Glutamine utilization by rat neutrophils: presence of phosphate-dependent glutaminase

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Glutamine utilization by rat neutrophils: presence of phosphate-dependent glutaminase. Am. J. Physiol. 273 (Cell Physiol. 42): C1124–C1129, 1997.—The capacity of rat neutrophils to utilize glutamine was investigated by 1) determination of oxygen consumption in the presence of glucose or glutamine, 2) measurement of maximal activity of phosphate-dependent glutaminase, 3) Northern blot, Western blot, and immunocytochemical detection of glutaminase, and 4) measurement of glutamine utilization and also production of ammonia, glutamate, aspartate, alanine, and lactate and decarboxylation of [U-14C]glutamine in cells incubated for 1 h. The rate of glutamine utilization was investigated by 1) determination of oxygen consumption in the presence of glucose or glutamine, 2) measurement of maximal activity of phosphate-dependent glutaminase, 3) Northern blot, Western blot, and immunocytochemical detection of glutaminase, and 4) measurement of glutamine utilization and also production of ammonia, glutamate, aspartate, alanine, and lactate and decarboxylation of [U-14C]glutamine in cells incubated for 1 h. The rate of glutamine utilization was investigated by 1) determination of oxygen consumption in the presence of glucose or glutamine, 2) measurement of maximal activity of phosphate-dependent glutaminase, 3) Northern blot, Western blot, and immunocytochemical detection of glutaminase, and 4) measurement of glutamine utilization and also production of ammonia, glutamate, aspartate, alanine, and lactate and decarboxylation of [U-14C]glutamine in cells incubated for 1 h. The rate of glutamine utilization was investigated by 1) determination of oxygen consumption in the presence of glucose or glutamine, 2) measurement of maximal activity of phosphate-dependent glutaminase, 3) Northern blot, Western blot, and immunocytochemical detection of glutaminase, and 4) measurement of glutamine utilization and also production of ammonia, glutamate, aspartate, alanine, and lactate and decarboxylation of [U-14C]glutamine in cells incubated for 1 h.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 180 g (~2 mo of age) were obtained from the Biomedical Institute (University of São Paulo, São Paulo, Brazil). The rats were maintained at 23°C under a 12:12-h light-dark cycle.

Several studies have shown that the rate of glutamine utilization by lymphocytes and macrophages is similar to or greater than the rate of glucose utilization. Neither glutamine nor glucose is fully oxidized by these cells. Glucose is mainly converted to lactate and most of the glutamine to glutamate, lactate, and aspartate (8, 9). A high rate of glutamine utilization, but only partial oxidation, is also characteristic of other cells (e.g., enterocytes, thymocytes, fibroblasts, and tumor cells) (22). Glutamine is an important precursor of nucleotides for RNA and DNA synthesis, and it has been recognized that this amino acid plays a key role in cell proliferation. Macrophages are terminally differentiated cells, but they also utilize glutamine at high rates (24). In these cells, glutamine has been assumed to take part in cytokine production (26). However, the significance of glutamine metabolism in macrophages remains to be established.

Neutrophils act as first-line-of-defense cells in the plasma and undergo phagocytosis alone or in cooperation with antigen-specific defenses. Neutrophils contain a characteristic lobulated chromatin-dense nucleus, which has given rise to the term polymorphonuclear leukocyte, and are 9–12 μm in diameter. At any time, >90% of the neutrophil population is located as newly differentiated cells within the bone marrow. The remaining neutrophils are distributed between the circulation and the vascular endothelium, where they are attached to marginated pools or located within specific tissues.

Along with the onset of phagocytosis of bacteria or tissue fragments by neutrophils, a number of different cellular processes, including motility, respiratory burst, and secretion of cytoplasmic (proteolytic) enzymes and immunomodulatory compounds, are initiated. The combination of these processes assists in the killing and digestion of the engulfed bacteria and, if prolonged, the development of a local inflammation. Increase of respiratory burst involves a sudden stimulus-induced increase in nonmitochondrial oxidative metabolism, which results in the production of the superoxide anion and associated reactive oxygen species (27). The processes of endocytosis, secretion of active compounds, and generation of reactive oxygen species have been assumed to be mostly dependent on glutamine metabolism in neutrophils (28). The rate of glutamine utilization was investigated by determination of 1) oxygen consumption in the presence of glucose or glutamine, 2) maximal activity and the Michaelis-Menten constant (Km) of phosphate-dependent glutaminase, 3) Northern blot, immunocytochemical detection, and Western blot of this enzyme, 4) inhibition of this enzyme activity by glutamate, oxoglutarate, and NH4Cl, and 5) glutamine utilization and production of ammonia, glutamate, aspartate, alanine, and lactate and decarboxylation of [U-14C]glutamine in cells incubated for 1 h.
Chemicals and enzymes. All chemicals and enzymes were obtained from Sigma Chemical (St. Louis, MO). [U-14C]glucose (295 mCi/mmol) and [U-14C]glutamine (229 mCi/mmol) were purchased from Amersham International (Buckinghamshire, UK).

