Continuous Administration of Organic Nitrate Decreases Hepatic Cytochrome P450

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ABSTRACT

We previously reported that cytochrome P450 (P450) is a key enzyme of organic nitrate biotransformation and that P450 levels of the heart and its vessels markedly decreased at the development of nitrate tolerance. Although our attention was mainly focused on the circulatory organs, most organic nitrates, including nitroglycerin (NTG), are metabolized in the liver, where nitric oxide (NO) is concomitantly produced from the organic nitrates. NO reacts with various molecules such as superoxide, heme, thiols, and oxygen. This paper examined whether hepatic P450 levels are also affected after organic nitrate administration, since the liver is the major organ of P450 related metabolism. Male Wistar rats were intravenously administrated NTG or isosorbide dinitrate (ISDN) for 24–96 h. We observed the level of hemeoxygenase-1 (HO-1) as the functional marker of hepatic P450, since one of the acute phase target proteins of NO induction is an inducible type of HO-1. Hepatic P450 was drastically decreased after 48 or 72 h of continuous NTG or ISDN infusion, when nitrate tolerance was observed, but it recovered 48 h after cessation of the NTG administration. HO-1 was induced within 24 h of continuous NTG infusion, but it returned to normal levels 48 h after cessation of NTG. The administration of sodium nitroprusside, an agent to which the animals showed no tolerance, did not induce HO-1 or P450 depletion. Chronic administration of organic nitrates significantly decreased hepatic P450. These results suggest that P450-dependent drug metabolism may be drastically affected after continuous organic nitrate administration.

The mechanism of nitrate tolerance is multifactorial. We previously reported that cytochrome P450 (P450) is a key enzyme in the biotransformation from organic nitrates to nitric oxide (NO) (Minamiyama et al., 1999). Nitrate tolerance is induced within 48 h after organic nitrate continuous infusion in rats. Plasma nitrate (NOx) initially rises with organic nitrate treatment and then decreases once nitrate tolerance develops (Minamiyama et al., 2001). NO reacts with various molecules such as superoxide, heme, thiols, and oxygen. NO forms nitrosyl-heme P450 reversibly in competition with oxygen. Nitrosyl-heme moiety is then dissociated from the protein, and the induced heme oxygenase clears this heme moiety (Ignarro et al., 2002). We have also reported that ONOO− decreased P450 activity (IC50 = 0.5 mM) through the modification of thiol residues without affecting P450 levels of hepatic microsome in vitro (Minamiyama et al., 1997a). In this process, NO was a stronger inhibitor of P450 activity than ONOO−, and nitrotyrosine was not detectable. Considering these results, we concluded that the NO-induced inhibition of P450 heme moiety is followed by the decomposition of heme (P450) by NO-induced HO-1. A functional and quantitative fall of P450 resulted in a decreased biotransformation of organic nitrate to NO. We have reported that nitrate tolerance is partially induced by NO-dependent P450 degradation in heart vessels (Minamiyama et al., 2001).

The liver is a main metabolic organ for xenobiotics, and organic nitrates are also catabolized in the liver (Osnes, 1984; Lalka et al., 1993; Bogaert, 1994). This process is P450-dependent (McGuire et al., 1998), and huge amounts of NO are produced via the degradation of organic nitrate. NO directly and indirectly inactivates and degrades P450 (Khat-

ABBREVIATIONS: P450, cytochrome P450; NTG, glyceryl trinitrate; SNP, sodium nitroprusside; ISDN, isosorbide dinitrate; NO, nitric oxide; HO-1, hemeoxygenase-1; MOPS, 4-morpholinepropanesulfonic acid; NOS, nitric-oxide synthase; eNOS, endothelial NOS.
senko et al., 1993; Shiro et al., 1995; Snyder et al., 1996; Minamiyama et al., 1997a; Demple, 1999; Takemura et al., 1999). Therefore, hepatic P450 may be inactivated along with the NO production pathway of organic nitrates. This study reports changes in P450 of the liver under continuous administration of ISDN or NTG.

