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Differential effects of pentoxifylline on the hepatic inflammatory response in porcine liver cell cultures Increase in inducible nitric oxide synthase expression

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Abstract

Pentoxifylline (PTX) has been shown to exert hepatoprotective effects in various liver injury models. However, little information is available about the effect of PTX on the hepatic acute phase response. In the present study, the effect of PTX on a lipopolysaccharide (LPS)-induced acute phase response in primary porcine liver cell cultures was examined. During 72 hr of incubation with or without LPS, the ability of PTX to influence the secretion of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), acute phase proteins, and nitric oxide (NO) was assessed. PTX completely inhibited LPS-induced TNF-α production and attenuated IL-6 only after 48 hr of incubation. In contrast, PTX potentiated NO production and the expression of inducible nitric oxide synthase (iNOS) in hepatocytes after stimulation with LPS. The increased expression of iNOS and concurrent production of NO was also observed when liver cell cultures were incubated with dibutyryl cyclic adenosine monophosphate. No effect of PTX on acute phase protein secretion was observed during 72 hr of incubation. The present results show that PTX differentially affects the endotoxin-induced inflammatory response in primary porcine liver cell cultures by suppressing TNF-α and IL-6 while potentiating NO production.

Keywords: Pentoxifylline; Cytokines; Inducible nitric oxide synthase; Cyclic adenosine monophosphate; Acute phase proteins; Hepatocytes

1. Introduction

The acute phase response is a host defense reaction initiated by various immunological and non-immunological events [1]. Its major symptoms are fever, leukocytosis, synthesis, and release of hepatic acute phase proteins. It is well known that proinflammatory cytokines released by monocytes or tissue macrophages play an important role in the regulation and control of the acute phase response [2,3].

In the liver, Kupffer cells represent the majority of fixed macrophages in the body. They are important cell types involved in the production of proinflammatory cytokines, reactive oxygen, or nitrogen species, and are involved in the regulation of hepatocyte functions [4–6]. In particular, TNF-α, IL-1, and IL-6 are responsible for the regulation of acute phase protein production, including that of α_1-acid glycoprotein, C-reactive protein, haptoglobin, and albumin [3,7,8]. However, high exposure of Kupffer cells to endotoxins can lead to high secretion of inflammatory mediators and ultimately to endotoxin-induced liver injury.

Different pharmacological compounds have been tested to reduce acute or chronic inflammatory responses, which may damage liver function. It was demonstrated in previous studies that phosphodiesterase inhibitors are protective in

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Abbreviations: TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; PTX, pentoxifylline; LPS, lipopolysaccharide; cAMP, cyclic adenosine monophosphate; pig MAP/ITIH4, pig major acute phase protein/inter-alpha-trypsin inhibitor heavy chain; iNOS, inducible nitric oxide synthase; NO, nitric oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and IgG, immunoglobulin G.

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various liver injury models [9,10]. PTX has been shown to exert a hepatoprotective effect by improving hepatic blood flow, inhibiting TNF-α production, and preventing LPS-induced down-regulation of cytochrome P450 enzyme activities [11–13]. Furthermore, antifibrotic effects of PTX have been described in an experimental model of hepatic fibrosis [14]. It is generally believed that PTX exerts its pharmacological mechanism of action by inhibiting phosphodiesterases, which leads to increased intracellular levels of cAMP [15,16]. Although PTX has been shown to inhibit TNF-α production by macrophages, little information is available about the effect of PTX on the hepatic acute phase response. The present study was designed to examine the effect of PTX on the hepatic inflammatory response. Therefore, an *in vitro* system was developed using crude porcine liver cells. Within this system, liver cells were exposed to LPS in the presence or absence of PTX. At various time points, the secretion of TNF-α, IL-6, both positive (pig MAP/ITIH4) and negative (albumin) acute phase proteins, and the expression of iNOS and concomitant production of NO were measured.