Peritoneal neutrophil preparation. The cells were obtained from 130- to 150-g Wistar rats killed by decapitation without anesthesia. Neutrophils were obtained by intraperitoneal lavage with 40 ml of sterile phosphate-buffered saline (PBS) 4 h after the intraperitoneal injection of 20 ml of 1% sterile oyster glycogen solution (type II, Sigma Chemical) in PBS. The cells were centrifuged (850 g for 8 min) three times in PBS. The number of viable cells, >95% neutrophils, was always counted in a Neubauer chamber using optical microscopy and 1% trypan blue solution.

Oxygen consumption. Oxygen consumption was monitored in an oxygen monitor (model 5300, Yellow Springs Instruments) for 10 min. In these experiments, 5 × 10⁶ cells/ml in PBS at 37°C in a final volume of 3.0 ml were always used. Oxygen consumption was measured in freshly obtained cells and neutrophils incubated for 18 h in PBS containing 2% defatted bovine serum albumin (BSA). The viability of the neutrophils incubated under these conditions was always >95%, as indicated by exclusion of trypan blue. The oxygen consumption procedure. Neutrophils were incubated (1.0 × 10⁶ cells/flask) at 37°C in 1 ml of Krebs-Ringer medium with 2% (wt/vol) defatted BSA in the presence of glucose (5 mM) or glutamine (2 mM). After 1 h of incubation, the cells were disrupted by addition of 0.2 ml of 25% (wt/vol) perchloric acid solution. The labeled CO2 was collected over 1 h in 1:1 (vol/vol) phenylethylamine-methanol, and the radioactivity was counted in a liquid scintillation counter (Beckman-LS 5000 TD, Beckman Instruments, Fullerton, CA).

Metabolite measurements. Neutralized samples of the incubation medium were used for measurements of glucose (3), glutamine (33), ammonia (33), glutamate (5), aspartate (4), alanine (32), and lactate (14). The content of glutamine and glutamate in the cells freshly obtained from the rats was also determined using the same methods. The production of NADH or NADPH was monitored at 340 nm using a recording spectrophotometer (Gilford Response).

Glutaminase assay. For the measurement of phosphate-dependent glutaminase activity in neutrophils, the cells were obtained by centrifugation at 4°C and then homogenized in an extraction medium containing 150 mM potassium phosphate, 1 mM EDTA, and 50 mM Tris·HCl at pH 8.6. Phosphate-dependent glutaminase (EC 3.5.1.2) was assayed as described by Currey and Lowry (11). The assay medium consisted of 50 mM phosphate buffer, 0.2 mM EDTA, 50 mM Tris·HCl, 20 mM glutamine, and 0.05% (vol/vol) Triton X-100, to which 100 µl of homogenate were added. The total volume was 1.0 ml at pH 8.6. Assay media, in duplicate, were incubated at 37°C.

The reaction was initiated by the addition of freshly prepared glutamine, promoting a 10-min linear reaction time course. The reaction was stopped by addition of 0.2 ml of 25% (wt/vol) perchloric acid solution and then neutralized. The amount of glutamate was determined as described by Bernt and Bergmeyer (5) at 340 nm in a Gilford Response spectrophotometer. The Kₘ of glutaminase was determined using 0.1, 0.5, 1.0, and 2.0 mM glutamine. The inhibiting effect of 2.0 mM oxoglutarate, 2.0 mM NH₄Cl, and 2.0 mM succinate on neutrophil glutaminase activity was also tested.

