Biochemical Pharmacology, Vol. 58, pp. 439–445, 1999.

ELSEVIER

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00102-1

Microsomal Formation of Nitric Oxide and Cyanamides from Non-physiological *N*-Hydroxyguanidines: *N*-Hydroxydebrisoquine as a Model Substrate

Bernd Clement,† Jean-Luc Boucher,‡ Daniel Mansuy‡ and Angela Harsdorf**

*Pharmazeutisches Institut, Christian-Albrechts-Universität, D-24118 Kiel, Germany; and ‡Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, URA 400 CNRS, Université Paris V, 75270 Paris Cedex 06, France

ABSTRACT. The microsomal oxidative transformation of a non-physiological *N*-hydroxyguanidine was demonstrated for the first time for *N*-hydroxydebrisoquine as a model substrate (Clement *et al.*, *Biochem Pharmacol* **46:** 2249–2267, 1993). The objective of the present work was to further compare this reaction with the analogous oxidation of arginine via *N*-hydroxyarginine to citrulline and nitric oxide. The oxidation of *N*-hydroxydebrisoquine by liver microsomes from rats pretreated with dexamethasone not only produced nitric oxide and the urea, but also the cyanamide derivative as the main metabolite. The low stability of the cyanamide derivative, which easily hydrolyzed to the urea derivative, was noted. The formation of all compounds required cosubstrate and the enzyme source. Experiments with catalase, superoxide dismutase, and H_2O_2 showed that the O_2^- formed from the enzyme and the substrate apparently participated in the reaction. While the *N*-hydroxylation of the guanidine involves the usual monooxygenase activity of cytochrome P-450 (Clement *et al.*, *Biochem Pharmacol* **46:** 2249–2267, 1993), the resultant *N*-hydroxyguanidine decoupled the monooxygenase. Nitric oxide was detected by the oxyhemoglobin assay. To examine the influence of enzymatically formed nitric oxide on the formation of the metabolites, the *N*-hydroxydebrisoquine was incubated with SIN-1 as nitric oxide donor under aerobic conditions. It was again possible to detect the cyanamide and urea derivatives, with the latter as main metabolite. It was concluded that the microsomal transformation of *N*-hydroxydebrisoquine produces a cyanamide and nitric oxide which reacts with *N*-hydroxydebrisoquine to form the urea derivative. The purely chemical reaction of the unsubstituted *N*-hydroxyguanidine with nitric oxide gave similar results (Fukuto *et al.*, *Biochem Pharmacol* **43:** 607–613, 1992). In conclusion, similarities (formation of a urea derivative) and differences (formation of a cyanamide derivative) between the physiological oxidation of *N*-hydroxy-L-arginine by nitric oxide synthases and non-physiological *N*-hydroxyguanidines by cytochrome P-450 were observed. Furthermore, non-physiological *N*-hydroxyguanidines can be regarded as nitric oxide donors. BIOCHEM PHARMACOL **58**;3:439–445, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *N*-hydroxydebrisoquine; nitric oxide; *N*-hydroxy-L-arginine; *N*-hydroxyguanidine; cytochrome P-450; cyanamides

In the past few years, NO§ has been found to be a biological mediator of major significance not only for blood pressure control and neurotransmission, but also for the immune defense [1]. Its biosynthesis in mammals involves a two-step oxidation of L-arginine [2]. The second step is an oxidative cleavage of the C=N〰OH bond of the intermediate N^{ω}-hydroxy-L-arginine, resulting in the formation of NO and citrulline. Both steps are catalyzed by NOS, a particular class of monooxygenases closely related to cytochromes P-450 [2, 3]. The three-electron oxidation in the second step is not a classical monooxygenase reaction and has also been found to be catalyzed by cytochrome P-450 [4]. Rat liver cytochromes P-450 oxidize N^{ω}-hydroxy-L-arginine to citrulline and NO, a reaction which depends to a great extent on the presence of superoxide [5]. In addition, it was also possible to detect NO from the P-450-mediated oxidation of benzamidoximes and *N*-hydroxyguanidines [5].

An *N*-hydroxyguanidine derived from debrisoquine was chosen as a model substrate to investigate the microsomal formation of NO from non-physiological *N*-hydroxyguanidines (for structures, see Fig. 1). Therefore, the influence of the nitric oxide produced on the formation of the other metabolites was examined as well as the role of reactive oxygen species. Due to the potential physiological relevance, we felt it worthwhile to investigate the similarities

† Corresponding author: Prof. Bernd Clement, Pharmazeutisches Institut, Christian-Albrechts Universität Kiel, Gutenbergstr. 76, 24118 Kiel, Germany. Tel. (+49) 431-880-1126; FAX (+49) 431-880-1352.

