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EVIDENCE THAT POLYMYXIN B IS A GLUCOSE TRANSPORT INHIBITOR

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Abstract—The effect of polymyxin B on 3-*O*-methylglucose transport was studied in isolated rat adipocytes and erythrocytes. Polymyxin B (300 μg/mL) inhibited basal transport and insulin-stimulated transport of 3-*O*-methylglucose in adipocytes by 26.1 and 40.1%, respectively. Polymyxin B at concentrations of 300 and 3000 μg/mL inhibited transport of 3-*O*-methylglucose in erythrocytes by 20.0 and 40.8%, respectively. Polymyxin E at a concentration of 3000 μg/mL also inhibited, by 40.6%, the transport of 3-*O*-methylglucose in erythrocytes but 300 μg/mL of polymyxin E did not inhibit it significantly. These results indicate that polymyxin B inhibits glucose transport *per se*, as well as the insulin-dependent stimulation of glucose transport.

Glucose transport in adipocytes and erythrocytes occurs by facilitated diffusion through glucose transporters on the cell surface [1–3] and the velocity of glucose transport depends on the number and function of the glucose transporters. Especially in adipocytes, regulation of glucose transport is considered to depend on the action of insulin in mobilizing the glucose transporters [4, 5]. Polymyxin B is a decapeptide antibiotic and has been recognized as a new agent inhibiting the action of insulin on glucose transport in isolated rat adipocytes [6] and in isolated rodent muscles [6–8], but a mechanism for the inhibition has not been determined. Although Amir *et al.* [6] reported that polymyxin B inhibited little non-insulin-stimulated (basal) uptake of hexose by adipocytes, we found that polymyxin B did inhibit the basal uptake of hexose into isolated rat adipocytes, as well as the insulin-stimulated uptake of hexose. This observation led us to examine whether or not polymyxin B inhibits glucose transport *per se*. To perform this experiment, we employed circulating erythrocytes which are insensitive to insulin [1] and with which we could evaluate accurately the effect of polymyxin B on glucose transport.

MATERIALS AND METHODS

Materials

3-*O*-methyl-D-[^{3}H]Glucose was purchased from New England Nuclear (Boston, MA, U.S.A.); polymyxin B sulfate (8090 units/mg) from Nacalai tesque (Kyoto, Japan); polymyxin E(Colistin) sulfate (min. 15,000 units/mg) from Wako Pure Chemical Industries (Osaka, Japan); porcine insulin, phloretin and bovine serum albumin (RIA grade) from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and collagenase (CLS 1) from the Worthington Biochemical Corp. (Freehold, NJ, U.S.A.).

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Experiments using adipocytes

Epididymal fat pads were removed from male Wistar rats weighing 200–210 g under anesthesia induced by intraperitoneal injection of 100 mg/kg of sodium pentobarbital and isolated adipocytes were prepared by the collagenase method [9]. The cell suspension in Krebs–Henseleit HEPES buffer [10] supplemented with 20 mg/mL of bovine serum albumin and 3 mM sodium pyruvate, pH 7.4 was adjusted to 21.3% in cytocrit value (20.0% in the net cell volume as 6% of the packed cell volume is occupied by extracellular water [11]). The glucose transport activity was assessed by measuring the rate of uptake of 3-*O*-methylglucose at 37°, based on the method of Toyoda *et al.* [10, 12]. In brief, the cells for polymyxin B treatment were mixed with 300 μg/mL polymyxin B 5 min before insulin stimulation. The cells for insulin stimulation were then exposed to 10 nM insulin for 10 min at 37°. At the end of the incubation, the glucose transport assay was initiated by adding 0.1 mM 3-*O*-methyl-D-[^{3}H]glucose. The insulin-stimulated cells were incubated with 3-*O*-methylglucose for 3 sec and the basal cells, for 15 sec. The uptake reaction was terminated by adding 1 mM phloretin and the cell suspension was transferred into a centrifuge tube containing dinonyl phthalate. The cells in the suspension were collected by the oil-flotation method and radioactivity was determined by the liquid scintillation counting method. The amount of 3-*O*-methylglucose in the extracellular water of the collected cell fraction was estimated by adding labeled hexose after phloretin to the corresponding cells incubated in parallel; the validity of this method has been verified by Toyoda *et al.* [10, 12]. All results were corrected for this factor and the values are shown in picomoles of hexose taken up specifically per min per mg of adipocytes. Polymyxin B (300 μg/mL) did not change the intracellular water space of the adipocytes, which was estimated by measuring an equilibrium space of 3-*O*-methylglucose.

