The polar head group of a novel insulin-sensitive glycophospholipid mimics insulin action on phospholipid methyltransferase

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A phospholipid has been purified from rat liver membranes which copurified with an insulin-sensitive glycophospholipid isolated from H35 hepatoma cells. The polar head group of this phospholipid was generated by treatment with a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus* and purified through a C18 extraction column. Like insulin, the addition of this polar head group to isolated rat adipocytes inhibited the stimulatory effect of isoproterenol on phospholipid methyltransferase. The polar head group was also active on a subcellular fraction. The addition of the polar head group to microsomes isolated from isoproterenol-treated adipocytes produced a time-dependent inactivation of phospholipid methyltransferase, approaching basal activity. It is proposed that the effects of insulin on phospholipid methyltransferase may be mediated by this polar head group.

Insulin *Glycophospholipid* *Phospholipase C* *Insulin mediator* *Adipocyte* *Phospholipid methyltransferase*

1. INTRODUCTION

Insulin is known to generate mediators of unknown chemical nature which mimick the effects of this hormone on a variety of enzymes (review [1]). Recently [2], two compounds, with M_r of about 1400, which modulate the high-affinity cAMP phosphodiesterase have been generated by treating liver membranes with either insulin or a phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*. It has been proposed [2] that these modulators of phosphodiesterase activity may be responsible for the regulation of the enzyme by insulin. A novel glycophospholipid containing non-*N*-acetylated glucosamine, which is sensitive to both insulin and a phospholipase C, has been identified in BC3H1 myocytes as the source of these modulators [3]. The concept that insulin generates chemical mediators by stimulating a phospholipase C acting on a novel glycophospholipid containing non-*N*-acetylated glucosamine was supported by similar findings from this laboratory using H35 hepatoma cells [4]. The insulin-sensitive glycophospholipid from H35 hepatoma cells was purified and it and its polar head group partially characterized.

Here, we report the insulin-like effects of the polar head group of this novel glycophospholipid, obtained by treating the purified phospholipid from rat liver membranes with *S. aureus* phospholipase C, on adipocyte phospholipid methyltransferase. This enzyme, which converts phosphatidylethanolamine into phosphatidylcholine by three successive *N*-methylations [5], is known to be inhibited by insulin in isolated hepatocytes [6,7] and adipocytes [8–10] from rats. It was previously demonstrated that an insulin-sensitive mediator could mimic the inhibitory ef-

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fect of insulin on phospholipid methyltransferase in adipocytes, but the chemical identity of this material was unknown [9]. The present results are compatible with insulin activating a phospholipase C which generates a polar head group from a novel glycophospholipid that inhibits phospholipid methyltransferase, thereby providing a mechanism for regulation of this enzyme by insulin.

2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase was from Worthington and albumin (fraction V) from US Biochemicals. Insulin was a gift from R. Chance of Eli Lilly. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine was from New England Nuclear. *S*-Adenosylmethionine was from Boehringer. Phosphatidyl-*N*-monomethylethanolamine was from Avanti Polar Lipids. Phosphatidylinositol-specific phospholipase C from *S. aureus* was a generous gift from Dr M. Low, Oklahoma Medical Research Foundation, Oklahoma City, OK. Silica gel G plates for TLC were from Merck.