Materials and Methods

Chemicals. NTG was obtained from Nihon Kayaku Co. (Tokyo, Japan) as a 0.5 mg/ml solution (Mirisrol) and a 500 mg/ml ethanol solution. ISDN was obtained from Eisai Co. (Tokyo, Japan) as a 1 mg/ml solution (Nitrol) and powder. Other reagents used were of analytical grade. Anti-catalase polyclonal antibody (Chemicon International, Temecula, CA), anti-HO-1 mouse-monoclonal antibody (StressGen Biotechnologies, San Diego, CA), and anti-eNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) were used for immunoblotting and immunohistochemistry.

Animals. Male Wistar rats (220–240 g; SLC, Co., Shizukuwa, Japan) were anesthetized with 50 mg/kg pentobarbital during a catheterization procedure. The catheter (polyethylene tubing) for drug injection was placed in the left femoral vein, filled with heparin (100 IU/ml), and externalized through the back skin. After catheter implantation, the rats were housed individually and exposed to a 12/12 h light/dark cycle with free access to standard rat chow and tap water. Once the animals had recovered from the surgery and reached an apparently healthy state, they were continuously infused with 0.5 mg/ml NTG, 1 mg/ml ISDN, 0.2 mg/ml sodium nitroprusside (SNP), or vehicle (n = 5 per group) at the constant flow rate of 1 ml/h during an ∼96 h period using a syringe pump (model CPY-3200; Nihon Koden, Tokyo, Japan). At the end of the infusion, to evaluate nitrate tolerance, additional NTG (bolus dose of 1 mg/kg) was injected through the femoral vein of some animals. To evaluate the recovery from nitrate tolerance, some animals were taken off the drug treatment after 48 h and then kept nitrate-free for an additional 48 h. Some of the rats were freely given 1 g/l ISDN or tap water ad libitum for −2 months. The animals (n = 5 per group) were sacrificed at the indicated times after treatment (0, 1, 2, 4, and 8 weeks). Urine was collected from the bladder. Blood was obtained through the abdominal aorta into a heparinized syringe and then ice-cold saline (50 ml) was perfused through the abdominal aorta. Liver tissues were immediately detached and frozen in liquid nitrogen, and some portions of the liver tissues were fixed with 5% buffered formalin.

The investigation conformed to the Guide for the Care and Use of Laboratory Animals approved by the authorities on experimental animal research at the local committee of the Osaka City University.

Nitrile and NOx Levels in Plasma and Urine. Each sample of plasma or 10-fold-diluted urine with H2O was treated with 100% methanol (1:1 volume) and centrifuged at 10,000g for 2 min. The supernatant was applied to an automated NO detector-HPLC system (ENO-10; Eicom, Kyoto, Japan) as described previously (Mi- namiyama et al., 2001). NTG and ISDN did not affect this detection. Urinary NOx was standardized with creatinine concentration. Creatinine levels of urine were evaluated by the enzymatic colorimetric method (Wako Pure Chemicals, Osaka, Japan).

Hepatic P450 Levels and NADPH-Dependent P450 Reductase Activities. Liver microsomes were prepared as described previously (Funae and Imaoka, 1985). P450 contents in isolated microsomes were determined by an absorption spectra of the reduced form and the reduced form of P450-OC complex as references (Omura and Sato, 1964). Activity of P450 reductase was also measured spectrophotometrically at 550 nm as described previously (Phillips and Langdon, 1962).

Western Blot of P450 Isoforms and HO-1. Cytosol fractions of the liver were centrifuged at 105,000g for 60 min at 4°C (Imaoka et al., 1987). The resulting microsomal fractions and various doses of purified P450 isoform standards were separated on 7.5% gels by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred electrophotically to polyvinylidene difluoride membranes. Blots were then probed with specific antibodies to various P450 isoforms (Imaoka et al., 1990), and immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Their density was semiquantitatively analyzed with an NIH imager (version 1.63), and the values were obtained by a standard curve.

Liver tissues for HO-1 were homogenized in cold lysis buffer on ice (150 mM NaCl, 50 mM Tris, pH 7.6; 1% SDS, 3% Nonidet P-40, 5 mM EDTA, 1 mM MgCl2, 2 mM 1,3-dichloroisocoumarin, 2 mM 1,10-phenanthroline, and 0.5 mM E-64). The homogenate was centrifuged at 10,000g for 10 min, and the supernatant was stored at −20°C for the measurement of protein concentration. The remaining supernatant was mixed with an equal volume of double-strength Laemmli sample buffer, divided into aliquots, and stored at −80°C. Electrophoresis and blots were performed as described above.

