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STIMULATION OF PHOSPHATIDYLCHOLINE SYNTHESIS BY INSULIN AND ATP IN ISOLATED RAT ADIPOCYTE PLASMA MEMBRANES[1]

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Summary. Added individually or together, insulin and/or ATP significantly and rapidly increased the concentration of phosphatidylcholine in an enriched plasma membrane preparation from rat adipocytes. The increase in phosphatidylcholine synthesis mediated by insulin or ATP was suppressed by the phospholipid methyltransferase inhibitor, S-adenosylhomocysteine. These results suggest that the activitiy of phospholipid methyltransferase from adipocyte plasma membranes may be increased by phosphorylation and that insulin may further increase the activity of the phosphorylated phospholipid methyltransferase by an alternative pathway.

Phosphatidylcholine (PtdChol) may be synthesized by two different pathways (1,2): the CDP-choline pathway and the successive methylation of phosphatidylethanolamine (PtdEtn). The transmembrane signal transmitted intracellularly following the binding of a ligand to its receptor may be directly related to modulation of phospholipid methylation within the membrane of a variety of cells (1-3). Most systems demonstrate an increase in phospholipid methylation following ligand binding, however, the timing of the reponse varies with the ligand and the cell type tested. The regulation of phospholipid methylation is not well-defined and may involve cAMP (1), calcium (1), phospholipase A_2 activity (3), cytosolic inhibitory proteins (4,5), unsaturated fatty acids (6), and phosphorylation (1,7,8). Varela et al. (7)

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Abbreviations Used: PtdChol, phosphatidylcholine; AdoHcy, S-adenosyl-L-homocysteine; PtdEtn, phosphatidylethanolamine; PtdEtn MeTase, phosphatidylethanolamine methyltransferase; ATP, adenosine 5′-triphosphate; MOPS, morpholinopropanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, 2-amino-2(hydroxymethyl)-1,3-propandiol; BSA, bovine serum albumin.

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have shown that partially purified phospholipid methyltransferase from rat liver microsomes are phosphorylated and activated by the addition of the catalytic subunit of cAMP-dependent protein kinase and MgATP.

Within 15 sec, insulin stimulated PtdEtn Metase in plasma membranes isolated from rat adipocytes as measured by the incorporation of [^{3}H-methyl]-S-adenosyl-L-methionine into the N-methylated derivativies of PtdEtn at pH 7.4 (9). To further investigate the magnitude and regulation of the effect of insulin on PtdChol synthesis, we incubated plasma membranes isolated from rat adipocytes in the presence and absence of insulin and/or ATP. PtdChol was measured enzymatically with modification of a previously published method (10,11) and the inhibitor of PtdEtn MeTase, S-adenosylhomocysteine (AdoHcy), was used to determine if an increased in PtdChol concentration was attributable to activation of PtdEtn MeTase.

Materials and Methods

Materials. Male Sprague Dawley rats (120g) were obtained from Harlan Sprague Dawley. Collagenase, morpholinopropanesulfonic acid (MOPS), albumin (Fraction V and Fraction IV), adenosine 5'-triphosphate (ATP) (disodium salt), phospholipase D (from cabbage; phosphatidylcholine phosphatidoohydrolase, EC 3.1.4.4), maleic acid, 4-aminoantipyrene, dipalmitoyl PtdChol (synthetic), Triton X-100, 3,3-dimethylglutarate and bovine serum albumin (BSA) were from Sigma. Sodium dodecyl sulfate (SDS) was from Polyscicence, Inc.; sodium 2-hydroxy-3,5-dichlorobenzenesulfonate was from Biosynth International or Research Organics, Inc. Choline oxidase (from Arthrobacter globiformis; choline: oxygen 1-oxoreductase; EC 1.1.3.17) and peroxidase (from horseradish; hydrogen peroxide oxidoreductace; EC 1.11.1.17) were from Finnsugar Biochemicals. Porcine insulin was a gift from W.R. Fields of Eli Lilly. The lots of collagenase and albumin (Fraction V) were chosen as previously described (9).

