Inflammation, Vol. 37, No. 1, February 2014 (© 2013)
DOI: 10.1007/s10753-013-9735-7

The Effects of Intestinal LPS Exposure on Inflammatory Responses in a Porcine Enterohepatic Co-culture System

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Abstract—A porcine enterohepatic co-culture system, with primary hepatocytes as bottom layer and IPEC-J2 epithelial cells as upper layer, was developed to study the effects of lipopolysaccharides (LPS) on the gene expression profile of pro-inflammatory cytokines (interleukin-8 (IL-8) and tumor necrosis factor-α) and CYP enzymes (CYP1A1, CYP1A2, CYP3A29). The barrier integrity of IPEC-J2 cells was investigated by transepithelial electrical resistance measurements and by fluorescein isothiocyanate–dextran-based test. Basolateral IL-8 production was significantly elevated in LPS-treated IPEC-J2 and primary hepatocyte mono-cultures as well as in the co-culture system, in a dose-independent manner. The LPS-induced changes in the expression of the CYP1A2 and CYP3A29 genes in hepatocyte mono-cultures differed from those in co-culture after LPS treatment on the apical side of the IPEC-J2 cell layer. CYP1A2 was downregulated by the LPS treatment in mono-cultures but upregulated at 10 μg/ml LPS in co-culture; gene expression of CYP3A29 showed no significant LPS-induced change in the hepatocyte mono-culture but was significantly downregulated in co-culture. The newly established co-culture system capable of mimicking enterohepatic interplay in LPS-induced inflammatory responses *in vitro* can be used in the future for reliable screening of potential anti-inflammatory compounds.

KEY WORDS: swine; enterocyte-liver co-culture; lipopolysaccharide (LPS); inflammatory cytokines; cytochrome P450 (CYP) enzymes.

INTRODUCTION

The intestinal epithelium provides a primary, selective barrier against enteral pathogens, but it can be considered as a communication organ as well, connecting as an interface the intestinal nutritional environment with the circulation of the host [1]. It maintains a predominant role in stress-induced bowel inflammation and mediates the innate and adaptive immune response of the gut-associated lymphoid tissue [2].

The lipopolysaccharide (LPS) of Gram-negative bacteria can induce inflammatory responses, predominantly mediated by activation of the NFκB pathway [3], contributing to pro-inflammatory cytokine overproduction [4, 5]. For instance, interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) can be secreted in elevated quantities following LPS challenge, providing a wide spectrum of pro-inflammatory activities, e.g., stimulation of migration and accumulation of leukocytes [6, 7]. These cytokines also play a key role in the crosstalk between intestinal epithelial cells, immune cells [2], and hepatocytes. Alleviating the production of these pro-inflammatory mediators is highly important to prevent excessive tissue injury as well as inflammation of the bowel and extraintestinal organs [8].

In contrast to the widely applied tumor cell lines (Caco-2, HT29), the non-cancerous IPEC-J2 swine jejunal epithelial cell line [9, 10] provides an optimal tool to study the function of the intestinal epithelium in gut-associated inflammatory processes due to the similar properties of cultured enterocytes to the *in vivo* situation

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[11]. Modeling enteral bacterial infections, LPS stimulation (from *Salmonella typhimurium*, 1 μg/ml, 2 h) of IPEC-J2 cells caused time-dependent elevation in the IL-8 and TNF-α mRNA levels [12]. Similarly, H_2O_2-induced oxidative stress increased the gene expression of these cytokines [13], which could be attenuated by the probiotic strain of *Lactobacillus plantarum* 2142 [13, 14]. IL-8 mRNA levels increased 2 h after exposure of HT-29 cells to 50 ng/ml LPS [15]. LPS-induced inflammation weakened membrane barrier integrity via enhancement of paracellular permeability of primary culture of colonic epithelial cells [16] and tumorigenic Caco-2 cells in low-glucose media [17]. However, the effects of apically added LPS on barrier function of IPEC-J2 mono-cultures have not been characterized yet.

LPS-evoked cytokine production of the intestinal epithelium is of special importance in the maintenance of pro-inflammatory pathways. Cytokines released to the portal system may constitute a potent stimulus to liver cells as well, possibly initiating an inflammatory cascade even in hepatocytes. It has been described that inflammation can downregulate the expression of different subfamilies of hepatic microsomal drug-metabolizing cytochrome P450 (CYP) enzymes [18, 19], possibly leading to alterations in the pharmacokinetics of xenobiotics. Numerous pro-inflammatory mediators are required in the LPS-induced suppression of CYP genes. However, the exact mechanisms of the involved signal transduction pathways have not been clarified yet [20].

To mimic more closely the *in vivo* conditions, several alternatives to one-dimensional setups have been developed [1]. Intestinal epithelial cells can be grown on microporous membrane inserts to modelize apical and basolateral transport mechanisms and functionality. In addition, enterocytes can be co-cultured with immune cells [21–23] or other, more specialized cell types, such as mucus producing Goblet cells [24]. Similarly to the gastrointestinal epithelia, hepatocytes can be cultured in certain 3D and sandwich cultures [25] or co-cultured with other liver-derived [26] and extrahepatic cell types, e.g., with fibroblasts or human aortic epithelial cells [27]. Moreover, tumor cell culture model systems have been developed, in an attempt to combine the functions of intestine and liver, like perfusion co-culture of Caco-2 with the HepG2 human hepatocellular carcinoma cell line [28–30]. However, co-cultures with non-transformed cells are highly desirable.

The aim of the present study was to establish a porcine enterohepatic co-culture, consisting of the IPEC-J2 small intestinal epithelial cell line, together with primary culture of porcine hepatocytes to modelize the *in vivo* inter-organ communication involved in systemic inflammatory processes and its effect on hepatic CYP expression. In our approach, we first investigated the LPS-induced inflammatory responses in separate cultures of IPEC-J2 cells and hepatocytes and then compared the LPS-induced effects with those in the newly established co-culture model. This is the first study in which a novel swine enterocyte-liver *in vitro* co-culture system was developed as an indispensable model to investigate the effects of intestinal LPS exposure on hepatic inflammatory responses.

MATERIALS AND METHODS

IPEC-J2 Cell Line and Culture Conditions

The non-transformed porcine intestinal epithelial cell line IPEC-J2, originally isolated from jejunal epithelia of a neonatal unsuckled piglet [9, 10], was a kind gift of Dr. Jody Gookin, Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA. IPEC-J2 cells were grown and maintained in complete medium, which consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (plain medium) supplemented with 5 % fetal bovine serum (FBS), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor, and 1 % penicillin–streptomycin (all from Fisher Scientific, USA). Cells were grown at 37 °C in a humidified atmosphere of 5 % CO_2. Cell cultures were tested by PCR and were found to be free of *Mycoplasma* contamination.

