Diabetes causes marked changes in lymphocyte metabolism

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Abstract

An enhanced susceptibility to infections is well known to occur in a poorly controlled diabetic state. Since glucose and glutamine are essential for lymphocyte function, we investigated whether their metabolism is changed in lymphocytes obtained from mesenteric lymph nodes of alloxan-induced diabetic rats (40 mg/kg body weight). The activities of hexokinase, phosphofructokinase, glucose-6-phosphate dehydrogenase (G6PDH), citrate synthase and phosphate-dependent glutaminase were determined. Decarboxylation of metabolites [U-14C]-, [1-14C]- and [6-14C]-glucose, [1-14C]- and [2-14C]-pyruvic acid, [U-14C]-palmitic acid and [U-14C]-glutamine was evaluated in incubated lymphocytes isolated from mesenteric lymph nodes. The measurements were carried out in cells following three experimental protocols: (1) lymphocytes freshly obtained from control and alloxan-induced diabetic rats, (2) lymphocytes from insulin-treated (2 U/rat per day) diabetic rats and (3) lymphocytes obtained from control and diabetic rats and cultured in the presence of insulin (1 mU/ml) for 6 h. The activities of hexokinase, G6PDH and citrate synthase were decreased by the diabetic state, whereas that of phosphofructokinase was raised. Decarboxylation of [U-14C]- and [6-14C]-glucose, [1-14C]- and [2-14C]-pyruvate and [U-14C]-glutamine were also decreased in lymphocytes from diabetic rats, whereas [U-14C]-palmitic acid decarboxylation was increased. Insulin administration in vivo or added to the culture medium reversed the changes observed in freshly obtained lymphocytes. Alloxan-induced diabetes did change lymphocyte metabolism and this may be an important mechanism leading to impairment of lymphocyte function.


Introduction

An enhanced susceptibility to infections is well known to occur in a poorly controlled diabetic state (Kraine & Tisch 1999). The incidence of a recognized group of rare infections is definitely high in diabetes mellitus or confined almost entirely to diabetic patients (Larkin et al. 1985). Infectious diseases, particularly tuberculosis, were a major cause of death among diabetic patients before the advent of insulin therapy (Eliopoulos 1995). Critical evaluations of the topic suggest that infections in general are more difficult to eliminate in the diabetic host (Garcia-Leme 1989). The reasons why diabetic patients present an increased susceptibility to frequent and prolonged infections, however, remain to be fully determined.

Mature lymphocytes recirculate via blood and lymph through lymphoid tissues in a relatively quiescent state until stimulated to proliferate during, for example, a bacterial or viral infection. T-cell blastic transformation stimulated by phytohaemagglutinin (Korfel et al. 1990) and plasma levels of immunoglobulins (Muller et al. 1989) are markedly reduced in patients with diabetes mellitus type 1, an effect reversed by insulin administration. In diabetic mice, the secretion of interleukin (IL)-4 is markedly reduced, in contrast to the secretion of IL-2 and interferon-γ, which is not affected (Wood et al. 1999). Type 2 diabetic patients show reduced thymidine uptake by lymphocytes, a reduced percentage of IL-2 receptor-positive cells and increased plasma levels of tumour necrosis factor-α when compared with controls (Pavelic et al. 1987, Chang & Shaio 1995, Pickup et al. 2000). On the other hand, production of IL-2, IL-6 and IL-10 is dose- and time-dependently suppressed by elevation in glucose concentrations. High glucose levels also inhibit proliferation of peripheral mononuclear cells (Reinhold et al. 1996). Despite these changes in immune function, the effect of the diabetic state on lymphocyte metabolism has not been addressed.

The metabolism of glucose and glutamine in lymphocytes obtained from alloxan-induced diabetic rats was investigated. These metabolites are well known major fuels for lymphocytes and play a key role in the biosynthesis of ATP, DNA, RNA and phospholipids (Newsholme et al. 1999). Key enzyme activities of glycolysis (hexokinase and phosphofructokinase), pentose-phosphate pathway (glucose-6-phosphate dehydrogenase;
Glucose (2.07 GBq/mmol), [1-14C]-pyruvic acid (9.25 GBq/mmol), [1-14C]-glucose (2.07 GBq/mmol), [2-14C]-pyruvic acid, [U-14C]-palmitic acid and [U-14C]-glutamine were evaluated in incubated lymphocytes isolated from mesenteric lymph nodes. The measurements were carried out in cells following three experimental protocols: (1) lymphocytes freshly obtained from control and alloxan-induced diabetic rats, (2) lymphocytes from insulin-treated diabetic rats and (3) lymphocytes obtained from control and diabetic rats and cultured in the presence of insulin.

