Inhibition of nuclear factor-κB activation by IRFI 042, protects against endotoxin-induced shock

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Abstract

Background: The aim of our study was to investigate the effect of IRFI 042, a novel dual vitamin E-like antioxidant, on nuclear factor-κB (NF-κB) activation, TNF-α gene priming and on the release of the mature protein during endotoxin shock. Methods: Endotoxin shock was produced in male rats by a single intravenous (i.v.) injection of 20 mg kg−1 of Salmonella enteritidis lipopolysaccharide (LPS). Survival rate, mean arterial blood pressure, serum TNF-α, and plasma malondialdehyde (MAL) levels were investigated. We then evaluated in the liver TNF-α mRNA levels, NF-κB binding activity and the inhibitory protein IκBα. Moreover we studied in LPS stimulated (50 μg ml−1) peritoneal macrophages (Mφ), NF-κB activation, cytoplasmic IκB-α degradation, the message for TNF-α, and TNF-α and MAL levels. Results: LPS administration reduced survival rate (0%, 72 h after LPS administration); decreased mean arterial blood pressure, augmented serum TNF-α (60±11 ng ml−1) and enhanced plasma malondialdehyde (MAL) levels (55±7.1 nmol l−1). LPS shocked rats also had increased TNF-α mRNA levels, augmented liver NF-κB binding activity in the nucleus and decreased levels of the inhibitory protein IκBα. In addition, in vitro LPS stimulation (50 μg ml−1) significantly induced NF-κB activation and cytoplasmic IκBα degradation in Mφ, enhanced TNF-α mRNA levels and increased Mφ TNF-α and MAL. Treatment with IRFI 042 (20 mg kg−1, i.v., 5 min after endotoxine challenge) protected against LPS-induced lethality (90% survival rate 24 h and 80% survival rate 72 h after LPS injection, respectively), reduced hypotension, blunted plasma MAL (9.0±0.9 nmol l−1) and decreased serum TNF-α (15±3 ng ml−1). The antioxidant also inhibited the loss of IκBα protein from the hepatic cytoplasm, blunted the increased NF-κB binding activity in the liver and decreased hepatic liver mRNA for TNF-α. Furthermore ‘in vitro’ IRFI 042 (50 μM) significantly inhibited activation of NF-κB through inhibition of IκBα degradation, reduced the amount of TNF-α mRNA, decreased LPS-induced TNF-α release and blunted lipid peroxidation (MAL) in LPS stimulated Mφ. Conclusions: These data suggest that IRFI 042 blocks the activation of NF-κB, reduces TNF-α mRNA levels, and finally reverses endotoxic shock. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cytokines; Endotoxins; Free radicals; Gene expression; Macrophages; Septic shock; Signal transduction

1. Introduction

Endotoxin shock is a leading cause of morbidity and mortality among hospitalized patients. The underlying pathobiocchemical alterations consist of a dramatic increase in eicosanoid and platelet activation factor production; a release of cytokines, in particular interleukin (IL)-1, IL-3, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF-α); an activation of the l-arginine–nitric oxide (NO) pathway; the formation of oxygen-centered free radicals; activation of
the plasmatic coagulation cascade, fibrinolysis and complement pathway [1]. All these substances represent key mediators of the multiple organ injury that occurs during endotoxin shock. They interact each other and mutually modulate their production and release. In particular endotoxin shock is characterized by a marked oxidant stress [2] and by a rapid production of different cytokines [3–6]: the production of these latter may be blunted by quenching oxidative stress. Nuclear factor-κB (NF-κB) is an intracellular messenger that could represent a good candidate to explain such an interaction.

NF-κB is a transcription factor which plays a central role in the modulation of the inflammatory and immune response and induces the expression of many genes codifying for cytokines involved in the pathogenesis of septic shock. In fact, it has been shown that bacterial endotoxin can cause cells to activate NF-κB thereby increasing the transcription, production and release of TNF-α which in turn stimulates the production of other mediators [7]. Alternatively the transcription factor may also be turned on by oxidative stress. NF-κB therefore represents an important target to develop new strategies to halt the inflammatory response.

