

Ferulaldehyde, a Water-Soluble Degradation Product of Polyphenols, Inhibits the Lipopolysaccharide-Induced Inflammatory Response in Mice¹⁻³

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Abstract

Antiinflammatory properties of polyphenols in natural products, traditional medicines, and healthy foods were recently attributed to highly soluble metabolites produced by the microflora of the intestines rather than the polyphenols themselves. To provide experimental basis for this hypothesis, we measured antiinflammatory properties of ferulaldehyde (FA), a natural intermediate of polyphenol metabolism of intestinal microflora, in a murine lipopolysaccharide (LPS)-induced septic shock model. We found that intraperitoneally administered FA (6 mg/kg) prolonged the lifespan of LPS-treated (40 mg/kg) mice, decreased the inflammatory response detected by T₂-weighted in vivo MRI, decreased early proinflammatory cytokines such as tumor necrosis factor- α and interleukin (IL)-1 β , and increased the antiinflammatory IL-10 in the sera of the mice. Additionally, FA inhibited LPS-induced activation of nuclear factor κ B transcription factor in the liver of the mice. According to our data, these effects were probably due to attenuating LPS-induced activation of c-Jun N-terminal kinase and Akt. Furthermore, FA decreased free radical and nitrite production in LPS plus interferon- γ -treated primary mouse hepatocytes, whose effects are expected to contribute to its antiinflammatory property. These data provide 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 antiinflammatory effects of polyphenol-containing healthy foods, natural products, and traditional medicines. *J. Nutr.* 139: 291–297, 2009.

Introduction

Sepsis is the leading cause of death in hospitalized patients and affects over 18 million people worldwide with an expected 1% increase of incidence per year. In Gram-negative bacterial

infection, lipopolysaccharide (LPS)⁶ plays a critical role in inducing sepsis (1). Chronic inflammation and infection leads to the upregulation of a series of enzymes and signaling proteins in affected tissues and cells. LPS induces the production of proinflammatory mediators, including tumor necrosis factor- α (TNF α) and several interleukins (IL) in endothelial and epithelial cells, neutrophils, macrophages, and lymphocytes (1).

Binding of LPS to the CD14 and toll-like receptor 4 (TLR4)/myeloid differentiation 2 (MD2) complexes (2,3) induces the activation of mitogen-activated protein kinase (MAPK) pathways, which includes extracellular signal-regulated kinase (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase (JNK), and p38 MAPK (4,5). Activation of MAPK can lead to the activation of the most important proinflammatory transcription factors: activator protein 1 (AP-1) and nuclear factor κ B (NF- κ B) (6,7). These transcription factors are critical to pathogen-associated molecular pattern-induced cytokine and chemokine gene expression (TNF α , IL-1, IL-8, IL-12, etc.) (7,8) as well as to the expression of cyclooxygenase (COX)-2, inducible nitric oxide (NO)-synthase (iNOS), and to the upregulation of cell adhesion molecules (9,10). Overexpression of COX-2 and iNOS can lead to

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³ Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.

⁶ Abbreviations used: AP-1, activator protein 1; COX, cyclooxygenase; CTRL, vehicle-treated group; ERK, extracellular signal-regulated kinase; FA, ferulaldehyde; IFN γ , interferon- γ ; IL, interleukin; iNOS, inducible nitric oxide-synthase; i.p., intraperitoneally; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LPS + FA, treated with LPS and FA; MAPK, mitogen-activated protein kinase; MD2, myeloid differentiation 2; MKK, mitogen-activated protein kinase kinase; NF- κ B, nuclear factor- κ B; NO, nitric oxide; PI3, phosphatidylinositol 3; PSS, physiological saline solution; ROS, reactive oxygen species; TLR4, toll-like receptor 4; TNF α , tumor necrosis factor α .

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reactive oxygen species (ROS) and NO production, which can contribute to systemic tissue damage. It was demonstrated that MAPK kinase (MKK)-3 and -6, upstream of p38, and MKK-4 and -7, upstream of the JNK, as well as the ERK-specific MKK-1 and -2, are key components of LPS signaling. Most recently it was shown that in the LPS-induced proinflammatory cytokine production, the activation of the MKK-4 and -7 in the JNK1 pathway plays a critical role. However, there are several possible candidates in pathogen-associated molecular pattern-induced MAPK signaling such as TNF receptor-associated factor, germinal center kinase, and mixed-lineage kinase-2 and -3. The exact mechanism of the process has not yet been defined (4).