§ *Abbreviations:* NO, nitric oxide; NOS, NO synthase; and SOD, superoxide dismutase.

Received 8 July 1998; accepted 26 January 1999.

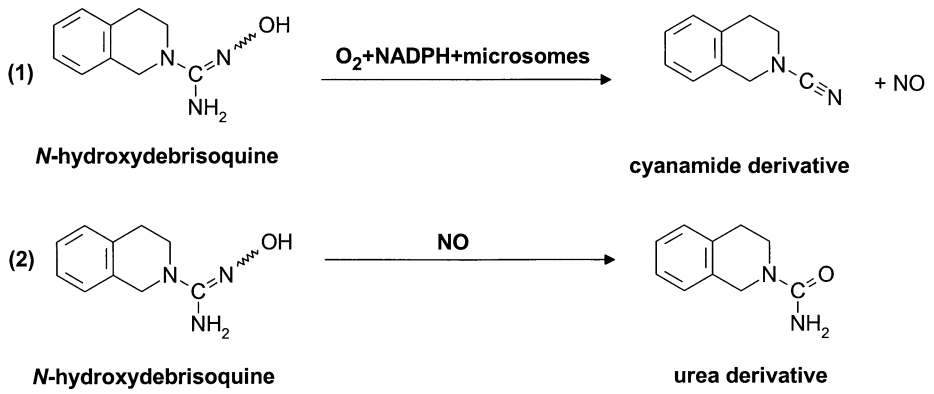


FIG. 1. Metabolism of *N*-hydroxydebrisoquine to the cyanamide derivative and NO by microsomal fractions of rat liver (reaction (1)) and chemical formation of the urea derivative by reaction of *N*-hydroxydebrisoquine with NO (reaction (2)).

and differences between the catalytic activity of NOS and cytochromes P-450.

MATERIALS AND METHODS

Chemicals and Reagents

Catalase, SOD, and human hemoglobin were purchased from Sigma Chemical Co. Oxyhemoglobin was prepared from hemoglobin as described previously [6] and SIN-1 was supplied by Alexis Co. NADPH (tetrasodium salt) as well as all other chemicals and solvents were from Merck unless otherwise stated.

Synthesis

The cyanamide and urea derivatives of debrisoquine were obtained by reaction of tetrahydroisochinoline and bromocyanogen or potassium cyanate, respectively, as described earlier [7]. *N*-Hydroxydebrisoquine was synthesized from the cyanamide by reaction with hydroxylamine [8]. The products were characterized in the usual manner. Configuration, E/Z-isomerism, and tautomerism of *N*-hydroxyguanidines were clarified by [^{15}N]NMR investigations [9].

Preparation of Microsomal Fractions

The livers of untreated rabbits (3.0–4.5 kg) and of male Wistar rats (300–400 g) which had been pretreated with dexamethasone were used as previously described [10]. The livers were perfused with KCl solution to remove blood residues and were then minced. All subsequent steps were performed as described in earlier publications [11].

Analytical Procedures

The protein content was determined by the method of Gornall *et al.* [12] (reagent kit, Merck). BSA was used as reference and the measurements were performed with a Beckmann DU 7000 diode array spectrophotometer. The content of cytochrome P-450 was analyzed using the method of Omura and Sato [13] and a Kontron Uvicon 930 spectrophotometer.

Incubations

N-OXIDATION OF N-HYDROXYDEBRISOQUINE WITH MICROSOMES FOR HPLC ANALYSIS. The standard incubation mixture (0.18 mL) contained 50 mM phosphate buffer (pH 7.4), 1 mM *N*-hydroxydebrisoquine, 0.5 mM NADPH, 3.3 mM $MgCl_2$, and microsomes equivalent to 0.17 nmol P-450. The reaction was started after 3-min preincubation at 37° by addition of substrate and NADPH. Incubation time was 20 min. Incubations were terminated by cooling on ice and lyophilization. The incubation mixtures were dissolved in methanol not more than 4 hr before measurement. Thereafter, they were immediately centrifuged at 15,000 *g* and 4° for 12 min. Aliquots of 10 μL of the supernatant were taken for HPLC analysis. Alternatively, *N*-hydroxydebrisoquine was incubated solely with 0.3 mM SIN-1 as NO donor for some experiments.

N-OXIDATION OF N-HYDROXYDEBRISOQUINE WITH MICROSOMES FOR THE OXYHEMOGLOBIN ASSAY. The incubation mixture (0.6 mL) contained the following: 50 mM phosphate buffer (pH 7.4), 8.7 μM oxyhemoglobin, 0.4 mM NADPH, and microsomes equivalent to 0.57 nmol P-450, which corresponds to the concentration of P-450 in the incubation mixtures for HPLC. The reaction was started by the addition of 1 mM *N*-hydroxydebrisoquine and was continued for 10 min. The blank consisted of the normal incubation mixture without substrate.

