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Inhibitory effect of nitric oxide on the metabolism of halogenated volatile anesthetics by cytochrome P-450

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Abstract

Purpose. This study was undertaken to examine the effect of nitric oxide (NO) on the metabolism of halogenated volatile anesthetics (HVA) by cytochrome P-450 (CYP) under both aerobic and anaerobic conditions using rat hepatic microsomes.

Methods. A microsomal fraction was prepared by centrifugation from normal and phenobarbital-treated male SD rats. The anaerobic metabolism of HVA by CYP was followed by measuring the formation of a halothane CYP complex spectrophotometrically. Aerobic CYP activity was determined using either the defluorination of sevoflurane or the demethylation of aminopyrine.

Results. The formation of the halothane-CYP complex was dose-dependently inhibited by NO. NO also decreased CYP defluorination of halothane in a dose-dependent manner. In phenobarbital-induced microsomes, the inhibition rates of both complex formation and the defluorination of halothane were the same as those seen in normal microsomes. Although the defluorination of sevoflurane and the demethylation of aminopyrine were inhibited by NO aerobically, the inhibition was much less than that of the metabolism of halothane under anaerobic conditions.

Conclusion. These results suggest that NO binds the heme of CYP and inhibits the metabolism of HVA, with the effect lasting for a prolonged period of time. Furthermore, the balance between NO and O_2 is important for NO to inhibit CYP.

Key words: Nitric oxide, Halogenated volatile anesthetics, Cytochrome P-450

Introduction

Nitric oxide (NO), first identified as an endothelium-derived relaxing factor [1], is considered a prominent messenger molecule with a wide range of biological functions. Besides its vasodilating action, NO has important roles such as neurotransmission [2] and cytotoxicity [3]. It has been demonstrated that hepatocytes also synthesize and release NO during chronic inflammation in vivo [4] and in response to the stimulation of cytokine in vitro [5]. However, the physiological significance of NO biosynthesis in the liver remains unknown.

The primary receptor for NO, soluble guanylyl cyclase (sGC), has been found to be a ferrous heme-containing enzyme. The interaction of NO with ferrous iron results in an alteration of the planar heme structure, producing activation of sGC [6] with subsequent production of cGMP.

NO is a radical and would be expected to have an extremely short half-life of less than a second [7]. It has been suggested that through the formation of *S*-nitrosothiols, NO is stabilized to prolong [8] its half-life and thereby preserves not only its primary biologic activity but its propensity to interact with nontarget sites such as other ferrous heme proteins.

The family of enzymes known as cytochrome P450 (CYP) are also ferrous heme-containing proteins. CYP has been shown to be exclusively responsible for the metabolism of halogenated volatile anesthetics (HVAs) [9]. HVAs are similar to NO in their great affinity for ferrous iron because of their specific halogen content and are metabolized by CYP because of this affinity [10]. Recently, the inhibitory effect of NO on CYP has been demonstrated in different mechanisms, such as binding to the prosthetic heme of CYP [11], increasing heme degradation [12] and decreasing the apoprotein of CYP [13].

This study was undertaken to examine the effect of NO on the metabolism of HVA by CYP under both aerobic and anaerobic conditions. The reductive metabolism of halothane and the oxidative metabolism of sevoflurane and aminopyrine were selected because halothane undergoes a unique reductive metabolism

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that is easily followed, and sevoflurane and aminopyrine are oxidatively metabolized, although at different rates, with the metabolism of sevoflurane being considerably slower than that of aminopyrine.

Materials and methods

Chemicals

NO gas (100%) was purchased from Puritan Bennett (Detroit, MI, USA). Halothane was purchased from Halocarbon Laboratories (Augusta, SC, USA), and sevoflurane was a gift from Maruishi Pharmaceutical (Osaka, Japan). All other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA).

Microsomal preparation

Male Sprague-Dawley rats (150–200 g) were fed a standard laboratory diet. Some were allowed access ad libitum to 0.2% sodium phenobarbital (PB) in their drinking water for 5 days. The rats were decapitated under ketamine anesthesia, and the liver was excised and immediately placed in ice-cold 20 mM Tris-HCl buffer containing 2 mM EDTA and 150 mM KCl (pH 7.4) and was homogenized. The homogenate was centrifuged for 10 min at 10000 *g*, and the supernatant was ultracentrifuged for 60 min at 105000 *g*. The resultant microsomal pellet was resuspented in 20 mM Tris-HCl buffer (pH 7.4). All of the above procedures were carried out at below 4°C. The hepatic microsomal protein concentration was determined by the method of Lowry [14], and the cytochrome P450 content (CYP) was measured by the method of Omura and Sato [15].

