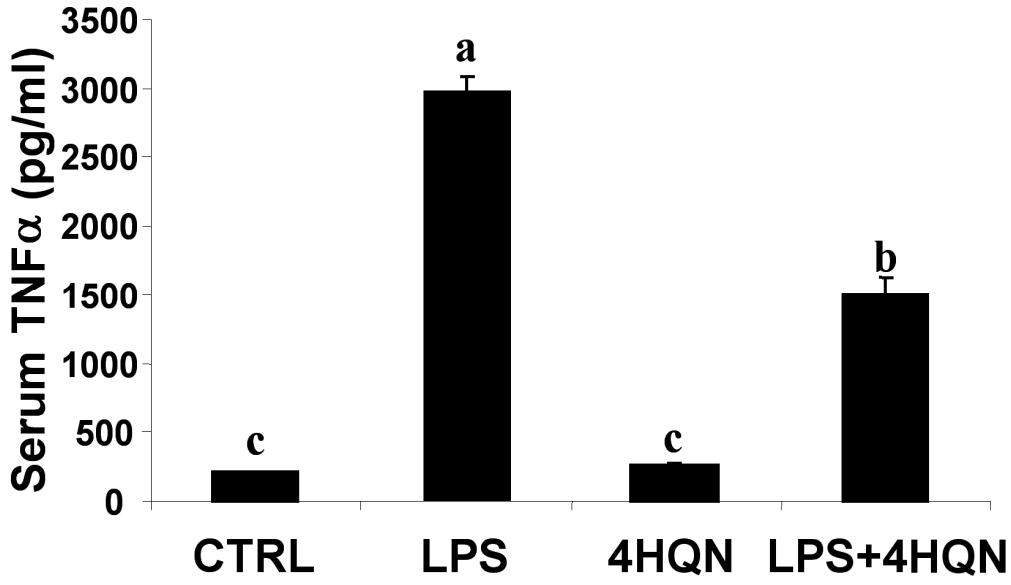


Fig. 8. **Effect of 4HQN on the serum TNFα concentrations of LPS-treated mice.** Serum TNFα concentrations were measured 1.5 hours after LPS injection. Control: injected with physiological saline; 4HQN: injected with 4HQN (3 × 100 mg/kg i.p.); LPS: injected with LPS (20 mg/kg i.p.); LPS + 4HQN: pre-treated with 4HQN (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. Means for a variable without a common letter differ, $P<0.05$.

7.1.4 Effect of 4HQN on LPS-induced phosphorylation of various kinases in liver, lung and spleen: Phospho-ERK1/2, 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 4HQN, and from 4HQN + LPS-treated mice.

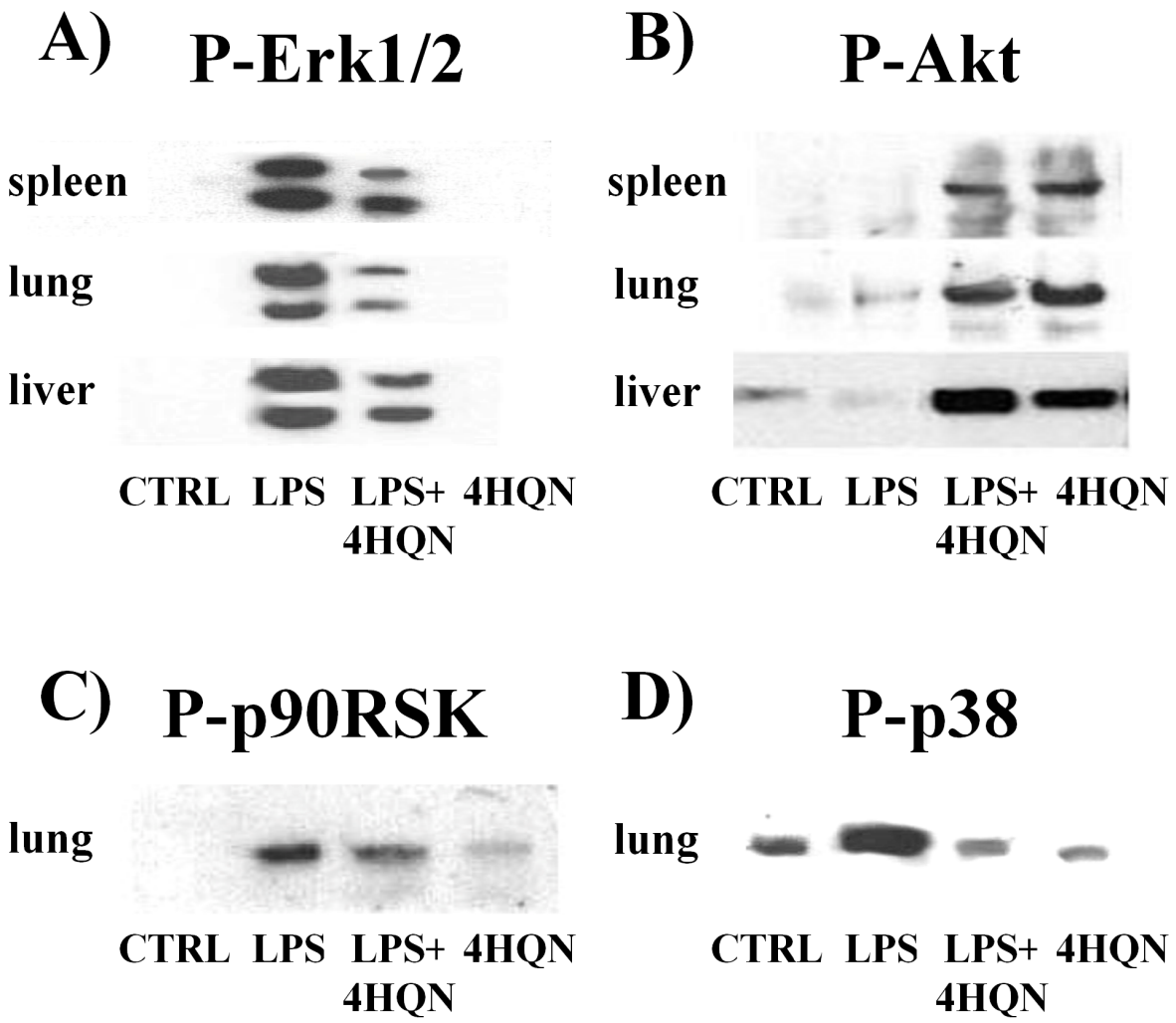


Fig. 9. **Effect of 4HQN on LPS-induced phosphorylation of various kinases in liver, lung and spleen:** *A) Inhibition of ERK1/2 MAPK by 4HQN pre-treatment, B) Activation of Akt by 4HQN pre-treatment,* C) *Effect of 4HQN on phosphorylation of p90RSK in lung, D) Inhibition of p38 MAPK by 4HQN pre-treatment in lung.* CTRL, injected with physiological saline); LPS, (20 mg/kg i.p.); LPS + 4HQN, pre-treated with 4HQN (3 × 100 mg/kg i.p.) and injected with LPS (20 mg/kg i.p.); 4HQN (injected with 100 mg/kg 4HQN alone).

Utilizing 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 4HQN and 4HQN + LPS treated mice (*Fig.9.*). Using an antibody against phospho Akt Thr^{308} region we did not find Akt activation neither with LPS nor with 4HQN 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 (Thr202/Tyr204) antibody. LPS-treatment resulted a marked increase in activation of ERK1/2 in the spleen, liver and in the lung. Pre-treatment with 4HQN significantly attenuated this activation in all three tissues. 4HQN treatment itself did not have any effect on the activation of phospho-ERK1/2 in the tissues we studied (*Fig.9.*). 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, which was attenuated by 4HQN pre-treatment (*Fig.9.C*). However, in spleen and in liver LPS did not induce the activation of p90RSK and 4HQN had no additional effect (*data not shown*). Phosphorylation of MAPK p38 was determined using an anti-phospho p38 antibody. As shown in *Fig.9.D*, LPS-stimulated p38 activation in the lung was attenuated by 4HQN pre-treatment. However, in spleen and in liver LPS did not induce the activation of p38 and 4HQN 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*).

7.1.5 Effect of 4HQN on LPS-induced NF-κB activation: Ninety minutes after LPS-treatment, NF-κB activation was assessed in spleen, lung and liver. LPS-treatment caused a significant increase in NF-κB activation in all three tissues. That was slightly but not statistically significantly attenuated by 4HQN pre-treatment in spleen (*Fig.10.A*). However, and in contrast to the spleen, NF-κB activation in the lung and in the liver was prevented by 4HQN pre-treatment (*Fig.10.B,C*). 4HQN treatment itself did not have any effect on NF-κB activation in the tissues studied.

