

Sphingosine, an inhibitor of protein kinase C, suppresses the insulin-like effects of growth hormone in rat adipocytes

(insulin action/lipogenesis/glucose uptake/phorbol esters)

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ABSTRACT Insulin, human growth hormone (hGH), and phorbol 12-myristate 13-acetate all stimulate lipogenesis in rat adipocytes preincubated without hGH for 4 hr. As previous data suggested that protein kinase C plays an important role in the action of insulin and in the insulin-like effects of hGH in rat adipocytes, we tested the effects of sphingosine, a potent inhibitor of protein kinase C, on the lipogenic activity of both hormones. At 50 μM , sphingosine had no effect on basal lipogenesis but completely abolished the action of phorbol 12-myristate 13-acetate and decreased by 65% and 89%, respectively, the effects of hGH and insulin. At higher concentrations (100 μM), sphingosine abolished both basal and hormone-stimulated lipogenesis; this effect was partially reversible after washing the cells. Similar effects of sphingosine on basal and stimulated glucose uptake were seen in parallel, suggesting that sphingosine inhibits lipogenesis at the glucose-uptake step in rat adipocytes. *N*-Acetylsphingosine and sphingomyelin, two analogs of sphingosine that are inactive on protein kinase C, did not inhibit lipogenesis induced by hGH, insulin, or phorbol 12-myristate 13-acetate. Sphingosine did not inhibit insulin binding to rat adipocytes at concentrations up to 200 μM but decreased hGH binding to its receptors by 44% at 50 μM . These data suggest a direct link between the inhibition of protein kinase C and that of lipogenesis and provide new evidence for the involvement of protein kinase C in the mechanism of action of growth hormone and insulin in rat adipocytes.

Human growth hormone (hGH) exerts a variety of effects on somatic growth, development, and metabolism (1–3). While most of growth hormone (GH) effects on skeletal growth are thought to be indirect and mediated through somatomedins such as insulin-like growth factor I (2, 4), GH actions on carbohydrate and lipid metabolism are probably direct (5). GH is classically an antagonist of insulin, with lipolytic and diabetogenic effects *in vivo*—but paradoxically GH also exerts acute and transient insulin-like effects *in vivo* and *in vitro* in models where endogenous GH has been suppressed (1, 2, 5). We recently showed that hGH, like insulin, stimulates lipogenesis in rat adipocytes preincubated without hormones during 4 hr, and the nonadditivity of the two maximal responses strongly suggested that the two hormones share a common subset of activated pathways (6).

Despite advances in our knowledge of the mechanisms of action of GH and insulin, we still do not understand at the molecular level how these hormones transduce their signals from their specific membrane receptors into the cell. The recent cloning of a putative GH receptor (7) has provided no clue as to a possible mechanism of action. The GH receptor sequence deduced from the cDNA is not related to known tyrosine kinases or to any other known protein except the prolactin receptor (8). Foster *et al.* (9) reported recently that

the GH receptors of 3T3-F442A fibroblasts and adipocytes are phosphorylated when occupied by GH, but the biological significance of this phosphorylation has still to be addressed.

In contrast, the mechanisms of action of insulin have been thoroughly investigated over the past decade. The advances in the molecular biology of the insulin receptor (10, 11), including site-directed mutagenesis, have helped build extensive circumstantial evidence for an important role of the receptor β -subunit tyrosine kinase (12), although specific intracellular substrates have yet to be identified. The insulin receptor has also recently been proposed to activate a phospholipase C that specifically hydrolyzes a glycosylphosphatidylinositol in the cell membrane (13). This glycosylphosphatidylinositol breakdown generates an inositol phosphate glycan (13) that mimics some intracellular effects of insulin and diacylglycerols (14), which are potential activators of protein kinase C. This kinase, an important intermediate in various pathways of signal transduction, hormone action, and cell regulation (15), has also been suggested to participate in insulin action. Indeed, insulin has been reported to provoke rapid increases in the synthesis and intracellular contents of phosphatidic acid, inositol phospholipids, and diacylglycerols in BC₃H1 myocytes and adipose tissue (13, 14, 16–25), although one recent work showed no effects of insulin on the breakdown of inositolphospholipids in rat adipocytes (26). Moreover, insulin stimulates the activity of protein kinase C in both cytosolic and membrane fraction of BC₃H1 myocytes and in rat diaphragm membranes (27). Finally, protein kinase C activators such as phorbol esters mimic some of the insulin effects in different cell types (20, 25, 28–45).