Spectrophotometry

The halothane-CYP complex was formed anaerobically by the methods of Van Dyke et al. [16]. The microsomal suspension was flushed with 100% nitrogen for 5 min and placed in a cuvette with an appropriate amount of crystalline sodium dithionite, halothane (2.6 mM), and the indicated concentration of NO. The final volume of microsomal suspension in a cuvette was 3 ml (protein concentration, 5 mg·ml^{-1}). The difference spectra in microsomes were determined using a Beckman DU-64 spectrophotometer. The cuvette containing microsomes and sodium dithionite was read as background. The samples were scanned between 430 and 530 nm. The absorbance of the halothane-CYP complex was derived from difference absorbance of 470–525 nm [17].

The NO solution was prepared by equilibrating 10 ml of pure NO gas and 95 ml of deoxygenated water, which was purged with 100% nitrogen for 40 min, in a gas-tight 100-ml bottle as reported by Moy et al. [18]. The concentration of NO in this saturated solution averaged 2.4 ± 0.1 mM [19].

To examine how NO influences the halothane-CYP complex, NO and halothane were added to the microsomal suspension in three sequences as follows: NO was added 10 min after halothane administration; NO and halothane were added simultaneously; NO was added 10 min before halothane administration.

Fluoride assay

The defluorination of HVA was assayed by measuring microsomal production of inorganic fluoride (F^-). Incubations were carried out for 30 min at 37°C using 3 ml of mixture containing 15 mg microsomal protein, 2 μmol of NADP, 5 μmol of glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase, with the indicated concentration of NO, 2.6 mM of halothane, and 1.7 mM of sevoflurane. Inorganic fluoride was determined using an ion electrode (Orion model 9409 fluoride electrode and 9002 reference electrode) with a Beckman IM 70 pH meter [20]. Defluorination of halothane was determined under anaerobic conditions; microsomal mixtures were purged with 100% nitrogen for 5 min before incubation.

Aminopyrine assay

The metabolism of aminopyrine was determined by measuring microsomal production of formaldehyde. The incubation was carried out at 37°C using 3 ml of mixture containing 3 mg of microsomal protein with 2 μmol of NADP, 10 μmol of glucose-6-phosphate, 1 unit of glucose-6-dehydrogenase, and 5 mM of aminopyrine. The reaction was terminated by adding 0.5 ml of saturated BaOH and 25% $ZnSO_4$ and cooling on ice. Following centrifugation for 30 min at 2500 *g*, the production of formaldehyde was assayed according to the methods of Nash [21] in aliquots of supernatant.

Statistical analysis

Statistical analysis of the data was performed by ANOVA with subsequent comparisons using Bonferroni's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Spectrophotometry

NO dose-dependently inhibited the formation of halothane-CYP complex in both normal and PB-