Preparation of adipocyte plasma membranes. Adipocytes were prepared from rat epididymal fat pads digested with collagenase and the isolated adipocytes were washed as in (12). Highly enriched plasma membranes were prepared as described by Kelly et al. (9). The cells were homogenized in 0.25 M sucrose, 10 mM MOPS, pH 7.4, at 4°C and fractionated on a discontinuous sucrose gradient of 0.8, 1.06, and 2.02 M sucrose. Plasma membranes were removed from the interface between 0.8 M and 1.06 M sucrose, washed, resuspended and frozen at -70°C in 50 mM potassium phosphate buffer, pH 7.4. Protein concentration was determined by the method of Lowry et al. (13).

Phosphatidylcholine assay. Plasma membranes (1.0-1.9 mg/ml) were incubated in the presence or absence of insulin and/or ATP. Insulin was added in the presence of 5% bovine serum albumin to slow the rate of insulin degradation (9). Control samples contained 5% BSA. Triplicate samples (50 ul) were removed at specific times, extracted with 800 ul of chloroform:methanol (2:1, by vol) (14), vortex mixed, centrifuged (1000xg) for 5 min. Then 100 ul water was added, vortex mixed, and centrifuged (1000xg) for 5 min. Chloroform layer (500 ul) was transferred to a glass tube and evaporated to

dryness with nitrogen. The sample for the PtdChol assay was redissolved in water containing 0.2% Triton X-100 and 0.9% SDS (w/v). The sample and standards were added to 100 ul of phospholipase D solution and 300 ul of acetate buffer solution, vortex mixed, and incubated at room temperature for 15 min. 500 ul of Tris-COD solution was added and incubated at 37°C for 15 min. The absorbance was measured against a reagent blank at 510 nm. Phospholipase D solution contains 10mM 3,3-dimethylglutarate buffer, pH 7.0, 11 kU/l phospholipase D and 1 g/l BSA. Acetate buffer solution consists of 100 mM acetate buffer, pH 5.5, 2 g/l Triton X-100, 8 mM 4-aminoantipyrene, 2 mM SDS and 91.7 mM $CaCl_2$. Tris-COD solution contains 400 mM Tris-HCl, pH 8.0, 2 g/l Triton X-100, 18 mM 2-hydroxy-3,5-dichlorobenzene sulfate, 2.8 kU/l choline oxidase and 102 kU/l peroxidase. Under the conditions of the assay, the reaction is specific for PtdChol, without significant interference from lysoPtdChol or sphingomyelin (10,11). Results were expressed as nmol of PtdChol per mg of plasma membrane protein ± SEM. Statistical significance was determined by use of the paired Student t test.

Results

Following the addition of 100 uU/ml insulin to plasma membranes isolated from rat adipocytes, the concentration of PtdChol was significantly increased compared to the control at 30 sec, 60 sec and 120 sec (Figure 1). The insulin induced increase in PtdChol concentration was 126 nmol/mg (28%), 30 sec; 114 nmol/mg (24%), 60 sec; 76 nmol/mg (15%), 120 sec. The basal level of PtdChol appeared to gradually increase over the 5 min incubation (Figure 1), however, this finding is not consistent (Figure 2). The addition of 0.5 mM ATP

