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Inhibition of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Stimulated *Cyp1a1* Promoter Activity by Hypoxic Agents

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ABSTRACT. Since hypoxia-inducible factor-1α (HIF-1α) and the arylhydrocarbon receptor (AhR) shared the AhR nuclear translocator (Arnt) for hypoxia- and AhR-mediated signaling, respectively, it was possible to establish the hypothesis that hypoxia could regulate cytochrome P450 1a1 (*Cyp1a1*) expression. In order to test this hypothesis, we undertook to examine the effect of hypoxia on *Cyp1a1* transcription in Hepa-I cells. Mouse *Cyp1a1* 5′-flanking DNA, 1.6 kb was cloned into pGL3 expression vector in order to construct pm*Cyp1a1*-Luc. Hepa-I cells were transfected with pm*Cyp1a1*-Luc and treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the presence or absence of various hypoxic agents such as 1–100 μM cobalt chloride, 1–100 μM picolinic acid, and 1–100 μM desferrioxamine. Luciferase activity of the reporter gene was measured from pm*Cyp1a1*-Luc-transfected Hepa-I 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 1-nM TCDD treatment in a dose-and time-dependent manner. Concomitant treatment of 150 μM ferrous sulfate with 1–100 μM desferrioxamine or 1–100 μM picolinic acid recovered luciferase activity from that inhibited by hypoxic agents or induced by TCDD. These data demonstrated that iron-chelating and hypoxic agents inhibited dioxin-induced *Cyp1a1* transcription in Hepa-I cells. Thus, we might suggest that hypoxia inhibits TCDD-induced *Cyp1a1* expression due to the competition between HIF-1α and the AhR for the Arnt in Hepa-I cells. BIOCHEM PHARMACOL **59**;12:1549–1556, 2000. © 2000 Elsevier Science Inc.

KEY WORDS: *Cyp1a1*; TCDD; hypoxia, Hepa-I cells

The cytochrome P450-dependent monooxygenase system catalyzes oxidative metabolism of a wide variety of substrates including endogenous as well as exogenous compounds. As a preliminary detoxification step, many compounds are first converted to polar metabolites by cytochrome P450, which facilitates their elimination. However, some compounds may also be inadvertently bioactivated by cytochrome P450 to reactive intermediates that produce adverse biological effects [1, 2]. For example, carcinogenic PAHs† such as benzo(*a*)pyrene (B*a*P) undergo metabolic activation by cytochrome P450 and epoxide hydrolase to chemically reactive ultimate carcinogen diol epoxides [3]. The *CYP1* family, which consists of at least three enzymes, *CYP1A1*, *CYP1A2*, and *CYP1B1*, has been shown to be important in the metabolism of several xenobiotics such as PAH and heterocyclic amines, and the expression of these enzymes is inducible by PAHs such as TCDD. TCDD induction of *CYP1* transcription is mediated by the cytosolic AhR, which is known as a ligand-activated transcription factor. Activation of the AhR involves ligand binding, dissociation of heat shock protein 90, nuclear translocation, and dimerization with the Arnt followed by binding to dioxin-responsive element (DRE, or XRE) enhancer elements in the 5′ non-coding region of the responsive gene [4–6]. The mechanism of action of this compound is to activate the AhR to a form that binds to specific gene regulatory sequence elements, called XREs, through heterodimerization with the Arnt [7–9]. The AhR and the Arnt have a similar overall structure and belong to the basic helix–loop–helix class of transcription factors [10, 11]. Members of this class of factors are characterized by a bHLH motif contiguous with a region, PAS (Per–Arnt–Sim), which is conserved between the *Drosophila* neural cell developmental regulator, Sim, the *Drosophila* circadian rhythm regulatory protein, Per, and Arnt [12]. Upon binding to XREs, the AhR–Arnt complex activates transcription of adjacent structural genes which encode enzymes that are involved in the oxidative metabolism of these compounds [13, 14]. HIF-1α has recently been reported to mediate transcrip-

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† *Abbreviations:* HIF-1α, hypoxia-inducible factor-1α; AhR, arylhydrocarbon receptor, Arnt, AhR nuclear translocator; CYP, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PAH, polycyclicaromatic hydrocarbon; XRE, xenobiotic-responsive element, also termed DRE; HRE, hypoxia-responsive element; and bHLH, basic helix–loop–helix.

