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PULMONARY MIXED-FUNCTION OXIDATION: STIMULATION BY GLUCOSE AND THE EFFECTS OF METABOLIC INHIBITORS*

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Abstract—Substrate requirements for pulmonary mixed-function oxidation of *p*-nitroanisole to *p*-nitrophenol were evaluated using the isolated perfused rabbit lung and a lung microsomal fraction. Addition of glucose (5 mM) to the lung perfusate (Krebs bicarbonate buffer) increased the mean rate of *p*-nitroanisole oxidation by 25–55 per cent; addition of pyruvate (5 mM) or palmitate (0.5 mM) gave similar results. Sucrose (5 mM) had no effect. Antimycin A, KCN, oligomycin, and bis-hexafluoroacetonyl acetone (an uncoupling agent) markedly depressed *p*-nitroanisole metabolism by the isolated lung. KCN also inhibited *p*-nitroanisole metabolism by lung microsomes, but antimycin A was without effect. These results indicate that pulmonary mixed-function oxidation requires substrate for intermediary metabolism as well as ATP, for maintenance of maximal rates. Glucose and mitochondrial substrates are equally effective in providing the energy requirements and the reducing potential for this reaction.

Although the liver has been most widely used to study drug metabolism, the mammalian lung also contains cytochrome P-450 and metabolizes a wide variety of agents by the mixed-function oxidation pathway [1–4]. A particularly convenient compound to evaluate this system is *p*-nitroanisole which is oxidatively demethylated to produce the yellow compound, *p*-nitrophenol. In recent studies, we have demonstrated that regulatory factors for the metabolism of *p*-nitroanisole can be evaluated with the intact, isolated, perfused lung [4, 5]. *p*-Nitroanisole *O*-demethylation in this model system was inhibited by CO and metyrapone, indicating a requirement for cytochrome P-450 [5]. The rate of *p*-nitroanisole metabolism was approximately two to three times greater on a weight basis in rabbit lung compared with rat lung, correlating with the relative microsomal cytochromes P-450 content for these species [6]. The rate of reaction was decreased by 50 per cent when alveolar O_2 pressure was decreased to 0.3 mm Hg, indicating a high affinity of this system for molecular O_2 [5]. In addition to cytochrome P-450 and molecular O_2, the mixed-function oxidase system in the intact organ requires the reactions of intermediary metabolism to generate NADPH. In the present study, we have examined the substrate requirements for generation of the NADPH required for pulmonary mixed-function oxidation. These studies have shown that metabolism of exogenous glucose could provide the reducing equivalents for metabolism of *p*-nitroanisole but that mitochrondrial substrates were equally effective.

METHODS

Lungs were isolated from male Dutch belt rabbits weighing 0.5 to 0.9 kg and were placed in the isolated lung perfusion chamber as described previously [4]. Lungs were ventilated continuously with 5% CO_2 in air, and were perfused at 30 ml/min in a recirculating system with Krebs–Ringer bicarbonate buffer (pH 7.4) aerated with 5% CO_2 in air and containing 5 mM glucose or other substrate as indicated. Metabolic inhibitors were dissolved in demethylformamide (DMF), and added to the perfusion medium, which gave a final concentration of DMF in the perfusate of 0.5 to 1% (v/v). After a 10–20 min stabilization period, 0.2 mM *p*-nitroanisole was added to the recirculating perfusate. Aliquots of the perfusate were taken at 5-min intervals and subsequently analyzed spectrophotometrically at 425 nm for *p*-nitrophenol content. The rate of *p*-nitroanisole *O*-demethylation was calculated from the linear increase in perfusate *p*-nitrophenol concentration as a function of time. Additional experiments were carried out with lungs from New Zealand White rabbits (2 to 2.5 kg), using a single pass perfusion

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system with continuous monitoring of the absorbancy of the effluent perfusate. These methods for measuring rates of *p*-nitroanisole *O*-demethylation in the perfused lungs have been described previously [4, 5].