Immunohistochemistry for P450 Isoforms and HO-1. Liver samples fixed with formalin were embedded in paraffin. The 4-μm thin sections were deparaffinized with xylene and ethanol, treated with 0.3% hydrogen peroxide in methanol, and then treated with normal serum (Vector Laboratories; Burlingame, CA; diluted 1:70). Each polyclonal antibody for several rat P450 isoforms (x300) or the monoclonal antibody for HO-1 (x1000) was layered on the sections overnight at 4°C. The specimens were then stained by the bridged immunoperoxidase method followed by nuclear staining.

Analyses of Catalase and eNOS. Catalase (EC 1.11.1.6) activity was assayed by following the decrease of H2O2 at 240 nm (Clairborne, 1985). For enzyme assays, frozen liver samples were homogenized on ice with a Teflon homogenizer in buffer (20 mM MOPS, 300 mM sucrose, and 0.1 mM EDTA, pH 7.2). The homogenate was centrifuged for 30 min at 13,000g, and the supernatant fraction was used for analysis of enzyme activities.

The conversion of [14C]arginine to [14C]citrulline was used to determine NOS activity. Liver tissue was homogenized in the lysis buffer of an NOS detect assay kit (Stratagene; La Jolla, CA) according to the manufacturer’s instructions.

Each catalase (x1000) or eNOS antibody (x2000) was used to detect the level of each protein in the liver by Western blotting, as described above.

Statistical Analysis. Unless otherwise stated, data are presented as mean ± S.E. (n = 5). Statistical analysis was performed by the Kruskal-Wallis test. Results were considered significant if p < 0.05.

Results

Hepatic P450 Levels and P450 Reductase Activities after NTG Administration. Hepatic P450 levels were significantly decreased by the NTG infusion, but they returned to their pretreatment levels 48 h after the NTG infusion was ceased (Fig. 1). NADPH-dependent P450 reductase did not change throughout the procedure (control group: 0.265 ± 0.06 U/mg protein). SNP had no affect on the levels of P450 and P450 reductase in the liver microsomes.

Western Blot of Hepatic P450 Isoforms After NTG Administration. CYP1A2 is one of the representative enzymes responsible for NTG biotransformation in rats (Minamiyama et al., 2001). Immunoreactivity of CYP1A2 became negative after 48 h of continuous infusion with NTG. 48 h after the NTG infusion was discontinued, the P450 immunoreactivity returned to the pretreatment level (Fig. 2). P450 reductase protein was not affected (data not shown). Figure 3 shows the Western blot of CYP isoforms of CYP1A2, −3A2, −2C11, and −2E1. Their immunoreactive signals decreased by −3A2, −2C11, and −2E1. Their immunoreactive signals decreased by 49%, 67%, and 63%, respectively, compared with the control group. Immunoreactivity returned to the pretreatment levels 48 h after NTG infusion. Therefore, P450 activity may be decreased along with the NO production pathway of organic nitrates. This study reports changes in P450 of the liver under continuous administration of ISDN or NTG.
returned to their pretreatment levels 48 h after the infusion was discontinued. SNP administration did not affect the hepatic levels of any isoforms.

**NOx Levels in Plasma and Urine after NTG or ISDN Infusion.** During the continuous and prolonged NTG and ISDN infusion, NOx in the plasma increased for the first 18 and 48 h, respectively, then decreased thereafter (Fig. 4). The levels of urine NOx peaked at 24 h during drug infusion in both groups and gradually decreased in spite of the continuous infusion of the drugs.

**Nitrate Tolerance and Hepatic P450 after NTG or ISDN Infusion.** NTG induced nitrate tolerance earlier than ISDN (Fig. 5). Hepatic P450 levels also decreased earlier by NTG than ISDN, but hepatic P450 levels returned to their pretreatment levels 48 h after the infusion was discontinued. Figure 6 shows immunostains of CYP1A2, -3A2, -2C11, -2B1, and -2E1 after ISDN infusion. Immunoreactive signals for each of the isoforms decreased after 72 h of continuous infusion of ISDN.

