Insulin and Epidermal Growth Factor Stimulate Glycolysis in Quiescent 3T3 Fibroblasts with no Changes in Key Glycolytic Enzyme Activities

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Abstract

In order to study the effect of insulin and epidermal growth factor (EGF) on glycolysis in quiescent 3T3 fibroblasts and their mechanisms of action, lactic acid produced by cells and activities of key glycolytic enzymes in cell extracts were determined. Insulin increased lactic acid production; the maximal stimulation occurred at the concentrations above 250 ng/ml and the half-maximal dose was 50 ng/ml. This effect of insulin appeared as early as one hour, and lactic acid production in the presence of insulin linearly increased up to 4 h. The 24-h pretreatment with insulin exhibited no significant effect on the production by cells afterward incubated either with or without insulin. Lactic acid production decreased as the concentration of phloridzin increased. However, insulin stimulation of the production still remained in the presence of phloridzin. Parahydroxymercuribenzoate reduced production only by the equivalent of the increase due to insulin. EGF also increased lactic acid production; this effect occurred at 1 ng/ml and was maximal at 100 ng/ml. The activities of hexokinase, phosphofructokinase and pyruvate kinase in quiescent cells were not increased by insulin, and the affinities for substrates of these enzymes remained unaltered. These findings suggest that glucose uptake is a rate-limiting step in glycolysis in quiescent 3T3 fibroblasts and that the stimulatory effect of insulin on glycolysis is mediated by enhanced glucose entry.
creased glucose uptake, but not with tumorigenicity.

With chick embryo fibroblasts, there have also been confusing observations on the mechanism of the stimulation of glycolysis. An activation of PFK after the addition of serum or after an increase in the pH of the medium in quiescent cultures of chick cells (Fodge and Rubin, 1973) and after virus-transformation (Singh et al., 1974) were reported in literature. On the other hand, Bissell (1973, 1976) and Kletzien and Perdue (1974) demonstrated that the rate of glucose uptake was the rate-limiting factor in glycolysis for cells in culture after transformation.

Many investigations were carried out under common conditions where cultured cells were transformed or proliferating after the addition of growth stimulants, and there were few experiments where cells were firmly arrested. This study was undertaken using quiescent cultures of untransformed 3T3 fibroblasts to examine the effects of insulin and EGF on glycolysis and the mechanisms of their actions. In this paper, we report the finding that the stimulated glycolysis in quiescent cells was probably due to increased glucose entry, and also that activities of key glycolytic enzymes remained unaltered under these conditions.

**Materials and Methods**

**Materials**

Materials were obtained from the following sources: Tissue-culture media from GIBCO (Gland Island, N.Y., USA) and the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan); fetal bovine serum from Flow Lab. (Rockville, Md., USA); \(^{131}\)I-labeled insulin (specific activity, 105 \(\mu Ci/\mu g\)) from New England Nuclear (Boston, Mass., USA); EGF from Collaborative Research (Waltham, Mass., USA); all enzymes from Boehringer (Mannheim, W.-Germany). Monocomponent porcine insulin was kindly supplied by Novo Industri A/S (Copenhagen, Denmark). Other reagents were analytical grade.

**Cell culture**

Swiss mouse 3T3 fibroblasts (Todaro and Green, 1963) purchased from American Type Culture Collection (Rockville, Md., USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin and 100 \(\mu g/ml\) streptomycin, and supplemented with 10% fetal bovine serum and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.3. Cells were subcultured every third day in 60-mm Corning tissue culture dishes with a 0.1% trypsin and 0.012% EDTA solution in calcium- and magnesium-free Dulbecco's phosphate buffered saline, and were grown at 37°C in a humidified atmosphere of 10% CO\(_2\) and 90% air. The growth medium was changed after 2 days.

**Measurement of lactic acid production**

After cells were inoculated in 60-mm dishes, the growth medium was changed twice at an interval of 2 days. The confluent, density-inhibited cultures of cells (approximately 2-3 \(\times\) 10\(^6\) cells/60-mm dish) were used 4-6 days after the second medium change for experiments on lactic acid production. To ensure that the cells would be arrested, the conditioned medium in which the cells were growing was diluted with serum-free DMEM to give a final serum concentration of 0.5% 20-30 h before experiments. In this procedure, insulin was added to the diluted conditioned medium in some experiments (insulin-pretreated).

