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The 21-aminosteroid U74389G prevents the down-regulation and decrease in activity of CYP1A1, 1A2 and 3A6 induced by an inflammatory reaction

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Abbreviations:
CYP, cytochrome P450 isoform
DFB, 3,4-difluorobenzyloxy-5,5-dimethyl-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one

ABSTRACT

In vivo, the 21-aminosteroid U74389G prevents the decrease in cytochrome P450 (P450) activity produced by a turpentine-induced inflammatory reaction (TIIR). To investigate the underlying mechanism of action, four groups of rabbits were used, controls receiving or not U74389G, and rabbits with the inflammatory reaction receiving or not U74389G. Hepatocytes were isolated 48 h later and incubated for 4 and 24 h with the serum of the rabbits. In vivo, the TIIR diminished CYP1A1/2 and 3A6 expression, and enhanced hepatic malondialdehyde (MDA) and nitric oxide ($NO^{\bullet}$) concentrations ($p < 0.05$). U74389G prevented the increase in MDA, as well as the decrease in CYP1A1/2 amounts and activity, but increased CYP3A6 expression by 40% ($p < 0.05$). In vitro, compared with serum from control rabbits (S_{CONT}), incubation of serum from rabbits with TIIR (S_{TIIR}) for 4 and 24 h with hepatocytes from rabbits with TIIR (H_{TIIR}) reduced CYP1A2 and CYP3A6 activity ($p < 0.05$) and increased the formation of $NO^{\bullet}$ and MDA. In rabbits with TIIR pretreated with U74389G, the S_{TIIR+U} failed to reduce CYP1A2 activity or to increase MDA, although increased $NO^{\bullet}$ and further reduced CYP3A6 activity. On the other hand, in hepatocytes harvested from rabbits with TIIR pretreated with U74389G, S_{TIIR} did not decrease CYP1A2 activity and did not enhance MDA, but still increased $NO^{\bullet}$. In vitro, the reduction of CYP1A2 and CYP3A6 activity by S_{TIIR} is not associated to NF-κB activation. In conclusion, U74389G prevents CYP1A1/2 down-regulation and decrease in activity by a double mechanism: hindering the release of serum mediators and by averting intracellular events, effect possibly associated with its antioxidant activity. On the other hand, U74389G up-regulates CYP3A6 but inhibits its catalytic activity.

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DFH,3-hydroxy-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one
1,3DMU, 1,3-dimethyluric acid
H_{CONT}, hepatocytes from a control rabbit
H_{TIIR}, hepatocytes from rabbits with a turpentine-induced inflammatory reaction
H_{TIIR+U}, hepatocytes from rabbits with a turpentine-induced inflammatory reaction and pretreated with U74389G for 72 h
IFN-γ, interferon-γ
IL, interleukin
L-NAME, N^{ω}-nitro-L-arginine methyl ester
LPS, lipopolysaccharide
MDA malondialdehyde
1MU, 1-methyluric acid
3MX, 3-methylxanthine
NF-κB, nuclear factor-κB
$NO^{\bullet}$, nitric oxide
NOS, nitric oxide synthase
P450, cytochrome P450
S_{CONT}, serum from control rabbits
S_{TIIR}, serum from rabbits with a turpentine-induced inflammatory reaction
S_{TIIR+U}, serum from rabbits with a turpentine-induced inflammatory reaction and pretreated with U74389G for 72 h
TIIR, turpentine-induced inflammatory reaction
TNF-α, tumor necrosis factor-α

1. Introduction

In humans, inflammatory reactions of any cause, such as viral and bacterial infections, decrease the clearance of xenobiotics because of the down-regulation and decrease in activity of multiple isoforms of the cytochrome P450 (P450) [1]. In animal models, infectious and non-infectious acute inflammatory reactions, such as those induced by endotoxin and turpentine, diminish the rate of metabolism of xenobiotics [1,2].

In rabbits, a turpentine-induced inflammatory reaction (TIIR) prompts a hepatic stress characterized by enhanced plasma antilipoperoxidant activity [3], increased liver lipid peroxidation, and decrease in reduced glutathione and in the activity of superoxide dismutase, catalase and glutathione peroxidase [4]. In vivo, N^{ω}-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, partially prevents the decrease in P450 activity produced by a TIIR [5]. Incubation of hepatocytes from rabbits with a TIIR (H_{TIIR}) with serum from rabbits with a TIIR (S_{TIIR}) for 4 h reduces P450 total content and activity, and increments lipid peroxidation in H_{TIIR}. The effect of S_{TIIR} on P450 content and activity is dose-dependently prevented by L-NAME and by antioxidants such as *N*-acetylcysteine and dimethylthiourea, and potentiated by inhibitors of superoxide dismutase, catalase and glutathione peroxidase [6].

In vivo, the decrease in P450 activity triggered by a TIIR is completely abrogated by the pretreatment of the rabbits with the 21-aminosteroid U74389G [7]. 21-Aminosteroids elicit an anti-lipid peroxidation probably by means of the inhibition of iron-catalyzed lipid peroxidation [8]. On the other hand, 21-aminosteroids are potent inhibitors of the synthesis of several pro-inflammatory cytokines, such as IL-1β, IL-2, IL-6, IL-8, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) [9,10], effect mediated by the inhibition of the activation of the nuclear factor-κB (NF-κB) transcription factor [11–13].

The decrease in activity and down-regulation of P450 elicited in vivo by a TIIR and in vitro by S_{TIIR} implicates serum mediators, e.g. IL-6 and IL-1β [14,15], and in the hepatocyte, reactive oxygen species (ROS) and nitric oxide ($NO^{\bullet}$) [5,6]. On the other hand, the induction of inducible NOS (NOS2) by IL-1β

and ROS and its inhibition by glucocorticoids are mediated by activation and inhibition of NF-κB, respectively [16,17]. The aim of this study was to document whether U74389G prevents the decrease in activity and down-regulation of hepatic P450 triggered by a TIIR by inhibiting the release of the serum mediators and/or by acting at the level of the hepatocyte preventing NF-κB nuclear translocation.