Materials and methods

Reagents and enzymes

All reagents for buffers, enzymes, bovine insulin and alloxan monohydrate were obtained from Sigma Chemical Company (St Louis, MO, USA). Neutral protamine hagedorn (NPH) insulin for rat treatment was obtained from Biobrás (Brazil, Brazil). Culture medium RPMI-1640 and supplements were purchased from Gibco BRL (Grand Island, NY, USA). [U-14C]-Glucose (11.3 GBq/mmol), [1-14C]-glucose (2.07 GBq/mmol), [6-14C]-glucose (2.07 GBq/mmol), [1-14C]-pyruvic acid (9.25 GBq/mmol), [2-14C]-pyruvic acid (9.36 GBq/mmol), [U-14C]-palmitic acid (9.257 MBq/mmol) and [U-14C]-glutamine (8.47 GBq/mmol) were obtained from Amersham International plc (Amersham, Bucks, UK).

Animals

Male Wistar rats, weighing 220 ± 20 g, obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, were used in this study. Animals were fed ad libitum and maintained in a room at 23 °C, with lights on from 0700 to 1900 h. The Institute of Biomedical Sciences Animal Experimental Committee, University of São Paulo granted ethical approval for these studies.

Induction of diabetes

The rats were either untreated (control group injected with saline, 0.9% NaCl) or intravenously injected with alloxan (dissolved in saline solution, pH 7) at a dose of 40 mg/kg body weight, after an overnight fasting period (Pereira et al. 1994). The diabetic rats used were those with glycaemia over 200 mg/dl, after 7 days of alloxan injection. A group of diabetic rats was also treated with NPH insulin through s.c. administration of 2 U/rat for 3 days.

Experimental procedure

Diabetic rats and matching controls were kept under similar conditions. After 7 days, the fed rats were killed by decapitation without anaesthesia between 0800 and 1100 h. Mesenteric lymph nodes were dissected and lymphocytes were prepared as previously described (Curi et al. 1988). After centrifugation at 1200 g for 10 min, lymphocytes were suspended in phosphate-buffered saline (PBS; 0.137 M NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, pH 7.4) and the determinations of metabolite decarboxylation and enzyme activities were determined in these cells.

The number of viable cells (>95%) was determined in a Neubauer chamber using an optical microscope (NikonYS2-H), following the addition of Trypan Blue aqueous solution (1% w/v).

Cell culture

Lymphocytes obtained from control and diabetic rats were cultured at 37 °C in an air/CO2 atmosphere at a density of 1 × 10^7 cells in 1 ml RPMI-1640 medium supplemented with 10% foetal calf serum, containing 5.6 mM glucose, 2 mM glutamine and antibiotics (100 units/ml streptomycin and 200 units/ml penicillin) in the presence of 1 μU/ml insulin for 6 h. This activity of insulin corresponds to the maximal biological response of skeletal muscle to insulin during a 1-h incubation (Ceddia et al. 1998). The cells were then collected, washed with PBS and used for enzymatic activity assays and/or measurements of glucose and glutamine oxidation. The cells obtained from rats or culture plates were disrupted through sonication (Vibra Cell, Manchester, CT, USA) as previously described (Otton et al. 1998).

Assay of enzyme activities

The activities of hexokinase (E.C. 2.7.1.1), G6PDH (E.C. 1.1.1.49), phosphofructokinase (E.C. 2.7.1.11), citrate synthase (E.C. 4.1.3.7) and phosphate-dependent glutaminase (E.C. 4.1.3.7) were determined as described...
in our previous studies (Cooney et al. 1986, Newsholme et al. 1986, Pereira et al. 1994, Otton et al. 1998) and in other studies (Mansour 1963).

Spectrophotometric conditions of the enzyme assays
Activities of hexokinase, G6PDH and phosphofructokinase were assayed at 25 °C and that of glutaminase at 37 °C. Citrate synthase activity was assayed by following the rate of change in absorbance at 412 nm and the remaining enzymes at 340 nm. The final volume of the assay mixtures in all cases was 1·0 ml. The production of NADH or NADPH was monitored in a Pharmacia Biotech spectrophotometer (model Ultrospec 3000).

Protein determination
The protein content of the cells was measured by the method of Bradford (1976), using bovine serum albumin as standard.

Statistical analysis
Enzyme activities are expressed as nmol substrate utilized/min per mg protein. The decarboxylation of [U-14C]-, [1-14C]- and [6-14C]-glucose, [1-14C]- and [2-14C]-pyruvic acid, [U-14C]-palmitic acid and [U-14C]-glutamine by incubated lymphocytes is expressed as nmol/h per 10^7 cells. The results are presented as means ± S.E.M. for five rats, as indicated in Fig. 1. P<0·05 was taken to indicate significant differences between groups using ANOVA.

