Effects of Streptozotocin-induced Diabetes and Insulin on Phospholipid Content of R3230AC Mammary Tumor Cells

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ABSTRACT

The influence of diabetes and insulin treatment on the phospholipid content of R3230AC mammary tumors, a hormonally responsive neoplasms, was studied. Diabetes was induced by administration of streptozotocin 3 days prior to tumor implantation. Protamine zinc insulin, 3 IU/rat twice daily, was administered to tumor-bearing rats for 3 days. Enzymatically dissociated tumor cells from diabetic animals showed significant increases in phosphatidyl choline, lysophosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and phosphatic acid, compared to controls. Diabetic animals treated with insulin displayed reductions in phosphatidyl choline, lysophosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and phosphatic acid to levels approximating those found in intact (control) animals. However, neither diabetes nor insulin treatment altered sphingomyelin levels.

MATERIALS AND METHODS

Female Fischer rats were obtained from Charles River Breeding Laboratory, Wilmington, MA. They were offered food and water ad libitum. The R3230AC tumors were implanted into animals weighing 90–100 g as described by Hilf et al. (9).

Induction of Diabetes and Insulin Treatment. Diabetes was induced by i.p. administration of streptozotocin 3 days prior to tumor implantation. Streptozotocin was dissolved in 0.9% sodium chloride, acidified to pH 4.5 with citrate, and injected within 5 min; the dose administered was 60–70 mg/kg. Animals were classified as diabetic if their serum glucose levels were 250 mg/100 ml or higher and urinary glucose, estimated by Clinistix (Ames Co., Inc., Elkhart, IN), was greater than 0.5 g/100 ml. Serum glucose was determined by glucose oxidase method (Worthington Biomedical Corp., Freehold, NJ) (10) and insulin was assayed using the magnetic solid phase RIA kit (Seron Diagnostics, Randolph, MA) (11, 12). Protamine zinc insulin (Eli Lilly and Co., Indianapolis, IN) was administered to diabetic rats at a dosage of 3 IU/rat, twice daily for 3 days.

Dissociation of Tumor Cells. Animals were sacrificed by cervical dislocation 3 weeks after tumor implantation. Tumors were removed quickly and placed in ice-cold 0.9% NaCl solution. After removal of connective tissue and areas of necrosis, approximately 3 g of tissue were minced on a Mcllwain tissue slicer (Brinkmann Instruments, Inc., Westbury, NY). The minced tissue was incubated with 10 ml of Hanks’ balanced salt solution (Ca2+- and Mg2+-free) containing 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) and 0.05% collagenase (Cooper Biomedical Inc., Malvern, PA) for 15 min at 37°C in a shaking water bath (50 cycles/min). The tissue was strained through a 100 mesh stainless steel strainer and the retained tissue was reincubated with 10 ml of fresh enzyme-containing solution for 60 min at 37°C. Dissociated cells were strained, collected, washed, and centrifuged four times at 500 x g for 5 min. Details of this procedure were published in 1976 (3). Cell viability was estimated by trypan blue dye exclusion; cell counting was performed with a hemocytometer.

Analytical Procedures. Dissociated cells (106) were suspended in 0.9% NaCl solution and homogenized. The proteins were precipitated by addition of 2.5 ml of cold trichloroacetic acid (10% w/v). The precipitate was washed once with 3 ml of 5% (w/v) trichloroacetic acid containing 1 mM EDTA and once with 3 ml of water. Phospholipids were extracted from the precipitate according to the procedure of Foehl et al. (13).
Insulin and Phospholipids in R3230AC Mammary Tumors

Glycerophosphoesters were separated on oxalated (1% potassium oxalate) thin layer chromatography plates using chloroform: acetone: methanol: acetic acid: water [40:15:15:12.6 (v/v)] or with chloroform:methanol:4 N ammonium hydroxide [9:7:2 (v/v)] solvent systems. The chromatograms were air-dried and sprayed with 1,6-diphenyl-1,3,5-hexatriene (2 to 5 mg/100 ml of hexane) and phospholipids were visualized under UV light. The ninhydrin reagent (100 mg ninhydrin in 50 ml of acetone plus 0.5 ml of lutidine) was used to detect aminophospholipids. A mixture of standard phospholipids, phosphatidyl choline, lysophosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, sphingomyelin, phosphatidic acid, phosphatidyl inositol, lysophosphatidyl ethanolamine, and cardiolipin were chromatographed along with the cell extracts each time. Standard phospholipids were obtained from Sigma Chemical Co. The recovery of phospholipids was between 90-100%. Since only traces of cardiolipin were detected during the separation procedures used, quantitation was not performed.