At the beginning of experiments, the confluent cultures of cells were washed twice with "pyruvate-free" DMEM which contained 450 mg/dl glucose and was supplemented with 0.1% bovine serum albumin (fraction V, Armour Pharmaceutical Co., Phoenix, Ariz., USA) and 20 mM HEPES buffer (pH 7.3). The cells in 60-mm dishes were incubated in 1.5 ml of this medium containing insulin and/or other test materials at 37°C in an atmosphere of 10% CO\(_2\) and 90% air. At the end of incubation, 1 ml of the medium was immediately mixed with 0.2 ml of 20% perchloric acid and then centrifuged. The supernatant was neutralized with 5 M K\(_2\)CO\(_3\) and processed for enzymatic determination of lactic acid by a modification of the method of Gut-
mann and Wahlefeld (1974).

After removal of the medium, the cells were washed three times with 0.9% NaCl and dissolved in 1 N NaOH. The solution was processed by the method of Lowry et al. (1951) for the determination of protein content.

Preparation of cell-free extract

After pretreatment with the diluted conditioned medium for 20–30 h, confluent cultures of cells in 100-mm dishes were washed twice with serum-free DMEM containing 100 mg/dl glucose, 0.1% bovine serum albumin and 20 mM HEPES buffer (pH 7.3), and incubated in 5 ml of serum-free medium with or without insulin at 37°C in a humidified CO₂ incubator. After 3 h incubation, aliquots of the medium were removed and processed for the measurement of lactic acid produced. The cells were washed twice with ice-cold 0.9% NaCl, scraped off into tubes with a rubber policeman, and centrifuged at 750 × g at 4°C for 5 min. The cell pellets were suspended in an ice-cold glycyglycine buffer (50 mM glycyglycine, pH 8.0; 5 mM EDTA; 5 mM dithiothreitol; 25 mM NaF), and disrupted by sonication with a Sonifier B-12 (Branson Sonic Power Co., Danbury, Conn., USA) at a dial setting of 3 for 20 sec. The homogenates were centrifuged at 15,000 × g at 4°C for 30 min, and the supernatants were used for the enzyme assays.

Assays of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK)

Enzyme activities were determined spectrophotometrically at 25°C by measuring the rate of appearance of NADPH or disappearance of NADH at 340 nm. HK was assayed by the method of Joshi and Jagannathan (1966). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM NADP, 1 mM ATP, 5 mM glucose and 2 units of glucose-6-phosphate dehydrogenase in a final volume of 1 ml. The standard assay of PFK was done as described previously (Kono, Uyeda and Oliver, 1973). The reaction mixture contained 50 mM glycyglycine (pH 8.1), 5 mM MgCl₂, 5 mM EDTA, 5 mM dithiothreitol, 10 mM ammonium sulfate, 0.2 mM NADH, 1 mM ATP, 1.7 mM fructose 6-phosphate (F6P) and auxiliary enzymes (0.4 unit of aldolase, 2 units of triosephosphate isomerase and 0.8 unit of α-glycerophosphate dehydrogenase) in a final volume of 1 ml. PK was assayed by the method of Bücher and Pfeiderer (1955). The reaction mixture of the standard assay contained 50 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 75 mM KCl, 0.2 mM NADH, 1.5 mM ADP, 2.5 mM phosphoenol pyruvate and 2 units of lactate dehydrogenase in a final volume of 1 ml. One unit of activity is defined as the amount of the enzyme that catalyzes the formation of 1 μmol of the product per min.

Insulin degradation

Degradation of insulin was determined by the method of Olefsky and Reaven (1974).

Statistical analysis

Student’s unpaired t-test was used for statistical analysis.

Results

Effects of insulin and EGF on lactic acid production by confluent and serum-starved 3T3 fibroblasts.

When serum-starved fibroblasts were incubated for 3 h, insulin stimulated lactic acid production. This effect was maximal (1.6-fold) at concentrations above 250 ng/ml. The half-maximal dose was 50 ng/ml (Fig. 1). The time courses of lactic acid production are depicted in Fig. 2. Lactic acid produced both in the presence and absence of insulin (500 ng/ml) almost linearly increased with incubation time up to 4 h (Fig. 2A). Insulin stimulation of the production appeared in the first hour of incubation (1.81-fold) and was slightly augmented afterward (2.11- to 2.13-fold). To examine the effect of prolonged exposure of fibroblasts to insulin, the cells were pretreated in the serum-starved medium containing 600 ng/ml of insulin for 24 h, and then incubated with or without 500 ng/ml of insulin for 1–4 h (Fig. 2B). Time courses of lactic acid production by insulin-pretreated cells both in the presence and absence of insulin were quite similar to those shown in Fig. 2A, indicating that 24-h insulin pretreatment of
cells had no effect on lactic acid production. Insulin degradation, determined by measuring trichloroacetic acid-unprecipitable radioactivity in the medium, was about 10% and 50% in 4 h and 24 h, respectively. These findings indicate that the stimulatory effect of insulin on lactic acid production disappeared immediately after removing insulin from the medium and was not enhanced by prolonged exposure for 24 h or more.

