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# ETHANOL AND LPS MODULATE NF-KAPPAB ACTIVATION, INDUCIBLE NO SYNTHASE AND COX-2 GENE EXPRESSION IN RAT LIVER CELLS *IN VIVO*

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## 1. ABSTRACT

Ethanol and LPS are immunomodulators, whose actions are associated with the activation of the transcription factor, NF-kappaB, that mediates the expression of a number of rapid response genes involved in the whole body inflammatory response to injury, including transcriptional regulation of iNOS and COX-2. We investigated modulation by acute ethanol (EtOH) intoxication, LPS and LPS tolerance of NF-kappaB activation in hepatocytes, Kupffer cells and sinusoidal endothelial cells (SEC), concurrent regulation of iNOS and COX-2 gene expression and the influence of gender on these mechanisms. *In vivo* EtOH alone or with LPS significantly activates NF-kappaB in Kupffer cells and SEC. iNOS gene expression in these cells is modulated by LPS+EtOH in a gender- dependent manner. Acute EtOH administration enhanced iNOS mRNA in hepatocytes and Kupffer cells.LPS tolerance decreased LPS-induced NF-kappaB activation in Kupffer cells, but markedly raised iNOS mRNA in all three cell types with gender differences (females being higher). In LPS tolerant rats EtOH decreased elevated iNOS mRNA in all cells studied. LPS tolerance significantly reduced LPS-induced COX-2 mRNA in SEC, but only moderately in Kupffer cells of females, and not at all in males. Since NO is a known scavenger of superoxide and therefore protective against oxidative injury associated with LPS and acute EtOH intoxication, the gender differential effect of LPS+EtOH on iNOS gene expression (reduced only in females) may contribute to the greater susceptibility of females to alcoholic liver disease. Suppression of COX-2 gene

expression in SEC may cause detrimental effects in the hepatic microcirculation, associated with cirrhosis.

## 2. INTRODUCTION

Nuclear factor-kappaB (NF-kappaB) plays an important role in regulating the expression of a variety of rapid response genes involved in the coordinated response to inflammation and sepsis (1). Alcohol abuse is associated with suppression of some immune and host defense mechanisms (2-4), and activation of some aspects of phagocytic cell functions, e.g. superoxide anion generation (5). Reactive oxygen species, in turn are thought to contribute to the activation of NF-kappaB (6). Acute ethanol treatment in vitro was reported to cause increased induction of the inhibitory, p50/p50 NF-kappaB homodimer and no induction of the p50/p65 transactivating heterodimer in human monocytes (7). Acute exposure of Kupffer cells to 100 mM ethanol in vitro was reported to lead to inhibition of LPS-mediated NF-kappaB activation. Administration of Salmonella enteritides endotoxin to male Fischer rats induced transient activation of NF-kappaB in the whole liver and one hour after the endotoxin treatment NF-kappaB was activated in all isolated liver cells (8).

Activation of NF-kappaB by pro-inflammatory mediators mediates transcriptional regulation of inducible nitric oxide synthase (iNOS) in rodent cells (9). iNOS is primarily regulated by transcriptional mechanisms (10), and iNOS expression leads to production of large amounts of nitric oxide (NO), an important effector molecule in sepsis and inflammatory conditions (11). Hepatic NO production is modulated by ethanol (EtOH) and LPS tolerance in a gender dependent manner (12). Furthermore, several aspects of the hepatic immune response (e.g. neutrophil infiltration, phagocytic activity of recruited neutrophils, TNF release by Kupffer cells, cytokine-induced neutrophil chemoattractant generation ) in EtOH- and/or LPs-treated rats is influenced by gender (13).

Stimulation of a wide variety of cell types with LPS, cytokines and other inflammatory stimuli results in the rapid induction of both iNOS and COX-2 (cyclooxygenase-2) (11,14,15). In cells expressing both inducible PGHS (prostaglandin endoperoxide synthase) and NOS, NO potentiates the formation of prostaglandins (16,17). Evidence has emerged recently showing that NF-kappaB is also involved in the induction of COX-2 expression in response to LPS in human and mouse macrophages (18,19).

In the present study we investigated *in vivo* LPS-mediated NF-kappaB activation in parenchymal and nonparenchymal liver cells, and the effect of acute ethanol (EtOH) administration and LPS tolerance on this process. We also examined the concurrent modulation of iNOS and COX-2 gene expression in Kupffer and sinusoidal endothelial cells and the influence of gender on these mechanisms.

## 3. MATERIALS AND METHODS

### 3.1. Animals

Male (300-330gm BW) Sprague-Dawley rats from Hilltop Lab, Inc. (Scottdale, PA) were given an i.v. bolus (1 mg/kg) of *Escherichia coli* endotoxin (lipopolysaccharide, LPS) injection. Endotoxin (*E. coli*:055:B5) prepared according to the Boivin method was from Difco, (Detroit, MI). Some rats were implanted with a jugular venous and carotid arterial catheter the day before experiments involving EtOH infusion, using aseptic surgical techniques. The rats were then fasted overnight with free access to water. The next morning the animals were infused intravenously with EtOH for 1, 3 or 5 hrs reaching a blood alcohol level of about 150-170mg/dl.