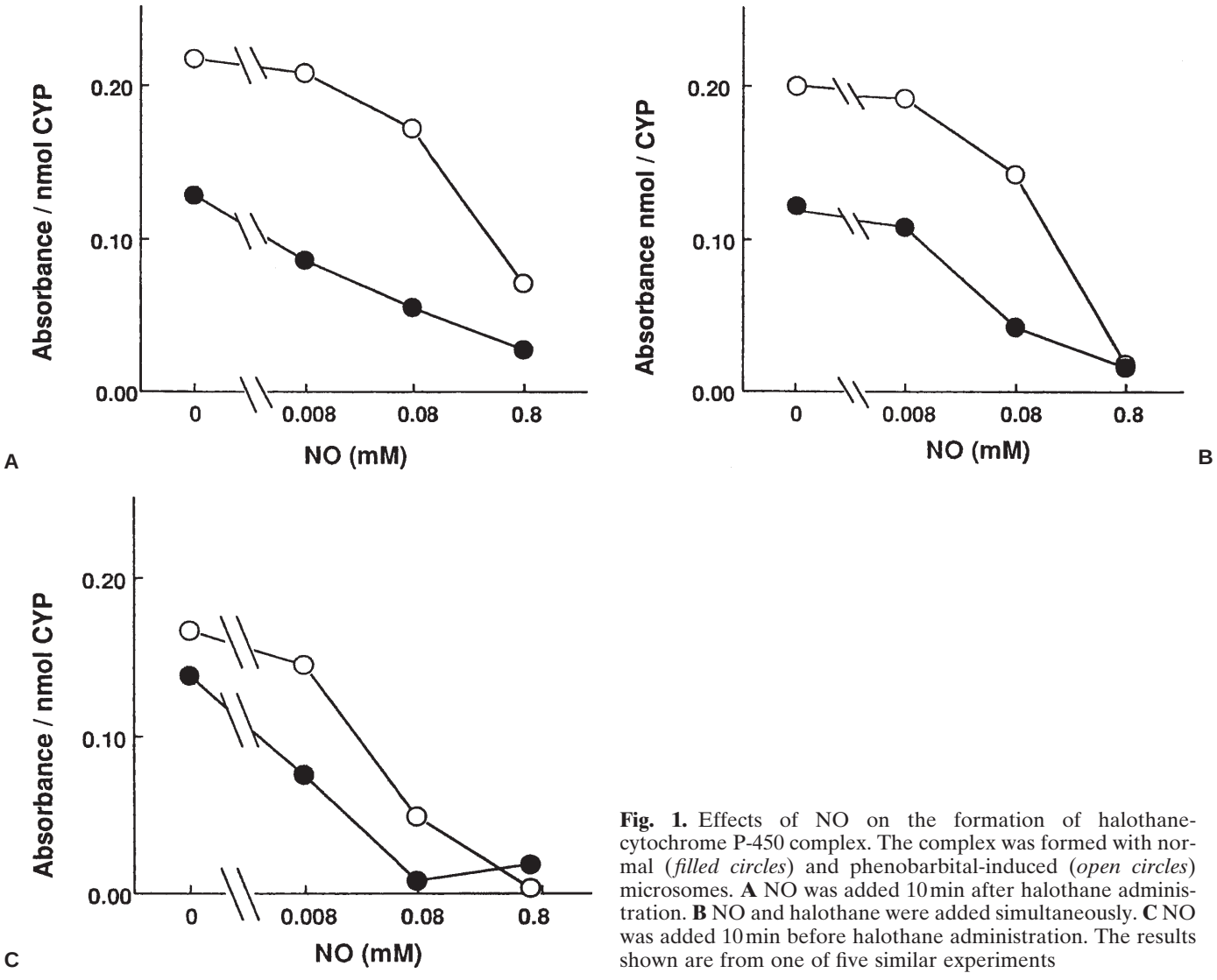

Fig. 1. Effects of NO on the formation of halothane-cytochrome P-450 complex. The complex was formed with normal (*filled circles*) and phenobarbital-induced (*open circles*) microsomes. **A** NO was added 10 min after halothane administration. **B** NO and halothane were added simultaneously. **C** NO was added 10 min before halothane administration. The results shown are from one of five similar experiments

induced microsomes regardless of the order of addition of NO (Fig. 1). PB-induced microsomes showed higher absorbance than did normal microsomes in all groups. However, when the percentage inhibition rates of NO at 0.08 mM were compared, there were no significant differences between normal and PB-induced microsomes (Table 1). The time course of complex formation relative to the order of NO addition is shown in Fig. 2. When halothane was added before NO, the formation of the complex was decreased by NO time-dependently. When halothane and NO were added simultaneously, a decreased amount of complex was produced, and this complex was stable. The complex formation was also stable when NO was added before halothane, but these values were much less than that shown when halothane and NO were added together.

Table 1. Inhibition rate (% of control) of NO on formation of the halothane-cytochrome P-450 complex in normal and phenobarbital-induced microsomes

Normal	Phenobarbital-induced
69.7 ± 4.2	74.2 ± 3.8

NO (0.08 mM) and halothane were added simultaneously and the absorbance was measured at 10 min. All values are means ± SE (n = 6).

Fluoride assay

NO dose-dependently inhibited the defluorination of halothane in both normal and PB-induced microsomes under anaerobic conditions (Fig. 3). These results were similar to those of the halothane-CYP complex inhibi-

