Can Plasma Glucose and Nonesterified Fatty Acid Be Regulators of Glucose Utilization in Skeletal Muscle?

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Abstract. The effect of plasma glucose and nonesterified fatty acid (NEFA) on basal and insulin-stimulated glucose utilization in skeletal muscle was assessed by perfused hindlimb preparations. Two-month-old male Wistar rats were divided into four groups: starved, glucose-loaded, hypoglycemic and control. Diabetic rats were made by means of streptozotocin, and divided into three groups: non-treated, insulin-treated normoglycemic and insulin-treated hyperglycemic. The effect of NEFA on glucose clearance was also investigated by adding palmitate to the perfusate.

Basal glucose utilization decreased with a rise in plasma glucose concentrations, and increased with a fall in them in each group. The available data strongly support the view that plasma glucose levels play an important role in the control of basal glucose utilization by the hindlimb muscle.

In contrast, continuous hyperglycemia in the diabetic state decreased insulin-stimulated glucose utilization by the skeletal muscle, whereas an acute rise in plasma glucose concentrations in the glucose-load state did not.

Palmitate stimulated basal glucose utilization, while it decreased insulin-stimulated glucose uptake. It was also clarified that it increased the affinity for glucose in the skeletal muscle in the basal state. This finding seems to indicate that NEFA has some influence on an increase in basal glucose utilization in starvation.

Key words: Glucose utilization, Skeletal muscle, Hindlimbs perfusion, Plasma NEFA, Plasma glucose.

PLASMA GLUCOSE and nonesterified fatty acid (NEFA) are paid much attention as the factors that effect peripheral glucose utilization and insulin sensitivity in diabetes. But there are few reports on their roles both in the absence and the presence of insulin separately in skeletal muscle.

The present study was undertaken to clarify the effect of acute and continuous hyperglycemia and hypoglycemia, in addition to the changes in plasma NEFA concentrations, on glucose clearance in the skeletal muscle of the rat both in the absence and the presence of insulin, using the isolated hindlimbs perfusion method.

Materials and Methods

Studies were carried out on two-month-old male Wistar rats weighing approximately 250 g. The animals were maintained on a 12-h light- (0700–1900 h) dark (1900–0700 h) cycle and fed a standard diet and water ad libitum. All experiments were initiated in the early afternoon 4 h after food withdrawal. All animal procedures were reviewed and approved by the Jikei University Animal Care Committee. Diabetes was induced by the intravenous injection of streptozotocin (STZ, Sigma Chemical Co., St. Louis, MO) 40 mg/kg, diluted in 0.01 M citrate buffer (pH 4.5). Blood was obtained from the tail vein 7 days later and analyzed for glucose. Rats with a glucose value over 21.1 mM were used for further study as diabetic. One hour
after the intraperitoneal injection of 4 U insulin (Novolin R, Novo Nordisk Pharma Co., Denmark) into the diabetic rats, the ones whose plasma glucose level was down within the range from 3.9 mM to 6.7 mM were chosen as normoglycemic, and the ones whose plasma glucose level remained over 20 mM were chosen as hyperglycemic.

In order to study the influence of acute change in plasma glucose levels, 2 g/kg oral glucose loading and 2 U intraperitoneal insulin injection were tried in normal rats 1 h before the hindlimbs perfusion. Moreover, the effect of NEFA on the glucose clearance in the skeletal muscle of the rats was also investigated by adding 1 mM palmitate to the perfusate. Rats were prepared for isolated hindlimbs perfusion by the techniques previously described by Mondon [1]. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium solution (Nembutal sodium solution, Abbott Laboratories, North Chicago, IL) 1 ml/kg. After a midline abdominal incision, the major abdominal branches of the great vessels were ligated and finally cannulations of the aorta and vena cava were undertaken. The perfusing medium consisted of a mixture of rat red blood cells and Krebs-Ringer bicarbonate buffer, containing 13.9 mM glucose, 45 g/liter dextran (Pharmacia Fine Chemicals, Uppsala, Sweden), 1 g/liter bovine serum albumin (Sigma Chemical Co., St. Louis, MO). In the case of the experiment to study the effect of palmitate on glucose clearance, 30 g/liter bovine serum albumin was added to the perfusate instead of 45 g/liter dextran and 1 g/liter bovine serum albumin. In order to study the influence of the glucose concentration in the perfusate on glucose uptake, the hindlimbs perfusion was also performed with 6.9 mM, 27.8 mM, 41.7 mM, and 55.6 mM glucose concentrations, in addition to the 13.9 mM glucose concentration in the absence of insulin in the fed and 24 h starved rats. The perfusion solution was equilibrated with 95% oxygen and 5% CO₂ at all times. The initial circulating perfusate volume during the perfusion of hindlimbs averaged 50 ml. The temperature of the solution was 37°C. The flow rate of the perfusate was adjusted to 5 ml/min. 100 µl blood samples were obtained from the reservoir flask every 10 min for 60 min in order to determine the glucose concentration. The uptake of glucose by the hindlimb was taken as the clearance of glucose from the perfusate medium.