Lung microsomes were prepared from rabbit lungs that were cleared of blood and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) [4]. The supernatant fraction from a 20,000 *g* centrifugation was spun at 100,000 *g* for 90 min and the pellet (microsomal fraction) was resuspended in 180 mM KH_2PO_4 (pH 7.4) buffer. Production of *p*-nitrophenol from *p*-nitroanisole by microsomes was measured using an NADPH regenerating system during a 30-min incubation at 37° in a metabolic shaking incubator. The method for microsome preparation and incubation has been described previously [4].

Results were evaluated by Student's *t*-test for independent samples, and the level of statistical significance was taken as $P < 0.05$.

The rabbits were obtained from Skippack Farms, Skippack, PA, and were maintained on food (Wayne Rabbit ration, Allied Mills, Chicago, IL) and water *ad lib.* *p*-Nitroanisole was obtained from the Eastman Kodak Co., Rochester, NY; inhibitors and substrates were obtained from the Sigma Chemical Co., St. Louis, MO. Bis-hexafluoroacetonyl acetone ("1799") was a gift of Dr. Peter Heylter, the Dupont Chemical Co., Wilmington, DE.

RESULTS

Effect of glucose and other substrates on p-*nitrophenol production.* When lungs were perfused with a single pass system in the absence of added glucose, a rapid increase occurred in optical density of the effluent perfusate, indicating production of *p*-nitrophenol from *p*-nitroanisole (Fig. 1). The peak rate of *p*-nitrophenol production was followed by a slow decline in rate over the next 5 min (Fig. 1). Upon addition of 5 mM glucose, the rate of *p*-nitrophenol production increased, and the subsequent rate of decline from the peak value was less rapid (Fig. 1A). In three experiments, the increase in the peak rate of *p*-nitrophenol production on addition of glucose was 26.6 ± 1.8 per cent (mean ± S.E.). Addition of

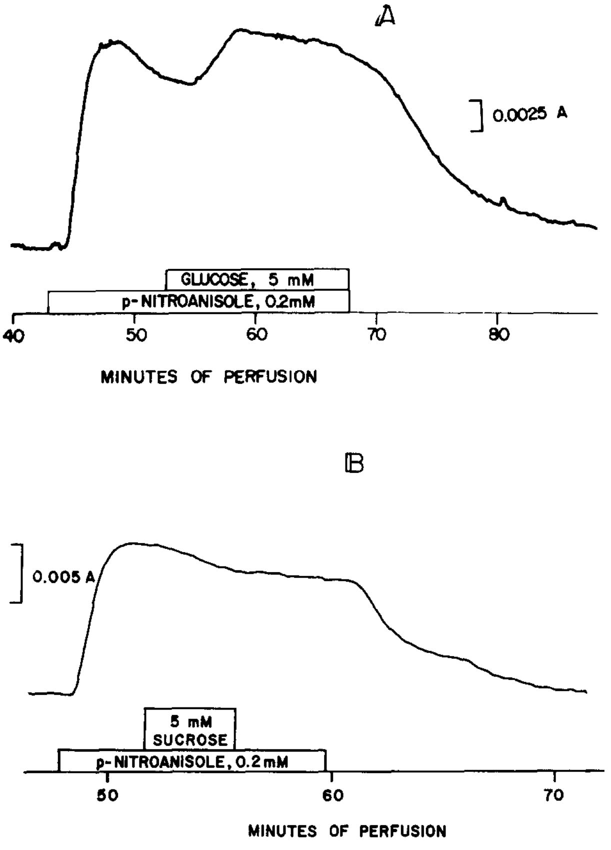

Fig. 1. Effects of glucose and sucrose on *p*-nitrophenol production from *p*-nitroanisole by the isolated New Zealand White rabbit lung. Absorbancy at 425 nm is indicated as a function of time. The lung was perfused at 40 ml/min with Krebs bicarbonate buffer (pH 7.4) and the absorbancy of the effluent was monitored continuously. *p*-Nitroanisole was added to the perfusion medium followed by glucose or sucrose as indicated by the horizontal bars. Panel A: addition of glucose; panel B: addition of sucrose.

equimolar sucrose to the glucose-free perfusate had no significant effect upon the rate of *p*-nitrophenol production (Fig. 1B), indicating that the effect of glucose was not due to osmotic or other non-specific influences. Base-line absorbance in the absence of *p*-nitroanisole was not altered by addition of either glucose or sucrose (data not shown).

