Relationship between Alveolar P_{O_2} and the Rate of *p*-Nitroanisole *O*-Demethylation by the Cytochrome P-450 Pathway in Isolated Rabbit Lungs

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ABSTRACT The relationship between alveolar P_{O_2} and the rate of *O*-demethylation of *p*-nitroanisole, a model substrate for cytochrome P-450-linked mixed-function oxidation, was evaluated in the isolated rabbit lung perfused with Krebs-Ringer bicarbonate buffer. The appearance of the product, *p*-nitrophenol, in the pulmonary perfusate was measured spectrophotometrically. The P_{O_2} of the ventilating gas was varied with an accurate gas mixing pump and measured with an electrochemical O_2 analyzer. In control lungs ventilated with 5% CO_2 in air, the rate of *p*-nitrophenol production was $\cong 3.1 \pm 0.04$ (mean±SE; $n = 9$) μmol/h per g dry wt. *p*-Nitrophenol production was unaltered when O_2 in the ventilating gas was decreased to 1%, but it was depressed reversibly when alveolar O_2 was 0.1% or less and was abolished during ventilation with 0.005% O_2. The rate of the reaction was inhibited by 50% when alveolar P_{O_2} was 0.3 mm Hg representing an intracellular $[O_2]$ of $\cong 0.4$ μM. In the presence of metyrapone (0.1–1 mM), an inhibitor of cytochrome P-450-dependent reactions, *p*-nitrophenol production was 0.07–0.19 μmol/h per g dry wt. Ventilation of lungs with varying CO concentration in 20% O_2 resulted in 50% inhibition of *p*-nitrophenol production when CO concentration was 10% ($CO/O_2 = 0.5$). These results indicated that *O*-demethylation of *p*-nitroanisole by the lung is a cytochrome P-450-dependent reaction and that its rate is not affected until alveolar P_{O_2} is <1 mm Hg.

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INTRODUCTION

The mammalian lung has the capacity to metabolize drugs and other foreign compounds by the microsomal mixed-function oxidation system (1–4). This system requires molecular oxygen and NADPH, and consists of a flavoprotein (NADPH-cytochrome P-450 reductase) and cytochrome P-450 which is the terminal oxidase (5). Although the precise physiological role of the cytochrome P-450-linked mixed-function oxidation pathway in the lung has not been defined, it may function in the biotransformation of toxic or other compounds that reach the pulmonary parenchyma through the airways or circulation. This biotransformation may inactivate some compounds (2) but in other cases may generate toxic (6) or carcinogenic species (7). It has been suggested recently that the cytochrome P-450 system may have additional functions in the lung to facilitate oxygen diffusion (8) or to mediate the pulmonary arterial response to hypoxia (9, 10). These latter potential roles would be expected to be critically dependent upon the affinity of the pulmonary cytochrome P-450 system for O_2. However, this information for the pulmonary cytochrome P-450 system is lacking. In the present study, we have used the intact rabbit lung to investigate the relationship between alveolar P_{O_2} and the rate of metabolism of *p*-nitroanisole to its product, *p*-nitrophenol. We have recently shown that this reaction can be used to study factors that influence the rate of mixed-function oxidation in the intact lung (3).

METHODS

Animals. Male weanling Dutch-Belt rabbits (Skippack Farms, Skippack, Pa.), weighing 0.4–0.9 kg, were allowed free access to food (Wayne Rabbit Ration, Allied Mills, Chicago, Ill.) and water. Rabbits were anesthetized with intravenous pentobarbital, 50 mg/kg body wt.

Lung perfusion. Lungs were removed from rabbits and placed in a water-jacketed incubation chamber maintained at 37°C as described previously (3). Lungs were ventilated at a frequency of 80/min and 3.5 ml tidal volume using an

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animal respirator (Harvard Apparatus Co., Inc., Millis, Mass.). An end-expiratory pressure of 2–3 cm of water was maintained. Lungs were perfused using a peristalic pump Model 1210, Harvard Apparatus Co., Inc. at 30 ml/min with Krebs-Ringer bicarbonate solution (pH 7.4) containing 5 mM glucose.

