NITRIC OXIDE INHIBITS DIOXIN ACTION FOR THE STIMULATION OF *Cyp1a1* PROMOTER ACTIVITY

Ki E. Jeong, Yeo W. Kim Ji-E. Kim and Yhun Y. Sheen

College of Pharmacy, Ewha Womans University, # 11-1, Daehyun-dong, Sudaemun-ku, Seoul, 120-750, Korea

Introduction

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] It was demonstrated that nitric oxide inhibited P450 dependent reaction when microsomal preparations were exposed to nitric oxide 2[)]. The effects of nitric oxide on biotransformation are not clearly understood although it was observed that *CYP1a1* expression was suppressed transcriptionally after inflammatory stimulation [3)]. As in many other cell types, an inducible nitric oxide synthase (*i*NOS) was identified in hepatocytes upon stimulation with cytokines and endotoxin[14, 19)]. The inducible nitric oxide synthase (*i*NOS) in hepatocytes was known to be regulated by lipopolysaccharide (LPS), interferon-γ (IFN-γ), tumor necrosis factor-β (TNF-β)[4)]. The gene coding *i*NOS has been cloned from human hepatocytes and expressed in heterologous system[5)]. Recently, it has been reported that human *i*NOS gene is transcriptionally regulated by nuclear factor-kappa B (NF-κB) dependent mechanism[6)]. The 5' untranslated region of the *i*NOS gene has been shown to contain three cis-elements, first of which is IFN-γ responsive region[7)], second of which is NF-κB responsive region[8)], and third of which is hypoxia responsive region, which is known as *i*NOS-HRE[9)]. It has been also reported that the transcriptional activation of *i*NOS by hypoxia requires HIF-1α/Arnt dimer protein binding to *i*NOS-HRE[10)]. This is distinct mechanism from the NF-κB mediated transcriptional activation of *i*NOS[11)]. Iron supressed LPS stimulation of *i*NOS, whereas iron chelator, desferrioxamine activated *i*NOS, which might be a strong indication of hypoxia-dependent activation of *i*NOS [12)]. Under the hypoxic condition, nitric oxide inhibited the HIF-1α activity eventhough, nitric oxide unchanged CYP1a1 mRNA level in HepG3 cells[13)]. Since 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 experimental approach by cloning the mouse *Cyp* 5' flanking DNA (1.6 kb) into pGL3 and used pm*Cyp1a1*-Luc to transfect into Hepa I cells. pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various *i*NOS inducers or hypoxic agents to study the *Cyp1a1* transcription and luciferase activities were measured.

Methods abd Materials.

Cell culture and

Hepa I (Hepa 1c1c7) mouse liver cell lines were transfected with pm*Cyp1a1*-Luc and LipofecTAMINE according to 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 DMSO as a vehicle and control cells were treated with 0.1% DMSO

Luciferase reporter assay

The luminescents were measured using luciferin.

Results and Discussion

LPS inhibition on TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (0.1μg/ml or 1μg/ml or 10μg/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 0.1μg/ml, 1μg/ml or 10μg/ml LPS treatment to 34%, 22%, or 5%, respectively. 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. 0.1μg/ml LPS treatment resulted in 34% that of the 10^{-9}M TCDD, and 1mM N^G-nitro-*l*-arginine concomitant treatment with 0.1μg/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.

SNP inhibition on TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were pretreated with various concentrations (10^{-6}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. This data shows that nitric oxide inhibits the TCDD induced luciferase activity with dose dependent manner. As shown in Fig 4, when 1mM N^G-nitro-*l*-arginine was pretreated along with different concentrations of SNP, the inhibitory effect of SNP was diminished. 10^{-6}M SNP alone pretreatment resulted in 57% that of 10^{-9}M TCDD treatment, however, 1mM N^G-nitro-*l*-arginine concomitant pretreatment with 10^{-6}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 reduce the power of TCDD stimulation on *Cyp1a1* gene expression. 10^{-5}M SNP or 10^{-4}M SNP alone pretreatment showed 11% or 3% that of 10^{-9}M TCDD treatment, whereas 1mM N^G-nitro-*l*-arginine 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 *Cyp1a1* promoter activity and endogenous *i*NOS activity being present in Hepa I cells. Thus, it seems that N^G-nitro-*l*-arginine suppresses endogenous nitric oxide production instead of antagonizing with SNP and net effect results in diminition of SNP inhibition on *Cyp1a1* expression.

N^G-nitro-*l*-arginine recovers cobalt chloride inhibition on the TCDD induced luciferase activity

pm*Cyp1a1*-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^{-9}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 increase the concentrations of cobalt chloride, the power of inhibition on TCDD induced luciferase activity was enlarged. When nitric oxide synthase inhibitor, N^G-nitro-*l*-arginine was administered together with cobalt chloride,

the inhibitory strength of cobalt chloride was weakened. 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 N^G-nitro-*l*-arginine, however, when 1mM N^G-nitro-*l*-arginine 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 39% that of 10^{-9}M TCDD induced, respectively. This result suggests that nitric oxide involves in the inhibition of the promoter activity of *Cyp1a1* by hypoxic agents.