The localized phospholipids were scraped off the chromatogram and digested with 0.3 ml of concentrated sulfuric acid plus 2 drops of concentrated nitric acid at 190°C for 30 min. The lipid phosphate was measured (14).

Proteins were determined by the method of Lowry et al. (15). DNA was extracted from the dissociated cells (16) and estimated according to the Dische procedure (17).

Results

Animals that had received 60-70 mg of streptozotocin/kg body weight exhibited weight loss, glucosuria, high serum glucose levels, and low serum insulin concentrations (Table 1). Administration of insulin to diabetic rats, at a dosage of 3 IU twice daily for 3 days, resulted in an increase in body weight and serum insulin levels, as well as a decrease in serum and urinary glucose levels, when compared to diabetic animals.

Effect of Insulin on DNA and Protein Content. DNA content from the dissociated mammary tumor cells from intact, diabetic, and insulin-treated animals was measured and is expressed as µg DNA/mg protein (Table 1). Mammary tumor cells from diabetic rats contained 63 ± 3 (SE) µg DNA/mg protein, a level that was significantly increased (P < 0.0125) above that observed in tumor cells from intact (control) animals. Administration of protamine zinc insulin (6 IU/day for 3 days) to diabetic animals lowered the DNA level to 48 ± 2 µg/mg protein, this decrease being significant (P < 0.005). The protein content was similar to that in tumor cells from intact, diabetic, and insulin-treated diabetic animals.

Phospholipid Composition. The effects of streptozotocin-induced diabetes and insulin treatment on the phospholipid content of R3230AC mammary tumor cells are illustrated in Chart 1. The phospholipid levels are expressed as nmol of phospholipid phosphorous/mg protein. Of the various phospholipids studied, tumor cells from diabetic animals showed a significant increase in PC (P < 0.0005), LPC (P < 0.0025), PE (P < 0.0005), PS (P < 0.0025), PI (P < 0.005), and PA (P < 0.05) when compared to controls. Administration of streptozotocin did not alter tumor cell LPE or SM levels from control. Diabetic animals treated with insulin, however, displayed reductions in the levels of PC, LPC, PE, PS, PI, and PA, these levels approximating those found in tumors from intact (control) animals. The significant decrease in phospholipids observed after insulin treatment was greatest for PC, LPC, PE, and PA (P < 0.0025), and somewhat less for PI (P < 0.0125) and PA (P < 0.05). Administration of insulin to diabetic animals lowered LPE levels but did not alter SM levels. Thus these data indicate that insulin regulated the amounts of certain phospholipids in this mammary tumor.

Discussion

The purpose of the present study was to ascertain whether (a) streptozotocin-induced diabetes and insulin treatment affected phospholipid content; and (b) whether phospholipid levels could be related to the growth behavior of the R3230AC mammary tumor. Mammary tumors transplanted and growing in diabetic animals showed an increase in DNA content/mg protein, whereas administration of insulin to these diabetic animals caused a decrease in DNA content. Previous studies in our laboratory with the R3230AC mammary adenocarcinoma demonstrated that this transplantable tumor grew somewhat faster in diabetic rats, and administration of exogenous insulin to diabetic animals resulted in decreased tumor growth (1). Increased growth of Zaj-Dela ascites hepatoma in diabetic rats has been reported by Weiser et al. (18). Thus it would appear that the changes in DNA content of the tumor in diabetic and insulin-treated animals were reflecting the altered cell growth under these experimental conditions.