The clearance constant K was calculated by means of the formula

$$K = \frac{\text{glucose uptake (nmol min}^{-1}\text{ muscle}^{-1})}{\text{glucose concentration (nmol/µl)}}$$

Glucose uptake between 0 and 10, 10 and 20, 20 and 30, 30 and 40, 40 and 50 and 50 and 60 min was calculated. The glucose concentration in the formula represents the mean concentration at the beginning and end of each period of measurement. The clearances for each period of measurement were averaged and the data expressed per gram hindlimb muscle. This was accomplished by removing gross fat from rat hindlimbs, boiling the carcass, and extracting the residual fat with ether. The recovered lipid was dried and weighed along with the residual bone and cartilage. Table 1 shows the amounts of fat, bone, cartilage and skeletal muscle in hindlimbs from 2-month-old Wistar rats. It should be noted that this does not include the epididymal fat pads, which are removed with the testis before perfusion. The perfused hindlimbs comprise 19% of total body weight. The sampling withdrawal and glucose utilization by erythrocytes were negligible.

In order to avoid the interference of oxygen in the perfusate with the measurement of glucose concentration, it was done by the hexokinase method.

The results are expressed as the mean±SEM, and statistical significance of differences was evaluated by Student’s t-test.

**Results**

*Plasma glucose and non-esterified fatty acid levels in various groups*

Tables 2 and 3 show the changes in plasma glucose and NEFA levels in various conditions. In the diabetic state the plasma glucose and NEFA concentrations increased to two times and four times, respectively. In normal rats, the plasma glucose level was decreased by the insulin injection, while it was increased by the glucose load. But plasma NEFA decreased in the same way under both conditions. As shown in Table 4, starvation led to a fall in plasma glucose and a rise in plasma NEFA. The degree of these changes was in proportion to the
The glucose clearance value (K value) increased with the rise in the insulin concentrations in a dependent manner up to the maximum level at 125 μU/ml insulin, but there was no further increase in the K value with a further rise in insulin concentrations (Fig. 1). In our hindlimbs perfusion system, the effect of insulin on glucose clearance by skeletal muscle can be investigated within the physi-

**Table 1. Tissue composition of hindlimbs from 2-month-old rats**

<table>
<thead>
<tr>
<th>Rat Wt, g</th>
<th>Skinned Hindlimb Wt, g</th>
<th>Skinned Hindlimb % Fat</th>
<th>Skinned Hindlimb % Bone</th>
<th>Skinned Hindlimb % Muscle</th>
<th>Skinned Muscle % body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>248±4</td>
<td>67.2±2.1</td>
<td>18.1±1.1</td>
<td>11.7±0.4</td>
<td>70.2±0.8</td>
<td>19.0</td>
</tr>
</tbody>
</table>

Values are the means±SEM for 12 rats.

**Table 2. Plasma glucose levels in various groups**

<table>
<thead>
<tr>
<th></th>
<th>Plasma glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>Untreated</td>
<td>22.6 ± 0.3</td>
</tr>
<tr>
<td>DM</td>
<td></td>
</tr>
<tr>
<td>Insulin inj.</td>
<td></td>
</tr>
<tr>
<td>Normoglycemic</td>
<td>22.1 ± 0.4</td>
</tr>
<tr>
<td>Hyperglycemic</td>
<td>22.4 ± 0.3</td>
</tr>
<tr>
<td>Glucose-loaded (2g/kg BW)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Insulin-induced hypoglycemic (2U ip)</td>
<td>5.6 ± 0.3</td>
</tr>
</tbody>
</table>

Values are the means±SEM for 12 rats in each group.

