

Rat liver slices as a tool to study LPS-induced inflammatory response in the liver

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Background/Aims: Inflammation in the liver is a complex interaction between parenchymal and non-parenchymal cells, and therefore can not be studied *in vitro* in pure cultures of these cells.

Methods: We investigated whether Kupffer cells in the liver slice are still responsive to an inflammatory stimulus of lipopolysaccharide (LPS), and evoke an inflammatory response in the hepatocytes.

Results: TNF α , IL-1 β and IL-10 were significantly elevated in culture medium of LPS-stimulated rat liver slices. Nitric oxide (NO) production of LPS-treated slices gradually increased from 5 to 24 h (24 h: $81 \pm 5 \mu\text{M}$ vs. $14 \pm 2 \mu\text{M}$ in control $P < 0.05$), paralleled by inducible nitric oxide synthase (iNOS) in the hepatocytes, iNOS mRNA was induced after 3 h. NO production but not iNOS induction was significantly inhibited by NOS inhibitors S-methylisothiourea and N^G-nitro-L-arginine methylester. Both pentoxifylline and dexamethasone inhibited TNF α and IL-1 β production, albeit to a different extent, iNOS induction and, as a result thereof, NO production.

Conclusions: These results imply that non-parenchymal cells in liver slices are viable and can be activated by LPS. In addition, it is concluded that the upregulation of iNOS in hepatocytes by LPS is caused by cytokines produced by Kupffer cells because inhibition of TNF α and IL-1 β production attenuated iNOS induction.

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1. Introduction

Lipopolysaccharide (LPS or endotoxin) a component of Gram-negative bacterial cell walls is associated with tissue injury and sepsis. One of the major features of endotoxic shock is the induction of nitric oxide synthase in the liver [1]. Inducible nitric oxide synthase (iNOS) induced by cytokines and LPS produces nitric oxide (NO) in large amounts [2]. NO is known to be a crucial factor in acute inflammation and sepsis. *In vivo*, NO has protective effects in inflammation and endotoxemia induced hepatic injury [3]. In addition, in response to endotoxin, proinflammatory cytokines including interleukines (IL-6 and IL-1 β) and tumor necrosis factor α

(TNF α) and anti-inflammatory cytokines such as IL-10, are produced by inflammatory cells. In the liver LPS activates the resident macrophages, which results in cytokine release [4]. Furthermore, LPS is cleared by the liver, mainly by Kupffer cells [5].

To further unravel the mechanisms underlying the inflammatory reactions in the liver, *in vitro* preparations will be indispensable, particularly to be able to study these phenomena in the human liver. LPS-induced iNOS expression in hepatocytes as observed *in vivo* [2] cannot be achieved in pure cultures of isolated hepatocytes. In fact, in pure hepatocyte cultures the induction of iNOS is found only after incubation with a mix of LPS and several cytokines [6–8]. Induction of iNOS by LPS alone can only be accomplished in co-cultures of hepatocytes and Kupffer cells [1]. These data indicate that induction of iNOS by LPS is mediated by cytokines released by the Kupffer cells in the liver.

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Therefore, to study the effects of LPS in the whole liver in vitro, ideally a system with all the different cell types present in the liver should be used. Co-cultures of hepatocytes and Kupffer cells have been proven useful [1,9]. However, the establishment of such co-cultures is technically complex while the cellular organisation as present in the intact liver is lacking. Precision-cut liver slices contain all cells in their original architecture and are easy to prepare and handle [10]. The use of precision-cut liver slices in metabolism and toxicological research steadily increased [11], this is predominately the function of the hepatocytes in the slice. There is abundant evidence now that hepatocytes in liver slices are properly functioning and are viable for at least 24 h [12–14]. Only very few studies have focused on the activity or the viability of the other cell types of the liver within the slice [15,16].

