Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 452-458

FORMATION OF NITRIC OXIDE BY CYTOCHROME P450-CATALYZED OXIDATION OF AROMATIC AMIDOXIMES

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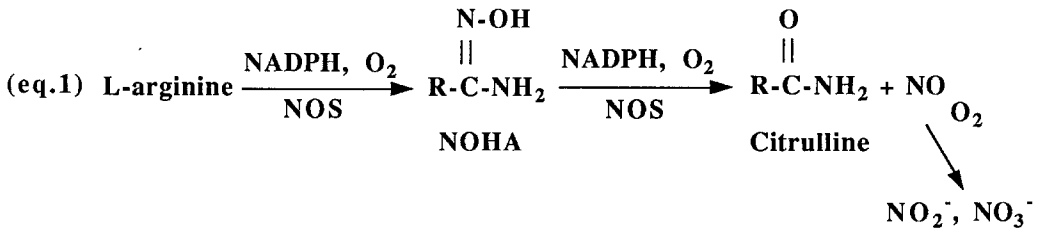
Received April 10, 1992

ABSTRACT : Rat liver microsomes catalyze the oxidation of para-hexyloxy-benzamidoxime **1** to the corresponding arylamide **2** and NO_2^-, by NADPH and O_2. Involvement of cytochromes P450 as catalysts of this reaction was shown by the strong inhibitory effects of CO and miconazole and the spectacular increase of the activity upon treatment of rats with dexamethasone, a specific inducer of cytochromes P450 of the 3A subfamily. Formation of NO during oxidation of **1** was shown by detection of the formation of cytochrome P450- and cytochrome P420-Fe(II)-NO complexes by visible and EPR spectroscopy. The formation of these complexes should be responsible, at least in part, for the fast decrease of the rate of microsomal oxidation of **1** with time. These results suggest that exogenous compounds containing amidine or amidoxime functions could act as precursors of NO *in vivo* after *in situ* oxidation by cytochromes P450.

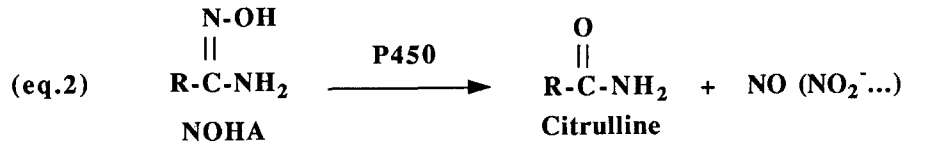
INTRODUCTION : Nitric oxide, NO, has been found recently to play very important functions in the physiology of cardiovascular, immune and nervous systems (1-3). Biosynthesis of NO involves an oxidation of L-arginine by NADPH and O_2 catalyzed by particular monooxygenases called NO-synthases (NOS) (4), with the intermediate formation of N^{ω}-hydroxy-L-arginine (NOHA) (eq.1) (5).



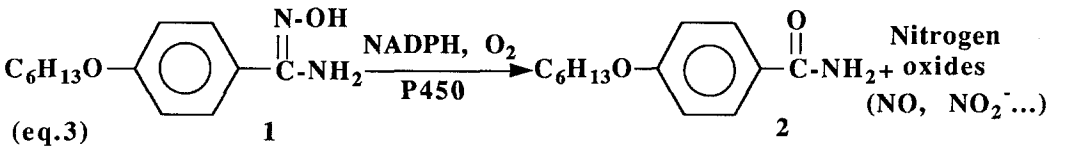
Abbreviations : NOHA : N^{ω}- hydroxy-L-arginine ; NOS : nitric oxide synthase ; DEX : dexamethasone ; 3MC : 3-methylcholanthrene ; βNF : β-naphthoflavone ; PB : phenobarbital ; CLO : clofibrate.

0006-291X/92 $4.00

Recently, it has been shown that microsomal liver cytochromes P450 are able to catalyze the second step of eq.1, the oxidative cleavage of a C-N bond of NOHA with formation of citrulline and nitrogen oxides (NO, NO_2^-...) (eq.2) (6).



