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Insulin and noradrenaline independently stimulate the translocation of glucose transporters from intracellular stores to the plasma membrane in mouse brown adipocytes

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The mechanism of the effect of noradrenaline on the transport of 3-*O*-methyl-D-[^{14}C]glucose ([^{14}C]-MG) was studied in mouse brown adipocytes. When cells were exposed to low concentrations (< 10^{-8} M) of insulin, the [^{14}C]-MG uptake by cells was enhanced by noradrenaline additively. The action of noradrenaline was mimicked by isoproterenol, and was completely blocked by propranolol. Exposing cells to noradrenaline induced both an increase in the transport activity of plasma membrane fractions and a decrease in that of microsomal fractions similar to insulin exposure, indicating that noradrenaline also induces the translocation of glucose transporters to the plasma membrane. The ratio of an increase in the transport activity of plasma membrane fraction to a decrease in the activity of microsomal fraction was lower in cells exposed to noradrenaline than in cells exposed to insulin. This quantitative disagreement suggests that there are at least two different modes involved in the regulation of the translocation of glucose transporters in mouse brown adipocytes.

Glucose transport; Insulin; Noradrenaline; Translocation; Cyclic AMP; Brown adipocyte

1. INTRODUCTION

Brown adipose tissue (BAT) is the major site of non-shivering thermogenesis activated by noradrenaline released from sympathetic nerve terminals. It has been suggested that glucose is an important fuel for thermogenesis as well as a source for synthesis of fatty acids [1]. Despite the fact that BAT contains glycolytic enzymes of high activity [2], the quantitative importance of glucose as thermogenic fuel is equivocal [2,4–8]. A number of studies on the regulation of glucose transport and utilization in BAT have been reported [3–12]. Recently, Slot et al. [11] have demonstrated histochemically that insulin brings about a facilitation of the translocation of glucose transporters (GLUT4) to the cell surface in brown adipocytes as well as in white adipocytes. On the other hand, the mechanism of the regulation of glucose transport by noradrenaline in BAT has not been established. The aim of the present study was to investigate the mechanism through which noradrenaline regulates glucose transport in mouse brown adipocytes. Present experiments revealed that insulin and noradrenaline independently stimulated the translocation of glucose transporters from intracellular stores to the plasma membrane.

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2. EXPERIMENTAL

2.1. *Preparation of brown adipocytes and membrane fractions*

Male ICR mice (aged 3–5 weeks), maintained at 25°C on a 12:12 h light–dark cycle and fed ad libitum, were used in the experiments. The animals were killed by cervical dislocation. The procedure for the isolation of brown adipocytes is similar to that reported by Marette and Bukowiecki [10]. The finally isolated cells were suspended in Krebs Ringer bicarbonate buffer (KRB) containing 4% bovine serum albumin (BSA) gassed with 95% O_2–5% CO_2 (pH 7.4), and were counted on the Bürker–Türk hemocytometer. All procedures were carried out in plastic vials or tubes.

After incubating with various test reagents, the cells were homogenized in 250 mM sucrose containing 1 mM EDTA and Tris-HCl (pH 7.4), then plasma and microsomal membrane fractions were prepared by the method described by Cushman and Wardzala [13]. Activities of 5'-nucleotidase, a marker enzyme of plasma membrane, and NADH oxidase, a marker enzyme of mitochondria, were measured by the method of Avruch and Wallach [14]. Protein was measured by the method of Bradford [15]. The yields and marker enzyme activities are shown in Table I.

2.2. *3-O-Methyl-D-glucose transport assay*

The glucose transport activity was estimated by measuring the uptake of 3-*O*-methyl-D-glucose (MG), a non-metabolic analogue of D-glucose, according to the modified method described by Vega and Kono [16]. The rate of labeled MG uptake was expressed as a unidirectional flux. Prior to the assay of MG transport, the cell suspensions of 5–10 × 10^5 cells/ml were preincubated with hormones or test reagents in KRB supplemented with 4% BSA for 25 min at 37°C. Control cells were incubated in KRB with 4% BSA under the same conditions. MG uptake was measured by adding 10 μl of MG (final concentration 0.1 mM) containing 3.7 kBq (0.1 μCi) of 3-*O*-methyl-D-[^{3}H]glucose and a tracer amount of [^{14}C]inulin (3.7 kBq) to 500 μl of cell suspension with or without 40 μM cytochalasin B. [^{3}H]Inulin added was a monitor

