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Kupffer cell-mediated differential down-regulation of cytochrome *P*450 metabolism in rat hepatocytes

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Abstract

Nonparenchymal cells, particularly Kupffer cells, might play an important role in the modulation of xenobiotic metabolism in liver and its pharmacological and toxicological consequences. This intercellular communication via the exchange of soluble factors was investigated in primary rat Kupffer cells and hepatocytes. Freshly isolated rat Kupffer cells were seeded onto cell culture inserts and cocultured with 5 day old serum-free rat hepatocyte monolayer cultures at a ratio of 1:1 for 2 days. Hepatocyte cultures, Kupffer cell cultures or cocultures were treated with 0.1 ng/ml–10 μg/ml lipopolysaccharide (LPS). Within this concentration range, no significant toxicity was observed in either cell type. In LPS-exposed cocultures, tumor necrosis factor α (TNFα) levels rose up to 5 ng/ml within 5 h; nitric oxide (NO) levels increased up to 70 μM within 48 h of treatment, both in a dose-dependent fashion. The release of negative (albumin) and positive (α1-acid-glycoprotein) acute phase proteins from the hepatocytes was strongly down- and up-regulated, respectively. The simultaneous treatment of the cocultures with phenobarbital and LPS (10 ng/ml) or 3-methylcholanthrene and LPS (10 ng/ml) resulted in a strong down-regulation (85%) of the phenobarbital-induced cytochrome *P*450 (CYP) isoform CYP2B1 in the hepatocytes whereas the 3-methylcholanthrene-induced isoform CYP1A1 was only weakly affected (15%). This specific down-regulation of CYP2B1 was mediated exclusively by TNFα, released from the Kupffer cells. It was not linked with NO release from or inducible NO synthase activity in the hepatocytes. The TNFα release was not affected by the two xenobiotics. Acetaminophen tested in these cocultures showed no direct interaction with the Kupffer cells. The use of liver cell cocultures is therefore a useful approach to investigate the influence of intercellular communication on xenobiotic metabolism in liver. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hepatocyte, rat; Kupffer cell; Coculture; TNFα (tumor necrosis factor α); CYP2B1; CYP1A1; Nitric oxide (NO); Acute phase response

1. Introduction

Chemically induced hepatotoxicity might often be caused by an interaction between xenobiotic-induced primary lesions and liver cell-mediated release of intercellularly acting regulatory factors. The Kupffer cells, the resident macrophages in the liver, are an important source of these factors such as cytokines, prostaglandins, leukotrienes, reactive oxygen species, platelet activating factors and nitric oxide (NO) (Brouwer et al., 1995; Decker, 1998). Some of the mediators are suspected to be involved in tissue homeostasis and in nongenotoxic carcinogenesis. Their release can be induced after exposure to bacterial endotoxins. It might be possible that these cellular-derived factors affect xenobiotic metabolism or vice versa, xenobiotics interact with the release of the factors. Whereas, the influence of individual cytokines on xenobiotic metabolism and toxicity is well documented (Roth et al., 1997), the mechanism of cellular interactions is as yet not well defined. In principle, three pathways are possible: (i) xenobiotics directly stimulate the release of Kupffer cell-derived mediators, in a fashion similar to LPSs, (ii) xenobiotics are first metabolised by hepatocytes into intermediates which are then able to stimulate the Kupffer cells or (iii) xenobiotics induce primary lesions in hepatocytes which are converted into cytotoxic lesions by contact with factors induced by constitutive levels of LPS. Additional cellular interactions might then take place in subsequent

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steps, e.g., by a direct contact with infiltrating blood cells (Sauer et al., 1996).

Complex cellular interactions, taking place either simultaneously or sequentially, are difficult to assess in whole animal studies. Therefore, we set up a coculture system combining the two most important partners in cellular interactions in the liver, namely hepatocytes and Kupffer cells. In these rat cell cocultures, the intercellular interactions, the actual concentration of signals, time sequence and type of cellular damage can be dissected.

Previous studies on cocultured rat liver cells often used mixed cultures of hepatocytes and Kupffer cells (Sauer et al., 1996; Steinhorn and Cerra, 1997). This allowed intercellular communication by direct cell–cell contact, a situation which does not correctly mimic the situation in the liver. Kupffer cells are located outside of the space of Disse and are physically separated from the hepatocytes by the sinusoidal endothelial cell layer. Therefore, as inducer of an immediate response after exposure to xenobiotics, the Kupffer cell or Kupffer cell via endothelial cell-derived soluble factors might play the most important role. During the subsequent steps, however, the direct cell–cell contact mediated interactions (including reactive oxygen species) and the recruitment of blood-derived cells, become more prominent. In the presently established coculture system, investigations are focused on the initial role of the Kupffer cells-derived soluble factors on hepatocyte toxicity. The freshly isolated hepatocytes are cultured as monolayers under defined, tissue-equivalent oxygen tensions (Ohno and Maier, 1994) and on a liver-specific, complete extracellular matrix (Saad et al., 1993a). The Kupffer cells are kept in culture plate inserts consisting of porous membranes. This assures free access of nutrients to the Kupffer cells and exchange of intercellular messengers between the two cell populations via the medium, while keeping the two cell types spatially separate. With this coculture set-up, the Kupffer cells can be added to the hepatocytes at given time points and for defined intervals and pre-exposures to chemicals or analysis of altered functions after cocultures can be carried out separately for both cell populations.

In the present experiments, first, the correct functional response of both cell types was tested. The Kupffer cells were exposed to a LPS (*Salmonella enteritidis*) and the hepatocytes to phenobarbital and methylcholanthrene. Combining the two cell populations in cocultures resulted in an intercellular interaction and in a differential down-regulation of induced cytochrome *P*450 (CYP) isoforms in the hepatocytes. The sequence of cellular events, the time-dependent release of mediators and the specific contributions of the two cell populations were analyzed. Acetaminophen, an analgesic and antipyretic drug suspected to induce liver toxicity by a Kupffer cell-mediated pathway (Laskin and Pilaro, 1986; Laskin et al., 1986; Blazka et al., 1995), was tested in this coculture system. No intercellular interaction nor release of inflammatory cytokines was detectable.

2. Materials and methods

2.1. Reagents

Bovine insulin, dexamethasone, egg-white trypsin inhibitor, aprotinin, bovine serum albumin, William's medium E (without L-glutamine and phenol red), 3-methylcholanthrene, LPSs from *Salmonella enteritidis*, 7-pentoxyresorufin, 5-ethoxyresorufin and dicumarol were purchased from Sigma (Buchs, Switzerland). Phosphate-buffered saline, collagenase, lactate assay and lactate dehydrogenase (LDH) kits were from Boehringer Mannheim (Rotkreuz, Switzerland). L-Glutamine, penicillin/streptomycin were purchased from Life Technologies (Basel, Switzerland). Porcine collagen type I was obtained from Pentapharm (Basel, Switzerland). Foetal calf serum for Kupffer cell cultures were from Amimed (Allschwil, Switzerland). The chemicals diethylpyrocarbonate, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 3-morpholinopropane-sulfonic acid (MOPS), guanidinium thiocyanate, sodium citrate, 2-mercaptoethanol, laurylsarcosine, ammonium acetate, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 5-ethyl-5-phenyl-barbituric acid sodium salt (phenobarbital), dimethylsulfoxide (DMSO) and selenium were purchased from Fluka (Buchs, Switzerland) and the inducible NO synthetase specific inhibitor L-N^6-(1-iminoethyl)-lysine (L-NIL) from ANAWA (Wangen, Switzerland). Rat recombinant tumor necrosis factor α (TNFα) was purchased from ImmunoKontact (Bioggio, Switzerland) and rat recombinant interferon-γ (IFN-γ) from Genzyme (Cambridge, MA, USA). All chemicals used were of reagent grade.

2.2. Antibodies

Western blots were analyzed with anti-rat CYP IIB1/2 and IA1 monoclonal antibodies from Amersham Life Sciences (Zurich, Switzerland; ECL Western Blotting Kits). Nonparenchymal cells were identified with the monoclonal antibodies OX-18, mouse anti-rat major histocompatibility complex I (Chemikon, Milan, Italy) and the Kupffer cells with the mouse anti-rat resident macrophages monoclonal antibodies ED2 (Bachem, Bubendorf, Switzerland). Goat monoclonal antibodies to mouse immunoglobulin G (IgG) and rabbit anti-rat albumin antibody, both conjugated with horseradish peroxidase, were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA) and Cappel (Turnhout, Belgium), respectively. Polyclonal rabbit anti-rat TNFα neutralising antibodies were obtained from ImmunoKontact and the rat TNFα ELISA kit from Biosource (Fleurus, Belgium).

