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Biochemical Pharmacology 66 (2003) 439–445
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effects of FK506 and rapamycin on generation of reactive oxygen species, nitric oxide production and nuclear factor kappa B activation in rat hepatocytes

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Received 8 January 2003; accepted 11 April 2003

Abstract

We investigated the effect of two immunosuppressant drugs, FK506 and rapamycin, on reactive oxygen species (ROS) generation, nitric oxide (NO) production, inducible nitric oxide synthase (iNOS) expression and nuclear factor kappa B (NF-κB) activation in lipopolysaccharide (LPS)-activated rat hepatocytes. Primary culture of rat hepatocytes was treated with LPS in the presence and absence of FK506 or rapamycin. LPS increased the release of lactate dehydrogenase (LDH) and nitrite into the culture medium. Western blot and reverse transcription–polymerase chain reaction analyses demonstrated increased levels of iNOS protein and mRNA. Both immunosuppressant agents inhibited the induction of iNOS mRNA and protein stimulated by LPS. ROS generation, assessed by flow cytometry using dichlorodihydrofluorescein diacetate, was significantly decreased by FK506 and rapamycin. Moreover, electrophoretic mobility shift assay experiments indicated that both drugs blocked the LPS-induced activation of NF-κB. Inhibitor kappa B protein levels were decreased by LPS and this effect was partly blocked by FK506 or rapamycin. In summary, both immunosuppressant agents decreased the intracellular generation of ROS and inhibited NO production and iNOS expression at mRNA level in association to NF-κB activation. In addition to its capacity to reduce acute allograft rejection, this study highlights the anti-inflammatory properties of FK506 and rapamycin.

Keywords: LPS; FK506; Rapamycin; Rat cultured hepatocytes; Nitric oxide; Nuclear factor-κB

1. Introduction

FK506 (tacrolimus) and rapamycin (sirolimus) are two structurally similar macrolide antibiotics derived from *Streptomyces* fungus with potent immunosuppressive properties that are currently used for the prevention of graft rejection in organ transplantation [1]. Immunosuppression results primarily from inhibition of T lymphocyte activation but, although both agents share the same intracellular binding proteins, FK506, like cyclosporin A, inhibits the Ca^{2+}-dependent serine/threonine protein phosphatase calcineurin while rapamycin inhibits G1/S cell cycle via a calcineurin-independent pathway [2]. FK506 presents a profile of adverse reactions, mainly alterations in kidney function and neurotoxicity [3] that appears to be a consequence of the calcineurin blockade in non-immune cells and the lack of this effect by rapamycin may render this drug less toxic [4]. Moreover, combination of rapamycin with either FK506 or cyclosporin results in a significant inhibition of lymphocyte proliferation or IL-2 expression compared with these drugs alone [5].

Most studies on the mechanism of action of FK506 or rapamycin have been concentrated on its inhibitory effect on T cell activation, T cell development and IL-2 production [6]. In addition, recent investigations have shown anti-inflammatory effects of immunosuppressive drugs with

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Abbreviations: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; IκB, inhibitor kappa B; IKK, IκB kinase; IL-2, interleukin-2; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffer saline; ROS, reactive oxygen species; RT–PCR, reverse transcription–polymerase chain reaction.

0006-2952/$ – see front matter
doi:10.1016/S0006-2952(03)00288-0

inhibition of the expression of inflammatory mediators [7,8]. NO plays a major role in regulating vascular tone, neurotransmission, killing of microorganisms and tumour cells and other homeostatic mechanisms and the increase in NO production following transplantation is associated to acute allograft rejection [9]. The inducible isoform of oxide nitric synthase (iNOS) is responsible for the overproduction of NO in inflammation and is induced in response to interferon-γ, LPS and a variety of pro-inflammatory cytokines [10]. Contradictory reports, however, exist on the relationship between NO production and immunosuppressant agents. Thus, while it has been indicated that in murine macrophages rapamycin but not FK506 is able to inhibit iNOS mRNA expression induced by LPS [8], it has also been shown that both compounds apparently inhibit NO production and iNOS expression in rat hepatocytes [7].