2. Materials and methods

2.1. Chemicals and drugs

Powdered Williams' medium E, glutamine, gentamicin, LPS (*Escherichia coli*, O111:B4), PTX, dibutyryl cAMP, dithiothreitol, EDTA, tergitol, MTT, alkaline phosphatase-conjugated goat anti-rabbit IgG, porcine albumin, and diethanol-amine were obtained from Sigma. Myoclone super plus fetal bovine serum (endotoxin less than 10 EU/mL) was obtained from Life Technologies. Recombinant porcine TNF-α, recombinant porcine IL-6, monoclonal antibody (mAb) anti-porcine TNF-α (clone 9B4, mouse isotype IgG1), and a porcine TNF-α ELISA were obtained from Endogen. Nutrilon premium blocking solution was purchased via Nutricia. Rabbit anti-iNOS was obtained from Cayman Chemical Co. Rabbit anti-human albumin polyclonal antibody, rabbit anti-mouse IgG/horseradish peroxidase, and peroxidase-conjugated goat anti-mouse were obtained from DAKO. Monoclonal antibodies raised against porcine macrophages (CVI-SWNL 517.2) were kindly provided by Dr. J.M.A. Pol (ID-DLO).

2.2. Origin and isolation of porcine liver cells

Liver cells were isolated from livers of 3 healthy castrated male pigs (Great Yorkshire $\times$ Dutch landrace), aged approximately 12 weeks and weighing between 28 and 35 kg. The animals were obtained from the University's breeding farm. The procedure for porcine liver cell isolation was based on Seglen's method [17] and described in detail by Monshouwer *et al*. [18]. The resultant cell suspension was washed three times with Williams' medium E (centrifuged at 100 *g*) without further purification and diluted to a final concentration of 1×10^6 viable cells/mL as assessed by trypan blue dye exclusion.

2.3. Cell cultures and incubations

Cells were cultured at a density of $2 * 10^6$ cells/well in 6-well macroplate culture dishes (Greiner) using Williams' Medium E, supplemented with 5% (v/v) fetal bovine serum, glutamine (1.67 mM), and gentamycin (50 μg/mL). After a 20-hr incubation at 37° and 5% CO_2, the medium was replaced with serum-free medium containing 0, 1, or 10 μg/mL of LPS with or without 1 mM PTX. After incubation periods of 1, 2, 4, 8, 24, 48, and 72 hr, the supernatants were harvested and stored at $-70°$ until used for analysis. Adherent cell monolayers were incubated with 2 mL tergitol buffer (pH 7.4, 125 mM PBS containing dithiothreitol, EDTA, 0.5% (v/v) tergitol, and 20% (v/v) glycerol). Cells were scraped from the plates and the total protein concentration was determined by Lowry's method.

2.4. 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*. [19]. TNF-α-induced cytotoxicity in PK-15 cells was determined using MTT. Prior to the experiments, the PK-15 bioassay was extensively validated by comparing it with a specific ELISA for porcine TNF-α (results not shown). As a positive control, threefold dilutions of recombinant porcine TNF-α were used and absorbance was measured at 590 nm. TNF-α in supernatants was quantified by comparing the calculated EC_{50} values from supernatants with those from the recombinant porcine TNF-α standard curve. Experiments were performed in duplicate for each sample from cells isolated from each pig.

2.5. 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*. [20]. IL-6-induced proliferation of B9 cells was determined using MTT. Samples were titrated in threefold dilutions, with threefold dilutions of recombinant porcine IL-6 being used as a positive control. Absorbance was then measured at 590 nm. IL-6 in tissue culture supernatants was quantified by comparing the 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.

2.6. Pig MAP/ITIH4 ELISA

Pig MAP/ITIH4 in liver cell culture medium was measured by using a double antibody sandwich ELISA as de-

scribed by Gonzalez-Ramon *et al*. [21]. The absorbance was measured at 405 nm and a quantified acute phase pig serum was used as pig MAP/ITIH4 standard solution. Samples were measured in duplicate.

2.7. Albumin ELISA

Albumin production was determined by using an antibody capture ELISA. Samples were diluted threefold ranging from 1:100 to 1:12,500 and added in duplicate, 100 μL/well, into high-binding capacity ELISA plates (Greiner) and incubated overnight at 4°. After washing once with PBS containing 0.05% Tween 20, 200 μL 5% Nutrilon premium blocking solution in PBS was added to each well and plates were incubated for another 4 hr at room temperature. Plates were then washed three times with PBS/Tween and incubated with 100 μL/well of rabbit anti-human albumin (0.5 μg/mL), which was highly cross-reactive with porcine albumin. After a 1-hr incubation, plates were washed again and incubated with 100 μL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 hr. The plates were again washed three times with PBS/Tween, and once with 1.0 M diethanol-amine buffer containing 10 mM $MgCl_2$ (pH 9.6). Finally, 100 μL of substrate buffer (1 mg/mL of 4-nitrophenylphosphate [Acros] in diethanol buffer) was added to the wells and, after a 30-min incubation at room temperature, the reaction was stopped by adding 50 μL 10% EDTA in H_2O. Absorbance was measured at 405 nm, and calculation of porcine albumin concentrations in samples was carried out by extrapolation from porcine albumin standard performed on each plate.