2.3. Hepatocyte cultures and treatment

Petriperm culture dishes (gas permeable hydrophilic Teflon membrane bottom, 20 cm^2, Heraeus, Zürich,

Switzerland) were coated with a mixture of 120 μg rat liver crude membrane fraction and 1.2 μg collagen type I each in 2 ml phosphate-buffered saline overnight at 4°C as described previously (Saad et al., 1993b). Hepatocytes were isolated by a two-step in situ liver perfusion from male Sprague–Dawley rats (ZUR/SIV, Institute of Laboratory Animal Sciences, University of Zürich, Zürich Switzerland; age 54 ± 2 days; 240–270 g b.w., anaesthesia with 50 mg/kg b.w. pentobarbital, i.p.) kept under controlled environmental conditions (12 h light–dark cycle, diet No. 890 NAFAG, Gossau, Switzerland, food and water ad libitum). Preparations with a cell viability of > 85% (assessed by Trypan Blue exclusion in samples without bovine serum albumin) were used. Contamination with nonparenchymal cells was below 0.5%. Some 3×10^6 hepatocytes were seeded on each Petriperm dish. The cells were incubated in serum-free culture medium (William's E medium without phenol red, supplemented with 100 nM dexamethasone, 10 nM insulin, 2 mM L-glutamine, 30 nM selenium, 1 μg/ml aprotinin and 100 IU/ml penicillin/streptomycin) under 13% O_2 (v/v; substitution of air with nitrogen) and 5% CO_2. The membrane bottom of the culture dishes provides a stable oxygen tension in the culture medium defined by the incubator atmosphere set to a periportal equivalent oxygen tension (13% v/v O_2) (Maier et al., 1994). The medium was replaced every 24 h. After 5 days in culture, dexamethasone was omitted from the medium and 10 U/ml rat recombinant interferon-γ (IFN-γ) was added together with LPSs and/or the corresponding test compound ('treatment medium'). The treatment period lasted for 48 h without medium change.

Phenobarbital, LPSs, L-NIL, neutralising antibodies and recombinant factors were dissolved in culture medium. 3-Methylcholanthrene was dissolved in DMSO and then diluted with medium; the final concentrations of DMSO did not exceed 0.1%.

2.4. Kupffer cell isolation, purification and cultures

Nonparenchymal cells were isolated from a separate rat by the collagenase–pronase method (Hendriks et al., 1990; Ammann and Maier, 1997). Briefly, the liver was cannulated, preperfused in situ with 60 ml Gey's solution, and subsequently perfused with 90 ml of a pronase E (Merck, Dietikon, Switzerland, 4×10^6 PU/g) solution (1.5 mg/ml Gey's buffer) followed by 150 ml of a mixture of pronase E (0.58 mg/ml) and collagenase (0.48 mg/ml) in Gey's buffer. The liver was then excised and the Glisson's capsule removed. The cell suspension was filtered through 250 μm nylon gauze (Nytal 250, Sefar, Thal, Switzerland) and incubated for 30 min in a mixture of pronase E (0.25 mg/ml) and collagenase (0.48 mg/ml) dissolved in Gey's buffer, at pH 7.4. The pellet resulting from the collagenase–pronase isolation protocol was resuspended in 10 ml of Gey's buffer. Red blood cells, fat storing cells and cellular debris were removed by density gradient centrifugation through a Nycodenz (Nyegaard and Co, Oslo, Norway) solution (28.7% w/v in NaCl free Gey's solution, 270 mOsm, 1500 × g for 15 min at 4°C). Lymphocytes, endothelial cells and Kupffer cells were collected at the interface Gey's–Nycodenz solution, resuspended in 50 ml Gey's buffer and filtered through a 61 μm nylon gauze (Nytal 61, Sefar, Thal, Switzerland) to remove aggregates. Kupffer cells were further purified by elutriation in a Beckman centrifuge (JE-6B rotor) at a constant speed of 3250 rpm using a Sanderson chamber and Gey's buffer at flow rate of 70 ml/min (in 150 ml) according to Juillerat et al. (1997). Lymphocytes (100 ml) and endothelial cells (150 ml) were previously collected at flow rates of 19 and 32 ml/min, respectively, and discarded. Purity, cell number and viability (Trypan Blue exclusion) were assessed by phase microscopy and cell volume measurements (Cell Analyzer System, CASY1, Schärfe System, Reutlingen, Germany). The purity of the Kupffer cell fraction ($62 \pm 16 \times 10^6$ cells per rat liver) after elutriation was 85 ± 11% and the viability 96 ± 2%.

Some 3×10^6 Kupffer cells were seeded onto Anopore culture inserts (diameter: 25 mm, pore size: 0.2 μm, Nunc, Roskilde, Denmark) in six-well plates for cell culture (Falcon, Basel, Switzerland) and cultured for 48 h with William's E medium without phenol red, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 10% foetal calf serum under 20% O_2 and 5% CO_2. After the first 48 h in culture, the purity of the Kupffer cells was over 95%.

The culture inserts with the Kupffer cells were then transferred either into hepatocyte cultures or into cell-free and coated Petriperm dishes (Fig. 1) and exposed to the corresponding chemicals or agents for 48 h (Fig. 2) in the treatment medium (13% O_2 and 5% CO_2).

2.5. Biochemical measurements

Cell homogenates were prepared from hepatocyte monolayers rinsed with ice-cold phosphate-buffered saline at pH 7.4, harvested by scraping off in 2 ml phosphate-buffered saline, homogenised in a Dounce homogeniser on ice (12 strokes/sample) and further disrupted by 30 pulses (500 ms) with a sonifier (Branson Sonifier 250) on ice. An aliquot of the homogenate was centrifuged for 10 min at 9000 × *g* and 4°C and the supernatant collected (S9-frac-

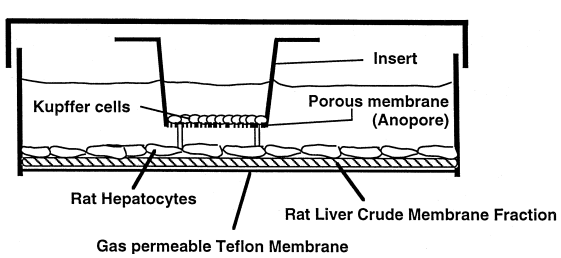

Fig. 1. Set-up of the coculture system.

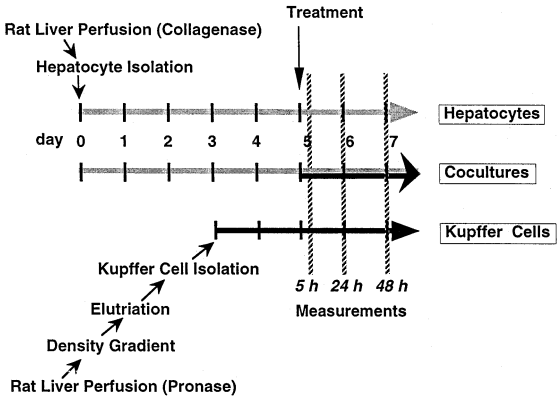

Fig. 2. Culture and treatment schedule.

tion). Protein content in cell-homogenates from individual dishes was determined with the Bradford protein assay (BIO-Rad, Glattbrugg, Switzerland; Bradford, 1976) using bovine serum albumin as standard. The LDH activity in cell homogenates and in culture media and the extracellular lactate content were determined spectrophotometrically (COBAS Fara, La Roche, Basel, Switzerland) with commercially available kits (Boehringer Mannheim). LDH release within 24 h was expressed in percentage of the total (extra- and intracellular) activity.

The viability of the Kupffer cells during culture was determined by the thiazolylblue tetrazoliumbromide (MTT) assay (Berridge and Tan, 1993). Some 1.2 mM (final concentration) of MTT in culture medium was given to the cell culture inserts. After incubation for 30 min to 1 h at 37°C, the medium was removed and replaced by 90% (v/v) ethanol in 50 mM HEPES-buffer (pH 8.0) in order to solubilize the chromophore. The quantity of MTT–formazan was estimated by measuring the absorbance at 550 nm on a Hamilton HR 7000 microplate reader.

TNFα levels in the supernatants of the cultures were determined in 50 μl aliquots according to the protocols of the Rat TNFα ELISA kit (Biosource) and the differential absorbance (405–600 nm) measured (Hamilton HR 7000 microplate reader).

Albumin levels in the supernatants of the cultures were determined in 100 μl aliquots, diluted 1:100 with phosphate-buffered saline, by ELISA using a rabbit anti-rat albumin antibody, conjugated with horseradish peroxidase.