2.2. *Methods*

2.2.1. Isolation of adipocytes and measurement of phospholipid methyltransferase activity

Isolated fat cells were prepared by the collagenase digestion method [11] from epididymal pads of fed male Sprague-Dawley rats (125–175 g) and preincubated for 30 min in a Krebs-Ringer bicarbonate buffer, pH 7.4, as described [8]. After 30 min preincubation, cells were stimulated with the test agents, after which an aliquot of the cells was removed and separated from the incubation medium by centrifugation. The incubation medium was aspirated and the cells disrupted by vortex-mixing vigorously in the homogenization buffer (125 mM Tris-HCl, 2 mM NaF, 2 mM EGTA, 2 mM EDTA, pH 8.5) as described [8]. Phospholipid methyltransferase activity was assayed in the resulting lipid-free infranatant as in [8] in the presence of 100 μM *S*-adenosylmethionine. In another set of experiments, microsomal membranes were isolated from the delipidated infranatant by centrifugation at 100000 × *g* for 60 min at 4°C. After centrifugation, microsomes were resuspended in 10 mM Tris-HCl, pH 7.4, and incubated at 37°C in the presence or absence of the various test agents. At different times aliquots were taken and phospholipid methyltransferase activity assayed in 125 mM Tris-HCl, pH 8.5, as mentioned above.

2.2.2. Generation and isolation of the polar head group

Two livers from Sprague-Dawley rats (125–175 g) were homogenized in 0.9% NaCl (40 ml/g liver). A membrane fraction was prepared by centrifugation at 100000 × *g* for 1 h at 4°C of the supernatant from a 12000 × *g* (10 min) spin. Membranes were extracted with acidified chloroform/methanol as described [12] and modified [13]. The organic extract was further purified by thin-layer chromatography on silica gel G. The plate was developed twice in chloroform/acetone/methanol/glacial acetic acid/water (50:20:10:10:5). A 1 cm region around the origin was eluted with methanol at 37°C, and this fraction rechromatographed in chloroform/methanol/NH_4OH/water (45:45:3.5:10). A [^{3}H]glucosamine-labeled insulin-sensitive glycophospholipid isolated from H35 cells [4] was run in parallel with the liver sample. The developed chromatogram was scraped into 1 cm fractions. The region of the liver lipids migrating with the same R_f as the ^{3}H-labeled glycophospholipid from H35 cells was scraped and eluted as described above. After elution, the purified sample was dried under a stream of nitrogen, resuspended in 25 mM Hepes/NaOH, pH 7.4, and incubated with 2.5 μg *S. aureus* phospholipase C for 60 min at 37°C [4]. The reaction was terminated by separating the polar head group from the phospholipase C on a Baker extraction C18 column equilibrated with water. The polar head group was eluted with water and after lyophilization, tested for bioactivity.

3. RESULTS

The addition of isoproterenol to isolated rat adipocytes caused a 2-fold elevation of phospholipid methyltransferase activity (table 1). Insulin at 100 μU/ml blocked the effect of isoproterenol. The polar head group isolated from glycophospholipid purified from liver membranes caused a similar effect to insulin on isoproterenol-stimulated phospholipid methyltransferase when added to adipocytes. This finding was similar to

Table 1

Effect of the polar head group and insulin on isoproterenol-stimulated phospholipid methyltransferase of isolated rat adipocytes

Addition	pmol/min per mg
None	23.0 ± 1.5
Isoproterenol (0.1 μM)	40.3 ± 2.0
Isoproterenol (0.1 μM) + PHG	26.8 ± 0.5
Isoproterenol (0.1 μM) + insulin (100 μU/ml)	26.9 ± 1.3

Rat adipocytes were incubated for 30 min in the presence or absence of the various additives. At the end of this period the adipocytes were homogenized and phospholipid methyltransferase activity determined as described in section 2. Results are means ± SE of a representative experiment performed in triplicate. PHG, polar head group

the effect on phospholipid methyltransferase of an unidentified mediator extracted from liver of insulin-treated rats when added to isoproterenol-treated adipocytes [9].