According to the most recent guidelines, activated protein C and corticosteroids are the only substances with proven efficacy in the therapy of severe sepsis and septic shock. Because these modern treatments have only a modest effect against sepsis (11), new therapeutic approaches are being explored. Recently, a number of natural products or ingredients of traditional medicines and healthy foods such as resveratrol, curcumin, and proanthocyanidins were extensively investigated and subjected to clinical trials as antiinflammatory agents (12,13). Because solubility of these compounds is limited, it is questionable whether their bioavailability could account for their pharmacological effect. Furthermore, recent publications show that polyphenols in healthy foods or drinks such as chocolate, red wine, or beer are readily metabolized to phenolic acids and aldehydes by the microflora of the intestines, raising the possibility that these metabolites, rather than the original natural products or food ingredients, are responsible for their antiinflammatory properties (14,15). Ferulaldehyde (FA) 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 (14,15). It is the reduced precursor of ferulic acid that was reported to stay in the blood longer than other antioxidants such as vitamin C (16) and have higher bioavailability than that of other dietary flavonoids and monophenolics studied so far (17). FA is a component of phenylpropanoid and polyphenol biosynthesis in plants and is a spontaneously forming product in aqueous solutions of curcumin (18). Furthermore, FA was reported to inhibit LPS-induced iNOS expression and NO synthesis in murine macrophage-like RAW 264.7 cells (19). Its oxidized form, ferulic acid, was found to attenuate LPS-induced production of proinflammatory molecules, including NO, macrophage inflammatory protein-2, TNF α , and prostaglandin E2 in murine macrophages (20–22). In this report, we studied in vivo antiinflammatory effects of FA in a murine septic shock model to provide direct evidence for the in vivo antiinflammatory effect of a degradation end-product of dietary polyphenols.

Materials and Methods

Materials. The FA was a generous gift of Prof. Kalman Hideg (Department of Organic and Pharmacological Chemistry, Faculty of Medicine, University of Pecs, Hungary). LPS from *Escherichia coli* 0127:B8 and all materials that are not specified elsewhere were purchased from Sigma-Aldrich. Anti-phospho-p44/42-MAPK^{Thr202/Tyr204}, anti-phospho-Akt^{Ser473}, anti-phospho-p38-MAPK^{Thr180/Tyr182}, and anti-phospho-JNK^{Thr183/Tyr185} primary antibodies were from Cell Signaling Technology.

Mice. C57BL/6 mice were purchased from Charles River Hungary Breeding. The mice were kept under standardized conditions. They consumed tap water and mouse nonpurified diet (maize, barley, wheat, milk powder, baker yeast, lime, soya) (CRLT/N, Szindbad Kft) ad libitum during the entire 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.

Cell culture. Primary hepatocytes of C57BL/6 mice (21–24 g body mass) were isolated according to Le Cam (23) with slight modifications. Briefly, livers were perfused in situ with 50 mL of physiological saline solution (PSS) containing 6 kU/L heparin and 0.66 mmol/L EGTA followed by 50 mL of PSS, then 35 mL of PSS containing 0.7 g/L collagenase H (Roche) and 10 mmol/L CaCl₂ at 37°C. Hepatocytes were seeded to 24- or 96-well plates coated with rat tail collagen type I (Sigma-Aldrich) in DMEM containing 1% minimum essential medium nonessential amino acid solution, 0.05% insulin, 0.1% penicillin-streptomycin, 10% fetal calf serum, and 0.1% dexamethasone.

Sepsis model. To induce murine endotoxic shock, C57BL/6 mice were injected intraperitoneally (i.p.) with a single dose of LPS (low dose, 20 mg/kg or high dose, 40 mg/kg). FA (6 mg/kg) was administered i.p. 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 (our unpublished data). FA only-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 hour for 96 h, after which time they were monitored 3 times/d for 1 wk. No late deaths were observed in any of the experimental groups. Experiments were repeated 3 times with 10 mice in each experimental group. Pooled survival rates of all 3 experiments ($n = 30$) are presented. Treatment groups were LPS, treated with LPS alone; LPS + FA, treated with LPS and FA; FA, treated with FA alone; CTRL, vehicle treated group.

