Bacterial Lipopolysaccharide Antagonizes Transforming Growth Factor β1–Induced Apoptosis in Primary Cultures of Hepatocytes

PALOMA MARTÍN-SANZ, MARÍA J. M. DÍAZ-GUERRA, MARTA CASADO, AND LISARDO BOSCÁ

Incubation of primary cultures of fetal hepatocytes with lipopolysaccharide (LPS) elicited the expression of nitric oxide (NO) synthetase as well as antagonized the apoptotic cell death evoked by treating the cells with transforming growth factor β1 (TGF-β1). In addition to LPS, exposure of the cells to chemical NO donors also protected against apoptotic cell death when assayed at concentrations in the low micromolar range. Treatment of hepatocytes with large concentrations of NO donors promoted both apoptotic and necrotic cell death. These results suggest that NO synthesis by hepatocytes might be involved in the protection against apoptotic death. (HEPATOLOGY 1996;23:1200-1207.)

Regulation of organ size and liver function is the result of a dynamic balance between stimulatory and inhibitory signals.[1-3] Under physiological conditions, senescent or damaged cells are removed by apoptosis, whereas the response to acute cell injury mostly involves a necrotic process.[4,5] Several growth factors and cytokines have been identified as inducers of hepatocyte apoptosis both *in vivo* and *in vitro,* among them a prominent role has been recognized for transforming growth factor β1 (TGF-β1) and for the engagement of the *Fas*/*Apo*-1 receptor.[6-8] Definition of apoptosis has been the subject of some controversy depending on the morphological or biochemical criteria used for its characterization. However, morphological alterations in the cellular and nuclear membranes correlate closely with the appearance of oligonucleosomal DNA fragments as a specific marker of apoptotic cell death.[4,9,10] In addition to receptor evoked apoptosis, it has been recently reported that exposure of several cell lines to nitric oxide (NO) is a sufficient condition for triggering apoptosis *per se* in macrophages and in neural cells.[11,12] In contrast to this ability to induce apoptosis, low concentrations of NO protect against apoptosis and cellular stress in the same way as occurs in neural cells and in various cell types of lymphoid origin.[12-14]

The role of NO in liver function under physiological and physiopathologic conditions is a subject of current interest; for instance, this molecule has been found to be involved in the regulation of the hyperdynamic circulation displayed by cirrhotic liver.[15,16] Indeed, inducible nitric oxide synthase (iNOS) is induced in liver in response to lipopolysaccharide (LPS) and to combinations of a panel of several proinflammatory cytokines, as well as under pathological conditions such as cirrhosis or liver regeneration after partial hepatectomy.[15-18] Like liver tissue, primary cultures of hepatocytes also express iNOS in response to various stimuli, therefore offering the possibility of analyzing the function of NO synthesis on hepatocyte physiology under defined *ex vivo* conditions.[17-19] Most of these data have been obtained using adult hepatocytes, and little information is available regarding fetal hepatocytes. Hepatocytes isolated from animals at 21 days gestation constitute an interesting model, because these cells express some receptors distinct from those expressed by adult hepatocytes (e.g., insulinlike growth factor I [IGF-I] receptor) and display a significant proliferative capacity when confronted with various growth factors.[20,21] For these reasons, in the present work we have investigated whether the release of NO by fetal hepatocytes can influence the response to cytokines that promote apoptotic death of these cells. Our results show that NO has a dual effect in the control of apoptosis in cultured fetal hepatocytes, i.e., that whereas a low synthesis of NO counteracts the apoptotic death induced by TGF-β, large amounts of NO have deleterious effects by itself, promoting both an apoptotic and necrotic response in these cells.

MATERIALS AND METHODS

Chemicals. Cytokines, growth factors, and enzymes were obtained from Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO). 3-morpholinosydnonimine (SIN-1) and mol-

Abbreviations: TGF-β1, transforming growth factor β1; NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IGF-I, insulinlike growth factor I; SIN-1, 3-morpholinosydnonimine; SIN-10, molsidomine; TNF-α, tumor necrosis factor α; PBS, phosphate-buffered saline; GSNO, *S*-nitroso glutathione; TdT, terminal deoxyribonucleotidyl transferase.

From the Instituto de Bioquímica (CSIC-UCM), Facultad de Farmacia, Universidad Complutense, Madrid, Spain.

Received July 28, 1995; accepted December 4, 1995.

Address reprint requests to: Lisardo Boscá, Ph.D., Instituto de Bio-química, Facultad de Farmacia, 28040 Madrid, Spain.

Supported by grant PB92/070 from the Comisión Interministerial de Ciencia y Tecnología and grant 92/0267 from the Fondo de Investigaciones Sanitarias de la Seguridad Social, respectively.

0270-9139/96/2305-0039$3.00/0

sidomine (SIN-10) were from Affinity Res. Products (Nottingham, England). Tissue culture dishes were from Costar (Cambridge, MA). Tissue culture media were from Biowhittaker (Walkersville, MD). Agarose and bacterial growth media were from Hispanagar (Barcelona, Spain). The endotoxin content of the TGF-β, tumor necrosis factor α (TNF-α), and IGF-I solutions was negligible using the *Limulus polyphemus* test (Sigma).