For the EtOH infusion, the rats received a bolus (20% v/v in saline, intravenously) at a dose of 1.75g/k body weight followed by a continuous infusion at a rate of 300 mg/kg/h. When both LPS and EtOH were administered, LPS was injected first, followed immediately by EtOH infusion. Acute endotoxin (LPS) tolerance was induced by i.v. injection of *E. coli* LPS (0.5 or 0.45 mg/kg) 48 hrs before the challenge dose of LPS (3 mg/kg, i.v.) (LPS-LPS group). Nontolerant controls received two saline injections 48 hours apart (sal-sal group). All animals received humane care, conforming to the guidelines of the National Institutes of Health.

### 3.2. Cell isolation

At 1, 3 or 5 h of LPS and/or EtOH treatment hepatocytes, Kupffer cells and sinusoidal endothelial cells were prepared by collagenase digestion followed by elutriation, as previously described (20).

### 3.3. Nuclear protein extraction and EMSA

Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) were performed as described by Essani *et al* (8). The gels were visualized by an AMBIS radioanalytic imaging system.

For competitive binding studies a 40-fold excess of unlabeled nucleotide was added 5 min before the addition of the radiolabeled probe. For supershift studies the samples were incubated at room temperature in the presence of the specific antibodies for 2 h prior to the addition of the radiolabeled probe.

### 3.4. iNOS and COX-2 mRNA analysis

For analysis of iNOS-mRNA, isolated hepatocytes, Kupffer and endothelial cells were plated on 35 mm 6 well tissue culture plates (Costar, Cambridge, MA). Cell densities were 1 x $10^6$ cells/3ml/well for hepatocytes, and 3 x $10^6$ cells/2.5 ml/well for Kuffer and endothelial cells.

mRNA of iNOS was measured by the reverse transcription polymerase chain reaction (RT-PCR). Total RNA from cells was isolated by TRIzol reagent (Gibco/BRL, Bethesda, MD) following the manufacturer's protocol. Total RNA was quantitated spectrophotometrically, measuring OD at 260 mm wavelength. The concentration of RNA was calculated based on the following formula: RNA (microg/ml) = $A_{260}$ x 40 x dilution factor). Reverse transcription was carried out in 40 microl reactions with 10 microg total RNA and a final concentration of 500 pM random hexamer as primer,

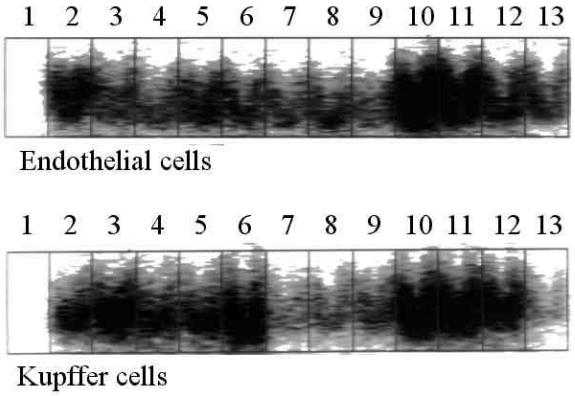


**Figure 1.** Electrophoretic mobility shift assay (EMSA) to demonstrate NF-kappaB activation in sinusoidal endothelial and in Kupffer cells of male and female rats 1 and 3 h after an *E. coli* LPS i.v. bolus (1 mg/kg) injection, or EtOH infusion, or a combination of LPS injection + EtOH infusion. Details of the procedures are given in the Materials and Methods section. Lane 1: probe; Lane 2: male LPS 1 hr; Lane 3: female LPS 1 hr; Lane 4: male LPS 3 hr; Lane 5: female LPS 3 hr; Lane 6: male EtOH 1 hr; Lane 7: female EtOH 1 hr; Lane 8: male EtOH 3 hr; Lane 9: female EtOH 3 hr; Lane 10: male LPS+EtOH 1 hr; Lane 11: female LPS+EtOH 1 hr; Lane 12: male LPS+EtOH 3 hr; Lane 13: female LPS+EtOH 3 hr.

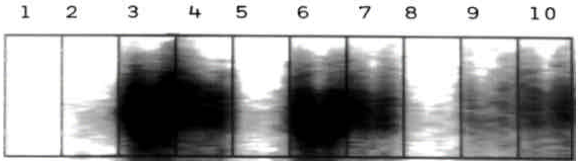


**Figure 2**. EMSA demonstration of levels of NF-kappaB activation in hepatocytes, endothelial and Kupffer cells of male rats after 1 h of LPS or EtOH treatment and after 5 h of EtOH treatment. Lane 1:Probe; Lane 2: LPS 1 Hr (Hepatocytes) H; Lane 3: LPS 1 Hr (Endothelial cells) E; Lane 4: LPS 1 Hr (Kupffer cells) K; Lane 5: EtOH 1 Hr (H); Lane 6: EtOH 1 Hr (E); Lane 7: EtOH 1 Hr (K); Lane 8: EtOH 5 Hr (H); Lane 9: EtOH 5 Hr (E); Lane 10: EtOH 5 Hr (K).