Vitamin E has been suggested to act as potential inhibitor of NF-κB activation [8]. Nevertheless the marked lipophilicity of this vitamin limits its therapeutic potential: acute administration results in fact in a very low circulating levels and poor distribution. A number of less lipophilic α-tocopherol analogues endowed with radical scavenging activity have been described in the literature. One of the vitamin E analogue IRFI-042 (±)-5-emisuccinoyl-2-[2-(acetylthio)ethyl]-2,3-dihydro-4,6,7-trimethylbenzofuran is more active than other previously investigated compounds [8]. The combination in the same molecule of a chain-breaking moiety (characteristic of phenols related to α-tocopherol) with the reducing ability of thiol groups (dual antioxidant) may result in powerful and peculiar biological actions, especially in those oxidative stress-mediated situations in which a significant depletion of endogenous thiols is observed. This compound shows no systemic toxicity even following high dosage (up to 1 g/kg). Recently it has been shown that IRFI-042 inhibits NF-κB activation and protects against myocardial ischaemia–reperfusion injury [9].

In light of these findings we investigated the effect of IRFI-042 on NF-κB activity and on the pathological sequelae associated with endotoxic shock.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health (NIH Publication No. 85-23, revised 1996).

2.1. Endotoxin shock procedure and survival evaluation

Male Sprague–Dawley rats (200–220 g), fed on a standard diet and with tap water ad libitum, were used. Environmental conditions were standardized, including a room temperature of 22±2°C and 12 h artificial lighting. Endotoxin shock was induced by administering a single intravenous (i.v.) dose of 20 mg kg−1 of Salmonella enteritidis lipopolysaccharide. Control rats received an equal volume of vehicle (0.9% NaCl). Five minutes after endotoxin injection, control rats received an i.v. bolus of vehicle (dimethylsulfoxide:NaCl 0.9%; 1 ml kg−1), and treated rats were injected with IRFI-042 (20 mg kg−1). Survival rate was evaluated in a first group of rats (n = 40) for 72 h after endotoxin administration.

2.2. Arterial blood pressure

A second group of rats (n = 12) was used to monitor blood pressure. Briefly, the animals were anesthetized with urethane (1.3 g kg−1) and a cannula (PE 50) was inserted into the left common carotid artery and connected to a pressure transducer. The pressure pulse triggered a carotidachometer, and arterial blood pressure, was monitored for 6 h and displayed on channels of a polygraph (Ugo Basile, Varese, Italy). Arterial blood pressure is reported as mean arterial pressure (MAP) in mmHg. These rats were treated as described above.

2.3. Isolated aortic rings

A third group of rats (n = 24) was used to evaluate vascular reactivity, and the circulating levels of TNF-α and malondialdehyde (MAL). These animals were treated as described above and they were sacrificed 3 h after endotoxin challenge. Thoracic aortae from control and IRFI-042-treated rats were removed 3 h after LPS injection and placed in cold Kreb’s solution of the following composition (mM): NaCl 118.4, KCl 4.7, MgSO4 1.2, CaCl2 2.5, KH2PO4 1.2, NaHCO3 25.0 and glucose 11.7. Then aortas were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. In some rings the vascular endothelium was removed mechanically by gently rubbing the luminal surface with a thin wooden stick. The rings were then placed under 1 g of tension in an organ bath containing 10 ml of Krebs’ solution at 37 °C and bubbled with 95% O2 and 5% CO2 (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μM) in order to exclude the involvement of prostaglandins and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Varese, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs’ solution at 15–20-min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine...
(PE, 100 nM). When the contraction was stable, the presence or absence of endothelium was assessed by administering acetylcholine (ACh, 100 nM). Concentration–response curves were obtained by cumulative concentrations of PE (1 nM–10 μM).

2.4. Malondialdehyde measurement

Determination of the MAL levels was carried out in plasma samples. Samples (0.2 ml) of arterial blood were drawn from the carotid catheter at 3 h following LPS injection. The blood was collected in polyethylene tubes to which had been added 10 μl of heparin solution (1000 i.u.). The plasma samples, obtained after centrifugation at 3000×g for 10 min at 4°C, were frozen at −70°C until the analysis. The assay was carried out by using a colorimetric commercial kit (Lipid peroxidation assay kit, cat. No. 437634, Calbiochem–Novabiochem, USA).

