Effect of a red wine compound on LPS-induced inflammatory processes *in vivo* and *in vitro*

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1. Contents

1. Contents 2

2. List of publications 4

3. Abbreviations 5

4. Introduction 6

4.1. Sepsis 6
4.1.1. Epidemiology of sepsis 6
4.1.2. Definitions of sepsis, severe sepsis and septic shock 7
4.1.3. Sepsis models 8

4.2. LPS, the public enemy 9

4.3. LPS signaling, LPS-induced inflammatory processes 11
4.3.1. TLR4 signaling 12
4.3.2. MAPK signaling, MKP-1 13
4.3.3. PI3K/Akt signaling 16
4.3.4. Transcription factors: NF- B and AP-1 17
4.3.5. ROS 19
4.3.5.1. ROS production in inflammation 19
4.3.5.2. Mitochondrial membrane potential and ROS production 20
4.3.6. Cytokines 21

4.4. Treatment of sepsis 22

4.5. Antioxidants 25
4.5.1. Polyphenols 25
4.5.2. Ferulic acid, ferulaldehyde 28

5. Aims of the study 31

6. Materials and Methods 32

6.1. Reagents 32

6.2. Animals 32

6.3. Cell cultures 32

6.4. Murine sepsis model 33

6.5. Magnetic resonance imaging (MRI) 33

6.6. Determination of ROS production 34

6.7. Measurement of nitrite concentration 34

6.8. Measurement of free radical scavenging activity 34

6.9. Western blot analysis 35
6.9.1. Treatments 35
6.9.2. Sample preparation from cells 35
6.9.3. Sample preparation from liver 35
6.9.4. SDS-PAGE 35

6.10. RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR) 36

6.11. Cytokine determination 36

6.12. Determination of NF- B activity 37
6.12.1. Using a Trans-AM[TM] Transcription Factor Assay Kits 37
6.12.2. Using NF- B luciferase assay 37

6.13. Measurement of mitochondrial membrane potential (m) 38

6.14. Mass spectrometry (MS) analysis 38

6.14. Statistical analysis 39

7. Results and Discussion 40

7.1. Ferulaldehyde reduced LPS-induced mortality in mice 40

7.2. Ferulaldehyde inhibited LPS-induced inflammatory response in vivo 41

7.3. Ferulaldehyde attenuated LPS-induced TNF-α, IL-1β but not IL-6 production, and enhanced IL-10 generation in the sera of mice 42

7.4. Feruladehyde inhibited LPS+IFN- -induced NO_2^- and ROS production in primary hepatocytes ... 45

7.5. Ferulaldehyde inhibited LPS-induced ROS and NO_2^- production in RAW 264.7 macrophages 46

7.6. Ferulaldehyde protected the mitochondrial membrane potential in RAW 264.7 macrophages 48

7.7. Ferulaldehyde negatively regulated LPS-induced phosphorylation of JNK and Akt, and activation of NF-κB in liver 50

7.8. Ferulaldehyde diminished LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages 53

7.9. Ferulaldehyde regulated LPS-induced MAPK activation via induction of MKP-1 expression 55

7.10. Ferulaldehyde inhibited LPS-induced NF- B activation in RAW 264.7 macrophages 56

7.11. Direct free-radical scavenging activity of ferulaldehyde 59

7.12. Inhibition of early inflammatory response in mice and in RAW 264.7 macrophages by ferulaldehyde 61

8. Conclusion 62

9. References 64

10. Acknowledgement 81

2. List of publications

This work is based on the following articles:

1. Tucsek Z, Radnai B, Racz B, Debreceni B, Priber K J, Dolowschiak T, Palkovics T, Gallyas F Jr, Sumegi B, Veres B.: Suppressing LPS-induced early signal transduction in macrophages by a polyphenol degradation product: a critical role of MKP-1. J Leukoc Biol. *(accepted)*

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2. 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.

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Further publications:

3. Szanto A, Hellebrand EE, Bognar Z, **Tucsek Z**, Szabo A, Gallyas F Jr, Sumegi B, Varbiro G.: PARP-1 inhibition-induced activation of PI-3-kinase-Akt pathway promotes resistance to taxol. Biochem Pharmacol. 2009 Apr 15;77(8):1348-57.

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3. Abbreviations

AP-1, activator protein-1, **APC**, activated protein C, **ATF-2**, activating transcription factor-2, **CAT**, catalase, **CRE**, cAMP-responsive element, **CREB**, cAMP-responsive element-binding protein, **COX-2**, cyclooxygenase-2, **DSP**, dual-specificity protein phosphatase, **dsRNA**, double-stranded RNA, **ELAM**, endothelial leukocyte cell adhesion molecule, **ERK1/2**, extracellular signal-regulated kinase, **FA**, ferulaldehyde, **GPI**, glycosylphosphatidylinositol, **GSH**, glutathione, **GSHPx**, glutathione peroxidase, **GSSGR**, glutathione reductase, **HMGB-1**, high-mobility group box-1 protein, **ICAM**, intercellular adhesion molecule, **IFN-** , interferon- , **I B**, inhibitor of NF- B, **IKK**, I B kinase, **IL**, interleukin, **iNOS**, inducible NO-synthase, **IRAK**, interleukin-1 receptor-associated kinase, **JNK**, c-Jun N-terminal kinase, **LBP**, lipopolysaccharide binding protein, **LPS**, lipopolysaccharide, **LY96**, TLR4-associated lymphocyte antigen 96, **M3K**, MKK kinase, **MAPK**, mitogen-activated protein kinase, **MDP**, muramyl dipeptide, **MKK**, MAPK kinase, **MKP**, MAP kinase phosphatase, **MyD88**, myeloid differentiation primary response gene 88, **MRI**, magnetic resonance imaging, **NAC**, N-acetyl-L-cysteine, **NF- B**, nuclear transcription factor-kappa B, **NIK**, NF- B-inducing kinase, **NO**, nitric-oxide, **PAMP**, pathogen-associated molecular pattern, **PI-3K**, phosphoinositide-3 kinase, **PIP2**, phosphatidylinositol (3,4)-bisphosphate, **PIP3**, phosphatidylinositol (3,4,5)-trisphosphate, **PRR**, pathogen recognition receptor, **PSS**, physiological saline solution, **PTEN**, phosphatase and tensin homologue, **RHR**, Rel homology region, **RNS**, reactive nitrogen species, **ROS**, reactive oxygen species, **SAPK**, stress-activated protein kinase, **Sir2**, silent information regulator 2, **SIRS**, systemic inflammatory response syndrome, **SIRT1**, sirtuin 1, **SOD**, superoxide dismutase, **ssRNA**, single-stranded RNA, **TAB**, TAK1-binding protein, **TAK1**, MAP3K transforming growth factor- -activated kinase1, **TICAM1**, toll-like receptor adaptor molecule 1, **TICAM2**, TIR domain-containing adapter molecule 2, **TIR**, Toll/IL-1 receptor, **TIRAP**, toll-interleukin 1 receptor domain containing adaptor protein, **TLR**, toll-like receptor, **TNF-** , tumor necrosis factor- , **TRAF-6**, TNF receptor-associated factor-6, **TRAM**, TRIF-related adaptor molecule, **TRE**, TPA-responsive element, **TRIF**, TIR-domain-containing adapter-inducing interferon- , **VCAM**, vascular cell adhesion molecule

4. Introduction

4.1. Sepsis

4.1.1. Epidemiology of sepsis

A tight regulation of the immune/inflammatory system is crucial for maintaining the balance between protective and tissue-damaging responses. Systemic inflammatory response syndrome (SIRS) and sepsis are characterized by a loss of control over inflammatory responses, which can be provoked by a variety of causative agents and severe clinical insults. Sepsis is the leading cause of death in hospitalized patients and its incidence is increasing worldwide. Lots of studies were published about the epidemiology of sepsis in different countries. One of the relevant studies reported an increase in the annual incidence from 73.6 to 175.9 per 100.000 of the population in the United States between 1979 and 1989, which represents up to 11% of all hospital admissions (Angus and Wax 2001). Harrison and colleagues examined trends in the incidence and mortality of severe sepsis in the UK between 1996 and 2004. 27% were identified as having severe sepsis in the first 24 h following admission. The percentage of admissions with severe sepsis during the first 24 h rose from 23.5% to 28.7%, but the mortality of these patients decreased from 48.3% to 44.7% in the nine-year long period (Harrison et al. 2006). In a German study 3877 patients were screened and the incidence of severe sepsis (including septic shock) was 11% and the incidence of sepsis was 12.4%. The hospital mortality of patients with severe sepsis was 48.4 and 55.2% (Engel et al. 2007). Sepsis remained the most common cause of death in intensive care units and affects over 18 million people worldwide with an expected 1% increase of incidence per year despite of use of more potent and broader-spectrum antibiotics, immunosuppressive agents and invasive technology (Ulloa and Tracey 2005). Developing of more effective drugs and clinical treatments needed to define the following terms: sepsis, severe sepsis and septic shock and determine their detailed physiological parameters.

4.1.2. Definitions of sepsis, severe sepsis and septic shock

American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference held in 1991, offered new definitions for SIRS, sepsis, severe sepsis, septic shock, hypotension, and multiple organ dysfunction syndrome which are in force since that time. SIRS describes a hyper-inflammatory state of the immune/inflammatory systems and it is defined by the combination of typical clinical symptoms in the presence (sepsis) or absence (SIRS) of microbial (bacterial, fungal, or parasitic) infection: hypothermia/hyperthermia (abnormalities of body temperature), tachycardia (heart rate), tachypnea (respiratory rate) and leukocytopenia/leukocytosis (white blood cell count) (Bone et al. 1992, Abraham et al. 2000, Matot and Sprung 2001). Originally, the diagnosis of sepsis and SIRS required the manifestation of at least two of the above mentioned clinical signs. Sepsis is defined as 'severe' when these symptoms are associated with hypotension, hypoperfusion, and different multiple organ dysfunctions, such as oliguria, lactic acidosis, elevated liver enzymes or altered cerebral function (Bone et al. 1992, Abraham et al. 2000, Matot and Sprung 2001). Severe sepsis is a less acute syndrome with a mortality rate of 30–70%, progressing over 7–14 days and this is the one of the two clinical syndromes which is traditionally associated with sepsis (Ulloa and Tracey 2005). Septic shock marked by hypotension despite fluid resuscitation along with the presence of perfusion abnormalities (Bone et al. 1992). Septic shock accompanied by ischemic necrosis and cardiovascular collapse is a highly lethal syndrome of cardiovascular shock that kills within 24–48 h after onset. In some cases, these two clinical syndromes, septic shock and severe sepsis represent two different stages of the progression of sepsis. Patients, who survived the acute phase of septic shock, usually develop a state of severe sepsis which is characterized by multiple organ dysfunctions. In spite of these data, not all the patients who have septic shock will accordingly develop severe sepsis, and some patients have an extended form of severe sepsis without ever developing septic shock (Ulloa and Tracey 2005). Because of the leading cause of sepsis and septic shock was considered to be an infection with gram-negative bacteria, we have to know the pathways by which the causative agents are recognized by the immune system of the body.

4.1.3. Sepsis models

Frequently used experimental models of sepsis include cecal ligation and puncture (CLP), colon ascendens stent peritonitis (CASP), and the i.p. or i.v. injection of bacteria or bacterial products (such as lipopolysaccharide, LPS). Many of these models mimic the pathophysiological changes in human sepsis through generation of systemic inflammation (Rittirsch et al. 2007).

The bacterial inoculum model features a known number of Escherichia coli (using with or without Bacteroides fragilis) which are infused i.p. (Nakatani et al. 1996, Mathiak et al. 2000). The mortality rate depends on the species of animals used and the number of bacteria administered, so that this model becomes more controllable and reproducible than the model of fecal pellet deposition which induced by i.p. administration of feces with the mixed aerobic and anaerobic flora. This fecal pellet model resembles the CLP and the CASP models in many aspects. Similar to CASP, an abdominal septic focus leads to a polymicrobial infection of the peritoneum in the model of CLP, eventually resulting in bacteremia, SIRS, sepsis, septic shock, and usually death. Despite the clinical relevance and widespread use of CLP model in sepsis research, the concern of it is its consistence. The outcome of CLP is strongly associated with several factors during the procedure, such as the length of the cecum ligation, the size of needles used for the puncture, the number of the punctures, and the fluid resuscitation (Singleton and Wischmeyer 2003). In another well established model, the systemic administration of endotoxin/LPS (which is part of the outer membrane of the cell envelope of gram-negative bacteria) into the abdominal cavity or its i.v. infusion causes the clinical features of sepsis. Although it turns out that the LPS model and sepsis in humans differ in several key points (especially in the profile of cytokine release) (Cavaillon et al. 2003), this experimental model is widely used and gold standard method in sepsis research, that is why we have chosen it in our experiments to determine the pathophysiology of endotoxemia.

4.2. LPS, the public enemy

Pathogen recognition is one of the most basic and important properties of the immune system. Organisms need to recognize pathogens and differentiate “self” from “non-self”. This process based on the existence of specific, structurally conserved components of potentially pathogenic microorganisms, such as fungal beta-glucans, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), peptidoglycan and its structural component muramyl dipeptide (MDP) of Gram-positive bacteria and lipopolysaccharide of Gram-negative bacteria. These motifs are known as pathogen-associated molecular patterns (PAMPs) (Iliev et al. 2005, Leon et al. 2008, Akira 2009). Endotoxin has been implicated as a major cause of Gram-negative infections and generalized inflammation (Rietschel et al. 1994, Leon et al. 2008). The cell wall of Gram-negative bacteria consists of a thin peptidoglycan layer and an outer membrane containing lipopolysaccharide. LPS can be found in the external part of the outer membrane while phospholipids build up its internal part. LPS consists of three distinct domains such as lipid A, a short and non-repeating core of oligosaccharide and a distal polysaccharide (O-antigen or O-specific chain) parts which differ in chemical structure and biological activity (Rietschel et al. 1994, Raetz et al. 2002, Leon et al. 2008) (Fig. 1).

O-specific chain is exposed on the very outer surface of the bacterial cell, and as a consequence, it is a target for recognition by host antibodies. The structure of the O-antigen is composed of repeating units consisting of one to eight glycosyl residues (Rietschel et al. 1994). Bacterial strains differ in the conformation of these repeating units and this structural variability determines the serological specificity of the LPS and of bacteria containing it, and therefore O-specific chain functions as an important surface antigen (Rietschel et al. 1994). The presence or absence of O-chains determines whether LPS is rough or smooth. Full length O-chains turn the LPS molecule smooth while the absence of O-chains makes the LPS rough (Leon et al. 2008). Bacteria with rough LPS usually have more penetrable cell membranes to hydrophobic antibiotics because of the hydrophobic property of the rough LPS molecule (Tsujimoto et al. 1999).

In bacteria that produce smooth LPS (for example the Enterobacteriaceae family), the core domain, which is located between the lipid A and the O-antigen, is divided into two regions: inner core (lipid A proximal) and outer core. The core domain is structurally more uniform than the 0-chain, its structural diversity primarily based on its outer core region. The outer core contains the common hexoses, D-glucose, D-galactose, and N-acetyl-D-

glucosamine, while the inner core is consists of the characteristic and LPS specific components, heptose, 3-deoxy-D-manno-octulosonic acid (Kdo) and other non-carbohydrate components (phosphate, amino acids, ethanolamine substituents) (Rietschel et al. 1994, Raetz et al. 2002).

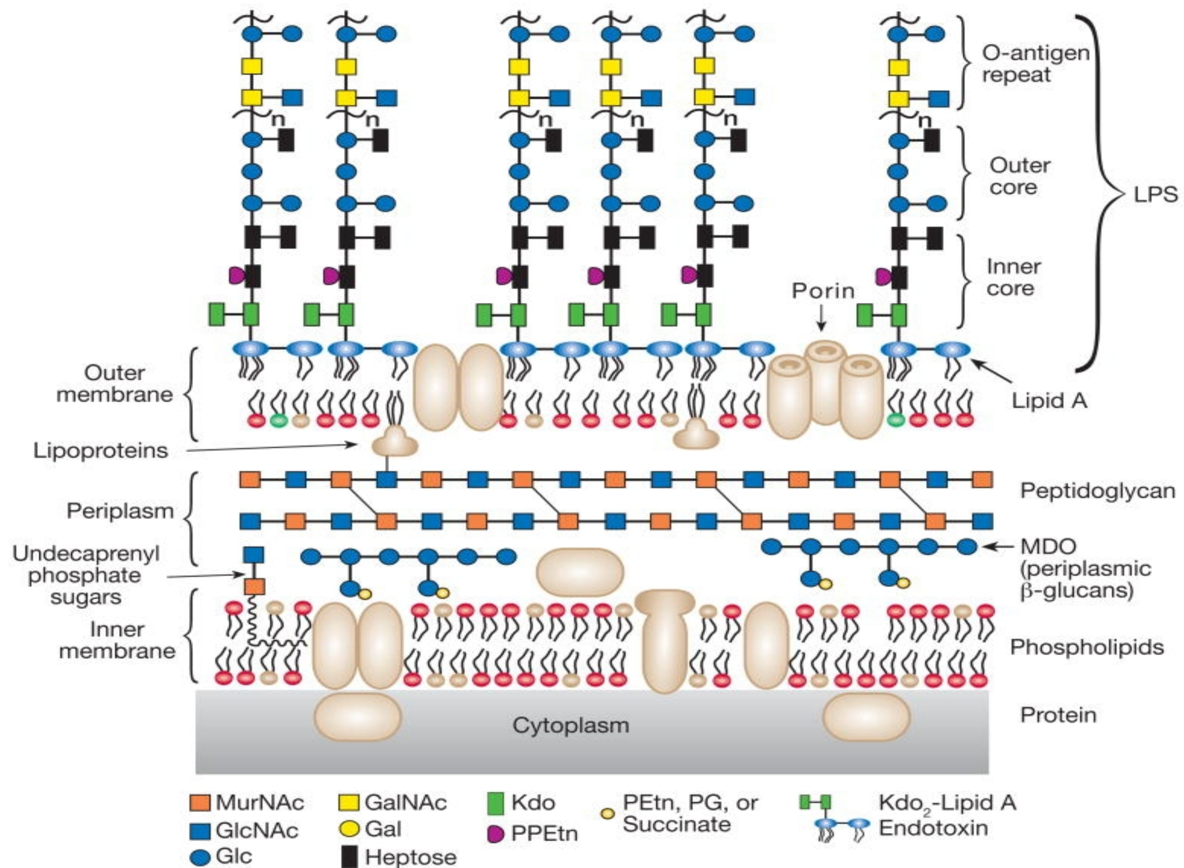


Fig. 1. Schematic representation of the cell wall of Gram-negative bacteria. *(Essentials of Glycobiology, Eubacteria and Archaea, Jeffrey D Esko, Tamara L Doering, and Christian RH Raetz)*

The glucosamine based phospholipid, lipid A anchors LPS to the peptidoglycan layer. The disaccharide glucosamine skeleton carries phosphoryl groups and (R)-3-hydroxy fatty acids. Variations in structure result from the type of hexosamine present, the degree of phosphorylation, the presence of phosphate substituents, and most notably, the nature, chain length, number, and location of acyl groups. Lipid A is the bioactive component of the LPS molecule and is recognized during human infection, thereby this part of the LPS molecule is responsible for the LPS-induced inflammatory processes (Raetz et al. 2002).