dNTPs (1 mM) (Pharmacia, Inc., Gaithesburg, MD), PCR buffer (50 mM KC1, 25 mM $MgCl_2$ and 10 mM Tris, pH8.3), dithiothreitol (1 mM), RNase inhibitor (20 units) and Moloney Murine Leukemia Virus-Reverse Transcriptase (400 units) (Gibco/BRL, Bethesda, MD). The same amount of cDNA was then analyzed and amplified using PCR with primers specific for murine-inducible NOSII. The primer sequences were as follows: NOSII-A 5'-AATGGCAACATCAGGTCGGCCATCACT-3', and NOSII-B: 5'-GCTGTGTGTCACAGAAGTCTCGAACTC-3'. The PCR product was quantified by electrophoresing an aliquot of the reaction mixture on a denaturing polyacrylamide gel and scanning the gel on a PhorsphorImager (Molecular Dynamics, Mountain View, CA). The volume of density (units obtained from the PhosphorImager) was used to evaluate the amount of cDNA for iNOS.

For COX-2 mRNA measurement freshly isolated Kupffer and endothelial cells were used. The procedures were the same as for iNOS mRNA, except for the primers specific for COX-2, which were as follows: COX-2A: 5'-ACTCACTCAGTTTGTTGAGTCATTC-3'; and COX-2B: 5'-TTTGATTAGTACTGTAGGGTTAATC-3'. These primer sets yield PCR product of 583 base pairs. Another difference from the iNOS mRNA measurement was that aliquots of PCR products were run on a 1% agarose gel in Tris borate/EDTA buffer.

### 3.5. Plasma IL-6 assay

Plasma IL-6 was measured in the laboratory of Dr. G. Bagby, of the Department of Physiology, LSU Health Sciences Center. A modified hybridoma growth assay in 96 cell microtiter plates was used (21).

## 4. RESULTS

### 4.1. *In vivo* EtOH treatment for 1 and 3 hrs in the absence and in the presence of LPS modulates NF-kappaB activation in non-parenchymal and parenchymal liver cells

Both EtOH and LPS activate NF-kappaB in Kupffer cells. After 1 h of EtOH infusion, NF-kappaB activation in Kupffer cells was greater than in Kupffer cells of rats treated with LPS for 1 h (figure 1). The dual insult, LPS + EtOH infusion resulted in greater activation of NF-kappaB in Kupffer cells both at 1 and 3 hours than activation by either agent alone (figure 1).

In sinusoidal endothelial cells (SEC), both LPS and EtOH activate NF-kappaB also, but 1 h of LPS treatment resulted in greater activation of NF-kappaB than *in vivo* exposure to EtOH for 1 h. As observed for Kupffer cells, the combined treatment with LPS + EtOH for 1 or 3 h activated NF-kappaB more than either LPS or EtOH singly (figure 1).

As can be observed in figure 2 the level of NF-kappaB activation by LPS or EtOH was greater in Kupffer and endothelial cells than in hepatocytes.

Thus, our results demonstrate - for the first time - modulation of *in vivo* LPS-induced NF-kappaB activation by *in vivo* treatment with EtOH.

### 4.2. Kinetics of activation of NF-kappaB in Kupffer cells and sinusoidal endothelial cells

The time course of NF-kappaB activation by LPS, EtOH and the combination of these two treatments is shown in figure 3. In both nonparenchymal cell types, greatest activation was achieved at 1 h post-treatment. In Kupffer cells the LPS-induced activation declines at a faster rate than in endothelial cells. Upon EtOH infusion, in Kupffer cells the rate of decline of activation is lower, whereas in endothelial cells it is greater than with LPS. The combined LPS + EtOH treatment results in Kupffer

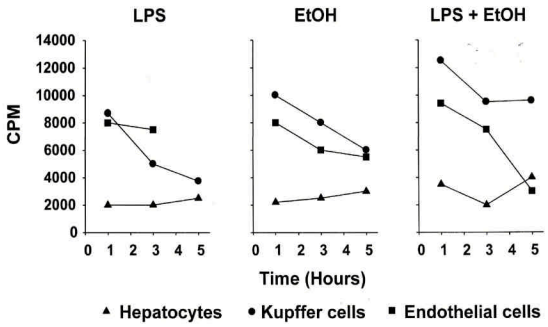


**Figure 3.** Time course of NF-kappaB activation by LPS, EtOH and LPS + EtOH in hepatocytes, Kupffer and sinusoidal endothelial cells. The data represent cpm calculated from a Radioanalytic Imager (AMBIS), obtained from 1 experiment out of 3, with similar results.

