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Flavocoxid, a dual inhibitor of COX-2 and 5-LOX of natural origin, attenuates the inflammatory response and protects mice from sepsis

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Abstract

Introduction: Cecal ligation and puncture (CLP) is an inflammatory condition that leads to multisystemic organ failure. Flavocoxid, a dual inhibitor of cyclooxygenase (COX-2) and 5-lipoxygenase (5-LOX), has been shown *in vitro* to possess antiinflammatory activity in lipopolysaccharide (LPS)-stimulated rat macrophages by reducing nuclear factor (NF)- κ B activity and COX-2, 5-LOX and inducible nitric oxide synthase (iNOS) expression. The aim of this study was to evaluate the effects of flavocoxid in a murine model of CLP-induced polymicrobial sepsis.

Methods: C57BL/6J mice were subjected to CLP or sham operation. In a first set of experiments, an intraperitoneal injection of flavocoxid (20 mg/kg) or vehicle was administered 1 hour after surgery and repeated every 12 hours. Survival rate was monitored every 24 hours throughout 120 hours. Furthermore, additional groups of sham and CLP mice were killed 18 hours after surgical procedures for blood-sample collection and the lung and liver were collected for biomolecular, biochemical and histopathologic studies.

Results: COX-2, 5-LOX, tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-10, extracellular-regulated-kinase 1/2 (ERK), JunN-terminal kinase (JNK), NF- κ B, and β -arrestin 2 protein expression were evaluated in lung and liver with Western blot analysis. In addition, leukotriene B₄ (LTB₄), prostaglandin E₂ (PGE₂), cytokines, and lipoxin A₄ serum content were measured with an enzyme-linked immunosorbent assay (ELISA). Flavocoxid administration improved survival, reduced the expression of NF- κ B, COX-2, 5-LOX, TNF- α and IL-6 and increased IL-10 production. Moreover, flavocoxid inhibited the mitogen-activated protein kinases (MAPKs) pathway, preserved β -arrestin 2 expression, reduced blood LTB₄, PGE₂, TNF- α and IL-6, and increased IL-10 and lipoxin A₄ serum levels. The treatment with flavocoxid also protected against the histologic damage induced by CLP and reduced the myeloperoxidase (MPO) activity in the lung and liver.

Conclusions: Flavocoxid protects mice from sepsis, suggesting that this dual inhibitor may represent a promising approach in such a life-threatening condition.

Introduction

Cecal ligation and puncture (CLP) is an experimental model of shock that reproduces all the pathologic sequelae of sepsis that occur in intensive care patients [1]. Despite a massive effort invested in developing potential therapies, to date, severe sepsis is still a common,

frequently fatal, and expensive pathologic condition [2]. Sepsis is a systemic inflammatory response to infection triggered by Gram-positive and/or -negative organisms, which can proliferate and/or release endotoxin and exotoxins that stimulate monocytes, macrophages, endothelial cells, and neutrophils to an overproduction and release of major inflammatory mediators, followed by tissue injury which in turn, culminates in multiple-system organ failure (MOF). The first step of this signaling pathway involves Toll-like receptors (TLRs) binding to the bacterial cell-wall components, which induces nuclear

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factor (NF)- κ B/I κ B system activation that exerts transcriptional regulation on proinflammatory genes and encodes various adhesion molecules, cytokines, and other proinflammatory mediators. In addition, TLRs activate MAP kinases, including extracellular-regulated-kinase 1/2 (ERK), JunN-terminal kinase (JNK), and p38 [3,4].

Recently, it was demonstrated that β -arrestins, adaptor proteins involved in G protein-coupled receptor (GPCR) desensitization, are also implicated in regulation of TLR signaling and proinflammatory gene expression. Furthermore, it has been suggested that β -arrestin 1 and 2 differentially regulate TLR4 signaling pathways and, in particular, β -arrestin 2 negatively regulates the inflammatory response in CLP-induced mortality [5,6].

During septic shock, eicosanoids, proinflammatory cytokines, such as TNF- α , IL-1 β , IL-3, IL-6, IL-8, and antiinflammatory mediators, such as IL-2, IL-4, and IL-10, are dramatically increased to block the infection and tissue damage [7-10]. Severe sepsis associated with hypotension, acute respiratory distress syndrome (ARDS), hepatic failure, disseminated intravascular coagulation, and organ dysfunction, is characterized by a poor prognosis; these changes are well documented first in the lung and then in the liver [11].

In addition, COX-2 and 5-LOX products derived from arachidonic acid, such as prostaglandins and leukotrienes, are responsible for the microvasculature failure, and are implicated as pathogenic mediators in endotoxemia [12]. Pharmacologic agents that modulate eicosanoid metabolism have been used to block the inflammatory response of various diseases, including septic shock [9,13-15]. Ito and co-workers [14] have shown that mice pretreated with a 5-LOX inhibitor have a reduced TNF- α production and attenuated liver injury during endotoxemia. Moreover, early survival improvement was found in endotoxin-challenged mice, but not in CLP mice, treated with a selective COX-2 inhibitor [15]. Conversely, it has been demonstrated that anti-TNF antibody and IL-1 receptor antagonist, as well as COX or LOX pathways single inhibitors, failed to protect patients with septicemia and septic shock. Altogether, these results support the effort to find a novel strategy; the development of dual inhibitors of COX-2 and 5-LOX pathways may represent new insights into the treatment of sepsis, thanks to the anti-inflammatory efficacy and the lower incidence of gastric toxicity [16].

Recently, we demonstrated that flavocoxid, which contains both the naturally occurring flavonoids baicalin and catechin isolated from *Scutellaria baicalensis* (*S. baicalensis*) and *Acacia catechu* (*A. catechu*), respectively, acts as a dual inhibitor of COX-2 and 5-LOX and blunts the proinflammatory phenotype in LPS-stimulated macrophages [17]; in addition, it holds immunomodulatory and antiinflammatory activities in experimental models

in vivo, as shown in a murine model of Duchenne muscular dystrophy [18] and in rats subjected to acute caerulein-induced pancreatitis [19].

To date, a dual inhibitor of both COX-2 and 5-LOX pathways has not been investigated in CLP-induced lethal sepsis in mice. Therefore, the aim of our study was to evaluate the potential therapeutic effect of flavocoxid, in a murine cecal-ligation puncture model of polymicrobial sepsis-induced mortality.

Materials and methods

Animals, experimental procedure, and treatments

All procedures complied with the standards for the care and use of animal subjects, as stated in the *Guide for the Care and Use of Laboratory Animals*, and were approved by the Committee on Animal Health and Care of Messina University. The 5-week-old male C57BL/6J mice (Charles River, Calco, LC, Italy), used for this study, had free access to a standard diet and tap water. They were maintained on a 12-hour light/dark cycle at 21°C.