MRI. Mice were treated exactly as for Western blot analysis. Six h after LPS treatment, T₂-weighted magnetic resonance images were taken as described previously (24). The intensities of the images were standardized to the signal of a 1-mm-i.d. tube filled with water:glycerol [9:1 (internal standard)], which was placed near the mice during the measurements. Despite the internal standard, we were not able to quantify accurately the T₂-weighted images due to individual differences among the mice and differences in their positioning inside the probe. Instead, we used qualitative scoring of the standardized T₂-weighted images performed by unbiased experts. Experiments were repeated 3 times.

Western blot analysis. For Western blot analysis, groups of C57BL/6 mice were pretreated with 6 mg/kg FA 1 h before LPS challenge (20 or 40 mg/kg). Livers were removed from the mice 1.5 h after the LPS treatment, frozen in liquid N₂, and processed exactly as described previously (25). We applied the primary antibodies at 4°C overnight at a dilution of 1:1000. The secondary antibody was horseradish peroxidase-conjugated rabbit IgG. Peroxidase labeling was visualized with the ECL Western blotting detection system (Amersham Biosciences). Experiments were repeated 3 times ($n = 9$).

TNF α , IL-1 β , IL-6, and IL-10 assays. Mice were treated exactly as for Western blot analysis. Blood samples were taken at 1.5 and 3 h after LPS administration and were processed as described previously (24). Selection of these time points was based on the published activation kinetics of the given cytokine. We used FA in a 6-mg/kg dose injected 1 h before the LPS, which was applied in a low dose of 20 mg/kg and a high dose of 40 mg/kg. Serum TNF α , IL-1 β , IL-6, and IL-10 concentrations were measured with the Quantikine MTNF α immunoassay kit (R&D Systems) and IL-1 β , IL-6, IL-10 ready-set-go kits (eBioscience). The ELISA kits were used in accordance with the protocol of the manufacturer. Three independent experiments with 3 mice in each experimental group were performed. Data were pooled ($n = 9$).

Determination of NF- κ B. Mice were treated exactly as for Western blot analysis. For nucleus isolation, liver was removed 1.5 h after the LPS treatment and homogenized immediately. Protein concentrations in nuclear extracts were determined using a bicinchoninic acid assay with bovine serum albumin as a standard (Sigma). To monitor NF- κ B activation in the liver, we used Trans-AM Transcription Factor Assay kits (Active Motif).

The ELISA kit was used in accordance with the protocol of the manufacturer. Three independent experiments with 3 mice in each experimental group were performed. Data were pooled ($n = 9$) and values are means \pm SEM.

Determination of ROS production. Culturing medium was replaced with a fresh one without dexamethasone and the primary hepatocytes were incubated in the presence of 5 mg/L LPS + 50 μ g/L interferon- γ (IFN γ) alone or together with FA (1–100 μ mol/L) for 24 h. Then 2,4-dichlorodihydrofluorescein-diacetate (C400, Invitrogen) at a final concentration of 2 mg/L was added to the medium for an additional 2 h. Fluorescence was measured at 485-nm excitation and 555-nm emission wavelengths by using a Fluostar Optima (BMG Labtechnologies) fluorescent microplate reader. All experiments were conducted in 6 parallels and repeated 3 times ($n = 18$).

Measurement of nitrite concentration. Culturing medium was replaced with a fresh one without dexamethasone and the primary hepatocytes were incubated in the presence of 5 mg/L LPS + 50 mg/L IFN γ alone or together with FA (1–100 μ mol/L) for 24 h. Then NO $_2^-$ production was measured by adding to a 50- μ L culture supernatant an equal volume of Griess-reagent (1% sulphanilamide, 0.1% naphthylethylenediamide in 5% phosphoric acid) and measuring light absorption at 550 nm using an Anthos 2010 (Rosys) microplate reader. All experiments were conducted in 6 parallels and repeated 3 times ($n = 18$).

Measurement of free radical scavenging activity. Oxidation of the redox dye dihydrorhodamine123 was induced by 10 μ mol/L H $_2$ O $_2$ and 60 μ mol/L EDTA Fe $^{2+}$ salt in the presence or absence of 0.25–50 μ mol/L of FA or resveratrol. Fluorescent intensity of the oxidized dye was measured at 494-nm excitation and 517-nm emission wavelengths using a LS50B spectrofluorimeter (Perkin-Elmer). Experiments were repeated 3 times ($n = 3$).

Statistical analysis. Values are presented as means \pm SEM. Data were analyzed using 1-way or 2-way ANOVA followed by Bonferroni's test. When an F-test indicated unequal variances, the Kruskal-Wallis test was used. For survival experiments, the Mantel-Cox's logrank test was used. Differences were considered significant at $P < 0.05$.

