Expression of Inducible Nitric Oxide Synthase in Endotoxemic Rat Hepatocytes Is Dependent on the Cellular Glutathione Status

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The inducible nitric oxide synthase (iNOS) promoter contains nuclear factor κB (NF-κB) binding sites. NF-κB activation is determined, in part, by the intracellular redox status. The aim of this study was to determine the importance of the cellular glutathione status in relation to NF-κB activation and iNOS expression in hepatocytes *in vivo* and *in vitro*. For *in vivo* experiments, rats were injected with endotoxin and sacrificed 6 hours later. Glutathione was depleted by diethylmaleate. For *in vitro* experiments, cultured hepatocytes from untreated rats were exposed to a cytokine mixture. Glutathione levels were depleted by diethylmaleate and restored by N-acetylcysteine. iNOS expression was assessed by Western blot, reverse transcription polymerase chain reaction, nitric oxide (NO) metabolites, and immunohistochemistry. NF-κB binding was assessed by electrophoretic mobility shift assay. Endotoxin-induced iNOS expression in rat liver was prominent in hepatocytes, Kupffer cells, and inflammatory cells, in particular neutrophils. Glutathione depletion prevented iNOS induction in hepatocytes, but not in inflammatory cells. iNOS protein levels were in accordance with iNOS messenger RNA and NO metabolites in plasma. Glutathione depletion did not affect neutrophil infiltration. Cytokines strongly induced iNOS in cultured hepatocytes. Induction was prevented by glutathione depletion and could be restored by addition of N-acetylcysteine. NF-κB binding correlated with iNOS induction. In conclusion, in this study we show that iNOS induction in hepatocytes *in vivo* and *in vitro* is dependent on the intracellular glutathione status and correlates with NF-κB binding. Glutathione-depletion has no effect on the expression of iNOS in inflammatory cells, nor on neutrophil infiltration. (HEPATOLOGY 1999;29:421-426.)

Nitric oxide radicals (NO) are synthesized by the enzyme nitric oxide synthase (NOS). Three isoforms of this enzyme encoded by distinct genes are known.[1,2] The constitutive isoforms are neuronal NOS (type I) and endothelial NOS (type III). Neuronal NOS is involved in neurotransmission, whereas NO derived from endothelial NOS has antithrombotic and vasorelaxing properties. Inducible NOS (type II, iNOS) is not expressed under normal conditions, but is induced by cytokines and endotoxin (lipopolysaccharide [LPS]) in various cell types including hepatocytes, macrophages, smooth muscle cells, and chondrocytes.[3]

iNOS-derived NO is an important component of the nonspecific host defense against invading microbial agents.[4] It has been shown that mice lacking a functional iNOS gene are more susceptible to infection with *Staphylococcus aureus*[5] and *Leishmania major*.[6] Furthermore, inhibition of viral replication by NO is shown by many laboratories by using NO donors, inhibiting NO production by NOS-inhibitors, or by using iNOS knockout mice.[7]

To induce iNOS messenger RNA (mRNA), activation of the transcription factor nuclear factor-κB (NF-κB) is essential[8-12] although probably not sufficient[13,14] for full iNOS induction. To induce gene transcription, NF-κB must be translocated from the cytoplasm to the nucleus.[15] Nuclear translocation of NF-κB is triggered by changes in the redox state.[16-18] Therefore, the intracellular glutathione (GSH) status may be a key determinant for the capacity of cells to express iNOS. Indeed, it has been shown that GSH depletion prevents iNOS induction and/or NO production in response to cytokines in cultured rat hepatocytes,[19,20] in the J774 macrophage cell line,[21] and in cultured macrophages.[22] To our knowledge, neither the relevance of the intracellular GSH status for the induction of iNOS in the liver *in vivo* nor the contribution of NF-κB in the GSH-dependent iNOS expression has been reported.

The aim of this study was to investigate the importance of the intracellular GSH status for the capacity of hepatocytes and inflammatory cells to express iNOS in response to endotoxin and/or cytokines *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals and Experimental Design in Vivo. Specified pathogen-free male Wistar rats (200-250 g) were purchased from Harlan-CPB, Zeist, the Netherlands. They were kept under routine laboratory

Abbreviations: NO, nitric oxide radical; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; mRNA, messenger RNA; GSH, glutathione; IP, intraperitoneally; PBS, phosphate-buffered saline; DEM, diethylmaleate; ALT, alanine transaminase; AST, aspartate transaminase, NOx, nitrite plus nitrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assay; NAC, N-acetylcysteine.