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for the estimation of the extracellular space. Incubation with tracers was performed for 20 s at 37°C. During this period the cell suspension was continuously shaken. After incubation, a 6-fold volume of ice-cold KRB containing 1 mM phloretin was added to the cell suspension, and the whole volume was filtered through a cellulose membrane filter (Millipore; pore size 0.45 μm). The filter was washed twice with the same buffer and dissolved in ACS-II scintillant (Amersham), and radioactivities were counted in a liquid scintillation counter. The amount of MG taken up by cells was calculated from the difference between the total radioactivity of [^{14}C]-MG on the filter and that in the extracellular inulin space [17]. The activities of MG transport in membrane fractions were estimated by the slightly modified method of Kashiwagi et al. [18]. In both cases the transporter-mediated transport activity was determined from the difference between the values in the presence and absence of 40 μM cytochalasin B. Statistical significance was evaluated by using the Student's *t*-test.

2.3. *Chemicals*

Collagenase was obtained from Wako Purechemical Industries (Osaka, Japan). Insulin, deoxyribonuclease I (DNase I), (−)-noradrenaline hydrochloride, N^6-2'-*O*-dibutyryl adenosine 3':5'-cyclic monophosphate sodium salt, cytochalasin B and 3-*O*-methyl-D-glucose were obtained from Sigma Chemical Co. Bovine serum albumin, DL-propranolol hydrochloride, phloretin, 3-iso-butyl-1-methylxanthine, DL-isoproterenol hydrochloride and all other reagents were purchased from Nacalai Tesque (Kyoto, Japan). 3-*O*-Methyl-D-[^{14}C]glucose and [^{3}H]inulin were obtained from Amersham.

Table I

Yields and marker enzyme activities in membrane preparations. Isolated brown adipocytes were incubated in test reagents for 25 min at 37°C prior to preparation of membrane fractions. The concentrations of insulin and noradrenaline are 10^{-9} M and 10^{-8} M, respectively. Data are given as mean ± S.E.M. for four experiments

Preparations	Yield and marker enzyme activities		
	Protein (mg/g of BAT)	5'-Nucleotidase (μmol/s/mg of protein)	NADH oxidase (μmol/min/mg of protein)
Control			
Homogenate	114.1 ± 2.0	2.4 ± 0.1	29.9 ± 7.5
Plasma membrane	1.3 ± 0.02	99.8 ± 5.3	153.2 ± 39.4
Microsomes	4.0 ± 0.1	2.0 ± 0.1	106.1 ± 27.5
Mitochondria	8.7 ± 0.2	1.6 ± 0.1	313.0 ± 15.8
Insulin-exposed			
Homogenate	93.2 ± 2.0	2.6 ± 0.2	32.7 ± 7.7
Plasma membrane	1.3 ± 0.02	102.4 ± 2.8	140.0 ± 22.2
Microsomes	3.2 ± 0.2	2.5 ± 0.1	108.7 ± 16.9
Mitochondria	8.9 ± 0.03	1.5 ± 0.1	314.3 ± 22.5
Noradrenaline-exposed			
Homogenate	100.0 ± 2.8	2.8 ± 0.2	31.2 ± 7.5
Plasma membrane	1.2 ± 0.03	105.5 ± 2.9	153.8 ± 20.0
Microsomes	2.8 ± 0.1	2.9 ± 0.2	123.9 ± 4.7
Mitochondria	7.7 ± 0.02	2.0 ± 0.2	342.0 ± 52.0
Insulin + Noradrenaline-exposed			
Homogenate	104.4 ± 2.8	2.8 ± 0.2	31.2 ± 7.5
Plasma membrane	1.3 ± 0.02	103.5 ± 1.5	140.2 ± 23.4
Microsomes	3.7 ± 0.2	2.9 ± 0.2	92.6 ± 11.1
Mitochondria	8.7 ± 0.2	1.8 ± 0.1	280.0 ± 46.0

3. RESULTS AND DISCUSSION

3.1. *Both insulin and noradrenaline stimulate MG transport in brown adipocytes*

The effects of insulin and noradrenaline on MG uptake by brown adipocytes are shown in Fig. 1A. Basal MG transport activity was approx. 10–18 pmol/s/10^6 cells. Exposing cells to insulin enhanced the transport activity in a dose-dependent manner. The half maximal concentration (1/2 V_{max}) for the stimulation was approx. 10^{-10} M of insulin, which was similar to the reported value in both brown and white adipocytes [10,19]. Beside the response to insulin, the MG uptake increased in response to a rise of the noradrenaline concentration in the range below 10^{-7} M (insert in Fig. 1A), being consistent with the reports in vivo [4–6] and in vitro [10,12]. A further elevation of noradrenaline concentration rather resulted in a suppression of MG