NO levels were determined in 50 μl aliquots of the culture supernatants mixed with 200 μl Griess reagent (Ding et al., 1988). The absorbance (550 nm) was measured after 15 min and NO_2^- concentrations were determined from a standard curve using 3–100 μM sodium nitrite in culture medium.

The activities of the 7-pentoxyresorufin-*O*-dependentylase (PROD) and the 5-ethoxyresorufin-*O*-deethylase (EROD) enzymes were determined in situ in hepatocyte monolayers incubated for 15 min with 1.5 ml of Hank's buffer solution supplemented with the corresponding substrate (7-pentoxyresorufin or 5-ethoxyresorufin) and 10 μM dicumarol as described by Wortelboer et al. (1990). Fluorescence in the supernatant was measured after 7.5 and 15 min with a Millipore CytoFluor 2350 microplate reader. In selected cases, at the end of incubation, the cells were scraped off in ice-cold phosphate-buffered saline, sonicated and the total fluorescence (intra- and extracellular content) was determined.

Denaturing polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). Up to 30 μg total protein per lane of the S9-fractions was separated on a mini-gel in a Mini-Protean-II gel chamber (BIO-Rad) at 15 mA per gel. Blotting on nitro-cellulose membranes (Protran BA 83, Schleicher and Schuell, Dassel) was performed in the same chamber at 4°C with 100 V for 80 min. Determination of CYP IA1 and IIB1/2 protein content on the blots was performed according to the protocols of the corresponding ECL western blotting kits and quantified with the gel analysis system GelPrint 2000i (MWG-Biotech and Zero-D/One-Dscan, Scanalytics, Billerica, MA, USA).

2.6. RNA isolation and Northern blot analysis

The medium in the culture dishes was removed and cells lysed in situ in 0.5 ml of an aqueous 0.1% diethylpyrocarbonate-solution (diethylpyrocarbonate–H_2O) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol and 0.5% laurylsarcosine. RNA was obtained after removal of proteins by phenol-chloroform extraction and precipitation with 0.1 volumes of 2.5 M ammonium acetate and 2.5 volumes of 95% ethanol. After washing in 70% ethanol, the final RNA pellets were resuspended in diethylpyrocarbonate–dH_2O, quantified by measuring the absorbance at 260 nm and stored at −80°C.

Total RNA was denaturated by a formaldehyde buffer (6.5% v/v in 20 mM MOPS-buffer) and 10 μg RNA aliquots were separated by formaldehyde–agarose gel electrophoresis and transferred to positively charged nylon membranes (Boehringer Mannheim). Equal RNA loading and transfer efficiencies were checked by ethidium bromide staining and quantified with the gel analysis system GelPrint 2000i. Rat cDNA probe α1-acid-glycoprotein (900 bp PstI fragment from Bluescript KS + -derived pBSrAGP, Baumann et al., 1986) was kindly provided by Dr. R. Wenger (Institute of Physiology, University of Zürich-Irchel, Zürich, Switzerland). Rat cDNA probes CYP2B1 (1.2 kbp, *Hin*dIII fragment from pBR322-derived pSV450) and CYP1A1 (2.2 kbp, PstI fragment from pUC19-derived pUC19/450IA1) were kindly provided by Dr. P. Bouis (Novartis-Pharma, Basel, Switzerland). They were labelled by using the Ready-To-Go DNA labelling beads (-dCTP) (Pharmacia Biotech, Uppsala, Sweden) with P^{32}-labelled CTP. Pre-hybridisation and hybridisation of the mRNA with the labelled CYP2B1 or CYP1A1 cDNA were per-

Table 1
Viability of hepatocytes and Kupffer cells after the 48 h LPS treatment period

LPS (ng/ml)	Hepatocytes (LDH release)		Kupffer cells (MTT reduction)	
	Cocultures	Monocultures	Cocultures	Monocultures
1	94±8	98±9	88±3	86±5
100	89±7	92±6	91±8	89±7
10,000	91±7	90±7	85±6	87±4

Values represent percentages of control values (see Section 3) from untreated cultures.
Data are expressed as means±S.D. of three independent experiments.

formed according to established protocols. The hybridisation signals of the corresponding CYP isoform mRNA were quantified with a PhosphorImager (BioRad, GS-250 Molecular Imager System).

3. Results

The cultured and cocultured hepatocytes maintained a high viability (LDH release, Table 1). The hepatocyte specific response to phenobarbital and 3-methylcholanthrene, as will be described later (inducible PROD activity; Fig. 6A,C, EROD activity; Fig. 6B,D), were preserved during the whole 7 day culture period. This was also the case for the cocultured Kupffer cells, which required a 2 day culture period in the presence of 10% foetal calf serum, in order to recover after the isolation procedure. During the following 2 day coculture period (Fig. 2), dexamethasone was removed from the medium, since steroids protect the liver from hepatic damage by bacterial LPSs (Levitin et al., 1956) and reduce the endotoxin-induced TNFα levels by half (Grewe et al., 1994). IFN-γ, as a well-established translation stimulating factor for cytokine production in macrophages and Kupffer cells was added at low concentrations (10 U/ml) to the coculture medium. At this concentration, the cytokine itself does not induce TNFα (Grewe et al., 1994) or NO release but increased the LPS-induced TNFα and NO release in our cocultures about four-fold (LPS 10 ng/ml without IFN-γ: TNFα = 723 pg/ml and NO = 8.6 μM; LPS 10 ng/ml + IFN-γ 10 U/ml: TNFα = 2982 pg/ml and NO = 29.6 μM).

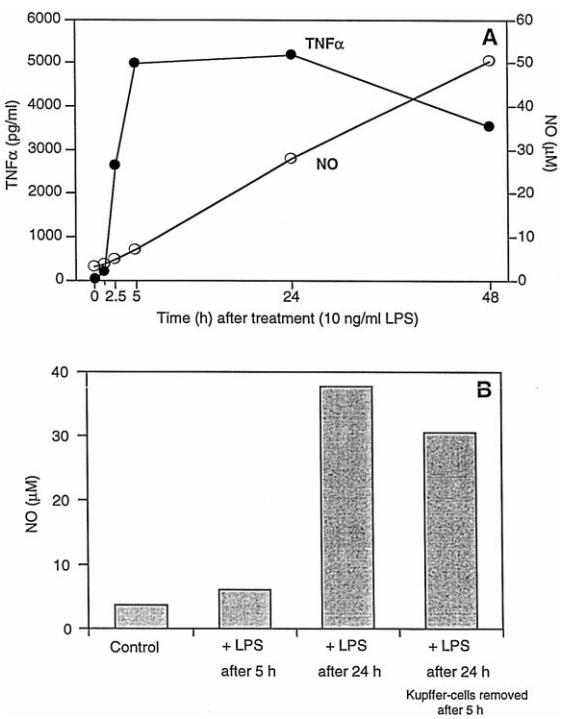

Fig. 3. (A) Time–response relationship of TNFα (●) and NO (○) release in LPS-treated (10 ng/ml) cocultures. (B) NO release in 24 h LPS-treated (10 ng/ml) cocultures. Kupffer cells were removed after 5 h without medium change. The values in A and B represent the means of two independent experiments.

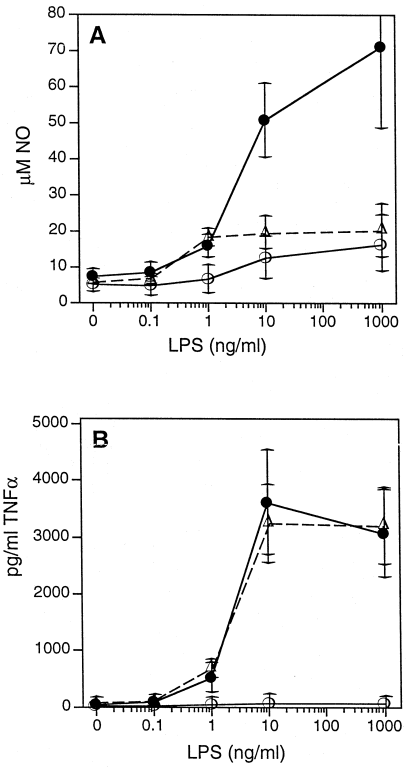

Fig. 4. LPS dose–response of (A) NO and (B) TNFα release in cocultures (●), hepatocyte cultures (○) and Kupffer cell cultures (△). Measurements were performed after a 48 h treatment period. The values represent the means±S.D. of three independent experiments.

