



# Inhibition of DNA synthesis in cultured hepatocytes by endotoxin-conditioned medium of activated stellate cells is transforming growth factor- $\beta$ and nitric oxide-independent

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**1** Activated hepatic stellate cells play a major role in the pathophysiology of chronic liver disease. They can influence the metabolism of hepatocytes by producing a variety of cytokines and growth factors. Upon stimulation with endotoxin, stellate cells also synthesize nitric oxide (NO), a potent mediator of growth of several cell types including hepatocytes.

**2** We investigated the effect of serum-free medium conditioned by activated stellate cells in the absence and presence of endotoxin on NO and DNA synthesis in hepatocytes. Stellate cells and hepatocytes were isolated by enzymatic digestion of the liver. Stellate cells were cultured for 10 days after which the majority exhibited  $\alpha$ -smooth muscle actin (a marker for activated cells); hepatocytes were used after overnight culture.

**3** While the medium conditioned by stellate cells in the absence of endotoxin stimulated DNA synthesis in hepatocytes, medium conditioned in its presence inhibited this process in an endotoxin concentration-dependent manner (10–1000 ng ml<sup>-1</sup>). Endotoxin-conditioned stellate cell medium also stimulated NO synthesis in hepatocytes; the effect was consistent with increased protein and mRNA expression of inducible NO synthase (iNOS). However, inhibition of DNA synthesis in hepatocytes caused by endotoxin-conditioned stellate cell medium was unaffected by the NOS inhibitor, L-N<sup>G</sup>-monomethylarginine (L-NMMA), guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and neutralizing antibodies for TGF- $\beta$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ .

**4** These results indicate that factors other than these cytokines produced by activated stellate cells upon stimulation with endotoxin or by hepatocytes challenged with endotoxin-conditioned stellate cell medium inhibit DNA synthesis in hepatocytes.

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**Keywords:** Cytokines; DNA synthesis; endotoxin; growth; hepatic stellate cells; hepatocytes; liver; nitric oxide

**Abbreviations:** EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbant assay; GBSS, Gay's balanced salt solution; HBSS, Hank's balanced salt solution; LPS, lipopolysaccharide; NO, nitric oxide, RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis

## Introduction

Hepatic stellate cells (fat-storing cells, perisinusoidal cells, Ito cells) are located in the space of Disse. The contractile stellate cells produce components of extracellular matrix and play a central role in the maintenance of sinusoidal blood flow and hepatic architecture (Blomhoff & Wake, 1991; Geerts *et al.*, 1994; Ramadori, 1994). During chronic liver injury, the quiescent stellate cells transform into proliferating  $\alpha$ -smooth muscle actin expressing cells (activated stellate cells) (Blomhoff & Wake, 1991; Geerts *et al.*, 1994; Ramadori, 1994). Apart from their strong fibrogenic activity and contractility, activated stellate cells also produce a variety of biologically active mediators such as transforming growth factor (TGF)- $\alpha$  and - $\beta$ , interleukin-6 (IL-6) and tumor necrosis factor (TNF)- $\alpha$ , but they lose the ability to express hepatocyte growth

factor (HGF) found in the quiescent stellate cells (Ramadori *et al.*, 1992; Schirmacher *et al.*, 1992). Endotoxin was recently reported to stimulate the expression of inducible nitric oxide synthase (iNOS) and the synthesis of nitric oxide (NO) in the stellate cells (Helyar *et al.*, 1994; Kawada *et al.*, 1998). The concentration of endotoxin is increased during acute and chronic liver injury (Triger *et al.*, 1978; Nolan, 1981; Lumsden *et al.*, 1988; Bomzon & Blendis, 1994). In Kupffer cells, endotoxin stimulated production of cytokines such as IL-1 $\beta$ , TNF- $\alpha$  and interferon (IFN)- $\gamma$  (Nathan, 1968; Adams & Hamilton, 1984; Decker, 1990; Klocker *et al.*, 2000) that cause induction of NO synthesis in hepatocytes (Curran *et al.*, 1990; Nussler *et al.*, 1992). It is conceivable that endotoxin exerts similar effect on hepatocytes *via* activated stellate cells. Thus the mediators produced by activated stellate cells with or without stimulation with endotoxin can be of critical significance in the regulation of the metabolism of hepatocytes during pathological developments. Therefore,

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the aim of this study was to investigate the effects of the medium conditioned by activated stellate cells in the absence and presence of endotoxin on hepatocytes. The results demonstrate that medium conditioned by activated stellate cells stimulates DNA but not NO synthesis in hepatocytes. Conversely, although the medium conditioned by stellate cells in the presence of endotoxin stimulates NO and inhibits DNA synthesis in hepatocytes, the latter effect is not mediated by NO or TGF- $\beta$ , TNF- $\alpha$ , IL-6 and IL-1 $\beta$ .

