Regulation of Glycolytic Enzyme Activity during Chronic Hypoxia by Changes in Rate-limiting Enzyme Content

USE OF MONOCLONAL ANTIBODIES TO QUANTITATE CHANGES IN PYRUVATE KINASE CONTENT

ALLAN J. HANCE, EUGENE D. ROBIN, LAWRENCE M. SIMON, SVETLANA ALEXANDER, LEONORE A. HERZENBERG, and JAMES THEODORE, *Departments of Medicine and Physiology, Stanford University School of Medicine, Stanford, California 94305; Palo Alto Veterans Hospital, Palo Alto, California 94306*

A B S T R A C T   Monoclonal antibodies were prepared against pyruvate kinase (PyKi; ATP: pyruvate phosphotransferase, EC 2.7.1.40) and used to quantitate PyKi content in L2 lung cells and WI-38 fibroblasts cultivated under hypoxic and normoxic conditions. After 96 h of hypoxic cultivation, PyKi activity was significantly increased in both cell types (L2: normoxia [Po_2 = 142 torr], 0.11 ± 0.01 [SD]; hypoxia [Po_2 = 14 torr], 0.25 ± 0.04 U/μg DNA, $P < 0.01$). PyKi content increased proportionately in both cell lines (L2: normoxia, 0.44 ±0.13; hypoxia, 0.94 ± 0.13 μg enzyme protein/μg DNA). Specific activity was not significantly different after 96 h (L2: normoxia, 261 ± 11; hypoxia, 261 ± 14 U/mg enzyme protein). These results indicate that regulation of glycolysis during chronic hypoxia occurs at the level of enzyme content. Chronic O_2 depletion leads to either an increased rate of biosynthesis or a decreased rate of biodegradation of PyKi, causing augmented glycolytic capacity. Monoclonal antibodies provide a highly specific, convenient approach to characterizing enzymes, as well as quantitating cellular enzyme content.

INTRODUCTION

Chronic O_2 depletion is an important clinical problem, but the cellular mechanisms responsible for adaptation to chronic hypoxia are obscure. The increased rate of glycolysis following acute hypoxic exposure was first described by Pasteur. The Pasteur effect has since been demonstrated in a wide variety of tissues, and maximal response occurs rapidly after hypoxic exposure. It is generally accepted that the Pasteur effect is related to acute increases in intracellular phosphofructokinase (PFK;[1] ATP: *d*-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) activity resulting from changes in the concentration of low molecular weight allosteric regulators, although other alterations in enzyme activities participate (1, 2).

We have previously demonstrated that chronic hypoxic exposure of a variety of tissues and cell systems results in substantial additional increases in the rate of glycolysis beyond those produced by the Pasteur effect. The rate of glycolysis in peritoneal macrophages (ambient Po_2 chronically <15 torr) is three times greater than in alveolar macrophages (ambient Po_2 = 100 torr); when alveolar macrophages are maintained under hypoxic conditions in vitro for several days, their glycolytic capacity exceeds that of peritoneal macrophages (3–5). This stimulation of glycolysis by chronic hypoxic exposure may also explain the bioenergetic differences observed between pulmonary artery endothelial cells (Po_2 = 40 torr) and aortic endothelial cells (Po_2 = 100 torr), as well as the differences observed in fetal and neonatal brain (6, 7). Increased activity of rate-limiting glycolytic enzymes has also been found in fibroblast, endothelial, and kidney cell lines cultured under hypoxic conditions. Therefore, the phenomenon appears to be a common cellular response to O_2 depletion.

Two lines of evidence suggest that the stimulation of glycolysis by chronic hypoxic exposure operates by a mechanism distinct from the Pasteur effect. The increase in glycolysis following acute hypoxic exposure (Pasteur effect) occurs within minutes; the activity of PFK and pyruvate kinase (PyKi; ATP: pyruvate phosphotransferase, EC 2.7.1.40), two rate-limiting enzymes

---

[1] *Abbreviations used in this paper*: FCS, fetal calf serum; PBS, phosphate-buffered saline; PFK, phosphofructokinase; PyKi, pyruvate kinase.

in glycolysis, are unchanged when assayed under conditions of substrate excess and in which low molecular weight regulators are present in constant amount. In contrast, the further increase in glycolysis seen after chronic hypoxic exposure is first demonstrable after 24 h of exposure, and requires several days to produce maximal effects. After chronic hypoxic exposure, PFK and PyKi enzyme activities are increased when assayed under conditions of substrate excess and in which the concentrations of low molecular weight regulators are constant.

