Irreversible Inhibition of Cytochrome P450 by Nitric Oxide

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ABSTRACT

Nitric oxide (NO) modulates various metabolisms through interaction with thiol proteins and hemoproteins. Although NO interacts reversibly with iron moieties of heme proteins, including cytochrome P450 (P450), dynamic aspects of the formation, catalytic functions and fates of NO-P450 adducts remain to be elucidated. When incubated with NOC7, which spontaneously and stoichiometrically releases NO within 5 min, microsomal P450 rapidly formed nitrosyl-heme adducts as determined by the electron spin resonance method. The signal intensity for the complex increased with time, peaking at 30 min and decreasing to below detectable levels by 60 min of incubation. In contrast, the microsomal levels of low-spin ferric forms of P450 (g = 2.26) rapidly decreased during the initial 30 min but recovered time-dependently thereafter. Analysis by differential spectra (reduced form/CO-reduced form) revealed that on incubation with NOC7, the form of microsomal P450 also changed in a biphase manner. To elucidate the mechanism for the decrease in the levels of P450, microsomal levels of P450 isozymes (CYPs) were determined by Western blot analysis using specific antibodies against CYP3A2 and CYP2C11, major isoforms found in male rat liver. Kinetic analysis revealed that no appreciable degradation of P450 proteins occurred during the incubation of microsomes with NOC7. The effect of NO on the catalytic activity of the enzymes was determined by using testosterone as substrate because hydroxylation of steroid hormones is one of the major functions of P450. When exposed to NO, the hydroxylation activity in microsomes rapidly decreased during the initial 10 min and then disappeared slowly. These results suggested that NO formed dissociable complexes with P450 isozymes and the catalytic functions of these isozymes were irreversibly inactivated after dissociation from their heme moiety.

P450 plays important roles in the metabolism of physiological substrates and xenobiotics, such as steroids, fatty acids, prostaglandins, environmental pollutants and carcinogens. NO reacts with various molecules, such as superoxide, iron, thiol compounds and various hemoproteins, including P450 (Henry et al., 1993; Nathan, 1992; Kim et al., 1995), at nearly diffusion-limited rates (Cassoly and Gibson, 1975; Doyle et al., 1988). These proteins might be the primary targets for NO. Although NO interacts reversibly with the heme iron of P450, dynamic aspects of the formation of NO-P450 adducts, their fate and catalytic functions remain to be elucidated. ESR spectra of a ferric form P450 reveal a typical feature in which low-spin signals (g = 2.43, 2.26 and 1.91) are resolved at a temperature of liquid nitrogen. P450 forms ferrous (Fe(2+)) -CO complexes that exhibit a Soret absorption at 447 nm. The interaction of NO with sulfhydryl compounds has been the focus of attention because of the relatively long lifetime of S-nitrosothiols and their reservoir function to release NO slowly. The catalytic functions of some enzymes, such as plasminogen activator, are enhanced by S-nitrosylation (McCall and Vallance, 1992). S-Nitrosylation of P450 may affect its catalytic functions because P450 contains four to nine free cysteinyl residues. Recent studies in this laboratory revealed that hepatic levels of P450 markedly decreased in endotoxemic rats, particularly when large amounts of NO were generated by inducible NO synthase (Takemura et al., 1996). To clarify the effect of NO on the rates and functions of P450 isozymes, we determined changes in the levels and activities of the isozymes with rat liver microsomes and purified P450 isozyme.

Materials and Methods

Reagents. NOC7, ONOO− and DMPO were obtained from Dojin Co. (Kumamoto). Testosterone and diame were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical (St. Louis, MO), respectively. Other reagents were of analytical grade from Wako Pure Chemical Co. (Osaka). NO solution was prepared at room temperature by bubbling 10 mM Tris-HCl, pH 7.4, buffer solution with argon gas for 10 min and then for 30 min with NO gas that

ABBREVIATIONS: P450, cytochrome P450; NO, nitric oxide; ESR, electron spin resonance; NOC7, [1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-3-aminopropyl] [3-methyl-1-triazene; DTT, dithiothreitol; ONOO−, peroxynitrite; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

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had been passed through a KOH column (2 × 2.5 cm) to remove nitrogen dioxide. The pH did not change after treatment with the NO gas solution. Aliquots of the NO-saturated solution (1.9 mM) were used for the experiments.

