Acceleration of Glycolysis in Erythrocytes by the Antidiabetic Agent M16209

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The effects of M16209 (1-(3-bromobenzozenofuran-2-ylsulfonyl)hydantoin), an antidiabetic agent and aldose reductase inhibitor, on glycolysis were studied in rat and human erythrocytes in vitro. M16209 increased lactate production from glucose when incubated with rat and human erythrocytes, and also increased glucose consumption in rat erythrocytes. The rates of production of lactate in rat erythrocytes treated with M16209 at 10, 25 and 50 μM were 113, 118 and 123%, respectively, of those in vehicle treated cells. Sorbinil (aldose reductase inhibitor), tolbutamide (sulfonylic amide), and binormine (bignanide) did not increase lactate production in rat erythrocytes when tested at 50 μM. On the other hand, M16209 did not affect lactate production from D-glyceric acid in erythrocytes. At 100 μM the agent decreased both glucose-6-phosphate and fructose-6-phosphate in rat erythrocytes, and increased fructose-1,6-bisphosphate; at 10 μM it also increased 6-phosphofructokinase activity in rat hemolysates. These findings suggest that M16209 accelerates glycolysis in erythrocytes via activation of 6-phosphofructokinase.

Key words 1-(3-bromobenzozenofuran-2-ylsulfonyl)hydantoin (M16209); glycolysis; erythrocyte; phosphofructokinase; D-glyceric acid; lactate production

We previously demonstrated that M16209 (1-(3-bromobenzozenofuran-2-ylsulfonyl)hydantoin) (see Fig. 1 for chemical structure), a potent aldose reductase inhibitor,1–19 exerted antihyperglycemic effects in rats with streptozotocin-induced mild diabetes via the augmentation of glucose-stimulated insulin secretion.4,5 In addition, M16209 exerted antihyperglycemic effects and also ameliorated hyperinsulinemia and insulin resistance in genetically obese, diabetic, and insulin-resistant Zucker fa/fa rats and C57BL/6j ob/ob mice.6,7 The inhibitor also accelerated insulin-stimulated glucose uptake and glycogen synthesis in oxidative muscle in these rodents.7 However, the mechanism by which M16209 lowers blood glucose concentrations has not been fully elucidated.

In the present study, we investigated the effect of M16209 on glycolysis in rat and human erythrocytes, in which almost all energy is produced by glycolysis.

MATERIALS AND METHODS

Drugs and Chemicals M16209 and sorbinil were synthesized in our laboratory. D-Glyceric acid was purchased from Aldrich Chemical Co., WI, U.S.A.. NADH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from leuconostoc mesenteroides, Type XXIV, glucose-6-phosphate isomerase (EC 5.3.1.9, from rabbit muscle, Type XI), 6-phosphofructokinase (EC 2.7.1.11, from rabbit muscle, Type III and from rabbit liver, Type VI), and fructose-bisphosphate aldolase (EC 4.1.2.13, from rabbit muscle, Type X) were obtained from Sigma Chemical Co., MO, U.S.A.. Glyceric acid-3-phosphate dehydrogenase (EC 1.2.1.12, from rabbit muscle), glyceric acid-3-phosphate dehydrogenase (EC 1.1.1.8, from rabbit muscle) and triosephosphate isomerase (EC 5.3.1.1, from rabbit muscle) were from Boehringer Mannheim GmbH (Mannheim, Germany). NAD was purchased from Oriental Yeast Co., Ltd. (Osaka, Japan). Determiner-LA was obtained from Kyowa Medex Co., Ltd. (Tokyo, Japan). Buffer hydrochloride, tolbutamide, dextran (MW 180–200 kDa), glucose C-Testwako© and hemo- globin B-Testwako© were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade available commercially.