For the experiments, IPEC-J2 cells between passages 42–48 were seeded onto six-well Transwell Polyester membrane inserts (Corning Inc., Corning, NY, USA), coated with 8 μg/cm^2 rat tail collagen type I (Sigma-Aldrich, Steinheim, Germany), at a density of 1.5×10^5 cells/ml (the volume of complete medium was 1.5 ml on the apical side and 2.5 ml on the basolateral side per well according to the manufacturer's instructions). Cells were allowed to adhere for 24 h before being washed and re-fed every other day until confluence was reached. Transepithelial electrical resistance (TEER) measurement of mono-cultures was performed on alternate days after seeding, from days 5 to 21 of culture, using an epithelial tissue volt/ohmmeter (World Precision Instruments, Berlin, Germany).

LPS Treatment of IPEC-J2 Cells

Before treatment, the confluent mono-cultures of IPEC-J2 cells were washed with plain medium. The applied LPS was derived from *Salmonella enterica* ser. typhimurium (Sigma-Aldrich, Steinheim, Germany). LPS solutions were prepared freshly prior to each experiment. LPS was added in plain medium at 1 μg/ml and 10 μg/ml on the apical side of the IPEC-J2 layer. After the incubation with LPS (1 h), cells were washed with plain medium without LPS and cultured for additional 24 h before being subjected to the subsequent procedures. TEER measurements were performed both before and after the LPS treatment.

Paracellular Permeability Assessment

IPEC-J2 cells were plated to confluence on six-well polyester membrane inserts without collagen coating, and they were allowed to form differentiated monolayers. LPS was added at 1 or 10 μg/ml concentration for 1 h, and TEER measurements were performed prior to and 2, 4, 24 h after LPS administration. In parallel to LPS, fluorescein isothiocyanate–dextran 4 kDa (FD4 obtained from Sigma-Aldrich, Munich, Germany) was added at 1 mg/ml to the apical compartment of IPEC-J2 cell mono-culture with different incubation times (2, 4, and 24 h). Samples of media from the basolateral chambers were collected, and tracer concentration was quantified by fluorescence at excitation 485 nm and emission 535 nm (Victor X2 2030 fluorometer).

Isolation and Cultivation of Hepatocytes

Hepatocyte isolation was performed according to a modified protocol of Puviani *et al.* [31] and Meng *et al.* [32]. Livers were obtained from clinically healthy male pigs of the Large White breed, weighing approximately 15 kg. Animals were anesthetized by intramuscular application of the combination of xylazine (2 mg/kg BW) and ketamine (20 mg/kg BW). After aseptic opening of the abdominal cavity by a midline incision, the liver was excised and the caudate lobe was isolated and exsanguinated by 300 ml chilled EDTA-containing buffer. The perfusion was continued with 300 ml pre-warmed (37 °C) EDTA-free buffer solution. Finally, 100 ml of this buffer, supplemented with 100 mg collagenase type IV and 4.8 mM $CaCl_2$, was applied and recirculated in order to disaggregate the liver and release the hepatocytes. Before application for perfusion, all solutions were oxygenated with carbogen (95 % O_2, 5 % CO_2, 1 l/min). Velocity of the perfusion was set to 100 ml/min. After collagenase digestion, the capsule was disrupted, and the digested parenchyma was filtered through a nylon mesh with 100 μm pore size (Millipore, Volketswil, Switzerland) to eliminate cell aggregates. Hepatocyte-enriched fractions were isolated and washed by low-speed centrifugation (50 g, 75 s) three times in Williams' medium E, supplemented with 10,000 IU/ml penicillin, 10 mg/ml streptomycin, 2 mM glutamine, 10 % FBS, and 0.2 IU/ml insulin. Cell viability, assessed by trypan blue exclusion, exceeded 90 % in all preparations. Yield of hepatocytes was determined by cell counting in Bürker chamber, and cell concentration was adjusted to 4×10^5/ml. Hepatocytes (1.5 ml cell suspension/well) were seeded onto six-well Costar TC cell culture dishes (well diameter: 34.8 mm; Corning International) and coated with collagen type I (10 μg/cm^2) according to the manufacturer's instructions. Cell cultures were incubated at 37 °C in humid atmosphere with 5 % CO_2; culture medium was changed 4 h after plating. Confluent mono-culture of hepatocytes was gained after 24 h incubation.

LPS Treatment of Mono-cultures of Hepatocytes

Twenty-four hours after isolation, culture medium of hepatocytes was changed to serum-free Williams' medium E and treated with LPS (1 μg/ml and 10 μg/ml) for 1 h. Medium was changed and hepatocytes were harvested after a 24-h incubation.

Preparation of Double-Layered Co-culture System. LPS Treatment of Epithelial Cells in the Co-culture Experiment

IPEC-J2 cells were used for experiments 18 days after plating when a confluent monolayer was formed expressing high TEER values (>1,200 Ω). A double-layered co-culture system (24 h after hepatocyte culturing) was prepared in six-well plates by placing inserts with confluent IPEC-J2 layer over the confluent layer of hepatocytes (see Fig. 1). The volume of the applied culture medium was 1.5 ml on the apical side (serum-free plain medium for IPEC-J2) and 2.5 ml on the basolateral side (Williams' medium E). After 1 h adaptation, LPS (1 μg/ml and 10 μg/ml) was applied to the apical side, and after 1 h further incubation, medium on the apical side was replaced by serum-free plain medium. IPEC-J2 cells and hepatocytes were harvested after 24 h.

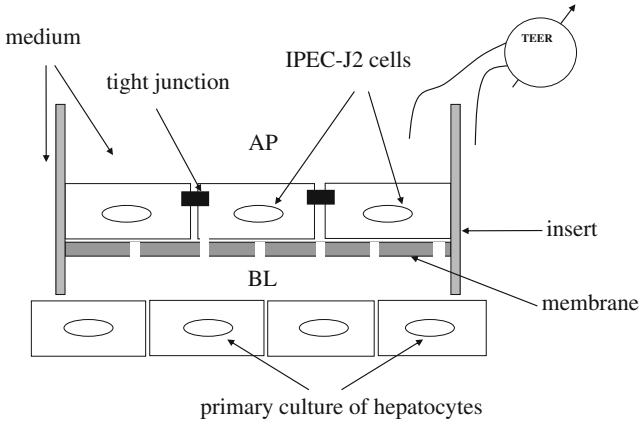

Fig. 1. Schematic diagram of the co-culture system. IPEC-J2 cells were seeded onto six-well Transwell polyester membrane inserts at a density of 1.5×10^5 cells/ml and cultured for 18 days. Freshly isolated hepatocytes were seeded onto six-well plates at a cell density of 6×10^5 cells/ml. After 24 h cultivation, when confluent mono-culture of hepatocytes was gained, the inserts with IPEC-J2 cells were placed into the wells over the hepatocytes. *AP* apical compartment, *BL* basolateral compartment.