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conditions at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals.

Rats were injected intraperitoneally (IP) with 5 mg/kg body weight endotoxin (LPS, *E. coli*, serotype 0127:B8, Sigma, St. Louis, MO) or the same volume of phosphate-buffered saline (PBS, control group, n = 7 for each experimental group). Five milligrams per kilogram is a sublethal dose, and no mortality was seen. GSH depletion was accomplished by administration of diethylmaleate (DEM, Sigma). DEM was dissolved in olive oil (1:1 vol/vol) and administered IP at a dose of 4 mmol/L/kg body weight 30 minutes before and 3 hours after LPS administration. Six hours after LPS administration, rats were anesthetized with pentobarbital (60 mg/kg, IP). Six hours after endotoxin-treatment was chosen because at this time-point iNOS protein induction is maximal and any inhibitory effects on iNOS expression would be most obvious at this time point. For determination of serum alanine transaminase (ALT) and aspartate transaminase (AST) activities and the NO metabolites nitrite plus nitrate (NOx), heparinized blood samples were obtained by cardiac puncture. The livers were perfused with PBS, removed, cut into small pieces, and snap-frozen in liquid nitrogen until further use. For routine histology, tissue specimens were fixed by immersion in 4% buffered paraformaldehyde and embedded in paraffin. For immunohistology, tissue specimens were frozen in isopentane.

Hepatocyte Isolation and Experimental Design in Vitro. Rat hepatocytes were isolated by two-step collagenase perfusion as described previously.[23] Hepatocytes were plated at a density of 100,000 cells per cm^2 in William's medium E and supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 mU/mL insulin (all from Life Technologies Ltd, Gaithersburg, MD). After 4 hours, the medium was changed and unattached cells were removed. Cells were used for iNOS induction 36 hours after attachment. At this time-point no indications of endotoxin-induced stimulation of hepatocytes were seen as evidenced by NF-κB activation, iNOS induction, and NOx accumulation. iNOS induction was achieved by adding a cytokine mixture to the hepatocytes composed of 20 ng/mL human recombinant interleukin-1β (R&D Systems, Abingdon, United Kingdom), 500 U/mL recombinant mouse tumor necrosis factor (Genzyme, Cambridge MA), 100 U/mL recombinant rat interferon-γ (Life Technologies Ltd) and 10 μg/mL LPS. Ten hours after the addition of cytokines, media were collected and the cell layer was harvested in distilled water containing 0.2 mmol/L PMSF and subsequently stored at −20°C. GSH was depleted with use of DEM. GSH levels were restored by addition of N-acetylcysteine (NAC, Sigma). DEM (1 mmol/L) and NAC (5 mmol/L) were added to the hepatocyte cultures 30 minutes before the cytokine mixture. DEM addition was repeated 5 hours after addition of the cytokines. Each experimental condition was performed in duplicate wells. Cell lysates were subjected to Western blotting, RNA isolation, and GSH determination as described later. Parallel experiments were performed in triplicate for determination of NF-κB activation. In these experiments, cell layers were used for preparation of nuclear extracts 2 hours after addition of cytokines. Each experiment was repeated twice, with use of hepatocytes from different isolations.

Antibodies. The rabbit polyclonal antibody against rat iNOS was developed in our laboratory and has been described before.[24] The His48 monoclonal antibody, which specifically recognizes rat neutrophils, was developed at our institute and has been described before.[25] All peroxidase-conjugated antibodies were from DAKO A/S (Glostrup, Denmark).

