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Signal transduction pathways implicated in the decrease in CYP1A1, 1A2 and 3A6 activity produced by serum from rabbits and humans with an inflammatory reaction

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Abstract

Incubation of serum from rabbits with a turpentine-induced inflammatory reaction and from humans with an upper respiratory viral infection with hepatocytes from rabbits with a turpentine-induced inflammatory reaction for 4 h reduces total cytochrome P450 content and activity of cytochrome P450 isoforms CYP1A1/1A2 and 3A6 without affecting the expression of these proteins. To document the signal transduction pathways implicated in the decrease in CYP1A1/1A2 and 3A6 activity, hepatocytes from rabbits with a turpentine-induced inflammatory reaction were incubated with serum from rabbits with a turpentine-induced inflammatory reaction, serum from individuals with a viral infection and interleukin-6 for 4 h in presence of inhibitors of protein kinases. The sera-induced decrease in CYP1A1/1A2 and 3A6 activity was partially prevented by the inhibition of Janus-associated protein tyrosine kinase, double-stranded RNA-dependent protein kinase, protein kinase C, and p42/44 mitogen-activated protein kinase. The serum from rabbits with a turpentine-induced inflammatory reaction increased the phosphorylation of Erk1/2, effect prevented by PD98059 but not by bis-indolylmaleimide, a specific inhibitor of protein kinase C. The results demonstrated that the decrease in total cytochrome P450 content and in CYP1A1/1A2 and 3A6 activity by sera and interleukin-6 involves the activation of protein tyrosine kinases, p42/44 mitogen-activated protein kinase and protein kinase C. Indirect evidence supported that nitric oxide is implicated in the decrease in activity of these enzymes.

Keywords: Cytochrome P450; Activity; Signal transduction pathways; Inflammation; Turpentine; Viral

1. Introduction

Infections and inflammatory reactions trigger an acute phase response that reduces the activity and total cytochrome P450 content [1]. In humans, bacterial pneumonia [2], influenza vaccines [3], and acute influenza infections [4] reduce monooxygenases activity. In animals, the injection of bacteria, viruses, parasites or inflammatory agents, such as turpentine or carrageenan, diminish the amount and drug-metabolizing activity of hepatic cytochrome 450 [5].

In vivo, the down-regulation of cytochrome P450 occasioned by turpentine is preceded by a reduction in cytochrome 450 activity [6]. In vitro, incubation of hepatocytes from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}) for 4 h with serum from individuals with an acute upper respiratory tract viral infection (HS_{URVI}) and serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) reduce the catalytic activity of

Abbreviations: CYP, apoprotein of the cytochrome P450; DFB, 3,4-difluorobenzyloxy-5,5-dimethyl-4-(4-methylsulfonyl phenyl)-(5*H*)-furan-2-one; DFH, 3-hydroxy-4-(4-methylsulfonyl phenyl)-(5*H*)-furan-2-one; 1,3DMU, 1,3-dimethyluric acid; Erk1/2, extracellular signal-related kinase 1/2; H_{CONT}, hepatocytes from a control rabbit; H_{TIIR}, hepatocytes from rabbits with a turpentine-induced inflammatory reaction; HS_{CONT}, serum from healthy volunteers; HS_{URVI}, serum from individuals with an upper respiratory tract viral infection; IFN-γ, interferon-γ; IL, interleukin; JAK, Janus-associated protein tyrosine kinase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; p42/44 MAPK, p42/44 mitogen-activated protein kinases; MDA, malondialdehyde; 1MU, 1-methyluric acid; 3MX, 3-methylxanthine; $NO^{\bullet}$, nitric oxide; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; PKR, double-stranded RNA-dependent protein kinase; PTK, protein tyrosine kinase; RS_{CONT}, serum from control rabbits; RS_{TIIR}, serum from rabbits with a turpentine-induced inflammatory reaction; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor-α

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CYP1A1, 1A2 and 3A6 without affecting their expression [7]. The serum mediators implicated in the decrease in cytochrome P450 activity by RS_{TIIR} and HS_{URVI} are interferon-γ (IFN-γ), interleukin-6 (IL-6) and IL-1β [8]. The signal transduction pathways and mechanisms underlying the decrease in cytochrome P450 activity produced by IFN-γ, IL-6 and IL-1β remain unknown.

Circumstantial evidence suggest that the expression of cytochrome P450 isoforms is modulated IFN-γ, IL-6 and IL-1β through the activation of several signal transduction pathways, such as Janus-associated protein tyrosine kinase (JAK) and the signal transducer and activator of transcription 3 (STAT3), protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), and p42/44 mitogen-activated protein kinases (p42/44 MAPK) [9–14]. Moreover, IFN-γ activates double-stranded RNA-dependent protein kinase (PKR) which is a major negative regulator of translation and consequently, an inhibitor of protein synthesis [15]. The aim of this study was to assess the signal transduction pathways activated by RS_{TIIR}, HS_{URVI}, and IL-6 leading to the decrease in total cytochrome P450 content and CYP1A1, 1A2 and 3A6 activity in hepatocytes.

2. Materials and methods

2.1. Hepatocyte isolation and culture conditions

Male New Zeeland rabbits (2–2.3 kg) (Ferme Cunicole, St. Valérien, Que., Canada) were housed in separate cages and fed water and chow ad libitum for at least 7 days before experiments started. The inflammatory reaction was provoked by local subcutaneous injections of turpentine distributed at four sites of the back of the rabbits (total volume injected 5 ml). The severity of the inflammatory reaction was assessed by taking the rectal temperature and by measuring the concentration of seromucoids [5]. All experiments were conducted according to the Canadian Council on Animal Care guidelines for use of laboratory animals.