HPLC

Separation and quantification were performed at 20° (column oven) using a Waters 510 HPLC pump equipped with a variable wavelength UV detector (Waters 486) set at 208 nm and connected to a Waters WISP 710 autosampler. The areas under the peaks were integrated by Waters W 820 Maxima Vers. 3.31 software. The mobile phase consisted of acetonitrile/KH_2PO_4 buffer (0.05 M, pH 3.0) (20:80, v/v) and the flow rate was 1 mL/min through a prepacked, reverse phase LiChrospher RP-select B-column (125 × 4 mm, particle size 5 μm; Merck). Solvents used in the analysis were filtered through a Sartolon membrane filter

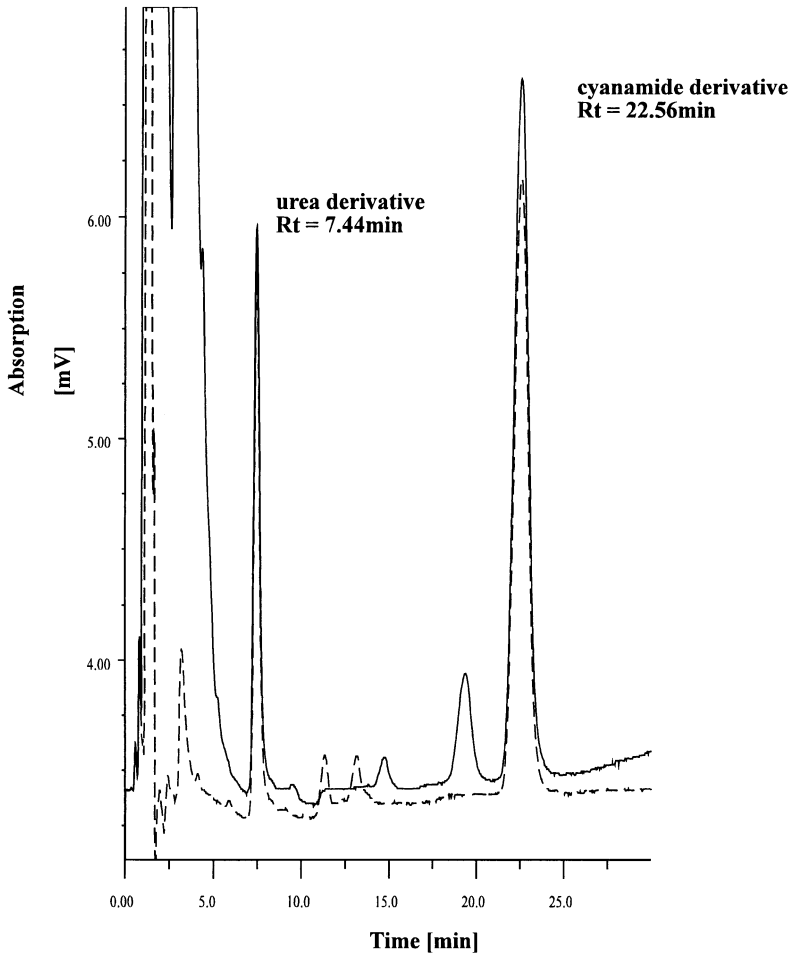


FIG. 2. Representative HPLC chromatogram of *N*-hydroxydebrisoquine metabolism with microsomes of rats pretreated with dexamethasone. Overlay of a complete incubation mixture and standards of the urea and cyanamide derivatives. See Materials and Methods for details.

(0.45 μm, Sartorius AG) and degassed by bubbling with helium. Standard curves (peak area) for the urea derivative of debrisoquine at the levels of 1.0, 2.5, 5.0, 10.0, and 15.0 μM solutions were found to be linear over this range with correlation coefficients of >0.9974. The curves were constructed by the addition of known amounts of the urea derivative to the usual incubation mixtures lacking cosubstrate and treated in the same manner as the experimental samples. The levels of the urea derivative in the samples were determined directly from these standard curves. The recovery rate after incubation and sample workup amounted to 107.1 ± 4.9% (N = 30) of that obtained from samples with the same amount of the urea derivative in phosphate buffer. The detection limit in the incubation mixture was 0.05 μM of the urea derivative. The retention time for the urea derivative was 7.9 ± 0.5 min.

