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The Role of Src Kinase in the Potentiation by Ethanol of Cytokine- and Endotoxin-Mediated Nitric Oxide Synthase Expression in Rat Hepatocytes

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SUMMARY

This study demonstrates that exposure of primary rat hepatocytes or mouse BNL Cl.2 liver cell line to ethanol causes potentiation of tumor necrosis factor-α (TNF-α)- and lipopolysaccharide (LPS)-stimulated nitrite accumulation. The potentiating effect of ethanol (0.02–2 mM) appears to be time and concentration dependent. Consistent with nitrite production, the amount of inducible nitric oxide synthase (iNOS) mRNA and protein is initially detected at 4 hr after treatment with TNF-α/LPS/ethanol. Furthermore, the capability of these agents to induce iNOS expression is primarily determined by the age of the animals. Interestingly, antioxidants such as *N*-acetylcysteine (NAC), ascorbic acid, or α-tocopherol fail to inhibit TNF-α/LPS/ethanol-induced increase in iNOS protein. In addition, several kinase inhibitors, including staurosporine, genistein, curcumin, and herbimycin A, were used to examine their effects on this induction. Among them, only herbimycin A potently inhibits the accumulation of nitrite and iNOS expression. *In vitro* kinase assay verifies that Src tyrosine kinase is rapidly activated with a peak at 1 hr after treatment with TNF-α/LPS/ethanol but is not activated by these agents singly or doubly. As expected, herbimycin A can block Src kinase activity under circumstances in which iNOS expression is also inhibited. However, our results do not indicate that the mitogen-activated protein kinase is activated after treatment with these agents. The study results suggest that Src tyrosine kinase plays a prominent role in transducing the signal to induce iNOS expression in hepatocytes treated with TNF-α/LPS/ethanol.

NO, the smallest known biologic mediator produced by mammalian cells, is involved in a diverse array of activities, including vasodilation, neurotransmission, and antimicrobial functions. NO is derived from L-arginine and can be produced by constitutively expressed NO synthase in cells such as endothelial cells (1), neurons (2), and cardiac myocytes (3) or by iNOS in cells such as macrophages (4), hepatocytes (5), and vascular smooth muscle cells (6). Because iNOS can produce a large amount of NO, its induction plays an influential role in cell death, tissue damage, and inflammation (7, 8). Maximal induction of iNOS depends on synergistically combining stimuli; the most effective stimuli vary with cell type. These stimulatory compounds include TNF-α, IL-1β, interferon-γ, and LPS. In hepatocytes, LPS, TNF-α, or IL-1β alone did not significantly stimulate NO formation, but double or triple combinations of LPS with the cytokines induced a significantly higher NO production than any of the agents alone (9). Evidence, although inconclusive, suggests that the induction mechanism by LPS and combined cytokines might include protein kinase C, phospholipase A_2, or protein tyrosine kinases in hepatocytes (9). A previous study postulated that reactive oxygen species participate in transducing the signal, thereby leading to activation of iNOS by TNF-α in rat hepatocytes (10). The mechanism underlying multiple cytokines or other agents inducing iNOS expression appears to be quite complicated and varies with different cell types.

Chronic inflammation has long been recognized as a risk factor for a variety of human cancers. Increasing evidence suggests that NO produced in inflamed tissues may contribute to the multistage carcinogenesis process (11). Epidemiological studies indicate that long term alcohol consumption can lead to hepatic injuries such as fatty liver, necrosis, inflammation, and fibrosis (12). However, whether NO plays a prominent role in initiating such alcoholic liver lesions remains unclear. In contrast, a previous study demonstrated

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ABBREVIATIONS: NO, nitric oxide; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NAC, *N*-acetylcysteine; MAPK, mitogen-activated protein kinase; IL-1β, interleukin-1β; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

that *in vivo* administration of NOS inhibitor increased the severity of liver injury, indicating that NO plays a protective role in alcohol liver disease (13). Taken together, although these results seem to be controversial, it implicates that NO is likely to be involved in ethanol-mediated modulation of the function of hepatic cells during alcohol intake. Another possible cause for increased NO production could be high levels of circulating endotoxin and TNF, which are markedly raised in acute alcoholic hepatitis (14). The aim of this study was to examine whether ethanol can stimulate NO production in primary rat hepatocytes in the presence of LPS and TNF-α and the signal transduction pathway responsible for the response.

Materials and Methods

Reagents. Recombinant human TNF-α (specific activity, 2.86×10^7 units/mg) was supplied by R and D Systems (Minneapolis, MN). Recombinant rat interferon-γ was purchased from GIBCO BRL (Gaithersburg, MD). LPS, herbimycin A, staurosporine, NAC, and genistein were obtained from Sigma Chemical (St. Louis, MO). Ethanol was purchased from Merck (Darmstadt, Germany).