2.8. Nitric oxide measurement

After 8, 24, 48, and 72 hr of incubation with or without LPS, samples were collected and stored at −70° until analysis. NO production was determined by measuring the amount of NO_2^- in culture supernatants according to the Griess reaction [22].

2.9. Immunohistochemical analysis

Cultures of primary isolated liver cells were plated on sterile No. 1 coverslips placed in 24-well dishes (Greiner). To clarify which cell type was responsible for NO production, a double antibody staining anti-iNOS and anti-porcine macrophage [23] was performed on liver cell cultures after 48 hr 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 the secondary antibody pig anti-rabbit alkaline phosphatase (AP) for 30 min. After washing three times with PBS (1% BSA and 0.5% saponin), cells were incubated with rabbit anti-mouse peroxidase for 30 min. Horseradish peroxidase and AP staining were performed consecutively, and cultures were analyzed by using light microscopy.

2.10. Western blot analysis for iNOS

After 48 hr of incubation with or without LPS in combination with PTX or dibutyryl cAMP, Western blot analysis of iNOS expression in liver cell cultures was performed. After washing the cells once in PBS, 8×10^6 liver cells were collected by cell scraping for preparation of total cell protein. Cells were centrifuged at 200 *g* and resuspended in 200 μL PBS. After homogenization by repeated freezing (−180°) and thawing (37°), samples were centrifuged at 13,000 rpm for 2 min in an Eppendorf centrifuge. Supernatants were collected and the protein contents determined using Lowry's method [24]. Total protein (10 μg) was loaded upon an SDS/polyacrylamide (8%) gel and blotted onto 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% (w/v) BSA and 0.3% (v/v) Tween 20. The primary antibody rabbit anti-mouse iNOS, which showed high cross-reactivity with porcine iNOS, was diluted 1:1000 in Tris-buffered saline (1% BSA, 0.3% Tween 20), and blots were incubated for 1 hr at room temperature. After washing 6 times with Tris-buffered saline for 5 min, the secondary antibody (pig anti-rabbit alkaline phosphatase), diluted 1:5000 in Tris-buffered saline (1% BSA and 0.3% Tween 20), was added. Blots were incubated for 1 hr and, after washing 6 times, were stained using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.11. Intracellular cAMP measurement

Cells were incubated with medium with or without 1 mM PTX. After a 30- or 90-min incubation at 37°, the culture medium was aspirated and cells were immediately placed on ice. Ice-cold 70% (v/v) ethanol was added (1 mL/well) to extract cAMP from the cells. Subsequently, the samples were transferred to tubes and ethanol was evaporated under a constant stream of N_2 at 45°. The residues in the tubes were dissolved in PBS, and cAMP was determined by ELISA using a commercially available cAMP assay kit (Cayman Chemical). Data are expressed as fmol cAMP per million cells.

2.12. Statistics

Unless stated otherwise, data are expressed as means ± SEM and were evaluated using a two-way ANOVA followed by a Dunnett test for comparison between two groups. A probability value $P < 0.01$ or $P < 0.05$ was considered statistically significant.