3.1. Response to LPS

In hepatocyte cultures exposed to 10 ng/ml LPS (always in presence of 10 U/ml IFN-γ), no significant release of TNFα was detectable, whereas in cocultures the levels rose quickly within 5 h and remained high during the following 43 h (Fig. 3A). In contrast, the NO level

increased steadily during the 48 h observation period (Fig. 3A). A similar time-dependent NO release was found in LPS-treated (10 ng/ml) cocultures in which the Kupffer cells were removed 5 h after beginning of treatment (Fig. 3B). When TNFα (1 ng/ml) was given to the hepatocyte cultures for the first 5 h and the medium subsequently exchanged, almost no NO release (5.3 ± 2.6 μM) was detectable during the following 43 h.

The LPS-induced NO release increased in a dose-dependent manner (Fig. 4A). With 1000 ng/ml LPS, the levels in cocultures were about four times higher than levels obtained with pure hepatocyte or Kupffer cell cultures. In cocultures, and to a lesser extent in Kupffer cell cultures, NO levels rose above control levels after as little as 0.1 ng/ml LPS and reached a plateau at 1 and 10 ng/ml LPS, respectively (Fig. 4A).

After exposure to LPS, TNFα levels rose with increasing LPS concentration in an identical fashion in cocultures and Kupffer cell cultures (Fig. 4B). The highest levels (approx. 100 × control values) were reached with 10 ng/ml LPS. Hepatocytes did not release significant amounts of TNFα, even at 1000 ng/ml LPS (Fig. 4B).

In cocultures, the production and release of hepatocyte-derived albumin, a representative negatively regulated acute phase protein, decreased rapidly with increasing concentration of LPS, up to 10 ng/ml LPS. In hepatocyte cultures, LPS induced only a slight dose-dependent decrease in albumin production (Fig. 5A).

A similar response was observed with RNA transcripts of α1-acid-glycoprotein, a representative of a positively responding acute phase protein. In cocultures, the mRNA levels reached highest values within 24 h after treatment with 10 ng LPS/ml and decreased with increasing concentration. Again, hepatocyte cultures showed almost no response (Fig. 5B).

3.2. Viability and metabolism

The viability of the hepatocytes and of the Kupffer cells was not affected by coculturing. In hepatocyte cultures, LDH release from day 4–5 in the presence of dexamethasone was $5.5 \pm 0.5\%$ of the total activity (intra + extracellular activity = 0.97 ± 0.04 U/mg protein). From day 5–6 and 6–7 without dexamethasone, LDH release was in the same range: in hepatocyte cultures, the extracellular activity was $11 \pm 2\%$ of the total ($= 1.48 \pm 0.13$ U/mg protein) and in cocultures $9 \pm 2\%$ (total = 1.35 ± 0.06 U/mg protein). The MTT formation in untreated Kupffer cells decreased daily by $5.5 \pm 1.5\%$ over the 48 h treatment period (days 5–7 in culture without serum).

The cells tolerated LPS from *Salmonella enteritidis* at levels of up to 10,000 ng/ml with no detectable signs of toxicity (as measured by the MTT-assay for Kupffer cells and extracellular LDH activity for hepatocytes, Table 1). Extracellular lactate content, however, did increase dose-

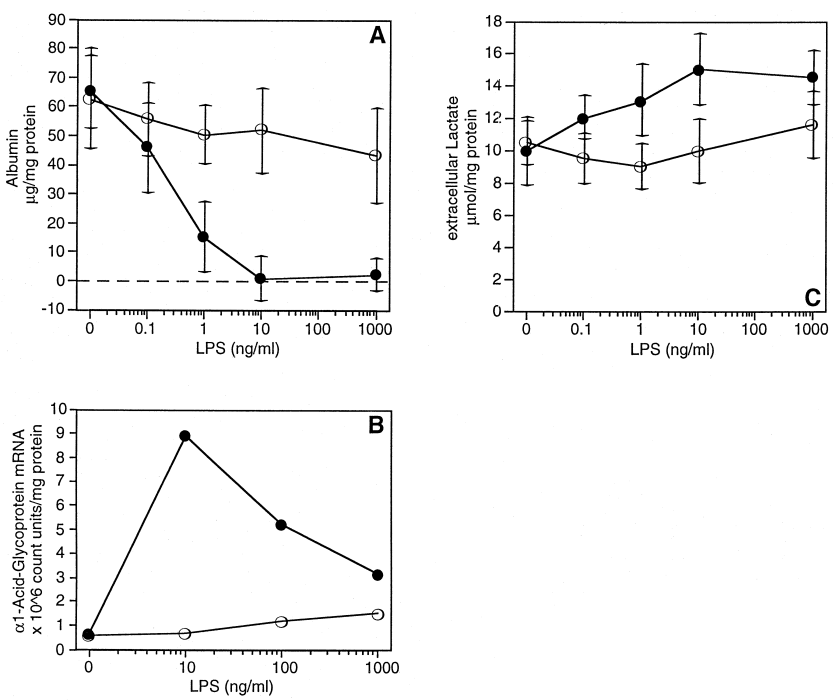

Fig. 5. LPS dose–response of (A) albumin release, (B) α1-acid-glycoprotein mRNA steady state content and (C) lactate release in cocultures (●) and hepatocyte cultures (○). Measurements were performed after a 48 h treatment period. The values in A and C represent the means ± S.D. of three independent experiments. The values in B represent the means of two independent experiments.

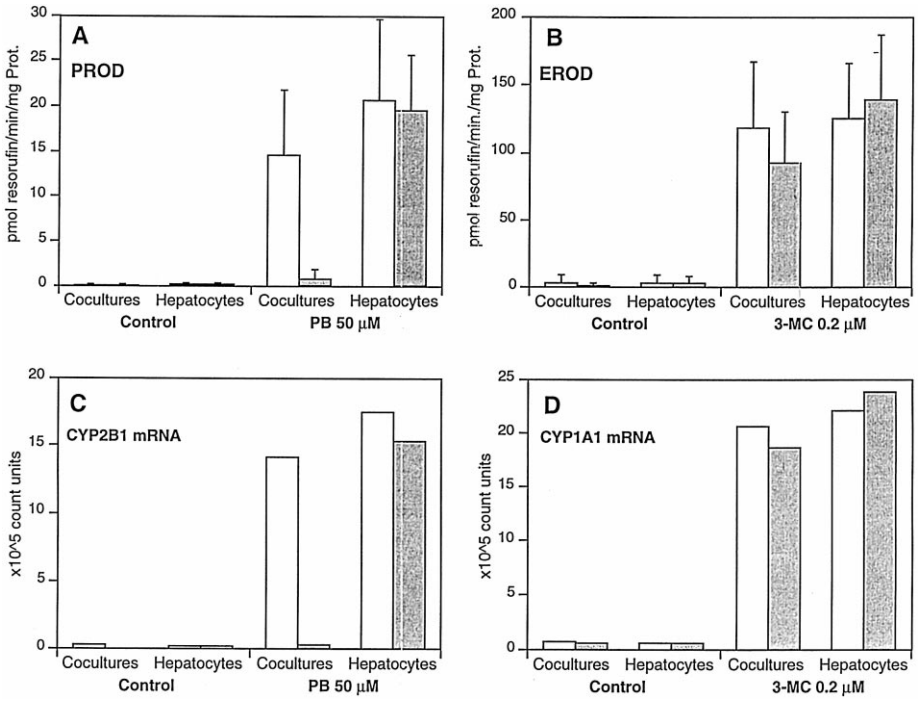

Fig. 6. LPS-mediated down-regulation of (A) PB-induced PROD and (B) 3-MC induced EROD activities and of (C) CYP2B1 and(D) CYP1A1 mRNA steady state contents. Measurements were performed after a 24 h treatment period with (▨) or without (□) LPS (10 ng/ml) in the presence of PB (50 μM) or 3-MC (0.2 μM). The values in A and B represent the means ± S.D. of three independent experiments. The values in C and D represent the means of two independent experiments.

dependently in cocultures over 48 h, but not in hepatocyte cultures (Fig. 5C).

3.3. Differential down-regulation of inducible xenobiotic metabolism

The two model CYP-enzyme inducers phenobarbital (50 μM) and 3-methylcholanthrene (0.2 μM) induced xenobiotic metabolism very efficiently and similarly in hepatocyte cultures and in cocultures (Fig. 6A–D). The 7-pentoxyresorufin-*O*-dependentylase activity (PROD, mainly catalysed by CYP2B1/2) and the 7-ethoxyresorufin-*O*-de-ethylase activity (EROD, predominantly catalysed by CYP1A1/2) (Nakajima et al., 1990) increased 20–50-fold within 24 h (Fig. 6A and B; Table 2). These are levels equivalent to that reported from animal experiments (Wortelboer et al., 1991). Enzyme activities in untreated cultures were very low and nearly identical in hepatocyte cultures and in cocultures (PROD: 0.2 ± 0.15 and 0.15 ± 0.1 pmol min^{-1} mg^{-1} protein; EROD: 5.7 ± 3.4 and 5.3 ± 3.2 pmol min^{-1} mg^{-1} protein).