A number of possibilities can be advanced to explain the increased activity of key glycolytic enzymes that apparently results in the increased rate of glycolysis seen in chronically hypoxic cells: (a) The enzymes may be subject to post-translational modification such as phosphorylation. Under hypoxic conditions, a form with a higher $\dot{V}_{max}$ might exist. (b) An inhibitor of enzyme activity may be present under normoxic conditions that is removed by hypoxic exposure. (c) Previously unrecognized allosteric regulators may be present whose concentration is altered by hypoxic exposure. (d) Multiple isozymes of both PyKi and PFK exist; hypoxic exposure may result in expression of equal amounts of a different isozyme type with a higher $\dot{V}_{max}$. (e) Cellular content of PFK and PyKi might be increased.

To investigate the mechanism by which the activity of PyKi is increased following chronic hypoxic exposure, we determined the isozyme type and content of PyKi present in a cloned cell line derived from rat lung (L2) and in WI-38 fibroblasts following hypoxic or normoxic cultivation. The results show an increase in the intracellular content of PyKi, with no significant change in specific activity (PyKi activity/PyKi content) or isozyme type present after hypoxic cultivation. This suggests that the availability of molecular O_2 modulates the content of key glycolytic enzymes, which in turn regulate the generation of ATP through glycolysis.

METHODS

Cell culture. L2 clone derived from rat lung was obtained from Dr. William Douglas in 36th population doubling (8) and maintained in cell culture by using Ham's F-12 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Confluent plates were trypsinized and replated weekly with a 1:4 split, and experiments were performed on cells between the 40th and 55th population doubling.

WI-38 fibroblasts were obtained from the American Type Culture Collection, Rockville, Md., in 17th passage. Cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged with a 1:4 split on a weekly basis.

The NS-1 variant of the P3 cell line, originally derived from MOPC-21 mouse myeloma tumor (9), was maintained in RPMI-1640 containing 15% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium).

Enzyme assays. PyKi activity was measured by the assay of Valentine and Tanaka (10). In studies using L2, 1 U of activity is defined as the amount of enzyme required to consume 1 μmol NADH/min at 37°C. In studies using WI-38 fibroblasts, PyKi activity was measured at 22°C. PyKi isozymes were determined electrophoretically by the technique of Susor et al. (11).

Development of monoclonal antibodies. Monoclonal antibodies were developed using a modification of the technique of Köhler and Milstein (9, 12, 13). Three 6-wk-old BALB/c mice were immunized by intraperitoneal injection of 0.2 ml of water-in-oil suspension of complete Freund's adjuvant containing 1 mg of rabbit muscle PyKi. 3 wk later animals were boosted by intraperitoneal injection of 0.2 ml of phosphate-buffered saline (PBS) containing 0.025 mg rabbit muscle PyKi.

3 d after boosting, the mice were sacrificed, the spleens removed, and 1.6×10^8 spleenocytes fused with 0.4×10^8 NS-1 myeloma cells by gentle stirring in 1 ml polyethylene glycol-1500 as previously described (13). Cells were plated in 96-well culture dishes and hybrid cells selected on the basis of growth in HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine in complete medium).

Detection of hybrids secreting antipyruvate kinase antibody. Anti-PyKi antibody secretion was assayed by solid-phase radioimmunoassay on 96-well u-shaped microtiter plates (14). 30 μl of a 0.1-mg/ml solution of rabbit muscle PyKi in PBS was pipeted into each well and allowed to stand at room temperature for 1 h. Wells were emptied and rinsed three times with 0.25 ml PBS containing 1% bovine serum albumin and 0.02% sodium azide (radioimmunoassay buffer). 20 μl of spent medium from individual primary culture wells was pipetted into each well of the microtiter plate and reacted at room temperature for 1 h. Unbound antibody was removed and each well rinsed three times with 0.25 ml of radioimmunoassay buffer. Finally, 20 μl of RPMI 1640 medium containing ^{125}I-labeled anti-mouse immunoglobulin (Ig) allotype antibodies (10,000 cpm each anti-Ig-1a and anti-Ig-4a) (15) was added to each well and reacted for 1 h at room temperature. Unbound antibody was removed, and the wells rinsed three times with 0.25 ml of radioimmunoassay buffer. ^{125}I bound was determined by cutting out and counting each well in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Wells corresponding to primary culture wells secreting anti-PyKi antibodies bound ^{125}I in excess of 5–10 times background (50–200 cpm).

Cloning of antibody-producing cells. Contents of wells in the primary culture that produced anti-PyKi antibodies were transferred to 24-well tissue culture plates in final volume of 1 ml complete medium containing 5×10^6 thymocyte feeder cells. After 7–10 d growth in 24-well plates, cells were cloned using a fluorescence-activated cell sorter (FACS-II) (16). A single, large, viable cell from each culture was dispensed into each well of a 96-well culture plate containing 0.1 ml complete medium and 1×10^6 thymocytes. Cultures were maintained in log-phase growth (0.5–2 $\times 10^6$ cells/ml) and expanded by transferring sequentially into 24-well tissue culture plates (2 ml), T-25 flasks (5 ml), and finally T-75 flasks (20–50 ml).