**Table 3. Plasma non-esterified fatty acid levels in various groups**

<table>
<thead>
<tr>
<th></th>
<th>Plasma NEFA level (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.75 ± 0.09</td>
</tr>
<tr>
<td>DM</td>
<td></td>
</tr>
<tr>
<td>Insulin inj.</td>
<td></td>
</tr>
<tr>
<td>Normoglycemic</td>
<td>1.79 ± 0.07</td>
</tr>
<tr>
<td>Hyperglycemic</td>
<td>1.63 ± 0.13</td>
</tr>
<tr>
<td>Glucose-loaded (2g/kg BW)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>Insulin-induced hypoglycemic (2U ip)</td>
<td>0.84 ± 0.06</td>
</tr>
</tbody>
</table>

Values are the means±SEM for 12 rats in each group.

**Effect of insulin on glucose clearance in normal rats**

The glucose clearance value (K value) increased with the rise in the insulin concentrations in a dependent manner up to the maximum level at 125 μU/ml insulin, but there was no further increase in the K value with a further rise in insulin concentrations (Fig. 1). In our hindlimbs perfusion system, the effect of insulin on glucose clearance by skeletal muscle can be investigated within the physi-

**Table 4. The effect of starvation on plasma glucose and non-esterified fatty acid levels**

<table>
<thead>
<tr>
<th></th>
<th>Plasma glucose (mM)</th>
<th>Plasma NEFA (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8±0.3</td>
<td>0.69±0.03</td>
</tr>
<tr>
<td>4h starved</td>
<td>5.2±0.2</td>
<td>0.83±0.04</td>
</tr>
<tr>
<td>12h starved</td>
<td>4.7±0.2</td>
<td>1.04±0.04</td>
</tr>
<tr>
<td>24h starved</td>
<td>3.8±0.2</td>
<td>1.32±0.04</td>
</tr>
</tbody>
</table>

Values are the means±SEM for 12 rats in each group.
ological range of insulin concentrations, from 0 to 125 µU/ml. Moreover, in addition to the change in insulin response, that of insulin sensitivity can also be examined. So it is quite useful for investigating the effect of insulin on the glucose metabolism in the skeletal muscle.

Change in glucose clearance in the diabetic state

As shown in Fig. 1, there was a decrease in the K value in the diabetic untreated group both in the absence and the presence of insulin, compared with the control. The maximum effect of insulin was attained at 125 µU/ml, as in the control, and a further rise in insulin concentrations failed to improve the K value (Fig. 1). The present data suggest that the STZ-induced diabetic state should bring about lower insulin responsiveness rather than decreased insulin sensitivity.

With the injection of insulin, there was no improvement in the K value in the absence or the presence of insulin in the hyperglycemic diabetic group. In the normoglycemic diabetic group, there was a significant increase in the K value in the absence of insulin (Fig. 2). In the presence of insulin, the K value still stayed at the lower level, compared with that in the control (Fig. 2).

Change in glucose clearance in glucose-loaded state and insulin-induced hypoglycemic state

In the glucose-loaded group, the K value decreased significantly in the absence of insulin, compared with the control (P<0.002, Fig. 3). In the insulin-induced hypoglycemic group, there was a significant increase in the K value (P<0.001, Fig. 3). On the other hand, in the presence of insulin, there was no significant change in the K value in either group, compared with the control (Fig. 3).

The effect of starvation on glucose clearance

As seen in Fig. 4, there was a stepwise increase in the K value in the absence of insulin with the extension of starved period, while in the presence of insulin, starvation did not alter the K value from that in the control.

Effect of palmitate on glucose clearance in the skeletal muscle

Palmitate increased the K value in the absence of insulin, compared with the control. In the presence of 125 µU/ml insulin, the K value was decreased

Mean ± SEM

Fig. 1. Effect of the insulin concentration on glucose clearance in normal and diabetic rats (*, NS; **, P<0.05; ***, P<0.02; ****, P<0.01; ***** P<0.001 compared with that in the absence of insulin, *P<0.001 compared with that in normal rats).
by palmitate, and a further rise in the insulin concentrations failed to improve a decrease in the $K$ value (Fig. 5). This suggests that palmitate decreases insulin responsiveness.

The effect of the glucose concentration in the perfusate on glucose uptake by skeletal muscle in the absence of insulin

In Fig. 6, the glucose uptake increased in a dependent manner in proportion to a rise in the glucose concentrations in the fed group. In the 24-h
starved group, the dose response curve shifted to the left, compared with that in the fed group.

As shown in Fig. 7, palmitate also shifted the dose response curve to the left compared with the control.

