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Inhibitory Effect of Nitric Oxide on the Induction of Cytochrome P450 3A4 mRNA by 1,25-Dihydroxyvitamin D_3 in Caco-2 Cells

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Cytochrome P450 (CYP)-dependent drug metabolism decreases *in vivo* and in cultured hepatocytes under various immunostimulatory conditions. Nitric oxide (NO) released during inflammation is presumed to be involved in this phenomenon. CYP3A4, which is abundant in the liver and small intestine and participates in the metabolism of various drugs, is known to be induced by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in the colon carcinoma cell line Caco-2. In this study we examined whether NO affected CYP3A4 gene expression induced by $1,25(OH)_2D_3$ in Caco-2 cells. Induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ was suppressed in a dose-dependent manner by treatment with the NO donors NOR-4 (15–500 μM) or S-nitroso-N-acetylpenicillamine (30 μM–1 mM), which spontaneously release NO. These results indicated that NO has an inhibitory effect on the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ in Caco-2 cells. Treatment with the guanylate cyclase inhibitor ODQ failed to prevent the inhibition of induction of CYP3A4 mRNA by $1,25(OH)_2D_3$. 8-Bromo cGMP had no effect on $1,25(OH)_2D_3$-induced CYP3A4 gene expression. Therefore, the suppression of CYP3A4 mRNA by NO might be mediated through a guanylate cyclase-independent pathway.

Keywords: Nitric oxide, cytochrome P450 3A4, 1,25-dihydroxyvitamin D_3, Caco-2

INTRODUCTION

Nitric oxide (NO) plays important bioregulatory roles in physiological processes such as regulation of vascular tone, platelet aggregation, neurotransmission and cytotoxicity of activated macrophages.[1,2] These physiological actions are based on modulation of enzyme activity through binding of NO to proteins with heme or non-heme iron cofactors. NO production increases in many cell types in response to immunological stimuli such as bacterial lipopolysaccharide (LPS) and cytokines.[3,4] Many studies has shown that cytochrome P450 (CYP)-mediated hepatic metabolism of many drugs is depressed during

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inflammation and bacterial infection.[5,6] CYPs are heme-containing enzymes that catalyze the oxidative metabolism of physiological substrates and xenobiotics.

The administration of inflammatory cytokines and LPS depresses CYP activities and its contents *in vivo* and *in vitro*.[7–10] NO synthase (NOS) inhibitors significantly prevent the cytokine- and LPS-mediated decreases in CYP protein levels and activities. In addition, it has been shown that exogenously added NO inhibits CYP activities and expression of CYP mRNAs.[11,12] These results indicated that NO plays an important role in transcriptional and post-transcriptional regulation of CYPs. It has been suggested that NO may inhibit CYP activities by binding to the heme group. However, the mechanism by which NO suppresses CYP gene expression remains unknown.

CYP3A is the most prominent CYP isozyme in the human liver and small intestine, and participates in the metabolism of many drugs. The expression of CYP3A is markedly induced in response to treatment with various compounds such as dexamethasone, rifampicin and phenobarbital.[13] Recently, it was reported that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) also up-regulates CYP3A4 gene expression in the human colon carcinoma cell line Caco-2, which has been extensively utilized as an experimental model of small intestinal cells.[14] Intestinal CYP3A4 has been suggested to contribute to first-pass drug metabolism,[15,16] since CYP3A accounts for 70% of the total CYPs in the human intestine. NO synthesis appears to be elevated in inflammatory bowel diseases.[17,18] Therefore, it is likely that the down-regulation of intestinal CYP3A4 expression caused by NO generated under intestinal inflammation may affect oral bioavailability of CYP3A4 substrates. However, whether NO affects CYP3A4 mRNA expression in the small intestine remains unknown. Thus, in the present study we examined the effects of NO on $1,25(OH)_2D_3$-induced expression of CYP3A4 using the colon carcinoma cell line Caco-2.

MATERIALS AND METHODS

Chemicals

$1,25(OH)_2D_3$ was purchased from Biomol Research laboratories, Inc. (Plymouth Meeting, PA, USA). (±)-N-(E)-{4-Ethyl-2-[(Z)-hydroxyimino]-5-nitro-3-hexene-1-yl}-3-pyridine carboxamide (NOR-4) and S-nitroso-N-acetylpenicillamine (SNAP) were purchased from Dojin Chemicals (Kumamoto, Japan). 1*H*-[1,2,4]Oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ) was purchased from Wako Pure Chemical Co. (Osaka, Japan).

