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Reduction of Nilutamide by NO Synthases: Implications for the Adverse Effects of This Nitroaromatic Antiandrogen Drug

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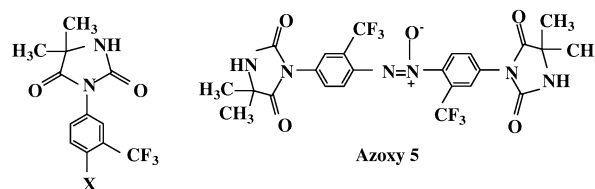
Received May 14, 2003

Nitric oxide synthases (NOSs) are flavohemoproteins that catalyze the oxidation of L-arginine to L-citrulline with formation of the widespread signal molecule NO. Beside their fundamental role in NO biosynthesis, these enzymes are also involved in the formation of reactive oxygen species and in the interactions with some xenobiotic compounds. Nilutamide is a nonsteroidal antiandrogen that behaves as a competitive antagonist of the androgen receptors and is proposed in the treatment of metastatic prostatic carcinoma. However, therapeutic effects of nilutamide are overshadowed by the occurrence of several adverse reactions mediated by toxic mechanism(s), which remain(s) poorly investigated. Here, we studied the interaction of NOSs with nilutamide. Our results show that the purified recombinant neuronal NOS reduced the nitroaromatic nilutamide to the corresponding hydroxylamine. The reduction of nilutamide catalyzed by neuronal NOS proceeded with intermediate formation of a nitro anion free radical easily observed by EPR, was insensitive to the addition of the usual heme ligands and L-arginine analogues, but strongly inhibited by O₂ and a flavin/NADPH binding inhibitor. Involvement of the reductase domain of nNOS in the reduction of nilutamide was confirmed by (i) the ability of the isolated reductase domain of nNOS to catalyze the reaction and (ii) the stimulating effect of Ca²⁺/calmodulin on the accumulation of hydroxylamine and nitro anion radical. In a similar manner, the recombinant inducible and endothelial NOS isoforms also displayed nitroreductase activity, albeit with lower yields. The selective reduction of nilutamide to its hydroxylamino derivative by the NOSs could explain some of the toxic effects of this drug.

Introduction

Nilutamide, **1** (Figure 1), is a nonsteroidal antiandrogen derivative that behaves as a competitive antagonist of the androgen receptors. This nitroaromatic compound is used in the treatment of metastatic prostatic carcinoma (1–3). After oral administration in humans, nilutamide is extensively metabolized in the liver, undergoing mainly reduction of its nitro group to the primary amine, **2** (RU 43866; Figure 1). In addition, nilutamide and the amine **2** also undergo hydroxylations of the aromatic ring and of the methyl groups of the heterocycle (4, 5).

The therapeutic effects of nilutamide are overshadowed by the occurrence of several adverse reactions. The most serious complications include drug-induced hepatitis and infiltrative lung disease. The absence of clear evidence for hypersensitivity manifestations suggests that nilutamide hepatitis is mediated by toxic mechanism(s).



- | | | |
|---|---------------------|---------------|
| 1 | X = NO ₂ | Nilutamide |
| 2 | X = NH ₂ | Amine |
| 3 | X = NHOH | Hydroxylamine |
| 4 | X = NO | Nitroso |

Figure 1. Structure of nilutamide **1** and compounds **2–5**.

However, these remain poorly known (6–8). Previous studies have shown that nilutamide inhibits human liver cytochromes P450 and may interfere with the oxidative metabolism of other drugs (9). Nilutamide inhibits the mitochondrial respiratory chain and ATP formation (10) and generates reactive oxygen species and free radicals, which may result in oxidative stress (11–13). Adverse effects of nilutamide are reminiscent of those observed with other nitroaromatic compounds, and the proposed mechanisms of toxicity involve initial one electron reduction of the nitro group to form the nitro anion radical (14). According to the dioxygen tension in the surrounding environment, the nitro anion radical may undergo

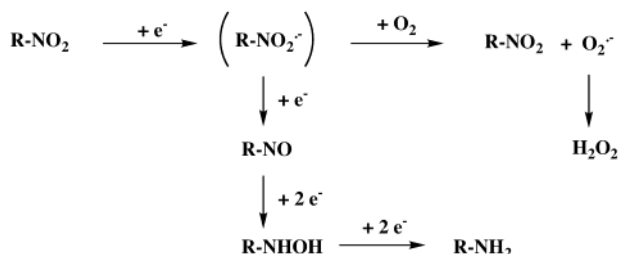
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Scheme 1. Possible Mechanisms for the Reduction of Nitroaromatic Compounds

two reactions: under anaerobic conditions, a further one electron reduction leads to the nitroso derivative, which can be further reduced by two electrons to the hydroxylamine, and, again by two electrons, to the corresponding amine (Scheme 1). The nitroso and the hydroxylamine derivatives are reactive species that can covalently bind to glutathione and/or alkylate nucleophilic sites on proteins and nucleic acids (15–17). Under aerobic conditions, molecular oxygen oxidizes the nitro anion radical, resulting in a redox cycle with regeneration of the nitro compound and production of superoxide anion, whose dismutation yields hydrogen peroxide (11–14). Therefore, the reduction of some nitroaromatic compounds can lead to the formation of reactive intermediates (nitroso and hydroxylamine) and/or potentially toxic reactive oxygen species (Scheme 1).

Recent data have indicated that nilutamide was reduced in the cytosol of rat lung. This reduction was oxygen sensitive, required the cofactors NADPH and FAD/FMN, and was induced by lipopolysaccharides (18). These results led us to hypothesize that NOSs¹ could be involved in the metabolism and the toxic effects of nilutamide.

NOSs are hemoproteins that catalyze the conversion of L-arginine to citrulline and nitric oxide. NO is a recently discovered mediator in mammals that plays key roles in a variety of physiological processes such as neurotransmission, vasorelaxation, platelet aggregation, and immune responses (19, 20). NOSs are homodimeric enzymes that consist of an NH₂-terminal oxygenase domain, which contains binding sites for the heme prosthetic group and the cofactor BH₄, and a CO₂H-terminal reductase domain containing binding sites for flavins, FMN, and FAD, and cofactor NADPH. These two domains are fused by a Ca²⁺-dependent CaM-binding sequence (21–23). CaM binding to NOSs activates both intra- and interdomain electron transfers and is required for maximal NO-forming activity (24). The reductase domain has a close homology to NADPH-cytochrome P450 reductase (25) and is able to catalyze the reduction of exogenous electron acceptors such as K₃FeCN₆, ferric cyt c, azo-dye methyl-red (26), and quinones including the antitumor quinones adriamycin and daunorubicin (27–30).

In the present study, we have investigated the reductive metabolism of nilutamide by purified recombinant

NOSs. We found evidence that this antiandrogen is reduced anaerobically by the three NOS isoforms with selective formation of its hydroxylamine and minor formation of the amine. This reduction occurs with formation of an intermediate nitro anion radical easily detected by EPR spectroscopy. This nitroreductive metabolism could explain some of the toxic effects observed with nilutamide in human therapeutics.