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tional responses to hypoxia by binding to the hypoxia-inducible enhancer motif (HREs) of target genes [15]. HRE core sequences are asymmetric E-box motifs that have been characterized in the erythropoietin (EPO) gene [16], genes encoding vascular endothelial growth factor (VEGF) [17, 18], and a number of glycolytic enzymes [19]. HIF-1α binds to the HRE motif as a heterodimeric complex, termed HIF-1, with the transcription factor Arnt [20]. Thus, the Arnt seems to be critical for both the dioxin receptor- and HIF-1α-mediated signaling pathways. In our effort to understand the mechanism of the regulation of *Cyp1a1* gene expression, we demonstrate here that hypoxic agents such as cobalt chloride, desferrioxamine, and picolinic acid inhibit TCDD-induced *Cyp1a1* promoter activity based on the determination of luciferase activity in Hepa-I cells transfected with pm*Cyp1a1*-Luc.

MATERIALS AND METHODS

Materials

TCDD was kindly provided by Dr. K. Chae from NIEHS (Research Triangle Park, NC). Desferrioxamine, picolinic acid, ferrous sulfate, and phenol were supplied by Sigma and cobalt chloride by Junsei Chem. Co. Agarose was purchased from FMC and LipofectAMINE and HindIII from Gibco BRL, while pGL3 basic vector and luciferase assay system were ordered from Promega.

Construction of Cyp1a1*-Luc*

Mouse *Cyp1a1* 5′-flanking DNA (−1642 ∼ +53) was cloned into pGL3 vector at HindIII site.

Cell Culture and Transfection

Hepa-I (Hepa 1c1c7) mouse liver cell lines were grown in Earle's balanced salt solution supplemented with 10% (v/v) fetal bovine serum and 100 units penicillin–streptomycin/mL. For the transfection of pm*Cyp1a1*-Luc, 50,000 Hepa-I cells were plated into each well of a 24-well plate and maintained at 37° in humidified 5% CO_2 for 24 hr. pm*Cyp1a1*-Luc (150 ng) and 1 μg of LipofecTAMINE were mixed in 50 μL of serum-free medium and incubated at room temperature for 45 min before being added to Hepa-I cells. Hepa-I cells were exposed to the DNA–lipid complexes in serum-free medium for at least 5 hr at 37° in humidified 5% CO_2 incubator before being maintained in normal minimum essential medium (MEM) containing 20% fetal bovine serum. The manufacturer's instructions were followed throughout.

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. Either cobalt chloride, desferrioxamine, or picolinic acid (all 1–100 μM) was administered in the presence or absence of 150 μM ferrous sulfate for 17 hr before a 24-hr 1 nM TCDD treatment.

Luciferase Reporter Assay

Luciferase activity was determined in cell extracts containing 2 μg of total protein. Twenty microliters of cell extracts was mixed with 100 μL of reporter assay reagent at 20–25°, and luminescence was measured using a liquid scintillation counter. Protein assay of cell extracts was carried out using the Micro bisinchoninic acid protein assay reagent kit and an ELISA Reader. Luciferase activity data are presented as the fold induction of control cells that were treated with 0.1% DMSO when control luciferase activity 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 the mouse *Cyp1a1* 5′-flanking region. Transfected Hepa-I cells were treated with various concentrations of TCDD (1 pM–1 nM) for 24 hr and lysed for luciferase activity measurement. As shown in Fig. 1, 1 pM TCDD increased luciferase activity 850-fold over that of control and as the TCDD concentration increased, luciferase activity was also increased in a dose-dependent manner. Maximal stimulation of luciferase activity upon 1-nM TCDD treatment was 6810-fold over that of control. This result is in agreement with previous data where 1 nM TCDD brought about maximal responses in stimulation of ethoxyresorufin deethylase activity and increases in *Cyp1a1* mRNA [21]. Thus, this reporter gene system may be a very useful tool for studying the mechanism of the regulation of *Cyp1a1* gene expression.

*Time-Dependent Induction of pm*Cyp1a1*-Luc Expression by TCDD*

A 1-nM TCDD concentration, which results in maximal biological response, was treated into Hepa-I cells that were transfected with pm*Cyp1a1*-Luc for various periods of time. Upon 6-hr treatment with TCDD, luciferase activity began to increase, reaching its maximal level at 24 hr. Forty-eight-hour treatment with TCDD brought the luciferase activity back to near the untreated level, while 72-hr and 96-hr treatments showed few luciferase activities, possibly due to cell death with TCDD treatment (Fig. 2). This result shows the time-dependent induction of *Cyp1a1* expression with 1-nM TCDD treatment up to 24 hr, with 24-hour treatment resulting in maximal stimulation.

