Enhanced Cholesterol Esterase Activity in the Pancreas of Rats with Streptozotocin-Induced Diabetes

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Diabetes was induced in rats by the intravenous administration of streptozotocin (STZ; 60 mg/ml). The activity of cholesterol esterase (C E a s e) in various tissues was determined by use of either a fluorogenic or radioactive substrate. Significant C E a s e activity was detected in extracts of pancreas. The specific activity of pancreatic C E a s e was considerably greater in pancreatic extracts from diabetic rats compared with normal rats. The activity of pancreatic C E a s e increased 6 d after the injection of STZ, but the difference was not statistically significant. It reached a maximum value approximately twice that of normal pancreas at 30 d with statistic significance. The highest specific activity of pancreatic C E a s e was found in the cytosolic fraction from diabetic rats, whereas the specific activity of the enzyme was lowest the same fraction from normal rats.

Keywords cholesterol esterase; pancreas; streptozotocin; absorption; hypercholesterolemia; hyperphagia

Diabetes mellitus (DM) is by far the most common serious metabolic disorder in man, with a worldwide prevalence estimated to be between 1 and 5%. All forms of diabetes, both inherited and acquired, are characterized by hyperglycemia, a relative or absolute lack of insulin, and development of diabetes-specific microvascular pathology in the retina and renal glomerules. Epidemiological studies in man have identified DM as one of the risk factors associated with typical atherosclerotic arterial disease.1-4) DM is frequently associated with hypercholesterolemia, which may be one of the important factors in the acceleration of atherosclerosis in diabetic patients.

It is well known that hyperphagia is a critical determinant of lipemia in diabetics, and severely diabetic rats whose food intake is restricted to normal have nearly normal plasma levels of cholesterol and triacylglycerol (TG).5,6) Even if insulin deficiency leads to decreased lipoprotein lipase (LPL) activity, and hence impaired clearance of plasma lipoproteins, it is evident that the activity of those enzymes involved in the clearance of cholesterol is adequate to handle normal levels of cholesterol.7-9) The fraction of dietary cholesterol absorbed by the small intestine has been shown to increase slightly in diabetic rats.5,10-13) Since increased dietary cholesterol appears to decrease the amount of whole-body synthesized cholesterol,14,15) the relative contribution of newly-synthesized cholesterol to total cholesterol is small. It is clear from this that hyperphagia contributes significantly to the hypercholesterolemia of diabetic rats with access to food ad libitum. The activity of cholesterol esterase (C E a s e) in the pancreas should be one of the important factors regulating the level of cholesterol in the blood, since cholesterol esters in food are absorbed by the small intestine, after their hydrolysis to free cholesterol by C E a s e.16,17) However, it is not known if pancreatic C E a s e is induced in diabetic rats because of the increased amounts of dietary cholesterol that accompany the diabetes-associated hyperphagia. The present studies were carried out to examine the effects of experimentally-induced DM on pancreatic C E a s e activity in rats.

MATERIALS AND METHODS

Materials Streptozotocin (STZ) was obtained from Sigma Chemical Co., St. Louis, MO. Cholesteryl-[¹⁴C]oleate (1.85 GBq/mmol) was from Amersham Japan, Tokyo. Sodium taurocholate was from Nakarai Tesque, Kyoto. Umbelliferyl palmitate was prepared by a conventional method from umbelliferone and palmitoyl chloride, and was crystallized twice from hot ethanol. Other chemicals were all of analytical grade and used without further purification.

Diabetes was induced by the intravenous administration of 60 mg/kg STZ to male Sprague-Dawley rats (150-180 g). The diabetic condition of the rats was monitored by collecting blood samples at one day after the injection for the analysis of plasma glucose. Diabetic rats were sacrificed with age-matched normal (non-diabetic) rats 2 months after the injection of STZ.

Individual pancreases from normal and diabetic rats were suspended in two volumes (v/w) of 0.05 M Tris–HCl (pH 7.4) containing 1 mM EDTA, 2 mM β-mercaptoethanol, chymostatin (10 μg/ml) and antipain (10 μg/ml). They were then homogenized in a motor-driven Teflon-glass homogenizer for 3 min in ice-water. Each homogenate was centrifuged at 8000 × g for 30 min and the supernatant was filtered through absorbent cotton to remove floating lipids solidified by the cold. To examine the subcellular localization of C E a s e, a pancreas was homogenized in 0.34 M sucrose in the above buffer, and centrifuged successively at 500 × g for 20 min, 8000 × g for 30 min and 105000 × g for 60 min.

