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Rat liver ischemia–reperfusion-induced apoptosis and necrosis are decreased by FK506 pretreatment

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Abstract

The aim of this study was to demonstrate that tacrolimus (FK506) has a hepatoprotective effect by reducing ischemia–reperfusion-induced apoptosis and necrosis, both of which lead to post-surgical liver dysfunction. An ischemia–reperfusion model and primary cultured rat hepatocytes subjected to hypoxic and reoxygenation phases, mimicking the surgical process, were used. c-*Jun* N-terminal kinase 1/stress-activated protein kinase 1 ($JNK_1/SAPK_1$) activation leads to caspase 3 activation, a trigger of apoptosis. The activation status of $JNK_1/SAPK_1$ was evaluated by immunoprecipitation or Western-blotting experiments. Apoptosis was assessed by measuring caspase activation and by TUNEL (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-biotin nick-end labeling) reaction. Necrosis was assessed histologically. Tacrolimus improved the survival rate of rats subjected to ischemia–reperfusion. After FK506 pretreatment, the liver necrosis rate was reduced, and ischemia–reperfusion-induced $JNK_1/SAPK_1$ activation and apoptosis were significantly decreased. In hypoxia-reoxygenation-subjected hepatocytes, tacrolimus reduced $JNK_1/SAPK_1$ and caspase 3 activation. In the liver, tacrolimus prevented ischemia-reperfusion-induced apoptosis and necrosis.

Keywords: FK506; Hypoxia-reoxygenation; $JNK_1/SAPK_1$; Apoptosis; Necrosis; Hepatocyte; (Rat)

1. Introduction

Ischemia–reperfusion injury is a main cause of primary dysfunction or non-function of the liver after resection or transplantation (Chien et al., 1997). Cellular injury is linked to activation of several inflammatory pathways, which include free radicals (Gonzalez-Flecha et al., 1993), cytokines (Colleti et al., 1996) and neutrophil infiltration (Jaeschke and Farhood, 1991). Ischemia–reperfusion injury results in apoptosis and necrosis, which may occur in parallel, both contributing to cell death in liver disease. We and others have previously demonstrated that apoptosis of hepatocytes and sinusoidal cells plays a central role in the pathogenesis of ischemia–reperfusion hepatic injury (Kohli et al., 1999; Natori et al., 1999; Crenesse et al., 2000a). Among several signal transduction pathways leading to apoptosis (Bradham et al., 1997), it has been shown that the activation of c-*Jun* N-terminal kinase 1/stress-activated protein kinase 1 ($JNK_1/SAPK_1$), a member of the Stress Activated Protein Kinase family, is crucial and leads to caspase 3 activation, which is involved in the triggering of apoptosis (Onishi et al., 1997; Crenesse et al., 2000a). $JNK_1/SAPK_1$ activation is reduced by oxygen-free radical scavengers (Crenesse et al., 2000b). FK506 (or tacrolimus) is a very frequently used immunosuppressant and can also protect the liver against ischemia–reperfusion injury (Sakr et al., 1991; Dhar et al., 1992). In addition to its inhibitory effect on the production of interleukin 2, FK506 possesses many other properties that may explain its protective effects: in particular, it has an inhibitory effect on cytokines and free radical production (Ishii et al., 1994;

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Garcia-Criado et al., 1997). The aim of the present study was to test the hypothesis that FK506 pretreatment provided protection through prevention of apoptosis. To this end, we developed two models of warm ischemia–reperfusion: an in vivo classic ischemia–reperfusion model and an in vitro study of primary cultured rat hepatocytes, isolated from other liver cells and blood flow, and subjected to hypoxic and reoxygenation phases mimicking conditions encountered during surgery.

2. Materials and methods

2.1. Reagents

All chemicals were obtained from Sigma (St. Quentin Fallavier, France) unless otherwise stated. Culture media were obtained from Eurobio (Les Ullis, France). Liberase RH, protease and phosphatase inhibitors were obtained from Boehringer Manheim (Meylan, France). ^{32}P isotopes were obtained from ICN (Irvine, CA).

2.2. In vivo experiments

2.2.1. Animal preparation and hepatic ischemia procedure

Male Wistar rats weighing 250–300 g (Charles River, Cleon, France) were used. All animal experiments were conducted in compliance with French regulations on animal handling. The rats, housed individually (14:10 h light–dark cycle) in a room with the temperature kept at 22 ± 1 °C, were allowed free access to food and water before and during ischemia. Normothermic ischemia of the liver, allowing the study of three experimental conditions on the same animal, was induced under ether anesthesia. Briefly, after laparotomy, ischemia was induced by occluding the blood vessels, including the bile ducts, to the median and left lateral lobes, with an atraumatic clamp. The caudate lobe was ligatured. After 120 min of warm ischemia, the caudate lobe was harvested, the vascular clamp was released and the abdomen was closed in two layers with silk. After operation, the animals were kept in individual cages. After 240 min of reperfusion, under ether anesthesia, the median and left lateral lobes were harvested as well as the right lateral lobe, which was used as a control lobe. The duration of ischemia and reperfusion was chosen to induce maximum apoptosis (Cursio et al., 1999).