Isolation and Incubation of Fetal Hepatocytes. Hepatocytes were prepared from 21-day-old fetuses of pregnant Albino Wistar rats (300-350 g) aged 3 months. Animals were fed a standard laboratory diet and killed over the course of 1 hour. Gestational age was assessed by standard criteria and fetuses were delivered by cesarean section.[22] A suspension of fetal hepatocytes was prepared by a nonperfusion collagenase dispersion method that involved incubation (3 g/flask) of chopped fetal liver for 30 minutes at 37°C with 30 mL of Ca^{2+}-free Krebs-bicarbonate buffer containing 0.5 mmol/L ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) under continuous gassing with carbogen (O_2/CO_2, 19/1).[23] The cell suspension was centrifuged (50*g* for 2 minutes), and the cell pellet was resuspended and incubated for 60 minutes in the presence of 2.5 mmol/L Ca^{2+} and 0.5 mg/mL of collagenase A (Boehringer). At the end of the incubation period, the cells were centrifuged at 50*g* for 5 minutes, and the resuspended cell pellet was progressively filtered through nylon membranes of 500-, 100-, and 50-μm mesh. The contamination of hematopoietic cells of the preparation was determined by microscopic observation and was below 5%. Cell viability was assessed by Trypan blue exclusion and was always higher than 90%. The hepatocyte suspension was washed twice with sterile Dulbecco's modified Eagle medium medium and then resuspended in this medium supplemented with 50 μg/mL of gentamicin, 50 μg/mL of penicillin G, and 50 μg/mL of streptomycin. Fetal hepatocytes were plated at 3 to 4 $\times$ 10^6 in 6-cm tissue-culture dishes, in a culture medium containing 2.5 mL of Dulbecco's modified Eagle medium supplemented with 10% of heat inactivated fetal calf serum. Two hours after seeding the cells, the medium was aspirated, and the plates were washed twice with phosphate-buffered saline (PBS) to remove the nonadherent cells. The cells were maintained in 2 mL of phenol red–free Dulbecco's modified Eagle medium containing 1% of heat-inactivated fetal calf serum. Additions were made so that the changes in the total incubation volume were less than 2%.

Synthesis of* S-*Nitroso Glutathione and Determination of* NO_x^- *Concentration. *S*-Nitroso glutathione (GSNO) was synthesized before use following a previous protocol.[24] GSH was dissolved with an equimolar concentration of HCl (0.7 mol/L) and kept in an ice bath. One volume of a 1.4 mol/L solution of sodium nitrite was added, and the mixture was gently stirred for 40 minutes. GSNO was precipitated in 2.5 volumes of acetone under continuous stirring. The precipitate was recovered by centrifugation and was washed with one volume of 80% acetone solution. The pellet was filtered through a Whatman 1 filter and extensively washed with 100% acetone and three times diethylether. After vacuum drying, an aliquot was characterized by UV spectroscopy. NO release was measured by the accumulation of nitrite and nitrate in the culture medium as previously described.[17] Briefly, nitrate was reduced to nitrite in the presence of 0.5 U of nitrate reductase, 50 μmol/mL of nicotinamide adenine dinucleotide phosphate, and 5 μmol/mL of flavin adenine dinucleotide. The nitrite concentration was determined using Griess reagent.[17]

RNA Extraction and Analysis. Total RNA (3-4 $\times$ 10^6 cells) was extracted following the guanidinium thiocyanate method.[25] After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde the RNA was transferred to a Nytran membrane (NY 13-N; Schleicher & Schuell, Germany) with 10 $\times$ SSC (10 $\times$ SSC is 1.5 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.4). The membrane was prehybridized and the level of iNOS messenger RNA was determined using an *Eco*RI-*Hin*dII fragment from the iNOS complementary DNA,[26] labeled (45% of efficiency) with [α-^{32}P]deoxycitidine triphosphate using the Random Primed labeling kit (Boehringer). The membranes were washed with 0.1 $\times$ SSC and 0.1% sodium dodecyl sulfate at room temperature for 10 minutes and twice at 50°C for 30 minutes, followed by exposure to micrograph film (Kodak X-OMAT). Different exposition times of the micrograph films were used to ensure that bands were not saturated. Quantification of the films was performed by laser densitometry (Molecular Dynamics), using the hybridization with a β-actin probe (0.6 kb *Eco*RI/*Hin*dIII fragment isolated from a VC 18 vector) as an internal standard.

Measurement of Lactatedehydrogenase Activity. To evaluate the degree of plasma membrane alteration the release of lactatedehydrogenase to the extracellular medium was measured. To do this, the medium was aspirated, centrifuged at 15,000*g* for 10 minutes and the lactatedehydrogenase activity present in the supernatant was assayed in the presence of 0.5 mmol/L pyruvate and 0.15 mmol/L nicotinamide adenine dinucleotide.[27]

Analysis of DNA Fragmentation. Internucleosomal DNA fragmentation was assessed by distinct independent methods including agarose gel electrophoresis analysis of fragmented DNA, and a cell death enzyme-linked immunosorbent assay kit based on the detection of mononucleosomes and oligonucleosomes in the cytosol (Boehringer). The cell layers (3-4 $\times$ 10^6) were washed twice with ice-cold PBS and lysis of the plasma membrane was achieved with 1 mL of 20 mmol/L ethylenediaminetetraacetic acid, 0.5% Triton X-100, 5 mmol/L Tris-HCl, pH 8.0. After incubation for 15 minutes at 4°C with gentle shaking, the nuclei were removed by centrifugation at 500*g* for 10 minutes and the resulting supernatant was centrifuged at 30,000*g* for 15 minutes. The oligonucleosomal DNA present in the soluble fraction was precipitated with 70% ethanol plus 2 mmol/L $MgSO_4$, and aliquots were treated for 1 hour at 55°C with 0.3 mg/mL of proteinase K. After two extractions with phenol/chloroform, the DNA was resuspended and analyzed in a 2% agarose gel after staining with 0.5 μg/mL of ethidium bromide. Alternatively, aliquots of the supernatants of lysed cells were analyzed using an enzyme-linked immunosorbent assay cell death kit (Boehringer) in which the enrichment of histone associated DNA fragments of mononucleosomes and oligonucleosomes in the cytosol was detected using a sandwich-enzyme immunoassay with antihistone and anti–DNA-peroxidase antibodies. The relative degree of apoptosis was determined quantitatively by measuring the peroxidase activity at 405 nm and calculating the ratio between the enzyme activity of a sample incubated for a given period of time, and the corresponding value at time 0 hour. The average A_{405} of samples from control cells was 0.15 optical density (enrichment factor). Also, an alternative quantitative approach was obtained by measuring the incorporation of fluorescein-12-deoxyuridine triphosphate (Boehringer) into DNA by terminal deoxyribonucleotidyl transferase (TdT) following a previous protocol.[28] Briefly, cells were kept in suspension after incubation for 10 minutes with PBS containing 2 mmol/L EGTA and 0.005% trypsin. The hepatocytes were fixed in 2% paraformaldehyde and permeabilized with 0.02% NP-40 for 5 minutes at 4°C. After

