Direct cell-to-cell contact between Kupffer cells and hepatocytes augments endotoxin-induced hepatic injury

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during infection, trauma, or an inflammatory response, liver functions are significantly altered. At the cellular level, these changes include production of acute phase proteins and a decrease in xenobiotic metabolizing enzyme systems (3, 23). Bacterial products such as endotoxins, including lipopolysaccharides (LPS), are among the agents that are able to induce these changes in liver functions either directly or indirectly via activation of Kupffer cells (KCs). KCs are among the first cells that respond to LPS and are considered to be the primary macrophages involved in the clearance of gut-derived bacteria or bacterial toxins (11). High portal levels of LPS can lead to a pronounced secretion of inflammatory mediators by KCs and ultimately to endotoxin-induced liver injury (14, 37).

KCs are located in the hepatic sinusoids and lie in between or on top of endothelial cells. However, they do have direct cell contact with parenchymatous hepatocytes (HCs) through their cytoplasmic extensions (38). Cellular communication between KCs and HCs is thought to occur mainly by production of cytokines and excretion of inflammatory mediators such as eicosanoids, nitric oxide (NO), and/or reactive oxygen species (ROS) (8). Proinflammatory cytokines, in particular tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 have been shown to be early and important mediators of the hepatic inflammatory response. These cytokines induce the synthesis of acute phase proteins and are also involved in the downregulation of xenobiotic metabolizing enzyme systems such as cytochrome P-450 (CYP) and UDP glucuronosyl transferase (UDPGT) (2, 24). Intercellular communication between KCs and HCs has been studied in various in vitro model systems. Most studies of intercellular communication are performed in cocultures consisting of KCs and HCs in direct contact (5, 34). Alternately, KC-conditioned culture medium was added to HC cultures or vice versa (5, 27, 34). Such approaches cannot discriminate between the effect on inflammatory responses concerning direct contact vs. indirect contact between KCs and HCs.

The present study was designed to dissect the LPS-induced inflammatory response of direct cellular contact between KCs and HCs from indirect contact via signaling molecules. In particular, we focused on the interaction between KCs and HCs in vitro, using separately isolated porcine HCs and KCs. To this end, HCs and KCs either were cultured as monocultures or used in two types of cocultures, either allowing direct contact (DC cocultures) or preventing direct cell-to-cell contact by using semipermeable membrane inserts (MI cocultures). In addition, direct responsiveness of HCs to LPS was examined using ultrapure HC cultures (UHC cultures).
MATERIALS AND METHODS

Chemicals and drugs. Powdered Williams’ medium E, glutamine, gentamicin, LPS (Escherichia coli, O111:B4), EDTA, 3,3’-diaminobenzidine, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), brefeldin A, saponin, alkaline phosphatase-conjugated goat anti-rabbit IgG, testosterone, 11β-hydroxytestosterone, 1-naphthol, 1-naphthyl glucuro-nide, and diethanol were obtained from Sigma Chemical (St. Louis, MO). Myoclon super plus fetal bovine serum (FBS); endotoxin <10 EU/ml was obtained from Life Technologies (Breda, The Netherlands). Recombinant porcine TNF-α, recombinant porcine IL-6, monoclonal antibody (MAb) mouse anti-porcine TNF-α (6Clone 3B4, isotype IgG1), and porcine TNF-α ELISA were obtained from Endogen (Cambridge, MA). N-acetyl-3,7-dihydroxyphenoxazin (A6550) and dichlo-rohydroxyfluorescein diacetate (H$_2$DCF-DA) were obtained from Molecular Probes Europe (Leiden, The Netherlands). Percoll was obtained from Pharmacia (Uppsala, Sweden). DAKO provided rabbit anti-mouse IgG/horseradish peroxi-dase, and rabbit anti-mouse inducible NO synthase (iNOS) was obtained from Cayman Chemical (Ann Arbor, MI). MAb anti porcine macrophage (CV1-SWNL 517.2) was a kind gift of Dr. J. M. A. Pol (ID-DLO, Lelystad, The Netherlands).

Origin of primary cells. HCs and KCs were isolated from livers of three castrated male pigs (Great Yorkshire × Dutch landrace) aged ~12 wk and weighing between 28 and 35 kg. The animals were obtained from the University’s breeding farm.