2.2.2. Experimental groups

Three groups ($N = 5$) were studied. Group 1 (control group): non-treated animals. Group 2: animals injected intravenously, via the dorsal penile vein, with tacrolimus (300 μg/kg) dissolved in NaCl 0.9% (Sakr et al., 1991), 24 h prior to induction of ischemia. Group 3 (Sham): animals underwent laparotomy with the same anesthesia duration but without liver ischemia.

2.2.3. Tissue preparation

At the end of the stress time, each liver lobe was immediately fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.2) for 3 h at 4 °C and gently shaken. Then the medium was changed for 20% sucrose in phosphate-buffered saline (PBS) and samples were gently shaken overnight at 4 °C. Subsequently each sample was immersed for 2 min in methyl-2 butane at − 35 °C and stored at − 80 °C until use.

2.2.4. Histological studies

Specimens were fixed in 10% formalin and embedded in paraffin. Sections at 3-μm intervals were stained with hematoxylin and eosin. At the end of each stress time, the extent of liver necrosis was semi-quantitatively assessed in 15 samples of caudate, median and left lateral, and right lateral lobes as follows: none = 0; single-cell necrosis = 1; up to 30% lobular necrosis = 2; up to 60% lobular necrosis = 3; more than 60% lobular necrosis = 4. Blind analysis was carried out for all histological studies (Cursio et al., 1999).

2.2.5. Mortality study

Three groups were studied ($N = 10$): group 1: 2-h ischemia; group 2: animals injected with tacrolimus and subjected to 2 h of ischemia; group 3: sham-operated animals. In groups 1 and 2, after ischemia, the non-ischemic lobe (i.e. right lateral lobe) was harvested before the beginning of reperfusion. After surgery, the animal was kept in an individual cage. Mortality rates were assessed on day 7.

2.3. In vitro experiments

2.3.1. Hepatocyte isolation and culture

Male Wistar rats weighing 250–300 g (Charles River, Cleon, France) were used. Hepatocytes were obtained according to methods previously described (Crenesse et al., 2000a). Briefly, fed rats were ether anesthetized and livers were perfused through the portal vein with liberase RH. Dissociated hepatocytes were then collected in William's culture medium (Eurobio) supplemented with 5% fetal calf serum and insulin (0.1 IU/ml). Cell viability was estimated to be superior to 90% by Trypan blue exclusion. Cells were adjusted to a density of 0.5×10^6 cells/ml and cultured in the presence of 95% air, 5% CO_2. The culture medium, containing dexamethasone (1 μM), was renewed 4 h later. After 16 h of culture, hepatocytes were used for experiments.

2.3.2. Hypoxic stress conditions

Culture plates were maintained at 37 °C in a hermetic bag (Bioblock, Illkirch, France) in which pO_2 was adjusted to 50 ± 10 mm Hg by N_2 supply and maintained constant. The culture medium used, Leibovitz medium (L15) (Eurobio) without added fetal calf serum and dexamethasone, was saturated with nitrogen. A sample of medium was left under the same conditions and immediately analyzed (Blood gas

analyzer Corning 2504, Cergy Pontoise, France) at the end of each experiment to control pO_2, pCO_2 and pH values. This latter was stable at 7.20 ± 0.02.

Six groups of three rats each were studied. Group 1 (control): hepatocytes were maintained at 37 °C in ambient air for a time equal to the total period of stress. Group 2: hepatocytes were maintained under a hypoxic atmosphere for 1 h at 37 °C. Group 3 corresponded to conditions of group 2 followed by 1 h for JNK measures or 2 h for caspase assay, in ambient air at 37 °C. In treated groups, hepatocytes were preincubated with tacrolimus (1 μM, dissolved in culture medium) for 4 h before the beginning of the stress. In groups 4, 5 and 6, hepatocytes were subjected to the same conditions as groups 1, 2 and 3, respectively.