Animals and cell culture. Study animals were 1-month-old male Wistar rats that were fed *ad libitum*. Hepatocytes were isolated from Wistar rats by collagenase perfusion (15) and purified by differential centrifugation to produce cultures of ≥90% viability and ≥95% purity. Hepatocytes were plated onto rat tail collagen-coated culture plates (Corning, Palo Alto, CA) in Weymouth's medium supplemented with 0.5 mM L-arginine, 10^{-6} M insulin, L-glutamine, penicillin, streptomycin, and 10% fetal calf serum. BNL Cl.2 mouse liver cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 units/mL penicillin.

Measurement of NO_2^-. Accumulation of NO_2^- in the medium was determined via the Greiss reagent (16) and was taken as an index of NO production. The measurement of NO_2^- accumulation in the medium was determined by mixing 0.5 ml of medium with an equal volume of Greiss reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% H_3PO_4) and incubation at room temperature for 15 min. Absorbance at 550 nm was measured in a spectrophotometer, and NO_2^- was determined with $NaNO_2$ as a standard.

Western blot analysis. Treated hepatocytes were washed and pelleted in PBS. Cellular lysates were prepared as previously described (17). A 50-μg sample of each lysate was subjected to electrophoresis on 8% and 15% SDS-polyacrylamide gels for detecting iNOS and MAPK, respectively. The samples were then electroblotted onto nitrocellulose paper. After blocking, blots were incubated with anti-iNOS (Transduction Laboratories, Lexington, KY) or anti-MAPK (Santa Cruz Biochemicals, Santa Cruz, CA) antibody in PBS/Tween 20 for 1 hr followed by two washes (15 min each) in PBS/Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL) for 30 min. After washing, the blots were incubated for 1 min with Western blotting reagent ECL, and chemiluminescence was detected by exposure of the filters to Kodak X-Omat films for 10 sec to 10 min. The specificity of this antibody to the 130-kDa iNOS was ensured by comparison with a standard macrophage iNOS.

RNA isolation and Northern blotting. The cDNA probe, derived from the mouse macrophage iNOS gene and having a length of ~1.8 kb, was purchased from Cayman Chemical (Ann Arbor, MI). The probe was random-primer labeled and used in a Northern blot analysis. Total RNA from the cells grown in cell culture dishes was isolated using 4 M guanidine isothiocyanate. Fifteen micrograms of RNA was used for Northern blotting as previously described (18).

$pp60^{c\text{-}src}$ kinase reaction. Kinase assays were performed essentially as described by Gould and Hunter (19). After treatment, hepatocytes were rinsed with cold PBS, lysed in 1 ml of cold RIPA buffer, and clarified at 15,000 × *g* for 1 hr at 4°. Next, $pp60^{c\text{-}src}$ was immunoprecipitated by incubating the lysate (300 μg) on ice for 1 hr with 1 μg of anti-c-Src antibody (Santa Cruz Biochemicals), followed by the addition of 40 μl of protein A-Sepharose beads (Santa Cruz Biochemicals) for an additional 4 hr, and immunocomplexes were collected by centrifugation at 4°. The immunocomplexes were washed three times with RIPA buffer and once with kinase buffer (20 mM HEPES, pH 7.0, 6 mM $MgCl_2$, 20 mM sodium orthovanadate) and suspended in 20 ml of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) followed by the addition of 10 μg of acid-denatured enolase (Sigma). The reaction was incubated for 10 min at 30° and terminated by the addition of 2× SDS sample buffer (0.5 M Tris·Cl, pH 8.8, 0.4% SDS, 20% glycerol; 2% mercaptoethanol, 1% Bromphenol Blue). The proteins were resolved on SDS-10% polyacrylamide gels. Wet gels were exposed for 30–60 min at room temperature. Later, the gels were stained, and bands were excised and quantified by scintillation counting.

Results

Ethanol potentiates TNF-α- and LPS-stimulated NO_2^- production. Primary rat hepatocytes and mouse BNL Cl.2 liver cell lines were treated with cytokines and LPS in combination to determine their production of NO_2^- (i.e., a stable oxidation product of NO). The 24-hr treatment of both cell systems with cytokines such as IL-1β (5 ng/ml), TNF-α (1 ng/ml), interferon-γ (10 ng/ml), or LPS (1 μg/ml) alone did not stimulate increased NO_2^- level. Although double combinations of LPS with the cytokines induced a higher NO_2^- production than any of the agents alone, we found the amount of NO_2^- was still difficult to detect (0.02–0.03 absorbance units by the Greiss reaction) (data not shown). Interestingly, the simultaneous addition of ethanol (0.02–2 mM) to both cell systems significantly increased LPS/TNF-α-induced NO_2^- production in a concentration-dependent manner (Fig. 1, A and B); in addition, the potentiating effect of ethanol in primary rat hepatocytes seemed more remarkable than that in mouse BNL Cl.2 cells. However, if the ethanol concentration exceeded 2 mM, enhancement of NO_2^- production by ethanol would be decreased in both cells. A time course of NO_2^- accumulation in hepatocytes after 1 mM ethanol plus LPS and TNF-α is showed in Fig. 1C. The result clearly shows that the induction of NO_2^- by ethanol/LPS/TNF-α appeared to be time dependent. The ethanol-mediated enhancement of this effect was significantly blocked by the concomitant addition of 100 μM *N*-nitro-L-arginine to both cells (Table 1).