Preparation of HC cultures. The procedure for isolation of porcine HCs was based on Seglen’s method (29), with some modifications as described by Monshouwer et al. (24). The obtained cell suspension was diluted in modified Hanks’ buffered salt solution (pH 7.65, 4°C, 9.2 mM HEPES, 9.91 g/l Hanks’ buffered salt solution without Ca$^{2+}$ and Mg$^{2+}$) and centrifuged at 200 g for 5 min. The supernatant was discarded, and cells were resuspended with PBS (pH 7.4) to a final volume of 100 ml and centrifuged for 2 min at 50 g. The supernatant, containing mainly nonparenchymal cells, was collected for isolation of KCs (see Preparation of KCs). Pellets containing mainly HCs were resuspended in Williams’ medium E and washed four times for 2 min at 50 g with Williams’ medium E. After the final wash step, cells were counted and diluted with Williams’ medium E supplemented with 5% (vol/vol) FBS, glutamine (2 mM), and gentamicin (50 µg/ml) to a final concentration of $10^6$ cells/ml. Viability of cultures was ~95% as assessed by trypan blue dye exclusion. FACs analysis and light microscopic observations revealed a purity of 90–95% HCs for HC cultures.

Preparation of UHC cultures. To examine whether HCs responded directly to LPS, HC cultures were further purified up to ~99% purity using Percoll, based on the method described by Smedsred et al. (31) plus an additional purification step by Sephadex and selective adhesion of KCs. Briefly, 4 ml of the HC suspension (see Preparation of HC cultures) was layered over 15 ml of 60% Percoll in PBS and centrifuged at 400 g for 15 min. Supernatants were discarded, and the pellet containing purified HCs was washed at 100 g for 5 min with Williams’ medium E. Cells were incubated on a 10-cm culture dish (Greiner, Alphen a/d Rijn, The Netherlands) for 1 h, resulting in selective attachment to the plate surface of contaminating cells but not of HCs. After 1 h, the unattached HCs were collected, counted, and diluted to a final concentration of $10^6$ cells/ml. Immunohistochemical and FACs analyses revealed a purity of ~99% for these UHC cultures.

Preparation of KCs. The procedure for KC isolation was based on the method of Smedsred et al. (31) with slight modifications. Supernatants (see Preparation of HC cultures) containing mainly nonparenchymal cells were transferred to four 50-ml Falcon tubes (Micronic, Lelystad, the Netherlands) followed by centrifugation at 50 g in a swing-out rotor at 4°C for 2 min. This procedure was repeated twice to discard the remaining HCs. After the final step, supernatants were centrifuged at 200 g in a swing-out rotor at 4°C for 10 min. Supernatants were discarded, and the resulting pellets were resuspended in PBS to a final volume of 40 ml. The nonparenchymal cell suspension was carefully placed on a two-step (60 and 25%) Percoll gradient and centrifuged for 15 min at 400 g (4°C). After centrifugation, the cell suspension, which was situated between the two layers of Percoll, was collected and diluted with PBS. The cell suspension, containing both KCs and endothelial cells, was centrifuged at 200 g for 10 min, and the resulting pellet was diluted with Williams’ medium E (without serum) and washed again. Hereafter, pellets consisting of 50% KCs and 50% endothelial cells were diluted in Williams’ medium E (without serum) to a final concentration of $2 \times 10^6$ cells/ml. To separate KCs from endothelial cells, cells were plated on tissue culture plates or membrane inserts at 37°C and 5% CO$_2$ for 30 min followed by a single wash step discarding the nonadherent endothelial cells. Viability of KCs was >95% as determined by trypan blue dye exclusion assay. Immunohistochemistry and microscopic observations revealed a purity of >85% KCs for KC cultures.

Cell cultures. HCs were cultured at a density of $0.5 \times 10^6$ cells/well in 24-well culture dishes (Greiner) using Williams medium E, supplemented with 5% (vol/vol) FBS, glutamine (2 mM), and gentamicin (50 µg/ml). KCs were cultured in Williams’ medium E containing 10% FBS, 2 mM glutamine, and 50 µg/ml gentamicin at a density of $5 \times 10^6$ cells/well in 24-well culture dishes.