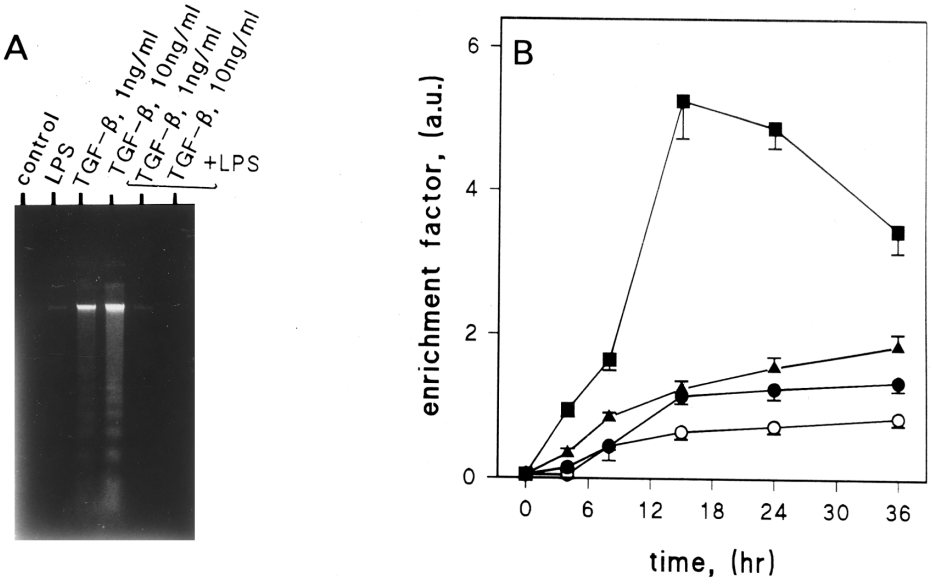

FIG. 1. LPS antagonizes the DNA degradation evoked by TGF-β in primary cultures of fetal hepatocytes. Hepatocytes (3×10^6 cells) were cultured for 24 hours with 0.5 μg/mL of LPS, with 1 or 10 ng/mL of TGF-β or a combination of LPS and TGF-β. The cell extracts were treated with proteinase K and ribonuclease and the DNA was analyzed in an agarose gel (A). Alternatively, samples (5×10^5 cells) were analyzed for the content in mononucleosomes and oligonucleosomes in the cytosol using a sandwich based immunoassay. (○) Control; (●) LPS, 0.5 μg/mL; (■) 1 ng/mL TGF-β; (▲) LPS + TGF-β (B). Results show a representative experiment out of three (A), or the means ± SEM of three experiments (B).

labeling with TdT (Boehringer), the cells were washed three times with PBS and the fluorescence was measured by cytofluorometry. Results were expressed as the mean channel fluorescence ratio with respect to control cultures (fluorescence ratio).

Data Analysis. The number of experiments is indicated in the corresponding figure. Statistical differences ($P < .05$) between mean values were determined by one-way ANOVA followed by Student's t test.

RESULTS

Apoptosis Triggering in Cultured Fetal Hepatocytes. Hepatocytes either *in vivo* or when kept in culture are relatively resistant to apoptosis in response to various cellular stresses. However, cytokines such as TGF-β have been recognized to be able to promote apoptosis, at least in adult hepatocytes.[6,7] In addition to cytokines, other substances that reach the intracellular space have been reported to induce apoptosis in various cell types. In this respect, high levels of NO constitute a condition that favors apoptosis in macrophages, neural cells, and some cancer cells.[12-14] To study whether this situation also applies to fetal hepatocytes, primary cultures were incubated with LPS to induce NO synthesis, and the rate of apoptotic death was investigated. As Fig. 1A shows, exposure of fetal hepatocytes to 0.5 μg/mL of LPS (a concentration that promotes NO release, see below) barely increased the apoptotic rate of these cells as compared with unstimulated controls. Incubation with TGF-β (1-10 ng/mL) promoted the DNA degradation expected, which exhibited the characteristic pattern of DNA laddering. However, simultaneous addition of LPS and TGF-β resulted in a blockage of the apoptotic death evoked by TGF-β. To have a more quantitative and detailed profile of the time course of DNA fragmentation, the appearance of oligonucleosomal moieties in the cytosol was observed using a DNA-histone immunoassay. As Fig. 1B shows, a peak of apoptosis was observed in cells treated for 15 to 24 hours with TGF-β, whereas in the presence of LPS this apoptotic response was abolished. Treatment of fetal hepatocytes with IGF-I did not affect the basal rate of apoptosis. However, in contrast to the protective effect exerted by LPS, IGF-I only partially blocked the apoptosis induced by TGF-β (Table 1).

Modulation by TGF-β of NO Release in LPS-Activated Fetal Hepatocytes. The protective effect exerted by LPS over the TGF-β induced apoptosis is compatible with the delivery of a signal in LPS treated hepatocytes that rescues the cells from apoptosis, and a likely candidate for this role is NO synthesis. To establish the relevance of NO in the control of cell fate, distinct conditions that modulate NO synthesis in these cells were tested. Exposure of primary cultures of fetal hepatocytes to LPS promoted iNOS expression and NO synthesis, a process that was sustained for at least 36

TABLE 1. Effect of IGF-I on the Apoptosis Induced by TGF-β in Fetal Hepatocytes

Addition	Enrichment Factor	Fluorescence Ratio
None	0.62 ± 0.58	1.0
IGF-I, 0.1 ng/mL	0.58 ± 0.04	1.1 ± 0.1
IGF-I, 1 ng/mL	0.60 ± 0.04	1.2 ± 0.1
IGF-I, 5 ng/mL	0.55 ± 0.06	1.0 ± 0.2
TGF-β, 1 ng/mL	5.7 ± 0.6	12.4 ± 1.1
+ IGF-I, 0.1 ng/mL	5.1 ± 0.4	9.2 ± 0.8
+ IGF-I, 1 ng/mL	4.6 ± 0.5	10.1 ± 1.0
+ IGF-I, 5 ng/mL	4.7 ± 0.5	9.7 ± 0.7

NOTE. Primary cultures of fetal hepatocytes were treated for 18 hours with various concentrations of IGF-I and in the absence or presence of TGF-β. The enrichment in nucleosomal particles in the cytosol or the fluorescence of *in situ* TdT-labeled cells were determined. Results shows the means ± SEM of three experiments.