Preparation of antibodies for radioimmunoassay. Purified monoclonal anti-Ig-1a and anti-Ig-4a were iodinated by a modification of the procedure of Fraker and Speck (17). Anti-PyKi monoclonal antibody was titered by using serial 1:2 dilutions of antibody (usually spent medium from cloned hybrid cells) in the solid-phase radioimmunoassay described below. A dilution of antibody resulting in 60–80% maximal binding of ^{125}I-anti-Ig-4a was used for quantitation of PyKi by radioimmunoassay (1:2 to 1:32 dilution for spent medium).

Preparation of solid-phase antibody. 5 mg of purified monoclonal antibody was dialyzed against 3 mM potassium phosphate buffer (pH 6.3) and coupled to 100 mg of $<10\text{-}\mu m$ diameter acrylamide beads (Immunobeads, Bio-Rad Laboratories, Richmond, Calif.) using 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide HCl (EDAC) according to the instructions provided by the manufacturer. The beads were washed extensively with PBS, 5 M guanidine-HCl in PBS (pH 7.2), and again in PBS, then resuspended in PBS at 10 mg beads/ml and stored at 4°C.

Quantitation of PyKi activity and content. PyKi in L2 was quantitated by a consumption radioimmunoassay. Confluent plates of cells were incubated under P_{O_2} 14 torr/P_{CO_2} 36 torr (hypoxia) or P_{O_2} 142 torr/P_{CO_2} 36 torr (normoxia) for 4 d.

For experiments on WI-38 fibroblasts, cells were trypsinized, plated at 500,000 cells/100-mm petri dish, and incubated under air/5% CO_2 for 2 d. Cells were then exposed to normoxia or hypoxia as described above for 96 h.

Cells were rinsed with 10 ml PBS and detached with a rubber policeman, and contents of the plates transferred to a test tube in a final volume of 1.0 ml. Contents were sonicated (Branson Sonicator, Branson Sonic Power Co., Danbury, Conn.; micro-tip; power setting = 3; 10 s) and centrifuged (10,000 g; 30 min). Aliquots of sonicate were assayed for DNA content (18). A 50-μl aliquot of the supernate was assayed for PyKi activity. No detectable PyKi activity was found in the pellet of cells cultivated under normoxic or hypoxic conditions, indicating that all PyKi activity was extracted and present in the supernate. To determine PyKi content, a series of 1:2 dilutions of supernate in RPMI-1640 between undiluted and 1:128 dilution was prepared. Rabbit muscle PyKi was used as a standard. A 25-μg/ml solution in PBS was prepared (concentration was determined spectrophotometrically) (19), and serial dilutions in RPMI-1640 containing 0.20–25 μg/ml were also prepared. 20-μl aliquots of serially diluted standard solutions or cell supernates were added to 20 μl of an appropriately diluted solution (see above) of anti-PyKi antibody (6B-H12) in a coated microtiter plate and incubated at 4°C overnight. Residual unbound antibody was assayed as described above. A standard curve was prepared (Fig. 2) and dilutions of L2 or WI-38 supernates resulting in binding values that fell on the linear portion of the standard curve were used to quantitate PyKi content. Each determination represents the average of two dilutions in the linear range, each assayed in duplicate.

Precipitation of radiolabeled proteins by anti-PyKi antibody and sodium dodecyl sulfate-acrylamide gel electrophoresis of immunoprecipitates. Newly synthesized proteins in L2 were radiolabeled by incubating a confluent plate in 5 ml of methionine-free Dulbecco's Modified Eagle's Medium containing 10% FCS and 60 μCi [^{35}S]methionine (1,050 Ci/mmol) for 15 h. Cells were rinsed, harvested in 0.5 ml PBS, sonicated, and centrifuged (10,000 g; 20 min).

100 μl of a solution containing 50 μg/ml anti-PyKi antibody (6B-H12) and 50 μg/ml BSA in PBS was placed in a well of a microtiter plate and allowed to stand at room temperature for 1 h (15). The solution was removed, and the well rinsed five times with 0.25 ml RIA buffer; 50 μl labeled cell supernate was added, and the solution allowed to stand for 1 h. Unbound radioactivity was removed by washing the well five times with radioimmunoassay buffer containing 0.3 M NaCl; the contents of the well (absorbed antibody and immunoprecipitated, labeled protein) were solubilized in 60 μl of 0.0625 M Tris–HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol, and boiled for 1 min. SDS-acrylamide gel electrophoresis was performed using the system of Laemmli (20) in 90 × 1.5-mm resolving slab gels (10% acrylamide/0.27% bisacrylamide), and fluorograms prepared by the method of Bonner and Laskey (21). Fluorograms were scanned at 560 nm.