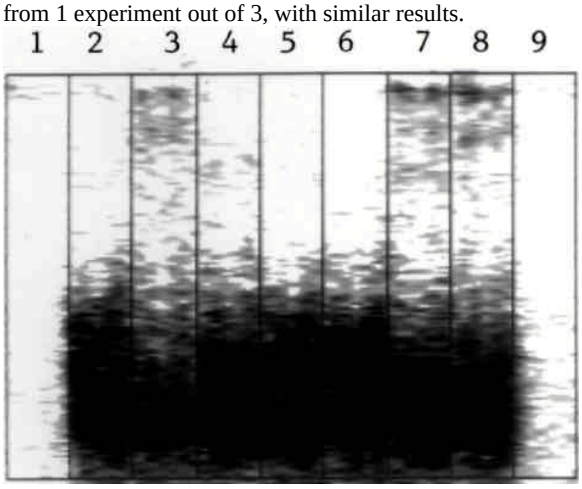


**Figure 4.** Supershifts and competitive binding in Kupffer cells of LPS + EtOH-treated rats as demonstrated by EMSA. Nuclear extracts were incubated with antisera generated against NF-kappaB p65, p50 and c-rel. Lane 1: Probe; Lane 2: LPS+EtOH 1 hr; Lane 3: LPS+EtOH 1 hr (p65); Lane 4: LPS+EtOH 1 hr (p50); Lane 5: LPS+EtOH 1 hr (c-rel); Lane 6: LPS+EtOH 1 hr (p52); Lane 7: LPS+EtOH 1 hr (p65 + p50); Lane 8: LPS+EtOH 1 hr (p65 + c-rel); Lane 9: LPS+EtOH 1 hr (competitive).

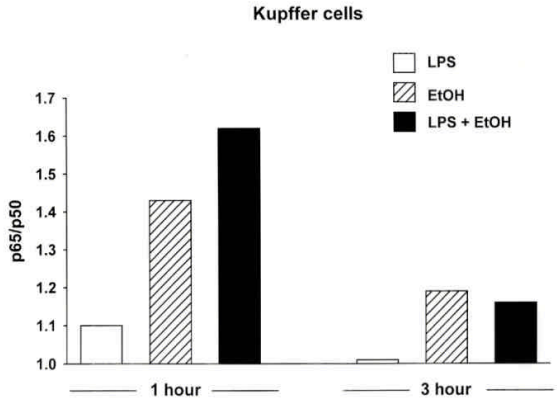


**Figure 5.** Modulation of subunit composition of activated NF-kappaB in Kupffer cells by LPS, EtOH and LPS + EtOH after 1 or 3 h of treatment.

cells in maintenance of 75% of the level of activation at 1 h, up to 5 hrs; in endothelial cells there is a precipitous decline in activation between 3 and 5 hrs. The low level of activation of hepatocyes by each of the three treatments is maintained up to 5 hrs.

**4.3. Specificity of binding and supershift**

Figure 4 demonstrates the specificity of binding in Kupffer cells of LPS + EtOH-treated rats by competitive binding (last lane). To determine the composition of the LPS + EtOH-induced NF-kppaB complex seen on EMSA, we utilized the ability of antibodies against various peptides of the transcription factor to alter the migration of the DNA-protein complex. The Figure also shows strong supershift with the p65 subunit, and with a combination of p65 + p50, and p65 + c-rel. A weak supershift can be seen with p50 and none with c-rel or p52. The pattern of supershifts and competitive binding in SEC of LPS + EtOH-treated rats is similar to that seen in Kupffer cells (data not shown).

Figure 5 shows that EtOH administration (with or without LPS) modifies the subunit composition of activated NF-kappaB in Kupffer cells by increasing the p65:p50 ratio in the activated transcription factor.

**4.4. LPS tolerance modulates NFkappaB activation in Kupffer cells**

The diminished activation of NFkappaB by LPS in Kupffer cells of LPS tolerant male and female rats is shown in figure 6. The altered pattern of supershift composition in Kupffer cells of tolerant male rats is demonstrated in figure 7. As can be observed, the decreased activation of NFkappaB by LPS is accompanied by a decreased ratio of p65 to p50, due mostly to the decreased p65 component. The same shift in subunit composition is seen in female rats (data not shown).

**4.4. Plasma IL-6 levels**

IL-6 is a major regulator of the acute phase response and a systemic administration of LPS results in rapid induction of circulating IL-6 (22). IL-6 plasma levels 1 h after LPS administration were increased over 30-fold relative to pre-LPS values, namely 10702 ± 2605 (n = 6) vs. 378 ± 99.7 (n = 6) pg/ml.

**4.5. iNOS mRNA in Kupffer and sinusoidal endothelial cells**

Available evidence suggests that induction of iNOS by LPS in some cell types involves NF-kappaB dependent transcription (23,24). Therefore, in conjunction with the NF-kappaB studies, we also measured iNOS gene expression in Kupffer and sinusoidal endothelial cells. Determination of iNOS mRNA expression in Kupffer and endothelial cells of LPS and LPS plus EtOH treated male rats revealed enhanced expression associated with LPS plus EtOH treatment (Density units at 1 hr: Kupffer cells LPS – 7900 ± 379 (n = 3), LPS + EtOH – 9375 ± 875 (average of 2 ± range), SEC LPS 5083 ± 300 (n = 3), LPS + EtOH – 7000 ± 0 (average of 2 ± range)), and reduced expression in identically treated female rats, relative to LPS treatment alone (Density units at 1 hr (average of 2 ± range in all

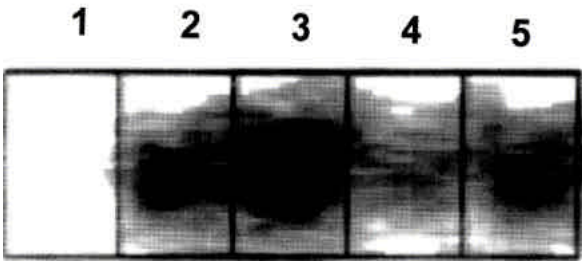


**Figure 6.** Diminished activation of NF-kappaB by LPS in Kupffer cells of LPS tolerant male and female rats as demonstrated by EMSA. Lane 1: probe; Lane 2: male non-tol.; Lane 3: female non-tol.; Lane 4: male tol.; Lane 5: female tol.

