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Characterization of the Lag Phase of Insulin Action on Glucose Transport in Rat Isolated Adipocytes*)

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ABSTRACT

Insulin action on glucose transport in isolated rat adipocytes was assessed using the method originally reported by Whitesell et al. Insulin increased the Vmax of glucose transport without affecting the Km. So far as concentration dependence of insulin action it had pH and temperature dependency, and analogue specificity quite similar to previous reports.

The lag phase (time necessary for full exhibition) of insulin action has never been paid much attention, since suitable system for studying it has never been developed, and since only recently post receptor processes of insulin actions were interested in detecting insulin resistance. Insulin action on glucose transport reached the maximum in 3 min with half maximal response at 1.8 min. Lowering the pH or temperature of incubation medium prolonged the period for half maximal response. On the other hand, various insulin analogues required comparable period with insulin for full exhibition of their actions. When isolated adipocytes were obtained from rats fasted for 48 hours, the period for half maximal response was markedly prolonged (12 min).

It might be suggested that in some insulin resistant conditions such as diabetic ketoacidosis and hunger, the delayed initiation of insulin action may be involved.

INTRODUCTION

In 1979, Whitesell et al.[17] have reported a reliable method to measure glucose transport using the oil floatation technique and 3-O-methyl-glucose which is transported very rapidly but not further metabolized. Since it takes less than 10 sec for 3-O-methyl-glucose to saturate intracellular space in the presence ot insulin, this method made it possible to assess the period required for insulin to exhibit full biological activity (insulin receptor-effector coupling time, lag phase of insulin action).

Until now, many investigations have dealt with insulin action by means of sensitivity and responsiveness in steady state[8,9,11].

In the present study, using a modified method[2] of Whitesell et al., we characterized our system for insulin action on glucose transport, and examined the situations in which the lag phase for insulin action was prolonged.

MATERIALS AND METHODS

Reagents

3-O-methyl-^{3}H-glucose (4.9 Ci/mmol) was purchased from Radiochemical Centre, Amersham, Searle, collagenase (type 1) was from Worthington Biochem. Co., and porcine crystal-

*) 久保敬二，小川潤一郎，小武家暁子，高見京子，ユーヌス・タンゴ，江草玄士，河瀬 徹，山本真一，原 均，西本幸男，石橋不可止：ラット遊離脂肪細胞の糖輸送におけるインスリン作用発現時間の遅延に関する検討

line insulin (lot 615-07J-256) was a generous gift from Eli Lilly Co.. Porcine proinsulin and fish insulin were generous gifts from Shimizu Pharm. Co.. 3-O-methyl-glucose, phloretin, bacitracin and fatty acid-free bovine serum albumin were obtained from Sigma Chem. Co..

Fat Cell Preparation

Rat isolated adipocytes were prepared according to Rodbell[13] using Wistar male rat (200–220 g) fed ad libitum unless stated otherwise. All incubations were performed in Krebs-Ringer HEPES buffer (pH 7.4) containing (in mM) Na^+, 140; K^+, 4.7; Ca^{2+}, 2.5; Mg^{2+}, 1.25; Cl^-, 142; $H_2PO_4/H_2PO_4^{2-}$, 2.5; SO_4^{2-}, 1.25; HEPES, 10, and 3.0 g/dl crystalline bovine serum albumin. Bacitracin (50 mg/dl) was added in order to prevent extracellular insulin degradation. All experiments were performed at 37°C.

3-O-methyl-^{3}H-glucose Transport

The transport of 3-O-methyl-^{3}H-glucose was assessed as described by Whitesell and Gliemann with some modifications[2]. Uptake of labeled 3-O-methyl-glucose was measured in round bottom polypropylene culture tubes. Albumin-free buffer (35 μl) with 0.1 μCi labeled 3-O-methyl-glucose was placed in the bottom of the tube. 80 μl aliquots of the cell suspension (35 %v/v) were squirted on to the isotope. The incubation was terminated at the indicated times by the addition of 400 μl of albumin-free buffer containing 0.3 mM phloretin. A 300 μl aliquot of the mixture was added to a microcentrifuge tube containing 100 μl of dinonyl phthalate. The microfuge tube was spun within 2 min in a Beckman microcentrifuge B for 30 sec, cut through the oil layer and the cell pellet was then added to scintillant for the determination of radioactivity.

The non-specific uptake was determined by adding phloretin before squirting cells, and net uptake was obtained by subtracting non-specific uptake from total uptake.

Saturated space by 3-O-methyl-glucose was obtained by incubating cells for 15 min with 3-O-methyl-^{3}H-glucose, and all results were presented as % saturation of distribution space.