Therefore, in the present study we established whether non-parenchymal cells are still viable in the rat liver slices and respond to LPS. Cytokine levels (TNF α , interleukine 1 β (IL-1 β) and interleukine 10 (IL-10)) were measured in the incubation medium as markers of non-parenchymal cell function. IL-1 β and IL-10 are almost exclusively produced by Kupffer cells [17]. Therefore, the production of these cytokines can be used as marker for the Kupffer cell function. In addition, the study was designed to elucidate the interaction between non-parenchymal and parenchymal cells in the liver after stimulation with LPS. Therefore, the mRNA of iNOS was determined by reversed transcriptase polymerase chain reaction (RT-PCR) and iNOS protein was studied immunohistochemically. The activity of iNOS was determined by measuring the rate of ^3H -L-citrulline formation from ^3H -L-arginine. Furthermore, the NO production was measured as nitrate/nitrite concentration (NO $_x$) in the culture medium. During incubation the viability of the slices was monitored using LDH, GPT and GOT levels in the culture medium. The possibility of pharmacological intervention during inflammation was studied through the incubation with the NOS inhibitors S-methylisothiourrea (SMT) and *N*^G-nitro-L-arginine methyl ester (L-NAME) [2]. In addition, pentoxifylline, a phosphodiesterase inhibitor, which blocks endotoxin-induced synthesis of TNF α [18] and the glucocorticoid dexamethasone, an inhibitor of cytokine production and iNOS expression, were included in the study [19].

This in vitro model, developed and validated with rat liver slices, can be applied to human liver slices as well, offering the unique possibility to study the inflammatory responses and pharmacological interventions in the *human* liver.

2. Material and methods

2.1. Material

Williams' medium E (WME) supplemented with Glutamax I and gentamicin was obtained from Gibco BRL (Paisley, Scotland). LPS *E-coli* serotype (B55:O55) and [L]-arginine-[2,3- ^3H] were purchased from Sigma

Chemical Company (St. Louis, MO, USA). 6-well culture plates were obtained from Greiner (Alphen a/d Rijn, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

2.2. Methods

2.2.1. Slice preparation

For preparation of liver slices, adult male Wistar rats (250–300 g) were obtained from Harlan PBC (Zeist, The Netherlands). Precision-cut liver slices (10–14 mg) were prepared as described earlier [11].

2.2.2. Incubations

After slicing, liver slices were kept in University of Wisconsin organ preservation solution (UW) on melting ice, until use (within 1 h) [13]. The culture medium was WME supplemented with D-glucose to a final concentration of 25 mM and 50 $\mu\text{g}/\text{ml}$ gentamicin, saturated with 95% O $_2$ /5% CO $_2$ (carbogen) at 37°C [12]. Slices were incubated for various periods, with or without 100 $\mu\text{g}/\text{ml}$ LPS. SMT (final concentration 0.05 mM), L-NAME (final concentration 0.5 mM) and pentoxifylline (final concentration 1.7 mM) were added simultaneously with LPS. Dexamethasone (final concentration 20 μM) was added 2 h before LPS addition. In pilot experiments no difference were observed between 2 h incubation of dexamethasone before LPS addition and simultaneously addition of LPS and dexamethasone.

2.2.3. Cytokine analysis

TNF α , IL-10 and IL-1 β and were analysed in the culture medium by sandwich ELISA, manufactured by respectively Pharmingen (Hamburg, Germany) and R& D systems (Minneapolis, MN, USA).

2.2.4. RT-PCR

Total RNA was isolated from frozen snap-frozen rat liver slices after different incubation periods with or without LPS. Single strand cDNA was synthesized from 10 μg RNA as described previously [20]. With the obtained cDNA, a PCR reaction was performed as was described by Vos et al. [20]. For every PCR reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Primer sequence for iNOS and GAPDH are described by Vos et al. [20]. Ten microliters of the PCR product was loaded on a 1% agarose gel and stained with ethidium bromide.

2.2.5. Immunohistochemistry

The slices were taken out of the incubation system, completely embedded in Tissue-tek and snap-frozen in isopentane (-80°C). They were stored at -80°C until sections of 4 μm were cut in a cryostat (-20°C) perpendicular to the surface of the slice. Sections were stained for the presence of iNOS protein according to Vos et al. [2].

2.2.6. Assays of NO formation and NOS activity

NO $_x$ concentrations in medium were measured according to Moshage et al. [21]. NOS activity was measured according to Vos et al. [2].

2.2.7. Viability

LDH, GOT and GPT were determined by routine clinical chemistry.

2.3. Statistics

Each experiment was performed with 3–5 livers using slices in triplo from each liver. Results were compared using two-tailed unpaired Student's *t*-test. A *P*-value <0.05 was considered significant.