Characterization of iNOS expression. In the experiment, Northern and Western blots were used to determine the amount of iNOS in primary rat hepatocytes treated with ethanol (1 mM)/TNF-α (1 ng/ml)/LPS (1 μg/ml) or with any of three agents alone. Fig. 2 (*top*) reveals that exposure of hepatocytes to ethanol/TNF-α/LPS for 4 hr resulted in an increase of iNOS mRNA, which was subsequently slightly increased in a time-dependent manner. Corroborating with the observation on mRNA increment, Western blotting analysis indicated that the level of iNOS protein was also initially detected after treatment for 4 hr and gradually increased thereafter (Fig. 2, *bottom*). No detectable signal of iNOS mRNA or protein appeared during treatment with TNF-α, LPS, or ethanol alone (data not shown).

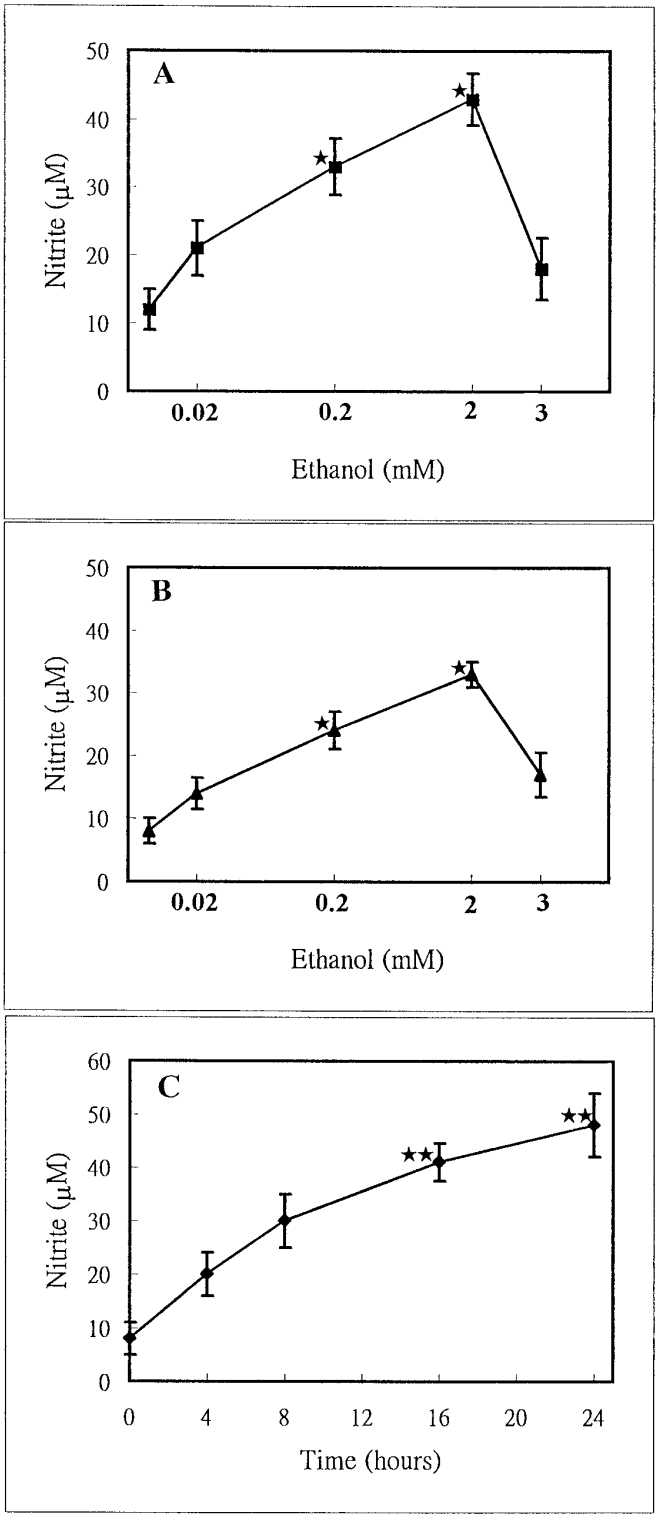

Fig. 1. Enhancing effect of ethanol on TNF-α/LPS-induced NO production in (A) primary rat hepatocytes or (B) mouse BNL Cl.2 liver cells. C, A time course of NO_2^- accumulation in rat hepatocytes after treatment with ethanol (1 mM)/TNF-α (1 ng/ml)/LPS (1 μg/ml). Cells were incubated with TNF-α (1 ng/ml)/LPS (1 μg/ml) and varying concentrations of ethanol (0–3 mM) for 24 hr or treated with ethanol (1 mM)/TNF-α (1 ng/ml)/LPS (1 μg/ml) for 0–24 hr; then, the NO_2^- level was determined by Greiss reagent as described in Materials and Methods. Results are mean ± standard error of five separate experiments. *, $p < 0.05$ versus control group at the same time point. **, $p < 0.01$ versus control group.