2.4. Kinase assays

2.4.1. p-JNK immunohistochemistry in liver specimens

Frozen sections were rehydrated through a graded series of ethanol (95%, 70%, 50%) and rinsed twice in PBS 1X. Then they were incubated in Tween 0.3% for 5 min and rinsed twice in PBS 1X. Sections were immersed in 0.3% hydrogen peroxide and PBS for 30 min, blocked with 1% goat serum (Vector Laboratories, Burlingame, CA) and 3% Triton X-100 for 2 h at room temperature, and rinsed in PBS 1X. Sections were incubated with the mouse monoclonal antibodies against JNKs, p-JNK (1/250e, Santa Cruz Biotechnology), overnight. After the primary incubation and three rinses in PBS 1 ×, sections were incubated with biotinylated horse antimouse antibody (Santa Cruz Biotechnology) for 2 h at room temperature. p-JNK was visualized with 3-amino-9-ethyl carbazole (AEC) staining using the VectaStain Avidin–Biotin Complex (ABC) kit (Vector Laboratories). All sections were washed a final time in PBS and distilled water, dried rapidly and mounted with Entelan (Merck, Fontenay-sous-bois, France). Sections were examined under a light microscope.

2.4.2. Kinase assays in cultured hepatocytes

At the end of the different stress conditions, the medium was aspirated, 1 ml of lysis buffer (150 mmol/l NaCl, 0.8 mmol/l $MgCl_2$, 5 mmol/l EGTA, 1% Nonidet P-40, 1 mmol/l pepstatin, 15 mg/ml leupeptin, 1 mmol/l phenyl-methanesulfonyl-fluoride (PMSF), 1 mmol/l Na_3VO_4 and 50 mmol/l HEPES at pH 7.5) was added, and the hepatocytes were shaken at 4 °C for 45 min. The crude lysates were transferred to Eppendorf tubes and centrifuged at 4 °C and 18,000 × *g* for 15 min. The supernatants (lysates) were removed and the protein concentrations were assayed using the Lowry method according to manufacturer's recommendations (Biorad, Ivry sur Seine, France). An equal amount of protein (1 mg) for each lysate was precleared with rabbit non-immune serum prebound to protein A-Sepharose (Pharmacia-LKB Biotechnologies), and Mitogen-Activated-Protein (MAP)-related kinases were immunoprecipitated from precleared lysates by incubation at 4 °C for 16 h with anti-JNK_1 anti-sera (1/200e) (Santa Cruz Biotechnology) bound to protein A-Sepharose (Crenesse et al., 2000a). Immunopellets were washed twice with lysis buffer, twice with MAP kinase buffer (30 mmol/l NaCl, 0.1% Nonidet P-40, 10% glycerol, 200 mmol/l Na_3VO_4, 30 mmol/l HEPES at pH 7.5) and resuspended in 50 μl of MAP kinase buffer containing 30 mmol/l Mg-acetate in the presence of 0.5 mg/ml of GST-ATF2, which was used as exogenous substrate for JNK_1. The kinase assay was initiated by addition of 25 mmol/l ATP and 100 μCi/ml [γ^{32}P]ATP. After incubation at 30 °C for 30 min, the reactions were stopped by addition of 9 × Laemmli sample buffer and boiling for 3 min. Immunocomplex reactions were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel), overnight at −80 °C, followed by blotting onto Immobilon P-membranes solution. The amount of JNK_1 was evaluated on autoradiography hyperfilms MP (Amersham).

2.5. Assessment of apoptosis in liver specimens

2.5.1. Caspase assay

After the above tissue preparations, sections were incubated with the polyclonal antibody anti-CPP32 (1/700e, rabbit anti-CPP32, Santa Cruz Biotechnology) overnight at 4 °C (Darmon et al.,1995). After the primary incubation and three rinses in PBS 1 ×, sections were then incubated with biotinylated horse antirabbit antibody (Vector Laboratories) for 2 h at room temperature. Caspase 3 was visualized using the VectaStain ABC kit (Vector Laboratories).

2.5.2. DNA nick-end labeling of liver sections

DNA nick-end labeling by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) reaction was used. In brief, frozen sections were rehydrated in ethanol (95%, 70% and 50%) followed by PBS and bathed for 30 min with 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase. Sections were then permeabilized with 0.3% Tween/PBS and washed twice in PBS before application of the TUNEL reaction mixture (F. Hoffmann-La Roche, Basel, Switzerland). All slides were incubated in a humid chamber at 37 °C for 2 h, washed twice in PBS and allowed to incubate overnight with the secondary antifluorescein-peroxidase conjugate, at 4 °C. On the following day, sections were washed three times with PBS and the peroxidase labeling was revealed with AEC by means of the VectaStain ABC kit (Vector Laboratories). After a final rinse in distilled water, sections were coverslipped. Two hundred hepatocytes on sections were counted and the number of TUNEL-positive hepatocytes per 100 hepatocytes was calculated.

