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A significant increase of lysophosphatidylinositol 4-phosphate with insulin in isolated rat fat cells

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We studied the effects of insulin on the incorporation of $^{32}P_i$ into phospholipids in rat fat cells. When the cells were treated with insulin, a new radioactive phospholipid was detected on thin layer chromatography. The substance migrated slower than phosphatidylinositol 4,5-bisphosphate and was hardly detectable in the absence of insulin. This effect of insulin was both time- and dose-dependent with half-maximal stimulation at 120 μU/ml. Pretreatment of insulin with anti-insulin antibody or the cells with anti-insulin receptor antibody inhibited the effect of insulin. The product of phosphatidylinositol 4-phosphate hydrolyzed by phospholipase A_2 was coincided with the substance on thin layer chromatography. Quinacrine inhibited the formation of the substance in a dose-dependent manner. These results suggested that insulin stimulates the generation of lysophosphatidylinositol 4-phosphate through the insulin-receptor interaction.

Insulin; Phosphoinositide; Phospholipase A_2; (Rat fat cell)

1. INTRODUCTION

Since the early observation that insulin stimulates the incorporation of $[^{32}P]P_i$ into phospholipids in fat tissue was reported [1], many studies have been reported about the effects of insulin on phosphoinositide metabolism [2–4]. Pennington et al. [5] reported that insulin stimulated the de novo synthesis of phosphoinositide, but had no effects on phosphoinositide breakdown in fat cells. Recently, Farese et al. [6] demonstrated that insulin had the effects in rat fat tissue of a transient activation of phospholipase C and a persistent increase in de novo phospholipid synthesis.

The actions of insulin are thought to be produced by the interaction of insulin with its receptors on the plasma membranes. It has been suggested that one or more chemical mediators are generated subsequent to this interaction and alter the activities of some insulin sensitive enzymes [7,8]. Several investigators suggest the possibility that insulin generated chemical mediators by activating an insulin-sensitive phospholipase C acting as a novel glycophospholipid [9–11].

In the course of our studies of the actions of insulin on phospholipid metabolism, a radioactive substance migrating slower than phosphatidylinositol 4,5-bisphosphate on thin layer chromatography (TLC) was detected in the phospholipid fraction of rat fat cells incubated with $[^{32}P]P_i$ in the presence of insulin. The examination of characteristics of this substance indicated that it might be lysophosphatidylinositol 4-phosphate.

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Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine

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2. EXPERIMENTAL

2.1. *Preparation of fat cells*

Fat cells were isolated by the method of Rodbell [12] with the following minor modifications. Epididymal fat pads (1 g/ml) from male Sprague-Dawley rats (140–180 g) were incubated with collagenase (3.5 mg/ml, Cooper Biomedical) for 45 min at 37°C. The incubation medium used throughout preparation was a modified Krebs-Ringer bicarbonate buffer, pH 7.4 (KRBB, 0.154 M NaCl, 0.154 M KCl, 0.055 M $CaCl_2$, 0.154 M KH_2PO_4, 0.154 M $MgSO_4$ and 1.3% $NaHCO_3$) supplemented with 2% bovine serum albumin (w/v) gassed with O_2/CO_2 (19:1). The digested fat pad was then filtered through a nylon mesh with 15 ml of KRBB buffer. After washing with KRBB buffer containing 10 mM glucose, isolated fat cells were preincubated for 20 min with the same buffer.

2.2. *Measurement of phosphoinositide metabolism*

Fat cells (4×10^5 cells/ml) were usually incubated with [^{32}P]P_i (25–40 μCi/ml, New England Nuclear) at 37°C for various times with or without insulin. The reaction was terminated by the addition of 2.5 ml of ice cold chloroform/methanol/12 N HCl (100:100:0.75, v/v) to 0.5 ml of incubation mixture [13]. The mixture was allowed to stand for 10 min at 0°C and then 0.5 ml of 0.6 N HCl was added. After centrifugation, the upper phase was removed and the lower phase was washed twice with 1 ml of chloroform/methanol/0.6 N HCl (3:48:47, v/v). An aliquot (0.25 ml) of the lower phase was dried under a stream of nitrogen.