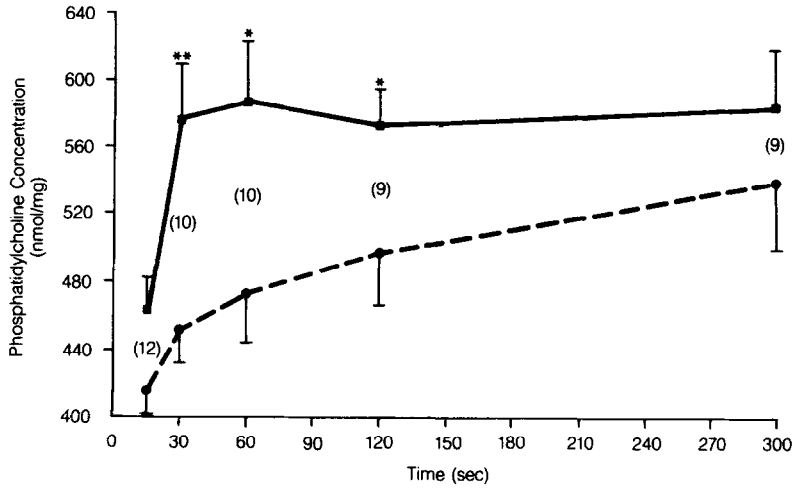

Figure 1: Phosphatidylcholine concentration in an enriched plasma membrane fraction prepared from rat adipocytes incubated in the presence of 100 uU/ml insulin and 5% BSA (■ — ■) or in 5% BSA (● - - ●). The bars indicate the SEM. The number in parenthesis represents the number of determinations. The paired Student t test revealed statistical significance at $p < 0.05$,*; and $p < 0.01$,**.

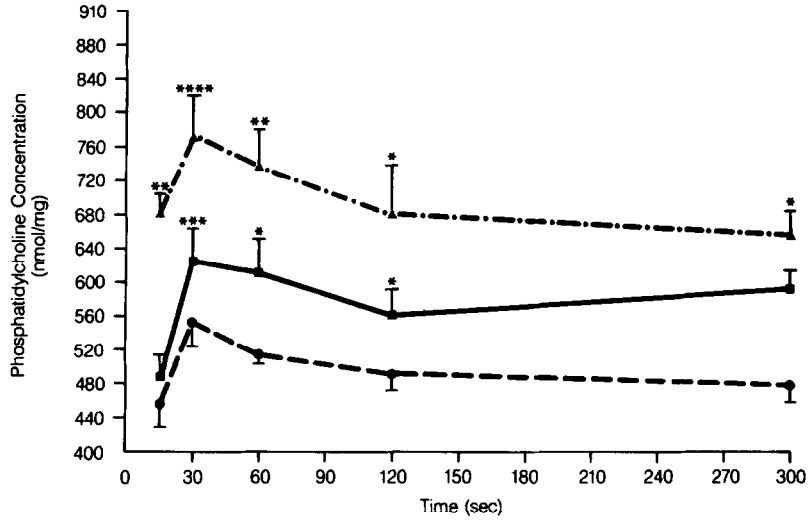

Figure 2: Phosphatidylcholine concentration in an enriched plasma membrane fraction prepared from rat adipocytes incubated in the presence of 0.5 mM ATP and 5% BSA (■ — ■); 5% BSA (● - - ●); 100 uM/ml insulin, 0.5 mM ATP, and 5% BSA (▲-●—●-▲). The results are from a representative experiment performed in triplicate. The bars indicate the SEM. The paired Student t test revealed statistical significance at $p < 0.05$,*; $p < 0.01$,**; $p < 0.025$,***; $p < 0.005$, ****.

significantly increased PtdChol concentration at three times compared to control: 76 nmol/mg (14%), 30 sec; 95 nmol/mg (18%), 60 sec; 72 nmol/mg (15%), 120 sec (Figure 2). The addition of both 0.5 mM ATP and 100 uU/ml insulin produced an apparent additive increase in PtdChol concentration if the individual changes for insulin (Figure 1) and ATP (Figure 2) are compared to the effect of both compounds (Figure 2): sum of individual changes vs both added together; 202 vs 227 nmol/mg, 30 sec; 209 vs 223 nmol/mg, 60 sec; 148 vs 189 nmol/mg, 120 sec. Maximal increase in PtdChol concentration for insulin,