2.6. Assessment of apoptosis in cultured hepatocytes

2.6.1. Caspase assay

Enzyme assays were performed at the end of different stress conditions. Briefly, the hepatocytes were lysed with

Table 1
Effect of Tacrolimus on the survival rate of ischemic rats

Group	Number of rats	Alive	Dead	% Survival
Control Group (1)	10	0	10	0
Tacrolimus-treated Group (2)	10	5	5	50[a]
Sham-operated (3)	10	10	0	100

Rats were subjected to ischemia (2 h) followed by reperfusion. Treated rats were injected intravenously, via the dorsal penile vein, with Tacrolimus (300 μg/kg), 24 h prior to induction of ischemia (group 2) and compared to untreated rats (group 1). A third group (group 3) of 10 sham-operated animals was also included in the protocol. Survival rate was assessed at day 7 for each group.

[a] $P < 0.001$ vs. group 1.

PBS 2 mM DL-dithiothreitol and sonicated 2 × 8 s (40 W). Cells were sedimented at 18,000 × *g* for 15 min at 4 °C. The supernatants were removed, and the protein concentrations were assayed using the Bradford method (Biorad), the used standard solution being bovine serum albumin solution. Cytosolic proteins (100 μg) were incubated at 37 °C with 200 μM DEVD-pNa (*N*-Acetyl-Asp-Glu-Val-Asp paranitroaniline) in the presence or in the absence of 200 μM Apoprotein-CPP32 inhibitor (Alexis, San Diego) in 96-well microtiter plates. At different times, hydrolytic activity was determined by measuring the absorbence of para-nitroaniline at 410 nm. Results are expressed as arbitrary fluorescence units (AFU)/mg protein/min.

2.7. Statistical analysis

Experimental values (means ± S.E.M.) are from independent measurements using different hepatocytes or rats. Differences between groups were analyzed by ANOVA (analysis of variance), with post-hoc Bonferroni test for multiple comparisons. Significance in mortality results was assessed using the χ^2 test. $P < 0.05$ was considered significant.

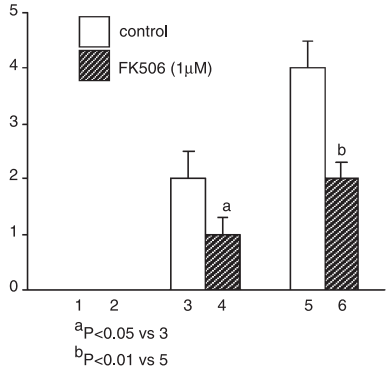

Fig. 1. Numerical degree of liver necrosis 6 h after reperfusion in control and tacrolimus-treated (300 μg/kg) groups. A significant difference was observed between the two groups: $P < 0.05$ in ischemic lobes (bars 3 and 4, respectively) and $P < 0.01$ in ischemia-reperfused lobes (bars 5 and 6, respectively). No necrosis areas were observed in lobes subjected to neither ischemia or reperfusion (bars 1 and 2, respectively). Each point is the mean ± S.E.M. of 15 determinations.

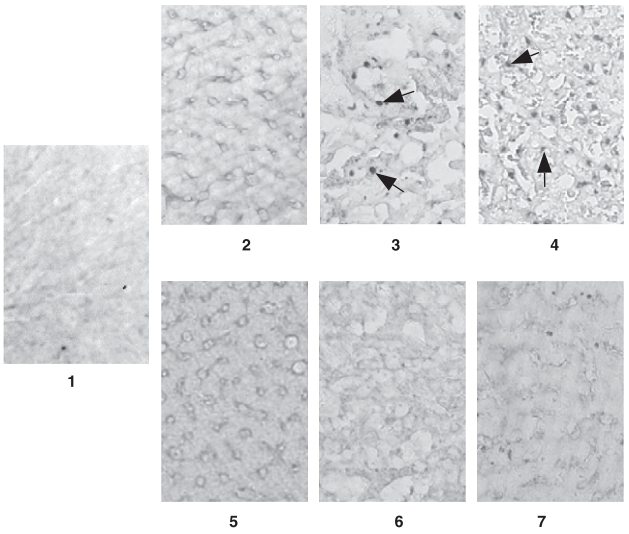

Fig. 2. Inhibition of ischemia–reperfusion-induced JNK activation by Tacrolimus. Immunohistochemical study showed the translocation of JNK to the nucleus (arrows) after 2-h ischemia (plate 3) and after 2-h ischemia followed by 4-h reoxygenation (plate 4). In tacrolimus-treated rats (300 μg/kg), JNK activation was strongly reduced after 2-h ischemia (plate 6) and after 2-h ischemia followed by 4-h reoxygenation (plate 7), and did not differ from non treated (plate 2) and treated (plate 5) non-ischemic lobes or sham group lobes (plate 1). (Original magnification: × 10). The figure shows a representative result from five independent experiments.