For the time course of 3-O-methyl-glucose uptake cells were incubated with 3-O-methyl-^{3}H-glucose for the indicated periods in the presence (10 ng/ml) or absence of insulin.

When substrate dependence of 3-O-methyl-glucose uptake was examined, cells were pulsed for 5 sec with 3-O-methyl-^{3}H-glucose containing varied concentrations of 3-O-methyl-glucose (0.5–20 mM).

When the time required for insulin to elicit maximal activation was assessed, 10 ng/ml insulin was added at zero time to fat cell suspension, and at indicated times 3-O-methyl-^{3}H-glucose uptake was measured by 5 sec pulse with labeled 3-O-methyl-glucose.

The concentration dependence of insulin action was determined by 5 sec pulse with 3-O-methyl-^{3}H-glucose in the presence of varied concentrations of insulin or insulin analogues.

RESULTS

The time course of 3-O-methyl-glucose uptake is shown in Fig. 1. 3-O-methyl-glucose

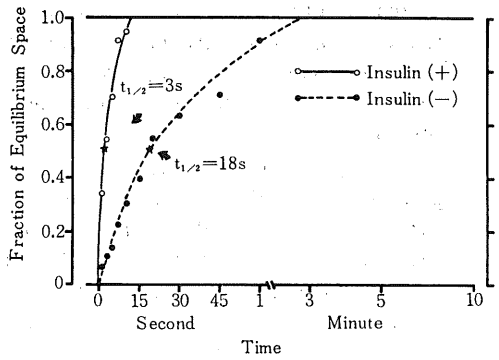

Fig. 1. Time course of 3-O-methyl-glucose uptake. The uptake was measured at 37°C as described "Materials and Methods" in the absence of insulin or in cells pretreated with 10 ng/ml insulin. The lines represent exponential curves with half-times as indicated. Values are mean of 4 experiments. SEMs of experiments were less than 7% of mean.

rapidly reached an equilibrium. Preincubation of fat cells with insulin (10 ng/ml) for 10 min increased the rate of uptake about 6 folds. In the presence of insulin this equilibrium reached in 7 sec with time for half maximal response (t 1/2) of 3 sec. On the other hand, the absence of insulin resulted in prolonged t 1/2 (18 sec).

Fig. 2 reveals the substrate dependence of 3-O-methyl-glucose equilibrium exchange. When the transport rate is analyzed using Hane's plot, insulin activates the transport by increasing the V_{max} (0.43×10^{-12} M/5 sec/100 μl cell to 1.5×10^{-12} M/5 sec/100 μl cell) without affecting the

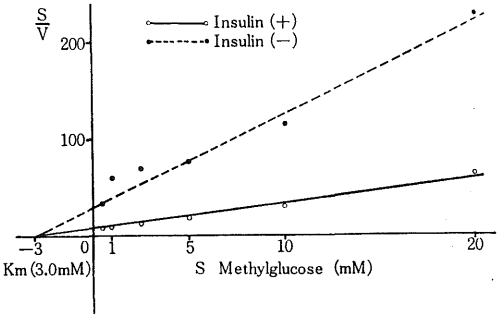

Fig. 2. Hane's plot of 3-O-methyl-glucose transport in basal and insulin-stimulated (10 ng/ml) state. Cells were preincubated for 30 min at 37°C with or without 10 ng/ml insulin. Methylglucose concentrations ranged from 0.5 to 20 mM. Values are mean of 4 experiments. SEMs of experiments were less than 5% of mean.

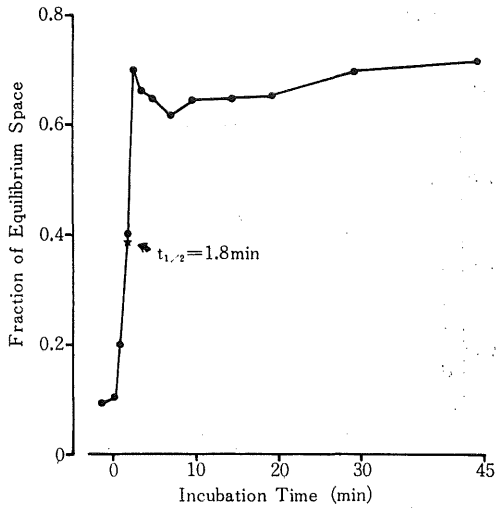

Fig. 3. Time course of insulin action on 3-O-methyl-glucose uptake. Insulin (10 ng/ml) was added at time 0 to fat cell suspension, and at the indicated times the transport was determined over 5 sec as described "Materials and Methods". Values are mean of 4 experiments. SEMs of experiments were less than 6% of mean.