Two different perfusion systems were used in these experiments. With one system, lungs were perfused in a once-through circuit as described previously (3). The perfusion medium was maintained in large reservoirs in a water bath at 37°C. The perfusate in the reservoirs were gassed for at least 30 min before use with fritted glass gas dispersion tubes (Arthur H. Thomas, Co., Philadelphia, Pa.). After passing through the lung, the perfusate dripped from the transected left atrium into a small collection chamber of 3–5-ml volume. A sample of the lung effluent was withdrawn continuously from the chamber for analysis (see below) and the remainder was discarded. The second method for lung perfusion was a recirculating system with a 60-ml perfusate volume. The effluent from the transected left atrium was collected in the incubation chamber, passed through an aerator, and then reinfused into the lung as described previously for the rat and guinea pig isolated lung preparations (11). Mixing of the lung effluent with the recirculating perfusate was found to be virtually complete in 2 min. The pressures required for lung ventilation and perfusion were continuously monitored with strain gauges (3). The P_{O_2} of the gases used for ventilation and of the expired gas from the perfused lung was measured with an O_2 analyzer (model S-3A, Applied Electrochemistry, Inc., Sunnyvale, Calif.) which had been calibrated with dry room air. The accuracy of the calibration was checked with analyzed low O_2 standards of 5 and 0.1% O_2 in N_2 (Airco Industrial Gases, Airco Inc., Murray Hill, N. J.). The measurement error was <3% of the meter reading. It should be noted that the electrical signal from this oxygen analyzer is a function of the difference between the unknown gas and room air and, therefore, it is well-suited to measure very low P_{O_2} values.

The gas used for ventilation of lungs and aeration of the perfusate under control conditions was 5% CO_2 in air. Low O_2 gas mixtures were prepared "on-line" from tanks of N_2, CO_2, and 5% O_2 in N_2 using an accurate gas-mixing pump (Dial-A-Gas model M3000, Calibrated Instruments Inc., Ardsley, N. Y.). A gas mixture of 50 ppm O_2 was obtained by adsorbing the major fraction of residual O_2 in the N_2 gas with an Oxy-Trap (Alltech Associates, Inc., Arlington Heights, Ill.). Gas mixtures with varying CO content were also prepared on-line by mixing varying proportions of tanks containing 75% N_2:20% O_2:5% CO_2 and 75% CO:20% O_2:5% CO_2. All ventilating gas mixtures contained 5% CO_2 in addition to other components.

Measurement of p-nitrophenol production. For once-through perfusion, two separate reservoirs were maintained with and without 0.2 mM *p*-nitroanisole. Perfusion was begun with the drug-free medium. A sample of the effluent perfusate from the lung was pumped from the collection chamber through a flow cell in a spectrophotometer (model 139, Hitachi America, Ltd., San Francisco, Calif.) where the optical density at 425 nm was measured continuously (3). After a stable base line of optical density was obtained with drug-free perfusate (≅20 min), the perfusate was switched to the solution containing *p*-nitroanisole. The rate of *p*-nitrophenol production was calculated from the lung perfusion rate and the change in absorbancy of the effluent using an extinction coefficient (pH 7.6) of 10.0×10^6 cm²/mol.

With the recirculating system, lungs were equilibrated for 10 min and then *p*-nitroanisole was added to the perfusate to give a concentration of 0.2 mM. Some experiments were carried out with metyrapone (CIBA Pharmaceutical Company, Div. of CIBA-GEIGY Corp., Summit, N. J.) added to the perfusion medium. 1-ml samples of perfusate taken at 5-min intervals after addition of *p*-nitroanisole were centrifuged, the supernate was adjusted to pH 11 with NaOH, and the absorbancy was measured in a spectrophotometer at 425 nM. The rate of *p*-nitrophenol production was calculated from the recirculating perfusate volume and its linear rate of increase in absorbance between 5 and 30 min of perfusion using an extinction coefficient (pH 11) of 12.4×10^6 cm²/mol.

Lungs were lyophilized to constant weight and results were expressed on the basis of dry weight.