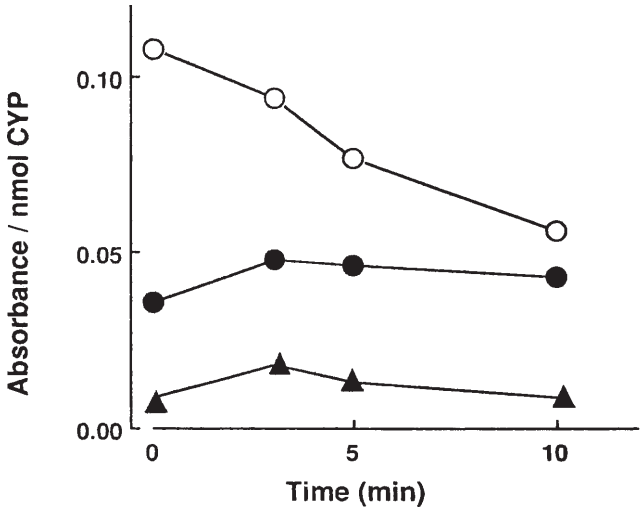

Fig. 2. Time course of formation of the halothane-cytochrome P-450 complex relative to order of NO addition. *Open circles*: NO (0.08 mM) was added 10 min after halothane administration. *Filled circles*: NO (0.08 mM) and halothane were added simultaneously. *Triangles*: NO (0.08 mM) was added 10 min before halothane administration. The absorbance was recorded at 0, 3, 5, and 10 min following addition of NO, NO and halothane, and halothane, respectively. The results shown are from one of five similar experiments

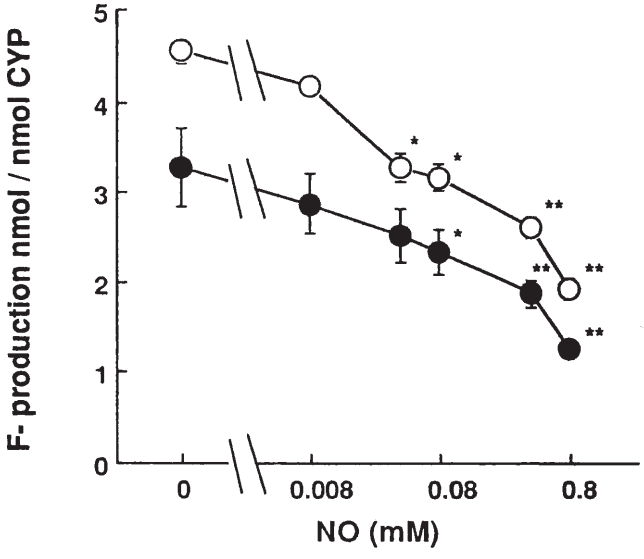

Fig. 3. Effects of NO on the defluorination of halothane. The incubations were carried out anaerobically for 30 min at 37°C using 3 ml of a mixture containing 15 mg of microsomal protein. *Filled circles*: normal microsomes; *open circles*: phenobarbital-induced microsomes. All values are means ± SE ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* NO 0 mM

tion, in that the individual values obtained from PB-induced microsomes were higher than those of normal microsomes, although the inhibition rate by NO in both microsomes was the same (Table 2). NO also tended to

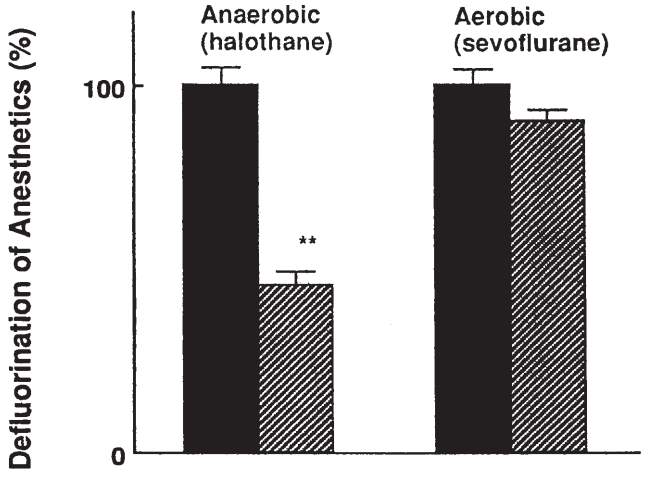

Fig. 4. Comparison of the effect of NO on the defluorination of anesthetics under anaerobic versus aerobic conditions. The incubations were carried out at 37°C for 30 min using 3 ml of a mixture containing 15 mg of microsomal protein with 1 µl with of halothane (anaerobic) and sevoflurane (aerobic). *Sodid bars*, control; *hatched bars*, NO (0.8 mM). All values are means ± SE ($n = 6$). $^{**}P < 0.01$ *vs* control

Table 2. Inhibition rate (% of control) of NO on defluorination in normal and phenobarbital-induced microsomes

Normal	Phenobarbital-induced
73.2 ± 3.3	72.3 ± 3.9

The incubation was carried out with NO (0.08 mM) for 30 min at 37°C using 3 ml of a mixture containing 15 mg microsomal protein. All values are means ± SE ($n = 6$).

inhibit the defluorination of sevoflurane under aerobic conditions (Fig. 4). However, these inhibitory effects of NO were less than those shown in halothane under anaerobic conditions and did not reach statistical significance.