Forty-eight hours after the injection of turpentine, a blood sample (15 ml) was withdrawn from the rabbits in a sterile Vacutainer Brand SST (Becton Dickinson, Mississauga, Ont., Canada). Thereafter, hepatocytes were isolated according to the two-step liver perfusion method [16] with minor modifications [7]. Harvested cells were centrifuged on isodensity Percoll to isolate viable liver cells (90% viability as assessed by trypan blue exclusion). Hepatocytes (3×10^6 in 3 ml of William's medium E supplemented with 10% calf serum) were plated in 12-well plastic culture plates (Falcon, Becton Dickinson Labware, Rutherford, NJ, USA) coated with type I rat tail collagen. Cell culture was conducted under sterile conditions and maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The medium was changed 2 h after plating, prior to the addition of serum or IL-6, and thereafter the hepatocytes were incubated for an additional 4 h.

2.2. Preparation of human serum

Human blood (20 ml) was withdrawn from volunteers, either healthy or presenting an inflammatory reaction secondary to an upper respiratory viral infection, at the apex of symptomatology, i.e. 24–48 h after the onset of clinical symptomatology (fever, rhinorrhea, sneezing, nasal congestion, sore throat, cough and systemic signs of malaise). Blood samples were allowed to clot at room temperature for 2 h, centrifuged at 2500 rpm for 5 min, and the serum was decanted and stored frozen at −80 °C in 1 ml aliquots until use. Preliminary studies have shown that when samples were handled as described, serum mediators conserve their activity for up to 12 months. Human sampling protocol was approved by the Comité d'éthique de la Recherché de la Faculté de Médecine of the University of Montréal (CERFM 28(00) 4#78).

Aliquots of 200 μl of serum from rabbits or from human volunteers were incubated for 4 h with hepatocytes from control rabbits (H_{CONT}) and H_{TIIR}, and the effect on cytochrome P450 was assessed by measuring total cytochrome P450 content and CYP1A1/1A2 and 3A6 activity. Since IL-6 is the serum mediator responsible for the decrease in cytochrome P450 activity in rabbits with a turpentine-induced inflammatory reaction [8], recombinant IL-6 was incubated with H_{CONT} and H_{TIIR} for 4 h.

2.3. Signal transduction pathways modulating CYP1A1 and 1A2 activity

There is indirect evidence suggesting that nitric oxide ($NO^{\bullet}$) is implicated in the reduction of CYP1A1/1A2 activity produced by the administration of lipopolysaccharide and by a turpentine-induced inflammatory reaction [17–19]. Therefore, signal transduction pathways leading to the production of $NO^{\bullet}$ were primarily investigated.

The signal transduction pathways implicated in the decrease in CYP1A1/1A2 activity induced by RS_{TIIR} ($n = 7$) were investigated by incubating H_{TIIR} with (a) genistein or 4′,5,7-trihydroxyisoflavone (90 μM), an unspecific inhibitor of JAK [20], (b) 2-aminopurine (0.5 μM), an adenine isomer that inhibits specific kinases that phosphorylate PKR [21], (c) bis-indolylmaleimide (100 nM), a specific inhibitor of PKC [22], (d) PD98059 (10 μM), a specific inhibitor of p42/44 MAPK [23], (e) pyrrolidine dithiocarbamate (10 μM), an antioxidant that specifically inhibits the nuclear factor-κB (NF-κB) pathway by scavenging reactive oxygen intermediates (ROI) and/or by inhibiting ubiquitin ligase activity towards phosphorylated IκBα [24], and (f) N^{ω}-nitro-L-arginine methyl ester (L-NAME; 1 mM), an inhibitor of nitric oxide synthase (NOS) and $NO^{\bullet}$ production. Preliminary studies

confirmed that genistein, 2-aminopurine, bis-indolylmaleimide, PD98059, pyrrolidine dithiocarbamate and L-NAME did not affect total cytochrome P450 content and CYP1A1, 1A2 and 3A6 activities.

In these experiments, hepatocytes were plated in 12-well plastic culture plates, the medium was changed 2 h after plating, and 30 min later the inhibitors were added. Following the incubation of the inhibitors and the hepatocytes for 30 min, 200 μl of RS_{TIIR} ($n = 7$) were added to the H_{TIIR} ($n = 3$) and further incubated for 4 h. Genistein, bis-indolylmaleimide and PD98059 were dissolved in dimethyl sulfoxide, and 2-aminopurine, pyrrolidine dithiocarbamanate and L-NAME in NaCl 0.9%, and 5 μl of the solutions were added to the hepatocytes in culture.

To ensure that the present experiments are in agreement with previous studies, when possible the activity of CYP1A2 and 1A1 was assessed by measuring the rate of formation of theophylline of each individual metabolite, e.g. 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU) [7,8,19,25]. Theophylline was dissolved in serum-free William's medium E, and 100 μl were added to each well containing hepatocytes to attain a final concentration of 176 μM. After 4 h incubation, the medium was collected and frozen at −20 °C until analysis of theophylline, 3MX, 1MU and 1,3DMU by HPLC [25].

In presence of some inhibitors, theophylline could not be used as substrate to assess CYP1A1/1A2 activity because dimethyl sulfoxide interferes with the HPLC assay of theophylline metabolites. Therefore, CYP1A1/1A2 activity was quantified by measuring the methoxyresorufin O-demethylation (MROD) to resorufin in intact cells, as described by Van Vleet et al. [26]. Growth media was removed and cells washed twice with 300 μl of Krebs solution. After removal of Krebs solution, 3.3 μM methoxyresorufin in 300 μl of Krebs solution was added to the wells containing the hepatocytes and incubated for 10 min at 37 °C. Thereafter, 100 μl of the supernatant were added to 100 μl of a solution of perchloric acid/glycine and 5.4% K_2CO_3 (2:1, v/v). Resorufin production was measured fluorimetrically at excitation and emission wavelengths of 530 and 584 nm, respectively, with a fluorescent plate reader (Wallac Victor2 1420 Multilabel Counter, Perkin Elmer).