3. Results

3.1. Tacrolimus increased the survival rate

Fifty percent of the Tacrolimus-treated rats survived to day 7 whereas 100% of rats in the control group died within the first 24 h after surgery ($P < 0.001$). In sham-operated animals, the 7-day survival rate was 100% (Table 1).

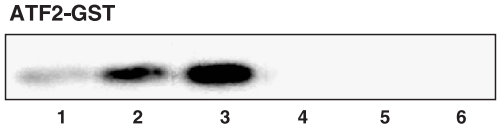

Fig. 3. Tacrolimus reduced hypoxia-reoxygenation-induced $JNK_1/SAPK_1$. $JNK_1/SAPK_1$ activities were performed, from cell lysates, by in vitro kinase assay using ATF2-GST as substrate after immunoprecipitation (IP) with specific Abs, as described under Materials and methods. Rat hepatocytes were incubated at 37 °C, 2 h under air (lane 1), 1 h under hypoxia (lane 2) or 1 h under hypoxia followed by 1 h under air (lane 3). In Tacrolimus-treated hepatocytes (1 μM), subjected to same stress conditions, $JNK_1/SAPK_1$ activation was totally abolished (lanes 4, 5 and 6, respectively). For each condition, the figure shows a representative result from three independent experiments.

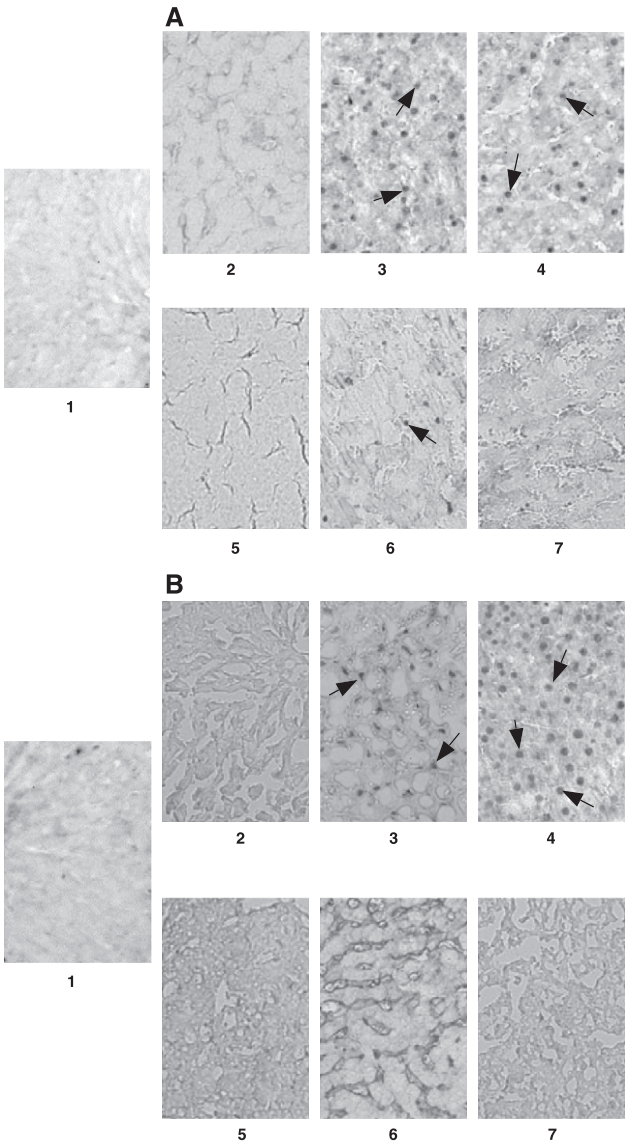

Fig. 4. Tacrolimus protection against apoptosis. (A) An immunohistochemical study using an anti-caspase 3 antibody was performed to examine the subcellular localization of this caspase. Compared to control lobes (plate 2) or sham group lobes (plate 1), caspase 3 activation was increased in liver lobes subjected to 2-h ischemia (plate 3) and to 2-h ischemia followed by 4 h-reperfusion (plate 4) whereas this activation was less important in tacrolimus-treated rats lobes subjected to ischemia (plate 6) and to ischemia–reperfusion (plate 7). (B) TUNEL-stained cells detected in lobes subjected to 2-h ischemia (plate 3) and in lobes subjected to 2-h ischemia followed by 4-h reperfusion (plate 4) were strongly increased compared to control lobes (plate 2) or sham group lobes (plate 1). In Tacrolimus-treated rats, TUNEL-stained cells were significantly less numerous (plates 6 and 7). Plate 5 represented control lobe in treated animals. (Original magnification: × 10). The figure shows a representative result from five independent experiments.

