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European Journal of Pharmacology 401 (2000) 317–328

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Lead stimulates intercellular signalling between hepatocytes and Kupffer cells

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Received 19 April 2000; received in revised form 27 June 2000; accepted 4 July 2000

Abstract

The role of intercellular signalling between liver cells in lead (Pb)[1]-induced liver toxicity was investigated in cocultures of freshly isolated and cultured rat hepatocytes and Kupffer cells. The Kupffer cells (seeded onto culture dish inserts), the hepatocytes or the two in cocultures were exposed to Pb acetate (2–50 μM) in combination with lipopolysaccharide (0.1–1000 ng/ml). In hepatocyte cultures, the combined Pb/lipopolysaccharide treatment induced no significant increase in the release of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) whereas in Kupffer cell cultures and in cocultures, at low lipopolysaccharide levels (0.1 and 1 ng/ml), TNF-α release was synergistically increased (up to 30-fold) when compared to lipopolysaccharide exposure alone. This stimulation of Kupffer cell-derived TNF-α release was specific for Pb or not detectable with mercury and cadmium. As a response to the Pb/lipopolysaccharide induced release of TNF-α, the cocultured hepatocytes increased their nitric oxide (NO) content sixfold when compared with lipopolysaccharide alone and downregulated the negatively regulated acute phase protein albumin. This downregulation was also detectable without lipopolysaccharide and without TNF-α release, indicating that Pb induces additional thus far unidentified Kupffer cell-derived factors, which interact with the cocultured hepatocytes. At the time of TNF-α release, the viability of the hepatocytes and the Kupffer cells was not affected. However, after a 48-h treatment period, Pb induced a Kupffer cell specific toxicity without affecting the hepatocytes. Loss of hepatocyte viability after lipopolysaccharide/Pb stimulation was only detectable in the presence of cocultured Kupffer cells together with human-derived granulocytes. It is concluded that Pb stimulates intercellular signalling between Kupffer cells and hepatocytes which is synergistically enhanced in the presence of low lipopolysaccharide levels. The released Kupffer cell-derived signals (e.g. cytokines) promotes most likely proteolytic hepatocyte killing in combination with a direct cellular interaction between the granulocytes and the hepatocytes.

Keywords: Lead; Hepatocyte, rat; Lipopolysaccharide; Kupffer cell; Coculture; TNF-alpha (tumor necrosis factor-α); Nitric oxide (NO); Nitric oxide (NO) synthase, inducible

1. Introduction

Hepatotoxicity is the outcome of a complex sequence of interactions between the toxicant and target macromolecules, between damage and intracellular repair systems and intercellular signalling among different cell types within the liver. It is well established that sinusoidal cells, in particular the Kupffer cells, release intercellularly acting mediators such as cytokines, prostaglandins, leukotrienes, reactive oxygen species, platelet activating factors and nitric oxide (NO) (Decker, 1998; Brouwer et al., 1995). These mediators can efficiently be induced by endotoxins such as the lipopolysaccharide. These factors affect cellular defence and repair systems and regulate a wide array of cellular functions within the liver as a whole. For example, the messengers control liver tissue homeostasis, which might be contributing factors in nongenotoxic carcinogenesis (Rose et al., 1997; Roberts et al., 1998), they affect xenobiotic metabolism in hepatocytes (Milosevic et al., 1999), and might be directly involved in liver toxicity of hepatotoxic compounds (Laskin et al., 1986; Sneed et al., 1997).

The interaction between hepatocytes and Kupffer cells is important in the context of individual sensitivity to hepatotoxic chemicals. Individuals are constitutively exposed to endotoxins deriving from the colonic flora or from bacterial infections (Van Deventer et al., 1988). If these provoke an inflammation in the liver, Kupffer cells will become activated and could modify an individual's

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00473-8

sensitivity toward hepatotoxins by mechanisms outlined above. Such a modulation of hepatotoxicity by intercellular signalling cannot be addressed in primary cultures of pure hepatocytes but requires cocultures between different liver cell types. In the present investigation, we combined primary Kupffer cells and hepatocytes in a most recently established coculture system (Milosevic et al., 1999).

Lead (Pb) is an environmental contaminant of continuing relevance. Blood Pb concentrations in the general population are in the order of 0.5–1 μM. Pb affects most organs, many biochemical pathways and enzyme systems. Striking effects in animals are the downregulation of the cell-mediated immune response, resulting in an enhanced susceptibility to bacterial and viral infections (Lawrence, 1985) and the stimulation of the mitogenic activity in liver (Ledda-Columbano et al., 1994), an attribute of nongenotoxic carcinogens.

Pb could be a xenobiotic which interferes with intercellular signalling between Kupffer cells and hepatocytes: lipopolysaccharide-induced liver injury was exacerbated by concomitant exposure to Pb in rodents (Selye et al., 1966; Calcamuggi et al., 1992). This effect was thought to be mediated by the cytokine tumor necrosis factor α (TNF-α, Honchel et al., 1991). Cultured mouse hepatocytes exposed to TNF-α and interferon-γ in combination with a non-toxic concentration of Pb exhibited a significantly higher cytotoxicity than hepatocytes treated with the cytokines alone (Sieg and Billings, 1997). Our goal was to investigate the role of intercellular signalling between cultured primary hepatocytes and Kupffer cells in the Pb-induced hepatocellular injury under realistic low, non-cytotoxic concentrations of Pb and lipopolysaccharide.

We used cocultures in which the rat hepatocytes preserve their liver specific metabolic competence up to 7–9 days in culture (Maier et al., 1994). The freshly isolated and cocultured Kupffer cells respond very efficiently to low lipopolysaccharide levels (Milosevic et al., 1999). The Kupffer cells are cultured on inserts with membrane filters which restrict the cell–cell communication between hepatocytes and Kupffer cells to soluble messengers. Furthermore, the insert allows the two cell populations to be separated for pretreatment or for biochemical analysis during or after the experiment. The simulation of subsequent events in a tissue, e.g. direct cell–cell interactions, can be investigated by the addition of other target cells, such as granulocytes, directly to the corresponding cell population.