RESULTS

Lungs perfused for the approximate 1-h duration of these experiments appeared normal with no evidence of alveolar edema. Ventilation and perfusion pressure each changed by <2 cm H_2O during perfusion. The ratio of dry weight to wet weight at the end of perfusion was >0.16. There were no differences in gross appearance, dry to wet weight ratio, ventilation pressure or perfusion pressure between experiments carried out under normoxic or hypoxic conditions, and perfusion pressure did not increase when the ventilating gas was made hypoxic. There was no detectable difference in O_2 concentration between the mixture used for ventilation and the expired gas from the isolated perfused lung even when lungs were ventilated with hypoxic gas mixtures.

Two experiments were carried out with the once-through perfusion system to determine the approximate relationship between oxygen concentration and *p*-nitrophenol production. As shown previously (3), infusion of *p*-nitroanisole into the isolated rabbit lung produced a rapid increase of optical density indicating production of *p*-nitrophenol from *p*-nitroanisole (Fig. 1). The peak rate of *p*-nitrophenol production in this experiment was 4.0 μmol/h per g dry wt. The rate of *p*-nitrophenol production did not change significantly as the O_2 concentration in the alveolar gas was decreased incrementally from 95 to 1% (Fig. 1). When the alveolar O_2 was

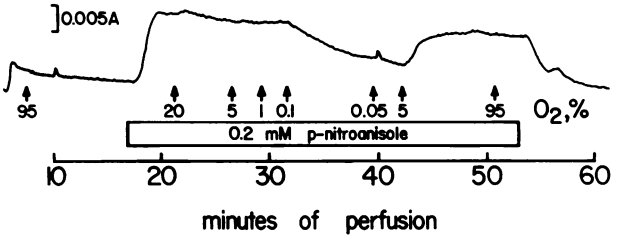

FIGURE 1 Effect of alveolar hypoxia on production of *p*-nitrophenol by isolated lungs from a Dutch-Belt rabbit perfused with a once-through system at 30 ml/min. The tracing indicates the absorbancy of the effluent perfusate during perfusion. *p*-Nitroanisole was added to the perfusate as indicated by the bar. The O_2 in the ventilating gas was changed as indicated by the arrows. In all cases, the ventilating gas in addition to O_2 contained 5% CO_2 with the balance N_2.

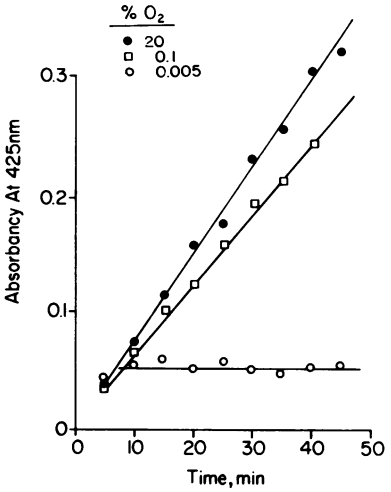

FIGURE 2 Production of *p*-nitrophenol during recirculating perfusion of isolated Dutch-Belt rabbit lungs. The absorbancy of the perfusate at 425 nm wavelength was measured in samples taken at 5-min intervals and adjusted to pH 11. Each set of points represents the result from one experiment carried out at the indicated O_2 concentration in the ventilating gas. The perfusate was equilibrated with the same gas-mixture used for ventilation. The rate of *p*-nitrophenol production was calculated from the slope of the line and the recirculating volume of perfusate (i.e., 60 ml).

TABLE I

Effect of Alveolar O_2 Concentration on p-Nitrophenol Production from p-Nitroanisole by Isolated Rabbit Lungs during Recirculating Perfusion

Alveolar O_2	*p*-Nitrophenol production	
%	*μmol/h/g dry wt*	%*
20	3.1±0.04 (9)	—
5	3.1±0.05 (3)	100
1	3.2±0.36 (4)	103
0.1	2.8±0.12 (4)	90
0.05	1.8±0.33‡ (3)	58
0.01	0.66±0.15‡ (3)	21
0.005	0±0‡ (3)	0

Results are mean standard error for number of experiments indicated in parentheses.

* Percent mean value for 20% O_2.

‡ $P < 0.05$ compared with 20% O_2 by *t* test for independent samples (12).