Assays of hexokinase, citrate synthase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase. The activities of hexokinase (EC 2.7.1.1), citrate synthase (EC 4.1.3.7), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were determined as previously described (10, 24). The extraction medium for hexokinase contained 50 mM Tris·HCl, 1 mM EDTA, 30 mM MgCl₂, and 20 mM β-mercaptoethanol at pH 7.4. The extraction medium for citrate synthase and glucose-6-phosphate dehydrogenase contained 50 mM Tris·HCl and 1 mM EDTA; the final pH values were 7.4 and 8.0, respectively. The extraction medium for lactate dehydrogenase consisted of 10 mM phosphate buffer at pH 7.4. The activity of lactate dehydrogenase was determined as previously described (10). For all enzyme assays, 0.05% (vol/vol) Triton X-100 was added to the assay system to complete the extraction of the enzyme.

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Western blot analysis. Immunoblot analysis was carried out essentially as described by Towbin et al. (31). The samples containing 50 µg of neutrophil protein and 10 µg of brain protein were subjected to electrophoresis on a preparative 10% (wt/vol) polyacrylamide slab gel in the presence of sodium dodecyl sulfate (18) and then transferred to nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) skim milk in Tris-buffered saline containing 0.1% (wt/vol) Tween 20 and then incubated with the primary antibody. The blots were then washed with Tris-buffered saline containing 0.1% (wt/vol) Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody. The blots were then washed with Tris-buffered saline containing 0.1% (wt/vol) Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody. The blots were then washed with Tris-buffered saline containing 0.1% (wt/vol) Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody.

Table 1. O₂ consumption by isolated neutrophils

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fresh cells</th>
<th>After 18 h in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>299.3 ± 25.1</td>
<td>336.6 ± 23.5</td>
</tr>
<tr>
<td>Glucose (5 mM)</td>
<td>328.2 ± 22.4</td>
<td>553.5 ± 19.3*</td>
</tr>
<tr>
<td>Glutamine (2 mM)</td>
<td>328.4 ± 25.2</td>
<td>548.0 ± 26.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 separate experiments. Neutrophils isolated from cavity of rats were incubated in the presence of glucose or glutamine. PBS, phosphate-buffered saline. *P < 0.05 compared with control.

Table 2. Maximal activities of phosphate-dependent glutaminase, HK, CS, G6PDH, and LDH of rat neutrophils

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Maximal Activity, nmol·min⁻¹·mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaminase</td>
<td>56.0 ± 1.4</td>
</tr>
<tr>
<td>HK</td>
<td>33.0 ± 0.6</td>
</tr>
<tr>
<td>CS</td>
<td>45.0 ± 5.0</td>
</tr>
<tr>
<td>G6PDH</td>
<td>68.0 ± 4.0</td>
</tr>
<tr>
<td>LDH</td>
<td>880.0 ± 22.0</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 separate experiments. Cells were obtained by lavage of intraperitoneal cavity 4 h after intraperitoneal injection of 1% glycogen solution. Enzyme activities were determined immediately after collection of the cells. HK, hexokinase; CS, citrate synthase; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase.
lose. After blocking with 20 mM Tris·HCl-500 mM NaCl, pH 7.4, containing 5% defatted milk (wt/vol), the nitrocellulose was incubated with affinity-purified antiglutaminase antibodies (gift from Dr. Norman Curthoys, Dept. of Biochemistry and Molecular Biology, Colorado State University, Ft. Collins, CO) (30). It was then incubated with a goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase conjugate and revealed by using the enhanced chemiluminescence detection system (Amersham Life Science).

Immunocytochemical analysis. Neutrophils were spread on clean, grease-free slide fixative solution (60% methanol-30% chloroform-10% acetic acid) at 4°C for 5 min. Slides were washed twice for 2 min in PBS and treated for 15 min with 0.3% H₂O₂ in PBS to block endogenous peroxidases. The slides were washed with PBS and incubated with goat serum in BSA-PBS for 30 min. Then the preparation was incubated overnight with affinity-purified antiglutaminase antibodies (1:100). The primary antibody was omitted in the control. Slides were washed three times for 10 min in PBS and incubated with goat anti-rabbit IgG-peroxidase conjugate (1:2,000) for 2 h at room temperature. After they were washed.

**Fig. 1.** Western blot analysis of phosphate-dependent glutaminase protein in rat neutrophils; 50 µg of protein were loaded in each lane. Bottom arrow, 65-kDa phosphate-dependent glutaminase protein. Lane a, rat brain; lanes b and c, rat neutrophils.