Quantitative Real-Time PCR

Twenty-four hours after the 1-h LPS treatment, culture medium was removed and 1 ml of ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to the hepatocyte mono-cultures or to the hepatocyte bottom layer of co-cultures. IPEC-J2 samples (from mono-culture) were handled similarly. After 1 h LPS treatment, culture medium was removed, and samples were collected and kept at −80 °C until further processing. Total RNA was isolated from the cells according to the manufacturer's instructions. To prevent DNA contamination, the isolated RNA (2 μg) was treated with AMP-D1 DNase I (Sigma). Quantity, A_{260}/A_{280} and A_{260}/A_{230} ratios of the extracted RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Quality and quantity control of the isolated RNA was carried out both before and after the DNase treatment.

Synthesis of the first strand of cDNA from 1,000 ng of total RNA was achieved using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's recommendations, using the random hexamer as a priming method. Quantitative real-time PCR (qRT-PCR) was performed using the iQ SYBR Green Supermix kit (BioRad, Hercules, CA, USA) on the MiniOpticon System (BioRad). The cDNA was diluted 5-fold, before equal amounts were added to duplicate qRT-PCR reactions. In hepatocytes, tested genes of interest were CYP1A1, CYP1A2, and CYP3A29, while in IPEC-J2 cell samples, the relative expression of IL-8 and TNF-α genes was determined. Hypoxanthine phosphoribosyl transferase and cyclophilin-A were used as reference genes in hepatocytes as well as IPEC-J2 cells. Primer sequences are listed in Table 1. For each PCR reaction, 2.5 μl cDNA was added directly to a PCR reaction mixture, set to a final volume of 25 μl, containing 1× concentrated iQ SYBR Green Supermix and 0.2 μM of the appropriate primers. The thermal profile for all reactions was 3 min at 95 °C, then 40 cycles of 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. At the end of each cycle, fluorescence monitoring was set for 10 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. In order to determine the efficiencies of the PCR reactions, standard curves were obtained for each target and reference gene, using serial dilutions of a reference cDNA. Real-time PCR efficiencies (E) were calculated according to the equation: $E=10^{(-1/\text{slope})}$. To determine the stability of the reference genes, the geNorm (version 3.5) was used.

IL-8 and Albumin ELISA

After the LPS treatments (1 h), IPEC-J2 cells, hepatocytes, or double-layered co-cultures were incubated for 24 h. Culture media were collected, centrifuged (245 g, 10 min), and diluted to measure the IL-8 and albumin protein concentrations. Level of IL-8 secretion was determined by a porcine-specific IL-8 ELISA kit (Invitrogen) according to the manufacturer's instructions. Albumin secretion of hepatocytes representing their viability in mono-cultures and double-layered co-cultures was determined 24 h after LPS treatment. Albumin levels were

Table 1. Sequence of Primer Sets Used for Quantitative Real-Time PCR

Gene symbol	Accession number	Primer sequences	Product size (bp)	Efficiency	R^2	Reference
CYP1A1	NM_214412.1	F 5′-CAGAGCTGCTTAGCCTTATCAACC-3′ R 5′-CTGGATGCTGGGATTTGTCACCAG-3′	386	2.09	0.970	[43]
CYP1A2	NM_001159614.1	F 5′-GTGAGGAGATGTTCAGCATCGTGAAG-3′ R 5′-CTTCTGTATCTCAGGATATGTCACA-3′	386	1.98	0.95	NCBI/Primer BLAST
CYP3A29	NM_214423.1	F 5′-TTCGTGCTTCACAGAGAGACCC-3′ R 5′-TACTAGGTGGGGGTGGATGG-3′	576	1.83	0.97	[44]
IL-8	NM_213867	F 5′-AGAGGTCTGCCTGGACCCCA-3′ R 5′-GGGAGCCACGGAGAATGGGT-3′	126	1.972	0.999	[13]
TNF-α	NM_214022	F 5′-TTCCAGCTGGCCCCTTGAGC-3′ R 5′-GAGGGCATTGGCATACCCAC-3′	146	1.873	0.982	[45]
HPRT	NM_001032376	F 5′-GGACTTGAATCATGTTTGTG-3′ R 5′-CAGATGTTTCCAAACTCAAC-3′	91	1.963	0.997	[46]
CycA	NM_214353	F 5′-GCGTCTCCTTCGAGCTGTT-3′ R 5′-CCATTATGGCGTGTGAAGTC-3′	160	1.907	0.998	[45]

HPRT hypoxanthine phosphoribosyl transferase, *CycA* cyclophilin-A, *F* forward, *R* reverse

determined using a porcine-specific albumin ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA).

Neutral Red Uptake Assay for Cell Viability

Influence of hepatocyte cellular metabolites on the viability of enterocytes in the co-culture system was tested. IPEC-J2 cells were seeded in a 96-well plate and incubated with undiluted and diluted (4- and 16-fold) hepatocyte supernatant (stem from a 24-h culture of swine hepatocytes, undiluted or diluted with serum-free cell culture medium). Viability of IPEC-J2 cells was measured by Neutral Red uptake assay as described by Repetto *et al.* [33].

Statistical Analysis

Relative gene expression levels of the genes of interest were calculated by the Relative Expression Software Tool 2009 Software (Qiagen GmbH, Hilden, Germany). Statistical analysis of other data was performed with R 2.14.0 software. Differences between means were evaluated by one-way or two-way ANOVA, with data of normal distribution, and homogeneity of variances was confirmed. To compare treated groups to controls, we used Dunnett post hoc test; for the comparison of different cultures, we used Tukey HSD test. Level of significance was set at $P<0.05$, P without subscript refers to ANOVA, and P values of post hoc tests are indicated as P_{Dunnett} and P_{Tukey}. All values were expressed as means±SEM.

RESULTS

Studies on IPEC-J2 Cell Mono-cultures Exposed to LPS Challenge

IPEC-J2 mono-cultures, exposed to LPS at 1 and 10 μg/ml, showed increased expression of IL-8 protein after 1 h of exposure ($P<0.001$) and also at both concentrations significantly increased mRNA encoding TNF-α ($P=0.025$ and $P=0.023$) (Fig. 2a, b). After incubation of IPEC-J2 cells with either concentration (1 and 10 μg/ml) of LPS, the IL-8 concentration was significantly higher in the basolateral culture media than in the apical compartment ($P<0.001$). The interaction of treatment and compartment was also significant ($P<0.001$). Results regarding IL-8 production of IPEC-J2 cells are presented in Fig. 2c.