Separation and quantification of the cyanamide derivative were performed in parallel and in the same manner. Standard curves at the levels of 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100.0 μM cyanamide derivative were found to be linear over this range with a correlation coefficient of >0.9967. The recovery of the cyanamide derivative in incubation mixtures was 85.2 ± 5.4% (N = 42) of that obtained using samples which contained the same amount of the cyanamide derivative dissolved in phosphate buffer. It was possible to detect a concentration of 0.5 μM of the cyanamide derivative, which corresponds to a reaction rate of 22 pmol/min/nmol P-450. The retention time for the cyanamide derivative was 23.6 ± 1.2 min.

Oxyhemoglobin Assay

The formation of NO was measured using an indirect difference spectrophotometric method [6]. The NO formation was quantified by measuring the decrease in the difference of absorbance at 420 nm. Values were expressed as initial kinetics calculated from the slope of the linear extinction versus the time gradient obtained shortly after the lag phase. The assay was carried out with a Beckman DU 7000 diode array spectrophotometer with temperature control at 37 ± 0.05°.

UV Spectra of the Metabolites

PREPARATIVE ENRICHMENT OF THE UREA AND CYNAMIDE DERIVATIVES OF DEBRISOQUINE FOR UV SPECTROSCOPY. For enrichment of the urea and cyanamide derivatives of debrisoquine, six of the usual incubation mixtures were run and worked up as usual. Thereafter, the supernatants were combined and evaporated to dryness, the residue was taken

TABLE 1. Metabolism of *N*-hydroxydebrisoquine by microsomal enzymes of rat liver and influence of NADPH and protein

Incubation mixture	Urea derivative nmol/min/ nmol P-450	Cyanamide derivative nmol/min/ nmol P-450
Complete/rat (dexamethasone-pretreated)	0.509 ± 0.052	1.883 ± 0.126
−NADPH	ND	ND
−Protein	ND	ND
Complete/control rat (not pretreated)	0.131 ± 0.021	0.485 ± 0.024

The rates ± SD were calculated from four different determinations with microsomes isolated from liver homogenates of 10 rats or 1 rabbit (ND = not detectable). All values statistically different from control (complete incubations) with $P < 0.001$ (Student's *t*-test).

up in 100 μL methanol and centrifuged, and 20-μL aliquots of the supernatant were taken for analysis.

UV SPECTRA OF THE UREA DERIVATIVE AND THE CYANAMIDE DERIVATIVE. The aliquots of six enriched microsomal incubation mixtures (see above) were analyzed on a high performance chromatograph (Waters 600 multisolvent delivery system, Waters Associates) coupled to a diode array detector (Waters 9919). The analytical conditions were as described above. The UV spectrum of the metabolite with a retention time of 7.9 min was identical to the spectrum of the synthesized urea derivative of debrisoquine, and the metabolite with a retention time of 23.6 min showed a UV spectrum identical to that of the synthesized cyanamide derivative.

RESULTS

Qualitative and Quantitative Analysis of the Urea and Cyanamide Derivatives of Debrisoquine

QUALITATIVE AND QUANTITATIVE ANALYSIS. The incubation of *N*-hydroxydebrisoquine with microsomes and NADPH resulted not only in the urea derivative [14], but also the cyanamide derivative (Fig. 1). The evidence for its formation was obtained unambiguously by HPLC. Addition of the reference substrate to the incubation mixture gave rise to an increase in the area of the metabolic peak, even when the eluent was varied (data not shown). A representative HPLC chromatogram recorded after the incubation of *N*-hydroxydebrisoquine with microsomes from rats pretreated with dexamethasone is shown in Fig. 2. Complete UV spectra of both metabolites were recorded by using a diode array detector. They were identical to the spectra of the reference substances recorded under the same conditions. Quantitative analysis of the urea and cyanamide derivatives was achieved by HPLC. The cyanamide was very unstable and the *N*-hydroxydebrisoquine also easily hydrolyzed to form the urea derivative. In order to analyze the exact ratio of cyanamide to urea formed during the incubation procedure, lyophilization was necessary. Although the cyanamide was relatively stable in buffer solution (pH 7.4), it decomposed when treated with acetonitrile or trichloroacetic acid. In methanol, the cyanamide and the *N*-hydroxydebrisoquine were stable for at least 4 hr (data not shown). Only by this method was a reasonable recovery rate (85%) of the cyanamide obtained.