2. Materials and methods

2.1. Reagents

The reagents used for hepatocyte and Kupffer cell isolation and cultures of the two were identical to those described in a previous publication (Milosevic et al., 1999). Lipopolysaccharides from *Salmonella enteritidis* and recombinant human endothelial growth factor (EGF) were purchased from Sigma (Buchs, Switzerland). Lactate dehydrogenase (LDH) kits were from Boehringer Mannheim (Rotkreuz, Switzerland). The chemicals Pb–acetate, mercury–acetate, cadmium–acetate, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetra-zolium bromide (MTT) and selenium, all of reagent grade, were purchased from Fluka (Buchs, Switzerland) and the specific inhibitor of inducible nitric oxide synthase (iNOS), L-N^6-(1-iminoethyl)-lysine (L-NIL), from ANAWA (Wangen, Switzerland). Rat recombinant TNF-α was purchased from ImmunoKontact (Bioggio, Switzerland) and rat recombinant interferon-γ from Genzyme (Cambridge, MA, USA). Hoechst 33342 dye was purchased from Calbiochem (La Jolla, CA).

2.2. Antibodies

Nonparenchymal cells were identified with the monoclonal antibody OX-18, mouse anti rat major histocompatibility complex I (MHC I; Chemikon, Milan, Italy) and the Kupffer cells with the mouse anti rat resident macrophages monoclonal antibody ED2 (Bachem, Bubendorf, Switzerland). Goat monoclonal antibody to mouse immunoglobulin G 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. Mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (PC-10) was purchased from DAKO (Glostrup, Denmark). Rat TNF-α ELISA kit was obtained from Biosource (Fleurus, Belgium).

2.3. Hepatocyte cultures and treatment

Hepatocytes were isolated by a two-step in situ liver perfusion (Milosevic et al., 1999) from male Sprague–Dawley rats SD (Crl/CD®), bred at the Institute of Toxicology, Swiss Federal Institute of Technology, Zürich, Switzerland (age 54 ± 2 days; 240–270 g b.w.), kept under controlled environmental conditions (12-h light–dark cycle, diet no. 3430 KLIBA, Kaiseraugst, Switzerland, food and water ad libitum); anaesthesia was with 50 mg/kg b.w. pentobarbital, i.p. 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%. Hepatocytes were cultured under controlled periportal equivalent oxygen tension (13% O_2 v/v incubator atmosphere; substitution of air with nitrogen and 5% CO_2 [Maier et al., 1994] and Petriperm culture dishes with gas-permeable hydrophilic Teflon membrane bottom, 20 cm^2, Heraeus, Zürich, Switzerland). The dishes were coated with a mixture of 120 μg rat liver crude membrane

fraction and 1.2 μg collagen type 1, each in 2 ml phosphate buffered saline (PBS) overnight at 4°C, as described previously (Saad et al., 1993). The hepatocytes ($143 \times 10^3/cm^2 = 3 \times 10^6$/dish) 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) and the medium was replaced every 24 h. After 4 days in culture, dexamethasone was omitted from the medium. Instead, rat recombinant interferon-γ was added at low concentrations (10 U/ml) as a well-established translation-stimulating factor for cytokine production in macrophages and Kupffer cells (mainly derived from T-cells after lipopolysaccharide exposure of an intact organism). Interferon-γ itself did not induce TNF-α but increased the lipopolysaccharide-induced cytokine release in our cocultures about fivefold (Milosevic et al., 1999). The test compounds (Pb acetate, lipopolysaccharide, L-NIL and recombinant factors) were dissolved in culture medium. The treatment period lasted 48 h (from days 4 to 6 after seeding) without medium change. This time interval was shown to be optimal for the hepatocyte-derived NO release (Milosevic et al., 1999) and the measurements of TNF-α levels.

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) as described in detail before (Ammann and Maier, 1997; Milosevic et al., 1999). Briefly, the liver was preperfused in situ with pronase E followed by a mixture of pronase E and collagenase. The obtained cell suspension was filtered, incubated in a mixture of pronase E and collagenase and the nonparenchymal cells isolated by density-gradient centrifugation. Kupffer cells were further purified by elutriation. Purity (immunohistochemistry), cell number, and viability (trypan blue exclusion) were assessed by phase microscopy and cell volume measurements. The purity of the Kupffer cell fraction ($60 \pm 16 \times 10^6$ cells per rat liver) after elutriation was 85% ± 11% and the viability 96% ± 2%.

Kupffer cells (3×10^6) were seeded onto 0.2 μm Anopore 25-mm culture inserts in 6-well plates for cell culture 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 more than 95%.

The culture inserts with the Kupffer cells were then transferred either into hepatocyte cultures or into cell-free and coated Petriperm dishes and exposed to the corresponding concentrations of Pb and lipopolysaccharide for 48 h in the medium, under the same conditions as described for the hepatocytes (13% O_2; 5% CO_2; without dexamethasone, with interferon-γ).

2.5. Polymorphonuclear leukocytes isolation

Isolation of blood neutrophils from rats is hampered by the animal's small total blood volume and low number of of neutrophils in circulation (Harkness and Wagner, 1995). Therefore, polymorphonuclear leukocytes were isolated from human blood. Fifteen milliliters of fresh human blood was layered on top of 10-ml Ficoll Paque (Pharmacia, Uppsala, Sweden) and allowed to sediment for 45 min at room temperature. Plasma was recovered and layered again on top of 10-ml Ficoll Paque. After centrifugation ($170 \times g$, 10 min at 4°C), the pelleted polymorphonuclear leukocytes were resuspended in 5 ml Gey's buffer. After a hypotonic lysis of the remaining erythrocytes for 60 s and centrifugation as above, the cells were washed twice with Gey's buffer and then resuspended in 3 ml of the medium as described for the hepatocytes (without dexamethasone, with interferon-γ) but without aprotinin. About 1.2×10^6 polymorphonuclear leukocytes with a viability of 98% (trypan blue exclusion) were isolated per milliliter of fresh human blood. Polymorphonuclear leukocytes were seeded (granulocytes/hepatocytes = 10:1 optimal for the granulocyte-mediated hepatocytotoxicity, Sauer et al., 1996) directly on top of the hepatocytes or on 0.2 μm Anopore 25-mm cell culture inserts in cocultures or hepatocyte cultures and exposed to the corresponding concentrations of Pb and lipopolysaccharide for 24 h in the medium as described for the hepatocytes (13% O_2; 5% CO_2; without dexamethasone, with interferon-γ, without aprotinin).

2.6. Biochemical measurements

The two cell population were separately investigated because the Kupffer cells were transferred to other vessels for analysis and measurements. Protein content, LDH activity, albumin release, bioreductive capacity (using the thiazolylblue tetrazoliumbromide (MTT) assay), TNF-α and NO levels were determined as has been described previously (Milosevic et al., 1999).