Assay of Pancreatic C E a s e Activity In the present study, we used two different methods for the determination of C E a s e activity: a convenient fluorometric assay and an assay with a specific radiolabeled substrate. For the former, the reaction mixture contained 40 μM umbelliferol palmitate, 1% dimethyl sulfoxide and the enzyme in 1 ml of 0.1 M Tris–HCl (pH 8.5), with or without 10 mM sodium taurocholate, in a quartz cell. After addition of the enzyme solution, the increase in fluorescence intensity at 460 nm, with excitation at 350 nm, was continuously recorded at
37°C. The difference between the activities, with and without taurocholate, was taken as a measure of CEase activity, which was expressed at nmol per min per mg protein. For the assay with radiolabeled substrate, the activity was measured isotopically using 1 nm cholesteryl-[14C]oleate and enzyme in 0.5 ml of 0.1 M Tris-HCl (pH 8.5) and incubating at 37°C for 10 min. The amount of radioactive fatty acid liberated was determined using the liquid-liquid partition system described by Belfrage and Vaughan. The total amount of free fatty acid released was calculated, after correction for the efficiency of extraction and quenching, and activities were expressed as nmol of fatty acid released per h per mg protein.

Protein was quantitated by the method of Lowry et al. with bovine serum albumin as the standard. Plasma glucose and total plasma cholesterol were determined using the appropriate diagnostic assay kits (Wako Pure Chemical Co., Osaka).

RESULTS

The plasma levels of glucose in rats, one day after the injection of STZ (60 mg/kg), were found to be 482 ± 73 mg/dl (n = 23), while in normal rats levels were 151 ± 21 mg/dl (n = 12). The plasma glucose levels of the STZ-treated rats were significantly higher than those of normal animals (p < 0.01), and were indicative of hyperglycemia in the treated rats one day after administration of STZ. The concentration of total cholesterol in plasma was 202 ± 23 mg/dl (n = 4) in diabetic rats, 2 weeks after injection of STZ, and 175 ± 18 mg/dl (n = 3) in age-matched normal rats. Although the plasma cholesterol levels were higher in diabetic rats than in normal rats, the difference was not statistically significant.

Pancreatic CEase is preferentially activated by cholate, while the activities of pancreatic lipase and other non-specific esterases are strongly inhibited by this detergent. The pancreatic extracts hydrolyzed fluorescein diacetate in the absence of taurocholate, but the rates of hydrolysis were not increased by the addition of the detergent (Table I). The hydrolysis of fluorescein diacetate by the pancreatic extract was increased approximately 4-fold in the presence of taurocholate. In addition, taurocholate markedly enhanced the rate of hydrolysis of umbelliferol palmitate, and cholesteryl-[14C]oleate by pancreatic extracts. Thus, the taurocholate-enhanced activity of the pancreatic extract is mostly due to CEase rather than to lipase and non-specific esterase activities. Therefore, we were able conveniently to measure CEase activity by a sensitive fluorometric method in the presence of 10 mm taurocholate. The activity measured in the absence of taurocholate was subtracted from the above activity to correct for the activities of non-specific esterases. Under such conditions, no CEase activity was detected in plasma or in extracts of several tissues (liver, kidney, adrenals and testis) from normal and diabetic rats, although low activities of non-specific esterases were detected in the extracts of these organs in the absence of taurocholate.

The supernatants obtained by centrifugation at 8000 x g of pancreases from normal and diabetic rats had significant

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity*&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without cholate</td>
<td>With cholate</td>
<td></td>
</tr>
<tr>
<td>Fluorometric assay (n = 4)</td>
<td>(nmol/min ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umbelliferol palmitate</td>
<td>74.9 ± 0.6</td>
<td>5498 ± 380</td>
<td></td>
</tr>
<tr>
<td>Fluorescein diurate</td>
<td>106.7 ± 1.6</td>
<td>458 ± 0</td>
<td></td>
</tr>
<tr>
<td>Fluorescein diacetate</td>
<td>650 ± 29</td>
<td>298 ± 8</td>
<td></td>
</tr>
<tr>
<td>Isotopic assay (n = 6)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cholesteryl oleate</td>
<td>0.25 ± 0.06</td>
<td>47.8 ± 9.2</td>
<td></td>
</tr>
</tbody>
</table>

* For the fluorometric assay, the reaction mixture contained 40 µl substrate, 1% dimethyl sulfoxide and enzyme in 1 ml of 0.1 M Tris-HCl (pH 8.5), with or without sodium taurocholate. For the isotopic assay, the reaction mixture containing 1 nmol cholesteryl-[14C]oleate (1.83 × 10<sup>9</sup> Bq) and enzyme in 0.5 ml of 0.1 M Tris-HCl (pH 8.5) was incubated for 10 min at 37°C, with or without 10 µM sodium taurocholate.