After exposure to LPS (10 ng/ml), given simultaneously with the two enzyme inducers to the hepatocyte cultures, the enzyme activities were slightly down-regu-

Table 2
Exposure-period dependent down-regulation of PROD and EROD activities

Enzyme	Exposure	12 h exposure		24 h exposure		48 h exposure	
		Cocu.	Hep.	Cocu.	Hep.	Cocu.	Hep.
PROD activity	PB (50 μM)	n.d.	n.d.	17.8 (100%)	22.8 (100%)	21.2 (100%)	24.3 (100%)
	+LPS (10 ng/ml)	n.d.	n.d.	0.7 (4%)	23.1 (101%)	0.4 (2%)	25.2 (104%)
EROD activity	3-MC (0.2 μM)	3.3 (100%)	6.2 (100%)	143.2 (100%)	134.2 (100%)	75.1 (100%)	70.5 (100%)
	+LPS (10 ng/ml)	4.7 (142%)	5.4 (87%)	112.3 (78%)	133.8 (100%)	63.3 (84%)	77.9 (110%)

n.d., not detectable; Cocu., cocultures; Hep., hepatocyte cultures.
The values represent the means of two independent experiments and are expressed in pmol min^{-1} mg^{-1} protein.

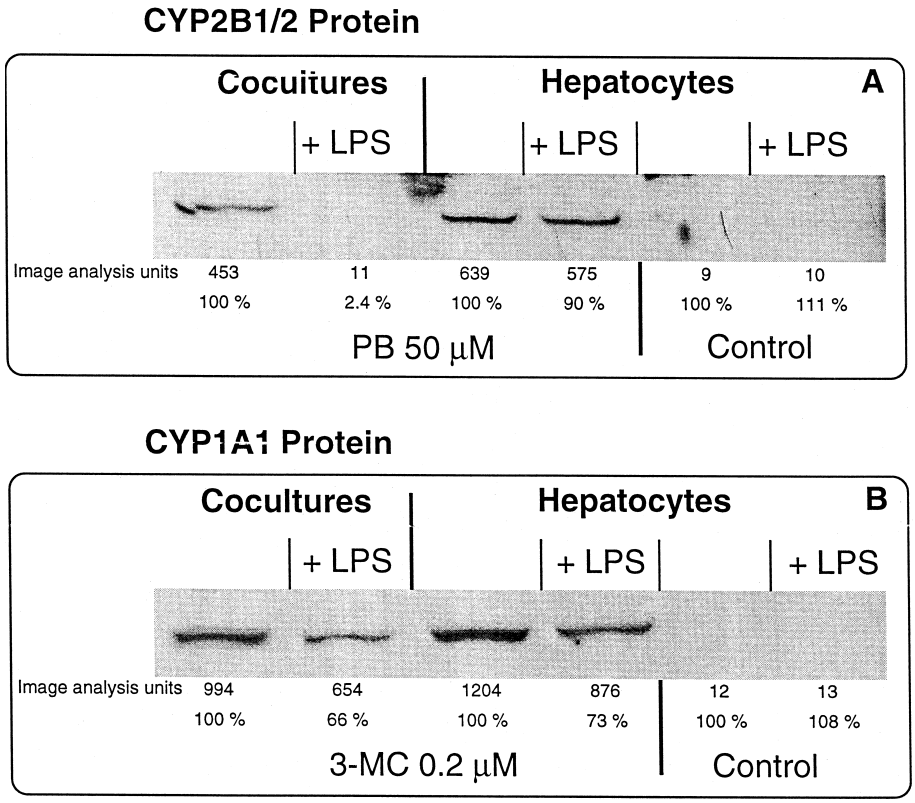

Fig. 7. Western blots of S9-extracts (20 μg total protein per lane) from 24 h (A) PB (50 μM) or (B) 3-MC (0.2 μM) treated cocultures and hepatocyte cultures with or without 10 ng/ml LPS. Blots were probed for (A) CYP2B1/2 or (B) CYP1A1 protein content with the corresponding ECL Western blotting kit. Image analysis was then performed with the Scanalytics One-D-Scan program.

lated by 15% (PROD) or even increased by 5% (EROD) (Fig. 6A and B). In cocultures, however, the LPS-mediated down-regulation was 85 and 15%, respectively. Phenobarbital and 3-methylcholanthrene did not significantly influence the LPS-induced NO and TNFα levels. The enzyme activities correlated well with the amount of the corresponding mRNA transcripts (Northern blot analysis: Fig. 6C and D) and proteins (Western blot analysis: Fig. 7A and B). A similar down-regulation was detectable when TNFα (500 pg/ml) was given to hepatocyte cultures simultaneously with or 12 h after the enzyme inducers (Table 3). In contrast, no down-regulation was observed when TNFα (500 pg/ml) was given 24 and 5 h prior to the enzyme inducers and then removed (medium change) (Table 3). Incubation with a rabbit anti-rat-TNFα neutralising polyclonal antibody during LPS treatment blocked the down-regulation of the CYP activities and the NO release in cocultures (Table 4). The addition of 50 μM L-NIL as a potent and selective inhibitor of the inducible NO synthase blocked the NO release but had no effect on the down-regulation of the CYP activities in cocultures (Table 4).

3.4. Response after exposure to acetaminophen

The CYP2B1-dependent direct acetaminophen-induced toxicity in rat hepatocytes has been well characterised (Jollow et al., 1973; Mitchell et al., 1973; Potter et al., 1973) whereas, the suspected role of macrophages in the chemically-induced tissue injury (Laskin et al., 1986) requires further elucidation as made with the present coculture system. Cocultures were exposed either to acetaminophen alone (1 and 10 mM) to acetaminophen together with LPSs or to acetaminophen following a 24 h pretreatment of the hepatocyte monoculture with 1 mM acetaminophen or 50 μM phenobarbital. No changes in NO and TNFα release were detectable under any of these conditions in comparison to untreated cultures. Only in cocultures treated simultaneously with 1 mM acetaminophen and 10 ng/ml LPS that a slight decrease of

Table 3
Influence of TNFα pre- and post-treatment on the PB or 3-MC induced PROD and EROD activity in hepatocyte cultures

Endpoints	rec. rat TNFα (500 pg/ml)			
	−24 to 0 h	−5 to 0 h	0 to 24 h	12 to 24 h
PROD activity[a]	92	91	15	35
EROD activity[b]	94	103	71	86

The values were determined after a 24 h treatment period and represent the means of two independent experiments.
[a] In percent (%) of PB (50 μM) treated (24 h) hepatocyte cultures without TNFα (20.7 ± 9.1 pmol min^{-1} mg^{-1} protein).
[b] In percent (%) of 3-MC (0.2 μM) treated (24 h) hepatocyte cultures without TNFα (124.6 ± 40.6 pmol min^{-1} mg^{-1} protein).

Table 4
Influence of the iNOS inhibitor L-NIL and the anti-TNFα antibody on the down-regulation of the PROD and EROD activities in PB or 3-MC treated cocultures or hepatocyte cultures

Endpoints	Treatment	Cotreatment	Cocultures		Hepatocyte cultures		
				+LPS (10 ng/ml)		+LPS (10 ng/ml)	+rec. rat TNFα (1 ng/ml)
PROD activity (pmol min^{-1} mg^{-1} protein)	PB (50 μM)	–	17.8	0.7	22.8	23.1	0.5
		anti-rat TNFα-IGg (5 μg/ml)	17.3	12.9	21.2	19.8	11.5
		L-NIL (50 μM)	18.5	1.3	19.6	21.5	0.8
EROD activity (pmol min^{-1} mg^{-1} protein)	3-MC (0.2 μM)	–	143.2	112.3	136.3	129.7	97.8
		anti-rat TNFα-IGg (5 μg/ml)	134.2	133.8	128.4	141.2	135.6
		L-NIL (50 μM)	121.7	103.7	136.4	133.1	94.3
NO (μM)	PB or 3-MC[a]	–	2.1	29.6	3.9	4.1	21.2
		anti-rat TNFα-IGg (5 μg/ml)	3.1	8.5	2.8	3.1	7.2
		L-NIL (50 μM)	2.9	2.1	3.9	3.8	3.5
TNFα (pg/ml)	PB or 3-MC[a]	–	6	2982	6	11	–
		L-NIL (50 μM)	5	3396	7	15	–

The values were calculated from measurements after a 24 h treatment period and represent the means of two independent experiments.
[a]For reduction, the corresponding values from PB (50 μM) or 3-MC (0.2 μM) treated cultures were pooled, because NO and TNFα release were not affected by both PB or 3-MC treatment.

the NO and TNFα release was seen in comparison to cocultures treated with 10 ng/ml LPS alone (Table 5). A similar anti-inflammatory effect of acetaminophen on the TNFα-release by hepatocytes was shown by Horbach et al. (1997).