Materials and Methods

Chemicals. Nilutamide **1**, NO₂-Arg, SEITU, and DPI came from Sigma. BH₄ came from Alexis (Coger, France). NADPH, NADH, and NADP⁺ were purchased from Boehringer. Amine **2** (RU 43866; Figure 1) was a gift from Aventis (Romainville, France). All other chemicals were purchased from Aldrich and Acros and were of the highest grade commercially available. Chemical reactions were monitored by TLC using Merck pre-coated silica gel 60F₂₅₄ (0.25 mm thickness) plates. Millipore SA silica gel 60 (35–70MY) was used for flash chromatography. All NMR experiments (¹H, ¹³C, HMQC, and HMBC) were carried out at room temperature on a Bruker ARX 250 MHz spectrometer. Chemical shifts are reported in ppm (δ) relative to TMS, and coupling constants (*J*) are reported in Hz. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer, and wavenumbers are reported in cm^{−1}. Melting points were determined on a Kofler apparatus and are uncorrected. Elemental analyses were performed at Service de Microanalyse, Pierre and Marie Curie University, Paris. Mass spectra were recorded at Ecole Normale Supérieure, Paris, on a RiberMag system with FAB and high-resolution capabilities.

Synthesis of 3-(4-Hydroxyamino-3-trifluoromethyl-phenyl)-5,5-dimethylimidazolidine-2,4-dione (3). Nilutamide was selectively reduced to hydroxylamine **3** by hydrogen using platinum oxide poisoned with triethyl phosphite, following a previously described protocol (31). Nilutamide (160 mg, 0.5 mmol) was dissolved in 2.5 mL of absolute ethanol containing 0.05 mL of P(OEt)₃ and 5 mg of PtO₂. The reaction mixture was placed under hydrogen atmosphere at room temperature until hydrogen uptake had ceased (about 5 h). The solution was filtered and concentrated under vacuum, and the crude product was purified by preparative TLC (CH₂Cl₂/acetone, 9:1, v/v) giving pure hydroxylamine **3** as a white solid (96 mg, 63%); mp 184–185 °C. IR (KBr): 3428, 3303, 3225, 1770, 1710, 830. ¹H NMR (*d*₆-Me₂SO): 8.78 (bs, 1H), 8.60 (bs, 1H), 8.49 (bs, 1H), 7.48–7.38 (m, 3H), 1.38 (s, 6H). ¹H NMR (D₂O): 7.44 (m, 3H), 1.36 (s, 6H). ¹³C NMR (*d*₆-Me₂SO): 176.7 (CO), 154.5 (N–CO–N), 148.6 (q, C₄, ³J_{CF} = 1.5), 132.1 (C₆), 124.8 (q, C₂, ³J_{CF} = 5.7), 124.1 (q, CF₃, ¹J_{CF} = 272.0), 123.0 (C₁), 114.2 (C₅), 111.8 (q, C₃, ²J_{CF} = 31.0), 57.9 (C(CH₃)₃), 24.8 (CH₃). MS (ESI, negative mode): *m/z* 302, 284, 241, 221. Anal calcd (C₁₂H₁₂F₃N₃O₃): C, 47.53; H, 3.99; N, 13.86. Found: C, 47.31; H, 4.21; N, 13.81. UV/vis (H₂O/CH₃CN 1:1): λ_{max} 249 nm, ε₂₄₉ = 18 400 M^{−1} cm^{−1}.

Synthesis of Azoxy Compound 5. Following a previously described method (31), air oxidation of hydroxylamine **3** in the presence of NaHCO₃ gave the azoxy-compound **5**. Hydroxylamine **3** (20 mg) was dissolved in a water:acetone (1:2 mL) mixture, and a saturated solution of NaHCO₃ was added until the pH reached 8. Air was bubbled through the solution for 2.5 h. During this time, the product was separated. After this time, the product was filtered. The product **5** (15 mg, 75%) was a yellow solid; mp > 265 °C. IR (KBr): 3230, 3110, 1786, 1732, 1400, 1287. ¹H NMR (*d*₆-Me₂SO): 8.73 (br s, 2H), 8.26 (d, 1H, *J* = 8.7), 8.16 (m, 2H), 8.06 (m, 2H), 7.94 (d, 1H, *J* = 8.7), 1.43 (s, 12 H). MS (ESI, negative mode): *m/z* 585, 474, 430, 403. MS (FAB, positive mode) 587.2, 571.2. High-resolution MS calcd for C₂₄H₂₁F₆N₆O₅, 587.1478; found, 587.1485. UV/vis (H₂O/CH₃CN 1:1): λ_{max} 316 nm, ε₃₁₆ = 12 400 M^{−1} cm^{−1}.

Preparation of Recombinant NOSs. Recombinant full length rat nNOS was isolated and purified from *Escherichia coli*

¹ Abbreviations: BH₄, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolidine-1-oxyl 3-oxide; cyt c, cytochrome c; DNB, dinitrobenzene; DPI, diphenyliodonium chloride; ImH, imidazole; NOS, nitric oxide synthase; nNOS, full length neuronal NOS; nNOS_{red}, reductase domain of nNOS; nNOS_{oxy}, oxygenase domain of nNOS; eNOS, endothelial NOS; iNOS, inducible NOS; NO₂-Arg, N^ω-nitro-L-arginine; SEITU, S-ethyl-*iso*-thiourea; ThioCit, thio-L-citrulline.

transformed with plasmid pCWNOS I containing rat brain NOS cDNA. The BL21(DE3) *E. coli* strain {F⁻, *ompT*, *hscS*_{B(r_B⁻m_B⁻)-gal dcm} was used as the host strain. Plasmid pCWori was kindly provided by Dr. M. Waterman (Vanderbilt University, Nashville, TN), and plasmid nNOS-V60 was described previously (32). A 305 bp DNA fragment was amplified from the ATG start codon to the *Apa* I restriction site of nNOS-V60 using a forward primer 5' TCAGCGCATATGGAAGAGAACACGTTT bearing the *Nde* I restriction site and a reverse primer 5' CCAGGAGTAAGACTCCCCGGACT bearing the *Apa* I restriction site. Twenty ng of nNOS-V60 was amplified by PCR (33) using 2.5 units of Taq polymerase on a PCR2400 thermocycler. The reaction was incubated at 95 °C for 5 min, followed by 30 cycles: 1 min at 95 °C, 30 s at 64 °C, and 1 min at 72 °C. The PCR fragment was digested by *Apa* I and *Nde* I and was coligated to the *Nde* I *Xba* I digested pCWori plasmid with the 4970 bp nNOS-V60 *Apa* I *Xba* I digested fragment. The ligation mixture was used to transform BL21(DE3) cells, and positive transformants were analyzed by the dideoxy chain terminating method (34). Growing of transformed *E. coli* and purification of full length nNOS were performed following previously described protocols (35).}

Recombinant full length murine iNOS, bovine eNOS, and the oxygenase (nNOS_{oxy}) and reductase (nNOS_{red}) domains of nNOS were overexpressed in *E. coli* and purified as previously described (36–38). Protein contents were measured using the Bradford reagent from Biorad and bovine serum albumin as a standard (39). They were more than 95% pure as judged by SDS–PAGE.

Anaerobic Incubations of Nilutamide in the Presence of NOSs. Usual incubations were performed in glass tubes previously purged with argon and stopped with a rubber septum. All of the compounds and buffer were cautiously degassed by bubbling argon for 30 min at 4 °C. Typical incubation mixtures (final volume, 500 μ L) contained 500 μ M nilutamide, 10 mM CaCl₂, 100 μ M BH₄, 10 μ g/mL CaM, and 30–300 μ g of NOS in 50 mM Hepes (pH 7.4). The reactions were started by the addition of NADPH (1 mM final concentration), shaken at 37 °C for 30 min, and quenched by the addition of 100 μ L of cold methanol containing 100 μ g/mL androstenedione (internal standard). The tubes were placed on ice and 2-fold extracted with 1 mL of CH₂Cl₂. The organic phases were dried over MgSO₄, filtered, and evaporated to dryness under nitrogen. The residues were redissolved in 1 mL of methanol, and 25 μ L aliquots were injected onto the HPLC system. Incubations in the presence of iNOS were performed similarly, but CaCl₂ and CaM were omitted. Yields for the recovery of nilutamide **1**, amine **2**, and hydroxylamine **3** following extraction by CH₂Cl₂ from the incubation mixtures were 95 \pm 5, 85 \pm 5, and 80 \pm 5%, respectively.