2. Materials and methods

2.1. *In vivo studies*

Male New Zealand rabbits (1.8–2.2 kg), were obtained from Ferme Charles River (St. Constant, Que., Canada). Rabbits were housed in separate cages and fed water and rabbit chow ad libitum for at least 7 days before being used. Rabbits were randomly allocated to one of four groups: (1) control rabbits ($n = 3$) given an intravenous injection (i.v.) of sterile NaCl 0.9% (1 ml) every 12 h for 72 h, and a subcutaneous (s.c.) injection of sterile NaCl 0.9% (5 ml) distributed over four distinct sites of the back, 24 h after the first i.v. injection of NaCl 0.9%; (2) rabbits ($n = 3$) receiving the 21-aminosteroid U74389G, 3 mg/kg i.v. every 12 h for 72 h, and sterile NaCl 0.9% (5 ml) s.c. distributed over four sites of the back, 24 h after initiating the regimen with U74389G; (3) rabbits ($n = 3$) given an i.v. injection of sterile NaCl 0.9% (1 ml) every 12 h for 72 h, and s.c. turpentine (Recochem, Montréal, Que.) (5 ml) injected over four distinct sites on the back of the rabbits to induce a TIIR [18], 24 h after the beginning of the injections of NaCl 0.9%; (4) rabbits ($n = 3$) receiving U74389G, 3 mg/kg i.v. every 12 h for 72 h, and s.c. turpentine (5 ml) injected over four distinct sites on the back of the rabbits to induce a TIIR, 24 h after the first injection of U74389G. Preliminary studies confirmed that a 3 mg/kg dose of U74389G injected every 12 h achieved a response similar to that described by Galal and du Souich [7]. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals.

Blood (10 ml) was withdrawn in a sterile Vacutainer Brand SST (Becton Dickinson, Mississauga, Ont., Canada) from the rabbits 72 h after initiating the administration of NaCl 0.9% or U74389G. Blood was left at room temperature for at least 2 h, and thereafter centrifuged at 2500 rpm for 5 min to obtain the serum. When serum samples are handled as described, serum mediators conserve the ability to depress P450 activity for up to 12 months.

Hepatocytes were isolated 48 h after the induction of the TIIR according to the two-step liver perfusion method of Seglen [19], with minor modifications [20]. Hepatocytes (4×10^6 ml^{-1}) were placed into 12-wells plastic culture plates (Falcon, Becton Dickinson Labware, NJ) coated with Type I rat-tail collagen (Sigma Chemical Company, St. Louis, USA); cells were suspended in WME supplemented with 10% calf serum and insulin 1 μM (Boehringer Mannheim GmbH, Germany). The plastic culture plates were incubated at 37 °C in a humidifier with 95% air and 5% CO_2. Viability was assessed before and after the incubation period by the trypan blue (0.2%) exclusion method; in all instances, viability was greater than 90%.

The in vivo effect of TIIR on P450 expression and activity was assessed with hepatocytes incubated with serum from control rabbits (S_{CONT}) for 4 h, since it has been demonstrated that under the present experimental conditions, S_{CONT} does not modify CYP1A1/2 and 3A6 expression or activity [14,20]. Thereafter, activity and protein expression of CYP1A1/2 and 3A6, as well as lipid peroxidation were assessed in the hepatocytes.

Activity of CYP1A1/2 was documented by incubating theophylline with the hepatocytes for 4 h, and measuring the formation of its metabolites, 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU). Theophylline was dissolved in serum-free William's medium E (WME), and 50 μl were added to each well containing the hepatocytes to attain a final concentration of 176 μM. Theophylline is primarily metabolized by CYP1A1/2 [21]. At time zero, 350 μl of the supernatant were collected from each well (control sample), and following 4 and 24 h of incubation, the remaining supernatant was collected and frozen at −20 °C until theophylline, 3MX, 1MU and 1,3DMU were assayed by HPLC [6,20].

The activity of CYP3A6 was determined by measuring the ability of the hepatocytes to convert 3,4-difluorobenzyloxy-5,5-dimethyl-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one (DFB) to 3-hydroxy-4-(4-methylsulfonyl phenyl)-(5H)-furan-2-one (DFH), its fluorescent metabolite [22,23]. The hepatocytes were incubated with 60 μM of DFB for 15 min, and an aliquot of the supernatant was transferred to a microtiter plate and quenched with an equal volume of acetonitrile containing 40% Tris buffer (0.05 M). The fluorescence of DFH was measured at excitation and emission wavelengths of 360 nm and 440 nm, respectively, using a fluorescent plate reader (Wallac Victor^2 1420 Multilabel Counter, Perkin-Elmer) and expressed in arbitrary units.

Nitric oxide was determined by measuring the nitrite and nitrate in the culture media using a colorimetric method based on the Griess reaction [24]. To reduce the nitrate, the samples were incubated at 37 °C in presence of 0.1 U/ml nitrate reductase, 50 μM NADPH and 5 μl FAD. To avoid any interference with the determination of nitrite, NADPH was oxidized by incubating the samples with 10 U/ml lactate dehydrogenase and 10 mM sodium pyruvate for 5 min at 37 °C. Because premixed Griess reagent results in an incomplete azo-dye formation at exposure to light and pH > 1, the following steps were observed: the samples were cooled at 4 °C, and sulfanilamide 1 mM, HCl 0.1 M, and naphthylethylene-diamine 1 mM were added. Nitrite was measured at 540 nm with a fluorescent plate reader (Wallac Victor^2 1420 Multilabel Counter, Perkin-Elmer).

The lipid peroxidation initiated by the TIIR in hepatocytes was assessed measuring the amount of malondialdehyde (MDA) formed by using the thiobarbituric acid reaction [18,25]. Protein content in the hepatocytes was determined by the method of Lowry et al. [26].