To study whether glycolysis stimulated by insulin bore some relation to glucose transport through cell membrane, the effects of inhibitors of glucose transport on lactic acid production were examined (Table 1). Phloridzin at 1–3 mM, a competitive inhibitor of sugar transport, added to the incubation medium significantly decreased lactic acid produced in 3 h (p < 0.05 at 1 mM and p < 0.001 at 3 mM) both with and without insulin. This inhibition was proportional to the concentration. However, insulin stimulation still remained at any concentration of phloridzin. The addition of 0.1–0.25 mM parahydroxymercuri-
Table 1. Effects of phloridzin and parahydroxymercuribenzoate (PHMB) on lactic acid production by serum-starved 3T3 cells.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Lactic acid (μmol/mg cell protein)</th>
<th>% stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin (500 ng/ml)</td>
</tr>
<tr>
<td>None</td>
<td>0.312±0.012 (4)</td>
<td>0.682±0.014** (4)</td>
</tr>
<tr>
<td>Phloridzin (mM)</td>
<td>0.306±0.010 (3)</td>
<td>0.682±0.014** (3)</td>
</tr>
<tr>
<td></td>
<td>0.257±0.006 (4)</td>
<td>0.607±0.016** (4)</td>
</tr>
<tr>
<td></td>
<td>0.159±0.006 (3)</td>
<td>0.343±0.011** (3)</td>
</tr>
<tr>
<td>PHMB (mM)</td>
<td>0.324±0.017 (3)</td>
<td>0.508±0.028* (3)</td>
</tr>
<tr>
<td></td>
<td>0.335±0.012 (3)</td>
<td>0.351±0.008 (3)</td>
</tr>
<tr>
<td></td>
<td>0.165±0.002 (3)</td>
<td>0.189±0.005 (3)</td>
</tr>
</tbody>
</table>

Cells were incubated for 3 h. Numbers of replicate dishes are given in parentheses. Values are means±S.E. * p<0.005 and ** p<0.001 versus Control.

Table 2. Dose responses of lactic acid production to epidermal growth factor (EGF) and insulin.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Lactic acid (μmol/mg cell protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0.410±0.008</td>
<td></td>
</tr>
<tr>
<td>EGF (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.518±0.032*</td>
<td>126%</td>
</tr>
<tr>
<td>10</td>
<td>0.551±0.014**</td>
<td>134%</td>
</tr>
<tr>
<td>10²</td>
<td>0.748±0.029**</td>
<td>182%</td>
</tr>
<tr>
<td>10³</td>
<td>0.752±0.016**</td>
<td>183%</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.438±0.031</td>
<td>107%</td>
</tr>
<tr>
<td>10</td>
<td>0.499±0.031*</td>
<td>122%</td>
</tr>
<tr>
<td>10²</td>
<td>0.523±0.023**</td>
<td>128%</td>
</tr>
<tr>
<td>10³</td>
<td>0.672±0.033**</td>
<td>164%</td>
</tr>
</tbody>
</table>

Triplicate dishes of cells were incubated for 3 h with EGF or insulin. Values are means±S.E. * p<0.05 and ** p<0.005 versus None (control).

Benzoate (PHMB), which inactivates facilitiated glucose transport (Renner, Plagemann and Bernlohr, 1972), significantly decreased lactic acid production (p<0.005 at 0.1 mM and p<0.001 at 0.25 mM) only in the presence of insulin, while the addition of 0.5 mM PHMB significantly decreased the production both with and without insulin (p<0.001).

Dose responses of lactic acid production to EGF and insulin are shown in Table 2. One ng/ml of EGF but not insulin significantly increased lactic acid production. EGF at 100 ng/ml or higher exhibited maximal stimulation, which was not significantly greater than that by 1,000 ng/ml of insulin. Table 3 shows the effect of PHMB on EGF stimulation of lactic acid production. PHMB at 0.1 mM significantly decreased the production only in the presence of EGF (p<0.005), and at 0.3 mM both in the presence and absence of EGF (p<0.001).