Results

FA reduced LPS-induced mortality in mice. Eighty percent of mice treated with a single high dose of LPS (40 mg/kg, i.p.) died within 36–48 h. Mice treated with FA 1 h before the LPS challenge (LPS + FA) and repeated every 12 h had a longer survival time compared with those that received PSS (Fig. 1). FA clearly delayed and attenuated the slope of the survival rate

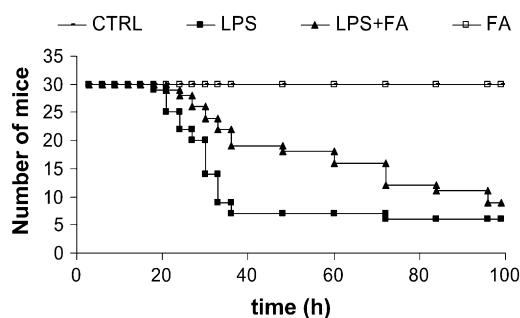


FIGURE 1 FA reduces LPS-induced mortality in mice. Mice were pretreated with FA (6 mg/kg i.p.) 1 h before LPS challenge (40 mg/kg, i.p.) and then FA treatment was repeated every 12 h. The survival rate was monitored for 96 h for CTRL (line), LPS (black square), LPS + FA (black triangle), and FA (white square) groups of 10 mice each. Data are pools of 3 independent experiments, each with 10 mice/group.

curve. At 36–48 h after the LPS challenge 70% of mice in LPS + FA group survived compared with 20% in the LPS-only group ($P < 0.05$). Subsequently, the difference between LPS + FA and LPS groups then decreased, indicating that FA had a protective effect on the early and intermediate phases of LPS-mediated inflammatory processes of septic shock but not on late-stage severe sepsis. FA treatment alone did not induce death or any obvious damage (Fig. 1). A lower dose of LPS (20 mg/kg, i.p.) resulted in an \sim 20% death rate within 72 h and therefore was not suitable for survival studies (data not shown).

FA inhibited LPS-induced inflammatory response in vivo. For demonstrating in vivo inflammatory response, T $_2$ -weighted images of CTRL, LPS, and LPS + FA-treated mice were taken 6 h after the LPS challenge (Fig. 2). The lower abdominal region of LPS-treated mice showed a marked increase of intensity (inflammation), especially in the lateral subcutaneous regions and the interintestinal cavities as well as around the kidneys. In mice treated with LPS + FA, the T $_2$ -weighted intensities were markedly lower, so the inflammatory response was significantly smaller. T $_2$ -weighted images of mice treated with FA alone were basically identical to images of untreated mice (data not shown).

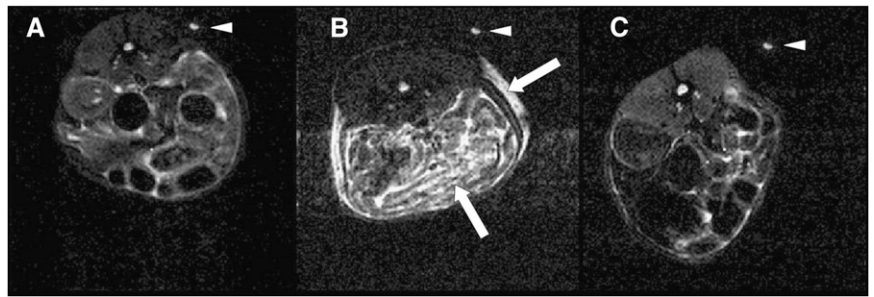
FA attenuated LPS-induced TNF α and IL-1 β but not IL-6 production and enhanced IL-10 generation in the sera. We measured the concentrations of various cytokines from sera of CTRL, LPS, LPS + FA, and FA-treated mice 1.5 or 3 h after the LPS challenge. LPS treatment caused a 16- to 20-fold increase in the serum TNF α concentration 1.5 h after the application of the LPS; this effect was attenuated by FA pretreatment. FA appeared to be more effective at the lower than at the higher dose of LPS in preventing TNF α induction (Fig. 3A). LPS-induced IL-1 β production was assessed 3 h after the LPS treatment. LPS induced a 5- to 9-fold increase in the serum IL-1 β concentration; this was decreased by FA at the lower dose of LPS (Fig. 3B). IL-6 production was measured 3 h after the LPS treatment. LPS, administered at 20 or 40 mg/kg, induced a dramatic increase in the IL-6 concentration. FA did not alter this at either dose (Fig. 3C). LPS caused a strong increase in IL-10 production 1.5 h after the LPS challenge. FA pretreatment increased IL-10 production in mice challenged with both doses of LPS (Fig. 3D), whereas FA alone had no effect (Fig. 3).

