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PENTOSE PATHWAY OF GLUCOSE METABOLISM IN ISOLATED GRANULAR PNEUMOCYTES

METABOLIC REGULATION AND STIMULATION BY PARAQUAT*

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Abstract—Activity of the pentose phosphate pathway of glucose metabolism was measured in isolated granular pneumocytes under a variety of metabolic conditions known to alter this pathway in intact lungs. Granular pneumocytes were isolated by trypsinization of rat lungs and maintained in primary culture for 24 hr before use. Cells were incubated for 1 hr at 37° with 5.5 mM glucose specifically labeled as 1-^{14}C, 6-^{14}C, U-^{14}C, or 5-^{3}H for determination of glucose utilization, pentose cycle activity, and partition of CO_2 production between mitochondrial and pentose pathways. With control cells, total glucose utilization was 111 ± 4.8 nmoles·hr^{-1}·(10^6 cells)$^{-1}$ (mean ± S.E., N = 19), and 2.2% was metabolized by the pentose cycle. Pentose cycle CO_2 production was 7.3 nmoles·hr^{-1}·(10^6 cells)$^{-1}$ representing 34% of total CO_2 production. Dinitrophenol (50 μM) stimulated mitochondrial CO_2 production 5-fold but had no effect on the pentose cycle activity. Phenazine methosulfate (5 μM) had no effect on mitochondrial activity but stimulated pentose cycle activity 15-fold. Antimycin A (0.4 μg/ml) markedly inhibited both pathways. After a 30-min preincubation with paraquat (3 mM), the pentose cycle CO_2 production increased to 107 nmoles·hr^{-1}·(10^6 cells)$^{-1}$ accounting for 39.6% of glucose utilization and 88.4% of CO_2 production. Mitochondrial CO_2 production was unchanged with paraquat. These studies demonstrate that the pentose cycle in resting granular pneumocytes accounts for a major fraction of the CO_2 production from glucose and that activity of this pathway is regulated by the utilization of cytoplasmic reducing equivalents. Paraquat produces marked stimulation of pentose cycle activity in granular pneumocytes, resulting in maximal utilization of cytoplasmic NADPH.

The pentose phosphate cycle of glucose metabolism has been demonstrated in the intact lung and is a major source of cellular NADPH generation [1]. This cytoplasmic reducing power is required for the specialized lung functions of synthesis of fatty acids and other surfactant-associated lipids [2, 3] and mixed-function oxidations by the cytochrome P-450 linked pathway [4]. Furthermore, tolerance to the toxic effects of hyperoxia is related, in part, to the generation of cytoplasmic reducing equivalents via the pentose pathway [5, 6]. Although activity may be present in most lung cells, the lung is cellularly heterogeneous and the distribution of the pentose phosphate cycle among cell types is not likely to be uniform. The granular pneumocyte (Type II alveolar epithelial cell), a major cell type of the alveolar septum, is the source of the lung surfactant lipids [7] and is the stem cell for regeneration of damaged epithelium [8]. The presence of relatively high activity of the pentose pathway in granular pneumocytes has been inferred through histochemical demonstration of glucose-6-phosphate dehydrogenase activity [9, 10], the enzyme catalyzing the initial step in the pentose pathway, and by direct measurement of this enzyme in isolated granular pneumocytes [11], although there has been no direct measurement of flux through this pathway in intact cells. In this study, activity of the pentose phosphate pathway was measured, and its control was investigated with metabolic inhibitors that are known to influence the rate of utilization of cytoplasmic reducing equivalents. In addition, the effect of paraquat on pentose cycle activity was measured since this agent requires metabolic activation by NADPH for its toxicity [12–14] and the granular pneumocyte is a known target cell [15].

MATERIALS AND METHODS

Granular pneumocytes were isolated from specific pathogen-free male Sprague–Dawley rats weighing 180–220 g (Charles River Breeding Laboratories, Wilmington, MA) by a previously described method [16]. Briefly, rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg body weight). Lungs were cleared of blood by perfusion through the pulmonary artery, removed from the rat, trimmed, minced, and incubated with 0.25% trypsin. The free cells were suspended in minimal essential medium (MEM), plated in plastic flasks gassed with 5% CO_2 in air, and maintained in primary culture at 37° for 24 hr. Cells were removed from attachment to plastic by sequential treatment with 0.02% EDTA and 0.25% trypsin. The cells were pelleted by centrifugation for 10 min at 550 *g* and resuspended in glucose-free MEM at a concentration of approxi-

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mately 5×10^7 cells/ml. The cell preparation consisted of more than 90% granular pneumocytes, and more than 90% of cells excluded the vital dye, erythrocin B.