The animals made diabetic by administration of streptozotocin displayed loss in body weight, glucosuria, hyperglycemia, and significant reductions in serum insulin levels. Administration of 3 IU insulin twice daily was effective in ameliorating these sequelae of diabetes, although this therapy did not return all of the parameters to those found in the intact animal. Insulin treatment increased body weight and prevented glucosuria, but serum glucose levels in the treated animals were below normal. Based on previous experience in our laboratory, the dose regimen of insulin used here was the best compromise since higher daily doses of insulin (8 IU) led to hypoglycemia and death, whereas lower daily doses (4 IU) did not improve body weight gain and animals remained hyperglycemic. The levels of insulin in serum of insulin-treated animals were within the range expected from these experimental conditions.

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To evaluate the effect of insulin and diabetes on phospholipid composition of the neoplasm we prepared enzymatically disso-

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of samples</th>
<th>Group 1 normal</th>
<th>Group 2 diabetic</th>
<th>Group 3 insulin treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>10</td>
<td>139 ± 3.0 b</td>
<td>91 ± 3.0 b</td>
<td>112 ± 4.0 c</td>
</tr>
<tr>
<td>(g/100 ml)</td>
<td>10</td>
<td>&lt;0.1</td>
<td>1.2</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Serum glucose</td>
<td>7</td>
<td>110 ± 11</td>
<td>460 ± 66 a</td>
<td>52 ± 4.0 a</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum insulin</td>
<td>5</td>
<td>27 ± 2.4</td>
<td>6.5 ± 1.4 a</td>
<td>233 ± 36.0 e</td>
</tr>
<tr>
<td>(µU/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular DNA level</td>
<td>7</td>
<td>52 ± 2</td>
<td>63 ± 2</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>(µg DNA/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein content</td>
<td>11</td>
<td>31 ± 1</td>
<td>31 ± 1</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>(mg protein/10^6 cells)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Mean ± SE from number of samples indicated.
* Significantly different versus normal (intact) animals.
* Significantly different versus diabetic animals.

The abbreviations used are: PC, phosphatidyl choline; LPC, lysophosphatidyl choline; PE, phosphatidyl ethanolamine; LPE, lysophosphatidyl ethanolamine; PS, phosphatidyl serine; SM, sphingomyelin; PA, phosphatidic acid; PI, phosphatidyl inositol.

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associated R3230AC mammary tumor cells, which were shown to possess insulin receptors (2) and display responses to insulin (3, 4). The present results demonstrate that growth of these tumors in diabetic animals was accompanied by increases in the mass of phospholipids, primarily in PC, LPC, PE, PS, PI and PA, whereas treatment with insulin prevented or reversed the effects of diabetes. An increase in the activity of enzymes that synthesize phospholipids and a decrease in the activity of enzymes that degrade phospholipids has been observed in the hepatic microsomal fraction of streptozotocin-induced diabetic rats; insulin treatment corrected these alterations (19). An increased incorporation of [32P]phosphate into phosphatidyl inositol-4,5-bisphosphate, probably due to an increased PI turnover, was observed in the sciatic nerve of streptozotocin-induced diabetic rats (20). Natarajan et al. (21) observed a decrease in phosphatidyl inositol (20 weeks after injection of 50 mg of streptozotocin/kg body weight) and a relative decrease in the rates of incorporation of phosphate into phosphatidyl inositol in nerves of both long term and acute (5 days after streptozotocin injection of 100 mg/kg body weight) diabetic rats. Decreased incorporation of inositol into rat sciatic nerve, probably due to impairment of inositol transport, was reported for streptozotocin- and alloxan-induced diabetes, and insulin treatment increased PI synthesis (22). Activity of the enzyme CDP diacylglycerol-inositol phosphatidyl transferase, an enzyme responsible for the synthesis of phosphatidyl inositol, was reported to be decreased in nervous tissue of diabetic rats (23).

Farese et al. (6) observed increased phospholipid synthesis in response to insulin for BC3H-1 cultured myocytes in vitro. In their experiments phospholipids of cultured myocytes were labeled to constant specific activity by prolonged incubation with [32P]phosphate and 32P measurement was used to reflect changes in phospholipid mass. Insulin was also shown to provoke dose-related increases in the concentration of PA and PI in rat adipose tissue both in vivo and in vitro (24). [32P]Phosphate incorporation into PC, LPC, SM, PI, and LPE was stimulated to varying degrees by insulin in cultures of MCF-7 human breast cells (25). Thus depending on the system under study, no clear picture of the effects of insulin on phospholipids has emerged. The effect of insulin on phospholipid metabolism in R3230AC mammary tumor cells would appear to be contrary to the effects usually seen with other polypeptide hormones (26–29). However, the results reported here, along with earlier studies of glucose and amino acid transport performed with R3230AC mammary tumor cells (3, 4), indicate that the growth inhibitory effects of insulin may be expressed as a decrease in these insulin-responsive parameters.