The amount of CYP1A1, 1A2 and 3A6 proteins was assessed in the hepatocytes by Western blot analysis. Fifty micrograms of cell extract was separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) under non-reducing conditions [27]. Separated proteins were electrophoretically transferred to a nitro-cellulose membrane using a semi-dry transfer process (Bio-Rad, Hercules, CA, USA). CYP1A1 and 1A2 were detected with a polyclonal anti-rabbit CYP1A1 (Oxford Biochemical Research, Oxford, MI, USA) diluted 1:100 in 5%

nonfat milk in PBS/0.1% Tween 20 and visualized with an alkaline phosphatase conjugated secondary antibody using nitro blue tetrazolium as substrate [28]. CYP3A6 was detected with a monoclonal anti-rat CYP3A1 (Oxford Biochemical Research, Oxford, MI, USA) diluted 1:500 in 5% nonfat milk in PBS/0.1% Tween 20 using a secondary antibody conjugated with chemiluminescence reagent (horseradish peroxidase enzyme) and visualized by autoradiography [29]. Each gel contained 50 μg of extract from the same control hepatocytes, which were used as reference proteins. The assay was linear in the range of protein amounts assessed under the present experimental conditions. The intensities of the bands were measured with the software Un-Scan-It-Gel (Silk Scientific Inc., Orem, UT) and are represented in arbitrary units.

2.2. *In vitro studies*

The in vitro studies aimed to document whether U74389G prevents the decrease in P450 activity by protecting the hepatocyte or by suppressing the release of serum mediators. It was postulated that U74389G will prevent the decrease in P450 activity (a) by means of an intracellular mechanism whenever S_{TIIR} does not decrease P450 activity in H_{TIIR} from rabbits treated with U74389G (H_{TIIR+U}), and/or (b) by preventing the release of serum mediators whenever S_{TIIR} from rabbits treated with U74389G (S_{TIIR+U}) do not decrease P450 activity of H_{TIIR}. To this purpose (1) hepatocytes from control rabbits pretreated with sterile NaCl 0.9% (H_{CONT}), (2) hepatocytes from control rabbits pretreated with U74389G (H_{CONT+U}), (3) hepatocytes from rabbits with a TIIR and pretreated with sterile NaCl 0.9% (H_{TIIR}), and (4) hepatocytes from rabbits with a TIIR and pretreated with U74389G (H_{TIIR+U}) were incubated for 4 and 24 h with 200 μl of (a) S_{CONT}, (b) serum from control rabbits pretreated with U74389G (S_{CONT+U}), (c) S_{TIIR}, and (d) S_{TIIR+U}. Each of the 16 experiments included $n = 9$. Activity of CYP1A1/2, $NO^{\bullet}$ and hepatic lipid peroxidation were assessed as described earlier.

The role NF-κB in the decrease of P450 activity induced by S_{TIIR} and the effect of U74389G was assessed with H_{CONT} ($n = 3$) and H_{TIIR} ($n = 4$). Hepatocytes were cultured in chamber slides for 24 h, and U74389G (5–20 μM) was added. One hour later, S_{CONT} (3–50 μl), S_{TIIR} (3–50 μl), IL-6 (20–80 ng/ml) and IL-1β (5–30 ng/ml) were added to the hepatocytes and incubated for 4 h. Thereafter, H_{CONT} and H_{TIIR} were rinsed with PBS at 4 °C once and fixed with 4% formaldehyde in phosphate-buffered saline for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 30 min at room temperature, and then washed three times with 0.1 M Tris–HCl buffer, pH 7.8. To block unspecific antigenic sites, the wells were incubated for 30 min with 5% nonfat dry milk in 0.1 M phosphate buffer, pH 7.8, at room temperature. After washing three times in 0.1 M Tris buffer, the hepatocytes were incubated overnight with 250 μl of rabbit anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:250 in 0.1 M phosphate buffer, pH 7.8, with 0.1% bovine serum albumin (fraction V; Sigma). The plates were washed three times in Tris buffer and incubated 60 min at room temperature, with 250 μl of the secondary antibody, 488 goat anti-rabbit IgG (H+L) Alexa Fluor antibody 1:250 (Invitrogen Molecular Probe, Burlington, Ont.) [30,31]. The plates are washed three times in Tris–HCl 0.1 M buffer and incubated with 1 μl/ml of 4,6-diamidino-2-phenylindole (DAPI) for 15 min. The plates are washed with water and mounted on slides in Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy on a Nikon Eclipse TE-200 inverted microscope. Images of immunofluorescent labelling were acquired using a Hamamatsu Orca-II digital cooled CCD camera and an Inovision workstation using Isee software (Inovision Corporation, Raleigh, NC, USA).

Nuclear translocation of NF-κB proteins, determined by immunofluorescence, was expressed as the ratio between the number of nuclei stained with secondary goat anti-rabbit IgG antibodies with regard to the total number of nuclei. This parameter was quantified by means of the nucleic acid staining technique with the blue fluorescent DAPI probe. In all cases, a minimum of five fields were examined and assessed.

2.3. *Materials*

Percoll gradient, William's medium E, calf serum, type I rat tail collagen, trypsin inhibitor, NaCl, KCl, KH_2PO_4, EGTA, glucose, theophylline and its metabolites, and L-NAME were purchased from Sigma Chemicals (Sigma, St. Louis, MO, USA), 21-aminosteroid U74389G (21-[4-(2,6-di-l-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione (2)-2-butenedioate; $C_{37}H_{50}N_6O_2{\cdot}C_4H_4O_4$), insulin, nitrate reductase, lactate dehydrogenase and sodium pyruvate from Boehringer Mannheim Biochemica (Mannheim, Germany). Collagenase A was acquired from Worthington Biochemical Corp. (Lakewood, NJ, USA). The 12- and 24-well plastic culture plates were obtained from Falcon, Becton Dickinson Labware (Rutherford, NJ, USA), turpentine from Recochem (Montréal, Que.). hrIL-6 and IL-1β were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Polyclonal anti-rabbit CYP1A1 and monoclonal anti-rat CYP3A1 were from Oxford Biochemical Research (Oxford, MI, USA). DFB and DFH were gracefully provided by Merck Frosst Canada (Kirkland, Que., Canada).