Cecal ligation and puncture (CLP) was performed in C57BL/6J mice as previously described [6]. Specifically, mice were anesthetized with ether, and a midline incision was made below the diaphragm to expose the cecum. The cecum was ligated at the colon juncture with a 4-0 silk ligature suture without interrupting intestinal continuity. The cecum was punctured once with a 22-gauge needle. The cecum was returned to the abdomen, and the incision was closed in layers with a 4-0 silk ligature suture. After the procedure, the animals were fluid-resuscitated with sterile saline (1 ml) injected subcutaneously (sc). Sham controls were subjected to the same procedures as were those with CLP without ligation and puncture of the cecum. Shams were treated with flavocoxid or vehicle. CLP animals were randomized to receive flavocoxid with and without gentamicin (3.2 mg/kg, sc), or baicalin (16 mg/kg, ip), or catechin (4 mg/kg, ip), or its vehicle (0.9% NaCl solution). On the basis of previous data, 20 mg/kg was chosen as the optimal dose of flavocoxid for these experiments [19]. The doses of baicalin and catechin reflected the ratio (4:1) of these two molecules in the formulation [17-19]. Flavocoxid intraperitoneal (ip) or oral treatment was started 1 hour after the CLP procedure and repeated every 12 hours. The same protocol was used for baicalin and catechin treatment. In a first experiment, mice were subjected to CLP ($n = 15$ per group) or sham operation ($n = 10$ per group), and survival was monitored every 24 hours for a total of 120 hours. In a second experiment, six mice per group were subjected to sham operation or CLP, and NF- κ B, MAP kinases, TNF- α , IL-6, IL-10, and β -arrestin 2 expression was determined in the lung and liver after 18 hours. In the same animals, a specimen

from lung and liver was also collected for MPO activity and histologic analysis, and blood was drawn for measuring serum cytokines, eicosanoids, and lipoxin A₄ levels.

Isolation of cytoplasmic and nuclear proteins

In brief, lung and liver samples were homogenized in 1 ml lysis buffer (25 mM Tris/HCl, pH 7.4, 1.0 mM ethylene glycol tetraacetic acid, 1.0 mM ethylenediamine tetraacetic acid, 0.5 mM phenylmethyl sulfonylfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 100 mM Na₃VO₄). The homogenate was subjected to centrifugation at 15,000 *g* for 15 minutes. The supernatant was collected and stored at -80°C. The concentration of total proteins was determined by using a Bio-Rad protein-assay kit (Milan, Italy).

Determination of β-arrestin 2, NF-κB, TNF-α, p-ERK 1/2, p-JNK, COX-2, 5-LOX, IL-6, and IL-10 with Western blot analysis

β-Arrestin 2, NF-κB, TNF-α, p-ERK 1/2, p-JNK, COX-2, 5-LOX, IL-6, and IL-10 expression was evaluated with Western blot, according to the method described previously [17]. Primary antibodies for NF-κB, TNF-α, IL-6, IL-10, COX-2, 5-LOX, and β-arrestin 2 were purchased from Abcam (Cambridge, UK), whereas primary antibodies for p-ERK 1/2 and p-JNK were purchased from Biovision (Mountain View, CA, USA). Secondary antibodies, peroxidase conjugated, were obtained from Pierce (Rockford, IL, USA). The protein signals were quantified by scanning densitometry by using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy) and were expressed as integrated intensity in comparison with those of control normal animals measured with the same batch [17].

TNF-α, IL-6, IL-10, and lipoxin A₄ production

TNF-α, IL-6, and IL-10 were measured by using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). Lipoxin A₄ production was analyzed with the ELISA kit (Oxford Biomedical Research, Oxford, MS, USA) and by following the manufacturer's instructions.

LTB₄ and PGE₂ production

Blood was collected, stored at -80°C, and analyzed for LTB₄ by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). Samples were run in duplicate, and the absorbance was read at 450 nm. The intensity of the color was proportional to the concentration of LTB₄ in the sample. PGE₂ production was assayed without purification by using the Cayman EIA kit (Cayman, Ann Arbor, MI, USA). Samples were run in duplicate, and the absorbance was read at 412 nm

and was directly proportional to the content of PGE₂ in samples.

Myeloperoxidase activity

MPO activity was determined in the lung and liver as an index of neutrophil accumulation, as previously described [6]. MPO activity of tissue was expressed in units per 100 mg of tissue.

Histologic evaluation

For light microscopy, lung and liver tissues were rapidly removed and fixed in 10% buffered formalin. Subsequently, they were embedded in paraffin, cut, and stained with hematoxylin and eosin (H&E). Assessment of tissue changes was carried out by an experienced pathologist who was blinded to the treatments. The histologic study of liver sections was based on the following parameters: infiltration of inflammatory cells, steatosis, necrosis, and ballooning degeneration. The parameters considered for scoring lung damage were infiltration of inflammatory cells, vascular congestion, and interstitial edema. All parameters were evaluated by the following score scale of values: 0, absent; 1, mild; 2, moderate; and 3, severe.

Drugs

Flavocoxid (Limbrel), baicalin, and catechin were a kind gift of Primus Pharmaceuticals, Inc. (Scottsdale, AZ, USA). Gentamicin sulfate was purchased from Sigma Aldrich (St. Louis, MO, USA). All the compounds were dissolved in 0.9% NaCl solution.

Statistical analysis

Data are expressed as the mean ± SD. All other data were analyzed with ANOVA followed by *post hoc* evaluation (Bonferroni test) by using GraphPad Prism Software, (San Diego, CA, USA). For the histologic results, statistical analysis was performed by using the Kruskal-Wallis test followed by the Mann-Whitney *U* test. Survival rates were analyzed with the Kaplan-Meier method, followed by the Log Rank test. A value of *P* < 0.05 was selected as the criterion for statistical significance.

Results

Effects of flavocoxid on CLP-induced mortality

For mortality studies, C57BL/6 mice subjected to CLP or sham operation were treated with flavocoxid (20 mg/kg/ip) or vehicle, 1 hour after CLP. All the CLP animals were fluid resuscitated with sterile 0.9% NaCl saline solution (1 ml/mouse). Flavocoxid treatment was repeated every 12 hours. Mouse survival was monitored for up to 120 hours. CLP-induced sepsis in mice produced severe mortality (*P* < 0.01) with respect to sham animals. Flavocoxid treatment did not affect survival in

sham animals, but significantly reduced mortality ($P < 0.01$) in CLP mice (Figure 1). Flavocoxid, administered orally at the same dosage, produced an overlapping effect on the survival rate (Figure 1). Furthermore, the combination of flavocoxid with gentamicin (administered subcutaneously at the dose of 3.2 mg/day throughout the experiment) caused a greater improvement in the survival rate (Figure 1). Neither baicalin (16 mg/kg/ip) nor catechin (4 mg/kg/ip), administered in the same percentage as in the 20 mg/kg/ip of flavocoxid, significantly modified survival (Figure 1).