Table I

Effect of S-adenosylhomocysteine on phosphatidylcholine concentration in adipocyte plasma membranes in the presence of insulin or ATP[1]

Condition	Phosphatidylcholine Concentration	Change
	nmol/mg	
control	460 ± 7	
+ 0.5 mM ATP	634 ± 9	+ 174
+ 0.5 mM ATP + 10 mM AdoHcy	468 ± 10	+ 8
+ 100 uU/ml insulin	568 ± 22	+ 108
+ 100 uU/ml insulin + 10 mM AdoHcy	494 ± 30	+ 34

[1]Following incubation for 30 sec in presence or absence (control) of insulin or ATP with or without AdoHcy, an inhibitor of phospholipid methylation, phospholipids were extracted from adipocyte plasma membranes (1 mg/ml) with chloroform:methanol (2:1, by vol). Phosphatidylcholine concentration was determined enzymically in triplicate as described in Methods and expressed as mean ± SEM. Change represents the difference from the control.

addition of an inhibitor of PtdEtn MeTase, AdoHcy. These findings confirm that adipocyte plasma membrane PtdEtn MeTase is activated by insulin and suggest that phosphorylation may also activate this enzyme. The insulin effect is consistent with the finding of increased incorporation of [^{3}H-methyl] from [^{3}H-methyl]-3-adenosylmethionine into all three methylated derivatives of PtdEtn (phosphatidyl-N-monomethyl-ethanolamine, phosphatidyl-N,N-dimethylethanolamine and PtdChol) in adipocyte plasma membranes 15 and 30 sec after the addition of 100 uU/ml insulin (9). This effect was also suppressed by AdoHcy (9). Phosphorylation by cyclic AMP-dependent protein kinases has been reported to activate rat liver PtdEtn MeTase (1,7,8). This phosphorylation reaction is also modulated by the ratio of S-adenosylmethionine/AdoHcy (8). Our data demonstrates a rapid increase in PtdChol concentration in the presence of ATP, suggesting that PtdEtn MeTase from adipocyte plasma membranes is activated by phosphorylation. The presence of both insulin and ATP resulted in a marked stimulation of PtdChol concentration compared to control which appeared to represent the sum of the individual effects: 23%, insulin; 14% ATP; 41%, insulin plus ATP, suggesting that insulin can stimulate the phosphorylated, activated PtdEtn MeTase. The mechanism of insulin's stimulation of PtdEtn MeTase may involve

phosphorylation or another mechanism. There are two pathways to elevate PtdChol concentration by increased flux through phospholipid methylation. The first is activation of PtdEtn MeTase through phosphorylation (1,7,8) and second involves an increase in the substrate for PtdEtn MeTase, PtdEtn, through other mechanisms, like increased decarboxylation of phosphatidylserine (15) or exchanges of head groups for ethanolamine.

It is not clear whether there is one or two PtdEtn MeTase enzymes (1,3). Adrenal medulla appears to contain two such enzymes (3). The first has a pH optimum of 7.0 and the second, 10.0 (3). Bansal and Kanfer (16) have shown that at alkaline pH, PtdEtn, either free or membrane bound, is methylated by [^{3}H-methyl]-S-adenosylmethionine both enzymatically and nonenzymatically. To reduce nonenzymatic methylation of PtdEtn, we performed our experiments at pH 7.4. Kelly *et al.* (17) has shown that insulin treatment of adipocytes resulted in a decrease in PtdEtn MeTase activity in a crude cellular extract which was assayed for PtdEtn MeTase activity at pH 9.2 using [^{3}H-methyl]-S-adenosylmethionine. It is possible that cystosolic proteins (4,5) or unsaturated fatty acids (6), which have been reported to inhibit PtdEtn MeTase, may be absent in our enriched plasma membrane preparation and present in the crude cellular extract of Kelly *et al.* (17). The role PtdEtn MeTase activity plays in the intracellular regulation of insulin action requires further investigation.

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