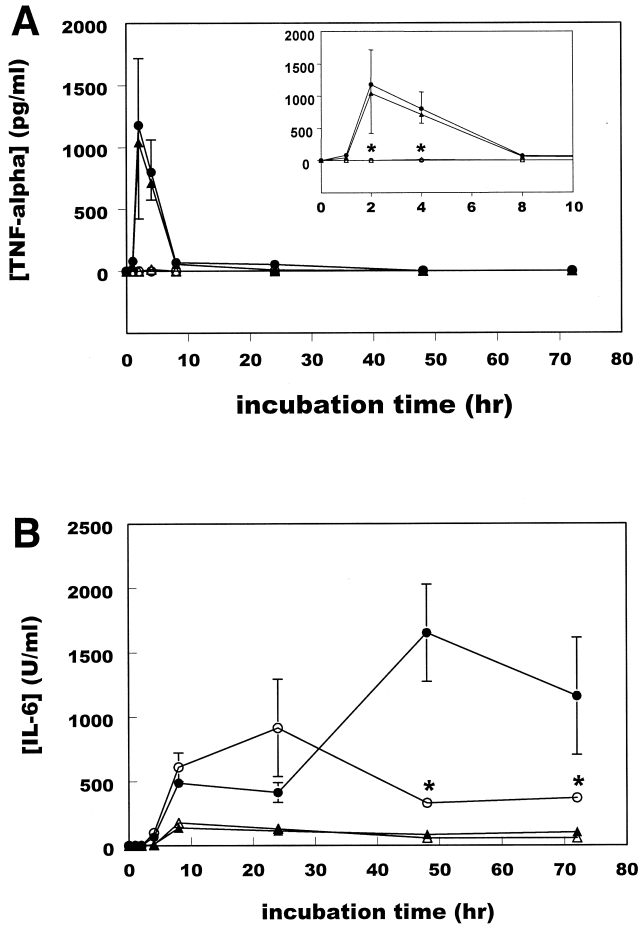

Fig. 1. TNF-α (A) and IL-6 (B) concentrations in tissue culture supernatant after exposure of primary liver cells to LPS. Cultures were untreated (not shown) or treated with either 1 or 10 μg/mL of LPS in the absence or presence of PTX. The mean values $\pm$ SEM of three separate experiments in duplicate are shown. (-▲- 1 μg/mL of LPS, -●- 10 μg/mL of LPS, -△- 1 μg/mL of LPS + PTX, -○- 10 μg/mL of LPS + PTX) *: Significant decrease in LPS-induced TNF-α or IL-6 production by PTX as compared to incubations with LPS alone ($P \leq 0.01$).

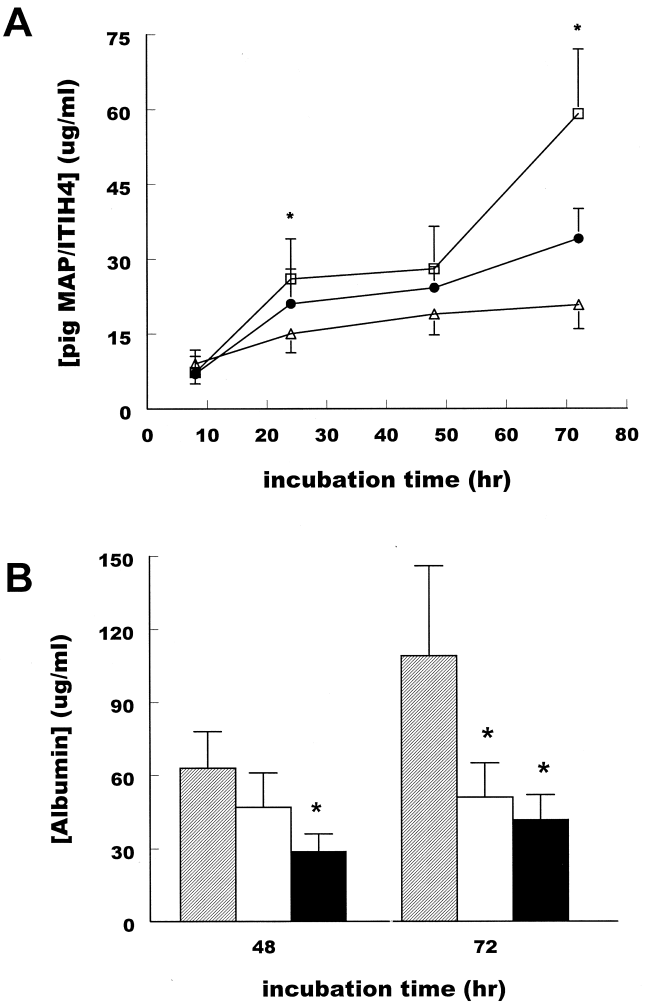

Fig. 2. Acute phase protein production by porcine liver cell cultures after stimulation with various doses of LPS. (A) Release of pig MAP/ITIH4 in tissue culture supernatant by liver cell cultures during a 72-hr time-course. Cells were non-treated (-△-) or treated with either 1 (-●-) or 10 μg/mL of LPS (-□-). (B) Concentration of albumin in tissue culture supernatant after 48 and 72 hr of incubation with or without LPS (▨ = control, □ = 1 μg/mL of LPS, ■ = 10 μg/mL of LPS). Values represent mean values $\pm$ SEM of three separate experiments in duplicate. *: Significant increase/decrease in acute phase protein production compared with control incubations ($P \leq 0.01$).