Preparation of Rat Erythrocytes Male Wistar rats, 7–10 weeks of age and 185–300 g, were used. They were supplied by Japan SLC Inc. (Shizuoka, Japan). The rats were maintained on laboratory chow and water ad libitum, and housed in a temperature- and humidity-controlled (22 ± 1°C, 50 ± 5%) room illuminated between 7:00–20:00. Heparinized blood samples were obtained from the jugular vein of rats anesthetized with ether. The blood samples were centrifuged (1500 × g), and the plasma and the buffy coat were removed. The erythrocytes were washed three times with a buffer (pH 7.4) consisting of 125 mm NaCl, 1.2 mm MgSO4, 1 mm CaCl2, 4 mm KCl, 15 mm NaH2PO4 and 5 or 0 mm glucose. Washed erythrocytes were suspended at about 10 g/dl hemoglobin in the same buffer. An erythrocyte suspension was prepared from one rat. In a dose–response study of M16209, suspensions prepared from 2–3 rats were pooled.

Preparation of Human Erythrocytes Human heparinized blood samples were obtained from three healthy men. The blood was mixed with a half volume of saline containing 6% (w/v) dextran, and kept at 37°C for 30 min. After centrifugation (1500 × g), the supernatant contain-

Fig. 1. Chemical Structure of M16209
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ing leukocytes and platelets was discarded, and washed erythrocyte suspensions were prepared as described above.

**Measurement of Lactate Production from Glucose**
M16209 dose–response studies for rat and human erythrocytes were performed as follows. Aliquots (390 μl) of the pooled erythrocyte suspensions (n = 9: rats, n = 4: human) with 5 mM glucose were mixed with 10 μl of 0–4 mM M16209 solutions (dimethylsulfoxide (DMSO) 20%/buffer 80%). These M16209 solutions were prepared as follows. M16209 was dissolved in DMSO and diluted with the buffer. Then the erythrocyte suspensions with M16209 solutions were incubated at 37°C with shaking (90 strokes/min). After 5 and 20 min, aliquots (100 μl) of the suspensions were removed and immediately mixed with 100 μl of 0.6 M HClO4. Denatured proteins were sedimented by centrifugation, and 150 μl of the supernatant was neutralized with ice-cold 3 M K2CO3. The precipitate was removed by centrifugation, and the supernatant liquid was analyzed for lactate by the lactate oxidase and peroxidase method using Determiner LA®. This supernatant liquid was also analyzed for glucose by the glucose oxidase method using glucose C-Testwako® in a glucose consumption study using six rat erythrocyte suspensions with M16209 (100 μM) or vehicle. The linearity of the lactate production of rat erythrocyte suspensions (n = 3) was confirmed during 125 min of incubation with M16209 (100 μM) or vehicle in the same fashion as above. A comparative study of several compounds was performed as follows. Aliquots (395 μl) of the erythrocyte suspensions (n = 5) with 5 mM glucose were mixed with 5 μl of 4 mM M16209, sorbinil, buformine or tolbutamide solution (DMSO 40%/buffer 60%). These solutions were prepared as follows. The compounds were dissolved in DMSO and diluted with the buffer. Just before and after 30 min of incubation, aliquots (100 μl) of the suspensions were removed, processed and measured for lactate in the same fashion as above.

**Measurement of Lactate Production from 0-Glyceraldehyde**
The rat erythrocyte suspensions (n = 4) without glucose were preincubated for 3 h at 37°C with shaking (90 strokes/min). The erythrocytes were collected by centrifugation and washed three times with the buffer containing 5 mM glucose or 2.5 mM 0-glyceraldehyde. Washed erythrocytes were resuspended in the same buffer. An aliquot (1 ml) of the suspension was added to 25 μl of 4 mM M16209 solution or vehicle solution (20% DMSO/80% buffer), and incubated for an hour at 37°C with shaking (90 strokes/min). At the start and end of incubation, aliquots of the suspensions were taken and immediately processed, and lactate was measured. The processing of the erythrocyte suspensions and the measurement of lactate were as described for the measurement of lactate production from glucose.