Materials. Tissue culture media, FCS, antibiotics, pyruvate, and trypsin were obtained from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.; Freund's adjuvant from Difco Laboratories, Detroit, Mich.; rabbit muscle PyKi, hypoxanthine, aminopterin, bovine serum albumin, and thymidine from Sigma Chemical Co., St. Louis, Mo.; polyethylene glycol from BDH Chemicals, Poole, England; 24- and 96-well plastic tissue culture plates from Costar Data Packaging, Cambridge, Mass.; microtiter plates from Cooke Lab Products, Alexandria, Va.; T-flasks from Corning Glass Works, Science Products Div., Corning, N. Y.; EDAC from Bio-Rad Laboratories; Iodo-gen from Pierce Chemical Co., Rockford, Ill.; and [^{35}S]methionine from Amersham Corp., Arlington Heights, Ill.

Anti-mouse immunoglobulin allotype antibodies (15) and NS-1 cells were obtained from Leonard Herzenberg, Stanford, Calif.

RESULTS

Production of anti-PyKi antibodies. Two cell-fusion experiments were performed on spleen cells obtained from three mice; six 96-well plates were plated and cells subjected to hypoxanthine:aminopterin:thymidine medium selection. When tested after 3 wk of growth, medium from 11/576 cells was found to contain anti-PyKi antibody. All positive wells were producing a γGl(Ig-4a) antibody type. Cells in each positive well were transferred to 24-well tissue culture plates, and after an additional week of growth, single, viable cells from each culture were cloned into 96-well tissue culture plates. When the cloning plates showed vigorous growth, clones were tested for anti-PyKi antibody production. In 8 of 11 cloning plates, 3–24 of the 96 clones were producing anti-PyKi antibody. Selected clones from each plate were expanded, and anti-PyKi antibody produced from these cell lines were used in the experiments described below. No anti-PyKi antibody-producing clones were obtained from three of our positive primary wells, and these cell lines were discarded.

Specificity of anti-PyKi monoclonal antibodies. To demonstrate that the anti-PyKi antibody (6B-H12) used was capable of reacting with all cellular PyKi, the following studies were performed. L2 were homogenized, centrifuged at 10,000 g, and the supernate assayed for PyKi activity. Preincubation of aliquots of this supernate with increasing amounts (0.5–2 mg) of acrylamide beads covalently linked to anti-PyKi monoclonal antibody resulted in a progressive decrease of PyKi activity remaining in the supernate. Addition of excess beads (4 mg) completely eliminated PyKi activity in the supernate.

