응용약물학회지, The Journal of Applied Pharmacology, 7, 315-321(1999)

Inhibition of TCDD Induced *Cyp1a1* Expression by SNP in Hepa I Cells

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(Received Noverber 11, 1999; accepted December 10, 1999)

Absract – Since it has been known that hypoxia increases inducible nitric oxide synthase (*i*NOS) gene expression through hypoxia responsive element, it was possible to establish the hypothesis that nitric oxide could be a mediator of hypoxia to inhibit *Cyp1a1* promoter activity. In order to test this hypothesis, we have undertaken the study to examine the effects of hypoxia and nitric oxide on *Cyp1a1* promoter activity in Hepa I cells. Mouse *Cyp1a1* 5' flanking DNA, 1.6 Kb was cloned into pGL3 expression vector in order to construct pmCyp1a1-Luc. Hepa I cells were transfected with pm*Cyp1a1*-Luc and were treated with 10^{-9} M TCDD and nitric oxide producing agents, such as lipopolysaccharide (LPS), sodium nitroprusside (SNP). Luciferase activity of reporter gene was measured from pm*Cyp1a1*-Luc transfected Hepa I cell lysate which contains 2 g total protein using luciferin as a substrate. Nitric oxide producing agents, such as lipopolysaccharide (LPS), sodium nitroprusside (SNP) showed inhibition of luciferase activity that was induced by 10^{-9}M TCDD treatment with dose dependent manner. Concomitant treatment of 1 mM N^G-nitro-*l*-arginine with 10^{-6}~10^{-4} M sodium nitroprusside recovered luciferase activity from the TCDD induced luciferase activity that was inhibited by nitric oxide producing agents. These data demonstrated that nitric oxide could be a mediator of inhibitors on dioxin induced *Cyp1a1* expression in Hepa I cells.

Keywords ☐ SNP, *Cyp1a1*, TCDD, Nitric oxide, N^G-nitro-*l*-arginine, Hepa I cells

Prolonged inflammation in liver usually results in hepatocellular dysfunction, which is associated with the suppression of synthetic performance and a profound inhibition of xenobiotic transformation. Since molecular basis of this phenomena is unknown, therapy is not available. However, a better understanding of the pathophysiology of the inflammed liver may result from the discovery of *l*-arginine-dependent nitric oxide (NO) production in hepatocytes and nonparenchymal liver cells (Billiar, T. R. *et al*., 1989; Curran. R. D., *et al*., 1989). As in many other cell types, an inducible NO synthase (*i*NOS) was identified in hepatocytes upon stimulation with cytokines and endotoxin (Curran, R. D. *et al*., 1990; Geller *et al*., 1993). The induction of *i*NOS was also observed in the course of parasitic infections, such as malaria (Nusslet *et al*., 1991). The gene coding *i*NOS has been cloned from human hepatocytes and expressed in heterologous system (Geller *et al*., 1993). Recently, it has been reported that human *i*NOS gene is transcriptionally regulated by nuclear factor-kappa B dependent mechanism (Nunokawa *et al*., 1996). NO brings about a number of biological functions (Moncada *et al*., 1991). NO can regulate the activity of important enzymes, such as the soluble guanylate cyclases or the enzyme complexes of the mitochondrial electron transport chain (Ignarro, L. J., 1990). NO inhibits vascular smooth muscle cell prolieration and neointima formation after balloon injury by the downregulation of Cdk 2 activity and cyclin A gene transcription (Guo *et al*., 1998). Many of these effects are based on the modulation of enzymatic activity through the binding of NO to prothetic iron complexes. In this context, it is interesting that NO was used for years as a spin-label probe to study the role of the heme groups in the catalytic centers of CYP enzymes (O'Keefe *et al*., 1978). Consequently, it was demonstrated that NO inhibits CYP dependent reaction when microsomal preparations were exposed to NO (Wink *et al*., 1993). Since CYP enzymes play a key role for the biotransformation of xenobiotics, the inhibition of these enzymes would affect hepatocellular detoxification. However, it is not clearly demonstrated that endogenously produced NO suppress CYP enzyme mediated metabolism of xenobiotics. Investigations of other effects of NO, particularly the inhibition of mito-