**Analysis of Glycolytic Intermediates**
Aliquots (2 ml) of the rat erythrocyte suspensions (n = 5) with 5 mM glucose were mixed with 50 μl of 4 mM M16209 solution or vehicle solution (20% DMSO/80% buffer), and incubated for 30 min at 37°C with shaking (90 strokes/min). After incubation, 4 ml of 1 M HClO4 was added, and the mixture was kept on ice for 15 min. Denatured proteins were sedimented by centrifugation, and 5 ml of the supernatant liquid was mixed with 0.4 ml of 1 M Hepes–NaOH buffer (pH 8.0) and 0.56 ml of 3 M K2CO3. The precipitate was removed by centrifugation, and the supernatant was analyzed for glycolytic intermediates. Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were measured by the fluorometric method of Segel et al.8)

**Analysis of 6-Phosphofructokinase Activity**
Hemolysates of the erythrocyte suspension (1 : 40 hemolysate) were prepared as follows. Aliquots (0.2 ml) of rat erythrocyte suspensions (n = 6) were mixed with 0.2 ml cold distilled water and 7.6 ml cold ME–EDTA solution (0.005% v/v mercaptoethanol, 0.1% w/v EDTA (pH 7.4) in distilled water). 6-Phosphofructokinase activities were measured using the method of Beutler et al.9) Compositions of assay mixtures were: 0.1 M Tris–HCl/0.5 mM EDTA, pH 8.0; 20 mM MgCl2; 0.2 mM fructose-6-phosphate; 0.3 mM adenosine diphosphate (ADP); 0.2 mM NADH; 0.4 U fructose-bisphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, hemolysates (1 : 1000) or purified rabbit 6-phosphofructokinase from muscle or liver; 1–100 μM M16209; DMSO 1%. After preincubation of this mixture at 37°C for 10 min, ATP (2 mM) was added and 6-phosphofructokinase activity was measured for 15 min by reduction of NADH. The 6-phosphofructokinase activity showed good proportionality to time and enzyme concentration. Purified rabbit muscle and liver 6-phosphofructokinase activities with the low-molecular-weight fraction (MW < 10000) of the hemolysate were measured as follows. The hemolysate (1 : 20) was subjected to ultrafiltration with Molcut II® (UFP1 LGC, Nihon Millipore Ltd., Tokyo, Japan), and the low-molecular-weight fraction (MW < 10000) of the hemolysate was obtained. This fraction was mixed with the same volumes of purified phosphofructokinases and incubated at 37°C for 30 min. These mixtures were used instead of hemolysates in the method of Beutler et al.9)

**Hemoglobin Assay**
Hemoglobin (Hb) concentration was measured in all the erythrocyte suspensions by the sodium lauryl sulfate method using hemoglobin B-Testwako®.

**Presentation of Results**
Values are expressed as means ± S.E. Statistical analysis was performed using analysis of variance (ANOVA) and the Bonferroni test for unpaired data, and by the paired t-test or two-way ANOVA for paired data. The mean for vehicle-treated erythrocytes was defined as 100% for unpaired data, and that for vehicle-treated erythrocytes was defined as 100% for paired data.

**RESULTS**
In rat erythrocytes, the lactate production shows good proportionality to time for up to 125 min of incubation with or without M16209 (100 μM). The lactate production rate was significantly higher (p < 0.01, Bonferroni test) in M16209 treated erythrocytes (183 ± 2 nmol/g Hb/min) than in vehicle-treated (141 ± 1 nmol/g Hb/min) (Fig. 2). When vehicle-treated rat erythrocytes were incubated for 15 min with glucose, the lactate production rate was
Fig. 2. Time Dependence of Lactate Production from Glucose in Rat Erythrocytes

Erythrocytes were incubated with M16209 (100 µM) or vehicle at 37°C for up to 125 min. Each value represents the mean ± S.E. of four experiments.

Fig. 3. Lactate Production from Glucose in Rat and Human Erythrocytes

Erythrocytes were incubated with M16209 at 37°C for 15 min. Lactate production rate (rat: 166 ± 7 nmol/g Hb/min; human: 141 ± 3) in vehicle-treated erythrocytes was defined as 100%. Each value represents the mean ± S.E. of nine experiments (rat) and four experiments (human). *p < 0.05, **p < 0.01, vs. vehicle-treated erythrocytes. Differences in values were analyzed by the Bonferroni test.