Activities of enzymes in the glycolytic pathway

Serum-starved cells in 100-mm dishes were incubated for 3 h in serum-free DMEM (glucose concentration, 100 mg/dl) with or without 970 ng/ml of insulin. The activities of HK, PFK and PK, the regulatory enzymes in the glycolytic pathway, in soluble fraction of homogenates of cells are shown in Table 4. HK activities (Vmax) in extracts of cells treated with and without insulin were 6.32 and 6.35 μU/mg protein, respec-
Table 3. Effect of parahydroxymercuribenzoate (PHMB) on lactic acid production by serum-starved 3T3 fibroblasts.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Lactic acid (μmol/mg cell protein)</th>
<th>EGF (100 ng/ml)</th>
<th>% stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EGF (100 ng/ml)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.400±0.011 (4)</td>
<td>0.767±0.019** (4)</td>
<td>+92%</td>
</tr>
<tr>
<td>PHMB (mM)</td>
<td>0.1</td>
<td>0.532±0.035* (3)</td>
<td>+29%</td>
</tr>
<tr>
<td></td>
<td>0.218±0.004 (3)</td>
<td>0.247±0.003* (3)</td>
<td>+13%</td>
</tr>
</tbody>
</table>

Fibroblasts were incubated for 3 h. Values are means±S.E. of the results with triplicate and quadruplicate dishes as given in parentheses. * p<0.05 and ** p<0.001 versus Control.

Table 4. Hexokinase, phosphofructokinase and pyruvate kinase activities in quiescent 3T3 fibroblasts.

| Enzyme activities were determined as described under “Materials and Methods”. Insulin concentration was 970 ng/ml. Values are averages of the results from two experiments. Percent of the maximal activity is given in parentheses. |

<table>
<thead>
<tr>
<th>Hexokinase</th>
<th>Glucose (mM)</th>
<th>Activity (mU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-treated</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.35</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>F6P (mM)</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>40.8 (98%)</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>27.5 (66%)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>21.3 (51%)</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>PEP (mM)</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>495 (69%)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>418 (58%)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>225 (31%)</td>
</tr>
</tbody>
</table>

Table 5. Kinetic analysis of phosphofructokinase at various pH.

<table>
<thead>
<tr>
<th>F6P (mM)</th>
<th>pH 6.9</th>
<th>pH 7.4</th>
<th>pH 8.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (-)</td>
<td>I (+)</td>
<td>I (-)</td>
</tr>
<tr>
<td>1.0</td>
<td>32.8</td>
<td>36.9</td>
<td>37.3</td>
</tr>
<tr>
<td>0.5</td>
<td>25.0</td>
<td>29.3</td>
<td>26.6</td>
</tr>
<tr>
<td>0.2</td>
<td>15.5</td>
<td>17.5</td>
<td>14.1</td>
</tr>
<tr>
<td>0.1</td>
<td>10.0</td>
<td>11.4</td>
<td>10.0</td>
</tr>
<tr>
<td>0.05</td>
<td>--</td>
<td>--</td>
<td>14.1</td>
</tr>
</tbody>
</table>

PFK activity in extracts of cells incubated for 3 h was determined as described under “Materials and Methods”. I (-), without insulin; I (+), with insulin (1 μg/ml).

PFK activity (Vmax) was not increased by exposure of the cells to insulin. When PFK activity was determined at a suboptimal concentration of F6P, the rate to the maximal activity of PFK was not changed by insulin exposure, either. Similar to PFK, PK activity (Vmax) or percent of the maximal activity was not altered by insulin (Table 4), indicating no change in the substrate affinities of these two enzymes. As the influence of allosteric effectors of PFK is diminished under standard assay
conditions at alkaline pH, kinetic analyses of PFK were carried out at low and neutral pH as well as high pH (Table 5). PFK activity at 1 mM F6P of the extract of cells exposed to insulin for 3 h was 1.09- to 1.18-fold of the activity of the extract of non-treated cells. By the analysis of the Lineeweaver-Burk plot, the Km for F6P in insulin-treated extract was nearly equal at all pH tested to that in non-treated extract; it was 0.4 mM at pH 6.9, 0.1 mM at pH 7.4 and 0.06 mM at pH 8.1.

Even when the glucose concentration in a test medium used in experiments on the activities of glycolytic enzymes was physiological (100 mg/dl), insulin also increased lactic acid production by 65%.