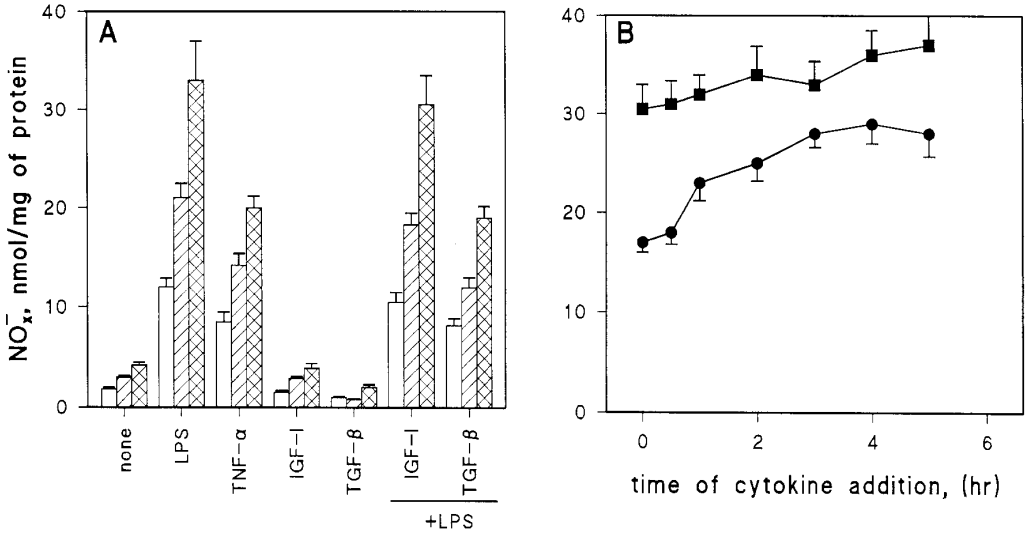

FIG. 2. NO synthesis in primary cultures of fetal hepatocytes. Cells were incubated with 0.5 μg/mL of LPS, 20 ng/mL of TNF-α, 1 ng/mL of TGF-β, 1 nmol/mL of IGF-I, or combinations of IGF-I plus LPS or TGF-β plus LPS. The production of nitrate plus nitrite was determined after 18 (□), 24 (▨) and 36 hours (☒) (A). The effect of 1 ng/mL of TGF-β (●) and 1 nM of IGF-I (■) on the NO release elicited by LPS after 36 hours of culture was determined as a function of the time after addition of the growth factors (B). Results show the means ± SEM of three experiments.

hours (Fig. 2A). In addition to LPS, TNF-α also promoted NO release to the medium, although this effect only represented a 55% of the response to LPS, therefore indicating that this proinflammatory cytokine cannot completely substitute for LPS in iNOS induction, at least in fetal hepatocytes. In contrast to this situation, TGF-β decreased the level of basal NO release, and when added simultaneously with LPS an important inhibitory effect (48% inhibition) was observed. Because fetal hepatocytes express the IGF-I receptor and IGF-I has also been reported to antagonize iNOS expression in several cell types,[29] the role of this growth factor in NO synthesis was investigated. As Fig. 2A shows, IGF-I by itself did not affect the basal synthesis of NO and failed to inhibit the NO synthesis significantly in response to LPS. Therefore, whereas TGF-β effectively antagonizes LPS-elicited NO release in fetal hepatocytes, IGF-I does not. To characterize the temporal pattern of the modulatory effect of TGF-β on LPS-induced NO synthesis, hepatocytes were incubated with LPS, and at different times TGF-β was added. As Fig. 2B shows TGF-β showed a biphasic effect on LPS-induced NO release; a marked inhibition was observed at times under 2 hours, whereas a 15% inhibition persisted when this cytokine was added after this period. As expected, IGF-I did not affect the NO release induced by LPS over a period of 5 hours after LPS stimulation. The inhibitory effect of TGF-β on NO synthesis well correlates closely with the levels of iNOS messenger RNA detected after 15 hours of culture (Fig. 3). A 44% decrease was observed when hepatocytes were treated with LPS and TGF-β simultaneously.

In addition to cytokines, NO can be released both intracellularly and extracellularly via chemical NO donors. Substances such as SIN-10 require hepatic metabolism to generate NO in the cell, whereas compounds such as SIN-1 or GSNO release NO in the absence of cellular metabolism. The NO release in primary cultures of hepatocytes treated with these substances was monitored as a function of time. Chemical NO-donors were administered to cells at 600 μmol/L, and as Fig. 4A shows, the rate of release of NO by GSNO was higher than by SIN-1, whereas SIN-10 displayed a progressive release lasting for at least 15 hours. These kinetics of NO production by NO donors allow the determination of the actual rate of NO synthesis in comparison with the amount of this substance produced in response to LPS at a given time and concentration. At the end of the incubation period (24 hours), samples were collected to analyze the DNA pattern, and as Fig. 4B shows, a clear and intense apoptosis was observed under these conditions. To determine whether a necrotic process accompanied the release of NO by NO donors, the presence of LDH in the incubation medium was assayed. As Fig. 4C shows, a striking parallelism between the rate of NO generation and the release of LDH to the medium was observed. In this respect, cells treated with GSNO released the highest LDH activity (27-fold with respect to the control), reflecting a more acute necrotic effect.