**Fig. 2.** Immunohistochemical analysis of neutrophils for phosphate-dependent kidney-type glutaminase. A: cells were immunostained with glutaminase primary antibody and goat anti-rabbit immunoglobulin G-peroxidase conjugate as secondary antibody. B: control; cells were incubated with secondary antibody only. Magnification ×1,100.
Fig. 3. Northern blot analysis of phosphate-dependent glutaminase mRNA in rat neutrophils. Arrow, 6.0-kb phosphate-dependent glutaminase mRNA. Lanes, a, rat kidney; lane b, rat neutrophils.

in PBS, the slides were incubated with diaminobenzidine-
H2O2 (Sigma Chemical) for 5 min at room temperature. Slides were washed in PBS for 10 min before they were stained with Mayer's hematoxylin for a few seconds.

Northern analysis. Total RNA was isolated from rat neutrophils using Trizol solution (GIBCO, Gaithersburg, MD). Aliquots containing 30 µg of the isolated RNAs were fractionated using Trizol solution (GIBCO, Gaithersburg, MD). Aliquots containing 30 µg of the isolated RNAs were fractionated by electrophoresis on a 1% agarose gel containing 3% formaldehyde with the DNA probes that were labeled (2 × 10^6 counts·min⁻¹·ml⁻¹) by random priming (15). The hybridized filters were exposed to Hyperfilm MP (Amersham Life Science). The results of glutaminase mRNA from neutrophils were compared with results from kidney, which are well established (17).

cDNA glutaminase probe. cDNA of phosphate-dependent glutaminase (2) was kindly provided by Dr. Norman P. Curthoys.

Protein determination. Protein content of neutrophils was measured by the method of Lowry et al. (19) using BSA as standard. The total protein content of neutrophils obtained under the conditions of this study was 0.64 mg/10^7 cells.

Expression of results and statistical analysis. The enzyme activities are expressed as nanomoles per minute per milligram of protein. The consumption of glucose and glutamine, the production of ammonia, glutamate, aspartate, alanine, and lactate from glutamine and lactate from glucose, and the decarboxylation of \([U-14C]\)glucose and \([U-14C]\)glutamine of neutrophils incubated for 1 h are expressed as nanomoles per hour per milligram of protein. Analysis of the significance of differences using a standard Student's t-test for P < 0.05 is given in Table 1.

RESULTS AND DISCUSSION

The rate of respiration by isolated neutrophils in the absence of added substrate was 5.0 nmol·min⁻¹·10^7 cells⁻¹, which was linear with respect to time for 1 h (results not shown). This rate is higher than that reported for lymphocytes and macrophages (21, 22). Incubation of neutrophils for 18 h in PBS did not modify the rate of oxygen consumption from endogenous substrate (Table 1). This finding may indicate that the intracellular concentration of metabolites in rat neutrophils is very high. Indeed, the intracellular concentrations of glutamine and glutamate in freshly obtained neutrophils were 12.9 and 17.5 mM, respectively (data not shown). The addition of 5 mM glucose to the incubation medium slightly raised oxygen consumption in freshly obtained cells but provoked a marked increase in neutrophils cultured for 18 h (64%). Similarly, the addition of glutamine also raised oxygen consumption in neutrophils cultured for 18 h (61%).

Maximal activity of phosphate-dependent glutaminase in neutrophils was 56 nmol·min⁻¹·mg protein⁻¹ in freshly obtained cells (Table 2). This glutaminase activity was higher than that reported for lymphocytes and macrophages (6, 22). The activity of glutaminase in neutrophils was comparable to that of hexokinase, citrate synthase, and glucose-6-phosphate dehydrogenase and much lower than that of lactate dehydrogenase. The differences may be partially a consequence of the temperature of the enzyme assays: 37°C for glutaminase and 25°C for the others.

The presence of glutaminase protein in neutrophils was confirmed by Western blot (Fig. 1) and immunocytochemical detection (Fig. 2). Rat neutrophils exhibited a positive immune reaction to a 65-kDa protein similar to that found in rat brain (16). The presence of the glutaminase mRNA in neutrophils was also evaluated by Northern blot (Fig. 3). Similar to results reported for kidney (29), neutrophils presented a significant amount of mRNA (6.0 kb) for glutaminase. The K₉ of neutrophil glutaminase for glutamine was determined to be 3.5 mM (data not shown). Addition of glutamate to the glutaminase assay inhibited this enzyme activity: 28% at 0.2 mM, 34% at 0.5 mM, and 70% at 2.0 mM. In addition to glutamate, other metabolites (at 2 mM) also inhibited this enzyme activity: 50% for 2-oxoglutarate, 32% for NH₄Cl, and 13% for succinate, as reported by Ardawi and Newsholme (1) for lymphocytes. These findings provide further evidence that the phosphate-dependent glutaminase found in rat neutrophils is a kidney-type glutaminase (12).