Labeled proteins immunoprecipitated from supernates of L2 by anti-PyKi antibody (6B-H12) were examined by SDS-acrylamide gel electrophoresis (Fig. 1). Greater than 95% of immunoprecipitated radioactivity migrated as a single sharp band with mobility identical
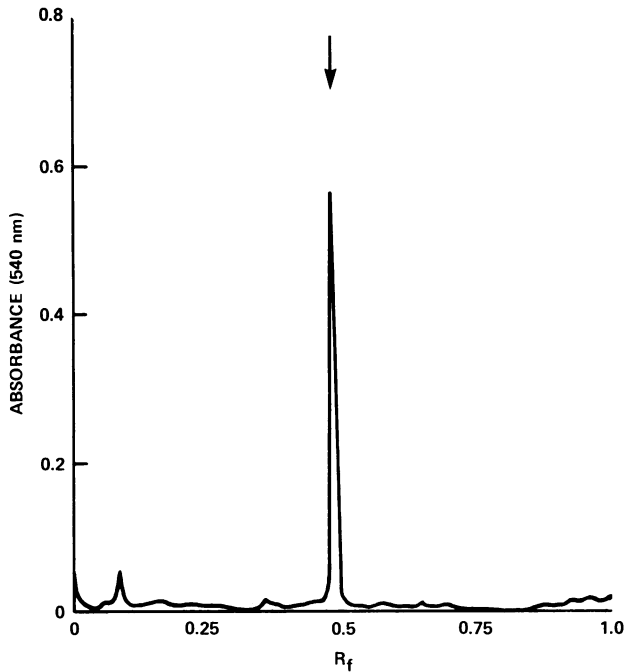
FIGURE 1 Electrophoresis of ^{35}S-labeled proteins from L2 precipitated by anti-PyKi antibody (6B-H12). L2 were incubated for 15 h in mediun containing 60 μCi ^{35}S, harvested, and sonicated, and immunoprecipitates prepared as described in Methods. The immunoprecipitates were electrophoresed, and fluorograms were prepared and scanned. R_f of 0 indicates top of gel and R_f of 1 corresponds to the migration of bromophenol blue dye. Arrow indicates migration of a rabbit muscle PyKi standard.
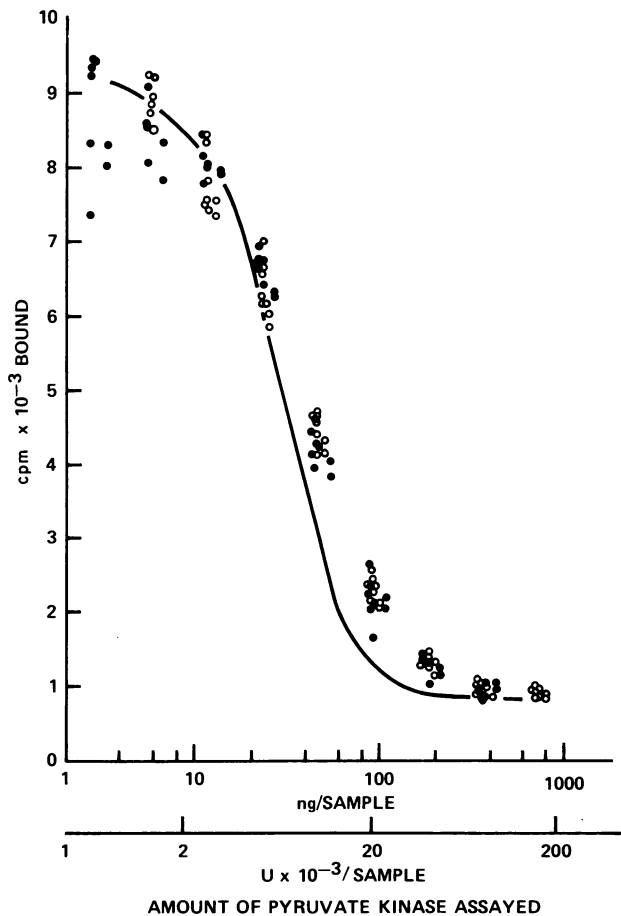

FIGURE 2 Standard curve for consumption radioimmunoassay of PyKi content. Varying amounts of rabbit muscle PyKi (solid line) and PyKi present in supernates from normoxically (filled circles) or hypoxically cultivated (open circles) L2 were incubated overnight with anti-PyKi antibody (6B-H12), and solutions assayed for residual antibody activity as described in Methods. Because the specific activity of the rabbit muscle and L2 PyKi were different, the abscissa indicating weight of sample (nanograms per sample) applies only to the rabbit muscle PyKi.

to that of a rabbit muscle PyKi standard. Thus, this antibody demonstrated high specificity toward PyKi.

The affinity of anti-PyKi monoclonal antibody 6B-H12 for rabbit muscle PyKi and PyKi present in the supernate of L2 are shown in Fig. 2. A fixed amount of antibody was preincubated with increasing amounts of rabbit muscle PyKi or supernate from L2, and residual anti-PyKi antibody was then assayed by solid-phase radioimmunoassay. Preincubation of antibody with 20 ng (0.0004 U) of rabbit muscle PyKi resulted in a 50% reduction in residual antibody activity; the linear portion of the standard curve ranged from 10 to 100 ng of PyKi.

The consumption of anti-PyKi antibody by PyKi present in supernates from L2 closely paralleled that produced by an equal amount of rabbit muscle PyKi enzyme activity (Fig. 2), although the curve is shifted slightly to the right (see below). These data also indicate that anti-PyKi antibody (6B-H12) has high specificity for PyKi. If supernates from L2 contained substances in sufficient quantity and high enough affinity to compete with PyKi for antibody consumption, the curve for supernates of L2 would be shifted to the left of that

obtained for highly purified rabbit muscle PyKi. In addition, the affinity of PyKi from L2 cultivated under normoxic conditions (filled circles) is similar to that of PyKi from cells grown under hypoxic conditions (open circles).

Effects of hypoxic exposure on PyKi activity and content in L2. Confluent plates of L2 were refed, incubated under P_{O_2} 142 torr/P_{CO_2} 36 torr or P_{O_2} 14 torr/ P_{CO_2} 36 torr for 4 d, then harvested, sonicated, and centrifuged. PyKi activity per cell in supernates from cultures grown under hypoxic conditions was more than double that of cells cultivated under normoxic conditions (0.11±0.01 [SD] and 0.25±0.04 U/μg DNA, respectively; $P < 0.01$), as we have previously

reported (4) (Fig. 3). Electrophoretic analysis demonstrated that both hypoxically and normoxically cultivated L2 contained only the M_2 isozyme of PyKi (data not shown).