Km (3.0 mM). This result is in agreement with other previous reports[10,17].

Fig. 3 shows the time course of insulin action on 3-O-methyl-glucose transport. The time required for insulin to cause maximal activation (lag phase) was about 3 min with t 1/2 of 1.8 min. The maximal stimulation occupied 71.5± 4.2% of the equilibrium space.

Dose related insulin action on 3-O-methyl-

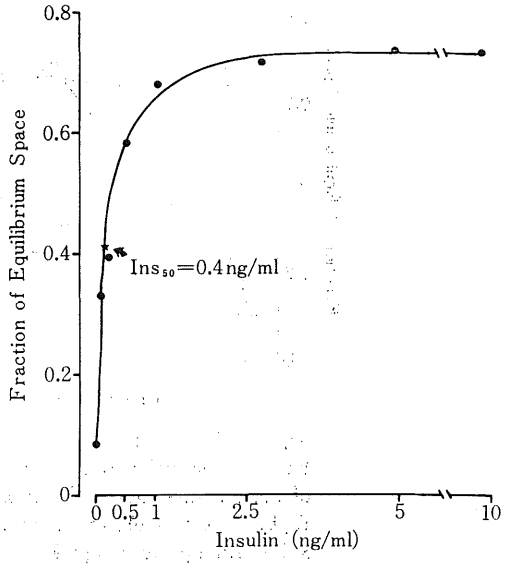

Fig. 4. Concentration dependence of insulin action on 3-O-methyl-glucose transport in fat cells. Fat cell suspension was incubated for 30 min at 37°C with insulin in the concentrations indicated on the abscissa. The uptake was measured by 5 sec pulse with labeled 3-O-methyl-glucose. Values are mean of 4 experiments. SEMs of experiments were less than 5% of mean.

glucose transport in steady state is demonstrated in Fig. 4. Half-maximal stimulation (Ins_{50}) was obtained at 0.4 ng/ml with the submaximal response at 1 ng/ml insulin.

Fig. 5 and Fig. 6 show the pH and temperature dependency on 3-O-methyl-glucose uptake, respectively. Lowering the pH of incubation medium from 7.4 to 7.2, 7.0, increased t 1/2 from 1.8 min to 11 min, 15 min, respectively. Insulin concentration for half-maximal stimulation (Ins_{50}) was increased from 0.4 ng/ml to 1.5 ng/ml, 1.6 ng/ml by lowering the pH from 7.4 to 7.2, 7.0, respectively (Fig. 5). Lowering temperature from 37°C to 32°C resulted in prolonged t 1/2 from 1.8 min to 3.6 min, but the Ins_{50} was not affected (0.3 ng/ml) (Fig. 6).

The effect of insulin analogues on 3-O-methyl-glucose transport is studied and shown in Fig. 7. When proinsulin was used instead of insulin, the t 1/2 did not alter (1.7 min), whereas the half-maximal effect of proinsulin ($Proins_{50}$) was obtained at 25 ng/ml. Fish insulin, similarly, required more peptide than porcine insulin for half maximal responce (Fish Ins_{50}; 32 ng/ml) without affecting t 1/2 (2.6

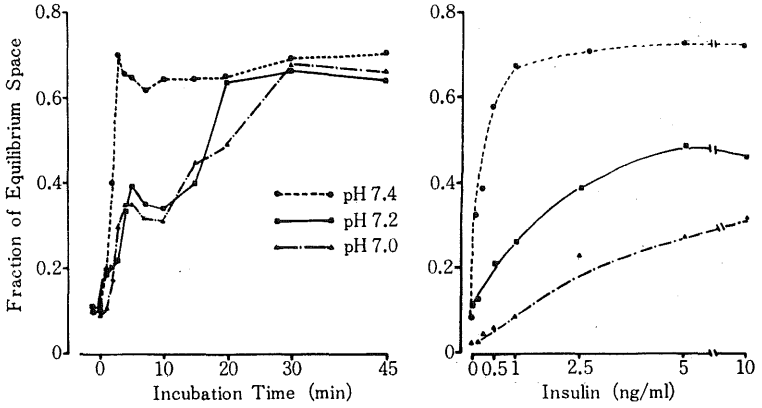

Fig. 5. Effect of pH on 3-O-methyl-glucose transport in fat cells. Fat cell suspension was incubated at various pH at 37°C. Left; Effect of pH on time course of insulin action on 3-O-methyl-glucose uptake. Right; Effect of pH on concentration dependence of insulin action on 3-O-methyl-glucose uptake. Values are mean of 4 experiments. SEMs of experiments were less then 6% of mean.