Because phorbol esters stimulate lipogenesis in rat adipocytes (36–38), we evaluated the possible involvement of protein kinase C in our postulated common pathway shared by insulin and hGH in these cells. We showed that phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, stimulated lipogenesis and that its effect was nonadditive to the effects of insulin or GH (43). Furthermore, downregulation of protein kinase C markedly inhibited the effects of both hormones (43). In the present work, we looked for a specific inhibitor of protein kinase C to further evaluate the potential role of protein kinase C in the actions of insulin and GH. Sphingosine, a sphingoid long-chain base, was recently shown to be a potent inhibitor of purified protein kinase C—and of phorbol dibutyrate binding *in vitro* and *in vivo* (46–48). We report here that sphingosine strongly inhibits GH-, insulin-, and PMA-stimulated lipogenesis and glucose uptake in rat adipocytes. These results are consistent with an involvement of protein kinase C in the mechanisms of action of GH and insulin.

MATERIALS AND METHODS

Reagents and Hormones. hGH (NIDDK hGH bulk at 2.4 international units per mg) was obtained from the National

Hormone and Pituitary Program, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIDDK) (S. Raiti, University of Maryland School of Medicine). Porcine insulin was purchased from Sigma; D-[3-³H]glucose was from DuPont/NEN; collagenase (Clis II, batch 67157M) was from Cooper Biomedical; bovine serum albumin (BSA) fraction V (batch 17F-0149), PMA, sphingosine, and sphingomyelin were from Sigma; and both 2,5-diphenyloxazole (PPO) and 1,4-bis-2(5-phenyloxazolyl)benzene (POPOP) were purchased from Eastman Kodak. *N*-Acetylsphingosine was prepared as reported (49). Other chemicals were of reagent grade and were obtained from J. T. Baker. The tubes used for lipogenesis assays were 7-ml high-density polyethylene S/P scintillation mini-vials from American Scientific Products (McGaw Park, IL).

Animals. Male Wistar rats (120–140 g) were obtained from Charles River Breeding Laboratories and were maintained in light/dark cycles of 12 hr and fed ad libitum.

Lipogenesis. The adipocyte isolation and lipogenesis assay were conducted as described (6). Briefly, dissected epididymal and retroperitoneal fat pads were digested by vigorous shaking at 37°C for 30 min with collagenase (1.0 mg/ml) in Krebs–Ringer–Hepes (KRH) buffer, pH 7.4/BSA (35 mg/ml)/0.27 mM glucose. After filtration through cheesecloth and four washes with KRH buffer/BSA (10 mg/ml), the adipocytes were preincubated for 4 hr at 37°C in the same buffer. Cells (8×10^4 cells per ml) were then incubated in triplicate (final volume, 0.5 ml per tube) for 10 min at 37°C with increased concentrations (0–100 μ M) of sphingosine, *N*-acetylsphingosine, or sphingomyelin before the addition of a maximally effective dose of insulin (100 ng/ml), hGH (1 μ g/ml), or PMA (100 ng/ml) and 9 nM D-[3-³H]glucose and maintained for 2 hr at 37°C. The incubation was interrupted by adding toluene scintillator (5 ml per tube; 1 liter of toluene containing 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 5 g of 2,5-diphenyloxazole) and shaking vigorously to disrupt the cells, after which a 1-hr interval allowed for complete extraction of lipids into the toluene scintillating phase before counting in a β counter. To prevent cytotoxic effects (50), sphingosine, *N*-acetylsphingosine, and sphingomyelin were dissolved in ethanol (100 mM) and then diluted in KRH buffer/1% BSA before administration to the cells. When used, PMA was dissolved in dimethyl sulfoxide (10 mg/ml) before dilution in KRH buffer/1% BSA. The maximal ethanol or dimethyl sulfoxide concentration used in lipogenesis experiments did not modify the cell response in a control lipogenesis assay.