Western Blot Analysis. Western blot analysis was performed exactly as described previously.[24]

Immunohistochemistry. For immunohistochemistry, 4μm cryostat sections were cut. After air-drying, sections were fixed in acetone for 10 minutes at room temperature and air-dried again. Subsequently, they were washed in PBS (pH 7.4) and incubated in the rat polyclonal iNOS antibody (1:300) in PBS containing 1% bovine serum albumin (BSA) or in undiluted His48 hybridoma supernatant for 60 minutes at room temperature. After incubation with the first antibody, endogenous peroxidase activity was blocked by incubating for 30 minutes in PBS containing 0.075% H_2O_2. For iNOS, secondary antibodies were peroxidase conjugated goat anti-rabbit antibody (1:50) and peroxidase conjugated rabbit anti-goat antibody (1:50). For His48, secondary antibodies were peroxidase conjugated rabbit anti-mouse antibody (1:50) and peroxidase conjugated goat anti-rabbit antibody (1:50). These antibodies were applied in PBS containing 1% BSA and 5% normal rat serum. The sections were finally incubated with filtered 3-amino-9-ethylcarbazole (10 mg/2.5 mL dimethylformamide in 50 mL 0.1 mol/L acetate buffer pH 5.0) containing 0.03% H_2O_2 for 10 minutes at room temperature. Counterstaining was performed with hematoxylin, and the slides were covered with Kaiser's glycerin-gelatin. After each incubation, the sections were rinsed with PBS. The number of His48-positive cells was analyzed blind, with use of an Olympus BX50 microscope (Olympus, Japan) equipped with a Sony DXC-950p camera (Sony, Japan) and then processed by an image analysis system (QWin version 2.0, Leica Imaging Systems, Cambridge, United Kingdom). Four microscopic fields per cryostat section were counted from four different rat livers per experimental group.

GSH Determination. Total GSH (the sum of oxidized and reduced GSH) in liver homogenates and cell lysates was determined by the enzymatic recycling procedure by using GSH reductase and 5,5′-dithiobis 2-nitrobenzoic acid as described by Griffith.[26] Values were normalized for protein content of the samples.

Determination of iNOS and GAPDH mRNA Expression. RNA was isolated from snap-frozen liver tissue (*in vivo*) and cell layers (*in vitro*) by using Trizol reagent (Life Technologies Ltd) according to manufacturer's instructions. RNA was reverse-transcribed into complementary DNA by using the Promega Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer's manual. Polymerase chain reaction (PCR) on complementary DNA was performed by using primers specific for rat iNOS[27] (sense: 5′-CGA GGA GGC TGC CCT GCA GAC TGG-3′; antisense: 5′-CTG GGA GGA GCT GAT GGA GTA GTA-3′), resulting in an amplified product of 1,383 base pairs (bp). Primers specific for glyceraldehyde-3-phosphate dehydrogenase[28] (GAPDH, sense: 5′-CCA TCA CCA TCT TCC AGG AG-3′; antisense: 5′-CCT GCT TCA CCA CCT TCT TG-3′), resulting in an amplified product of 576 bp, were used as control for the RT-PCR procedure. The tubes were incubated in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT) at 95°C for 5 minutes to denature the primers and complementary DNA. The cycling program was 95°C for 40 seconds, 60°C for 40 seconds, 72°C for 40 seconds, and for 1 minute in the last cycle, and comprised 30 cycles for both primer sets. Taq polymerase was from Pharmacia Biotech, Uppsala, Sweden.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts of cultured hepatocytes were prepared by using a final concentration of Nonidet P-40 of 0.25%. EMSA for NF-κB were performed as described previously.[29] Equal amounts of nuclear extract protein were used for EMSA. As controls, competition experiments were performed with excess nonlabeled NF-κB probe and an excess unrelated nonlabeled probe (AP-1), as well as super-shift assays, by using specific antibodies against the NF-κB p50 and p65 subunits (Santa Cruz Biotechnology, Santa Cruz, CA).

Analytical Procedures. ALT and AST were determined by routine clinical chemistry. NOx concentrations in plasma and media were measured according to Moshage et al.[30]

Statistical Analysis. The data resulting from each experimental group were expressed as the mean ± SEM. Statistical analysis was performed by using two-way–analysis of variance.