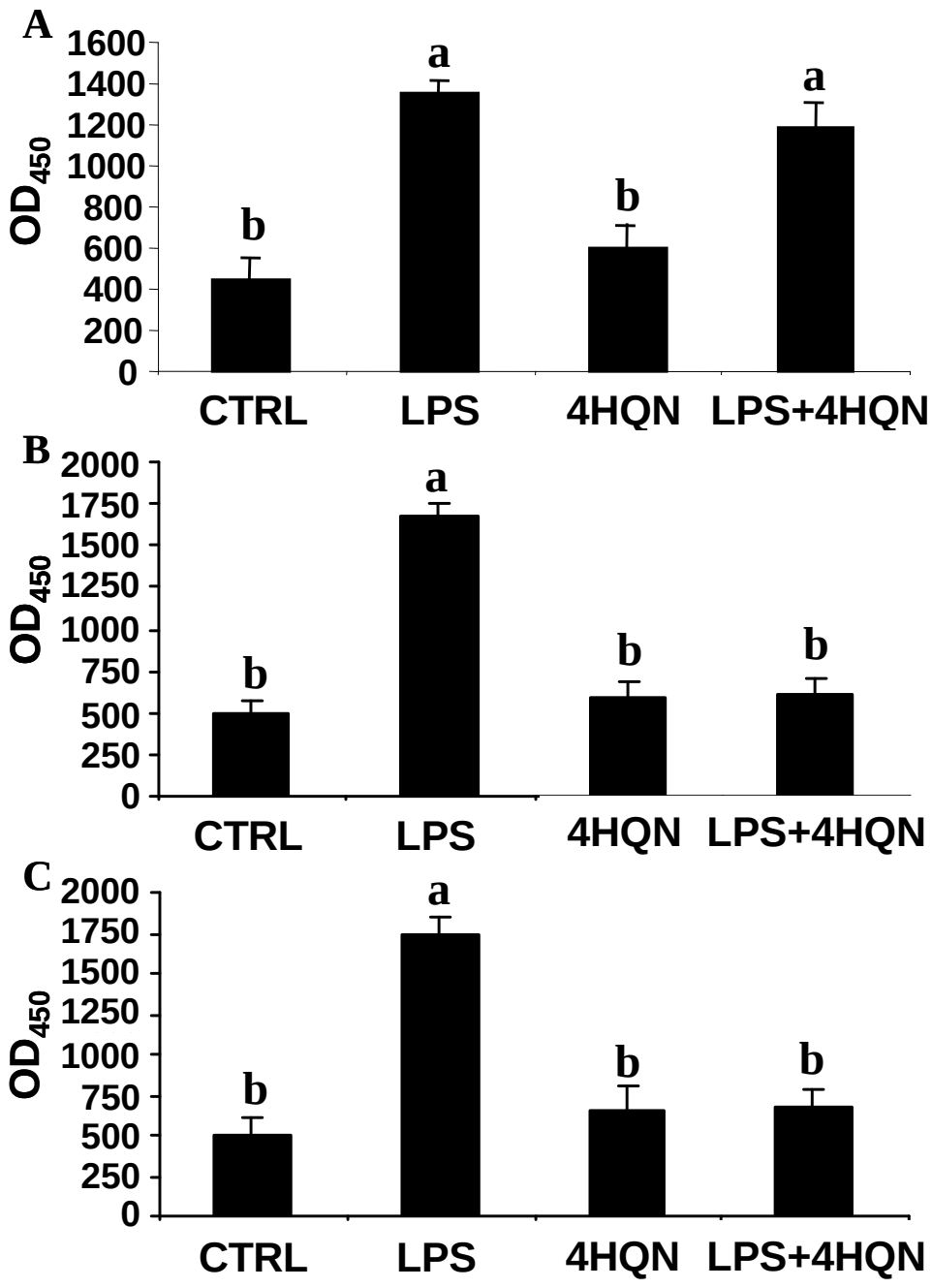


Fig.10. **Effect of 4HQN on NF-κB activation in the spleen (A), lung (B) and liver (C) in mice:** 1.5 hours after LPS injection, NF-κB activation was measured, with and without 4HQN pre-treatment. CTRL, injected with physiological saline; LPS, injected with LPS (20 mg/kg i.p.); 4HQN, injected with 4HQN (3 × 100 mg/kg i.p.); LPS + 4HQN, pre-treated with 4HQN (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. Means for a variable without a common letter differ, $P<0.05$.

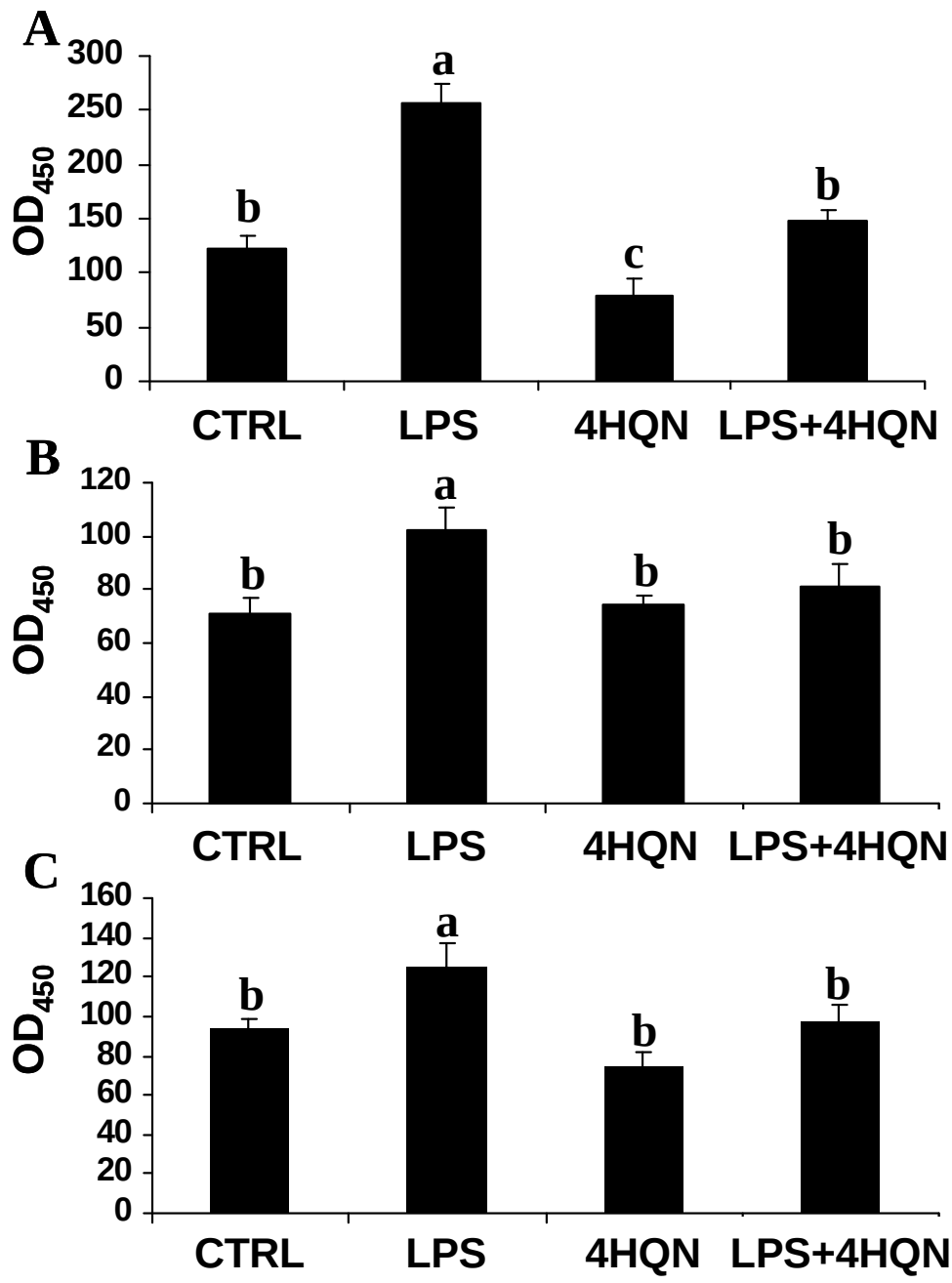


Fig.11. **Effect of 4HQN on AP-1 (c-Fos) activation in the spleen (A), lung (B) and liver (C) in mice:** 1.5 hours after LPS injection, AP-1 activation was measured with and without 4HQN pre-treatment. Control: injected with physiological saline; 4HQN: injected with 4HQN (3 × 100 mg/kg i.p.); LPS: injected with LPS (20 mg/kg i.p.); LPS + 4HQN: pre-treated with 4HQN (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. Means for a variable without a common letter differ, $P<0.05$.