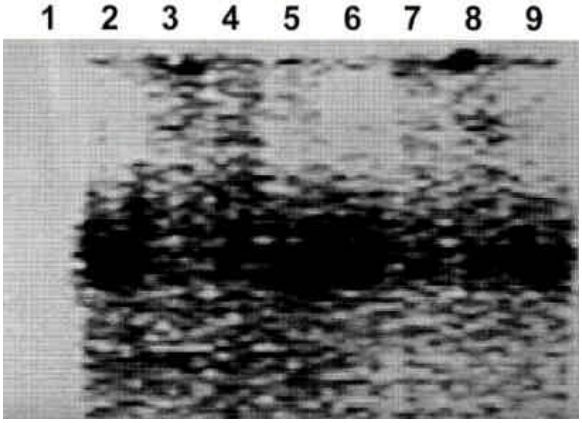


**Figure 7.** Altered pattern of the activated NF-kappaB complex composition in Kupffer cells of tolerant male rats as demonstrated by EMSA. p65/p50:1.03 in nontolerant and 0.871 in tolerant male rats. In female rats (data not shown) p65/p50: 0.978 in nontolerant and 0.814 in tolerant animals. Lane 1: probe; Lane 2: non-tol.; Lane 3: non-tol. p65; Lane 4: non-tol. p50; Lane 5: non-tol. c-Rel; Lane 6: tol.; Lane 7: tol. p65; Lane 8: tol. p50; Lane 9: tol. c-rel.

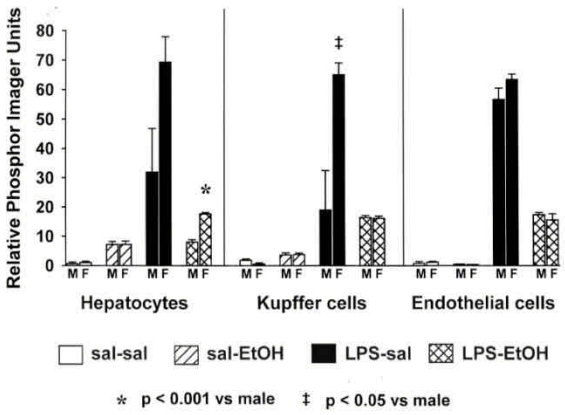


**Figure 8.** The effect of EtOH and LPS tolerance on iNOS mRNA expression. Polyacrylamide gel electrophoresis of the polymerase chain reaction (PCR) product was followed by scanning the gel on a Phosphor Imager. Details of all the procedures are presented under Materials and Methods.

cases): Kupffer cells LPS – 14,500 ± 4000, LPS + EtOH – 8000 ± 0, SEC LPS – 10,375 ± 625, LPS + EtOH – 7178 ± 322. Thus in Kupffer and endothelial cells of male rats iNOS gene expression and NF-kappaB activation were affected the same way by LPS plus EtOH treatment. In the same cells of female rats, LPS plus EtOH treatment resulted in divergent effects on NF-kappaB activation (increase) and iNOS gene expression (decrease), compared to LPS treatment alone.

### 4.6. Modulation of iNOS mRNA expression by LPS tolerance and EtOH

The effects of LPS tolerance and EtOH on iNOS mRNA expression in hepatocytes, Kupffer cells and endothelial cells are presented in figure 8. iNOS mRNA was expressed only weakly in hepatocytes, Kupffer cells or endothelial cells taken from control (sal-sal) rats (figure 8). Acute EtOH administration enhanced the expression of iNOS mRNA both in hepatocytes and Kupffer cells and no differences were observed between males and females. Hepatocytes, Kupffer cells and endothelial cells taken from LPS-tolerant rats exhibited strong expression of iNOS mRNA. In Kupffer cells there was a marked difference between cells taken from females (being much higher) and cells taken from male animals. This difference in hepatocytes did not quite reach statistical significance, and in endothelial cells there was no difference observed. EtOH administration in LPS-tolerant rats (LPS-EtOH) markedly decreased the elevated iNOS mRNA expression in all three cell types. Under these conditions (LPS-EtOH) a gender difference was also observed in hepatocytes, but not in Kupffer or endothelial cells. Figure 9 demonstrates the results of a representative experiment of iNOS mRNA expression by the three hepatic cell types under the various experimental conditions employed.

It is of interest to note that the LPS-tolerant state is accompanied by a marked induction of iNOS mRNA in all three cell types in both male and female rats, with gender differences present in hepatocytes and Kupffer cells. An inhibitory effect of EtOH on this induction in all three cell types is clearly shown in Figure 8 and is in contrast to the EtOH-induced enhancement of iNOS mRNA in hepatocytes and Kupffer cells of non-tolerant male and female rats. Both hepatocytes and Kupffer cells responded to EtOH administration in the opposite manner in the LPS-tolerant as compared to the non-tolerant states. This response also showed gender differences.