RESULTS

Effect of GSH Depletion on iNOS Protein and mRNA Expression in Rat Liver After LPS Induction. As shown by Western blotting, normal rat liver does not contain iNOS (Fig. 1). Six hours after LPS administration (5 mg/kg, IP), iNOS protein was strongly induced. The GSH levels of LPS-treated rat liver were 94% compared with normal rat liver, whereas the GSH levels of DEM-treated and LPS + DEM–treated rat livers were 25% and 21%, respectively. GSH depletion was accompanied by a strongly reduced iNOS expression in endotoxemic rat liver (Fig. 1). The changes in iNOS protein were regulated at the level of gene transcription (Fig. 2). iNOS mRNA is undetectable in control liver and induced after LPS administration. GSH depletion almost completely abolished iNOS mRNA expression in livers of LPS-treated rats. Under all experimental conditions, RT-PCR for the house-keeping gene GAPDH resulted in a clear signal (Fig. 2). iNOS induction was accompanied by a significant increase in the plasma NOx concentration (Fig. 3). In accordance with iNOS protein and mRNA expression, GSH depletion resulted in significantly reduced plasma NOx concentrations in GSH-depleted endotoxemic rats compared with endotoxemic rats without GSH depletion (Fig. 3). However, NOx concentrations in plasma of GSH-depleted endotoxemic rats were still significantly increased compared with nonendotoxemic rats (Fig. 3). AST and ALT levels were significantly ($P < .05$) increased in LPS-treated rats (AST: 261 ± 53; ALT: 219 ± 66 U/L) and GSH-depleted endotoxemic rats (AST: 356 ± 61U/L; ALT: 196 ± 49 U/L) compared with control rats (AST: 80 ± 9 U/L; ALT: 43 ± 1 U/L) but not significantly increased in DEM-treated rats (AST: 165 ± 19 U/L; ALT: 61 ± 3 U/L).

Immunohistochemistry. Liver tissue of control animals showed a normal architecture and no inflammatory cells in the portal tracts nor in the parenchyma. LPS-treated rats displayed moderate inflammatory activity with sporadic acidophilic necrotic hepatocytes (data not shown). DEM-treatment did not affect the inflammatory activity in LPS-treated rats (data not shown). In accordance with the Western blot results, no iNOS staining was observed in sections of normal rat liver (Fig. 4A). Six hours after LPS administration, iNOS staining is prominent in hepatocytes, Kupffer cells, and inflammatory cells (Fig. 4B). The reduced expression of iNOS observed in GSH-depleted endotoxemic rats was a result of a complete absence of iNOS expression in hepatocytes. Expression of iNOS in inflammatory cells was not compromised by GSH depletion (Fig. 4C). Few infiltrating neutrophils are detected in normal rat liver, as shown by His48 staining (Fig. 4D). Six hours after LPS-treatment, massive infiltration of neutrophils into the liver parenchyma is observed (Fig. 4E), which is not affected by GSH depletion (Fig. 4F).

Neutrophil infiltration was quantified by counting the number of His48-positive cells per microscopic field. Control sections as well as sections of DEM-treated animals displayed only a few neutrophils per microscopic field (12 ± 4 and 17 ± 2, respectively). LPS-treatment led to a significant increase of neutrophil infiltration (201 ± 19), which was not significantly influenced by glutathione depletion (203 ± 22).

Double staining revealed that the majority of neutrophils (90%) were positive for iNOS 6 hours after LPS administration. This pattern was not changed by GSH depletion.

iNOS Expression in Cultured Rat Hepatocytes. To gain more insight in the GSH-dependent expression of iNOS, studies were performed with cultured rat hepatocytes. iNOS expres-

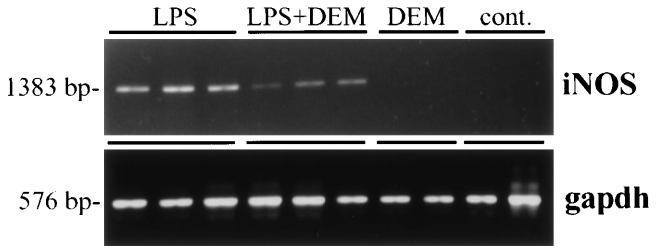

FIG. 2. iNOS and GAPDH mRNA expression in LPS-treated rat liver (LPS, n = 3), GSH-depleted, LPS-treated rat liver (LPS + DEM, n = 3), GSH-depleted rat liver (DEM, n = 2), and normal rat liver (cont., n = 2). mRNA levels were determined by RT-PCR as described in Materials and Methods. Treatments are indicated above the figure. Representative RT-PCR reactions from n = 7 per experimental group are shown.