LPS-triggered partial disruption of cell monolayer integrity caused increased paracellular transport of the

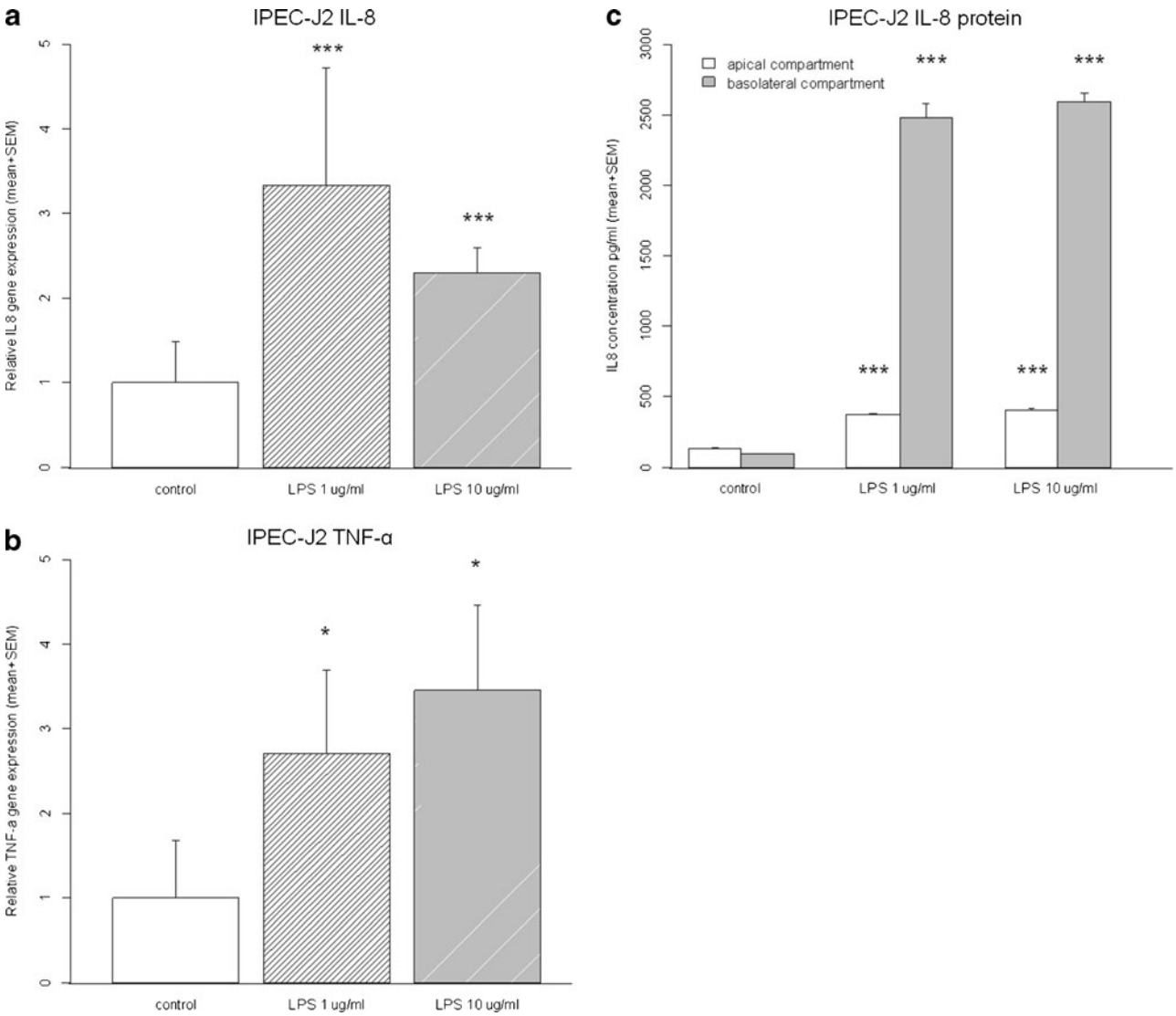

Fig. 2. Relative gene expression (mRNA) of **a** IL-8, **b** TNF-α, and **c** protein concentration of IL-8 in the apical and basolateral culture medium of IPEC-J2 cells exposed to LPS treatment (at 1 and 10 μg/ml, 1 h) compared to untreated groups (n=6/group, *P<0.05, ***P<0.001 according to Dunnett multiple comparison of means). Data are shown as means+SEM.

hydrophilic tracer FD4 from the apical chamber to basolateral compartment. After 2, 4, and 24 h incubation with LPS (1 and 10 μg/ml), fluorescence intensity of FD4 on the basolateral side was significantly higher compared to controls (P<0.001).

Incubation time also affected the fluorescence intensity (P<0.001), and there was a significant interaction (P=0.017) between treatment concentrations and incubation time. In the basolateral compartment of untreated IPEC-J2 cells, only negligible amount of FD4 was detected indicating an intact cell monolayer before application of LPS (Fig. 3a). In addition, TEER values prior to and post-treatment were compared to check if the polarized cell monolayer integrity was influenced by the presence of LPS. As shown in Fig. 3b, LPS application did not affect TEER values significantly (P=0.874).

Studies on Mono-cultures of Porcine Hepatocytes Exposed to LPS Challenge

Albumin production of the liver cells was investigated in order to characterize the mono-culture of porcine