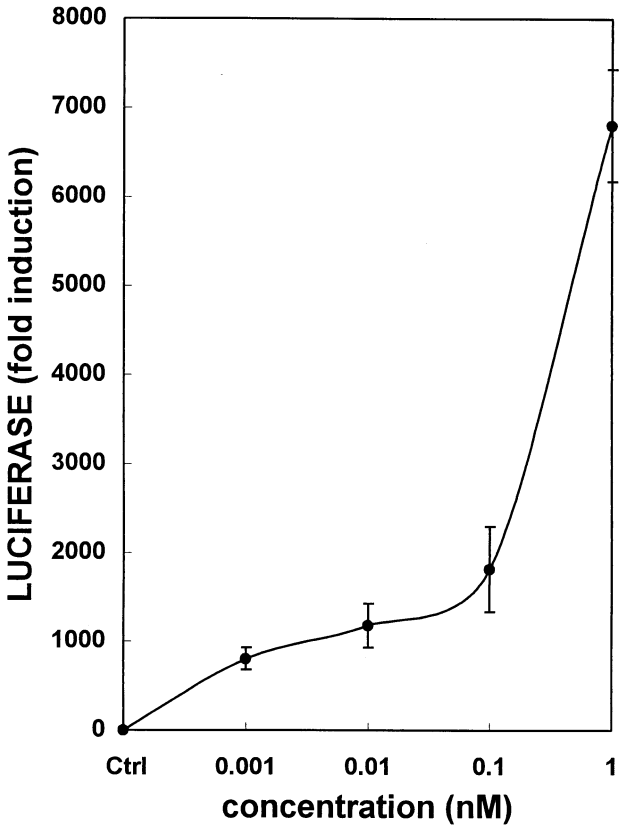

FIG. 1. The dose–effect of TCDD on luciferase activity in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were treated with 0.1% DMSO for control or 0.001, 0.01, 0.1, or 1 nM TCDD for 24 hr. Luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when the luciferase activity of control cells was set at 1, and the data represent means + SE. (N = 4).

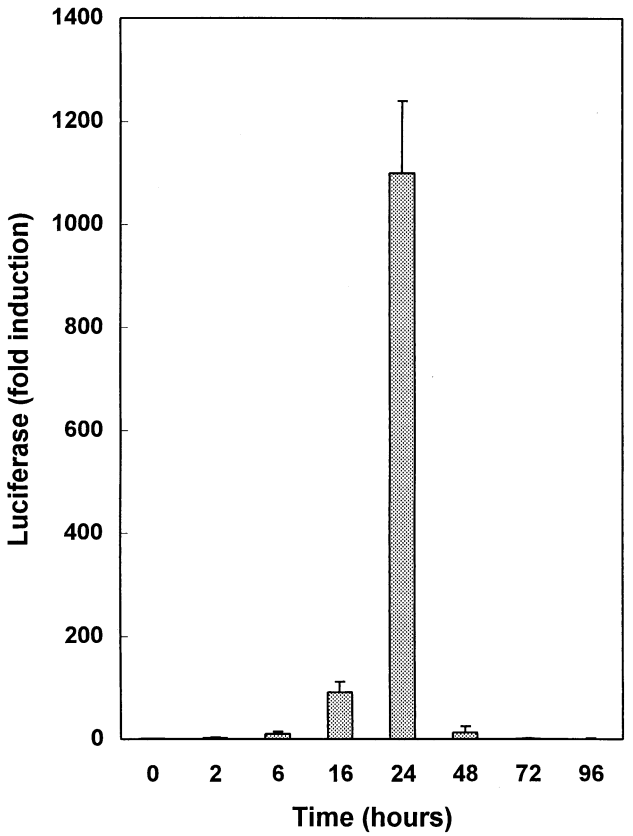

FIG. 2. The time–course effect of TCDD on luciferase activity in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were treated with 1 nM TCDD for 0, 2, 6, 16, 24, 48, 72, or 96 hr. Luciferase activity was assayed in cell lysate containing 2 μg of total protein as described in Materials and Methods. Luciferase activity shows the fold induction of control cells when the luciferase activity of control cells was set at 1, and the data represent means + SE. (N = 4).