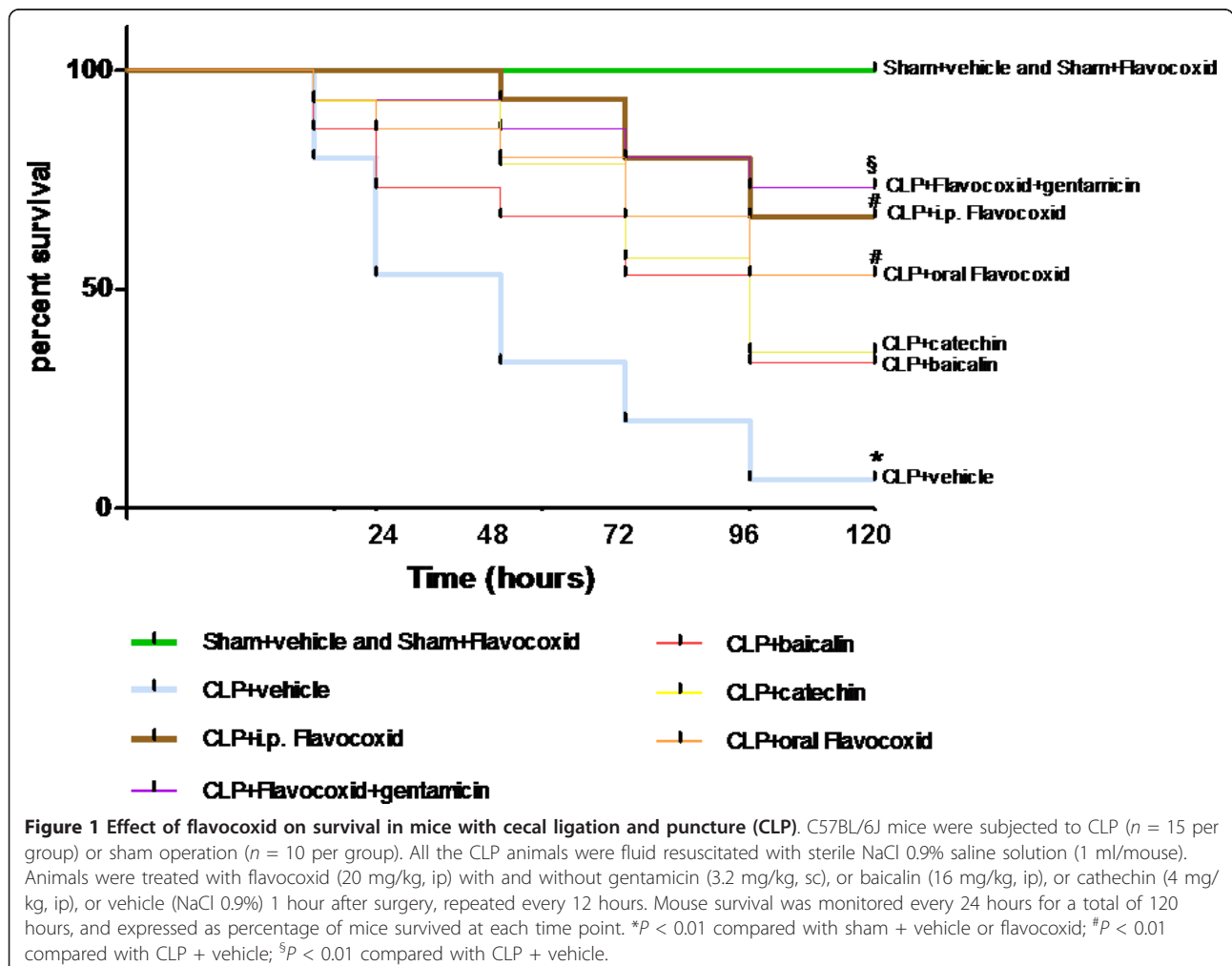
Effects of flavocoxid on TNF- α , IL-6, and IL-10 expression

Inflammatory cytokines have a crucial role in CLP-induced sepsis and mortality. Therefore, we studied the effects of flavocoxid on CLP-induced TNF- α , IL-6, and IL-10 expression in lung and liver of mice. Basal protein levels of TNF- α , IL-6, and IL-10 were measured in lung (Figure 2a-c) and liver (Figure 2d-f) of sham mice. CLP mice showed a significant increase in these cytokines (p

< 0.005) compared with sham (Figure 2a-f). Flavocoxid did not change protein expression in sham animals, but markedly inhibited the expression of TNF- α and IL-6 in CLP animals ($P < 0.005$); moreover, IL-10 protein expression was further enhanced after treatment in both lung (Figure 2a-c) and liver (Figure 2d-f) tissues of septic animals.

Flavocoxid reduces COX-2 and 5-LOX CLP-induced expression, serum cytokines, LTB₄ and PGE₂, and increases lipoxin A₄

To verify *in vivo* the protective effect of flavocoxid, we first evaluated COX-2 and 5-LOX expression in lung and liver of both CLP and sham mice. Sham mice showed a low expression of COX-2 and 5-LOX in both lung and liver (Figure 3a-d), and no differences were found between sham animals given either flavocoxid or vehicle. In contrast, the expression of both enzymes was increased in lung and liver of CLP mice, with respect to sham mice. Flavocoxid treatment significantly reduced



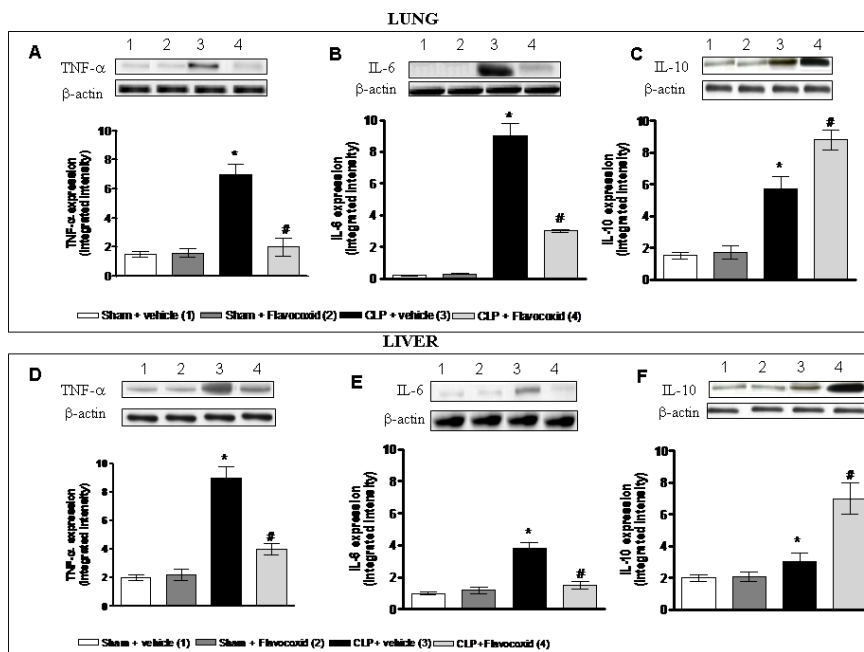


Figure 2 Western blot analysis of TNF- α , IL-6, and IL-10 in the lung (a through c) and liver (d through f) of CLP and sham mice. Animals were treated with flavocoid (20 mg/kg, ip) or vehicle (NaCl 0.9%) 1 hour after surgery, repeated every 12 hours. Bars represent the mean \pm SD of six animals. * $P < 0.005$ compared with sham + vehicle or flavocoid; # $P < 0.005$ compared with CLP + vehicle.