7.1.6 Effect of 4HQN on LPS-induced AP-1 (c-Fos) activation: Ninety minutes after LPS-treatment, phosphorylation of AP-1 family member c-Fos and thereby the activation of this transcription factor was assessed in spleen, liver and lung. LPS-treatment caused a significant increase in c-Fos activation in all three tissues. 4HQN pre-treatment significantly inhibited this activation in the tissues we studied (*Fig.11.*). 4HQN treatment itself did not have any effect on c-Fos activation in the studied tissues.

7.2 Effect of FA on LPS-induced inflammatory processes *in vivo* and *in vitro*

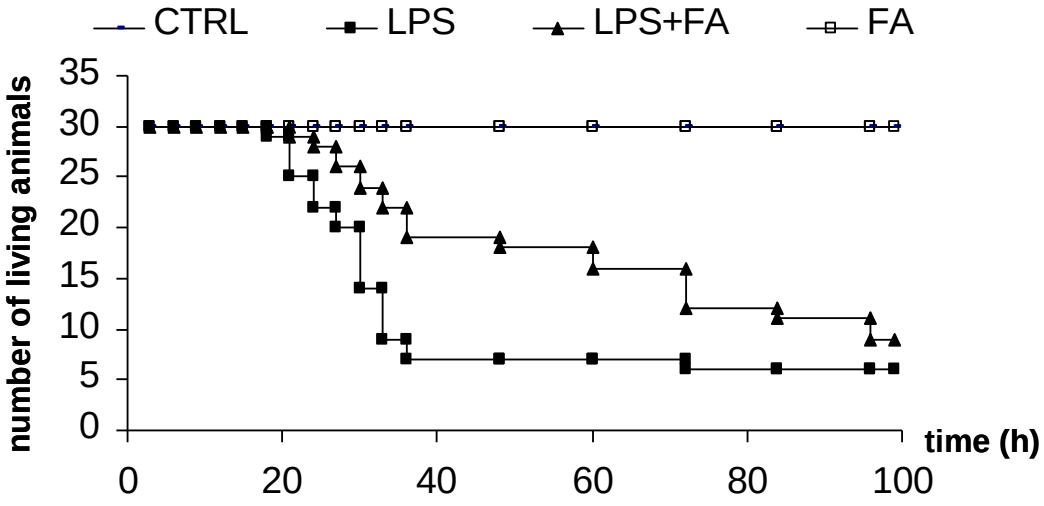


Fig.12. **FA prolongs survival time of LPS-treated mice**. Mice were pretreated with FA (6 mg/kg i.p.) 1 h before LPS challenge (40 mg/kg i.p.), and repeated every 12 h. Survival rate was observed up to 96 h for CTRL (line), LPS (filled square), LPS + FA (filled triangle) and FA (open square) groups having 10 mice each. Pooled data from three independent experiments (n = 30) are presented.

7.2.1 FA prolongs the survival time of LPS-treted mice: 80% of mice treated with a single high dose of LPS (40 mg/kg, i.p.) died within 36-48 h. Mice treated with FA 1 h before the LPS challenge (LPS + FA) and repeated every 12 h had a longer survival time compared to those that received PSS (*Fig.12.*). FA clearly delayed and attenuated the slope of the survival rate curve. 36-48 h after the LPS challenge 70% of mice in LPS + FA group survived vs. 20% in the LPS only group ($P<0.05$). Then, the difference between LPS + FA and LPS groups decreased indicating that FA had protective effect on early and intermediate phase of LPS-mediated inflammatory processes of septic shock, but not on late stage severe sepsis. FA

treatment alone did not induce death or any obvious damage (*Fig.12.*). A lower dose of LPS (20 mg/kg, i.p.) resulted in about 20% death within 72 h, and therefore was not suitable for survival studies (*data not shown*).

7.2.2 FA inhibits LPS-induced inflammatory response in vivo: For demonstrating *in vivo* inflammatory response, T_2-weighted images of CTRL, LPS and LPS + FA-treated mice were taken 6 h after the LPS challenge (*Fig.13.*). The lower abdominal region of LPS-treated mice showed marked increase of intensity *(inflammation)* especially in the lateral subcutaneous regions and the interintestinal cavities as well as around the kidneys. In mice treated with LPS + FA, the T_2-weighted intensities were markedly lower, so the inflammatory response was significantly smaller. T_2-weighted images of mice treated with FA alone were basically identical to images of untreated mice (*data not shown*).

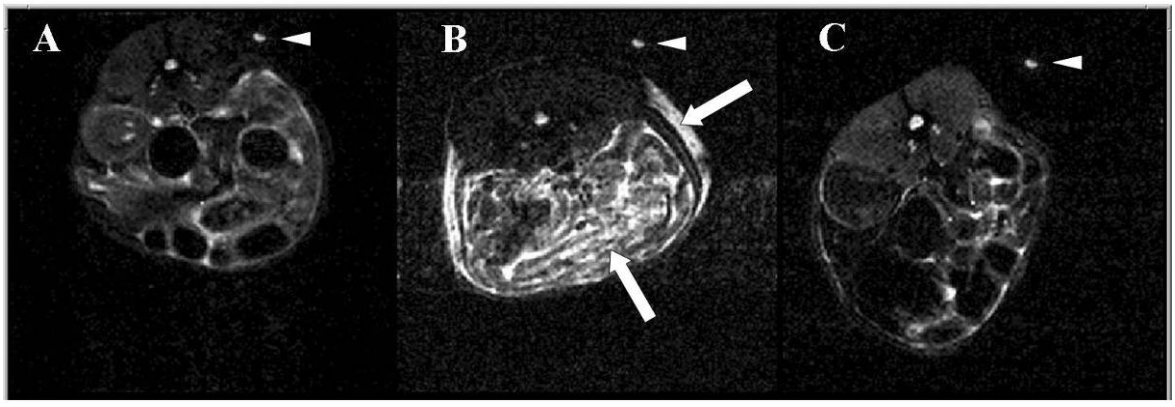


Fig.13. In vivo **inflammatory responses in untreated mice (A) and in those treated with LPS (B) or LPS + FA (C).** Lower abdominal region of LPS treated mice showed marked increase of intensity representing inflammatory response (arrows) especially in the lateral subcutaneous regions and in the interintestinal cavities. T_2-weighted images of mice treated with FA alone were basically identical to images of CTRL mice. Intensities were normalized to the internal standard (arrow heads). Results of the three sets of independent experiments were basically identical.

7.2.3 FA attenuates LPS-induced TNFα, IL-1β but not IL-6 production, and enhances IL-10 generation in the sera: We measured concentration of various cytokines from sera of CTRL, LPS, LPS + FA and FA-treated mice 1.5 or 3 h after the LPS challenge.

LPS treatment caused a 16-20 times increase of serum TNFα concentration 1.5 h after the application of the LPS that was attenuated by FA pre-treatment. FA was more effective in the case of the lower than of the higher dose of LPS in preventing LPS-induced TNFα induction (*Fig.14.A*). LPS-induced IL-1β production was assessed 3 h after the LPS treatment. LPS induced a 5-9 times increase in serum IL-1β concentrations that was decreased in the case of the low dose of LPS (*Fig.14.B*). IL-6 production was measured 3 h after the LPS treatment. LPS, administered in 20 or 40 mg/kg, induced a dramatic increase in IL-6 concentration but FA showed no reducing effect in either case (*Fig.14.C*). LPS caused a strong increase in IL-10 production 1.5 h after the LPS challenge. FA did not cause a significant alteration in the IL-10 production of mice receiving 20 mg/kg LPS. However and in contrast, FA pre-treatment increased IL-10 production in mice challenged with the higher dose of LPS (*Fig.14.D*). FA alone did not exert any effect on serum concentration of any of the cytokines (*Fig.14.*).

7.2.4 FA inhibits LPS+IFNγ-induced NO_2^- and ROS production in primary hepatocytes: Because of the proven role of NO production in inflammation-induced cell damage and death, we studied the effect of FA on LPS + IFNγ-induced NO production in primary hepatocytes by measuring NO_2^- in the culturing medium following 24 h incubation. Activation of hepatocytes by LPS + IFNγ increased NO_2^- concentration that was reduced by FA in a concentration dependent manner. FA, at the highest concentration used (100 µmol/L), reduced NO_2^- to the level of untreated control (*Fig.15.A*). During a 24 h incubation period, LPS + IFNγ increased ROS production in primary hepatocytes that was completely abolished by FA at the concentration of 50 µmol/L. Higher concentrations of FA decreased ROS production below the level of the untreated control (*Fig.15.B*). FA concentration in the culture medium did not decrease significantly during the incubation period (*data not shown*).