TLC was performed by the method of Jolles [13] using silica gel G plate (Merck). Before use, the plates were developed in methanol/water (2:3, v/v) containing 1% potassium oxalate and activated at 110°C for 30 min. The lipid extracts were applied to the plates and developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v). Lipids were visualized by iodine staining and radiolabelled spots were detected by autoradiography on Fuji X-ray film. The radioactive spots were scraped from the plate and counted in a liquid scintillation spectrophotometer.

2.3. *Phospholipase A_2 treatment*

Phospholipase A_2 (from *Naja naja* venom, Sigma) in 0.1 M borate buffer, pH 8.9, containing 20 mM calcium acetate (100 U/ml) was added to a lipid extract suspended in ether/methanol (98:2, v/v) and then the mixture was incubated for 2 h at room temperature [14]. The reaction was stopped by the addition of chloroform/methanol/12 N HCl (200:100:0.75, v/v).

2.4. *Other methods*

Protein was determined by the method of Lowry et al. [15]. Deacylation was based on the method of Creba et al. [16]. Anti-insulin IgG was prepared from guinea pig anti-insulin serum by ammonium sulfate precipitation and DEAE-cellulose column chromatography [17]. Control IgG was prepared from non-immunized guinea pig serum. Anti-insulin receptor serum (B5) was a kind gift from Dr Kasuga of Tokyo University.

3. RESULTS AND DISCUSSION

3.1. *Effect of insulin on the incorporation of [^{32}P]P_i into phospholipids*

Insulin caused an increase of the incorporation of [^{32}P]P_i into total and each phospholipid fraction (i.e. PS + PC, PI, PIP, PIP_2) of rat fat cells. These effects of insulin agreed with the result of Farese et al. [6]. In addition, a substance which migrated slightly slower than PIP_2 on TLC, was detected as shown in fig.1.

Fig.2A shows that the incorporation of [^{32}P]P_i into the substance was enhanced by insulin in a dose-dependent manner. The half-maximal activation was approx. 120 μU/ml of insulin.

Fig.2B shows the results of time course experiments in the presence or absence of insulin (1 mU/ml). The rate of increase of [^{32}P]P_i incorporation into the substance by insulin was much higher than that into PI, PIP and PIP_2 (not shown).

After incubation of fat cells with [^{32}P]P_i and insulin for 30 min in phosphate-free buffer, [^{32}P]P_i was removed from the medium by repeated washing with KRBB buffer and the incubation was continued in the presence of insulin. The radioactivity of the substance became hardly detectable within 30 min (not shown). The results of these ex-

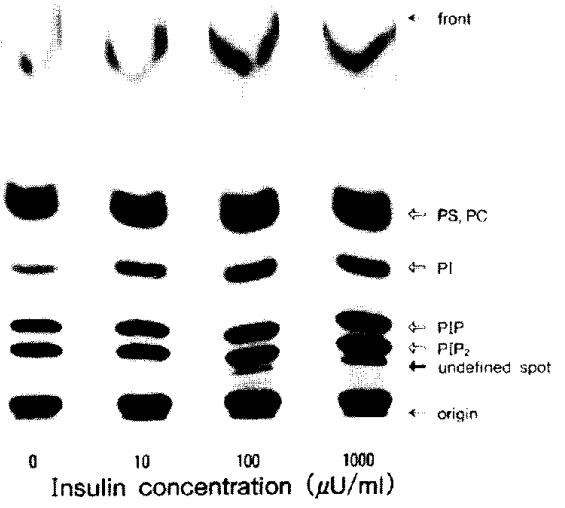

Fig.1. Separations of [^{32}P]P_i-labelled phospholipids in rat fat cells. Fat cells were incubated with [^{32}P]P_i (25 μCi/ml) in the presence of insulin at the indicated concentrations for 60 min at 37°C. Samples were extracted and separated by TLC. Radioactive spots were located by autoradiography.