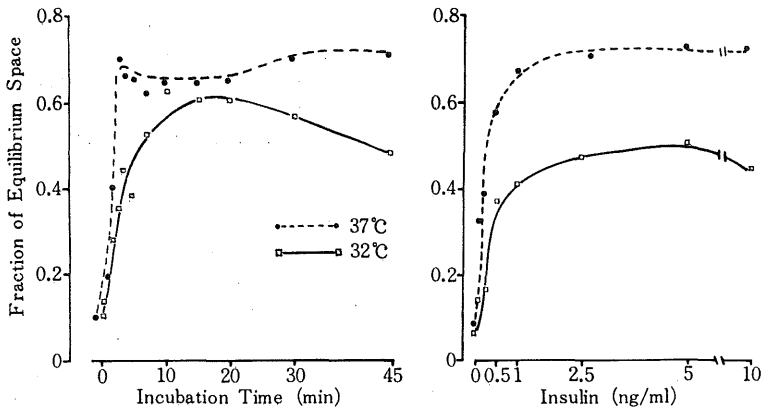

Fig. 6. Effect of low temperature on 3-O-methyl-glucose transport in fat cells. Fat cell suspension was incubated at 37°C or 32°C. Left; Effect of low temperature on time course of insulin action on 3-O-methyl-glucose uptake. Right; Effect of low temperature on concentration dependence of insulin action on 3-O-methyl-glucose uptake. Values are mean of 4 experiments. SEMs of experiments were less than 5% of mean.

min).

Fig. 8 shows the effect of fasting on 3-O-methyl-glucose transport. When adipocytes were isolated from rats fasted for 48 hrs, fasting markedly prolonged the t 1/2 (12 min). Fasting, however, did not affect insulin sensitivity (Ins_{50}; 0.5 ng/ml).

DISCUSSION

3-O-methyl-glucose is used to investigate the insulin action on the glucose transport system in rat adipocytes. 3-O-methyl-glucose is transported rapidly into adipocytes in the presence of insulin and reaches an equilibrium within 7 sec with t 1/2 of 3 sec. Under these conditions, insulin stimulates 3-O-methyl-glucose transport in a dose-dependent manner with Ins_{50} of 0.4 ng/ml. Thus, physiological levels of insulin can stimulate glucose transport in our assay system. As in other reports[15,17], a Hane's plot

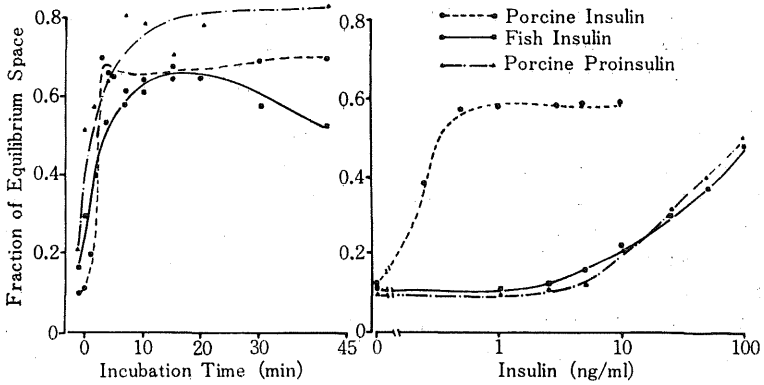

Fig. 7. Time course and concentration dependence of action of insulin analogues on 3-O-methyl-glucose transport in fat cells. Fat cell suspension was incubated with insulin analogues instead of insulin. Left; Time course of insulin analogues stimulation of 3-O-methyl-glucose uptake. Right; Concentration dependence of action of insulin analogues on 3-O-methyl-glucose uptake. Values are mean of 4 experiments. SEMs of experiments were less than 7% of mean.