**Interaction of microsomes with NO.** Freshly prepared microsomes (4 mg of protein/ml) were suspended in argon-saturated phosphate-buffered saline (pH 7.4) and incubated with NOC7 for varying times at 37°C. After incubation, samples were immediately frozen under liquid nitrogen and subjected to ESR analysis. Aliquots of incubated samples were also used for the analyses described below.

**Determination of P450 contents in hepatic microsomes.** Liver microsomes were prepared from male Wistar rats (220–250 g) (SLC Co., Shizuoka, Japan) as described previously (Funae and Imaoka, 1985). P450 contents in isolated microsomes were determined by measuring the intensity of low-spin heme (g = 2.26) using ESR and by absorption spectra of the reduced form vs. the reduced form of P450 CO complex (Omura and Sato, 1964a). The freshly prepared microsomes (400 μl of 4 mg of protein/ml were put into ESR tubes (4-mm inner diameter), quickly frozen in liquid nitrogen and analyzed by ESR at 110°K using a JES-RE1X spectrometer (JEOL, Tokyo) with 100-kHz field modulation. ESR analysis was conducted with microwave power at 8 mW at a frequency of 9.108 GHz, 325 conducted with microwave power at 8 mW at a frequency of 9.108 GHz, 325°C. ESR analysis was conducted with microwave power at 8 mW at a frequency of 9.108 GHz, 325°C. ESR analysis was conducted with microwave power at 8 mW at a frequency of 9.108 GHz, 325°C. ESR analysis was conducted with microwave power at 8 mW at a frequency of 9.108 GHz, 325°C.

**Measurement of free radicals from microsomes.** One hundred micrograms of microsomal protein were used for the detection of free radicals. Microsomes were incubated for 5 min at 37°C in 0.1 M potassium phosphate buffer, pH 7.5; 0.2 M DMPO was added; and the mixture was measured after 1 min at room temperature using ESR spectrometer. ESR conditions were magnetic field of 357.5 mT, microwave power of 8.0 mW, modulation frequency of 100 kHz, modulation amplitude of 0.1 mT, sweep width of 5.0 mT, sweep time of 10 mT/min, response time of 0.03 sec and receiver gain of ×500. Some experiments, 5 mM NADPH or 5 units/ml of SOD was added to the reaction mixture.

**Statistical analysis.** Unless otherwise stated, data are presented as mean ± S.E.M. One-way analysis of variance was used where appropriate, and a value of P < .05 was considered significant.

**Results**

**Effect of NO on P450 contents.** When microsomes were incubated with 100 μM NOC7, which spontaneously and stoichiometrically releases NO within 5 min, P450 rapidly formed NO adducts at the heme moiety as determined by an ESR method. ESR spectra revealed a specific signal with a g value of 2.0 representing the nitrosyl-iron complex (fig. 1). The signal intensity increased time-dependently, peaked at 30 min after incubation and then decreased thereafter, disappearing completely by 60 min of incubation (fig. 2). In contrast, the microsomal

**Fig. 1.** Effect of NO on the formation of P450-NO complexes in the rat microsomes. Hepatic microsomes were incubated at 37°C with NOC7 (10 and 100 μM), which spontaneously and stoichiometrically releases NO within 5 min. After incubation at 10 min, 400 μl of the sample was quickly frozen with liquid nitrogen and set into an ESR spectrometer. ESR spectra revealed low-spin heme (g = 2.43, 2.26 and 1.91) and a specific signal with three-line hyperfine structure at g value of 2.0 responsible for the nitrosyl-iron complex.
Effect of NO on total contents of hepatic microsomal P450. Hepatic microsomes were incubated with NOC7 as described in the legend of figure 1. At indicated times, microsomes were reduced by Na2S2O4 and bubbled with CO gas. Analysis by differential spectra (reduced form/CO-reduced form) revealed the biphasic changes in the microsomal levels of P450. The signal responsible for the ferric form of P450 recovered time-dependently. In contrast, the microsomal levels of the low-spin ferric P450 rapidly decreased during the initial 10 and 30 min by ~60% and ~90%, respectively. Thereafter, the signal responsible for the ferric form of P450 recovered time-dependently (70% at 60 min).