2.7. Mitosis and apoptosis

Mitotic cells were identified by immunostaining of the hepatocytes for PCNA, an auxiliary protein of DNA polymerase δ, 48 h after the beginning of treatment as described earlier (Fasciati and Maier, 1997). Staining was performed with the metal-enhanced diaminobenzidine substrate (Pierce, Rockford, Ireland) and PCNA-expressing nuclei examined by light microscopy. Subsequently, the nuclei were stained with Hoechst 33342 (2 μg/ml in PBS) and apoptotic cells (condensed and fragmented nuclei) identified by fluorescence microscopy (E_x/E_m 340/460 nm) as reported earlier (Ohno et al., 1995).

2.8. Pb analysis

The distribution of Pb in the cultures was analysed after 5- and 24-h exposure to 50 μM Pb acetate. Concentrations were determined in the supernatant and the hepatocytes of the cultures with and without pretreatment with 5 μM rotenone (a mitochondrial complex I inhibitor resulting in cell death) for 12 h. The samples were taken from 200 μl of the culture supernatants and from homogenised hepatocytes scraped off the dishes into 1 ml 1% Triton X 100 and collected in plasticware pretreated by submersion for 48 h in 1 M nitric acid and washing with distilled water. Culture supernatant samples and cell homogenates were then air-dried and analysed at the Swiss Federal Laboratories for Materials Testing and Research (by R. Figi, EMPA, Duebendorf, Switzerland). The samples were dissolved in nitric acid > 65% w/w (TraceSelectUltra quality) under pressure (135 bar) and high temperature (300°C) in a high pressure asher (model HPA-S, A. Paar, A-Graz). The solution was colorless and clear. The determination of Pb was performed by graphite tube atomic absorption with standard addition procedures according to standard operation procedures established for Pb analysis (personal comunication, R. Figi).

3. Results

The cell type-specific changes detectable after exposure to lipopolysaccharide, Pb or a combination of both were determined in hepatocyte cultures, in Kupffer cell cultures and Kupffer cell/hepatocyte cocultures. Selected dose response data are given in Figs. 1–3. An overview and comparison of the effects in the different cell types at one concentration of Pb acetate and lipopolysaccharide (50 μM and 0.1 ng/ml, respectively) is found in Tables 1 and 2.

In studies such as these, it is imperative that the individual cultures be free of contaminating nonparenchymal cells. The contamination of the hepatocyte cultures by Kupffer cells was extremely small. This is shown by the following: (i) the number of nonparenchymal cells in the hepatocyte cultures was very low (< 0.5%) due to the 20-g sedimentation force used for hepatocyte isolation (Maier et al., 1994); (ii) no corresponding high TNF-α level was detectable after lipopolysaccharide treatment (Table 1); (iii) without foetal calf serum at the beginning of the culture period (foetal calf serum is not added to the hepatocyte cultures), Kupffer cells do not thrive beyond 4 days in culture (Fig. 4B). The coculture experiments however were carried out between 4 and 6 days after hepatocyte seeding.

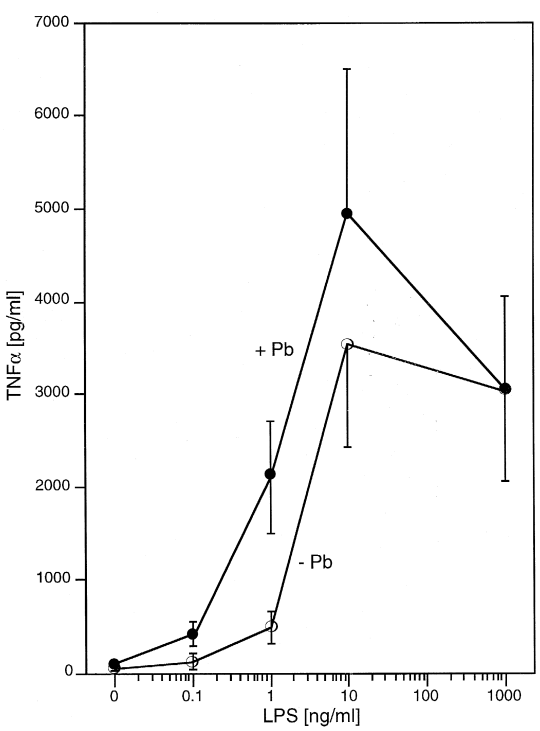

Fig. 1. TNF-α release in cocultures after a 24-h treatment period with LPS and with (●) or without (○) Pb acetate (50 μM). The values represent the mean ± S.D. of three independent experiments.

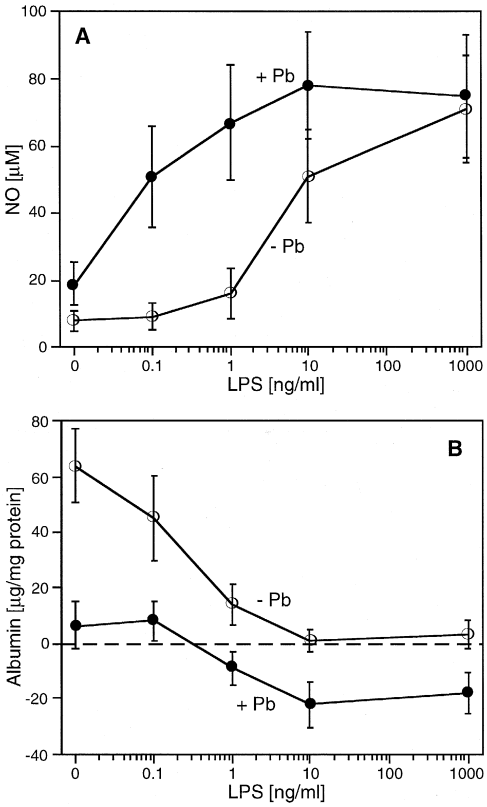

Fig. 2. Acute phase response in cocultures after LPS treatment with (●) or without (○) Pb acetate (50 μM). (A) NO levels were determined after a 24-h treatment period in culture supernatants. (B) Albumin release is expressed as the difference of albumin levels at 48 h minus albumin levels at 24 h after beginning of treatment in culture supernatants. All values represent the mean ± S.D. of three independent experiments.