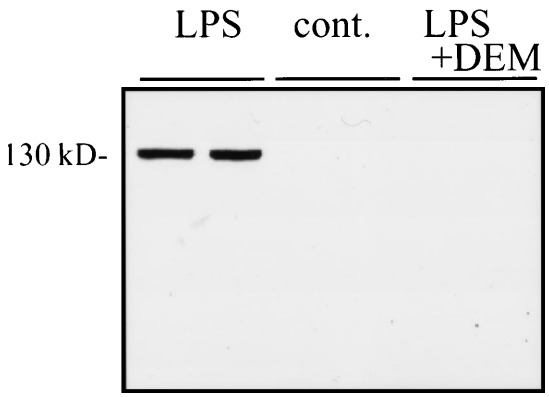

FIG. 1. iNOS protein level in LPS-treated rat liver (LPS), normal rat liver (cont.), and GSH-depleted, LPS-treated rat liver (LPS + DEM). Crude cell lysates (50 μg) were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and immunostained with antiserum against iNOS (dilution 1:2,000). Two representative rat livers from n = 7 per experimental group are shown.

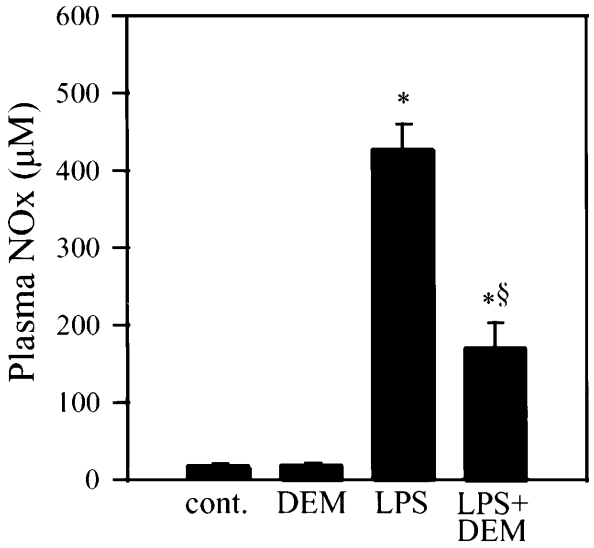

FIG. 3. NOx levels in plasma of control rats (cont.), GSH-depleted rats (DEM), LPS-treated rats (LPS), and GSH-depleted, LPS-treated rats (LPS + DEM). *, significant from control rats and DEM-treated rats ($P < .01$); §, significant from LPS-treated rats ($P < .01$). The data represent mean ± SEM (n = 7).