Reverse Phase HPLC Separation of Metabolites from NOS-Catalyzed Reduction of Nilutamide. Separations of metabolites from nilutamide were performed at room temperature on a 250 mm \times 4.6 mm Hypersil MOS column set on a Thermo-Finnigan HPLC system. The mobile phase was a mixture of acetonitrile and methanol in water (40:55, v/v) run isocratically at a flow rate of 1 mL/min. The absorbance was monitored at 250 nm and recorded with the Borwin data acquisition system. Calibration curves were made from mixtures containing various concentrations of nilutamide **1**, amine **2**, hydroxylamine **3**, and azoxy-compound **5**, in the presence of androstenedione. For further identification of metabolites, the HPLC system was connected to an Esquire-LC mass spectrometer (Bruker, Bremen, Germany) fitted with an ESI source. Detection was done using both the negative and the positive modes. The MS parameters were optimized by flow injection at a rate of 800 μ L/h. All spectra were recorded in full scan mode (*m/z* range 50–500), and maximal accumulation time was set at 200 ms.

NOS Activities Assays. [³H]L-Citrulline Formation. NOS-dependent oxidation of L-arginine to L-citrulline was determined according to a previously described protocol (40). Briefly,

enzymatic reactions were conducted at 37 °C for 5 min in 50 mM Hepes (pH 7.4) containing 5 mM DTT, 1 mM NADPH, 1 mM CaCl₂, 10 μ g/mL CaM, 20 μ M BH₄, 4 μ M FAD, 4 μ M FMN, about 500 000 cpm [2,3,4,5-³H]L-arginine, and 10 μ M L-arginine. Final incubation volumes were 100 μ L. Nilutamide was commonly added to the incubation mixtures as 5 μ L portions dissolved in Me₂SO; control incubations contained similar amounts (5%) of Me₂SO without nilutamide. The reactions were started by the addition of protein and terminated by the addition of 500 μ L of cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA). Aliquots (500 μ L) were applied to glass columns containing 1 mL of Dowex AG 50W-X8 (Na⁺ form), preequilibrated with stop buffer, and a total of 1.5 mL of stop buffer was added to eluate [³H]L-citrulline. Aliquots were mixed with Pico-Fluor 40 and counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer. Control samples without NOS or NADPH were included for background determinations.

NADPH Consumption by nNOS. NADPH consumption by nNOS was followed at 37 °C on a Kontron 940 spectrophotometer by monitoring the decrease in absorbance at 340 nm and quantitated using an extinction coefficient of 6.2 mM⁻¹ cm⁻¹. Cuvettes containing 200 μ M NADPH, 10 mM CaCl₂, 10 μ g/mL CaM, 100 μ M BH₄, and various amounts of nilutamide in 150 μ L of Hepes buffer were allowed to stay at 37 °C for 2 min prior the addition of 10–20 μ g of nNOS to the sample cuvette.

Cyt c Reductase Activity of nNOS. The initial rates of flavin-dependent reduction of cyt c by nNOS were quantitated spectrophotometrically at 550 nm, using an extinction coefficient of 21 mM⁻¹ cm⁻¹. The reaction mixtures contained 10 mM CaCl₂, 200 μ M NADPH, and 50 μ M cyt c in Hepes buffer (without DTT). When required, assays contained 10 μ g/mL CaM and various amounts of nilutamide. The reactions were run at 37 °C and were initiated by the addition of 1–2 μ g of nNOS.

EPR Experiments. Room temperature EPR experiments were performed on mixtures containing 200–300 μ g/mL nNOS, 2 mM nilutamide, 10 mM CaCl₂, 10 μ g/mL CaM, 100 μ M BH₄, and 1 mM NADPH in argon-purged 50 mM Hepes buffer (pH 7.4). The anaerobic reaction mixture was transferred via a Teflon capillary tube to a Bruker Aqua-X cell inserted in a shq 0011 cavity (Bruker). EPR spectra were recorded on a Elexsys E 500 EPR spectrometer using the following instrument settings: microwave frequency, 9.82 GHz; field modulation frequency, 100 kHz; sampling time, 0.16 s; field sweep, 7 mT; field modulation amplitude, 0.32 or 0.032 mT; number of scans, 4–20; and microwave power, 20 mW. To estimate the concentration of the radical anion derived from nilutamide, we used the stable water soluble nitronyl-nitroxide radical CPTIO (41). The EPR spectra of known amounts of CPTIO were recorded under identical nonsaturating conditions, and quantitation of the spectra was performed by double integration. Simulations were performed with the XSophe software (Bruker).

Results

Metabolism of Nilutamide by nNOS. Preliminary experiments had shown that nilutamide was not transformed in incubations run in the presence of purified nNOS containing all of its cofactors, under normal atmosphere. However, when nNOS was incubated anaerobically with nilutamide, two new peaks were detected on the HPLC profile (Figure 2). The first one displayed the same retention time (4.2 min) and UV (λ_{max} = 249 nm) and LC/MS characteristics as authentic hydroxylamine **3**. LC/MS studies showed that this compound could be detected in both the positive and the negative detection modes with *m/z* of 304 and 302, respectively, indicating a molecular mass of 303. Spiking the samples with authentic **3** increased both the height and the area of this peak, confirming that it was indeed the hydroxylamino derivative of nilutamide. The second metabolite

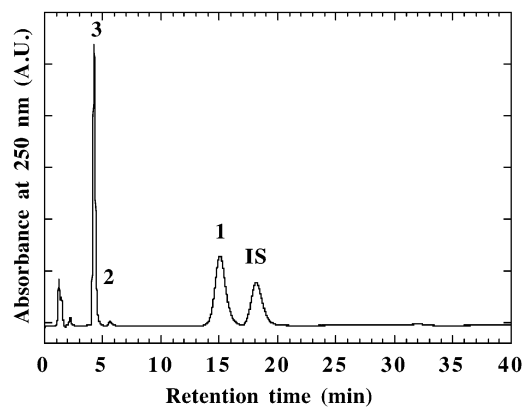


Figure 2. Products generated by the reduction of nilutamide **1** catalyzed by nNOS. Reversed phase HPLC profile of an extract from a typical anaerobic incubation of 500 μ M nilutamide **1** in the presence of 50 μ g of nNOS, 10 mM CaCl_2 , 100 μ M BH_4 , and 5 μ g of CaM in 50 mM deoxygenated Hepes buffer (final volume, 500 μ L). Following 2 min of preincubation at 37 $^\circ\text{C}$, the reaction was started by the addition of NADPH and stopped after 30 min by the addition of 100 μ L of cold methanol containing 0.1 mg/mL androstenedione (IS, internal standard). The resulting mixture was extracted with CH_2Cl_2 , the extracts were concentrated, and a 25 μ L aliquot was analyzed on the HPLC system, as described in the Materials and Methods. Elution times of the authentic compounds were as follows: nilutamide **1**, 15.0 min; amine **2**, 5.7 min; hydroxylamine **3**, 4.2 min; androstenedione (IS), 18.0 min; and azoxy **5**, 31.8 min.

had the same retention time (5.7 min) and UV ($\lambda_{\text{max}} = 247$ nm) and LC/MS (m/z 286, negative mode) characteristics as the authentic amine **2**. In some incubations, low amounts of another metabolite with the same retention time (31.8 min) and UV ($\lambda_{\text{max}} = 316$ nm) and LC/MS ($m/z = 585$, negative mode) characteristics as the azoxy-compound **5** could also be observed. No other peak could be detected in these incubation mixtures.