Experiments using erythrocytes

Erythrocyte preparation. Blood (5–6 mL) was

Table 1. Effect of 300 μg/mL polymyxin B on basal and insulin-stimulated uptake of 3-*O*-methylglucose by isolated rat adipocytes

	3-*O*-Methylglucose uptake (pmol/min/mg cells)	
	Basal	Insulin-stimulated
Control cells	2.72 ± 0.054	14.88 ± 0.55
Polymyxin B-treated cells	2.01 ± 0.085*	8.92 ± 0.61*

The actual uptake time was 3 sec in the insulin-stimulated cells and 15 sec in the basal cells. The uptake values were calculated to picomoles of 3-*O*-methylglucose taken up per min per mg cells. The concentration of insulin was 10 nM.
Values are means ± SE, N = 6. * $P < 0.005$ vs controls.

drawn from the abdominal aorta of male Wistar rats weighing 210–280 g into a heparinized syringe under anesthesia induced by the intraperitoneal injection of 80 mg/kg sodium pentobarbital and isolated erythrocytes were prepared by the Ficoll–Hypaque method [13], as described by Gambhir *et al.* [14]. The collected erythrocytes were washed once with physiological saline and then washed twice with Krebs–Henseleit HEPES buffer, pH 7.4. After the first and second washes, the cells were incubated for 5 min at 37° to remove intracellular glucose, as described by May [15]. Following washes the cell suspension was adjusted to 20% in hematocrit value.

Glucose transport. The glucose transport activity was assessed by measuring the initial rate of uptake of 3-*O*-methylglucose at 22°, based on the same principle of hexose uptake, as described above for adipocytes. Unless otherwise stated, the cells for polymyxin B and polymyxin E treatment were mixed with 300 or 3000 μg/mL of agent. After incubation for 15 min at 22°, the glucose transport assay was initiated by adding 0.1 mM 3-*O*-methyl-D-[^{3}H]-glucose. The cells were incubated with 3-*O*-methylglucose for 1 min at 22°. The uptake reaction was terminated by adding 1 mM phloretin and the cell suspension was transferred into a centrifuge tube containing dibutyl phthalate. The cells in the suspension were collected by the oil-centrifugation method, as described by Gambhir *et al.* [14]. The middle layer of dibutyl phthalate was cut with a knife and a portion of cell pellet was put into a counting vial. The cell pellet was dissolved in 0.2 mL of 6% KOH, neutralized with acetic acid and dissolved further in ACS II (Amersham Corp., Arlington Heights, IL, U.S.A.). The radioactivity of ^{3}H was counted in a liquid scintillation counter Tri-Carb 300C (Packard Instrument Co., Downers Grove, IL, U.S.A.). The extracellular trap of 3-*O*-methylglucose in each collected cell fraction was estimated by adding labeled hexose after phloretin to the corresponding cells incubated in parallel. All results were corrected for this factor and the values are shown in picomoles of hexose taken up specifically per min per 10 μL of erythrocytes.

Evaluation of intracellular water space. The intracellular water space of erythrocytes was assessed by measuring an equilibrium space of 3-*O*-methylglucose at 37°. Basically the same method was used as for the glucose transport assay described above, except for incubation time and temperature. The cells were preincubated with 0 or 3000 μg/mL of polymyxin B or polymyxin E for 15 min at 22° and were incubated further with 0.1 mM 3-*O*-methyl-D-[^{3}H]glucose for 30, 60 or 120 min at 37. The subsequent procedure was the same as that for the glucose transport assay. The results show the ^{3}H activity taken up specifically by erythrocytes as a percentage of the total ^{3}H activity of the assay system.

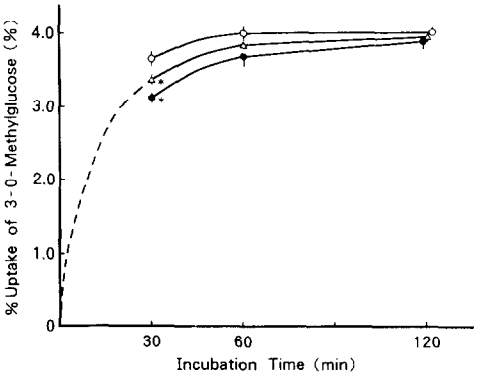

Fig. 1. Evaluation of intracellular water space of erythrocytes untreated (○) or treated with 3000 μg/mL polymyxin B (●) or 3000 μg/mL polymyxin E (△) by measuring an equilibrium space of 3-*O*-methylglucose at 37° at the indicated times. Values are means ± SE, N = 4. * $P < 0.05$ vs corresponding control.

Statistical analysis

All results were expressed as means ± SEM. The two-tailed and one-tailed Mann–Whitney U test and the one-tailed unpaired *t*-test were applied as appropriate.