Briefly, 0.65 ml of 10.3 mM N-methyl-2-phenyl-indole in acetonitrile were added to 0.2 ml of samples. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well and closed with a tight stopper NF-vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well and closed with a tight stopper for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total proteins in the samples was determined by a commercially available protein assay reagent. To estimate possible contamination of the nuclear extracts with the cytoplasmatic extracts, when preparing the nuclear and cytoplasmatic proteins, lactate dehydrogenase (LDH) activity was determined by a commercially available kit for the quantitative kinetic determination of LDH activity (Sigma, St. Louis, MO). Values were expressed as LDH activity units per milligram of protein. To establish that the nuclear extracts contained mainly nuclear proteins, 40 μg of nuclear protein preparations were subjected to Western blot analysis for histone H3, a nuclear protein, with anti-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).

2.5. Plasma TNF-α levels

Blood (750 μl) was drawn 3 h following endotoxin challenge. Plasma TNF-α concentrations were determined by an ELISA kit (Genzyme).

2.6. Isolation of nuclear and cytoplasmatic proteins

A fourth group of rats (n=18) was used to study NF-κB activity. Animals were treated as described above. Liver sections were obtained 1 h after endotoxin challenge. Briefly 70 mg of pulverized liver samples were homogenized in 0.8 ml ice-cold hypotonic buffer (10 mM Hepes, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT); protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, aprotinin, pepstatin, leupeptin (10 μg/ml each); and phosphatase inhibitors: 50 mM NAF, 30 mM β-glycerophosphate, 1 mM Na3VO4, and 20 mM p-nitrophenyl phosphate). The homogenates were centrifuged for 30 s at 2000 rpm at 4°C to eliminate any unbroken tissues. The supernatants were incubated on ice for 20 min, vortexed for 30 s after addition of 50 μl of 10% Nonidet P-40 and then centrifuged for 1 min at 4°C in an Eppendorf centrifuge. Supernatants containing cytoplasmatic protein were collected and stored at −80°C. The pellets after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors, and phosphatase inhibitors), incubated on ice for 30 min, mixed frequently, and centrifuged for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C.

The concentration of total proteins in the samples was determined by a commercially available protein assay reagent. To estimate possible contamination of the nuclear extracts with the cytoplasmatic extracts, when preparing the nuclear and cytoplasmatic proteins, lactate dehydrogenase (LDH) activity was determined by a commercially available kit for the quantitative kinetic determination of LDH activity (Sigma, St. Louis, MO). Values were expressed as LDH activity units per milligram of protein. To establish that the nuclear extracts contained mainly nuclear proteins, 40 μg of nuclear protein preparations were subjected to Western blot analysis for histone H3, a nuclear protein, with anti-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).

2.7. Electrophoretic mobility shift assay

NF-κB binding activity was performed in a 15-μl binding reaction mixture containing 1% binding buffer (50 μg/ml of double-stranded poly(dI–dC), 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl2, and 10% glycerol), 15 μg of nuclear proteins, and 35 fmol (50,000 cpm, Cherenkov counting) of double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) to eliminate any unbroken tissues. The binding reaction mixture was incubated at room temperature for 30 min and analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. After electrophoresis, the gels were dried using a gel-drier and exposed to Kodak X-ray films at −70°C. The binding bands were quantified by scanning densitometry of a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). The results of each group were expressed as relative integrated intensity compared with the sham operated group liver measured in the same batch because the integrated intensity of group samples from different electrophoretic mobility shift assay (EMSA) batches would be affected by the half-life of the isotope, exposure time, and background levels.

2.8. Western blot analysis of IκBα in cytoplasm

Cytoplasmatic proteins (40 μg) from each sample were mixed with 2X SDS sample buffer (62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003%...
bromophenol blue), heated at 95 °C for 5 min, and separated by SDS–polyacrylamide gel electrophoresis. After electrophoresis on 12.5% polyacrylamide gels, the separated proteins were transferred from the gels into Hybond electrochemiluminiscence membranes (Amersham) using a Bio-Rad semidy transfer system (Bio-Rad) for 2 h. The membranes were blocked with 5% not-fat dry milk in TBS–0.05% Tween for 1 h at room temperature, washed three times for 10 min each in TBS–0.05% Tween 20, and incubated with a primary IκBα antibody (Santa Cruz Biotechnology) in TBS–0.05% Tween 20 containing 5% not-fat dry milk for 1–2 h at room temperature. After being washed three times for 10 min each in TBS–0.05% Tween 20, the membranes were incubated with a second antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) for 1 h at room temperature. After washing, the membranes were analyzed by the enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham). The IκBα protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil, Celpio, Milan, Italy). The results from each experimental group were expressed as relative integrated intensity compared with normal liver measured in the same batch.