LPS Antagonizes the Apoptosis Induced by TGF-β. To examine the relative roles of NO and LPS on the protection against the apoptosis evoked by TGF-β, primary cultures of fetal hepatocytes were incubated in arginine-free Dulbecco's modified Eagle medium supplemented with 50 μmol/L arginine or in the presence of 0.8 mmol/L of L-nitromethylarginine. As Fig. 5A shows, in the presence of the iNOS substrate analogue inhibitor apoptosis is increased after LPS treatment and only a partial protection is observed when cells are treated with both LPS and TGF-β (83% versus 63% in the absence of NO synthesis, respectively). The concentration of nitrite plus nitrate under these conditions is shown in Fig. 5B. These results suggest that NO synthesis, in the range elicited after LPS stimulation protects against apoptosis. To investigate further the protective role of NO on the apoptosis evoked by TGF-β primary cultures of fetal hepatocytes were incubated

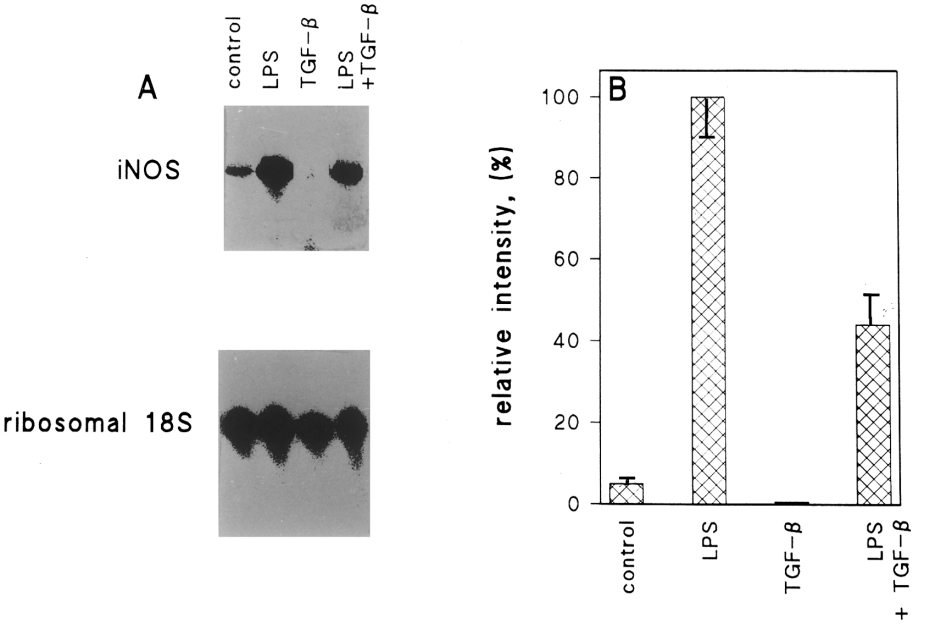

FIG. 3. iNOS expression in primary cultures of fetal hepatocytes. Fetal cells (3×10^6) were incubated for 15 hours with 0.5 μg/mL of LPS, 1 ng/mL of TGF-β, or a combination of both. The RNA was analyzed by Northern blot using a probe specific for the cytokine inducible isoenzyme. After normalization of the blot, the relative messenger RNA content was expressed as percentage of the LPS-treated cells' condition. Results show a representative experiment. (B) Means ± SEM of 3 experiments.

with various concentrations of the NO donor SIN-10 and in the presence of TGF-β. As Fig. 6A shows, an effective protection against DNA fragmentation was observed at low SIN-10 concentration (2-100 μmol/L), whereas deleterious effects were observed at concentrations higher than 200 μmol/L. These results were confirmed when the release of oligonucleosomes to the cytosol were quantified (Fig. 6B).

DISCUSSION

The use of primary cultures of hepatocytes constitutes a good experimental model for the study of the signaling pathways that control proliferation, growth arrest, and apoptotic cell death, and in general, close agreement exists between the results obtained using this *ex vivo* system and in intact liver. One example of this situation is the response to TGF-β that evokes apoptotic cell death both in liver and in cultured hepatocytes.[6,30] The ability of TGF-β to antagonize cytokine-induced hepatocyte proliferation and to induce apoptosis has been widely reported and discussed.[31,32] An important *in vivo* apoptosis has been observed in developing human liver (including hepatocytes), possibly reflecting remodeling events in the tissue.[33] However, when fetal hepatocytes are isolated and kept in culture the rate of apoptosis is quite low, probably because of the process of isolation and attachment to the plate. Indeed, hepatocytes can be maintained in culture for several days without significant changes in the number of cells.

The initial aim of this work was to investigate the effect of LPS, as an agonist that induces iNOS expression, on the viability of primary cultures of fetal hepatocytes. Previous results from our group indicated that NO synthesis could be involved in the regenerative process of liver after partial hepatectomy.[17] Several inde-