RESULTS

In isolated rat adipocytes treated with 300 μg/mL polymyxin B, the insulin-stimulated and basal uptakes of 3-*O*-methylglucose were decreased significantly ($p < 0.005$) by 40.1 and 26.1%, respectively, compared with those in control cells (Table 1).

In control rat erythrocytes, as shown in Fig. 1, an

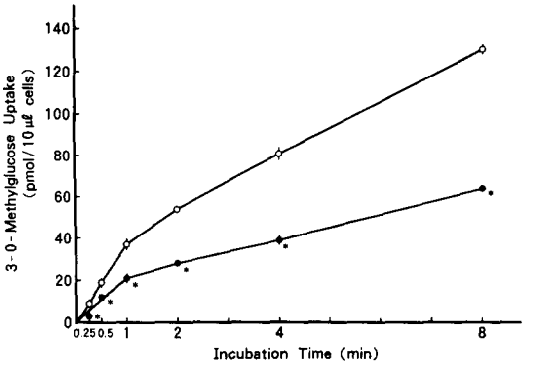

Fig. 2. Time course of 3-*O*-methylglucose uptake at 22° by erythrocytes preincubated with 0 μg/mL (○) or 3000 μg/mL polymyxin B (●) at 22° for 15 min. Values are means ± SE, N = 3. The equilibrium value of 3-*O*-methylglucose uptake was 385.4 ± 2.6 pmol/10 μL cells, 3.85% of the total radioactivity added to the assay system, in this experiment and the uptake value after 1 min in the control cells, 36.9 ± 2.5 pmol/10 μL cells, was 9.57% of the equilibrium value. * $P < 0.05$ vs corresponding control.

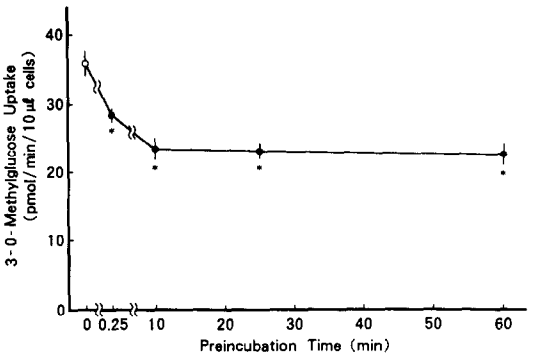

Fig. 3. Time course of preincubation of erythrocytes with 3000 μg/mL polymyxin B at 22°. After preincubation for the indicated periods, the rate of 3-*O*-methylglucose uptake by erythrocytes was measured at 22° for 1 min. Values are means ± SE, N = 4. * $P < 0.02$ vs zero-time controls.

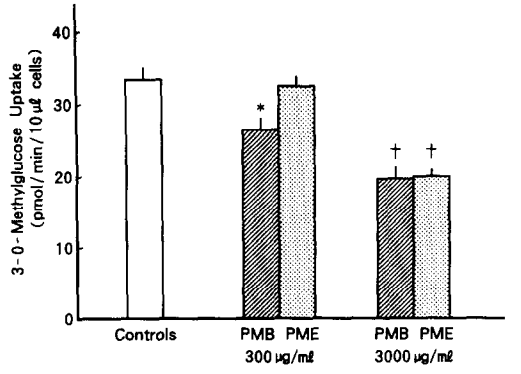

Fig. 4. Effect of 300 and 3000 μg/mL of polymyxin B (PMB) or polymyxin E (PME) on 3-*O*-methylglucose uptake by erythrocytes. The cells were preincubated with or without the indicated agent at 22° for 15 min and the rate of 3-*O*-methylglucose uptake was measured at 22° for 1 min. Values are means ± SE, N = 4. * $P < 0.05$ vs controls; † $P < 0.02$ vs controls.