For metabolic studies, 1.9 ml of medium containing 11 μmoles glucose (to give a final glucose concentration of 5.5 mM) with either 1 μCi [^{14}C]glucose (labeled either uniformly or in the first or sixth position) or 2 μCi [5-^{3}H]glucose was placed in a capped 25-ml Erlenmeyer flask in a Dubnoff incubator at 37° and shaken at 100 cycles/min. The reaction was started by addition of 0.1 ml of cell suspension and generally terminated after 60 min. Parallel incubations were used to measure the rate of formation of $^{14}CO_2$ and ^3H_2O. For studies with metabolic inhibitors, antimycin A, dinitrophenol, or phenazine methosulfate was dissolved in dimethylformamide (5 μl) and added at the start of incubation. Paraquat, dissolved in MEM, was added to cells along with glucose 30 min before the addition of radio-chemicals. All incubations were carried out in duplicate, and the individual results were averaged.

To measure $^{14}CO_2$ production, 0.3 ml hyamine hydroxide was added to a plastic well suspended inside the flask from the rubber cap. The cell reaction was stopped by addition of 0.2 ml of 12% (w/v) perchloric acid, and incubation was continued for an additional 45 min to trap the $^{14}CO_2$. The entire well was then added to 10 ml of scintillation fluid (Betafluor, National Diagnostics, Somerville, NJ), and radioactivity was determined in a scintillation counter using quench corrections based on internal standards. Recovery of radioactivity from labeled $H^{14}CO_3^-$ added to the incubation flask exceeded 90%.

To measure $^{14}CO_2$ production, 0.3 ml hyamine was stopped by centrifuging cells for 2 min at 7500 *g*. A 0.2-ml aliquot of the supernatant fraction was applied to a column containing Dowex-1 borate prepared from Dowex-1 chloride × 8 (200–400 mesh) (Bio-Rad Laboratories, Richmond, CA) by treatment with 0.05 M $Na_2B_4O_7$ [17]. The column was washed with 2 ml H_2O, and the effluent was assayed for radioactivity using Liquiscint (National Diagnostics). Recovery of authentic ^3H_2O placed on the column was approximately 80%, while elution of labeled glucose was less than 3%.

Total CO_2 production from glucose was measured in incubations with [U-^{14}C]glucose. Pentose phosphate cycle activity was estimated as a fraction of total glucose utilization from the formulation of Katz and Wood [18] based on $^{14}CO_2$ production from glucose labeled in the first and sixth positions and the rate of glucose utilization [1]. The latter was estimated from the rate of ^3H_2O production from [5-^{3}H]glucose [19–22].

To calculate CO_2 production via the pentose cycle, each mole of glucose oxidized by the pentose cycle was assumed to generate 3 moles CO_2 [18]. Mitochondrial CO_2 production was calculated as the difference between total CO_2 production and pentose phosphate cycle CO_2 production; this presumably includes reactions of pyruvate dehydrogenase and those of the tricarboxylic acid cycle.

Minimal essential medium was obtained from Flow Laboratories (McLean, VA) and glucose-free minimal essential medium from GIBCO (Grand Island, NY). Radiochemicals were purchased from the New England Nuclear Corp. (Boston, MA). Inhibitors and substrates were obtained from the Sigma Chemical Co. (St. Louis, MO). Paraquat was recrystallized from ethanol before use.

RESULTS

Production of $^{14}CO_2$ or ^3H_2O by cells was initially measured as a function of time of incubation (Fig. 1). ^3H_2O production increased linearly during a 75-min incubation, indicating a constant rate of glycolytic activity. CO_2 production from [U-^{14}C]glucose and from [1-^{14}C]glucose also showed linear increase dur-

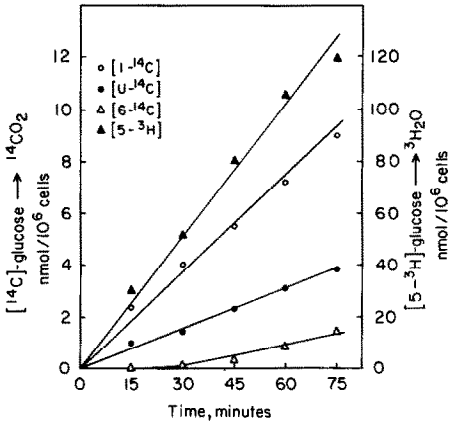

Fig. 1. Metabolism of specifically labeled glucose by isolated granular pneumocytes as a function of time. Results show glucose oxidation to $^{14}CO_2$ for glucose labeled with ^{14}C in the first or sixth positions or uniformly. Also shown is the production of ^3H_2O from [^{3}H]glucose labeled in the fifth position. ^3H_2O production was taken as an index of total glucose utilization via glycolysis. Each point represents the mean value for a separate incubation carried out in duplicate.