### 4.8. COX-2 mRNA expression in Kupffer and sinusoidal endothelial cells

Figure 10 demonstrates COX-2 mRNA expression in endothelial and Kupffer cells of male rats 4 h after LPS (3.0 mg/kg) administration. Endothelial cells from control (saline treated) rats do not show demonstrable COX-2 mRNA expression. LPS treatment induces COX-2 mRNA in endothelial cells, which is significantly reduced in LPS tolerant animals. In Kupffer cells LPS treatment is associated with upregulated COX-2 mRNA expression, that is not diminished by LPS tolerance.

In female rats endothelial cells express COX-2 mRNA more strongly than Kupffer cells both after 2 and 4 h of LPS treatment. In LPS-tolerant female rats COX-2 mRNA expression was diminished substantially in

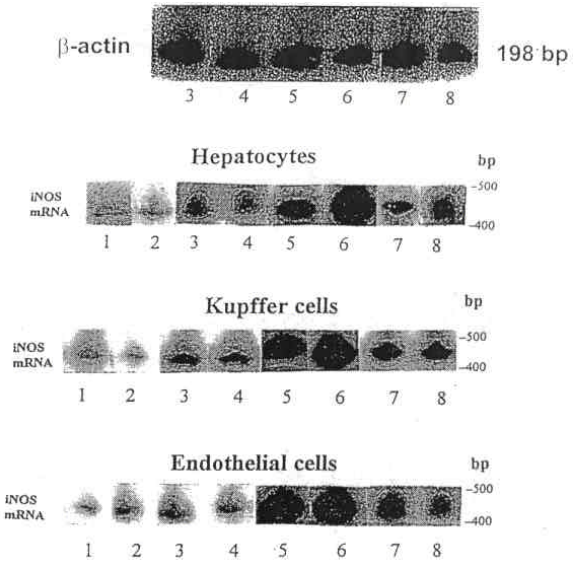


**Figure 9.** Northern blot analysis of iNOS mRNA in hepatic cells of male and female rats. The various treatments were as follows: sal-sal – control rats, receiving 2 saline injections 48 h apart. Sal-EtOH – saline injection 48 h prior to 1 h EtOH infusion. LPS-sal – tolerant rats, receiving a low dose of LPS 48 h prior to saline injection. LPS-EtOH – tolerant rats given a low dose LPS injection 48 h prior to a 1 h EtOH infusion. Details of procedures given in the Materials and Methods section. Lane 1: male (sal-sal); Lane 2: female (sal-sal); Lane 3: male (sal-EtOH); Lane 4: female (sal-EtOH); Lane 5: male (LPS-sal); Lane 6: female (LPS-sal); Lane 7: male (LPS-EtOH); Lane 8: female (LPS-EtOH).

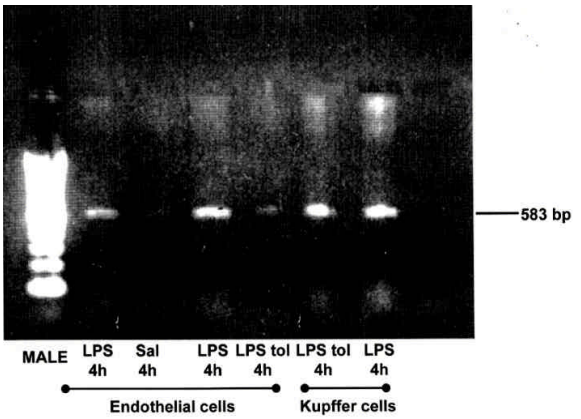


**Figure 10.** Northern blot analysis of LPS-induced COX-2 mRNA expression in Kupffer and sinusoidal endothelial cells of male LPS tolerant and nontolerant rats. LPS was injected i.v. 4 h before sacrifice.

endothelial cells, while the blunting effect of tolerance appears to be more moderate in Kupffer cells (figure 11).

## 5. DISCUSSION

The transcription factor, nuclear factor kappa B, mediates the expression of a number of inflammatory genes involved in the whole body inflammatory response to injury. NF-kappaB participates both directly and indirectly in the transcription of genes producing inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), TNF-?and interferon-?(IFN-?). IL-6 induces production of virtually all of the acute phase proteins by the liver (1,25). Both IL-1 and TNF-? induce the production of prostaglandins and cell adhesion molecules, induce growth and differentiation of lymphocytes and draw cells to the site of inflammation by chemotaxis.

NF-kappaB activation is modulated by a number of factors, among them LPS, oxygen derived free radicals and EtOH. LPS was shown to activate NF-kappaB in a variety of conditions. A two-fold increase in NF-kappaB activation in hepatic macrophages was reported in rats intragastrically fed with a high fat diet plus alcohol, compared with cells from pair-fed control rats (26). Other investigators found no increase in hepatic NF-kappaB activation 45 min after acute EtOH injection into male Wistar rats (27). NF-kappaB activation increased after partial hepatectomy in rats peaking at about 30 min. Acute EtOH consumption accelerates this process while chronic EtOH consumption inhibits induction of NF-kappaB binding activity in the regenerating liver (28).