2.4. Signal transduction pathways modulating CYP3A6 activity

H_{TIIR} were incubated for 30 min with (a) phloretin (500 μM), a chalcone derivative intermediate precursor for all flavonoid compounds, which is an non-specific inhibitor of protein tyrosine kinase (PTK) and PKC [27,28], (b) 2-aminopurine (0.5 μM), (c) bis-indolylmaleimide (100 nM), and (d) PD98059 (10 μM). Phloretin, bis-indolylmaleimide and PD98059 were dissolved in dimethyl sulfoxide, and 2-aminopurine in NaCl 0.9%, and 5 μl of the solutions were added to the hepatocytes in culture. Thirty minutes later, 200 μl of RS_{TIIR} ($n = 7$), 200 μl of HS_{URVI} ($n = 4$), and 20 ng of interleukin-6 were added to the H_{TIIR} ($n = 8$) and further incubated for 4 h. The concentration used of IL-6 was similar to that assayed in the plasma of rabbits with a turpentine-induced inflammatory reaction [29].

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)-(5*H*)-furan-2-one (DFB), a CYP3A probe, to 3-hydroxy-4-(4-methylsulfonyl phenyl)-(5*H*)-furan-2-one (DFH), its fluorescent metabolite [30]. Incubations were performed according to a published procedure [31]. Briefly, 60 μM of DFB were incubated for 20 min with the hepatocytes that had been previously incubated with the inhibitors and the serum. An aliquot of the supernatant was then transferred to a microtiter plate and quenched with an equal volume of acetonitrile containing 40% Tris buffer (0.05 M). The fluorescence metabolite DFH was measured at excitation and emission wavelengths of 360 and 440 nm, respectively, using a fluorescent plate reader (Wallac Victor2 1420 Multilabel Counter, Perkin Elmer).

2.5. Effect of $NO^{\bullet}$ on CYP1A1, 1A2 and 3A6 activity

To assess the effect of $NO^{\bullet}$ on CYP1A1, 1A2 and 3A6 activity, H_{TIIR} ($n = 6$) were incubated with 0.5 mM of sodium nitroprusside [19] in presence and in absence of the inhibitors previously mentioned. Control experiments were conducted by incubating H_{CONT} with RS_{TIIR}, HS_{URVI}, IL-6, sodium nitroprusside, and the kinase inhibitors at the above-mentioned concentrations and total cytochrome P450 content was assessed 4 h later.

2.6. Measure of total cytochrome P450 content and $NO^{\bullet}$

Total cytochrome P450 content was assessed spectrophotometrically by its ability to bind carbon monoxide [32]. Protein content in hepatocytes was measured by the method of Lowry et al. [33]. Nitric oxide was determined by measuring nitrite and nitrate in the culture media using a colorimetric method based on the Griess reaction [34]. To reduce nitrate to nitrite, samples were incubated at 37 °C in the presence of 0.1 U/ml nitrate reductase, 50 μM NADPH and 5 μM FAD. Following nitrate reduction, 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. All along the manuscript, it has been assumed that the concentration of nitrite reflected that of nitric oxide and the results are expressed as concentration of $NO^{\bullet}$.

2.7. Measure of the expression of CYP1A1, 1A2 and 3A6 proteins

The amount of CYP1A1, 1A2 and 3A6 proteins in H_{TIIR} incubated for 4 h with serum and interleukin-6 was assessed by Western blot analysis. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (7.5% polyacrylamide) [35]. Proteins were electrophoretically transferred to a nitro-cellulose membrane using a semidry transfer process (Bio-Rad, Hercules, CA, USA). CYP1A1 and 1A2 proteins were detected with polyclonal anti-rabbit CYP1A1 and visualized with an alkaline phosphatase conjugated secondary goat antibody using blue tetrazolium as the substrate [36]. CYP3A6 protein was detected with a monoclonal anti-rat CYP3A1, with cross-reactivity to rabbit's CYP3A6, and a horseradish peroxidase-conjugated secondary antibody. Chemiluminescence was visualized by autoradiography [37]. In each gel, 50 μg of proteins extracted from the same batch of H_{CONT}, with constant amounts of CYP1A1, 1A2 and 3A6, 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, USA), and the results are presented as the ratio to the reference protein.

2.8. Immunoblot analysis of Erk1 and Erk2

The H_{TIIR} were incubated with RS_{CONT} and RS_{TIIR} in absence and in presence of various doses of PD98059 (10–100 μM) and of bis-indolylmaleimide (100–200 μM). Protein extracts were prepared by homogenization of hepatocytes in lysis buffer. Equal amounts of protein were resolved by SDS–polyacrylamide gel electrophoresis and transferred to nylon membrane. The membrane was blocked in TBS, 0.1% Tween 20, 4% non-fat dried milk, and probed with antibody 1cp44 (1:1000 dilution), which recognizes Erk1 and Erk2 isoforms [38]. To control for protein loading, the blot was stripped and reprobed with anti-β-actin (1:10,000 dilution). We also used an Erk2 N-terminal-specific polyclonal antibody (AB3055; Chemicon) to confirm the absence of expression of N-terminal fragments of Erk2. The anti-Erk1/2 phosphothreonine 183, phosphotyrosine185 (ppErk1/2) E10 monoclonal antibody was purchased from Cell Signaling Technology and was diluted 1:1000 according to manufacturer's instructions.