equilibrium of 3-*O*-methylglucose diffusion was obtained after 30–60 min incubation at 37°. In polymyxin B (3000 μg/mL) and polymyxin E (3000 μg/mL) treated cells, the reaction was not quite at equilibrium after 30 min but reached a plateau after 60–120 min. There was no difference in the equilibrium space attained after 120 min at 37° between the control (4.02 ± 0.04%, N = 4), polymyxin B-treated (3.91 ± 0.08%, N = 4) and polymyxin E-treated cells (3.97 ± 0.04%, N = 4). As this experiment clarified that these agents do not change the intracellular water space, the rates of uptake of 3-*O*-methylglucose into erythrocytes were compared directly without correction for this factor in the following experiments. Figure 2 shows the rate of uptake of 3-*O*-methylglucose at various time points. The uptake appeared to be biphasic in both the control and the 3000 μg/mL polymyxin B-treated cells. Although the reason for the biphasic uptake was not clear, the uptake rate was regarded as being linear for at least 1 min in both groups, therefore, 1 min was taken as the uptake time providing an initial rate of reaction in the following experiments. The rate of 3-*O*-methylglucose uptake in this experiment was decreased significantly ($P < 0.05$) in the 3000 μg/mL polymyxin B-treated cells at every time point, compared with that in the control cells (Fig. 2). Figure 3 shows that the inhibitory effect of 3000 μg/mL polymyxin B on 3-*O*-methylglucose uptake was constant after 10 min preincubation with the agent and, accordingly, 15 min of preincubation time was appropriate for the evaluation of this effect. Figure 4 shows the effect of polymyxins on 3-*O*-methylglucose uptake. The rate of 3-*O*-methylglucose uptake was decreased significantly ($P < 0.02$ and $P < 0.05$) by 40.8 and 20.0% in the 3000 μg/mL polymyxin B-treated cells (19.74 ± 1.35 pmol/min/10 μL cells, N = 4) and the 300 μg/mL polymyxin B-treated cells (26.68 ± 1.52 pmol/min/10 μL cells, N = 4), respectively, compared with that in the control cells (33.35 ± 1.64 pmol/min/10 μL cells, N = 4). In addition, the rate of 3-*O*-methylglucose uptake was decreased significantly ($P < 0.02$) by 40.6% in the 3000 μg/mL polymyxin E-treated cells (19.80 ± 1.01 pmol/min/10 μL cells, N = 4) but was not decreased significantly in the 300 μg/mL polymyxin E-treated cells (32.56 ± 1.23 pmol/min/10 μL cells, N = 4).

DISCUSSION

In the present study, we have shown that polymyxin B inhibits basal transport of glucose by measuring 3-*O*-methylglucose uptake by isolated rat adipocytes (Table 1), while previous studies using isolated rat adipocytes [6] or isolated rodent muscles [6–8] have shown little or no inhibitory effect of polymyxin B on the basal uptake of glucose. The reason for this discrepancy is unclear but higher concentrations of

polymyxin B may be required to inhibit the basal glucose transport than those used by previous investigators of 100–250 μg/mL [6–8]. We used 300 μg/mL polymyxin B in the present study because this is the maximal concentration having an inhibitory effect on the insulin-stimulated uptake of glucose but not changing the intracellular or the extracellular water space of adipocytes. In general, inhibitory agents can reduce the intrinsic activity of glucose transporters, inhibit the maximal effect of insulin and/or inhibit the sensitivity of the glucose transport system to insulin. The present study focused on the effect of polymyxin B on glucose transport *per se* because of our observation of basal glucose transport inhibition, as stated in the Introduction. We demonstrated clearly that polymyxin B did inhibit glucose transport *per se* by using circulating rat erythrocytes (Figs 1–4). Although a mechanism for the inhibitory effect of polymyxin B on glucose transport has not been determined, we speculate that some peptides might inhibit the function of glucose transporters, since polymyxin E (3000 μg/mL), a decapeptide which has a similar sequence of amino acids to polymyxin B, also inhibited glucose transport (Fig. 4) and the dipeptide metalloendoprotease substrates, can inhibit glucose transport through some effect on the glucose transporters, as shown by Aiello *et al.* [16] and Wheeler [17]. In order to investigate this possibility, further studies are needed.

The possibility that erythrocytes were destroyed by polymyxin B or polymyxin E and that the level of glucose transport was thus decreased is unlikely because the intracellular water space estimated as an equilibrium space of 3-*O*-methylglucose was comparable between the agent-treated cells and the control cells (Fig. 1).

The effect of polymyxin B on the insulin-dependent stimulation of glucose transport in adipocytes should be evaluated carefully under the present conditions. From Table 1, 300 μg/mL polymyxin B inhibited the basal transport of glucose by 26% while it inhibited the insulin-stimulated transport of glucose by 40%. If the glucose transporters recruited by insulin and those at basal state are inhibited equally by polymyxin B, the insulin-dependent stimulation of glucose transport would appear to be inhibited by 14% by this concentration of polymyxin B. Alternatively, if polymyxin B does not affect the insulin action itself but inhibits the function of the glucose transporters recruited by insulin to a greater extent than that of those at basal state, the present result is simply explained and is substantially compatible with the result obtained by Amir *et al.* [6], i.e. that polymyxin B inhibits the insulin-stimulated uptake of 2-deoxyglucose by isolated rat adipocytes to a much greater extent than the basal uptake of 2-deoxyglucose. However, it is still uncertain whether polymyxin B inhibits the action of insulin itself or the glucose transport system already stimulated by insulin. This problem is under investigation in our laboratory using isolated rat adipocytes.

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