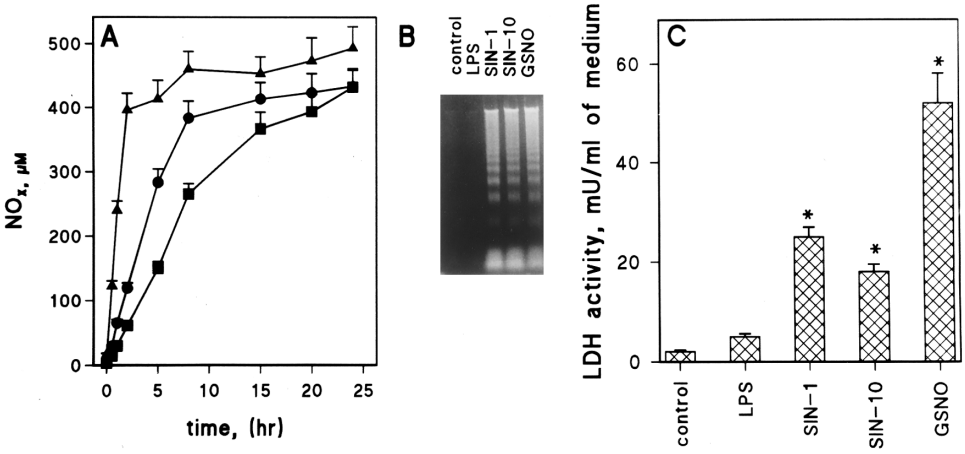

FIG. 4. Release of NO by chemical donors. Hepatocyte cultures were incubated with 600 μmol/L of GSNO (▲), SIN-1 (●) or SIN-10 (■), and at different times the concentration of nitrate and nitrite in the medium was assayed (A). After 24 hours of culture, the DNA fragmentation was analyzed in agarose gel electrophoresis (B). The release of LDH activity to the incubation medium was measured after 24 hours of culture with 0.5 μg/mL of LPS or with NO-donors (C). Results show the means ± SEM of three experiments (A and C), or a representative experiment (B).

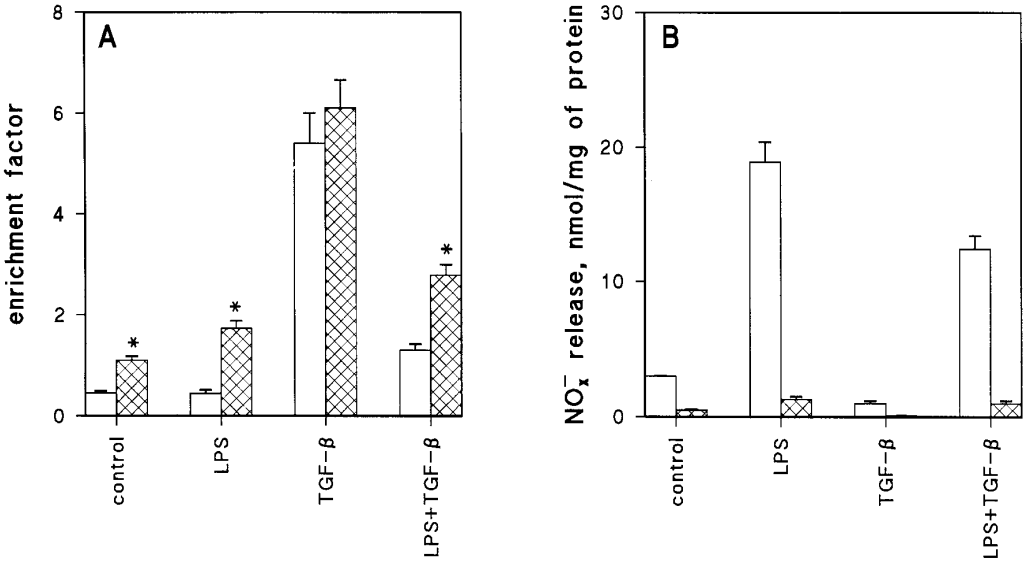

FIG. 5. Effect of NO synthesis inhibition on DNA fragmentation. Hepatocytes were incubated with Dulbecco's modified Eagle medium containing only 50 μmol/L arginine and in the absence (open bars) or presence of 0.8 mmol/L L-NMA (hatched bars) and stimulated with 0.5 μg/mL of LPS or 1 ng/mL of TGF-β. The release of nucleosomes to the cytosol was measured as the ratio between the A_{405} at 18 and 0 hours. The average A_{405} at 0 hours was 0.14 $\pm$ 0.02 Units per day (A). The synthesis of NO_x^- species was measured at the end of the culture period (B). *, $P < .01$ vs. in the absence of inhibitor.

pendent groups have shown that iNOS is both expressed and catalytically active after partial hepatectomy[17,34,35] and that inhibition of NO synthesis with arginine analogues results in a marked alteration in the pattern of ploidy characteristic of regenerating liver.[17,36] These results suggest that hepatic NO synthesis, at least in this experimental model, does not have a deleterious effect *per se* as is the case for instance in macrophage cell lines in which apoptosis follows after activation with LPS or inflammatory cytokines.[11,37] However, in primary cultures of hepatocytes our results show that the NO released after treatment of the cells with LPS does not promote DNA degradation and, interestingly, is sufficient to prevent the DNA fragmentation evoked by TGF-β. The protection against apoptosis exerted by NO once released after LPS triggering contrasts with the marked apoptotic and necrotic effects observed when delivered at high concentrations by means of chemical donors. These results confirm the data obtained using the RAW 264.7 macrophage cell line, in which addition of 1 mmol/L GSNO elicits an intense apoptosis that correlates with an up-regulation of p53 expression.[38] From our data, we have calculated that the maximal linear rate of NO release after LPS activation was about 9 to 11 nmol per hour and milligram of protein, which is equivalent to the rate of NO obtained in cells treated with 100 to 120 μmol/L SIN-10 under the same experimental conditions. In agreement with these data, concentrations of SIN-10 in the 10-100 μmol/L range also antago-