3. Results

3.1. Effects of PTX on cytokine production

The effects of PTX on cytokine production by primary pig liver cells are shown in Fig. 1. During the control incubation (no LPS) with or without PTX, no detectable TNF-α or IL-6 levels could be measured (results not shown). Stimulation of liver cells with 1 or 10 μg/mL of LPS resulted in maximal TNF-α peak concentrations of $\pm$1100 pg/mL for both concentrations (Fig. 1A). At 8 hr, TNF-α concentrations were already decreased and after 48 hr of incubation with LPS, no TNF-α secretion could be determined as measured by the PK-15 bioassay. PTX completely abolished TNF-α production in liver cell cultures after incubation with LPS (Fig. 1A).

IL-6 production in the liver cell cultures was observed after 8 hr of incubation and, when compared to TNF-α, showed a different kinetic pattern. Addition of LPS to these cultures resulted in a dose-dependent IL-6 production (Fig. 1B). Eight hours after exposure to 10 μg/mL of LPS, IL-6 levels reached a first plateau followed by a more pronounced second peak at 48 hr (Fig. 1B). PTX did not affect or even elevate IL-6 levels in the first phase, but did significantly lower IL-6 production after 48 hr of incubation with LPS (Fig. 1B).

3.2. Effects of PTX on acute phase protein production

To determine the effect of PTX on acute phase protein production at various time points, concentrations of pig MAP/ITIH4 and albumin were measured during the control

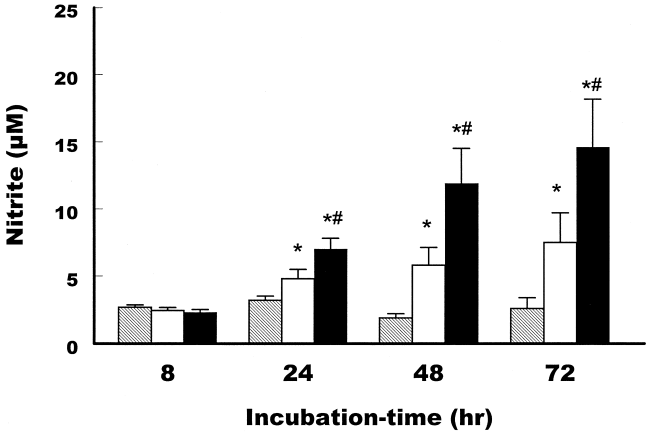

Fig. 3. NO production after 8, 24, 48, and 72 hr of control incubation (▨) or after incubation with 1 (□) or 10 µg/mL (■) of LPS. NO secretion was determined by measuring the amount of NO_2^- present in tissue culture supernatant according to the Griess reaction. Values represent mean values ± SEM of three separate experiments in duplicate. *,#: Significant increase in NO_2^- in tissue culture supernatant compared with control incubations (*) or incubations with 1 µg/mL of LPS (#) ($P \leq 0.05$).

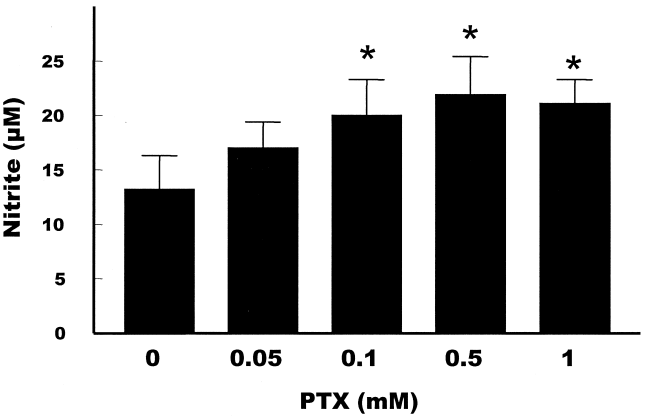

Fig. 5. Dose–response effect of PTX on LPS-induced NO production after 48 hr of incubation. Liver cell cultures were incubated with 10 µg/mL of LPS and various concentrations of PTX. NO secretion was determined by measuring the amount of NO_2^- in tissue culture supernatant using the Griess reaction. Values represent mean values ± SEM of three separate experiments in duplicate. *: Significant increase in NO_2^- in tissue culture supernatant compared with LPS incubations in the absence of PTX ($P \leq 0.01$).

incubation or upon stimulation with LPS (Fig. 2, A and B). Fig. 2A shows concentration–time curves of pig MAP/ITIH4 in culture supernatants. During control incubation (without LPS or PTX), low but significant levels of pig MAP/ITIH4 were measured in tissue culture supernatants. After 24 hr of incubation with 10 µg/mL of LPS, pig MAP/ITIH4 levels were significantly increased compared to the control group or after stimulation with 1 µg/mL of LPS.