Culture and Treatment of Cells

Caco-2 cells were grown in DMEM supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids, 100 units/ml penicillin G and 0.1 mg/ml streptomycin in a 5% CO_2/95% air incubator at 37°C. Cells were grown to 80% confluence in 10 cm culture dishes and were maintained for 2 days in serum-free DMEM containing 5 ng/ml insulin, 5 ng/ml transferrin, 5 ng/ml sodium selenite and 0.1 mM non-essential amino acids. The serum-free medium was renewed, and cells were treated with various reagents for 24 h. $1,25(OH)_2D_3$ dissolved in ethanol was added to cells in culture at concentrations from 15 to 500 nM. To test the effects of NO on the $1,25(OH)_2D_3$-inducible CYP3A4 expression, cells were exposed to various concentrations of the NO donors NOR-4 or SNAP in the presence of $1,25(OH)_2D_3$ at the concentrations indicated in the figure legends. The soluble guanylate cyclase inhibitor ODQ was co-administrated at a final concentration of 10 μM with 250 μM NOR-4 in the presence of $1,25(OH)_2D_3$. NO donors and ODQ were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in each culture was $< 0.2\%$.

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted with TRIzol reagent (Gibco BRL, Grand Island, NY, USA) from the treated cells. First-strand cDNA was generated from 10 μg of total RNA. Reverse-transcription reaction was carried out for 60 min at 37°C in a 50 μl volume containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate (dNTP), 1.5 μg random primer, 1 unit of RNase inhibitor and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Gibco BRL). Aliquots of the reverse-transcription reaction mixture (1 μl) were amplified with primers specific for human CYP3A4 (forward primer, 5′-CCTTACACATACACACCCTTTGGAAGT-3′; reverse primer, 5′-AGCTCAATGCATGTACAGAATCCCCGGTTA-3′)[19] and β-actin (forward primer, 5′-CAAGAGATGGCCACGGCTGCT-3′; reverse primer, 5′-TCCTTCTGCATCCTGTCGGCA-3′). The 25-μl PCR reaction mixtures contained 25 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.125 units of Taq DNA polymerase (Gibco BRL). For amplification of CYP3A4, PCR was carried out as follows; 2 min at 94°C, one cycle; 40 s at 94°C, 40 s at 60°C, 1 min at 72°C, 35 cycles. For amplification of β-actin, PCR was performed as follows; 2 min at 94°C, one cycle; 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, 18 cycles. The linear range of PCR amplification products was established by gradually increasing the cycle numbers. Aliquots of the PCR mixture were separated on 3% agarose gel (agarose : NuSieve agarose, 2 : 1) and stained with ethidum bromide.

Measurement of Nitrite/Nitrate Concentration

Various concentrations of NOR-4 or SNAP were added to cells maintained for 2 days in serum-free medium in 24-well culture plates, followed by incubation for 24 h. The nitrite/nitrate concentrations in the medium were measured using NO_2/NO_3 Assay Kit-C (Dojin Chemicals, Japan) according to the manufacturer's protocol.

Measurement of mRNA stability

Cells were cultured in serum-free medium for 24 h, and were incubated in the presence of 1,25-$(OH)_2D_3$ for 24 h. The medium were renewed, and cells were treated with 10 μg/ml actinomycin D and 250 μM NOR-4 for 6, 10, 12 and 24 h. CYP3A4 and β-actin mRNA levels were determined by RT-PCR as described above. Densitometric measurements of PCR products were obtained with NIH Image.

RESULTS

Induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ in Caco-2 Cells

As described previously,[14] we used $1,25(OH)_2D_3$ as an inducer of CYP3A4 gene expression in the colon carcinoma cell line Caco-2, since the level of CYP3A4 mRNA is very low in these cells (Figure 1). A dose-response experiment was carried out to determine the optimal concentration of $1,25(OH)_2D_3$ for CYP3A4 gene expression under our culture conditions, since

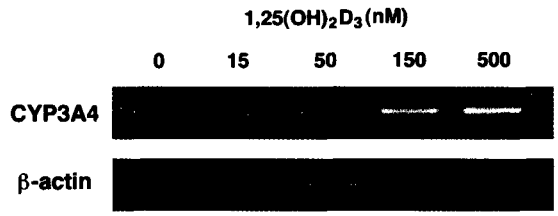

FIGURE 1 Dose-dependent effect of $1,25(OH)_2D_3$ on CYP3A4 gene expression in Caco-2 cells. Caco-2 cells were cultured in serum-free medium for 48 h, and were then treated with ethanol or 15, 50, 150 or 500 nM $1,25(OH)_2D_3$ for 24 h. Total RNA was prepared and semi-quantitative RT-PCR was performed using specific primer sets for CYP3A4 and β-actin as described in Materials and Methods. PCR products of CYP3A4 and β-actin corresponded to 381 and 275 bp, respectively. A typical result of two independent experiments is shown.