PyKi content determined by consumption radioimmunoassay was also more than twice as high in hypoxically cultivated cells than in cells grown under normoxic conditions (0.94±0.13 [SD] and 0.44±0.01 μg enzyme protein/μg DNA, respectively; $P < 0.01$). Because the activity and content increased proportionately, the specific activity of the PyKi in L2 under the conditions of our assay was similar in normoxic- and hypoxic-exposed cells (261±11 and 261±14 U/mg enzyme protein, respectively; P not significant). Further, the specific activity of PyKi in L2 was similar to the specific activity of rabbit muscle standard (214 U/mg enzyme protein).

Effects of hypoxic exposure on PyKi activity and content in WI-38 human lung fibroblasts. In similar experiments, WI-38 fibroblasts were exposed to PO_2 142 torr/PCO_2 36 torr or PO_2 14 torr/PCO_2 36 torr for 96 h, and measurements of PyKi activity, content, and specific activity were made as described above. Hypoxic cultivation resulted in a twofold increase in PyKi activity compared with air-grown cells (0.147±0.019 and 0.079±0.002 U/μg DNA, respectively; $P < 0.01$). Enzyme content increased proportionately (hypoxic cultivation, 2.97±0.30 μg PyKi/μg DNA; normoxic cultivation, 1.58±0.08 μg PyKi/μg DNA; $P < 0.05$). Specific activity did not change significantly (hypoxia, 48.8 ±3.7; normoxia, 51.1±11.1). Specific activity of the rab-

FIGURE 3   Effects of hypoxic and normoxic cultivation on activity, content, and specific activity of PyKi in L2. L2 were cultivated under PO_2 142 torr/PCO_2 36 torr (normoxia, open bars) or PO_2 14 torr/PCO_2 36 torr (hypoxia, shaded bars) for 96 h and then assayed for enzyme activity (units per microgram DNA; left), enzyme content (micrograms PyKi per microgram DNA; center), and specific activity (units per milligram enzyme protein; right).

bit muscle standard assayed under these conditions was 53 U/mg protein.

DISCUSSION

The bioenergetic responses to chronic O_2 depletion are of obvious clinical and biologic importance. Chronic, but not acute, hypoxic cultivation of L2 and WI-38 fibroblasts results in an increase in PyKi activity measured under conditions of substrate excess and in which the concentrations of known allosteric regulators are constant. In our study, cellular PyKi enzyme content was found to increase proportionately, indicating that the increased enzyme activity observed in chronically hypoxic cells was entirely explained by an increase in enzyme content. In previous studies, we have shown that chronic hypoxic exposure of a wide variety of cell types is associated with increased PyKi and PFK activity. These increases in measured activity are associated with increased aerobic and anaerobic rates of glycolysis measured under standard conditions (3–5). Thus, the increases in enzyme activity and content appear to have functional significance. In cell-culture systems, maximal increases in activity and in content of PyKi and PFK require ~96 h of hypoxic cultivation, and thus this form of regulation may be regarded as a chronic, rather than acute, regulatory mechanism (such as the Pasteur effect). As there is an increase in enzyme content with hypoxic exposure, the changes must occur as a result of some combination of increased biosynthesis or decreased biodegradation of PyKi as a function of decreased O_2 availability. It appears that the activity of molecular O_2 regulates glycolytic rate by regulating the content of rate-limiting enzymes. This regulation appears to be a phenomenon found in cells generally, although judging by measurements of enzyme activity, a variety of cancer cells lack this form of regulation (22).

An important issue involves the relevance of these in vitro changes to bioenergetic regulation in vivo. Chronic hypoxic exposure of several intact cellular systems results in stimulation of glycolysis beyond that produced by acute hypoxia. We have shown that freshly obtained intimal strips from calf pulmonary artery (ambient $PO_2 \simeq 40$ torr) have higher activities of PyKi than aortic intimal strips (ambient $PO_2 \simeq 100$ torr) (6). Freshly harvested alveolar macrophages (ambient $PO_2 \simeq 100$ torr) have lower activities of PyKi and lower rates of lactate production than freshly harvested peritoneal macrophages (ambient $PO_2 < 15$ torr). Exposure of alveolar macrophages to low ambient PO_2 results in increased PyKi and PFK activity (3). These models may represent in vivo examples of chronic hypoxic exposure that results in increased content of key glycolytic enzymes and thereby stimulates glycolysis.