2.9. Materials

Percoll gradient, William's medium E, calf serum, type I rat tail collagen, trypsin inhibitor, NaCl, KCl, KH_2PO_4, EGTA, glucose, theophylline, 3MX, 1MU acid and 1,3DMU, genistein, 2-aminopurine, bis-indolylmaleimide, pyrrolidine dithiocarbamate and L-NAME were purchased from Sigma Chemicals (Sigma, St. Louis, MO, USA), insulin, nitrate reductase, lactate dehydrogenase and sodium pyruvate from Boehringer Mannheim Biochemica (Mannheim, Germany). Collagenase A was acquired from Worthington Biochemical Corp. Six-well plastic culture plates were obtained from Falcon, Becton Dickinson Labware, (Rutherford, NJ, USA), turpentine from Recochem (Montreal, Que., Canada). Phloretin, PD98059 and hrIL-6 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).

2.10. Statistical analysis

All results are reported as means ± 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 pairwise multiple comparison procedures Tukey test. The differences were considered significant when $P < 0.05$.

3. Results

3.1. Effect of RS_{TIIR}, HS_{URVI} and IL-6 on total cytochrome P450 content and activity

In control rabbits ($n = 11$), mean rectal temperature was 38.7 ± 0.5 °C, and average seromucoids concentration was 21.4 ± 2.8 mg/dl. Whereas in rabbits with a turpentine-induced inflammatory reaction ($n = 11$), rectal temperature was 41.1 ± 0.7 °C ($P < 0.05$) and average seromucoids concentration was 69.8 ± 2.3 mg/dl ($P < 0.05$).

Compared with H_{CONT}, in H_{TIIR} total cytochrome P450 content was diminished ($P < 0.05$) (Table 1), the activity of CYP1A1 and 1A2 was decreased by 30–70% depending upon the metabolite of theophylline considered ($P < 0.05$), and the activity of CYP3A6 was diminished by 41% ($P < 0.05$). Moreover, compared with H_{CONT}, in H_{TIIR}, the amount from CYP1A1 and 1A2 isoforms was reduced by 24 and 35% ($P < 0.05$) and that of CYP3A6 by around 85% (Fig. 1).

In H_{CONT}, total cytochrome P450 content and activity of CYP1A1/1A2 and 3A6 were not affected by 4 h incubation with serum from control rabbits (RS_{CONT}), RS_{TIIR}, serum from healthy volunteers (HS_{CONT}), and HS_{URVI} (data not shown). In H_{TIIR}, these parameters were not affected by RS_{CONT} and HS_{CONT} (data not shown). However, compared with the RS_{CONT}, incubation of H_{TIIR} with RS_{TIIR} for 4 h further decreased total cytochrome P450 content ($P < 0.05$), and the activity of CYP1A1, 1A2 and 3A6 ($P < 0.05$) (Table 1). Similarly, compared with HS_{CONT}, incubation of HS_{URVI} with H_{TIIR} for 4 h, reduced total cytochrome P450 content, as well as the activity of CYP1A1, 1A2 and 3A6 ($P < 0.05$), as reflected by the

Table 1
Effect of a turpentine-induced inflammatory reaction (TIIR), serum from rabbits with a TIIR (RS_{TIIR}) and from individuals with an upper respiratory viral infection (HS_{URVI}), interleukin-6 (IL-6) and sodium nitroprussiate (SNP) on total cytochrome P450 content and activity of CYP1A1, 1A2 and 3A6 in hepatocytes from control (H_{CONT}) and rabbits with a TIIR (H_{TIIR})

	Cytochrome P450 (nmol/mg protein)	CYP1A1/1A2 activity[a]			CYP3A6 activity[a]
		3MX	1MU (ng/ml/min)	1,3DMU	DFH (arbitrary units)
H_{CONT}	0.24 ± 0.06	0.108 ± 0.013	0.275 ± 0.054	6.13 ± 0.85	5160 ± 90
H_{TIIR}	0.14 ± 0.05[b]	0.075 ± 0.038[b]	0.138 ± 0.038[b]	1.86 ± 0.15[b]	3246 ± 141[b]
H_{TIIR} + RS_{CONT}	0.15 ± 0.02	0.092 ± 0.013	0.146 ± 0.050	1.61 ± 0.12	3682 ± 163
H_{TIIR} + RS_{TIIR}	0.10 ± 0.04[c]	0.063 ± 0.010	0.058 ± 0.025[c]	1.18 ± 0.05[c]	2990 ± 72[c]
H_{TIIR} + HS_{CONT}	0.13 ± 0.03	0.096 ± 0.025	0.150 ± 0.033	1.58 ± 0.21	3214 ± 118
H_{TIIR} + HS_{URVI}	0.08 ± 0.03[c]	0.075 ± 0.025	0.083 ± 0.038[c]	1.13 ± 0.31[c]	2720 ± 68[c]
H_{TIIR} + IL-6	0.10 ± 0.03[d]	0.088 ± 0.017	0.067 ± 0.010[d]	1.17 ± 0.34[d]	2600 ± 112[c]
H_{TIIR} + SNP	0.08 ± 0.04[d]	0.043 ± 0.022[d]	0.080 ± 0.022[d]	1.02 ± 0.08[d]	1164 ± 59[d]

Values are mean ± S.E.M. RS_{CONT} and HS_{CONT} are serum from control rabbits and healthy volunteers, respectively.

[a] The activity of CYP1A1/1A2 is determined by the rate of production of each individual metabolite of theophylline, e.g. 3-methylxanthine (3MX), 1-methyluric acid (1MU) and 1,3-dimethyluric acid (1,3DMU). The activity of CYP3A6 was determined by the production of 3-hydroxy-4-(4-methylsulfonyl phenyl)-(5*H*)-furan-2-one (DFH) a fluorescent metabolite.

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

[c] $P < 0.05$ compared with H_{TIIR} + RS_{CONT} or H_{TIIR} + HS_{CONT}.