3.2. Tacrolimus reduced the necrosis rate

The degree of liver necrosis was greater in lobes subjected to ischemia–reperfusion than in ischemic or non-ischemic lobes. These lesions were diminished in treated animals (Fig. 1). The localization of necrosis was heterogeneous, predominating in centrolobular areas. Histological lesions were absent in sham-operated animals (data not shown).

3.3. In vivo, tacrolimus reduced ischemia–reperfusion-induced JNK activation

Our original model showed that the translocation of JNK to the nucleus was important in liver lobes subjected to ischemia only (Fig. 2, plate 3) and in lobes subjected to ischemia followed by reperfusion (Fig. 2, plate 4). When rats were treated with tacrolimus, activation of the kinase was significantly reduced (Fig. 2, plates 6 and 7) and did not differ from that of control non-ischemic lobes (Fig. 2, plates 2 and 5, respectively) or sham group lobes (Fig. 2, plate 1).

3.4. Tacrolimus reduced hypoxia-reoxygenation-induced $JNK_1/SAPK_1$ activation in isolated hepatocytes

Under our stress conditions, 1 h of hypoxia was able to induce $JNK_1/SAPK_1$ activation but to a lesser extent than that observed after 1 h of hypoxia followed by 1 h of reoxygenation (Fig. 3, lanes 2 and 3, respectively). There was no $JNK_1/SAPK_1$ activation in tacrolimus-treated hepatocytes (Fig. 3, lanes 5 and 6, respectively).

3.5. Tacrolimus, in vivo, reduced ischemia–reperfusion-induced apoptosis

Ischemia for 2 h alone (Fig. 4A, plate 3) and for 2 h followed by 4 h of reperfusion (Fig. 4A, plate 4) induced caspase 3 activation. This activation was much lower in rats treated with tacrolimus (Fig. 4A, plates 6 and 7, respectively).

Tissue was analyzed by TUNEL staining for apoptotic cells. TUNEL-positive hepatocytes were counted, and the number of TUNEL-positive hepatocytes per 100 hepatocytes was calculated (Table 2). As in the sham model (Fig. 4B,

Table 2
Effect of Tacrolimus on ischemia–reperfusion-induced apoptosis of hepatocytes

Group	Number of rats	TUNEL-positive hepatocytes rate (%)
Control group (1)	5	
C lobes		0
I lobes		50
I/R lobes		80
Tacrolimus-treated group (2)	5	
C lobes		0
I lobes		5[a]
I/R lobes		20[a]
Sham-operated (3)	5	0

C lobes: non-ischemic lobes; I lobes: lobes subjected to 2 h ischemia; I/R lobes: lobes subjected to 2 h ischemia followed by 4 h reperfusion.
[a] $P < 0.01$ vs. group 1.

plate 1), apoptotic cells were not observed in the lobe subjected to neither ischemia–reperfusion (Fig. 4B, plate 2). In the lobe subjected to 2 h of ischemia, the apoptosis rate was 50% (Fig. 4B, plate 3), and in the lobe subjected to 2 h of ischemia followed by 4 h of reperfusion, this rate reached 80% (Fig. 4B, plate 4). When rats were treated with tacrolimus, the apoptosis rate was significantly reduced (5% and 20%, respectively), compared to that observed in non-treated animals (Fig. 4B, plates 6 and 7, respectively). Positive control was obtained by pre-incubation of a section with DNase I (results not shown).

3.6. Tacrolimus reduced hypoxia-reoxygenation-induced caspase 3 activation in isolated hepatocytes

As proteases encoded by the caspase gene family are required for the initiation and execution of phases of apoptosis (Patel et al., 1996), we tested the effect of hypoxia-reoxygenation stress on caspase activity in rat hepatocytes. As shown previously (Crenesse et al., 2000a) and compared to control conditions under air (Fig. 5, bar 1), caspase activity increased after 1 h of hypoxia (by a factor of 1.39) (Fig. 5, bar 3) but to a lesser extent compared to that observed after 1 h of hypoxia followed by 2 h of reoxygenation (by a factor of 1.90, $P<0.05$ vs. control) (Fig. 5, bar 5). In tacrolimus-treated hepatocytes, stress-induced caspase activity was significantly ($P<0.0001$) decreased and results were even lower than those for control (Fig. 5, bars 4 and 6, respectively). The involvement of caspase 3 in this process was previously demonstrated by incubation of the lysates with DEVD-CHO (*N*-Acetyl-Asp-Glu-Val-Asp-aldehyde), a caspase inhibitor (Alexis Biochemical, Paris, France) (Crenesse et al., 2000a).