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chondrial respiration, revealed that major differences between exogenously applied and endogenously produced NO can be observed (Stadler *et al.*, 1991). In light of these observations, hepatocytes seem to have a capacity to resist metabolic inhibition by endogenous NO. In addition, the effects of NO on biotransformation are not clearly understood based on the fact that transcriptional suppression of CYP expression was observed after inflammatory stimulation (Morgan, 1989; Barker *et al.*, 1992). The responsible mediators were found to be the same that induce hepatocellular *i*NOS. In an attempt to clarify the mechanism of NO inhibition of CYP transcription, we have designed experimental approach as following. Mouse *Cyp* 5' flanking DNA (1.6 Kb) was cloned into pGL3 and resulted pm*Cyp1a1*-Luc. This expression plasmid was transfected into Hepa I cells that have been treated with various chemicals to study the *Cyp* transcription and luciferase activities were measured.

MATERIALS AND METHODS

Materials

2,3,7,8-tetrachloro-p-dioxin was kindly provided by Dr. K. Chae from NIEHS, (Research Triangle Park, NC, U.S.A.). N^G-nitro-*l*-arginine, *l*-arginine, and lipopolysaccharide were supplied by Sigma (St. Louise, MO, U.S.A.); sodium nitroprusside (SNP) by Showa chemical Inc. (Japan); LipofectAMINE, Hind III by Gibco BRL (Gaithersburg, MD, U.S.A.); pGL3 basic vector, luciferase assay substrate, luciferase assay buffer, reporter lysis buffer by Promega (Madison, WI, U.S.A.).

Construction of *Cyp1a15*-Luc

Mouse *Cyp1a1* 5' flanking DNA (−1642~+53) was cloned into pGL3 vector at Hind III site.

Cell culture and transfection

Hepa I (Hepa 1c1c7) mouse liver cell lines were grown in Eagles (EBSS) supplemented with 10% (v/v) fetal bovine serum, 100 units penicillin-streptomycin/ml (Gibco BRL, Gaithersburg, MD, U.S.A.). For the transfection, 50,000 Hepa I cells were plated into each well of 24 well plate (Falcon, Lincoln Park, NJ, U.S.A.) and maintained at 37°C with humidified 5% CO_2 for 24 hours. 150 ng of pm*Cyp1a1*-Luc and 1 μg of LipofecTAMINE were mixed in 50 μl of serum-free medium and incubated at room temperature for 45 min before adding it into a well of Hepa I cells. And Hepa I cells were exposed to the DNA-lipid complexes diluted with 200 μl of serum-free medium for 5 hours at 37°C in humidified 5% CO_2 incubator. The details were followed as supplier's manual. After that period, normal MEM medium containing 20% fetal bovine serum were added and incubated for 2 hours.

Chemical treatment

Hepa I cells were rinsed with serum-free medium twice for administration of various chemicals. Cells were pretreated with chemical alone (N^G-nitro-*l*-arginine, *l*-arginine, sodium nitroprusside, or lipopolysaccharide) or N^G-nitro-*l*-arginine, or NG-nitro-*l*-arginine + *l*-arginine was concomitantly treated with sodium nitoprusside or lipopolysaccharide. After incubation for 17 hours, 1 nM of TCDD were administered and incubated for 24 hours.

