Nitric Oxide Inhibits Dioxin Action for the Stimulation of Cyp1a1 Promoter Activity

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Since it is known that hypoxia increases inducible nitric oxide synthase (iNOS) gene expression through the hypoxia responsive element, it was hypothesized that nitric oxide could be a mediator of hypoxia to inhibit Cyp1a1 promoter activity. In order to test this hypothesis, we have undertaken a study to examine the effects of hypoxia and nitric oxide on Cyp1a1 promoter activity in Hepa 1 cells. Mouse Cyp1a1 5′ flanking DNA, 1.6 kb, was cloned into pGL3 expression vector in order to construct pmCyp1a1-Luc. Hepa 1 cells were transfected with pmCyp1a1-Luc and were treated with 10⁻⁵M 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the presence or absence of various hypoxic agents such as 10⁻⁸–10⁻⁴M cobalt chloride or 10⁻⁸–10⁻⁴M picolinic acid or 10⁻⁸–10⁻⁴M desferrioxamine. The luciferase activity of the reporter gene was measured from pmCyp1a1-Luc transfected Hepa 1 cell lysate which contains 2 μg total protein using luciferin as a substrate. Hypoxic agents such as cobalt chloride, picolinic acid, and desferrioxamine showed inhibition of luciferase activity that was induced by 10⁻³M TCDD treatment in a dose dependent manner. Concomitant treatment of 1 mM N⁵-nitro-l-arginine with 10⁻⁴–10⁻⁴M cobalt chloride or 10⁻⁴–10⁻⁴M desferrioxamine or 10⁻⁴–10⁻⁴M picolinic acid or 10⁻³–10⁻⁴M sodium nitroprusside recovered luciferase activity from the TCDD induced luciferase activity that was inhibited by hypoxic agents. These data demonstrated that nitric oxide might be a mediator of iron chelating agents and hypoxic agents to inhibit dioxin induced Cyp1a1 promoter activity in Hepa 1 cells.

Key words Cyp1a1; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); nitric oxide, N⁵-nitro-l-arginine; hypoxia; Hepa 1 cell

Nitric oxide is a short lived inter- and intracellular second messenger generated by a family of enzymes known as nitric oxide synthases (NOSs) and brings about a number of biological functions.¹⁻⁴ Nitric oxide can regulate the activity of important enzymes, such as the soluble guanylate cyclases or the enzyme complexes of the mitochondrial electron transport chain or glycolytic enzymes, aldolase A, endolase-1, lactate dehydrogenase A, phosphoglycerate kinase-1 or heme oxygenase.⁵⁻⁹ Many of these effects are based on the modulation of enzymatic activity through the binding of nitric oxide to prosthetic iron complexes. In this context, it is interesting that nitric oxide has been used for years as a spin-label probe to study the role of the heme groups in the catalytic centers of P450 enzymes.¹⁰ Consequently, it was demonstrated that nitric oxide inhibited P450 dependent reactions when microsomal preparations were exposed to nitric oxide.¹¹ Since P450 enzymes play a key role for the bio- transformation of xenobiotics, the inhibition of these enzymes would affect hepatocellular detoxification.¹²⁻¹⁵ However, it is not clearly demonstrated that endogenously produced nitric oxide suppresses P450 enzyme mediated metabolism of xenobiotics. Investigations of other effects of nitric oxide, particularly the inhibition of mitochondrial respiration, revealed that major differences between exogenously applied and endogenously produced nitric oxide could be observed.¹⁶ In light of these observations, hepatocytes seem to have a capacity to resist metabolic inhibition by endogenous nitric oxide. In addition, the effects of nitric oxide on bio-transformation are not clearly understood although it was observed that Cyp1a1 expression was suppressed transcriptionally after inflammatory stimulation.¹⁷,¹⁸ As in many other cell types, an inducible nitric oxide synthase (iNOS) was identified in hepatocytes upon stimulation with cytokines and endotoxin.¹⁹ The iNOS in hepatocytes was known to be regulated by lipopolysaccharide (LPS), interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β),²⁰⁻²² The gene coding iNOS has been cloned from human hepatocytes and expressed in heterologous system.²³ Recently, it has been reported that human iNOS gene is transcriptionally regulated by a nuclear factor-kappa B (NF-κB) dependent mechanism.²⁴ The 5′ untranslated region of the iNOS gene has been shown to contain three cis-elements, the first of which is the IFN-γ responsive region,²⁵ the second of which is the NF-κB responsive region,²⁶ and the third of which is the hypoxia responsive region, which is known as NOS-HRE.²⁷ It has been also reported that the transcriptional activation of iNOS by hypoxia requires HIF-1α/Arnt dimer protein binding to NOS-HRE.²⁸ This is a distinct mechanism from the NF-κB mediated transcriptional activation of iNOS.²⁹,³⁰ Iron suppressed LPS stimulation of iNOS, whereas the iron chelator, desferrioxamine (DFX), activated iNOS, which might be a strong indication of hypoxia-dependent activation of iNOS.²⁹,³⁰ Under the hypoxic condition, nitric oxide inhibited the HIF-1α activity even though nitric oxide did not change the Cyp1a1 mRNA level in HepG3 cells.²⁸,³¹ Since the hypoxia and dioxin signal pathways are known to be in competition to recruit Arnt at the level of transcription, it is possible that nitric oxide and hypoxia cross-talk might exist for the regulation of Cyp1a1 expression. In an attempt to examine the cross-talk between hypoxia and nitric oxide on the inhibition of Cyp1a1 transcription, we have designed an experimental approach by cloning the mouse Cyp 5′ flanking DNA (1.6 kb) into pGL3 and used pmCyp1a1-Luc to transfect into Hepa 1 cells. pmCyp1a1-Luc transfected Hepa 1 cells were treated with various iNOS inducers or hypoxic agents to study the Cyp1a1 transcription and luciferase activities were measured.