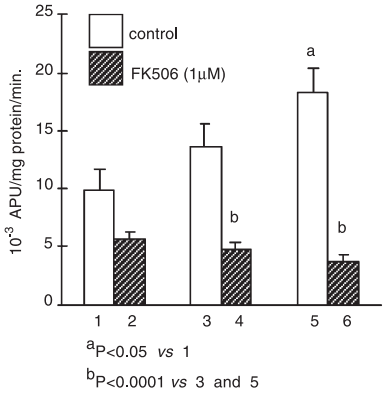

Fig. 5. Tacrolimus reduced apoptosis in cultured hepatocytes. Apoptosis was evaluated by measuring the stimulation of caspase 3 activity. The involvement of caspase 3 was assessed by measuring hydrolysis of the chromogenic CPP32-like substrate DEVD-pNA. Compared to control condition (bar 1), caspase activity increased after 1-h hypoxia (bar 3) but to a lesser extent compared to that observed after 1-h hypoxia followed by 2-h reoxygenation ($P<0.05$) (bar 5). When rat hepatocytes were treated by Tacrolimus (1 μM), caspase 3 activation was considerably reduced ($P<0.0001$) (bars 4 and 6). The figure shows a representative result (mean ± S.E.M.) from five independent experiments.

4. Discussion

The mechanism by which ischemia–reperfusion leads to liver injury is partially understood. It results in necrosis and apoptosis, which may occur in parallel, both contributing to cell death and liver disease (Jaeschke and Farhood, 1991; Kohli et al., 1999). In the present study, we demonstrated that both hepatocyte apoptosis and necrosis were decreased in tacrolimus-treated rats.

During ischemia–reperfusion, Kupffer cells are activated and release cytokines (especially Tumor Necrosis Factor-α, TNF-α) which activate chemokine secretion leading to neutrophil sequestration. Several hours later, neutrophil-induced injury and oxidant stress lead to hepatocellular necrosis (Colleti et al., 1996). The central role of hepatocyte and sinusoidal cell apoptosis in the pathogenesis of ischemia–reperfusion liver injury has been demonstrated (Bradham et al., 1997; Garcia-Criado et al., 1997; Borghi-Scoazec et al., 1997; Kohli et al., 1999) but the exact mechanism inducing apoptosis, probably multifactorial, is still unknown. Apoptosis can be induced by TNF-α (Hewitt et al., 1995), Fas/Fas ligand interaction (Pinkoski et al., 2000) and by oxygen free radical release (Gasbarrini et al., 1998) alone or in combination.

In a model of warm ischemia similar to that used in the present work, we demonstrated the critical importance of apoptosis to liver viability, as the treatment of rats with caspase inhibitor fully protected them from by ischemia–reperfusion-induced death (Cursio et al., 1999). However, apoptosis inhibition might have deleterious effects such as induction of malignant diseases (Rocken and Carl-Mc Grath, 2001), especially after transplantation because long-term immunosuppressive treatment is necessary (Cacciarelli et al., 1998; Sunyecz et al., 1996). In our study, this malignancy risk is probably very low because only one tacrolimus injection was used (24 h before ischemia) and the half-life of tacrolimus is approximately 12 h (Wallemacq and Verbeeck, 2001).

We observed the tacrolimus-induced improvement of survival previously demonstrated in rats (Okano et al., 1994), dogs (Dhar et al., 1993) and pigs (Kim et al., 1994) after warm ischemia–reperfusion of the liver. Several mechanisms have been suggested to explain these protective effects: in addition to its well-known inhibitory effect on the production of interleukin 2, tacrolimus can significantly reduce TNF-α, interleukin 1, interleukin 6 levels (Ishii et al., 1994) and decrease $ICAM_1$ induction (Otto et al., 1998). FK506 can also inhibit the production of liver tissue oxygen free radicals and liver infiltration by neutrophils (Garcia-Criado et al., 1997). It is well known that glucocorticoids are able to induce apoptosis. Ishizuka et al. (1997) have shown that FK506 has no effect on dexamethasone-induced apoptosis in a T lymphoblastoid cell line whereas Ellouk-Achard et al. (1997) have demonstrated that dexamethasone increases FK506 toxicity. Induction was achieved with dexamethasone (200 mg/kg per day for 4 days) before hepatocyte culture. Our

results were obtained in vivo without dexamethasone induction, and in vitro dexamethasone was used only during the overnight attachment period. All plates were subjected to the same culture conditions, and for every oxygenation state results were compared among FK506-treated or non-treated samples. Hypoxia-reoxygenation stress was performed without this drug and the tacrolimus concentration was lower (1 μM vs. 5–150 μM) than that used by Ellouk-Achard et al. (1997). Likewise, FK506 enhanced apoptosis induced by antigen in peripheral T cells (Migita et al., 1999) but these results were obtained with a different cell stress model and with a higher dose of tacrolimus (2 mg/kg vs. 300 μg/kg). In our study, the generally used therapeutic dose was selected.