Table 1. Glucose utilization and oxidation to CO_2 by rat granular pneumocytes*

	(A) [5-^{3}H]Glucose → ^3H_2O [nmoles·hr^{-1}·(10^6 cells)$^{-1}$]	(B) [U-^{14}C]Glucose → $^{14}CO_2$ [nmoles·hr^{-1}·(10^6 cells)$^{-1}$]	B/A × 100
Control	111 ± 4.8 (19)	3.6 ± 0.13 (12)	3.2
Antimycin A, 0.4 μg/ml	57.8 ± 8.3 (6)	0.5 ± 0.1 (6)	0.9
Dinitrophenol, 50 μM	86.5 ± 11.5 (4)	12.1 ± 1.1 (4)	14.0
Phenazine methosulfate, 5 μM	169 ± 23 (2)	21.0 ± 3.7 (3)	12.4
Paraquat, 3 mM	88.9 ± 10.2 (3)	20.3 ± 1.3 (3)	22.8

* Values are mean ± S.E. for the number of incubations given in parentheses or mean ± range for N = 2.

Table 2. Oxidation of glucose labeled in the 1 and 6 positions to $^{14}CO_2$ by rat granular pneumocytes*

	[1-^{14}C]Glucose → $^{14}CO_2$ [nmoles·hr^{-1}·(10^6 cells)$^{-1}$]	[6-^{14}C]Glucose → $^{14}CO_2$ [nmoles·hr^{-1}·(10^6 cells)$^{-1}$]	Pentose cycle† (%)
Control (10)	8.0 ± 0.78	1.0 ± 0.12	2.2
Antimycin A, 0.4 μg/ml (3)	0.95 ± 0.15	0.005 ± 0.004	0.55
Dinitrophenol, 50 μM (3)	13.1 ± 1.4	8.3 ± 0.71	2.1
Phenazine methosulfate, 5 μM (4)	79.3 ± 4.4	3.2 ± 0.09	22.0
Paraquat, 3 mM (3)	60.0 ± 3.1	3.1 ± 0.25	39.6

* Values are mean ± S.E. for the number of incubations given in parentheses.
† Percent of glucose utilization calculated from specific yields of CO_2 [18].

ing 75 min of incubation. On the other hand, there was a lag of approximately 30 min in appearance of $^{14}CO_2$ from [6-^{14}C]glucose, suggesting a delay in the equilibration of this substrate with precursor pools in the cells. Consequently, single point analysis at 60 min of incubation provided accurate estimates of the rate of production of ^3H_2O and of $^{14}CO_2$ from [U- and 1-^{14}C]glucose but underestimated the rate from [6-^{14}C]glucose by approximately 35%.

^3H_2O production by control cells was relatively reproducible with a large number of incubations (Table 1). Assuming that this value represents the glycolytic rate, approximately 3% of the glucose metabolized by normal cells was oxidized to CO_2 (Table 1). In the presence of the mitochondrial inhibitor antimycin A, glucose utilization decreased significantly and CO_2 production was nearly abolished. With the mitochondrial uncoupler dinitrophenol, CO_2 production was stimulated approximately 3.5-fold, while glucose utilization was essentially unchanged. Phenazine methosulfate caused an even greater increase in glucose oxidation to CO_2 along with an increase in the glycolytic rate.

CO_2 produced from glucose labeled in the first and sixth positions and ^3H_2O generation were used to calculate pentose cycle activity. Calculated mean pentose cycle activity in control cells was 2.2% of glucose utilized (Table 2). The pentose cycle activity as percent of glucose utilized was inhibited by antimycin A, unchanged with dinitrophenol, and stimulated 10-fold with phenazine methosulfate.