## Methods

### *Preparation of stellate cells*

The experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee in accordance with the guidelines of the National Institutes of Health. Stellate cells were prepared from the livers of male Sprague–Dawley rats (450–500 g) as described previously (Gabriel *et al.*, 1998). Briefly, following the collagenase/protease digestion of the liver and removal of the hepatocytes and cell debris by low speed centrifugation, stellate cells were purified from other nonparenchymal cells by density gradient centrifugation using 8.12% (w v<sup>-1</sup>) Nycodenz. Stellate cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 250 u ml<sup>-1</sup> penicillin, 250  $\mu$ g ml<sup>-1</sup> streptomycin and 10% foetal bovine serum/10% horse serum, and plated in 24-well culture plates at a density of  $1 \times 10^6$  cells well<sup>-1</sup>. The viability of the cells was greater than 95% as determined by Trypan blue exclusion. The purity of the cells was determined by phase contrast microscopy, vitamin A autofluorescence, and immunohistochemically using specific markers for endothelial cells (antibody to factor VIII related antigen; DAKO, Carpinteria, CA, U.S.A.), stellate cells (antibody to desmin; DAKO), Kupffer cells (clone ED2; Serotec, Indianapolis, IN, U.S.A.) and epithelial cells (clone AE1/AE3; Boehringer Mannheim, Indianapolis, CA, U.S.A.) as described previously (Gandhi *et al.*, 1999). Briefly, the cells were washed with phosphate buffered saline (PBS), fixed in 95% ethanol and antibodies were applied. Biotinylated IgGs were used as secondary antibodies, and avidin-biotin complex (Vectorstain, Burlingame, CA, U.S.A.) and peroxidase chromogen kit (Biomedica Corp. Foster City, CA, U.S.A.) were used for detection as described (Gandhi *et al.*, 1999). The purity and the plating efficiency of the cells were greater than 95 and 75% respectively. Cells were used on day 10 of culture when more than 80% expressed  $\alpha$ -smooth muscle actin as determined by immunohistochemistry (Gabriel *et al.*, 1998).

### *Preparation of hepatocytes*

Hepatocytes were prepared by collagenase (type CLSI, 0.05%) digestion of the liver as described previously (Kuddus *et al.*, 2000). The cells were separated in ice-cold Hank's balanced salt solution (HBSS). The suspension was filtered and the filtrate centrifuged at  $50 \times g$  for 1 min at 4°C. The cell pellet was washed three times with PBS containing 1 mM CaCl<sub>2</sub> and suspended in this medium at  $5 \times 10^6$  cells ml<sup>-1</sup>. The suspension was mixed with equal volume of 90% percoll in HBSS and centrifuged at  $50 \times g$  for 10 min at 4°C. The

pellet containing viable hepatocytes (>95%) was washed ( $2 \times$ ), suspended in William's medium E supplemented with 2 mM L-glutamine, 250 u ml<sup>-1</sup> penicillin, 250  $\mu$ g ml<sup>-1</sup> streptomycin, 10% foetal bovine serum,  $10^{-6}$  M insulin (Eli Lilly Co., Indianapolis, IN, U.S.A.) and 10 mM HEPES, and plated in 24-well plates ( $0.125 \times 10^6$  cells well<sup>-1</sup>). The medium was renewed after a 3 h attachment period and the cells were used for experiments after an overnight incubation. The purity of the cells, determined immunohistochemically using specific markers for various nonparenchymal cells as described above in the preparation of stellate cells, was greater than 98%.

### *Determination of the effects of stellate cell-conditioned medium on hepatocytes*

Stellate cells were washed and placed in serum-free DMEM containing 0.1% BSA (w v<sup>-1</sup>) and the test agents at concentrations indicated in the figure legends. After 24 h, the medium was filtered (0.2  $\mu$ m; Gelman Laboratory, Ann Arbor, MI, U.S.A.) and used for NO determination. Medium from another set of stellate cells was transferred to the overnight culture of hepatocytes. Appropriate controls were used to determine the effects of the agents and unconditioned medium on hepatocytes. Hepatocytes were incubated for 24 h with the test agents or the medium conditioned by stellate cells after which various determinations were made.

### *Determination of nitric oxide*

The concentration of NO end products nitrite and nitrate was determined by Griess method (Green *et al.*, 1982) using a colorimetric assay kit (Canyan Chemicals, Ann Arbor, MI, U.S.A.). Briefly, Griess reagent was added to the culture supernatant after treatment with nitrate reductase. The optical density was determined spectrophotometrically at 550 nm. A standard curve was developed using concentrations of sodium nitrate (0 and 35  $\mu$ M) treated similarly.

### *Determination of DNA synthesis*

After aspirating the medium from stellate cells or hepatocytes, fresh medium containing 1  $\mu$ Ci ml<sup>-1</sup> [<sup>3</sup>H]-thymidine was added to the culture wells. Following an incubation of 4 h at 37°C, the cells were washed with ice-cold HBSS containing 0.1% BSA, treated with ice-cold 10% trichloroacetic acid (TCA) for 10 min, and washed once with TCA followed by 95% ethanol. The cells were then digested with 5% (w v<sup>-1</sup>) SDS and the radioactivity was determined.

### *Western blot analysis for iNOS*

Cells were scraped from the plate in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, containing 0.1 M NaCl, 1 mM EDTA, 0.5 mM PMSF and 1 mM aprotinin). Following homogenization in an ice bath, the homogenate was kept in ice for 30 min and then centrifuged at  $15,300 \times g$  for 10 min at 4°C. The supernatant was aspirated and its protein concentration was determined by Lowry's procedure (Lowry

*et al.*, 1951). The lysate containing 10  $\mu\text{g}$  protein was subjected to SDS-PAGE on a 8% acrylamide gel and the separated proteins were transferred on to a Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membrane was treated with a rabbit monoclonal anti-rat iNOS antibody (Transduction Laboratories Co., Lexington, KY, U.S.A.). After washing, the membrane was incubated with peroxidase-linked anti-rabbit IgG (Amersham-Pharmacia), and detection was achieved using an ECL chemiluminescence kit (Amersham-Pharmacia).