Several signal transduction pathways leading to apoptosis have recently been described. These pathways link surface receptors such as the Fas or TNF-α receptor with a family of caspases responsible for the initiation of programmed cell death. Colletti et al. (1990) have shown that liver ischemia results in the rapid release of TNF-α. Recently, Rüdiger and Clavien (2002) identified TNF-α as a crucial inducer of apoptotic cell death in the murine ischemic liver. Oxidative stress could be a "facilitator" of TNF-α-mediated hepatocytes apoptosis. Stress-Activated-Protein-Kinases (SAPKs), activated by tyrosine/threonine phosphorylation, are one of the targets of environmental stress and cytokines and are associated with the induction of apoptosis in various cells (Chen et al., 1996). We have demonstrated, in hepatocytes subjected to hypoxia-reoxygenation, that $JNK_1/SAPK_1$ activity was significantly increased and induced apoptosis (Crenesse et al., 2000a,b). Inhibition of $JNK_1/SAPK_1$ activation led to a decrease in apoptosis (Crenesse et al., 2000a). During post-anoxic reoxygenation, oxygen free radical release has been demonstrated (Caraceni et al., 1995; Rauen et al., 1999), and under our stress conditions oxygen free radical inhibition reduced $JNK_1/SAPK_1$ activation (Crenesse et al., 2000b).

The FK506-induced inhibition of $JNK_1/SAPK_1$ activation occurs in neuronal cultures (Klettner et al., 2001; Yardin et al., 1998) but this present study demonstrates for the first time, in both in vivo and in vitro liver experiments, the protective ability of tacrolimus against $JNK_1/SAPK_1$ activation. In the ischemia–reperfusion model, the protective effect of FK506 was associated with a decrease in apoptosis, measured by the TUNEL method, and by a decrease in caspase 3 activation, 4 h after reperfusion. In addition, in cultured hepatocytes isolated from other liver cells (especially Kupffer cells releasing TNF-α), tacrolimus pretreatment reduced $JNK_1/SAPK_1$ activation and subsequent apoptosis. These data were in agreement with those of Herr et al. (1999) in neurons. In ischemia–reperfusion brain damage, Herr et al. (1999) have shown that tacrolimus decreases apoptosis by inhibiting ceramide release.

The intracellular mechanisms by which tacrolimus protects hepatocytes against ischemia–reperfusion-induced apoptosis and necrosis remain unclear. In the whole liver subjected to ischemia–reperfusion, tacrolimus reduces TNF-α release, which contributes to apoptosis and necrosis (Jaeschke and Farhood, 1991; Kohli et al., 1999). But we demonstrated that tacrolimus has a protective effect directly on isolated hepatocytes. Two other mechanisms may be involved: (i) Tacrolimus pretreatment prevents the cellular Ca^{2+} overload during reperfusion following ischemia in dogs (Dahr et al., 1996) and we have recently shown that Ca^{2+} inhibitors (especially diltiazem) reduce the $JNK_1/SAPK_1$ activation which leads to apoptosis (Crenesse et al., 2002); (ii) FK506 pretreatment decreases oxygen free radical production (Garcia-Criado et al., 1997). Oxygen free radicals are known to cause hepatocellular necrosis (Jaeschke and Farhood, 1991) and we have shown, in primary cultured hepatocytes, that oxygen free radical scavengers reduce $JNK_1/SAPK_1$ activation (Crenesse et al., 2000b). In this way, we speculate that FK506 pretreatment could reduce deleterious ischemia–reperfusion effects by decreasing oxygen free radical production.

In conclusion, this study has shown that both in an in vivo model of ischemia–reperfusion of the liver and in primary cultured hepatocytes, FK506 pretreatment reduces $JNK_1/SAPK_1$ and caspase 3 activation and subsequent apoptosis. Tacrolimus, which is considered to be indispensable for the prevention of organ rejection after transplantation, could improve, by a single intravenous injection, postoperative liver function by reducing hepatocyte apoptosis and necrosis.

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