DC cocultures consisted of $0.5 \times 10^6$ attached KCs in 24-well tissue culture plates with the addition of $0.5 \times 10^6$ HCs in direct contact. MI cocultures were prepared by culturing $0.5 \times 10^6$ HCs at the bottom of 24-well tissue culture plates and $0.5 \times 10^6$ KCs plated on membrane inserts with a pore size of 0.45 µM (Becton Dickinson). For both coculture systems, Williams’ medium E supplemented with 5% (vol/vol) FBS, glutamine (2 mM), and gentamicin (50 µg/ml) was used. After an attachment period of 4 h, medium was replaced by fresh medium in all culture types.

Experimental design. After a recovery period of 24 h at 37°C and 5% CO$_2$, medium was replaced by medium containing 0, 1, or 10 µg/ml LPS. Because HCs are known to produce various important serum compounds such as LPS-binding protein, medium of KC cultures still contained 10% FBS. After 2, 4, 8, and 24 h of incubation, tissue culture supernatants were collected for analysis of cytokines and NO and performed as described by Openshaw et al. (26) using a four 50-ml Falcon tubes (Micronic, Lelystad, the Netherlands) followed by centrifugation at 50 g in a swing-out rotor at 4°C for 2 min. This procedure was repeated twice to discard the remaining HCs. After the final step, supernatants were centrifuged at 200 g in a swing-out rotor at 4°C for 10 min. Supernatants were discarded, and the resulting pellets were resuspended in PBS to a final volume of 40 ml. The nonparenchymal cell suspension was carefully placed on a two-step (60 and 25%) Percoll gradient and centrifuged for 15 min at 400 g (4°C). After centrifugation, the cell suspension, which was situated between the two layers of Percoll, was collected and diluted with PBS. The cell suspension, containing both KCs and endothelial cells, was centrifuged at 200 g for 10 min, and the resulting pellet was diluted with Williams’ medium E (without serum) and washed again. Hereafter, pellets consisting of 50% KCs and 50% endothelial cells were diluted in Williams’ medium E (without serum) to a final concentration of $2 \times 10^6$ cells/ml. To separate KCs from endothelial cells, cells were plated on tissue culture plates or membrane inserts at 37°C and 5% CO$_2$ for 30 min followed by a single wash step discarding the nonadherent endothelial cells. Viability of KCs was >95% as determined by trypan blue dye exclusion assay. Immunohistochemistry and microscopic observations revealed a purity of >85% KCs for KC cultures.

Immunohistochemical analyses. HC cultures, KC cultures, and DC cocultures were plated on sterile no. 1 coverslips placed in 24-well plates. Intracellular TNF-α labeling was performed as described by Openshaw et al. (26) using a specific MAb for porcine TNF-α. Cells were stimulated with various concentrations of LPS in the presence of 10 µg/ml brefeldin A to inactivate the Golgi apparatus, resulting in an intracellular TNF-α assemblage. After 4 h of stimulation, cells were fixed with 2% paraformaldehyde in PBS containing 10 µg/ml brefeldin A. Fixed cells were washed three times with PBS containing 1% BSA and 0.5% saponin (pH 7.4).
Cells were labeled for 45 min with a MAb directed against porcine TNF-α. After being washed three times for 5 min with PBS (1% BSA and saponin) cells were incubated for another 45 min with rabbit anti-mouse FITC (Molecular Probes Europe). Cells were washed and immediately used for fluorescence microscopy.

To clarify which cell type was responsible for NO production, a double antibody staining anti-iNOS and anti-porcine macrophage (10) was performed on KC cultures, HC cultures, and DC cocultures after 24 h of incubation with or without LPS. After incubation with rabbit anti-mouse iNOS and mouse anti-porcine macrophage for 45 min, cultures were washed and incubated for 30 min with a secondary antibody pig anti-rabbit alkaline phosphatase for 30 min. After being washed three times with PBS (1% BSA and 0.5% saponin), cells were incubated with rabbit anti-mouse peroxidase for 30 min. Consecutively, horseradish peroxidase and alkaline phosphatase staining were performed, and cultures were analyzed by light microscopy.

**TNF-α bioassay.** TNF-α concentrations in cell culture supernatants were measured with a cytotoxicity assay using a porcine kidney cell line (PK-15) according to the method of Bertoni et al. (4). Cytotoxicity was determined by measuring the decrease in mitochondrial activity using MTT. Before the experiments, the PK-15 bioassay was validated by comparison with a specific ELISA for porcine TNF-α (results not shown).