[d] $P < 0.05$ compared with H_{TIIR}.

production of theophylline metabolites and of DFH (Table 1). However, incubation of RS_{TIIR} and HS_{URVI} with H_{TIIR} for 4 h did not affect the expression of CYP1A1, 1A2, and 3A6 apoproteins (Fig. 1).

Compared with H_{TIIR} alone, incubation of IL-6 with H_{TIIR} for 4 h reduced total cytochrome P450 content ($P < 0.05$), and the activity of CYP1A1, 1A2, and 3A6 ($P < 0.05$) as reflected by the production of theophylline

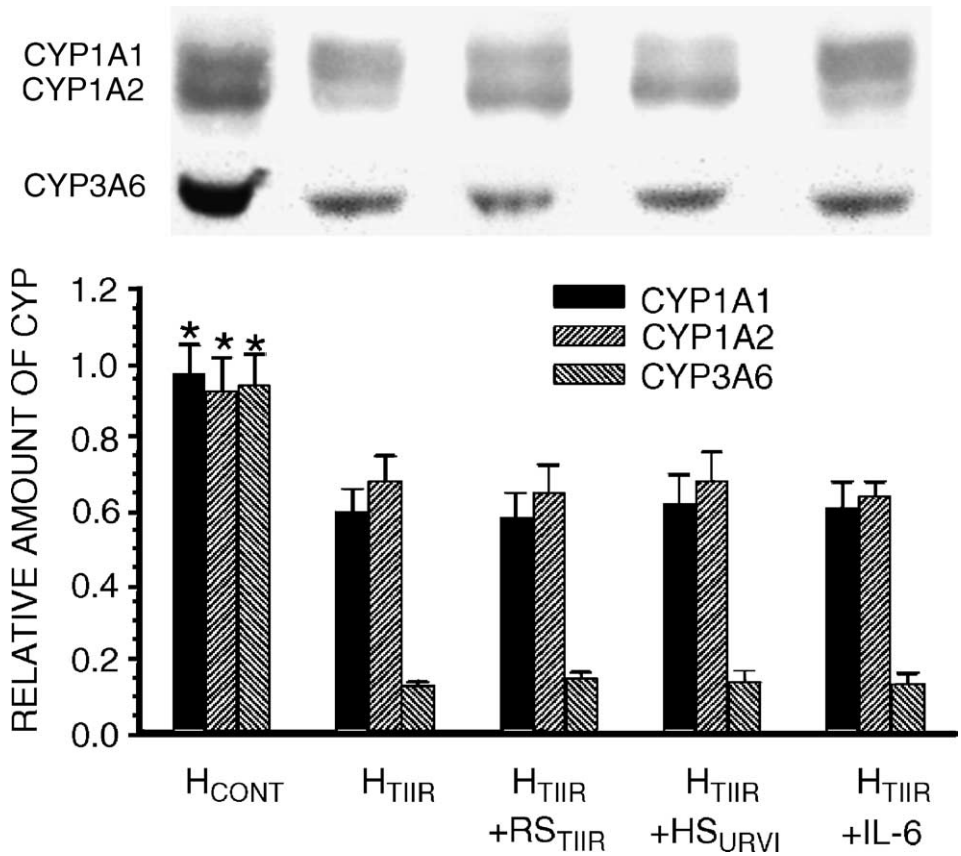

Fig. 1. Amount of CYP1A1, 1A2 and 3A6 apoproteins in hepatocytes from control rabbits (H_{CONT}) and rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}), following 4 h of incubation with NaCl 0.9%, serum of rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}), serum from individuals with an upper respiratory viral infection (HS_{URVI}), and interleukin-6 (IL-6).

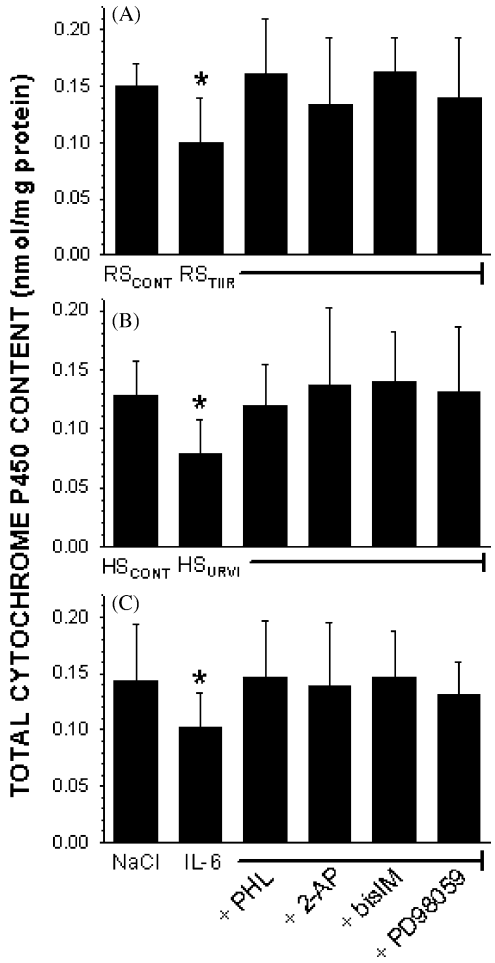

Fig. 2. Total cytochrome P450 content of hepatocytes harvested from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}) following 4 h of incubation with serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) (panel A), serum from individuals with an upper viral respiratory infection (HS_{URVI}) (panel B), and interleukin-6 (IL-6) (panel C) in absence or in presence of phloretin (PHL), 2-aminopurine (2-AP), bis-indolylmaleimide (bisIM), and PD98059. RS_{CONT} is serum from control rabbits. $^{*}P < 0.05$ compared with RS_{CONT} or NaCl. Vertical bars are S.E.M.

metabolites and of DFH (Table 1), although it did not affect the expression of CYP1A1, 1A2 and 3A6 (Fig. 1).