Albumin levels were measured at 8, 24, 48, and 72 hr of incubation with or without PTX and/or LPS. Albumin levels were only significantly decreased after 48 and 72 hr of incubation with LPS (Fig. 2B). After 72 hr of incubation, albumin concentrations in tissue culture supernatants were decreased by 53% and 62% for incubation with 1 and 10 µg/mL of LPS, respectively (Fig. 2B). PTX had no effect on the production of either pig MAP/ITIH4 or albumin protein levels at 8, 24, 48, or 72 hr of incubation with or without LPS.

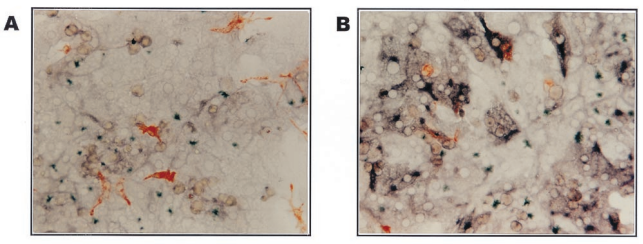

Fig. 4. Light microscopic observations of immunohistochemical double antibody staining for Kupffer cells (red stain) and iNOS (blue stain) in liver cell cultures after 48 hr of control incubations (A) or after incubation with 10 µg/mL of LPS (B) (magnification 600×). Cells were fixated with paraformaldehyde and used for double antibody staining as described in the Materials and Methods section.

3.3. Measurement of NO_2^- and iNOS expression

LPS induced a dose-dependent production of NO after 24 hr of incubation with LPS (Fig. 3). The LPS-induced NO response was most pronounced at 72 hr of incubation. In the absence of LPS, no significant levels of NO_2^- in tissue culture supernatants were detected. After 48 hr of incubation with or without LPS, the cultures were examined immunohistochemically by staining with a specific monoclonal antibody for porcine macrophages (red staining) and a polyclonal rabbit anti-mouse iNOS (blue staining). Fig. 4 shows a representative picture of liver cell cultures after 48 hr of control incubation (panel A) and after 48 hr of LPS incubation (panel B). The percentage of Kupffer cells present in the liver cell cultures was approximately 5%. After exposure of the cell cultures to LPS, an increased staining for iNOS was observed. In addition, these results showed that the hepatocytes were positively stained for iNOS.

3.4. Effect of PTX on NO_2^- production and iNOS expression

Incubation of the liver cell cultures with LPS (10 µg/mL) and various concentrations of PTX resulted in a significant increase in NO production in liver cell cultures (Fig. 5) as compared to LPS incubations alone. PTX significantly increased NO production at 24, 48, and 72 hr of incubation. Only the results at 48 hr of incubation are shown (Figs. 5 and 6). The potentiating effect of PTX on NO production could be mimicked when cells were incubated with 500 µM dibutyryl cAMP (Fig. 6A). Western blot analysis of iNOS expression in liver cell cultures showed an increased expression of iNOS when cells were stimulated with LPS.

