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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 anti-androgen

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Abstract—The pulmonary metabolism of nilutamide, a nitroaromatic anti-androgen drug leading to pulmonary lesions in a few recipients, has been investigated in rats. Incubation of nilutamide (1 mM) with rat lung microsomes and NADPH under anaerobic conditions led to the formation of the nitro anion free radical, as indicated by ESR spectroscopy. The steady state concentration of this radical was not decreased by CO or SKF 525-A (two inhibitors of cytochrome P450), but was decreased by $NADP^+$ (10 mM) or *p*-chloromercuribenzoate (0.47 mM) (two inhibitors of NADPH-cytochrome P450 reductase activity). Anaerobic incubations of [^{3}H]nilutamide (0.1 mM) with rat lung microsomes and a NADPH-generating system resulted in the *in vivo* covalent binding of [^{3}H]nilutamide metabolites to microsomal proteins; covalent binding required NADPH; it was decreased in the presence of $NADP^+$ (10 mM), or in the presence of the nucleophile glutathione (10 mM), but was unchanged in the presence of carbon monoxide. Under aerobic conditions, in contrast, the nitro anion free radical was reoxidized by oxygen, and its ESR signal was not detected. Covalent binding was essentially suppressed. Instead, there was consumption of NADPH and oxygen, and production of superoxide anion and hydrogen peroxide. We conclude that nilutamide is reduced by rat lung microsomes NADPH-cytochrome P450 reductase into a nitro anion free radical. In anaerobiosis, the radical is reduced further to covalent binding species. In the presence of oxygen, in contrast, this nitro anion free radical undergoes redox cycling, with the generation of reactive oxygen species.

Nilutamide is a non-steroidal anti-androgen derivative behaving as a competitive antagonist of the androgen receptor [1, 2]. This nitroaromatic compound is proposed in the treatment of metastatic prostatic carcinoma in association with castration [3, 4]. Therapeutic effects of nilutamide are overshadowed by the occurrence of several adverse drug reactions, including pulmonary interstitial fibrosis [5–7] and drug-induced hepatitis. Lung lesions occur in about 1.5% of recipients (Cassenne Laboratories, personal communication).

Toxicity of some nitroaromatic compounds such as nitrofuran and nitroimidazole derivatives depends on the reduction of the nitro group [8, 9]. The initial one-electron reduction of the nitro group forms a nitro anion free radical. Under anaerobic conditions, further reductions lead successively to the nitroso, the hydroxylamine and the amine metabolites. The nitroso and the hydroxylamine are reactive species which can covalently bind to glutathione and cellular macromolecules. Under aerobic conditions, however, molecular oxygen oxidizes the nitro anion free radical resulting in a redox cycle with regeneration of the nitroarene compound and formation of reactive oxygen species.

We have reported previously that rat liver microsomes catalyse the one-electron reduction of nilutamide to its corresponding nitro anion free radical [10]. It remains unknown, however, whether this radical is also formed in the lung. In the present study, we have investigated the metabolism of nilutamide by rat lung microsomes and have provided evidence that this anti-androgen is reduced to a nitro anion free radical capable of generating toxic reactive species both under aerobic and anaerobic conditions.

Materials and Methods

Materials. Male Sprague–Dawley Crl:CD (SD) BR rats, weighing 250–300 g were purchased from Charles River (Saint-Aubin-les-Elbeuf, France). Animals were fed *ad lib.*

on a standard diet (Autoclavé 113, UAR, Villemoissoin-sur-Orge, France).

Nilutamide and [^{3}H]nilutamide (sp. act. 11 Ci/mmol) were generous gifts from Cassenne Laboratories (Paris, France). Other products were from the Sigma Chemical Co. (Poole, U.K.).

Preparation of lung microsomes. Rats were killed by decapitation and lungs were perfused via the inferior vena cava with at least 100 mL of saline solution. Lungs were removed and microsomes were prepared as described previously [11]. Protein concentration was determined by the method of Lowry *et al.* [12].