Because of the important biological effects of NO, it seems interesting to know whether stable exogenous molecules could act as precursors of NO in mammals after an *in situ* oxidation catalyzed by endogenous enzymes. Since not only NO-synthases (5) but also ubiquitous cytochromes P450 (6) are able to catalyze the oxidative cleavage of a C-N bond of NOHA with formation of NO (and other nitrogen oxides) and citrulline, it was tempting to know whether compounds containing an amidoxime function, analogous to the N-hydroxy-guanidine function of NOHA, were also oxidized by cytochromes P450 with an oxidative cleavage of a C-N bond and formation of nitrogen oxides. This communication describes a new reaction of liver microsomal cytochromes P450, the oxidation of the arylamidoxime, **1**, with formation of NO (and other nitrogen oxides) and the corresponding arylamide **2** (eq.3), under physiological conditions (liver microsomes with NADPH and O_2). It also shows that liver microsomal cytochromes P450 from rats pretreated by dexamethasone are the most efficient catalysts for this reaction.



MATERIALS AND METHODS

Chemicals. NADPH was purchased from Boehringer. β-Naphthoflavone, 3-methylcholanthrene, miconazole, 4-hydroxy-benzonitrile, 4-hydroxy-benzoic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride and dexamethasone were obtained from Janssen. Other compounds were purchased from sources as indicated : clofibrate (Fluka), phenobarbital (Merck), PICB7 (Waters-Millipore). All solvents and other reagents were of the highest purity commercially available.

Spectroscopic measurements.

NMR spectra were recorded on a Bruker 250 MHz NMR spectrometer. The samples were dissolved in $CDCl_3$ and the chemical shifts are reported in ppm relative to tetramethylsilane as an internal standard. The coupling constants (J) are reported in Hz and multiplicity of the signals is noted : s = singlet, d = doublet, t = triplet, q = quadruplet and m = multiplet. Infra-Red (IR) spectra were recorded on a Perkin Elmer 783 spectrophotometer and the characteristic absorption frequencies are reported in cm^{-1}. Melting points (m.p.) were determined on a Büchi apparatus using capiliary tubes and are uncorrected. Mass spectra (MS) were recorded on a Riber-Mag R10.10C spectrometer operating (at an ionising potential of 70eV) on the electron impact mode. UV-Visible spectroscopy was performed at 37°C using a Kontron 940 spectrophotometer. Detection of P420-Fe(II)-NO formation was made by addition of O_2

to an anaerobic suspension of rat liver microsomes (2μM P450) containing 200 μM **1** and 1 mM NADPH. EPR spectroscopy was recorded at 77 K using a Varian E109 spectrometer. Microwave power was 10 mW, klystron frequency 9.18 GHz, modulation amplitude 10G and time constant 0.5s. Detection of P420-Fe(II)-NO was made by addition of O_2 to an anaerobic suspension of rat liver microsomes (75 μM P450) containing 100 μM **1** and 5 mM NADPH.

Synthesis of the arylamidoxime 1 and amide 2.

Compound **1** was prepared from (4-cyano-phenoxy)-1-hexane by a classical method described for the synthesis of arylamidoximes (7). Compound **2** was obtained from 4-hydroxy-benzoic acid using classical steps. The detailed synthesis of **1** and **2** will be reported elsewhere. Their following characteristics are in complete agreement with the proposed structure : Compound **1 : m.p.** 112°C ; **^{1}H NMR** : 7.55 (d, 2H, J=8) ; 6.90 (d, 2H, J=8) ; 4.80 (s, 3H) ; 3.98 (t, 2H, J=7.5) ; 1.78 (m, 2H) ; 1.45 and 1.35 (m, 6H) ; 0.90 (t, 3H, J=7.5) ; **IR** : 3450, 3250, 3060, 1650, 830 ; **MS** : 236 (M^+) ; 219 ; 152 ; 135 (100%) and 120. Compound **2 : m.p.** : 154°C ; **^{1}H NMR** : 7.78 (d, 2H, J=8) ; 6.92 (d, 2H, J=8) ; 5.78 (m, large, 2H) ; 4.0 (t, 2H, J=7) ; 1.79 (m, 2H) ; 1.45 and 1.32 (m, 6H) ; 0.92 (t, 3H, J=7.5) ; **MS** : 221 (M^+), 137, 121 (100%).