2.4. *Statistical analysis*

All results are reported as mean ± S.E. The comparison of the results from the various experimental groups and their corresponding controls was carried out using a one way analysis of variance (ANOVA) followed by Newman–Keuls post hoc tests. The differences were considered significant when $p \leq 0.05$.

3. Results

3.1. *In vivo effect of U74389G on the expression and activity of CYP1A1/2 and 3A6*

Pretreatment of control rabbits with U74389G for 72 h did not modify hepatic expression of CYP1A1/2, although increased the expression of CYP3A6 by 255% ($p < 0.05$) (Fig. 1). By comparison with H_{CONT}, in H_{TIIR}, the expression of CYP1A1/2 was reduced ($p < 0.05$) and as a consequence, the output of 3MX, 1MU and 1,3DMU was diminished ($p < 0.05$) (Table 1). On

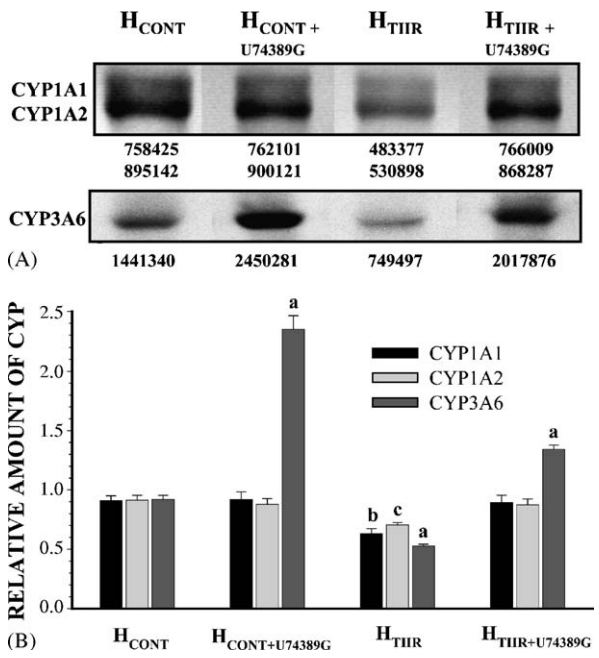

Fig. 1 – (A) Representative bands reflecting the amount of CYP1A1/2 and 3A6 apoproteins in hepatocytes from control rabbits (H_{CONT}), from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}), and rabbits control ($H_{CONT+U74389G}$) and with a turpentine-induced inflammatory reaction ($H_{TIIR+U74389G}$) but pretreated with U74389G for 72 h. Numbers are densitometric values of the bands in arbitrary units. (B) Average amounts of CYP1A1/2 and 3A6 in H_{CONT} and H_{TIIR} from rabbits with or without pretreatment with U74389G relative to a standard protein. Vertical bars are S.E.M. of $n = 3$. Where (a) $p < 0.05$ compared with CYP3A6 in H_{CONT}, (b) $p < 0.05$ compared with CYP1A1 in H_{CONT}, and (c) $p < 0.05$ compared with CYP1A2 in H_{CONT}.

Table 1 – Effect of the 21-aminosteroid U74389G on the biotransformation of theophylline to 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) in hepatocytes from control rabbits or rabbits with a turpentine-induced acute inflammatory reaction receiving or not U74389G and incubated for 4 h with serum from control rabbits or rabbits with a turpentine-induced inflammatory reaction receiving or not U74389G

	3MX	1MU (ng/ml)	1,3DMU
H_{CONT}+			
S_{CONT}	72 ± 10	111 ± 6	1154 ± 55
S_{TIIR}	44 ± 2	101 ± 7	911 ± 31
S_{CONT+U}	74 ± 1	106 ± 6	1038 ± 47
S_{TIIR+U}	73 ± 9	112 ± 5	1047 ± 90
H_{CONT+U}+			
S_{CONT}	70 ± 2	125 ± 4	1002 ± 34
S_{TIIR}	70 ± 3	119 ± 4	1037 ± 45
S_{CONT+U}	72 ± 2	114 ± 8	939 ± 30
S_{TIIR+U}	76 ± 3	123 ± 5	924 ± 21
H_{TIIR}+			
S_{CONT}	34 ± 1[a]	76 ± 2[a]	765 ± 33[a]
S_{TIIR}	25 ± 1[b]	57 ± 1[b]	579 ± 12[b]
S_{CONT+U}	35 ± 2	720 ± 3	752 ± 34
S_{TIIR+U}	35 ± 1	71 ± 16	732 ± 16
H_{TIIR+U}+			
S_{CONT}	71 ± 2	107 ± 1	992 ± 17
S_{TIIR}	66 ± 2	107 ± 4	931 ± 20
S_{CONT+U}	73 ± 2	108 ± 4	943 ± 27
S_{TIIR+U}	71 ± 3	107 ± 4	948 ± 14

H_{CONT} are hepatocytes from control rabbits pretreated with vehicle; H_{CONT+U} are hepatocytes from control rabbits pretreated with U74389G; H_{TIIR} are hepatocytes from rabbits with a turpentine-induced inflammatory reaction pretreated with vehicle; H_{TIIR+U} are hepatocytes from rabbits with a turpentine-induced inflammatory reaction pretreated with U74389G; S_{CONT}, S_{TIIR}, S_{CONT+U}, and S_{TIIR+U} are serum from control rabbits and from rabbits with a turpentine-induced inflammatory reaction without or with pretreatment with U74389G.

[a] $p < 0.01$ compared with H_{CONT} incubated with S_{CONT}.

[b] $p < 0.05$ compared with H_{TIIR} incubated with S_{CONT}.

the other hand, in H_{TIIR} the expression of CYP3A6 was diminished by 43% ($p < 0.05$) and its activity by 80%, e.g. 14551 ± 678 versus 2743 ± 105 (arbitrary units) ($p < 0.05$). Pretreatment of the rabbits with a TIIR with U74389G for 72 h abrogated the down-regulation of CYP1A1/2 and increased CYP3A6 expression by 46% (Fig. 1).