TNF-α was determined in tissue culture supernatants after 4 h of incubation with various concentrations of LPS. As positive control, threefold dilution of recombinant porcine TNF-α was used. Absorbance was measured at 590 nm. TNF-α in supernatants was quantified by comparison of calculated EC_{50} values from supernatants with EC_{50} values from the recombinant porcine TNF-α standard curve. Experiments were performed in duplicate for each sample from cells isolated from each pig.

**IL-6 bioassay.** Porcine IL-6 was measured with a murine hybridoma B9 cell line in 96-well plates according to the method of Helle et al. (16). Proliferation was determined by measuring the increase in mitochondrial activity using MTT. Samples were titrated in threefold dilutions, and as positive control threefold dilutions of recombinant porcine IL-6 were used. Absorbance was measured at 590 nm. IL-6 in tissue culture supernatants was quantified by comparison of calculated EC_{50} values from supernatants with EC_{50} values from the recombinant porcine IL-6 standard curve. Experiments were performed in duplicate for each sample from cells isolated from each pig.

**NO measurement.** After 24 h of incubation with or without LPS, samples were collected and stored at −70°C until analysis. The NO production was determined by measuring the amount of nitrite in culture supernatants according to the Griess reaction (12).

**Western blot analysis for iNOS.** After 24 h incubation with or without LPS, Western blot analysis of iNOS expression in KC cultures, HC cultures, and DC cocultures was performed. After the cells were washed once in PBS, protein for Western blotting was prepared from 8 × 10^6 KCs, HCs, or DC coculture using a rubber policeman. Cells were centrifuged at 200 g and resuspended in 200 μl PBS. After homogenization by repeated freezing (−180°C) and thawing (37°C), samples were centrifuged at 13,000 rpm for 2 min in an Eppendorf centrifuge. Supernatants were collected, and the protein contents were determined using the method of Lowry. Total protein (10 μg) was loaded onto an SDS/polyacrylamide (8%) gel and blotted on to nitrocellulose membranes after electrophoresis. Membranes were blocked for 30 min by Tris-buffered saline (10 mM Tris·HCl, 150 mM NaCl, pH 8) containing 1% (wt/vol) BSA and 0.3% (vol/vol) Tween 20. The primary antibody rabbit anti-mouse iNOS, which strongly cross-reacts with porcine iNOS, was diluted 1:1,000 in Tris-buffered saline (1% BSA, 0.3% Tween 20), and blots were incubated for 1 h at room temperature. After 6 washes with Tris-buffered saline for 5 min, the secondary antibody (pig anti-rabbit alkaline phosphatase) was added in a 1:5,000 dilution in Tris-buffered saline (1% BSA and 0.3% Tween 20). Blots were incubated for 1 h, and, after being washed 6 times, blots were stained using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Biotransformation enzyme assays.** After 24 h incubation of cultures in medium with or without LPS, HC functionality was determined by measuring β- and 25-testosterone hydroxylation rates (both marker activities for CYP3A) and glucuronidation of 1-naphthol (marker for UDPG enzyme activity).

Testosterone hydroxylation rates were determined by incubation of cell cultures with 250 μM testosterone (1 ml/well) in 24-well plates for 24 h. The formation of different testosterone metabolites was quantified by using the HPLC method as described by Wortelboer et al. (40).

The glucuronidation rate of 1-naphthol was determined by incubation of the cultures with 1.25 mM 1-naphthol (1 ml/well) in 24-well plates for 1 h. The formation of 1-naphthyl glucuronide was determined using the HPLC method as described by Wortelboer et al. (39).

**Measurement of ROS.** For the measurement of intracellular ROS production, oxidation of the fluorescent probe H_{2}DCF-DA was measured following the method of Trayner et al. (33) with some slight modifications. Briefly, KCs and HCs were plated in 96-well plates at a density of 1 × 10^4 cells/well. Cocultures consisted of 1 × 10^4 KCs and 1 × 10^4 HCs per well. After incubation overnight at 37°C and 5% CO_2, medium was removed and cells were washed with 100 μl/well Krebs-Ringer-buffered glucose (KRBO) (10 mM glucose, 10 mM HEPES, 140 mM NaCl, 4.86 mM KCl, 1.8 mM CaCl_2, and 1 mM MgCl_2, pH 7.4). Cells were loaded with 10 μM H_{2}DCF-DA for 30 min at 37°C and 5% CO_2. After 30 min, cells were washed with medium followed by addition of medium with or without various concentrations of LPS. After 4 h of incubation at 37°C and 5% CO_2, fluorescence was determined using a Cytoflour model 2300 microplate fluorometer (Millipore, Bedford, MA) using an excitation wavelength of 485 nm and emission wavelength of 538 nm. Experiments were performed in quadruplicate for cells derived from each pig, and values were normalized to control incubations for each culture type and depicted as relative signal intensity.