TABLE 1

Effects of antioxidants and protein kinase inhibitors on TNFα/LPS/ethanol-stimulated nitrite production in rat hepatocytes

Treatment	Concentration[b]	Nitrite (μM)
None		12 ± 5[c]
TNFα/LPS/ethanol[a]		47 ± 8
+ NAC	5 mM	43 ± 6
+ Ascorbic acid	100 μM	49 ± 10
+αTocopherol	25 μM	45 ± 9
+Curcumin	10 μM	51 ± 11
+Staurosporine	1 μM	45 ± 3
+Herbimycin A	5 μM	11 ± 3*
+Genistein	20 μM	42 ± 10
+ N-nitro-L-arginine	100 μM	13 ± 4*

[a] Hepatocytes were incubated with TNFα (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) or plus above modulators for 24 h. Media samples were collected and assayed for total nitrite and nitrate accumulation.

[b] The concentration of these inhibitors and antioxidants used here was non-cytotoxic to hepatocytes under this experimental condition.

[c] Values are mean ± standard error of triplicate samples in two independent experiments. *Statistically significant decrease in nitrite production compared with ethanol/TNF-α/LPS-treated cells ($P < 0.05$).

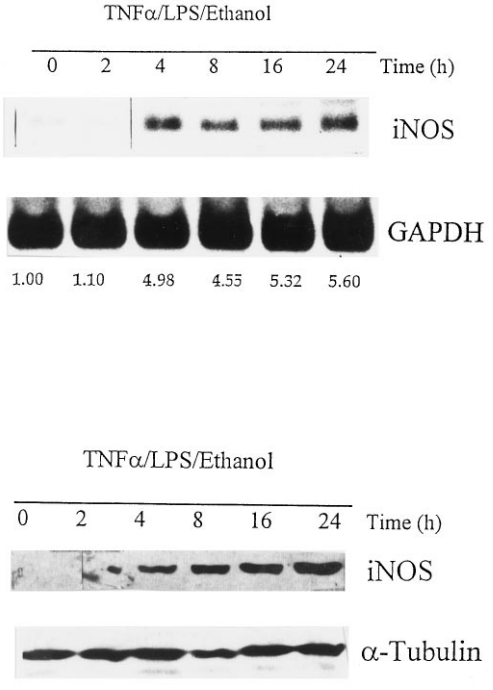

Fig. 2. Characterization of TNF-α/LPS/ethanol-induced iNOS gene expression. *Top*, kinetics of iNOS mRNA induction by TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM). Total RNA was extracted from 10^7 hepatocytes at different time points after the combined agents treatment as indicated in the figure. The signal of glyceraldehyde-3-phosphate dehydrogenase as an internal control to normalize the level of iNOS in respective sample. The number indicates the fold-change of iNOS transcripts of treated cells from control cells. *Bottom*, kinetics of immunoreactive iNOS protein expression by TNF-α/LPS/ethanol. Total protein was collected from treated or untreated rat hepatocytes and assayed for iNOS protein by Western blotting with a specific anti-iNOS antibody as described in Materials and Methods. Similar findings were observed in three separate experiments.

Assuming that the age of the animals may affect the ethanol-enhanced LPS/TNF-α-induced increase of iNOS, we examined the level of iNOS protein in ethanol/LPS/TNF-α-treated hepatocytes from different ages of rats. Western blotting reveals that hepatocytes isolated from rats <2 months old exhibited a high induction of iNOS protein when exposed to the combined agents. However, if hepatocytes

were derived from rats >2 months old, the level of iNOS protein became nearly undetectable under the same experimental condition (Fig. 3, *top*). These results were corroborated when the propagation of mouse BNL Cl.2 liver cell lines was higher than passage 13; no signal for iNOS protein could be detected after ethanol/TNF-α/LPS stimulation (Fig. 4, *top*). The age-dependent inductions were similarly obtained for assays of NO_2^- production by Greiss reaction in primary rat hepatocytes or mouse liver cell lines, respectively (Figs. 3, *bottom*, and 4, *bottom*).