3.2. Signal transduction pathways regulating total cytochrome P450 content, and CYP1A1/1A2 and 3A6 activity

Incubation of phloretin, 2-aminopurine, bis-indolylmaleimide, and PD98059 with H_{TIIR} prevented the decrease in total cytochrome P450 content produced by RS_{TIIR}, HS_{URVI} and IL-6 (Fig. 2). Compared with H_{TIIR} incubated with RS_{CONT}, incubation with RS_{TIIR} reduced the activity of CYP1A1/1A2 by 14% ($P < 0.05$) as measured by the demethylation of MROD (Fig. 3). The inhibitors of PTK, PKR, PKC, and p22/44 MAPK, as well as the NF-κB inhibitor pyrrolidine dithiocarbamanate and L-NAME partially prevented the reduction in CYP1A1/1A2 activity

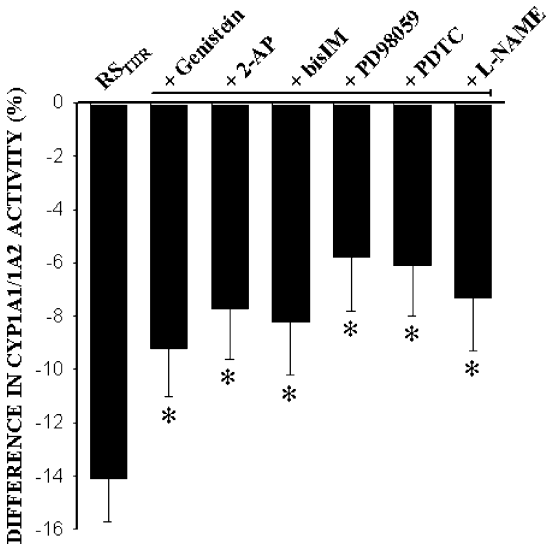

Fig. 3. Decrease in CYP1A1/1A2 activity in hepatocytes of rabbits with a turpentine-induced inflammatory reaction following 4 h incubation with serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}), in absence and presence of genistein, 2-aminopurine (2-AP), bis-indolylmaleimide (bisIM), PD98059, pyrrolidine dithiocarbamate (PDTC) and N^{ω}-nitro-L-arginine methyl ester (L-NAME). CYP1A1/1A2 activity was assessed by means of the O-demethylation of methoxyresorufin. Values are presented as percentage difference with baseline CYP1A1/1A2 activity in hepatocytes of rabbits with a turpentine-induced inflammatory reaction. $^{*}P < 0.05$ compared with RS_{TIIR}. Vertical bars are S.E.M.

produced by RS_{TIIR}. Pre-incubation of H_{TIIR} with phloretin, 2-aminopurine, bis-indolylmaleimide, and PD98059 did not completely prevent the decrease in CYP3A6 activity produced by RS_{TIIR}, and HS_{URVI} (Fig. 4). In H_{TIIR}, ppErk1/2 were barely detectable (Fig. 5), and as expected,

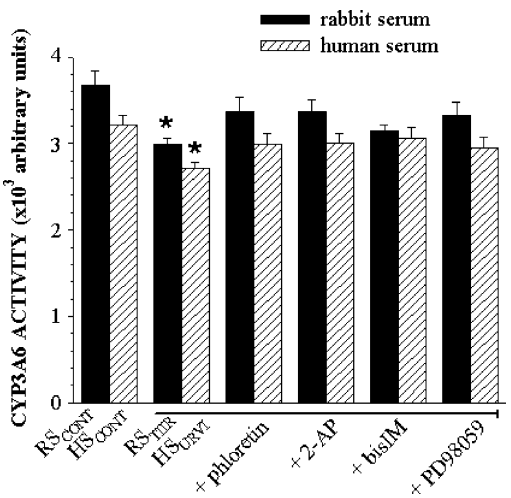

Fig. 4. Activity of CYP3A6 in hepatocytes from rabbits with a turpentine-induced inflammatory reaction incubated for 4 h with serum of rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) and with serum from individuals with an upper viral respiratory infection (HS_{URVI}) in absence and presence of phloretin, 2-aminopurine (2-AP), bis-indolylmaleimide (bisIM), and PD98059. $^{*}P < 0.05$ compared with serum from control rabbits (RS_{CONT}) and humans (HS_{CONT}). Vertical bars are S.E.M. of arbitrary units.

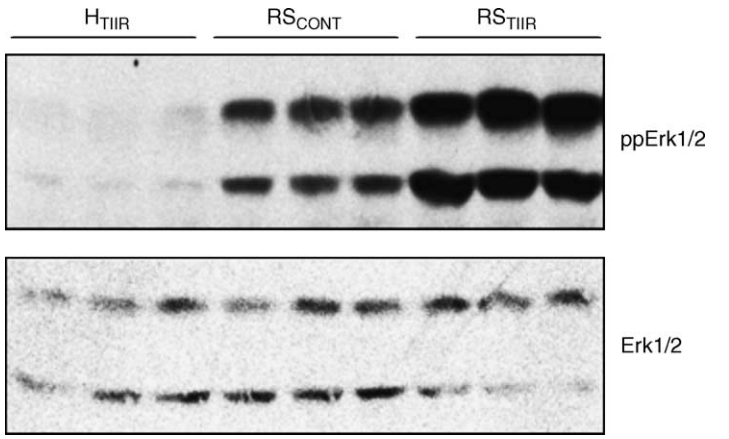

Fig. 5. Effect of serum from control rabbits (RS_{CONT}) and from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) on total extracellular signal-related kinase 1/2 (Erk1/2) and on phosphorylated Erk1/2 (ppErk1/2) in hepatocytes harvested from rabbits with a turpentine-induced inflammatory reaction (H_{TIIR}).

the addition of RS_{CONT} increased the amount of ppErk1/2, although the increase in ppErk1/2 was much more pronounced when H_{TIIR} was incubated with RS_{TIIR} (Fig. 5). PD98059 inhibited dose-dependently the phosphorylation of Erk1/2 induced by RS_{TIIR}, although bis-indolylmaleimide did not impede the phosphorylation of Erk1/2 indicating that the activation of Erk1/2 is independent of PKC (data not shown).