Luciferase reporter assay

Luciferase activity was determined using liquid scintillation counter. 20 μl of cell extracts including about 2 μg of total protein were mixed with 100 μl of reporter assay reagent at 20°!25°C and luciferase activity was counted in scintillation counter (Beckman, CA, U.S.A.). Protein assay was performed using Micro BCA protein assay reagent kit (Pierce, Rockford, Illinois, U.S.A.) and Elisa Reader (BioRad, CA, U.S.A.). Data represent the fold induction of luciferase activity by calculating the means of four experiments when the luciferase activity of

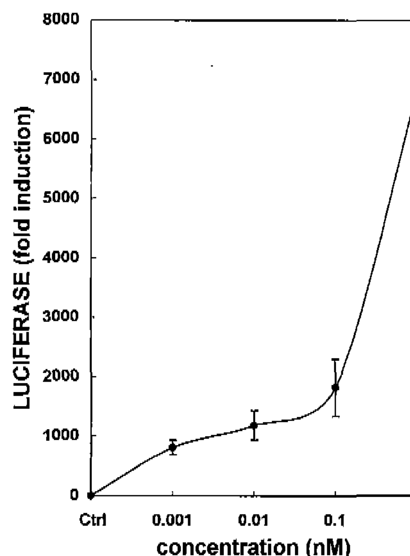

Fig. 1. The dose effect of TCDD on the luciferase activity in Hepa I cells transfected with pm*Cyp1a1*-Luc. After Hepa I cells were transfected, cells were treated with 0.1% DMSO for control or 0.001 nM or 0.01 nM or 0.1 nM or 1 nM TCDD for 24 hours. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when luciferase activity of control cells was set at 1 and the data represent mean±S.E. (n=4).

control treated with 0.1% of DMSO is set at 1.

RESULTS

TCDD concentration dependent induction of pm*Cyp1a1*-Luc expression

Mouse hepa I cells were transfected with pm*Cyp1a1*-Luc construct containing 1.6 Kb DNA of mouse *Cyp1a1* 5' flanking region. Transfected Hepa I cells were treated with various concentration of TCDD (1 pM~1 nM) for 24 hours and lysed for luciferase activity measurement. As shown in Figure 1, 1 pM TCDD increased luciferase activity 850 fold over that of control and as the TCDD concentration increased, luciferase activity was also increased with dose dependent manner. The maximal stimulation of luciferase activity with 1 nM TCDD treatment was 6810 fold over that of control. This result is in agreement with the previous data that 1 nM TCDD brought about maximal responses in stimulation of ethoxyresorufin deethylase activity, and *Cyp1a1* mRNA increases (Ahn M. R. and Sheen Y. Y., 1998). Thus, this reporter gene system can be very useful model to study the mechanism of the regulation of *Cyp1a1* gene expression.

Time dependent induction of pm*Cyp1a1*-Luc expression by TCDD

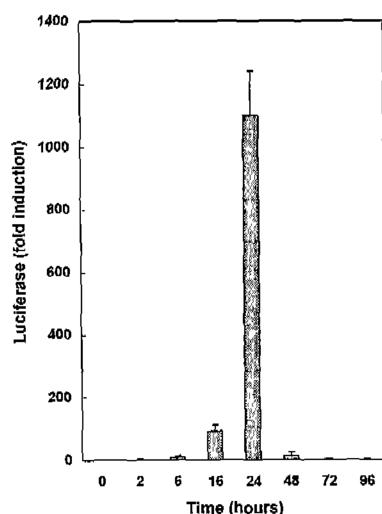

Fig. 2. The time course effect of TCDD on the luciferase activity in Hepa I cells transfected with *pmCyp1a1*-Luc. After Hepa I cells were transfected, cells were treated with 1nM TCDD for 0 or 2 or 6 or 16 or 24 or 48 or 72 or or 96 hours. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when luciferase activity of control cells was set at 1 and the data represent mean±S.E. (n=4).

1 nM TCDD which results in maximal biological response was treated into Hepa I cells that were transfected with pm*Cyp1a1*-Luc for various periods of time. From the 6 hour treatment with TCDD, luciferase activity began to increase and reached the maximal level at 24 hour treatment with TCDD. 48 hour treatment with TCDD brought the luciferase activity back to near the untreated level and 72 and 96 hour treatments show little luciferase activities possibly due to the cell death with TCDD treatment (Fig. 2). This data shows the time dependent induction of *Cyp1a1* expression with 1nM TCDD treatment.