Effects of signal transduction modulators on ethanol/TNF-α/LPS-induced iNOS expression. To gain further insight into the mechanism by which ethanol potentiates TNF-α/LPS-stimulated iNOS expression, we used various kinds of signal transduction modulators to examine such an event. Based on the hypothesis that the oxidation/reduction status may regulate TNF-α- or ethanol-caused effects (10), hepatocyte cultures were treated with antioxidants NAC, ascorbic acid, and α-tocopherol to examine their effects on NO_2^- production induced by ethanol/TNF-α/LPS. According to the results presented in Table 1, none of these antioxidants significantly inhibited ethanol/TNF-α/LPS-stimulated NO_2^- production. Consequently, several kinase inhibitors, including staurosporine (protein kinase C inhibitor), genistein (tyrosine kinase inhibitor), curcumin (nonspecific kinase inhibitor), and herbimycin A (Src-related kinase inhibitor), were used to examine their effects on the stimulation. Among them, only herbimycin A completely inhibited the NO_2^- production induced by the combination of agents.

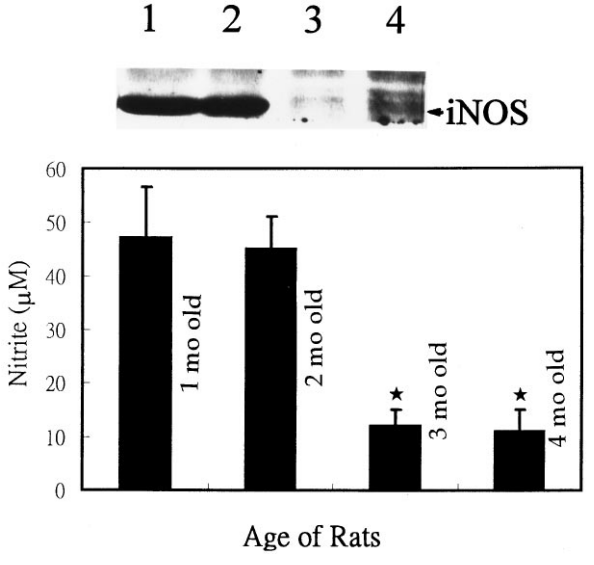

Fig. 3. *Top*, effect of age on the level of iNOS protein in rat hepatocytes treated with TNF-α/LPS/ethanol. Hepatocytes isolated from 1-month-old (*lane 1*), 2-month-old (*lane 2*), 3- month-old (*lane 3*), or 4-month-old (*lane 4*) rats were treated with TNF-α (1 ng/ml)/LPS(1 μg/ml)/ethanol (1 mM) for 24 hr; Then, iNOS proteins were determined by Western blotting as described in Materials and Methods. *Bottom*, effect of age on the accumulation of NO_2^- in rat hepatocytes treated with TNF-α/LPS/ethanol. The NO_2^- production from different ages of rat hepatocytes treated with TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) was determined by Greiss reagent as described in Material and Methods. Experimental conditions were identical to those in Fig. 3A. Values are mean ± standard error of triplicate samples from two to four experiments. *, $p < 0.05$ versus hepatocytes from young rats (1 and 2 months old).

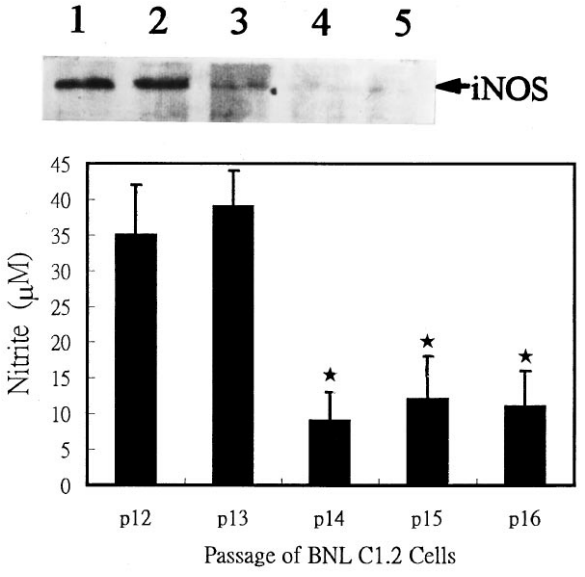

Fig. 4. Effect of passage number on iNOS expression in mouse BNL Cl.2 liver cell line by TNF-α/LPS/ethanol. *Top*, Western blot analysis of iNOS. Total proteins were collected after 24-hr treatment of TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) and assayed for iNOS protein by immunoblotting. *Lane 1*, passages 12. *Lane 2*, passages 13. *Lane 3*, passages 14. *Lane 4*, passages 15. *Lane 5*, passages 16. *Bottom*, nitrite production in different passages of BNL Cl.2 liver cell lines treated with TNF-α/LPS/ethanol. Mouse BNL Cl.2 cells were treated as described above. Media samples were collected and assayed for total NO_2^- and nitrate accumulation. Values are mean ± standard error of duplicate samples in two experiments. *, $p < 0.05$ versus early passages of BNL Cl.2 cells (passages 12 and 13).

The concentrations of modulators used herein were noncytotoxic to rat hepatocytes.