Oxidative demethylation of *p*-nitroanisole was also demonstrated with the recirculating perfusion system. The rate of *p*-nitrophenol production in those perfusions containing 5 mM glucose was 55 per cent greater than in those carried out in the absence of added substrate (Table 1). Addition of sucrose to the perfusion medium had no effect on the rate of *p*-nitrophenol production.

Several additional substrates were evaluated for their effects on *p*-nitroanisole *O*-demethylation. Lungs, perfused in the recirculating system in the absence of glucose but with added pyruvate or palmitate, oxidized *p*-nitroanisole at rates similar to those obtained in the presence of glucose (Table 1). These results indicate that mitochondrial substrates as well as glucose can support maximal rates of *p*-nitroanisole *O*-demethylation.

Effects of mitochondrial inhibitors on p*-nitrophenol production.* To further evaluate the requirement for oxidative metabolism to support pulmonary mixed-function oxidation, *p*-nitrophenol production was measured in lungs perfused in the recirculating system with antimycin A, an inhibitor of mitochondrial electron transport, with oligomycin, an inhibitor of respiration-linked ATP synthesis, or with bis-hexafluoroacetonyl acetone ("1799"), an uncoupler of oxidative phosphorylation. Control studies showed that the vehicle (DMF) had no significant effect on the rate of *p*-nitrophenol production from *p*-nitroanisole (Table 2). Antimycin A in the absence of glucose inhibited *p*-nitrophenol production by 83 per cent (Table 2); inhibition was slightly but significantly less when glucose was present in the perfusion medium ($P < 0.01$ for comparison of antimycin A plus, and minus, glucose). Addition of "1799" resulted in a dose-dependent inhibition of *p*-nitrophenol production by 40–60 per cent. Oligomycin at a concentration of 5 μg/ml inhibited *p*-nitrophenol production by 33 per cent; no further inhibition was observed when the dose was doubled.

The inhibitory effect of antimycin A on *p*-nitroanisole *O*-demethylation was confirmed during single pass perfusion. The addition of antimycin A (30 μg/ml) resulted in a rapid and nearly complete but reversible decrease in the rate of *p*-nitrophenol production (Fig. 2A). In this experiment, antimycin A was dissolved in ethanol, resulting in a perfusate alcohol concentration of approximately 100 mM. A control infusion of ethanol at the same concentration

Table 1. Effects of added substrate on *p*-nitroanisole *O*-demethylation by isolated perfused rabbit lungs*

Additions†	N	*p*-Nitrophenol production $[\mu\text{moles} \cdot \text{hr}^{-1} \cdot (\text{g dry wt}^{-1})]$
None	5	2.0 ± 0.16‡
Glucose, 5 mM (control)	9	3.1 ± 0.040§
Sucrose, 5 mM	4	2.0 ± 0.095‡
Pyruvate · Na, 5 mM	4	3.2 ± 0.070
Palmitate · Na, 0.5 mM	4	2.9 ± 0.13

* Results are means ± S.E. for N experiments.

† Basic medium was Krebs bicarbonate buffer (pH 7.4).

‡ Significantly decreased ($P < 0.05$) compared with control.

§ Data reported previously [5].

Table 2. Effects of metabolic inhibitors on *p*-nitroanisole *O*-demethylation by isolated perfused rabbit lungs*

Additions to medium†	p-Nitrophenol production $[\mu\text{moles} \cdot \text{hr}^{-1} \cdot (\text{g dry wt})^{-1}]$	% of Control
Control	3.0 ± 0.021	
Antimycin A, 30 μg/ml		
−Glucose	0.52 ± 0.026	17
+Glucose	0.68 ± 0.018	23
"1799"		
5 μg/ml	1.8 ± 0.075	60
10 μg/ml	1.2 ± 0.062	40
Oligomycin		
5 μg/ml	2.0 ± 0.10	67
10 μg/ml	2.0 ± 0.032	67

* Results are means ± S.E. for three experiments under each condition.