2.9. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

A fifth group of rats (n=18) was used to study hepatic mRNA for TNF-α. These animals were treated as described before. Total cellular RNA was extracted from liver sections 2 h after endotoxin challenge. The methods used in the current study have been described elsewhere [10]. In brief, approximately 100 mg of liver was homogenized with 800 μl RNAZOL STAT (Teltest, Firenswood, TX) in a microfuge tube, after which 80 μl chloroform was added. After vortexing and centrifugation, the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA recovered by precipitation by chilling at −80 °C for 15 min. The pellet was washed with cold ethanol 70%, centrifuged, dried in speed vacuum, centrifuged a second time and then dissolved in 20 μl of buffer. A 2-μg portion of total RNA was subjected to first strand cDNA synthesis in a 20 μl reaction mixture containing the AMV reverse transcriptase (Superscript II; BRL, USA), each dNTP, the specific primers, Tris–HCl and MgCl₂.

After dilution of the product with distilled water, 5 μl were used for each polymerase chain reaction (PCR) which contained the Taq polymerase (Perkin-Elmer), the buffer as supplied with the enzyme, each dNTP and the specific primers, designed to cross introns and to avoid confusion between mRNA expression and genomic contamination.

The following oligonucleotide pairs were used (5’ oligo/ 3’ oligo), each sequence as 5’ to 3’:

- TNF-α: CACGCTTCTGTTTACTGG/GGACTCTGTGAGTCTAAGT
- GAPDH: ACCACATGGAGAAGTTCGG/CTCAGTGATCCCAGGATGCC

The optimal cycle number for TNF-α was 25 and we used a PCR-negative and a PCR-positive control without cDNA or with a known cDNA, respectively. A portion of the PCR product was electrophoresed and transferred to a nylon membrane which was prehybridized with oligonucleotide probes, radiolabeled with [32P]ATP by a T4 oligonucleotide kinase. After an overnight hybridization at 55 °C, filters underwent the autoradiography in a darkroom with a fixed camera. The captured image, sent to an image analysis software (Bio-Profil, Celpio, Milan, Italy) was subjected to densitometric analysis.

2.10. Macrophage culture

Peritoneal macrophages were harvested from control normal rats by washing the abdominal cavity with RPMI 1640. The cells were centrifuged twice and suspended again in the same medium at a concentration of 1×10⁶ cells ml⁻¹. Peritoneal macrophages were obtained after 2 h adhesion to plastic Petri dishes (Nunc, Denmark) at 37 °C in an atmosphere of 5% CO₂ in air. The homogeneity and the viability of macrophages were greater than 98% as determined by differential staining and trypan blue exclusion. In order to study the effects of IRFI 042 on TNF-α production and on macrophage MAL, peritoneal macrophages were incubated for 4 h with S. enteritidis LPS (50 μg ml⁻¹) alone or together with IRFI 042 (12, 25 and 50 μM). TNF-α production and MAL content were evaluated as reported above.

The activation of NF-κB and the degradation of cytoplasmic IκB-α were evaluated in macrophages following 1 h of LPS stimulation in the presence or absence of IRFI 042 (50 μM). NF-κB activity was investigated as reported above.

2.11. Drug

IRFI 042 was supplied by Biomedica Foscama Research Centre, Ferentino (FR), Italy. The compound was dissolved in dimethylsulphoxide:NaCl 0.9% (1:1, v/v) and prepared fresh daily.

2.12. Statistical analysis

Data are expressed as means±S.E. mean and were analyzed by analysis of variance for multiple comparison of results. Duncan’s multiple range test was used to compare group means. In all cases, a probability error of less than 0.05 was selected as criterion for statistical significance.
Table 1
Effects of IRFI 042 on survival rate in rats subjected to endotoxin shock (LPS)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hours after endotoxin challenge (surviving animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + vehicle (1 ml kg⁻¹)</td>
<td>10/10 10/10 10/10 10/10 10/10</td>
</tr>
<tr>
<td>Control + IRFI 042 (20 mg kg⁻¹)</td>
<td>10/10 10/10 10/10 10/10 10/10</td>
</tr>
<tr>
<td>LPS + vehicle (1 ml kg⁻¹)</td>
<td>10/10 10/10 10/10 10/10 10/10</td>
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<tr>
<td>LPS + IRFI 042 (20 mg kg⁻¹)</td>
<td>10/10 10/10 10/10 10/10 10/10</td>
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Animals received IRFI 042 or vehicle, 5 min after LPS injection. *P<0.01 versus control + vehicle, **P<0.001 versus LPS + vehicle. Each point represents the mean±S.E. mean of six experiments.