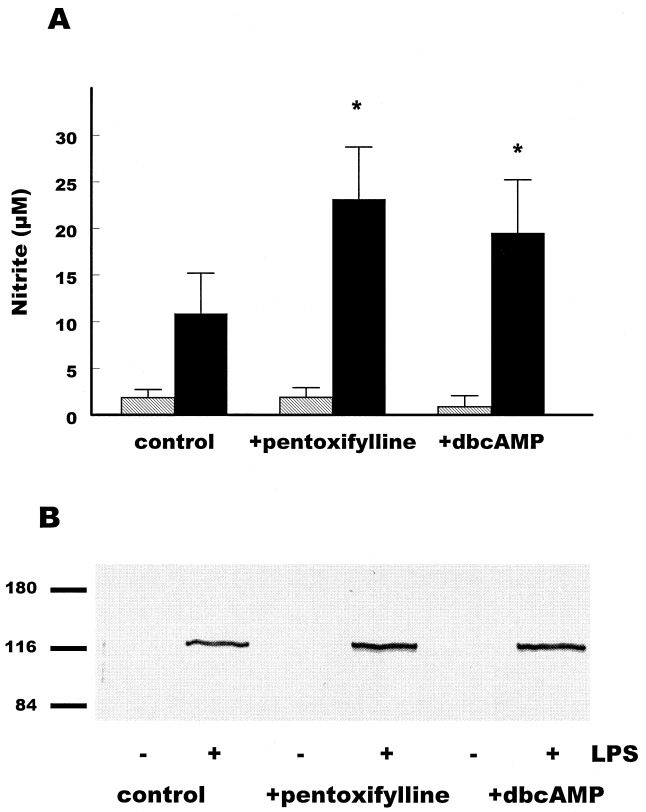

Fig. 6. Effect of PTX (1 mM) and dibutyryl cAMP (500 μM) on NO production by liver cell cultures after 48 hr of incubation without (▨) or with 10 μg/mL (■) of LPS (A). Values represent mean values ± SEM of three separate experiments in duplicate. *: Significant increase in NO production compared to control incubations ($P \leq 0.05$). (B) Western blot analysis of LPS-induced iNOS expression in liver cell cultures during 48 hr of control incubations or incubations with LPS (10 μg/mL) in the presence of 1 mM PTX or 500 μM dibutyryl cAMP.

Compared to LPS stimulation, 2.1- and 2.4-fold increases in iNOS expression were observed when the cells were exposed to LPS in combination with PTX or dibutyryl cAMP, respectively, as determined by densitometry analysis (Fig. 6B).

3.5. Effect of PTX on intracellular cAMP levels

The effect of PTX on intracellular cAMP levels is depicted in Fig. 7. PTX significantly increased intracellular cAMP levels in liver cells already at 30 min after addition of PTX. At 90 min of incubation, intracellular cAMP levels in PTX-treated cells were more significantly increased and resulted in intracellular cAMP levels up to 2000 fmol per million cells compared to 600 fmol per million cells for control incubations (Fig. 7).

4. Discussion

The acute phase response is known to impair liver function. This inflammatory reaction cannot only be triggered by

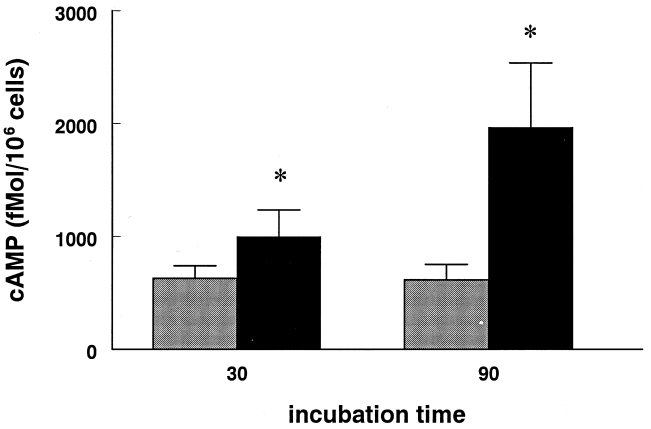

Fig. 7. Intracellular cAMP levels in porcine liver cells during control incubations (▨) or incubation with 1 mM PTX (■) after 30 or 90 min of incubation. Values are expressed in fmol/10^6 cells and represent mean values ± SEM of a representative experiment in triplicate. *: Significant increase in intracellular cAMP production compared to control incubations ($P \leq 0.05$).

infectious agents but also by various non-infectious stimuli including hypoxia or toxicants, which can ultimately result in chronic liver disease or liver fibrosis. PTX has been widely proposed as a therapeutic agent for these indications. Several studies have shown that PTX has a hepatoprotective effect during liver injury induced by various forms of stress. To study the effects of PTX on liver cell function, we used primary porcine liver cells without further purification of cultures. Immunohistochemical studies revealed that these liver cell cultures contained approximately 5% Kupffer cells, which are known to be of major importance for the onset of the inflammatory response through the production of proinflammatory cytokines such as TNF-α and IL-6. The present study shows that these cultures were capable of mimicking the hepatic inflammatory response with regard to production of proinflammatory cytokines, acute phase proteins, and the secretion of NO.