Cobalt Chloride Inhibition of TCDD-Induced Luciferase Activity

pmC*yp1a1*-Luc-transfected Hepa-I cells were treated with various concentrations (1, 10, and 100 μM) of cobalt chloride for 17 hr before treatment with 1 nM TCDD for 24 hr. The treatment with 1 nM TCDD resulted in a 4170-fold induction of luciferase activity, whereas 1-, 10-, or 100-μM cobalt chloride treatment decreased TCDD-stimulated luciferase activity in a dose-dependent manner when it was pretreated (Fig. 3). pmC*yp1a1*-Luc-transfected Hepa-I cells were treated with 100 μM cobalt chloride for various times followed by either 0.1% DMSO treatment for control or 1-nM TCDD treatment for 24 hr. As shown in Fig. 4, 1-hr pretreatment with 100 μM cobalt chloride showed 34% inhibition of 1-nM TCDD-induced luciferase activity, and as the time of pretreatment of cobalt chloride increased, the luciferase activity was inhibited. As a 17-hr pretreatment with 100 μM cobalt chloride showed 89% inhibition of 1-nM TCDD-induced luciferase activity, this same 17-hr pretreatment with cobalt chloride was used for the dose–response study. These data show that cobalt chloride inhibits TCDD-induced luciferase activity and strongly suggest that an iron-chelating (or hypoxic) agent such as cobalt chloride may inhibit TCDD induction of *Cyp1a1* expression.

Desferrioxamine Inhibition of TCDD-Induced Luciferase Activity

pmC*yp1a1*-Luc-transfected Hepa-I cells were treated with various concentrations (1, 10, and 100 μM) of desferrioxamine for 17 hr before treatment with 1 nM TCDD for 24 hr. The treatment with 1 nM TCDD resulted in a 7250-fold induction of luciferase activity, which was decreased dose dependently when 1, 10, or 100 μM desferrioxamine was pretreated for 17 hr (Fig. 5). pmC*yp1a1*-Luc-transfected Hepa-I cells were treated with 100 μM desferrioxamine for various times followed by either 0.1% DMSO treatment for control or 1-nM TCDD treatment for 24 hr. As is shown in Fig. 6, as the time of desferrioxamine pretreatment rose, the luciferase activity was increasingly inhibited: 8-hr pretreatment with 100 μM desferrioxamine showed 35% inhibition

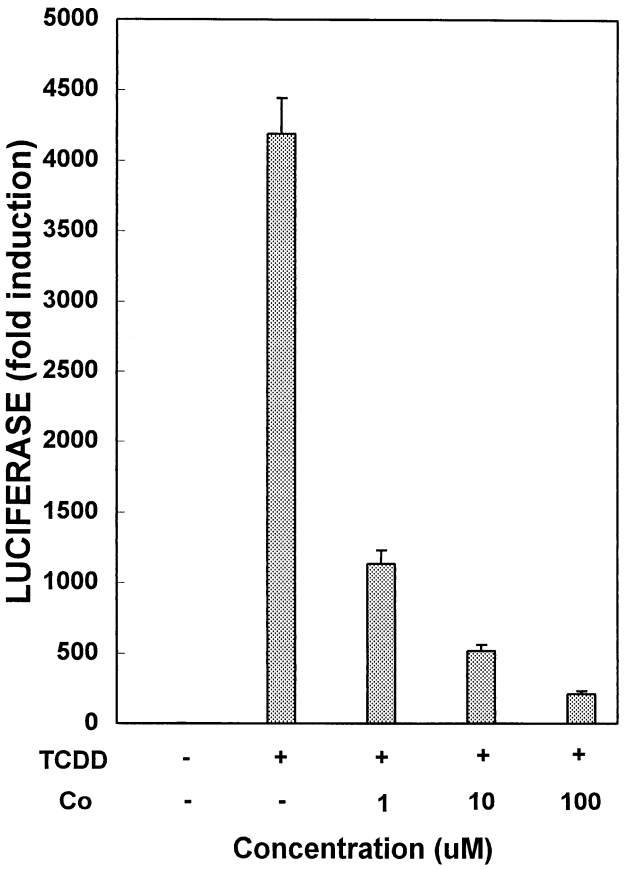

FIG. 3. The dose–effect of cobalt chloride on luciferase activity induced by TCDD in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with 1, 10, or 100 μM cobalt chloride (Co) for 17 hr before 0.1% DMSO (for control) or 1-nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