Extracellular release of ROS was determined using a horseradish peroxidase substrate A6550, which becomes highly fluorescent on horseradish peroxidase-catalyzed H_2O_2 oxidation. The experiments were performed as described by Mohanty et al. (22). Briefly, KCs and HCs were plated in 96-well plates at a density of 1 × 10^4 cells/well. Cocultures consisted of 1 × 10^4 KCs and 1 × 10^4 HCs per well. After incubation overnight at 37°C and 5% CO_2, cells were washed with KRBO and incubated with 100 μl/well of 10 μM A6550 and 1 U/ml horseradish peroxidase in KRBO with various doses of LPS. After 4 h incubation at 37°C and 5% CO_2, fluorescence was determined using a Cytoflour model 2300 microplate fluorometer and an excitation wavelength of 590 nm and emission wavelength of 645 nm. Experiments were performed in quadruplicate, and values were quantified by comparison with a standard curve of H_2O_2 for each experiment.
Viability. After 24 h incubation with or without LPS, cell viability was assessed in all cultures by measuring mitochondrial activity using 3,3′-diaminobenzidine and MTT. Briefly, cells were incubated with 0.5 ml MTT (0.5 mg/ml) in Williams’ medium E. After 4 h of incubation, medium was discarded and cells were lysed with 0.5% SDS in isopropanol. After lysis, absorbances were measured at 590 nm.

Statistics. Unless stated otherwise, data are expressed as means ± SE or SD and evaluated using a two-way ANOVA followed by Dunnett’s test for comparison between two groups. P < 0.01 was considered statistically significant.

RESULTS

Proinflammatory cytokine production after stimulation with LPS. Production of TNF-α and IL-6 was measured after incubation of HC and KC cultures and MI and DC cocultures in medium with or without LPS. A time course study showed that maximal cytokine levels were observed after 4 h for TNF-α and 24 h incubation for IL-6, respectively (data not shown). Maximal TNF-α and IL-6 levels as measured in different culture types are depicted in Fig. 1. Stimulation of MI cocultures and KC cultures with LPS resulted in comparable levels of both TNF-α and IL-6. However, in DC cocultures the amount of TNF-α was significantly increased up to 10-fold after stimulation with LPS (Fig. 1A). The effect of direct cell-to-cell contact was even more pronounced on IL-6 levels in DC cocultures, and an increase of 100- to 1,000-fold in IL-6 was observed compared with other culture types (Fig. 1B). Exposure of HC cultures to various concentrations of LPS resulted in the release of small amounts of TNF-α and IL-6 (Fig. 1). In LPS-stimulated UHC cultures, this response was even less pronounced and no significant release of TNF-α or IL-6 could be measured (results not shown).

Intracellular TNF-α labeling with a fluorescent probe in KC cultures and DC cocultures after control and LPS incubation are shown in Fig. 2. Only after stimulation with LPS could a significant fluorescence be detected in both culture types (Fig. 2B and D). Intracellular TNF-α in DC cocultures was allocated to KCs only and could not be detected in HC cultures (Fig. 2D). Both cultures revealed a comparable amount of KCs stained for intracellular TNF-α (Fig. 2, B and D).

Release of NO and expression of iNOS. LPS addition to HC cultures and both cocultures resulted in a dose-dependent formation of nitrite as measured by Griess reaction (Fig. 3A). NO production was significantly increased in DC cocultures as well as MI cocultures during a 24-h incubation period with LPS. Nitrite levels in supernatants of DC cocultures were already significantly increased when cells were exposed to 10 ng/ml LPS (Fig. 3B). No significant differences were found with regard to nitrite formation in UHC cultures compared with HC cultures. Exposure of both cultures to 10 μg/ml LPS for 24 h resulted in concentrations up to 12 μM nitrite in culture supernatants for both culture types (Fig. 3B). In KC cultures, no significant nitrite levels could be measured (Fig. 3A).