Aminopyrine assay

The microsomal production of formaldehyde time-dependently increased and NO significantly inhibited this production at 3, 5, 10 min (Fig. 5). The inhibitory effect of NO on aminopyrine metabolism was also less than that shown on halothane under anaerobic conditions. However, it should be noted that the inhibition by NO at 3 and 5 min was uniform and did not show a decrease with time of incubation until 5 min, which would have been expected because of the rapid NO oxidation.

Discussion

The present study offers evidence that NO inhibited the metabolism of HVA by CYP. It is also obvious that the

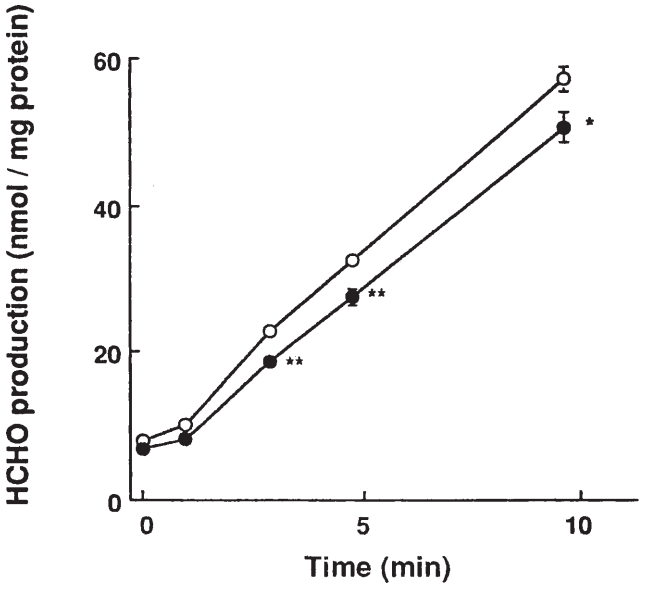

Fig. 5. Effects of NO on the metabolism of aminopyrine. The incubations were carried out at 37°C using 3 ml of a mixture containing 3 mg of microsomal protein. *Open circles*: without NO; *filled circles*: with NO (0.8 mM). All values are means ± SE ($n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$ *vs* without NO

interaction of NO with CYP and the degree of inhibition of HVA metabolism were dependent on the presence or absence of oxygen. That NO interacts with ferrous heme protein is well known, because its receptor, sGC, is a ferrous hemeprotein that reacts rapidly with NO [6], and other ferrous heme proteins, such as myoglobin and oxyhemoglobin, are excellent traps for NO and have been used to inhibit NO-stimulated reactions [22].

Previous studies demonstrated that in the process of reductive metabolism of halothane by CYP, a complex is formed between halothane and CYP that results from the two-electron reduction of halothane [17]. This complex is unique and has several important characteristics: its formation is rapid, spectrally observable at 470 nm, and quite stable, and when it dissociates, it immediately releases chlorodifluoroethylene [23]. Furthermore, the complex was reported to be CYP isozyme-specific, i.e., two PB-inducible CYP isozymes were able to form this complex to a greater extent than others [16,24]. The actual structure of the complex has not been determined, but it is believed that both the iron center and the protein are involved in complex formation; thus, NO could be interfering with either site to inhibit the formation of the halothane-CYP complex. However, if NO bound to any site other than the ferrous iron, it would show isozyme specificity. Since NO showed no inhibitory differences between PB-induced and -uninduced CYP, we conclude that the site NO interferes with is most likely the iron atom.

In several CYP isozymes, it has been suggested that NO inhibits CYP activity by binding to the ferrous iron of this enzyme [11,25]. Incubation of liver microsomes isolated from rats treated with pyrasole to induce CYP2E1, with gaseous NO or NO donors, resulted in a loss of CYP2E1 catalytic activity with specific substrates [26]. Because the NO-inactivated CYP2E1 was not degraded, as shown by Western blot analysis, and the formation of a stable heme-NO complex with CYP2E1 was identified by ESR, this study indicated that the inhibitory effect of NO on CYP2E1 can probably be explained, at least in part, by NO binding to the oxygen-binding site. These results are quite consistent with our data.