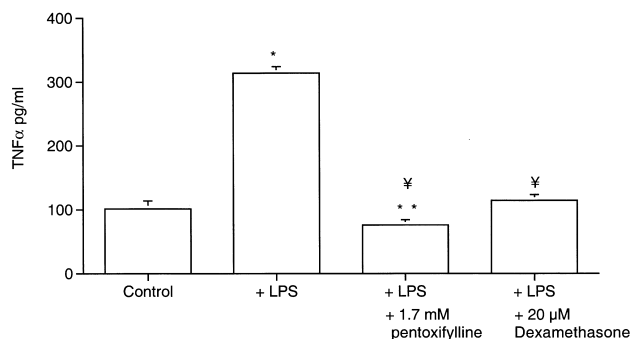


Fig. 1. Production of TNF α by liver slices in pg/ml in culture medium after 24 h of stimulation with 100 μ g/ml LPS alone and in the presence of pentoxifylline and dexamethasone. Data are the mean of 3–5 experiments \pm SEM. * P < 0.005 vs. control. ** P < 0.05 vs. in the presence of dexamethasone. ‡ P < 0.005 vs. +100 μ g/ml LPS.

3. Results

3.1. Release of cytokines

TNF α , IL-1 β and IL-10 concentration in the culture medium after 24 h with 100 μ g/ml LPS, were significantly increased compared to control incubations (Figs. 1–3). Pentoxifylline and dexamethasone significantly decreased the TNF α and IL-1 β release induced by endotoxin (Figs. 1 and 2).

3.2. iNOS induction

Incubation with different concentrations of LPS revealed that NO production, as measured by nitrite and nitrate, is dependent on LPS concentration (Fig. 4). To assess the different phenomena of LPS stimulation the highest concentration of LPS (100 μ g/ml) was used in all experiments. NO $_x$ concentration in the medium was significantly elevated after incubation for 5 h with 100 μ g/ml LPS versus control and increased further during 24 h of incubation (Fig. 5).

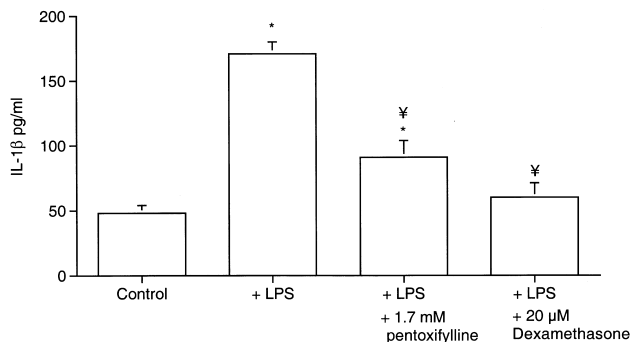


Fig. 2. Production of IL-1 β by liver slices in pg/ml in culture medium after 24 h of stimulation of with 100 μ g/ml LPS alone and in the presence of pentoxifylline and dexamethasone. Data are the mean of 3–5 experiments \pm SEM. * P < 0.005 vs. control. ‡ P < 0.005 vs. +100 μ g/ml LPS.

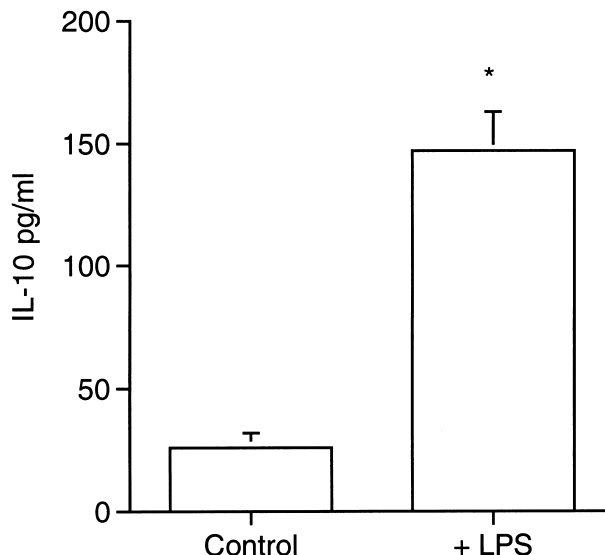


Fig. 3. Production of IL-10 by liver slices in pg/ml in culture medium after 24 h of stimulation with 100 μ g/ml LPS. * P < 0.005 vs. control.

iNOS mRNA expression in slices stimulated with LPS was up-regulated after 3 h of incubation versus control. iNOS mRNA expression was elevated up to 24 h in rat liver slices stimulated with LPS (Fig. 6). In the control liver slices iNOS mRNA expression was visible after 9 h (Fig. 6). The housekeeping gene GAPDH remained constant in control and LPS stimulated slices during 24 h of incubation (Fig. 6).