166 ± 7 nmol/g Hb/min. M16209 accelerated this lactate production dose-dependently, and this acceleration was significant above the concentration of 10 µM (Fig. 3). In human erythrocytes as well as in those of rats, M16209 enhanced lactate production significantly at concentrations above 25 µM, and the lactate production rate of vehicle-treated erythrocytes was 141 ± 3 nmol/g Hb/min (Fig. 3). In rat erythrocytes incubated for 60 min with glucose, M16209 (100 µM) accelerated the consumption of glucose as well as the production of lactate (Table 1). In rat erythrocytes incubated for 60 min with glucose or D-glyceraldehyde after preincubation in glucose-free buffer for 3 h, M16209 at a concentration of 100 µM accelerated the production of lactate from glucose, but did not affect the production of lactate from D-glyceraldehyde (Fig. 4).

The lactate production rates of vehicle-treated erythrocytes incubated with glucose and D-glyceraldehyde were 183 ± 8 and 195 ± 15 nmol/g Hb/min, respectively.

The levels of glycolytic intermediates in rat erythrocytes treated with vehicle and 100 µM M16209 for 30 min were as follows (nmol/g Hb): glucose-6-phosphate 320.7 ± 18.2 and 256.0 ± 15.5, fructose-6-phosphate 87.3 ± 3.4 and 72.4 ± 4.9, fructose-1,6-bisphosphate 10.0 ± 0.5 and 12.3 ± 0.7, and dihydroxyacetone phosphate 15.5 ± 1.4 and 15.9 ± 1.1, respectively. M16209 significantly decreased the levels of glucose-6-phosphate and fructose-6-phosphate, but significantly increased the levels of fructose-1,6-bisphosphate (Fig. 5). Glyceraldehyde-3-phosphate was not detected in erythrocytes in this study.

In rat hemolysates, M16209 above the concentration of 10 µM significantly and dose-dependently stimulated 6-phosphofructokinase activity (Fig. 6). In purified rabbit muscle and liver phosphofructokinase, M16209 at 100 µM did not activate 6-phosphofructokinase with or without

Table 1. Glucose Consumption and Lactate Production in Rat Erythrocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose consumption (µmol/g Hb/min)</th>
<th>Lactate production (µmol/g Hb/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.119 ± 0.003</td>
<td>0.195 ± 0.007</td>
</tr>
<tr>
<td>M16209 (100 µM)</td>
<td>0.147 ± 0.003**</td>
<td>0.251 ± 0.007**</td>
</tr>
</tbody>
</table>

Erythrocytes were incubated with M16209 at 37°C for 60 min. Each value represents the mean ± S.E. of six rats. * * p < 0.01, vs. vehicle-treated erythrocytes. Differences in values were analyzed by the paired t-test.

Fig. 4. Effect of M16209 on Lactate Production from Glucose and D-Glyceraldehyde (D-GA) in Rat Erythrocytes

Erythrocytes were incubated with 100 µM M16209 at 37°C for 60 min. The level in vehicle-treated erythrocytes was defined as 100%. Each value represents the mean ± S.E. of four rats. * p < 0.05, vs. vehicle-treated erythrocytes. Differences in values were analyzed by the paired t-test. Lactate production rates from glucose and D-GA in vehicle-treated erythrocytes were 183 ± 8 and 195 ± 15 nmol/g Hb/min, respectively.

Fig. 5. Change in Levels of Some Glycolytic Intermediates by M16209 in Rat Erythrocytes

Erythrocytes were incubated with 100 µM M16209 at 37°C for 30 min. The level in vehicle-treated erythrocytes was defined as 100%. Each value represents the mean ± S.E. of six rats. ** p < 0.01, vs. vehicle-treated erythrocytes. Differences in values were analyzed by the paired t-test. Abbreviations and the values (nmol/g Hb) of intermediates in vehicle-treated erythrocytes: G6P, glucose-6-phosphate (320.7 ± 18.2); F6P, fructose-6-phosphate (87.3 ± 3.4); F1,6BP, fructose-1,6-bisphosphate (10.0 ± 0.5); DHAP, dihydroxyacetone phosphate (15.5 ± 1.4).
the low-molecular-fraction (MW < 10000) of the hemolysate (Table 2).