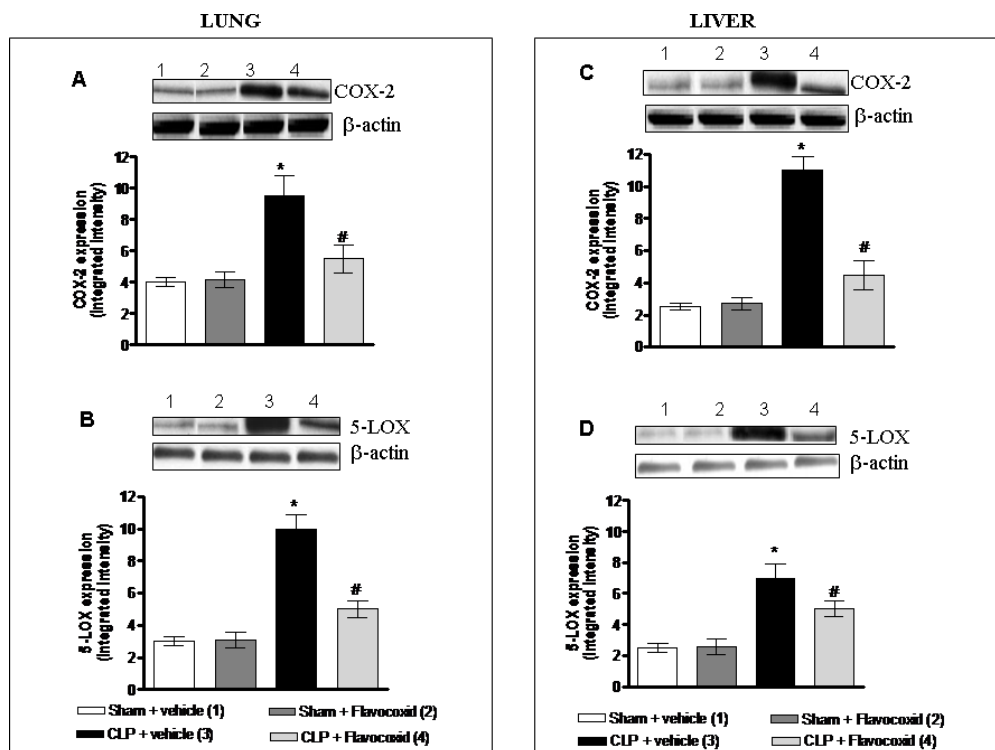


Figure 3

Figure 3 Western blot analysis of COX-2 and 5-LOX in the lung (a and b) and liver (c and d) of CLP and sham mice. Animals were treated with flavocoid (20 mg/kg, ip) or vehicle (NaCl 0.9%) 1 hour after surgery, repeated every 12 hours. Bars represent the mean \pm SD of six animals. * $P < 0.005$ compared with sham + vehicle or flavocoid; # $P < 0.005$ compared with CLP + vehicle.

COX-2 and 5-LOX expression either in lung and in liver of CLP mice (Figure 3a-d).

Circulating levels of TNF- α , IL-6, and IL-10 were also measured 18 hours after surgery. Serum cytokines were significantly augmented in the bloodstream of CLP mice (Figure 4a-c). Flavocoxid treatment markedly blunted the serum concentration of TNF- α (Figure 4a) and IL-6 (Figure 4b), but increased serum IL-10 (Figure 4c).

Serum PGE₂ and LTB₄ measurements were also determined in CLP and sham mice, 18 hours after surgery. Figure 5a and 5b shows a significant increase of both PGE₂ and LTB₄ in serum of CLP mice. As a consequence of COX-2 and 5-LOX inhibition by flavocoxid treatment, PGE₂ and LTB₄ serum levels were significantly reduced in CLP mice ($P < 0.005$) (Figure 5a and 5b). Lipoxin A₄ was also enhanced in the serum of CLP mice 18 hours after surgery (Figure 5c). Flavocoxid administration caused a greater increase in the circulating antiinflammatory lipid mediator, thus suggesting

that this antiinflammatory mechanism may concur with the protective effect of flavocoxid (Figure 5c).

Flavocoxid regulates CLP-induced expression of NF- κ B, p-ERK, p-JNK, and β -arrestin 2

NF- κ B is a transcription factor that is triggered by TLR activation and causes the nuclear activation of inflammatory genes involved in sepsis. Nuclear NF- κ B was markedly expressed in the lung (Figure 6a) and in the liver (Figure 6c) of CLP mice, 18 hours after surgery. Flavocoxid treatment blunted the increased nuclear expression of NF- κ B in both the lung (Figure 6a) and the liver (Figure 6c). To determine whether β -arrestin 2 plays a key role in modulating the TLR signaling pathway in CLP-induced inflammation, β -arrestin 2 protein expression in lung and liver of sham and CLP mice was studied. Basal levels of β -arrestin 2 were measured in lung and liver of sham animals (Figure 6b and 6d). As shown in Figure 6b and 6d, β -arrestin 2 expression was significantly reduced in lung and liver of mice subjected