In control incubations, almost no iNOS was found immunohistochemically after 24 h treatment with LPS. The intensity of immunohistochemical staining of iNOS in liver slices

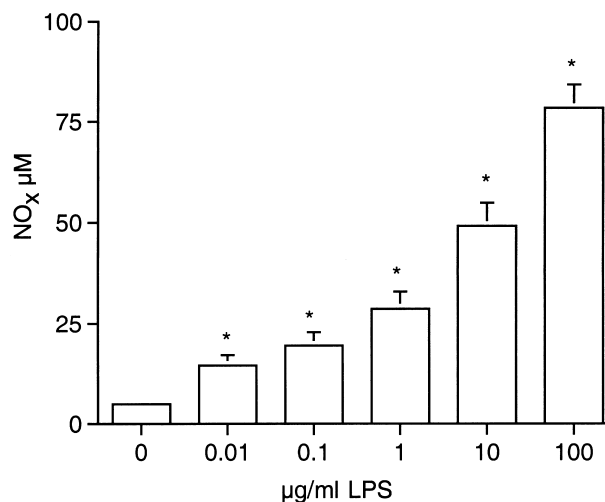


Fig. 4. NO production of rat liver slices incubated with concentrations from 0–100 μ g/ml LPS after 24 h of incubation. The NO production is measured as nitrate/nitrite (NO $_x$) concentrations in medium (μ M). Data are expressed as mean \pm SEM of six experiments. * P < 0.005 represents a significant increase of NO production by liver slices compared to the lower concentrations of LPS.

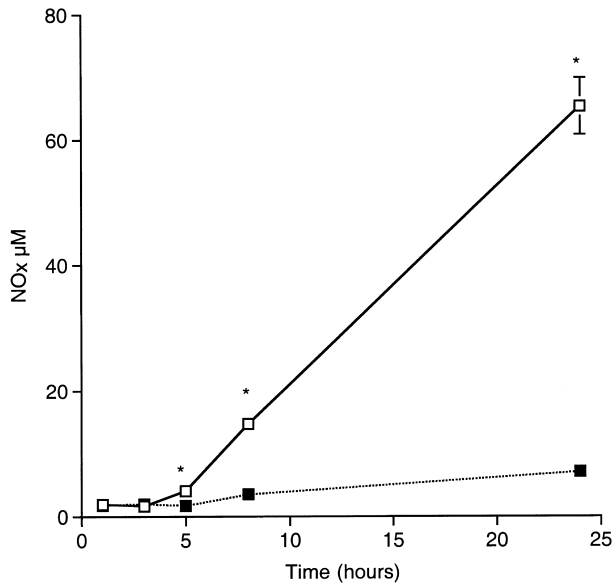


Fig. 5. NO production of rat liver slices during incubation with and without 100 µg/ml LPS. The NO production is measured as nitrate/nitrite (NO_x) concentrations in medium (µM). Control (□) and +100 µg/ml LPS (■). Data are expressed as mean ± SEM of four experiments. * $P < 0.005$ represents a significant increase of NO production by liver slices due to stimulation by LPS.

induced by LPS followed the same time course as the rise in NO_x concentration in the culture medium (data not shown); iNOS was clearly detected in the liver slices after 5 h incubation with LPS and increased further until 24 h. The staining of iNOS in liver slices after incubation with LPS (Fig. 7) showed a scattered pattern and was distributed throughout the liver slice with no signs of acinar heterogeneity. Staining was predominantly detected in the hepatocytes. Staining of Kupffer cells was also found. The hepatocytes that were stained had a weak cytoplasmatic staining and a strong chicken wire-like membranous staining.