4. Discussion

Nonparenchymal liver cells are suspected to contribute to the toxicity of hepatotoxic compounds. They also might be a critical target in nongenotoxic carcinogenesis since Kupffer cell-derived factors are involved in liver tissue homeostasis. Bearing in mind that hepatotoxicity and hepatocarcinogenesis are the outcomes of multistep processes which cannot be dissected in detail in whole animal studies, the present coculture system offers a valuable opportunity to elucidate the mode of action of hepatotoxic xenobiotics. Prerequisites are that hepatocytes express a high metabolic competence and that the cocultured Kupffer cells respond to realistic levels of endotoxins (corresponding to those found in vivo). Both are fulfilled with the present approach. Data obtained demonstrates that a crosstalk between Kupffer cells and hepatocytes takes place and this interaction affects xenobiotic metabolism.

4.1. In vivo equivalent cocultures and sensitivity of the Kupffer cells

In the present set-up of the cocultures, as mentioned before, the interaction between the two liver cell populations is restricted to soluble messengers responsible for the first insults after exposure to endotoxins via paracrine or autocrine pathways. The use of culture dish inserts with membrane filters (Fig. 1) facilitates access to nutrients, the release of mediators through the apical and basolateral surface of the cells and the expression of a possible polarised location of cell surface receptors. The possible separation of the two cell populations during the experiments allows the contribution of each of them to be determined (e.g., NO release, Fig. 3B). It also permits pretreatment of the hepatocytes (e.g., phenobarbital, acetaminophen, Table 5), as well as the separate analysis of the two cell populations after coculturing (e.g., viability; Table 1). The mimicry of subsequent events, e.g., due to direct cell–cell interactions, cannot be investigated in the present approach. The Kupffer cells require a 2 day culture period in presence of fetal calf serum for their down-regulation and recovery from the isolation procedure. The addition of serum to the hepatocyte cultures, however, down-regulates the liver specific gene expression. Similarly, the removal of the Kupffer cells from the cell culture inserts by means of proteases causes a dramatic loss of cell specific functions and viability.

The Kupffer cells in the present cultures are highly sensitive to endotoxin stimuli (Fig. 4A and B). This sensitivity allows the detection of the effects of low constitutive

Table 5
Effect of APAP and LPS treatment on NO release, TNFα release and hepatocyte viability (LDH release) in cocultures

Treatment (0 h to 24 h)	Pretreatment (−24 h to 0 h)	NO (μM)	TNFα (pg/ml)	Viability (% of control)
Control		3.1 ± 1.2	5 ± 2	100
LPS (10 ng/ml)		28.2 ± 7.4	2869 ± 532	86 ± 11
APAP (1 mM)		3.6 ± 2.3	6 ± 3	94 ± 13
APAP (10 mM)		4.1 ± 2.8	4 ± 1	88 ± 8
APAP (1 mM) + LPS (10 ng/ml)		21.3 ± 8.2	2212 ± 465	92 ± 12
LPS (10 ng/ml)	APAP (1 mM)	24.2 ± 5.6	2534 ± 497	102 ± 18
APAP (1 mM)	APAP (1 mM)	3.3 ± 1.7	7 ± 4	93 ± 11
APAP (1 mM) + LPS (10 ng/ml)	APAP (1 mM)	27.5 ± 8.4	2872 ± 626	89 ± 7
LPS (10 ng/ml)	PB (50 μM)	30.2 ± 9.1	3011 ± 564	91 ± 10
APAP (1 mM)	PB (50 μM)	4.3 ± 1.9	5 ± 3	99 ± 15
APAP (1 mM) + LPS (10 ng/ml)	PB (50 μM)	26.4 ± 7.6	2733 ± 435	95 ± 12

All values were determined after a 24 h treatment period and are expressed as means ± S.D. of three independent experiments.

LPS levels as released from the normal colonic flora in the intestine (Van Deventer et al., 1988).

In vivo, the distribution of Kupffer cells expressing TNFα is homogeneous throughout the liver lobules and showed no zonal preference (Hoffmann et al., 1994). In accordance, periportal or pericentral equivalent pO_2 levels and insulin concentrations (Ohno and Maier, 1994) had no significant influence on the endotoxin induced TNFα release in our present investigation whereas the hepatic NO formation was reduced by half under low periportal equivalent pO_2 (4% O_2) similar to that previously reported in hepatocytes exposed to TNFα (Ohno and Maier, 1995). Therefore, the present investigations were restricted to 13% O_2 and 2 nM insulin.

4.2. Time- and dose-dependent response after LPS exposure

In animals, LPS and TNFα are rapidly eliminated resulting in a quick response with a peak 1–2 h after treatment (Hartung et al., 1996). The measured TNFα release represents the integral of the released cytokine at the time measured because TNFα is relatively stable in biological fluids (Cruz Ruiz et al., 1996). The rapid response seen within 5 h after exposure (Fig. 3A) approximates quite well the quick response in whole animals. The amount of the cytokine released (2–3 ng/ml) with the *Salmonella* LPS (10 ng/ml) is comparable to that reported (5 ng/ml) with a 1000× higher concentration of *Escherichia coli* LPS (10 μg/ml) by Hartung and Wendel (1991). The plateau reached, despite increasing LPS levels and increased exposure times, suggests that either no additional TNFα is released by the Kupffer cells (feedback mechanism) and no degradation takes place or the amount of degraded and released TNFα is in steady-state (Fig. 3A). Based on the half-life of TNFα the former is more likely the case. The dose-dependent increase and prolonged elevating NO release (Fig. 3AFig. 4A) is most likely due to an activation of the inducible NO synthase in the hepatocytes as shown in the experiment with the removal of the Kupffer cells after 5 h (Fig. 3B). Moreover, it demonstrates that after LPS exposure, approx. 80% of NO in the coculture medium derive from the hepatocytes. The release can be completely blocked by the specific inducible NO synthase inhibitor L-NIL. In contrast, after carbon tetrachloride-mediated and obstructive hepatic injury, inducible NO synthase mRNA and nitrite was detected in nonparenchymal cells (Rockey and Chung, 1997).

Induction of hepatic inducible NO synthase is maintained only in the presence of TNFα, since after a short-term exposure to TNFα (5 h) and subsequent removal, no increase in NO release was found (see Section 3.1). In addition, the main outcome of the acute phase response in liver (increase of positive and decrease of negative acute phase protein levels, increase in lactate release as a consequence of enhanced glucose uptake; Fig. 5A–C) after exposure to proinflammatory stimuli such as LPSs or cytokines (Andus et al., 1988; Baumann and Gauldie, 1994) was well detectable in the present coculture system. The declining expression of the positively regulated α1-acid-glycoprotein at LPS exposure levels higher than 10 ng/ml might be due to an exhaustive response of the Kupffer cells, because the LDH release, mainly deriving from the hepatocytes, is not further increased.

4.3. Liver injury and cytotoxicity in cocultures

In animal experiments, endotoxin-induced liver injury can be attributed to an initial activation of hepatic macrophages and TNFα release (Fujita et al., 1995), followed by a synergistic interaction between endotoxin-stimulated Kupffer cells and granulocytes (Sauer et al., 1996). In the present approach without granulocytes, LPS levels chosen had no direct cell-damaging effect on hepatocytes (LDH release) or on Kupffer cells (MTT-assay) (Table 1). A reduced hepatocyte viability was reported only during the first 24 h in culture and in presence of serum (Hartung and Wendel, 1991), conditions which are

in favor of an undifferentiated phenotype of the hepatocytes. The present investigations were carried out after 5–7 days in culture and without serum in order to preserve the liver specific metabolic competence.