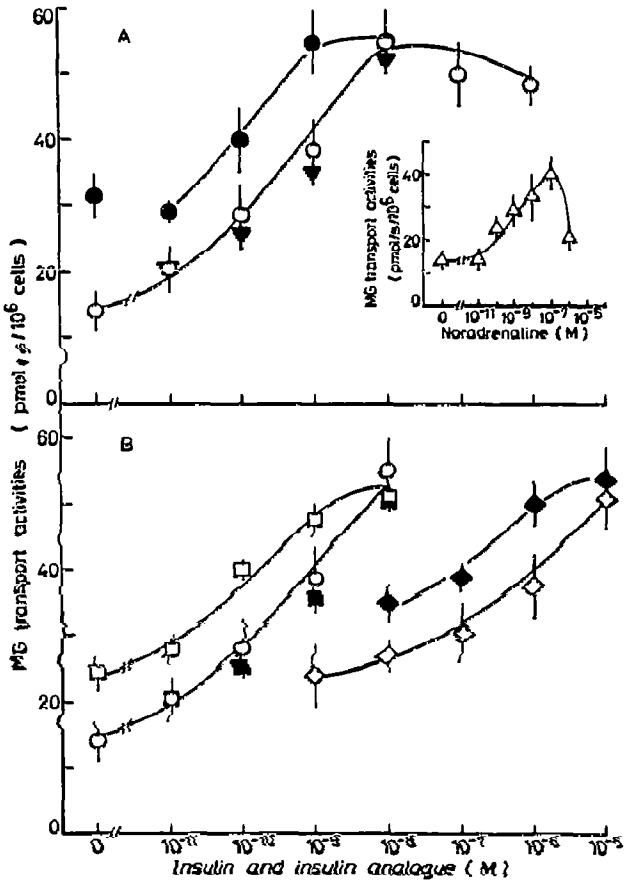

Fig. 1. Effects of insulin, noradrenaline, isoproterenol and propranolol on MG transport in brown adipocytes. Isolated brown adipocytes were incubated in various test reagents for 25 min at 37°C prior to the assay of MG transport. Conditions of the incubation solutions are as follows: A. ○, insulin; ●, insulin and 10^{-5} M noradrenaline; ▼, insulin, 10^{-8} M noradrenaline and 10^{-6} M propranolol. B. △, insulin and 10^{-7} M isoproterenol; ▲, insulin, 10^{-7} M isoproterenol and 10^{-5} M propranolol; ◇, [Leu]$^{B-24}$-insulin; ◆, [Leu]$^{B-24}$-insulin and 10^{-5} M noradrenaline. The insert in A shows the relationship between MG transport and concentration of noradrenaline. Vertical bars indicate S.E.M. for four experiments.

transport activity. This is compatible with the report that the high concentration of noradrenaline (10^{-6} M) has no influence on glucose transport [8]. The effect of noradrenaline on insulin-stimulated glucose transport was also examined. At any concentrations of insulin lower than 10^{-8} M, noradrenaline (10^{-8} M) stimulated MG transport. However, when the concentration of insulin was 10^{-8} M, noradrenaline had no effect on the MG transport activity. Propranolol (10^{-6} M), β-adrenergic antagonist, completely suppressed the stimulatory effect of noradrenaline on MG transport at all insulin concentrations examined. Isoproterenol (10^{-7} M), β-adrenergic agonist, well mimicked the action of noradrenaline on insulin-stimulated MG transport (Fig. 1B). Propranolol of 10^{-6} M completely blocked the stimulatory effect of isoproterenol. Phenylephrine, α-adrenergic agonist, had no significant effects on the MG uptake (data not shown). Fig. 1B shows that MG transport in brown adipocytes were also stimulated by [Leu]B^{24}-insulin, a semisynthesized insulin analogue. This finding accords with the previous observation in skeletal muscle [20]. Again, noradrenaline of 10^{-8} M enhanced MG transport at concentrations of the analogue lower than 10^{-5} M.

To know whether this effect of noradrenaline depends on cyclic AMP, dibutyryl cyclic AMP (DBcAMP, a membrane-permeable cyclic AMP analogue) and 3-isobutyl-1-methylxanthine (IBMX, an inhibitor of phosphodiesterase) were examined. As listed in Table II, both reagents stimulated the MG transport. Adding insulin at 10^{-9} M to the incubation solution containing one of these reagents caused a further increase in MG transport. Further addition of propranolol did not give any statistically significant effects on the levels of MG transport stimulated by insulin and either DBcAMP or IBMX.