Effect of NO on the structure of P450. To elucidate the mechanism for the decrease in the levels of reduced form P450, structural changes in microsomal P450 were studied by Western blot analysis of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis using specific antibodies against CYP3A2 and 2C11. The antigenic activity and molecular size of P450 isozymes in the microsomal samples remained unchanged during the experiments (fig. 4).

Effect of NO on testosterone hydroxylation activity of P450. Effect of NO on the catalytic activity of P450 was determined by using testosterone as a substrate because hydroxylation of steroid hormones is one of the major functions of P450. On incubation of liver microsomes at 37°C under atmospheric conditions, the catalytic activity of 2C11 and 3A2 isozymes spontaneously decreased. When incubated with 100 μM NOC7, the hydroxylation activity of the membranes for 2α-, 16α-, 2β- and 6β-OH testosterone rapidly decreased by 23%, 23%, 11% and 20%, respectively, during the initial 10 min and slowly disappeared (fig. 5). The inhibitory effect depended on the concentration of NO; the decrease in the catalytic activity was enhanced at NO concentrations of >10 μM. The presence of 1 mM (final concentration) of substrate testosterone did not affect the inhibitory effect of NO (fig. 6).

Effect of NO on microsomal thiol levels and iron status. Free SH group is one of the important targets for NO. Incubation of microsomes with NOC7, an NO donor, decreased their levels of free thiogroup in a concentration-dependent manner (fig. 7). At NOC7 concentrations of 100 μM and 1 mM, microsomal SH levels decreased by ~22% and ~85%, respectively.

The level of iron associated with microsomes was 0.13 μg/mg of protein. Incubation of microsomes with NOC7 released the membrane-associated iron only slightly, at concentrations of <100 μM. However, at a higher dose of NOC7 (300 μM), significant fractions of membrane-bound iron were released (fig. 8).

Effect of thiol oxidation on testosterone hydroxylation activity. Diamide selectively oxidizes free SH groups. This compound decreased free thiol levels in microsomes dose-dependently (fig. 9), and this correlated well with the decrease in the hydroxylation activity of the microsomes. When NOC7-treated microsomes were incubated with DTT, the catalytic activity was enhanced dose-dependently (fig. 10), and this correlated well with the increase in the hydroxylation activity of the microsomes.

Fig. 2. Time course of the formation and disappearance of low-spin heme (g = 2.26) and iron-nitrosyl complexes (g = 2.0). Hepatic microsomes were incubated with NOC7 and thereafter subjected to ESR as described in the legend of figure 1. The g value of 2.0 signal intensity increased time-dependently, peaked at 30 min after incubation and then decreased thereafter, disappearing completely by 60 min of incubation (A). Thereafter, the signal responsible for the ferric form of P450 recovered time-dependently. In contrast, the microsomal levels of the low-spin ferric P450 rapidly decreased during the initial 30 min (B). ○ control; ◦, NOC7 (10 μM); ◦, NOC7 (100 μM). Data are mean ± S.E.M. (n = 3). **P < .01 compared with control.

Fig. 3. Effect of NO on total contents of hepatic microsomal P450. Hepatic microsomes were incubated with NOC7 as described in the legend of figure 1. At indicated times, microsomes were reduced by Na2S2O4 and bubbled with CO gas. Analysis by differential spectra revealed the biphasic changes in the microsomal levels of P450. ○ control; ◦, NOC7 (10 μM); ◦, NOC7 (100 μM). Data are mean ± S.E.M. (n = 3). **P < .01 compared with control.

Fig. 4. Western blot analysis for P450 forms. Hepatic microsomes were incubated with NOC7 as described in the legend of figure 1. Microsomes (5 μg protein) were electrophoresed 7.5% polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane. The nitrocellulose was stained enzymochromically. Antibodies of P450 isozymes were used for anti-CYP2C11 and anti-CYP3A2 antibodies. These isozymes are major forms in hepatic microsomes of male rats.
3). The microsomal testosterone hydroxylase activity was measured in the incubation mixture containing 100 μg of microsomal protein, 0.2 μmol of NADPH and 0.5 μmol of testosterone in 0.5 ml of 0.1 M of potassium phosphate buffer, pH 7.4. Testosterone metabolites were extracted with ethyl acetate and analyzed by high-performance liquid chromatography.  The microsomal testosterone hydroxylase activity decreased concentration-dependently by a mechanism that was fully recovered by DTT (fig. 11). Both 16α- and 16β-hydroxylation activities were decreased by ONOO⁻ decreased microsomal thiol levels to the same degree, but NO-induced inhibition of testosterone hydroxylase activity was greater than that of ONOO⁻ (fig. 13).