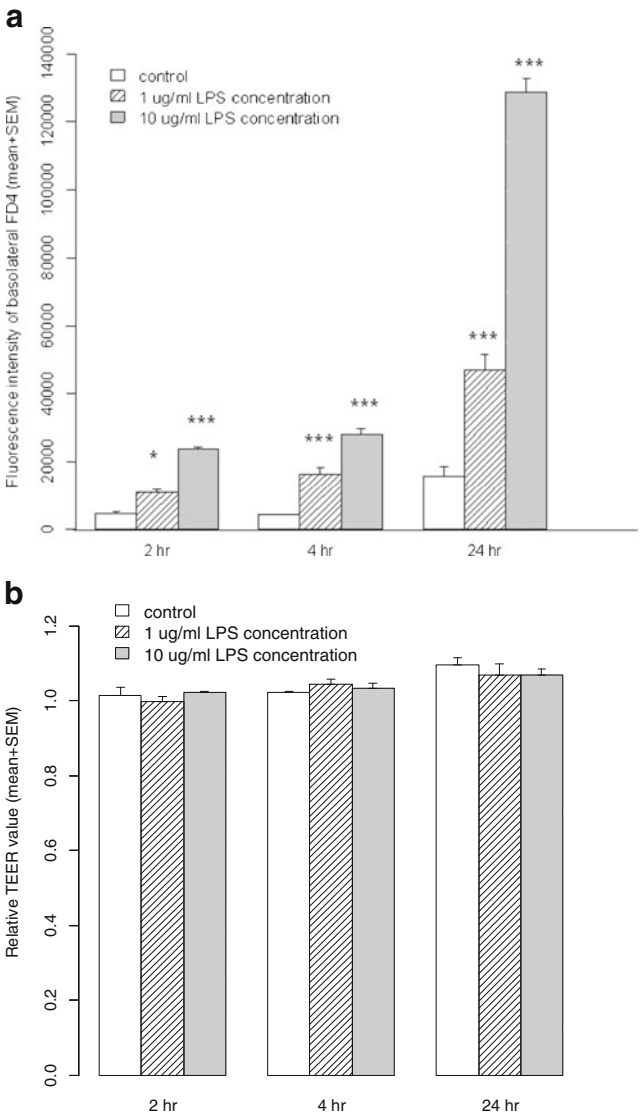

Fig. 3. **a** Penetration of the fluorescent FITC-dextran 4 (*FD4*) from the apical to the basolateral compartment of IPEC-J2 cells as a result of LPS treatment. Significant increase in basolateral FD4 concentration was measured up to 24 h incubation time of IPEC-J2 cells after LPS treatment (1 h, 1 and 10 μg/ml) compared to untreated control (n=6/ group, *P<0.05, ***P<0.001 according to Dunnett multiple comparison of means). Data are shown as means+SEM. **b** Transepithelial electrical resistance (*TEER*) values between apical and basolateral chambers of IPEC-J2 cell mono-cultures did not change significantly by administration of 1 and 10 μg/ml LPS (P>0.05). Data are shown as relative TEER±SEM.

hepatocytes and to assess viability of the cultured cells. Albumin concentration corresponding to intact hepatocytes was significantly reduced 24 h after LPS treatment with either of the two concentrations of LPS (P=0.035, at 1 and 10 μg/ml P_{Dunnett}=0.038 and P_{Dunnett}=0.041, respectively) (Fig. 4a). IL-8 production of hepatocytes was not significantly altered at the higher LPS dose (P= 0.107) (Fig. 4b).

Regarding the results of the quantitative real-time PCR examinations, CYP1A1 and CYP1A2 gene expression of hepatocytes was significantly decreased 24 h after the 1-h exposure to 1 μg/ml LPS (P=0.009 and P=0.046, respectively), but under this treatment condition, CYP3A29 was not altered (P=0.278). No significant difference could be found between 10 μg/ml LPS-stimulated and control cells concerning the expression

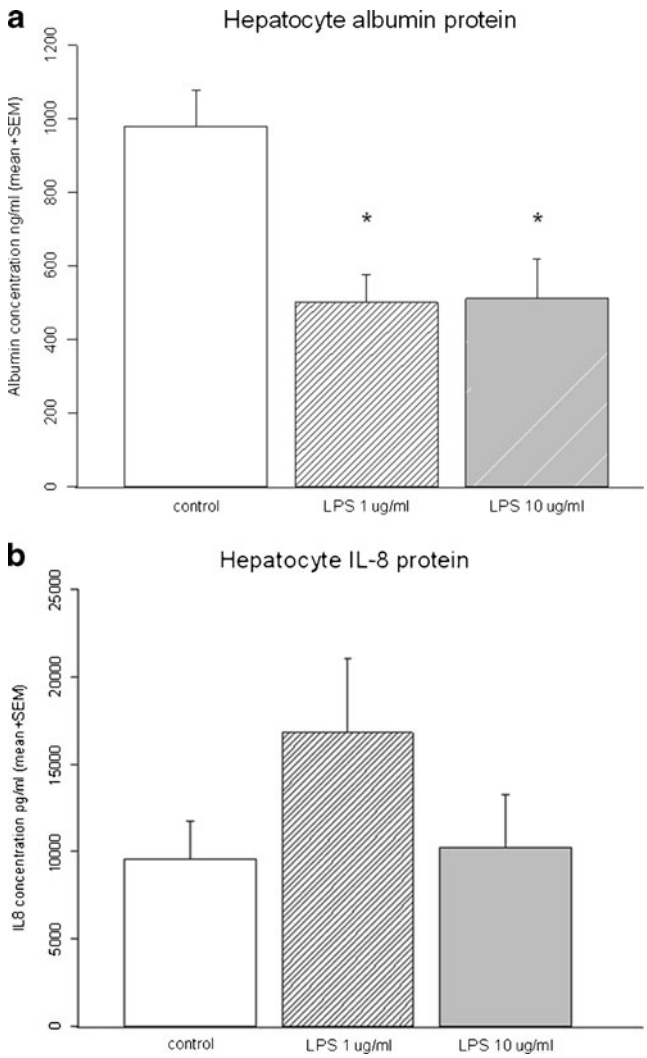

Fig. 4. **a** Albumin and **b** IL-8 protein concentrations in the medium of mono-cultures of porcine hepatocytes, exposed to LPS treatment (at 1 and 10 μg/ml, 1 h) compared to untreated groups (n=6/group). Data are shown as means+SEM.

of hepatic CYP1A1 and CYP3A29 genes (P=0.811 and P=0.172, respectively); however, CYP1A2 was downregulated after treatment with the applied higher LPS concentration (P=0.021) (Fig. 5a–c).

Studies on the Double-Layered Co-culture Model Exposed to LPS Challenge

To confirm the integrity of the established double-layered co-culture, TEER values of the IPEC-J2 cells, and albumin secreted by hepatocytes (basolateral compartment), were measured. Co-culture experiments were performed with confluent polarized IPEC-J2 cells with high TEER values after 24 h of co-cultivation (TEERs of control and to 1 μg/ml LPS-exposed cells were 2,157±178 and 1,980±482 Ω, respectively. TEER of 10 μg/ml LPS-treated cells was 2,290±185 Ω proving that integrity of the polarized IPEC-J2 monolayer was not altered (P=0.109). Viability of hepatocytes in the co-culture was assessed by monitoring albumin production, which matched the requirements of a successful hepatocyte cultivation. Albumin production in co-culture was lower than in the hepatocyte mono-culture, and no significant difference in albumin production was detected by comparing wells with or without LPS treatment after 24 h (P=0.617) (Fig. 6a). Result of Neutral Red uptake assay showed that there was no significant difference in number of