7.2.5 Direct free-radical scavenging activity of FA: We tested direct free-radical scavenging activity of FA by measuring H_2O_2-induced oxidation of fluorescent redox dye dihydrorhodamine123 in the presence and absence of the substance. FA at the concentration range of 0.25 to 50 µmol/L attenuated oxidation of the dye in a concentration dependent manner. The effect of FA and resveratrol did not differ significantly (*Fig.16.*).

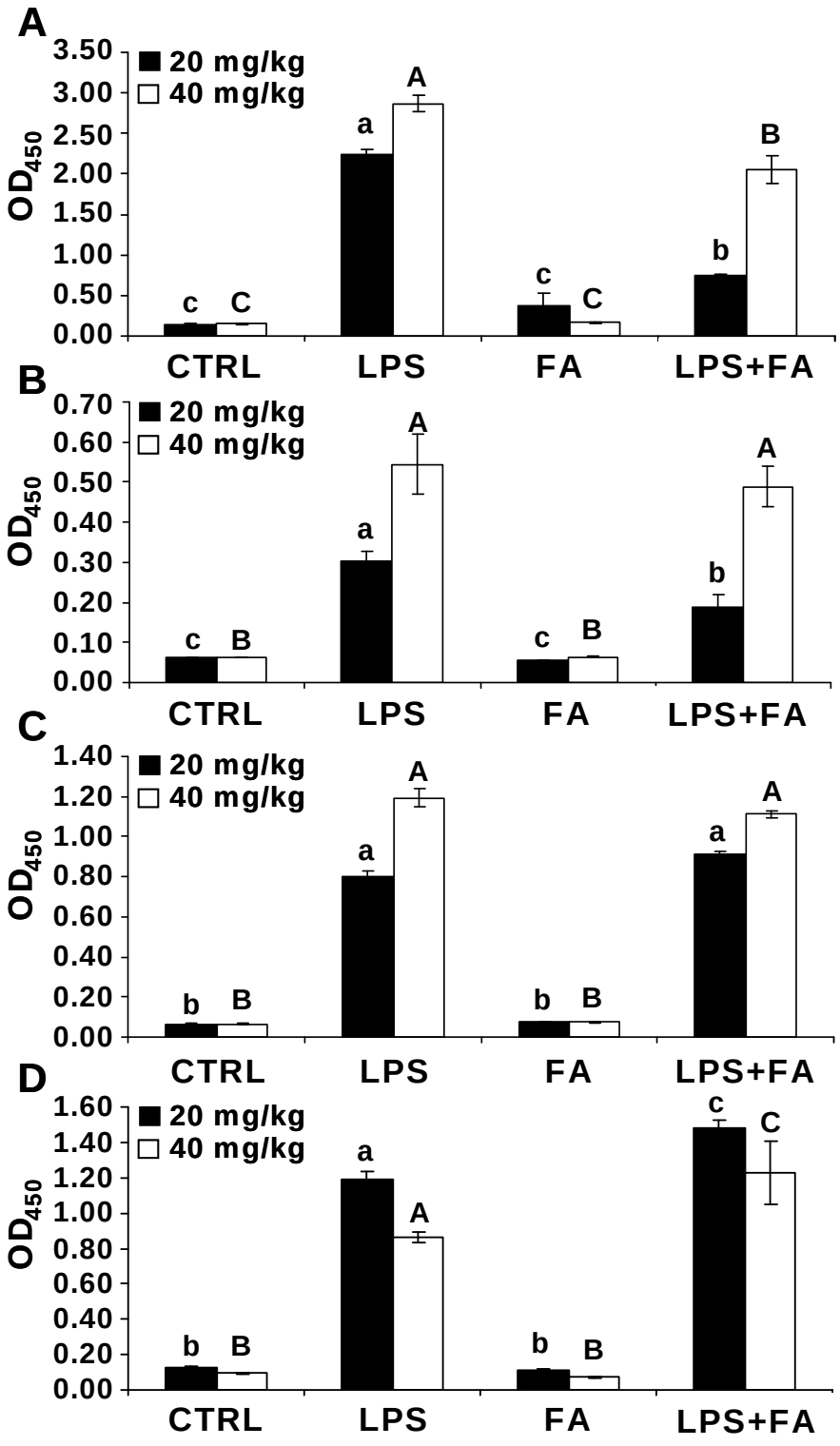


Fig.14. **Effect of FA on LPS-induced TNFα (A), IL-1β (B), IL-6 (C) and IL-10 (D) production in the sera of LPS treated mice.** Cytokine level was measured 1.5 (A, D) or 3 (B, C) h after LPS administration from the sera of CTRL, LPS, LPS + FA and FA mice. Three independent experiments with three mice in each experimental group were performed. Data were pooled (n = 9) and values are means ± SEM. Means for a variable without a common letter differ, $P<0.05$.

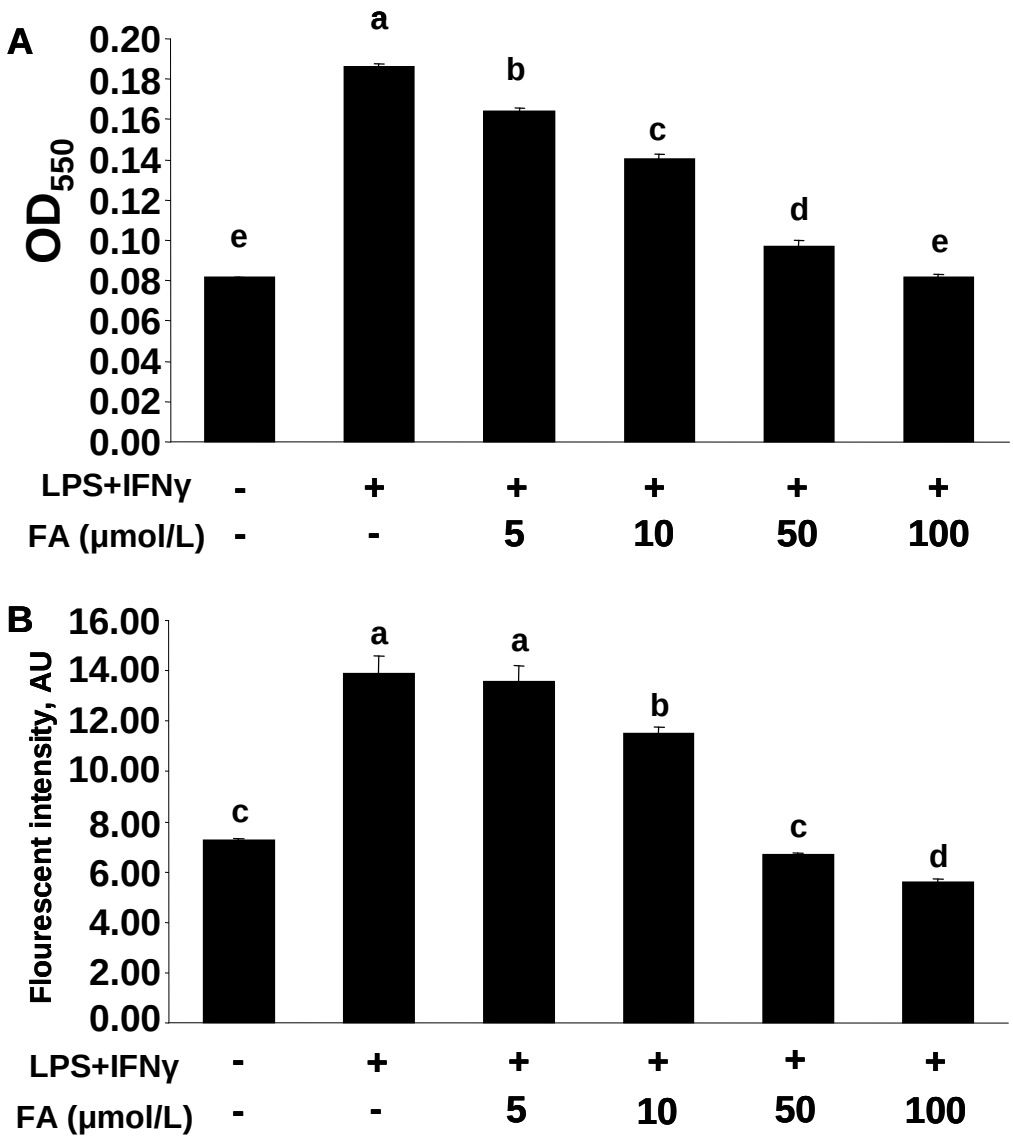


Fig.15. **FA reduces LPS + IFNγ induced NO_2^- (A) and ROS (B) production in primary hepatocytes.** Primary hepatocytes were incubated in the presence (+) and absence (-) of 5 mg/L LPS + 50 μg/L IFNγ alone (LPS+IFN**γ)**, and together with 1-100 μmol/L FA (actual concentration is indicated) for 24 h before measuring NO_2^- (A) and ROS (B) content in the culture supernatant as it was described in Materials and Methods. Data are expressed as mean ± SEM. All experiments were run in six parallels and repeated three times (n = 18). Means for a variable without a common letter differ, $P<0.05$.