Results
The effect of alloxan-induced diabetes on the metabolism of freshly obtained lymphocyte is presented in Table 1. Diabetes decreased decarboxylation of [U-14C]- (27%) and [6-14C]-glucose (50%), [1-14C]- (23%) and [2-14C]-pyruvate (36%) and [U-14C]-glutamine (33%) in 1-h incubated lymphocytes, whereas the decarboxylation of [1-14C]-glucose was not affected. [U-14C]-palmitic acid decarboxylation, on the other hand, was higher (50%) in lymphocytes obtained from diabetic rats. Hexokinase activity was decreased (20%) in lymphocytes from diabetic rats as compared with control. Diabetes also decreased G6PDH activity (18%) and increased that of phosphofructokinase (53%). Citrate synthase activity was decreased.

Figure 1 Maximal enzyme activities of 6-h cultured lymphocytes obtained from control and alloxan-induced diabetic rats. The control (cont) and diabetic (diab) cells were cultured in the absence (−) or presence (+) of insulin (1 mU/10^7 cells). The enzyme activities are expressed as nmol/min per mg protein. The values are presented as means ± S.E.M. of ten determinations from five rats per group.

aP<0·05 for comparison between the respective diabetic and control groups. bP<0·05 due to the effect of insulin.
(40%), whereas that of phosphate-dependent glutaminase activity was not modified in diabetic lymphocytes.

Insulin treatment (2 U/day) of the rats was performed in order to abolish the metabolic changes induced by diabetes (data not shown). The decrease in [U-14C]-glucose decarboxylation induced by diabetes was fully reversed by insulin administration, the value obtained in the treated group being 29% higher than in control rats. The decrease in the activity of G6PDH in diabetic lymphocytes was also abolished by insulin treatment, being 30% higher than in control cells. The decrease in citrate synthase activity of diabetic lymphocytes was partially recovered by insulin injection, whereas the increase in phosphofructokinase activity caused by diabetes was abolished by the hormonal treatment.

Experiments using lymphocytes in culture were performed to evaluate whether the metabolic alterations observed in freshly obtained lymphocytes were due to the lack of insulin only and whether the addition of this hormone to the medium would be able to fully reverse the changes induced by diabetes. Results of the enzyme activities obtained from lymphocytes cultured for 6 h in the presence or absence of 1 mU insulin are shown in Fig. 1. Maximum activities of hexokinase, G6PDH and citrate synthase returned to control values after the addition of insulin to the medium. This hormone, however, did not affect phosphofructokinase activity. Similarly, the presence of insulin fully reversed the alterations observed in the decarboxylation of [U-14C]- and [6-14C]-glucose, [1-14C]- and [2-14C]-pyruvate and [U-14C]-glutamine induced by diabetes.

**Discussion**

The purpose of this study was to examine the effect of the diabetic condition on lymphocyte metabolism. This was the reason for choosing the model of alloxan-induced diabetes. Alloxan-diabetic rats present low plasma levels of insulin with no change in the activation state of the immune cells. Leukocyte activation is well known to occur in spontaneously diabetic Bio Breeding rats (Field et al. 1990) and patients (Conroy et al. 2000) and is the major cause of the pathogenesis of diabetes. Therefore, the use of lymphocytes from genetically diabetic rats and diabetic patients was not considered appropriate for the purpose of this study.

The results presented herein indicate that lymphocytes obtained from alloxan-induced diabetic rats show marked changes in glucose and glutamine metabolism. Impairment in the ability of lymphocytes to utilize glucose and glutamine could significantly affect their capacity to respond to immune stimuli. The important role of insulin for lymphocyte metabolism was confirmed by the fact that insulin treatment either *in vivo* or *in vitro* reversed most of the changes observed in Table 1.

In addition to glucose decarboxylation, the measurement of glucose utilization and lactate production was also performed in 1-h incubated cells. Glucose utilization and lactate production were raised by 20% and 2.2-fold respectively in diabetic lymphocytes (data not shown). Despite the increase in glucose uptake, diabetic lymphocytes were not able to oxidize this metabolite efficiently. Similar observations have been previously reported in thymus lymphocytes from streptozotocin-induced diabetic rats (Moreno-Aurioles et al. 1996).