In all these experiments, the decomposition products of NOC7 or NOx (nitrite or nitrate) did not affect the results (data not shown).

## Discussion

The present work demonstrates that NO can interact with P450 in two ways: NO reversibly binds to the heme moiety of P450, forming iron-nitrosyl complexes, and it irreversibly inactivates P450 via the thiol modification pathway. ESR analysis revealed that, on incubation with either NO or an NO donor, the ferric form of P450 (g = 2.26) decreased with concomitant increase in the five-coordinated ferrous state.

The hydroxylation activity recovered fully to that of intact microsomes (fig. 10).

Purified CYP2C11 showed 4.54 ± 0.25 and 6.27 ± 0.12 nmol/min/nmol of 2α- and 16α-hydroxylation activities, respectively. The purified P450 is fairly unstable and degraded during the incubation. When exposed to NO, the hydroxylase activity of the enzyme decreased dose-dependently by a mechanism that was fully recovered by DTT (fig. 11).

Effect of ONOO⁻ on P450 structure and levels, thiol levels and testosterone hydroxylase activity. ONOO⁻ (∼0.6 mM) did not affect P450 levels or structural changes in microsomal P450 analyzed by Western blotting for CYP3A2 and CYP2C11. Microsomal levels of free thiols and testosterone hydroxylase activity decreased concentration-dependently (fig. 12). Both 16α- and 6β-hydroxylation activities were decreased by ONOO⁻ in the same manner. NO or ONOO⁻ decreased microsomal thiol levels to the same degree, but NO-induced inhibition of testosterone hydroxylase activity was greater than that of ONOO⁻ (fig. 13).
The nitrosyl form ($g = 2.0$), similar to that of the inactive form, P420 (O'Keefe et al., 1978). When incubated with NO in the presence of NADPH, NO-dependent decrease in the ferric form of P450 in microsomes was inhibited with a concomitant increase in the level of ferrous-NO complex (data not shown). These observations suggested that both ferrous (ESR-detectable) and ferric forms (ESR-silent) of NO-P450 complexes were generated in microsomes. This reduction mechanism of ferric to ferrous forms of P450 is still unclear. However, in this study, microsomes spontaneously generated hydroxyl radicals as detected by the spin-trapping method (fig. 14). These signals were increased by the addition of NADPH but were abolished by superoxide dismutase, suggesting that these signals were derived from superoxide. These observations suggest that one possible mechanism is the reduction by superoxide or some other unknown reductants in microsomes (fig. 15).

The decrease in testosterone hydroxylation activity seems to correlate with the decrease in P450 contents during the initial 30 min of incubation. Although 90% of P450 was apparently recovered spectrophotometrically 60 min after incubation with NOC7, its catalytic function remained activated. The inhibitory effect of NO was not affected by the presence of testosterone. Hence, the critical site(s) for modification by NO might be localized at site(s) other than the heme moiety and substrate-binding sites of P450.

The present work also demonstrates that NO decreased the free thiol levels in microsomes in a concentration-dependent manner. However, microsomal electron transport system was not affected by NO because NADPH-cytochrome c reductase activity remained unchanged during the experiments. In contrast, oxidation of free thiols in microsomes and purified CYP2C11 by either NO or diamide significantly de-
increased the testosterone hydroxylation activity of P450 by a mechanism that was recovered by DTT. Thus, the free cysteinyl residue(s) in P450 might play a role in the maintenance of its catalytic activity. P450-catalyzed O-dealkylation of benzyloxyresorufin was inhibited by NO by some mechanism that was presumably suppressed by the formation of albumin-S-NO (Wink et al., 1993). Serum albumin has a free sulfhydryl group (Cys34) that might have reacted with NO to form albumin-S-NO, thus liberating the sulfhydryl group of P450 and recovering its catalytic activity.