Compared with H_{CONT}, in H_{TIIR} lipid peroxidation, assessed by the production of MDA, increased two-fold ($p < 0.05$; Table 2). Pretreatment of the rabbits with U74389G prevented the increase in lipid peroxidation in H_{TIIR}. On the other [hand, in H_{CONT}, the concentration of $NO^{\bullet}$ was 0.18 ± 0.03 μM/mg protein, and increased to 2.11 ± 0.40 μM/mg protein in H_{TIIR} ($p < 0.05$), increase not reverted by pretreatment with U74389G, e.g. $NO^{\bullet}$ was 2.05 ± 0.32 μM/mg protein.

3.2. *In vitro effect of U74389G*

3.2.1. *Effect of serum of rabbits pretreated with U74389G (S_{CONT+U} and S_{TIIR+U}) on hepatocytes*

3.2.1.1. *Incubation for 4 h.* Incubation of H_{CONT} with S_{TIIR} for 4 h did not reduce the activities of CYP1A1/2 and CYP3A6 (Tables 1 and 3). The presence of U74389G did not influence the activity of CYP1A1/2 but reduced the activity of CYP3A6 by 50% in H_{CONT} (Table 3). On the other hand, incubation of H_{TIIR} with S_{TIIR} for 4 h reduced the activity of CYP1A1/2 and CYP3A6 by around 25 and 40%, respectively ($p < 0.05$). The effect of S_{TIIR} on CYP1A1/2 activity was totally averted when the rabbits with a TIIR were pretreated with U74389G, and S_{TIIR+U} was incubated with H_{TIIR}. In contrast, in presence of U74389G, S_{TIIR} produced a greater reduction of CYP3A6 activity than alone (Table 3). Incubation of H_{CONT} and H_{TIIR} with S_{TIIR} for 4 h increased hepatic MDA ($p < 0.05$) (Table 2), although S_{TIIR+U} did not enhance MDA concentrations.

3.2.1.2. *Incubation for 24 h.* Incubation of H_{CONT} and H_{TIIR} with S_{TIIR} for 24 h diminished the activity of both CYP1A1/2 and CYP3A6 ($p < 0.05$) (Tables 3 and 4). However, when H_{CONT} and H_{TIIR} were incubated with S_{TIIR+U} for 24 h, the rate of biotransformation of theophylline was not modified. On the other hand, when S_{TIIR} was incubated with H_{CONT} and H_{TIIR} in presence of U74389G, CYP3A6 activity decreased further (Table 3). The expression of CYP1A1/2 and CYP3A6 in H_{CONT}

Table 2 – Formation of malondialdehyde (pmol/mg protein) following the incubation of sera from rabbits with hepatocytes of rabbits for 4 and 24 h

	S_{CONT}	S_{CONT+U}	S_{TIIR}	S_{TIIR+U}
H_{CONT}				
4 h	50 ± 3	50 ± 3	70 ± 3[a]	52 ± 3
24 h	115 ± 5	116 ± 4	154 ± 6[a]	115 ± 5
H_{CONT+U}				
4 h	56 ± 4	60 ± 3	62 ± 3	61 ± 3
24 h	118 ± 6	119 ± 6	123 ± 3	112 ± 6
H_{TIIR}				
4 h	107 ± 4[b]	100 ± 4[b]	134 ± 5[a,b]	111 ± 5[b]
24 h	215 ± 7[b]	201 ± 4[b]	233 ± 6[a,b]	216 ± 7[b]
H_{TIIR+U}				
4 h	51 ± 2	57 ± 3	66 ± 3[a]	54 ± 2
24 h	126 ± 5	123 ± 4	131 ± 5	125 ± 5

S_{CONT}, S_{CONT+U}, S_{TIIR}, R_{TIIR+U} are serum from control rabbits, rabbits pretreated for 72 h with U74389G, with a turpentine-induced inflammatory reaction and pretreated with U74389G pretreated for 72 h with U74389G, respectively. H_{CONT}, H_{CONT+U}, H_{TIIR}, H_{TIIR+U} are hepatocytes from control rabbits, rabbits pretreated for 72 h with U74389G, with a turpentine-induced inflammatory reaction and pretreated with U74389G pretreated for 72 h with U74389G, respectively.

[a] $p < 0.05$ compared with S_{CONT}.

[b] $p < 0.05$ compared with H_{CONT}.

and H_{TIIR} was not modified by the 24 h incubation with S_{CONT}, S_{CONT+U}, S_{TIIR}, and S_{TIIR+U} (data not shown).

By reference to S_{CONT}, incubation of H_{CONT} and H_{TIIR} with S_{TIIR} for 24 h increased the concentration of malondialdehyde ($p < 0.05$) (Table 2). Although, in rabbits pretreated with U74389G, S_{TIIR+U} did not increase malondialdehyde concentrations in H_{CONT} and H_{TIIR}.

In H_{CONT} incubated with S_{CONT} for 24 h, $NO^{\bullet}$ concentration was 0.38 ± 0.03 μM/mg protein, and after incubation with S_{TIIR}, $NO^{\bullet}$ concentration increased to 0.73 ± 0.02 μM/mg protein ($p < 0.05$). The increase in $NO^{\bullet}$ produced by S_{TIIR} was not prevented by pretreatment with U74389G, e.g. S_{TIIR+U} increased $NO^{\bullet}$ to values of 0.82 ± 0.01 μM/mg protein. In H_{TIIR}, $NO^{\bullet}$ mean concentration was almost 10-fold greater than in H_{CONT}, e.g. 2.55 ± 0.64 μM/mg protein ($p < 0.05$). Incubation of H_{TIIR} with S_{TIIR} further augmented the concentration of $NO^{\bullet}$ (Fig. 2), increase not prevented by the pretreatment of the rabbits with U74389G, e.g. S_{TIIR+U} enhanced $NO^{\bullet}$ concentration by 67% ($p < 0.05$) in H_{TIIR}.