These results show that nNOS can anaerobically reduce nilutamide with predominant formation of its corresponding hydroxylamine **3** and very minor amounts of the amine **2**. To the best of our knowledge, the hydroxylamine **3** has been identified by LC/MS from urines of rats, dogs, and humans treated with nilutamide (**4**) but has never been previously synthesized and fully chemically characterized. Hydroxylamine **3** was prepared in 63% yield from reduction of nilutamide with H_2 in the presence of poisoned PtO_2 (**31**). It was found unaltered after anaerobic incubation in the presence of nNOS under usual conditions and thus seems relatively stable. However, air exposure of methanolic solutions of **3** resulted in the formation of azoxy-compound **5** after a few hours. Oxidation of hydroxylamine **3** by ferric chloride following a usual protocol (**42**) led to the HPLC detection of azoxy **5** and of a new compound, which could be the nitroso compound **4**. However, all of our attempts to extract it from the reaction mixture resulted in the isolation of the azoxy **5**. These results suggest that the nitroso **4** (an intermediate in the reduction of nilutamide to hydroxylamine **3**) is too reactive to be synthesized under those conditions.

Effect of Incubation Conditions on the Reduction of Nilutamide by Purified nNOS. The formation of the amine **2** and hydroxylamine **3** during the reduction of nilutamide catalyzed by nNOS was linear in the 0–15 min time range (Figure 3) and was dependent upon the amounts of purified protein (in the 5–300 μ g/mL range, data not shown). Formation of hydroxylamine **3** was

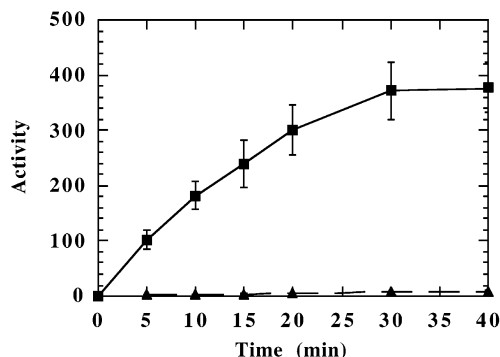


Figure 3. Time-dependent formation of amine **2** (▲) and hydroxylamine **3** (■) during the anaerobic reduction of nilutamide catalyzed by nNOS containing all of its cofactors. The incubation mixture (final volume, 3.0 mL) contained 500 μ M nilutamide, 300 μ g of nNOS, 10 mM CaCl_2 , 100 μ M BH_4 , and 30 μ g of CaM in 50 mM deoxygenated Hepes buffer, pH 7.4. Following a 2 min preincubation at 37 $^\circ\text{C}$, the reaction was started by the addition of NADPH (final concentration, 1 mM). At the required times, 0.5 mL aliquots were removed, mixed with 100 μ L of cold methanol containing 0.1 mg/mL IS, extracted with CH_2Cl_2 , and analyzed by HPLC as described in the Materials and Methods. Data represent means \pm SD of three separate experiments. Activity expressed in nmol mg prot $^{-1}$.

dependent upon the concentration of nilutamide, and the apparent kinetic constants for its anaerobic reduction catalyzed by nNOS were 340 ± 50 μ M and 20.5 ± 3.5 nmol min $^{-1}$ mg protein $^{-1}$. Amine **2** was formed at a much lower initial rate (0.07 ± 0.03 nmol min $^{-1}$ mg protein $^{-1}$), and its kinetic constants could not be accurately determined. Azoxy-compound **5** was detected in very low amounts and probably arose from the air oxidation of the hydroxylamine **3** during the workup of the reaction mixtures.

The reduction of nilutamide to hydroxylamine **3** required the presence of active nNOS and NADPH and was completely inhibited in the presence of dioxygen or of DPI, a strong inhibitor of flavin-dependent enzymes (Table 1). The omission of CaM to the incubation mixtures reduced 3-fold the reaction (Table 1), whereas the addition of FMN, FAD, or FMN + FAD (all 5 μ M) had no effect (data not shown). The reduction of nilutamide was also independent upon the presence of BH_4 , a cofactor required for optimal NO synthesis from L-arginine, and similar amounts of **3** were formed in incubations of nNOS supplemented with 100 μ M BH_4 (usual conditions) or without any addition of BH_4 (Table 1). Furthermore, the addition of classical NOS inhibitors such as ThioCit, NO_2 -Arg, and SEITU (L-arginine analogues that bind at the active site) (**19–23**) or of the heme ligands, ImH and CO, had minimal effects on the reaction (Table 1).

Effect of Nilutamide on the Activities of nNOS.

We investigated the effects of nilutamide on the aerobic activities of nNOS by measuring the oxidation of L-arginine to L-citrulline and of NADPH to NADP^+ in the presence of increasing concentrations of nilutamide. The nNOS-catalyzed oxidation of L-arginine and of NADPH occurred with basal initial rates of 320 ± 40 and 1175 ± 220 nmol min $^{-1}$ mg protein $^{-1}$, respectively. The addition of nilutamide slightly reduced the NADPH oxidation rate of nNOS ($15 \pm 5\%$ at 500 μ M) but had no significant effect on the oxidation of L-arginine (up to 2 mM, data not shown). The nNOS-catalyzed reduction of ferric cyt *c* to ferrous cyt *c* in the presence and in the absence of CaM

Table 1. Effects of Incubation Conditions on the Anaerobic Reduction of Nilutamide Catalyzed by nNOS

conditions	% CS ^a
CS ^b	100
– nNOS	<0.1
– NADPH	<0.1
+ O ₂ ^c	<0.1
+ DPI (100 μ M)	<0.1
– CaM	36 \pm 6
– BH ₄	88 \pm 8
+ ThioCit (1 mM)	91 \pm 10
+ NO ₂ -Arg (1 mM)	113 \pm 12
+ SEITU (1 mM)	87 \pm 15
+ ImH (10 mM)	102 \pm 8
+ CO ^d	125 \pm 10

^a The complete system (CS) contained 500 μ M nilutamide, 50 μ g of nNOS, 10 mM CaCl₂, 100 μ M BH₄, and 5 μ g of CaM in 50 mM deoxygenated Hepes buffer (final volume, 500 μ L). After 2 min at 37 $^{\circ}$ C, the reactions were started by the addition of NADPH (final concentration, 1 mM) and stopped after 30 min by the addition of 100 μ L of cold methanol containing 0.1 mg/mL androstenedione. The resulting mixtures were extracted and treated as described in the Materials and Methods. ^b Results are expressed as % of the maximal activity of reduction of nilutamide catalyzed by nNOS (18.5 \pm 3.5 nmol min^{–1} mg prot^{–1}) and are means \pm SD from three independent experiments. As very low amounts of amine **2** were formed, only hydroxylamine **3** was reported in this table. ^c Experiments were performed in aerated 50 mM Hepes buffer, pH 7.4. ^d Experiments were performed in 50 mM Hepes buffer previously deoxygenated by bubbling argon and then saturated by bubbling CO.