These two compounds gave satisfactory elemental analyses (C, H, N).

Preparation of rat liver microsomes : Male Sprague-Dawley rats (200-250g) we provided laboratory chow and water ad libitum. After 10 days of adaptation, animals were treated either with 3MC (20 mg/kg, in corn oil, i.p. for 4 days), βNF (50 mg/kg, in corn oil, i.p. for 4 days), PB (80 mg/kg, in 0.9% saline, i.p. for 4 days), DEX (50 mg/kg, in corn oil, i.p. for 4 days) or CLO (500 mg/kg, in corn oil, i.p. for 4 days). The control animals were treated with corn oil (0.5 ml). Microsomes were prepared as reported (8) and stored at -80°C until use. Protein concentrations were determined by the method of Lowry (9) with bovine serum albumine as standard. Cytochrome P-450 contents were determined as described by Omura and Sato (10).

Incubation procedures : The standard incubation mixtures (total volume : 0.5 m contained the following components : 100 nmol of substrate **1** and 0.5 mg of microsomal proteins in 0.1 M phosphate buffer, pH 7.4. After preincubation 2 min at 37°C, the reaction was started by addition of 500 nmol NADPH. A 10 min incubation time was routinely employed. Incubations were stopped by mixing thoroughly with 0.5 ml acetonitrile and the proteins were separated by centrifugation at 3000 rpm for 20 min.

Determination of NO_2^- : Nitrite ion formation was measured spectrophotometrically as previously described (11). Aliquots (0.25 ml) of the above supernatants were mixed with 0.25 ml of 1% sulfanilamide in 0.4N HCl and with 0.25 ml of 0.1% N-(1-naphthyl)ethylenediamine in 0.4N HCl and their absorption measured at 543 nm. Quantitations of NO_2^- formation were determined from standard curves which were run in the same way as the experimental samples.

HPLC analysis of the incubates : Aliquots (0.25 ml) of the incubation supernatants wei mixed with 0.25 ml of the mobile phase and separations of **1** and **2** were obtained on a 250 x 4.6 mm column of ODS silica (5 μm, Nucleosil C_8, Société Française Chromato Colonne-Shandon, France). The mobile phase was a mixture of water and acetonitrile (550 : 450) containing 4 mM sodium heptanesulfonate (PIC B7) and 5 mM phosphoric acid. Flow rate was 1 ml/min and the UV absorbance was monitored at 254 nm. The retention times were 8 min for **1** and 17 min for **2**. Quantitations were done by comparison with calibration curves obtained by introducing known amounts (in the range between 0.1 nmol and 200 nmol) of authentic samples into the usual incubation mixture at 0°C and treating the resulting mixture as an experimental sample. Molar absorption coefficients were 11500 and 18500 $mol^{-1}cm^{-1}$ at 254 nm for **1** and **2** respectively.

Identification of the metabolite, isolated from a preparative incubation (100ml) of **1** (200μM) and NADPH (1mM) with microsomal proteins (1mg per ml), to an authentic sample of **2** was done by comparison of their HPLC retention times and their ^{1}H NMR spectra.