#### Determination of mRNA expression of iNOS and cytokines

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay was employed to assess the relative levels of the mRNA expression of iNOS, IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IFN $\gamma$  in LPS-stimulated and unstimulated stellate cells as described previously (Gabriel *et al.*, 1998; 1999). Levels of  $\beta$ -actin mRNA were determined to ascertain the efficiency of cDNA synthesis and reverse transcription. The PCR primers specific for iNOS cDNA: 5'-AGAATGTTCCAGAATCCCTCCCTGGACA-3' (F) and 5'-GAGTGAGCTGGTAGGTTCTGTTG-3' (R) [product size, 356 bp (Kosuga *et al.*, 1994)]; TNF- $\alpha$  cDNA: 5'-CACGCTCTTCTGTCTACTGA-3' (F) and 5'-GGACTC-CGTGATGTCTAAGT-3' (R) [product size, 543 bp (Uemura *et al.*, 2000)]; IL-1 $\beta$  cDNA: 5'-GAAGCTGTGGCAGC-TACCTATGTCT (F) and CTCTGCTTGAGAGGTGCT-GATGTACC-3' (R) [product size, 522 bp (Uemura *et al.*, 2000)]; IL-6 cDNA: 5'-GAAAGTCAACTCCATCTGCC-3' (F) and 5'-CATAGCACACTAGGTTTGCC-3' (R) [product size, 681 bp (Uemura *et al.*, 2000)]; IFN- $\gamma$  cDNA: 5'-ATC-TGGAGGAACTGGCAAAGGACG-3' (F) and 5'-CCT-TAGGCTAGATTCTGGTGACAGC-3' (R) [product size, 288 bp (Tian *et al.*, 1997)]; and  $\beta$ -actin cDNA: 5'-TTCTA-CAATGAGCTGCGTGTG-3' (F) and 5'-TTCATGGATGC-CACAGGATTC-3' (R) [product size, 561 bp (Nudel *et al.*, 1983)] were used. PCR amplification reaction was performed essentially as described previously (Gabriel *et al.*, 1998; 1999). PCR products were resolved in a 1.5% agarose gel and stained with 1  $\times$  SYBR Green I (FMC Biproduct, Rockland, ME, U.S.A.). The gels were scanned under blue fluorescence light using a phosphorimager and the band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

#### Determination of cyclic GMP

Stellate cells or hepatocytes were treated with 1  $\mu\text{g ml}^{-1}$  LPS for 24 h. Likewise, hepatocytes were incubated with stellate cell medium conditioned in the absence or presence of LPS for 24 h. During the last 30 min of incubation, 1 mM 3-isobutyl-1-methylxanthine was added to the medium to inhibit endogenous phosphodiesterase activity. At the end of the incubation period, the medium was aspirated and cells were scraped in 1 ml ice-cold ethanol and left at room temperature for 5 min. The suspension was centrifuged at 1500  $\times g$  for 10 min at 4°C, and the supernatant was dried by vacuum centrifugation. The residue was reconstituted in phosphate buffer and cyclic GMP concentration was determined by ELISA using a commercial kit as per the

instructions of the manufacturer (Cayman Chemicals, Ann Arbor, MI, U.S.A.). The assay is based on the competition between free cyclic GMP and a cyclic GMP tracer (cyclic GMP linked to acetylcholinesterase molecule) for a limited number of cyclic GMP-specific rabbit antiserum binding sites. Free acetylcholinesterase is then reacted with Ellman's reagent that contains the substrate for acetylcholinesterase, and the distinct yellow color of the reaction is read at 412 nm. The assay was performed in duplicate in 96 well plates using 50  $\mu\text{l}$  of the reconstituted cell extract. For developing a standard curve, 0.23–30 pmol ml $^{-1}$  cyclic GMP solution was used. Appropriate conditions for nonspecific binding and blank were also used. Cyclic GMP acetylcholinesterase tracer was added to all wells except the blanks. Cyclic GMP antiserum was added to the wells containing cell extracts and standards and the plate was incubated for 18 h at room temperature. The plate was emptied, and rinsed five times with the wash buffer provided by the manufacturer. Ellman's reagent was added to each well and the absorbance of the resultant colour was read at 412 nm.

#### Materials

The following were purchased from the indicated sources: Protease type XIV, Nycodenz, bovine serum albumin (fraction V) (BSA), N-[2-hydroxyethyl] piperazine-N'-[ethanesulfonic acid] (HEPES), endotoxin (*Escherichia coli* lipopolysaccharide, serotype 0111:B4) (Sigma Chemical Co., St. Louis, MO, U.S.A.), collagenase type IV and type CLS1 (Worthington Biochemical Corporation, Freehold, NJ, U.S.A.), Dulbecco's modified Eagle medium, William's medium E, penicillin G, streptomycin, foetal bovine serum and horse serum, transforming growth factor (TGF)- $\beta$  (Life Technologies, Grand Island, N.Y., U.S.A.); monoclonal antibody for TGF- $\beta$  (Genzyme Corporation, Cambridge, MA, U.S.A.); neutralizing anti-TGF- $\alpha$  polyclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.); L-N $^G$ -monomethylarginine monoacetate (L-NMMA) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Alexis Biochemicals, San Diego, CA, U.S.A.); [methyl- $^3\text{H}$ ]thymidine (3.18TBq/mmol) (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.).