Mediators in the inflammatory response activate NF-κB that leads to the induction of expression of many genes encoding for cytokines, growth factors, cell adhesion molecules or inflammatory enzymes [11]. The promoters of murine and human genes encoding iNOS contain a consensus sequence for the binding of NF-κB, which is necessary to confer inducibility by cytokines and LPS [12], and it has been demonstrated that FK506 inhibits the induction of iNOS during NF-κB activation [7]. Oxidative stress generated by ROS has been reported to be a potent activator of NF-κB [11]. ROS are generated under numerous pathological conditions including inflammation and may inflict direct damage to vital cell constituents such as lipids, proteins and DNA in addition to the modulation of the pattern of gene expression through functional alterations of transcription factors [13]. Moreover, there are massive synergism and redundancy in the pathways of inflammation, i.e. NO may react with ROS such as the superoxide radical to yield the highly reactive oxidant species peroxynitrite [14] which further contributes to oxidative stress.

The purpose of our study was to investigate the effects of FK506 and rapamycin on generation of ROS, NO production, iNOS expression and NF-κB activation in LPS-treated hepatocytes.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–250 g were housed in a room maintained at 22° with humidity ranging from 45 to 55% and a 12-hr dark/light cycle. The animals had free access to food (standard diet from Panlab) and water, and were not starved before experiments. All study protocols were reviewed and approved by the University of Leon Animal Care Committee and were in accordance with the indications of the Guide to the Care and Use of Experimental Animals.

2.2. Hepatocyte isolation

Rats were injected intraperitoneally (i.p.) with 5 mg/kg body weight endotoxin (LPS, *E. coli*, serotype 0127:BB, Sigma Chemical Co.) or the same volume of PBS (control group). Two hours after LPS or PBS administration hepatocytes were isolated by a non-recirculating *in situ* collagenase (Sigma) perfusion of liver through the portal vein. Hepatocytes were separated from nonparenchymal cells by differential centrifugation four times at 50 *g*. Hepatocyte purity assessed by microscopy was >98% and cell viability consistently exceeded 95% by trypan blue exclusion.

2.3. Cell culture and treatment

The isolated hepatocytes were suspended in culture medium at $5.5-6.0 \times 10^5$ cell/mL, seeded onto plastic dishes (2 mL/dish, 35 mm × 10 mm:9 cm^2, Falcon Plastic) and then cultured at monolayers in a 5% CO_2 humidified incubator at 37°. The culture medium used was Williams' medium E (Gibco) supplemented with 10% fetal calf serum, Hepes (5 mM), penicillin (100 units/mL), streptomycin (0.1 mg/mL) and insulin (10 nM). After 4 hr, the medium was changed to include LPS (10 μg/mL) with or without FK506 (10 μM) or rapamycin (10 μM). The concentration of both immunosuppressant agents was higher than those used in the clinical setting, but coincides with previous studies focused to test their anti-inflammatory properties *in vitro* [7,15]. After the timing of the incubations culture medium and hepatocytes were collected and frozen at −80°.

2.4. LDH release

LDH activity in the culture medium was measured by incubation with β-NADPH (0.2 mM) and pyruvic acid (0.4 mM) diluted in PBS. LDH activity in the sample was proportional to the β-NADPH consumption measured as the linear decrease in the absorbance at 334 nm. LDH release was calculated using a commercial standard (Merck).

2.5. Nitrite determination

Accumulation of nitrite in the medium was used as a measure of NO formation. Nitrite was determined by the Griess method, adapted from Green *et al.* [16]. Briefly, 100 μL of the culture medium was incubated with 100 μL of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine-dihydrochloride in 5% phosphoric acid) at room temperature for 10 min. The absorbance at 541 nm was then measured, and nitrite concentration was determined using a curve calibrated with sodium nitrite standards.

2.6. Intracellular generation of ROS

Production of peroxides was monitored by flow cytometry using DCFH-DA (Sigma). This dye is a stable

nonpolar compound that readily diffuses into cells and yields DCFH. Intracellular H_2O_2 or low-molecular-weight peroxides, in the presence of peroxidases oxidise DCFH to the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxides produced by the cells. At the end of the incubation periods, cells were washed with PBS and immediately detached with trypsin/EDTA, then incubated for 30 min in 2 mL of PBS containing 5 μM DCFH-DA at 37°. The cells were washed twice with PBS to remove the extracellular DCFH-DA, followed by analysis on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) (excitation 488 nm and emission 525 nm for DCF). Quantification of fluorescence intensity of M2 peaks was expressed as percentage of control values.