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MATERIALS AND METHODS

Materials  2,3,7,8-Tetrachloro-\(\rho\)-dioxin was kindly provided by Dr. K. Chae from NIEHS, (Research Triangle Park, NC, U.S.A.), \(N^2\)-Nitro-\(L\)-arginine (NNR), \(L\)-arginine, LPS, DFX, picolinic acid (PA), ferric sulfate and phenol were supplied by Sigma (St. Louis, MO, U.S.A.), sodium nitroprusside (SNP) purchased from Showa Chemical Inc. (Japan), cobalt chloride was purchased from Junsei Chem. Co. (Japan), and agargose was purchased from FMC (Rockland, ME, U.S.A.). LipofectAMINE and Hind III were ordered from by Gibco BRL (Gaithersburg, MD, U.S.A.), pGL3 basic vector and luciferase assay system were purchased from Promega (Madison, WI, U.S.A.).

Construction of \textit{Cyp1a1-Luc} Mouse \textit{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 earle's balanced salt solution (EBSS) supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin-streptomycin (Gibco BRL, Gaithersburg, MD, U.S.A.). For the transfection of pm\textit{Cyp1a1-Luc}, 50000 Hepa I cells were plated into each well of a 24 well plate (Falcon, Lincoln Park, NJ, U.S.A.) and maintained at 37°C with humidified 5% CO\(_2\) for 24 h. 150 ng of pm\textit{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 to Hepa I cells. All Hepa I cells were exposed to the DNA-lipid complexes in serum-free medium for at least 5 h at 37°C in a humidified 5% CO\(_2\) incubator, before cells were maintained in normal minimum essential medium (MEM) containing 20% fetal bovine serum. The details were followed as described in the supplier's manual.

Chemical Treatment  Hepa I cells were rinsed with serum-free medium twice before the administration of various chemicals in serum free medium. Stock solutions of chemicals were made in dimethyl sulfoxide (DMSO) as a vehicle and control cells were treated with 0.1% DMSO. 0.01—1 mM NNR or 10 mM L-arginine was administered for 17 h before the 10\(^{-9}\) M TCDD treatment for 24 h. And 10\(^{-6}\)—10\(^{-4}\) M sodium nitroprusside or 0.1—10 µg/ml LPS or 10\(^{-6}\)—10\(^{-4}\) M cobalt chloride or 10\(^{-6}\)—10\(^{-4}\) M DFX or 10\(^{-6}\)—10\(^{-4}\) M PA was administered in the presence or absence of 1 mM NNR for 17 h before the 10\(^{-9}\) M TCDD treatment for 24 h.