they were different from those reported previously.[14] CYP3A4 mRNA was detected by semi-quantitative RT-PCR using a specific primer set for CYP3A4 to avoid cross-detection of closely related CYP3A isoforms, CYP3A5 and CYP3A7, which are expressed in Caco-2 cells. After 48 h of culture in serum-free medium, Caco-2 cells were exposed to various concentrations of $1,25(OH)_2D_3$ (15–500 nM) for 24 h. As shown in Figure 1, CYP3A4 mRNA level was very low in untreated cells and was significantly increased by $1,25(OH)_2D_3$ in a dose-dependent manner. Significant expression of CYP3A4 mRNA was obtained at $1,25(OH)_2D_3$ concentrations >150 nM. Thus, we treated cells with 250 nM $1,25(OH)_2D_3$ for 24 h in the following studies.

Effects of NO Donors on $1,25(OH)_2D_3$-Induced Expression of CYP3A4 mRNA

We investigated the effects of NO donors on $1,25(OH)_2D_3$-induced expression of CYP3A4 mRNA. After 48 h of culture in serum-free medium, cells was exposed to various concentrations of NOR-4, which causes spontaneous release of NO, for 24 h in the presence of $1,25(OH)_2D_3$. As shown in Figure 2A, $1,25(OH)_2D_3$-induced expression of CYP3A4 mRNA was reduced by NOR-4 in a dose-dependent manner, and was completely inhibited at 150 μM NOR-4. We also examined the effects of another structurally different NO donor, SNAP, on $1,25(OH)_2D_3$-induced expression of CYP3A4 mRNA. $1,25(OH)_2D_3$-induced CYP3A4 gene expression was also inhibited by SNAP in a dose-dependent manner (Figure 2B), and complete suppression was observed at 1 mM SNAP. The expression of β-actin was unchanged by the addition of NOR-4 or SNAP.

To confirm that NO inhibited $1,25(OH)_2D_3$-induced CYP3A4 gene expression, we examined whether NO donors released NO into the culture medium. As shown in Figure 3, the concentration of nitrite/nitrate, which are stable end products of NO oxidation, in culture medium was increased in proportion as the amounts of

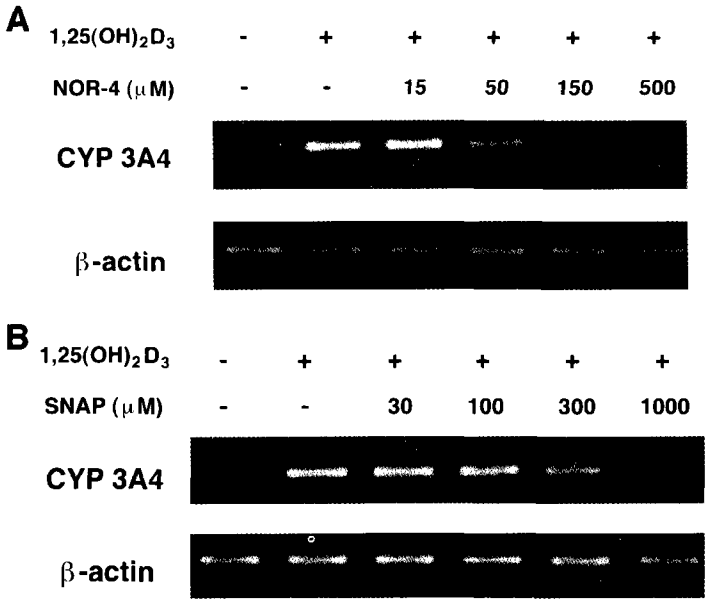

FIGURE 2 Effects of NO donors, NOR-4 (A) and SNAP (B) on the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$. Caco-2 cells were cultured in serum-free medium for 48 h, and were then treated with 0, 15, 50, 150 or, 500 μM NOR-4 (A) or 0, 30, 100, 300 or 1000 μM SNAP (B) in the presence of 250 nM $1,25(OH)_2D_3$ for 24 h. Total RNA was prepared and RT-PCR was performed as described in Materials and Methods. Typical results of two independent experiments are shown.