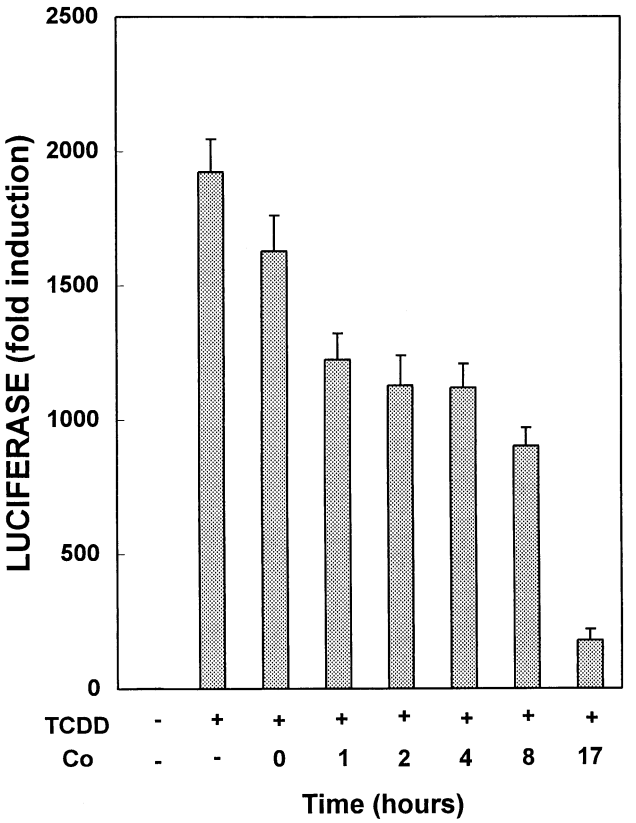

FIG. 4. The time–course effect of cobalt chloride on luciferase activity induced by TCDD in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with cobalt chloride (Co) for 0, 1, 2, 4, 8, or 17 hr before 0.1% DMSO (for control) or 1 nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

and 17-hr pretreatment showed 94% inhibition of 1-nM TCDD-induced luciferase activity. These data demonstrate that desferrioxamine inhibits TCDD-induced luciferase activity and strongly suggest that a hypoxic agent such as desferrioxamine may inhibit TCDD induction of C*yp1a1* expression.

Picolinic Acid Inhibition of TCDD-Induced Luciferase Activity

pmC*yp1a1*-Luc-transfected Hepa-I cells were treated with various concentrations (1, 10, and 100 μM) of picolinic acid for 17 hr before treatment with 1nM TCDD for 24 hr. This treatment with TCDD resulted in a 2680-fold induction of luciferase activity, which was decreased with 1-, 10-, or 100-μM picolinic acid pretreatment to 85%, 37%, and 17%, respectively, that of 1-nM TCDD-treated luciferase activity (Fig. 7). These data show that picolinic acid is one of the hypoxic agents capable of inhibiting TCDD-induced luciferase activity in a dose-dependent manner and suggest that picolinic acid can inhibit TCDD-induced C*yp1a1* gene expression.

The Effect of Ferrous Sulfate on Luciferase Activity Inhibited by either Desferrioxamine or Picolinic Acid Treatment

Various concentrations of desferrioxamine (1, 10, and 100 μM) in the presence or absence of 150 μM ferrous sulfate were administered into Hepa-I cells that were transfected with pmC*yp1a1*-Luc for 17 hr before treatment with either 0.1% DMSO or 1 nM TCDD for 24 hr. As shown in Fig. 8, desferrioxamine inhibited TCDD-induced luciferase activity dose dependently, and this inhibition was mostly blocked when cells were treated simultaneously with the ferrous sulfate. This result shows that iron-chelating agents inhibit TCDD-induced C*yp1a1* gene expression. Likewise, in the case of picolinic acid treatment, as shown in Fig. 9, the inhibition of luciferase activity by picolinic acid was also mostly recovered with ferrous sulfate treatment. These

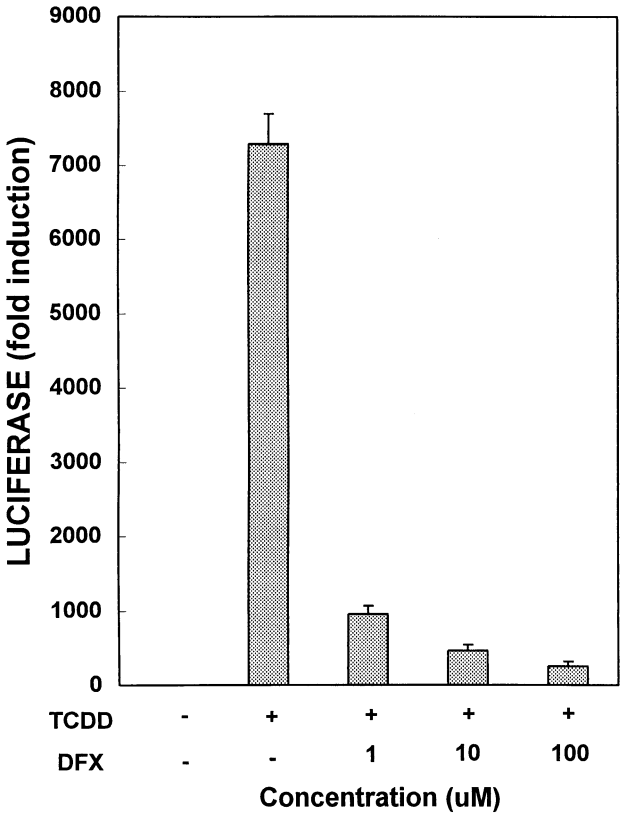

FIG. 5. The dose–effect of desferrioxamine on luciferase activity induced by TCDD in Hepa-I cells transfected with pm*Cyp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with 1, 10, or 100 μM desferrioxamine (DFX) for 17 hr before 0.1% DMSO (for control) or 1-nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