FA inhibited LPS+IFN γ -induced NO $_2^-$ and ROS production in primary hepatocytes. Because of the proven role of NO production in inflammation-induced cell damage and death, we studied the effect of FA on LPS + IFN γ -induced NO production in primary hepatocytes by measuring NO $_2^-$ in the culturing medium following a 24-h incubation. Activation of hepatocytes by LPS + IFN γ increased NO $_2^-$ concentration that was reduced by FA in a concentration-dependent manner. FA, at the highest concentration used (100 μ mol/L), reduced NO $_2^-$ to the level of untreated control (Fig. 4A).

During a 24-h incubation period, LPS + IFN γ increased ROS production in primary hepatocytes that was completely abolished by FA at the concentration of 50 μ mol/L. Higher concentrations of FA decreased ROS production below the level of the untreated control (Fig. 4B). FA concentration in the culture medium did not decrease significantly during the incubation period (data not shown).

Direct free radical-scavenging activity of FA. We tested direct free radical-scavenging activity of FA by measuring H $_2$ O $_2$ -induced oxidation of fluorescent redox dye dihydrorhodamine123 in the presence or absence of the substance. FA at the concentration

FIGURE 2 In vivo inflammatory responses in untreated mice (A) and in those treated with LPS (B) or LPS + FA (C). The lower abdominal region of LPS-treated mice had a 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 3 sets of independent experiments were basically identical.



range of 0.25–50 $\mu\text{mol/L}$ attenuated oxidation of the dye in a concentration-dependent manner. The effect of FA and resveratrol did not differ significantly (Fig. 5).

FA inhibited LPS-induced NF- κ B activation in liver. Because the NF- κ B pathway plays an important role in TLR4 signal transduction and in transcription of proinflammatory cytokines,

we investigated the role of FA in NF- κ B activation and nuclear translocation of CTRL, LPS, LPS + FA, and FA-treated mice. We found a nearly 4-fold activation of NF- κ B 1.5 h after LPS challenge compared with the control liver. LPS-induced activation and nuclear translocation of NF- κ B was strongly inhibited in the liver of FA-pretreated mice (Fig. 6). FA alone did not affect the activation of NF- κ B in our experimental model.

FA negatively regulated LPS-induced phosphorylation of JNK and Akt in liver. Previously, we demonstrated that MAPK and Akt play important roles in LPS signaling in our septic shock model (24,25). To study whether these kinases were involved in the protective effect of FA, we measured phosphorylation of JNK, ERK1/2, p38 MAPK, and Akt from the liver of CTRL, LPS, LPS + FA, and FA-treated mice 1.5 h after the LPS challenge. Phosphorylation and thereby activation of all the kinases studied, except p38 MAPK, increased following LPS stimulation. FA significantly prevented this activation in the case