Glutamine was utilized by 1-h-incubated neutrophils at a rate of 12.8 nmol·min⁻¹·mg protein⁻¹ when added to the medium at 2 mM, which is three to four times higher than the physiological concentration (Table 3).

Table 3. Utilization of glucose and glutamine, production of lactate, glutamate, aspartate, alanine, and ammonia, and decarboxylation of \([U-14C]\)glucose and \([U-14C]\)glutamine in 1-h-incubated neutrophils

<table>
<thead>
<tr>
<th>Utilization/Production of</th>
<th>Glucose</th>
<th>Glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>550.0±20.0</td>
<td>320.0±23.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>250.0±15.0</td>
<td>68.0±14.0</td>
</tr>
<tr>
<td>Aspartate</td>
<td>13.5±2.0</td>
<td>430.0±39.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.4±0.1</td>
<td>6.5±0.8</td>
</tr>
<tr>
<td>Ammonia</td>
<td>460.0±100.0</td>
<td>770.0±120.0</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 incubations expressed as nmol·h⁻¹·mg protein⁻¹. Cells were obtained by lavage of intraperitoneal cavity of rats 4 h after an intraperitoneal injection of 1% oyster glucogen solution. Consumption and production of metabolites were determined after 1 h of incubation.

Table 2. Utilization of glucose and glutamine, production of lactate, glutamate, aspartate, alanine, and ammonia, and decarboxylation of \([U-14C]\)glucose and \([U-14C]\)glutamine in 1-h-incubated neutrophils

<table>
<thead>
<tr>
<th>Glucose</th>
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<tr>
<td>Lactate</td>
<td>550.0±20.0</td>
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</tr>
<tr>
<td>Alanine</td>
<td>2.4±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE of 7 incubations expressed as nmol·h⁻¹·mg protein⁻¹. Cells were obtained by lavage of intraperitoneal cavity of rats 4 h after an intraperitoneal injection of 1% oyster glucogen solution. Consumption and production of metabolites were determined after 1 h of incubation.
In the presence of 0.5 mM glutamine, the amino acid was utilized at a rate of 2.9 ± 0.1 (SE) nmol·min⁻¹·mg protein⁻¹ (n = 5 incubations). The addition of 0.5 mM glutamate to the incubation medium reduced glutamine utilization by neutrophils by ~70%; 0.9 ± 0.4 (SE) nmol·min⁻¹·mg protein⁻¹ (n = 5 incubations). Therefore, the discrepancy between the capacity to hydrolyze glutamine and glutamine utilized was possibly due to the suppression caused by the intracellular concentration of glutamate. Also, these findings led us to speculate that the inhibition of glutamine utilization by extracellular glutamate may occur in neutrophils under certain pathophysiological conditions, such as acquired immune deficiency syndrome (13).

Glucose was utilized at 7.7 nmol·min⁻¹·mg protein⁻¹ when cells were incubated in 5 mM glucose. The conversion of glucose to lactate was high (60% of the amount utilized), whereas [U-¹⁴C]glutamine to ¹⁴CO₂ was very low: ~1% was totally oxidized. The formation of ammonia was ~27% of glutamine utilization in neutrophils. The conversion of glutamine to glutamate, aspartate, alanine, and lactate accounted for ~84.6% of the total amino acid utilized by these cells.

Glutamine supplementation improves survival rates in septic mice and prevents sepsis-induced multiple system organ failure (20). A complex interrelationship in septic mice and prevents sepsis-induced multiple system organ failure (20). A complex interrelationship exists among infection, endotoxin, cytokines, and inter-organ glutamine metabolism. The utilization of this amino acid by immune cells has been assumed to play an important role in the function of the immune system (21, 23). In fact, glutamine is actively utilized by lymphocytes and macrophages (22). In this study, evidence is presented that neutrophils also utilize glutamine at significant rates. Ogle et al. (25) showed that glutamine enhances the bactericidal function of neutrophils from normal subjects and burn patients. Further studies are necessary to fully address the significance of glutamine metabolism for neutrophil function.

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