ESR measurements. ESR studies were performed at 20°, using a Bruker ER 200 spectrometer equipped with a TM 110 rectangular mode cavity. Lung microsomes in 0.1 M sodium–potassium phosphate buffer, pH 7.4 were gassed with nitrogen or CO before initiating the reaction with NADPH and transferring the sample anaerobically into the ESR cell.

Oxygen consumption. Oxygen consumption by lung microsomes (5 mg protein/mL) was measured with a Gilson oxygraph at 37° and using a Clark electrode, after initiating the reaction with 1 mM NADPH.

Production of superoxide anion. Formation of superoxide anion was determined by the adrenochrome assay by measuring the absorption at 480 minus 575 nm [13]. The incubation mixture contained lung microsomes (4.5 mg protein/mL) plus 1 mM epinephrine. The initial rate (30 sec) of adrenochrome formation at 37° was recorded after initiating the reaction with 0.5 mM NADPH, using an absorption coefficient of 2.96 $mM^{-1}\,cm^{-1}$. Some incubations were carried out in the presence of superoxide dismutase.

Formation of hydrogen peroxide. After a preincubation of 15 min at 37° in the presence of 0.5 mM sodium azide (an inhibitor of catalase), the suspension of lung microsomes (6 mg protein/mL) was incubated for 20 min at 37° in the presence of the following NADPH-generating system: NADP (0.4 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate-dehydrogenase (2 e.u./mL) and $MgCl_2$ (6 mM). The reaction was stopped by adding 5% trichloroacetic acid. After centrifugation (22,000 *g* for 10 min), formation of hydrogen peroxide was measured by the method of Green and Hill [14].

In vitro *covalent binding.* Lung microsomes (10 mg protein/mL), [^{3}H]nilutamide (20 mCi/mL; 0.1 mM) and the NADPH-generating system described above were incubated with shaking at 37° for 15 min under various atmospheres (nitrogen, CO or air). After precipitation with 10% perchloric acid, covalent binding to proteins was measured as described previously [10].

Results

ESR study. Anaerobic incubation of nilutamide with rat lung microsomes generated a multiline ESR spectrum characteristic of the nilutamide nitro anion free radical (Fig. 1). Analysis of the nuclear hyperfine parameters of the radical agreed well with those determined for the radical anion generated in rat liver microsomes (Fig. 1). No ESR signal was detected when incubations were carried out in the absence of either nilutamide, or NADPH, or microsomes, or when the incubations were performed in the presence of air (not shown). The steady state concentration of the nitro anion radical was decreased by either $NADP^+$ (Table 1), a competitive inhibitor of NADPH-cytochrome P450 reductase, or by *p*-chloromercuribenzoate (Table 1), which inhibits electron transport in liver microsomes during NADPH oxidation. In contrast, inhibitors of cytochrome P450, such as CO or SKF 525-A, did not modify the steady state concentration of the nitro anion radical (Table 1).

NADPH oxidation, oxygen consumption and formation of reactive oxygen species. Incubation of rat lung microsomes