4.4. Mode of action of CYP down-regulation

The down-regulation of CYP in animals (Chen et al., 1992) and isolated hepatocytes (Peterson and Renton, 1986; Barker et al., 1992) including human hepatocytes (Muntané-Relat et al., 1995) by inflammatory stimuli such as LPSs or cytokines is well established. Using pentoxifylline (which suppresses TNFα gene expression), it was shown that the reduction of CYP1A2 and CYP2B content is linked to the presence of TNFα (Monshouwer et al., 1996a). In the present investigation, we found an isoform-specific down-regulation: specifically, the CYP2B1 isoform was affected at the transcriptional level, as indicated by the parallel loss of mRNA transcripts (Fig. 6C,D), protein content (Fig. 7) and catalytic activity (Fig. 6A,B). This specific down-regulation was independent of the exposure period (Table 2). The slight down-regulation of CYP1A1, however, was found in LPS exposed cocultures and hepatocyte cultures (Fig. 7) and is therefore not mediated by the Kupffer cells. Such an isoform specific but different pattern of down-regulation was also reported to occur in cultured human hepatocytes after exposure to individual cytokines (Muntané-Relat et al., 1995). The observed down-regulation in the present experiments is not linked to the TNFα-dependent up-regulation of the inducible NO synthase. Accordingly, NO is not involved in the CYP2B1 down-regulation process. This was shown with the TNFα neutralising antibody which inhibited the down-regulation of the induced CYP2B1 and the hepatocyte-derived NO release whereas the inhibition of the inducible NO synthase by L-NIL was without effect (Table 4). NO-independent down-regulation of CYP isoforms by different cytokines and LPS was also shown for constitutive CYP2C11 mRNA expression in primary rat hepatocyte cultures (Sewer and Morgan, 1997) and for constitutive 2β and 6β testosterone hydroxylase activities in primary pig hepatocyte cultures (Monshouwer et al., 1996b). In contrast, NO was often suspected to contribute to the decrease in CYP-dependent monooxygenases (Khatsenko et al., 1993). Such a NO-dependent down-regulation was reported for constitutive protein levels of CYP isoforms 1A2, 2C11, 2B1/2 and 3A2 by TNFα and IL-1β in primary rat hepatocyte cultures (Carlson and Billings, 1996). However, quite often, the contamination of primary hepatocyte cultures with Kupffer cells is not well defined.

The LPS-initiated and TNFα-mediated down-regulation is not due to a competitive inhibition of the active site on CYP, because TNFα given for 24 or 5 h before exposition to phenobarbital or 3-methylcholanthrene (Table 3), had no down-regulating effect.

4.5. Acetaminophen

In the liver of animals exposed to acetaminophen, macrophages accumulated and became activated in areas which subsequently showed signs of toxicity (Laskin and Pilaro, 1986). Serum levels of proinflammatory cytokines (IL-1α and TNFα) were increased in animals after treatment with acetaminophen (Blazka et al., 1995). It is well established that rats are not very susceptible to the hepatotoxic effects of acetaminophen (LD_{50}: 3.7 g acetaminophen/kg b.w.) in comparison to mice and humans (Boyd and Bereczky, 1966). The LD_{50} for rats is approximately equivalent to 24.4 mM in cell cultures. This concentration cannot be achieved in the culture medium without the use of a solvent. In vivo, susceptibility can be increased by phenobarbital pretreatment (Mitchell et al., 1973), which indicates that CYP2B1/2 is involved in the hepatotoxic activity of acetaminophen. Pretreatment of the hepatocytes with phenobarbital or acetaminophen in the present experiments was without effect (Table 5). Acetaminophen also failed to stimulate the release of TNFα in Kupffer cells. Together, the data indicate that the observed activation of Kupffer cells in animals (Laskin, 1989) is a consequence rather than the cause of acetaminophen-induced liver toxicity. In fact, an initial stimulation of Kupffer cells would lead to a preferential down-regulation of the CYP isoform (CYP2B1/2) which is involved in the direct hepatotoxicity. This is most likely the explanation for the reduction of acetaminophen induced liver injury in rats after Kupffer cell stimulation with *Corynebacterium parvum* (Raiford and Thigpen, 1994). Moreover, acetaminophen induces severe hepatic necrosis even in TNFα/lymphotoxin-α (LT-α) knockout mice (Boess et al., 1998). Further evidence for the predominant role of the biotransformed metabolite is given by the use of CYP1A2 and CYP2E1 double-null mice, which are protected against acetaminophen toxicity (Zaher et al., 1998). Together, it can be concluded that acetaminophen induce primary lesions in hepatocytes which are converted into cytotoxic lesions by contact with factors induced by constitutive levels of LPS, followed by recruitment and activation of Kupffer cells, macrophages and other blood cells (Horbach et al., 1997). Direct cell adhesion, including the adherence of leukocytes to the sinusoidal cells (Fujita et al., 1995), might then provide conditions for the synergistic interaction with stimulated liver macrophages, leading to the proteolytic killing of hepatocytes (Sauer et al., 1996).

In summary, the data obtained demonstrate that intercellular interactions between hepatocytes and Kupffer cells take place and can be well modelled in the present coculture system. The results are relevant to the in vivo situation with respect to concentration– and time–response relationships. Accordingly, this approach will be suitable for investigating the impact of intercellular communication on drug metabolism or drug-induced alterations in intercellular communication.

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References

Ammann, P., Maier, P., 1997. Preservation and inducibility of xenobiotic metabolism in long-term cultures of adult rat liver cell aggregates. Toxicol. In Vitro 11, 43–56.

Andus, T., Geiger, T., Hirano, T., Kishimoto, T., Heinrich, P.C., 1988. Action of recombinant human interleukin 6, interleukin 1β and tumor necrosis factor α on the mRNA induction of acute-phase proteins. Eur. J. Immunol. 18, 739–746.

Barker, C.W., Fagan, J.B., Pascos, D.S., 1992. Interleukin-1β suppresses the induction of *P*4501A1 and *P*4501A2 mRNAs in isolated hepatocytes. J. Biol. Chem. 267, 8050–8055.

Baumann, H., Gauldie, J., 1994. The acute phase response. Immunol. Today 15, 74–80.

Baumann, H., Hill, R.E., Sauder, D.N., Jahreis, G.P., 1986. Regulation of major acute-phase plasma proteins by hepatocyte-stimulating factors of human squamous carcinoma cells. J. Cell Biol. 102, 370–383.

Berridge, M.V., Tan, A.S., 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT): subcellular localization, subtrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophys. 303, 474–482.

Blazka, M.E., Wilmer, J.L., Holladay, S.D., Wilson, R.E., Luster, M.I., 1995. Role of proinflammatory cytokines in acetoaminophen hepatotoxicity. Toxicol. Appl. Pharmacol. 133, 43–52.

Boess, F., Bopst, M., Althaus, R., Polsky, S., Cohen, S.D., Eugster, H.P., Boelsterli, U.A., 1998. Acetaminophen hepatotoxicity in tumor necrosis factor/lymphotoxin-alpha gene knockout mice. Hepatology 27, 1021–1029.

Boyd, E.M., Bereczky, G.M., 1966. Liver necrosis from paracetamol. Br. J. Pharmacol. 26, 606–614.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brouwer, A., Parker, S.G., Hendriks, H.F.J., Gibbons, L., Horan, M.A., 1995. Production of eicosanoids and cytokines by Kupffer cells from young and old rats stimulated by endotoxins. Clin. Sci. Colch. 88, 211–217.

Carlson, T.J., Billings, R.E., 1996. Role of nitric oxide in the cytokine-mediated regulation of cytochrome *P*450. Mol. Pharmacol. 49, 796–801.

Chen, Y.L., Florentin, A., Batt, A.M., Ferrari, L., Giroud, J.P., Chauvelot-Moachon, L., 1992. Effects of interleukin-6 on cytochrome *P*450-dependent mixed-function oxidases in the rat. Biochem. Pharmacol. 44, 137–148.

Cruz Ruiz, M., Recio Quijano, F., Lopez Cortes, L.F., Hebles Duvison, M., Vazquez Rubio, R., 1996. Determination of shelf life and activation energy for tumor necrosis factor-α in cerebrospinal fluid samples. Clin. Chem. 42, 670–674.

Decker, K., 1998. The response of liver macrophages to inflammatory stimulation. Keio J. Med. 47, 1–9.

Ding, A.H., Nathan, C.F., Stuehr, D.J., 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J. Immunol. 141, 2407–2412.

Fujita, S., Arii, S., Monden, K., Adachi, Y., Funaki, N., Higashitsuji, H., Furutani, M., Mise, M., Ishiguro, S., Kitao, T., Nakamura, T., Imamura, M., 1995. Participation of hepatic macrophages and plasma factors in endotoxin-induced liver injury. J. Surg. Res. 59, 263–270.

Grewe, M., Gausling, R., Gyufko, K., Hoffmann, R., Decker, K., 1994. Regulation of the mRNA expression for tumor necrosis factor-α in rat liver macrophages. J. Hepatol. 20, 811–818.