Discussion

It is clear that in the brain, glucose transport is not accelerated by insulin itself. The brain and the muscle have different glucose transport isozymes. The former has GLUT 1, while the latter has GLUT
which is translocated by insulin from intracellular membranes to the plasma membrane. So the result in the brain cannot be directly applied to the appreciation of that in the muscle. It may, however, be useful in considering the effect of plasma glucose on glucose transporter in the plasma membrane even in muscle. It is reported that in in vivo experiments with streptozotocin or alloxan-induced rats, glucose transport into the brain decreased [2, 3]. This defect is improved by prolonged treatment with insulin, but not acutely altered by insulin administration [3], so no doubt continuous hyperglycemia itself impaired the glucose transport capacity of the brain.

Concerning the problem of whether or not the correction of hyperglycemia in diabetic rats without changing the plasma insulin concentration can improve insulin resistance, there are some reports...
Phlorizin can normalize hyperglycemia in diabetic rats without altering insulin secretion by inhibiting renal tubular glucose reabsorption and facilitating glucose excretion. Rossetti et al. examined the effect of phlorizin treatment on insulin sensitivity in partially pancreatectomized rats by means of the euglycemic hyperinsulinemic clamp technique [5]. It is cleared that phlorizin treatment of diabetic rats completely normalized insulin sensitivity. Kahn et al. demonstrated that the normalization of plasma glucose levels in diabetic rats with phlorizin restored insulin-stimulated glucose transport in adipose cells and insulin-mediated glucose disposal in vivo [5]. Judging from these reports, it is possible that continuous hyperglycemia itself impairs insulin-stimulated glucose transport.

We tried to make it clear in the present study whether or not any changes in plasma glucose and NEFA concentrations can have any effect on glucose utilization in the skeletal muscle when hindlimbs perfusion techniques are used. The present data are summarized in Table 5. The direction of the arrow for each factor under various conditions is depicted, compared with the corresponding factor in the control.

In the absence of insulin, an acute rise in the plasma glucose concentration, such as in the glucose-loaded state, and continuous hyperglycemia, such as in the diabetic state, brought about a significant decrease in the glucose clearance by the hindlimb muscle. An acute fall in plasma glucose concentration for example in the insulin-induced hypoglycemic condition and the insulin-treated normoglycemic state of the diabetic rat, and continuous lowering of plasma glucose levels, such as in starvation, increased glucose clearance by the hindlimb muscle in the absence of insulin. These data support the view that plasma glucose plays an important role in the control of glucose utilization by hindlimb muscle in the absence of insulin.

According to Unger’s hypothesis [6] reduced glucose utilization is supposed to be the consequence of a generalized down-regulation and/or occupation of glucose transporters caused by hyperglycemia. Our present data tend to support his concept. The target site of hyperglycemia might be the glucose transporter in the plasma membrane. The normalization of plasma glucose by phlorizin in diabetic rats without the amelioration of intracellular glucose metabolism by insulin succeeded in improving glucose utilization. Glucose toxicity might be explained by an increase in glucose-occupied transporters and a decrease in available glucose transporters.

In glucose-loaded normal rats, basal glucose clearance decreased significantly, but there was no change in insulin-stimulated glucose clearance. The majority of glucose transporters are located intracellularly and translocated to the plasma membrane by insulin. By glucose loading, glucose transporters in the plasma membrane are occupied by glucose, so that basal glucose clearance decreases. In contrast, insulin-enhanced translocation of glucose transporters from intracellular membranes to the plasma membrane resulted in an increase in the number of available glucose transporters. Therefore, insulin-stimulated glucose clearance was not affected by an increase in the glucose-occupied transporters in the plasma membrane.

In diabetic rats, basal glucose clearance increased remarkably with the normalization of

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**Table 5. Plasma glucose, NEFA, and insulin levels with glucose clearance in the absence and presence of insulin under various conditions**

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starved</td>
<td>Glucose loaded</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Plasma NEFA</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Plasma insulin</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Glucose clearance</td>
<td>insulin (−)</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>insulin (+)</td>
<td>→</td>
</tr>
</tbody>
</table>

↑ Increase; ↓ Decrease; → No change.
plasma glucose. Insulin-stimulated glucose clearance was, however, not improved completely, compared with that in the control. These findings could be explained by the impairment of translocation of glucose transporters and glucose metabolism induced by insulin resistance in diabetic state. Glucose toxicity is not a synonym of insulin resistance, but a part of it in addition to impaired translocation of glucose transporters and decreased glucose metabolism. The amelioration of insulin resistance, therefore, should be brought about by continuous normalization of plasma glucose based on these improvements.