Table 2. Comparison of the Activities of the Anaerobic Reduction of Nilutamide by NADPH Catalyzed by nNOS, nNOS_{red}, nNOS_{oxy}, iNOS, and eNOS

conditions ^a	hydroxylamine 3 ^b	amine 2 ^b
nNOS	18.5 \pm 3.5	0.05 \pm 0.02
nNOS _{red}	15.7 \pm 3.5	<0.02
nNOS _{red} – CaM	8.8 \pm 2.5	<0.02
nNOS _{oxy}	0.12 \pm 0.02	<0.02
iNOS	4.5 \pm 1.0	0.09 \pm 0.03
eNOS	2.7 \pm 0.7	<0.02

^a Conditions are the same as in Table 1, except that CaCl₂ and CaM were omitted in the experiments with iNOS. ^b Results are expressed in nmol min^{–1} mg prot^{–1} and are means \pm SD from three to four independent experiments.

occurred with initial rates of 2500 \pm 300 and 280 \pm 50 nmol min^{–1} mg protein^{–1}, respectively, and were not significantly modified by the addition of nilutamide (up to 2 mM, data not shown).

Reduction of Nilutamide Catalyzed by the Oxygenase and Reductase Domains of nNOS and by the Full Length iNOSs and eNOSs. To determine the major site of nNOS responsible for the reduction of nilutamide, we performed anaerobic incubations in the presence of the purified reductase domain (nNOS_{red}) of nNOS. The nNOS_{red} catalyzed the selective reduction of nilutamide to hydroxylamine **3** by NADPH with an activity that matches the activity of full-length nNOS (15.7 \pm 3.5 nmol min^{–1} mg protein^{–1}; Table 2). As previously observed in the presence of full length nNOS, the reduction of nilutamide by NADPH catalyzed by nNOS_{red} was inhibited by O₂ and DPI, insensitive to SEITU, and 2–3-fold decreased in the absence of CaM (Table 2). Interestingly, the nNOS_{oxy} also catalyzed the reduction of nilutamide to hydroxylamine **3** but with a 100-fold lower activity than full length nNOS (Table 2). These results clearly showed that the nitroreduction of nilutamide was mostly dependent upon the flavin-containing nNOS_{red}.

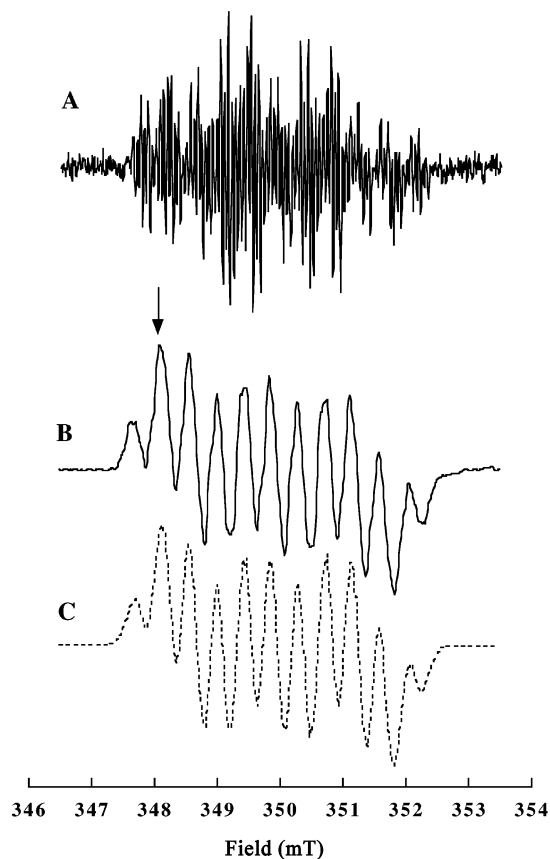


Figure 4. EPR spectra of the nilutamide anion free radical. Trace A shows the spectrum of nilutamide anion free radical recorded during the anaerobic incubation of 1 mM nilutamide in the presence of 1 mM NADPH, 10 μ g/mL CaM, 10 mM CaCl₂, 100 μ M BH₄, and 300 μ g of nNOS in 50 mM Hepes buffer, pH 7.4. The microwave power was 20 mW, and the modulation amplitude was 0.032 mT. Trace B shows the nilutamide anion radical recorded under the same conditions as in A, except that the modulation amplitude was 0.32 mT. Trace C shows the computer simulation of the nilutamide radical anion spectrum. The hyperfine coupling constants were $a_{\text{NO}_2}^{\text{N}} = 1.292$ mT, $a_{\text{CF}_3}^{\text{F}} = 0.465$ mT, $a_{\text{ortho}}^{\text{H}} = 0.312$ mT, $a_{\text{meta}}^{\text{H}} = 0.098$ mT, and $a_{\text{N}}^{\text{N}} = 0.048$ mT, and the line width was 0.3 mT.

Given these data, we wondered whether the inducible iNOS and constitutive endothelial eNOS might also reduce nilutamide under similar anaerobic conditions. Analyses of the incubations performed with these isoforms clearly showed that iNOS and eNOS reduced nilutamide to its hydroxylamine **3** with the concomitant formation of very low amounts of amine **2**. The rate of reduction catalyzed by iNOS was about 3–4-fold less than in the presence of nNOS whereas the endothelial eNOS was 8–10-fold less active than nNOS (Table 2). Reduction of nilutamide catalyzed by iNOS and eNOS was abolished by the addition of O₂ or DPI but was unaffected by the addition of SEITU or NO₂-Arg (data not shown).

Generation of Free Radicals during the Reduction of Nilutamide Catalyzed by nNOS. Introduction of an anaerobic mixture containing purified nNOS, CaCl₂, BH₄, CaM, NADPH, and nilutamide into the aquaX cell fitted into the EPR spectrometer cavity caused the immediate appearance of a free radical species (Figure 4). This radical displayed hyperfine coupling constants $a_{\text{NO}_2}^{\text{N}} = 1.292$ mT, $a_{\text{CF}_3}^{\text{F}} = 0.465$ mT, $a_{\text{ortho}}^{\text{H}} = 0.312$ mT, $a_{\text{meta}}^{\text{H}} = 0.098$ mT, and $a_{\text{N}}^{\text{N}} = 0.048$ mT very similar to those previously described for the anion radical derived

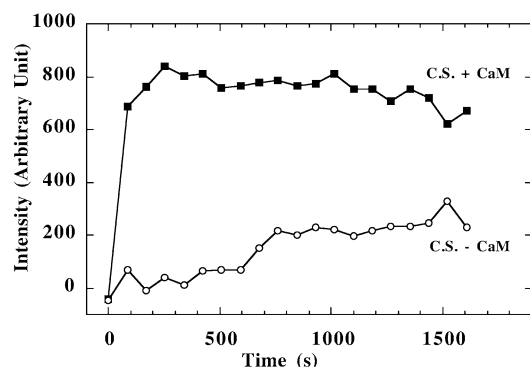


Figure 5. Time dependence of the EPR signal amplitude of the nilutamide radical anion. Amplitude was determined at the field position indicated by an arrow in Figure 4B. The anaerobic incubation mixture contained 1 mM nilutamide, 1 mM NADPH, 10 mM CaCl_2 , 100 μM BH_4 , and 300 μg of nNOS in 50 mM Hepes buffer without CaM (\circ) or in the presence (\blacksquare) of 10 μg /mL CaM.

from one electron reduction of nilutamide (12, 13). Maximal intensity of the radical signals was reached about 5 min after the introduction of the mixture in the EPR cell (Figure 5), and a steady state radical concentration was observed for about 20 min. The intensity of these signals then slowly decreased and became almost undetectable after 40 min (data not shown). Addition of superoxide dismutase to these experiments modified neither the signals of the anion radical observed nor its rate of appearance and its steady state intensity (data not shown). At the opposite, introduction of dioxygen in the incubation mixture abolished the EPR signals of the radical (data not shown).