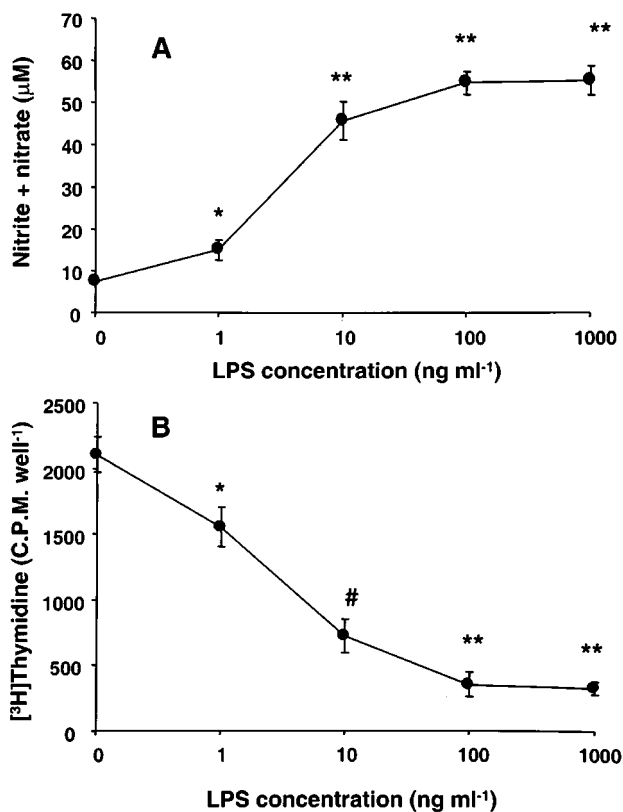
#### Data analysis

The values are presented as averages of triplicate determinations  $\pm$  s.e.mean. Each experiment was repeated at least three times using cells from different animals. Student's *t*-test was employed for statistical comparison between the groups. A *P* value of <0.05 was considered statistically significant.

## Results

#### Effect of LPS on NO and DNA synthesis in stellate cells

LPS stimulated NO synthesis in stellate cells in a concentration-dependent manner (Figure 1A). The effect was significant at the lowest concentration of LPS tested (1 ng ml $^{-1}$ ). LPS produced concentration-dependent inhibition of DNA synthesis in stellate cells, the effect being significant at the lowest concentration tested (1 ng ml $^{-1}$ ) (Figure 1B).



**Figure 1** Effect of LPS concentration on NO and DNA synthesis in stellate cells. Stellate cells were incubated in serum-free medium for 24 h in the presence of indicated concentrations of LPS. The concentration of nitrite+nitrate in the medium (A) and DNA synthesis in the cells (B) were determined. \* $P < 0.05$  vs control; # $P < 0.01$  vs control; \*\* $P < 0.001$  vs control.

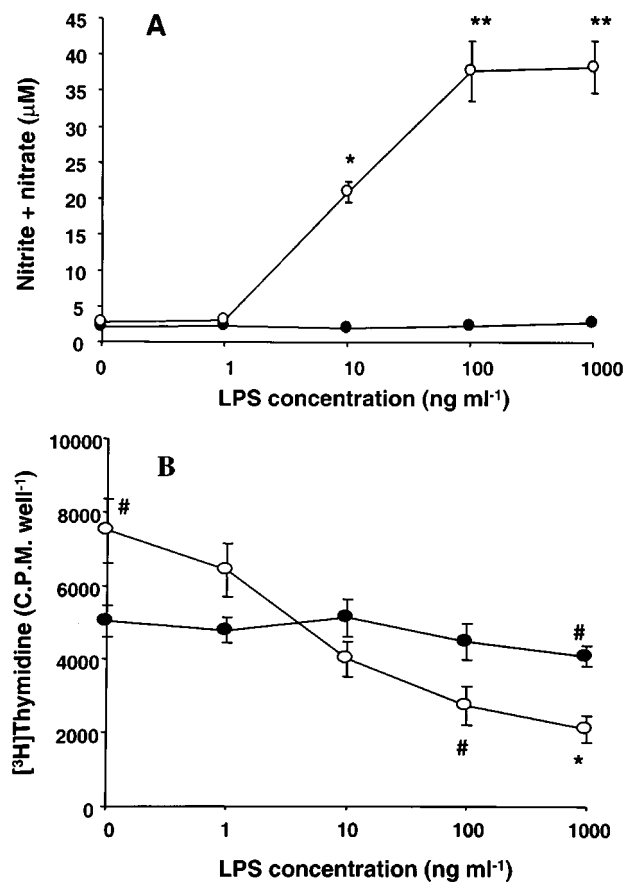
#### Effect of medium conditioned by stellate cells with LPS on NO and DNA synthesis in hepatocytes

Cultured hepatocytes and hepatic endothelial cells spontaneously release NO, such release being 100 times greater in the former cell type (Zhang *et al.*, 1997). We also observed considerable spontaneous release of NO by hepatocytes. This basal release of NO by hepatocytes was unaffected by LPS and medium conditioned by stellate cells in the absence of LPS (Figure 2A). However, exposure of hepatocytes to stellate cell medium conditioned with LPS caused an increase in the production of NO at LPS concentration of 10 ng ml<sup>-1</sup> and was maximal at 100 ng ml<sup>-1</sup> LPS.

Incubation of hepatocytes with stellate cell-conditioned medium produced significant increase in the DNA synthesis (Figure 2B). In contrast, incubation of hepatocytes with medium conditioned by stellate cells in the presence of increasing concentrations of LPS produced progressive inhibition of DNA synthesis. LPS by itself caused some inhibition of DNA synthesis in hepatocytes at the highest (1000 ng ml<sup>-1</sup>) concentration tested.