Pyruvate is a common product of glucose and glutamine metabolism in lymphocytes (Curi et al. 1988). However, the capacities for both glycolysis and glutaminolysis are very much greater than the capacity for pyruvate oxidation (Curi et al. 1988). In fact, pyruvate metabolism is partially directed to lipid synthesis in lymphocytes (Curi et al. 1999).

The rate of pyruvate oxidation in intact cells is usually measured following the conversion of [1-14C]-pyruvate into 14CO2. However, it has been pointed out that there is pyruvate carboxylase activity in rat mesenteric lymphocytes (Curi et al. 1988). Therefore, these cells can convert [1-14C]-pyruvate into 14CO2 through pyruvate dehydrogenase and the TCA cycle via pyruvate carboxylase reaction (Curi & Newsholme 1989). In order to estimate the contribution of both decarboxylation sites of glucose (PDH and TCA cycles), measurements of 14CO2 production from [1-14C]- and [2-14C]-pyruvate were carried out. [2-14C]-Pyruvate is decarboxylated through

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**Table 1** Metabolic parameters evaluated in lymphocytes freshly obtained from control and alloxan-induced diabetic rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>14CO2 from [U-14C]-glucose</td>
<td>13·1 ± 0·81</td>
<td>9·50 ± 0·59*</td>
</tr>
<tr>
<td>14CO2 from [1-14C]-pyruvate</td>
<td>7·52 ± 0·22</td>
<td>5·81 ± 0·39*</td>
</tr>
<tr>
<td>14CO2 from [2-14C]-pyruvate</td>
<td>6·04 ± 0·47</td>
<td>3·83 ± 0·43*</td>
</tr>
<tr>
<td>14CO2 from [1-14C]-glucose</td>
<td>0·79 ± 0·06</td>
<td>0·98 ± 0·13</td>
</tr>
<tr>
<td>14CO2 from [6-14C]-glucose</td>
<td>0·70 ± 0·08</td>
<td>0·35 ± 0·07*</td>
</tr>
<tr>
<td>14CO2 from [U-14C]-glutamine</td>
<td>13·1 ± 1·08</td>
<td>8·69 ± 0·54*</td>
</tr>
<tr>
<td>14CO2 from [U-14C]-palmitic acid</td>
<td>0·76 ± 0·08</td>
<td>1·56 ± 0·24*</td>
</tr>
<tr>
<td>Hexokinase activity</td>
<td>33·8 ± 13·6</td>
<td>28·6 ± 9·99*</td>
</tr>
<tr>
<td>G6PDH activity</td>
<td>23·2 ± 10·3</td>
<td>19·1 ± 1·30*</td>
</tr>
<tr>
<td>Citrate synthase activity</td>
<td>16·3 ± 11·5</td>
<td>98·6 ± 51·5*</td>
</tr>
<tr>
<td>Phosphofructokinase activity</td>
<td>90·9 ± 6·53</td>
<td>139 ± 116*</td>
</tr>
<tr>
<td>Glutaminase activity</td>
<td>24·4 ± 1·76</td>
<td>25·1 ± 1·68</td>
</tr>
</tbody>
</table>

*P<0·05 compared with control rats.
the TCA cycle only in opposition to [1-14C]-pyruvate which produces 14CO2 in both sites as mentioned (Curi et al. 1988). The decarboxylation of [1-14C]- and [2-14C]-pyruvate was decreased in diabetic lymphocytes as compared with the control group. Based on these findings, we can assume that the flux of metabolites through pyruvate dehydrogenase and the Krebs cycle was affected by the diabetic state. After in vitro insulin administration, decarboxylation of both [1-14C]- and [2-14C]-pyruvate returned to control levels (data not shown). An increased oxidation of fatty acids (Table 1) and so the production of acetyl-CoA may be an important mechanism to inhibit PDH activity in diabetic lymphocytes (Kelley & Mandarino 2000).