RESULTS

Amidoxime **1** was prepared from para-hexyloxy-benzonitrile by a method previously described for other arylamidoximes (7). Incubation of 200µM **1** with liver microsomes from DEX-pretreated rats in the presence of 1mM NADPH led to the formation of the arylamide **2** which was detected by HPLC. Identification of this metabolite to an authentic sample of **2** was done by comparison of their HPLC retention times and ^1H NMR spectra (see Materials and Methods). Formation of **2** was linear for only 3 min with a rate of about 4 nmol of **2** formed per mg protein per min (Fig.1). The reaction was clearly enzymatic as boiled microsomes were inactive (Table 1). It required both NADPH and O_2 as incubations in the absence of NADPH or under anaerobic conditions failed to give any formation of **2**. These microsomal incubations of **1** with NADPH and O_2 led to concomitant formation of **2** and NO_2^- (Fig.1) which was detected by a reported spectrophotometric method (11). The NO_2^-/**2** molar ratio was inferior to 1 but we did not try to detect the formation of other stable nitrogen oxides like NO_3^- which is usually formed with NO_2^- in NO-synthase-catalyzed reactions (2). Formation of NO_2^- was also suppressed in incubations in the absence of O_2 or NADPH or in the presence of boiled microsomes. Good inhibitors of microsomal cytochromes P450, like CO and miconazole (10µM) (12), markedly inhibited microsomal oxidation of **1** to **2**, and 50µM miconazole led to an almost complete inhibition of this reaction. Implication of cytochromes P450 in this reaction was further supported by results obtained with microsomes from rats pretreated with various cytochrome P450 inducers (Fig.2). Pretreatment of rats with 3MC or βNF, PB and CLO, which are typical inducers of the P450 families 1A, 2B and 4B (13) respectively, only led to small variations of the activity of control microsomes. On the contrary, pretreatment of rats by DEX, an inducer of the P450 3A subfamily (13), led to a spectacular 10-fold increase of this activity.

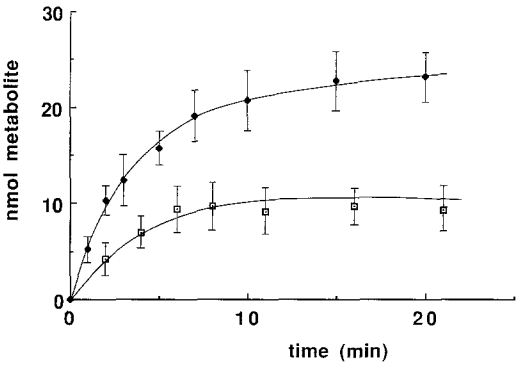


Fig.1 . Time course formation of 2 and NO_2^- by oxidation of 1 by rat liver microsomes, NADPH and O_2.

nmol **2** (◆) and nmol NO_2^- (⊡) formed per mg protein (conditions : 200 µM **1**, 1 mM NADPH and 1 mg liver microsomal protein from rats treated by DEX (2 nmol P450 per mg protein), in phosphate buffer pH 7.4 at 37°C ; mean values ± SD from three experiments).

Table 1 . Effects of various factors on the oxidation of 1 to 2 by rat liver microsomes

Incubation mixture	Activity (a)	Activity (b)
Complete system(c)	18.1	(100)
" - O_2(d)	< 0.1	(0)
" - NADPH	< 0.1	(0)
" + Miconazole (10 mM)	4.7	(48)
" + Miconazole (50 mM)	1.4	(8)
" +CO (CO : O_2 = 1 : 1)	5.5	(30)
" using boiled microsomes(e)	< 0.1	(0)

(a) nmol **2** formed per mg protein per 10 min mean values ± 15% from 3 independent determinations. (b) % activity relative to that of the complete system. (c) incubations of 200 µM **1** in phosphate buffer pH 7.4 containing about 1 mg liver microsomal proteins (1.8 nmol P450.mg protein^{-1}) from rats treated by dexamethasone. (d) complete system made anaerobic by argon bubbling. (e) conditions as in (a) but using microsomes heated at 100°C for 5 min.

In fact, a study of the interaction of **1** with cytochromes P450 in the various microsomes by difference visible spectroscopy showed that only microsomes from DEX-treated rats gave significant difference spectra characterized by a peak at 390 nm and a

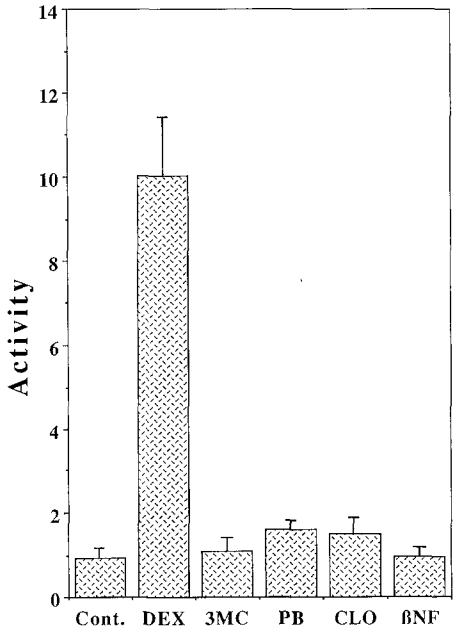


Fig.2 . Effects of treatment of rats by inducers on the microsomal oxidation of 1 to 2 by NADPH and O_2.