Luciferase Reporter Assay  Luciferase activity was determined in cell extracts containing 2 µg of total protein. Twenty microliters of cell extracts were mixed with 100 µl of reporter assay reagent at 20—25°C and luminescents were measured using liquid scintillation counter (Beckman, CA, U.S.A.). Protein assay of cell extracts was carried out using Micro BCA protein assay reagent kit (Pierce, Rockford, IL, U.S.A.) and ELISA Reader (BioRad, CA, U.S.A.). Luciferase activity data is presented as the fold induction of control cells that were treated with 0.1% DMSO when control luciferase activity is set at 1.

RESULTS

LPS Inhibition on TCDD Induced Luciferase Activity

pm\textit{Cyp1a1-Luc} transfected Hepa I cells were treated with various concentrations (0.1 or 1 or 10 µg/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, 1 or 10 µg/ml LPS treatment to 34%, 22%, or 5%, respectively (Fig. 1). When 1 mM NNR was pretreated along with different concentrations of LPS, the inhibitory effect of LPS was smaller than that without NNR (Fig. 2). 0.1 µg/ml LPS treatment resulted in 34% that of the 10\(^{-9}\) M TCDD, and 1 mM NNR concomitant treatment with 0.1 µg/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 \textit{Cyp1a1} promoter activity.

SNP Inhibition on TCDD Induced Luciferase Activity

pm\textit{Cyp1a1-Luc} transfected Hepa I cells were pretreated with various concentrations (10\(^{-7}\) M, 10\(^{-5}\) M, 10\(^{-4}\) M) of SNP for 17 h before the treatment of 10\(^{-9}\) M TCDD for 24 h. The treatment with 10\(^{-9}\) M TCDD resulted in 8110-fold induction of luciferase activity, which was decreased with 10\(^{-6}\) M, 10\(^{-5}\) M, and 10\(^{-4}\) M SNP pretreatment to 37%, 11%, and 3% that of 10\(^{-9}\) M TCDD treatment, respectively (Fig. 3). This data shows that nitric oxide inhibits the TCDD induced luciferase activity in a dose dependent manner. As shown in Fig. 4, when 1 mM NNR was pretreated along with different concentrations of SNP, the inhibitory effect of SNP was diminished. 10\(^{-8}\) M SNP alone pretreatment resulted in 57% that of 10\(^{-9}\) M TCDD treatment, however, 1 mM NNR concomitant pretreatment with 10\(^{-8}\) M SNP resulted in 186% that of 10\(^{-9}\) M TCDD treatment. This suggested that there might be endogenous nitric oxide in Hepa I cells which might re-

![Fig. 1. The Dose Effect of LPS on the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc](image-url)
Fig. 2. The Effect of NNR on the Various Concentrations of LPS That Inhibits the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with 0.1, or 1, or 10 μg/ml LPS in the absence or presence of 1 mM NNR for 17 h before being treated with 0.1% DMSO or 1 mM TCDD for 24 h. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the percent of 10^{-7} M TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

Fig. 3. The Dose Effect of SNP on the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with 10^{-5} M or 10^{-4} M SNP for 17 h before being treated with 0.1% DMSO for control or 10^{-7} M TCDD for 24 h. 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).

Fig. 4. The Effect of NNR on the Various Concentrations of SNP That Inhibits the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with 10^{-5} M or 10^{-4} M or 10^{-3} M SNP in the absence or presence of 1 mM NNR for 17 h before being treated with 0.1% DMSO for control or 10^{-7} M TCDD for 24 h. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the percent of 10^{-7} M TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

duce the power of TCDD stimulation on Cyp1a gene expression. 10^{-5} M SNP or 10^{-4} M SNP alone pretreatment showed 11% or 3% that of 10^{-7} M TCDD treatment, whereas 1 mM NNR concomitant pretreatment with either 10^{-5} M SNP or 10^{-4} M SNP showed 36% or 9% that of 10^{-9} M TCDD treatment, respectively. This data indicates that nitric oxide inhibits Cyp1a promoter activity and endogenous iNOS activity being present in Hepa I cells. Thus, it seems that NNA suppresses endogenous nitric oxide production instead of antagonizing with SNP and the net effect results in diminution of SNP inhibition on Cyp1a expression.