The pentose-phosphate pathway oxidizes glucose-6-phosphate to intermediates of the glycolytic pathway, generating NADPH and ribose-5-phosphate for fatty acids and nucleotide synthesis respectively. This pathway can be divided into two portions: (1) an oxidative phase where glucose-6-phosphate is oxidatively decarboxylated to a pentose, ribose-5-phosphate and (2) the non-oxidative portion where intermediates of glycolytic pathway are formed. To examine the role of the pentose-phosphate pathway in the reduction of glucose oxidation found in diabetic lymphocytes, we determined the G6PDH activity and the decarboxylation of [1-14C]- and [6-14C]-glucose. The productions of 14CO2 from [1-14C]- and [6-14C]-glucose were compared with estimates of the upper limit of the pentose-phosphate pathway activity in mesenteric lymph node lymphocytes as described for other cell types (Larrabee 1989). The production of 14CO2 from [1-14C]-glucose in the diabetic group was 0.98 compared with 0.78 nmol/h per 107 cells for control lymphocytes. The difference between 14CO2 production from [1-14C]- and [6-14C]-glucose in diabetic lymphocytes was 0.63 nmol/h per 107 cells compared with control lymphocytes which was 0.09 nmol/h per 107 cells. Insulin administration in vitro returned the decarboxylation of [1-14C]- and [6-14C]-glucose to control levels (data not shown). These findings support the proposition that the activity of the pentose-phosphate pathway is markedly raised in lymphocytes from diabetic rats. This occurred despite the fact that G6PDH activity was slightly lower (only 18%) in lymphocytes from diabetic rats. In fact, the activity of this enzyme is at least 180-fold higher than the rates of glucose decarboxylation and so low changes do not affect the flux of substrates. A more pronounced decrease in G6PDH activity was reported by Costa-Rosa et al. (1996) in peritoneal macrophages obtained from alloxan-induced diabetic rats.

The presence of glucose, high activities of membrane glucose transport and of both Krebs cycle and glycolysis has been demonstrated to be necessary to maintain DNA synthesis and to sustain lymphocyte proliferation. The TCA cycle is an important source of ATP and precursors for macromolecule biosynthesis (Curi et al. 1999). Citrate synthase activity, which is an important enzyme of the TCA cycle (Newsholme et al. 1987) was significantly lower (40%) in lymphocytes from diabetic rats (Table 1). The maximal activity of phosphofructokinase, on the other hand, was increased by the diabetic state, indicating that the capacity of glycolysis is markedly in excess of that for glucose oxidation. Citrate is an allosteric inhibitor of phosphofructokinase (Reinhold et al. 1996) and so its decrease may partially explain the increase in this enzyme activity. Although citrate synthase activity returned to control levels after insulin treatment, the same was not observed for phosphofructokinase (Fig. 1). Therefore, the mechanism involved in phosphofructokinase stimulation under these conditions remains to be elucidated. Increases of fructose 2,6-bisphosphate levels and of phosphofructokinase activity have also been found in thymocytes obtained from streptozotocin-induced diabetic rats (Moreno-Aurioles et al. 1996).

Glutamine is both an oxidative substrate and an important source of nitrogen for de novo synthesis of pyrimidine and purine nucleotides and amino sugars in lymphocytes (Curi & Newsholme 1989, Curi et al. 1999). Glutamine is well known to be required for both lymphocyte proliferation and cytokine production (Pallavicini & William 1976, Calder & Yaqoob 1999, Newsholme et al. 1999). We have reported here that glutamine oxidation was decreased in diabetic lymphocytes (33%, Table 1), whereas glutaminase activity was not modified. After insulin treatment, decarboxylation of [U-14C]-glutamine in lymphocytes from the diabetic group was similar to that of the control group (data not shown). Glutamine is mainly oxidized through the left-hand side of the TCA cycle (Curi et al. 1999). Therefore, the lower activity of citrate synthase (the first step on the right-hand side of the TCA) in diabetic lymphocytes may not explain the reduced capacity of the Krebs cycle to oxidize glutamine. In fact, decreased glutamine uptake (32%) and glutamate production (22%) in incubated lymphocytes obtained from diabetic rats were also found in this study (data not shown). Although glutaminase activity was not modified, a reduction in glutamine transport may occur in diabetic lymphocytes leading to a decrease in glutamine oxidation (Table 1).

Since glucose and glutamine oxidation was low, we investigated whether fatty acids could be an important source of ATP for diabetic lymphocytes. It is well known that other metabolites can enter the TCA cycle via acetyl CoA in lymphocytes, e.g. fatty acids and ketone bodies. Evidence has previously been obtained that [14C]-oleate is converted to 14CO2 by lymphocytes (Ardawi & Newsholme 1984). Calder et al. (1994) demonstrated that lymphocytes utilize fatty acids originated from triacylglycerols due to the activity of lipoprotein lipase. As reported herein, lymphocytes from diabetic rats presented a greater capacity of [U-14C]-palmitic acid decarboxylase than those from the control group. Diabetic rats and patients
present a high plasma level of free fatty acids and triacylglycerols (Boden 1999, Kelley & Mandarino 2000). Therefore, lymphocytes from diabetic rats may replace glucose and glutamine by an increase in fatty acid oxidation.

Changes in leukocyte metabolism, although critically important to their function, have received little attention in immunodeficient states, such as diabetes. The metabolic changes reported herein demonstrate that this could well be one of the mechanisms for the impaired immune function observed in diabetic patients.

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