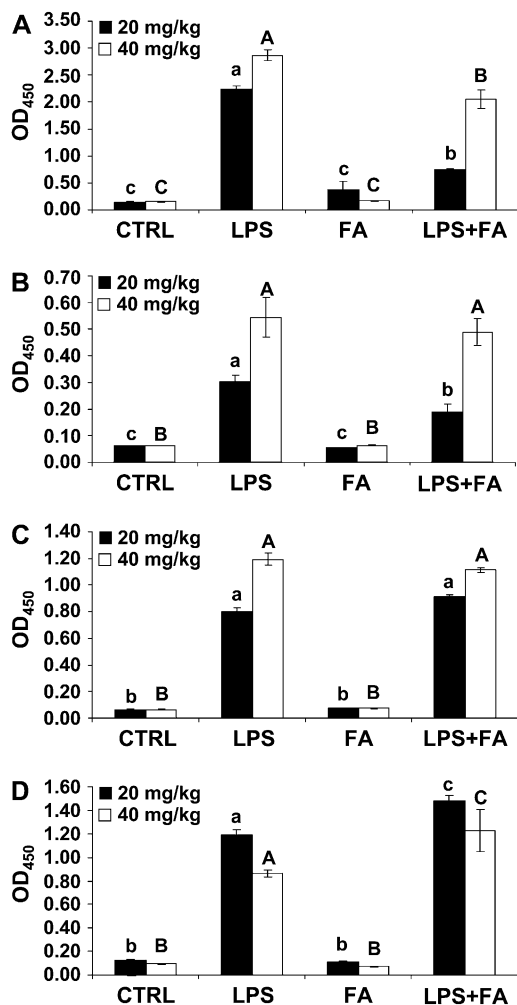


FIGURE 3 Effects of FA (6 mg/kg) and LPS (20 or 40 mg/kg) on serum TNF α (A), IL-1 β (B), IL-6 (C), and IL-10 (D) concentrations in mice. Cytokine concentrations were measured 1.5 (A,D) or 3 (B,C) h after LPS administration. Values are means \pm SEM, $n = 9$ (3 independent experiments, each with 3 mice/group). Within a dose of LPS, means without a common letter differ, $P < 0.05$.

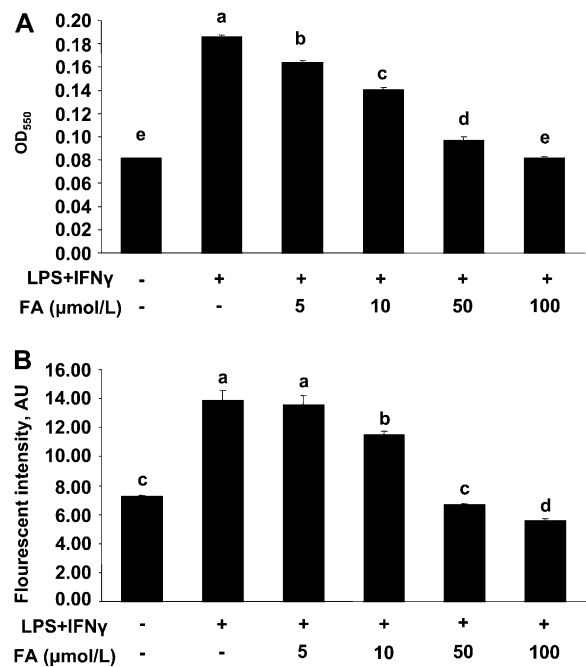


FIGURE 4 FA reduces LPS + IFN γ -induced NO₂⁻ (A) and ROS (B) production in mouse primary hepatocytes. Primary hepatocytes were incubated in the presence (+) or absence (-) of 5 mg/L LPS + 50 $\mu\text{g/L}$ IFN γ and 1–100 $\mu\text{mol/L}$ FA for 24 h. Data are means \pm SEM, $n = 18$ (6 replicates repeated 3 times). Means for a variable without a common letter differ, $P < 0.05$.

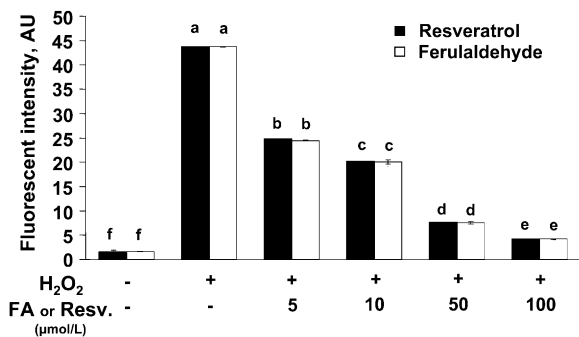


FIGURE 5 Antioxidant effect of FA and resveratrol in a cell-free system. Oxidation of the redox dye dihydrorhodamine123 was induced by 10 mmol/L H₂O₂ in the presence (actual concentrations are indicated) or absence (-) of FA or resveratrol. Data are expressed as means ± SEM. All experiments were conducted in 6 parallels and repeated 3 times (*n* = 18). Means for a variable without a common letter differ, *P* < 0.05.

of JNK and Akt but did not attenuate LPS-induced activation of ERK1/2 (Fig. 7; Supplemental Fig. 1). FA alone did not affect the phosphorylation of the kinases studied in our experimental model.