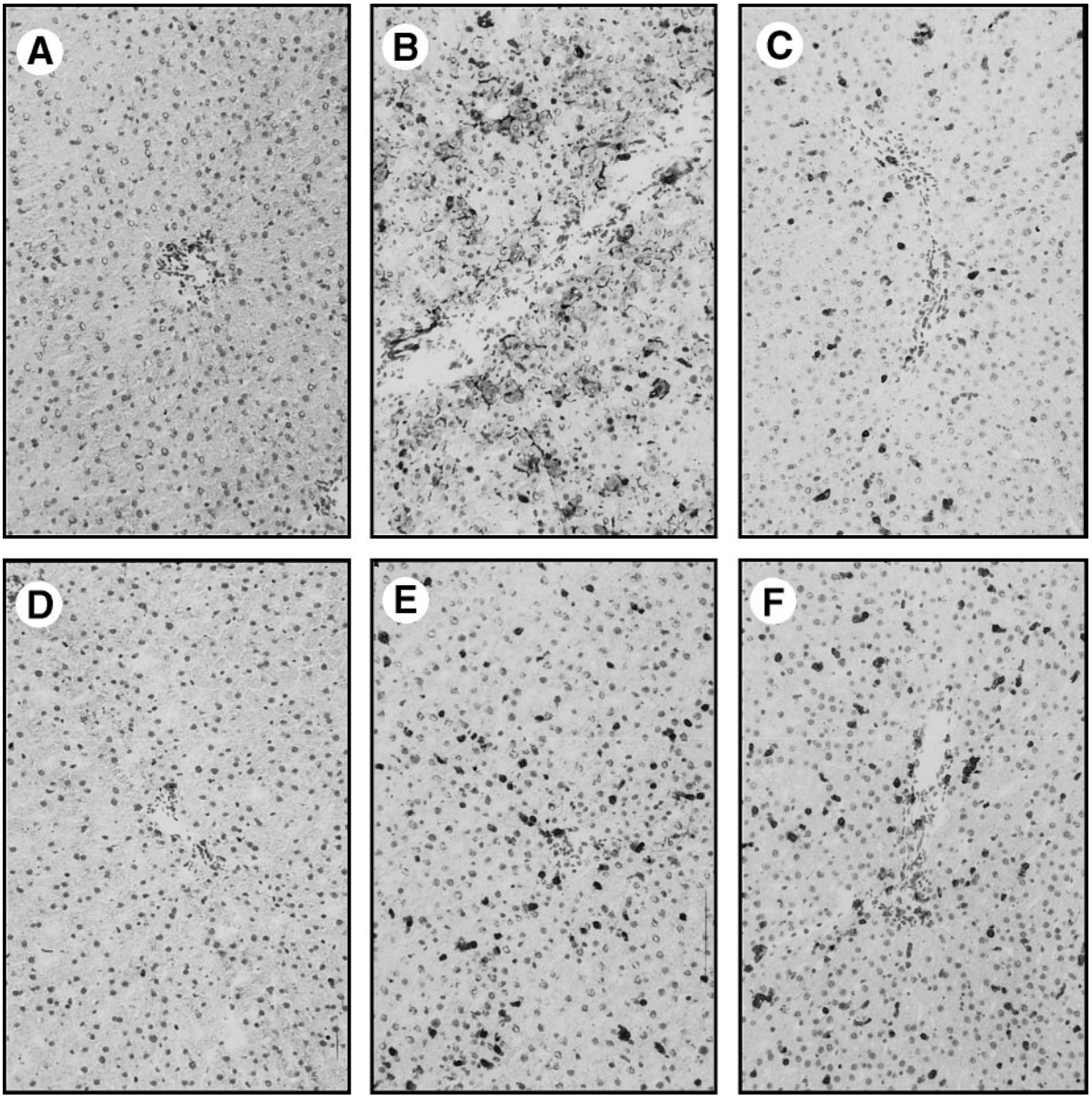

FIG. 4. Immunohistochemistry of normal rat liver, LPS-treated rat liver and GSH-depleted LPS-treated rat liver. (A, B, and C) Distribution of iNOS; (D, E, and F) distribution of His48-positive neutrophils. Normal rat liver, panel A and D; LPS-treated rat liver, B and E; GSH-depleted, LPS-treated rat liver, C and F.

sion was strongly induced in cultured rat hepatocytes after addition of cytokines (Fig. 5). DEM effectively depleted GSH levels both in control and cytokine-stimulated hepatocytes to 16% to 20% compared with GSH levels in control hepatocytes. GSH levels were completely restored by treatment with NAC (80%-120% compared with control levels). Cytokine-induced expression of iNOS was not detectable in GSH-depleted hepatocytes (Fig. 5). The inability of GSH-depleted hepatocytes to express iNOS was reversed by the addition of the GSH precursor NAC (Fig. 5). As shown in Fig. 6, iNOS protein levels were in accordance with iNOS mRNA expression: iNOS mRNA was not detected in nonstimulated hepatocytes, was strongly induced in cytokine-stimulated hepatocytes, and GSH depletion almost completely abolished iNOS mRNA expression in cytokine-stimulated hepatocytes. The expression of iNOS mRNA in GSH-depleted, cytokine-stimulated hepatocytes was restored by NAC (Fig. 6). The