The iNOS activity was determined after 24 h of LPS exposure (± 100 µg/ml) in the crude lysate of the liver slices by ³H-L-citrulline formation from ³H-arginine. iNOS activity was 9-fold higher in the LPS-stimulated slices than in

control slices (25 dpm/mg wet weight vs. 227 dpm/mg wet weight respectively).

NO production of LPS-stimulated liver slices measured after 24 h was significantly reduced by the NO synthase inhibitors SMT and L-NAME (Fig. 8). SMT and L-NAME did not influence the immunohistochemical staining for iNOS after LPS induction (data not shown).

Pentoxifylline decreased LPS-induced NO production to about 70% whereas dexamethasone inhibited NO production completely (Fig. 8). The staining for iNOS after endotoxin stimulation was attenuated in the presence of pentoxifylline and absent in the presence of dexamethasone (data not shown).

3.3. Viability parameters

In the presence of LPS, the levels of LDH, GOT and GPT in the slice medium were somewhat increased during 24 h (Fig. 9) compared to control incubation. Addition of SMT, L-NAME or dexamethasone with endotoxin did not change LDH, GOT or GPT leakage during 24 h (Fig. 10). However, pentoxifylline significantly decreased LDH and GOT leakage after LPS induction, GPT leakage was also attenuated, but the difference was not statistically significant ($P = 0.059$) (Fig. 10).

4. Discussion

4.1. Release of cytokines

LPS stimulation causes a release of cytokines by liver slices. These cytokines are measured in the medium and the concentration found is probably an underestimation of the amount released, because the cytokines are produced *in* the slice and they will consequently and specifically bind to receptors on the hepatocyte, internalized and degraded and only partly released in the medium [22,23]. After LPS stimulation of the liver, Kupffer cells are the main producers of TNFα, IL-1β and IL-10 [24]. Whereas, the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10 are almost exclusively produced (>95%) by Kupffer

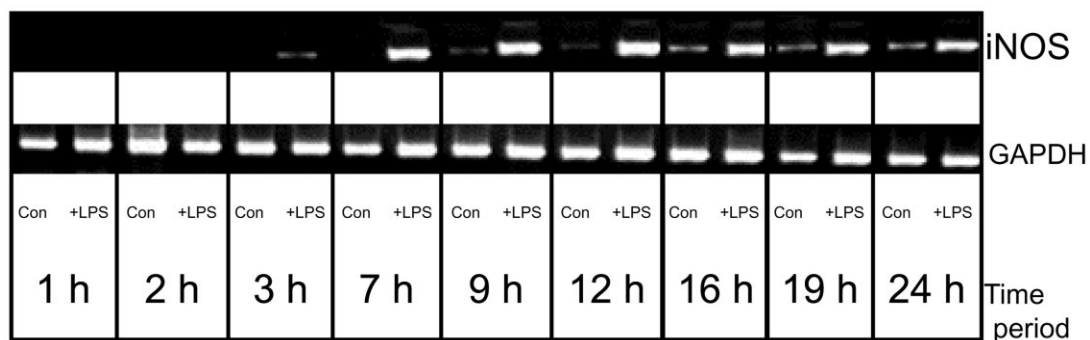


Fig. 6. iNOS and GAPDH mRNA expression in rat liver slices during incubation with and without 100 µg/ml LPS. mRNA levels were determined by RT-PCR as described in Materials and methods. Representative experiment of $n = 4$ per time point.

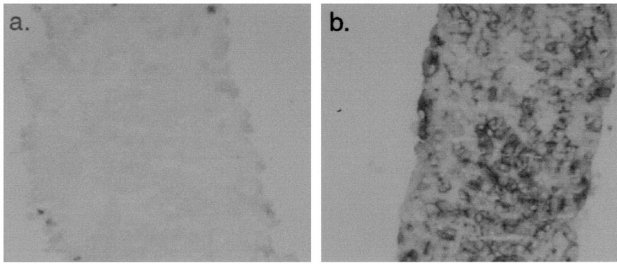


Fig. 7. Immunohistochemistry with α iNOS. (a) Control liver slice after incubation for 24 h; (b) Liver slice after incubation for 24 h with 100 μ g/ml LPS.