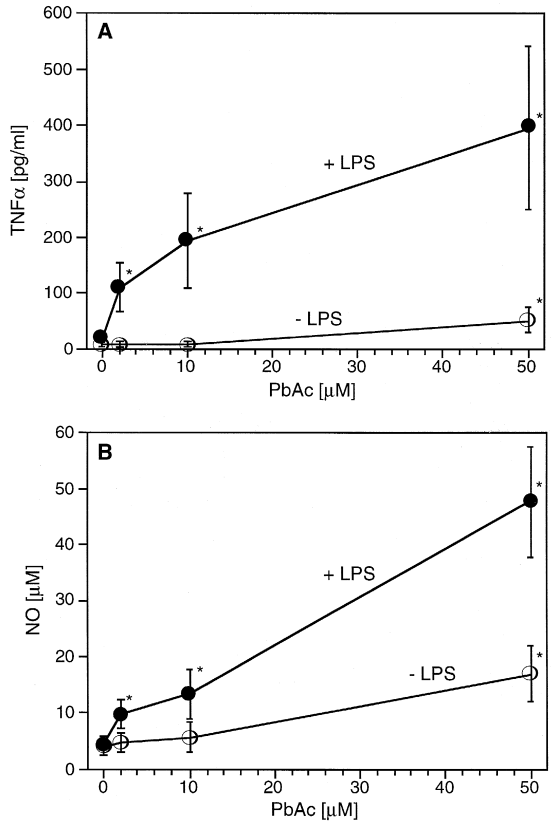

Fig. 3. Dose response of Pb acetate with (●) or without (○) LPS (0.1 ng/ml) on (A) TNF-α and (B) NO release in cocultures. (A) TNF-α levels were determined after a 24-h and (B) NO levels after a 48-h treatment period. All values represent the mean ± S.D. of three independent experiments. * Values are significantly different from controls ($p < 0.05$).

3.1. In Kupffer cell cultures, Pb and lipopolysaccharide synergistically stimulated the release of TNF-α

In Kupffer cell cultures, both lipopolysaccharide and Pb acetate treatments produce dose-dependent increases in TNF-α release (data no shown) in a manner similar to that observed in cocultures (Fig. 1). Lowest effective concentrations were 2 μM Pb acetate and 0.1 ng/ml lipopolysaccharide. In combination, the two treatments produced a synergistic enhancement of TNF-α release. For example, whereas lipopolysaccharide alone (0.1 ng/ml) brought about a twofold increase and Pb acetate (50 μM) an 11-fold increase, in combination they stimulated TNF-α release by more than 80-fold (Table 1). This synergistic effect was most effective at low concentrations of lipopolysaccharide (Fig. 1).

In contrast, NO release was not significantly stimulated in Kupffer cells by Pb acetate (data not shown) not even with the most effective concentration of 50 μM in cocultures (Fig. 3B). A weak dose response was seen with lipopolysaccharide, with a maximum of 16 ± 7 μM NO at 1 ng/ml lipopolysaccharide (threefold increase over controls). The combined treatment of lipopolysaccharide and Pb produced no increase of NO release in Kupffer cells (Table 2).

3.2. Hepatocytes are less responsive than Kupffer cells to the effects of lipopolysaccharide and Pb

The cultured hepatocytes showed no altered release of the proinflammatory protein TNF-α after treatment with lipopolysaccharide or Pb up to concentrations of 1000 ng/ml (Milosevic et al., 1999) and 50 μM, respectively (Table 1). Similarly, exposure to Pb caused no release of NO in the dose range tested (Table 2). NO release could be induced in hepatocytes by lipopolysaccharide (10-fold increase over controls), but only at very high concentrations (1000 ng lipopolysaccharide/ml; Milosevic et al., 1999; Saad et al., 1995).

High concentrations of albumin in the supernatant of hepatocyte cultures is indicative of normal metabolic functioning of the cells. On the other hand, albumin production is reduced when the cells are stressed, as a part of the so-called acute phase response. Neither Pb (50 mM) nor lipopolysaccharide (0.1 ng/ml, highest effect in cocultures), nor the combination of the two caused a reduction in albumin production by hepatocytes when cultured alone (Table 2).

Table 1
Pb synergistically enhances release of TNF-α in Kupffer cells

TNF-α (pg/ml)	Hepatocyte cultures	Kupffer cell cultures	Cocultures
Control	4 ± 1	5 ± 3	6 ± 3
LPS (0.1 ng/ml)	4 ± 2 (1 ×)	13 ± 7[a] (2.5 ×)	12 ± 5[a] (2 ×)
Pb (50 μM)	5 ± 3 (1 ×)	55 ± 20[a] (11 ×)	43 ± 16[a] (7 ×)
Pb (50 μM) + LPS (0.1 ng/ml)	6 ± 3 (1 ×)	420 ± 80[a] (84 ×)	400 ± 100[a] (67 ×)

LPS = lipopolysaccharide; TNF-α levels were determined 24 h after beginning of treatment. The values represent the mean ± S.D. of three independent experiments. The numbers within parentheses indicate the factors related to the corresponding controls.

[a] Values are significantly different from controls ($P < 0.05$).

Table 2
In cocultures LPS and Pb synergistically induce the Kupffer cell-mediated acute phase response in hepatocytes

	Hepatocyte cultures	Kupffer cell cultures	Cocultures
NO (μM)			
Control	5 ± 2	5 ± 2	6 ± 3
LPS (0.1 ng/ml)	4 ± 3 (1×)	6 ± 3 (1×)	8 ± 4 (1×)
Pb (50 μM)	4 ± 1 (1×)	4 ± 2 (1×)	19 ± 7[a] (3×)
Pb (50 μM) + LPS (0.1 ng/ml)	5 ± 2 (1×)	5 ± 2 (1×)	48 ± 15[a] (8×)
Albumin (μg / mg protein)			
Control	61 ± 17		64 ± 14
LPS (0.1 ng/ml)	55 ± 13 (1×)		45 ± 15 (1×)
Pb (50 μM)	43 ± 14 (1×)		6 ± 8[a] (0.1×)
Pb (50 μM) + LPS (0.1 ng/ml)	53 ± 16 (1×)		8 ± 7[a] (0.1×)

LPS = lipopolysaccharide; nitric oxide (NO) levels were determined 48 h after beginning of treatment and albumin release is expressed as the difference of albumin levels at 48 h minus albumin levels at 24 h after beginning of treatment. The values represent the mean ± S.D. of three independent experiments. The numbers within parentheses indicate the factors related to the corresponding controls.

[a]Values are significantly different from controls ($P < 0.05$).

3.3. Intercellular signalling increases the sensitivity of hepatocytes to Pb and lipopolysaccharide in coculture

In cocultures, the release of TNF-α after exposure to lipopolysaccharide, to Pb or to lipopolysaccharide and Pb (Table 1) was nearly identical to that found in Kupffer cells alone. The synergistic stimulation by Pb was again most prominent at low lipopolysaccharide concentrations (0.1–1 ng/ml; Fig. 1), increasing in a dose-dependent manner and levelling off at 10 ng/ml.