Activities in nmol **2** formed per nmol P450 per 10 min ; mean values ± SD from three experiments (P450 contents of the different microsomes : 1.1, 1.8, 1.8, 2.6, 1.7 and 1.6 nmol P450 per mg protein respectively for control rats and rats treated by DEX, 3MC, PB, CLO and βNF).

trough at 420 nm as expected for the formation of a P450-substrate complex, with an apparent dissociation constant of 50μM (data not shown). Addition of **1** to liver microsomes from DEX-treated rats in the presence of O_2 and NADPH led to the appearance , in difference visible spectroscopy, of a peak at 423nm. Similar experiments followed by EPR spectroscopy led to the appearance of a triplet centered at g = 2.011 (a_H = 17 gauss) which is characteristic of the cytochrome P420-Fe(II)-NO complex (14, 15). Almost identical spectra were obtained upon treatment of microsomes by 1mM isobutyl nitrate and 5mM cysteine, a system known to produce NO (16). As P450-Fe(II)-NO complexes are known to be easily transformed to P420-Fe(II)-NO complexes (14, 15), these results indicate that cytochrome P450-Fe(II)-NO complexes are formed transiently during microsomal oxidation of **1** and led eventually to P420-Fe(II)-NO complexes.

DISCUSSION

The aforementioned results describe a new reaction of microsomal cytochrome P450, the oxidative denitration of the amidoxime function with formation of the corresponding amide function and nitrogen oxides like NO and NO_2^-. Formally, this corresponds to the replacement of a C=N-OH function by a C=O function, a reaction which has been already observed in the cytochrome P450-dependent oxidation of N^{ω}-hydroxy-L-arginine to citrulline and nitrogen oxides (6). Formation of NO_2^- occurs at the same time as that of **2** (Fig.1) and formation of NO was shown by detection of cytochrome P420-Fe(II)-NO complexes by visible and EPR spectroscopy. The NO_2^- : **2** molar ratio, which was found clearly smaller than 1, is expected for such oxidative denitrations (2) since other nitrogen oxides like NO and NO_3^- should be also formed. Implication of cytochrome P450-dependent monooxygenases in the microsomal oxidation of **1** to **2** and nitrogen oxides, is clearly shown by the absolute requirement of NADPH and O_2 and the strong inhibitory effects of classical P450 inhibitors like CO and miconazole (Table 1). Interestingly, rat liver cytochromes P450 of the 3A subfamily, which are induced in rats treated with dexamethasone, exhibit a particularly high activity for oxidation of **1** (Fig.2). The rate of microsomal oxidation of **1** to **2** and nitrogen oxides rapidly decreases with time (Fig.1). This should be due to denaturating effects of nitrogen oxides on microsomal proteins and, more particularly, to the inactivation of cytochromes P450 by formation of P450-Fe(II)-NO and P420-Fe(II)-NO complexes after a certain number of turnovers.

This ability of microsomal cytochromes P450 to catalyze the oxidation of the arylamidoxime **1** with formation of **2** and nitrogen oxides suggests that **NO could be formed *in vivo* by oxidation of exogenous compounds containing an amidoxime or a related function.** In that respect, it is noteworthy that several drugs involving amidine or guanidine functions are known (17). Moreover, it has been shown that arylamidines are oxidized to the corresponding arylamidoximes by microsomal cytochromes P450 (18). Thus, drugs containing amidine (or related) functions could be oxidized *in vivo* by

microsomal cytochromes P450 with formation of NO in two steps. This NO formation could participate in the pharmacological or toxicological effects of these drugs.

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