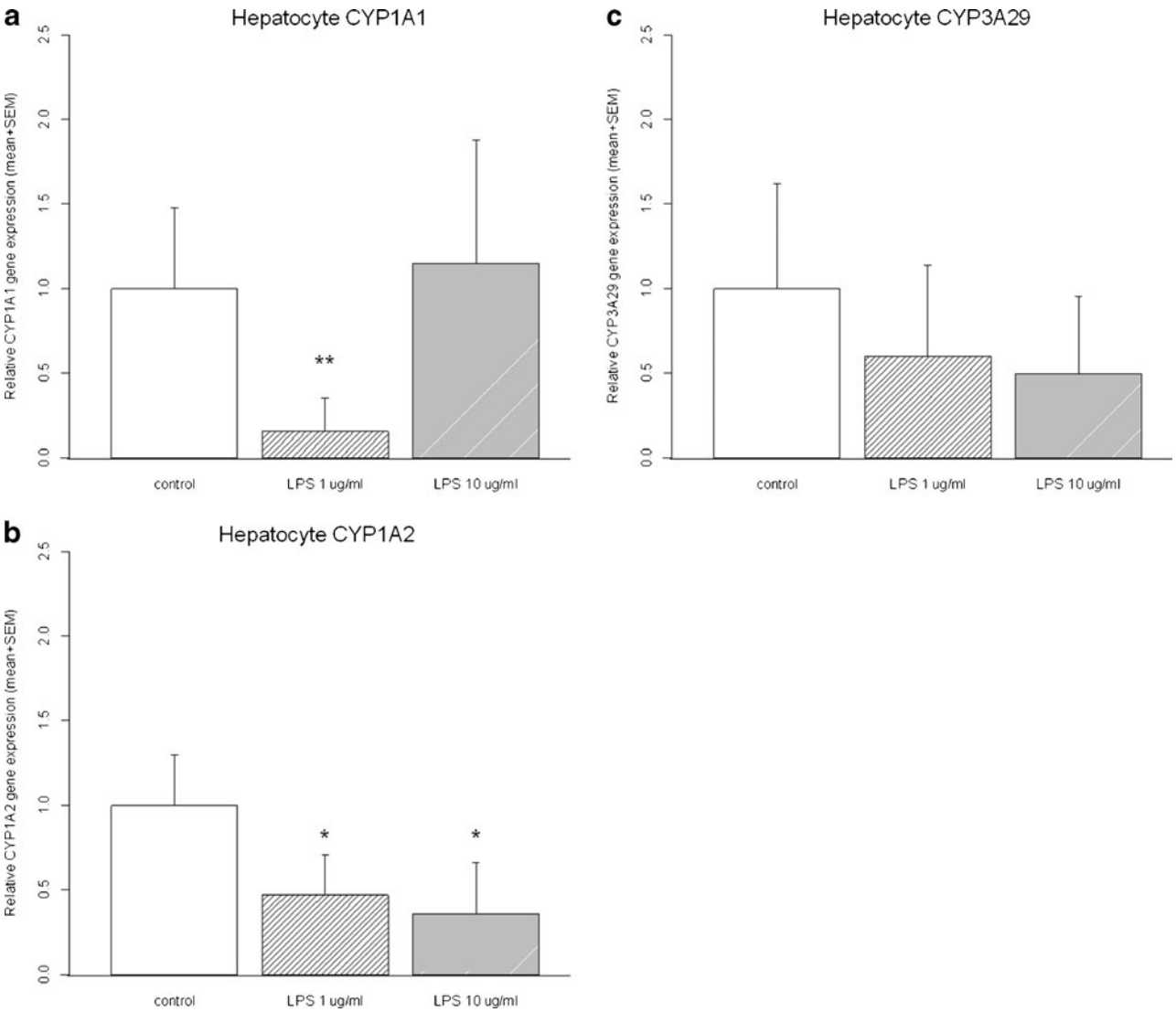

Fig. 5. Relative mRNA expression of **a** CYP1A1, **b** CYP1A2, and **c** CYP3A29 genes in mono-cultures of porcine hepatocytes, exposed to LPS treatment (at 1 and 10 μg/ml, 1 h), compared to untreated groups (n=6/group, *P<0.05, **P<0.01 according to Dunnett multiple comparison of means). Data are shown as means+SEM.

viable IPEC-J2 cells incubated with hepatocyte supernatant for 24 h compared to control cells (data not shown).

After incubation at the higher LPS concentration (10 μg/ml), IL-8 protein level was increased in the culture media in the compartment between the two cell layers ($P_{Dunnett}$=0.012), while the difference did not reach significance at the lower dose of LPS (1 μg/ml) ($P_{Dunnett}$=0.592) (Fig. 6b). Expression of the examined hepatic CYP genes (CYP1A1, CYP1A2, and CYP3A29) was significantly suppressed at the lower concentration (1 μg/ml) of LPS (P=0.01, P=0.007, P=0.007, respectively). However, LPS exposure to the higher concentration (10 μg/ml) did not suppress the relative gene expression of CYP1A1 (P=0.243) and CYP3A29 (P=0.106), and CYP1A2 was significantly upregulated by this LPS treatment (P=0.039) (Fig. 7a–c).

DISCUSSION

IPEC-J2 cells showed optimal TEER values prior to the treatments as well as after the 24-h incubation period. The LPS-induced inflammatory response resulted in increased rate of apicobasolateral transport of FD4