Next, we checked whether the inhibition of herbimycin A on ethanol/TNF-α/LPS-stimulated NO_2^- accumulation reflects the level of iNOS protein. Western blotting reveals that herbimycin A clearly abolished ethanol/TNF-α/LPS-induced iNOS protein expression but not other modulators or inhibitors (Fig. 5, *top*). The inhibitory effect of herbimycin A was also found on iNOS mRNA level (Fig. 5, *bottom*), suggesting that the inhibition may occur before transcriptional events or at the level of signal transduction.

Activation of $pp60^{c\text{-}src}$ but not MAP kinase by ethanol/TNF-α/LPS. Due to the potent and specific inhibitory action of herbimycin A on Src tyrosine kinase (20), whether the kinase is involved in the capability of ethanol to enhance TNF-α/LPS-mediated iNOS expression in hepatocytes remains unclear. To address this issue, Src immune complex kinase assay was performed in rat hepatocytes exposed to ethanol/TNF-α/LPS for varying periods of time. As Fig. 6 (*top*) depicts, the $pp60^{c\text{-}src}$ kinase activity (phosphorylation of enolase) reached a maximal level at 1 hr after treatment of the agents. The kinase activity then gradually decreased and returned to a control level by 6 hr. As Fig. 6 (*bottom*) reveals, herbimycin A at the concentration that inhibited iNOS expression also prevented $pp60^{c\text{-}src}$ kinase activation. Similar to the observation on iNOS induction, none of the other kinase modulators were capable of inhibiting $pp60^{c\text{-}src}$ kinase. Table 2 shows that 1–5 μM herbimycin A exerted a dose-dependent inhibition on ethanol/TNF-α/LPS-induced Src kinase activity and NO_2^- accumulation but that genistein did not affect either event.

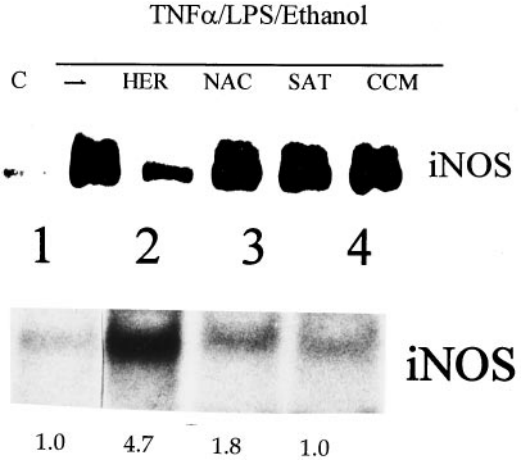

Fig. 5. *Top*, effect of protein kinase inhibitors and antioxidants on iNOS protein expression by TNF-α/LPS/ethanol in rat hepatocytes. Hepatocytes (5×10^6) were incubated in the absence (C) or presence of TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) (–) or plus various inhibitors such as herbimycin A (*HER*, 5 μM), NAC (5 mM), staurosporine (*SAT*, 1 μM), or curcumin (*CCM*, 10 μM) for 24 hr; then, the iNOS protein level of total cell lysates was determined by Western blotting. *Bottom*, inhibition of herbimycin A on TNF-α/LPS/ethanol-induced iNOS mRNA expression. Hepatocytes were treated as follows. *Lane 1*, untreated. *Lane 2*, TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) for 6 hr. *Lane 3*, TNF-α/LPS/ethanol plus herbimycin A (5 μM) for 6 hr. *Lane 4*, herbimycin A (5 μM) for 6 hr. Methods for total RNA isolation and Northern blotting are described in Materials and Methods. *Number*, fold-change of iNOS transcripts of treated cells from control cells.