4.3. LPS signaling, LPS-induced inflammatory processes

Inflammation is a self-limiting and protective response of the body in which lymphocytes, monocytes, and macrophages produce cytokines under control to eliminate infectious agents. However, aberrant innate immune response to infection has been implicated into triggering dysregulated hyper-inflammatory responses in sepsis (Bone 1996, Russell 2006, Shimaoka and Park 2008). Recognition of PAMPs (e.g. LPS) is mediated by genotypically encoded pathogen recognition receptors (PRRs), for example the toll-like receptors (TLRs) (Iliev et al. 2005, Akira 2009). Different members of the TLR family can transmit signals through activating distinct intracellular signaling cascades that may result in pathogen specific cellular responses (Hirschfeld et al. 2001, Takeda and Akira 2005). In macrophages and monocytes, LPS binding to TLR4 has been shown to initiate multiple intracellular signaling events, including the activation of I B kinase (IKK)/ nuclear transcription factor (NF)- B pathway; mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (p42/44 MAPK or ERK1/2), p38 MAP kinase and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK); and the phosphoinositide-3 kinase (PI-3K)/Akt pathway (Cario et al. 2000, Dumitru et al. 2000, Fang et al. 2007, Zhong and Kyriakis 2007) (Fig. 2). These signaling pathways in turn activate a variety of transcription factors that include NF- B (p50/p65) and activator protein-1 (AP-1, c-Fos/c-Jun) (Bone et al. 1997, Bozinovski et al. 2002, Kim et al. 2005, Luyendyk et al. 2008). The activation of these transcription factors ultimately leads to the synthesis and release of diverse mediators of inflammation, pro-inflammatory cytokines and chemokines (tumor necrosis factor- (TNF-), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), high-mobility group box-1 protein (HMGB-1) etc.) (Beutler and Cerami 1988, Dinarello 1991, Lakhani and Bogue 2003, Murakami et al. 2005, Luyendyk et al. 2008), as well as the expression of cyclooxygenase-2 (COX-2), inducible NO-synthase (iNOS) and the upregulation of cell adhesion molecules (intercellular adhesion molecules (ICAMs), endothelial leukocyte cell adhesion molecules (ELAMs, E-selectin), platelet endothelial cell adhesion molecule-1 (P-selectin) and vascular cell adhesion molecules (VCAMs)) (Woo and Kwon 2007, Chiu and Lin 2008). Overexpression of COX-2 and iNOS can lead to the production of reactive oxygen species (ROS) and nitric-oxide (NO) respectively, as prime tools of antimicrobial function of activated macrophages (Chiu and Lin 2008) (Fig. 2). Secretion of pro-inflammatory cytokines, upregulation of cell adhesion molecules and production of ROS and NO lead to a continued stimulation of epithelial and

endothelial cells that in turn might activate the NF- B transcription factor. Thus, a positive autoregulatory loop might be established that can amplify the inflammatory response and finally leads to enhanced systemic inflammation, endothelial dysfunction and organ failure.

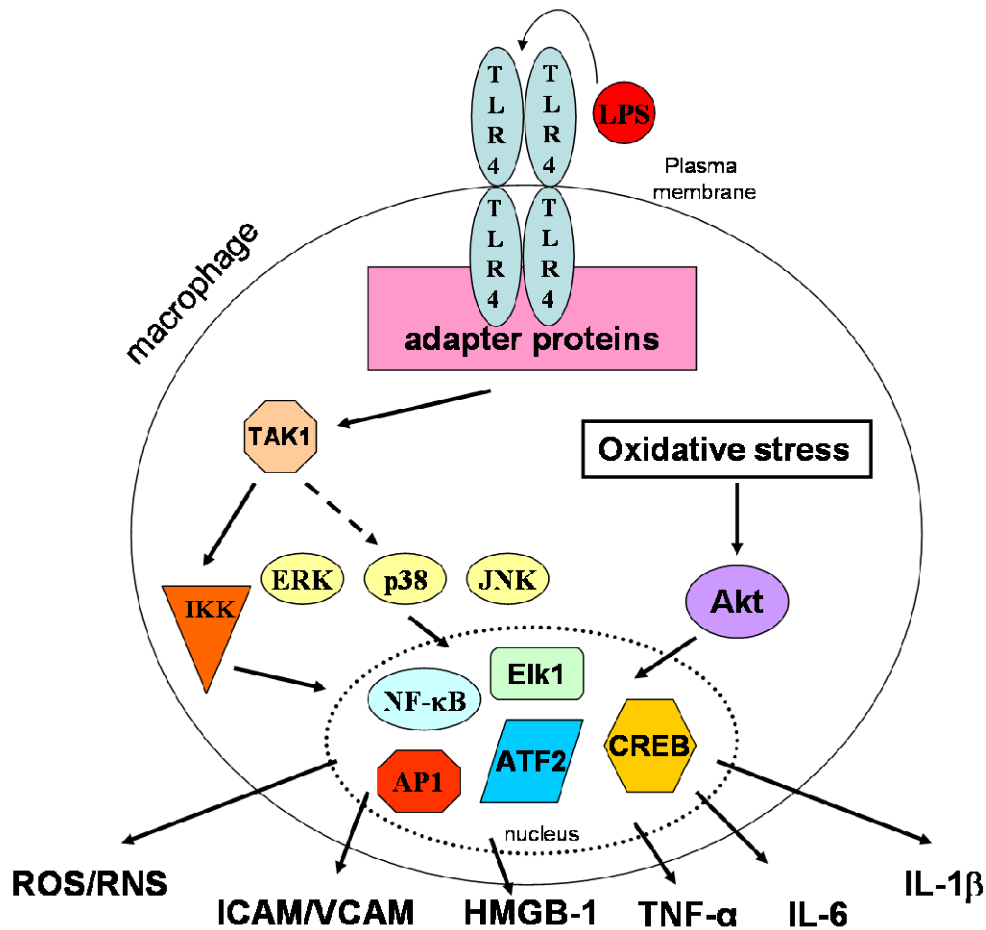


Fig. 2. LPS signaling: from the receptor to the inflammatory mediators.

4.3.1. TLR4 signaling

TLRs play a major role in the recognition of PAMP present on bacteria and bacterial products (Kopp and Medzhitov 2003, Sabroe et al. 2003). At present, fourteen different TLRs have been discovered which belong to the interleukin-1 receptor/toll-like receptor superfamily and are described as type 1 transmembrane receptors containing an N-terminal extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor (TIR) domain (Takeda et al. 2003, Zhang et al. 2004) (Fig. 3). Besides being the first to be identified, mammalian TLR4 is by far the best functionally characterized member of the TLR family (Medzhitov et al. 1997)

and is functioned as a cellular receptor for bacterial LPS, a central Gram-negative bacterial cell wall component (Fenton and Golenbock 1998, Cario and Podolsky 2000, Viriyakosol et al. 2000, Abreu et al. 2002, 2003, Hausmann et al. 2002, Lorenz et al. 2002, Backhed and Hornef 2003, Fan and Malik 2003).

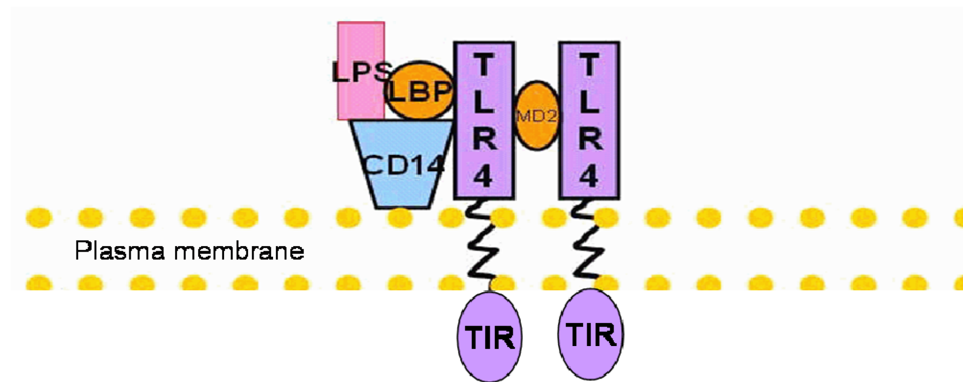


Fig. 3. LPS binding to receptor TLR4.

LPS-induced signaling occurs after a series of binding interactions. First LPS is recognized by the lipopolysaccharide binding protein (LBP) which is an acute phase serum protein that is a member of the lipid transfer - LT/LBP family (Schumann et al. 1990, Mathison et al. 1992). In turn, LBP facilitates the transfer of LPS to CD14 (Hailman et al. 1994), which is a glycosylphosphatidylinositol (GPI) - linked receptor that may be present on the cell surface or exist as a soluble form in the serum (Fenton and Golenbock 1998, Viriyakosol et al. 2000). The LPS CD14 binding is followed by relocation of monomeric LPS to TLR4-associated lymphocyte antigen 96 (LY96, also called MD2 protein). MD2 surface expressed protein associates with the extracellular domain of TLR4 and LPS which results in homodimerisation and activation of TLR4 (Gioannini et al. 2004, 2005) (Fig. 3).

4.3.2. MAPK signaling, MKP-1

MAPK transduction cascades are evolutionarily ancient signaling pathways that take part in many physiological processes, including a prominent role in defense against infection. Binding of LPS to the TLR is followed by recruitment of TIR domain containing intracellular adapter molecules, such as myeloid differentiation primary response gene 88 (MyD88) (Burns et al. 1998), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) (Horng et

al. 2002), toll-like receptor adaptor molecule 1 (TICAM1), also called TIR-domain-containing adapter-inducing interferon- (TRIF) (Yamamoto et al. 2002, Oshiumi et al. 2003) and TIR domain-containing adapter molecule 2 (TICAM2), also called TRIF-related adaptor molecule (TRAM) (Fitzgerald et al. 2003, Bin et al. 2003); and activation of a string of signal transduction factors most notably, interleukin-1 receptor-associated kinase-1 and 4 (IRAK-1 and IRAK-4), TNF receptor-associated factor-6 (TRAF-6), and IKK / / (Cao et al. 1996, Suzuki et al. 2002, Palsson-McDermott and O'Neill 2004) (Fig. 4). The differential recruitment of these adaptor proteins to the TLRs form the basis for specificity in the signaling processes activated by TLRs. MyD88 associates with the intracellular TIR domain of the TLRs and upon binding of ligand to the receptor MyD88 recruits IRAK-4 and facilitates IRAK-4-mediated phosphorylation of IRAK-1 (Fig. 4). Activation of IRAK-1 molecules results in recruitment of TRAF-6, leading to dissociation of the IRAK-1/IRAK-4/TRAF-6 from the receptor complex (Cao et al. 1996, Li et al. 2002, Suzuki et al. 2002). TRAF-6 and IRAK-1 interact with a membrane bound pre-associated complex of MAP3K transforming growth factor- -activated kinase1 (TAK1) and TAK1-binding proteins (TAB1/2) (Fig. 4). TRAF-6 ubiquitinates, and in this way activates itself and its substrate TAK1 (Wang et al. 2001). The activated TAK1 functions as the initiator of both NF- B and MAPK pathways (Deng et al. 2000, Takaesu et al. 2001, Wang et al. 2001, Jiang et al. 2002) (Fig. 4).

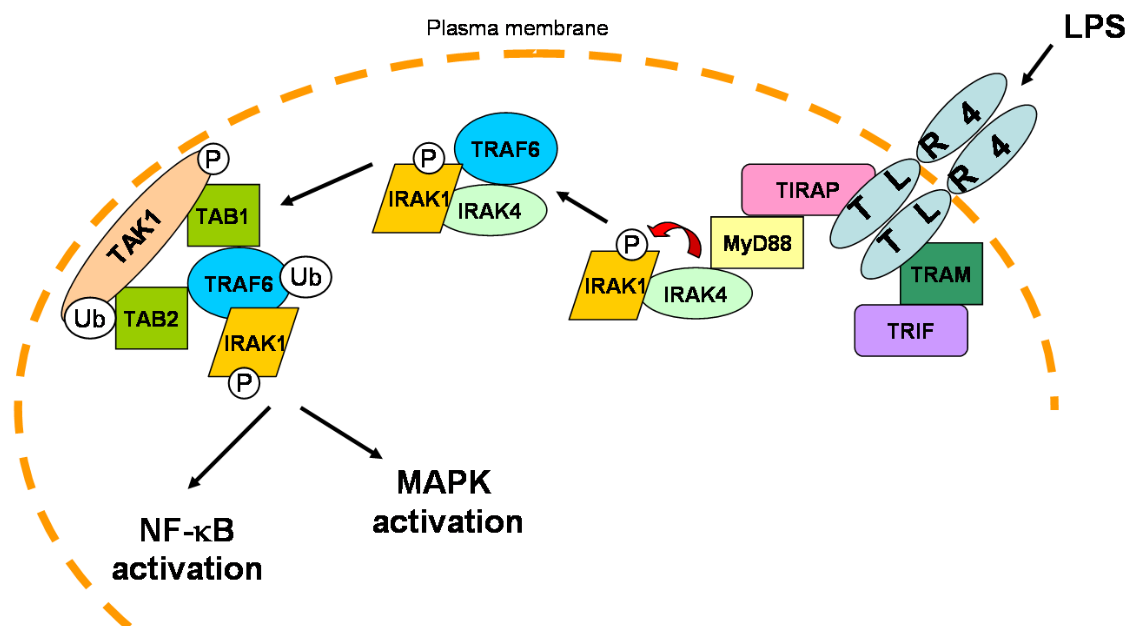


Fig. 4. TLR4 signaling.

The MAPK-signal transduction pathways are highly conserved cascades important in diverse aspects of the immune response. They form a family of protein kinases that include ERK, p38 and JNK. MAPKs may be present in cytosolic, mitochondrial and nuclear compartments. MAPKs are activated by upstream MAPK kinases (MKKs). The MKKs in turn are activated by MKK kinases (M3Ks), a prominent one of which is TAK1 (Rao 2001, Torres and Forman 2003, Denkers et al. 2004). Upon activation, MAPKs activate transcription factors including Elk-1, activating transcription factor-2 (ATF-2), cAMP-responsive element-binding protein (CREB), and AP-1 (Bozinovski et al. 2002, Luyendyk et al. 2008) (Fig. 5).

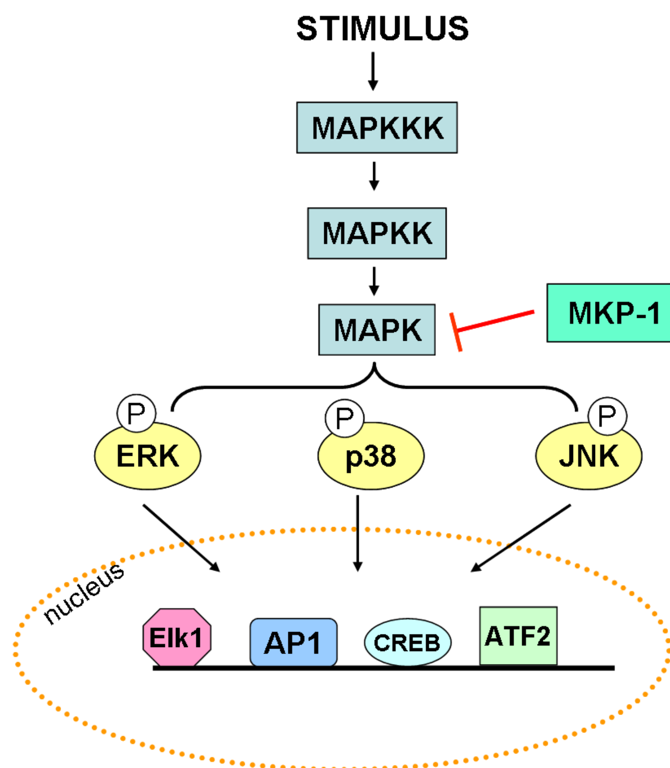


Fig. 5. MAPK signaling pathway.

While activation of these signal transduction cascades is critical for mounting an aggressive immune response to eliminate invading pathogens, deactivation of the signaling pathways restrains the potentially devastating actions of the immunological system on the host, thus preventing self destruction. These negative regulators modulate the strength and duration of the transduced signals. MAP kinase phosphatases (MKPs) have a central role in

the restraint of innate immune response and the prevention of septic shock syndrome during pathogenic microbial infection (Zhao et al. 2006, Hammer et al. 2006, Salojin et al. 2006, Chi et al. 2006, Wang and Liu 2007). In mammalian cells, MKPs, also called dual-specificity (tyrosine and serine/threonine) protein phosphatases (DSPs) are the primary phosphatases responsible for dephosphorylation and deactivation of MAP kinases (Wu 2007, Wang et al. 2007, Chi and Flavell 2008) (Fig. 5). To date, at least eleven MKPs have been identified in mammalian cells, with MKP-1 being the archetype (Dickinson and Keyse 2006) which is a MAP kinase-selective protein phosphatase (Sun et al. 1993). The activity of MKP-1 can be regulated at multiple levels. First, MKP-1 can be regulated at transcriptional level. The expression of MKP-1 can be robustly induced by extracellular stimulation (growth factors and stress) (Keyse 2000). In addition to the transcriptional mechanism, MKP-1 expression may also subject to posttranscriptional and post-translational regulation. MKP-1 activity can be modulated through stabilization of the protein and catalytic activation. ERK can phosphorylate MKP-1, in this way increases the half-life of the protein and inhibits its ubiquitin-mediated degradation (Brondello et al. 1999). In addition to increasing stability of MKP-1, its interaction with its substrate MAP kinases increases its catalytic activity (Hutter et al. 2000, Slack et al. 2001) and the overactivated MAPKs can promote their own deactivation and dephosphorylation through phosphorylation and activation of MKP-1.

MKP-1 is a critical negative regulator of macrophage signaling in response to inflammatory stimuli and through deactivation of MAKPs it can switch off the production of pro-inflammatory cytokines *in vitro* and *in vivo* (Chen et al. 2002, Chi et al. 2006).