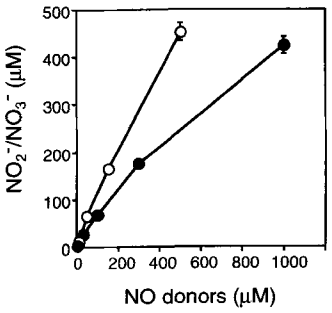

FIGURE 3 NO production from NO donors. Caco-2 cells were cultured in serum-free medium for 48 h, and were then treated with various concentrations of NOR-4 (○) or SNAP (●) for 24 h. The concentration of nitrite/nitrate in the medium was measured. Values indicate means ± SE of three independent experiments.

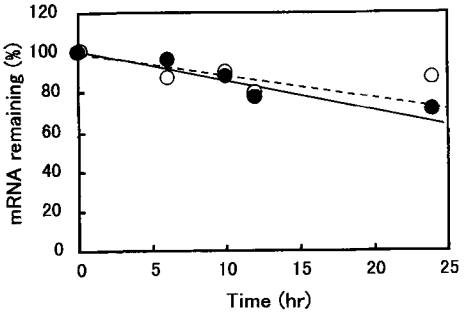

FIGURE 4 Effect of NOR-4 on CYP3A4 mRNA stability. Caco-2 cells were treated with 250 nM $1,25(OH)_2D_3$ for 24 h. After this incubation period, 250 μM NOR-4 (○) or vehicle (●) was administrated to the cells in the presence of actinomycin D (10 μg/ml). Total RNA was prepared at the indicated times after administration of actinomycin D. RT-PCR was performed as described in Materials and Methods. The mRNA amounts were quantified by densitometric analysis and the ratio CYP3A4/β-actin in each lane was calculated. A representative of two independent experiments is shown.

NO donors added. This observation indicated that NO was generated by the NO donors under our experimental conditions. Figure 3 also shows that NOR-4 spontaneously generated a larger quantity of NO than SNAP at the same concentration. This agreed with the results shown in Figure 2 indicating that NOR-4 inhibited 1,25-$(OH)_2D_3$-induced gene expression of CYP3A4 at lower concentrations than SNAP.

Effect of NO on the Stability of CYP3A4 mRNA

In order to determine whether NO donor, NOR-4, affected the steady-state level of CYP3A4 mRNA by increasing its rate of degradation, we measured CYP3A4 mRNA in the presence of actinomycin D. However, as shown in Figure 4, NOR-4 did not affect the rate of degradation of CYP3A4 mRNA. This result indicates that NO could inhibit the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ at transcriptional level.

Effects of Soluble Guanylate Cyclase on Inhibition of $1,25(OH)_2D_3$-Induced CYP3A4 Gene Expression

To confirm whether this inhibition involved cGMP produced due to activation of soluble guanylate cyclase by NO, we first examined the

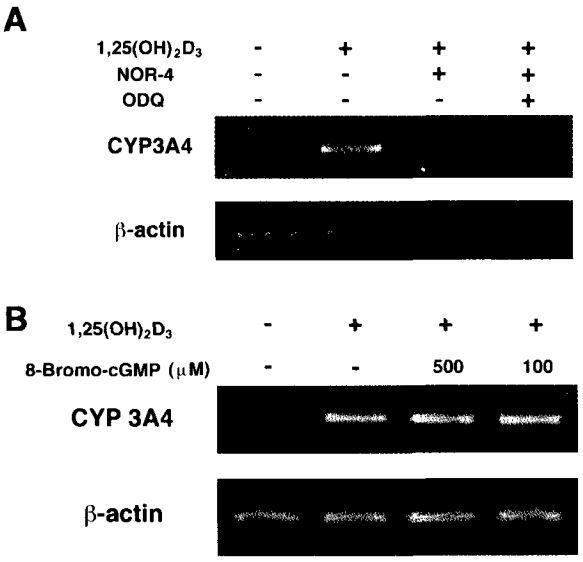

FIGURE 5 Effects of a soluble guanylate cyclase inhibitor (A) and cGMP analog (B) on the suppression of $1,25(OH)_2D_3$-inducible CYP3A4 mRNA by NOR-4. (A) Caco-2 cells were cultured in serum-free medium for 48 h, and were then treated with 10 μM ODQ and 250 μM NOR-4 in the presence of 250 nM $1,25(OH)_2D_3$ for 24 h. (B) Caco-2 cells were cultured in serum-free medium for 48 h, and were then exposed to 100 or 500 μM 8-bromo cGMP in the presence of 250 nM $1,25(OH)_2D_3$ for 24 h. Total RNA was prepared and RT-PCR was performed as described in Materials and Methods. Typical results of two independent experiments are shown.