The inhibiting effect of PTX on TNF-α production in various cell types has been previously shown *in vivo* as well as *in vitro* [25,26]. This inhibitory effect was confirmed in the present study, where LPS-induced TNF-α production was completely inhibited in the presence of PTX. The effect of PTX on IL-6 secretion has been described previously [27,28], and was found to be more complex than with TNF-α. It was shown that IL-6 production either remained the same, following the addition of PTX, or was inhibited. In this study, the first phase of the curve up to 24 hr of incubation appeared to be unaffected or even slightly increased by the presence of PTX, whereas the second phase was significantly inhibited. That PTX inhibited only the late IL-6 response suggests that the kinetic profile of IL-6 could be caused by a combination of an initial direct induction by LPS and an indirect induction via LPS-induced proinflammatory cytokines such as TNF-α. However, these results also show that the secondary IL-6 peak cannot be mediated by TNF-α alone, since stimulation with 1 or 10 μg/mL of

LPS resulted in a similar TNF-α production but a different IL-6 response, suggesting involvement of additional proinflammatory mediators.

Although LPS strongly influenced both albumin and pig MAP/ITIH4 production as negative and positive acute phase proteins, respectively, no effect of PTX was observed during 72 hr of incubation. However, we cannot exclude the possibility that the effect of PTX becomes apparent after 72 hr of incubation. In a study performed in a rat model, Voisin and colleagues have shown that PTX treatment had no effect on *Escherichia coli*-induced down-regulation of albumin synthesis during the first 6 days of infection [29]. Furthermore, they showed that PTX treatment of TNF-α-independent acute phase proteins such as fibrinogen and α_2-macroglobulin were affected only at day 6 postinfection. These results are in agreement with those from our study and suggest a complex and more impeded regulation with regard to acute phase protein production.

PTX potentiated both iNOS expression and NO production after incubation of liver cell cultures with LPS. Although we cannot exclude the possibility that a small unidentified cell population is partly involved in iNOS expression, immunohistochemical studies revealed that hepatocytes in particular and not Kupffer cells were responsible for iNOS expression after exposure to LPS. Several lines of evidence suggest that there are considerable differences in iNOS expression and regulation in the liver between species. For instance, in mouse and rat macrophages iNOS expression can be induced by LPS [30,31], while in human monocytes and macrophages it has been difficult to demonstrate iNOS expression [32]. The latter observation is in accordance with our study, where iNOS expression could not be demonstrated in Kupffer cells. In addition, both rat and mouse hepatocytes have been shown to express high levels of iNOS in response to TNF-α, IL-1β, and interferon-γ as a single stimulus, while human hepatocytes respond to LPS alone [33]. In our study, exposure of hepatocytes to LPS in combination with PTX resulted in the expression of high levels of iNOS, while the TNF-α response was completely abolished. This latter observation suggests that porcine hepatocytes are able to respond to LPS alone without additional exposure to TNF-α. The response to LPS in porcine liver thus resembles that in human liver.

The effect of PTX on iNOS expression and NO production has been described for various cell types and seems to be cell type-dependent. In rat astrocytes and a murine fibrosarcoma cell line (L929), PTX proved to potentiate cytokine- or LPS-induced iNOS expression, while in most macrophages PTX was shown to inhibit iNOS expression and NO production [34–36]. Cyclic AMP has been described as an important mediator in the regulation of iNOS expression by either stimulating or inhibiting this expression, depending on the cell type (reviewed by Galea and Feinstein [37]). Harbecht *et al*. have shown that glucagon inhibits IL-1-induced iNOS expression in rat hepatocytes and that the observed inhibition seemed to be mediated by cAMP [38]. In the present study, PTX potentiated LPS-induced hepatocyte iNOS expression and NO production. Furthermore, the potentiating effect on iNOS could be mimicked when liver cells were incubated with dibutyryl cAMP, suggesting an important role for cAMP in the regulation of iNOS expression in porcine hepatocytes. Cyclic AMP has been reported to stabilize iNOS mRNA [39,40] and has in mesengial cells also been suggested to enhance iNOS promotor activity via the protein kinase A signaling pathway [41]. Whether these mechanisms indeed play an important role in the regulation of iNOS expression in porcine hepatocytes remains to be solved and deserves further attention.

Although PTX is known to be a potent inhibitor of TNF-α production in various cell types, this study shows that after LPS stimulation, PTX exerted cell-specific changes in the inflammatory responses and potentiated both iNOS expression and NO production in hepatocytes. Even though NO exerts both cytoprotective and cytotoxic properties, the increased iNOS expression could, to a certain extent, be of pharmacological value, since a decreased hepatic blood flow in cirrhotic patients is observed. However, the use of PTX as a therapeutic agent for the treatment of liver fibrogenesis or other liver diseases could be critical and should be investigated more thoroughly with regard to hepatic iNOS expression.

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