In contrast, the pattern of NO release was quite different in cocultures than in the monocultures: 50 μM Pb alone produced a threefold increase in NO release. NO production was further stimulated by the addition of increasing concentrations of lipopolysaccharide (Fig. 2A). An eightfold induction over controls was observed in cocultures at 0.1 ng lipopolysaccharide/ml (Table 2, Fig. 2A). Thus, the induction of NO release requires the simultaneous presence of both cell types in response to Pb (50 mM) and/or lipopolysaccharide (0.1 ng/ml).

Similarly, albumin production was significantly downregulated in cocultures by both lipopolysaccharide and Pb acetate alone or in combination (Table 2 and Fig. 2B). The negative values presented in Fig. 2B indicate a net albumin decay during the second day of culture in comparison to the first 24-h culture period. It can be concluded that the downregulation of the negatively regulated acute phase protein albumin in hepatocytes depends on the release of soluble factors from the cocultured Kupffer cells. The downregulation of albumin without lipopolysaccharide is stronger than that induced by the equivalent concentrations (Table 1) of TNF-α alone (Milosevic et al., 1999).

The net effect of the Kupffer cells is an increase in the sensitivity of cocultured hepatocytes towards Pb (Table 1 and 2). In the presence of low concentrations of lipopolysaccharide (0.1 ng/ml), this sensitivity is further increased down to Pb concentrations as low as 2 μM Pb (Fig. 3A,B). As the two cell types are not in direct contact with one another, the increase in sensitivity must be mediated by the release of soluble factors that serve as intercellular signals.

3.4. Mercury and cadmium induce no intercellular signalling

The effects described above were specific for Pb and could not be reproduced with other metals of environmental concern. Neither mercury–acetate (2–50 μM) nor cadmium–acetate (0.1–2 μM) at equitoxic concentrations induced the release of TNF-α or NO in Kupffer cell cultures, in hepatocyte cultures or in cocultures (data not shown). These control experiments also ruled out the possibility that the acetate counter ion was responsible for the Pb-specific effects.

3.5. Kupffer cells-specific cytotoxicity after a 48-h exposure to Pb

The question remains whether the induced TNF-α release was a consequence of a cytotoxic event in Kupffer cells. Whereas hepatocytes showed no toxicity towards Pb at up to 50 μM concentrations (as measured by LDH release), the Kupffer cells were more sensitive (measured by the MTT-test) (Fig. 4A). A significant toxicity towards Pb was seen after the 48-h exposure period: the bioreductive capacity of the Kupffer cells dropped to 30% of the initial values (Fig. 4B). This cytotoxicity was not influenced by lipopolysaccharide (0.1 ng/ml).

The time-course of the Pb-induced cytotoxicity in Kupffer cells is shown in Fig. 4B. This time-course was established with the highest Pb concentration (50 μM). At the time of TNF-α release (within 5–12 h after stimulation;

Milosevic et al., 1999), no prominent toxicity is yet detectable.

3.6. NO does not protect hepatocytes in cocultures

Although the hepatocytes were clearly more sensitive to the effects of Pb and lipopolysaccharide in coculture, responding with the acute phase reaction, no concommitant loss of viability was observed (Table 3/Fig. 4A). Hepatocyte death would have been expected on the basis of the observed Pb-induced liver toxicity in animal experiments (Selye et al., 1966). It might well be that in stationary cultures the released NO protects the hepatocytes from the toxic activity of lipopolysaccharide/Pb exposure. NO is produced by the inducible enzyme iNOS. A selective iNOS inhibitor (L-NIL) was added to cocultures and to hepatocyte cultures treated with Pb acetate (50 μM) and lipopolysaccharide (0.1 ng/ml). As expected, NO release was efficiently inhibited without affecting the release of TNF-α. However, the viability of the hepatocytes (extracellular LDH activity; Table 3) was not reduced. Thus, NO was not exerting a cytoprotective activity on the cocultured hepatocytes.

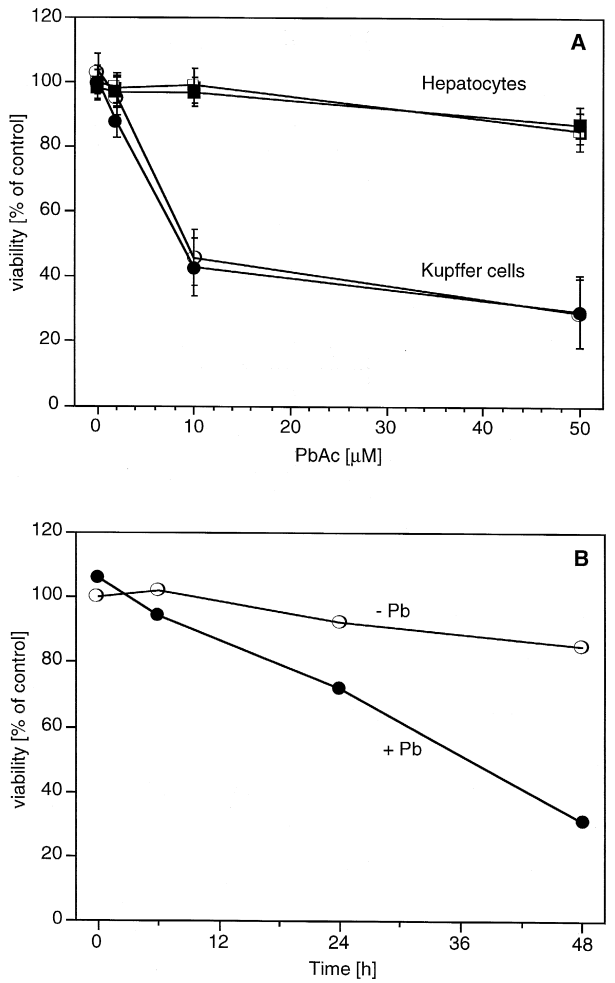

Fig. 4. Effects of Pb acetate treatment on the survival of cultured Kupffer cells and hepatocytes. (A) Dose response of Pb acetate with (●,■) or without (○,□) LPS (0.1 ng/ml) on the survival of Kupffer cells (●,○) and hepatocytes (■,□) in cocultures. After a 48-h treatment period the LDH release (extracellular LDH activity in percent of total [intra- and extracellular] activity) was determined for the hepatocytes and the MTT-reducing activity was determined for the Kupffer cells. The values are expressed as viability in percent of control (Kupffer cells or hepatocytes from cocultures without Pb acetate and LPS) and represent the mean ± S.D. of three independent experiments. (B) Viability (MTT-reducing activity) of pure Kupffer cell cultures was measured at different time-points during culture with (●) or without (○) 50 μM Pb acetate in absence of LPS. The values are expressed as viability in percent of control (untreated Kupffer cell cultures at time-point 0 h) and represent the mean of two independent experiments.