NNR Recovers Cobalt Chloride Inhibition on the TCDD Induced Luciferase Activity pmCyp1a1-Luc transfected Hepa I cells were treated with various concentrations (10^{-6} M, 10^{-5} M, 10^{-4} M) of cobalt chloride for 17 h before the treatment of 10^{-9} M TCDD for 24 h. The treatment with 10^{-7} M TCDD resulted in 4170-fold induction of luciferase activity, whereas the pretreatment of 10^{-6} M, 10^{-5} M or 10^{-4} M cobalt chloride decreased the TCDD stimulated luciferase activity to 37%, 9%, or 5% that of 10^{-9} M TCDD treatment, respectively. As concentration of cobalt chloride increased, the power of inhibition on TCDD induced luciferase activity was enlarged (Fig. 5). When the NOS inhibitor, NNR was administered together with cobalt chloride, the inhibitory strength of cobalt chloride was weakened (Fig. 5). 10^{-6} M, 10^{-5} M or 10^{-4} M cobalt chloride respectively showed 37%, or 9%, or 5% that of 10^{-9} M TCDD stimulated luciferase activity without NNR, however, when 1 mM NNR was added concomitantly with 10^{-6} M, or 10^{-5} M or 10^{-4} M cobalt chloride, the luciferase activity was 120%, or 78%, or
Fig. 5. The Effect of NNR on the Various Concentrations of Cobalt Chloride Which Inhibits the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1α-Luc

After the transfection, cells were pretreated with $10^{-7} \text{M}$ or $10^{-5} \text{M}$ or $10^{-4} \text{M}$ cobalt chloride (Co) in the absence or presence of $1 \text{mM}$ NNR and incubated for 17 h before being treated with $0.1\%$ DMSO for control or $10^{-5} \text{M}$ TCDD for 24 h. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the percent of $10^{-7} \text{M}$ TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean ± S.E. (n=4).

39% that of $10^{-7} \text{M}$ TCDD induced, respectively. This result suggests that nitric oxide is involved in the inhibition of the promoter activity of Cyp1α by hypoxic agents.

**NNR Recovers DFX Inhibition on the TCDD Induced Luciferase Activity** pmCyp1α-Luc transfected Hepa I cells were treated with various concentrations ($10^{-6} \text{M}$, $10^{-5} \text{M}$, $10^{-4} \text{M}$) of DFX for 17 h before the treatment of $10^{-9} \text{M}$ TCDD for 24 h. The treatment with $10^{-5} \text{M}$ TCDD resulted in 7250-fold induction of luciferase activity, which was decreased in a dose-dependent manner when $10^{-6} \text{M}$ or $10^{-5} \text{M}$ or $10^{-4} \text{M}$ DFX was pretreated for 17 h to 13%, or 7%, or 3% that of $10^{-9} \text{M}$ TCDD treatment, respectively (Fig. 6). Concomitant treatment of NNR with $10^{-6} \text{M}$ or $10^{-5} \text{M}$ or $10^{-4} \text{M}$ DFX to pmCyp1α-Luc transfected Hepa I cells showed 81%, or 64%, or 57% that of $10^{-9} \text{M}$ TCDD treated luciferase activity, respectively. This data demonstrated that DFX inhibited luciferase activity in a dose-dependent manner and the iNOS inhibitor, NNR reversed the effect of DFX on Cyp1α promoter activity. This result suggested that nitric oxide might be a mediator of inhibitory effect of DFX on Cyp1α expression by TCDD.

**NNR Recovers PA Inhibition on the TCDD Induced Luciferase Activity** pmCyp1α-Luc transfected Hepa I cells were treated with various concentrations ($10^{-6} \text{M}$, $10^{-5} \text{M}$, $10^{-4} \text{M}$) of PA for 17 h before the treatment of $10^{-9} \text{M}$ TCDD for 24 h. The treatment of $10^{-9} \text{M}$ TCDD resulted in 2680-fold induction of luciferase activity, which was decreased with $10^{-6} \text{M}$ or $10^{-5} \text{M}$ or $10^{-4} \text{M}$ PA pretreatment to 85%, or 37%, or 17% that of $10^{-9} \text{M}$ TCDD treated luciferase activity, respectively (Fig. 7). When NNR was treated along with $10^{-6} \text{M}$ or $10^{-5} \text{M}$ or $10^{-4} \text{M}$ PA, the luciferase activity was 99%, or 87%, or 71%, respectively (Fig. 7). This data showed PA, a hypoxic agent, inhibited the TCDD induced luciferase activity in a dose-dependent manner and NNR recovered the effect of PA. Thus these data suggest that PA inhibits the TCDD induced Cyp1α gene expression through nitric oxide.