In rat erythrocytes incubated for 30 min, butyformine, tolbutamide and sorbinil at a concentration of 50 μM each had no effect on lactate production (102, 100 and 100% respectively, vehicle = 100%), but M16209 increased it (121%, p < 0.01, paired t-test). In vehicle-treated erythrocytes, the lactate production rate was 185 ± 5 nmol/g Hb/min.

DISCUSSION

Erythrocytes extract energy from glucose almost entirely via an anaerobic pathway, and produce lactate as the final product of glycolysis. In this study, M16209 accelerated lactate production from glucose in rat and human erythrocytes, suggesting that the agent activates glycolysis in erythrocytes. On the other hand, d-glyceraldehyde was used as the substrate by erythrocytes, there was no acceleration of lactate production by M16209. d-Glyceraldehyde is known to be metabolized by triokinase to glyceraldehyde-3-phosphate, which is used for glycolysis in erythrocytes.

Concerning the levels of glycolytic intermediates prior to the glyceraldehyde-3-phosphate step in the glycolytic pathway, M16209 lowered the levels of both glucose-6-phosphate and fructose-6-phosphate, but increased fructose-1,6-bisphosphate. These findings indicate that M16209 activated 6-phosphofructokinase and, in fact, augmented this activity in rat hemolysates. 6-Phosphofructokinase is known to be a key regulatory enzyme in the glycolytic pathway, suggesting that its activation by M16209 leads to acceleration of lactate production in erythrocytes.

It is well known that 6-phosphofructokinase includes three types of isozymes: muscle-, liver- and C-type, and that much of the isozyme in erythrocytes is a mixture of the muscle-type and the liver-type. M16209 did not activate purified rabbit muscle (muscle-type) or liver (liver-type) 6-phosphofructokinase, but did activate 6-phosphofructokinase in hemolysate. In erythrocytes, 6-phosphofructokinase is a hybrid-tetramer of muscle-type and liver-type. The sensitivity of allosteric inhibition of this hybrid by ATP, citrate and 2,3-diphosphoglycerate is reported to be intermediate between muscle-type and liver-type homo-tetramer, and not simply the same as a mixture of equal quantities of the two types of homo-tetramer.

Therefore, this difference of molecular components might be associated with the activation of 6-phosphofructokinase by M16209. 6-Phosphofructokinase activity is reported to be regulated by several compounds, and, moreover, the low-molecular-fraction (MW < 10000) of the hemolysate did not induce activation of 6-phosphofructokinase by M16209 in this study. This demonstrates that activation of 6-phosphofructokinase by M16209 requires the high-molecular-fraction (MW > 10000) of hemolysate. It is unknown which agents modulate the effects of M16209; however, it is possible that the action of M16209 involves enzymes associated with the synthesis or degradation of attenuators of 6-phosphofructokinase activity and band-3 protein, which binds and activates 6-phosphofructokinase and others. In addition, it cannot be denied that the activities of enzymes which synthesize or degrade the attenuators of 6-phosphofructokinase activity may be affected by the low-molecular-fraction (MW < 10000). So, it is possible that the action of M16209 is due to the cooperation of the low-molecular-fraction (MW < 10000) and the high-molecular-fraction (MW ≥ 10000) of hemolysate.

M16209 has both an antidiabetic effect and an aldose reductase inhibiting effect. Sorbinil, an aldose reductase inhibitor which inhibits aldose reductase activity in vitro at a concentration of 50 μM, did not increase lactate production. These findings suggest that the enhancement of lactate production by M16209 is not due to the aldose reductase inhibitory effect. Neither butyformine, a biguanide, nor tolbutamide, a sulfonylurea, accelerated lactate production at a concentration of 50 μM.

In conclusion, the findings indicate that M16209 increases glycolysis via activation of 6-phosphofructokinase, suggesting that M16209 may accelerate glycolysis in other tissues as well as erythrocytes. Consequently, the acceleration of glycolysis by M16209 plays a role in the production of energy and the formation of lactate.
mechanism of antihyperglycemia, particularly by increasing glucose uptake in peripheral tissues.

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