Quantitation of the amount of the anion radical under steady state conditions was performed with the use of the stable water soluble nitronyl-nitroxide radical CPTIO (41). The steady state radical concentration observed 15 min after introduction in the EPR cavity was found to be $10.5 \pm 0.5 \mu\text{M}$. In the absence of CaM, the same radical species appeared in the anaerobic incubations but with a much slower rate (Figure 5), and quantitation of its steady state level 15 min after the addition of nNOS was found to be $4.0 \pm 0.5 \mu\text{M}$. The incubations performed in the EPR cell were extracted and analyzed by HPLC as described previously. About a 3-fold higher amount of hydroxylamine **3** accumulated in the experiments performed in the presence of CaM as compared to the experiments without CaM. These results indicated a direct correlation between anion radical generation and hydroxylamine formation.

Discussion

This study demonstrates for the first time that under anaerobic conditions nNOS catalyzes the reduction of the antiandrogen nilutamide with selective formation of its corresponding hydroxylamine **3**. Chemical synthesis of hydroxylamine **3** allowed us to unambiguously identify and quantitate this putative metabolite that had not been completely described previously (4).

This new reaction of nNOS requires NADPH and is strongly inhibited by O_2 and DPI and thus displays strong similarities with previously known dioxygen sensitive flavin-dependent class II nitroreductases (43). The nitroreduction of nilutamide catalyzed by nNOS mostly involves the reductase domain of the enzyme as shown

by (i) the inability of heme ligands, ImH and CO, to inhibit the reaction, (ii) the lack of effect of the usual L-arginine analogues, $\text{NO}_2\text{-Arg}$, ThioCit, and SEITU, and (iii) the strong effects of the NADPH-cytochrome P450 reductase inhibitor, DPI. This was confirmed by the ability for purified nNOS_{red} to catalyze this nitroreduction, albeit with a lower efficiency than full length nNOS (Table 2). This could suggest that synergistic effects between nNOS_{oxy} and nNOS_{red} could be required to obtain the maximal nitroreductase activity of nNOS. Surprisingly, purified nNOS_{oxy} itself displayed a small nitroreductase activity forming selectively the hydroxylamine **3**, although 150-fold lower than nNOS. The nitroreduction of nilutamide is also catalyzed by recombinant iNOS and, to a lesser extent, eNOS (Table 2). However, strong differences are observed among the three NOS isoforms, with nNOS leading to the highest reduction rate (in the presence of CaM) and eNOS to the lowest one (Table 2). These differences can be explained by the known structural changes in regulatory elements contained in the reductase domain of the three isoforms that result in different abilities to transfer electrons from NADPH to FAD and FMN, eNOS being the less efficient one (21–23, 44). The nitroreductase activity of nNOS is low by comparison to its ability to reduce cyt c, but this reaction occurs with kinetic data close to those observed for the reduction of nilutamide by liver microsomes (12, 13).

Anaerobic reduction of nitroaromatic compounds catalyzed by microsomal preparations from liver and lung containing NADPH-cytochrome P450 reductase is a well-known reaction believed to proceed only to the hydroxylamine whereas further reduction to the amine would be mediated by cytochrome P450 (14, 45). Our results show that full length NOSs and nNOS_{red} selectively catalyze the reduction of **1** by NADPH to the hydroxylamine **3** with very low amounts of amine **2** and thus support this assertion. However, preliminary experiments with rat liver microsomes suggest that more complex reactions may occur and that the selectivity of the nitroreduction could also be dependent upon the cytochrome P450 isoform (manuscript in preparation).

Intermediates in the six electron reduction of the nitro moiety to the corresponding primary amine usually include the nitro anion radical, the nitroso species, and the hydroxylamine (Scheme 1). We clearly observed that reduction of nilutamide catalyzed by nNOS proceeded with the formation of an intermediate nitro anion free radical easily detected by EPR spectroscopy. This nitro anion radical is stable enough to accumulate and be detected without the use of a spin-trap and is unaffected by the addition of SOD, ruling out the involvement of the superoxide anion radical in its formation. Last, simulation of the EPR spectra shows that this radical displays all of the characteristics of the anion radical of nilutamide previously observed in the presence of microsomal proteins and NADPH (12, 13). Anaerobic incubation of nNOS containing NADPH but in the absence of CaM resulted in the accumulation of the same nitro anion radical, but its rate of formation was 2–3-fold reduced by comparison to incubations containing CaM, and there was a direct relationship between EPR detection of this radical and hydroxylamine accumulation. These results are consistent with the known activating effect of CaM on the electron transfer in the reductase domain of constitutive nNOS (27). However, the absence of this coenzyme was less detrimental on nitroreduction of

nilutamide than on formation of NO from L-arginine, which is completely abolished in the absence of CaM (24). The nitro anion radical of nilutamide was not detected under aerobic conditions, a result attributed to the fast oxidation of the nitro anion radical by O_2 that regenerates the parent nitro compound **1** with concomitant formation of the superoxide anion (Scheme 1) (14). A feature common to compounds undergoing reductive metabolism is their ability to shuttle back and forth between the reduced (nitro anion radical, $R-NO_2^{\cdot-}$) and the native oxidized state ($R-NO_2$). During this futile metabolism, a flow of electrons can be transferred to dioxygen, thus generating superoxide anion. Previous studies showed that when certain redox active compounds such as quinones (28–30) and DNBs (46) are incubated with NOS, $O_2^{\cdot-}$ could be simultaneously produced with L-citrulline and NO. NO and $O_2^{\cdot-}$ then combine to produce peroxynitrite, $ONOO^-$, at a near diffusion-limited rate (47). As previously shown in stopped-flow kinetic studies (48), the rate of electron flux through the nNOS reductase is more than sufficient to support both NO and L-citrulline production, as well as simultaneous electron transfer to some exogenous electron acceptors. DNBs have been reported to induce an increase in nNOS activity with a shift in product formation from L-citrulline and NO, to L-citrulline and $ONOO^-$ (46). This originates from a redox cycling by the nitrocompounds that lead to superoxide anion and, also, from the particular ability of DNBs to stimulate NADPH oxidation by yet unknown mechanism(s) (46). DNBs increase the electron flux through the nNOS_{red} to its oxygenase domain and thus increase formation of L-citrulline and NO. NO and superoxide then combine and form high amounts of $ONOO^-$. Our results suggest that nilutamide behaves in a different manner than DNBs as it slightly decreases NADPH oxidase activity of nNOS and has almost no effect on citrulline formation. This could originate from differences in the redox potentials of nilutamide and DNBs (49) or in their mode(s) of interaction(s) with the protein. It is well-known that the electronic properties of substituents para to the nitro moiety greatly influence the reactivity and product formation from nitroaromatic compounds (49, 50). For example, flutamide, another nitroaromatic with antiandrogen properties that differs from nilutamide by the nature of its para substituent, does not lead to the formation of a nitro anion radical in the presence of liver microsomes and NADPH (51). In addition, strong differences have been observed in the rates of reduction of isomeric DNBs by rat isolated hepatocytes and liver microsomes (52, 53). In the case of nNOS, the importance of the nature of the para substituent on the electron transfer has been already described and it has been observed that 1,4-DNB more efficiently stimulated NADPH consumption than 1,2- and 1,3-DNBs (46). Further studies are under investigation to identify the key factors involved in the reduction of nitrocompounds by NOSs and their putative effects on the generation of $O_2^{\cdot-}$ and $ONOO^-$ by nNOS.