Because of the lag in $^{14}CO_2$ production from [6-^{14}C]glucose, pentose cycle activity under control conditions was also estimated using the linear rates of $^{14}CO_2$ production (Fig. 1). In this instance, pentose cycle activity calculated from rates of CO_2 production represented 1.8% of glucose utilization whereas pentose cycle activity using the single point values at 60 min for this experiment was 2.1%. Therefore, use of our standard method resulted in a 14% overestimation of the pentose cycle activity for control cells.

Using the results for percentage pentose cycle activity and glucose utilization, CO_2 production was partitioned between pentose cycle and mitochondrial pathways. Note that total CO_2 production is the glucose oxidized to CO_2 (Table 1) times 6. In control cells, approximately $\frac{1}{3}$ of the CO_2 was produced by the pentose cycle and $\frac{2}{3}$ by mitochondrial pathways (Fig. 2). Both pathways were inhibited significantly in the presence of antimycin A. Dinitrophenol resulted in a 5-fold increase in mitochondrial CO_2 production with no change in pentose cycle activity, while phenazine methosulfate had no effect on mitochondrial activity but stimulated the pentose cycle by approximately 15-fold. In the presence of phenazine methosulfate, the pentose cycle accounted for nearly 90% of the CO_2 production.

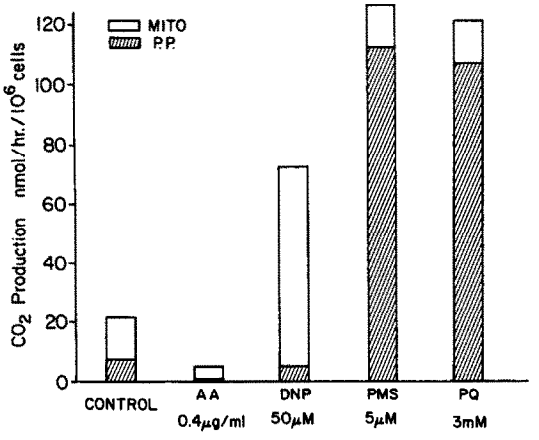

Fig. 2. Partition of CO_2 production between pentose cycle and mitochondrial pathways for granular pneumocytes incubated under various metabolic conditions. The height of the bar indicates total CO_2 production from [U-^{14}C]glucose. P.P. is the estimated CO_2 production by the pentose cycle calculated from glucose utilization and percent pentose cycle activity. Mito represents mitochondrial CO_2 production calculated as the difference between total and P.P. CO_2 production. AA = antimycin A; DNP = dinitrophenol; PMS = phenazine methosulfate; and PQ = paraquat.

Cells preincubated with paraquat showed no change in glucose utilization but a large increase in glucose oxidation to CO_2 (Table 1). This was due entirely to stimulation of pentose activity (Table 2) since mitochondrial oxidation was unchanged (Fig. 2). Pentose cycle CO_2 production with paraquat was similar to that with phenazine methosulfate (Fig. 2), while the percent pentose cycle activity was even greater (Table 2).

DISCUSSION

The present results showing significantly greater rates of CO_2 production from glucose labeled in the first as compared with the sixth position indicate that isolated granular pneumocytes catabolize glucose via

the pentose phosphate shunt pathway. We have estimated the flux of glucose through this pathway using the model proposed by Katz and Wood [18]. This model requires measurement of glucose utilization in order to estimate the percent contribution of the pentose cycle. In the present study, estimation of glucose utilization from production of ^3H_2O by control granular pneumocytes gave values very similar to previous results based on glucose assay [16]. This value is also compatible with our previous measurements of 2-deoxyglucose (DOG) uptake by isolated granular pneumocytes [23]. In this latter study, the initial rate of DOG uptake was approximately 80 nmoles·hr^{-1}·(10^6 cells)$^{-1}$ while the affinity of the transport system for glucose was approximately 1.5 times that for DOG. Glycogen synthesis or the utilization of pentose for nucleic acid synthesis may result in the underestimation of glucose utilization by the ^3H_2O production method, but this effect appears to be minor. The high rates of glucose utilization by the cells reflect, in part, the high rates of lactate and pyruvate production in the lactate-free medium. Consequently, the pentose cycle in the isolated cells accounts for a relatively small percent of total glucose utilization.