Glucose Uptake. Adipocytes were isolated and then preincubated 4 hr at 37°C in KRH buffer/BSA (10 mg/ml)/0.27 mM glucose as described in the lipogenesis assay procedure. For glucose-uptake measurements, isolated adipocytes (16×10^4 cells per ml) were incubated according to the lipogenesis procedure with increased concentrations of sphingosine (0–100 μ M) during 10 min at 37°C before the addition of a maximally effective dose of insulin (100 ng/ml), hGH (1 μ g/ml), or PMA (100 ng/ml) and 9 nM D-[3-³H]glucose for 2 hr at 37°C. Incubation was interrupted by adding successively 3 ml of ice-cold 0.9% NaCl and 1 ml of dinonylphthalate per tube. The tubes were immediately centrifuged at 4°C, $3000 \times g$ for 5 min. Floating cells were recovered with a 500- μ l pipet fitted with a truncated tip, transferred in a scintillation vial containing 10 ml of universal scintillating fluid (Aquasol-2, NEN Research Product, Boston), and thoroughly shaken to break the cells before counting.

Hormone Binding. The binding of insulin and GH to their receptors was studied as described (6). We prepared ¹²⁵I-labeled insulin (labeled on tyrosine A14) and ¹²⁵I-labeled hGH as described (51, 52). Isolated adipocytes (8×10^5 cells per ml for insulin binding; 16×10^5 cells per ml for hGH binding) were preincubated in duplicate (final volume, 0.5 ml per tube) in binding buffer [KRH buffer, pH 7.4/5% BSA (wt/wt)/0.27 mM glucose/bacitracin at 0.5 mg/ml/aprotinin at 0.5 μ g/ml] and various concentrations of sphingosine (0–200 μ M) for 10 min at 37°C before adding ¹²⁵I-labeled insulin (30,000 cpm per tube corresponding to 30-pM final concentration) or ¹²⁵I-labeled hGH (50,000 cpm per tube corresponding to 45-pM final concentration). Incubation time was 45 min at 37°C for insulin and 75 min at 37°C for hGH. Incubations were interrupted by adding successively 3 ml of ice-cold 0.9% NaCl and 1 ml of dinonylphthalate per tube. The tubes were immediately centrifuged at 4°C at $3000 \times g$ for 5 min. Floating cells were recovered with a 500- μ l pipet fitted with a truncated tip, and the cell-associated radioactivity was counted (total binding). Binding of ¹²⁵I-labeled insulin and ¹²⁵I-labeled hGH in the presence of 10 μ g/ml of unlabeled insulin and hGH, respectively, was considered nonspecific binding.

RESULTS

Effect of Sphingosine on the Lipogenesis Stimulated by hGH and Insulin. Fig. 1 shows the effects of sphingosine on the incorporation of tritiated glucose into lipids in isolated rat adipocytes. At 50 μ M (Fig. 1 *Right*), sphingosine had no