NO could decrease apoprotein of CYP and interfere with the enzymatic activity [13,27]. In the human hepatocytes, NO, which was released in response to interferon-γ (INF-γ), decreased specific CYP activity [28]. Since INF-γ also produced a reduction in CYP apoprotein and mRNA levels, it was suggested that the change in apoprotein level caused by NO was responsible for the decreased CYP activity. It has been proposed that the changes in apoprotein are largely due to changes in corresponding mRNA levels [13]. Although it is not certain whether NO decreased the level of CYP apoprotein directly in microsomes, our microsomal incubation did not include any protein synthesis system and was only carried out for approximately 5–30 min. Therefore, the apoprotein level did not change in our experimental conditions, and the inhibitory effect of NO in the present study is not related to the level of apoprotein.

There was a difference in the inhibition depending on the order of addition of NO and halothane. Once the halothane-CYP complex had been formed, NO time-dependently degradated the complex by displacing halothane, whereas adding NO first completely blocked complex formation. These results indicate that the affinity of NO for the ferrous iron of CYP is stronger than that of halothane. The firm binding of NO to ferrous iron was demonstrated in sGC [29] and CYP [26]. In the presence of nanomolar concentrations of NO, CO would be competitively prevented from heme binding of sGC. Moreover, NO from NO donors inhibited formation of the characteristic CO-binding spectrum of CYP. Thus, NO could bind to the ferrous iron of CYP and decrease the anaerobic metabolism of halothane because of this great affinity.

The effect of NO on the aerobic activity of CYP was not as pronounced as that on anaerobic activity. An immediate question is whether the effective NO concentration was instantly decreased by the rapid oxidation of NO in an aerobic system. Although this is a strong possibility and one that cannot be entirely ruled out, there is also the possibility that O_2 and NO compete

for the same ferrous heme site on CYP, thereby attenuating the inhibitory effect. Although NO has been reported to have an extremely short half-life in vivo [7], endothelium-derived NO has a second-order half-life as it diffuses from one cell to another [8]. This prolonged half-life suggests that NO is stabilized in some manner by a carrier molecule. This carrier molecule is thought to be a protein containing a large number of reduced thiol groups. Studies with bovine serum albumin have shown that the half-life of the biologic activity of NO can be prolonged significantly through formation of *S*-nitrosothiols. Spontaneous action of NO with reduced thiols may result in the release of NO over a longer period of time [30]. In the present study, a prolonged presence of NO in microsomes was confirmed even after aerobic incubation for 5 min with NADPH, conditions that are considered to be optimal for NO oxidation. Furthermore, a great affinity of O_2 for ferrous iron is demonstrated from its easy binding to hemoglobin. Therefore the fact that NO inhibited CYP less in the presence of oxygen than in its absence may be related to the relative affinities of NO and O_2 for the ferrous iron of CYP. This is consistent with the report suggesting that the reversible inhibitory effect of NO on CYP most likely represents the prevention of binding of O_2 [11].

Hepatocytes have been reported to produce NO in response to cytokine [5] and endotoxin [31]. NO produced by hepatocytes will bind to CYP and decrease the metabolism of HVA in conditions such as chronic inflammation and endotoxin shock. Then these conditions will deepen the level of anesthesia and prolong recovery from anesthesia. In addition, it should be noted that this situation could be enhanced by hypoxia. The decreased activity of CYP caused by NO would also be important in understanding the altered pharmacokinetics of drugs other than HVA, because CYP is a key enzyme in the catalysis of oxidative reactions in the biotransformation of most xenobiotics [32]. Therefore, inhibition of CYP could affect hepatocellular detoxication. On the contrary, it has been demonstrated that NO may prevent the toxicity of agents that require bioactivation by CYP isoforms such as CYP2E1 and in the generation of reactive intermediates by CYP2E1 [26]. Although the clinical significance of the inhibition of CYP by NO has not yet been determined, NO might play an important role in the regulation of CYP activity.

In summary, NO inhibited the metabolism of HVA by CYP under both aerobic and anaerobic conditions. This inhibition was rapid and complete under anaerobic conditions with less aerobic inhibition. These results suggest that NO binds to the ferrous iron of heme and attenuates the activity of CYP. Furthermore, the inhibitory effect of NO on the metabolism of HVA was minimized in the presence of O_2 under normal physiologic conditions. If the balance between O_2 and NO is disturbed either by hypoxia or by any other conditions that increase the release of NO, the inhibition will become evident under conditions of relative shortage of O_2.

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