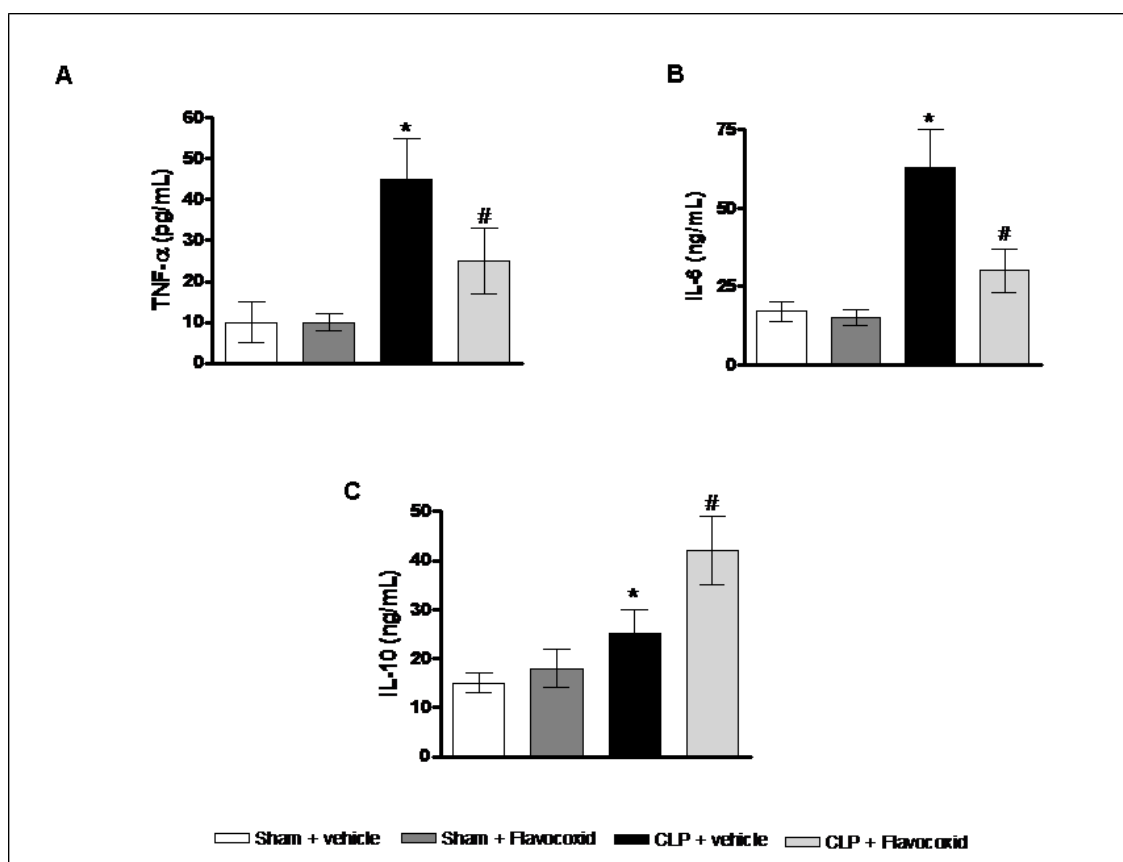


Figure 4 Serum TNF- α (a), IL-6 (b), and IL-10 (c) levels obtained from CLP and sham mice. Animals were treated with flavocoxid (20 mg/kg, ip or vehicle (NaCl 0.9%) 1 hour after surgery, repeated every 12 hours. Bars represent the mean \pm SD of six animals. * $P < 0.005$ compared with sham + vehicle or flavocoxid; # $P < 0.005$ compared with CLP + vehicle.

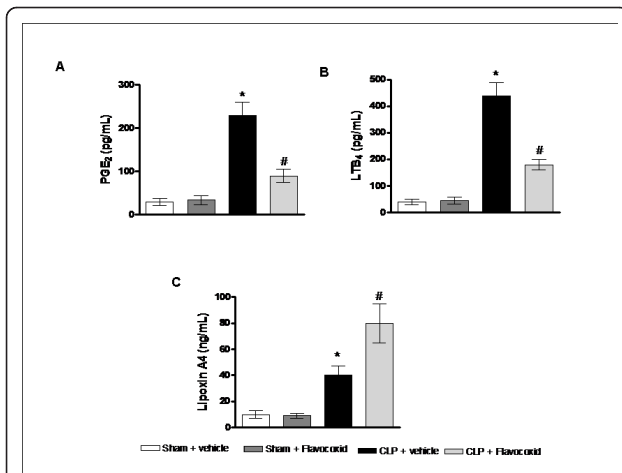


Figure 5 Serum PGE₂ (a), LTB₄ (b), and lipoxin A₄ (c) levels obtained from CLP and sham mice. Animals were treated with flavocoxid (20 mg/kg, ip) or vehicle (NaCl 0.9%) 1 hour after surgery, repeated every 12 hours. Bars represent the mean ± SD of six animals. **P* < 0.005 compared with sham + vehicle or flavocoxid; #*P* < 0.005 compared with CLP + vehicle.

to CLP (*P* < 0.005) compared with sham-operated animals. Treatment with flavocoxid preserved β-arrestin 2 expression in both lung and liver of CLP mice (Figure 6b and 6d), but it did not affect basal values of β-arrestin 2 in sham animals.

Furthermore, to better characterize the mechanism of action of flavocoxid, p-ERK 1/2 and p-JNK were evaluated. A low expression of either p-ERK (Figure 7a and 7c) or p-JNK (Figure 7b and 7d) was determined in both lung and liver of sham mice. No significant differences were found in MAPKs expression in sham animals administered flavocoxid. CLP mice showed a significant increase in the expression of MAPKs with respect to sham animals (Figure 7a to 7d). In contrast, flavocoxid treatment significantly reduced (*P* < 0.005) p-ERK and p-JNK expression in lung and liver of CLP animals (Figure 7a to 7d), suggesting that β-arrestin 2 regulates sepsis-induced MAPK activity *in vivo*.

Effects of flavocoxid on histologic changes and myeloperoxidase activity

Sham animals treated with either vehicle or flavocoxid showed a normal architecture of lung and liver (Figure 8a and 8d; Tables 1 and 2). CLP induced damage in lung and liver, characterized by microscopic alterations, hemorrhagic areas, and marked leukocyte infiltration (Figure 8b and 8e; Tables 1 and 2). In CLP mice, lung histologic examination showed ample inflammatory infiltrate, interstitial edema, and marked vascular congestion (Figure 8b and Table 1). The histologic picture showed that liver damage was characterized by a high degree of inflammatory infiltrate, steatosis, necrosis, ballooning degeneration, as well