Although the exact contribution of NOSs in nilutamide metabolism in lung and other tissues remains to be evaluated in vivo, this study strongly supports the hypothesis of a key role for NOSs in the nitroreduction of nilutamide (18). Partial anaerobiosis is observed in certain tissues and could be compatible with the nitroreductase activity of NOSs. The reduction of nilutamide catalyzed by NOSs may have relevance to the pulmonary

toxicity of **1** by at least two different mechanisms. First, the generation of nitro anion radical from one electron reduction of nilutamide and its trapping by proteins or DNA may account for the toxic effects. Previous studies have shown that nitro anion radicals derived from several nitroaromatic compounds, including nilutamide, are non-reactive toward biological nucleophiles (54), but one cannot completely exclude its involvement in adverse reactions. Our results highlight a second, much more plausible, origin for the toxic effects of nilutamide. This involves the particular ability for NOSs to selectively reduce nilutamide to hydroxylamine **3**. Arylhydroxylamines have demonstrated a great reactivity toward biological molecules. Well-known pathways involve disproportionation to the corresponding amino and nitroso derivatives, addition of nucleophiles to the nitroso, and acetylation of the NHOH group of hydroxylamines (55). The *N*-acetoxy derivative may then form a highly reactive nitrenium intermediate that alkylates proteins or DNA (56). These hypotheses must be investigated, and the chemical reactivity of the hydroxylamine **3** and its cell toxicity are currently under study.

Acknowledgment. This work was supported by a grant from the Ministère de la Recherche et de la Technologie (to P.C.). We thank Aventis Pharma (Romainville, France) for their gift of RU 43866 and Dr. P. Dansette (UMR 8601 CNRS, Paris) and Dr. E. Sémon (INRA, UMR Arômes, Dijon, France) for their help in performing LC/MS spectra.

References

- (1) Desai, A., Stadler, W. M., and Vogelzang, N. J. (2001) Nilutamide: Possible utility as a second-line hormonal agent. *Urology* **58**, 1016–1020.
- (2) Yin, D., He, Y., Perera, M. A., Hong, S. S., Marhefka, C., Stourman, N., Kirkovsky, L., Miller, D. D., and Dalton, J. T. (2003) Key structural features of nonsteroidal ligands for binding and activation of the androgen receptor. *Mol. Pharmacol.* **63**, 211–223.
- (3) Mahler, C., Verhelst, J., and Denis, L. (1998) Clinical pharmacokinetics of the antiandrogens and their efficacy in prostate cancer. *Clin. Pharmacokinet.* **34**, 405–417.
- (4) Creaven, P. J., Pendyala, L., and Tremblay, D. (1991) Pharmacokinetic and metabolism of nilutamide. *Urology* **37**, 13–19.
- (5) Camus, P., Coudert, B., D'Athis, P., Foucher, P., Delchambre, J., Dupront, A., and Jeannin, L. (1991) Pharmacokinetics and metabolism of nilutamide in the isolated rat lung. *J. Pharmacol. Exp. Ther.* **259**, 1247–1255.
- (6) Gomez, J. L., Dupont, A., Cusan, L., Tremblay, M., and Labrie, F. (1992) Simultaneous liver and lung toxicity related to the nonsteroidal antiandrogen nilutamide (Anandron): A case report. *Am. J. Med.* **92**, 563–566.
- (7) Gram, T. E. (1997) Chemically reactive intermediates and pulmonary xenobiotic toxicity. *Pharmacol. Rev.* **49**, 297–341.
- (8) McLeod, D. G. (1997) Tolerability of nonsteroidal antiandrogens in the treatment of advanced prostate cancer. *Oncologist* **2**, 18–27.
- (9) Babany, G., Tinel, M., Letteron, P., Freneaux, E., Berson, A., Larrey, D., and Pessayre, D. (1989) Inhibitory effects of nilutamide, a new androgen receptor antagonist, on mouse and human liver cytochrome P-450. *Biochem. Pharmacol.* **38**, 941–947.
- (10) Berson, A., Schmets, L., Fisch, C., Fau, D., Wolf, C., Fromenty, B., Deschamps, D., and Pessayre, D. (1994) Inhibition by nilutamide of the mitochondrial respiratory chain and ATP formation. Possible contribution to the adverse effects of this anti-androgen. *J. Pharmacol. Exp. Ther.* **270**, 167–176.
- (11) Fau, D., Berson, A., Eugene, D., Fromenty, B., Fisch, C., and Pessayre, D. (1992) Mechanism for the hepatotoxicity of the antiandrogen, nilutamide. Evidence suggesting that redox cycling of this nitroaromatic drug leads to oxidative stress in isolated hepatocytes. *J. Pharmacol. Exp. Ther.* **263**, 69–77.
- (12) Berger, V., Berson, A., Wolf, C., Chachaty, C., Fau, D., Fromenty, B., and Pessayre, D. (1992) Generation of free radicals during