4.3.3. PI3K/Akt signaling

PI3K/Akt pathway plays a crucial role in controlling numerous biological processes, including cellular survival, proliferation, growth and motility (Cantrell 2001, Katso et al. 2001, Vanhaesebroeck et al. 2001, Cantley 2002). LPS can activate PI3K/Akt cellular signaling pathway in monocytes and macrophages (Díaz-Guerra et al. 1999, Guha and Mackman 2002, Lee et al. 2007). PI3K family is divided into four classes: IA, IB, II and III (Cantley 2002). LPS activation of Akt is mediated by class IA PI3K in monocytic cells (Lee et al. 2007). IA PI3K catalyzes the phosphorylation of phosphatidylinositol (3,4)-bisphosphate (PIP2) to the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Binding of PDK-1 and Akt to PIP3 leads to the phosphorylation of Akt (Fig. 6). Several data

indicate that the PI3K-Akt pathway negatively regulates LPS signaling and gene expression (Díaz-Guerra et al. 1999, Guha and Mackman 2002, Martin et al. 2003, Fukao and Koyasu 2003, Martin et al. 2005, Luyendyk et al. 2008), however, a number of studies have shown that the PI3K-Akt pathway is required for LPS induction of gene expression in monocytes and macrophages (Park et al. 2002, Ojaniemi et al. 2003, Kim et al. 2005, Kuo et al. 2006).

PI3K/Akt pathway is negatively regulated by the phosphatase and tensin homologue (PTEN) which converts PIP3 to PIP2 (Leslie et al. 2003, Luyendyk et al. 2008, Oudit and Penninger 2009). Oxidative stress can inactivate PTEN and this inactivation leads to an increased level of cellular PIP3 and the activation of signaling pathways downstream of this lipid second messenger, for example the Akt pathway (Leslie et al. 2003) (Fig. 6).

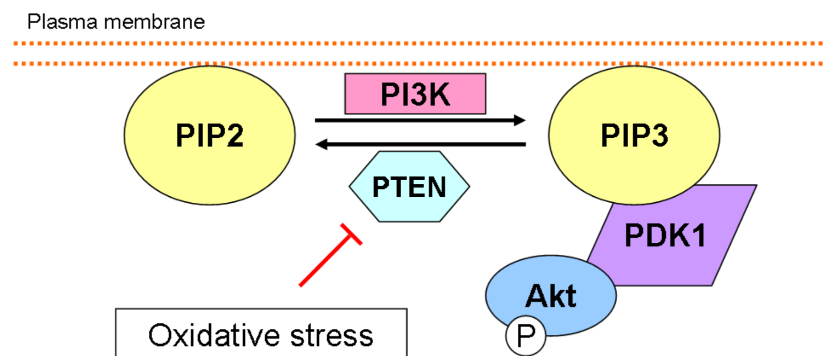


Fig. 6. PI3K/Akt signaling pathway.

4.3.4. Transcription factors: NF- B and AP-1

NF- B is involved in the control of a variety of genes activated upon inflammation, and it plays a central role in the inflammatory response to infection. Binding of LPS to TLR triggers a cascade of cellular signals, culminating in the eventual activation of NF- B and AP-1 transcription factors, which bind to a discrete nucleotide sequence in the upstream regions of genes that produce pro-inflammatory cytokines, such as TNF- , IL-1 , IL-6 and IL-12, thereby regulating their expression (Baeuerle and Henkel 1994, Bone et al. 1997, Bozinovski et al. 2002, Kim et al. 2005, Luyendyk et al. 2008).

Reticuloendotheliosis (Rel)/NF- B molecules are a diverse collection of heterodimers consisting of RelA/p65, RelB, c-Rel, NF- B1 (p50 and its precursor p105), and NF- B2 (p52 and its precursor p100). Each of these molecules possesses a conserved Rel homology region (RHR). RHR promotes dimerization, DNA binding, and interaction with inhibitor of NF- B (I B) molecules. Only RelA/p65 and c-Rel possess strong transcriptional activating (TA) domains (Schmitz and Baeuerle 1991, Ghosh and Karin 2002). These TA domains contains serine residues which are phosphorylated in unstimulated cells (Naumann and Scheidereit 1994), but this basal phosphorylation can be increased by a broad range of stimuli (hydrogen peroxide, TNF- , IL-1, LPS and CpG DNA) (Naumann and Scheidereit 1994, Bird et al. 1997, Sakurai et al. 1999, Wang et al. 2000). This increased phosphorylation is required for transactivation of gene expression. NF- B dimers lacking transcriptional activating domains (e.g. p50) may be more important as negative regulators of NF- B (Baer et al. 1998). The inhibitory molecules I B , I B , and I B are related to p100 and p105 and they function to maintain NF- B in an inactive state in the cytoplasm by masking nuclear localization sequences of the latter. However, following inflammatory stimuli, such as LPS, I B molecules are phosphorylated by IKK and are subsequently degraded by the proteasome. Beside to activating of MAPKs, TAK1 can also phosphorylate and activate its downstream target IKK promoting NF- B translocation to the nucleus, where it triggers the expression of pro-inflammatory cytokines (Mercurio et al. 1997, Régnier et al. 1997, Woronicz et al. 1997, Zandi et al. 1997, Karin and Ben-Neriah 2000) (Fig. 7).

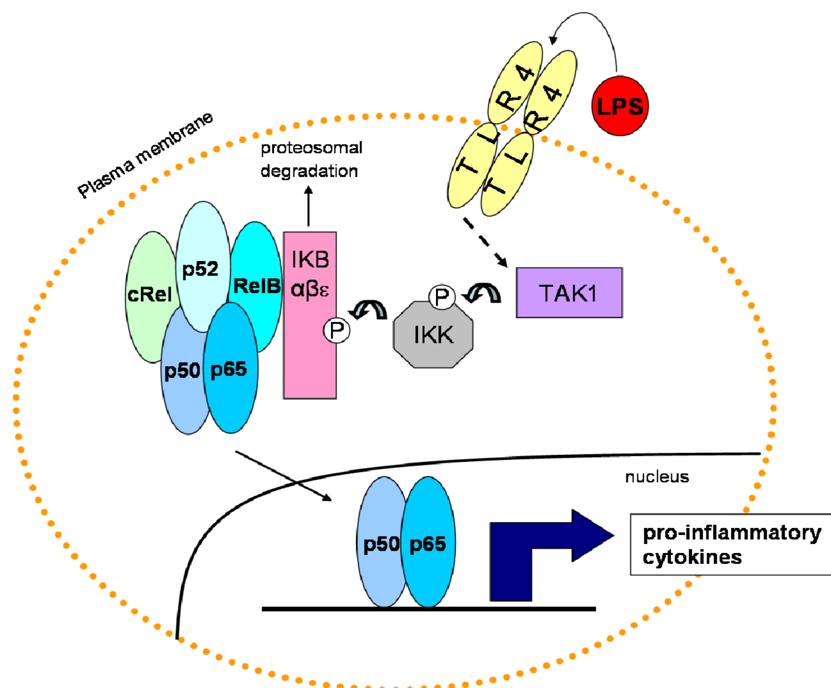


Fig. 7. NF- B signaling.

Similar to NF- B, AP-1 is dramatically activated by many pathophysiological stimuli, including LPS, cytokines, and ROS (Foletta et al. 1998). Members of the MAPK family JNK and p38 kinases, are important in the regulation of AP-1 to mediate expression of inducible genes, including ICAM-1 and COX-2 (Ballantyne et al. 1992, Karin 1995, Wadleigh et al. 2000). AP-1 is a ubiquitous regulatory protein complex belongs to a large family of dimeric transcription factors. AP-1 binds to TPA-responsive elements (AP-1/TREs) or cAMP-responsive elements (CREs) DNA motifs found in many gene promoters and enhancers (Gomard et al. 2010). AP-1 is involved in inflammatory processes and innate immunity. It exists either homodimeric c-Jun complex or as a c-Jun/c-Fos heterodimer that is regulated by transcription and direct phosphorylation (Angel and Karin 1991, Shaywitz and Greenberg 1999, Chinenov and Kerppola 2001).

4.3.5. ROS

4.3.5.1. ROS production in inflammation

Beside the inflammatory processes, oxidative stress is also involved in the pathomechanism of sepsis and reactive oxygen and nitrogen species (ROS/RNS) are important mediators of cellular injury during endotoxemia (Cadenas S and Cadenas AM 2002). iNOS is expressed and continuously active during inflammation, where it is involved in host-defense against pathogens. iNOS generates NO which can be converted to its stable products, nitrite and nitrate (Evans et al. 1993, Gomez-Jimenez et al. 1995). ROS are generated during normal cellular metabolism. The respiratory chain in mitochondria is the major source of oxygen radicals. They are very unstable, as they possess one or more unpaired electrons which can make the species highly reactive (Halliwell and Gutteridge 1999). In inflammatory processes, beside the mitochondrial ROS production there are other possible sources of ROS such as metabolic cascade of arachidonic acid (via COX-2), protease-mediated enzyme xanthine-oxidase and membrane-bound enzyme complex NADPH oxidase (Victor et al. 2005). ROS and RNS cause peroxidation of membrane phospholipids, oxidation of proteins and DNA damage (Pattanaik and Prasad 1996). These processes lead to altering of membrane fluidity, loss of cellular integrity, decrease of energy levels in the cell and finally cell and tissue damages. Furthermore oxidative damage has been implicated in playing a crucial role in pathogenesis of a number of diseases including neurodegenerative

disorders, such as Alzheimer's disease, cardiovascular alterations, sepsis and septic shock (Parratt 1998, Titheradge 1999). ROS can regulate the production of cytokines in macrophages through NF- B-dependent mechanisms. LPS, which stimulates the production of TNF- , induces ROS generation via the activation of NF- B in macrophages (Sanlioglu et al. 2001). In turn various stimulants of NADPH oxidase can also trigger NF- B activation (Kang et al. 2000). Beside the transcription factor NF- B, MAPKs are involved and related to ROS production during inflammation. It was also published that increased intracellular production of ROS activates ERK (Irani 2000).

Aerobic organisms have developed an array of defense mechanisms against ROS damage, which represent a fine balance between ROS generation and antioxidant defense in cells. This system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx), and glutathione reductase (GSSGR), which protect by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species. Non-enzymatic defense system includes compounds of intrinsic antioxidant properties, such as vitamins C and E, glutathione, and ß-carotene (Victor et al. 2005). Glutathione plays a key role in maintaining the physiological balance between prooxidants and antioxidants. N-acetyl-L-cysteine (NAC), a precursor of glutathione (GSH), vitamin E and β-carotene can protect the rat lung and brain against endotoxin-mediated oxidative stress and decrease the mortality of the animals (Sprong et al. 1998, Kheir-Eldin et al. 2001). In addition, NAC has been shown to decrease free radical production *in vitro* in macrophages from mice treated with a lethal dose of endotoxin (Victor and De la Fuente 2002). A variety of antioxidants have been reported to potently suppress the activation of NF-κB both *in vivo* (Blackwell et al. 1996, Sprong et al. 1997) and *in vitro* (Schreck et al. 1992) in response to most inducing conditions. Considered together, these results suggest that antioxidants may be beneficial in preventing endotoxin-induced oxidative damage.

4.3.5.2. Mitochondrial membrane potential and ROS production

Most of the reactive oxygen species are generated in cells by the mitochondrial respiratory chain. Mitochondrial ROS production is modulated largely by the rate of electron flow through respiratory chain complexes. During respiration, most of the oxygen consumed is reduced to water. However, an estimated 1–2% of oxygen consumed during respiration is not completely reduced to water but is instead partially reduced to superoxide ($O_2^{\bullet -}$) (Cadenas

et al. 1977) which can be converted to hydrogen peroxide (H_2O_2) (Boveris et al. 1972) and the highly reactive hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$ reacts easily with free radicals, including nitric oxide ($NO^{\bullet}$) - which is mainly produced by the enzyme NOS - to produce peroxynitrite ($ONOO^{-}$) (Beckman and Koppenol 1996). Endogenously produced $ONOO^{-}$ and $O_2^{\bullet-}$ are important mediators of the inhibition of the mitochondrial respiration through the deactivation of mitochondrial aconitase (Castro et al. 1994) in cultured monocytic macrophages (Szabó and Salzman 1995, Szabó et al. 1996). Decreased rate of oxygen consumption is attached to highly reduced electron carriers, high mitochondrial membrane potential (m) and increased rate of ROS production (Echtay 2007, Poyton et al. 2009).

4.3.6. Cytokines

During Gram-negative infection NF- B plays a major role in producing pro-inflammatory cytokines and chemokines especially TNF- , IL-1, IL-6, IL-8, IL-12, ICAM-1, E-selectin, P-selectin, VCAM-1 and HMGB-1 by endothelial and epithelial cells, as well as by neutrophils, macrophages and lymphocytes (Woo and Kwon 2007, Luyendyk et al. 2008, Chiu and Lin 2008).

TNF- plays a pivotal role in the early phase of immune response against infections and in part in the organ dysfunction related to septic shock (Bone 1996, Ulloa and Tracey 2005, Russell 2006). Plasma levels of TNF- and IL-1 peak at the early stage within minutes after infection, but after 3–4 h secretion stops and their levels become almost undetectable in the late stage (in severe sepsis) (Tracey and Cerami 1993, Lotze and Tracey 2005, Russell 2006). TNF- enhances the expression of adhesion molecules ICAM-1 and VCAM-1 as well as chemokines in endothelial cells. TNF- also activates neutrophils by promoting extravasation to the lungs, liver, gut and other organs. TNF- and IL-1 induce an aberrant expression of tissue factor, which causes blood coagulation in subendothelial cells upon vascular injury. Extravasated neutrophils damage tissues by releasing oxygen free radicals and proteases. In addition, TNF- amplifies inflammatory cascades in an autocrine and paracrine manner by activating macrophages and monocytes to secrete other pro-inflammatory cytokines (Shimaoka and Park 2008).

HMGB-1 protein has been identified as a late mediator of sepsis because macrophages secrete HMGB-1 20 h after activation and serum HMGB-1 level is detectable between 20 and 72 h in a prolonged plateau phase (Ombrellino et al. 1999, Wang et al. 1999, Czura et al.

2003). HMGB-1 was originally described as a nuclear DNA-binding protein which plays a critical role in stabilizing nucleosome formation and in regulating transcription (Lotze and Tracey 2005). HMGB-1 upregulates ICAM-1 and VCAM-1 expression, and has been implicated in the enhanced accumulation of leukocytes (Fiuza et al. 2003). Furthermore, HMGB1 increases the permeability of gut epithelial cells, in this way plays a role in the prolongation of inflammation and induction of endothelial and epithelial damage.

Sepsis is characterized by a surge of the pro-inflammatory cytokines TNF- and IL-1 at the early stage. However, as the disease progresses, this early stage coverts to the anti-inflammatory state, marked by decreased levels of TNF- and increased levels of IL-10 (Scumpia and Moldawer 2005, Rice and Bernard 2005, Russell 2006). The increased production of IL-10 in the late phase of sepsis is believed to contribute to 'immunosuppression'.

By contrast, early cytokines, such as TNF- and IL-1, which are produced within minutes after infection, HMGB-1 is a late mediator of sepsis that might be a potential therapeutic target to treat 'established' sepsis.

4.4. Treatment of sepsis

Sepsis is the most common cause of death in intensive care units worldwide and despite the extensive research we do not fully understand the cellular and molecular mechanisms that are involved in triggering and propagation of septic injury. A number of different approaches have been investigated to try to treat and/or prevent septic shock associated with infections caused by Gram-negative bacteria. Antibiotics constitute a necessary part of the treatment of sepsis, but antibiotics alone, even used optimally, are not sufficient to dramatically reduce the mortality of septic patient, because antibiotics cannot control the complex systemic inflammation and dysregulated host responses. For this reason, considerable efforts have been expended in developing non-antibiotic forms of treatment. There are three main categories: improvements in supportive care, treatments aimed at bacterial virulence factors, and treatments aimed at host mediators (Cohen 2009).

The name of the strategy, which has been developed in the 1980s was ‘goal-directed therapy’ included fluid optimization and oxygenation/ventilation strategies. Subsequently, the generalizability of this approach has been challenged, and a further clinical trial is currently underway to determine whether the findings can be confirmed.

Bacterial virulence factors became highly attractive therapeutic targets, because they initiate the pathological effects of the infection. LPS was probably the very first target for the development of a non-antibiotic treatment for sepsis. Animal models where LPS was removed or neutralized and animals which were genetically resistant to LPS were essentially protected from its lethal effects. However, there is a commercially available tool in the Japanese market to neutralize LPS, its beneficial effects in sepsis and septic shock have not been confirmed yet worldwide. For this reason a clinically relevant anti-endotoxin strategy is still to be developed (Vincent et al. 2005).

Bacteria (through their PAMPs) initiate the septic response, which is the dysregulated host immune response with excessive production of pro-inflammatory cytokines that amplifies the process and causes the cellular injury that ultimately leads to the characteristic picture of multiple organ failure (Riedemann et al. 2003, Remick 2007, LaRosa and Opal 2008). Two different approaches exist: ‘immunosuppression’, aimed at several points in the inflammatory pathway, and ‘immunotherapy’, in which drugs are aimed at specific key elements.

Preclinical data from the 1980s had suggested that many of the manifestations of sepsis were due to excess inflammation, and that anti-inflammatory doses of steroids would be beneficial. In animal experimental models these high-dose steroids were not effective in preventing death from sepsis, or, indeed, were perhaps even harmful, increasing the risk of superinfection (Bone et al. 1987). A more attractive strategy is the targeted immunotherapy. The most important targets of the immunotherapy are TLRs, cytokines (TNF- , IL-1 and HMGB-1) and coagulation proteins.

1. TLRs

TLRs recognize PAMPs to indicate their distinct roles in infection, inflammation and tissue damage. TLR4 is one of the candidates in blocking the innate immune system, but only two TLR4 antagonists have made so far into the clinical phase (Leon et al. 2008).

2. Cytokines

Experimental strategies neutralizing the early phase cytokines (monoclonal antibodies against TNF, IL-1-receptor antagonists) are successful therapeutic approaches against several inflammatory disorders, including rheumatoid arthritis and Crohn’s disease (Van Assche and

Rutgeerts 2000, Feldmann 2002), but these strategies have produced modest effects in clinical trials (Abraham et al. 2001). A possible explanation is that some patients enrolled in these clinical trials suffer from severe sepsis, and TNF appears to be a mediator of septic shock but not of severe sepsis. Thus, agents directed against early pro-inflammatory cytokines are ineffective in large clinical trials against severe sepsis, which might be characterized by specific 'late' appearing cytokines (such as HMGB-1) produced during the slow progression of this syndrome. Thus, HMGB-1 is a therapeutic target in experimental models of severe sepsis and inhibition of HMGB-1 secretion or action (using neutralizing antibodies, antagonists, ethyl pyruvate and nicotinic stimulation) can prevent endotoxin-induced multiple organ failure and rescue animals from established sepsis. (Wang et al. 1999, 2004, Ulloa et al. 2002, Yang et al. 2004, Fink 2004, Matthay and Ware 2004).