2.7. Western blot analysis

For Western blot analysis of iNOS and IκB protein formation, hepatocytes were homogenised with 140 mM NaCl, 15 mM EDTA, 10% glycerol, 20 mM Tris and a protease inhibitor cocktail. The mixture was centrifuged for 30 min at 13,000 *g* and 4°. The supernatant was kept as hepatocyte extracts. Samples containing 75 μg of protein were separated by SDS–polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose. Non-specific binding was blocked by preincubation of the nitrocellulose in PBS containing 5% bovine serum albumin for 1 hr. The nitrocellulose was then incubated overnight at 4° with polyclonal anti-iNOS or anti-IκB antibodies (Santa Cruz Biotechnology). Bound primary antibody was detected using a peroxidase conjugated secondary antibody (DAKO) by chemiluminiscence using the ECL kit (Amersham). The density of the specific iNOS (130 kDa) and IκB (36 kDa) bands were quantitated with an imaging densitometer.

2.8. RT–PCR

Total RNA was extracted by using guanidium thiocyanate (QuickPrep Total RNA Extraction kit, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Later, single-stranded cDNA was synthetised from 2 μg RNA using 5 μM random primers (Ambion) and 10 U AMV Reverse Transcriptase (Promega). PCR on complementary DNA was performed by using primers purchased from Biosource International. The PCR-primer sequences for rat iNOS were (sense) 5′-CTC TGA AGA AAT CTC TGT TC-3′ and (antisense) 5′-TTG AGG TCT AGA GAC TCT GG-3′. The mRNA levels were normalised against β-actin mRNA. The amplified products for iNOS and β-actin contained 352 and 457 base pairs (bp). After amplification PCR products were subjected to electrophoresis in 1% agarose gel and visualised by means of ethidium bromide staining. Fragments were then photographed using a Gelprinter plus photodocumentation system (TDI).

2.9. Electrophoretic mobility shift assay (EMSA)

Activation of NF-κB was examined in nuclear extracts using consensus oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Probes were labelled by T4 polynucleotide kinase as described [13]. Binding reactions included 10 mg of nuclear extracts in incubation buffer (10 mM Tris–HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA and 4% glycerol and 1 μg poly(dI–dC)). After 15 min on ice, the labelled oligonucleotide (30,000 cpm) was added and the mixture incubated 20 min at room temperature. For competition studies, 3.5 pmol of unlabelled NF-κB oligonucleotide were mixed 15 min before the incubation with the labelled oligonucleotide. The mixture was electrophoresed through a 6% polyacrylamide gel for 90 min at 150 V. The gel was then dried and autoradiographed at −70° overnight. Signals were densitometrically analysed.

2.10. Statistical analysis

Means and SEMs were calculated. Significant differences between means were evaluated by ANOVA and Newman–Keul's test. A difference was considered significant when P was less than 0.05.

3. Results

3.1. LDH release

LPS increased LDH activity in the culture medium (+104%). This effect was markedly reduced in the presence of FK506 or rapamycin (Fig. 1).

3.2. Generation of ROS

We investigated the generation of peroxides by hepatocytes using the DCFH-DA probe. Figure 2 shows the intracellular generation of peroxides in hepatocytes incubated in the presence of LPS with or without FK506 or rapamycin. Figure 2A shows the histograms in which the fluorescence, detected with the FL1-H channel, is plotted against the relative number of cells. Figure 2B shows the quantification of the M2 peaks of Fig. 2A as percentage of control values; the increase induced by LPS was significantly blocked by both FK506 and rapamycin.

3.3. Nitrite release and iNOS expression

LPS increased the release of nitrite ions (oxidation products of NO) into the culture medium (+117%). Simultaneous addition of FK506 or rapamycin resulted in an inhibition of nitrite ions release that did not significantly differ from control values (Fig. 1). Both immunosuppressive drugs inhibited the induction of iNOS mRNA and protein stimulated by LPS in hepatocytes, although to a greater extent in the case of rapamycin (Figs. 3 and 4).