Table II

Effects of DBcAMP, IBMX, insulin and propranolol on MG transport in brown adipocytes. Isolated brown adipocytes were incubated in test reagents for 25 min at 37°C prior to the assay of MG transport. Data are given as mean ± S.E.M. for four experiments

Incubation conditions			MG transport (pmol/s/10^6 cells)
DBcAMP (10^{-3} M)	Insulin (10^{-9} M)	Propranolol (10^{-6} M)	
−	−	−	19.0 ± 4.0
+	−	−	37.0 ± 5.4
+	+	−	66.0 ± 2.4
+	+	+	66.5 ± 2.4
IBMX (10^{-4} M)	Insulin (10^{-9} M)	Propranolol (10^{-6} M)	
+	−	−	37.3 ± 4.3
+	+	−	71.9 ± 7.8
+	+	+	68.4 ± 7.8

To see if noradrenaline affects the process of the translocation of glucose transporters which is stimulated by insulin, brown adipocytes were incubated with insulin in the presence or absence of noradrenaline for various periods of time before adding [^{14}C]-MG to the cell suspension. Fig. 2A shows the time courses of the increase in glucose transport activity induced by insulin of 10^{-9} M with and without noradrenaline. The transport activity stimulated by insulin alone did not reach a steady level even after 30 min of the starting incubation. In the presence of noradrenaline the stimulation by insulin seemed to be accelerated. Fig. 2B shows the time course of the increase in MG transport activity when only noradrenaline was added to preincubation solution. The difference between two curves shown in Fig. 2A showed good accordance with the curve in Fig. 2B. This observation suggests that noradrenaline does not facilitate the translocation induced by insulin of glucose transporters, and that noradrenaline stimulates the transport activity of the plasma membrane via its own pathway.

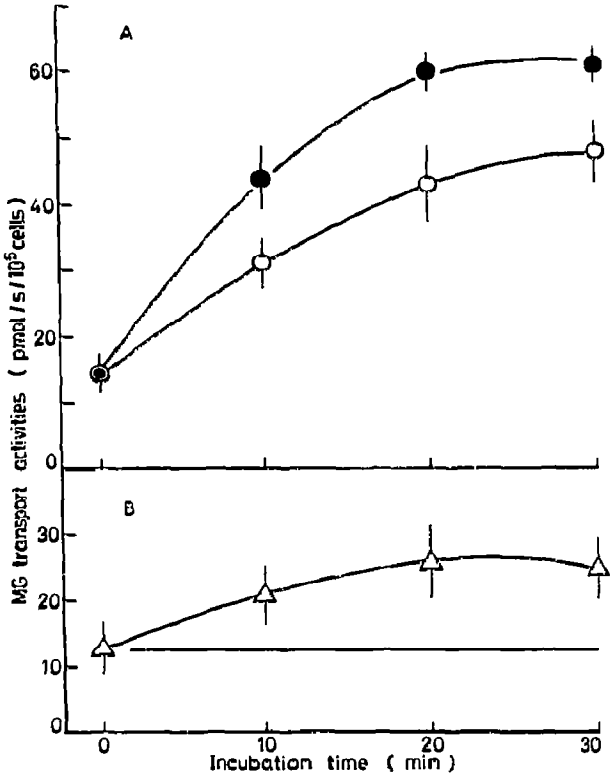

Fig. 2. Time course of the stimulation of MG transport in brown adipocytes by insulin and noradrenaline. A. Isolated brown adipocytes were incubated in insulin in the presence and absence of noradrenaline for the various periods at 37°C prior to the assay of transport. Conditions of the incubation solutions are as follows: A. ○, 10^{-9} M insulin; ●, 10^{-9} M insulin and 10^{-8} M noradrenaline. B shows the time course of the change in MG transport activity in brown adipocytes after adding noradrenaline into the incubation solution. Vertical bars indicate S.E.M. for four experiments. A straight line represents the back ground level of transport activity.