SNP inhibition on TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations of SNP for 17 hours before the treatment of 1 nM TCDD for 24 hours. The treatment with 1 nM TCDD resulted in 8110 folds induction of luciferase activity, which was decreased to 37%, 11%, 3% with 1 μM or 10 μM or 100 μM SNP treatment respectively when TCDD effect was set at 100% (Fig. 3). This data shows NO inhibits the TCDD induced luciferase activity with dose dependent manner. When 1 mM NG-nitro-*l*-arginine was pretreated along

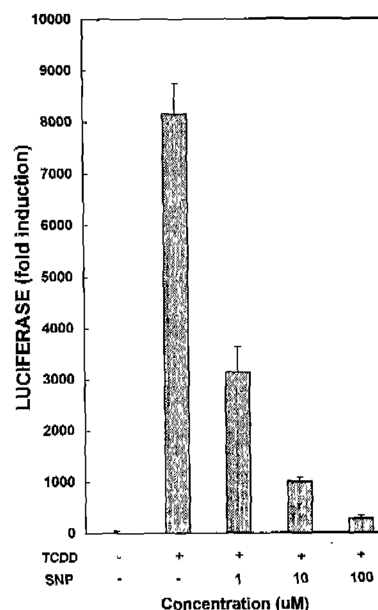

Fig. 3. The dose effect of sodium nitroprusside on the luciferase activity induced by TCDD in Hepa I cells transfected with pm*Cyp1a1*-Luc. After the transfection, cells were pretreated with 1M, 10M, or 100M sodium nitroprusside (SNP) for 17 hours before 0.1% DMSO for control or 1 nM TCDD was treated for 24 hours. The luciferase activity was assayed in cell lysate containing 2 g of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when luciferase activity of control cells was set at 1 and the data represent mean±S.E. (n=4).

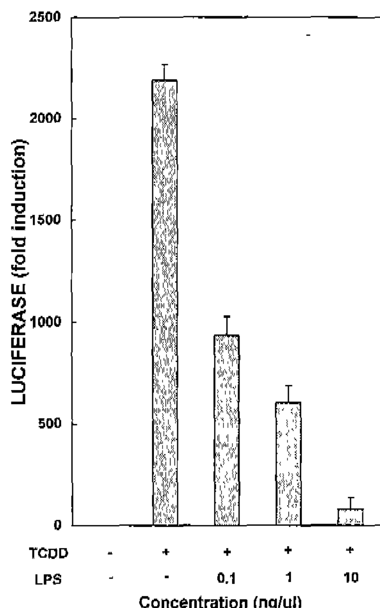

Fig. 4. The effect of N^G-nitro-*l*-arginine on the various concentrations of sodium nitroprusside that inhibits the luciferase activity induced by TCDD in Hepa I cells transfected with pm*Cyp1a1*-Luc. After the transfection, cells were pretreated with 1 M, 10 M, or 100 M sodium nitroprusside (SNP) in the absence or presence of 1 mM N^G-nitro-*l*-arginine for 17 hours before 0.1% DMSO for control or 1 nM TCDD was treated for 24 hours. The luciferase activity was assayed in cell lysate containing 2 g of total protein as described in Materials and Methods. Luciferase activity shows the percent of 1nM TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