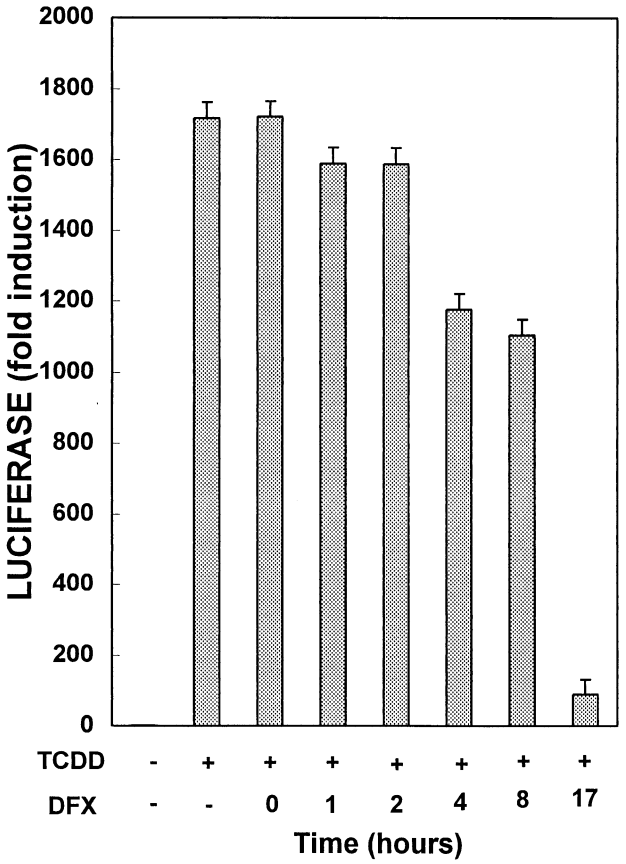

FIG. 6. The time–course effect of desferrioxamine on luciferase activity induced by TCDD in Hepa-I cells transfected with pm*Cyp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with desferrioxamine (DFX) for 0, 1, 2, 4, 8, or 17 hr before 0.1% DMSO (for control) or 1-nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

data strongly suggest that iron chelation inhibits TCDD-induced *Cyp1a1* gene expression.

DISCUSSION

The results of this study show that hypoxic agents inhibited TCDD-induced *Cyp1a1* transcription and that this inhibitory effect was dependent on the concentration of these agents. This study also demonstrates that iron-chelating agents inhibited TCDD-induced *Cyp1a1* transcription and that ferrous sulfate treatment recovered luciferase activity from the inhibition of *Cyp1a1* transcription by iron chelation. TCDD is known as a strong inducer of *Cyp1a1* gene expression [22, 23], and the induction of *Cyp1a1* transcription is mediated by a soluble protein designated as the AhR [24, 25]. Prior to binding with an inducer, the AhR exists in the cytoplasm as part of a complex that has a molecular mass of 280 kDa. This complex is composed of the AhR, two molecules of the 90 kDa heat shock protein (Hsp 90), and possibly other proteins [26]. After binding with a ligand, the AhR dissociates from the above complex and translocates to the nucleus, where it heterodimerizes with the Arnt [27]. The heterodimer AhR–Arnt complex binds to several copies of short sequence, termed XREs, located within the 5′-flanking region of the *Cyp1a1* gene to stimulate the synthesis of the *Cyp1a1* protein and several other proteins involved in xenobiotic metabolism [28]. Thus, the induction of *Cyp1a1* is regulated exclusively at the transcriptional level [29]. The AhR and Arnt proteins have two domains necessary for the TCDD-induced activation of the *Cyp1a1* gene. One of these domains is the Per-Arnt-Sim region, composed of approximately 300 amino acids, that mediates the ligand binding and dimerization of the AhR and Arnt toward these amino termini [30]. The bHLH motif has been seen in a number of transcriptional factors. Most transcriptional factors, such as dimers to the specific DNA sequence (CACGTG), are termed the E-box [31]. Recently, it has been reported that the Arnt constitutively binds as a homodimer to the E-box motif of AdMLP [32]. On the other hand, the AhR–Arnt