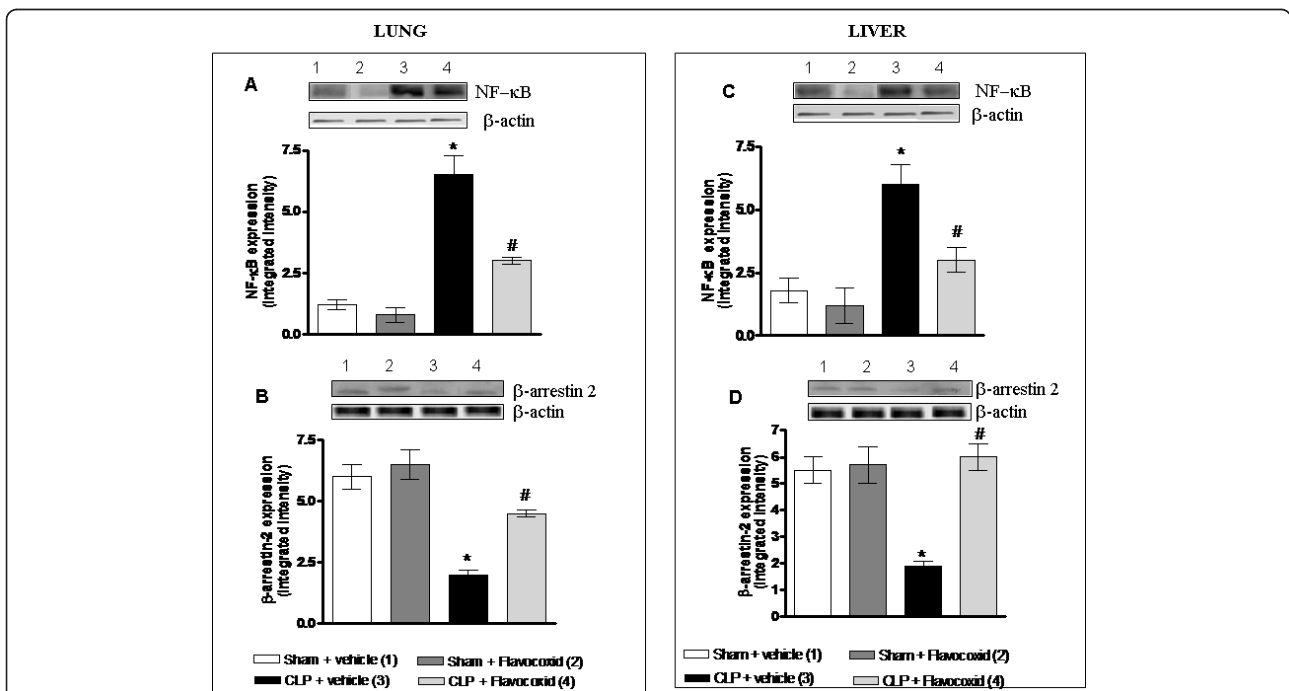


Figure 6 Western blot analysis of NF-κB and β-arrestin 2 in lung (a, b) and liver (c, d) of CLP and sham mice. Animals were treated with flavocoxid (20 mg/kg, ip) or vehicle (NaCl 0.9%) 1 hour after surgery, and repeated every 12 hours. Bars represent the mean ± SD of six animals. **P* < 0.005 compared with sham + vehicle or flavocoxid; #*P* < 0.005 compared with CLP + vehicle.

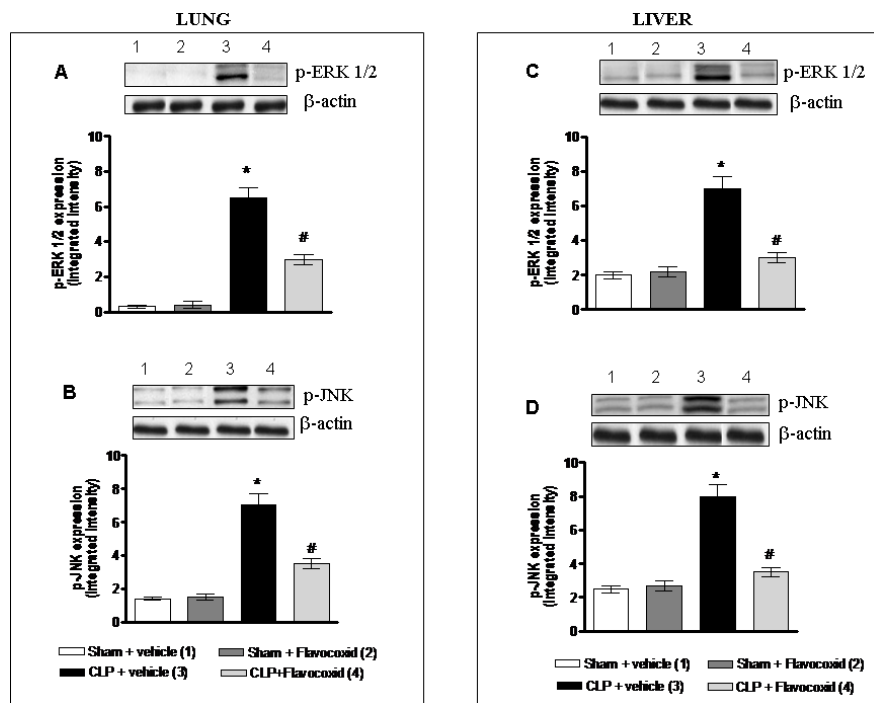


Figure 7 Western blot analysis of p-ERK 1/2 and p-JNK in lung (a, b) and liver (c, d) of CLP and sham mice. Animals were treated with flavocoxid (20 mg/kg, ip) or vehicle (NaCl 0.9%) 1 hour after surgery, repeated every 12 hours. Bars represent the mean ± SD of six animals. * $P < 0.005$ compared with sham + vehicle or flavocoxid; # $P < 0.005$ compared with CLP + vehicle.

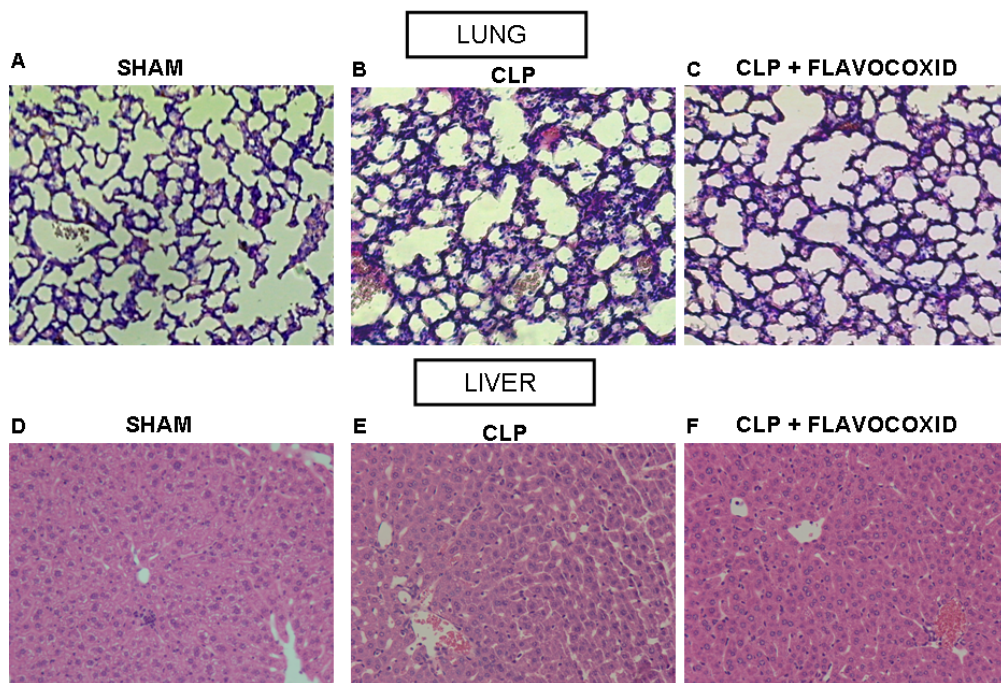


Figure 8 Histology of treated and untreated mice. (a) Normal histology of lung obtained from sham mice. (b) Representative cecal ligation and puncture (CLP)-induced lung damage. (c) Representative lung of CLP mice treated with flavocoxid (20 mg/kg, ip, administered 1 hour after surgery, repeated every 12 hours). (d) Normal histology of liver obtained from sham mice. (e) Representative CLP-induced liver damage. (f) Representative liver of CLP mice treated with flavocoxid (20 mg/kg, ip, administered 1 hour after surgery, repeated every 12 hours). Sham and CLP mice were killed 18 hours after surgery. Histopathology was performed on lung and liver. Original magnification, $\times 20$.