Table 3 – Effect of the 21-aminosteroid U74389G on the activity of CYP3A6 in hepatocytes from control rabbits or rabbits with a turpentine-induced acute inflammatory reaction

	In vitro 4 h incubation		In vitro 24 h incubation	
	H_{CONT}	H_{TIIR}	H_{CONT}	H_{TIIR}
S_{CONT}	14023 ± 387	2592 ± 365	6721 ± 135	1652 ± 27
S_{TIIR}	14781 ± 354	1612 ± 172[a]	5946 ± 232[a]	1550 ± 16[a]
S_{TIIR+U}	6812 ± 376[a]	1048 ± 42[b]	3779 ± 458[b]	1023 ± 58[b]

H_{CONT} are hepatocytes from control rabbits pretreated with vehicle; H_{TIIR} are hepatocytes from rabbits with a turpentine-induced inflammatory reaction pretreated with vehicle. S_{CONT}, S_{TIIR}, and S_{TIIR+U} are serum from control rabbits and from rabbits with a turpentine-induced inflammatory reaction with pretreatment with U74389G.

[a] $p < 0.01$ compared with S_{CONT}.

[b] $p < 0.05$ compared with S_{CONT} and S_{TIIR}.

3.2.2. *Effect of serum on hepatocytes of rabbits pretreated with U74389G (H_{CONT+U} and H_{TIIR+U})*

3.2.2.1. *Incubation for 4 h.* In H_{CONT+U} and in H_{TIIR+U}, S_{TIIR} did not affect CYP1A1/2 activity (Table 1). In H_{TIIR+U} incubated with S_{CONT}, the concentration of MDA was similar to that measured in H_{CONT+U} incubated with S_{CONT} (Table 2). However, when S_{TIIR} was incubated with H_{TIIR+U}, MDA increased by around 25% ($p < 0.05$).

3.2.2.2. *Incubation for 24 h.* In H_{CONT+U} and in H_{TIIR+U}, S_{TIIR} and S_{TIIR+U} did not modify the output of theophylline metabolites (Table 4), and the activity of CYP3A6 was too low to be reliably

Table 4 – Effect of the 21-aminosteroid U74389G on the biotransformation of theophylline to 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) in hepatocytes from control rabbits or rabbits with a turpentine-induced acute inflammatory reaction receiving or not U74389G and incubated for 24 h with serum from control rabbits or rabbits with a turpentine-induced inflammatory reaction receiving or not U74389G

	3MX	1MU (ng/ml)	1,3DMU
H_{CONT}+			
S_{CONT}	150 ± 4	190 ± 12	4520 ± 79
S_{TIIR}	140 ± 5	120 ± 11[a]	3580 ± 24[a]
S_{CONT+U}	140 ± 5	180 ± 12	4000 ± 73
S_{TIIR+U}	150 ± 6	200 ± 11	4310 ± 98
H_{CONT+U}+			
S_{CONT}	140 ± 3	170 ± 8	4150 ± 135
S_{TIIR}	140 ± 2	150 ± 3	3960 ± 77
S_{CONT+U}	140 ± 3	170 ± 8	4090 ± 121
S_{TIIR+U}	140 ± 2	160 ± 7	3870 ± 259
H_{TIIR}+			
S_{CONT}	50 ± 1[a]	110 ± 5[a]	2210 ± 79[a]
S_{TIIR}	40 ± 2[b]	70 ± 1[b]	1460 ± 24[b]
S_{CONT+U}	50 ± 3	100 ± 4	2220 ± 73
S_{TIIR+U}	50 ± 2	100 ± 9	2150 ± 99
H_{TIIR+U}+			
S_{CONT}	140 ± 4	170 ± 8	4250 ± 37
S_{TIIR}	130 ± 3	150 ± 2	4150 ± 37
S_{CONT+U}	130 ± 2	160 ± 4	4160 ± 54
S_{TIIR+U}	140 ± 4	170 ± 7	4180 ± 64

H_{CONT} are hepatocytes from control rabbits pretreated with vehicle; H_{CONT+U} are hepatocytes from control rabbits pretreated with U74389G; H_{TIIR} are hepatocytes from rabbits with a turpentine-induced inflammatory reaction pretreated with vehicle; H_{TIIR+U} are hepatocytes from rabbits with a turpentine-induced inflammatory reaction pretreated with U74389G. S_{CONT}, S_{TIIR}, S_{CONT+U}, and S_{TIIR+U} are serum from control rabbits and from rabbits with a turpentine-induced inflammatory reaction without or with pretreatment with U74389G.

[a] $p < 0.01$ compared with H_{CONT} incubated with S_{CONT}.

[b] $p < 0.05$ compared with H_{TIIR} incubated with S_{CONT}.

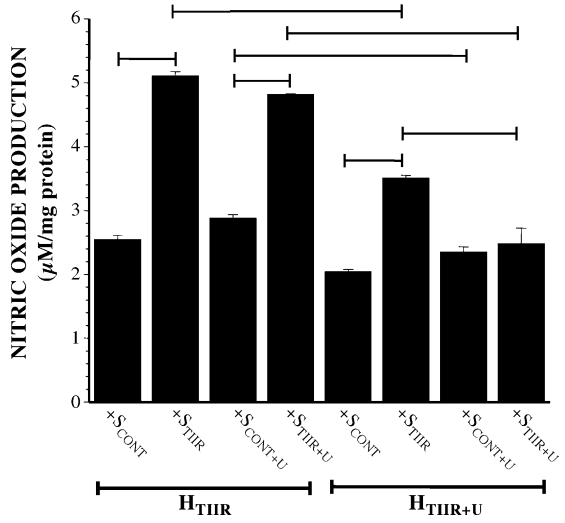

Fig. 2 – Concentration of nitric oxide in the supernatant of hepatocytes from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}) and in hepatocytes from rabbits with a turpentine-induced inflammatory reaction and pretreated with U74389G (H_{TIIR+U}) incubated for 24 h with serum from control rabbits (S_{CONT}) and serum from rabbits with a turpentine-induced inflammatory reaction (S_{TIIR}) without or with pretreatment with U74389G (S_{CONT+U}; S_{TIIR+U}). Upper horizontal bars indicate group differences $p < 0.05$. Vertical bars are S.E.M.

measured. The amounts of CYP1A1/2 and 3A6 in H_{CONT+U} or H_{TIIR+U} were not altered following 24 h of incubation with S_{CONT} or S_{TIIR} (data not shown). The amount of MDA formed in H_{CONT+U} and in H_{TIIR+U} after 24 h of incubation with S_{TIIR} or S_{TIIR+U} remained unchanged (Table 2). By comparison with S_{CONT}, incubation of H_{TIIR+U} with S_{TIIR} for 24 h raised the concentration of $NO^{\bullet}$ ($p < 0.05$), however significantly less than in H_{TIIR} ($p < 0.05$) (Fig. 2). In addition, in H_{TIIR+U}, S_{TIIR+U} did not increase the concentration of $NO^{\bullet}$.