Concerning the acute rise in glucose clearance in the insulin-induced hypoglycemic state and the insulin-treated normoglycemic state of diabetic rats, it is possible that this increase could be caused by an in vivo insulin effect with insulin injection. Glucose transporters could be translocated to the plasma membrane by in vivo insulin effect, so that increased available glucose transporters might accelerate glucose clearance.

But Hansen et al. [7] reported that 2-h preperfusion with insulin and glucose did not cause any rise in glucose uptake in the absence of insulin in rat skeletal muscle perfusion. Garvey et al. [8] also made it clear that 24-h preincubation with insulin and glucose did not increase glucose uptake in the absence of insulin in cultured rat adipocytes. Moreover, in glucose-loaded normal rats, insulin secretion was stimulated by glucose load, so some in vivo insulin effect should have resulted. In these cases, however, glucose was loaded at the same time as insulin was secreted and this could increase glucose-occupied transporters. This might mask in vivo insulin effects.

In the present series of experiments, diabetic rats were divided into two groups: hyperglycemic and normoglycemic. There was no difference between hyperglycemic and non-treated diabetic rats in insulin-stimulated glucose clearance. Therefore, hyperglycemic diabetic rats should not be selected for insulin resistant in the muscle. The difference between hyperglycemic and normoglycemic groups would rather be induced by the difference in insulin resistance in the liver. In hyperglycemic diabetic rats, too, the in vivo insulin effect might be masked by hyperglycemia.

Clinically it is sometimes found that in insulin-treated patients, an increase in fasting plasma glucose causes a worsening of the profile of daily plasma glucose levels, which leads to an increase in the insulin dosage. This phenomenon might be able to be explained by our present glucose occupied transporter theory.

A continuous hypoglycemic state caused by starvation increased basal glucose clearance in skeletal muscle. According to Putten et al. [9] in cultured fat cells 24-h glucose deprivation results in a 500% increase in basal 2-deoxyglucose uptake. Our present results are consistent with their data. Concerning the glucose deprivation-induced increase in glucose clearance, it is supposed in their report to be due to a decrease in the rate of degradation, internalization, or inactivation of glucose transporters rather than an increase in de novo synthesis, insertion, or the activation of glucose transporters. In the present study, palmitate accelerated glucose clearance in the absence of insulin. Moreover, in dose response experiments on perfusate glucose, it is indicated that the stimulatory effect of palmitate on it should have some relationship with an increase in the affinity for glucose in skeletal muscle.

Joost et al. made it clear in their study [10] that NEFA increased basal glucose utilization in fat cells. Their results suggest that the affinity of glucose transporter should be increased by fatty acid. By forearm intralipid infusion, Yki-Järvinen et al. demonstrated that an increase in plasma NEFA significantly increased forearm glucose uptake in the basal state [11]. They supposed that the utilization of more glucose for reesterification in the face of increased NEFA influx would be a logical explanation for the increase in glucose uptake. There are also many reports [11-17] indicating that NEFA inhibits insulin-stimulated glucose utilization. Their results are consistent with our present data. In our experiments, too, palmitate decreased insulin-stimulated glucose clearance in skeletal muscle.

In the untreated diabetic rat, there was a rise in plasma NEFA concentrations. In the absence of insulin, however, there was a decrease in glucose clearance rather than an increase. So the stimulating effect of NEFA on glucose clearance in the absence of insulin seemed to be masked by the inhibiting effect of glucose. On the other hand, there was a decrease in insulin-stimulated glucose clearance in the untreated diabetic. In order to examine whether or not plasma NEFA plays a role in this decrease, the same experiment was tried in the insulin-injected hyperglycemic rat, in which plasma
NEFA decreased without changing the hyperglycemic state. The result was that there was no improvement in insulin-stimulated glucose clearance. It is indicated that to maintain the normal level of plasma NEFA continuously might be necessary to improve its decrease caused by a rise in the plasma NEFA concentration in the diabetic state.

In starvation, the plasma NEFA level increased in proportion to its duration. In the absence of insulin, glucose clearance also had the same tendency with the plasma NEFA level. There are few reports proposing the mechanism for an increase in basal glucose uptake in muscle in starvation. In the dose response study of perfusate glucose in the absence of insulin in 24-h starved rats, it was revealed in the present study that starvation shifted the dose response curve of perfusate glucose to the left by supposedly increasing the affinity for glucose in the skeletal muscle. Therefore, there is a strong possibility that plasma NEFA plays a role in increasing glucose clearance in the absence of insulin in starvation.

References