These results were confirmed by Western blot analysis (Fig. 3C), which resulted in identification of a 125- to 130-kDa protein band specific for iNOS. In KC cultures, no iNOS was expressed, whereas in HC cultures and especially DC cocultures iNOS was significantly induced (Fig. 3B) after stimulation with LPS.

To clarify which cell type was responsible for the LPS-induced NO production, HC and KC cultures and DC cocultures were examined for expression of iNOS combined with a specific staining for KCs. The results from DC cocultures are depicted in Fig. 4. During control incubations, hardly any iNOS could be detected in KC cultures and DC cocultures (Fig. 4A). After incubation with LPS, only HCs were positively stained for iNOS, whereas KCs remained unstained (Fig. 4B).

Production of extracellular and intracellular ROS. Stimulation of different cultures with LPS resulted in a dose-dependent release of H2O2 as measured extra-
cellularly during 4 h incubation (Fig. 5A). This release was significantly higher in DC cocultures and HC cultures compared with KC cultures. However, only minor differences were observed between the LPS-induced release of ROS in HC cultures and DC cocultures.

In addition, intracellular ROS was measured with H$_2$DCF-DA (Fig. 5B). In all culture types, an LPS-induced increase of intracellular ROS was observed. However, no significant differences were recognized. Furthermore, exposure of UHC cultures resulted in a comparable intracellular ROS response to that observed for HC cultures (results not shown).

**Biotransformation capacity of HC cultures and cocultures.** Testosterone hydroxylation rates (CYP3A) and naphthol glucuronidation rates (UDPGT) were measured to determine HC functionality in different cultures. CYP3A and UDPGT enzyme activities after 24 h stimulation with LPS are shown in Table 1. Compared with HC cultures, both cocultures showed a significant decrease in testosterone hydroxylation rate during control incubations, but this decrease was most abundant in DC cocultures. After stimulation of DC cocultures with LPS, CYP3A activity was almost completely suppressed, whereas in MI cocultures CYP3A activity was still 50–60% of control levels when stimulated with 1 or 10 µg/ml LPS.

For UDPGT-dependent enzyme activities, comparable results were obtained (Table 1). Again, DC cocultures showed a decreased UDPGT enzyme activity, down to 45% of control levels in HC cultures. After exposure to LPS, hardly any measurable glucuronidation of 1-naphthol was observed.

**Viability.** To determine the LPS-induced cytotoxicity in the various culture types, viability was assessed using MTT after 24 h incubation with or without LPS. Although LPS stimulation in all cultures decreased viability, this decrease was most apparent in DC cocultures (Fig. 6).

**DISCUSSION**

In the intact liver, HC are in direct contact with KCs. The present experiments were designed to assess whether direct contact between KCs and HCs is of influence for the outcome of an LPS-induced inflammatory response. When comparing the production of TNF-α and IL-6 in the different culture types, direct contact between KCs and HCs seems to be essential, because an ~10- and 500-fold increase of TNF-α and IL-6, respectively, could be observed in DC cocultures. Results from intracellular TNF-α labeling indicated that TNF-α in these cultures is exclusively produced by
KC and not by HCs. Furthermore, these results show that the increased TNF-α production is due to an increased activation of KCs in DC cocultures and not the result of a decreased viability of KCs in KC cultures. Although cytokine levels in KC cultures were markedly lower than those observed in cocultures, incubation of KC cultures with 10 μg/ml LPS still resulted in an abundant cytokine response of 3,300 pg/ml TNF-α and 3,100 U/ml IL-6. Although LPS-induced cytokine expression in cocultures or KC cultures has been extensively studied (21, 36), in this study we have shown that direct contact between KCs and HCs significantly increases TNF-α production by porcine KCs.

The LPS-induced NO release in monocultures vs. cocultures is in agreement with results published by the group of Billiar et al. (5, 6). However, our data show that NO production in porcine liver cell cultures is due to an LPS-induced iNOS expression only by HCs and not KCs. Evidence is accumulating that there are considerable species differences in iNOS expression and regulation in the liver. For instance, in mouse and rat macrophages iNOS expression can be induced by LPS, whereas in human monocytes and macrophages it has been difficult to demonstrate iNOS expression (1, 9). The latter observation is in accordance with our study, in which iNOS expression could not be demonstrated in KCs, indicating that porcine KCs more closely resemble human macrophages. In addition, both rat and mouse HCs have been shown to express high levels of iNOS in response to TNF-α, IL-1β, and interferon-γ as a single stimulus, whereas human HCs responded to LPS with a decreased expression of iNOS (1, 9).