#### Protein and mRNA expression of iNOS in stellate cells and hepatocytes

LPS did not affect the very low protein and mRNA expression of iNOS found in hepatocytes (Figure 3). Stellate

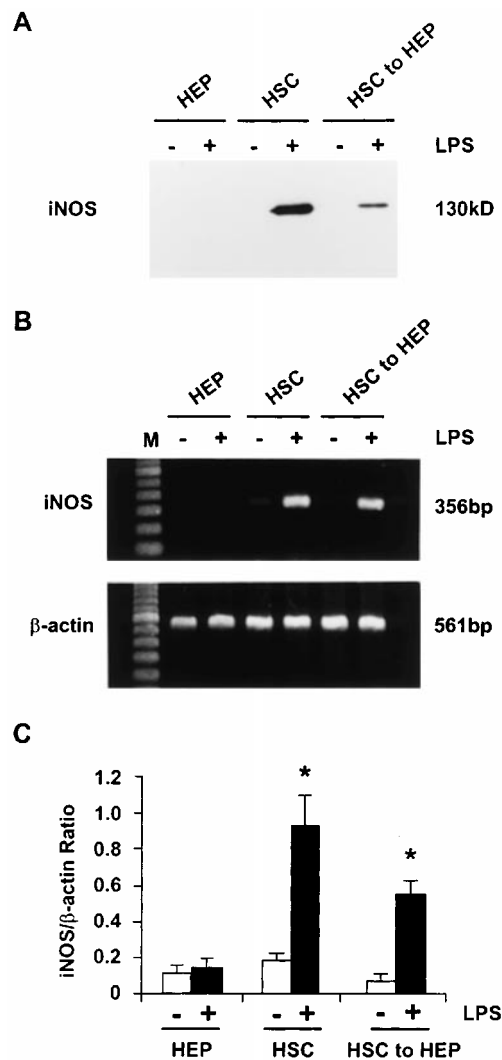


**Figure 2** Effect of stellate cell medium conditioned with increasing concentrations of LPS on NO and DNA synthesis in hepatocytes. Stellate cells were incubated with indicated concentrations of LPS for 24 h in serum-free medium. The medium was then transferred to hepatocytes incubated in serum-free medium for 24 h. For control experiments, hepatocytes were incubated in serum-free medium with LPS. After 24 h, the concentration of nitrite+nitrate in the medium (A) and DNA synthesis in the cells (B) were determined. Solid symbols, hepatocytes; hollow symbols, hepatocytes incubated with stellate cell-conditioned medium. \* $P < 0.01$  vs control; \*\* $P < 0.001$  vs control; # $P < 0.05$  vs control.

cells did not contain detectable level of iNOS protein (Figure 3A) although the mRNA expression was feebly detectable (Figure 3B). Stimulation of stellate cells with LPS caused dramatic increase in the expressions of iNOS protein (Figure 3A) and mRNA (Figure 3B, C). Stellate cell-conditioned medium did not cause significant change in the expression of iNOS protein or mRNA in hepatocytes (Figure 3A–C). However, consistent with the stimulation of the production of NO, stellate cell medium conditioned with LPS produced robust increase in the expression of both iNOS protein and mRNA in hepatocytes (Figure 3).

#### Effect of L-NMMA and ODQ on NO and DNA synthesis

A specific inhibitor of nitric oxide synthase, L-NMMA, was used to assess whether the effect of LPS on stellate cells and of stellate cell medium conditioned with LPS on hepatocyte DNA synthesis was mediated by NO. L-NMMA caused complete inhibition of LPS-induced production of NO by stellate cells (Figure 4A). L-NMMA did not alter the basal

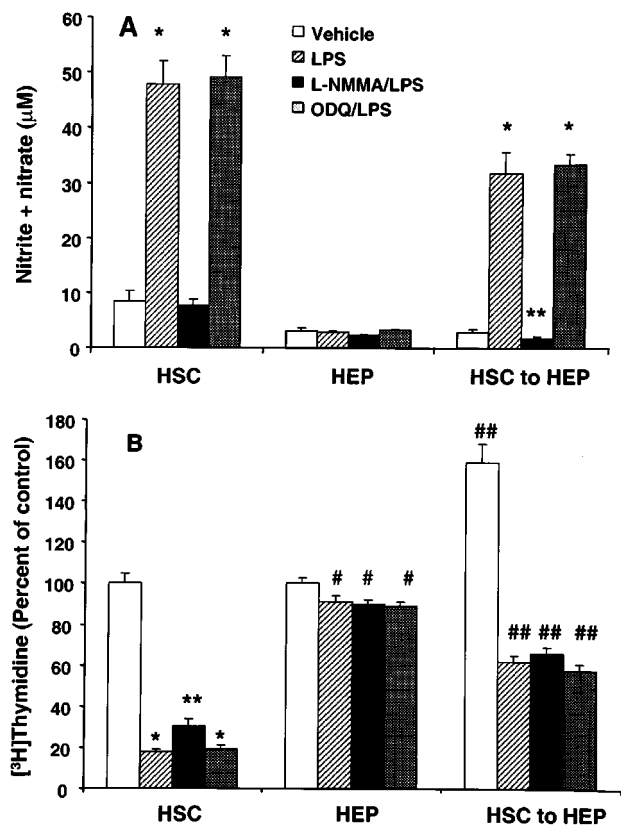


**Figure 3** Protein and mRNA expression of iNOS in stellate cells and hepatocytes. Stellate cells were incubated in serum-free medium with or without  $1 \mu\text{g ml}^{-1}$  LPS for 24 h. The medium was then transferred to hepatocytes and incubation was continued for 24 h. In parallel, hepatocytes were incubated in serum-free medium with or without LPS. The cells were then utilized for determination of iNOS protein expression by Western analysis (A) or for mRNA expression by semiquantitative RT-PCR (B). (C) The values show mRNA expression of iNOS standardized relative to the expression  $\beta$ -actin mRNA. HEP, hepatocytes; HSC, hepatic stellate cells; HSC to HEP, hepatocytes incubated with medium conditioned by stellate cells. \* $P < 0.01$  vs values in the absence of LPS.

synthesis of NO in hepatocytes, but inhibited that stimulated by LPS-conditioned stellate cell medium (Figure 4A).