Table 1 Flavocoxid effects on lung histologic damage in CLP mice

Parameter	Sham	Sham + flavocoxid	CLP	CLP + flavocoxid
Inflammatory infiltrate	0	0	2.27 ± 0.59	1.15 ± 0.57 ^a
Vascular congestion	0	0	2.12 ± 0.20	1.25 ± 0.27 ^a
Interstitial edema	0	0	2.33 ± 0.41	1.25 ± 0.52 ^a

Histologic grading as reported in Methods; six mice per group treated with flavocoxid (20 mg/kg, ip). ^aP < 0.05 versus CLP.

as morphologic alterations of the nuclei (Figure 8e and Table 2). In contrast, treatment with flavocoxid significantly reduced the histopathologic alterations in the lung (Figure 8c) and liver (Figure 8f) of CLP mice. Lung histology revealed a reduced inflammatory infiltrate, vascular congestion, and interstitial edema (Figure 8c and Table 1). After flavocoxid treatment, liver inflammatory infiltrate and steatosis were less evident, necrosis was absent, and normal cellular architecture was restored (Figure 8f and Table 2).

To confirm the antiinflammatory effects of flavocoxid, we evaluated myeloperoxidase (MPO) activity, a marker of neutrophil infiltration in both the lung (Figure 9a) and the liver (Figure 9b) of CLP mice 18 hours after surgery. MPO content was markedly increased in the lung (Figure 9a) and in the liver (Figure 9b) of septic mice. The dual inhibitor of COX-2 and 5-LOX markedly reduced leukocyte infiltration in the lung (Figure 9a) and in the liver (Figure 9b) of CLP mice.

Discussion

Flavonoids have been used in traditional remedies for the treatment of a variety of diseases, including inflammatory conditions [20-22]. Flavocoxid, marketed as Limbrel, is an FDA-regulated prescription, medical food, for the clinical dietary management of OA in United States, which contains the flavonoids, baicalin and catechin [23].

We previously showed that flavocoxid reduced the expression of both COX-2 and 5-LOX enzymes and inhibited the activation of the transcription factor NF- κ B in LPS-stimulated macrophages *in vitro* [17]. Therefore, we evaluated the effects of flavocoxid in an *in vivo*

murine model of polymicrobial sepsis, which better reproduces human sepsis.

Li *et al.* [9] demonstrated that NS-398, a selective inhibitor of COX-2, ameliorates cytokine imbalance and decreases liver injury in septic rats. Additionally, Reddy *et al.* [15] showed that treatment with a COX-2 inhibitor improves early survival in murine endotoxemia but not in CLP mice. Our study demonstrates for the first time that a dual COX-2 and 5-LOX inhibitor exerts beneficial effects in a murine CLP model of abdominal sepsis. In the present study, flavocoxid significantly improved survival in CLP mice; this protective effect was strongly associated with reduced expression and production of proinflammatory cytokines TNF- α and IL-6, as well as with increased antiinflammatory IL-10 protein level.

Recently, different studies have shown that β -arrestin 2, a member of the arrestin family originally known for its prominent role in desensitization process of G protein-coupled receptors, plays an important function in the signal-transduction pathway of TLR4 [5,24,25]. We found that β -arrestin 2 expression was significantly reduced in lung and liver of CLP mice compared with sham mice, and, in parallel experiments, CLP-induced TNF- α , IL-6, and IL-10 expression was significantly higher in control mice. Our findings, therefore, are in agreement with those of other studies, which indicate β -arrestin 2 as a negative regulator of the CLP-induced inflammatory response [5,6,26,27]. It was demonstrated that β -arrestin 2 downregulates inflammatory responses in polymorphonuclear leukocytes [26] as well as in splenocytes after CLP induction [6]. An altered cytokine production was also found by Wang *et al.* [27] in bone marrow-derived macrophages from β -arrestin 2-deficient mice, in response to LPS. All these data suggest that β -arrestin 2 is an essential negative regulator of sepsis-induced inflammation via TLR signaling and may represent an innovative target for antisepsis drugs.

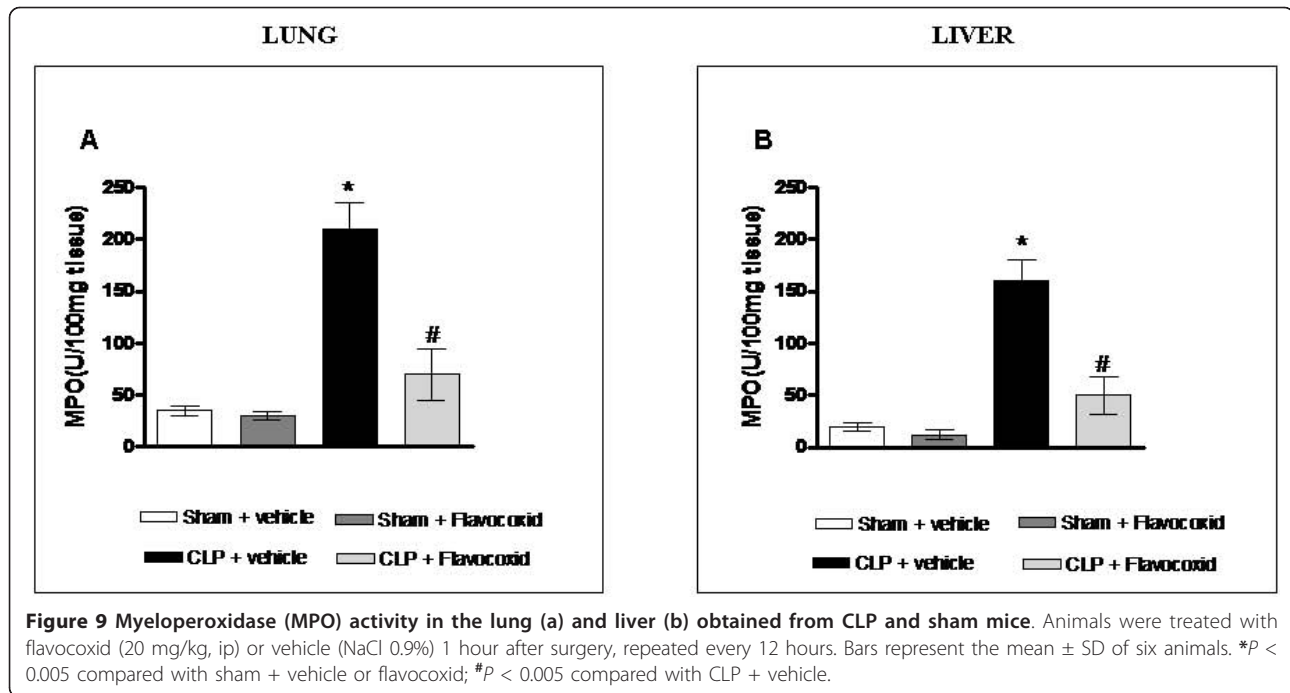
However, conflicting results were obtained by Porter *et al.* [28] in regulating inflammatory cytokines production in beta arrestins knockout (KO) mice during endotoxic shock; the observed differences might be explained by the use of a different shock model.