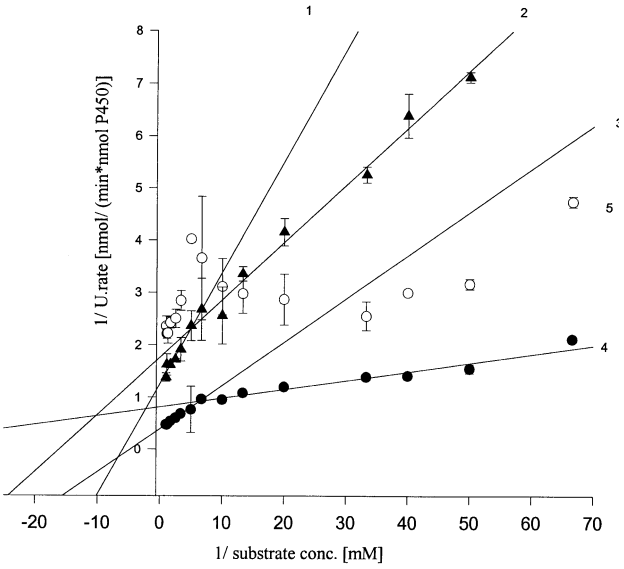
SUBCELLULAR DISTRIBUTION AND COFACTOR REQUIREMENTS. The reaction rates obtained under different conditions with rat enzymes are listed in Table 1. The formation of all metabolites was dependent on the presence of the enzyme source and the cosubstrate. A protein concentration of 1 μmol P450 in the incubation mixture proved to be optimal with regard to sample workup and analysis. Incubations with liver microsomes from rats which had been pretreated with dexamethasone showed a remarkable increase in reaction rates. Pretreatment with 3-methylcholanthrene and phenobarbital gave a lower increase in rates (data not shown). Enzyme preparations other than microsomes showed no marked enzyme activity (data not shown).

EFFECT OF SOD, CATALASE, AND ACTIVE OXYGEN SPECIES. Addition of SOD (IC_{50} less than 1 U/mL) lowered the occurrence of all metabolites including nitric oxide, but catalase had no effect. The addition of active oxygen species to incubation mixtures lacking cosubstrate led to minor amounts of the metabolites (Table 2).

TABLE 2. Oxidation of *N*-hydroxydebrisoquine by microsomal enzymes of rats pretreated with dexamethasone and influence of SOD and KO_2

Incubation mixture	Urea derivative nmol/min/ nmol P-450	Cyanamide derivative nmol/min/ nmol P-450
Complete/rat (dexamethasone-pretreated)	0.509 ± 0.052	1.883 ± 0.126
+SOD (500 U/mL)	0.047 ± 0.009	0.045 ± 0.009
+KO_2 (100 μM)/-NADPH	0.076 ± 0.006	0.074 ± 0.024

The rates ± SD were calculated from four different determinations with microsomes isolated from liver homogenates of 10 rats. All values statistically different from control (complete incubation mixture) with $P < 0.001$ (Student's *t*-test).



Km_1= 169 µM v_{max}= 0.797 nmol/ (min*nmol P450)
Km2= 62 µM v_{max}= 0.567 nmol/ (min*nmol P450)
Km_3= 267 µM v_{max}= 2.683 nmol/ (min*nmol P450)
Km_4= 20 µM v_{max}= 1.227 nmol/ (min*nmol P450)

FIG. 3. Lineweaver–Burk plot of the oxidation of *N*-hydroxydebrisoquine. Each point is the mean of four different determinations with microsomes isolated from liver homogenates of 10 rats ± SD. Formation of NO (1, 2 ▲), the cyanamide derivative (3, 4 ●), and the urea derivative (5 ○).

KINETICS OF THE OXIDATION OF N-HYDROXYDEBRISOQUINE. The reaction rate of the formation of the urea and cyanamide derivatives was linear up to 10 min. All experiments were performed with an incubation time of 20 min in order to obtain a sufficient amount of both metabolites. The formation of the cyanamide derivative showed a biphasic course in the Lineweaver–Burk plot. The kinetic parameters thus obtained are presented in Fig. 3. For the urea derivative, no clear kinetic data could be determined.

REACTION OF N-HYDROXYDEBRISOQUINE WITH NO. The incubation of *N*-hydroxydebrisoquine with SIN-1 as a spontaneous NO donor led to the formation of the urea derivative with just a small amount of the cyanamide derivative. Addition of SOD lowered the formation of the cyanamide derivative significantly (Table 3).

Qualitative and Quantitative Analysis of NO Formed by Microsomal Oxidation of N-*Hydroxydebrisoquine*

QUALITATIVE AND QUANTITATIVE ANALYSIS. The incubation of *N*-hydroxydebrisoquine with microsomes and NADPH led to the production of NO. The development of NO was monitored indirectly by the oxyhemoglobin assay.

SUBCELLULAR DISTRIBUTION AND COFACTOR REQUIREMENTS AND INFLUENCE OF ACTIVE OXYGEN SPECIES. The reaction rates obtained under different conditions with rat and rabbit enzymes are listed in Table 4. The formation of NO required the enzyme source and NADPH as cofactor. Experiments with microsomes of rats which had been pretreated with dexamethasone showed a 3-fold increase in reaction rate. Addition of SOD led to lower reaction rates, while in incubation mixtures lacking cofactor the addition of active oxygen species led to minor amounts of NO (Table 5).