3.3. *Effect of U74389G on NF-κB nuclear translocation*

Incubation of S_{CONT} with H_{CONT} (Fig. 3, panel A) and H_{TIIR} (Fig. 3, panel B) did not activate the nuclear translocation of NF-κB as shown by the absence of nuclear fluorescence. Similarly, S_{TIIR} and IL-6 incubated with H_{TIIR} did not activate NF-κB nuclear translocation (Fig. 3, panels C and D). On the other hand, IL-1β dose-dependently activated NF-κB nuclear translocation (Fig. 3, panel E), effect partially prevented by U74389G at the concentrations assayed (Fig. 3, panel F), e.g. the number of fluorescent nuclei were reduced by around 60% ($p < 0.05$) in presence of U74389G.

4. Discussion

The present study shows that in vivo pretreatment of rabbits with the 21-aminosteroid U74389G prevents the down-regulation of CYP1A1/2 and CYP3A6 provoked by a TIIR; moreover, U74389G increases CYP3A6 expression. In vitro, incubation of H_{CONT} and H_{TIIR} with S_{TIIR} for 24 h reduces the activity of CYP1A1/2 and CYP3A6 without affecting their expression, and enhances $NO^{\bullet}$ and MDA concentrations; although, S_{TIIR+U} did not reduce CYP1A1/2 activity in H_{CONT} and H_{TIIR}. In contrast, S_{TIIR+U} decreased CYP3A6 activity in H_{CONT} and H_{TIIR} to a greater degree than did S_{TIIR} alone. S_{TIIR+U} increased $NO^{\bullet}$ concentrations as did S_{TIIR}, but did not enhance intrahepatic MDA concentrations. On the other hand, in H_{TIIR+U}, S_{TIIR} did not reduce CYP1A1/2 activity, even if MDA and $NO^{\bullet}$ concentrations were slightly but significantly increased, albeit less than in H_{TIIR} by S_{TIIR}. These results show that the presence U74389G modulates the effect of an inflammatory

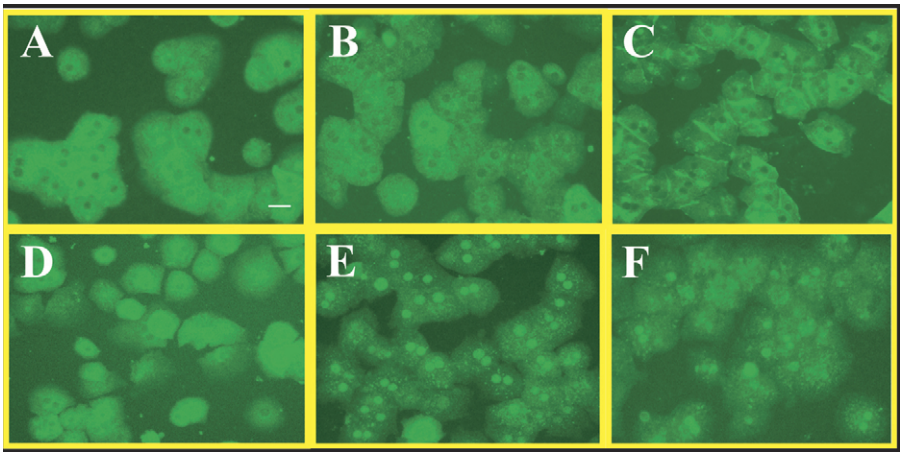

Fig. 3 – Fluorescent micrographs representing p65 NF-κB in hepatocytes (20×). In control hepatocytes (panel A) as well as in hepatocytes harvested from rabbits with the turpentine-induced inflammatory reaction, alone (panel B) or incubated for 4 h with serum from rabbits with a turpentine-induced inflammatory reaction (50 μl) (panel C), and IL-6 (20 ng/ml) (panel D) nuclei do not depict fluorescence indicating that under these experimental conditions the nuclear translocation of NF-κB is not activated. Incubation of hepatocytes harvested from rabbits with the turpentine-induced inflammatory reaction with IL-1β (15 ng/ml) activates NF-κB nuclear translocation (panel E), effect partially abrogated by the pre-incubation with U74389G (10 μM) (panel F). Scale bar in (A): 20 μm.

reaction on CYP1A1/2 and CYP3A6 differently, e.g. U74389G prevents the decrease of expression and activity of CYP1A1/2, and increases the expression of CYP3A6 but reduces its catalytic activity. The response to U74389G is secondary to an effect at the level of the serum mediators and at the level of the hepatocyte.

In vitro, S_{TIIR} and IL-6 do not activate NF-κB nuclear translocation, indicating that the reduction in P450 activity and the increase in $NO^{\bullet}$ concentrations produced by S_{TIIR} are not mediated by NF-κB. On the other hand, IL-1β did activate NF-κB translocation, effect abrogated by U74389G. These observations agree with the report of Iber et al. [32] who showed that a mutation of NF-κB prevented the down-regulation of CYP2C11 by IL-1β but did not impede IL-6 to down-regulate CYP2C11, indicating that IL-1β but not IL-6 effect on *CYP2C11* gene was mediated by NF-κB.