The effect of the polar head group was tested in a subcellular system to determine where in the pathway of isoproterenol action the polar head group inhibited phospholipid methyltransferase. Microsomes from control and isoproterenol-treated adipocytes were incubated at 37°C in the presence or absence of the polar head group. At various times, an aliquot was taken and phospholipid methyltransferase activity determined. As shown in fig.1, phospholipid methyltransferase activity in microsomes isolated from isoproterenol-treated cells was about 2.5-fold higher than that observed in control membranes. Incubation of the microsomes isolated from isoproterenol-treated adipocytes produced a time-dependent inactivation of phospholipid methyl-

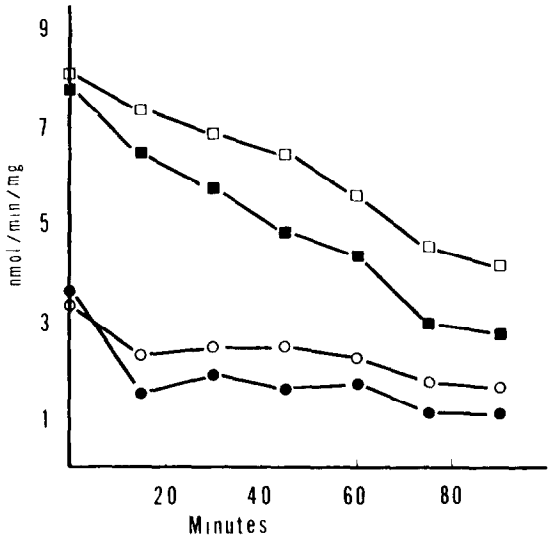

Fig.1. Effect of the polar head group on phospholipid methyltransferase inactivation. Rat adipocytes were incubated for 30 min in the presence (□, ■) or absence (○,●) of 0.1 μM isoproterenol, after which microsomes were prepared as described. Microsomes were then incubated at 37°C in the presence (■,●) or absence (□,○) of the polar head group. At various times aliquots were taken and phospholipid methyltransferase activity determined as mentioned in section 2. Results shown are from a representative experiment performed in triplicate.

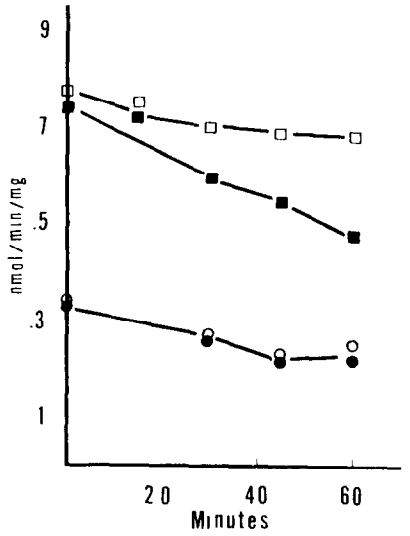

Fig.2. Effect of the polar head group on phospholipid methyltransferase inactivation in the presence of NaF/EDTA/EGTA. Conditions were as described in the legend to fig.1 except that microsomes were resuspended in 25 mM Tris-HCl in the presence of 2 mM NaF, 2 mM EDTA and 2 mM EGTA at pH 7.4. Incubations were carried out in the presence (■,●) or absence (□,○) of the polar head group. (□, ■) Microsomes obtained from isoproterenol-treated adipocytes; (○,●) microsomes obtained from control adipocytes. Results shown are from a representative experiment performed in triplicate.

transferase which was markedly accelerated by the addition of the polar head group. Since there is evidence indicating that phospholipid methyltransferase is modulated by reversible phosphorylation (review [16]), the effect of NaF/EDTA/EGTA addition on the inactivation of this enzyme was also tested. As shown in fig.2, the addition of NaF/EDTA/EGTA prevented the inactivation of phospholipid methyltransferase in microsomes isolated from isoproterenol-treated adipocytes when incubated at 37°C. However, these additives did not prevent the inactivation of phospholipid methyltransferase mediated by the polar head group.