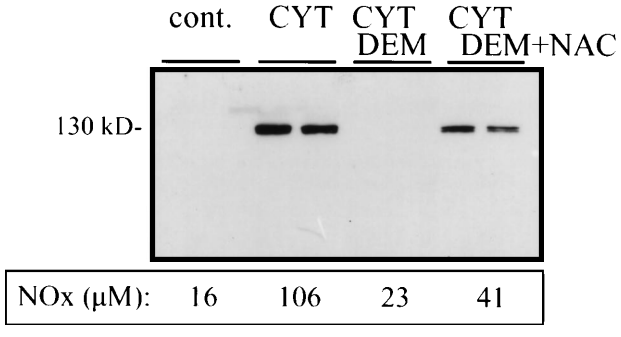

FIG. 5. iNOS protein and NOx levels in cultured rat hepatocytes. Crude cell lysates (50 μg) were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and immunostained with antiserum against iNOS (dilution 1:2,000). Treatments are indicated above the Western blot (cont., control; CYT, cytokine mixture; DEM, diethylmaleate; NAC, N-acetylcysteine), NOx levels in medium below. Each experimental condition was performed in duplicate wells.

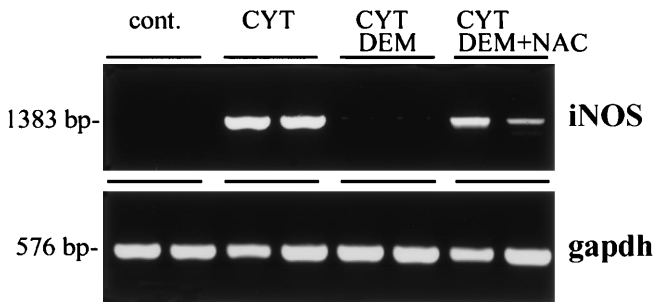

FIG. 6. iNOS and GAPDH mRNA expression in cultured hepatocytes. mRNA levels were determined by RT-PCR as described in Materials and Methods. Treatments are indicated above the figure (cont., control; CYT, cytokine mixture; DEM, diethylmaleate; NAC, N-acetylcysteine). Each experimental condition was performed in duplicate wells.

expression levels of iNOS in cultured hepatocytes correlated very well with NOx concentrations in culture media (Fig. 5).

NF-κB Activation. Because induction of iNOS gene expression is predominantly mediated by the transcription factor NF-κB, the relationship between iNOS gene expression, GSH status and NF-κB activation was investigated. As shown in Fig. 7, NF-κB was not activated in any of the control conditions. Stimulation by cytokines resulted in NF-κB activation, which was completely prevented by GSH depletion and restored by addition of NAC. The NF-κB EMSA signal disappeared by addition of an excess nonlabeled NF-κB probe (Fig. 7, lane 1), but remained unchanged in the presence of an excess nonlabeled unrelated probe (AP-1, Fig. 7, lane 2). Moreover, the presence of the NF-κB p50 (Fig. 7, lane 3) and p65 (Fig. 7, lane 4) subunits were shown by supershift assays by using specific antibodies against these subunits.

DISCUSSION

In primary cell cultures and cell lines it has been shown that the induction of iNOS by cytokines is dependent on the cellular GSH status. In this study, we show that this phenomenon also holds up for hepatocytes *in vivo*.

Six hours after endotoxin administration, iNOS expression is maximally induced in hepatocytes, Kupffer cells, and inflammatory cells, notably neutrophils. Double staining revealed that most neutrophils (90%) were also iNOS-positive.

In GSH-depleted endotoxemic rats, iNOS protein and iNOS mRNA content in liver tissue were strikingly diminished as were plasma NOx levels compared with endotoxemic rats not subjected to GSH depletion. This was because of a decreased expression of iNOS in hepatocytes. GSH depletion did not compromise iNOS expression in inflammatory cells (neutrophils and Kupffer cells), nor did it affect neutrophil infiltration. Therefore, the NOx present in plasma of GSH-depleted endotoxemic rats is most likely derived from NO produced by iNOS-positive neutrophils and Kupffer cells in the liver and/or iNOS-positive cells from extrahepatic sites. These results were confirmed by *in vitro* experiments with use of primary cultures of rat hepatocytes. The effect of GSH-depletion on iNOS induction and NO production could be reversed by the GSH precursor NAC. Although we cannot exclude the possibility that DEM does not deplete GSH in neutrophils *in vivo*, we consider this possibility unlikely because DEM has been used successfully for the purpose of GSH depletion in neutrophils *in vitro*.[31,32]