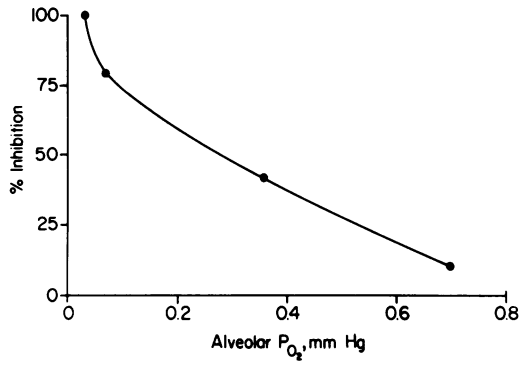

FIGURE 3 Percent inhibition of the rate of *p*-nitrophenol production as a function of the calculated P_{O_2} in the ventilating gas. Data points represent the mean values from Table I.

lowered to 0.1%, however, there was a rapid decrease in the absorbancy of the effluent perfusate indicating an approximate 50% decrease in the rate of *p*-nitrophenol production. A further decrement in the rate of *p*-nitrophenol production occurred with 0.05% O_2. When 5% O_2 was reintroduced, the rate of *p*-nitrophenol production increased rapidly indicating that the inhibitory effect of low O_2 concentration was reversible (Fig. 1). The results with the second experiment using once-through perfusion were similar.

29 experiments were carried out with the recirculating perfusion system. The absorbancy of the perfusate at 425 nm under both control and hypoxic conditions increased linearly with time during 45 min of perfusion (Fig. 2) indicating a constant rate of *p*-nitrophenol production. The rate of *p*-nitrophenol production was not different from control when lungs were ventilated with 5 or 1% O_2 but it decreased progressively during ventilation with O_2 concentrations below 0.1% and was inhibited totally when the ventilating gas contained 0.005% (50 ppm) O_2 (Fig. 2 and Table I). 50% inhibition of the mixed-function oxidation reaction occurred when alveolar O_2 was ≅0.04% (Fig. 3). This corresponds to a gas phase P_{O_2} of ≅0.3 mm Hg and an O_2 concentration in solution of ≅0.4 μM.

Two additional series of experiments were carried out to confirm the involvement of cytochrome P-450 in *p*-nitroanisole *O*-demthylation in the lung. In the first series, two experiments using the once-through perfusion system evaluated the relationship between alveolar CO concentration and the rate of *p*-nitrophenol production from *p*-nitroanisole. The alveolar O_2 concentration was held constant at 20% and the alveolar CO concentration was varied from 0 to 40%. The rate of *p*-nitrophenol production was inhibited by ≅50% when the alveolar CO was 10% ($CO/O_2 = 0.5$) and by 90% when the CO/O_2 was 2 (Table II). In

TABLE II

Effect of CO in Ventilating Gas on p-Nitrophenol Production from p-Nitroanisole by Isolated Rabbit Lungs during Once-Through Perfusion

CO*	$CO‡/O_2$	p-Nitrophenol production§		
		Expt. 1	Expt. 2	Mean
%		μmol/h/g dry wt		
0	0	6.9	6.5	6.7
5	0.25	4.7	5.1	4.9
10	0.5	3.4	3.7	3.6
20	1	1.8	1.8	1.8
40	2	0.7	0.7	0.7

* Percent CO in the ventilating gas which, in addition, contained 20% O_2, 5% CO_2, and the balance N_2.

‡ Ratio of CO to O_2 in the ventilating gas.

§ In experiment 1, the CO concentration was progressively increased, and in experiment 2, CO concentration was progressively decreased.

the second series, five experiments with the recirculating system evaluated the effect of metyrapone on *p*-nitrophenol production. In the presence of 1 mM metyrapone (three experiments), *p*-nitrophenol production was 0.10±0.01 μmol/h per g dry wt. In two experiments with 0.1 mM metyrapone, *p*-nitrophenol production was 0.19 and 0.16 μmol/h per g dry wt.