In R3230AC mammary tumor cells from intact rats (controls), PE represented about 10–12%, LPC around 15%, and LPE was approximately 14% of total phospholipids (Chart 1A). In normal
cells such as mononuclear leukocytes, PE and LPE contributed 29 and 3%, respectively, to the total phospholipid content (30). Lysophosphatidyl choline of normal rat mammary tissue during pregnancy, early lactation, lactation, and postweaning periods was 4, 6, 7, and 8%, respectively, of total phospholipids (31). The level of LPC and LPE observed in R3230AC mammary tumor cells appears to be higher than that usually observed in normal cells. Increased levels of lysophospholipids were reported in mammary carcinomas induced by dimethylbenz(a)anthracene (32). The higher level of lysophospholipids in this mammary tumor may have resulted from either an increase in phospholipase A2 activity, an inhibition of lysophospholipid acyl transferase activity (31), or both. The observed LPE and LPC levels might have arisen from cleavage of the vinyl ether bond of ethanolamine and choline plasmalogens, respectively, under acid conditions. Using conventional Folch's extraction procedure for tumor cells and separating lysophospholipids with basic solvent systems [chloroform:methanol:4 N ammonium hydroxide (9:7:2) or chloroform:methanol:ammonium hydroxide (65:25:4)], we confirmed that the R3230AC tumor cells possess these higher levels of lysophospholipids. The levels of both PC and LPE increased in the tumor cells of the diabetic host. Administration of insulin to diabetic animals showed reduction in the levels of PC and LPE, approximating those found in tumors from intact (control) animals. Although the PE level was increased in tumors from diabetic animals, the LPE level was not altered significantly from that of controls. This suggests a possible synthesis of PE from PS by phosphatidyl serine decarboxylase (33, 34). However, since administration of insulin lowered both PC and LPE levels significantly, we speculate that this response was probably due to an effect on the synthesis of these compounds.

The results observed from the effects of diabetes and their reversal by insulin treatment suggest that the phospholipid levels in this mammary tumor are regulated by the presence or absence of insulin. Shafie et al. (35) demonstrated that insulin receptors in R3230AC mammary tumors were subjected to down regulation in vivo; insulin binding increased in tumor cells from diabetic rats and decreased after insulin treatment. Down regulation of insulin receptors by insulin was confirmed in primary cultures of these tumors in vitro (36). Alterations in fatty acid composition of cells may result in significant effects on the properties of hormone receptors, including the insulin receptor (37, 38). Changes in fatty acid composition of phospholipids have been observed in mammary tumor cells, from diabetic hosts. Therefore we suggest that an altered phospholipid composition resulting from diabetes may contribute to altered insulin-binding characteristics under these experimental conditions.

Turnover of some phospholipid species is influenced by growth conditions (39, 40) and degradation of PI is one parameter that appears to correlate with cell growth (41). Hormones, growth factors, and many other biologically active substances are thought to induce cellular proliferation through activation of protein kinase C (42). Though PS appeared to be more effective in activating protein kinase C (43), other phospholipids, such as PA and PI, also activate this enzyme in bovine luteal and rat ovarian cytosol preparations (44). The order of potency for various phospholipid species to stimulate this enzyme activity, as reported in mouse brain to be as follows: PS > PA > PI > PE > SM > PC (45). The mechanistic significance of the effects of diabetes and insulin on phospholipid content in R3230AC mammary tumor cells is as yet unknown. However, the concordant increases in DNA content and phospholipid mass (mainly PC, PE, PS, PI, and PA) in tumors from diabetic animals suggest that the increased level of phospholipids may be of importance for the activation of protein kinase C. Work is currently under way to test this hypothesis and to better define the role of phospholipids in mediating the effects of diabetes and insulin in R3230AC mammary tumor growth.

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