Hartung, T., Wendel, A., 1991. Endotoxin-inducible cytotoxicity in liver cell cultures. Biochem. Pharmacol. 42, 1129–1135.

Hartung, T., Hermening, S., Sauer, A., Wendel, A., 1996. Freisetzung eines Botenstoffes der Entzündung (TNFα) aus Lebermakrophagen in Perfusions–Zellkultur. ALTEX 13, 17–23.

Hendriks, H.F.J., Brouwer, A., Knook, D.L., 1990. Isolation, purification and characterization of liver cell types. Methods Enzymol. 190, 49–58.

Hoffmann, R., Grewe, M., Estler, H., Schulze-Specking, A., Decker, K., 1994. Regulation of tumor necrosis factor-α-mRNA synthesis and distribution of tumor necrosis factor-α-mRNA synthesizing cells in rat liver during experimental endotoxemia. J. Hepatol. 20, 122–128.

Horbach, M., Gerber, E., Kahl, R., 1997. Influence of acetaminophen treatment and hydrogen peroxide treatment on the release of a CINC-related protein and TNFα from rat hepatocyte cultures. Toxicology 121, 117–126.

Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gilette, J.R., Brodie, B.B., 1973. Acetaminophen-induced hepatoc necrosis: II. Role of covalent binding in vivo. J. Pharmacol. Exp. Ther. 187, 195–202.

Juillerat, M., Marceau, N., Coeytaux, S., Sierra, F., Kolodziejczyk, E., Guigoz, Y., 1997. Adult rat liver cells form aggregates and express organ specific structures and functions. Toxicol. In Vitro 11, 57–69.

Khatsenko, O.G., Gross, S.S., Rifkind, A.B., Vane, J.R., 1993. Nitric oxide is a mediator of the decrease in cytochrome *P*450-dependent metabolism caused by immunostimulants. Proc. Natl. Acad. Sci. U.S.A. 90, 11147–11151.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680–685.

Laskin, D.L., 1989. Potential role of activated macrophages in chemical and drug induced liver injury. In: Wisse, E., Knook, D.L., Decker, K. (Eds.), Cells of the Hepatic Sinusoid, Vol. 2. Kupffer Cell Foundation, Amsterdam, pp. 284–287.

Laskin, D.L., Pilaro, A.M., 1986. Potential role of activated macrophages in acetaminophen hepatotoxicity, Isolation and characterization of activated macrophages from rat liver. Toxicol. Appl. Pharmacol. 86, 204–215.

Laskin, D.L., Pilaro, A.M., Ji, S., 1986. Potential role of activated macrophages in acetaminophen hepatotoxicity. Mechanism of macrophage accumulation and activation. Toxicol. Appl. Pharmacol. 86, 216–226.

Levitin, H., Kendrick, M.L., Kass, E.H., 1956. Effect of route of administration on protective action of corticosterone and cortisol against endotoxin. Proc. Soc. Exp. Biol. Med. 93, 306–309.

Maier, P., Saad, B., Schawalder, H.P., 1994. Periportal- and centrilobular-equivalent oxygen tension affect liver specific functions and xenobiotic metabolism in long-term rat hepatocyte cultures. Toxicol. In Vitro 8, 423–435.

Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R., Brodie, B.B., 1973. Acetaminophen-induced hepatoc necrosis: I. Role of drug metabolism. J. Pharmacol. Exp. Ther. 187, 185–194.

Monshouwer, M., McLellan, R.A., Delaporte, E., Witkamp, R.F., Van

Miert, A.S., Renton, K.W., 1996a. Differential effect of pentoxifylline on lipopolysaccharide-induced downregulation of cytochrome *P*450. Biochem. Pharmacol. 52, 1195–1200.

Monshouwer, M., Witkamp, R.F., Nijmeijer, S.M., Van Amsterdam, J.G., Van Miert, A.S., 1996b. Suppression of cytochrome *P*450- and UDP glucuronosyl transferase-dependent enzyme activities by proinflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. Toxicol. Appl. Pharmacol. 137, 237–244.

Muntané-Relat, J., Ourlin, J.C., Domergue, J., Maurel, P., 1995. Differential effects of cytokines on the inducible expression of CYP1A1, CYP1A2 and CYP3A4 in human hepatocytes in primary culture. Hepatology 22, 1143–1153.

Nakajima, T., Elovaara, E., Park, S.S., Gelboin, H.V., Hietanen, E., Vainio, H., 1990. Monoclonal antibody-directed characterization of benzene, ethoxyresorufin and pentoxyresorufin metabolism in rat liver microsomes. Biochem. Pharmacol. 40, 1255–1261.

Ohno, K., Maier, P., 1994. Cultured rat hepatocytes adapt their cellular glycolytic activity and adenylate energy status to tissue oxygen tension: influences of extracellular matrix components, insulin and glucagon. J. Cell. Physiol. 160, 358–366.

Ohno, K., Maier, P., 1995. Tumor necrosis factor α differentially regulates the cellular response of rat hepatocytes in periportal- and perivenous-equivalent cultures. Eur. J. Pharmacol. 292, 205–214.

Peterson, T.C., Renton, K.W., 1986. Kupffer cell factor mediated depression of hepatic parenchymal cell cytochrome *P*-450. Biochem. Pharmacol. 35, 1491–1497.

Potter, W.Z., Davis, D.C., Mitchell, J.R., Jollow, D.J., Gilette, J.R., Brodie, B.B., 1973. Acetaminophen-induced hepatic necrosis: III. Cytochrome *P*-450-mediated covalent binding in vitro. J. Pharmacol. Exp. Ther. 187, 203–210.

Raiford, D.S., Thigpen, M.C., 1994. Kupffer cell stimulation with *Corynebacterium parvum* reduces some cytochrome *P*450-dependent activities and diminishes acetaminophen and carbon tetrachloride-induced liver injury in the rat. Toxicol. Appl. Pharmacol. 129, 36–45.

Rockey, D.C., Chung, J.J., 1997. Regulation of inducible nitric oxide synthase and nitric oxide during hepatic injury and fibrogenesis. Am. J. Physiol. 273, 124–130.

Roth, R.A., Harkema, J.R., Pestka, J.P., Ganey, P.E., 1997. Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agent?. Toxicol. Appl. Pharmacol. 147, 300–311.

Saad, B., Schawalder, H.P., Maier, P., 1993a. Crude liver membrane fractions maintain liver specific functions in long term, serum free rat hepatocyte cultures. In Vitro Cell Dev. Biol. 29A, 32–40.

Saad, B., Scholl, F.A., Thomas, H., Schawalder, H.P., Streit, V., Waechter, F., Maier, P., 1993b. Crude liver membrane fractions and extracellular matrix components as substrata regulate differentially the preservation and inducibilty of cytochrome *P*-450 isoenzymes in cultured rat hepatocytes. Eur. J. Biochem. 213, 805–814.

Sauer, A., Hartung, T., Aigner, J., Wendel, A., 1996. Endotoxin-inducible granulocyte-mediated hepatocytotoxicity requires adhesion and serine protease release. J. Leukocyte Biol. 60, 633–643.

Sewer, M.B., Morgan, E.T., 1997. Nitric oxide-independent suppression of *P*450 2C11 expression by interleukin-1β and endotoxin in primary rat hepatocytes. Biochem. Pharmacol. 54, 729–737.

Steinhorn, D.M., Cerra, F.B., 1997. Comparative effects of lipopolysaccharide on newborn versus adult rat hepatocyte and nonparenchymal cell cocultures. Crit. Care Med. 25, 121–127.

Van Deventer, S.J.H., Ten Cate, J.W., Tytgat, G.N.J., 1988. Intestinal endotoxemia: clinical significance. Gastroenterology 94, 825–831.

Wortelboer, H.M., De Kruif, C.A., Van Iersel, A.A.J., Falke, H.E., Noordhoeck, J., Blaauboer, B.J., 1990. The isoenzyme pattern of cytochrome *P*450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and intact monolayers. Biochem. Pharmacol. 40, 2525–2534.

Wortelboer, H.M., De Kruif, C.A., Van Iersel, A.A.J., Falke, H.E., Noordhoek, J., Blaauboer, B.J., 1991. Comparison of cytochrome *P*450 isoenzyme profiles in rat liver and hepatocyte cultures. The effect of model inducers on apoproteins and biotransformation activities. Biochem. Pharmacol. 42, 381–390.

Zaher, H., Buter, J.T.M., Ward, J.M., Bruno, M.K., Lucas, A.M., Stern, S.T., Cohen, S.D., Gonzalez, F.J., 1998. Protection against acetaminophen toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol. Appl. Pharmacol. 152, 193–199.