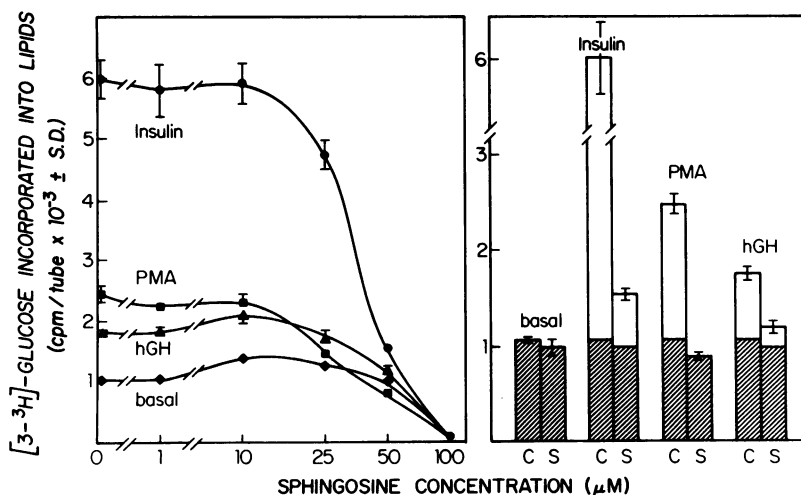


FIG. 1. Effect of sphingosine on the lipogenesis stimulated by hGH and insulin. After 4-hr preincubation, rat adipocytes (4×10^4 cells per tube) were incubated in triplicate for 2 hr at 37°C as described without (basal) or with a maximally effective dose of insulin (100 ng/ml), PMA (100 ng/ml), or hGH (1 μ g/ml) and 9 nM D-[3-³H]glucose in the presence of increased concentrations of sphingosine (*Left*) or at 0 (C bars) and 50 μ M (S bars) of sphingosine (*Right*). Results (mean \pm 1 SD), expressed in cpm per tube, are plotted as a function of sphingosine concentration.

significant effect on basal lipogenesis, but it completely inhibited the stimulation of lipogenesis by PMA. Sphingosine also markedly decreased the enhancement of lipogenesis by GH (by 65%) and insulin (by 89%) (Fig. 1 Right). At higher concentration (usually 100 μM), sphingosine completely abolished lipogenesis (Fig. 1 Left). The steep dose-response curve of the effects of sphingosine seen in rat adipocytes is in the range described by other investigators for the inhibition of protein kinase C *in vitro* as well as *in vivo* on different cell types (46, 48, 53). Viability of the adipocytes, assessed by the trypan blue exclusion method, remained unchanged in 200 μM sphingosine. In a control experiment, the effect of sphingosine (100 μM) on basal lipogenesis and on GH- and PMA-stimulated lipogenesis was totally reversible after washing the cells, whereas insulin-stimulated lipogenesis recovered 87% of control values.

Specificity of the Effect of Sphingosine on Lipogenesis. We tested the specificity of the action of sphingosine by comparing its effects on lipogenesis with those of sphingomyelin. The critical structural features of lysosphingolipids required for a specific inhibition of protein kinase C are a primary amine and the hydrophobic character of the long chain (46). In contrast with sphingosine, sphingomyelin, which has an amide-linked fatty acid at the 2-amino position of the sphingoid base, is inactive on protein kinase C and did not inhibit either basal lipogenesis, PMA-, or hormone-stimulated lipogenesis at concentrations up to 100 μM (Fig. 2). Similar results were obtained with another analog inactive on protein kinase C, *N*-acetyl sphingosine, at 100 μM (Fig. 2). Thus, the inhibition of lipogenesis had the same specificity as protein kinase C inhibition. Another argument for a specific effect on protein kinase C is that 100 μM sphingosine completely inhibited the activity of purified protein kinase C *in vitro*, as described (46) but had no effect on the tyrosine kinase activity of the purified insulin receptor (Y. Fujita-Yamaguchi, personal communication).