The influence of in vitro exposure of cells to EtOH on NF-kappaB activation was also studied. EtOH was reported to activate NF-kappaB and AP-1 in E47 cells but not in HepG2 cells (29). Acute EtOH treatment of human monocytes *in vitro* increased DNA binding of NF-kappaB. However, this NF-kappaB complex was a p50/p50 homodimer which inhibits gene transcription (30).

Inflammation within the liver is a complication of both chronic (31) and acute EtOH exposure (32). The potential role of LPS in hepatic injury due to chronic EtOH exposure has been demonstrated experimentally (33) as has been shown in earlier studies of chronic liver injury (34). Other studies with chronically EtOH fed rats have suggested that Kupffer cells may be directly activated by EtOH (35). When Kupffer cells were pulsed with 100 mM EtOH for 90 min then washed and stimulated with physiologically relevant doses of LPS in the absence of EtOH, LPS-mediated NF-kappaB activation was inhibited. At the same time the steady state levels of TNF-?and TNF-secretion were also depressed (36).

In our experiments using a primed continuous infusion of EtOH for 1 h into male Sprague-Dawley rats resulted in considerable NF-kappaB activation in Kupffer cells and sinusoidal endothelial cells (SEC). Hepatocytes showed only minimal activity. Kupffer cells have been shown to have constitutive NF-kappaB binding activity, while hepatocytes have only inducible NF-kappaB (37). Other investigators found no evidence for NF-kappaB binding activity in intact control rat livers, but demonstrated massive hepatic NF-kappaB activation 1 h after LPS administration *in* vivo (8).

The kinetics of activation varied considerably among the three cell types we studied and among the three *in vivo* treatments. In Kupffer cells LPS-induced activation

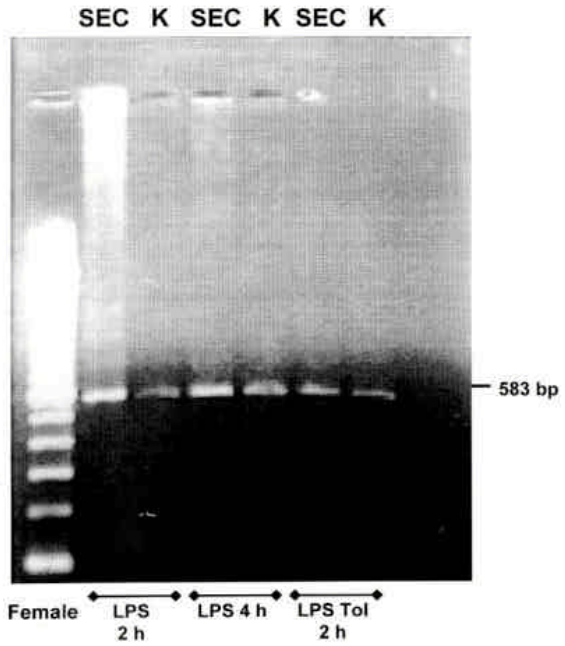


**Figure 11.** Northern blot analysis of LPS-induced COX-2 mRNA expression in Kupffer (K) and sinusoidal endothelial cells (SEC) of female LPS-tolerant and nontolerant rats 2 h after LPS administration.

declined faster than in SEC. EtOH infusion resulted in the maintenance of NF-kappaB activation at a higher level at 3 and 5 h in Kupffer cells and at a lower level in SEC at 3 h than upon LPS treatment. The combined LPS and EtOH treatment resulted in Kupffer cells in the maintenance of 75% of the level of activation at 1 h, up to 5 h, whereas in SEC a precipitous decline in activation occurred between 3 and 5 h. The low level activation in hepatocytes was only minimally attenuated up to 5 h. These findings suggest that there is differential regulation of the kinetics of activation in these three cell types and the responses to the various treatments are also not uniform. A predominant activation of NF-kappaB in Kupffer cells earlier than in hepatocytes was reported (38).

NF-kappaB is composed of two DNA-binding subunits, p50 and p65 (Re1A) (39). In our experiments p65, and p50 could be identified separately and in combination as well as p65 + c-rel in combination in LPS or LPS + EtOH treated rats' Kupffer cells and SEC. Heterodimers of p65/p50 and p65/cRel were shown to be major inducers of gene transcription (1,40). Supershift assays with antibodies to p65, p50 and cRel suggest that these subunits are involved in binding to the iNOS and COX-2 promoters.

LPS tolerance is characterized by a diminished production of TNF during prolonged exposure to LPS, brought about in part by increased expression of the p50 subunit of NF-kappaB (41). Limiting NF-kappaB activation may be beneficial in decreasing host-derived tissue injury and organ dysfunction in disease states such as SIRS, MODS and ARDS. Induction of endotoxin tolerance is one of the specific interventions that may suppress NF-kappaB activation. Thus NF-(kappaB activation may be an attractive target for therapeutic intervention.

One of the key regulators of LPS-induced gene expression is the transcription factor NF-kappaB (42,43). To determine whether EtOH-induced increase in NF-kappaB activation resulted in any significant biological consequence, we measured the effect of EtOH treatment on iNOS and COX-2 mRNA levels.