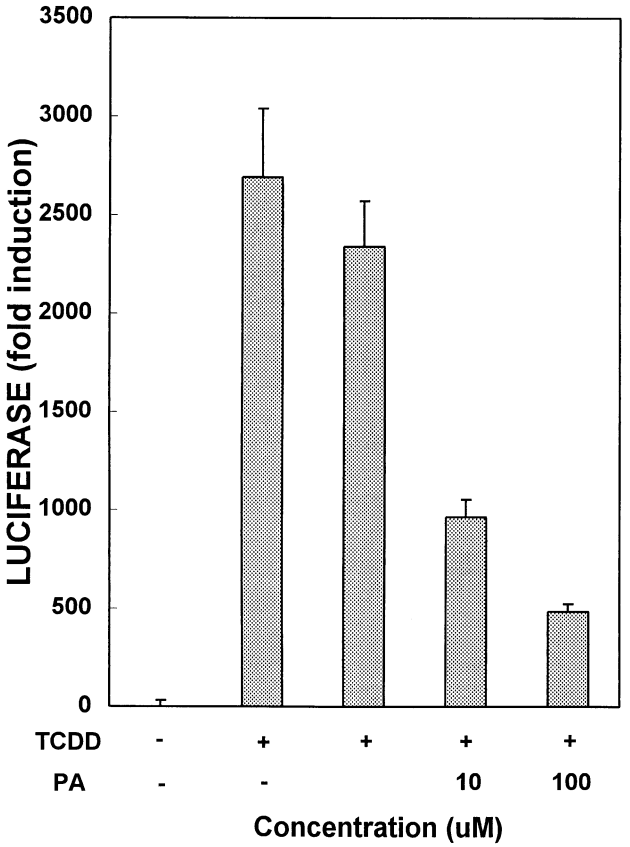

FIG. 7. The dose–effect of picolinic acid on luciferase activity induced by TCDD in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with 1, 10, or 100 μM picolinic acid (PA) for 17 hr before 0.1% DMSO (for control) or 1-nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

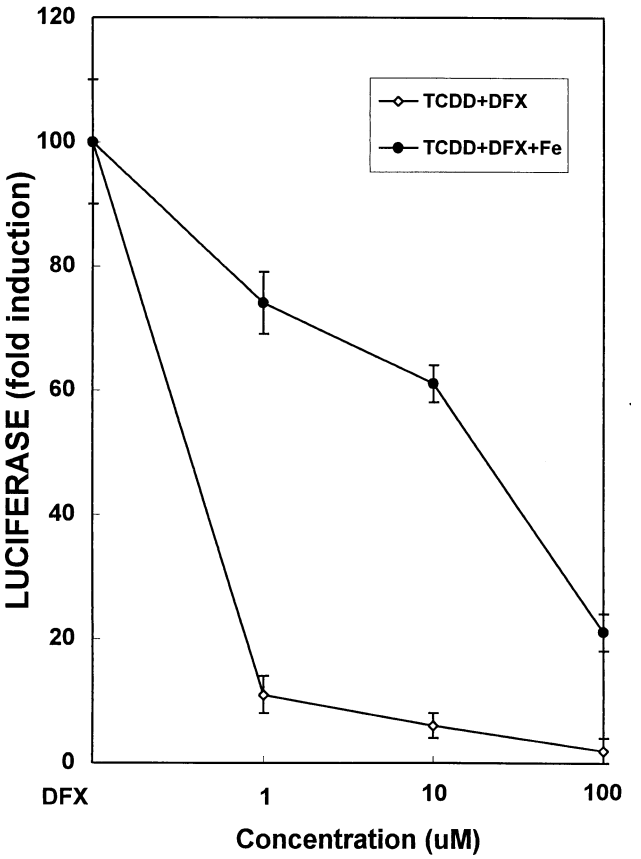

FIG. 8. The effect of ferrous sulfate on the desferrioxamine inhibition of luciferase activity induced by TCDD in Hepa-I cells transfected with pmC*yp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with 1, 10, or 100 μM desferrioxamine (DFX) in the absence or presence of 150 μM ferrous sulfate for 17 hr before 0.1% DMSO or 1-nM TCDD treatment for 24 hr. 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 means + SE. (N = 4).

heterodimer recognizes an asymmetrical XRE sequence that only partially resembles the E-box. The consensus sequence of XRE has been identified as 5′-(T/G)NGCGTG(A/C)(G/C)(A/T)-3′ [33]. Four core nucleotides (5′-CGTG-3′) within XRE are absolutely required for the binding with the AhR–Arnt heterodimer. The Arnt binds to the thymidine in the 5′-CGTG-3′ core identical to an E-box half-site (GTG), whereas the AhR binds to 5′ proximal to the 5′-CGTG-3′ core in the XRE [34].