Effect of Sphingosine on Hormone Binding to Rat Adipocytes. Because sphingosine accumulates in the cell membrane, it was important to verify that its inhibitory effects on the stimulation of lipogenesis were not from an inhibition of the binding of the hormones to their receptors. The binding experiments were conducted under conditions as close as possible to those used to measure lipogenesis (6). Sphingosine had no significant effect on insulin binding at concentrations up to 200 μM , except for a small increase in non-

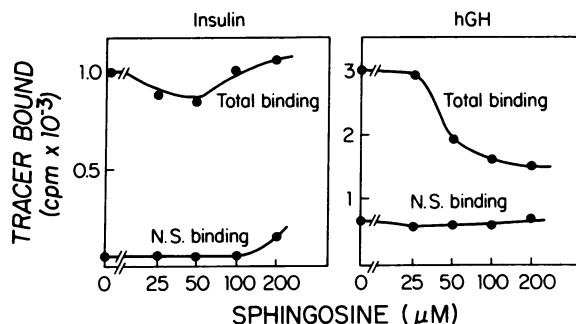


FIG. 3. Effect of sphingosine on hormone binding to rat adipocytes. (Left) ^{125}I -labeled insulin (30,000 cpm/tube) was incubated in duplicate for 45 min at 37°C with isolated adipocytes (4×10^5 cells per tube) without (total binding) or with unlabeled insulin (10 $\mu\text{g}/\text{ml}$) (N.S. binding) in the presence of increased concentrations of sphingosine. (Right) ^{125}I -labeled hGH (50,000 cpm per tube) was incubated in duplicate for 75 min at 37°C with isolated adipocytes (8×10^5 cells per tube) without (total binding) or with unlabeled hGH at 10 $\mu\text{g}/\text{ml}$ (N.S. binding) in the presence of increased concentrations of sphingosine. The ^{125}I -labeled hormone binding, expressed in cpm per tube, is plotted as a function of sphingosine concentration.

specific binding at 200 μM (Fig. 3 Left). In contrast, GH binding to its receptor appears to be sensitive to sphingosine at high concentration. The binding of GH progressively decreased with increased concentrations of sphingosine, with maximal inhibition of 60% at 200 μM sphingosine (Fig. 3 Right). However, GH-specific binding did not decrease below 43% of its control value at a sphingosine concentration (100 μM) that inhibited lipogenesis completely. Thus, the effect of sphingosine on GH- or insulin-stimulated lipogenesis is not explained by inhibition of hormonal binding to respective receptor.

Parallel Effects of Sphingosine on Stimulated Lipogenesis and Glucose Uptake. Lipogenesis is a complex metabolic pathway with numerous steps potentially susceptible to inhibition. Interestingly, GH and PMA, like insulin, stimulate glucose uptake in different cell types (5, 28). To determine the site(s) of action of sphingosine, we studied its effect on the first step of lipogenesis, glucose uptake. In experiments conducted simultaneously on the same batch of adipocytes, sphingosine inhibited lipogenesis and glucose uptake stimulated by GH, insulin, or PMA to the same extent at each

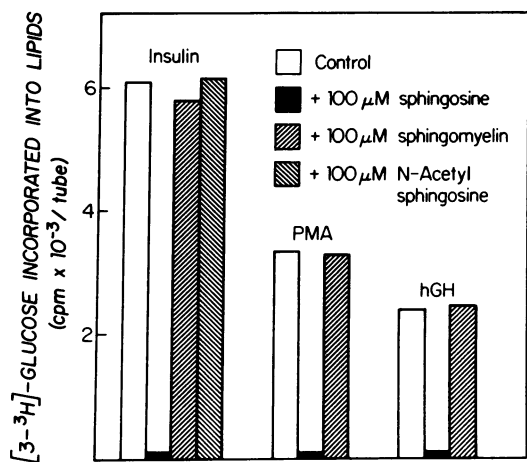


FIG. 2. Specificity of the effect of sphingosine on lipogenesis. The incorporation of D-[3- ^3H]glucose into lipids in preincubated rat adipocytes (4×10^4 cells per tube) was measured in triplicate in the presence of a maximally effective dose of insulin (100 ng/ml), PMA (100 ng/ml), or hGH (1 $\mu\text{g}/\text{ml}$) and without (control) or with 100 μM of sphingosine, sphingomyelin, or *N*-acetylsphingosine.