- the reductive metabolism of nilutamide by lung microsomes: Possible role in the development of lung lesions in patients treated with this antiandrogen. *Biochem. Pharmacol.* **43**, 654–657.
- (13) Berson, A., Wolf, C., Berger, V., Fau, D., Chachaty, C., Fromenty, B., and Pessayre, D. (1991) Generation of free radicals during the reductive metabolism of the nitroaromatic compound, nilutamide. *J. Pharmacol. Exp. Ther.* **257**, 714–719.
 - (14) Mason, R. P., and Holtzman, J. L. (1975) The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* **14**, 1626–1632.
 - (15) Reeve, I. T., and Miller, M. G. (2002) 1,3-Dinitrobenzene metabolism and protein binding. *Chem. Res. Toxicol.* **15**, 352–360.
 - (16) Reeve, I. T., Voss, J. C., and Miller, M. G. (2002) 1,3-Dinitrobenzene metabolism and GSH depletion. *Chem. Res. Toxicol.* **15**, 361–366.
 - (17) Eisenbrand, G., Muller, N., Denkel, E., and Sterzel, W. (1986) DNA adducts and DNA damage by antineoplastic and carcinogenic N-nitrosocompounds. *J. Cancer Res. Clin. Oncol.* **112**, 196–204.
 - (18) Ask, K., Heydel, J.-H., Piton, A., Foucher, P., Bonniaud, P., and Camus, P. (2000) Inducible nitric oxide synthase is implicated in the reductive metabolism of nilutamide in lung tissues. *Nitric Oxide Biol. Chem.* **4**, 316.
 - (19) Kerwin, J. F., Lancaster, J. R., and Feldman, P. L. (1995) Nitric oxide: A new paradigm for second messengers. *J. Med. Chem.* **38**, 4343–4362.
 - (20) Pfeiffer, S., Mayer, B., and Hemmens, B. (1999) Nitric oxide. Chemical puzzles posed by a biological messenger. *Angew. Chem., Int. Ed.* **38**, 1714–1731.
 - (21) Masters, B. S. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. (1996) Neuronal nitric oxide synthase, a modular enzyme formed by convergent evolution: Structure studies of a cysteine thiolate-ligated heme protein that hydroxylates L-arginine to produce NO as a cellular signal. *FASEB J.* **10**, 552–558.
 - (22) Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001) Nitric oxide synthases: Structure, function and inhibition. *Biochem. J.* **357**, 593–615.
 - (23) Stuehr, D. J. (1999) Mammalian nitric oxide synthases. *Biochim. Biophys. Acta* **1411**, 217–230.
 - (24) Abu-Soud, H. M., Yoho, L. L., and Stuehr, D. J. (1994) Calmodulin controls neuronal nitric oxide synthase by a dual mechanism. *J. Biol. Chem.* **269**, 32047–32050.
 - (25) Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**, 714–718.
 - (26) Miyajima, M., Sagami, I., Daff, S., Migita, C. T., and Shimizu, T. (2000) Azo reduction of Methyl Red by neuronal nitric oxide synthase. The importance of FMN in catalysis. *Biochem. Biophys. Res. Commun.* **275**, 752–758.
 - (27) Klatt, P., Heinzel, B., John, M., Kastner, M., Bohme, E., and Mayer, B. (1992) Ca²⁺/Calmodulin-dependent cytochrome c reductase activity of brain nitric oxide synthase. *J. Biol. Chem.* **267**, 11374–11378.
 - (28) Kumagai, Y., Nakajima, H., Midorikawa, K., Homma-Takeda, S., and Shimojo, N. (1998) Inhibition of nitric oxide formation by neuronal nitric oxide synthase by quinones: Nitric oxide synthase as a quinone reductase. *Chem. Res. Toxicol.* **11**, 608–613.
 - (29) Vázquez-Vivar, J., Martásek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., and Kalyanaram, B. (1997) Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. *Biochemistry* **36**, 11293–11297.
 - (30) Garner, A. P., Paine, M. J. I., Rodriguez-Crespo, I., Chinje, E. C., Ortiz de Montellano, P., Stratford, I. J., Tew, D. G., and Wolf, C. R. (1999) Nitric oxide synthases catalyze the activation of redox cycling and bioreductive anticancer agents. *Cancer Res.* **59**, 1929–1934.
 - (31) Uetrecht, J. P., Sweetman, B. J., Woosley, R. L., and Oates, J. A. (1984) Metabolism of procainamide to a hydroxylamine by rat and human hepatic microsomes. *Drug Metab. Dispos.* **12**, 77–81.
 - (32) Renodon, A., Boucher, J.-L., Sari, M.-A., Delaforge, M., Ouazzani, J., and Mansuy, D. (1997) Strong inhibition of neuronal nitric oxide synthase by the Calmodulin antagonist and anti-estrogen drug Tamoxifen. *Biochem. Pharmacol.* **54**, 1109–1114.
 - (33) Saiki, R. K., Gelfand D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
 - (34) Sanger F., Nicklen S., and Coulson A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467.
 - (35) Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999) Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. *Biochemistry* **38**, 15689–15696.
 - (36) Wu, C., Zhang, J., Abu-Soud, H., Ghosh, D. K., and Stuehr, D. J. (1996) High-level expression of mouse inducible nitric oxide synthase in *Escherichia coli* requires coexpression with calmodulin. *Biochem. Biophys. Res. Commun.* **222**, 439–444.
 - (37) Ghosh, S., Gachhui, R., Crooks, C., Wu, C., Lisanti, M. P., and Stuehr, D. J. (1998) Interaction between caveolin-1 and the reductase domain of endothelial nitric oxide synthase. *J. Biol. Chem.* **273**, 22267–22271.
 - (38) Abu-Soud, H.M., Gachhui, R., Raushel, F. M., and Stuehr, D. J. (1997) The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydrobiopterin on its stability. *J. Biol. Chem.* **272**, 17349–17353.
 - (39) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–252.
 - (40) Bredt, D. S., and Snyder, S. H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 682–685.
 - (41) Akaike, T., Yoshida, M., Miyamoto, Y., Sato, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S., and Maeda, H. (1993) Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. *Biochemistry* **32**, 827–832.
 - (42) Entwistle, I. D., Gilkerson, T., Johnstone, R. A. W., and Telford, R. P. (1978) Rapid catalytic transfer reduction of aromatic nitro compounds to hydroxylamines. *Tetrahedron* **34**, 213–215.
 - (43) Workman, P. (1992) Bioreductive mechanisms. *Int. J. Radiat. Oncol. Biol. Phys.* **22**, 631–637.
 - (44) Santolini, J., Adak, S., Curran, C. M. L., and Stuehr, D. J. (2001) A kinetic simulation model that describes catalysis and regulation in nitric oxide synthase. *J. Biol. Chem.* **276**, 1233–1243.
 - (45) Gillette, J. R., Kamm, J. J., and Sasame, H. A. (1968) Mechanism of p-nitrobenzoate reduction in liver: The possible role of cytochrome P-450 in liver microsomes. *Mol. Pharmacol.* **4**, 541–548.
 - (46) Miller, R. T. (2002) Dinitrobenzene-mediated production of peroxynitrite by neuronal nitric oxide synthase. *Chem. Res. Toxicol.* **15**, 927–934.
 - (47) Huie, R. E., and Padmaja, S. (1993) The reaction of NO with superoxide. *Free Radical Res. Commun.* **18**, 195–199.
 - (48) Miller, R. T., Martasek, P., Omura, T., and Masters, B. S. S. (1999) Rapid kinetic studies of electron transfer in the three isoforms of nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **265**, 184–188.
 - (49) Orna, M. V., and Mason, R. P. (1989) Correlation of kinetic parameters of nitroreductase enzymes with redox properties of nitroaromatic compounds. *J. Biol. Chem.* **264**, 12379–12384.
 - (50) Cenas, N., Nemeikaite-Ceniene, A., Sergedienė, E., Nivinskas, H., Anusevicius, Z., and Sarlauskas, J. (2001) Quantitative structure–activity relationships in enzymatic single-electron reduction of nitroaromatic explosives: Implications for their cytotoxicity. *Biochim. Biophys. Acta* **1528**, 31–38.
 - (51) Berson, A., Wolf, C., Chachaty, C., Fisch, C., Fau, D., Eugene, D., Loeper, J., Gauthier, J.-C., Beaune, P., and Pompon, D. (1993) Metabolic activation of the nitroaromatic antiandrogen flutamide by rat and human cytochromes P-450, including forms belonging to the 3A and 1A subfamilies. *J. Pharmacol. Exp. Ther.* **265**, 366–372.
 - (52) Cossum, P. A., and Rickert, D. E. (1985) Metabolism of dinitrobenzenes by rat isolated hepatocytes. *Drug Metab. Dispos.* **13**, 664–668.
 - (53) Mceuen, S. F., and Miller, M. G. (1991) Metabolism and pharmacokinetics of 1,3-dinitrobenzene in the rat and the hamster. *Drug Metab. Dispos.* **19**, 661–666.
 - (54) Kedderis, G. L., and Miwa, G. T. (1988) The metabolic activation of nitroheterocyclic therapeutic agents. *Drug Metab. Rev.* **19**, 33–62.
 - (55) Weisburger, J. H., and Weisburger, K. W. (1973) Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Rev.* **25**, 1–66.
 - (56) Streeter, A. J., and Hoener, B. A. (1988) Evidence for the involvement of a nitrenium ion in the covalent binding of nitrofurazone to DNA. *Pharm. Res.* **5**, 434–436.