3. Results

3.1. Survival rate

Table 1 shows the ratio of animals surviving in each group to the total number of animals throughout the experimental period. The endotoxin shocked rats had 1 and 0 survivors out of 10, 48 and 72 h after LPS challenge, respectively. IRFI 042 (20 mg kg⁻¹) administered 5 min after LPS injection significantly protected against endotoxin induced lethality.

3.2. Arterial blood pressure

Rats injected with endotoxin showed a sharp and long-lasting decrease in mean arterial blood pressure (Fig. 1). IRFI 042 (20 mg kg⁻¹, 5 min after LPS injection) significantly blunted the sustained decrease in MAP.

3.3. Contractile response to phenylephrine

Fig. 2 represents the contractile response to phenylephrine (PE; 1 nM–10 μM) of intact and endothelium denuded aortic rings obtained from control or endotoxin shocked rats. Aortic rings obtained from endotoxin shocked rats showed a decreased responsiveness to phenylephrine (Fig. 2) with respect to control rats. Administration of IRFI 042 (20 mg kg⁻¹, 5 min after LPS) significantly improved the constrictor response to PE in aortic rings obtained from endotoxin shocked rats.

3.4. Plasma MAL analysis and serum TNF-α

Determination of plasma malonylaldheyde (MAL) was performed to evaluate free radical damage on biological membranes after LPS injection. Table 2 shows a significant increase of MAL concentration in plasma obtained 3 h following endotoxin challenge. The administration of IRFI 042 (20 mg kg⁻¹, 5 min after LPS) significantly decreased plasma MAL levels (Table 2).

3.5. Activation of NF-κB in the liver

NF-κB activation in the nuclear extracts of liver was determined by EMSA 1 h after endotoxin challenge. The top of Fig. 3a shows representative EMSA picture indicating activation of NF-κB. The bottom of the figure shows quantitative data. NF-κB binding activity was present at very low levels in sham shocked animals. NF-κB was, in contrast, markedly increased in the liver of LPS treated animals (Fig. 3a). The administration of IRFI-042 markedly reduced NF-κB binding activity (Fig. 3a).

3.6. Loss of IκBα protein in the liver cytoplasm

NF-κB activation was also indirectly investigated by studying its inhibitory protein IκBα in the liver cytoplasm. The top of Fig. 3b shows representative Western blot analysis indicating reduction of IκBα protein in the liver cytoplasm.
cytoplasm of liver obtained from sham shocked animals and rats subjected to endotoxin shock and treated with vehicle or IRFI 042. The bottom of Fig. 3b represents quantitative data. IκBα levels showed a significant reduction in the liver cytoplasm of septic rats treated with vehicle. The administration of IRFI 042 blunted the consistent loss of IκBα protein from the cytoplasm (Fig. 3b).

3.7. Liver TNF-α mRNA expression

Liver TNF-α mRNA was evaluated 2 h after LPS administration. The top of Fig. 4 shows representative autoradiograms highlighting mRNA expression for liver TNF-α in rats subjected to endotoxin shock and treated with vehicle or IRFI 042. The bottom of Fig. 4 depicts quantitative data and indicates relative amount of liver TNF-α mRNA in septic rats treated with vehicle or the antioxidant.

Liver mRNA levels for TNF-α were significantly elevated in endotoxin rats treated with vehicle.

Administration of IRFI-042 (Fig. 4) blunted hepatic TNF-α mRNA expression in LPS shocked rats.

3.8. Activation of NF-κB in peritoneal macrophages

NF-κB binding activity in nuclear extracts of peritoneal macrophages was determined by EMSA. Peritoneal macrophages were stimulated with LPS (50 μg ml⁻¹) alone or together with IRFI 042 (50 μM) for 1 h. As shown in Fig. 5, un-stimulated macrophages express low basal levels of...
NF-κB activity. NF-κB binding activity was significantly increased in peritoneal macrophages challenged with LPS. IRFI 042 significantly reduced NF-κB activity (Fig. 5).