TABLE 3. Reaction of N-hydroxydebrisoquine with SIN-1

Incubation mixture	Urea derivative nmol/incubation	Cyanamide derivative nmol/incubation
Complete	6.2 ± 0.40	1.16 ± 0.05
+SOD (500 U/mL)	9.02 ± 1.87	0.53 ± 0.11*

The rates ± SD were calculated from three different determinations.
* Statistically different from control (complete incubation mixture) with $P < 0.01$ (Student's *t*-test).

KINETICS OF THE FORMATION OF NO. The formation of NO obeyed Michaelis–Menten kinetics and showed a biphasic course (see Fig. 3).

DISCUSSION

Metabolism of N-*Hydroxydebrisoquine*

The microsomal incubation of *N*-hydroxydebrisoquine produced not only the urea but also the cyanamide derivative as the main metabolites. The formation of a cyanamide was unexpected since during previous studies, also with amidoximes, similar metabolites were not identified [5]. Very recently, oxidation of various *N*-hydroxyguanidines and amidoximes by rat liver microsomes was found to lead to the corresponding cyanamides and nitriles, respectively*. Moreover, investigations on the purely chemical transformation of other *N*-hydroxyguanidines showed that some agents also oxidize those compounds to cyanamides [15]. In the case of peracids, the urea derivative was described as being the main metabolite, with small amounts of cyanamide derivative also observed [16]. As pointed out earlier, the cyanamide easily hydrolyzes, especially in acid medium, to form the urea derivative. Therefore, the oxidation via peracids was expected to produce the urea derivative as the stable final product irrespective of what the product was initially.

Incubation mixtures with microsomes from rats pretreated with dexamethasone produced a 4-fold increase in the reaction rates of both metabolites compared with the results of control rats, demonstrating that the cytochrome P-450 isoenzymes 3A1 and 3A2 are mainly involved in this reaction (Table 1). The fact that the kinetics showed a biphasic course both for the formation of NO and of the cyanamide derivative indicates that two isoenzymes with different K_m values are possibly involved in the oxidation of *N*-hydroxydebrisoquine. On the other hand, the formation

* Jousserandot A, Boucher JL, Henry Y, Niklaus B, Clement B and Mansuy D, Manuscript submitted for publication.

TABLE 4. *In vitro* oxidation of *N*-hydroxydebrisoquine to NO by microsomal enzymes of rat liver and influence of NADPH and protein

Incubation mixture	NO nmol/min/ nmol P-450
Complete/rat	0.747 ± 0.088
−NADPH	0.012 ± 0.007*
−protein	0.007 ± 0.006*

The rates ± SD were calculated from three different determinations with microsomes isolated from liver homogenates of 10 rats.

* Statistically different from control (complete incubation mixture) with $P < 0.001$ or $+P < 0.01$ (Student's *t*-test).

of the urea derivative did not show clear kinetics (Fig. 3). Thus, it does not appear to be formed enzymatically, although both enzyme source and cofactor are required for its generation (Table 1). Moreover, the formation of the urea derivative was too rapid to be a purely chemical hydrolysis (data not shown), as was demonstrated by investigations with the cyanamide in buffer solution. Thus, a side reaction seems to be involved. Pharmacological studies by Zembowicz and co-workers [17–19] indicated an interaction of *N*-hydroxy-L-arginine with NO resulting in another potent, as yet unidentified, vasoactive species. They also found that the *N*-hydroxyguanidine function was responsible for the generation of this species and not *N*-hydroxy-L-arginine in particular [17]. Another study investigated the chemical interactions between NO and *N*-hydroxyguanidines [20]. The alkyl-substituted analog of *N*-hydroxyguanidine formed both the cyanamide and the urea derivative, the latter being the main metabolite apart from N_2O. Therefore, it is most likely that the urea derivative of debrisoquine is the product of a side reaction of *N*-hydroxydebrisoquine with the enzymatically formed NO. Several mechanisms are feasible for the oxidation of *N*-hydroxyguanidines by NO [20].