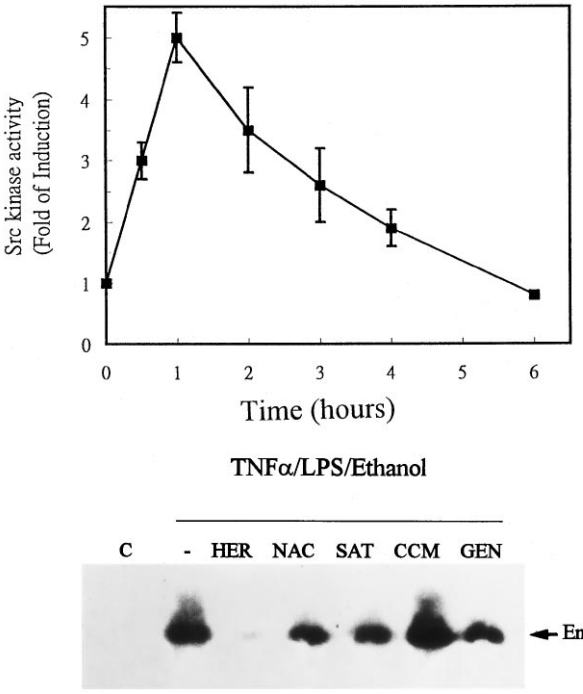

Fig. 6. *Top*, kinetics of Src kinase activity induced by TNF-α/LPS/ethanol in rat hepatocytes. Hepatocytes (5×10^6) were incubated in the presence of TNF-α (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) for different time points as indicated in this figure. Equal amount (100 μg) of cell lysates were immunoprecipitated with anti-c-Src antibody; subsequently, the immunocomplexes were assayed for c-Src kinase activity using enolase as a substrate. The fold increase in c-Src kinase activity was quantified by scintillation counting of the enolase band. The indicated values are the averages of three experiments. *Bars*, mean $\pm$ standard error. *Bottom*, herbimycin A inhibits TNF-α/LPS/ethanol-elicited c-Src activity in rat hepatocytes. Hepatocytes were concomitantly treated with TNF-α/LPS/ethanol (–) and various modulators, such as herbimycin A (*HER*, 5 μM), NAC (5 mM), staurosporine (*SAT*, 1 μM), curcumin (*CCM*, 10 μM), or genistein (*GEN*, 20 μM) for 1 hr. Methods of immunoprecipitation and c-Src kinase assay are described in Materials and Methods.

Because activation of 44-kDa mitogen-activated protein kinases (e.g., MAPK, ERK1) is essential for induction of iNOS in response to IL-1β and interferon-γ in myocytes and endothelial cells (21), whether the kinase is activated in our system remains an unresolved question. Immunoblotting with anti-ERK1 antibody indicates that MAPK is not activated in rat hepatocytes during exposure to ethanol/TNF-α/LPS, as demonstrated by a lack of changes in band mobility on gel compared with controls (Fig. 7). However, the addition of 10 ng/ml hepatocyte growth factor to the rat hepatocytes resulted in activation of ERK1 through phosphorylation (Fig. 7, *lane 6*).

Discussion

This study clearly demonstrates that a low ethanol concentration potentiates TNF-α- plus LPS-stimulated NO_2^- production and iNOS expression in both primary rat hepatocytes and mouse liver cell line. Nitrite level in the two hepatic cell systems is not detectable when treating with TNF-α alone. In contrast, another study (10) indicated that treatment of rat hepatocytes with TNF-α can stimulate a significant increase of NO_2^- level. Such a discrepancy may be due to the cytokine concentration (1 ng/ml) used, which is markedly lower than that used in others (1 μg/ml). However, the concentration used in this study is closer to the physiological condition.

This study shows that a sustained level of iNOS mRNA was detected at 24 hr after treatment with TNF-α/LPS/ethanol. However, previous studies have shown that treatment of rat hepatocytes with various cytokines plus LPS induced iNOS mRNA with a peak at 6–8 hr and then declined slightly by 24 hr (22, 23). This discrepancy is possibly due to the synergizing effect of ethanol to produce prolonged induction of iNOS mRNA. Several agents, such as cAMP, cycloheximide, and 12-*O*-tetradecanoylphorbol-13-acetate, have been found to affect the stability of iNOS mRNA that led to a synergetic induction of iNOS mRNA (24, 25). This raises a

TABLE 2

Effects of herbumycin A and genistein on ethanol/TNFα/LPS-induced Src activity and nitrite production

Treatment	Concentration[b]	Src activity[c] (%)	Nitrite[d] (μM)
None		0	11 $\pm$ 3
TNFα/LPS/ethanol[a]		100	50 $\pm$ 8
+ Herbimycin A	1 μM	87	45 $\pm$ 7
+ Herbimycin A	2.5 μM	56	31 $\pm$ 4*
+ Herbimycin A	5 μM	11	13 $\pm$ 5*
+ Genistein	10 μM	95	48 $\pm$ 9
+ Genistein	20 μM	91	45 $\pm$ 5

[a] Hepatocytes were incubated with TNFα (1 ng/ml)/LPS (1 μg/ml)/ethanol (1 mM) or plus with herbimycin A or genistein for 1 h or 24 h for assaying Src kinase activity or nitrite production.

[b] The maximally subcytotoxic dose of herbimycin A and genistein is 5 μM and 20 μM, respectively.

[c] The $pp60^{c\text{-}src}$ kinase activity is determined by phosphorylating its substrate enolase as described in Materials and Methods.

[d] Values are mean $\pm$ standard error of triplicate samples in two independent experiments. *Statistically significant decrease in nitrite production compared with TNFα/LPS/ethanol-treated group ($P < 0.05$).