HIF-1α has recently been reported to mediate the transcriptional response to hypoxia by binding to enhancer motifs (HREs) of target genes [35, 36]. HRE core sequences are asymmetric E-box motifs that have been characterized in the erythropoietin (EPO) gene, genes encoding vascular endothelial growth factor (VEGF), and a number of glycolytic enzymes [37]. HIF-1α binds HRE motifs as a heterodimeric complex, termed HIF-1, with the transcription factor Arnt [38]. Both HIF-1α and Arnt are members of a novel subclass of the bHLH family of transcriptional factors. Members of this class of factors are characterized by a bHLH motif contiguous with a region, PAS (Per–Arnt–Sim), which is conserved between the *Drosophila* neuronal cell developmental regulator Sim, the *Drosophila* circadian rhythm regulatory protein Per, the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) (Ah) receptor, and the Arnt. HIF-1α and the intracellular dioxin receptor mediate hypoxia and dioxin signaling, respectively. Both proteins are conditionally regulated bHLH transcriptional factors that, in addition to the bHLH motif, share a PAS region of homology and form heterodimeric complexes with the common bHLH/PAS partner factor Arnt. Both the bHLH and PAS motifs of the Arnt were critical for dimerization with HIF-1α, and HIF-1α exhibited very high affinity for the Arnt, resulting in competition with the ligand-activated dioxin receptor for recruitment of the Arnt [39–41]. In addition, the activation of the HIF-1α function *in vivo* or the overexpression of HIF-1α inhibited ligand-dependent induction of DNA binding activity by the dioxin receptor.

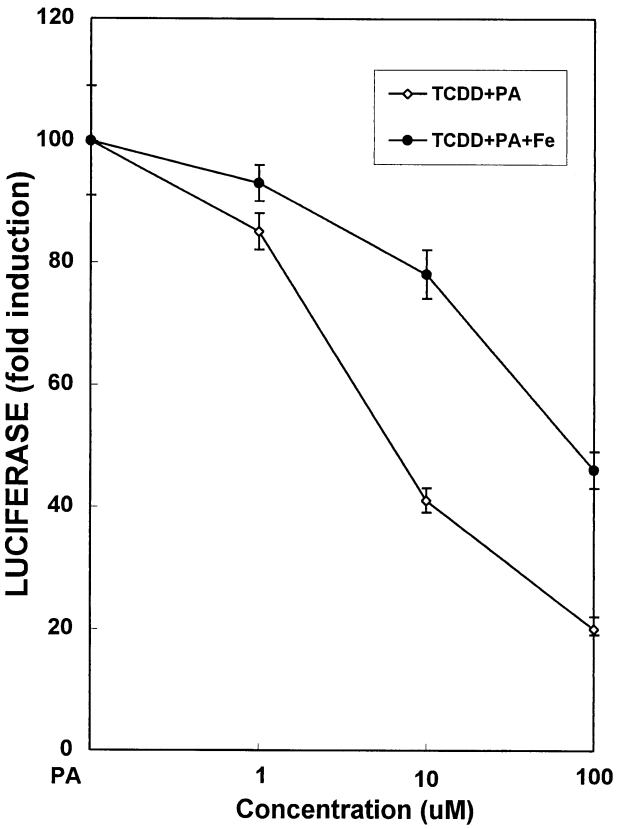

FIG. 9. The effect of ferrous sulfate on picolinic acid inhibition of luciferase activity induced by TCDD in Hepa-I cells transfected with pm*Cyp1a1*-Luc. After Hepa-I cells were transfected, cells were pretreated with 1, 10, or 100 μM picolinic acid (PA) in the absence or presence of 150 μM ferrous sulfate for 17 hr before 0.1% DMSO or 1-nM TCDD treatment for 24 hr. 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 + SE. (N = 4).

However, the activation of the dioxin receptor function did not impair HIF-1α-dependent induction of target gene expression [42, 20]. Based on these observations, it seems that HIF-1α- and dioxin receptor-mediated signaling pathways were not mutually exclusive. The results of this study are in agreement with previous reports wherein hypoxia inhibits TCDD-induced gene expression possibly due to the competition between HIF-1α and the AhR for the Arnt, as explained above.

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