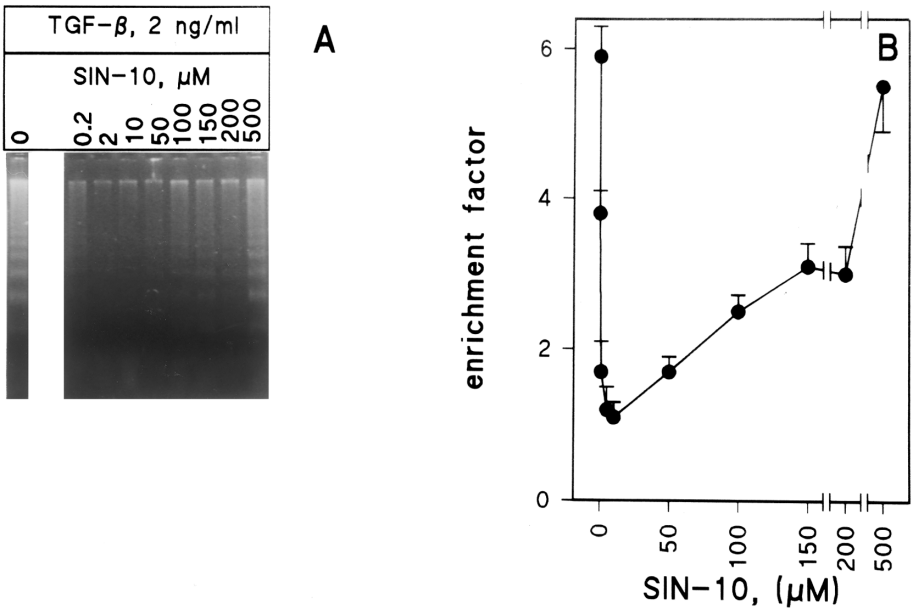

FIG. 6. Low concentrations of NO protect against the apoptosis elicited by TGF-β. Cultured fetal hepatocytes were treated for 18 hours with 1 ng/mL of TGF-β and the indicated concentrations of SIN-10. DNA was analyzed by agarose gel electrophoresis (A), and by the release of nucleosomes to the cytosol (B). Results show a representative experiment of DNA laddering and the means $\pm$ SEM of the enrichment in nucleosomes of the cytosol from three experiments.

nized the DNA fragmentation induced by 1 ng/mL of TGF-β.

The protective effect of LPS against TGF-β–mediated apoptosis seems to be caused by the engagement of distinct pathways, because in the absence of NO synthesis, only a partial protection is observed accounting for 63% of the maximal effect. Indeed, these results confirm previous observations suggesting that given an experimental model (e.g., macrophages) apoptosis can be achieved through different pathways; this is the case of resident peritoneal macrophages that display apoptotic features when incubated with LPS or with lipopeptides derived from the bacterial cell wall, regardless of NO synthesis.[37] A protective role of NO against apoptosis has been described in distinct cell types such as neutrophils and human and murine B lymphocytes.[12-14,39] However, the mechanisms involved in this anti-apoptotic effect of NO are still poorly characterized; in neural and human B lymphocytes, a control over the redox state has been proposed,[12,13] whereas in murine lymphocytes NO synthesis correlates with sustained levels of *Bcl*-2, therefore offering a possible anti-apoptotic effect through an up-regulation of the levels of this proto-oncogene.[14] In hepatocytes, the mechanism by which NO acts is more elusive because the identification of pathways intended to support hepatocyte protection against cell death is still in progress. The levels of *Bcl*-2 and *Bax* in hepatocytes have been assessed by immunohistochemistry and seem to be quite low.[40] However, different data suggest that a redox mechanism might be involved in the control of apoptosis, because treatment of hepatocytes with TGF-β suppresses the expression of antioxidizing enzymes such as Mn^{2+}-, Cu^{2+}-, and Zn^{2+}-superoxide dismutase, and catalase.[41] Moreover, substances that promote peroxisome proliferation also blocked the apoptosis evoked by TGF-β.[42] Signaling through LPS involves the engagement of CD14, and in liver, it has been shown that LPS activates among other Stat3 transcription factor, in a way similar to that elicited after EGF activation,[43] therefore suggesting an additional form of protection against apoptosis. We also investigated the role of IGF-I as a possible candidate for both NO synthesis modulation and apoptotic rescue. In fact, the antiapoptotic effects of IGF-I have been described in several systems; therefore, it offers an NO-independent protection.[45,46] However, to our surprise, not only did IGF-I not affect the release of NO elicited by LPS, but also the protection it gave against the apoptosis induced by TGF-β was minimal.

Finally, our results suggest an antiapoptotic role for NO in hepatocytes, and in this sense, this molecule might contribute to the complex signaling pathways involved in the dynamic control of cell growth and arrest in this tissue, which have been recently discussed in the context of liver regeneration.[46]

Acknowledgment: We thank Dr. I. Fabregat for fruitful discussions, O. G. Bodelón for technical support, D. A. Gilson for help in the preparation of the manuscript, and Dr. Q-w. Xie and C. F. Nathan for the generous gift of the iNOS complementary DNA probe.

REFERENCES

1. Michalopoulos GK. Liver regeneration: molecular mechanisms of growth control. FASEB J 1990;4:176-187.
2. Fausto N. Hepatic regeneration. In: Zakim D, Boyer TD, eds. Hepatology: a text book of liver disease. Philadelphia: Saunders, 1990:49-62.
3. Webber EM, Godowski PJ, Fausto N. In vivo response of hepatocytes to growth factors requires a priming stimulus. HEPATOLOGY 1994;14:489-497.
4. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine Rev 1993;14:133-151.
5. Chien KR, Abrams J, Serroni A, Martin JT, Farber JL. Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischemic liver cell injury. J Biol Chem 1978;4809-4817.
6. Oberhammer F, Bursch W, Parzefall W, Breit P, Erber E, Stadler M, Schulte-Hermann R. Effect of transforming growth factor β on cell death of cultured rat hepatocytes. Cancer Res 1991;51: 2478-2485.
7. Bursch W, Oberhammer F, Jirtle RL, Askari M, Sedivy R, Grasl-Kraupp B, Purchio AF, et al. Transforming growth factor-β1 as a signal for induction of cell death by apoptosis. Br J Cancer 1993;67:531-536.
8. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992; 356:314-317.
9. Hockenbery D. Defining apoptosis. Am J Pathol 1995;146:16-19.
10. Marx J. Cell death studies yield cancer clues. Science 1993;259: 760-761.
11. Albina JE, Cui S, Mateo RB, Reichner SJ. Nitric-oxide mediated apoptosis in murine peritoneal macrophages. J Immunol 1993; 150:5080-5085.
12. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, et al. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 1993;364:626-632.
13. Mannick JB, Asano K, Izumi K, Kieff E, Stamler JS. Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein-Barr virus reactivation. Cell 1994;79:1137-1146.
14. Genaro AM, Hortelano S, Alvarez A, Martínez-A. C, Boscá L. Nitric oxide rescues B cells from programmed cell death. J Clin Invest 1995;95:1884-1890.
15. Stark ME, Szurszewski JH. Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology 1992; 103:1928-1949.
16. Bomzon A, Blendis LM. The nitric oxide hypothesis and the hyperdynamic circulation in cirrhosis. HEPATOLOGY 1994;20:1343-1350.
17. Hortelano S, Dewez B, Genaro AM, Díaz-Guerra MJM, Boscá L. Nitric oxide is released in regenerating liver after partial hepatectomy. HEPATOLOGY 1995;21:776-786.
18. Curran RD, Billiar TR, Stuehr DJ, Hofmann K, Simmons RL. Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. J Exp Med 1989;170:1769-1774.
19. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, Billiar TR. Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci U S A 1993; 90:522-526.
20. deJuan C, Benito M, Fabregat I. Regulation of albumin expression in fetal rat hepatocytes cultured under proliferative conditions: role of epidermal growth factor and hormones. J Cell Physiol 1992;152:95-101.
21. de Juan C, Benito M, Alvarez A, Fabregat I. Differential proliferative response of cultured fetal and regenerating hepatocytes to growth factors and hormones. Exp Cell Res 1992;202:495-500.