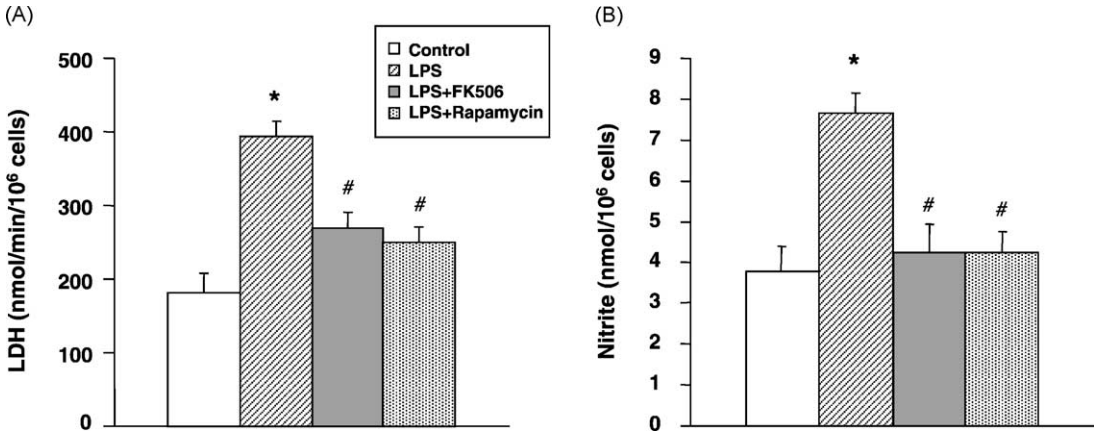

Fig. 1. LDH activity (A) and nitrite concentration (B) in culture medium. The cells were treated with LPS only or LPS + FK506 or rapamycin for 24 hr. Data are means ± SEM of five different observations. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

3.4. NF-κB Activation and IκB expression

As shown in Fig. 5, incubation of hepatocytes with LPS induced a marked activation of NF-κB that was significantly reduced in hepatocytes treated with either FK506 or rapamycin (Fig. 5). Since it has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκB, we assessed protein levels of IκB. Protein levels were decreased by LPS and this effect was partly blocked by both immunosuppressants (Fig. 6).

4. Discussion

Although recent investigations have shown that the immunosuppressive drugs FK506 and rapamycin inhibit the expression of different inflammatory mediators [7,8], the anti-inflammatory functions of these drugs are not well established, contradictory reports exist on the relationship with NO production, and effects on important mediators of the inflammatory response such as ROS or NF-κB inhibitors are unknown.

Increased free radical production during inflammation gives place to an increase in oxidative stress, which would contribute to the hepatic damage. Previous studies have demonstrated that LPS triggers the synthesis of ROS such as superoxide and oxidative damage plays a role in its cytotoxicity [17]. By using the fluorescence probe DCFH-DA and flow cytometry techniques, we found increases in DCF fluorescence, indicating overproduction of peroxides, when rat hepatocytes were incubated in the presence of LPS. The increase of emitted DCF fluorescence reached levels more than three times higher than those found in non-treated cells and these changes were in agreement with the cytotoxicity and membrane lysis observed by LDH leakage. Addition of FK506 or rapamycin resulted in a