L-NMMA partially reversed LPS-induced inhibition of DNA synthesis in stellate cells (Figure 4B). However, the inhibition of DNA synthesis in hepatocytes caused by LPS-conditioned stellate cell medium was unaltered by L-NMMA (Figure 4B).

Several effects of NO on the cellular processes are mediated by cyclic GMP (Schmidt *et al.*, 1993). The concentration of cyclic GMP increased in stellate cells stimulated with LPS and in hepatocytes challenged with LPS-conditioned stellate cell medium (Table 1). Therefore, ODQ, an inhibitor of soluble guanyl cyclase (Garthwaite *et*



**Figure 4** Effect of L-NMMA and ODQ on LPS-induced NO (A) and inhibition of DNA synthesis (B) in stellate cells and hepatocytes. Stellate cells were incubated with  $1 \mu\text{g ml}^{-1}$  LPS  $\pm 0.5 \text{ mM}$  L-NMMA or  $5 \mu\text{M}$  ODQ for 24 h in serum-free medium. The medium was used for measurement of nitrite + nitrate concentration and the cells for determination of DNA synthesis. Medium from another set of stellate cells was transferred to cultured hepatocytes and incubation as continued for 24 h. Nitrite + nitrate (medium) concentration and DNA synthesis (cells) were then determined. For control experiment, hepatocytes were incubated with LPS  $\pm$  L-NMMA or ODQ in unconditioned medium. HSC, hepatic stellate cells; HEP, hepatocytes; HSC to HEP, hepatocytes incubated with medium conditioned by stellate cells. (A) \* $P < 0.001$  vs vehicle; \*\* $P < 0.05$  vs vehicle. (B) \* $P < 0.001$  vs vehicle; \*\* $P < 0.001$  vs vehicle or  $P < 0.01$  vs LPS; # $P < 0.05$  vs vehicle; ## $P < 0.01$  vs hepatocytes with unconditioned medium.

*et al.*, 1995) was used to determine whether the effects of LPS on stellate cells and those of stellate cell-conditioned medium on hepatocytes were mediated by cyclic GMP. ODQ did not affect LPS-induced synthesis of NO in stellate cells (Figure 4A). Additionally, no difference was observed between the NO production by hepatocytes incubated with stellate cell medium conditioned with LPS in the presence and absence of ODQ. ODQ also did not affect LPS-induced inhibition of DNA synthesis in stellate cells and caused by LPS-conditioned stellate cell medium in hepatocytes (Figure 4B).

#### *Effect of antiTGF- $\alpha$ antibody on DNA synthesis in hepatocytes stimulated by stellate cell-conditioned medium*

A neutralizing antibody for TGF- $\alpha$  did not affect the stimulation of DNA synthesis in hepatocytes caused by

stellate cell-conditioned medium suggesting that this effect was not mediated by TGF- $\alpha$  (Table 2).

*Effect of neutralizing antibodies for TGF- $\beta$ , TNF- $\alpha$ , IL-1 $\beta$  and IL-6 on inhibition of DNA synthesis in hepatocytes caused by LPS-conditioned stellate cell medium*

LPS treatment caused increased mRNA expression of IL-1 $\beta$ , IL-6, but not of TNF- $\alpha$  (Figure 5). No expression of IFN- $\gamma$  mRNA was observed in unstimulated or LPS-stimulated stellate cells (Figure 5). Therefore, we determined whether neutralizing antibodies against TGF- $\beta$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$  reversed the inhibition of DNA synthesis in hepatocytes caused by LPS-conditioned stellate cell medium. As shown in Figure 6A, anti-IL-1 $\beta$  and TNF- $\alpha$  antibodies partially reversed NO production in hepatocytes stimulated by LPS-conditioned stellate cell medium. However, anti-TGF- $\beta$ - and anti-IL-6 antibodies did not affect this process. None of these antibodies affected the inhibition of DNA synthesis in hepatocyte caused by LPS-conditioned stellate cell medium (Figure 6B).

## Discussion

This study demonstrates that activated stellate cells produce growth mediators that influence hepatocyte DNA synthesis.

**Table 1** Cyclic GMP concentration in LPS-treated stellate cells and in hepatocytes treated with LPS-conditioned stellate cell medium

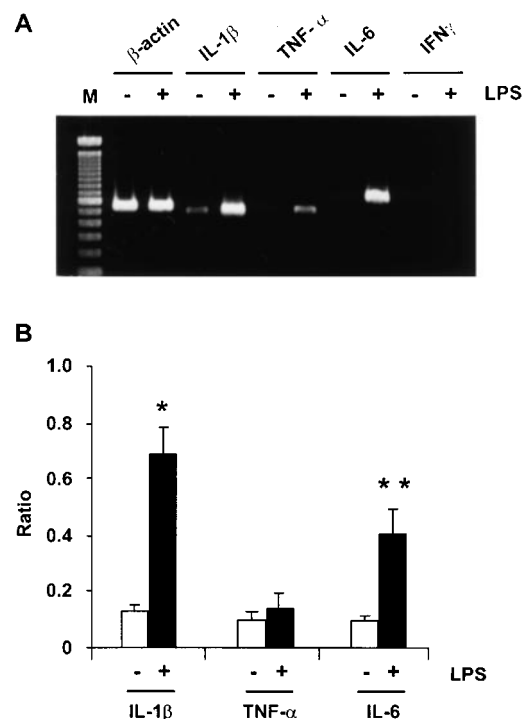
	HSC	Hepatocytes	HSC to Hepatocytes
Vehicle	1.22 $\pm$ 0.07	0.039 $\pm$ 0.005	0.045 $\pm$ 0.03
LPS	2.36 $\pm$ 0.12*	0.053 $\pm$ 0.006	0.271 $\pm$ 0.051**