The expression of iNOS in cells taken from control animals was minimal or non-existent. The tolerant animals' (LPS-sal) iNOS expression was elevated in all three cell types studied. Yet, under these conditions there was a relatively small increase of NO production in these cells (12). One possible reason for that could be the inability of NF-kappaB to be activated and to act as a transcription factor. In fact, NF-kappaB activation was decreased in tolerance. Ziegler-Heitbrock (44) demonstrated that LPS-tolerance is accompanied by the predominance of p50p50 homodimers in the NF-kappaB transcription factor which do not transactivate, unlike the normally present p50p65 heterodimers.

The iNOS mRNA data for LPS-tolerant EtOH-treated rats are in concordance with concurrent iNOS activity, i.e. NO production observed by hepatocytes of both male and female rats (12). The existence of an inducible NO synthase in hepatocytes that is different from that in macrophages may account for the differences in convergence between message and enzyme activity in parenchymal and nonparenchymal cells. Such discrepancies were previously reported in a study of the attenuation by EtOH of LPS-induced iNOS mRNA (45). It is also possible that the translation rate or activation of the two isozymes is differentially affected by the various treatments the rats have undergone, and/or the gender differences demonstrated in TNF and cytokine-induced neutrophil chemoattractant (CINC) production in acutely EtOH-intoxicated LPS-treated rats[13]. Furthermore, it was shown by Nussler *et al* that once iNOS is synthesized, it is relatively stable and the enzyme activity does not decline in keeping with the decreasing mRNA levels (46).

Our results demonstrate that LPS-induced NF-kappaB activation is associated in addition to upregulation of iNOS mRNA expression, with upregulation of another aspect of the inflammatory response, namely COX-2 mRNA expression in Kupffer cells and SEC of both male and female rats. Furthermore, LPS tolerance modulates the expression of COX-2 mRNA in these cells in a gender dependent manner. It is interesting to note that Kupffer cells and SEC isolated from LPS tolerant rats showed strong expression of iNOS mRNA; in Kupffer cells of female animals, the gene expression was much higher than in Kupffer cells of males. On the other hand, the LPS tolerant state was associated with suppressed COX-2 mRNA expression in SEC and no change in Kupffer cells of male and female rats.

Many of the cytokines and signaling molecules that induce NO formation during immune and inflammatory states also promote prostaglandin biosynthesis (47). Treatment of LPS-stimulated macrophages with either L-NMMA or aminoguanidine (AG) prevents the formation of NO and $PGE_2$ (16). Nitric oxide synthase (NOS) inhibitors also inhibit NO and $PGE_2$ formation in an *in vivo* model of acute inflammation (48). Studies in rat mesangial cells suggest that NO may regulate prostaglandin biosynthesis through alterations in COX-2 gene expression (49).

Although numerous *in vitro* and *in vivo* studies support the concept of NO-induced changes in PG synthesis, the mechanisms underlying these changes have not been fully elucidated. Results obtained in a murine macrophage cell line (ANA-1) revealed that, while endogenous NO is not required for *de novo* COX-2 mRNA and protein expression, NO is necessary for maintaining prolonged COX-2 gene expression (50). However, other studies have shown that L-NMMA enhances $PGE_2$ formation in cultured rat Kupffer cells (51). Differences between cell types, different PG-promoting stimuli utilizing different signal transduction pathways to modulate COX-2 gene expression, and different redox potentials of the various cell types are likely contributors to the variability of reports of how NO influences COX-2 gene expression.

Taken together, our results demonstrate – for the first time – modulation of *in vivo* LPS-induced NF-kappaB activation in various liver cells by *in vivo* treatment with EtOH. LPS-induced NF-kappaB activation is associated with upregulation of iNOS and COX-2 gene expression in Kupffer cells and SEC of both male and female rats. In female rats LPS-induced COX-2 gene expression is stronger in SEC than in Kupffer cells. Acute EtOH intoxication enhanced the expression of iNOS mRNA in hepatocytes and Kupffer cells.

LPS tolerance is associated with diminished activation of NF-kappaB by LPS in Kupffer cells of both male and female rats, accompanied by a decreased ratio of p65 to p50 subunit, due mostly to the decreased p65 component. Hepatocytes, Kupffer cells and SEC of LPS tolerant rats exhibited strong expression of iNOS mRNA, with gender differences in Kupffer cells (cells from females being higher), and EtOH administration markedly decreased this elevated iNOS mRNA expression in all three cell types. LPS tolerance reduced LPS-induced COX-2 mRNA expression in SEC, but not in Kupffer cells. Since endothelial cells produce primarily $PGI_2$, a vasodilator (52), suppression of their production in LPS tolerance may have detrimental effects on the intrahepatic microcirculation, associated with cirrhotic livers (53).

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**Abbreviations:** LPS, lipopolysaccharide, NF-kappaB, nuclear factor kappa B, NO, nitric oxide, COX-2, cyclooxygenase-2, iNOS, inducible nitric oxide synthase, EtOH, ethanol, SEC, sinusoidal endothelial cells, mRNA, messenger ribonucleic acid, PGHS, prostaglandin endoperoxide synthase, EMSA, electrophoretic mobility shift assay, IL-6, interleukin-6, DNA, deoxyribonucleic acid, L-NMMA, $N^G$-monomethyl-L-arginine, $PGE_2$, prostaglandin $E_2$, TNF, tumor necrosis factor

**Key Words:** Gender Differences, Hepatic Inflammatory Responses

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