**Fig. 3.** A: concentration of nitrite after 24 h incubation with or without LPS in tissue culture supernatant of different culture types as measured by Griess reaction. Control, hatched bars; 1 μg/ml LPS, open bars; 10 μg/ml LPS, black bars. Values are means ± SE (n = 6). *P < 0.01 for nitrite concentration in DC and MI cocultures vs. HC and KC cultures after 1 or 10 μg/ml LPS. B: formation of nitrite by various concentrations of LPS (ng/ml) in DC cocultures (dot-dash line) and HC (dashed line) and ultrapure HC cultures (UHC cultures) (solid line). Values are means ± SE (n = 6). *P < 0.01 for nitrite concentration in DC, HC, and UHC vs. control. C: Western blot analysis of inducible nitric oxide synthase (iNOS) expression in KC and HC cultures and DC cocultures after 24 h incubation with (+) or without (−) LPS (10 μg/ml). MW, molecular weight.

**Fig. 4.** Light microscopic observations of immunohistochemical double antibody staining for KCs (red) and iNOS (blue) in DC cocultures. After 24 h incubation with or without LPS (10 μg/ml), cells were fixed with paraformaldehyde and used for double antibody staining as described in MATERIALS AND METHODS. In control incubations, KCs were morphologically recognized as stellate cells with large extensions, stretched in direct contact with HCs (A). After incubation with 10 μg/ml LPS (B), the number of attached KCs was significantly decreased and loss of the large extensions was observed.
Cultures, MI cocultures show an increased production of various cell types (7, 13, 30, 32). Compared with HC cultures and DC cocultures (dashed line) as measured by dichlorodihydroxyfluorescein diacetate and N-acetyl-3,7-dihydroxyphenoxazine fluorescence probes during 4 h incubation with various doses of LPS. Values are means ± SE (n = 6).

LPS alone without exposure to additional stimuli (25). In our study, exposure of porcine UHC cultures to LPS resulted in increased ROS and NO productions, whereas LPS exposure did not result in increased levels of TNF-α and/or IL-6 in these cultures. These results suggest that in porcine HCs LPS alone, without further involvement of KCs and/or cytokines, initiates NO production. Together with the observation that in porcine KCs no iNOS expression could be demonstrated, these findings suggest that the regulation and expression of iNOS in porcine liver closely resemble the human liver physiology.

The relatively high NO concentrations measured in MI cocultures suggest that NO production could be further induced by soluble factors capable of transporting through the highly porous membrane of the inserts and is not dependent on direct cell-to-cell contact. Several studies have shown that TNF-α is at least in a synergistic way able to induce iNOS expression in various cell types (7, 13, 30, 32). Compared with HC cultures, MI cocultures show an increased production of TNF-α. Time course studies performed with these cultures reveal an increased production of TNF-α preceding the NO production, suggesting that increased TNF-α levels could be involved in the increased NO production, as observed in MI cocultures.

LPS stimulation of DC cocultures resulted in significant cytotoxicity, whereas the cell viability in the other culture systems remained less affected. It seems unlikely that the observed cytotoxicity was mediated by release of extracellular ROS since LPS stimulation did not result in a significant difference between ROS formation in HC cultures and DC cocultures. However, the observed cell death in DC cocultures could be further involvement of KCs and/or cytokines, initiates NO production, as observed in MI cocultures.

Table 1. Effect of LPS on biotransformation capacity in HC cultures, DC cocultures, and MI cocultures

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Data are expressed as percentage of untreated hepatocyte cultures (control, 100%), and each value is a mean ± SE (n = 6). After 24 h incubation with 0.1 or 10 µg/ml lipopolysaccharide (LPS), cultures were incubated with either testosterone or 1-naphthol to determine phase I and phase II biotransformation capacity. HC, hepatocyte; DC, direct contact; MI, membrane insert; 6β-OH, 6β testosterone hydroxylation (100% = 364 pmol/min × 100% of hepatocytes –1); 2β-OH, 2β testosterone hydroxylation (100% = 105 pmol/min × 100% of hepatocytes –1); 1-NG, 1-naphthol glucuronidation (100% = 3.60 µmol/min × 100% of hepatocytes). *Significant decrease of biotransformation capacity after stimulation with LPS compared with control incubation for corresponding culture. †Significant decrease of biotransformation activity compared to control in HC cultures.