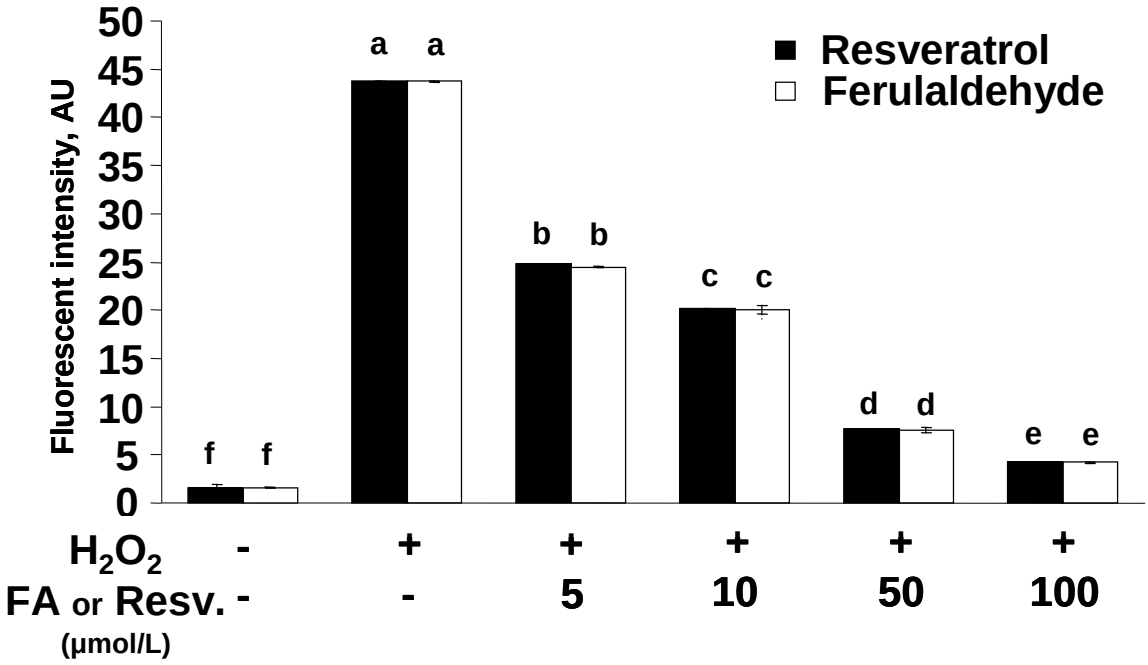


Fig.16. **Antioxidant effect of FA in cell-free system.** ROS content was measured in a cell free system. Oxidation of the redox dye dihydrorhodamine123 was induced by 10 µmol/L H_2O_2 in the presence (actual concentration is indicated) and absence (-) of 5 to 100 µmol/L of FA or resveratrol. Data are expressed as means ± SEM. All experiments were run in six parallels and repeated three times (n = 18). Means for a variable without a common letter differ, $P<0.05$.

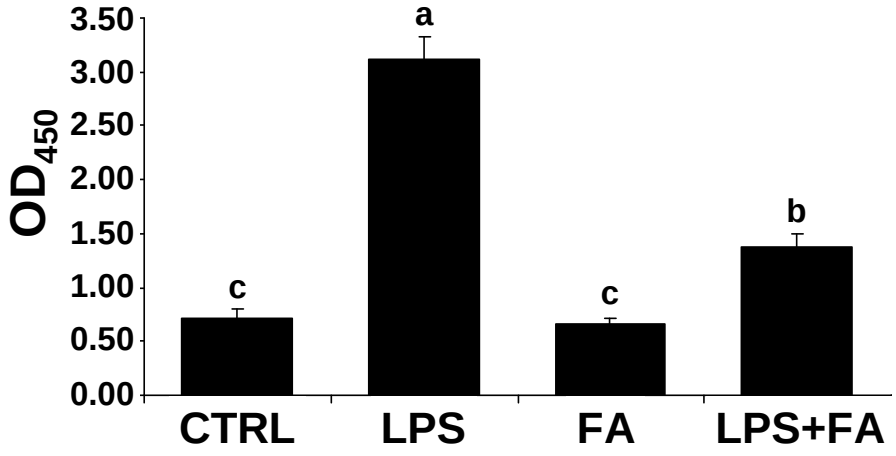


Fig.17. **FA inhibits NF-κB activation in the liver of LPS-treated mice.** Activation and nuclear translocation of NF-κB was measured 1.5 h after LPS injection from the nuclear fraction of the liver of CTRL, LPS, LPS + FA and FA mice. Data are expressed as means ± SEM of three mice per group. The experiment was repeated three times (n = 9). Means for a variable without a common letter differ, $P<0.05$.

7.2.6 FA inhibits LPS-induced NF-κB activation in liver: Since NF-κB pathway plays an important role in TLR4 signal transduction and in transcription of proinflammatory cytokines, we investigated the role of FA in NF-κB activation and nuclear translocation in CTRL, LPS, LPS + FA and FA-treated mice. We found a nearly four-fold activation of NF-κB 1.5 h after LPS challenge as compared to liver of control animals. LPS-induced activation and nuclear translocation of NF-κB was strongly inhibited in the liver of FA-pretreated mice (*Fig.17.*). FA alone did not exert any effect on the activation of NF-κB in our experimental model.

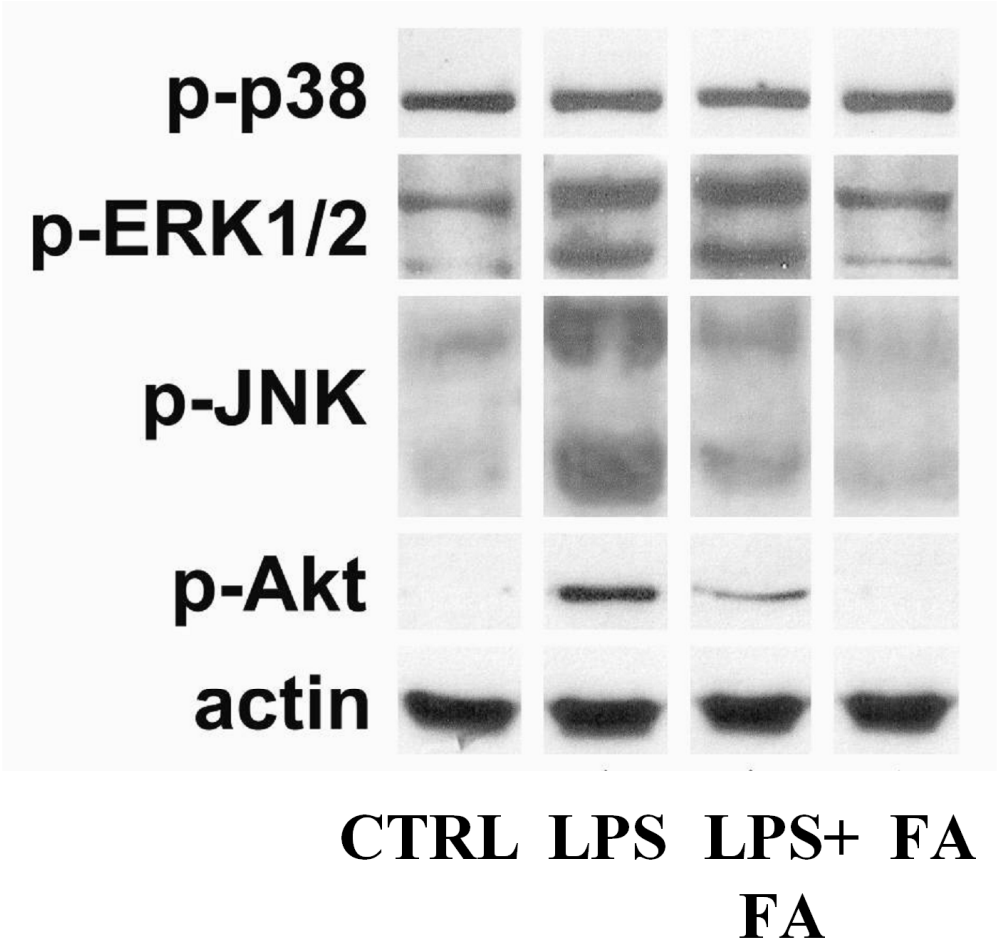


Fig.18. **Effect of FA on kinase activation in the liver of LPS-treated mice.** Activation of Akt, p38, ERK1/2 and JNK was demonstrated 1.5 h after LPS injection from liver homogenates of CTRL, LPS, LPS + FA and FA treated mice by using phosphorylation specific primary antibodies and immunoblotting. Representative immunoblots are presented. Experiments with three mice per group were repeated three times with basically identical results (n = 9).