DISCUSSION

The present experiments were undertaken to investigate the relationship between oxygen concentration in the alveolar gas and the rate of oxidative demethylation of *p*-nitroanisole by the pulmonary microsomal mixed-function oxidation pathway. This relationship was evaluated by determining the effect of graded alveolar hypoxia on the rate of *p*-nitrophenol production from *p*-nitroanisole by the isolated, perfused rabbit lung. A once-through perfusion system was utilized to find the approximate range of alveolar oxygen tensions that would inhibit *p*-nitrophenol production. In subsequent experiments, a recirculating system was utilized to assure steady-state conditions and to control for the duration of perfusion on the mixed-function oxidation reaction. The results for the two types of experiments were essentially the same and indicated that the rate of *p*-nitroanisole *O*-demthylation was not inhibited until very low levels of alveolar P_{O_2} were reached. Inhibition was not observed until alveolar P_{O_2} was <1 mm Hg and 50% inhibition required a calculated alveolar P_{O_2} of ≅0.3 mm Hg (intracellular $[O_2]$ ≅0.4 μM).

The most likely mechanism for inhibition of *p*-nitrophenol production by low oxygen tension is the decreased availability of molecular oxygen for cytochrome P-450, the terminal oxidase of the mixed-function oxidation system. It should be noted that molecular oxygen is a reactant in *p*-nitroanisole *O*-demthylation (3) as well as in other mixed-function oxidation reactions (5). Other potential mechanisms for the hypoxic effect such as reduction of ATP supply or damage to lung parenchyma are unlikely because ATP is not involved in these reactions and the effect of hypoxia on *p*-nitrophenol production was rapidly reversible.

There are several lines of evidence indicating that *p*-nitroanisole is metabolized in the lung by a cytochrome P-450-linked pathway. First, subcellular fractionation studies indicate that *p*-nitroanisole *O*-demethylase specific activity is greatest in the microsomal fraction (3). Second, activity appears to be inducible by pretreatment of animals with a phenothiazine drug (3). Third, *p*-nitroanisole *O*-demethylase activity is much greater in rabbit lungs compared with rat lungs (3), a finding which correlates with their relative cytochrome P-450 contents (13). Fourth, *p*-nitroanisole *O*-demethylation was inhibited in the presence of metyrapone, an inhibitor of cytochrome P-450 dependent reactions (14). Finally, *p*-nitroanisole *O*-demethylation was inhibited by 50% when the CO/O_2 was ≅0.5 (Table II); this degree of inhibition by CO is compatible with the known relative affinities of cytochrome P-450 for CO vs. O_2 of ≅1–2 (15).

The relationship between P_{O_2} and cytochrome P-450-linked mixed-function oxidation reactions has been studied previously with isolated microsomes and with perfused liver. The K_m for O_2 in isolated microsomes were found to be <1 μM (16). On the other hand, the rate of *p*-nitroanisole *O*-demethylation in the isolated perfused liver was found to decrease when arterial P_{O_2} was lowered below 200 mm Hg (17). However, the venous outflow P_{O_2} in these latter experiments was zero so that the actual intracellular P_{O_2}, which could not be determined, was probably quite low. Although the liver, because of its high rate of drug metabolism, has been widely used to study cytochrome P-450 reactions, the lung presents a unique opportunity to evaluate the relationship between oxygen and intracellular metabolic events in an intact organ since the lung cells are in close proximity to their source of oxygen (i.e., the alveolar space). In the present experiments, there should have been no uptake of O_2 from the alveolar space into the perfusate because the latter was aerated with the same gas used for lung ventilation. O_2 consumption by the normal rabbit lung parenchyma is ≅0.04 ml/min (13) which would represent an insignificant fraction (<2%) of O_2 delivered to the alveoli via ventilation until the O_2 content of the ventilating gas is reduced below 1%. Therefore, the approximate magnitude of the

intracellular P_{O_2} is well-defined at least until very low values of alveolar O_2 are reached.

The results presented in this study provide some evidence to define whether cytochrome P-450 may function physiologically as a carrier to facilitate oxygen transport or as a sensor of alveolar hypoxia as proposed by other investigators (8–10). These functions require the effects of O_2 to be manifest at P_{O_2} levels approximately 100-fold greater than those found to inhibit *p*-nitroanisole *O*-demethylation in the rabbit lung. If the affinity of the cytochrome P-450 system for O_2 is similar with *p*-nitroanisole and other substrates, then cytochrome P-450 could not fulfill this proposed physiological role. Therefore, the influence of P_{O_2} on the metabolism of other substrates for mixed-function oxidation (e.g. endogenous lipids and steroids) will have to be investigated before such a conclusion may be generalized.

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