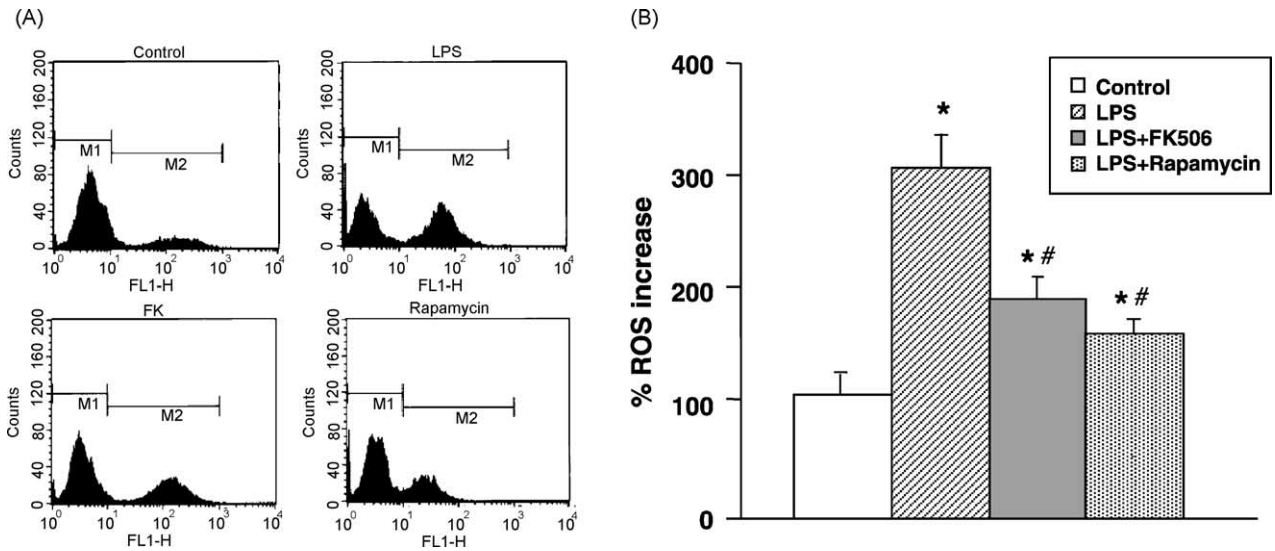

Fig. 2. Intracellular peroxide generation in culture of hepatocytes incubated with LPS with or without FK506 or rapamycin for 24 hr. (A) A representative histogram in which the fluorescence (FL1-H) is plotted against the number of cells. (B) The quantification of M2 peaks referred by fluorescence intensity as percentage of control values. Data are means ± SEM of five different observations. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

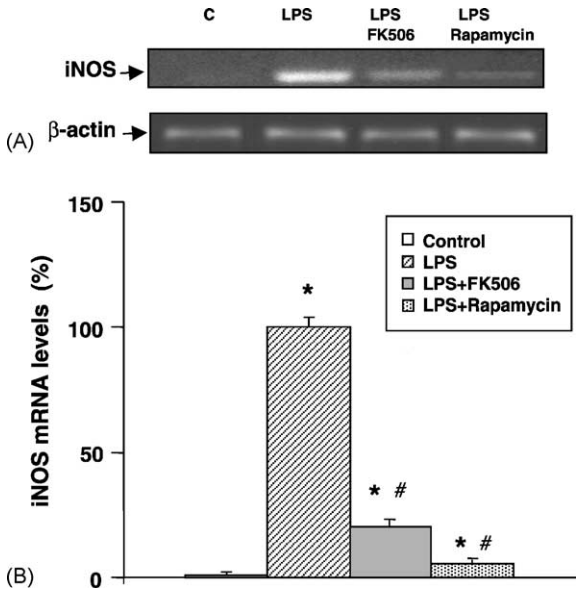

Fig. 3. iNOS mRNA levels (RT–PCR) in cultured hepatocytes from the different experimental groups. After stimulation with LPS or LPS + FK506 or rapamycin for 8 hr, total RNA was extracted and RT–PCR reaction on complementary DNA was performed by using rat iNOS specific primers. (A) A representative RT–PCR reactions. (B) Mean values ± SEM, expressed as percentage of LPS values, normalised to β-actin mRNA of five different observations. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

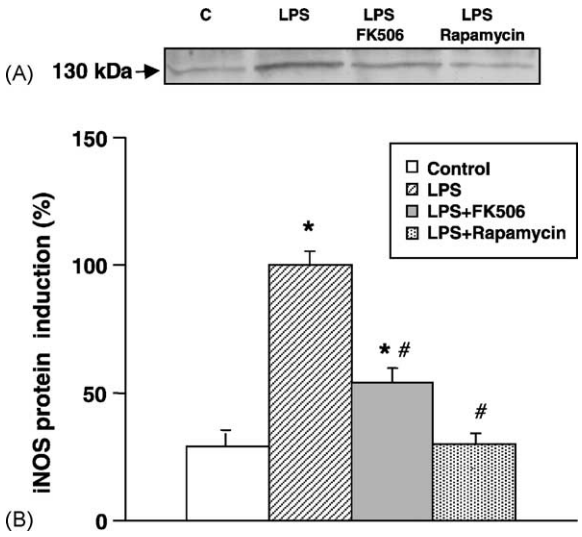

Fig. 4. Western blot analysis of iNOS protein in cultured hepatocytes following LPS or LPS + FK506 or rapamycin exposure for 12 hr. Total cellular protein was separated on 12% SDS–polyacrylamide gels and blotted with anti-iNOS antibodies. (A) A representative Western blot photographs. (B) Mean values ± SEM, expressed as percentage of LPS values, of five different observations. Equal loading was confirmed by Ponceau S staining. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