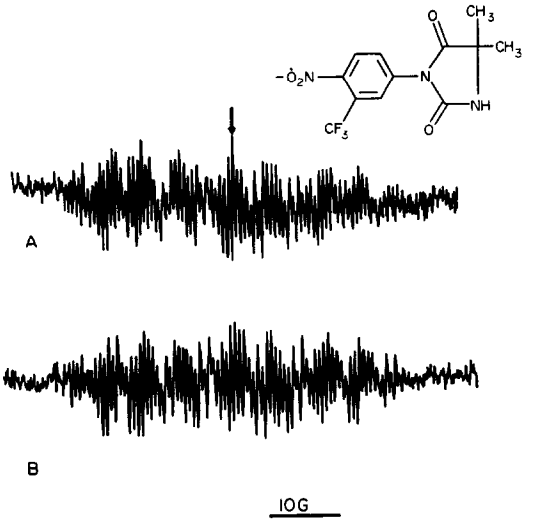

Fig. 1. ESR spectrum of the nilutamide anion free radical. Tracing A shows the spectrum of the nilutamide anion free radical recorded during the anaerobic incubation of 1 mM nilutamide with rat lung microsomes (10 mg protein/mL) and 5 mM NADPH. The microwave power (band X) was 20 mW and the modulation amplitude 0.32 G. The arrow indicates the field position used to determine the ESR signal amplitude in subsequent experiments (Table 1). Tracing B shows the spectrum of the nilutamide anion free radical recorded during the anaerobic incubation of 1 mM nilutamide with rat liver microsomes (10 mg protein/mL) and 5 mM NADPH. The instrument settings were the same as in tracing A. This spectrum is characterized by the following splitting constants:

$a^N_{NO_2} = 13.02$; $a^F_{CF_3} = 4.65$ G; $a^H_{ortho} = 3.12$ G;

$a^H_{meta} = 0.97$ G; $a^N_N = 0.48$ G.

Table 1. Steady state signal amplitude of the nilutamide nitroanion free radical under various conditions

	Relative amplitude (% of control)
Control	100 ± 21
$NADP^+$ (10 mM)	0*
p-Chloromercuribenzoate (0.47 mM)	0*
Carbon monoxide	105 ± 5
SKF 525-A (3 mM)	85 ± 1

In the control experiment, the incubation mixture containing 10 mM nilutamide and lung microsomes (14.6 mg/mL) was gassed under nitrogen for 10 min before anaerobic transfer to the ESR cell. In other experiments, various additions were made, as indicated; other samples were gassed under carbon monoxide. The reaction was initiated by adding 10 mM NADPH. Microwave power was 20 mW; modulation amplitude was 2.5 G. The amplitude of the peak indicated by an arrow in Fig. 1 was monitored as a function of time.

Results (mean ± SD for three incubations) are expressed as per cent of the mean control value.

* Significantly different from control incubation ($P < 0.05$).

Table 2. Effect of nilutamide on NADPH oxidation, oxygen consumption and production of reactive oxygen species by rat lung microsomes

	NADPH oxidation (nmol/mg protein/min)	Oxygen consumption (natom O/mg protein/min)	Superoxide anion formation (nmol/mg protein/min)	Hydrogen peroxide formation (nmol/mg protein/min)
Control	0.69 ± 0.14	1.96 ± 0.09	0.4 ± 0.08	0.03 ± 0.01
Nilutamide (1 mM)	1.27 ± 0.24*	2.73 ± 0.01*	1.03 ± 0.14*	0.38 ± 0.01*

Results are means ± SD for at least three experiments.
* Significantly different from control values ($P < 0.05$).

in the presence of nilutamide increased NADPH oxidation, oxygen consumption and formation of reactive oxygen species (Table 2).

Covalent binding. Incubation of [^{3}H]nilutamide with rat lung microsomes and an NADPH-generating system under anaerobic conditions resulted in the irreversible binding of radioactive products to microsomal proteins (Table 3). Covalent binding was decreased markedly when incubations were carried out aerobically, or when the NADPH-generating system was omitted (Table 3). The extent of covalent binding was not modified by CO, an inhibitor of cytochrome P450, but was decreased by $NADP^+$, an inhibitor of NADPH-cytochrome P450 reductase (Table 3). Covalent binding was decreased by the addition of the nucleophile GSH* (Table 3).

Discussion

This study shows that lung microsomes reduce the nitro compound nilutamide to the corresponding nitro anion free radical under anaerobic conditions, as detected by ESR spectroscopy (Fig. 1). Formation of the nitro anion free radical was not inhibited by inhibitors of cytochrome P450 like CO or SKF 525-A (Table 1). In contrast, formation of the nitro anion free radical required NADPH and was inhibited by either $NADP^+$, a competitive inhibitor of NADPH-cytochrome P450 reductase or by *p*-chloromercuribenzoate, which inhibits electron transport in microsomes during NADPH oxidation (Table 1). These results suggest that lung NADPH-cytochrome P450 reductase is involved in the one-electron reduction of nilutamide to the corresponding nitro anion free radical.