Discussion

Multiple organ failure leading to death could be the result of either early phase septic shock characterized by rapidly increased expression of proinflammatory cytokines or late-stage severe sepsis associated with elevated high mobility group-1 (1). Under our experimental conditions, FA increased the survival time of mice challenged with a high dose of LPS, showing that FA can positively influence the complex processes leading to LPS-induced oxidative stress, inflammatory response, and death. This protective effect of FA was more pronounced on the early phase of LPS-mediated inflammatory processes of septic shock rather than on late-stage severe sepsis. In accordance with this time profile, we used a noninvasive *in vivo* MRI method to have real-time insight into early phase inflammatory signals in the affected tissues and organs. T₂-weighted MR-images of LPS-challenged mice showed that LPS-induced inflammation was well detected in the abdominal regions of the mice and that FA attenuated the overall abdominal inflammatory response.

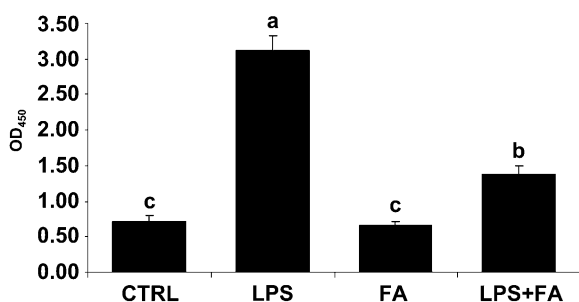


FIGURE 6 FA inhibits NF-κB activation in the liver of LPS-treated mice. Activation of NF-κB was measured 1.5 h after LPS injection from the nuclear fraction of the liver of CTRL, LPS, LPS + FA, and FA mice. Data are expressed as means ± SEM of 3 mice per group. The experiment was repeated 3 times (*n* = 9). Means for a variable without a common letter differ, *P* < 0.05.

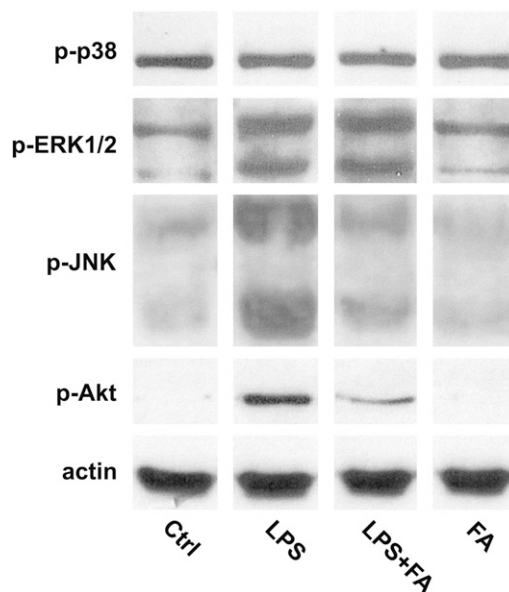


FIGURE 7 Effect of FA on Akt, p38, ERK1/2, and JNK activation in the liver of LPS-treated mice. Activation of the kinases was determined 1.5 h after LPS injection from liver homogenates of untreated (CTRL) mice, or mice treated with 20 mg/kg LPS (LPS), combination of 20 mg/kg LPS and 6 mg/kg FA (LPS + FA) or 6 mg/kg FA alone (FA) by using phosphorylation specific primary antibodies. Representative immunoblots are presented. Experiments with 3 mice per group were repeated 3 times with basically identical results (*n* = 9). Results of a complete representative experiment are presented (Supplemental Fig. 1).

TNFα is a primary mediator of the innate immune system and is crucial for the induction of a local protective immune response against infection, trauma, and ischemia. However, excessive TNFα production can be lethal in itself, because it spreads in the bloodstream and produces cardiovascular collapse. TNFα is a sufficient and necessary mediator of early phase septic shock (1). In our LPS-induced endotoxic shock model, even the lower LPS dose that caused only negligible death rate among the mice induced significant induction of all the proinflammatory cytokines tested. FA attenuated the LPS-induced increase of serum TNFα and IL-1β for both LPS doses; however, it was more effective in reducing proinflammatory cytokine production in the lower LPS dose. In several previous reports, a direct link between TNFα and IL-1β as well as IL-10 was established (24). However, contradictory results appeared about the role of IL-6 in the inflammatory response (26). Our finding that FA could not attenuate the LPS-induced elevation of the serum IL-6 level indicates that IL-6 was not involved among the antiinflammatory mechanisms of FA in our model. The link between the proinflammatory TNFα and antiinflammatory 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. This indicates that FA attenuated the LPS-induced *in vivo* inflammatory response not only by attenuating TNFα-linked proinflammatory cytokine production but also by enhancing antiinflammatory IL-10 production.