marked decrease in the intracellular generation of peroxides. Although the generation of ROS has been presumed to play a major role in cyclosporin A and FK506-induced toxicity [18], we have previously shown that markers of oxidative stress do not change in FK506-treated rats [19] and are significantly induced by cyclosporin A only in aged animals [20]. Moreover, there is a lack of evidence for oxidative damage in plasma of liver transplant patients treated with either cyclosporin A or FK506 and it has been reported that FK506 down-regulates free radical levels in rats subjected to ischemia and reperfusion [21]. The protective effect of the immunosuppressant agents may be due to different factors. Thus, it is known that FK506 decreases oxidative phosphorylation of mitochondria by inhibiting both complex III, where ROS are generated, and complex V, where ATP is depleted by ATPase activation [22]. In addition, immunophilin ligands have been reported to protect against H_2O_2-induced cell damage in NG108-15 cells by increasing intracellular glutathione concentration [23].

During inflammation, hepatocytes respond to cytokines by expressing iNOS [24], and it is known that in addition to the production of ROS, LPS triggers the synthesis and release of NO in Kuppfer cells and hepatocytes [25]. NO may react spontaneously with ROS such as the superoxide radical to form the potent and versatile oxidant peroxynitrite [14] and it has been suggested that NO itself or through one of its metabolites may be a major determinant of oxidant stress in LPS-induced liver injury [25]. Our *in vitro* experi-

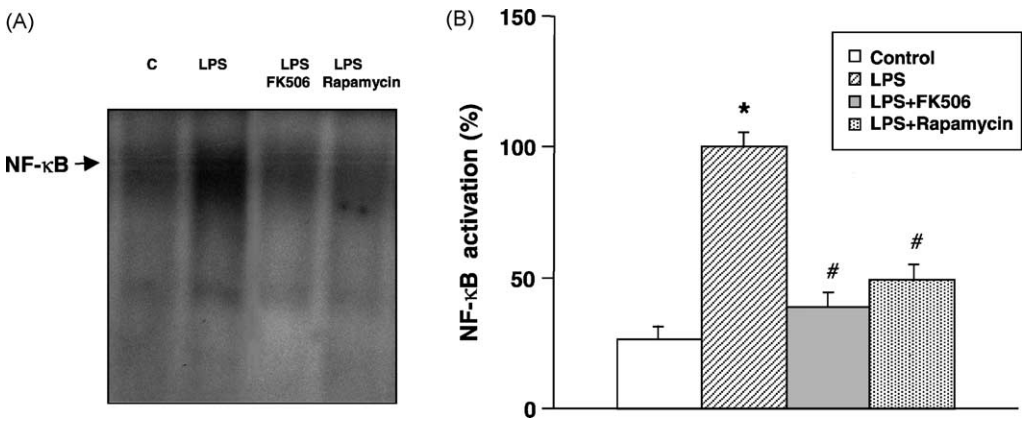

Fig. 5. Nuclear factor κB activation in culture of hepatocytes stimulated with LPS with or without FK506 or rapamycin for 1 hr. For the EMSA assay nuclear extracts were incubated with ^{32}P-labelled consensus oligonucleotide, followed by electrophoresis and analysis by autoradiography. (A) A representative EMSA. (B) Mean values ± SEM expressed as percentage of LPS values of five different observations. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

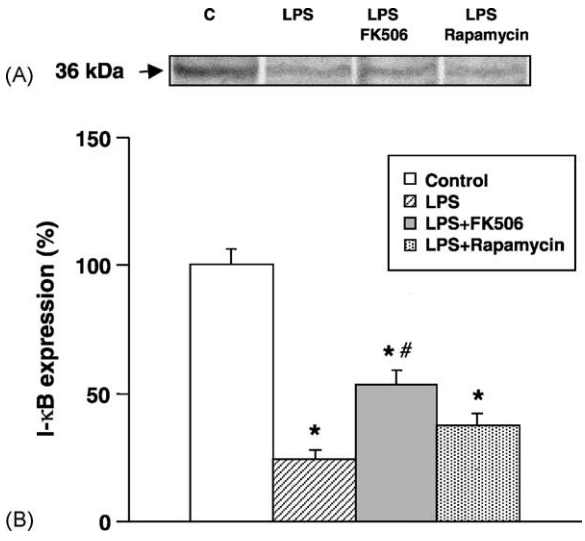

Fig. 6. Western blot analysis of IκB protein in cultured hepatocytes incubated with LPS with or without FK506 or rapamycin for 30 min. Total cellular protein was separated on 12% SDS–polyacrylamide gels and blotted with anti-IκB antibodies. (A) A representative Western blot photographs. (B) Mean values ± SEM, expressed as percentage of control values, of five different observations. Equal loading was confirmed by Ponceau S staining. (*) $P < 0.05$ vs. control, (#) $P < 0.05$ vs. LPS.