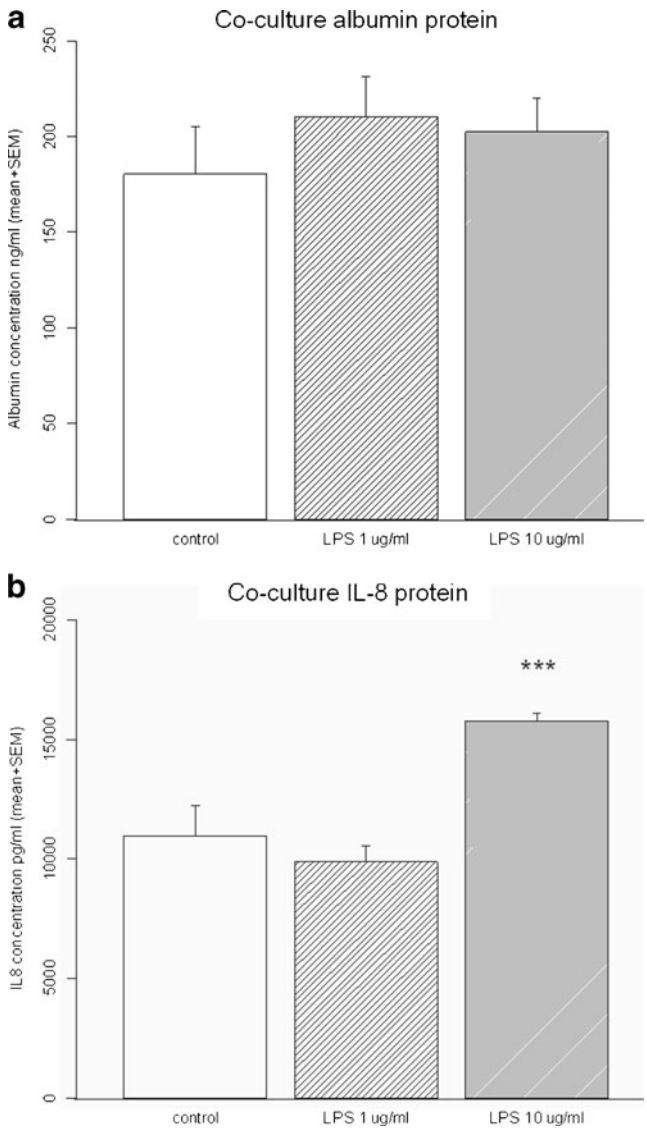

Fig. 6. **a** Albumin and **b** IL-8 protein levels in the basolateral medium of co-cultures of IPEC-J2 cells with porcine hepatocytes, exposed on the apical side of IPEC-J2 cells to LPS (1 and 10 μg/ml, 1 h, and incubated for 24 h), compared to untreated groups (n=6/group, ***P<0.001). Data are shown as means+SEM.

without affecting transepithelial electrical resistance of polarized IPEC-J2 cell monolayers suggesting that two paracellular pathways exist: ionic charge-selective small pore-system carrying most of the electrical current (reflected in TEER) and larger barrier discontinuities lacking charge and size discrimination in epithelium [34].

Disruption of the intestinal epithelium integrity contributes to facilitated translocation of bacteria and bacterial products from the intestine to the liver via the portal vein causing impaired liver homeostasis and enhanced liver inflammation [35, 36]. In this study, a novel double-layered porcine enterohepatic co-culture system consisting of IPEC-J2 small intestinal epithelial cells and primary cultured hepatocytes was successfully established enabling in-depth characterization of each cell mono-culture and their interaction. Co-cultured layers compared to *in vitro* hepatocyte mono-cultures appear to be a reliable system to describe gut-mediated changes in gene expression of hepatic CYP enzymes, acting as crosstalk model between the liver and the gut. Treatment of hepatocyte mono-culture with 1 μg/ml LPS decreased the relative gene expression of CYP1A1 and CYP1A2,

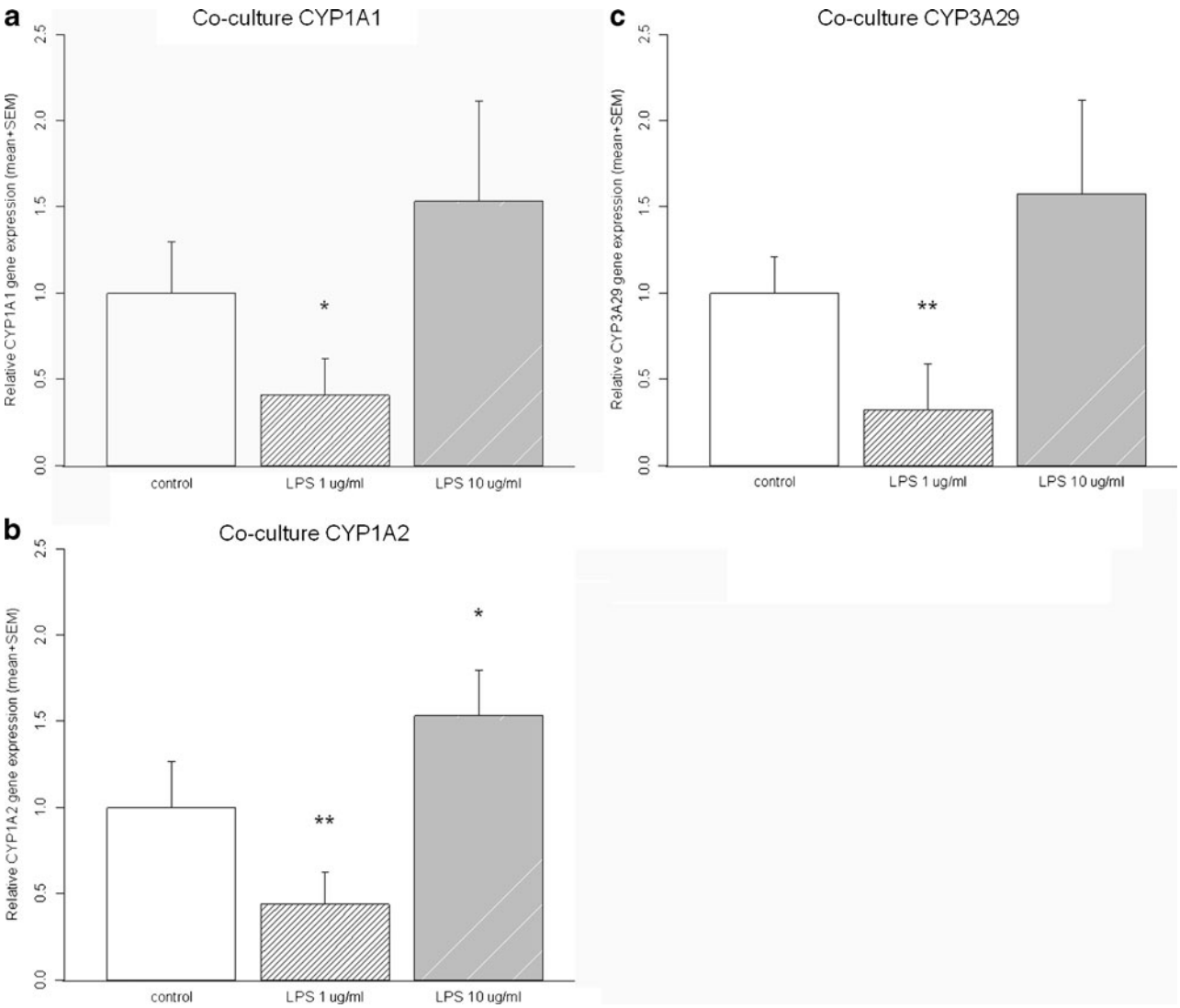

Fig. 7. Relative expression of **a** CYP1A1, **b** CYP1A2, and **c** CYP3A29 genes in porcine hepatocytes co-cultured with IPEC-J2 cells, exposed on the apical side of the IPEC-J2 cell layer to LPS (at 1 and 10 μg/ml, 1 h) compared to untreated groups (n=6/group, *P<0.05, **P<0.01). Data are shown as means+SEM.

but not that of CYP3A29 expression. At the higher LPS dose (10 μg/ml), relative gene expression of CYP1A1 and CYP3A29 did not change, but that of CYP1A2 decreased significantly.