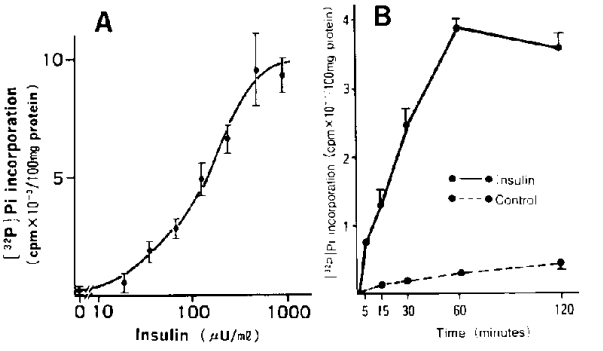

Fig.2. Effect of insulin on the incorporation of [^{32}P]P_i into the substance in rat fat cells. (A) Fat cells were incubated with [^{32}P]P_i (40 μCi/ml) and insulin (0–1000 μU/ml) for 60 min in phosphate-free KRBB. After extraction and separation on TLC, the radioactive spot was scraped and counted. (B) Fat cells were incubated with [^{32}P]P_i (25 μCi/ml) in the presence (——) or absence (– – –) of insulin (1 mU/ml). Samples were removed at the times indicated and lipids were extracted as described in section 2. Data are expressed as the mean ± SD of triplicate determinations. Similar results were obtained with 3 separate fat cell preparations.

periments indicate the substance to be metabolized.

3.2. *Effect of anti-insulin antibody and anti-insulin receptor antibody*

When fat cells were incubated with insulin in the presence of anti-insulin antibody, or anti-insulin receptor antibody, the effect of insulin on the incorporation of [^{32}P]P_i into the substance decreased to 19% and 37%, respectively (table 1). These results suggest that the activation of the incorporation of [^{32}P]P_i into the substance is due to the action of insulin through the insulin receptor.

3.3. *Characteristics of the substance*

The substance had the following characteristics: (i) the ratio of the radioactivity of PIP_2/substance on TLC was not changed by repeated washing (2–5 times) with methanol/1.2 N HCl (1 : 1, v/v). (ii) The radioactivity of the substance was recovered in the water phase after deacylation with mild alkali. (iii) When extracted lipids were applied to a silicic acid column following the method of Rouser et al. [18], the radioactivity of the substance was eluted with methanol, but not with chloroform. These characteristics suggest that the substance is a phospholipid.

When fat cells were incubated with D-*myo*-[^{3}H]inositol (New England Nuclear) in the presence of insulin for 2 h, no significant incorporation of [^{3}H]inositol into the substance was observed in spite of good incorporation of that into the PI. It might be due to a much smaller amount of radioactivity coming from [^{3}H]inositol-labelled PI to the substance for this incubation time.

Fat cells were incubated with insulin in the presence of quinacrine (0–1 mM), an inhibitor of phospholipase A_2. The radioactivity of substance was reduced in a dose-dependent manner (table 2). This result suggests that the substance is lysophospholipid.

To determine the possibility as mentioned above, we examined the effect of phospholipase A_2 treatment, on labelled phospholipids that were extracted from fat cells (fig.3). The autoradiogram shows that the radioactivity of phospholipid

Table 1

Effects of anti-insulin antibody and anti-insulin receptor antibody on the incorporation of $[^{32}P]P_i$ into the substance in rat fat cells

Treatments	Incorporation of $[^{32}P]P_i$ (cpm × 10^{-3}/100 mg protein)
Anti-insulin antibody (−)	4.84 ± 0.25
Anti-insulin antibody (+)[a]	0.92 ± 0.06
Anti-insulin receptor antibody (−)[b]	4.59 ± 0.26
Anti-insulin receptor antibody (+)	1.71 ± 0.18

[a] After insulin was incubated with or without anti-insulin antibody in KRBB buffer containing 10 mM glucose for 60 min at room temperature, fat cells and $[^{32}P]P_i$ (25 μCi/ml) were added to the medium. The incubations were continued for 30 min at 37°C

[b] Fat cells were preincubated for 30 min with or without anti-insulin receptor antibody, and then incubated with $[^{32}P]P_i$ (25 μCi/ml) in the presence of insulin (1 mU/ml) for 30 min at 37°C. Phospholipids were extracted and separated by TLC as described in section 2. Each value is mean ± SD of 4 determinations

products hydrolyzed by the enzyme increased in the area corresponding to the substance, which coincided with the product of labelled PIP treated with the enzyme on TLC. This suggests that the substance is the lyso form of PIP.