Further metabolism of the nitro anion free radical of nilutamide differs in anaerobic and in aerobic lung microsomes. Under *anaerobic conditions*, the nitro anion free radical probably undergoes dismutation, forming the nitroso derivative, which is subsequently reduced to the hydroxylamine [10]. Indeed, the reductive metabolism of nilutamide by lung microsomes resulted in covalent binding of reactive species to microsomal proteins under such anaerobic conditions (Table 3). Covalent binding was decreased by GSH (Table 3), suggesting that the reactive species involved in covalent binding to lung proteins could react with GSH. It is thought that nitro anion free radicals themselves are unreactive towards GSH [9]. Indeed, the steady state concentration of the nitro anion free radical of nilutamide was not modified by the addition of GSH in hepatic microsomes [10]. These observations suggest that the reactive species involved in the covalent binding to lung proteins is not the nitro anion free radical itself, but, most likely, the nitroso or the hydroxylamine derivatives, as already suggested for several other nitroaromatic compounds [15].

* Abbreviation: GSH, reduced glutathione.

Under *aerobic conditions*, in contrast, the nitro anion free radical of nilutamide ($Ar\text{–}NO_2^{\cdot}$) rapidly reacts with oxygen, to regenerate nilutamide ($Ar\text{–}NO_2$) and form the superoxide anion free radical ($O_2^{\cdot}$):

$$Ar\text{–}NO_2^{\cdot} + O_2 \rightarrow Ar\text{–}NO_2 + O_2^{\cdot}.$$

Indeed, under such aerobic conditions, the steady state concentration of the nitro anion free radical was so low as to be undetectable by ESR (Results). This low steady state concentration prevented dismutation of the nitro anion free radical to the nitroso derivative, and the subsequent formation of the hydroxylamine metabolite, explaining that oxygen essentially suppressed covalent binding to microsomal proteins (Table 3). In contrast, repeated reduction of nilutamide to the nitro anion free radical, followed by its immediate reoxidation by oxygen (with generation of a superoxide anion), resulted in a marked redox cycle, as attested by increased NADPH and oxygen consumption, and by increased formation of superoxide anion and hydrogen peroxide (Table 2).

Administration of nilutamide produces lung lesions in a few human recipients. The metabolism of nilutamide also involves reduction of the nitro group in humans, as shown by the appearance of the amine derivative (Cassenne Laboratories, personal communication). Like nilutamide,

Table 3. Covalent binding of [^{3}H]nilutamide metabolites to rat lung microsomal proteins

	Covalent binding (pmol/mg protein/min)
Under nitrogen	
Standard system	12.5 ± 0.9
Without cofactors	6.8 ± 1.1*
With $NADPH^+$ (10 mM)	0*
With GSH (10 mM)	7.09 ± 0.2*
Under air	
Standard system	0.6 ± 0.2*
Under carbon monoxide	
standard system	11.8 ± 0.5

In the standard system, rat lung microsomes (10 mg protein/mL) were incubated at 37° for 15 min with [^{3}H]-nilutamide and a NADPH-regenerating system under various atmospheres. In some flasks, cofactors were deleted; in other flasks, $NADPH^+$ or GSH was added. Results are means ± SD for at least three experiments.
* Significantly different from incubations made with the standard system under nitrogen ($P < 0.01$).

the nitroaromatic drug, nitrofurantoin produces lung lesions in humans [16]. The toxicity of nitrofurantoin has been ascribed to the reduction of the nitroaromatic group [17]. From these observations, it is tempting to speculate that production of reactive species during the reductive metabolism of nilutamide may account for nilutamide-induced lung toxicity in humans. Toxicity may arise either by formation of reactive nilutamide species reacting with cellular proteins, and/or by an oxidative stress due to the formation of reactive oxygen species. In view of the high partial pressure of oxygen in the lung, it is tempting to speculate that lung toxicity may be due mainly to the latter mechanism.

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