cells [17]. TNF α can also be produced by other cell types in the liver such as hepatocytes and endothelial cells [17]. In our experiments, TNF α , IL-1 β and IL-10 are abundantly detected in the incubation medium of liver slices upon LPS stimulation indicating that the Kupffer cells in the slice are still viable. These results are in line with those of Luster et al. [15] who found that also in human and mouse liver slices Kupffer cells are viable and respond to a LPS stimulus with a cytokine release. These results are also in line with our previous studies in which we have shown that modified proteins, which are specifically taken up via receptor mediated endocytosis by Kupffer or endothelial cells, accumulate in the incubated liver slices [25]. In addition, we found that dexamethasone coupled to human serum albumin, which is predominantly taken up by non-parenchymal cells, effectively inhibited LPS-induced TNF α production [26]. We infer from these data that Kupffer cells, and also other non-parenchymal cells, are still active and viable after preparation and incubation of rat liver slices and can be activated to produce cytokines.

4.2. iNOS induction

NO production was significantly increased in liver slices in the presence of LPS already after 5 h. Since iNOS expression, as determined immunohistochemically, paralleled this

increase in NO production it is likely that this is due to iNOS induction. This increased expression of iNOS protein is preceded by increased mRNA expression of iNOS in the liver slices stimulated with LPS which was already increased after 3 h. iNOS mRNA expression was elevated up to 24 h, indicating that induction of iNOS mRNA was continuously triggered by the inflammatory response of the LPS stimulus. In the control slices also some iNOS mRNA expression could be found. As it is almost impossible to work in LPS free environment, a low amount of LPS that is always present may have triggered an inflammatory response leading to iNOS mRNA expression.

A pattern of scattered positive hepatocytes with chicken-wire iNOS staining was found in the liver slices. Staining of some Kupffer cells was also found. This pattern suggests staining of bile canaliculi of the hepatocytes and possibly of the plasma membranes, as reported in previous studies [2]. This pattern excludes mainly Kupffer and endothelial associated staining. The NO production and iNOS protein expression steadily increased up to 24 h. Moreover, after 24 h, the differences in activity of iNOS in homogenates of LPS-stimulated slices versus control slices, determined by the production of 3 H-L-citrulline, showed that the induced enzyme iNOS was still active. The NOS inhibitors SMT and L-NAME both inhibited this NO production. These results show that SMT and L-NAME can enter the slice and can inhibit LPS-induced NO production as has been previously described in vivo [2]. This occurred without down-regulation of the expression of iNOS, as was confirmed by immunohistochemistry. In contrast, pentoxifylline and dexamethasone down-regulated the iNOS expression.

In contrast to liver slices, addition of LPS alone to hepatocytes in pure cultures did not induce iNOS expression [6–8]. Only an incubation with LPS together with a mix of cytokines is reported to increase iNOS expression in hepatocytes [6–8] and consequently induces NO production. The initial time course (up to 8 h) of iNOS protein and mRNA expression and NO production found in liver slices stimulated with LPS alone is quite similar to what was observed

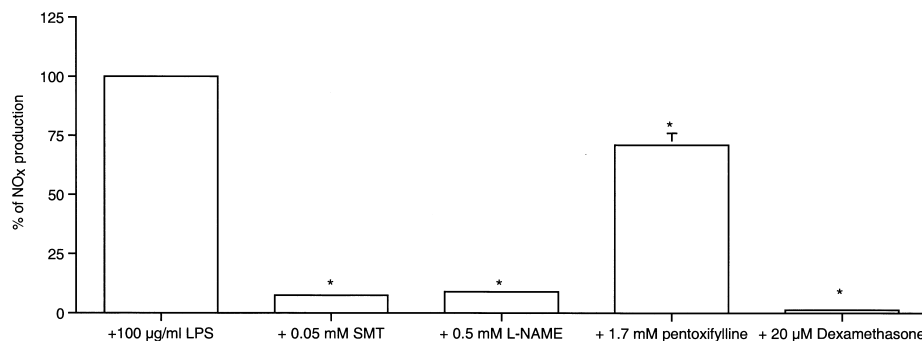


Fig. 8. NO_x production after 24 h of culture of rat liver slices with 100 μ g/ml LPS in the presence of SMT, L-NAME, pentoxifylline or dexamethasone vs. rat liver slices incubated with 100 μ g/ml LPS alone. Data are expressed as % of NO_x production of liver slices incubated with 100 μ g/ml LPS for 24 h. The concentration values of the 100% NO_x production range between 75–200 μ M. Data are the mean of 3–5 experiments \pm SEM. * P < 0.005 vs. +100 μ g/ml LPS.