Calculation of pentose cycle activity from the model of Katz and Wood [18] requires several assumptions including the complete equilibration of the hexose phosphate pool and low activity of fructose-1,6-diphosphatase and glucose-6-phosphatase. These assumptions have not yet been critically evaluated for the granular pneumocytes. Furthermore, our calculations slightly overestimated activity of the pentose pathway because of delayed equilibration of $^{14}CO_2$ from [6-^{14}C]glucose. Consequently, the present results more reliably reflect changes with metabolic conditions rather than absolute values for flux through this pathway.

The results obtained with metabolic inhibitors provide evidence for metabolic control of the pentose cycle in the isolated granular pneumocytes. As expected, antimycin A, an inhibitor of the respiratory chain at the second phosphorylation site, markedly inhibited CO_2 production by the mitochondria. Pentose cycle activity was also inhibited markedly, presumably due to inhibition of the utilization of cytoplasmic reducing equivalents. A similar effect on pentose cycle activity has been noted previously for the isolated perfused lung using carbon monoxide as a mitochondrial inhibitor [1]. Dinitrophenol, an uncoupler of oxidative phosphorylation, resulted in marked stimulation of mitochondrial CO_2 production but had no effect on pentose phosphate cycle activity. On the other hand, phenazine methosulfate, an agent that interacts with cytoplasmic pyridine nucleotides [21, 24], markedly stimulated the pentose phosphate cycle but had no effect on mitochondrial metabolism. These results provide evidence that the pentose phosphate cycle in the isolated granular pneumocytes is controlled by the $NADP^+$ concentration in the cytosol and indicate that the granular pneumocyte preparation conforms to accepted models for control of this metabolic pathway.

Previous histochemical studies of the lung have suggested that the pentose cycle may be preferentially located in the granular pneumocyte [9, 10]. This localization is consistent with the functions of the granular pneumocyte in fatty acid and phospholipid synthesis [7] and in cytochrome P-450 linked mixed-function oxidations [25] since these reactions require NADPH. Our previous assays have indicated that glucose-6-phosphate dehydrogenase activity, the initial enzyme in the pentose phosphate cycle, is enriched approximately 2-fold compared with the whole lung (normalized for soluble protein or DNA) [11]. Based on the previously reported DNA content of lung cells [16], estimated pentose cycle flux in the granular pneumocyte is 1 μmole of CO_2 produced·hr^{-1}·(mg DNA)$^{-1}$. The previously reported value for pentose cycle flux in the control perfused rat lung is approximately 0.25 μmole CO_2 produced·hr^{-1}·(mg DNA)$^{-1}$ [1]. By this comparison, pentose cycle flux in granular pneumocytes is approximately four times greater than that for the average lung cell. However, the possibility cannot be excluded that metabolic properties of the granular pneumocytes are altered during primary culture. Comparison of the present results for granular pneumocytes with those previously obtained for the isolated perfused lung [1] indicate qualitatively similar responses to agents that stimulate or inhibit the pentose cycle, although the degree of stimulation with phenazine methosulfate and paraquat was much greater with the isolated cells.

We have demonstrated previously that paraquat is accumulated by isolated granular pneumocytes against a concentration gradient by a time-dependent process [26]. Therefore, we preincubated cells for 30 min prior to studying the effect of paraquat on granular pneumocyte metabolism. Based on previous results [26], the estimated paraquat concentration in the cell cytoplasm at the initiation of the metabolic measurements would have been approximately 1.5 mM, a value approximately ten times the measured K_m for paraquat-dependent NADPH oxidation. Consequently, the incubation conditions likely resulted in maximal effects due to paraquat which is compatible with the apparent maximal stimulation of measured pentose cycle activity. Stimulation of pentose cycle activity by paraquat has been demonstrated previously in lung slices [27] and perfused lung [14]. This study shows stimulation of the pentose cycle by paraquat in the granular pneumocytes, target cell for paraquat toxicity.

The mechanism for paraquat stimulation of the pentose cycle activity is likely related to the increased utilization of cytoplasmic reducing equivalents in the presence of this agent. Paraquat is reduced in an NADPH-requiring reaction that results in a product which can auto-oxidize in the presence of O_2 with the generation of superoxide anion [13]. Detoxification of superoxide or its products by reduction, mainly via glutathione-dependent reactions, further utilizes cytoplasmic reducing equivalents. This redox cycling has been demonstrated to cause a critical depletion of NADPH in the isolated perfused lung for a cytochrome P-450-dependent reaction [28] or in the alveolar macrophage for the respiratory burst [29]. The possibility that NADPH depletion by paraquat impairs critical function of the granular pneumocytes has not yet been evaluated with the isolated cells.

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