3. Coagulation proteins

Complex interactions between inflammation and coagulation are involved in the pathogenesis of sepsis. Inflammatory cytokines both activate the coagulation cascade and inhibit fibrinolysis. In turn, components of the coagulation and fibrinolytic systems have pro-inflammatory effects. Disseminated intravascular coagulation, one of the most feared complications of sepsis, is a manifestation of the dysregulation of coagulation (Levi et al. 2003). Protein C is a soluble, vitamin K–dependent, plasma serine protease that's activated form plays a central role in endogenous anticoagulation (Griffin et al. 2007). Pro-inflammatory cytokines such as TNF- and IL-1 decrease the generation of activated protein C (APC) (Nawroth et al. 1986, Gerlach et al. 1989), and this reduced level of APC in patients with sepsis have been correlated with an increase risk of death (Fourrier et al. 1992, Boldt et al. 2000). These observations led to the hypothesis that the administration of APC might be beneficial in patients with sepsis. APC acting as an antithrombotic factor and beside its negative role on the regulation of blood coagulation, APC possesses anti-inflammatory properties (Zeerleder et al. 2005, Slofstra et al. 2006, Griffin et al. 2007). It inhibits the production of inflammatory cytokines and the transduction of NF- B signaling in monocytes stimulated by LPS and thrombin (Yuksel et al. 2002). APC also inhibits nitric oxide–induced vascular dysfunction and suppresses endothelial permeability as well as the transendothelial migration of neutrophils (Zeng et al. 2004).

Despite the intense researches and hopeful results of animal experiments, a new drug that is effective in reducing mortality in sepsis is still awaited. By this time only some of the licensed drugs are available for the treatment of sepsis.

4.5. Antioxidants

4.5.1. Polyphenols

A wide variety of dietary plants including grains, berries, legumes, tea, beer, grape/wine, olive oil, chocolate/cocoa, coffee, walnuts, peanuts, spices, fruits, vegetables etc. contain polyphenols (Bravo et al. 1998). Polyphenols, with 8000 structural variants, are characterized by the presence of aromatic rings bearing one or more hydroxyl moieties, which have a pivotal role in mediating of its antioxidant properties. As antioxidants, polyphenols are normally produced by plants for their antibiotic and antifungal features (Leiro et al. 2004). Polyphenols, which are generally divided into six major groups: hydroxybenzoic acids, phenolic alcohols, hydroxycinnamic acids, lignans, flavonoids and stilbenes (e.g. resveratrol) (D'Archivio et al. 2007) (Fig. 8).

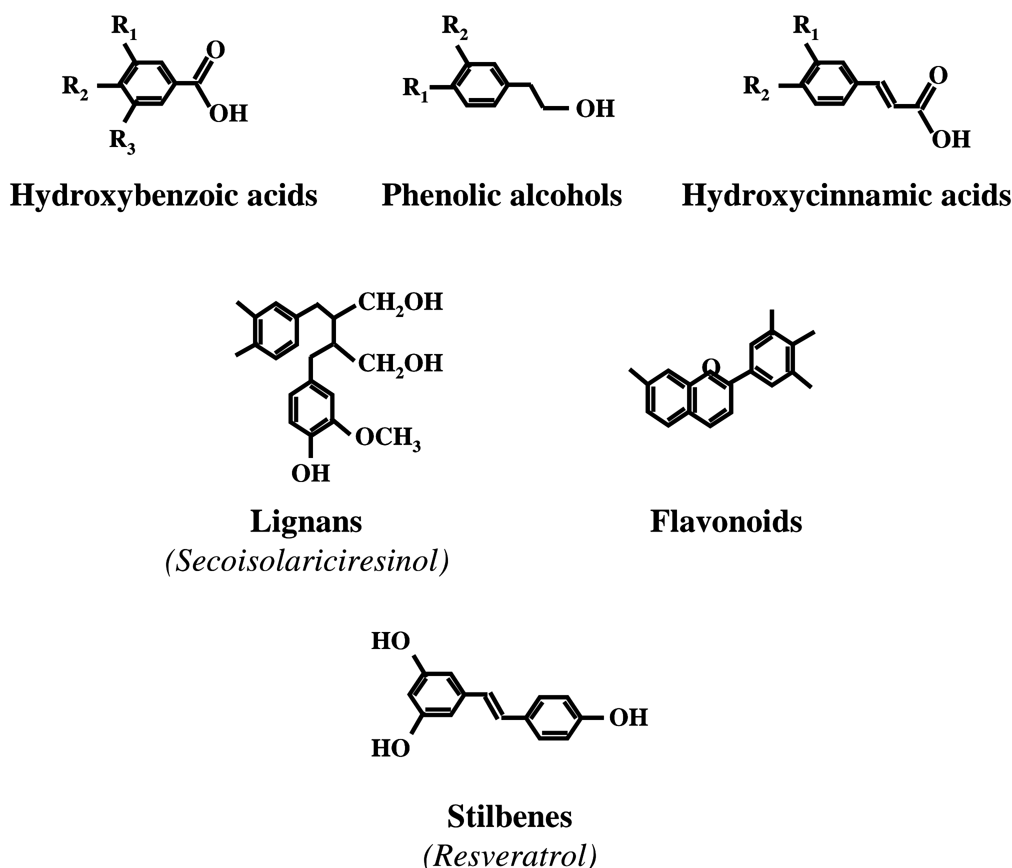


Fig. 8. Classification of polyphenols.

Recently, a number of natural products or ingredients of traditional medicines and healthy foods such as resveratrol, curcumin, and catechins were extensively investigated and subjected to clinical trials as anti-inflammatory agents (Hatcher et al. 2008). Although the knowledge of absorption, bioavailability and metabolism of polyphenols is not entirely known, it appears that some polyphenols are bioactive and are absorbed in their native or modified form. After the metabolization of polyphenols by the microflora of the intestines, their absorbed forms may be detected in plasma in nanomolar concentration (Rahman et al. 2006).

The active components of dietary phytochemicals (e.g. curcumin, resveratrol, capsaicin, catechins, vitamins, beta carotene and dietary fiber) are believed to suppress the inflammatory processes, moderate cell signaling pathways, proliferation, apoptosis, redox balance and most often appear to be protective against cancer, neurodegenerative disorders and cardiovascular diseases. (Aggarwal and Shishodia 2006, Rahman et al. 2006). Polyphenols can exert their anti-inflammatorical properties at multiple levels, through the modulation of MAPK, Akt and NF- B signaling pathways, inhibition the production of inflammatory cytokines and chemokines, suppressing the activity of COX and iNOS and decreasing the production of ROS/RNS. MAPKs which play critical roles in inflammation are inhibited by catechins in macrophages (Ichikawa et al. 2004). Other dietary phytochemicals, namely curcumin (Cho et al. 2005), resveratrol (Shih et al. 2002) and green tea polyphenols (Katiyar et al. 2001) have been shown to modulate the MAP kinases and it was dependent on cell type and on the polyphenol used. Akt plays crucial roles in mammalian cell survival signaling and has been shown to be activated in various cancers (Clarke 2003, Chang et al. 2003). Activated Akt promotes cell survival by activating NF- B signaling pathway (Romashkova and Makarov 1999). Several phytochemicals including genistein (Li and Sarkar 2002), curcuminoids (Aggarwal et al. 2006) and catechins (Tang et al. 2003) are known to suppress the activation of Akt, in this way inhibit cancer cell growth. Beside and in consequence of processes suppressing of Akt, anticancer properties of polyphenols are exerted at multiple levels including inhibition of NF- B and AP-1 expression (Singh and Aggarwal 1995, Manna et al. 2000), inhibition of angiogenesis, inhibition of metastasis, and suppression of cell proliferation (Shankar et al. 2008). Almost all cell types, when exposed to TNF- , LPS or other stimuli, activate NF- B and AP-1 transcription factors, leading to the expression of inflammatory genes, such as COX-2, iNOS, cell adhesion molecules, inflammatory cytokines and chemokines. Thus, all the dietary agents that can suppress these transcription factors have the potential of inhibiting the expression of COX-2, iNOS, cell

adhesion molecules, TNF-   and interleukins. Several dietary components including green tea catechins (Gerhäuser et al. 2003), curcumin (Plummer et al. 1999), and resveratrol (Subbaramaiah et al. 1998) have been shown to suppress COX-2 and in this way to decrease the production of reactive oxygen species. iNOS, which is responsible for the release of free radical nitric oxide, was suppressed by several phytochemicals and dietary agents in RAW 264.7 macrophage cell line, stimulated with LPS and interferon-   (IFN-) (Kim et al. 1998). Other sources of the antioxidant properties of polyphenols is their free radicals scavenger features, which is based on their structure (Joe and Lokesh 1994, Babu and Liu 2008). Furthermore, several polyphenols suppress lipid peroxidation through to maintain the cellular status of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (Reddy and Lokesh 1992, Labinskyy et al. 2006). Due to the NF- B suppressing effect of polyphenols, some of them (e.g. curcumin, resveratrol, quercetin and green tee polyphenols) have been shown to decrease the expression of chemokines and cytokines (Hidaka et al. 2002, Kowalski et al. 2005).

One of the most investigated and potent polyphenolic compound that is found in highest concentration in the skin of grapes and regulate the inflammation is a stilbene, called resveratrol. In the 80’s, interest in the possible health benefits of resveratrol in wine was spurred by discussion of the “French paradox” which estimated the state of health of wine drinkers in France. These data suggest that nutritional intake of resveratrol and other polyphenol compounds may contribute to a relatively low incidence of cardiovascular diseases in the Mediterranean population (de Lorgeril et al. 1999, Zern and Fernandez 2005). Resveratrol (and other natural plolyphenol compounds) are thought to have diverse anti-atherogenic activities such as the inhibition of LDL oxidation, platelet aggregation, regulation of vascular smooth muscle proliferation and modulation of NO production. There is accumulating evidence that resveratrol can exert antioxidant, anti-inflammatory and cytoprotective effects via inhibition of signal transduction pathways including MAP kinases (Kirk et al. 2000) and polyphosphoinositide signaling (Olas et al. 2005), down-regulation of NF- B and AP-1 transcription factors (Manna et al. 2000), decreasing the expression of endothelial VCAM, ICAM-1 (Carluccio et al. 2003) and iNOS (Donnelly et al. 2004). Resveratrol has also been shown to be a potent activator of the protein sirtuin 1 (SIRT1) in vitro (Alcaín and Villalba 2009) and in vivo (Baur et al. 2006) and this property of resveratrol has been proposed to account for its anti-aging effects (Labinskyy et al. 2006). SIRT1 functions as a NAD^+-dependent protein deacetylase which is capable of deacetylating and suppressing the transcription activity of a wide variety of transcription factors including p53

(Zhang et al. 2010) and NF- B (Chen et al. 2002). SIRT1 knockout or knockdown leads to the activation of NF- B and AP-1 and release of pro-inflammatory cytokines whereas activation of SIRT1 by resveratrol (or other polyphenols) inhibits the releasing of NF- B-mediated inflammatory cytokines and chemokines in vitro and in vivo suggesting the role of SIRT1 in regulation of inflammation. These data suggest that polyphenols would be an approach for the intervention of various chronic inflammatory diseases (Yeung et al. 2004, Milne et al. 2007, Rajendrasozhan et al. 2008).

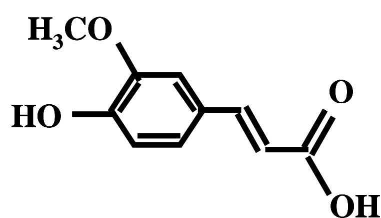
Polyphenols have been reported to bring benefits in lowering inflammation and oxidative stress trough affecting of multiple cell signaling pathways. Furthermore, natural products or ingredients of traditional medicines and healthy foods exert positive effects in cancer and cardiovascular and chronic inflammatory diseases, so these agents can be used in combination with existing therapy.

4.5.2. Ferulic acid, ferulaldehyde

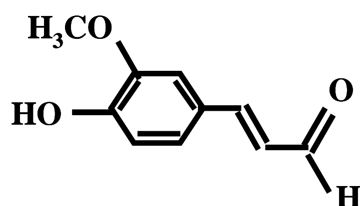
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid) is a product of the phenylalanine and tyrosine metabolism, and it is produced by the shikimate pathway in plants (Fig. 9). It is commonly found in fruits like orange and in vegetables, such as tomato, carrot, sweet corn and rice bran. The wide spectrum of beneficial effects of ferulic acid for human health due to its antibacterial, anti-inflammatory, hepatoprotective, anticancer, antidiabetic, neuroprotective, anti-atherogenic and antioxidant activity (Srinivasan et al. 2007).

The antioxidant potential of ferulic acid can usually be attributed to its structural characteristic. Three distinctive motifs (3-methoxy and 4-hydroxy groups on the benzene ring, and the carboxylic acid group) of ferulic acid are responsible for its free radical scavenging capability (Fig. 9). The methoxy and hydroxy groups terminate the free radical chain reaction due to their electron donating capacity, and C-C double bonds can provide attack sites for free radicals. Highly reactive radicals blundering against ferulic acid and easily abstract a hydrogen atom to form phenoxy radical, which accounts for the potent antioxidant activity of ferulic acid. 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 (Graf 1992).

Partially, because of its antioxidant and anti-inflammatory activity, ferulic acid is considered as a potential therapeutic agent (together with other naturally phenolic compounds) against various diseases like cancer, diabetes, cardiovascular dysfunction, inflammatory diseases and neurodegenerative diseases (Soobrattee et al. 2005). Namely, ferulic acid and its derivatives decreased the levels of inflammatory mediators, e.g., TNF- (Han et al. 2007) and inhibited the release of ROS and RNS via suppression of iNOS (Jiang et al. 2009) and COX-2 (Hirata et al. 2005, Ronchetti et al. 2009) in LPS-stimulated macrophages. It was reported that ferulic acid significantly decreases the activation of NF- B and inhibits the nuclear translocation of its p65 subunit in RAW 264.7 macrophages stimulated by LPS (Islam et al. 2009). Furthermore, ferulate can suppress the redox-sensitive, pro-inflammatory NF- B activation via NF- B-inducing kinase (NIK)/IKK and MAPKs by reducing oxidative stress in aged rats. Ferulate exhibited its antioxidative action by maintaining redox regulation, suppressing NF- B activation and modulating the expression of NF- B-induced, pro-inflammatory mediators, such as COX-2, iNOS, VCAM-1 and ICAM-1 (Jung et al. 2009). These evidences strongly suggest that ferulic acid has a potential as an anti-inflammatory drug against chronic inflammatory diseases.



Ferulic acid



Ferulaldehyde

Fig. 9. Molecular structure of ferulic acid and ferulaldehyde.

The structural characteristic of ferulic acid and its reduced form, ferulaldehyde (FA) mainly resembles, the difference is one functional group (Fig. 9). Due to this structural similarity and the presence of the reactive aldehyde group (which can be easily oxidized to carboxylic group), FA is thought to have very similar or maybe better biological activity as ferulic acid. Numerous publications were reported which revealed the effects of ferulic acid under different experimental conditions, but the effects of FA remain still unclear. However it was established that ferulaldehyde inhibits the expression of iNOS and the synthesis of NO in murine macrophage-like RAW 264.7 cells stimulated by LPS and IFN- (Kim et al. 1999), and to have a good antioxidant activity in about the same degree as ferulic acid (Nenadis et al. 2003).

Recently, the anti-inflammatory properties of natural products or ingredients of traditional medicines and healthy foods were extensively investigated, but the solubility of these compounds is limited. Because of it, it is questionable whether their bioavailability could account for their pharmacological effect. 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 et al. 2003). Ferulaldehyde is a water-soluble end-product of dietary polyphenol degradation, because it was found at a high concentration in human urine after red wine and chocolate consumption ((Rios et al. 2003, Gonthier et al. 2003), and its oxidized form ferulic acid 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 1998).

5. Aims of the study

1. Using *in vitro* experimental systems, some recent publications raised the possibility that the anti-inflammatory properties of polyphenols in natural products, traditional medicines and healthy foods based on highly soluble metabolites produced by the microflora of the intestines rather than the polyphenols themselves. To provide *in vivo* experimental basis for this theory, our first aim was to investigate the anti-inflammatory features of ferulaldehyde, a natural end-product of polyphenol metabolism of intestinal microflora, in a murine LPS-induced septic shock model as well as in primary hepatocytes activated by LPS and IFN- . Also, we intended to identify signaling mechanisms, transcription factors and inflammatory cytokines involved in the anti-inflammatory effects of FA.

2. Our *in vivo* data provide direct evidences that ferulaldehyde, a water-soluble end-product of dietary polyphenol degradation, exerted its beneficial anti-inflammatory effects during the early phase of inflammation. Macrophages represent the first defense line against bacterial infection and play a crucial role in early inflammatory response, therefore our second aim was to identify the effects of ferulaldehyde on the signaling mechanisms in an *in vitro* model, utilizing LPS-induced RAW 264.7 macrophage cells.

3. LPS treatment and oxidative stress trigger MAPK activation. MKP-1 was reported to dephosphorylate all three MAP kinases and it was found to be a critical negative regulator in the innate immune response to LPS. Based on these facts our third aim was to investigate the role of MKP-1 activation in regulation of early inflammatory response in RAW 264.7 macrophage cells.

4. Since *in vivo* and *in vitro* experimental systems have fundamental differences in several aspects we wanted to investigate and compare the signaling mechanisms in our models, especially focusing on regulation of MAPK pathways.

6. Materials and Methods

6.1. Reagents

Ferulaldehyde was a kind gift from Prof. Kalman Hideg (Institute of Organic and Medicinal Chemistry, Faculty of Medicine, University Pecs, Hungary). LPS from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich Co, (Budapest, Hungary).

6.2. Animals

C57BL/6 mice were purchased from Charles River Hungary Breeding LTD. The mice were kept under standardized conditions; tap water and mouse nonpurified diet (CRLT/N, Szindbad Kft, Hungary) were 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. Registration number of the Permission For Animal Experiments: BA02/2000-34/2001.

6.3. Cell cultures

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 Diagnostics, Germany) 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 Co, Budapest, Hungary) 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.

RAW 264.7 murine macrophage cells (ECACC, Salisbury, UK) were cultured in 5% CO_2 at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM–endotoxin tested) with 10% fetal calf serum and L-Glutamine (Sigma-Aldrich Co, Budapest, Hungary). Cells were seeded at $2x10^4$ or 10^5 cells/well in a 96-well plate or $2x10^6$ cells/well into a 6-well plate.

6.4. Murine sepsis model

To induce murine endotoxic shock, C57BL/6 mice were injected i.p. with a single dose of LPS (20 mg/kg being low or 40 mg/kg being high dose). FA (6 mg/kg) was administered i.p. in every 12 h; the first injection was given 1 h before the LPS treatment. The 6 mg/kg FA dose was chosen based on our preliminary *in vivo* experiments (unpublished data). FA alone treated mice 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 h for 84 h, after which time they were monitored three times a day for 1 week. No late deaths were observed in any of the experimental groups. Experiments were repeated three times with 10 mice in each experimental group. Pooled survival rates of all three experiments (n = 30) were presented. Treatment groups: LPS, treated with LPS alone; LPS + FA, treated with LPS and FA; FA, treated with FA alone; CTRL, vehicle treated group.