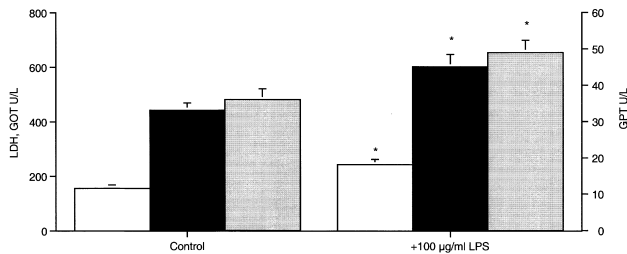


Fig. 9. Viability of liver slices measured as leakage of GOT, LDH and GPT. GOT (□), LDH (■) (in U/L, on the left axis) and GPT (▨) (in U/L, on the right axis) after 24 h culture of rat liver slices with 100 µg/ml LPS. Data are the mean of 6 experiments ± SEM. * $P < 0.005$ vs. control.

in cultures of hepatocytes stimulated with LPS and a mix of cytokines [27] as well as in vivo studies in the rat [2,28]. Nussler et al. showed that with 10 mg/ml LPS and a mix of cytokines, cultured hepatocytes produce 363 nmol $\text{NO}_x/10^6$ hepatocytes during 24 h, this is in line with the results obtained with our liver slices; slices incubated with 100 µg/ml of LPS produced 256 nmol $\text{NO}_x/10^6$ hepatocytes NO within 24 h (assuming that 1 g of liver contains 100 million hepatocytes [11]). The liver slices stimulated with LPS produce an adequate amount of cytokines which resulted in a similar induction of iNOS as was found in hepatocytes stimulated with LPS and a cytokine mix. Therefore, this preparation seems to be an appropriate in vitro liver system that can mimic the cytokine release of the liver in vivo. Moreover, it can be concluded that the hepatocytes in the slice retain the ability to respond to cytokines with induction of gene expression.

Pentoxifylline, a phosphodiesterase inhibitor, was previously shown to inhibit $\text{TNF}\alpha$ synthesis induced by LPS in cultured macrophages, as well as in vivo in human volunteers and laboratory animals [18]. In the present study we show that pentoxifylline is capable of reducing the release of $\text{TNF}\alpha$ and IL-1 β in LPS-stimulated liver slices. Dexamethasone also reduced the release of both cytokines, but it reduced IL-1 β to a greater extend than pentoxifylline

did. Interestingly, NO production in the presence of pentoxifylline was not decreased to control levels indicating that the concentration of IL-1 β in the presence of LPS is sufficiently elevated to induce iNOS. These results are in line with results reported by Kitade et al. [29]. They showed in rat hepatocytes that IL-1 β is mainly responsible for iNOS induction: stimulation with LPS and $\text{TNF}\alpha$ raised NO_x production only slightly whereas addition of IL-1 β increased NO_x production considerably. The absence of NO_x production in dexamethasone-treated liver slices stimulated with LPS is in line with previously reported results [19], showing that dexamethasone inhibits the expression of cytokine genes as well as the iNOS gene, due to inhibition of NF- κ B activation [19]. In addition, Melgert et al. [26] showed that dexamethasone both free and coupled to human serum albumin is effectively inhibiting LPS-induced NO_x and $\text{TNF}\alpha$ production in precision-cut liver slices.

4.3. Viability parameters

The LDH levels found in the medium of the control slices are in agreement with data presented in a previously performed investigation [12]. The culture medium used in previously published reports contained fetal calf serum and insulin, however, in the present study these additions were omitted to avoid interference of these compounds with the LPS effect. These results imply that fetal calf serum and insulin are not necessary to maintain the viability of the liver slice during 24 h of culture [12]. Pilot experiments showed that ATP-levels in liver slices remain constant during at least 48 h (manuscript in preparation).