7.2.7 FA negatively regulates LPS-induced phosphorylation of JNK and Akt in liver: Previously, we demonstrated that MAPK and Akt play important role in LPS signaling in our septic shock model (*Veres et al., 2004; Veres et al., 2003*). In order to study whether these kinases were involved in the protective effect of FA, we measured phosphorylation of JNK, ERK1/2, p38 MAPK and Akt from the liver of CTRL, LPS, LPS + FA and FA-treated mice 1.5 h after the LPS challenge. Phosphorylation and thereby activation of all the kinases studied, except p38 MAPK, were increased following LPS stimulation (*Fig.18.*). FA significantly prevented this activation in the case of JNK and Akt, but failed to attenuate LPS-induced activation of ERK1/2 (*Fig.18.*). FA alone did not exert any effect on the phosphorylation of the kinases studied in our experimental model.

Discussion

Despite of numerous hopeful *in vivo* and *in vitro* experimental studies, sepsis remains a leading cause of death, and there are just a few successful attempts in the development of potent anti-inflammatory drugs for human therapy. In this work, we demonstrated the effect and mechanism of action of two basically different compounds; 4-hydroxyquinazoline, a synthetic PARP inhibitor, and ferulaldehyde, a naturally occurring antioxidant and degradation product of some other polyphenols, in an *in vivo* model of LPS-induced endotoxic shock. In addition, we studied antioxidant effect of FA, in LPS+IFNγ-treated primary hepatocytes. Both of the used compounds inhibited NF-κB, one of the most important inflammatory transcription factor, but the regulation of signaling mechanisms leading to the activation and nuclear translocation of NF-κB suggested two fundamentally different mechanisms.

In previous works, PARP-1 "knock out" mice were shown to be highly resistant against LPS-induced endotoxic shock. In order to determine whether PARP-1 inhibition with 4HQN prevents LPS-induced lethality in mice and interferes with early or late mediators of endotoxic shock, we performed survival studies in which we administered 4HQN in pre-treatment as well as in post-treatment 1 or 6 hours after the LPS-challenge (*Fig.6.*). Within 2 hours after LPS-injection, the mice exhibited signs of hypomotility, hypothermia, tremor, diarrhea and a characteristic crouching gait. We have found that 4HQN pre-treatment protected the BALB/c mice in about 80%, had a protective effect of 30% when it was added 1h after, but had no effect when it was added 6 hours after the LPS (20mg/kg) injection suggesting that 4HQN interfered preferably with early mediators of the LPS-induced septic shock (*Fig.6.*). To support this observation, 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, liver or spleen and 4HQN did not have any additional effect on these factors (*data not shown*).

In our next work, we used FA a naturally occurring antioxidant at the lowest possible concentration (6mg/kg), at which the compound still had protective effect against septic shock in order to match the physiological concentrations that were found after consuming polyphenol-rich food. Also, in the experiments with FA, we used LPS in two different concentrations; in a 20mg/kg (*low dose*) for determining the molecular mechanism of the drug and a 40mg/kg (*high dose*) for survival experiments. Furthermore, since we investigated the

effect of a potent antioxidant, we needed an alternative mice strain, namely C57BL/6, which produces a stronger immunresponse including a robust oxidative stress after LPS-injection than the BALB/c mice used for the experiments with the PARP-inhibitor. Many comparative studies suggest that acute inflammation develops in a strain-specific manner in mice (*McCaskill et al., 2006*). C57BL/6 and BALB/c mice are prototype hosts for studying resistance and susceptibility to several infectious diseases. In experiments with *Propionibacterium acnes* (PA), an anaerobic bacterium implicated as a putative etiologic agent of sarcoidosis, C57BL/6 and BALB/c mice were intraperitoneally sensitized and intratracheally challenged with heat-killed bacteria. C57BL/6 mice treated with PA developed a strong cellular immune response characterized by elevations in Th1 cytokines/chemokines, increased numbers of lymphocytes and macrophages in lung lavage fluid, and peribronchovascular granulomatous inflammation composed of T- and B-lymphocytes and epithelioid histiocytes. T-lymphocytes in the lung lavage fluid showed a marked CD4+ cell predominance. In contrast, BALB/c mice challenged with PA, showed only a modest induction of Th1 cytokines, less pulmonary inflammation, and no granulomatous changes in the lung (*McCaskill et al., 2006*). These results showed, that in contrast to BALB/c mice, C57BL/6 mice developed a more powerful inflammatory response, with the activation of the innate immunity and possesses a significantly higher resistance to bacterial infection. Due to this strong immunresponse, C57BL/6 mice were found to be more resistant to infections than BALB/c mice under numerous experimental conditions, including LPS or IFNγ induction, Gram-positive bacterial infection *(Propionibacterium acnes, Staphylococcus epidermidis* (*McCaskill et al., 2006*)) or even the infection with a nematode parasite of wild rodents *(Angiostrongylus costaricensis* (*Geiger et al., 2001*)*)* .

The high dose of LPS used for survival studies resulted in about 80% death of the C57BL/6 animals (*Fig.12.*). Although not able to prevent eventual death, FA significantly increased survival time of C57BL/6 mice (*Fig.12.*) showing that it was able to positively influence the complex processes leading to LPS-induced oxidative stress and inflammatory response. This protective effect of FA was more pronounced on the early phase of LPS-mediated inflammatory processes of septic shock rather than on late stage severe sepsis. The different efficacy of 4HQN and FA on the survival of LPS-treated mice was probably due to (i) the relatively low concentration of FA used in our experiments, (ii) strain differences of the mice used and (iii) the presumptively distinct molecular mechanism of the two drugs.

Sepsis is characterized by end-organ dysfunction, which is poorly detectable by pathohistological techniques in the early phase of sepsis. With our method, based on the

phenomenon of nuclear magnetic resonance, we obtained a real time insight into inflammatory processes in living animals. The *in vivo* response to lipopolysaccharide-induced endotoxic shock was detected by MR-Imaging techniques. Six hours after LPS-administration, we observed markedly increased intensities in the thoracic and lower abdominal region of the animals on T_2-weighted MR-images. 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.7.*) similar to those found in a porcine endotoxic shock model (*Oldner et al., 1999*). The importance of these tracts in the mediation of sepsis and multiple organ failure is well documented (*Boulares et al., 2003; Standiford et al., 1995*). Pre-treatment of the BALB/c mice with a potent PARP-1 inhibitor 4HQN diminished the LPS-induced thoracic and abdominal inflammatory responses as revealed by T_2 imaging (*Fig.7.*). T_2 weighted MR-images of LPS-challenged C57BL/6 mice showed, that LPS-induced inflammation was well detectable in the abdominal regions of the mice, and that FA significantly attenuated the overall abdominal inflammatory response (*Fig.13.*).

TNFα is a substantial early mediator of endotoxemia since the production of this cytokine returned to a normal level 4 hours after LPS treatment (*James et al., 2002; Zanetti et al., 1992*). It is a primary mediator of the innate immune system and is crucial for the induction of a local protective immune response against infection, trauma and ischemia. However, excessive TNFα production can be lethal in itself, because it spreads in the bloodstream and produces cardiovascular collapse. TNFα is a sufficient and necessary mediator of early phase septic shock. When administered before LPS-treatment (20mg/kg to BALB/c mice), 4HQN attenuated the LPS-induced elevation in the serum TNFα concentrations by approximately 50 % (*Fig.8.*), consistent with the notion that this PARP-1 inhibitor, similarly to PJ34, partially inhibits the expression of TNFα gene. In our other LPS-induced endotoxic shock model with C57BL/6 mice, even the lower LPS dose (20mg/kg), that caused only negligible death rate among the mice, induced significant induction of TNFα and all the proinflammatory cytokines tested. FA attenuated the LPS-induced increase of serum TNFα and IL-1β for both LPS doses; however, it was significantly more effective in reducing proinflammatory cytokine production in case of the lower LPS dose (*Fig.14.A,B*). In several previous reports, a direct links between TNFα and IL-1β as well as IL-10 were established (*Xing et al., 1998*). However, contradictory results appeared about the role of IL-6 in the inflammatory response (*Xing et al., 1998*). Our finding that FA could not attenuate

LPS-induced elevation of serum IL-6 level indicates that IL-6 was not involved among the anti-inflammatory mechanisms of FA in our murine septic shock model (*Fig.14.C*). The link between the proinflammatory TNFα and anti-inflammatory IL-10 was most probably due to some compensatory mechanism. In agreement with this view, FA did not affect any of the cytokines in unstimulated mice. FA enhanced the LPS-stimulated IL-10 production (*Fig.14.D*), which indicates that FA attenuated the LPS-induced *in vivo* inflammatory response not only by attenuating TNFα-linked proinflammatory cytokine production but also by enhancing anti-inflammatory IL-10 production.