In contrast to the hepatocyte mono-culture, expression of all three investigated hepatic CYP genes was down-regulated after 1 μg/ml LPS treatment of IPEC-J2 cells in co-culture. It was described earlier that several CYP genes could be downregulated by LPS-induced inflammatory processes [20], which is of special importance from a pharmacological point of view due to the possibly altered kinetics of the simultaneously applied xenobiotics. In this study, the suppressive effect of LPS-treated intestinal epithelial cells on the expression of hepatic CYP enzymes is for the first time demonstrated in an *in vitro* enterohepatic model. Since low-dose LPS-provoked suppression of the CYP3A29 gene could not be detected in mono-cultured hepatocytes, the applied co-culture system seems to be indispensable to elicit new details of systemic inflammatory processes in association with the gut. The observed findings at the higher LPS dose (10 μg/ml) in co-cultures are in good agreement with systemic infection-caused significant change in the levels and activities of certain hepatic CYP isoforms in mice and rats [37]. The signal transduction

pathways leading to downregulation of CYPs are not described yet in detail. It was reported that LPS stimuli decreased the relative protein expression level and activity of hepatic CYP1A, CYP2B, CYP3A, and CYP4A enzymes in wild-type and TNF-α-receptor-deficient mice to similar extents [38]. Deletion of IL-6 gene did not rescue the lowered CYP gene expression after LPS treatment [39], but IL-6 exerted pan-suppression of the most important CYPs in human hepatocytes [40]. Colonic bacterial colonization and subsequent large bowel inflammation caused downregulation of CYP4A, but increased expression of CYP4F and CYP2D in the liver of mice, in correlation with elevated serum IL-6 and TNF-α levels [41]. In our co-culture model, IL-8 concentration of the basolateral culture media was not increased, but hepatic CYPs were downregulated after 1 μg/ml LPS treatment of enterocytes, while 10 μg/ml LPS led to elevated IL-8-production without CYP suppression at the same time. Based on these findings, it can be hypothetized that IL-8 is not primarily involved in the signaling of LPS-triggered alterations of hepatic CYPs.

The measured albumin concentration of the basolateral culture media of co-cultured hepatocytes was lower than that in mono-cultures of hepatocytes ($P<0.001$), but still matched the requirements of the successful hepatocyte cultivation [42], thus indicating the viability of the cultured hepatocytes. In addition, it was not influenced by any of the LPS treatments. The effect of LPS administration on albumin secretion of hepatocyte mono-culture was different compared to that of hepatocytes in co-culture system (interaction between culture and treatment $P=0.01$).

LPS treatment of IPEC-J2 cells strongly influenced the gene expression as well as protein levels of IL-8 at 1 and 10 μg/ml LPS exposure. These data are in good agreement with a recent study, revealing that 1 μg/ml LPS upregulated the IL-8 and TNF-α genes in jejunal epithelial cells [12]. The gut mucosal epithelium serves as a barrier against enteral pathogens, playing a key role in the mediation of bacterial LPS-induced systemic inflammatory processes. Hence, treatment of cultured hepatocytes with bacterial lipopolysaccharide mimics the *in vivo* LPS effect on the liver less closely than LPS exposure in an enterohepatic co-culture system. In this way, the cytokine-mediated pathways of LPS-induced inflammation seemed to be essential in order to achieve an elevated IL-8 response of the liver. In case of inflammatory bowel disease, hepatocytes have also direct contact with LPS derived from the gut due to the consequences of the leaky epithelium. Hence, the investigation of impact of LPS on hepatocyte mono-cultures can greatly contribute to the better understanding of *in vivo* processes under intestinal inflammatory conditions. In the newly established co-culture system, 1 μg/ml LPS exposure of the enterocytes on the apical side did not change the IL-8 concentration in the basolateral compartment compared to control, but IL-8 was significantly elevated after incubation with 10 μg/ml LPS. In the experiments with IPEC-J2 cells alone, baseline IL-8 secretion of enterocytes into the basolateral compartment was quite low and increased even at the lower dose of LPS (interaction between culture and treatment $P=0.002$). In comparison, IL-8 concentration in the basolateral medium of co-cultures was quite high even in wells with untreated cells, possibly due to the additional enteral and hepatic cytokine production (difference between co-culture and IPEC-J2 mono-culture $P_{Tukey}<0.001$, between co-culture and hepatocyte mono-culture $P_{Tukey}<0.477$). These results demonstrate the usefulness of the developed co-culture system. LPS did not show a direct stimulatory effect on IL-8 production in mono-cultures of hepatocytes, but the basolateral IL-8 concentration of co-cultures was elevated after apical 10 μg/ml LPS exposure modeling the *in vivo* conditions of the enterocyte-liver axis (interaction between culture and treatment $P=0.066$).

However, the lack of immune and enteroendocrine cells from the intestinal layer partly limits the model's applicability to the *in vivo* conditions. The main impact of this study is to provide a novel *in vitro* system to investigate the role of pure enterocytes in the mediation of LPS-triggered systemic inflammation.

In conclusion, the newly developed porcine enterocyte-liver co-culture model has clear benefits over intestinal and hepatocyte mono-cultures to provide a tool to study the inflammatory responses related to the enterohepatic system. It seems to be a more complete model to investigate the effects of apical LPS exposure of enterocytes on basolateral cytokine production and on hepatic detoxification function, including gene expression of microsomal drug-metabolizing CYP enzymes. This crosstalk model between enterocytes and liver may mimic the *in vivo* interactions in different biological processes even in other than inflammation and can serve by this way as a more general, promising future tool to study certain enterohepatic pathways.

ACKNOWLEDGMENTS

The research described here was supported by the Hungarian Scientific Research Fund (OTKA grants no. 100701 and no. 105718). The study was sponsored by the

TÁMOP-4.2.2.B-10/1 and TÁMOP-4.2.1.B-11/2/KMR-2011-0003 projects. This paper is dedicated to Prof. Dr. Péter Gálfi on the occasion of his 60th birthday for establishment of the IPEC-J2 and hepatocyte co-culture system. We are indebted to Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA) for providing the IPEC-J2 cells. Special thanks have to be given to Hedvig Fébel (Research Institute for Animal Breeding and Nutrition, Herceghalom, Hungary) for providing the animals used for hepatocyte isolation. The excellent assistance of Janka Petrilla and Melinda Szabó (Faculty of Veterinary Science, Szent István University, Budapest, Hungary) is gratefully acknowledged.

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