**Discussion**

This report describes the effect of insulin on the production of lactic acid and three key enzyme activities in the glycolytic pathway in cultured 3T3 fibroblasts. As lactic acid is primarily the end product of glucose metabolism in cultured fibroblasts (Cristofalo an Kritchevsky, 1965), the increased production of lactic acid by insulin and EGF observed in the present study denotes stimulation of glycolysis. To analyze the mechanisms of their actions in the stimulation of glycolysis in quiescent 3T3 fibroblasts, we examined the effects of inhibitors of glucose transport and prolonged exposure of cells to insulin on the production of lactic acid, and the effect of insulin on the activities of key glycolytic enzymes.

Both phloridzin and PHMB exhibited an inhibitory effect on lactic acid production (Table 1 and 3). However, their modes of inhibition would not be the same. The addition of phloridzin to the incubation medium decreased lactic acid produced by cells both in the presence and absence of insulin. Although the decrease in the production was proportional to the concentration of phloridzin, insulin still stimulated glycolysis at any concentration of the inhibitor. As phloridzin competes with glucose in the transport through cell membrane, it seems reasonable to conclude that insulin stimulation of glycolysis was not eliminated by phloridzin if insulin enhances glucose entry by increasing carrier protein in 3T3 cells. The precise relationship, however, between glycolysis and glucose transport in the presence of insulin in 3T3 fibroblasts remains to be clarified.

As shown in Table 1, PHMB at 0.25 mM or less reduced only the insulin-increased portion of lactic acid production, while 0.5 mM PHMB decreased the production both with and without insulin. These results may imply that relatively low concentrations of PHMB selectively erase the effect of insulin at the site of insulin action and that a higher concentration of PHMB also exerts its effect at a site other than that of insulin action. From the fact that PHMB inactivates not only facilitated glucose transport (Renner, Plagemann and Bernlohr, 1972) but also some sulfhydryl enzymes, two possible sites of insulin action, glucose transport and sulfhydryl enzyme(s) in glycolysis, are proposed. However, the exact site of insulin action and the mechanism of PHMB interference in insulin action could not be determined from the results of the present study.

The molecular weight of EGF is 6,045 and nearly equal to that of insulin. Though EGF was more potent than insulin, in a minimally effective concentration, in increasing lactic acid production (1 ng/ml with EGF and 10 ng/ml with insulin), the maximal stimulation by EGF was not significantly different from that by insulin (Table 2). From the experiment using PHMB (Table 3), the site and the mode of EGF action in 3T3 cells appear to be analogous to those of insulin action.

No activation of key glycolytic enzymes including PFK in cell extract by insulin...
was demonstrated in the present study (Table 4 and 5). However, the possible existence of the intracellular effector generated by insulin and exerting an activation of the enzyme(s) only in intact cells could not be denied, because only a little knowledge of the mechanism of insulin action has been accumulated. To clarify the mechanism of insulin action in quiescent 3T3 cells, further investigation on various insulin effects on chemical mediator, cyclic AMP or adenylate cyclase, carrier protein of glucose and so on would be required.

In the experiments in which time courses of lactic acid production were examined, insulin stimulation of the production appeared as early as one hour (Fig. 2A) and disappeared soon after the removal of insulin (Fig. 2B). The stimulation by insulin was not augmented even after 24-h pretreatment. Relatively small production in the first hour of incubation, which was observed under any cell conditions, would be due to the time lag of the release of lactic acid from cells to the medium. Our results contrast with the reports by Rozengurt and his colleagues (Diamond et al., 1978; Schneider, Diamond and Rozengurt, 1978) who described that the production rate gradually increased in a few hours with a simultaneous increase in PFK activity using quiescent 3T3 cells, logarithmically growing 3T6 cells and simian virus 40-transformed 3T3 cells. The population density of untransformed 3T3 cells which they used was much lower than that in the present study (4 × 10^4 versus 10–14×10^4 cells/cm^2). Their findings on PFK activation by serum, insulin or EGF could be related to cell multiplication, while stimulation of glycolysis by insulin observed in this study would not be involved in the sequence of the early metabolic events leading to the initiation of DNA synthesis; it may simply reflect a metabolic action of the hormone.

It may not be conflicting that both an increase in glucose uptake and an activation of some glycolytic enzymes were observed in transformed cells (Bissell, 1976; Singh et al., 1974). In such a case, it could not be easily determined which is more regulatory in glucose metabolism. In untransformed 3T3 cells, however, our findings suggest that glucose entry rather than the PFK step is rate-limiting in the glycolytic pathway, and that insulin stimulation of glycolysis is mediated by increased glucose entry, though direct measurement of glucose transport was not performed.

Acknowledgements

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