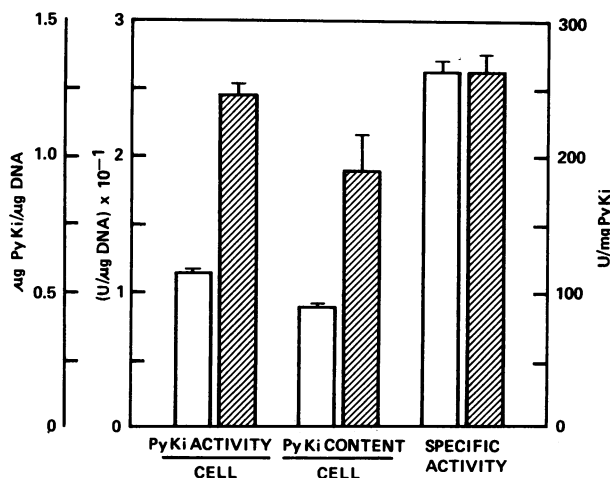Several limitations of the present study should be stressed. These studies do not investigate the precise

role of pyruvate kinase in regulation of glycolysis in hypoxic cells. Under a variety of conditions in vivo, the reactions catalyzed by PyKi and PFK are furthest from equilibrium, and presumably are rate limiting (23). Following acute hypoxic exposure, the activity of PyKi *in situ* increases with that of PFK because the increases in fructose 1,6-diphosphate levels, resulting from increased PFK activity, allosterically stimulate both M_2 and L PyKi isozyme types (23). Following chronic hypoxic exposure, PFK and PyKi activity also increase in tandem. Enzyme content of PyKi is increased in chronically hypoxic L2 and WI-38 fibroblasts. Presumably similar alterations in the content of PFK occur.

We used purified rabbit muscle PyKi as the antigen. This form of PyKi is the M_1 isozyme and thus is different from the M_2 PyKi found in the cell types studied. As a result, our estimates of content are in error by the differences in mass between the two purified enzymes. Other workers have reported molecular masses for both M_1 and M_2 isozymes of 210,000–250,000 daltons (19, 24–27). Rabbit muscle PyKi and PyKi precipitated from supernates of L2 by antibody covalently linked to acrylamide beads had identical mobility when examined by SDS-polyacrylamide gel electrophoresis; therefore, errors resulting from differences in molecular mass must be quite small.

In addition, if the affinity of our antibody for rabbit muscle PyKi and cellular PyKi differed, a systematic error would be introduced. As mentioned earlier, the consumption of antibody by equal activity of the two PyKi types was similar; however, the curve for L2 PyKi was shifted slightly to the right (Fig. 2). This finding is consistent with the interpretation that our antibody had a higher affinity for rabbit muscle PyKi (the original antigen) than for L2 PyKi. A more likely explanation is that the extensive purification procedure used for isolation of rabbit muscle PyKi resulted in inactivation of a fraction of enzyme that retained antigenic properties. In experiments quantitating PyKi content, therefore, we used a standard curve based on absorbance of a rabbit muscle PyKi solution, which would more closely reflect total enzyme present. The apparent specific activity of L2 PyKi determined by this assay (261 U/mg enzyme) was higher than that of rabbit muscle PyKi and similar to values reported by other workers. These considerations would not influence conclusions regarding the relative content of PyKi in hypoxic and normoxic cells, since affinity of PyKi for antibody was not influenced by hypoxic exposure.

We have not studied whether the increase in enzyme content is related to increased biosynthesis or decreased biodegradation, or have we studied the problem of how such alterations are produced by changes in O_2 availability. Both problems can be approached by using monoclonal antibodies.

The potential usefulness of monoclonal antibodies directed against enzymes has been indicated by Milstein (28). There are several advantages of monoclonal antibodies when compared with polyvalent antibody preparations conventionally employed in studies of this type. The specificity of the antibodies obtained (probably detecting a single antigenic determinant) is not dependent upon the purity of the antigen used for immunization, and therefore extensive purification of enzyme is not necessary for antibody production. Similarly, the need for absorption of antisera is obviated. The antibody can be produced in almost unlimited quantity, and cell lines or antibody can be made available to other workers.[2]

Despite these limitations, it is clear that chronic hypoxia regulates the rate of glycolysis by direct alteration of enzyme content. It appears likely that this mechanism is not only present in cell culture systems, but is a major determinant of the rate of glycolysis in a variety of in vivo systems as well.