Table 3
The lack of direct cytotoxicity after treatment with LPS and Pb acetate is not due to NO release

	Cocultures		Hepatocyte cultures	
		+ L-NIL		+ L-NIL
Intercellular signals:				
TNF-α (pg/ml)	374 (10)	359 (12)	7 (6)	5 (8)
NO (μM)	46 (6)	4 (3)	5 (6)	3 (3)
Cytotoxicity:				
LDH release (% of total activity)	14 (10)	14 (11)	14 (11)	15 (10)

The iNOS inhibitor L-NIL (50 μM) was added to cocultures and hepatocyte cultures at the beginning of treatment with Pb acetate (Pb 50 μM) and in the presence of 0.1 ng/ml lipopolysaccharide. After 24 h TNF-α release and after 48 h NO and LDH release were determined. The values represent the mean of two independent experiments. Corresponding control values from cultures without Pb are indicated in parenthesis.

3.7. The potential mitogenic activity of TNF-α is not preventing hepatocyte toxicity in cocultures

The question was then asked whether TNF-α was masking putative cytotoxic effects (the ratio between extra- and intracellular LDH content) in the hepatocyte cultures, due to its suspected influence on tissue homeostasis (Rose et al., 1997), e.g. inhibition of apoptotis (determined by nuclear fragmentation) or stimulation of mitosis (PCNA positive nuclei). A dose range-finding study with TNF-α was carried out in hepatocyte monocultures treated for 48 h with increasing concentrations (up to 1 and 100 ng/ml) in the presence or absence of Pb acetate (50 μM) (Table 4). Pb alone increased the number of apoptotic nuclei by a factor of 2.5 and inhibited the spontaneous low rate of mitosis by half. The hepatocytes responded efficiently to the mitogenic stimulus of EGF used as positive control (sevenfold increase in PCNA positive nuclei). TNF-α doubled control values at concentrations of 100 ng/ml (mean of two experiments). This concentration is 1000-fold higher than those found in cocultures. In combination with Pb,

Table 4
Influence of TNF-α and Pb on apoptotic and PCNA positive nuclei

	Apoptotic nuclei (%)	PCNA + nuclei (%)
	Mean	Mean
Without Pb		
Control	1.0 ± 0.1	2.0 ± 0.2
TNF-α (1000 pg/ml)	0.8 ± 0.2	2.8 ± 0.2
TNF-α (105 pg/ml)	0.9 ± 0.1	5.3 ± 0.3
With 50 μM Pb		
Control	3.1 ± 0.3	1.2 ± 0.3
TNF-α (1000 pg/ml)	2.4 ± 0.2	1.5 ± 0.1
TNF-α (10^5 pg/ml)	2.6 ± 0.4	2.6 ± 0.3
Positive control (without TNF-α and Pb)		
EGF (10 ng/ml)	0.8 ± 0.1	12.6 ± 0.5
EGF (100 ng/ml)	0.8 ± 0.2	15.3 ± 0.7

EGF = epidermal growth factor; the values were determined 48 h after beginning of treatment. They represent the mean ± the range of two independent experiments and are expressed as percentage of total nuclei.

these effects were even less pronounced. Therefore, it is highly unlikely that the Pb/lipopolysaccharide treatment-induced TNF-α release is masking the loss of hepatocytes by compensatory hepatocyte growth or prevention of Pb-induced apoptosis.

3.8. Granulocytes mediate a Pb-induced hepatocyte toxicity in cocultures

The Pb-mediated liver toxicity observed in animal experiments could not be adequately explained by the results presented so far. Therefore, in further mechanistic experiments, we investigated a possible cell–cell mediated toxicity. Granulocytes were added directly to the hepatocytes in coculture at the beginning of treatment with lipopolysaccharide and Pb. Under these conditions, lipopolysaccharide and Pb drastically increased the hepatocyte-derived extracellular LDH-activity (Fig. 5). Toxicity was not observed when the granulocytes were physically separated from the hepatocytes by adding them to the cocultures on a second cell culture insert; neither was toxicity evident in the absence of Kupffer cells (Fig. 6). The granulocyte-mediated toxicity was also abolished by the addition of 10 μg aprotinin (protease inhibitor) (Fig. 6). From this, it was concluded that in our experiments the mechanism involved proteolytic hepatocyte killing in combination with a direct cellular interaction between the granulocytes and the hepatocytes.

3.9. Effective Pb concentrations in the supernatant of hepatocyte cultures

In order to compare effective Pb concentrations in vivo and in vitro, it is necessary to know the actual concentra-

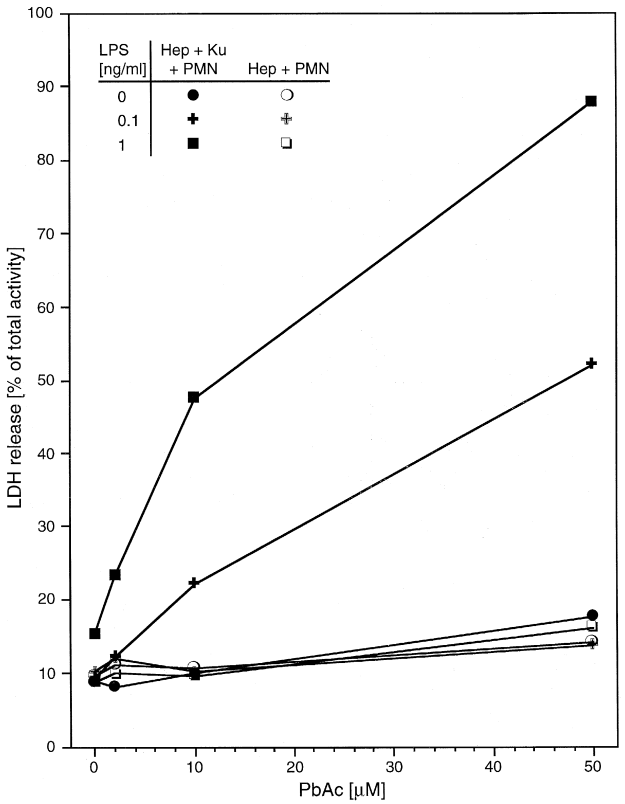

Fig. 5. LDH release (extracellular LDH activity in percent of total [intra- and extracellular] activity) in cocultures of hepatocytes and Kupffer cells (Hep + Ku) and hepatocyte cultures (Hep) cultured with freshly isolated granulocytes (PMN), which were added to the cultures (granulocytes to hepatocytes ratio of 10:1) at the beginning of treatment with Pb acetate and LPS. The values were determined after a 24-h treatment period and represent the mean of two independent experiments.