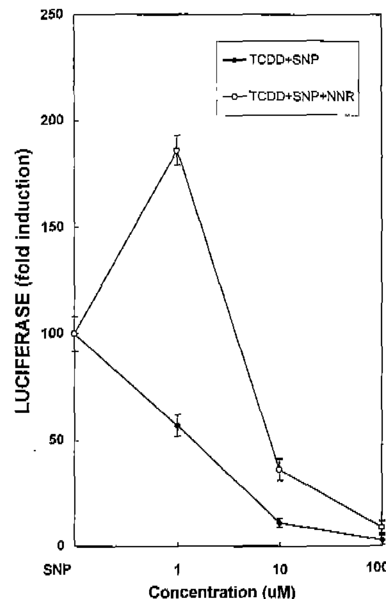

Fig. 5. The dose effect of lipopolysaccharide on the luciferase activity induced by TCDD in Hepa I cells transfected with pm*Cyp1a1*-Luc. After the transfection, cells were pretreated with 0.1 ng/l, 1 ng/l, or 10 ng/l lipopolysaccharide (LPS) for 17 hours before 0.1% DMSO for control or 1nM TCDD was treated for 24 hours. The luciferase activity was assayed in cell lysate containing 2 g of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when luciferase activity of control cells was set at 1 and the data represent mean±S.E. (n=4).

with different concentrations of SNP, the inhibitory effect of SNP was recovered to 186%, 36%, 9% with 1 μM or 10 μM or 100 μM SNP respectively when TCDD stimulated luciferase activity was set at 100%(Fig. 4). This data indicates that endogenous *i*NOS activity being present in Hepa I cells. N^G-nitro-*l*-arginine suppresses endogenous NO production instead of antagonizing with SNP and net effect results in recovery of SNP inhibition on *Cyp1a1* expression.

LPS inhibition on TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (0.1 g/ml or 1 g/ml or 10 g/ml) of LPS for 17 h before the treatment of 10^{-9} M TCDD for 24 h. The treatment with 10^{-9} M TCDD resulted in 2180-fold induction of luciferase activity over control cells, which was decreased with 0.1 g/ml or 1 g/ml or 10 g/ml LPS treatment to 34%, or 22%, or 5%, respectively (Fig. 5). When 1mM N^G-nitro-l-arginine was pretreated along with different concentrations of LPS, the inhibitory effect of LPS was smaller than that without N^G-nitro-*l*-arginine (Fig. 6). 0.1 g/ml LPS treatment resulted in 34% that of the 10^{-9} M TCDD, and 1 mM N^G-nitro-*l*-arginine concomitant treatment with 0.1 g/ml LPS showed 90% that of 10^{-9} M TCDD treatment. These data strongly suggests that nitric oxide may mediate the inhibition of TCDD stimulated *Cyp1a1* promoter activity.

The effect of *l*-arginine and N^G-nitro-*l*-arginine on TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were pretreated with *l*-arginine or N^G-nitro-*l*-arginine in the presence or absence of *l*-arginine for 17 h before the treatment of 10^{-9} M TCDD for 24 h. 10^{-9} M TCDD alone treatment resulted in about 6540-fold induction of luciferase activity over that of control, and *l*-arginine pretreatment decreased the TCDD stimulated luciferase activity to 8% that of 10^{-9} M TCDD treatment. This inhibitory effect of *l*-arginine was not changed with the concomitant treatment of N^G-nitro-*l*-arginine that was known to inhibit *i*NOS. N^G-nitro-*l*-arginine pretreatment resulted in further stimulation of the luciferase activity to 430% that of 10^{-9} M TCDD treatment (Fig. 7). This data indicates the presence of endogenous nitric oxide inhibiting TCDD stimulated luciferase activity somewhat, and an inhibition of *i*NOS enhances stimulatory