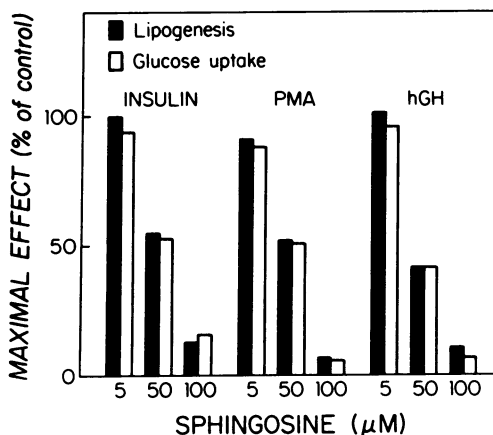


FIG. 4. Inhibition of lipogenesis and glucose uptake by sphingosine in rat adipocytes. Lipogenesis and glucose uptake stimulated by maximally effective doses of insulin (100 ng/ml), PMA (100 ng/ml), or hGH (1000 ng/ml) were measured in parallel on the same batch of adipocytes in the presence of increased doses of sphingosine. Results, normalized in percent of the effect measured without sphingosine, were plotted as a function of the sphingosine concentration.

concentration used (Fig. 4). These data imply that the observed sphingosine effect on lipogenesis is located at the glucose-uptake step. The possible involvement of protein kinase C in the intracellular effects of insulin and GH in rat adipocytes may thus be closely related to glucose transport.

DISCUSSION

Several lines of evidence suggest that protein kinase C plays a role in some effects of insulin in rat adipocytes. Indeed, phorbol esters mimic insulin actions on the phosphorylation of different intracellular substrates, on glucose transport, on glycolysis and on lipogenesis (30, 31, 36–38, 43, 45, 54), whereas protein kinase C downregulation significantly decreased insulin effects in these cells (43, 54). However, the exact role of protein kinase C in insulin action and the exact nature of the interactions between phorbol esters and insulin are rather complex and remain controversial (26, 39, 55). We recently reported that protein kinase C may also be involved in the insulin-like activity of GH in rat adipocytes (43).

In the present study we used sphingosine, a protein kinase C inhibitor, to further evaluate the potential role of protein kinase C in the action of insulin and GH. Our data clearly showed that glucose transport and, consequently, lipogenesis are progressively inhibited by sphingosine in the same concentration range that has been reported for the inhibition of protein kinase C *in vitro* as well as *in vivo* (46, 48, 53). We also found that the lipogenic effect of vasopressin, a hormone known to act through the activation of protein kinase C (56), is abolished in a similar way by sphingosine (J.S., S. Kathuria, and P.D.M., unpublished work). Therefore, our results strongly suggest a direct link between the inhibition of protein kinase C and that of the studied hormonal effects and bring new arguments for the involvement of protein kinase C in the mechanism of action of insulin and GH in rat adipocytes.

Insulin (and presumably GH) stimulates the conversion of glucose into lipids through two types of actions, glucose transport and activation of lipogenic enzymes such as pyruvate dehydrogenase and acetyl CoA carboxylase. At physiological concentrations of glucose, both pathways are important. There is no evidence at this point that sphingosine inhibits the latter pathway. In agreement with the present results, Robertson *et al.* showed in a preliminary communication* that 25 μM sphingosine partially inhibited stimulation of hexose transport and glucose oxidation in rat adipocytes but not in cultured human fibroblasts; they did not report effects of higher sphingosine concentrations. Nelson and Murray (57) also reported that 40 μM sphingosine partially inhibited insulin and PMA effects on 2-deoxyglucose uptake in 3T3-L1 fibroblasts, whereas sphingomyelin was ineffective at the same concentration. Civan *et al.* (58) recently showed that D-sphingosine (100 μM) reduces the Na^+ -transporting stimulation of insulin on frog skin, suggesting that at least part of its effects on the membrane Na^+ permeability and Na^+ , K^+ -exchange pump are mediated by protein kinase C.