Table 2

Effect of quinacrine on the incorporation of $[^{32}P]P_i$ into the substance in rat fat cells treated with insulin

Quinacrine (mM)	Incorporation of $[^{32}P]P_i$ (cpm × 10^{-3}/100 mg protein)
0	2.56 ± 0.14
0.1	1.81 ± 0.19
0.5	1.07 ± 0.08
1.0	0.59 ± 0.09

Fat cells were preincubated with $[^{32}P]P_i$ (25 μCi/ml) for 30 min. Then the cells were treated with insulin (1 mU/ml) in the presence of quinacrine (0–1 mM) for 15 min. Lipids were extracted and separated by TLC as described in section 2. Each value is mean ± SD of 4 determinations

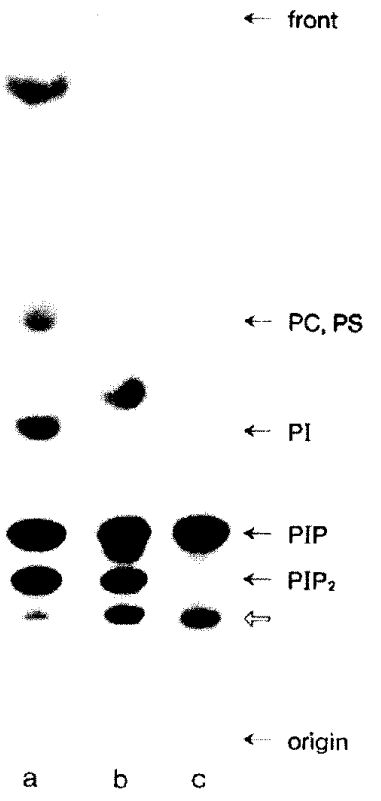

Fig.3. Autoradiograms of phospholipids treated with phospholipase A_2. (a) Fat cells were incubated with $[^{32}P]P_i$ (25 μCi/ml) in the presence of insulin (1 mU/ml). (b) Fat cells were incubated with $[^{32}P]P_i$ in the absence of insulin. Extracted lipids were treated with phospholipase A_2. The treatment of phospholipase A_2 is described in section 2. (c) Fat cells were labelled with $[^{32}P]P_i$. Labelled PIP purified by TLC was treated with phospholipase A_2.

If insulin stimulates phospholipase A_2, other lysophospholipids should be increased with insulin. We examined the effect of insulin using another basic solvent system (chloroform/methanol/NH_4OH/water, 90:70:11:11, v/v). The incorporation of $[^{32}P]P_i$ into other lysophospholipids was not effected by insulin. Therefore, insulin presumably stimulates only the phospholipase A_2 acting on PIP.

Gumber et al. [19] reported that a product formed from PIP in the presence of bivalent metal ions and ATP showed an R_f value very close to that

of PIP_2 on TLC with an acidic solvent and that the product was phosphatidylinositol 4-pyrophosphate. The possibility that the substance observed under our labelling conditions is phosphatidylinositol 4-pyrophosphate was excluded from the finding that hydrolysis of the substance in 0.05 M H_2SO_4 at 100°C for 7 min resulted in no release of radioactivity as orthophosphate.

Batty et al. [20] showed that inositol 1,3,4,5-tetraphosphate accumulates in rat brain stimulated with carbachol. But there is no proof of the existence of a parent lipid from which this may be derived (i.e. phosphatidylinositol triphosphate). This question has yet to be clarified.

Saltiel et al. [9,10] and Mato et al. [11] demonstrated the possibility that insulin generated chemical mediators by activating an insulin-sensitive phospholipase C acting on a novel glycophospholipid. Although this glycolipid migrated between PIP and PI, our undefined phospholipid migrated slower than PIP_2 by the similar solvent system on TLC.

Further examination is needed for the determination of the chemical identity and physiological significance of this phospholipid.

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