6.5. Magnetic resonance imaging (MRI)

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

MRI measurements were performed on a Varian UNITYINOVA 400 spectrometer (Varian, Inc., Paolo Alto, CA, USA) 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, USA). After tuning, shimming (1H linewidth 150 Hz) and RF calibration, the slice of interest was selected using a T1-weighted multislice spin-echo sequence (4.0 ms sinc pulses, TR = 1000 ms, TE = 12 ms, slice thickness = 1 mm, FOV = 30 mm × 30 mm, acquisition matrix 128 × 128). T2-weighted images were recorded using a multi-slice spin-echo sequence (parameters were like at T1-weighting, except TR = 3000 ms and TE = 50 ms). One average was taken and images were reconstructed as 256 × 256 matrices. The intensities of the images were standardised to the signal of a 1 mm inner diameter tube filled with water : glycerol = 9 : 1, which was placed near the animal during the measurements. Despite the internal standard, we were not able to quantify accurately the T2-weighted images due to the individual differences among the animals and differences in their positioning inside the probe. Instead, we used qualitative scoring of the standardized T2-weighted images performed by experts who were blind to the experiment. Experiments were repeated 3 times.

6.6. Determination of ROS production

Intracellular ROS were determined using the oxidation-sensitive 2,4 dichlorodihydrofluorescein-diacetat (C-400) fluorescent dye. Cells were seeded into 96-well plates at a concentration of 10^5 cell/well and cultured overnight. 24 h after the appropriate treatment (indicated in the figure legends), medium was removed and the cells were labeled with the fluorescent dye containing 1mg/ml C400 dissolved in DMSO. Incubation was continued for an additional 1 h. Fluorescence was excited at 485 nm, and the evoked emission was measured at 555 nm using a Fluostar Optima (BMG Labtechnologies, Heidelberg, Germany) fluorescent microplate reader. All experiments were run in at least 6 parallels and repeated three times.

6.7. Measurement of nitrite concentration

Cells were seeded at a concentration of 10^5 cells/well into a 96-well plate and cultured overnight before the appropriate treatment (indicated in the figure legends). After 24 h incubation NO_2 production was determined by adding to 50 µl medium equal volume Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) and absorption was measured with an Anthos Labtech 2010 enzyme-linked immunosorbent assay reader (Rosys, Wiena, Austria) at 550 nm wavelength. All experiments were run in at least 6 parallels and repeated three times.

6.8. 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 the absence of 0.25 to 50 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.

6.9. Western blot analysis

6.9.1. Treatments

Two times 10^6 cells/well were seeded into a 6-well plate and treated or not with 100 ng/ml LPS with or without 50 μM ferulaldehyde for 10 or 30 min. C57BL/6 mice were pretreated 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.

6.9.2. Sample preparation from cells

Cells were harvested in the ice-cold lysis buffer containing 0.5 mM sodium metavanadate, 1 mM EDTA, and protease inhibitor mixture and phosphate-buffered saline. The proteins were precipitated by trichloroacetic acid, washed three times with -20°C acetone. Pellets were resuspended in sample buffer and subjected to SDS-PAGE.

6.9.3. Sample preparation from liver

Tissue samples (50 mg) were homogenized in 50 mM TRISZ (pH=8) with ultraturax. After boiling and centrifuging in 2x sample buffer, supernatants were subjected to SDS-PAGE.

6.9.4. SDS-PAGE

Proteins (20 μg/lane) were separated on 12% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at 4°C overnight. Primary antibodies anti-phospho-p44/42-MAPK, anti-phospho-p38-MAPK, anti-phospho-SAPK/JNK, anti-phospho-Akt (Ser 473) anti-phospho-NF- B p65 (Ser536) (all from Cell Signaling Technology, Boston, MA, USA), and MKP-1 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1:1000 in blocking solution. Horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Sigma-Aldrich Co, Budapest, Hungary) were used for 2 h at room temperature in 1:5000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence (ECL) using the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL, USA). The developed films were scanned, and the pixel volumes of the bands were determined using NIH Image J software. All experiments were repeated four times.

6.10. RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from RAW 264.7 cells using TRIZol reagent (Sigma-Aldrich Co, Budapest, Hungary), according to manufacturer's protocol. RNA (1 µg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MuLV RT, RevertAidTM First Strand cDNA Synthesis Kit) (Fermentas, Burlington, Ontario, Canada) for 1 h at 42°C final volume was 20 µl. 1 µl of cDNA was used for real-time PCR. Real-time PCR runs were performed on Corbett Rotor-gene 3000. PCR was conducted over 45 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s three-step thermal cycling preceded by an initial 95°C for 7 s using iQ SYBR Green Supermix Kit (Bio-Rad, Hercules, CA, USA).

PCR was performed using the following primers:

MKP-1 Fwd 5'-TAACCACTTTGAGGGTCACTACC-3'

MKP-1 Rev 5'-TTCACAAACTCAAAGGCCTCG-3'

GAPDH Fwd 5'-ATTGTGGAAGGGCTCATGACC-3'

GAPDH Rev 5'-ATACTTGGCAGGTTTCTCCAGG-3'

Statistical analysis of relative expression of the target gene based on Ct values with efficiency correction was made with the Relative Expression Software Tool (Corbett Research, Germantown, MD, USA) normalized to the housekeeping gene GAPDH.

6.11. Cytokine 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 h at room temperature before centrifugation for 20 min at 2000 x g. 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 TNF- , IL-1 , IL-6 and IL-10 concentrations were determined with the Quantikine M TNF- immunoassay kit (R&D Systems, Minneapolis, MN, USA) and IL-1 , IL-6, IL-10 ready-set-go kits (eBioscience, San Diego, CA, USA). 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.

6.12. Determination of NF- B activity

6.12.1. Using a Trans-AM$^{TM.}$Transcription Factor Assay Kits

Mice were treated exactly as for Western blot analysis. For nucleus isolation, liver was removed 1.5 h after the LPS treatment and was homogenized immediately. Protein concentrations in nuclear extracts were determined using a bicinchoninic acid assay with bovine serum albumin as a standard (Sigma-Aldrich Co, Budapest, Hungary). To monitor NF- B activation in the liver, we used Trans-AM$^{TM.}$Transcription Factor Assay Kits (Active Motif, Rixensart, Belgian). The kit consists of 96-well plates into which oligonucleotides containing the NF- B (5'-GGGACTTTCC-3') are bound. The active form of NF- B in the nuclear extract specifically binds to the consensus site and it is recognized by primary antibody. A horseradish peroxidase-conjugated secondary antibody provides the basis for the colorimetric quantification. 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 means ± SEM of 12 independent values (three independent experiments with 4 mice in each group).

6.12.2. Using NF- B luciferase assay

RAW 264.7 macrophages were transiently cotransfected with either NF- B luciferase (NF- B) or control (TA-Luc) (Panomics, Santa Clara, CA, USA), and SV-β-galactosidase (pSV-β-gal) (Promega Corporation, Madison, WI, USA) plasmids by using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. 24 h after the transfection cells were treated as indicated, and another 24 h later cell lysates were collected. Cellular proteins were assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA, Luciferase Assay System Technical Bulletin TB281). The ratio of luciferase to β-galactosidase activity served to normalize the luciferase activity to correct for any differences in transfection efficiencies.

6.13. Measurement of mitochondrial membrane potential (m)

m was measured using the mitochondrial-specific dual-fluorescence probe, JC-1 (Molecular Probes, Eugene, OR, USA). RAW 264.7 cells were seeded to glass cover slips, treated as indicated, washed twice in ice-cold PBS, and loaded with JC-1 for 15 min at 37°C. When excited at 488 nm, the dye emits green fluorescence when the mitochondria are depolarized, and red for normal m. Cells were imaged with a Zeiss Axiovert 25 fluorescent microscope equipped with a ProgRes C12 Plus CCD camera using a 63 x objective and epifluorescent illumination. The same microscopic field was imaged in the red then the green channel, then the images were merged. In control experiments, we did not observe considerable bleed-through between the red and green channels.

Alternatively, after the indicated treatment, RAW 264.7 cells were washed twice in ice-cold PBS, incubated with 2 μM of JC-1 for 30 min then assayed by flow cytometry (FacsCalibur, BD Biosciences, San Jose, CA, USA). Results were analyzed by Cellquest software (BD Biosciences, San Jose, CA, USA). Fluorescence emission shift from red (590 nm) to green (529 nm) was monitored at 488 nm excitation. Mitochondrial depolarization is indicated by decrease in the red/green fluorescence intensity ratio.

6.14. Mass spectrometry (MS) analysis

1 μL of standard solutions and of wine samples were applied to a Bruker 384 ground steel plate without matrix. To promote crystallization, 1 μL of 0.1% trifluoroacetic acid solution was added for 30 sec to the samples, then the solution was removed. Analysis was performed on a Bruker Autoflex II MALDI TOF TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument used a 337 nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH., Berlin, Germany). The ions were accelerated under delayed extraction conditions (80 ns) in positive ion mode with an acceleration voltage of 17.05 kV and a reflector voltage of 20.00 kV. The Bruker FlexControl 2.4 software was used for control of the instrument and the Bruker FlexAnalysis 2.4 software for spectra evaluation. Measurements were repeated three times.

6.14. Statistical analysis

Values are presented as means ± SEM. 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.

7. Results and Discussion

Multiple organ failure leading to death could be the result of either early phase septic shock characterized by rapidly increased expression of pro-inflammatory cytokines or late stage severe sepsis associated with elevated high mobility group-1 (Ulloa and Tracey 2005). In this work, we investigated the effect and molecular mechanism of ferulaldehyde, a water-soluble end-product of dietary polyphenol degradation, on LPS-induced inflammation *in vivo* and *in vitro* and we found that ferulaldehyde exerted its beneficial anti-inflammatory effects during the early phase of inflammation. Ferulaldehyde is the reduced form and assumed physiological precursor of ferulic acid that was reported to have anti-inflammatory, anti-atherogenic, anti-diabetic, antiaging, neuroprotective, radioprotective and hepatoprotective effects (Srinivasan et al. 2007). We used C57BL/6 mice for *in vivo* and RAW 264.7 macrophage cells for *in vitro* experiments.

7.1. Ferulaldehyde reduced LPS-induced mortality in mice

In our *in vivo* experiments we used FA at 6 mg/kg concentration, at which the compound still had protective effect against septic shock in order to match the physiological concentrations that were found after consuming pholyphenol-rich food. Also, in our *in vivo* experiments with FA, we used LPS in two different concentrations; in a 20 mg/kg (*sublethal or lower*) dose for determine the molecular mechanism of the drug and a 40 mg/kg (*lethal or higher*) dose for survival experiments. In our survival study the higher dose of LPS (40 mg/kg, i.p.) resulted in about 80% death of the animals within 36-48 h (Fig. 10). C57BL/6 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. 10). 36-48 h after the LPS challenge 70% of mice in LPS + FA group survived compared with 20% in the LPS only group. Although FA was not able to prevent eventual death, it significantly increased the survival time of mice (Fig. 10) 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 then on the late stage severe sepsis. FA treatment alone did not induce death or any obvious damage (Fig. 10). A lower dose of LPS (20 mg/kg, i.p.) resulted in an approximately 20% death rate within 72 h and therefore was not suitable for survival studies (data not shown).

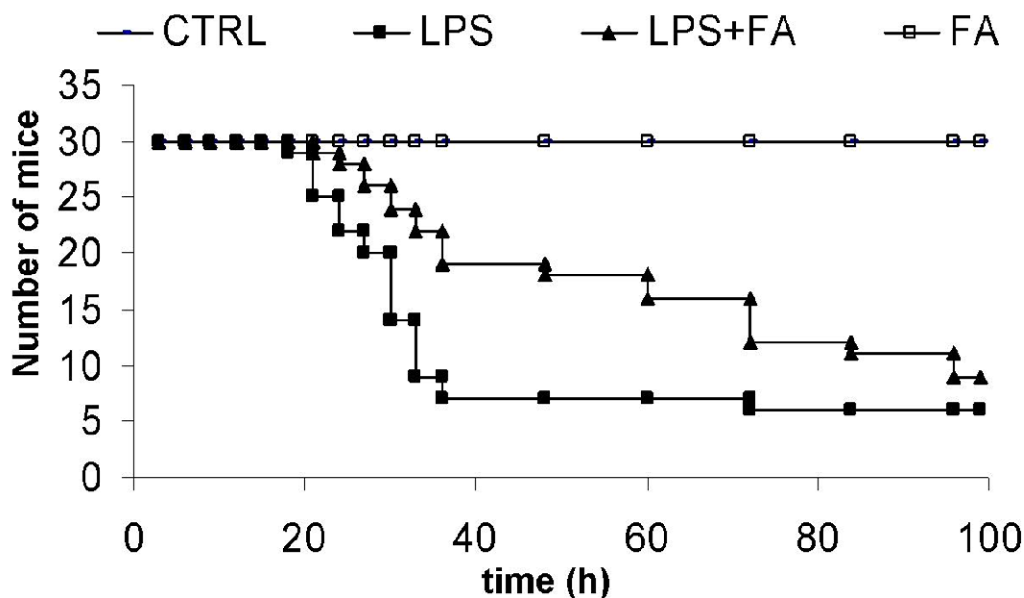


Fig. 10. Ferulaldehyde (FA) reduces lypopolysaccharide (LPS)-induced mortality in mice. Mice were pretreated with FA (6 mg/kg i.p.) 1 h before LPS challenge (40 mg/kg i.p.), and repeated every 12 h. Survival rate was observed up to 96 h for CTRL (line), LPS (filled square), LPS + FA (filled triangle)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

7.2. Ferulaldehyde inhibited LPS-induced inflammatory response in vivo

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 LPS-induced endotoxic shock was detected by MR-Imaging techniques. T_2-weighted images of CTRL, LPS and LPS + FA-treated mice were taken 6 h after the LPS challenge (Fig. 11). 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, similar to those found in another experimental endotoxic shock model (Veres et al. 2004). The importance of these tracts in the mediation of sepsis and multiple organ failure is well documented (Standiford et al. 1995, Boulares et al. 2003). In mice treated with LPS + FA, the T_2-weighted intensities were markedly lower, so the inflammatory response was significantly smaller (Fig. 11). T_2-weighted images of mice treated with FA alone were basically identical to images of untreated mice (data not shown).

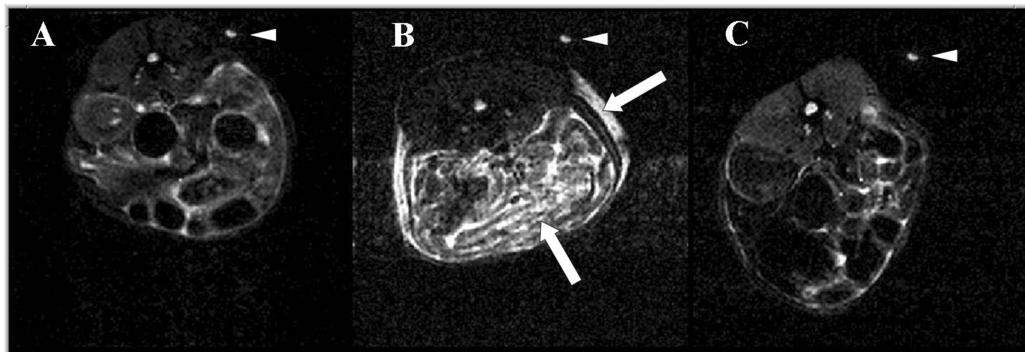


Fig. 11. In vivo inflammatory responses in untreated mice (A) and in those treated with lypopolysaccharide (LPS) (B) or LPS + ferulaldehyde (FA) (C). Lower abdominal region of LPS treated mice showed marked increase of intensity representing inflammatory response (arrows) especially in the lateral subcutaneous regions and in the interintestinal cavities. T2-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.

7.3. Ferulaldehyde attenuated LPS-induced TNF-α, IL-1β but not IL-6 production, and enhanced IL-10 generation in the sera of mice

TNF- is a substantial early mediator of endotoxemia since the production of this cytokine returned to a normal level 4 h after LPS treatment (Zanetti et al. 1992, James et al. 2002). TNF- 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 TNF- production can be lethal in itself, because it spreads in the bloodstream and produces cardiovascular collapse. TNF- is a sufficient and necessary mediator of early phase septic shock (Ulloa and Tracey 2005). In several previous reports, a

direct link between TNF-α and IL-1 as well as IL-10 was established (Veres et al. 2004). However, contradictory results appeared about the role of IL-6 in the inflammatory response (Xing et al. 1998). In our LPS-induced endotoxic shock model even the lower LPS dose (20mg/kg), that caused only negligible death rate among the mice (data not shown), induced significant induction of all the pro-inflammatory cytokines tested (Fig. 12). We measured the concentration of various cytokines from sera of CTRL, LPS, LPS + FA and FA-treated mice 1.5 or 3 h after the LPS challenge using enzyme-linked immunosorbent assay (ELISA)-based kits. LPS treatment caused a 16-20 times increase of serum TNF-α 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 TNF-α induction (Fig. 12A). 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 by FA in the case of the low dose of LPS (Fig. 12B). 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. 12C). 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. 12D). FA alone did not exert any effect on serum concentration of any of the cytokines (Fig. 12). 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. The link between the pro-inflammatory TNF- 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. However, FA enhanced the LPS-stimulated IL-10 production (Fig. 12D), and this enhancement reached the level of statistical significance in case of the higher LPS dose. This indicates that FA attenuated the LPS-induced *in vivo* inflammatory response not only by attenuating TNF- -linked pro-inflammatory cytokine production but also by enhancing anti-inflammatory IL-10 production.