N^G-nitro-*l*-arginine recovers desferrioxamine inhibition on the TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (10^{-6}M, 10^{-5}M, 10^{-4}M) of desferrioxamine for 17 h before the treatment of 10^{-9}M TCDD for 24 h. The treatment with 10^{-9}M TCDD resulted in 7250-fold induction of luciferase activity, which was decreased with dose dependent manner when 10^{-6}M or 10^{-5}M or 10^{-4}M desferrioxamine was pretreated for 17 h to 13%, or 7%, or 3% that of 10^{-9}M TCDD treatment, respectively. Concomitant treatment of N^G-nitro-*l*-arginine with 10^{-6}M or, 10^{-5}M or 10^{-4}M desferrioxamine to pm*Cyp1a1*-Luc transfected Hepa I cells showed 81%, or 64%, or 57% that of 10^{-9}M TCDD treated luciferase activity, respectively. This data demonstrated that desferrioxamine inhibited luciferase activity with dose dependent manner and *i*NOS inhibitor, N^G-nitro-*l*-arginine reversed the effect of desferrioxamine on *Cyp1a1* promoter activity. This result suggested that nitric oxide might be a mediator of inhibitory effect of desferrioxamine on *Cyp1a1* expression by TCDD.

N^G-nitro-*l*-arginine recovers picolinic acid inhibition on the TCDD induced luciferase activity

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (10^{-6}M, 10^{-5}M, 10^{-4}M) of picolinic acid for 17 h before the treatment of 10^{-9}M TCDD for 24 h. The treatment of 10^{-9}M TCDD resulted in 2680-fold induction of luciferase activity, which was decreased with 10^{-6}M or 10^{-5}M or 10^{-4}M picolinic acid pretreatment to 85%, or 37%, or 17% that of 10^{-9}M TCDD treated luciferase activity, respectively. When N^G-nitro-*l*-arginine was treated along with 10^{-6}M or 10^{-5}M or 10^{-4}M picolinic acid, the luciferase activity was 99%, or 87%, or 71%, respectively. This data showed picolinic acid, one of hypoxia agents inhibited the TCDD induced luciferase activity with dose dependent manner and N^G-nitro-*l*-arginine recovered the effect of picolinic acid. Thus these data suggest that picolinic acid can inhibit the TCDD induced *Cyp1a1* gene expression might be through nitric oxide.

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. These data indicate the presence of endogenous nitric oxide inhibiting TCDD stimulated luciferase activity somewhat, and an inhibition of *i*NOS enhances stimulatory effect of TCDD on luciferase activity. In order to examine the dose effect of N^G-nitro-*l*-arginine, different concentrations (0.01mM, 0.1mM, 1mM) 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 9, N^G-nitro-*l*-arginine pretreatment increased TCDD stimulated luciferase activity to 150%, 275%, 800% with 0.01mM, 0.1mM, 1mM 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.

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.

References

1. Moncada S., Paln . R. M., Higgs E. A., *Pharmacol. Rev.*, **43,** 109-142 (1991).
2. Guo K., Andres V., Walsh K., *Circulation,* **97,** 2066-2072 (1998).
3. Stuehr D. J., Nathan C. F., *J. Exp. Med.,* **169,** 1543-1555 (1989).
4. Gonzalez F. J., *Pharmacol. Rev.*, **40,** 244-288 (1989).
5. Morgan E. T., *Mol. Pharmacol.*, **36,** 699-707 (1989).
6. Barker C., Fagan J., Pasco D., *J. Biol. Chem.*, **267,** 8050-8055 (1992).
7. Geller D. A., Nussler A. K., Di S. M., Lowenstein C. J., Shapiro R. A., Wang S. C., Simmons R. L Billiar T. R., *Proc. Natl. Acad. Sci. USA,* **90,** 522-552 (1993).
8. Xie Q., Cho H. J., Calaycay J., Mumford R. A., Swiderek K. M., Lee T. D., Ding A., Troso T., Nathan C., *Science,* **256,** 225-228 (1992).
9. Nathan, C., Xie Q., *J. Biol.Chem.,* **269,** 13725-13728 (1994).
10. Geller D. A., Lowenstein C. J., Shapiro R. A., Nussler A. K., Di S. M., Wang S. C., Nakayama D K., Simmons R. L., Snyder S. H., and Billiar T. R., *Proc. Natl. Acad. Sci. USA* , **9,** 3491-3495 (1993)
11. Nunokawa Y., Oikawa S., Tanaka S., *Biochem. Biophys. Res. Commun.*, **223,** 347-352 (1996).
12. Xie Q., Kashiwabara Y., Nathan C., *J. Biol. Chem.,* **269,** 4705-4708 (1994).