Signaling mechanisms leading to systemic tissue damages induced by the binding of LPS to the CD14 and TLR4/MD2 complexes involve activation of ERK1/2, JNK, p38 MAPK, and the proinflammatory transcription factors AP-1 and NF-κB. The

antiinflammatory effects of dietary polyphenols and natural products were attributed to their modulating effects on these signaling mechanisms (27–29). Previously, we found that the most prominent pathological changes were in the liver of LPS-treated mice (24,25). Accordingly, we determined the effect of FA on MAPK signaling and NF- κ B activation from the livers of LPS-treated mice. We found ERK1/2, JNK, and NF- κ B, but not p38, activation in response to LPS. JNK plays a crucial role in LPS-induced NF- κ B activation (30); therefore, activation of this kinase pathway is consistent with both NF- κ B activation and proinflammatory cytokine production in the liver and blood of LPS-treated mice. FA inhibited the LPS-induced NF- κ B and JNK activation but did not affect ERK1/2 and p38 MAPK pathways. LPS-induced Akt activation was also suppressed by FA, which could have contributed to its antiinflammatory effect. It has been suggested that, independently from JNK pathway, phosphatidylinositol 3 (PI3)K-Akt-mammalian target of rapamycin can activate LPS-induced NF- κ B activation and nuclear translocation (31). Suppressing both LPS-induced JNK and Akt activation, FA inhibited the most important pathways leading to NF- κ B activation, namely the LPS-TLR4-JNK and LPS-PI3K-Akt-mammalian target of rapamycin pathways. Similar mechanisms could account for the antiinflammatory properties of one of the most deeply studied polyphenols, trans-resveratrol (32), which was reported to inhibit PI3K (33), the upstream activator of Akt. Our observation that FA did not affect the LPS-induced activation of ERK1/2 and p38 MAPK pathways indicates that FA's inhibitory target(s) is necessarily downstream of the TLR4 receptors.

AP-1 and NF- κ B activation is critical to the expression of COX-2 and iNOS that can lead to ROS and NO production. 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 (34). Eliminating or inhibiting the production of these highly reactive radicals has been shown to attenuate inflammatory damages. Previously, it was found that FA suppressed the LPS + IFN γ -induced iNOS expression in RAW264.7 macrophage cells (19). The pathological changes that we previously observed in the liver of LPS-treated mice could result from ROS and NO produced by the hepatocytes themselves. Therefore, we determined FA's effect on LPS + IFN γ -induced NO $_2^-$ and ROS production in primary hepatocytes. Typical LPS concentrations used for the activation of macrophages are in the 100 μ g/L to 1 mg/L range; however, primary hepatocytes proved to be less sensitive. Therefore, we used 5 mg/L LPS combined with 50 μ g/L IFN γ to induce full activation of the hepatocytes. We found that FA inhibited the NO production induced by LPS + IFN γ in a concentration-dependent manner in the hepatocytes similarly to RAW264.7 macrophages. Furthermore, FA attenuated LPS + IFN γ -induced ROS production in the hepatocytes. To determine whether this antioxidant property of FA was due to its free radical-scavenging activity, we used a cell-free in vitro system. As we found, FA demonstrated a free radical-scavenging activity that was about the same as that of resveratrol.

Published effects of resveratrol on cytokine profile, NF- κ B translocation, and kinase signaling (28,29) 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 urine after consumption of red wine (15); furthermore, FA is present in red wine as a natural component or degradation product at a concentration comparable to that of resveratrol (our unpublished result). Bioavailability of ferulic acid was reported to be higher

than that of other dietary flavonoids and monophenolics (17) and we found the plasma concentration of FA to be 22 times higher than that of resveratrol 1.5 h after equimolar i.p. administration of the 2 drugs to the mice (unpublished result). Curcumin, another widely studied antioxidant and antiinflammatory natural product, rapidly and spontaneously degrades to FA among other substances in solution (18). Again, there was a high similarity between published effects of curcumin on cytokine profile, NF- κ B activation, and kinase signaling (29,35) 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 antiinflammatory effects of resveratrol and curcumin and in a broader sense to that of dietary polyphenols and natural compounds.

In conclusion, we provided direct in vivo experimental evidence, supporting the assumption that a highly soluble degradation product, rather than its poorly soluble precursor, is responsible for the beneficial effects of natural products and healthy food ingredients. Pursuing this assumption may provide the basis for developing new antiinflammatory agents for the therapy of severe sepsis and septic shock.

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