Why is GSH essential to induce iNOS in hepatocytes and why not for the induction of iNOS in inflammatory cells? iNOS induction is controlled to a large extent by NF-κB activation. Several studies have shown that inhibition of NF-κB prevents the induction of iNOS. However, the regulation of NF-κB activation may be cell-type specific. For example, dexamethasone inhibits NF-κB activation and iNOS expression in cultured rat hepatocytes,[33] but has no effect on LPS-induced NF-κB activation in rat Kupffer cells.[34] In addition to a cell type specific response of NF-κB activation to anti-inflammatory agents or antioxidants, there might also be a cell-type specific response to LPS/cytokines. This is supported by a study of Freedman et al.[35] reporting on constitutive expression of NF-κB in Kupffer cells and LPS-induced activation of NF-κB in hepatocytes. A differential regulation of NF-κB activation by GSH, or more general, antioxidants, in inflammatory cells (neutrophils and Kupffer cells) and hepatocytes, and thereby a differential regulation of iNOS induction would explain our finding that GSH depletion prevents iNOS induction in hepatocytes but not in inflammatory cells.

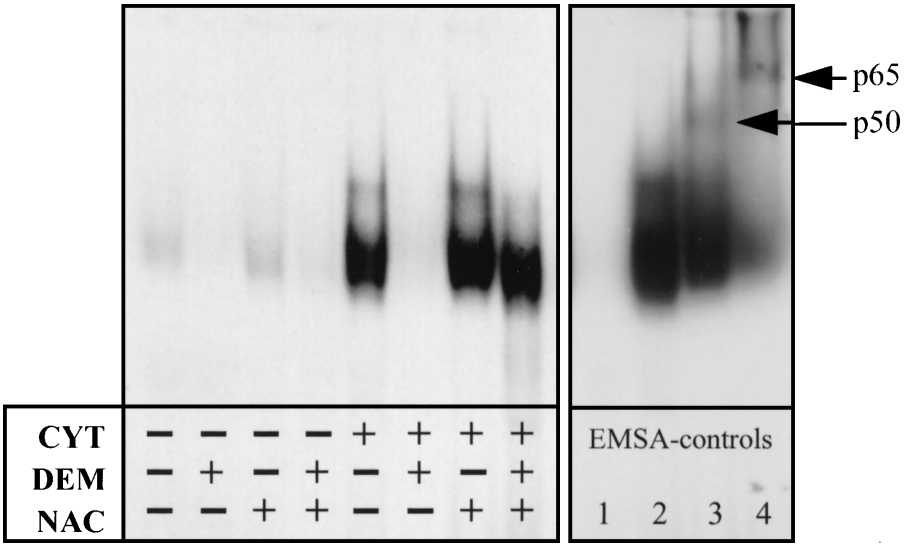

FIG. 7. NF-κB Electrophoretic mobility shift assay. EMSA was performed as described in Materials and Methods. Additions to the hepatocyte medium are indicated: (−) absent from medium, (+) present in medium. One representative result of n = 3 per experimental group is shown. As EMSA-controls, competition experiments were performed with an excess non-labeled NF-κB probe (*lane 1*) and an excess unrelated nonlabeled AP-1 probe (*lane 2*). Moreover, supershift assays were performed by using antibodies against the NF-κB p50 subunit (*lane 3*) and p65 subunit (*lane 4*).

Although there is a strong correlation between NF-κB activation, cellular GSH status and iNOS expression, we can not rule out the possibility that other transcription factors are required for iNOS expression.

The differential iNOS regulation may be of importance considering the role of inflammatory cells as protecting cells. Their first priority is their role in the host defense, and this requires full iNOS activation, independent of the cellular GSH status. In contrast, hepatocytes are the cells that need protection. They have the ability to produce NO, but their main function is the maintenance of metabolic processes. Thus, when fully repleted with glutathione, they have the ability to mount some defense against, for example, invading microorganisms, but when the redox state of hepatocytes becomes compromised, their first priority is the maintenance of liver metabolism for which all available GSH is needed.

Our results suggest that depletion of cellular GSH stores, which occurs during aging, starvation, alcohol abuse, viral infections, and chronic liver diseases, reduces the ability of the hepatocyte to produce NO and therefore contributes to the impairment of the host defense.

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