A peak of NO-P450 complex was slower than that of NOC7-
released NO. Because NO and its intermediate metabolites rapidly form nitrosothiols, a slow releaser of NO, in the presence of oxygen, the delay of the peak may be due to the nitrosothiols slowly releasing NO, which secondarily reacted with heme to form an NO-P450 complex for a fairly long time.

NO dose-dependently inhibited the hydroxylation activity of P450, and the inhibitory effect was more pronounced with CYP2C11 (~1.5-fold) than with CYP3A2. Although CYP2C11 and CYP3A2 have nine and six cysteinyl residues, respectively (Yoshioka et al., 1987; Miyata et al., 1994), critical cysteinyl residues required for their activity remain unclear. In consideration of these results, possible mechanisms of P450 inactivation are shown in figure 15.

Ferric (No. 1) and ferrous (No. 2) forms of P450 coexist in microsomes. Ferrous forms of P450 (No. 2) are partially produced by superoxide and unknown reductants-induced reduction of No. 1. NO reacts with both 1 and 2 to produce 3 and 4, respectively, and the NO-heme reaction is fairly rapid at nearly diffusion-limited rates (Cassoly and Gibson, 1975; Doyle et al., 1988). Although the ferric-NO form (No. 4) is relatively more stable than ferrous-NO (No. 3), the reduction process from 4 to 3 takes place in the presence of another NO and OH− (Hoshino et al., 1996). No. 3 releases its heme-bound NO in the presence of oxygen (No. 5) and results in the formation of nitrate and an ESR-detectable oxidized form (No. 6) (Yoshida et al., 1980). The NO-thiol (NO−S−) reaction is relatively slower (k = 6 × 106 M−2·sec−1) (Goldstein and Czapski, 1996) than that of NO-heme. However, the rate at which NO is released from S-nitrosothiols differs among targets for NO. For example, S-nitrosothiol of low molecular proteins (cysteine, glutathione, and so on) has a shorter half-life than that of high molecular proteins (albumin, and so on), with the half-life of albumin-S-NO being >10 hr (Arnelle and Stamler, 1995). Thus, the half-life of P450-S-NO (P450 is a higher molecular protein) may be speculated to be fairly long, resulting in the irreversible inactive forms. Hence, No. 1 apparently transforms to No. 3 and is completely recovered as No. 6 for the ESR-detectable forms, but the reactivity is not recovered.

Because inducible NO synthase is strongly induced in the liver of endotoxemic subjects, high concentrations of de novo synthesized NO might irreversibly inhibit the activity of P450. Reactive intermediates of NO and oxygen enhance the oxidation and nitrosation of various molecules (Nguyen et al., 1992; Wink et al., 1991). In fact, tyrosine residues in various proteins are oxidized by nitrogen dioxide (k = 5 × 109 M−1·sec−1) (Prutz et al., 1985). Thus, nitration of tyrosine residues might underlie the mechanism for the inhibition of their catalytic activity by NO. However, under the present experimental conditions, nitrotyrosine was not detectable in NO-treated microsomes as determined by Western blot analysis using anti-nitrotyrosine antibody (data not shown). Although the same concentration of ONOO− as NO decreased free thiol levels in microsomes, NO-induced inhibition of testosterone hydroxylation activity was stronger than that of ONOO−. These results suggest that the sites of thiol modification are different for NO and ONOO−. However, the reactivity of NO and free thiol is ~1000-fold faster compared with ONOO−. Therefore, considering the diffusion rate, reactivity of ONOO− may not be much affected in vivo.

Levels of P450-NO adducts increased in the liver of endotoxemic rats with concomitant decrease in P450 activity. The
presence of endotoxia and decrease in hepatic GSH levels and the enhancement of GSH turnover caused a condition of high oxidative stress. The occurrence of oxidation of SH in endotoxic liver may be due to some NO-dependent mechanism. The endogenous NO generation may negatively regulate steroidogenesis through interaction with P450. In view of the results of this study, the mechanism of P450 inactivation by NO and the importance of NO-P450 interaction in the pathogenesis of liver injury in endotoxia should be further studied.

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