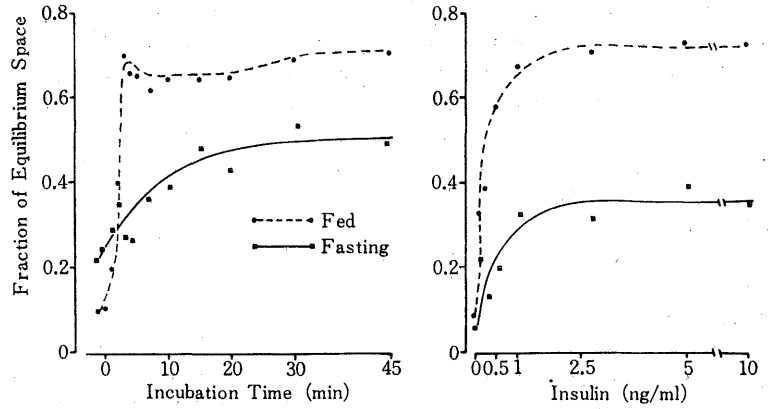

Fig. 8. Effect of fasting on 3-O-methyl-glucose transport in fat cells. The transport was determined with cells from fed or 48 hr-fasted rats. Left; Effect of fasting on time course of insulin action on 3-O-methyl-glucose uptake. Right; Effect of fasting on concentration dependence of insulin action on 3-O-methyl-glucose uptake. Values are mean of 4 experiments. SEMs of experiments were less than 8% of mean.

demonstrates that insulin enhances the V_{max} of 3-O-methyl-glucose transport but does not influence the Km. PH and temperature dependency is shown in the steady state insulin dose-responce relationship on 3-O-methyl-glucose transport. Ciaraldi et al.[1] and Pedersen & Gliemann[12] reported this dependency in recent observations. In order to examine the specificity of insulin action on glucose transport, potencies of various insulin analogues are compared. The order of potencies for stimulation of glucose transport is similar to those reported previously[4,6]. When adipocytes from rats fasted for 48 hrs are examined, dose-responce relationship on glucose transport reveals decrease in responsiveness without affecting sensitivity, which is in agreement with the results of Olefsky[11] and of Kasuga et al[8].

Previous in vitro studies on insulin action in rat adipocytes have focused on sensitivity and

responsiveness to insulin in steady state[8,9,11], and insulin resistance have been discussed only at the level of insulin receptors. On the other hand, much interest is concentrating on post-receptor defects in insulin resistance. In this study, we examined time required for insulin to elicit maximal effect (lag phase). As shown in Fig. 3, under physiological condition (pH 7.4 and 37°C), it passed about 3 min before the full effect of insulin was achieved, and the half of the full effect was obtained at 1.8 min. Since time required for insulin to cause maximal effect represents the processes which couple receptor binding and glucose transport activity, we can suggest the mechanisms of insulin resistance at postreceptor level. Then, we examine the lag phase in various conditions, and discuss the mechanism of insulin resistance in pathological states, such as diabetic ketoacidosis and hunger.

The lag phase of insulin is pH and temperature dependent. Previous study[9] demonstrated that insulin receptor affinity decreased with decreasing pH. This has been proposed as a mechanism for the insulin resistance of diabetic ketoacidosis[7]. In this study we show that low pH also prolongs the lag phase. Therefore, not only low insulin receptor affinity but also postreceptor defects may play a role in the mechanisms of insulin resistance at diabetic ketoacidosis. Similarly, low temperature prolongs the lag phase, as previously reported[5]. Vinten[16] found that low temperature decreased the V_{max} of glucose transport without affecting the Km. This finding implies that numbers of functional glucose carriers decrease in low temperature. It might be suggested that decreased number of glucose carrier cause prolongation of the lag phase. On the other hand, when insulin analogues are used instead of insulin, the lag phase is not affected. The difference in peptide concentrations for half maximal stimulation may reflect the binding specificity of these analogues to insulin receptor[4,9]. Because insulin analogues have the same post receptor system as insulin for their biological action, it is clear that insulin analogues do not affect the lag phase.

Fasting is known as a state of insulin resistance. Olefsky[11] showed that in adipocytes from fasted rats insulin binding was increased but insulin stimluated 2-deoxy-glucose uptake was significantly decreased. There seem to be disorders in coupling insulin receptor and glucose transport in fasting[8]. In our present study, fasting definitely prolongs the lag phase. These observations indicate that insulin resistance in fasting is due to disorders at the levels of postreceptor. Recently, Suzuki & Kono[14] have reported "translocation theory" that insulin stimulates recruitment of glucose transport carriers from an intracellular pool to the plasma membrane. Although the lag phase of insulin action on glucose transport involves many unknown processes, we speculate that the inhibition of recruitment of glucose carriers may play a role in disturbed postreceptor mechanisms of insulin action.

In this study, we find the fact that there exist several conditions in which the lag phase of insulin action on glucose transport is prolonged without affecting insulin sensitivity. In conclusion, the prolonged lag phase of insulin action on glucose transport may play a role in mechanism of insulin resistance such as diabetic ketoacidosis and hunger, and characterization of the lag phase of insulin action will be a useful tool to study the postreceptor transmission of insulin action.

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