† Perfusion medium was Krebs bicarbonate buffer (pH 7.4) with 1% (v/v) dimethylformamide and, except where indicated, 5 mM glucose.

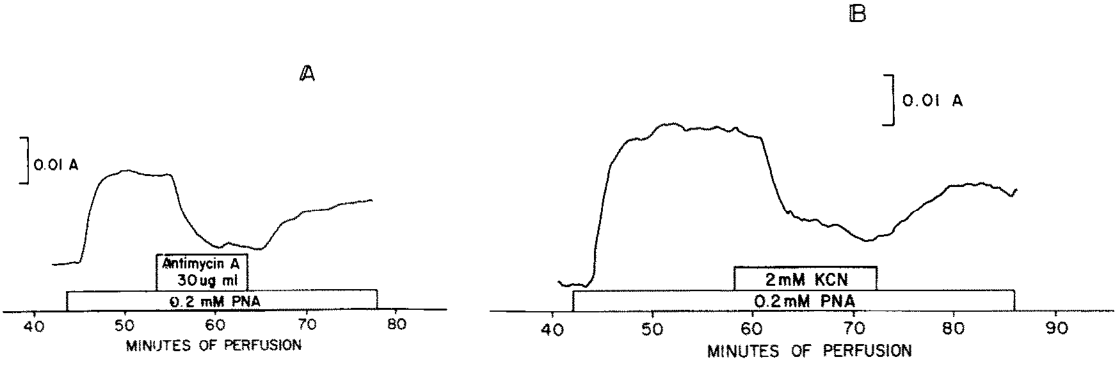

Fig. 2. Effects of metabolic inhibitors on *p*-nitrophenol production from *p*-nitroanisole by the isolated perfused rabbit lung. Perfusion conditions were as described in the legend of Fig. 1 except that 5 mM glucose was present in the perfusion medium. PNA = *p*-nitroanisole. Panel A: addition of antimycin A; panel B: addition of KCN.

inhibited the rate of *p*-nitrophenol production by less than 30 per cent. Addition of KCN (2 mM) to the perfusate also inhibited *p*-nitrophenol production markedly (Fig. 2B).

To evaluate possible effects of these inhibitors on the electron transport system of the endoplasmic reticulum, we measured oxidative demethylation of *p*-nitroanisole by isolated lung microsomes in the presence and absence of antimycin A or KCN. These additions had no significant effects on the pH of the incubation medium. Antimycin A in concentrations up to 40 µg/ml had no significant effect on the rate of *p*-nitrophenol production (Fig. 3A). On the other hand, KCN caused progressive inhibition of *p*-nitrophenol production (Fig. 3B); with 2 mM KCN, the inhibition was approximately 70 per cent. Therefore, the effect of KCN on *p*-nitroanisole demethylation in the perfused lung system could have resulted from inhibition of the microsomal electron transport system, although this explanation cannot hold for the data obtained in the perfused lung with antimycin A.

DISCUSSION

Glucose added to the perfusion medium accelerated the rate of pulmonary *p*-nitroanisole *O*-demethylation. A similar effect of glucose has been shown for liver from fasted rats, although omission of glucose had no significant effect on the rate of mixed-function oxidation by the perfused liver from fed rats [7]. This difference in the effect of glucose between the lung and the liver of the fed animal is probably related to glycogen stores which are considerable in the adult rat liver (approximately 200 mg/g dry wt) [7] but minimal in the fasted liver and in the lung (5–12 mg/g dry wt) [8]. Therefore, the lung, unlike the well-fed liver, cannot rely on glycogen breakdown to supply glucose. There are three possible mechanisms by which glucose may stimulate the rate of pulmonary *p*-nitroanisole oxidation. First, glucose may provide substrate for the pentose phosphate shunt pathway that, in turn, directly generates NADPH for mixed-function oxidation [9]. Second, glycolysis may provide substrate