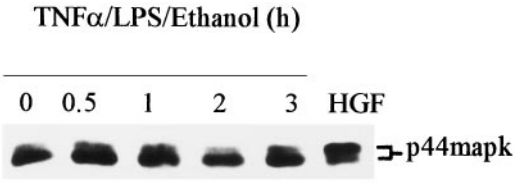

Fig. 7. Effect of TNF-α/LPS/ethanol on MAPK in rat hepatocytes. Hepatocytes (5×10^6) were treated with TNF-α/LPS/ethanol for various time points or treated with hepatocyte growth factor (10 ng/ml) for 1 hr. The cell lysates were subjected to electrophoresis and electrotransferred to nitrocellulose papers. The $p44^{mapk}$ protein on blots was detected as described in Materials and Methods. *Tick marks*, positions of the hyperphosphorylated (*bottom*) and hypophosphorylated (*top*) $p44^{mapk}$.

possibility that the potentiating effect of ethanol on iNOS mRNA induction may mediated through an affect on its stability. Although ethanol enhances the expression of iNOS, its potentiating effects on TNF-α/LPS-stimulated NO_2^- production (~2–4-fold) is lower than that on iNOS mRNA and protein levels (~4–7-fold). A possibility arises that released NO moiety into a culture medium may be neutralized by cellular constituents or nutrients, leading to an underestimation of the actual NO_2^- level. Our current finding that ethanol can potentiate iNOS expression in cultured rat hepatocytes contrasts with a recent study in which it was demonstrates that the *in vivo* administration of ethanol attenuated iNOS expression in a rat liver (26). Reasons for the contradictory results of the two studies remain unknown. However, several possible explanations are available. First, the ethanol concentration used *in vivo* is higher than that in our *in vitro* systems. As mentioned earlier, our results indicate that the potentiating effect of ethanol requires a concentration ranging from 0.02 to 2 mM because the addition of an ethanol concentration of >2 mM decreases the NO_2^- level. This finding suggests that ethanol, under different concentrations, possibly exerts a complicated effect in regulating NO release. Second, the rats used in *in vivo* study were older than the rats used in the current study. In addition, according to our results, the potentiating effect of ethanol fails when hepatocytes are obtained from rats >2 months old. This finding clearly suggests that the potentiation of ethanol on iNOS expression is critically determined by the age of the animals. Finally, the *in vivo* administration of ethanol may result in its biotransformation in the liver to secondary metabolites that can inhibit iNOS induction, which are not formed in our *in vitro* culture systems.

Our current results reveal that the induction of iNOS mRNA by TNF-α/LPS/ethanol in rat hepatocytes is age dependent. It is well documented that many physiological functions or signaling pathways in rat hepatocytes could be affected during the aging process. For example, the expression of heat shock protein 70, epidermal growth factor-stimulated DNA synthesis, or insulin-induced glucosyl-phosphatidylinositol signaling could be reduced or attenuated in rat hepatocytes from old rats (27–29). The biological role of NO_2^- induced by TNF-α/LPS/ethanol in hepatocytes from young rats is unclear. However, previous studies have suggested that adaptive NO synthesis in the hepatic cells is beneficial or protective for the liver in the presence of toxic insults, such as LPS or ethanol (13, 30). In this context, we thus propose that the preferential induction of NO_2^- in hepatocytes from young rats may implicate the more actively protective mechanism existing in these cells.

The nonreceptor tyrosine kinase $pp60^{c\text{-}src}$ plays a critical role in modulating cell signals in response to several growth factor, such as platelet-derived growth factor (31), epidermal growth factor (32), and interleukin-3 (33). In addition to growth factors, oxidative stress initiated by ultraviolet irradiation has been found to potentially activate Src tyrosine kinase, thereby leading to trigger of activator protein-1 activity (34). However, this is the first time it has been demonstrated that activation of Src tyrosine kinase is critically involved in inducing iNOS through ethanol/TNF-α/LPS treatment. Because treatment of cells with these agents alone did not activate Src kinase, the diverse signals triggered by these single agents are likely a prerequisite to interact and finally converge on Src kinase. As well documented, exposure to ethanol, TNF-α, or LPS alone can produce reactive oxygen species in various cell or animal systems (35, 36). Nevertheless, in this study, we failed to decrease both Src kinase activity (Fig. 6B) and iNOS expression (Fig. 5A) by using some well-known antioxidants. This suggests that reactive oxygen species do not contribute to Src-associated iNOS induction by ethanol/TNF-α/LPS. A more recent study indicated that iNOS gene expression is regulated differently in response to specific cytokines in each cell type (21). For instance, IL-1β induces iNOS in cardiac myocytes and endothelial cells, whereas interferon-γ induces iNOS in myocytes but not in endothelial cells.

Activation of MAPK is importantly attributed to iNOS induction by IL-1β or interferon-γ in its respective cell context. In hepatocytes, we did not find an alteration in MAPK activity after exposure to ethanol/TNF-α/LPS (Fig. 7).

In conclusion, this study demonstrates that clinically relevant concentrations of ethanol modulate the expression of iNOS in primary hepatocytes by potentiating the TNF-α- and LPS-stimulated elevation of iNOS mRNA and protein. Further evidence suggests that Src tyrosine kinase plays an essential role in regulating iNOS expression by ethanol/TNF-α/LPS, but the actual mechanisms by which these combined agents activate Src kinase remain unknown and require further study.

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