**Changes in Hepatic P450 Levels and Plasma NOx after Oral ISDN Administration.** Results obtained by oral ISDN administration are shown in Fig. 7. The rats were given tap water mixed with 1 g/l ISDN, and each animal drank about 20 ml of the solution per day. Hepatic P450 levels had significantly decreased to about 70% at 1 week ($p < 0.05$), and low levels were maintained thereafter (8 weeks) (Fig. 7). The plasma NOx levels gradually declined after their peak at week 4 of ISDN administration. NADPH-dependent P450 reductase did not change throughout the procedure.

**Effect of NTG Administration on Hepatic HO-1 Levels.** Figure 8 shows hepatic HO-1 levels in animals given NTG infusion. In normal liver, HO-1 was observed only in nonparenchymal cells such as Kupffer cells. Continuous infusion of NTG induced HO-1 in liver parenchymal cells, but the HO-1 returned to its pretreatment levels 48 h after the NTG infusion was ceased (Fig. 8). SNP did not affect HO-1 levels in the liver microsomes.

**Effect of NTG Administration on Hepatic Catalase and eNOS.** Continuous NTG or SNP infusion did not affect the activities of hepatic catalase (control, 486 ± 24 U/mg protein) and Ca-dependent NOS (eNOS; control, 7.8 ± 2.1 nmol/min/g protein) (Fig. 9).

**Discussion**

We previously reported that tolerance of organic nitrates was the result of P450 degradation induced by the continuous exposure to NO and that this tolerance could be prevented by the maintaining P450 activity (Minamiyama et al., 2001). Although our attention was mainly focused on the circulatory organs, most organic nitrates are metabolized in the liver, where NO is concomitantly produced from organic nitrates. The present study showed the effect of nitrate administration
Fig. 3. Changes in CYP isoforms after 48 h NTG infusion. Rats were treated as described in Fig. 1. Microsomal samples (0.05–5 μg of protein) and purified isoform standards were each subjected to SDS (2%)-polyacrylamide gel (7.5%) and electrophoresed. Data are mean ± S.E. (n = 5). *, p < 0.05 as compared with the control group.

Fig. 4. NO₂⁻ and NO₃⁻ levels in plasma and urine under continuous NTG and ISDN infusions. Rats were administered 0.5 mg/ml NTG (E) or 1 mg/ml ISDN at a rate of 1 ml/h for up to 72 h. At the indicated time, plasma and urine were collected and treated with methanol as described under Materials and Methods. Data are mean ± S.E. (n = 5). ○, NTG infusion; ●, ISDN infusion.

Fig. 5. Changes in blood pressure to a bolus injection of NTG following continuous NTG or ISDN infusion and their hepatic levels of P450. Rats were administered 0.5 mg/ml NTG (○) or 1 mg/ml ISDN (●) at a rate of 1 ml/h for up to 72 h. At the indicated time, NTG (1 mg/kg) was injected through the femoral vein. At the indicated times, hepatic P450 levels after NTG (open column) or ISDN (dotted column) infusion were measured as described under Materials and Methods. 48 h after the cessation of the NTG (48 h) or the ISDN (72 h), P450 levels returned to pretreatment levels. Values are mean ± S.E. (n = 5). *, p < 0.05; **, p < 0.01 as compared with control.
on hepatic levels of P450 and the continuous bioactivation of organic nitrates by P450 and HO-1 induction in the liver. Hepatic P450 is known to play important roles in the external drug metabolism as well as endogenous metabolism. Organic nitrates are also metabolized by hepatic P450. Dudenhoefer et al. (2002) reported that NTG induced less vasorelaxation than NO donor in cirrhotic liver and that NOx production in cirrhotic livers was lower than in normal livers during NTG administration. Other studies on cirrhotic livers demonstrated low levels of hepatic P450 and an altered pattern of P450 expression (Farrell and Zaluzny, 1985; George et al., 1995; Chalasani et al., 2001). We reported that P450 is important for bioactivation of organic nitrates (Minamiyama et al., 1999). Nonenzymatic NO donor produced the same levels of NOx in both normal and cirrhotic livers, and the NO level did not depend on the hepatic function (Chalasani et al., 2001). Generated NO rapidly reacts with P450 enzymes and inhibits P450 function (Khatsenko et al., 1993; Shiro et al., 1995; Snyder et al., 1996; Minamiyama et al., 1997a). NTG was associated with tolerance earlier than ISDN (Fig. 5). The clinical dose of ISDN (40–80 mg) is 2–3-fold of NTG (5–10 mg), and the duration of efficacy of ISDN (24–48 h) is longer than NTG (12 h). Thus, because they differ in potency and in pharmacokinetics (Osnes, 1984), we used the different doses of NTG and ISDN infusion.