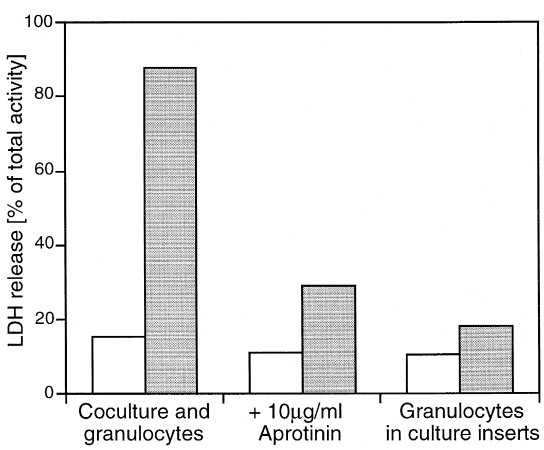

Fig. 6. Effects of aprotinin (10 μg/ml) addition and physical separation of the granulocytes from the hepatocytes (granulocytes added into Anopore culture inserts) on the granulocyte-enhanced LDH release (extracellular LDH activity in percent of total [intra- and extracellular] activity) in cocultures (hepatocytes and Kupffer cells) treated with (B) or without (G) Pb acetate (50 μM) and in presence of LPS (1 ng/ml). Freshly isolated granulocytes were added (granulocytes to hepatocytes ratio of 10:1) to the cocultures at the beginning of treatment. The values were determined after a 24-h treatment period and represent the mean of two independent experiments.

Table 5
Pb distribution between hepatocytes and culture supernatants

Pb (50 μM)	Supernatants	Cells (homogenate)
	Total Pb (μg)	Total Pb (μg)
5-h exposure		
	2.88	19.7
+ Rotenon (5 μM)	3.6	20.8
24-h exposure		
	2.64	22.8

The values represent the mean of two independent experiments and are expressed as the total Pb amount (μg) recovered per well in culture supernatants or cell homogenates after treatment of the cultures with 3 ml 50 μM PbAc each, which equals 31.5 μg Pb per well. Corresponding control values from cultures without Pb averaged 0.5 mg in supernatants and 0.06 mg in cell homogenates. The cytotoxic compound rotenon (respiratory chain blocker) was added, where indicated, 12 h before exposure of the cultures to Pb acetate.

tion of Pb at the target cells, in this case at the cocultured Kupffer cells in the supernatant. Therefore, the distribution of Pb between the hepatocytes and the supernatant was investigated in hepatocyte cultures treated for 5 or 24 h with 3 ml of medium containing 50 μM Pb acetate, corresponding to a total of 31.5 μg Pb per dish. Two thirds of the administered Pb was found in the hepatocytes (Table 5) within 5 h, whereas the Pb concentration of the supernatant remained stable in dishes without cells over the whole treatment period (data not shown). This reduced the Pb concentration in the supernatant fivefold. This accumulation of Pb was due to a passive absorption rather than to active accumulation, since living and dead hepatocytes (rotenone pretreatment, resulting in cell death) showed the same Pb accumulation.

4. Discussion

Investigations about the mechanisms involved in the toxicity of xenobiotics in man often use cell culture models. They have the advantage that a broad pattern of changes in functions and structures can be analysed under defined conditions (e.g. time and concentration). Crucial for the use of cell cultures is the selection of the relevant target cells and appropriate culture conditions under which, the cells preserve and express their organ specific functions. As the present work shows, the possibility of intercellular signalling should be incorporated in cell culture studies in order to more closely mimic the situation in the organ. In the present investigation dealing with liver toxicity, we focused on the two most interacting players: Kupffer cells and hepatocytes.

4.1. Simulation of liver conditions in vitro

The TNF-α-expressing hepatic Kupffer cells are homogeneously distributed throughout the liver lobules, showing no zonal preference (Hoffmann et al., 1994). In our cultures, neither pO_2 levels (periportal or pericentral equivalent) nor insulin concentrations had a significant influence on endotoxin-induced TNF-α release (Milosevic et al., 1999). NO release on the other hand was stronger in cultures kept under 13% O_2 (periportal conditions) than under 4% O_2 (pericentral conditions) (Ohno and Maier, 1995). Accordingly, the present investigations were carried out under periportal equivalent oxygen tension (13% O_2) and with low (2 nM) insulin concentration, conditions which preserve the liver specific metabolism for up to 9 days in culture (Maier et al., 1994). During treatment, dexamethasone was omitted from the medium, since steroids protect the liver from hepatic damage by bacterial lipopolysaccharides (Levitin et al., 1956) and reduce endotoxin-induced TNF-α levels (Grewe et al., 1994).

4.2. Dissection of mechanisms of intercellular signalling in vitro

Highest TNF-α levels in the supernatant of Kupffer cells or in cocultures, as reported earlier (Milosevic et al., 1999), were found within 5 h after beginning of lipopolysaccharide treatment and levelled off after 24–48 h. This quick response mirrors the situation in whole animals. The efficiency of low lipopolysaccharide concentrations acting synergistically with Pb suggests that this process might occur at constitutive lipopolysaccharide levels found in the body, e.g. derived from the colonic flora in the intestine (Van Deventer et al., 1988).

The Pb- or lipopolysaccharide-mediated increase in NO release in the present cocultures derives mainly from an iNOS in hepatocytes which can be induced, e.g. by Kupffer cell-derived TNF-α (Milosevic et al., 1999). The observed synergistic Pb/lipopolysaccharide-mediated increase in NO release is therefore the result of intercellular signalling between the Kupffer cells and the cocultured hepatocytes. Although Kupffer cells are also capable of producing NO (Table 2), this effect was inhibited rather than stimulated by Pb (data not shown). A similar inhibition of NO production by Pb has been reported in murine macrophages (Tian and Lawrence, 1996).

The use of intact, differentiated liver cells in the present cocultures makes it possible to recognize that TNF-α is an important but not the only factor which causes the acute phase response in cocultured hepatocytes. The Pb-mediated upregulation of hepatocyte-derived NO (Fig. 2A) and the downregulation of the negatively responding acute phase protein albumin (Fig. 2B) required the presence of cocultured Kupffer cells, but was also effective without lipopolysaccharide and irrespective of TNF-α levels (Tables 1 and 2). Clearly, the Pb-induced release of messengers at the cellular level involved more than a single cytokine. The multifunctional cytokine IL-1β might belong to this intercellularly acting factors. It is released from Kupffer cells (Aono et al., 1997) and initiates iNOS

expression in rat hepatocytes but not in Kupffer cells (Kitade et al., 1996).