ACKNOWLEDGMENTS

REFERENCES

1. Krebs, H. A. 1972. The Pasteur effect and relations between respiration and fermentation. *Essays Biochem.* **8:** 1–34.
2. Racker, E. 1980. From Pasteur to Mitchell: a hundred years of bioenergetics. *Fed. Proc.* **39:** 210–215.
3. Simon, L. M., E. D. Robin, J. R. Phillips, J. Acevedo, S. G. Axline, and J. Theodore. 1977. Enzymatic basis for bioenergetic differences of alveolar versus peritoneal macrophages and enzyme regulation by molecular O_2. *J. Clin. Invest.* **59:** 443–448.
4. Simon, L. M., E. D. Robin, T. A. Raffin, J. Theodore, and W. H. J. Douglas. 1978. Bioenergetic pattern of isolated Type II pneumocytes in air and during hypoxia. *J. Clin. Invest.* **61:** 1232–1239.
5. Simon, L. M., J. Theodore, and E. D. Robin. 1979. Regulation of biosynthesis/biodegradation of oxygen-related enzymes by molecular oxygen. In Extrapulmonary Manifestations of Pulmonary Disease. E. D. Robin, editor. Marcel Dekker, Inc., New York. 151–169.
6. Cummiskey, J. M., L. M. Simon, J. Theodore, U. Ryan, and E. D. Robin. 1979. The effects of variable oxygen tensions on bioenergetic enzymes of isolated pulmonary artery and aortic endothelial cells. *Clin. Res.* **27:** 396A.

---

[2] Investigators interested in obtaining anti-PyKi antibody should contact the authors.

7. Phillips, J. R., J. Theodore, and E. D. Robin. 1975. Comparative enzymatic maturation of postnatal rabbit brain and heart: relation to organ development and resistance to hypoxia. *Clin. Res.* **23:** 351A.

8. Douglas, W. H. J., and M. E. Kaighn. 1974. Clonal isolation of differentiated rat lung cells. *In Vitro.* **10:** 230–237.

9. Köhler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **6:** 511–519.

10. Valentine, W. N. and K. R. Tanaka. 1966. Pyruvate kinase: clinical aspects. *Methods Enzymol.* **9:** 469–473.

11. Susor, W. A., E. Penhot, and W. J. Rutter. 1975. Fructose-diphosphate aldolase, pyruvate kinase and puridine nucleotide-linked activities after electrophoresis. *Methods Enzymol.* **41:** 66–73.

12. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells. *Nature (Lond.).* **256:** 495–497.

13. Herzenberg, L. A., L. A. Herzenberg, and C. Milstein. 1978. Cell hybrids of myelomas with antibody forming cells and T-lymphomas with T cells. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 3rd edition. 25.1–25.7.

14. Tsu, T. T., and L. A. Herzenberg. 1979. Solid-phase radioimmune assays. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman & Company Publishers, San Francisco, Calif. 18.1–18.7.

15. Oi, V. T., and L. A. Herzenberg. 1979. Localization of murine Ig-1b and Ig-1a (IgG$_{2a}$) allotypic determinants detected with monoclonal antibodies. *Mol. Immunol.* **16:** 1005–1017.

16. Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 1962–1966.

17. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* **80:** 849–857.

18. Richards, G. M. 1974. Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal. Biochem.* **57:** 369–376.

19. Cardenas, J. M., R. D. Dyson, and J. J. Strandholm. 1973. Bovine pyruvate kinases. *J. Biol. Chem.* **248:** 6931–6937.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:** 680–685.

21. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:** 83–88.

22. Simon, L. M., E. D. Robin, T. A. Raffin, and J. Theodore. 1979. Differences in oxygen mediated enzyme regulation between tumor (fibrosarcoma) and normal (fibroblast) cells. *Clin. Res.* **27:** 493A.

23. Ramaiah, A. 1974. Pasteur effect and phosphofructokinase. *Curr. Top. Cell. Regul.* **8:** 297–345.

24. Imamura, K., K. Taniuchi, and T. Tanaka. 1972. Multimolecular forms of pyruvate kinase. *J. Biochem.* **72:** 1001–1015.

25. Saheki, S., K. Saheki, and T. Tanaka. 1978. Peptide mapping by limited proteolysis of four pyruvate kinase isozymes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **93:** 25–28.

26. Cottam, G. L., P. F. Hollenberg, and M. J. Coon. 1969. Subunit structure of rabbit muscle pyruvate kinase. *J. Biol. Chem.* **244:** 1481–1486.

27. Harkins, R. N., J. A. Black, and M. B. Rittenberg. 1977. M_2 isozyme of pyruvate kinase from human kidney as the product of a separate gene: its purification and characterization. *Biochemistry.* **16:** 3831–3837.

28. Milstein, C. 1978. Monoclonal antibodies: a new opening in biochemistry. Presented June 5, 1978, Federation of American Societies for Experimental Biology, Atlanta, Georgia.