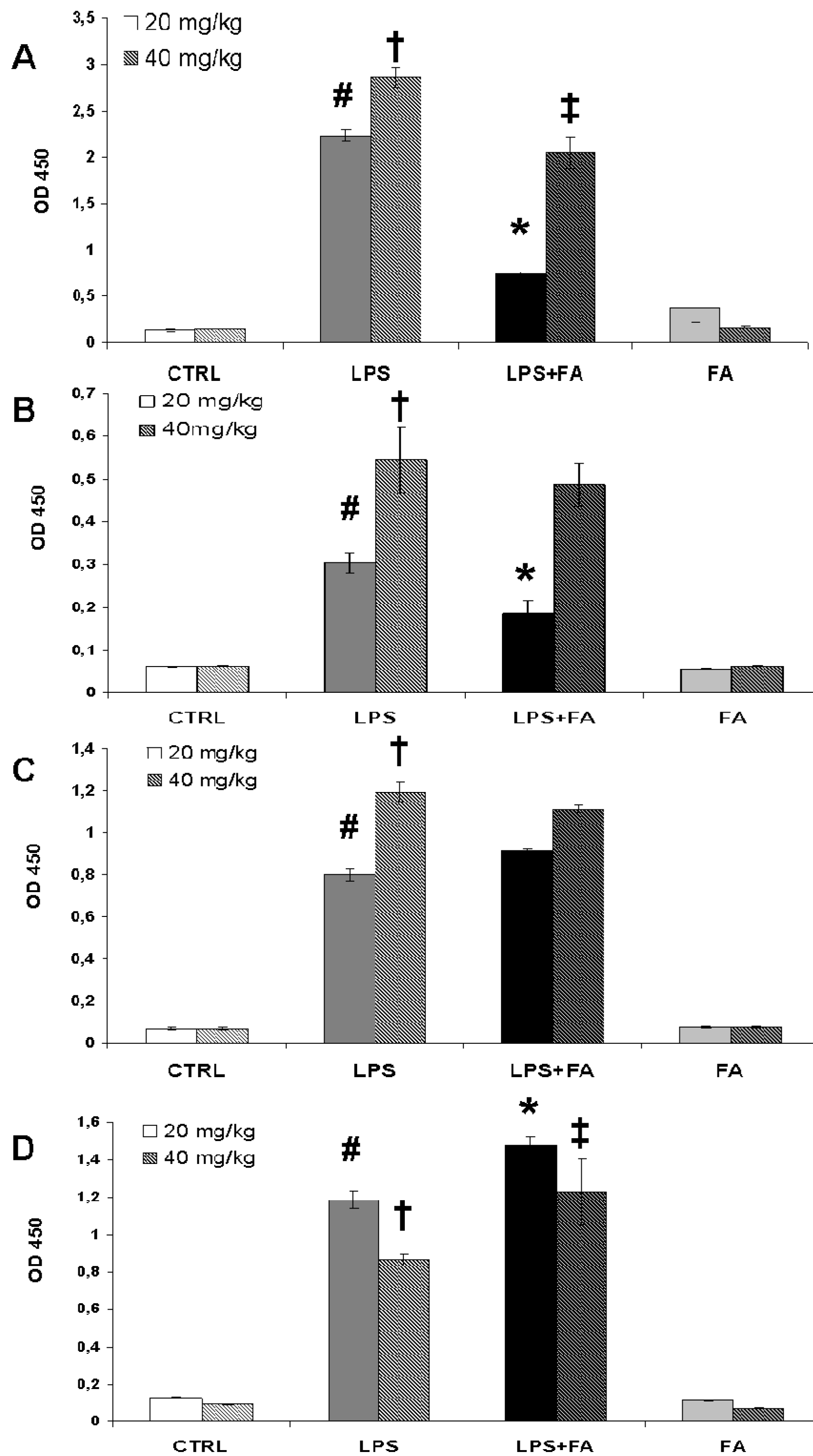


Fig. 12. Effect of ferulaldehyde (FA) on lypopolysaccharide (LPS)-induced TNF- (A), IL-1 (B), IL-6 (C) and IL-10 (D) production in the sera of LPS treated mice. Cytokine level was measured 1.5 (A, D) or 3 (B, C) h after LPS administration from the sera of CTRL, LPS, LPS + FA and FA mice. Three independent experiments with three mice in each experimental group were performed. Data were pooled (n = 9) and values are means ± SEM. ∗ $P < 0.05$ LPS (20 mg/kg) + FA vs. LPS (20 mg/kg), # $P < 0.05$ CTRL vs. LPS (20 mg/kg), ‡ $P < 0.05$ LPS (40 mg/kg) + FA vs. LPS (40 mg/kg), † $P < 0.05$ CTRL vs. LPS (40 mg/kg).

7.4. Feruladehyde inhibited LPS+IFN- -induced NO_2^- and ROS production in primary hepatocytes

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. It was found that FA suppressed the LPS + IFN- -induced iNOS expression in RAW 264.7 macrophage cells (Kim et al. 1999). However, the pathological changes that were previously observed in the liver of LPS-treated mice (Veres et al. 2004) could be resulted from ROS and NO produced by the hepatocytes themselves. Therefore we determined FA's effect on LPS + IFN- -induced ROS and NO_2^-production in primary hepatocytes by measuring these in the culturing medium following a 24-h incubation using a fluorescent redoxi dye and a Griess reagent based assay, respectively. 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 IFN- in order to induce full activation of the hepatocytes. During a 24-h incubation period, LPS + IFN- increased ROS production in primary hepatocytes 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. 13A), similarly to RAW 264.7 macrophages (Fig. 14A). Activation of hepatocytes by LPS + IFN- increased NO_2^- concentration 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. 13B) and the effect of FA was similar then in the macrophage model (Fig. 14B). FA concentration in the culture medium did not decrease significantly during the incubation period (data not shown).

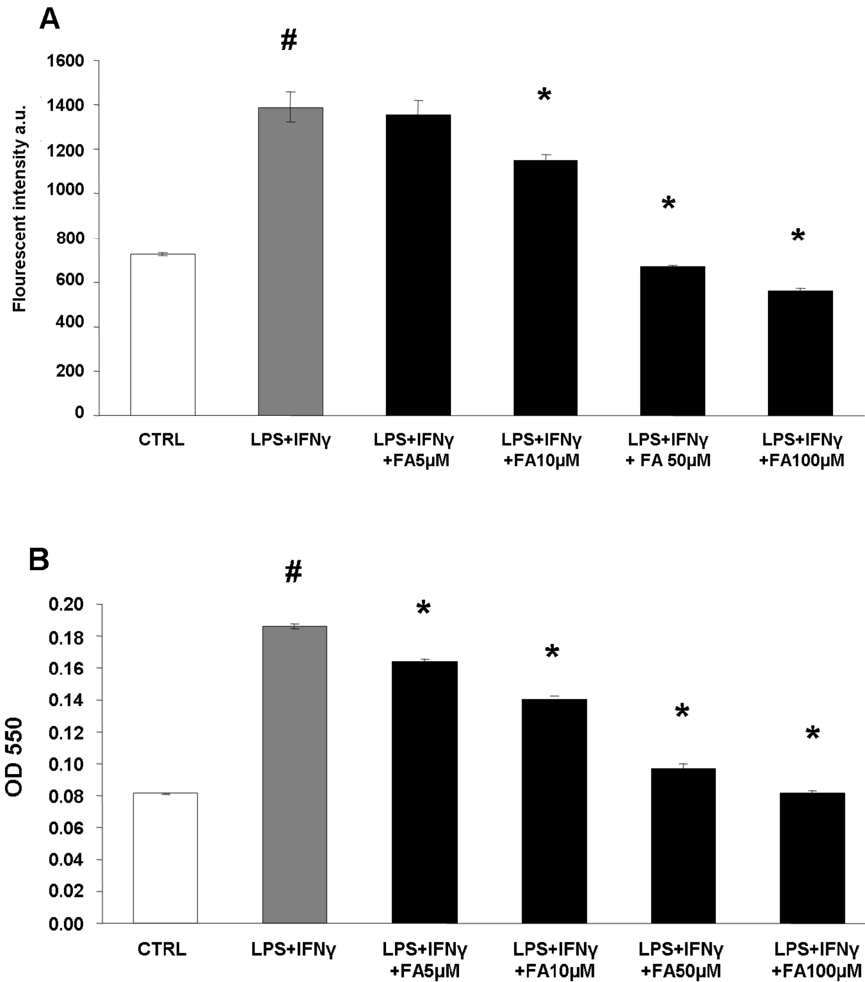


Fig. 13. Ferulaldehyde (FA) reduces lipopolysaccharyde (LPS) + interferon- (IFN-)-induced ROS (A) and NO2$^-$ (B) production in primary hepatocytes. Primary hepatocytes were incubated in the presence and absence of 5mg/L LPS + 50 µg/L IFN- alone (LPS+IFN), and together with 1-100 µmol/L FA (actual concentration is indicated) for 24 h before measuring ROS (A) and NO2$^-$ (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). ∗ $P < 0.05$ LPS + FA vs. LPS, # $P < 0.05$ CTRL vs. LPS.

7.5. Ferulaldehyde inhibited LPS-induced ROS and NO_2^- production in RAW 264.7 macrophages

Macrophages represent the first defense line against pathogens and play a crucial role for the induction of the protective response to eliminate infectious agents producing pro-inflammatory cytokines, reactive oxygen and nitrogen species. However overproduction of these mediators could cause tissue and cell destruction (Huet et al. 2007). Therefore, we

determined FA's effect on LPS-induced ROS and NO production in RAW macrophage cells by using a fluorescent redoxi dye and a Griess reagent based assay, respectively. We induced ROS and NO production by treating the cells with 100 ng/ml LPS for 24 h that was found not to have considerable cytotoxic effect under these conditions (data not shown). Similarly to a previous report (Kim et al. 1999) and our finding in the model of primary hepatocytes (Fig. 13), we found that the amount of both reactive oxygen and nitrogen species induced by LPS was reduced by FA in a concentration-dependent manner (Fig. 14).

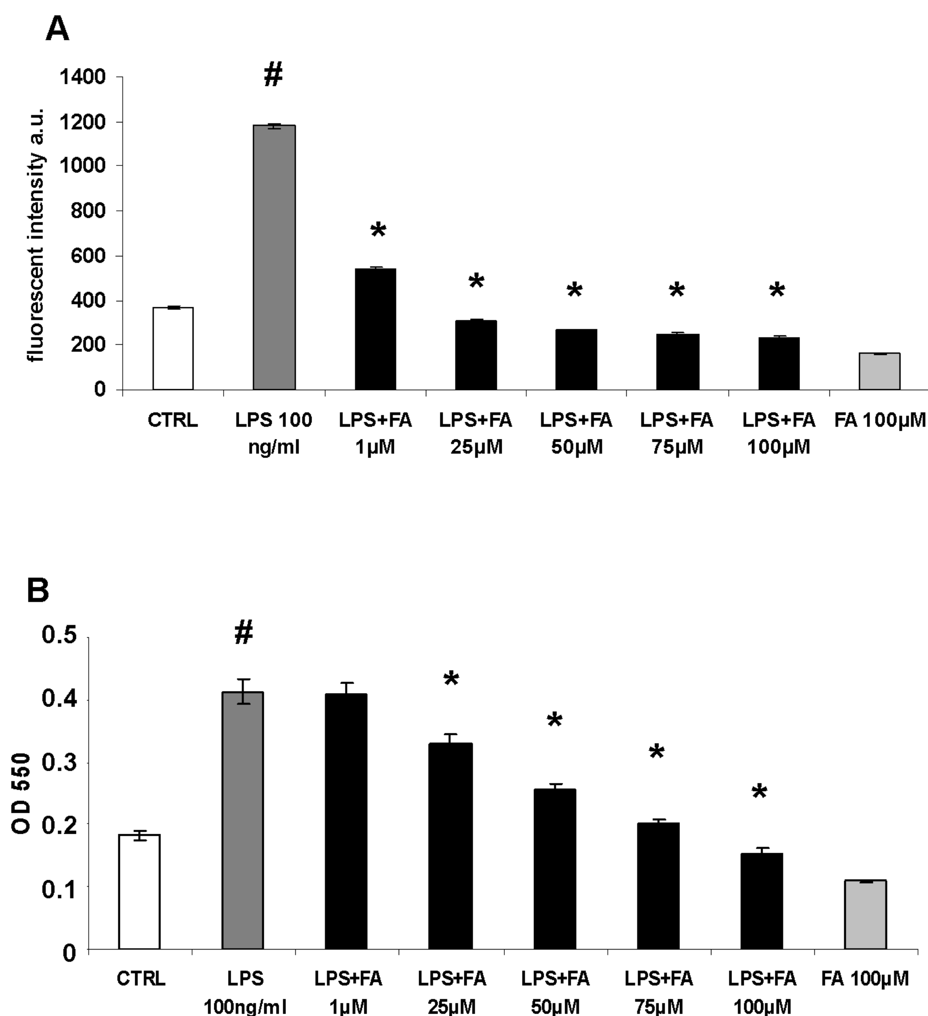


Fig. 14. Ferulaldehyde (FA) inhibited the ROS and NO_2^- production of lipopolysaccharyde (LPS)-activated RAW 264.7 macrophages in a dose dependent manner. The production of ROS (**A**) and NO_2^- (**B**) was measured 24 h after LPS challenge from the media of treated or control cells. Ctrl: untreated; LPS: treated with LPS (100 ng/ml); LPS + FA: treated with FA (1-100 μM) 5 min before LPS (100 ng/ml) challenge; FA: treated with FA (100 μM). Data are expressed as mean ± SEM of 3 independent experiments running in 6 parallels (n = 18). ∗ $P < 0.01$ LPS + FA vs. LPS, # $P < 0.01$ CTRL vs. LPS.

This finding is consistent with the notion that eliminating or inhibiting the production of the highly reactive radicals during the early phase of inflammatory response attenuates inflammatory damages (Lee et al. 2009). Since at 50 µM concentration FA diminished LPS-induced ROS and nitrite accumulation in the cells to below and close to the control level, respectively; and this concentration was comparable to the one (6 mg/kg~33.6 µM) we used *in vivo*, we applied FA at a concentration of 50 µM in all *in vitro* experiments.

7.6. Ferulaldehyde protected the mitochondrial membrane potential in RAW 264.7 macrophages

Excessive intracellular reactive oxygen species can be generated by mitochondria, and mitochondrial oxidative stress damage and dysfunction contribute to a number of cell pathologies that manifest themselves in a range of conditions including sepsis (Brealey and Singer 2003, Zapelini et al. 2008). In turn, increased ROS leads to simultaneous collapse of m and a transient increased ROS generation by the electron transfer chain (Zorov et al. 2000, Brady et al. 2006). We investigated the mitochondrial membrane potential using a cell-permeable voltage-sensitive fluorescent mitochondrial dye, JC-1 that emits green fluorescence when the mitochondria are depolarized, and red for normal m when excited at 488 nm. Flow cytometry analysis revealed that LPS-induced substantial mitochondrial depolarization at all time points of the experiment. It was significant as early as 5 min, and was maximal at 10 and 30 min after 100 ng/ml LPS challenge (Fig. 15A,B). FA diminished this effect of LPS on m at all of the time points we used (Fig. 15A,B). Massive depolarization of mitochondria by LPS indicated by faint green fluorescence was also demonstrated by fluorescent microscopy performed 30 min after 100 ng/ml LPS challenge (Fig. 15C). However, m was preserved by 50 µM FA as indicated by appearance of red fluorescence emitted by JC-1 aggregates in the mitochondria (Fig. 15C). Protection of mitochondria against LPS-induced rapid and massive membrane depolarization by FA indicate the importance of mitochondrial integrity in early inflammatory response. This notion is supported by previous findings that one of the most thoroughly studied polyphenols, trans-resveratrol inhibited mitochondrial membrane depolarization and ATP depletion (Leiro et al. 2004, Dave et al. 2008, Shin et al. 2009) thereby exerted cytoprotective effects (Ungvari et al. 2009, 2010).

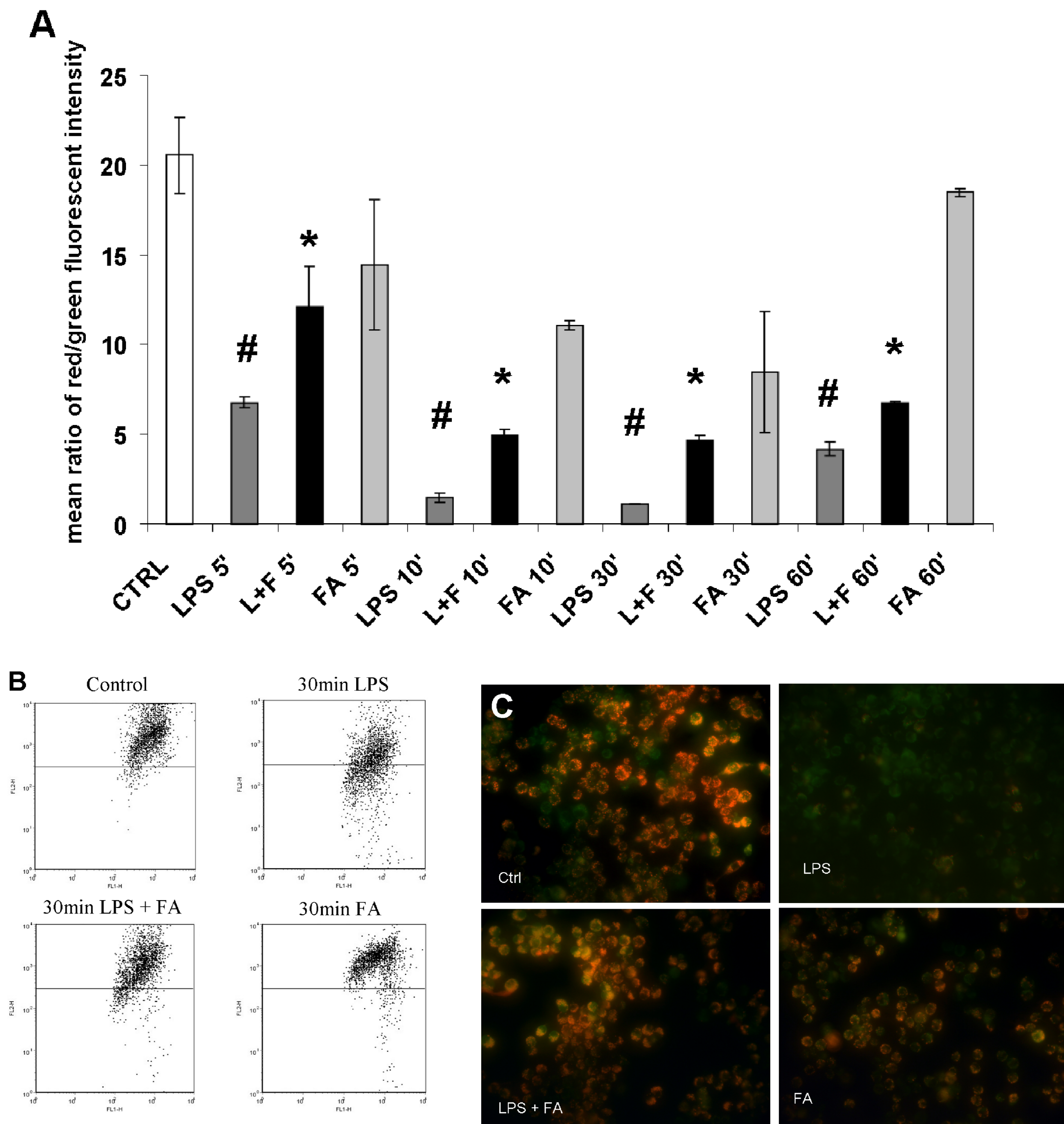


Fig. 15. Detection of mitochondrial membrane potential by flow cytometry and microscopy using JC-1 dye. RAW 264.7 cells were treated or not (CTRL) for 5, 10, 30 or 60 min with 100 ng/ml lipopolysaccharide (LPS) together with or without 50 μM ferulaldehyde (FA), stained with JC-1, then assayed by flow cytometry. Fluorescence emission shift from red (590 nm) to green (529 nm) representing mitochondrial membrane depolarization was monitored at 488 nm excitation. Data for all time points are expressed as ratio of red to green fluorescent intensity (**A**), mean ± SEM of 3 independent experiments running in 6 parallels (n = 18). * $P < 0.01$ LPS + FA vs. LPS, # $P < 0.01$ CTRL vs. LPS. Representative dot-plots (**B**) acquired 30 min after the LPS challenge are also presented. FL1-H and FL2-H axes represent green and red fluorescent intensity, respectively. Ctrl, untreated; LPS: treated with LPS (100 ng/ml); LPS + FA: treated with FA (50 μM) 5 min before LPS (100 ng/ml) challenge; FA: treated with FA (50 μM). Alternatively, green and red fluorescent images were taken of the same microscopic field after 30 min of LPS treatment. Treatment groups were the same as in the previous experiment. Representative merged images **(C)** of three independent experiments are presented.