Active oxygen species also play an important role, since the addition of SOD to the incubation mixtures decreased the reaction rates. This was confirmed in experiments in which these oxygen species were added to incubation mixtures lacking cosubstrate (Tables 2 and 5). Superoxide anion efficiently performs the oxidative cleavage of C=N〰OH bonds [21]. Apparently, the oxidase activity of cytochrome P-450 plays a major role in the transformation of *N*-hydroxydebrisoquine, although some activity remained even in the presence of high SOD concentrations. This strongly suggests that both $O_2^{\cdot-}$ and its Fe(III) complex are able to interact with C=N(OH) bonds. As *N*-hydroxydebrisoquine seems to be badly positioned in the active site of cytochrome P-450, the reaction would appear to be too slow to be able to compete with the P-450 Fe(III)-$OO^{\cdot}$ decomposition and $O_2^{\cdot-}$ formation. *N*-Hydroxy-L-arginine is likely to be a high-affinity substrate for NOS, which explains why SOD has less influence on the reaction rate but a great effect on the transformation of *N*-hydroxy-L-arginine by cytochrome P-450 [5]. The mechanism of the formation of the cyanamide could be by two one-electron oxidations [15]. The electron acceptor should be mainly $O_2^{\cdot-}$. The involvement of oxaziridines has been proposed for the purely chemical oxidation of *N*-hydroxyguanidines [16].

TABLE 5. *In vitro* oxidation of *N*-hydroxydebrisoquine to NO by microsomal enzymes of rats pretreated with dexamethasone and influence of SOD and KO_2

Incubation mixture	NO nmol/min/ nmol P-450
Complete/rat (dexamethasone-pretreated)	0.747 ± 0.088
+SOD (500 U/mL)	0.397 ± 0.154*
+KO_2 (100 μM)/-NADPH	0.195 ± 0.01

The rates ± SD were calculated from three different determinations with microsomes isolated from liver homogenates of 10 rats.

* Statistically different from control (complete incubation mixture) with $P < 0.01$ (Student's *t*-test).

Reaction of N-Hydroxydebrisoquine with Nitric Oxide

To examine the influence of enzymatically formed NO on the formation of the metabolites, *N*-hydroxydebrisoquine was incubated with NO under aerobic conditions. SIN-1 was used as NO donor, since in aqueous solution it spontaneously decomposes to NO and superoxide anion [22]. It was again possible to detect the cyanamide and urea derivatives; the reaction of the unsubstituted *N*-hydroxyguanidine with NO gave similar results [20]. The fact that the urea derivative was the main metabolite and that SOD had a slight promoting effect on its production (Table 3) is remarkable, as it differs from the results with microsomes (Table 1). On the other hand, the addition of SOD decreased the formation of the cyanamide derivative. Therefore, if NO is provided directly, the formation of the urea derivative is independent of superoxide anion, whereas the generation of the cyanamide derivative is still dependent on it. The fact that SIN-1 decomposes to NO and $O_2^{\cdot-}$ is responsible for the rest activity of cyanamide formation. Addition of SOD to the incubation mixture of *N*-hydroxydebrisoquine and SIN-1 eliminates most of the produced superoxide anion and less cyanamide derivative is thus obtained. Incubation of the cyanamide derivative with SIN-1 with or without SOD showed no reaction, thereby excluding the incidence of side effects.

This work on the oxidation of *N*-hydroxydebrisoquine has demonstrated that NO and the cyanamide derivative are the main metabolites of the enzymatic reaction. On the other hand, the formation of the urea derivative is mainly due to a chemical side reaction of *N*-hydroxydebrisoquine with the generated NO. This finding explains the requirement of the enzyme source and cofactor for NO generation. Less NO is obtained than the cyanamide derivative because some NO interacts with the substrate to form the urea derivative. As a result, it seems likely that two moles of NO are needed to form one mole of urea derivative.

In conclusion, similarities (formation of a urea deriva-

tive) and differences (formation of a cyanamide derivative) between the physiological oxidation of *N*-hydroxyarginine by NOS and non-physiological *N*-hydroxyguanidines by cytochrome P-450 can be observed. A cyanamide (N^{δ}-cyanoornithine) is only formed from *N*-hydroxy-L-arginine by NOS when hydrogen peroxide is present instead of NADPH as was reported recently [23]. Further studies have also implicated the oxidative cleavages of C=N(OH) bonds in different classes of compounds with respect to the formation of structures with the CN-moiety*. It is also possible that the reactivity of NO with *N*-hydroxyguanidines is dependent on the structure of the *N*-hydroxylated compound. Furthermore, non-physiological *N*-hydroxyguanidines can be regarded as NO donors, as in this study the formation of NO was demonstrated by the oxyhemoglobin assay.

The financial support of the "Fonds der Chemischen Industrie" is gratefully acknowledged.