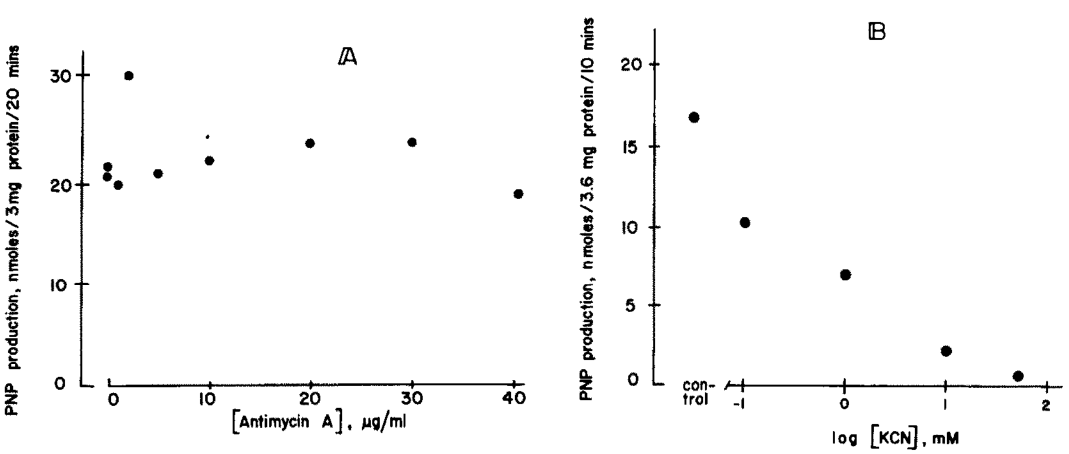

Fig. 3. Effects of antimycin A and KCN on *p*-nitrophenol production from *p*-nitroanisole by isolated rabbit lung microsomal fraction. The increase of optical density (at 436 nm) of the deproteinized incubation medium after a 10–20 min incubation was measured as a function of inhibitor concentration. Panel A: antimycin A; panel B: KCN.

for mitochondrial oxidations that apparently can generate sufficient NADPH to maintain maximal rates of pulmonary mixed-function oxidation; this possibility is supported by the observation that glucose could be replaced with pyruvate or palmitate, which are not metabolized by the pentose shunt pathway (Table 1). The precise mechanisms by which reducing equivalents may be transferred from the mitochondrial compartment to the cytoplasm of lung cells have not yet been investigated. A third possible role for glucose in mixed-function oxidation is to provide substrate for cytoplasmic and mitochondrial generation of ATP. Although ATP is not required directly for mixed-function oxidation in isolated systems, it may have an indirect role in the intact cell. This potential role for glucose was evaluated in the perfused lung with several inhibitors that, by different mechanisms, decrease intracellular ATP. Each of these inhibitors (antimycin A, 1799, oligomycin, and KCN) depressed the rate of *p*-nitrophenol production from *p*-nitroanisole. Although the effect of KCN on the pulmonary mixed-function oxidases is most likely a non-specific one, the other three inhibitors appear to act specifically by lowering intracellular ATP. Thus, these data are consistent with the hypothesis that mitochondrial ATP generation is necessary for pulmonary mixed-function oxidation. A possible role for mitochondrial ATP is energy-dependent transmembrane transport of substrate (*p*-nitroanisole). This seems unlikely since *p*-nitroanisole is lipid soluble and, in one experiment (data not shown), ouabain (0.1 mM) added to the perfusate had no effect on *p*-nitrophenol production, suggesting that the Na^+–K^+ activated ATPase was not involved in this reaction. An alternative and unifying possibility is that ATP is required for energy-dependent transhydrogenation and/or transfer of reducing equivalents from the mitochondria into the cytoplasm. The precise role for mitochondrial NADPH generation in mixed-function oxidation remains undefined, although a requirement similar to that previously shown with the perfused rat liver [10], has now been demonstrated in the intact rabbit lung.

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