3.9. Effects of IRFI 042 on IκBα degradation in peritoneal macrophages

NF-κB activation was also indirectly studied by the reduction of its inhibitory protein IκBα. Cytoplasmic proteins were extracted and analyzed by Western blot analysis using an anti-IκBα antibody. As shown in Fig. 6, LPS induced a significant degradation of the protein but, in contrast, loss of cytoplasmic IκBα was blocked by IRFI 042 (Fig. 6).

3.10. Effects of IRFI 042 on macrophage TNF-α and MAL

Fig. 7 shows representative autoradiograms of mRNA expression for TNF-α in peritoneal macrophages stimulated with LPS or H₂O₂ in the presence or absence of IRFI 042 for 4 h. Increased mRNA levels of TNF-α were found in macrophages incubated with LPS (50 μg ml⁻¹) or H₂O₂ (250 μM). Treatment with IRFI 042 (50 μM) markedly suppressed macrophage TNF-α expression.

As shown in Table 3, in vitro LPS induced a significant release of TNF-α by macrophages. IRFI 042 added in vitro (12, 25 and 50 μM) reduced the cytokine levels in macrophage supernatants. In vitro LPS also caused a marked production of MAL that was suppressed in a dose-dependent manner by IRFI 042 (Table 3).

4. Discussion

In the last few years, several studies have been carried out in order to find a new therapeutic target for the treatment of endotoxic shock. It has been demonstrated that treatment with antioxidants, corticosteroids, proteasome inhibitors, and the induction of endotoxin tolerance can suppress TNF-α gene up-regulation and the production of other pro-inflammatory mediators through inhibition of NF-κB activation [11–13]. Modulation of NF-κB activation may be an important strategy for the treatment of endotoxin-induced multiple organ injury [14]. There is, in fact, increasing evidence suggesting that NF-κB is an important mediator in the pathophysiology of disease states characterized by elevated levels of cytokines and reactive oxygen intermediates (ROI) such as in sepsis and in inflammation. LPS activates nuclear translocation of NF-κB.
antioxidant agents have been tested in experimental models of endotoxin shock.

In a murine model of endotoxic shock, green tea polyphenols blocked TNF-α gene expression by modulating NF-κB activation through their antioxidant activity [15]. Moreover, U-74389G, a new lazaroid with chain-breaking properties, reduced TNF-α production through inhibition of NF-κB activation and it has been proposed for the treatment of endotoxic shock [16]. Furthermore another antioxidant, N-acetyl cysteine has been shown to suppress NF-κB and TNF-α production in peritoneal macrophages activated with endotoxin [17]. Finally pyrrolidine dithiocarbamate, an antioxidant that selectively inhibits NF-κB activation [18], prevented in vivo expression of pro-inflammatory genes following LPS injection [19].

Theoretically vitamin E could also represent a good candidate to inhibit NF-κB activation. However, its poor pharmacokinetic profile precludes the use in pathological situations that require an acute administration. This justifies the search for alternative analogues that could overcome this problem. Experimental evidence suggests that vitamin E analogues such as its derivative penta-Fig. 5. Effects of IRFI 042 on different activators (H2O2, LPS) of NF-κB. Macrophages were preincubated for 120 min at 37 °C with IRFI 042 (50 μM) or RPMI and then stimulated with H2O2 (250 μM) or LPS (50 μg/ml). Each value represents the mean±S.E. mean of five experiments. *P<0.01 versus RPMI.

κB either indirectly (via the production of a strong oxidant stress) or directly by modifying its inhibitory subunit, IκB. As a direct consequence of this observation, several

Fig. 6. Effects of IRFI 042 on IκBα loss from the cytoplasm induced by different substances (H2O2, LPS). Macrophages were preincubated for 120 min at 37 °C with IRFI 042 (50 μM) or RPMI and then stimulated with H2O2 (250 μM) or LPS (50 μg/ml). Each value represents the mean±S.E. mean of five experiments. *P<0.001 versus RPMI.

IRFI-042 restored the vascular failure and the hyporeac-
Fig. 7. Effects of IRFI 042 on TNF-α mRNA levels stimulated by H₂O₂ or LPS. Macrophages were pre-incubated for 120 min at 37 °C with IRFI 042 (50 μM) or RPMI and then stimulated with H₂O₂ (250 μM) or LPS (50 μg/ml). Each value represents the mean ± S.E. mean of five experiments. *P<0.005 versus RPMI.

Inflammation response during endotoxin shock and sequential multiple organ failure/dysfunction syndrome (MOF/MODS).

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