4. DISCUSSION

A glycophospholipid containing non-*N*-acetylated glucosamine isolated from BC3H1 myocytes [3] was recently demonstrated to be the source of a compound(s) with 'insulin-like' activity [3]. A polar head group(s) generated from this same glycophospholipid in liver membranes after treatment with insulin or phospholipase C from *S. aureus* [2,3] mimicked the effect of insulin to stimulate high-affinity cAMP phosphodiesterase [2]. The complete structure of the polar head group(s) remains to be determined but it is known to be a phospho-carbohydrate with a size of about 1400 Da [2]. This laboratory recently demonstrated the generation of a polar head group from a purified glycophospholipid from H35 hepatoma cells [4] treated with *S. aureus* phospholipase C. The polar head group of this phospholipid is probably identical or similar to the modulator of cAMP phosphodiesterase [2,3]. That is, both can be generated from a glycophospholipid in response to *S. aureus* phospholipase C, both contain non-*N*-acetylated glucosamine and inositol, and they are of similar size [2–4]. The present work demonstrated that like insulin, this polar head group inhibited phospholipid methyltransferase, suggesting that it may be an intracellular mediator for the effect of insulin on phospholipid methyltransferase. This study was the first to demonstrate that the polar head group can act on an intact cell system as well as in a subcellular system, consistent with the effects reported for the insulin mediator from rat liver and skeletal tissue [1,9]. It remains to be determined whether the polar head group of this novel glycophospholipid is the same material as that previously identified as 'insulin mediator'.

The activity of phospholipid methyltransferase is regulated by phosphorylation and dephosphorylation. A variety of hormones including glucagon [6,14], isoproterenol [7,9,10], human chorionic gonadotropin [15] and ACTH [8] stimulated this enzyme. In addition, other agents which elevate intracellular cAMP levels, like cAMP analogues and forskolin, have also been reported to stimulate phospholipid methyltransferase in a variety of tissues including rat adipocytes (review [16]). Insulin has been shown to inhibit the activation of phospholipid methyltransferase in response to glucagon [6], isoproterenol [7,9,10] and ACTH [8]. The enzyme from rat liver has been purified and found to be phosphorylated and activated by the cAMP-dependent protein kinase [17]. Phospholipid methyltransferase has also been phosphorylated in intact hepatocytes treated with glucagon [18] and insulin prevented glucagon-dependent phosphorylation of the enzyme [19]. These results strongly indicated that phospholipid methyltransferase was modulated by insulin. Because insulin can modulate the activities of both phosphatases and kinases (reviews [1,9]), it would seem that insulin inhibits phospholipid methyltransferase by preventing phosphorylation and/or activating dephosphorylation of the enzyme.

The present study showed that the purified polar head group from rat liver membranes could mimic the inhibitory effect of insulin on phospholipid methyltransferase in intact cells. This effect could have been mediated by inhibition of phosphorylation, activation of dephosphorylation or both. Although both possibilities are likely, evidence to support a role for activation of a phosphatase was obtained from subcellular studies. There was a time-dependent loss of isoproterenol-stimulated phospholipid methyltransferase activity in microsomes incubated at 37°C that was inhibited by NaF/EDTA/EGTA. These data suggested that the microsomes contained a phosphatase that could decrease phospholipid methyltransferase activity. Incubation of microsomes with the polar head group enhanced deactivation of phospholipid methyltransferase. The effect of the polar head group was greater on isoproterenol-stimulated microsomes, and was not prevented by the addition of NaF/EDTA/EGTA. These data suggested that the polar head group activated an NaF-resistant phos-

phoprotein phosphatase resulting in a loss of phospholipid methyltransferase activity due to dephosphorylation of the enzyme.

The results of these studies suggested that the inhibitory effect of insulin on phospholipid methyltransferase is mediated by the polar head group of a novel insulin-sensitive glycophospholipid. Further studies are needed to determine if this polar head group can inhibit phospholipid methyltransferase by interfering with phosphorylation of the enzyme in addition to stimulating its dephosphorylation. Of particular interest in terms of insulin action is the possibility that the effect of insulin to modulate phosphorylation of other insulin-sensitive enzymes may also be mediated by the polar head group of this novel glycophospholipid.

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