Fig. 5. Extracellular (A) and intracellular (B) reactive oxygen species production in HC (dotted line) and KC (solid line) cultures and DC cocultures (dashed line) as measured by dichlorodihydroxyfluorescein diacetate and N-acetyl-3,7-dihydroxyphenoxazine fluorescent probes during 4 h incubation with various doses of LPS. Values are means ± SE (n = 12).

Fig. 6. LPS-induced cytotoxicity as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in HC and KC cultures and DC and MI cocultures after 24 h incubation with various doses of LPS. Control, filled bars; 1 µg/ml LPS, open bars; 10 µg/ml LPS, hatched bars. Values are means ± SE (n = 6). 100% values in optical density: KC = 0.26, HC = 0.44, DC = 0.67, and MI = 0.63. *P < 0.01 vs. control incubations. †P < 0.01 for DC cocultures vs. other cultures after stimulation with LPS.
mediated by the excessive amounts of TNF-α produced in DC cocultures on LPS stimulation. Various studies in vivo as well as in vitro have shown that TNF-α is involved in induction of direct hepatotoxicity through apoptosis (15, 17, 20). Although the significance of TNF-α production in the observed cell death seems apparent, several studies have shown that KC-mediated cytotoxicity against HCs or hepatoma cells can occur through production of NO (5, 28). Furthermore, Kurose et al. (19) showed that the increased production of NO by KCs was CD-18/intracellular adhesion molecule-1-dependent and correlated with an increased oxidative activation of nuclear factor-kB.

An important functional parameter of HCs is their biotransformation capacity. Although our results show that KCs strongly suppress the activity of at least the enzymes CYP3A and UDPGT, even in the absence of LPS, the exact mechanism underlying this process is still not clear. These results are in agreement with studies performed by Simmons and co-workers (34, 35), who showed that cocultures of KCs and HCs in direct cell-to-cell contact resulted in a decreased protein synthesis by HCs. Furthermore, they showed that the decrease in HC protein synthesis was NO dependent (5, 7). With regard to CYP, evidence is accumulating that both NO and proinflammatory cytokines are important mediators involved in the regulation of CYP activity (18, 24). The observed decrease in biotransformation capacity after stimulation with LPS may result from an increased production of NO and/or proinflammatory cytokines as observed in DC cocultures. However, a decrease in biotransformation capacity of HCs was already observed during control incubations in DC cocultures as well as MI cocultures, whereas no significant TNF-α, IL-6, or NO could be detected in the culture medium. This implies that alternate KC-derived signaling molecules, capable of transporting through the membrane insert, might contribute to the observed decrease in biotransformation capacity.

Results obtained from MI and DC cocultures clearly demonstrate that different types of cocultures consisting of KCs and HCs can lead to disparate results. Cocultures from KCs and HCs separated by cell culture membrane inserts showed comparable proinflammatory cytokine responses as observed for KC cultures. In contrast, DC cocultures with direct cell-to-cell contact showed an increased cytokine production. Initial experiments had shown that the high-density membranes used in the experiments did not limit the diffusion of cytokines or other signaling molecules through the membrane (results not shown). However, the distance between KCs and HCs in these cocultures could be of importance for the distribution of reactive oxygen and/or nitrogen species. Since these small size molecular mediators have a short half-life, they will not reach the target sites if the two cell types are clearly separated. Therefore, these small reactive mediators are less effective in MI cocultures.

The increased proinflammatory cytokine production in DC cocultures clearly suggests involvement of mechanisms via direct cell-to-cell contact. Whether these mechanisms include adhesion molecules and/or short-life small-size molecular mediators remains to be elucidated. The present results provide evidence that the degree of the LPS-induced cytokine response is mediated by direct cell-to-cell contact between KCs and HCs. At the same time, DC cocultures reflect the mutual interaction between HCs and KCs in vivo more closely. To understand the mechanisms involved in the imposed LPS response in DC cocultures, future experiments should be directed to unravel all contributing signaling molecules. The insight into these mechanisms is essential for the identification of molecular targets in the treatment of a fulminating hepatic inflammatory response or endotoxin-induced liver injury.

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REFERENCES
LPS RESPONSE IN KUPFFER CELL HEPATOCYTE COCULTURES