7.7. Ferulaldehyde negatively regulated LPS-induced phosphorylation of JNK and Akt, and activation of NF-κB in liver

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 pro-inflammatory transcription factors: AP-1 and NF-κB. Previous works had shown that LPS did induce activation of MAPK pathways in different cell lines, and these pathways played key role in the transduction of the LPS signal between the cell membrane receptor and the cytoplasmic and nuclear response resulting in activation of gene expression (Dumitru et al. 2000, Bozinovski et al. 2002). Anti-inflammatory effects of dietary polyphenols and natural products were attributed to their modulating effects on these signaling mechanisms (Stali ska et al. 2005, Kutuk et al. 2006, Gonzales and Orlando 2008). Previously, it was found that the most prominent pathological changes were in the liver of LPS-treated mice so we focused to this organ in our study (Veres et al. 2003, 2004).

In accordance with these results, we determined the effect of FA on MAPK signaling and NF- B activation from the livers of LPS-treated mice. 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 by Western-blotting. Phosphorylation and thereby activation of all the kinases studied (Fig. 16A,B,D), except p38 MAPK (Fig. 16C), were increased following LPS stimulation. FA significantly prevented this activation in the case of JNK (Fig. 16B) and Akt (Fig. 16D), but failed to attenuate LPS-induced activation of ERK1/2 (Fig. 16A). FA alone did not exert any effect on the phosphorylation of the kinases studied in our experimental model.

NF-κB activation and nuclear translocation was examined from the liver of CTRL, LPS, LPS + FA and FA-treated mice 1.5 h after the LPS challenge by transcription factor assay kit. We found a nearly four-fold activation of NF-κB after LPS challenge as compared to the control liver. 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.

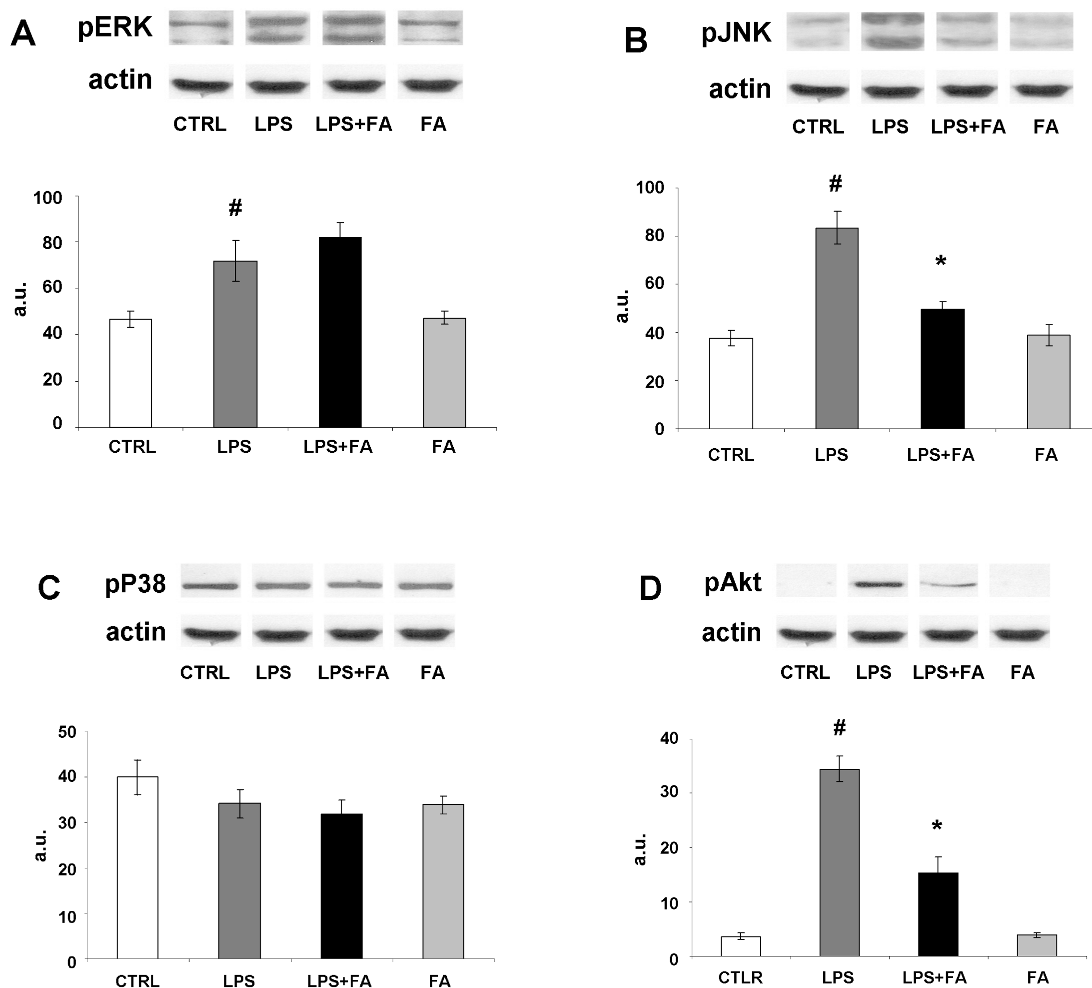


Fig. 16. Effect of ferulaldehyde (FA) on kinase activation in the liver of lipopolysaccharide (LPS)-treated mice. Activation of Akt, p38, ERK1/2 and JNK was demonstrated 1.5 h after LPS injection from liver homogenates of CTRL, LPS, LPS + FA and FA mice by using phosphorylation specific primary antibodies and immunoblotting. Representative immunoblots are presented. Experiments with three mice per group were repeated three times with basically identical results (n = 9). $*$ $P < 0.05$ LPS + FA vs. LPS, # $P < 0.05$ CTRL vs. LPS.

To summarize, we found ERK1/2, JNK and NF- B, but not p38 activation in response to LPS. JNK plays a crucial role in LPS-induced NF-κB activation (Sanchez-Tillo et al. 2007); therefore, activation of this kinase pathway is consistent with both NF-κB activation (Fig. 17) and pro-inflammatory cytokine production (Fig. 12) in the liver and blood of LPS-treated mice, respectively. FA inhibited the LPS-induced NF-κB (Fig. 17) and JNK (Fig. 16B) activation, but did not affect ERK1/2 (Fig. 16A) and p38 (Fig. 16C) MAPK pathways. LPS-induced Akt activation was also suppressed by FA (Fig. 16D), which could have contributed

to its anti-inflammatory effect. It has been suggested that, independently from JNK pathway, PI-3K–Akt-mammalian target of rapamycin can activate the 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 and LPS/PI-3K/Akt pathways. Similar mechanisms could account for the anti-inflammatory properties of one of the most deeply studied polyphenols, trans-resveratrol (Das S and Das DK 2007) which was reported to inhibit PI-3K (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.

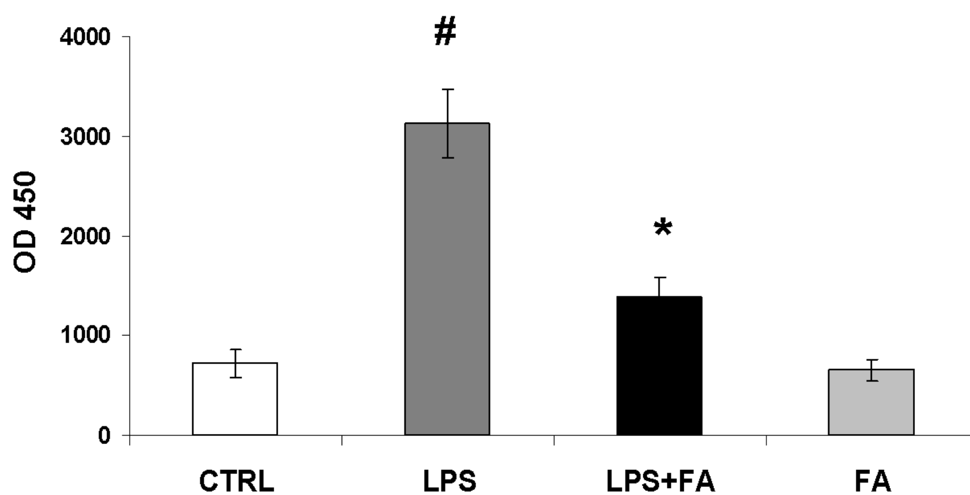


Fig. 17. Ferulaldehyde (FA) inhibits NF- B activation in the liver of lipopolysaccharide (LPS)-treated mice. Activation and nuclear translocation of NF- B was measured 1.5 h after LPS injection from the nuclear fraction of the liver of CTRL, LPS, LPS + FA and FA mice. Data are expressed as means ± SEM of three mice per group. The experiment was repeated three times (n = 9). ∗ $P < 0.05$ LPS + FA vs. LPS, # $P < 0.05$ CTRL vs. LPS.

7.8. Ferulaldehyde diminished LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages

LPS treatment (Veres et al. 2003, 2004) and cellular oxidative stress (Tapodi et al. 2005) trigger MAPK activation, which, in turn, leads to augmented production of pro-inflammatory cytokines (Zhao et al. 2006). To study whether MAPKs were involved in the protective effect of FA in RAW macrophages, we measured phosphorylation of p38, ERK1/2 and JNK from CTRL, LPS, LPS + FA and FA-treated cells 10 and 30 min after 100 ng/ml LPS challenge by Western-blotting utilizing phosphorylation-specific primary antibodies. Phosphorylation and thereby activation of all the kinases were increased following LPS stimulation at both time points except for JNK at 10 min (Fig. 18). Ferulaldehyde alone did not exert any effect on the phosphorylation of the MAPKs. We found that FA inhibited LPS-induced NF- B (Fig. 17) and JNK (Fig. 16B) activation, but did not affect ERK1/2 (Fig. 16A) and p38 (Fig. 16C) MAPK pathways in liver of mice. However, unlike the *in vivo* model, FA attenuated LPS-induced activation of all three MAPKs except for ERK1/2 at 10 min (Fig. 18) suggesting a uniform regulation of MAPK activation in LPS-stimulated macrophages.

To summarize, in the investigation of signaling mechanisms in the livers of mice and in macrophages, we found a modified kinase activation pattern induced by the same type of LPS molecule. The main differences in MAPKs pattern were found in the activation of ERK1/2 and p38 in our models. These results may arise from the differences of the inflammatory models used, and cell- and tissue-specificity of the LPS-induced processes (Veres et al. 2004). Macrophages represent the first defense line against pathogens and are intensely exposed to the inflammation caused by infectious agents in this way they are involved in the induction of a stronger protective response to eliminate causative agents. However, liver which was the objective of our *in vivo* experiments, is a target organ for bacterial infection and as such it shows another type of protective response for pathogens.

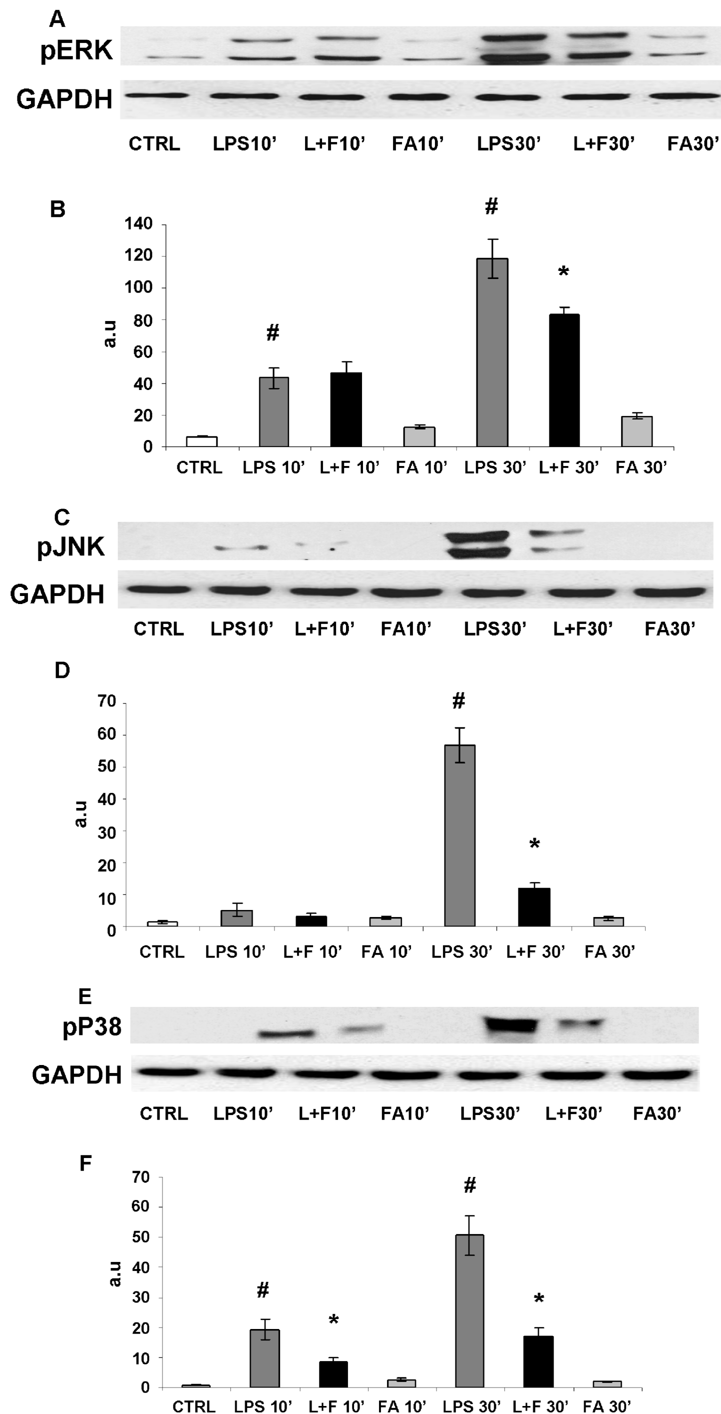


Fig. 18. Effect of ferulaldehyde (FA) on lipopolysaccharide (LPS)-induced activation of MAP kinases in LPS-treated RAW 264.7 macrophages. The phosphorylation of ERK1/2 (**A, B**), JNK (**C, D**) and p38 (**E, F**) was measured 10 and 30 min after LPS challenge of treated and control cells. GAPDH was used as a loading control. Representative blots of three independent experiments are presented (**A, C, E**). Quantification of band intensities was performed by densitometric analysis using ImageJ software (**B, D, F**). Ctrl: untreated; LPS: treated with LPS (100 ng/ml); LPS + FA: treated with FA (50 μM) 5 min before LPS (100 ng/ml) challenge; FA: treated with FA (50 μM). Data are expressed as mean ± SEM of 4 independent experiments running in 3 parallels (n = 12). ∗ $P < 0.01$ LPS + FA vs. LPS, # $P < 0.01$ CTRL vs. LPS.

7.9. Ferulaldehyde regulated LPS-induced MAPK activation via induction of MKP-1 expression

MKP-1 was reported to dephosphorylate all three MAP kinases (Wu 2007, Wang et al. 2007, Chi and Flavell 2008). Furthermore, it was found to be a critical negative regulator in the innate immune response to LPS. In peritoneal macrophages, MKP-1 is induced by LPS and plays a critical role in the attenuation of MAPKs. More specifically, induction of MKP-1 60 min after LPS treatment was reported, which was accompanied by attenuation of MAPK phosphorylation (Shepherd et al. 2004, Zhao et al. 2006). However, MKP-1 was reported to have a fast turnover rate (Zhao et al. 2006), therefore, we were interested how LPS and FA affected MKP-1 expression as early as 10 and 30 min after LPS treatment. In contrast to the previous notion that LPS monotonously induces MKP-1 expression, we found by Q-RT-PCR that MKP-1 mRNA level dropped to about half of the control value within 10 min after 100 ng/ml LPS application before increasing and reaching control level in 30 min (Fig. 19). LPS-induced decrease of MKP-1 mRNA level was significantly diminished by FA 10 min after LPS treatment, and we found dramatically elevated MKP-1 mRNA expression levels in cells treated with FA alone or in combination with LPS at 30 min (Fig. 19). In agreement with findings of other groups (Shepherd et al. 2004, Zhao et al. 2006), we found elevated expression of MKP-1 60 min after the LPS challenge in RAW macrophages at the protein level (data not shown). However, as we found by Western-blotting, MKP-1 protein concentrations reflected changes of its mRNA levels in a delayed fashion. Namely, we found markedly decreased MKP-1 protein concentration 30 min after 100 ng/ml LPS treatment that was prevented by FA (Fig. 20). In complete accordance with these data, FA inhibited LPS-induced early activation of all three MAPKs (Fig. 18). To summary, MAPK activation is followed by increased MKP-1 expression probably as a compensatory regulatory mechanism. In our *in vitro* experimental model, we found that FA shifted increased expression of MKP-1 forward in time which in turn attenuated activation of MAPKs.

Recently, it was found that MKP-2$^{-/-}$ mice have a survival advantage over wild-type ones in various septic shock models (Cornell et al. 2010). MKP-2$^{-/-}$ bone marrow-derived macrophages showed increased phosphorylation of ERK1/2, decreased phosphorylation of JNK and p38, and increased induction of MKP-1 upon LPS stimulation. Furthermore, knocking down MKP-1 in these macrophages increased their capacity for cytokine production (Cornell et al. 2010). All these data suggested a regulatory role for MKP-2 by targeting ERK1/2 deactivation, thereby decreasing MKP-1 (Cornell et al. 2010). MKP-2 could account

for the early suppression of MKP-1 that we observed 30 min after LPS stimulation (Fig.20). However, LPS increased ERK1/2 activation at 10 min and significantly enhanced this increase at 30 min after its application (Fig. 18A,B) that suggest a more complex regulation of MKP-1 expression upon LPS stimulation in our *in vitro* model.