The explanation of why the levels of P450 did not parallel with changes of plasma NOx levels will be that NO reacts with various biological molecules such as iron, thiols, and heme (Moncada and Higgs, 1991; Moncada et al., 1991). For example, the interaction of NO with sulphydryl compounds has been the focus of attention because of the relatively long lifetime of S-nitrosothiols and their reservoir function to release NO slowly. In fact, a peak of plasma NOx (8–12 h) appeared later than that of inducible NOS expression (5 h) (Takemura et al., 1999). NO and its derivatives form nitrosothiol complexes with heme, hemoglobin, albumin, or other proteins. Since these complexes are slow releasers of NO in the presence of oxygen, the delay of NO peak may be due to the slowly released NO from nitrosoproteins, which secondarily generated NOx in plasma for a fairly long time (Minamiyama et al., 1996, 1997a, b). Other reasons include the biotransformation of NTG by other molecules and enzymes. Chen et al. (2002) have reported that the biotransformation of NTG also occurred in mitochondria through a novel reductase action of mitochondrial aldehyde dehydrogenase. This should be studied further in our model.

In this study, continuous administration of organic nitrates markedly decreased P450 enzymes and simulta-
neously induced HO-1. NO is a well known inducer of HO-1 mRNA (Hartsfield et al., 1997; Demple, 1999; Bouton and Demple, 2000), and HO-1 has been shown to suppress the levels of P450 proteins in a tissue-specific manner with concomitant effects on their activity (Botros et al., 2002). Furthermore, HO-1 induction was reported to decrease various isoforms of P450 enzymes (Da Silva et al., 1994; Iba et al., 1999; Kobayashi et al., 2000). However, SNP, a nonenzymatic NO donor, did not elicit any tolerance or HO-1 induction. These results therefore indicate that NO-induced P450 degradation under HO-1 induction occurs in very localized places, such as in microsomes, where NO generation from organic nitrates takes place. We discovered that NO from organic nitrates had no effect on heme enzymes existed in other compartments such as NO synthase (cytosol) and catalase (peroxisome).

P450 and HO-1 recovered to their original levels 48 h after the cessation of continuous organic nitrates infusion. Preliminary studies in situ hybridization suggested that this is the result of newly synthesized P450 mRNA. This observation was in good agreement with the finding that P450 proteins reappear at a relatively early time after stopping infusion, and clinically a 12–24-h nitrate-free interval is sufficient to prevent nitrate tolerance (Kosmicki et al., 2000). During in vitro studies, rat hepatocytes treated with S-nitroso-N-acetylpenicillamine exhibited increased expression of CYP2E1 (Zamora et al., 2001). Our result showed that SNP administration did not elicit any effect on hepatic P450 levels. The result seems to contradict the finding of the S-nitroso-N-acetylpenicillamine study. However, as NO release from SNP takes place in blood vessels in vivo, the released NO binds to huge amounts of hemoglobin. Thus, the NO released from SNP might not have any direct effect on the hepatocytes. In fact, the SNP administration in our study also failed to induce hepatic HO-1.

In summary, we have demonstrated that the continuous administration of organic nitrates decreased hepatic P450 enzymes. This phenomenon suggests that P450-dependent drug metabolism might be drastically affected under continuous organic nitrate administration and that the endogenous metabolic processes such as steroidogenesis may also be affected under the same condition. Patients who are receiving long-term organic nitrates should be carefully watched.

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