22. Di Marco PN, Ghisalberti AV, Pearce PH, Oliver IT. Postnatal changes in blood glucose, phosphopyruvate carboxylase and tyrosine aminotransferase after normal birth and premature delivery in the rat. Biol Neonate 1976;30:205-215.
23. Martin-Sanz P, Cascales M, Bosca L. Glucagon-induced changes in fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase in cultured rat foetal hepatocytes. Biochem J 1989;257:795-799.
24. Hart, TW. Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. Tetrahedron Lett 1985;26:2013-2016.
25. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiociate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
26. Xie Q-w, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan CF. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256:225-228.
27. Bergmeyer HU, Graβl M, Walter H-E. In: Bergmeyer HU, ed. Methods of enzymatic analysis. Vol II, Ed 3. Weinheim: Verlag Chemie, 1984:232-233.
28. Kroemer G, Boscá L, Zamzami N, Marchetti P, Hortelano S, Martínez-A C. Detection of apoptosis and apoptosis associated alterations. In: Lefkovits I, ed. The immunology methods manual" 1995. NY: Academic Press,
29. Schini VB, Catovsky S, Schary UB, Busse R, Vanhoutte PM. Insulin-like growth factor I inhibits induction of nitric oxide synthase in vascular smoothe muscle cells. Circ Res 1994;74:34-32.
30. Oberhammer F, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. Proc Natl Acad Sci U S A 1992;89:5408-5412.
31. Kiso S, Kawata S, Tamura S, Ito N, Takaishi K, Shirai Y, Tsushima H, Matsuzawa Y. Alteration in growth regulation of hepatocytes in primary culture obtained from cirrhotic rat: poor response to transforming growth factor-beta 1 and interferons. HEPATOLOGY 1994;20:1303-1308.
32. Fausto N. Hepatocyte apoptosis: is transforming growth factor β-1 the kiss of death? HEPATOLOGY 1993;18:1536-1537.
33. Terada T, Nakanuma Y. Detection of apoptosis and expression of apoptosis-related proteins during human intrahepatic bile duct development. Am J Pathol 1995;146:67-74.
34. Obolenskaya MY, Vanin AF, Mordvintcev PI, Mülsch A, Decker K. EPR evidence of nitric oxide production by regenerating rat liver. Biochem Biophys Res Commun 1994;202:571-576.
35. Obolenskaya M, Schulze-Specking A, Plaumann B, Frenzer K, Freudenberg N, Decker K. Nitric oxide production by cells isolated from regenerating liver. Biochem Biophys Res Commun 1994;204:1305-1311.
36. Mossin L, Blankson H, Huitfeldt H, Seglen PO. Ploidy-dependent growth and binucleation in cultured rat hepatocytes. Exp Cell Res 1994;214:551-560.
37. Terenzi F, Díaz-Guerra MJM, Casado M, Hortelano S, Leoni S, Boscá L. Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. J Biol Chem 1995;270:6017-6021.
38. Meβmer UK, Ankarcrona M, Nicotera P, Brüne B. p53 expression in nitric oxide-induced apoptosis. FEBS Lett 1994;355:23-26.
39. Beauvais F, Michel L, Dubertret L. The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. FEBS Lett 1995;361:229-232.
40. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol 1994; 145:1323-1336.
41. Kayanoki Y, Fujii J, Suzuki K, Kawata S, Matsuzawa Y, Taniguchi N. Suppression of antioxidative enzyme expression by transforming growth factor β1 in rat hepatocytes. J Biol Chem 1994;269:15488-15492.
42. Bayly AC, Roberts RA, Dive C. Suppression of liver cell apoptosis in vitro by the non-genotoxic hepatocarcinogen and peroxidsome proliferator nafenopin. J Cell Biol 1994;125:197-203.
43. Ruff-jamison S, Zhong Z, Wen Z, Chen K, Darnell JEjr, Cohen S. Epidermal growth factor and lipopolysaccharide activate Stat3 transcription factor in mouse liver. J Biol Chem 1994;269:21933-21935.
44. Sell C, Baserga R, Rubin R. Insulin-like growth factor and the IGF-I receptor prevent etoposide-induced apoptosis. Cancer Res 1995;55:303-306.
45. Harrington EA, Bennett MR, Fanidi A, Evan GI. c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J 1994;13:3286-3295.
46. LaBrecque D. Liver regeneration: a picture emerges from the puzzle. Am J Gastroenterol 1994;89:S86-S96.