ments using cultured rat hepatocytes confirm the LPS-mediated induction of iNOS expression in parenchymal cells and demonstrate that both FK506 and rapamycin inhibit the induction of iNOS mRNA and protein. Regulation of iNOS expression and NO production may play an important role in graft rejection during organ transplantation. Plasma concentrations of NO increase during acute allograft rejection and correlate with rejection severity [26], and decreased NO production is associated with reduced frequency of acute rejection in patients receiving immunosuppressants [9]. However, effects of immunosuppressant drugs on NO formation are not uniform and tissue/cell specific effects having been reported. Thus, FK506 has been shown to inhibit iNOS expression induced by IL-1β in hepatocytes [7] but not in LPS-stimulated macrophages [8]. Our data indicate that inhibition of NO formation may explain part of the favourable effect of immunosuppressants in inflammatory conditions. Additionally, the marked attenuation of iNOS expression by rapamycin confirms that a calcineurin-dependent pathway does not mediate this effect. An interesting fact is that both iNOS mRNA and protein were still higher compared to the control after rapamycin or, to a higher degree, FK506 treatment, while the amount of nitrite was similar. This could be explained by the only partial normalisation in the formation of peroxynitrite, of which the formation of DCF is a sensitive indicator [27].

One pathway by which ROS and NO can contribute to liver injury is activation of inflammatory cascades through the NF-κB, resulting in inflammation manifested by cytokine expression [12]. NF-κB may be an important signal orchestrating the inflammatory response and, when activated, translocates into the nucleus, where it binds to the promoter region of target genes and results in the subsequent activation of inflammatory mediators, with up-regulation of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and induction of iNOS gene [28]. NF-κB may therefore represent an important target for therapeutic blockade of inflammation and liver injury.

It has been shown that challenge of rats with LPS activates NF-κB in lungs, heart and liver, suggesting that NF-κB activation is a critical mechanism mediating LPS-induced organ injury [29]. Activation of NF-κB, in turn, is induced by phosphorylation of IκB, in response to diverse stimuli including ROS, which leads to its degradation and results in unmasking of nuclear localisation signals that allow NF-κB to be translocated into the cell nucleus [30]. Results in this study indicate that in LPS-treated hepatocytes proteolysis of IκB is coincident with activation and nuclear translocation of NF-κB and this is accompanied by iNOS gene up-regulation. Data obtained, however, do not clearly allow to know whether both immunosuppressants have similar effects on IκB degradation and, because the key regulatory step in NF-κB activation by proinflammatory stimuli is the activation of IKKs [31], which in turn phosphorylate IκBs, an analysis of IKKs should be necessary to get a further insight into the mechanisms of action of both immunosuppressant drugs. In any case, given the fact that NF-κB is a component of a multipartite regulatory circuit that is activated by ROS and delicately balanced by a controlled inflammatory reaction, disruption of this balance by the increase of the oxidant overload during LPS challenge may contribute to NF-κB activation. Our data confirm the previous report that FK506 inhibits IL-1β-stimulated iNOS expression at the transcriptional stage by the blockade of NF-κB activation and translocation [7] and provides further insight into the mechanisms of action of immunosuppressant drugs.

In conclusion, results obtained show that FK506 and rapamycin have potent protective effects against damage caused by LPS in rat hepatocytes, preventing oxidative stress and iNOS over-expression. Although the causal relationship between the events here described remains to be established, the activation of NF-κB constitutes one mechanism by which inflammation promotes liver injury and NF-κB activation was markedly attenuated by both immunosuppressants. Our data indicate that both FK506 and rapamycin, although with some quantitative differences, modulate key responses of the hepatocytes to inflammatory mediators. Further studies should be required to identify the anti-inflammatory potential of FK505 or rapamycin in the clinical setting.

Acknowledgments

This work was supported by grants from the Fondo de Investigación Sanitaria (FIS), Spain and the Spanish Network for Cooperative Research G03/015.

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