In vivo, the decrease in CYP1A1/2 expression and activity by a turpentine-induced inflammatory reaction is mediated by circulating IL-6 and IL-1β [15,33]. The 21-aminosteroid U74389G is an antioxidant and reduces or prevents lipid peroxidation [7,8,34,35]. There is evidence that the antioxidant effect elicited by U74389G suppresses the degradation of IκB proteins and consequently, prevents the activation NF-κB nuclear translocation [11–13] and as a consequence, prevents the up-regulation of pro-inflammatory cytokines IL-1β and TNF-α [36-38], required to increase the hepatic production of IL-6 and IL-1β [9,11,12,39]. Therefore, in vivo U74389G may have prevented the down-regulation of CYP1A1/2 by diminishing the serum mediators. The fact that in H_{TIIR} harvested 48 h after the injection of turpentine there was no evidence of increased nuclear NF-κB translocation (Fig. 3, panel B) does not deter the possibility that the decrease of serum mediators may be associated to a reduction in NF-κB nuclear translocation. This is because the inflammatory reaction triggered by turpentine increases NF-κB nuclear translocation in the liver and in other organs, although transiently, and nuclear NF-κB returns to baseline levels after 12 h [40–42]. In vitro, the decrease in CYP1A1/2 activity produced by S_{TIIR} in H_{TIIR} depends primarily upon IL-6 [14]. The fact that S_{TIIR+U} does not reduce CYP1A1/2 activity, suggests that U74389G prevents the release of IL-6.

There is evidence that $NO^{\bullet}$ is involved directly or indirectly in the decrease in activity of P450 isoforms in septic and aseptic inflammatory reactions [43,44]. Perfusion of sodium nitroprusside into isolated rat livers reduce many P450 isoforms [45]. Moreover, pretreatment of the rabbits in vivo with L-NAME diminishes the decrease in CYP1A1/2 activity produced by a TIIR [5,46]. In vitro, L-NAME reduces dose-dependently the decrease in P450 activity produced by S_{TIIR} in H_{TIIR} [6]. It is interesting to note that S_{TIIR+U} did not reduce CYP1A1/2 activity in H_{TIIR}, in spite of an increase in $NO^{\bullet}$, and that S_{TIIR} did not affect CYP1A1/2 activity in H_{TIIR+U} also in spite of an increase in $NO^{\bullet}$; however, in both experimental conditions, lipid peroxidation was decreased or absent. This observation suggests that the increase in $NO^{\bullet}$ is not directly associated with decrease in CYP1A1/2 activity.

To reconcile the published evidence and the actual observation that U74389G prevents the S_{TIIR}-induced decrease in CYP1A1/2 activity despite increased concentrations of $NO^{\bullet}$, U74389G may have prevented the formation of peroxynitrite by diminishing reactive oxygen species, as supported by the decrease in hepatic lipid peroxidation. Peroxynitrite reduces P450 activity primarily by nitration of tyrosine at residues 190 and 203 with concomitant loss of P450 isoforms [47–49]. However, this does not appear to be the mechanism underlying the S_{TIIR}-induced reduction in P450 activity, since TIIR nitrates tyrosine of CYP1A1/2 and CYP3A6 in H_{CONT} as well as in H_{TIIR} without loss of activity in the former [50]. On the other hand, direct phosphorylation of serine residues by protein kinase C (PKC) or other kinase will abrogate P450 enzyme catalytic activity [51–53]. Peroxynitrite is capable to activate several kinases, e.g. the phosphoinositide 3-kinase/Akt pathway and Erk1/2 [54,55]. Since bisindolylmaleimide, an inhibitor of PKC, and PD98059, an inhibitor of Erk1/2, partially prevent the decrease in CYP1A1/2 activity produced by S_{TIIR} [46,50], we are tempted to hypothesize that U74389G protected CYP1A1/2 activity from S_{TIIR} by means of its antioxidant properties and by preventing the formation of peroxynitrite.

It is noteworthy that U74389G not only prevents CYP3A6 down-regulation but it increases the expression of CYP3A6, even in presence of TIIR. The expression of CYP3A6 is modulated by several nuclear receptors, such as constitutive androstane receptor (CAR), pregnane × receptor (PXR), retinoid × receptor (RXR), hepatocyte nuclear factor 4 (HNF-4), and glucocorticoid receptor (GR) [56]. Contrary to other CYP3A6 inducers, e.g. dexamethasone, U74389G does not bind or increase the expression of the GR [57]. There is no information concerning the effect of U74389G on CAR, PXR, RXR, and HNF-4. However, being U74389G a pregnatriene derivative, we might speculate that U74389G activates PXR since this nuclear receptor binds numerous pregnane derivatives known to be CYP3A4 inducers [58,59].

On the other hand, U74389G reduces CYP3A6 activity. It is of interest that other antioxidants, such as pyrrolidine dithiocarbamate, an inhibitor of NF-κB, reduces CYP3A6 activity without changing its expression [50]. Moreover, numerous antioxidants are inhibitors of CYP3A4 catalytic activity, such as silymarin, polyphenols, isoflavans, quercetin and ginsenoside, peppermint oil, menthol, menthyl acetate, and ascorbyl palmitate [60–63]. The mechanism underlying CYP3A6 inhibition by antioxidants is unknown, but two mechanisms, not mutually exclusive, could be proposed, firstly antioxidants diminish the availability of ROS necessary for CYP3A6-dependent substrate oxidation [64], and secondly, antioxidants also inhibit NADPH-cytochrome P450 reductase and the transfer of electrons required for P450 catalytic activity [65].

In conclusion, pretreatment of rabbits in vivo with U74389G prevents the down-regulation of CYP1A1/2 triggered by a TIIR. The mechanism of action underlying the effect of U74389G is double; on the one hand, U74389G modulates serum mediators and on the other hand, it regulates the cellular response. Since the in vitro reduction of CYP1A1/2 activity by S_{TIIR} is not associated to NF-κB activation, the mechanism of action underlying the protection conferred by U74389G on H_{TIIR} is possibly associated with its antioxidant activity. Finally, in vivo, U74389G not only prevents the down-regulation of CYP3A6 produced by the TIIR but it is a potent enzyme inducer of CYP3A6, however it inhibits its activity.

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