References

1. Moncada S, Palmer RM and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–142, 1991.
2. Feldman PL, Griffith OW and Stuehr DJ, The surprising life of nitric oxide. *Chem Eng News* **71:** 26–38, 1993.
3. Marletta MA, Nitric oxide synthase structure and mechanism. *J Biol Chem* **268:** 12231–12234, 1993.
4. Boucher JL, Genet A, Vadon S, Delaforge M, Henry Y and Mansuy D, Cytochrome P-450 catalyzes the oxidation of N^{ω}-hydroxy-L-arginine by NADPH and O_2 to nitric oxide and citrulline. *Biochem Biophys Res Commun* **187:** 880–886, 1992.
5. Mansuy D, Boucher JL and Clement B, On the mechanism of nitric oxide formation upon oxidative cleavage of C=N(OH) bonds by NO-synthases and cytochromes P-450. *Biochimie* **77:** 661–667, 1995.
6. Feelisch M and Noack EA, Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* **139:** 19–30, 1987.
7. Bamberger E and Dieckmann W, Über das Tetrahydrür des Isochinolins. *Ber Dtsch Chem Ges* **26:** 1205–1221, 1893.
8. Bailey DM, De Grazia CG, Lape HE, Frering R, Fort D and Skulan T, Hydroxyguanidines. A new class of antihypertensive reagents. *J Med Chem* **16:** 151–156, 1973.
9. Clement B and Kämpchen T, ^{15}N-NMR-Studien an 2-Hydroxyguanidinen und Amidoximen. *Chem Ber* **118:** 3481–3491, 1985.
10. Kremers P, Beaune P, Cresteil T, Graeve JD, Columelli S, Leroux JP and Gielen JE, Cytochrome P-450 monooxygenase activities in human and rat liver microsomes. *Eur J Biochem* **118:** 599–606, 1981.
11. Clement B, Demesmaeker M and Linne S, Microsomal catalyzed *N*-hydroxylation of guanabenz and reduction of the *N*-hydroxylated metabolite: Characterization of the two reactions and genotoxic potential of guanoxabenz. *Chem Res Toxicol* **9:** 682–688, 1996.
12. Gornall AG, Bardawill CJ and David MM, Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177:** 751–766, 1949.
13. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239:** 2370–2378, 1964.
14. Clement B, Schultze Mosgau MH and Wohlers H, Cytochrome P-450-dependent *N*-hydroxylation of a guanidine (debrisoquine), microsomal catalysed reduction and further oxidation of the *N*-hydroxy-guanidine metabolite to the urea derivative. Similarity with the oxidation of arginine to citrulline and nitric oxide. *Biochem Pharmacol* **46:** 2249–2267, 1993.
15. Fukuto JM, Wallace GC, Hszieh R and Chaudhuri G, Chemical oxidation of *N*-hydroxyguanidine compounds. Release of nitric oxide, nitroxyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem Pharmacol* **43:** 607–613, 1992.
16. Fukuto JM, Stuehr DJ, Feldman PL, Bova MP and Wong P, Peracid oxidation of an *N*-hydroxyguanidine compound: A chemical model for the oxidation of N^{ω}-hydroxy-L-arginine by nitric oxide synthase. *J Med Chem* **36:** 2666–2670, 1993.
17. Zembowicz A, Chlopicki S, Radziszewski W, Vane JR and Gryglewski RJ, N^{ω}-hydroxy-L-arginine and hydroxyguanidine potentiate the biological activity of endothelium-derived relaxing factor released from the rabbit aorta. *Biochem Biophys Res Commun* **189:** 711–716, 1992.
18. Zembowicz A, Swierkosz TA, Southan GJ, Hecker M, Gryglewski RJ and Vane JR, Mechanisms of the endothelium-dependent relaxation induced by N^{ω}-hydroxy-L-arginine. *J Cardiovasc Pharmacol* **20** (Suppl 12): S57–S59, 1992.
19. Zembowicz A, Hecker M, Macarthur H, Sessa WC and Vane JR, Nitric oxide and another potent vasodilator are formed from N^{ω}-hydroxy-L-arginine by cultured endothelial cells. *Proc Natl Acad Sci USA* **88:** 11172–11176, 1991.
20. Yoo J and Fukuto JM, Oxidation of *N*-hydroxyguanidine by nitric oxide and the possible generation of vasoactive species. *Biochem Pharmacol* **50:** 1995–2000, 1995.
21. Sennequier N, Boucher JL, Battioni P and Mansuy D, Superoxide anion efficiently performs the oxidative cleavage of C = NOH bonds of amidoximes and *N*-hydroxyguanidines with formation of nitrogen oxides. *Tetrahedron Lett* **36:** 6059–6062, 1995.
22. Feelisch M, Ostrowski J and Noack E, On the mechanism of NO release from sydnonimines. *J Cardiovasc Pharmacol* **14** (Suppl 11): S13–S22, 1989.
23. Clague MJ, Wishnok JS and Marletta MA, Formation of N^{δ}-cyanoornithine from N^{ω}-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: Implications for mechanism. *Biochemistry* **36:** 14465–14473, 1997.

* Jousserandot A, Boucher JL, Henry Y, Niklaus B, Clement B and Mansuy D, Manuscript submitted for publication.