The Effect of l-Arginine and NNR on TCDD Induced Luciferase Activity pmCyp1α-Luc transfected Hepa I cells were pretreated with l-arginine or NNR in the presence or absence of l-arginine for 17 h before the treatment of $10^{-9} \text{M}$ TCDD for 24 h. $10^{-9} \text{M}$ 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 $10^{-9} \text{M}$ TCDD treatment. This inhibitory effect of l-arginine was not changed with the concomitant treatment of NNR that is known to inhibit iNOS. NNR pretreatment resulted in further stimulation of the luciferase activity to 430% that of $10^{-9} \text{M}$ TCDD treatment (Fig. 8). These data indicate the presence of endogenous nitric oxide inhibiting TCDD stimulated luciferase activity somewhat, and an inhibition of iNOS enhances the stimulatory effect of TCDD on luciferase activity. In order to examine the dose effect of NNR, different concentrations (0.01, 0.1, 1 mM) of NNR were administered into Hepa I cells containing pmCyp1α-Luc for 17 h before the
Fig. 7. The Effect of NNR on the Various Concentrations of PA Which Inhibits the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with $10^{-4}$ M or $10^{-5}$ M or $10^{-6}$ M PA in the absence or presence of 1 mM NNR and incubated for 17 h before being treated with 0.1% DMSO for control or $10^{-7}$ M TCDD for 24 h. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the percent of $10^{-7}$ M TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

Fig. 8. The Effect of NNR and L-Arginine on the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with 1 mM NNR and 10 mM L-arginine (R) for 17 h before being treated with 0.1% DMSO for control or $10^{-7}$ M TCDD for 24 h. 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).

Fig. 9. The Effect of L-Arginine on the Various Concentrations of NNR that Enhances the Luciferase Activity Induced by TCDD in Hepa I Cells Transfected with pmCyp1a1-Luc

After the transfection, cells were pretreated with 0.01, 0.1, or 1 mM NNR in the absence or presence of 10 mM L-arginine for 17 h before being treated with 0.1% DMSO for control or $10^{-7}$ M TCDD for 24 h. The luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the percent of $10^{-7}$ M TCDD treated cells when luciferase activity of TCDD cells was set at 100 and the data represent mean±S.E. (n=4).

10^{-8} M TCDD treatment for 24 h. As shown in Fig. 9, NNR pretreatment increased TCDD stimulated luciferase activity to 150%, 275%, 800% with 0.01, 0.1, 1 mM NNR, respectively when $10^{-9}$ M TCDD stimulated luciferase activity was set at 100%. This NNR 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, pmCyp1a1-Luc transfected Hepa I cells were a 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 iNOS inhibitor, NNR. 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 and that other heme proteins, such as the heme oxygenase or the lipoxygenase, were
also inhibited by nitric oxide. However, another study showed that predominantly nonheme iron-nitrosyl complexes were formed by nitric oxide in hepatocytes based on electron paramagnetic resonance (EPR). 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. In accordance with previous reports, results of this study demonstrated that nitric oxide producing agents, such as LPS, SNP decreased TCDD induced luciferase activity in a dose dependent manner (Figs. 1, 3). And NNR treatment with either LPS or SNP concomitantly recovered the inhibition of LPS or SNP on TCDD induced luciferase activity. NNR alone treatment augmented the luciferase activity that was induced by TCDD. It was known that LPS stimulated iNOS by activating NF-κB which in turn interacted with iNOS-HRE. Results of this study suggested that nitric oxide produced upon stimulating iNOS 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. Furthermore, hypoxic agents such as cobalt chloride, DFX, PA inhibited TCDD induced Cyp1a1 promoter activity (Figs. 5—7), and these inhibitory effects were decreased by NNR. However, it was reported that nitric oxide did not change the Cyp1a1 mRNA level that was stimulated by 3MC in human HepG3 cells. 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, iNOS gene has been studied extensively and known to contain HRE at 5' flanking region where HIF-1α/Arnt dimer binds to transactivate iNOS gene. Based on these reports, it was possible to hypothesize that hypoxia activated iNOS via HIF-1α/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 Arh for Arnt but also by nitric oxide production by iNOS activation. In conclusion, our results indicated that nitric oxide producing signal could be one of main regulatory mechanisms of downregulating Cyp1a1 gene expression.

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