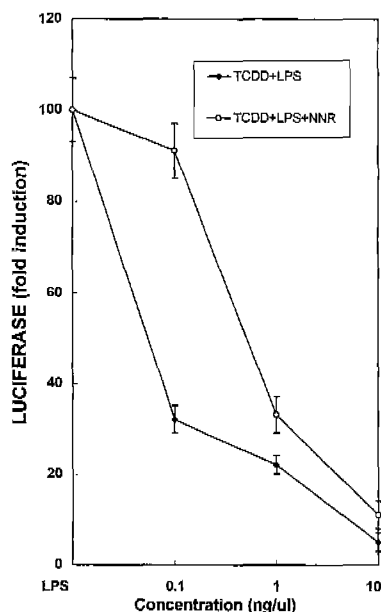

Fig. 6. The effect of N^G-nitro-*l*-arginine on the various concentrations of lipopolysaccharide that inhibits the luciferase activity induced by TCDD in Hepa I cells transfected with pm*Cyp1a1*-Luc. After the transfection, cells were pretreated with 0.1 ng/l, 1 ng/l, or 10 ng/l lipopolysaccharide (LPS) in the absence or presence of 1 mM N^G-nitro-*l*-arginine for 17 hours before 0.1% DMSO or 1 nM TCDD was treated for 24 hours. The luciferase activity was assayed in cell lysate containing 2 g of total protein as described in Materials and Methods. Luciferase activity shows the percent of 1 nM TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

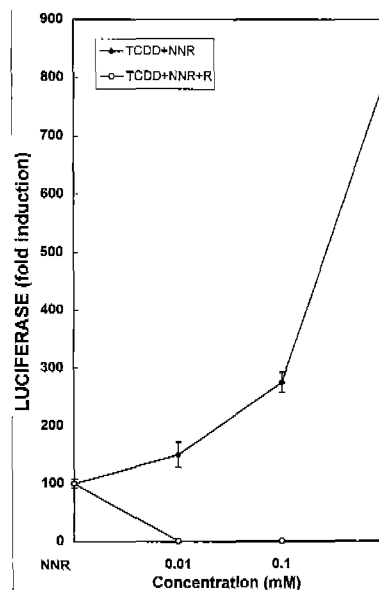

Fig. 7. The effect of *l*-arginine on the various concentrations of NG-nitro-*l*-arginine that enhances the luciferase activity induced by TCDD in Hepa I cells transfected with pm*Cyp1a1*-Luc. After the transfection, cells were pretreated with 0.01 mM, 0.1 mM, or 1 mM N^G-nitro-*l*-arginine (NNR) in the absence or presence of 10 mM *l*-arginine for 17 hours before 0.1% DMSO for control or 1 nM TCDD was treated for 24 hours. The luciferase activity was assayed in cell lysate containing 2 g of total protein as described in Materials and Methods. Luciferase activity shows the percent of 1 nM TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

effect of TCDD on luciferase activity. In order to examine the dose effect of N^G-nitro-*l*-arginine, different concentrations (0.01 mM, 0.1 mM, 1 mM) of N^G-nitro-*l*-arginine were administered into Hepa I cells containing pm*Cyp1a1*-Luc for 17 h before the 10^{-9} M TCDD treatment for 24 h. As shown in Fig. 7, N^G-nitro-*l*-arginine pretreatment increased TCDD stimulated luciferase activity to 150%, 275%, 800% with 0.01 mM, 0.1 mM, 1 mM N^G-nitro-*l*-arginine, respectively when 10^{-9} M TCDD stimulated luciferase activity was set at 100%. This N^G-nitro-*l*-arginine effect was completely abolished with concomitant treatment of *l*-arginine. These data strongly suggested that nitric oxide played an inhibitory role for TCDD stimulation of *Cyp1a1* expression.

DISCUSSION

In this study, it was demonstrated that nitric oxide might affect hepatocellular biotransformation by inhibiting *Cyp1a1* promoter activity. Due to the complex regulation of P450 metabolism, it is difficult to investigate the effects on specific P450 enzymes using hepatocytes or other cells from natural sources. For such purpose, pm*Cyp1a1*-Luc transfected Hepa I cells were good experimental model. After an induction of nitric oxide biosynthesis with LPS, a dramatic decrease in TCDD induced *Cyp1a1* promoter activity was significantly recovered by an *i*NOS inhibitor, N^G-nitro-l-arginine. This data suggested that endogenous nitric oxide had the same inhibitory effect on *Cyp1a1* promoter activity as exogenously applied nitric oxide. At this point, the molecular basis of the inhibitory effect of nitric oxide on P450 enzymes is not clear. It is most likely that inhibition of enzyme activity is from binding of nitric oxide to the heme group in the catalytic center. In this context, it was demonstrated that nitric oxide bound to P450 heme groups with high affinity (O'Keefe D. *et al.*, 1978) and that other hemoproteins, such as the heme oxygenase or the lipoxygenase, were also inhibited by nitric oxide (Stadler J. *et al.*, 1993). However, another study showed that predominantly nonheme iron-nitrosylcomplexes were formed by nitric oxide in hepatocytes based on electron