The results expressed as pmol cyclic GMP mg<sup>-1</sup> protein are means of triplicate determinations  $\pm$  s.e.mean. Similar results were obtained in two separate experiments. HSC: hepatic stellate cells; HSC to hepatocytes: hepatocytes incubated with medium conditioned by stellate cells. \* $P$  < 0.01 vs vehicle; \*\* $P$  < 0.05 vs hepatocytes incubated with medium conditioned by stellate cells or with LPS alone as determined by Student's *t*-test.

**Table 2** Effect of TGF- $\alpha$  antibody on DNA synthesis in hepatocytes stimulated by stellate cell-conditioned medium

	[ <sup>3</sup> H]-thymidine incorporation (c.p.m. well <sup>-1</sup> )
Control	2981 $\pm$ 164
Stellate cell-conditioned medium	4789 $\pm$ 283*
Stellate cell-conditioned medium + antiTGF- $\alpha$ AB	4670 $\pm$ 180*

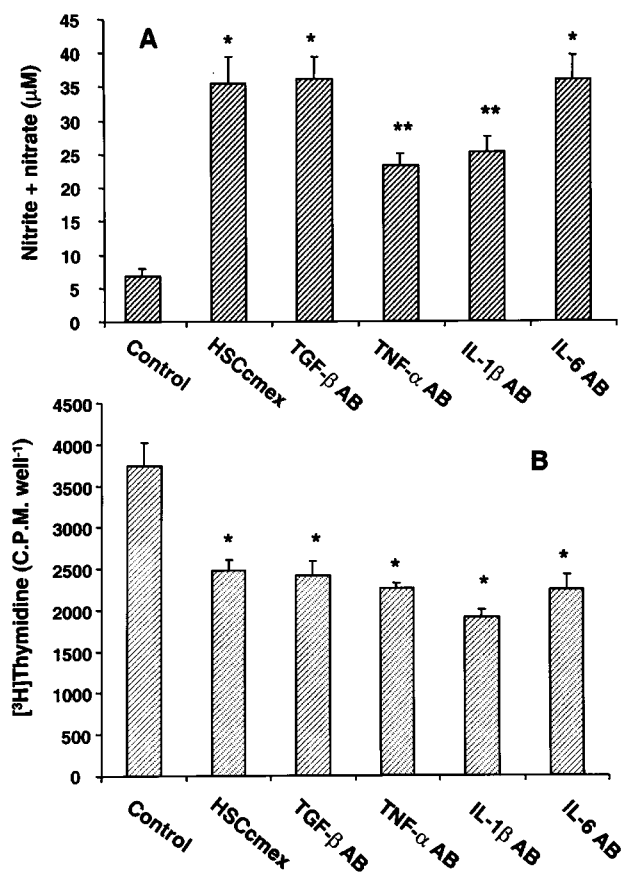
After an overnight culture, hepatocytes were placed in serum-free medium for 24 h. The medium was then replaced with fresh medium, or with stellate cell-conditioned medium  $\pm$  antiTGF- $\alpha$  antibody (10  $\mu$ g ml<sup>-1</sup>). After 24 h, [<sup>3</sup>H]-thymidine incorporation was determined. The values shown are C.P.M.  $\pm$  s.e.mean of triplicate determinations. This experiment was repeated three more times with essentially similar results. \* $P$  < 0.01 vs control.



**Figure 5** Effect of LPS on the mRNA expression of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IFN $\gamma$  in activated stellate cells. (A) Cells were incubated with 1  $\mu$ g ml<sup>-1</sup> LPS for 24 h. RNA was then extracted and cDNA was prepared. Semiquantitative RT-PCR analysis was performed to determine the concentrations of mRNA for the cytokines. (B) mRNA expression of the cytokines standardized relative to the expression of  $\beta$ -actin mRNA. \* $P$  < 0.01 vs vehicle; \*\* $P$  < 0.05 vs vehicle.

While medium conditioned by activated stellate cell stimulated DNA synthesis in hepatocytes, medium conditioned in the presence of endotoxin caused strong inhibition of this process. Physiologically, hepatocytes are quiescent with 1/20,000 undergoing mitosis at a given time (Fausto, 1990). During chronic injury, they regenerate in a unique fashion in nodules encapsulated by fibrous tissue produced mainly by activated stellate cells. Activated stellate cells have been shown to synthesize several potent growth mediators and cytokines (Blomhoff & Wake, 1991; Geerts *et al.*, 1994; Ramadori, 1994). However, the precise mechanisms by which stellate cells modulate the growth of hepatocytes remain inconclusive. Since activated stellate cells do not produce HGF (Ramadori *et al.*, 1992; Schirmacher *et al.*, 1992), HGF cannot be responsible for the stimulation of DNA synthesis in hepatocytes caused by the medium conditioned by activated stellate cells. Our results showing inability of a neutralizing TGF- $\alpha$  antibody to inhibit DNA synthesis in hepatocytes stimulated by medium conditioned by activated stellate cells indicate that TGF- $\alpha$  may not be a major factor responsible for this effect.