7.10. Ferulaldehyde inhibited LPS-induced NF- B activation in RAW 264.7 macrophages

Elevated ROS production and activation of MAP kinases in turn lead to nuclear translocation of NF- B. We observed NF- B activation in livers of LPS-treated mice (Fig. 17) accompanied by elevated serum cytokine levels (Fig. 12). LPS-induced NF- B activation triggers the expression of inflammatory genes including COX-2 and iNOS (Woo and Kwon 2007, Chiu and Lin 2008). Overexpression of COX-2 and iNOS can lead to ROS and NO production, which can contribute to systemic tissue damage and can cause impairment of mitochondrial membrane integrity. Therefore, we were interested whether modulation of MAPK pathways by LPS and FA was reflected in NF- B activation in RAW 264.7 macrophages. We assessed NF- B activation in the cells by determining phosphorylation of its p65 subunit by Western blotting (Fig. 21A,B) 10 min after 100 ng/ml LPS treatment, and accumulation of NF- B-dependent expression of luciferase (Fig. 21C) from an NF- B-luciferase reporter plasmid for 24 h in 5 or 50 ng/ml LPS-treated macrophages. We used 5 and 50 ng/ml concentration of LPS since in the NF- B-luciferase assay 50 ng/ml LPS induced maximal activation of NF- B, and we wanted to demonstrate that a 10 times lower concentration of LPS could cause substantial effect. We found that even 5 ng/ml LPS induced a strong activation of NF- B that was strongly attenuated by 50 µM FA (Fig. 21C). FA alone did not exert any effect on the activation of NF- B in our *in vitro* model, however, markedly reduced the effect of each 5, 50 and 100 ng/ml LPS (Fig. 21).

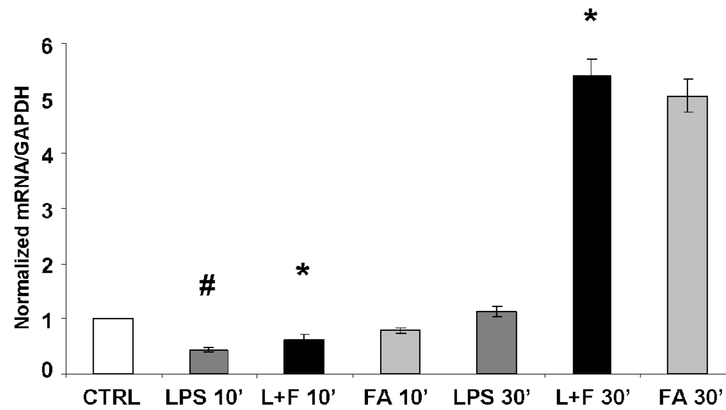


Fig. 19. Q-RT-PCR analysis of mouse MKP-1 mRNA expression in RAW 264.7 cells. Level of mouse MKP-1 mRNA was measured 10 and 30 min after lipopolysaccharyde (LPS) challenge of treated and control cells. GAPDH was used as a housekeeping control gene. Three independent experiments running in three parallels were performed. Ctrl: untreated; LPS: treated with LPS (100 ng/ml); LPS + FA: treated with FA (50 μM) 5 min before LPS (100 ng/ml) challenge; FA: treated with FA (50 μM). Specific primer sequences and PCR conditions are described under "Materials and Methods." Data are expressed as mean ± SEM (n = 9). ∗ $P < 0.01$ LPS + FA vs. LPS, # $P < 0.01$ CTRL vs. LPS.

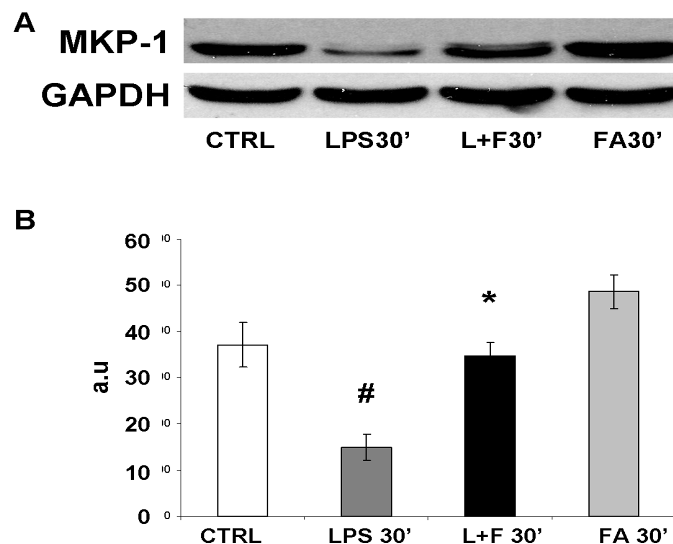


Fig. 20. LPS-induced decreased MKP-1 level was elevated by ferulaldehyde (FA) treatment. 30 min after lipopolysaccharide (LPS) treatment MKP-1 level of RAW 264.7 cells was detected by Western blot analysis. GAPDH was used as a loading control. Representative blots (**A**) of 4 independent experiments are presented. Quantification of band intensities (**B**) was performed by densitometric analysis using ImageJ software. Ctrl: untreated; LPS: treated with LPS (100 ng/ml); LPS + FA: treated with FA (50 μM) 5 min before LPS (100 ng/ml) challenge; FA: treated with FA (50 μM). Data are expressed as mean ± SEM of 4 independent experiments running in 3 parallels (n = 12). ∗ $P < 0.01$ LPS + FA vs. LPS, # $P < 0.01$ CTRL vs. LPS.

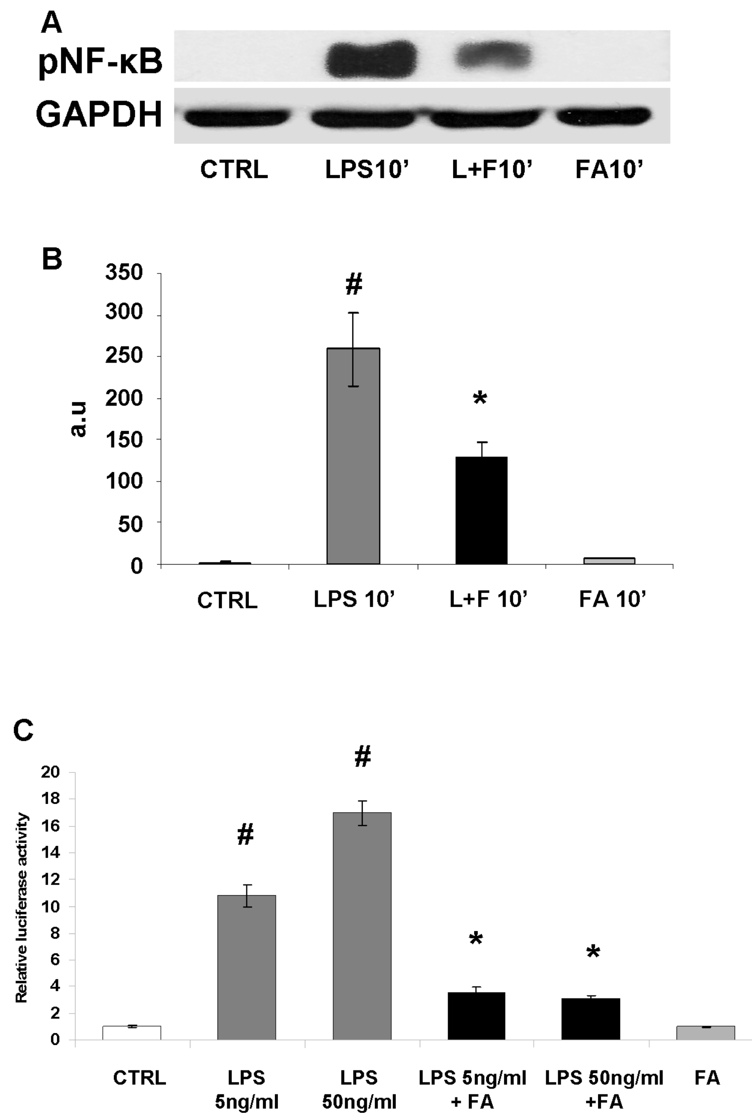


Fig. 21. Activation of NF- B was inhibited by ferulaldehyde (FA) in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Phosphorylated form of p65 subunit of NF- B was detected 10 min after LPS treatment by Western blot analysis. GAPDH was used as a loading control. Representative blots of 4 independent experiments are presented (**A**). Band intensities were quantified by densitometric analysis using ImageJ software (**B**) and were expressed as mean ± SEM of 4 independent experiments running in 3 parallels (n = 12). ∗ *P* < 0.01 LPS + FA vs. LPS, # *P* < 0.01 CTRL vs. LPS. Ctrl: untreated; LPS*:* treated with LPS (100 ng/ml); LPS + FA*:* treated with FA (50 μM) 5 min before LPS (100 ng/ml) challenge; FA*:* treated with FA (50 μM).

Alternatively, activation of NF- B was assessed by a luciferase reporter assay (**C**) after exposing the cells to 5 or 50 ng/ml LPS with or without 50 μM FA for 24 h. Data are expressed as mean ± SEM of 4 independent experiments running in 3 parallels (n = 12). ∗ *P* < 0.01 LPS + FA vs. LPS, # *P* < 0.01 CTRL vs. LPS.

7.11. Direct free-radical scavenging activity of ferulaldehyde

Since we found that FA inhibited NO_2^- and ROS production induced by LPS plus IFN- in primary hepatocytes (Fig. 13) similarly to RAW macrophages (Fig. 14), we determined whether the antioxidant property of FA was due to its free-radical scavenging activity. To this end we used a cell-free *in vitro* system. We tested direct free-radical scavenging activity of FA by measuring H_2O_2-induced oxidation of fluorescent redox dye dihydrorhodamine 123 in the presence and absence of the substance. FA at the concentration range of 5 to 100 µmol/L attenuated oxidation of the dye in a concentration dependent manner and it demonstrated a free-radical scavenging activity that was about the same as that of a known antioxidant, resveratrol (Fig. 22). Published effects of resveratrol on cytokine profile, NF- B translocation and kinase signaling (Kutuk et al. 2006, Gonzales and Orlando 2008) were very similar to those effects 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, Rios 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 (Fig. 23). Bioavailability of ferulic acid was reported to be higher than that of other dietary flavonoids and monophenolics (Beecher 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 (our 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- B activation and kinase signaling (Kim et al. 2007, Gonzales and Orlando 2008) and of our FA results. 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.

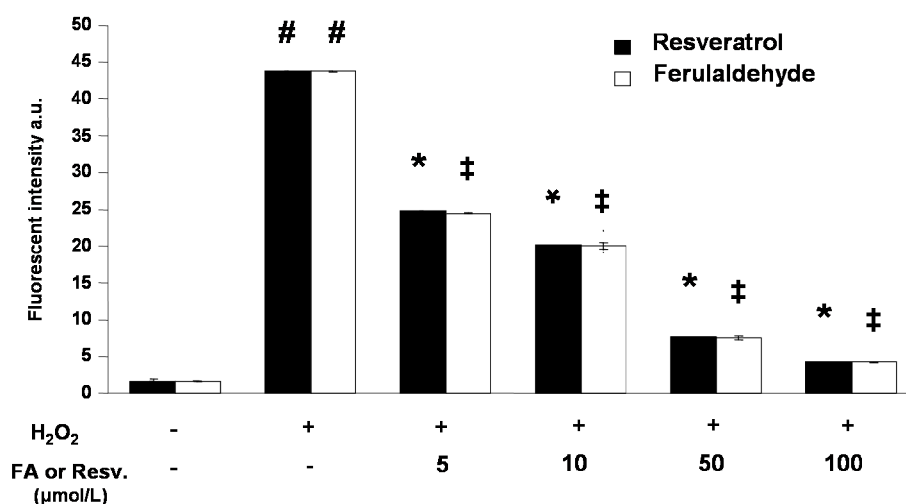


Fig. 22. Antioxidant effect of ferulaldehyde (FA) in cell-free system. ROS content was measured in a cell free system. Oxidation of the redox dye dihydrorhodamine123 was induced by 10 μmol/L H_2O_2 in the presence (actual concentration is indicated) and absence (-) of 5 to 100 μmol/L of FA or resveratrol. Data are expressed as means ± SEM. All experiments were run in six parallels and repeated three times (n = 18). ∗ $P < 0.05$ H_2O_2 + resveratrol vs. H_2O_2, # $P < 0.05$ CTRL vs. H_2O_2, ‡ $P < 0.05$ H_2O_2 + FA vs. H_2O_2.

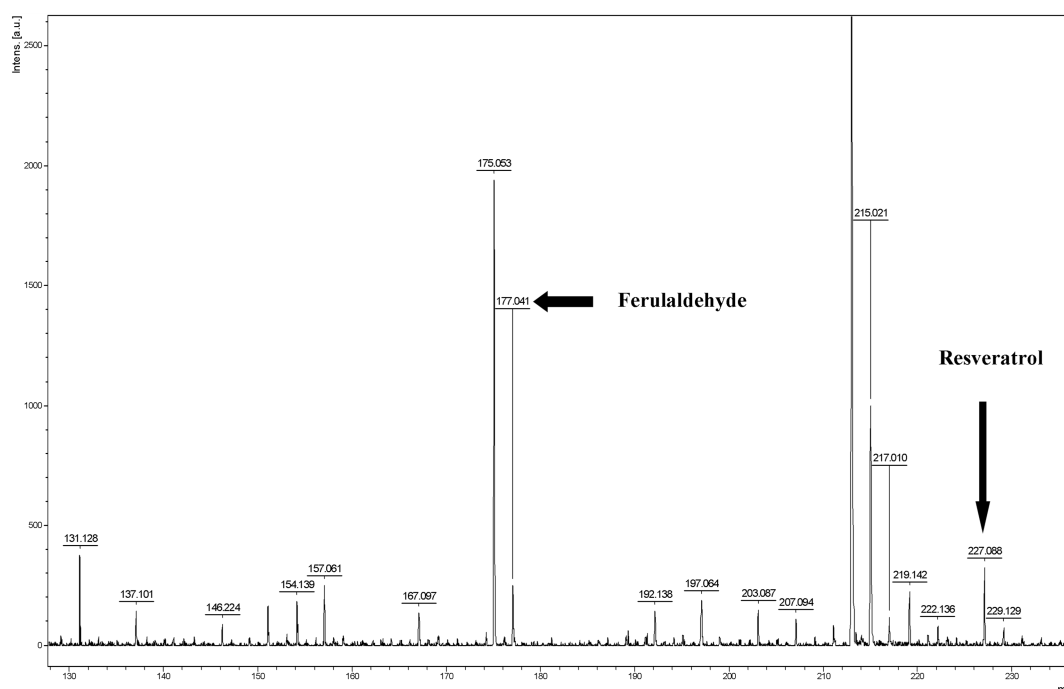


Fig. 23. Presence of ferulaldehyde (FA) in red wine. Representative MALDI TOF TOF mass spectrum of a Hungarian red wine that was previously found rich in resveratrol. Mass spectrometry was performed as it is described in Materials and Methods. X axis represents mass of the molecule-ions, y axis demonstrates ion-intensity in arbitrary units. Measurements were repeated three times.

7.12. Inhibition of early inflammatory response in mice and in RAW 264.7 macrophages by ferulaldehyde

Taken together, increased ROS production and early activation of MAPKs by LPS treatment lead to nuclear translocation of NF- B that triggers expression of NF- B-dependent genes including cytokines, COX-2 and iNOS mediating early inflammatory response in mice and macrophages. Ferulaldehyde inhibited the transcription factor NF-κB via the inhibition of JNK and Akt pathways in the livers of LPS-treated mice. This positive effect on modulating of these important kinase cascades could be the consequence of the antioxidant property of FA. Since the anti-inflammatory features of FA are thought to not exclusively attributed to their antioxidant capacity, we investigated further the effects of FA in an LPS-induced inflammation model in macrophages. In this study, we found that FA shifted increased expression of MKP-1 forward in time which in turn attenuated activation of MAPKs as well as of NF- B-dependent gene expression thereby protecting mitochondrial membrane integrity and polarization, and preventing the generation of mitochondrial ROS in the macrophages. This mechanism is in line with *in vivo* findings of accelerated mortality accompanied with increased cytokine production, COX-2 and iNOS expression; as well as impaired bactericidal activities of the innate immune system, and marred metabolic response to stress in Mkp-1-/- mice in various rodent septic shock models (Zhao et al. 2005, 2006, Wang et al. 2007, Frazier et al. 2009). All these data indicate importance of regulating MKP-1 expression and suggest it as a potential therapeutic target in early inflammatory processes.

8. Conclusion

In our experiments we investigated the anti-inflammatory effects of ferulaldehyde, a potent antioxidant, on LPS-induced inflammatory processes in C57BL/6 mice and RAW 264.7 macrophage cells.

1. In our *in vivo* experiments we found that ferulaldehyde, a microbial end-product of several polyphenols has anti-inflammatory effect via decreasing early pro-inflammatory cytokines such as TNF- , IL-1β and increasing the anti-inflammatory IL-10 in the sera of the LPS-treated mice. Additionally, FA inhibited LPS-induced activation of NF- B transcription factor in the liver of mice. According to our data, these effects were probably due to attenuating LPS-induced activation of JNK and Akt. Furthermore, FA decreased ROS and RNS production in LPS plus IFN- -treated primary mouse hepatocytes, whose effects are expected to contribute to its anti-inflammatory property. These data provide the first direct *in vivo* evidence that a water soluble degradation product of polyphenols could be responsible for, or at least could significantly contribute to, the beneficial anti-inflammatory effects of polyphenol containing healthy foods, natural products and traditional medicines.

2. In our macrophage model we found that ferulaldehyde reduced ROS and RNS formations and protected mitochondria against LPS-induced rapid and massive membrane depolarization, which indicate the importance of mitochondrial integrity in early inflammatory response. Additionally, FA suppressed the activation of JNK, ERK and p38 MAPKs, thereby inhibited NF- B activation in LPS-treated RAW 264.7 cells.

3. In our *in vitro* model, in contrast to the previous notion that LPS monotonously induces MKP-1 expression, we found that LPS induced early decrease of MKP-1 that was accompanied by activation of MAPKs. Additionally, we found that FA shifted elevated MKP-1 mRNA expression and protein levels forward in time which in turn attenuated activation of MAPKs in macrophage cells. All these data indicate importance of regulating MKP-1 expression and suggest it as a potential therapeutic target in early inflammatory processes.

4. Signaling mechanisms in the liver of mice and in macrophages showed a modified kinase activation pattern induced by the same type of LPS molecule. We found that FA inhibited LPS-induced JNK activation, but did not affect ERK1/2 and p38 MAPK pathways in liver of mice. However, unlike our *in vivo* model, FA attenuated LPS-induced activation of all three MAPKs except of ERK1/2 at 10 min suggesting a uniform regulation of MAPK activation in LPS-stimulated macrophages. These results may arise from the differences of the inflammatory models used, and cell- and tissue-specificity of the LPS-induced processes. Macrophages represent the first defense line against pathogens and are intensely exposed to the inflammation caused by infectious agents in this way they are involved in the induction of a stronger protective response to eliminate causative agents. However, liver which was the objective of our *in vivo* experiments, is a target organ for bacterial infection and as such it shows another type of protective response for pathogens.

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