Signaling mechanisms leading to systemic tissue damages induced by binding of LPS to the CD14 and TLR4/MD2 complexes involve activation of ERK1/2, JNK, p38 MAP kinases and the proinflammatory transcription factors: AP-1 and NF-κB. Due to the importance of the functional state of different organs during septic shock, we investigated in lung, liver and spleen of BALB/c mice 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 ERK1/2 and other MAP kinases in different cell lines (*Guha et al., 2002; Dumitru et al., 2000; Xiao et al., 2002*). In agreement with these results, we found in BALB/c mice, that ERK1/2 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 ERK1/2 and JNK was reported that was not detectable in our BALB/c sepsis model. However, we have found that p90RSK, a downstream target of ERK1/2, showed a similar activation pattern in lung as did ERK1/2 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 MAPK are a key element of the signal transduction that regulates the LPS-mediated transcription factor activity, inhibition of these kinases can contribute to reducing the endotoxin-induced inflammatory responses. Under our experimental conditions in BALB/c mice, ERK1/2 was activated (*Fig.9.A*) by LPS in all the tissues studied (liver, lung and spleen) but p38 MAP kinase (*Fig.9.D*) had detectable activation only in lung indicating that the signaling from cell surface (CD14-TLR4/MD2)-LPS receptor complex to the activation of the different branches of MAP kinases has tissue specific components (*Fig.9.*).

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 ERK1/2 and JNK pathways in these tissues in our experimental system of LPS-induced BALB/c mice. Interestingly, the phosphorylation of the ERK1/2 substrate p90RSK (*Fig.9.C*) also showed tissue specificity indicating that ERK1/2 activation does not necessarily trigger the activation of p90RSK and the activation of p90RSK-dependent transcription factors. LPS induced the activation of ERK1/2, p90RSK and p38 MAP kinase but not JNK in lung, and there was a significant increase in the activation of NF-κB (*Fig.10.*) and c-Fos (*Fig.11.*) transcription factors showing that gene expressions in lung are regulated by ERK1/2 or p38 MAP kinase but not by JNK pathway in the investigated mice strain. In liver and spleen, out of the MAP kinases, only the ERK1/2 pathway was activated by LPS in the BALB/c mice, and it is likely that this pathway activated the c-Fos and NF-kB transcription factors. In the absence of PARP-1 inhibitor, LPS did not affected PI3K/Akt (*Fig.9.B*) pathway, therefore, MAP kinase pathways had to play the major role in activation of Ap-1 and NF-kB in BALB/c mice.

The clearest effect of PARP-1 inhibitor on the kinase cascades was the activation of Akt in the absence and in the presence of LPS in all studied tissues (*Fig. 9.B*). In monocytes, it was found that inhibition of PI3K/Akt pathway can activate MAP kinases (*Guha and Mackman, 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-kB 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.9.*).

Since Akt activation can be induced by structurally different PARP-1 inhibitors, such as quinazoline derivates, phenantridine derivates (*Veres et al., 2003*) and carboxamino benzimidazol derivates (*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. Oxidative stress can activate both the PARP enzyme and Akt, but it is also known, that the activation of PARP suppresses the phosphorylation, i.e. activation of Akt. Accordingly, there is a fine balance between induction and suppression, which can be pushed by the PARP inhibitors toward the activation.

In spite of the lack of the precise molecular mechanism, the finding that PARP-1 inhibitors reverse the inflammatory processes and organ damages (*Fig.7.)*(*Veres et al., 2003*) probably via the activation one of the most important protective kinase cascades, the

PI3K/Akt pathway, provides a novel possibility to prevent multiorgan failure in septic shock. While the Akt activation seems to occur in all studied tissues (*Fig.9.*), 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 could suppress NF-kB activation in lung and in liver but not in spleen (*Fig.10.*), while c-Fos activation was suppressed in all studied tissues (*Fig.11.*) in BALB/c mice.

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

Not just PARP inhibitors, but dietary polyphenols and natural products were shown to modulate the aforementioned signaling mechanisms in different tissues and cells (*Stalińska et al., 2005; Kutuk et al., 2006; Sanchez-Tillo et al., 2007*). In the pathomechanism of inflammation ROS and RNS are strongly involved. Since these high reactive agents can affect strand breaks in the DNA and therefore PARP activation, the elimination of free radicals can prevent the induction of PARP enzyme. This phenomenon proposes the possibility to use antioxidants as an indirect inhibitor of PARP in some experimental models. Accordingly, in the further experiments with C57BL/6 mice, we determined the effect of FA on MAPK and Akt signaling and NF-κB activation only in one organ namely in the liver of LPS-treated mice. Our preference was based on previous experiments with primary hepatocytes derived from the same type of mice. After LPS+IFNγ-treatment, we measured an increased ROS and nitrite production of hepatocytes, which was significantly attenuated by FA in a concentration dependent manner (*Fig.15.A,B*), showing an important role of FA in the abrogation of inflammatory processes in the liver. Interestingly, we found altered kinase activation pattern in the liver of C57BL/6 mice in contrast to the activation pattern found in the same organ of BALB/c mice. We found ERK1/2 and JNK, but not p38, activation in response to LPS *(Fig.18.)*. JNK plays a crucial role in LPS-induced NF-κB activation (*Sanchez-Tillo et al., 2007*); therefore, upon our observation, activation of this kinase pathway is consistent with

both NF-κB activation and proinflammatory cytokine production in the liver and blood of LPS-treated C57BL/6 mice. FA inhibited the LPS-induced JNK activation, but did not affect ERK1/2 and p38 MAPK pathways *(Fig.18.)*. LPS-induced Akt activation was also suppressed by FA *(Fig.18.)* that could have contributed to its anti-inflammatory effect since it has been suggested that, independently from JNK pathway, PI3K–Akt-mTOR can activate LPS-induced NF-κB activation and nuclear translocation (*Dos Santos et al., 2007*). Suppressing both LPS-induced JNK and Akt activation, FA inhibited the most important pathways leading to NF-κB activation, namely the LPS-TLR4-JNK, LPS-PI3K-Akt-mTOR pathways and NF-κB itself. Similar mechanisms could account for the anti-inflammatory properties of one of the most profoundly studied polyphenols, trans-resveratrol (*Das et al., 2007*) which was reported to inhibit PI3K (*Fröjdö et al., 2007*), the upstream activator of Akt. Our observation that FA did not show any effect on the LPS-induced activation of ERK1/2 and p38 MAP kinase pathways indicates that FA's inhibitory target(s) is necessarily downstream of the TLR4 receptors.

In the investigation of signaling mechanisms in the livers of mice derived from different strains, we found not just an alteration in the effect of 4HQN and FA, but a modified kinase activation pattern induced by the same type of LPS molecule. These findings can potentially explain the proven difference in the inflammatory response produced by the different type of mice. Interestingly, the main difference in the kinase pattern found in the liver was the activation of JNK and induction of Akt pathway by LPS in C57BL/6 mice, in contrast to BALB/c mice, in which we were not able to measure neither JNK nor Akt phosphorylation after LPS injunction. JNK is known as a proinflammatory pathway, which leads via AP-1 and NF-κB activation to the production of proinflammatory cytokines and to inflammation. The previously observed phenomenon that C57BL/6 mice produce a more powerful inflammatory response than BALB/c mice, can be explained by our observation of the induction of the proinflammatory JNK pathway in C57BL/6 but not in BALB/c mice.