Flavocoxid, given curatively, restored β -arrestin 2 to basal levels and blunted CLP-induced increase in TNF-

Table 2 Flavocoxid effects on liver histologic damage in CLP mice

Parameter	Sham	Sham + flavocoxid	CLP	CLP + flavocoxid
Inflammatory infiltrate	0	0	2.52 ± 0.52	1.55 ± 0.46 ^a
Steatosis	0	0	2.33 ± 0.41	1.47 ± 0.26 ^a
Necrosis	0	0	2.77 ± 0.23	0 ^b
Ballooning degeneration	0	0	2.17 ± 0.26	0.21 ± 0.12 ^b

Histologic grading as reported in Methods; six mice per group treated with flavocoxid (20 mg/kg, ip). ^aP < 0.05 versus CLP; ^bP < 0.001 versus CLP.



α and IL-6, but not IL-10 production in both lung and liver tissues. In addition, flavocoxid reduced serum levels of PGE₂ and LTB₄ as a consequence of a downregulation of COX-2 and 5-LOX expression in lung and liver of mice subjected to CLP, implicating the same mechanism of action of flavocoxid as reported in our previous studies [17-19,23]. These findings are further supported by histologic examinations: flavocoxid treatment restored organ damage specifically in lung and liver that are profoundly involved in the pathophysiologic changes that occur during sepsis. Interestingly, besides acting as a dual inhibitor of COX-2 and 5-LOX, flavocoxid might stimulate β -arrestin through some yet-unrecognized mechanism.

It has been suggested that β -arrestin 2 acts as scaffold for ERK and JNK cascades, thus leading to modulation of transcriptional events [24,25]. In our experimental model, CLP-induced MAPKs expression was significantly increased in both lung and liver tissues compared with that in sham mice. Reduced p-ERK1/2 and p-JNK expression was found after flavocoxid treatment of CLP mice, thus suggesting that the compound may upregulate β -arrestin 2 through a MAPKs-dependent mechanism.

All these data, taken together, confirm that β -arrestin 2 plays a critical role in a clinically relevant model of sepsis, as already shown by Fan *et al.* [6], and support the hypothesis that β -arrestin 2 improved expression is protective in a model of abdominal sepsis.

However, additional mechanism(s) underlying flavocoxid beneficial effects in experimental sepsis might be

taken into account. NF- κ B is an early signal that activates an inflammatory cascade leading to the expression of cell-adhesion molecules and cytokines and finally culminating in the deleterious accumulation of leukocytes in peripheral organs such as lung and liver [29]. Because of this, CLP animals showed an increased nuclear NF- κ B expression and an enhanced content of MPO, a marker of neutrophil accumulation, in both lung and liver. Flavocoxid administration succeeded in reducing these steps of the inflammatory cascade, thus suggesting that the dual inhibitor of COX-2 and 5-LOX may work as an NF- κ B inhibitor. Furthermore, lipoxin A₄, another lipid mediator that is endogenously derived from arachidonic acid metabolism, has been shown to possess marked anti-inflammatory and pro-resolving effects [30]. Our results suggest that flavocoxid treatment augmented the levels of lipoxin A₄: therefore, this effect may concur in the overall protective activity of this natural anti-inflammatory drug.

Finally, we established for the first time that a natural derived dual inhibitor of COX-2 and 5-LOX activity is able to improve survival and restore the histologic architecture of lung and liver of mice subjected to polymicrobial sepsis. However, further studies will be necessary to characterize better whether other mediators contribute in the mechanism of action of flavocoxid in this model of sepsis.

Conclusions

This is the first report demonstrating that flavocoxid, a dual COX-2 and 5-LOX inhibitor of natural origin,

exerts an antiinflammatory response and protects mice from sepsis in a murine model of CLP. Therefore, on the basis of efficacy and safety profile [17-19,23,31], flavocoxid represents a novel antiinflammatory therapy and a potential approach for future clinical trials in patients with sepsis.

Key messages

- Despite a massive effort invested in developing potential therapies, to date, severe sepsis is still a common, frequently fatal, and expensive pathologic condition.
- Because of the enhanced antiinflammatory efficacy and the lower incidence of gastric toxicity, the development of dual inhibitors of COX-2 and 5-LOX pathways may represent a new strategy to fight severe sepsis.
- Flavocoxid, which contains both the naturally occurring flavonoids baicalin and catechin isolated from *S. baicalensis* and *A. catechu*, respectively, acts as a dual inhibitor of COX-2 and 5-LOX and blunts the proinflammatory phenotype in LPS-stimulated macrophages.
- Cecal ligation and puncture (CLP) is an experimental model of shock that reproduces all the pathologic sequelae of sepsis that occur in intensive care patients. β -Arrestin 2 is an essential negative regulator of sepsis-induced inflammation via TLR signaling and may represent an innovative target for antisepsis drugs. Flavocoxid reduced the inflammatory cascade associated with sepsis, enhanced β -arrestin 2, and improved the survival rate.
- By inhibiting both COX-2 and 5-LOX expression and stimulating the negative regulator β -arrestin 2 through an MAPKs-dependent mechanism, flavocoxid may represent a potential new approach to the treatment of sepsis.

Abbreviations

5-LOX: 5-lipoxygenase; ARDS: acute respiratory distress syndrome; CLP: cecal ligation and puncture; COX-2: cyclooxygenase 2; ELISA: enzyme-linked immunosorbent assay; FDA: Food and Drug Administration; GPCR: G-protein-coupled receptor; H&E: hematoxylin and eosin; IL: interleukin; iNOS: inducible nitric oxide synthase; ip: intraperitoneal; KO: knockout; LPS: lipopolysaccharide; LT_{B4}: leukotriene B₄; MAPK: mitogen-activated protein kinase; MOF: multiple-system organ failure; MPO: myeloperoxidase; NF- κ B: nuclear factor κ B; OA: osteoarthritis; p-ERK: phospho-extracellular-regulated kinase; p-JNK: phospho-Jun N-terminal kinase; PGE₂: prostaglandin E₂; TLRs: Toll-like receptors; TNF- α : tumor necrosis factor α ; sc: subcutaneous.

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Authors' contributions

AB carried out the *in vivo* studies and drafted the manuscript, LM carried out the *in vitro* studies and drafted the manuscript, AD participated in the design of the study, NI and MR carried out ELISA and biomolecular measurements, FSV drafted the manuscript, FS participated in the design of the study, and DA designed studies, analyzed data, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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