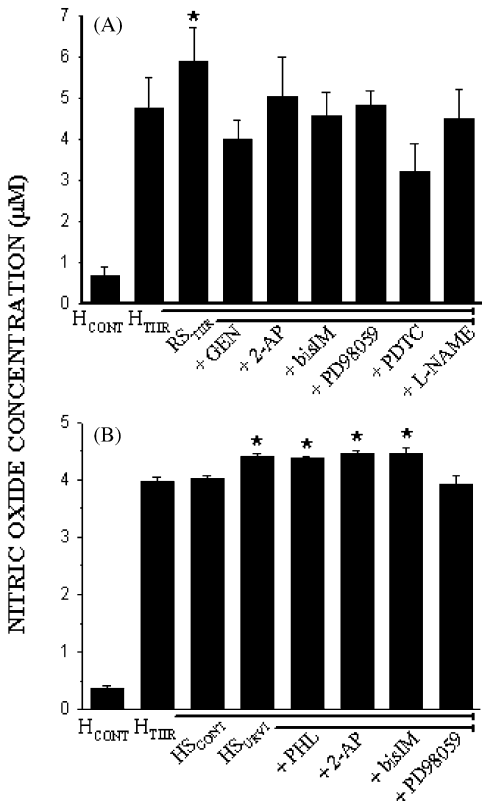

Fig. 6. Nitric oxide concentration in the supernatant of hepatocytes from control rabbits (H_{CONT}) and in hepatocytes from rabbits with a turpentine-inflammatory reaction (H_{TIIR}) incubated for 4 h with serum from rabbits with a turpentine-induced inflammatory reaction (RS_{TIIR}) (panel A) and with serum from individuals with an upper tract viral respiratory infection (HS_{URVI}) (panel B), in absence or in presence of genistein (GEN), 2-aminopurine (2-AP), bis-indolylmaleimide (bisIM), PD98059, pyrrolidine dithiocarbamate (PDTC) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), and phloretin (PHL). Total nitrate was estimated by the Griess reaction and assumed to reflect nitric oxide. $^{*}P < 0.05$ compared with H_{TIIR}. Vertical bars are S.E.M.

3.3. Effect of protein-kinase inhibitors on $NO^{\bullet}$ concentrations in H_{TIIR} and on sodium nitroprusside-induced decrease in cytochrome P450 content

By comparison with H_{CONT}, average concentration of $NO^{\bullet}$ was approximately 10-fold greater in the supernatant of H_{TIIR} (Fig. 6). Incubation of RS_{TIIR} and HS_{URVI} with H_{TIIR} for 4 h raised the concentration of $NO^{\bullet}$ ($P < 0.05$). The increase in $NO^{\bullet}$ induced by RS_{TIIR} was prevented by pre-incubating the hepatocytes with the inhibitors of PTK, PKR, PKC, and p22/44 MAPK, as well as with pyrrolidine dithiocarbamanate and L-NAME. On the other hand, the increase in $NO^{\bullet}$ induced by HS_{URVI} was prevented only by the inhibition of p42/44 MAPK (Fig. 6).

Exposure of H_{TIIR} to sodium nitroprusside decreased total cytochrome P450 content by 43% ($n = 6$) ($P < 0.05$) (Table 1). In addition, sodium nitroprussiate reduced the activity of CYP1A1/1A2 by around 45% and that of CYP3A6 by 64% ($P < 0.05$) as reflected by the production of theophylline metabolites and of DFH. Sodium nitroprusside reduced total cytochrome P450 content in H_{TIIR} from 0.128 ± 0.036 nmol/mg to 0.073 ± 0.038 nmol/mg of protein ($P < 0.05$) and pre-incubation with phloretin, 2-aminopurine, bis-indolylmaleimide and PD98059 did not prevent this decrease, e.g. cytochrome P450 content was 0.095 ± 0.039 nmol/mg, 0.081 ± 0.042 nmol/mg, 0.084 ± 0.041 nmol/mg and 0.087 ± 0.025 nmol/mg of protein ($P > 0.05$), respectively.

4. Discussion

The above experiments provide evidence that incubation of RS_{TIIR}, HS_{URVI} and IL-6 with hepatocytes from rabbits

with a turpentine-induced inflammatory reaction for 4 h reduce total cytochrome P450 content and CYP1A1, 1A2 and 3A6 activity, without changes in the amount of CYP1A1, 1A2 and 3A6 isoforms. Non-specific inhibition of PTK and PKC by phloretin, inhibition of PKR by 2-aminopurine, and specific inhibition of PKC and p42/44 MAPK in H_{TIIR} prevent the decrease in total cytochrome P450 content produced by the sera from rabbits and humans, and IL-6. The fact that each inhibitor prevents the decrease in total cytochrome P450 content suggest that the kinases inhibited are part of a cascade of dependent phenomena, rather than parallel redundant pathways. On the other hand, these inhibitors did not prevent completely the reduction in CYP1A1, 1A2 and 3A6 activity produced by the sera and IL-6. These results suggest that the mechanisms underlying the decrease in total cytochrome P450 content and the activity of cytochrome P450 isoforms may differ. Based on these results, we may propose that the effect of rabbit and human sera and IL-6 involves the parallel activation of p42/44 MAPK and PKC [39–42]. This postulate is consistent with the signal transduction pathways activated by the serum mediators responsible for the decrease in cytochrome P450 activity, e.g. IL-6 in RS_{TIIR}, and IFN-γ, IL-1β and IL-6 in HS_{URVI} [8].