Endotoxin produced by intestinal bacteria entering the circulation is taken up and destroyed by Kupffer cells in the liver (Nolan, 1981). During chronic liver injury, endotoxin concentration increases as a consequence of increased intestinal translocation (Triger *et al.*, 1978; Lumsden *et al.*, 1988), reduced hepatic clearance due to intra- and extra-hepatic shunts and impaired activity of the reticuloendothelial



**Figure 6** Effect of antibodies to TGF- $\beta$ , TNF- $\alpha$ , IL-1 $\beta$  and IL-6 on the stimulation of NO and inhibition of DNA synthesis in hepatocytes induced by LPS-conditioned stellate cell medium. Hepatocytes were incubated with LPS-conditioned stellate cell medium in the presence of 15  $\mu\text{g ml}^{-1}$  anti-TGF- $\beta$ , 2.5  $\mu\text{g ml}^{-1}$  anti-TNF- $\alpha$ , 2  $\mu\text{g ml}^{-1}$  anti-IL-1 $\beta$ , 0.4  $\mu\text{g ml}^{-1}$  and anti-IL-6 antibodies. After 24 h, concentration of nitrite + nitrate in the medium (A) and DNA synthesis in the cells (B) were determined. (A) \* $P < 0.01$  vs control; \*\* $P < 0.01$  vs control or  $P < 0.05$  vs hepatocytes incubated with LPS-conditioned stellate cell medium (HSCcmex). (B) \* $P < 0.05$  vs control.

system (Bomzon & Blendis, 1994). In such situations, it has been suggested that endotoxin may exert effects on other hepatic cell types including stellate cells (Nolan, 1981). Indeed, endotoxin stimulated iNOS expression and NO synthesis, and inhibited DNA synthesis in the activated stellate cells. The effect of endotoxin on DNA synthesis in stellate cells appears to be partially mediated by NO as evident by its incomplete inhibition by L-NMMA. Experiments of the present investigation were performed in serum-free medium. Since the presence of serum is required for providing LPS-binding protein (LBP) in the initiation of binding of LPS to its receptor CD14 (Martin *et al.*, 1992), our findings suggest that either activated stellate cells may produce LBP or that LBP is not required for the action of LPS on stellate cells.

Interestingly, in contrast to the stimulatory effect of stellate cell medium conditioned in the absence of LPS, medium

conditioned in its presence strongly inhibited DNA synthesis in hepatocytes. Since LPS did not alter DNA synthesis in hepatocytes, it is apparent that mediators produced by LPS-stimulated stellate cells are the responsible factors. NO has been shown to inhibit DNA synthesis in several cell types such as vascular smooth muscle cells (Paul *et al.*, 1997), stellate cells (Helyar *et al.*, 1994; Kawada *et al.*, 1998), cardiac myocytes (Calderone *et al.*, 1998), fibroblasts (Calderone *et al.*, 1998) and renal mesangial cells (Rupprecht *et al.*, 2000). A strong induction of iNOS activity in hepatocytes treated with LPS-conditioned stellate cell medium suggested that the effect on DNA synthesis may be mediated by NO. However, inhibition of NOS and guanylyl cyclase respectively by L-NMMA and ODQ did not alter inhibition of DNA synthesis in hepatocytes caused by LPS-conditioned stellate cell medium. These results indicate that factor(s) other than NO are most probably responsible for the effect of LPS-conditioned stellate cell medium on hepatocytes.

Cultured nonparenchymal cells (a mixture of endothelial and Kupffer cells) isolated from regenerating rat liver, but not the unmodified liver, were found to release factor(s) that inhibited DNA synthesis in hepatocytes (Boulton *et al.*, 1997). Endotoxin causes increased production of several cytokines, such as IFN $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$ , in macrophages including Kupffer cells (Nathan, 1968; Adams & Hamilton, 1984; Decker, 1990; Klocker *et al.*, 2000), that are known to modulate DNA and NO synthesis in hepatocytes (Feingold *et al.*, 1988; Nakamura *et al.*, 1988; Beyer *et al.*, 1990; Kuma *et al.*, 1990; Webber *et al.*, 1998; Curran *et al.*, 1990; Nussler *et al.*, 1992). Activated stellate cells are also capable of producing such cytokines as well as TGF- $\beta$  (Blomhoff & Wake, 1991; Geerts *et al.*, 1994; Ramadori, 1994). In the present study, LPS increased the mRNA expression of IL-6 and IL-1 $\beta$ , but not of TNF- $\alpha$ , and no expression of IFN- $\gamma$  was found in unstimulated or LPS-stimulated stellate cells. However, antibodies to TNF- $\alpha$ , IL-6, IL-1 $\beta$  as well as to TGF- $\beta$  failed to exert any effect on the inhibition of DNA synthesis in hepatocytes induced by LPS-conditioned stellate cell medium. These results indicate that LPS stimulates the synthesis of an as yet unidentified growth modulator(s) by activated stellate cells that is capable of inhibiting DNA synthesis in hepatocytes.

The knowledge of the factors responsible for the abnormal liver growth as well as of those that prevent this condition is incomplete. The results of this study indicate that activated stellate cells are capable of producing both types of mediators. It is apparent that an imbalance in the production of such mediators, and modulation of their synthesis by endotoxin play an important role in hepatic pathology. Identification of these factors is, therefore, critical in formulating effective therapies to prevent abnormal liver growth.

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