paramagnetic resonance (EPR) (Stadler J. *et al.*, 1993). It still might be speculated that functional inhibition of P450 by nitric oxide is not attributable to heme binding but is attributable to other effects on the enzymes, such as oxidation of critical amino acids within the molecule (Firth J. D. *et al.*, 1995). In accordance with previous reports (Barker C *et al.*, 1992; Fukuda Y. *et al.*, 1992), results of this study demonstrated that nitric oxide producing agents, such as LPS, SNP decreased TCDD induced luciferase activity with dose dependent manner (Fig. 3, 4). And N^G-nitro-*l*-arginine treatment with either LPS or SNP concomitantly recovered the inhibition of LPS or SNP on TCDD induced luciferase activity. N^G-nitro-*l*-arginine alone treatment augmented the luciferase activity that was induced by TCDD. It was known that LPS stimulated *i*NOS by activating NF-B which in turn interacted with *i*NOS-HRE (Xie Q. *et al.*, 1994). Results of this study suggested that nitric oxide produced upon stimulating *i*NOS by LPS inhibited TCDD induced *Cyp1a1* gene expression. And also results of this study with SNP confirmed previous reports that showed nitric oxide inhibited P450 enzymatic activity and *Cyp1a1* gene expression as well (Firth J. D. *et al.*, 1995). Furthermore, hypoxic agents such as cobalt chloride, desferrioxamine, picolinic acid inhibited TCDD induced Cyp1a1 promoter activity (Kim J. E. and Sheen Y. Y., 1999), and these inhibitory effects were decreased by N^G-nitro-*l*-arginine. However, it was reported that nitric oxide unchanged CYP1a1 mRNA level that was stimulated by 3MC in human HepG3 cells (Sogawa K. *et al.*, 1998). Our unpublished data (Kim, Y. W. and Sheen, Y. Y.) showed SNP inhibited TCDD stimulated CYP1a1 mRNA level in mouse Hepa cells. At this moment, the nature of the discrepancy between human HepG3 and mouse Hepa cells is not known. These data strongly suggested the presence of cross-talk between hypoxia and nitric oxide on the regulation of *Cyp1a1* gene expression. Recently, *i*NOS gene has been studied extensively and known to contain HRE at 5' flanking region where HIF-1/Arnt dimer binds to transactivate *i*NOS gene (Melillo G. *at al.*, 1997). Based on these reports, it was possible to hypothesize that hypoxia activated *i*NOS via HIF-1/Arnt dimer binding to HRE which in turn increased nitric oxide production, and then it downregulated *Cyp1a1* promoter activity. Therefore, hypoxia possibly down regulates *Cyp1a1* gene expression not only by competition with Ahr for Arnt but also by nitric oxide production by *i*NOS activation. In conclusion, our results indicated that nitric oxide producing signal could be one of main regulatory mechanisms of downregulating *Cyp1a1* gene expression. These findings may help to understand the problem of impaired detoxification by the inflamed liver because human hepatocytes have been shown to produce large amounts of nitric oxide under inflammatory stimulation (Kuo P. C. and Slivka A., 1994).

ACKNOWLEDGEMENTS

The present study was supported by the grant # 1998-019-F0074 from ministry of education and HMP-98-B-3-0015 from ministry of health and welfare in Korea.

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