The other important pathway, which is able to modulate inflammation, is the PI3K/Akt pathway, a "two-edged sword". In general, Akt is thought to be a survival pathway making the cells to survive and to proliferate under numerous conditions. But many papers provide evidence about the role of Akt in potentiating inflammation via inducing iNOS and NF-κB. Many reports suggest that the resistance of C57BL/6 is due to the production of nitric oxide by macrophages in response to IFNγ and TNF-α (*Santos et al. 2006*). These cytokines are mainly secreted by Th1 cells and macrophages against the invading bacteria. BALB/c,

usually unable to give rise to Th1 lymphocytes, does not control certain infections. Furthermore, C57BL/6 derived macrophages were shown to be far more sensitive to the stimulus of LPS+IFNγ and produce definitely more NO than, macrophages of BALB/c. The differential amount of NO produced by the macrophages was found to be correlated with the accumulation of iNOS mRNA and protein, which shows that the expression of iNOS is differentially regulated in the cells derived from differential strains of mice (*Santos et al. 2006*). Many papers indicate an essential role of Akt in regulating the production of NO via activating iNOS. According to these, the observed phosphorylation of Akt in the liver of C57BL/6 (*Fig.18.*) found in our experiments, can lead to an enhanced iNOS activity and to elevated NO production, in contrast to BALB/c, which lacks Akt activation and therefore a powerful NO production after LPS treatment. These published results, together with our findings can potentially explain the different immune response in C57BL/6 and BALB/c mice via the found distinct molecular mechanisms.

In our experiments, we used two fundamentally different compounds to inhibit inflammatory processes: FA, a naturally occurring antioxidant and 4HQN a synthetic PARP inhibitor, which does not possess a direct antioxidant activity. This feature might probably be the basis of the differential mechanism by which these molecules regulate the inflammation. The main difference between the effect of FA and 4HQN was the regulation of Akt. We found, that 4HQN activated Akt with or without LPS treatment and FA inhibited Akt phosphorylation induced by LPS-challenge. The regulatory mechanism of FA is probably due to its antioxidant activity. PTEN, a highly ROS/RNS sensitive protein-phosphatase is able to modulate Akt phosphorylation. High levels of ROS/RNS can damage the PTEN-protein which leads to loss of function, namely to the loss of phosphatase activity, so PTEN is unable to dephosphorylate PI3K, an upstream activator of Akt. This phenomenon is in agreement with our findings, i.e. the found Akt activation in the liver after LPS-injection. We hypothesize that by its antioxidant activity, FA was able to eliminate reactive oxygen and nitrogen species and PTEN could dephosphorylate PI3K preventing phosphorylation of Akt as we found in the liver after LPS+FA treatment. About the effect of PARP inhibitors on Akt activation is less known, the identification of possible molecular targets and the mechanism by which an inhibitor of a nuclear enzyme can modulate the activation of the cytosolic Akt needs further investigation.

Since macrophages of C57BL/6 mice were shown to produce remarkably more NO than macrophages from BALB/c after LPS+IFNγ-treatment (*Santos et al. 2006*), and liver is one of the main organs affected in septic shock, we measured the effect of FA on ROS and

RNS production in primary hepatocytes derived from C57BL/6 and induced by LPS+IFNγ treatment. Reactive oxygen and nitrogen species are strongly involved in the pathomechanism of the LPS-induced inflammatory response, primarily among cellular components of the blood and endothelial cells (*Huet et al., 2007*). Eliminating or inhibiting the production of these highly reactive radicals has been shown to attenuate inflammatory damages. Previously, it was found that FA suppressed the LPS+IFNγ-induced iNOS expression in RAW264.7 macrophage cells (*Kim et al., 1999*). However, the pathological changes in the liver of LPS-treated mice could be resulted from ROS and NO produced by the hepatocytes themselves. Therefore, we determined FA's effect on LPS+IFNγ-induced NO_2^- and ROS production in primary hepatocytes. Typical LPS concentrations used for activation of macrophages are in the 100 μg/L to 1 mg/L range; however, primary hepatocytes proved to be less sensitive. Therefore, we used 5 mg/L LPS combined with 50 μg/L IFNγ in order to induce full activation of the hepatocytes. We found that FA inhibited the NO production induced by LPS plus IFNγ (*Fig.15.A*) in a concentration dependent manner in the hepatocytes similarly to RAW264.7 macrophages. Furthermore, FA attenuated LPS+IFNγ-induced ROS production in the hepatocytes (*Fig.15.B*). In order to determine whether this antioxidant property of FA was due to its free-radical scavenging activity, we used a cell-free *in vitro* system. As we found, FA demonstrated a free-radical scavenging activity that was about the same as that of resveratrol (*Fig.16.*). Published effects of resveratrol on cytokine profile, NF-κB translocation and kinase signaling (*Das et al., 2007; Fröjdö et al., 2007*) were very similar to those we found for FA. However, being a phenolic compound, resveratrol possesses a low bioavailability and most importantly, a rapid clearance from the plasma. Ferulic acid, the oxidized form of FA, was a major degradation product in the urine after consumption of red wine (*Gonthier et al., 2003*), furthermore, FA is present in red wine as a natural component or degradation product at a concentration comparable to that of resveratrol (*unpublished result*). Bioavailability of ferulic acid was reported to be higher than that of other dietary flavonoids and monophenolics (*Beecher et al., 1998*), and we found plasma concentration of FA to be 22 times higher than that of resveratrol 1.5 h after equimolar i.p. administration of the two drugs to the mice (*unpublished result*). Curcumin, another widely studied antioxidant and anti-inflammatory natural product, rapidly and spontaneously degrades to FA among other substances in solution (*Wang et al., 1997*). Again, there was a high similarity between published effects of curcumin on cytokine profile, NF-κB activation and kinase signaling (*Gonzales et al., 2008; Kim et al., 2007*) and of our results on FA. All these data suggest that highly soluble degradation end products could be responsible for or contribute to the

antioxidant and anti-inflammatory effects of resveratrol and curcumin, and in a broader sense to that of dietary polyphenols and natural compounds.

Taken together, LPS induces a different extent of MAP kinase activation in different organs and even in the same organ of mice derived from different strains. MAPK can activate Ap-1 and NF-κB transcription factors in a tissue specific manner, which induces activation of pro-inflammatory genes (TNFα, IL-1β; IL-6) 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 PI3K/Akt pathway, which can inhibit MAP kinase (p38, Erk1/2) and p90RSK activation, can attenuate transcription factor activation (NF-κB, AP-1), were able to inhibit cytokine production (TNFα) and prevent inflammatory tissue damage in a tissue specific manner. FA, as a well known antioxidant inhibited the same transcription factor the NF-κB, but via a different mechanism, namely with the inhibition of JNK and Akt pathways. This positive effect on modulating important kinase cascades can be due to the elimination of ROS and RNS.

Conclusion

In our experiments we investigated the effect of 4HQN, a PARP inhibitor with negligible antioxidant properties, and FA, a potent antioxidant with no effect on PARP, on LPS-induced inflammatory processes in BALB/c and C57BL/6 mice, respectively.

1. Our experiments underlined that PARP activation is critically involved in the pathogenesis of sepsis and inhibition of this nuclear enzyme is able to reduce the activation of the most important sepsis related transcription factor, the NF-κB via modulating signal transduction pathways such as PI3K/Akt as well as ERK and p38-MAPK in a tissue specific manner in mice.

2. We found that FA, a microbial metabolite of several polyphenols has anti-inflammatory effect via modulation of kinase cascades such as JNK and Akt, the transcription factor NF-κB and some inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-10 in mice. Furthermore, elimination of ROS and RNS contributed to this anti-inflammatory effect as we demonstrated in primary hepatocytes and in a cell free system. Accordingly, phenolic acids and aldehydes, as potential microbial degradation products of several polyphenols, with higher bioavailability than their parent molecules can at least contribute to the anti-inflammatory effect of their parent polyphenols.

3. According to our data, FA, an antioxidant and 4HQN, a PARP inhibitor both inhibited LPS-induced inflammatory processes, but via a completely different mechanism. 4HQN inhibited PARP, the transcriptional co-activator of NF-κB, and enhanced LPS-induced overactivation of Akt thereby increased the activity of this cytoprotective pathway. On the other hand, FA prevented nuclear translocation of NF-κB by scavenging ROS and RNS as well as by inhibiting the pro-inflammatory JNK signaling.

4. We found that LPS did induce Akt activation in C57BL/6 while did not at all affected it in BALB/c mice. Also, JNK was activated by LPS in C57BL/6 while unchanged in BALB/c mice. Considering the importance of these kinase signaling pathways in the

inflammatory process, these findings can explain the altered sensitivity toward LPS of these mouse strains.

These results about the effect of 4HQN and FA in LPS-induced endotoxic shock can confirm the overall picture about the role of NF-κB in inflammation and gives another insight into the complex world of signaling mechanism leading to the modulation of this transcription factor.

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