Stimulation of liver slices with LPS leads to moderate tissue damage as was established by the elevated levels of LDH, GOT and GPT. This was also found in cultured hepatocytes stimulated with LPS and a mix of cytokines [30]. Stadler et al. [31] postulated that liver injury by LPS is mediated by $\text{TNF}\alpha$. Liang et al. [30] however, argue that liver damage is mediated by IFN γ . In our experiments, pentoxifylline decreased $\text{TNF}\alpha$ production after LPS treatment, and also reduced LDH, GPT and GOT leakage.

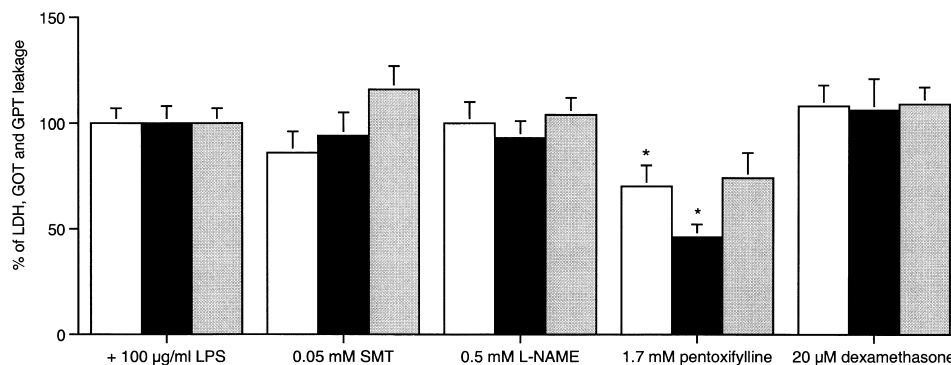


Fig. 10. Viability of liver slices measured after 24 h of incubation with 100 µg/ml LPS in the presence of SMT, L-NAME, pentoxifylline and dexamethasone. Data are expressed as percentage of GOT (□), LDH (■) and GPT (▨) leakage after 24 h incubation of rat liver slices with 100 µg/ml LPS. Data are the mean of 3–5 experiments ± SEM. * $P < 0.05$ vs. with 100 µg/ml LPS.

However, dexamethasone also decreased TNF α production, albeit to a lesser extent than pentoxifylline but had no effect on leakage of LDH, GPT and GOT. To establish whether LPS-induced injury in the liver is primarily mediated by TNF α , more experiments, e.g. with anti-TNF α antibodies, should be performed. We also conclude that the damage as indicated by LDH and GOT release is not related to NO production because SMT and L-NAME did not influence these parameters. This is in agreement with the results found in cultured hepatocytes [30]. Direct hepatotoxic effects of NO have only been found in vivo, which were related to bloodflow, e.g. with hemorrhagic shock [6].

5. Conclusion

Our experiments provide evidence that the induction of iNOS in hepatocytes by LPS is caused by the cytokines produced by Kupffer cells. They confirm the observation that iNOS induction in cultured hepatocytes can only be achieved by the combination of cytokines and LPS and not by LPS alone. We conclude that the complex cell-cell interactions after an inflammatory stimulus are retained in the slice and show that the liver slice model may be used to elucidate mechanisms of inflammation. One example is that LPS causes cholestasis and a down-regulation of transporters such as the Na⁺-taurocholate transporter (ntcp), the multidrug resistance protein 2 (mrp2) in vivo in the rat, these same phenomena are found with rat liver slices stimulated with LPS [32]. In addition, we found that human liver slices can produce acute phase proteins during liver inflammation [33]. Another example is the involvement of the CD14 receptor on Kupffer cells in the uptake of LPS, which is currently under study in liver slices (manuscript in preparation). Studies on LPS uptake by CD14 receptor in the liver is hampered by the fact that the CD14 receptor disappears from the Kupffer cells during the isolation procedure [34]. Therefore, liver slices seem the ideal in vitro system to study these phenomena, because no enzymatic digestion is necessary to prepare liver slices. Furthermore, liver slices can be used to study the effects of anti-inflammatory agents and to elucidate their mechanism of action. Moreover, if human liver slices were to be used this would provide the unique opportunity to study these aspects of inflammation in man. Preliminary studies with human liver slices in our laboratory also indicate LPS-induced cytokine release (manuscript in preparation). Therefore, liver slices can be employed to study potential anti-inflammatory agents designed for therapy of human liver diseases.

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