4.3. Mechanism of Pb-induced liver toxicity

Despite the use of cocultures, a direct hepatotoxicity of Pb toward the parenchymal cells, as suggested by results from animal experiments, was not detectable (Table 3, Fig. 4A). NO was not a protective agent against oxidative stress and subsequent cytotoxicity (Kuo et al., 1996) as could be shown with the specific iNOS inhibitor L-NIL (Table 3). TNF-α-induced cytotoxicity in hepatocyte cultures depends on cell density (Hartung and Wendel, 1991). Low densities ($10–30 \times 10^3$ hepatocytes/cm^2; Sieg and Billings, 1997) make the hepatocytes more sensitive. Hepatocytes in the present cultures were kept at a high densities (143×10^3 hepatocytes/cm^2), mimicking more closely the cell–cell interactions between parenchymal cells in liver tissue and preserving more efficiently liver-specific metabolism (Maier et al., 1994). Accordingly, we conclude that the hepatotoxic activity in animal experiments is not caused directly by the released messengers (TNF-α and/or NO). In accordance with these conclusions are findings from animal studies about lipopolysaccharide-induced liver toxicity (Hewett et al., 1993), in which it was demonstrated that TNF-α contributes to liver injury but insufficiently in the absence of circulating polymorphonuclear leucocytes (Hewett et al., 1993).

Endotoxin-induced liver injury is attributed to an initial activation of hepatic macrophages and TNF-α release (Fujita et al., 1995), followed by an infiltration of activated neutrophilic granulocytes (Schlayer et al., 1989; Hewett et al., 1993). Subsequent steps include adhesion between polymorphonuclear leukocytes and hepatocytes. This results in a synergistic interaction between endotoxin-stimulated Kupffer cells and granulocytes (Sauer et al., 1996) and the proteolytic killing of the parenchymal cells. A similar pathway seems to be responsible for Pb toxicity as shown in the experiments with granulocytes (Fig. 5). A 10-fold increase in hepatocyte toxicity was observed when granulocytes were added to the coculture system. It might well be that after long-term exposure to Pb combined with low lipopolysaccharide levels, selective cytotoxicity might occur in the Kupffer cells (as found after long-term (48 h) treatment). This would attract the infiltration of polymorphonuclear leukocytes into the liver tissue and initiate proteolytic cell killing of the hepatocytes. This is supported in the present investigation by the facts that (a) no toxicity was inducible when the granulocytes were cultured in a second insert, physically separated from the hepatocytes, similar to the Kupffer cells (Fig. 6), and (b) the cytotoxic activity was inhibited by the protease inhibitor aprotinin (Fig. 6), most likely by the reported inhibition of the polymorphonuclear leukocyte-derived serine proteases (Sauer et al., 1996).

4.4. Effective Pb concentration in vitro and relevance for human exposure

Effective Pb concentrations in our culture system seem rather high when compared to blood plasma levels found during chronic exposure (0.68 μM: Roberts et al., 1985). However, a more detailed analysis demonstrates that this is not the case: Pb concentration in blood reflects only recent exposure. The half-life of Pb in the general circulation is relatively short (Cook et al., 1981; Gregus and Klaassen, 1986). In the sinusoids of the liver, much higher concentrations can be reached. In vivo, 20–70% of a total injected Pb dose accumulated in the liver of the animals. Active transport mechanisms for heavy metals together with the enterohepatic circulation result in a 100-fold higher concentration in the bile than in the blood plasma. On the other hand, the cocultured Kupffer cells in our system seems to be exposed to much lower concentrations than indicated by the Pb concentration given to the cultures (Table 5). Following absorption by the hepatocytes, the equilibrium concentration of Pb available in the supernatant to enter the Kupffer cells at the highest Pb concentrations (50 μM) was approximately 9.7 μM, corresponding to a fivefold lower effective concentration. Put together, the accumulation of Pb in the enterohepatic circulation in vivo and the reduction of effective concentration to which Kupffer cells are exposed in cocultures, suggest that Pb concentrations in the present investigations are relevant to the in vivo situation in humans.

4.5. Possible role of intercellular signalling in nongenotoxic liver carcinogenesis

TNF-α released from Kupffer cells and the resulting NO release from hepatocytes can alter hepatic functions, e.g. they downregulate xenobiotic metabolism in the hepatocytes (Milosevic et al., 1999), inhibit apoptosis (Rolfe et al., 1997), stimulate DNA synthesis and liver regeneration (Bojes et al., 1997; Akerman et al., 1992). Together, it might well be that Kupffer cell-derived factors are ultimately responsible for the mitogenic effect of nongenotoxic carcinogens in rat liver. For TNF-α, this has been reported with peroxisome proliferators (Bojes et al., 1997) or with Pb-treated rats (Columbano et al., 1996; Shinozuka et al., 1996). In cultured rat hepatocytes, recombinant TNF-α increased DNA synthesis two- to threefold (Rolfe et al., 1997) at a concentration range of 1000–5000 U/ml, values comparable to that found in the present cocultures after exposure to 1–10 ng/ml lipopolysaccharide in combination with Pb (3.5–16 ng/ml). In our experimental setup, however, using high cell densities, a 1000-fold higher concentration of TNF-α was necessary to cause a significant mitotic stimulus in hepatocytes (Table 4). Pb even counteracted the effects of TNF-α by promoting apoptosis and inhibiting mitosis. In order to achieve a

tumor-promoting activity with TNF-α alone, it must be assumed that in vivo, a mitotic response occurs in one and the Pb-induced apoptosis in another susceptible hepatocyte subpopulation. Concomitantly, the released NO reduces the TNF-α-induced accumulation of polymorphonuclear leucocytes in liver and protects cells toward the hepatocyte-polymorphonuclear leukocyte-mediated toxicity (Dalmau et al., 1996; Kim et al., 1997).

With respect to the detection of nongenotoxic liver carcinogens, the modulation of intercellular signalling might be a crucial step. Further investigations are necessary in cell coculture systems that offer a unique method to identify potential nongenotoxic carcinogens and their mode of action.

In summary, the cocultures between two liver cell populations have enabled the mode of action of intercellular communication to be elucidated and its modulation by a xenobiotic to be studied. The approach allows the potential broad range of responses between intact cells to be analysed and their consequences to be estimated. The results also point to the potential role of altered intercellular signalling pathways between liver cells in nongenotoxic carcinogenesis. Furthermore, the results underline the importance of bacterial endotoxin as modifier of xenobiotic metabolism (Milosevic et al., 1999) and toxicity by xenobiotics (Roth et al., 1997), again with possible consequences in nongenotoxic carcinogenesis.

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