3.2. *Insulin and noradrenaline independently induce both an increase in the MG transport activity of plasma membranes and a decrease in the activity of microsomes*

In order to determine whether noradrenaline causes the redistribution of glucose transporters in brown adipocytes, MG transport activities in membrane fractions prepared under a variety of conditions were examined. Transport activities are summarized in Table III, which shows that only 22% of the total MG transport activity was present in the plasma membrane fraction prepared from the control brown adipocytes. In insulin-exposed cells, MG transport activity of the plasma membrane fraction was higher than its control value. The plasma membrane fraction prepared from noradrenaline-exposed cells also showed an increase in the transport activity. Similar to the cells exposed to noradrenaline, cells exposed to isoproterenol (10^{-7} M) also showed both an increase in the MG transport activity of the plasma membrane fraction and a decrease in the activity of the microsomal fraction (data not shown). As expected from the curves shown in Fig. 1A, the increased induced by noradrenaline at 10^{-8} M in the transport activity of the plasma membrane fraction was smaller than the increase induced by 10^{-9} M insulin. The MG transport activity of the plasma membrane fraction prepared from the cells exposed to both insulin (10^{-9} M) and noradrenaline (10^{-8} M) was 315.0 ± 13.8 pmol/s/g whereas its control value was 69.2 ± 7.2 pmol/s/g. The difference between these two values was 245 pmol/s/g which was nearly equal to the sum of the increases in the transport activity induced by exposing the cells to insulin (228.7 pmol/s/g) and by exposing the cells to noradrenaline (32.8 pmol/s/g), separately. On the other hand, in the corresponding microsomal fractions the MG transport activity was seen to be decreased in a mirror image fashion to the events in the plasma membrane fractions. These results suggest that noradrenaline induces the translocation of glucose transporters to the plasma membrane independently of the stimulation by insulin. The increase in MG transport activity of the plasma membrane fraction of insulin-exposed cells is 7.1-fold (228.7/32.2) of that of noradrenaline-exposed cells. However, the decrease in MG transport activity of the microsomal fraction by insulin is only 2.2-fold (147.8/66.6) of that by noradrenaline. This quantitative disagreement implies that the mechanisms of translocation of transporters may be different between stimulations by insulin and noradrenaline.

Insulin has been reported to be capable of causing the redistribution of not only glucose transporters but also of other membrane proteins in various tissues [9,11,15,21–23]. It has been reported that catecholamines inhibit insulin-stimulated glucose uptake in white adipocytes [24,25] and that exogenous adenosine modifies insulin sensitivity in white adipocytes [8,10]. In contrast with the reported action of catecholamines on white adipocytes, present experiments revealed that noradrenaline stimulated glucose uptake in brown adipocytes by recruiting glucose transporters to the plasma membrane from intracellular stores even in the presence of insulin. This finding supports the view that a procedure activating thermogenesis may involve the stimulation of glucose transport [12]. It is reasonable to consider that BAT utilizes a readily available energy source during the period of activated thermogenesis.

In conclusion, noradrenaline activates the translocation of glucose transporters through a cyclic AMP-dependent pathway which is different from that involved in the activation induced by insulin. Taking into account the observation that the maximum stimulation by insulin of Mg transport activity was not affected by noradrenaline, it may be said that the number of translocated transporters is limited with the depletion

Table III

MG transport in plasma and microsomal membrane fractions prepared from insulin-, noradrenaline- and both of insulin and noradrenaline-exposed brown adipocytes. Isolated brown adipocytes were incubated in test reagents for 25 min at 37°C prior to prepare membrane fractions. The concentrations of insulin and noradrenaline are 10^{-9} M and 10^{-8} M, respectively. Data are given as mean ± S.E.M. for four experiments

Incubation conditions	MG transport (pmol/s/g BAT)			
	Plasma membrane fraction (pmf)		Microsomal fraction (mf)	
	M	$\Delta(M)_{pmf}$	M	$\Delta(M)_{mf}$
Control	69.2 ± 7.2	0	247.6 ± 14.3	0
Insulin-exposed	297.9 ± 8.0**	228.7	99.8 ± 10.5**	−147.8
Noradrenaline-exposed	101.4 ± 6.8**	32.2	180.7 ± 7.3**	−66.9
Insulin + Noradrenaline-exposed	315.0 ± 13.8**	245.8	76.2 ± 1.8*	−171.4

** and * represent the statistical significance of the difference between test and control groups evaluated by using the Student's t-test (**$P < 0.01$ and *$P < 0.05$).

$\Delta(M)_{pmf} = (M - M_{control})_{pmf}$, $\Delta(M)_{mf} = (M - M_{control})_{mf}$

of the transporters stored in primary available intracellular sites, and that noradrenaline may play a role when blood insulin level is low.

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