Total cytochrome P450 content decreased without the reduction in the expression of CYP1A1, 1A2 and 3A6 isoforms. This apparent contradiction may be explained by the fact that the spectrophotometric assay of total cytochrome P450 content is based on the binding of carbon monoxide to reduced iron (Fe^{2+}) of the heme moiety at the binding site of O_2. The spectrophotometric measure of total cytochrome P450 content will decrease whenever Fe^{2+} binding site is not available. Immunoquantitation of an isoform by using an antibody to a specific epitope is independent of the availability of the binding site on Fe^{2+}. Therefore, we may speculate that the decrease in total cytochrome P450 content is associated with the reduction in Fe^{2+} binding sites. The decrease in cytochrome P450 content because of the down-regulation of isoforms other than CYP1A1, 1A2 and 3A6, following the incubation of hepatocytes with sera and IL-6 for 4 h is difficult to reconcile with the long half-life of cytochrome P450 isoforms [43], and the fact that CYP3A6 constitutes almost 50% of total cytochrome P450 content.

The catalytic activity of CYP1A1, 1A2 and 3A6 was decreased by RS_{TIIR}, HS_{URVI} and IL-6. This decrease in CYP1A1, 1A2 and 3A6 activity by RS_{TIIR} is partially prevented by the inhibition of PTK, PKR, PKC, and p22/44 MAPK. Direct phosphorylation of the apoprotein will denature it with loss of catalytic activity [44]. In vitro, CYP2B1/2 [45], CYP2B4 [46], CYP2C [47], CYP2E1 [48], and CYP3A1 [49] can be phosphorylated on serine residues by PKC [12]. Not all cytochrome P450 isoforms are equally sensitive to phosphorylation, CYP1A1 and 1A2 are less prone to be phosphorylated by PKC [50,51]. Another mechanism that may explain the decrease in CYP1A1, 1A2 and 3A6 activity could involve $NO^{\bullet}$ [17,52]. $NO^{\bullet}$ can reduce the activity of cytochrome P450 isoforms by two mechanisms. In first place, $NO^{\bullet}$ interacts with the Fe^{2+}- and Fe^{3+}-heme at the active center of cytochrome P450 isoforms, resulting in a reversible inactivation of the enzyme [53]. Secondly, $NO^{\bullet}$ can nitrate tyrosine residues in the proximity of the active site culminating in the irreversible inactivation of the isoforms [54]. Probably the two mechanisms of inactivation are sequential, with an early binding to Fe^{2+}/Fe^{3+} and a later nitration of tyrosine residues [55].

There are several pieces of evidence indirectly supporting that $NO^{\bullet}$ contributes to the reduction of the activity of CYP1A1, 1A2 and 3A6 produced by sera and IL-6. The catalytic activity of cytochrome P450 isoforms depends upon binding of O_2 to Fe^{2+} to form the cytochrome P450-dioxygen complex required to transfer an oxygen atom to the substrate. Therefore, the decrease in cytochrome P450 content without changes in expression is compatible with the hypothesis that $NO^{\bullet}$ binds to Fe^{2+} and reduces its catalytic activity, as well as carbon monoxide binding for its spectrophotometric measure. Moreover, the present study provides evidence that an excess of $NO^{\bullet}$ generated by sodium nitroprussiate reduces the activity of CYP 1A1, 1A2, and 3A6. Finally, RS_{TIIR} increased $NO^{\bullet}$ production, and L-NAME prevented the increase in $NO^{\bullet}$ and partially the reduction in CYP1A1/1A2 activity. These last results are in agreement with reports showing that in vivo L-NAME partially prevents the reduction in CYP1A1/1A2 activity produced by a turpentine-induced inflammatory reaction [6]. Moreover, in vitro, L-NAME dose-dependently hinders the decrease in CYP1A1 and 1A2 activity and in total cytochrome P450 content triggered by RS_{TIIR} and HS_{URVI} [19].

Circumstantial evidence further supports the hypothesis that $NO^{\bullet}$ and ROI are implicated in the modulation of CYP1A1/1A2 activity. Inhibition of NF-κB by the antioxidant pyrrolidine dithiocarbamate elicits almost the same effect as PD98059 and L-NAME. This is because p42/44 MAPK and ROI activate the nuclear translocation of NF-κB and binding to NOS promoter, resulting in a rapid (less than 4 h) increase in NOS expression, and $NO^{\bullet}$ production [56–58]. Moreover, it has been reported that ROI can increase the formation of $NO^{\bullet}$ in the matter of minutes due to an enhanced transport of L-arginine into the cell, which depends upon p42/44 MAPK [59–62]. The decrease in CYP1A1 and 1A2 activity in H_{TIIR} by sera is closely associated to the presence of ROI because on the one hand, the antioxidants *N*-acetylcysteine and dimethylthiourea prevent dose-dependently the effect of RS_{TIIR} and HS_{URVI} on cytochrome P450 activity; on the other hand, the addition of diethyldithiocarbamate, an inhibitor of superoxide dismutase, of DL-buthionine-(*R*,*S*)-sulfoximine, an inhibitor of glutathione peroxidase, and of 3-amino-1,2,4-triazole, and inhibitor of catalase,

potentiate the effect of sera on CYP1A1, 1A2 and 3A6 activity and total cytochrome P450 content [19].

In conclusion, the present study demonstrates that the process leading to the decrease in CYP1A1, 1A2, and 3A6 activity triggered by RS_{TIIR}, HS_{URVI} and IL-6 involves the activation of PTK, p42/44 MAPK and PKC. Indirect evidence supports that $NO^{\bullet}$ is implicated in the decrease in activity of these enzymes, although in the case of CYP1A1/1A2 several mechanisms may contribute to regulate its activity.

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