effects of a guanylate cyclase inhibitor, ODQ, on the inhibition of $1,25(OH)_2D_3$-induced expression of CYP3A4 mRNA by NOR-4. Cells were treated with $1,25(OH)_2D_3$ and NOR-4 (250 μM) in the presence or absence of ODQ (10 μM). As shown in Figure 5A, this treatment failed to prevent the inhibition of CYP3A4 gene expression by NOR-4. Next, cells were incubated with $1,25(OH)_2D_3$ and the cGMP analog 8-bromo cGMP. This treatment also had no effect on $1,25(OH)_2D_3$-induced CYP3A4 gene expression (Figure 5B). These results suggested that the suppression of $1,25(OH)_2D_3$-induced expression of CYP3A4 mRNA was mediated through a soluble guanylate cyclase-independent pathway. The mechanism by which $1,25(OH)_2D_3$-induced CYP3A4 gene expression is inhibited by NO remains unknown at present.

DISCUSSION

Decreases in hepatic drug metabolism during infection or inflammatory stress arises from the reduced activity and expression of CYP isoenzymes.[5,6] Suppression of hepatic metabolism by CYP prolongs the duration and intensity of action of drugs and endogenously produced substances. Recently, NO released during inflammation was suggested to be involved in this phenomenon. In the present study, we demonstrated that CYP3A4 gene expression induced by $1,25(OH)_2D_3$ in Caco-2 cells was inhibited in dose-dependent manner by NO donors, NOR-4 or SNAP. These results indicated that NO plays an important role of the suppression of $1,25-(OH)_2D_3$-induced CYP3A4 gene expression in Caco-2 cells.

Although CYP enzymes are primarily concentrated in the liver, they are also known to be present in extrahepatic tissues such as the small intestine. Recent studies suggested that the small intestine may also contribute significantly to first-pass metabolism.[15,16] CYP3A4 is the most abundant of all CYP isoforms in the liver and small intestine. Expression of CYP3A4 in the villus epithelium of the small intestine is thought to result in significant first-pass metabolism of many clinically important drugs such as cyclosporine and verapamil.[20,21] Some studies have shown that the down-regulation of constitutive and inducible hepatic CYP enzymes involves NO. However, although NO synthesis appears to be elevated in inflammatory bowel diseases,[17,18] whether NO also affects CYP expression in the small intestine remains unknown. Our observation that the suppression of inducible intestinal CYP3A4 expression is caused by NO suggested that NO may affect oral bioavailability of CYP3A substrates. Since basal CYP3A4 expression level in Caco-2 cells is very low, it is of interest to determine whether constitutive expression of CYP3A4 is down-regulated by NO.

There are at least two possible mechanisms to explain the inhibition of CYP systems by NO; binding of NO to the heme group in the catalytic center of these enzymes, or the down-regulation of constitutive and inducible CYP mRNA by NO. In the present study, we demonstrated that the expression of CYP3A4 mRNA induced by $1,25(OH)_2D_3$ was inhibited by NO donors at the transcriptional level. We carried out experiments to exclude the possibility of chemical inactivation of $1,25(OH)_2D_3$ by NO and the inhibition of $1,25(OH)_2D_2$ induced expression of CYP3A4 by the product derived from reaction of NO. $1,25(OH)_2D_2$ was incubated with or without NOR-4 for 16 h at 37°C in cell-free assay medium. The active form of NOR-4 and NO could not be present in the medium since the half-lives of NOR-4 and NO in aqueous solution are about 60 min and a few seconds, respectively. When cells were treated with the preincubated medium, the CYP3A4 expression induced by $1,25(OH)_2D_3$ was not inhibited (data not shown). These observations suggested that the inhibition of $1,25-(OH)_2D_3$-induced expression of CYP3A4 mRNA by NO donors was due to the direct action of NO. Since the soluble guanylate cyclase inhibitor

ODQ did not prevent this inhibition and cGMP analog had no effect on $1,25(OH)_2D_3$-induced CYP3A4 gene expression, this phenomenon was thought to be modulated through a soluble guanylate cyclase-independent pathway.

In conclusion, NO inhibits the inducible expression of CYP3A4 in Caco-2 cells used as an experimental model of the small intestine. Our results indicate that the suppression of CYP3A4 by NO may impair the first-pass metabolism of various drugs in the small intestine under intestinal inflammatory stimulation. The down-regulation of CYP systems by NO is a clinically important problem for the pharmacotherapy of patients with inflammatory and infectious diseases.

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