Although our results are highly suggestive of a role of protein kinase C in the actions of insulin and hGH in rat adipocytes, several elements appear to complicate this interpretation. (i) Phorbol esters have been reported to partially inhibit the stimulation of lipogenesis by insulin (36, 37) and the activation of the insulin receptor tyrosine kinase in freshly isolated adipocytes (59). However, as we showed before, this inhibition of insulin actions is not apparent anymore once adipocytes have been preincubated 4 hr under the conditions reported here (43). (ii) Although sphingosine, a natural component of the cellular membrane, could alter

membrane structure at the concentrations used and, as a consequence, impair cellular functions associated with the membrane (e.g., glucose transport), this hypothesis appears unlikely given the lack of effect of sphingomyelin, a similar component of cell membrane and of *N*-acetylsphingosine, an even closer structural analog of sphingosine. (iii) Various methods known to inhibit protein kinase C, although effective to some extent, are not equally potent in antagonizing insulin and GH effects. For example, downregulation of protein kinase C in rat adipocytes or treatment with isoquinoline sulfonamide H-7, which blocks the ATP binding site of protein kinase C (60), only partially blocked lipogenesis stimulated by hGH or insulin.[†] In contrast, sphingosine (this report) and staurosporine (J.S., S. Kathuria, and P.D.M., unpublished work) caused complete inhibition. We have also shown that acridine orange, which Hannun and Bell have also shown to inhibit protein kinase C (61), completely blocked insulin and GH stimulation of lipogenesis, whereas 9-acridinecarboxylic acid, inactive on protein kinase C, is devoid of any effect (62). An explanation for the differential effects seen may reside in the involvement of multiple members of the protein kinase C family (63), which may not all be as sensitive to various inhibitors. (iv) The specificity of sphingosine derivatives with respect to protein kinase C may not be absolute. In fact, sphingosine has recently been shown to inhibit several calmodulin-dependent enzymes (64). The fact that we see inhibition of the effects of insulin and GH with a number of chemically unrelated inhibitors of protein kinase C, however, favors the hypothesis that protein kinase C is involved, at least in part, in the common pathway shared by GH and insulin in rat adipocytes. There is no evidence presented here that allows us to infer a direct hormonal activation of protein kinase C, but our data show, at least, that active protein kinase C is necessary for the expression of this action of insulin and hGH in adipocytes.

Finally, one striking implication of our results concerns the metabolic status of patients with inborn disorders of sphingolipid metabolism (for review, see ref. 65). The accumulation of glucosylsphingosine in the brain and spleen of patients with Gaucher disease has been estimated to reach levels as high as 150 μM (66), and galactosylsphingosine could reach 140 μM in affected tissues of patients with Krabbe disease (66). These concentrations are well in the range that is postulated to inhibit protein kinase C (46) and which we show abolishes the effects of insulin. Hannun and Bell (66) have suggested that inhibition of protein kinase C plays an important role in the pathogenesis of the sphingolipidoses. The antagonism of the effects of insulin reported here, if effective *in vivo* on insulin-sensitive tissues, would create widespread insulin resistance in such patients. Even if limited only to fat tissue, blockade of the anabolic effects of insulin could contribute to the wasting seen in many such patients. Insulin resistance in those diseases has, to our knowledge, not been reported, and our results suggest that this possibility is worth investigating.

Note Added in Proof. After submission of this work, a paper appeared showing that GH stimulates *c-fos* gene expression in preadipose cells by means of protein kinase C without increasing inositol lipid turnover, while protein kinase C does not appear to be involved in induction by GH of insulin-like growth factor I mRNA (67).

*Smal, J., Fujita-Yamaguchi, Y. & De Meyts, P., 70th Annual Meeting of the Endocrine Society, June 8–11, 1988, New Orleans, p. 84 (abstr. 253).

*Robertson, D. G., Longo, N., Newby, F. D., Di Girolamo, M., Merrill, A. H. & Lambeth, J. D., 69th Annual Meeting of the Endocrine Society, June 10–12, 1987, Indianapolis, p. 39 (abstr. 71).

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