Table II. Increase in Pancreatic Cholesteryl Esterase Activity in Rats with STZ-Induced Diabetes

<table>
<thead>
<tr>
<th>Activity*&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fluorometric (nmol/min/mg protein)</th>
<th>Isotopic (nmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.1 ± 3.1 (n = 9)</td>
<td>2.13 ± 0.41 (n = 6)</td>
</tr>
<tr>
<td>Diabetic</td>
<td>16.3 ± 3.9 (n = 6)</td>
<td>5.71 ± 0.65 (n = 4)</td>
</tr>
</tbody>
</table>

The source of the enzyme was the supernatant obtained by centrifugation of cell homogenate at 8000 x g. Assays were performed as described in the legend to Table I.

Fig. 1. Time-Course of Induction of Pancreatic Cholesteryl Esterase in Rats after the Administration of STZ

The pancreas was removed from rats on the indicated day after injection of STZ and also from age-matched normal rats and the CEase activity was measured as described in the text. The source of the enzymes used was the supernatant obtained by centrifugation of cell homogenate at 8000 x g. The specific activity for diabetic rats as a percentage of that for normal rats was plotted against days after injection of STZ.

CEase activity (Table II). The specific activities of CEase per mg protein were considerably greater in STZ-induced diabetic rats than in normal rats. Radiolabeled cholesteryl oleate was hydrolyzed by the supernatants from diabetic rats at a much higher rate than by the supernatants from normal rats.

Enhancement of pancreatic CEase activity was time-dependent with respect to the injection of STZ (Fig. 1).
TABLE III. Subcellular Distribution of Pancreatic Cholesteryl Esterase from Diabetic Rats 2 Weeks after the Injection of STZ

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Normal (n=3)</th>
<th>Diabetic (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific-activity (nmol/min/ mg protein)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Homogenate</td>
<td>92 ± 38</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>51 ± 10</td>
<td>90</td>
</tr>
<tr>
<td>(500 × g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>66 ± 5</td>
<td>111</td>
</tr>
<tr>
<td>(8000 × g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>18 ± 18</td>
<td>11</td>
</tr>
<tr>
<td>(105000 × g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td>81 ± 18</td>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 2. Subcellular Distribution of Pancreatic Cholesteryl Esterase from Diabetic Rats (n=4) 2 Weeks after the Injection of STZ Together That from Age-Matched Normal Rats (n=3)

Subcellular fractions were obtained as described in the text, and CЕase activity was measured isotonically. Open and solid bars indicate normal and diabetic rats, respectively. 1, homogenate; 2, supernatant (500 × g); 3, supernatant (8000 × g); 4, supernatant (105000 × g).

CЕase activity increased with time after the intravenous administration of STZ reaching a plateau after 30 d.

Since CЕase is localized in the zymogen granules in the exocrine pancreas, it seemed possible that differences in terms of CЕase activity between diabetic and normal rats might be due to an abnormal subcellular distribution of the enzyme. The subcellular distribution of pancreatic CЕase activity from diabetic and normal rats is shown in Table III. The CЕase activity in the initial homogenate of diabetic pancreas was about twice that of normal pancreas. Therefore, it is unlikely that the higher activity of CЕase in the supernatant obtained by centrifugation at 8000 × g from the diabetic pancreas, compared with the normal pancreas, is due to differences in the extractability of the enzyme. Of four fractions from the diabetic pancreas, the specific activity of CЕase was highest in the cytosol fraction, while it was lowest in that fraction from normal pancreas. Somewhat more than half of the total CЕase activity in the pancreatic homogenate was found in the cytosol fraction from the diabetic pancreas, whereas only about one-tenth of the total activity was found in this fraction from the normal pancreas.

To confirm the above observations, pancreatic CЕase activity was measured isotonically using cholesteryl-[14C]oleate as substrate (Fig. 2). Of the four fractions from both diabetic and normal pancreas the specific activity of CЕase was highest in the cytosol fraction. Moreover, recoveries of activity in the cytosolic fraction were 50% and 45% that of the cell homogenate from diabetic and normal rats, respectively (data not shown).

All the results obtained indicate that the increase in pancreatic CЕase activity in rats with STZ-induced diabetes is not due to abnormal zymogen granules in the pancreas.

**DISCUSSION**

Since severe hypercholesterolemia is caused by high levels of dietary exogenous cholesterol in both normal and diabetic rats,24-26 it appears that the absolute amounts of cholesterol absorbed from the diet are the primary determinants of plasma cholesterol in rats supplied with food ad libitum. The present study demonstrates that plasma cholesterol levels in rats with STZ-induced diabetes are higher than those in normal rats. Our result confirms previous observations reported by others.24-26 It is well known that pancreatic CЕase is essential for the absorption of dietary cholesterol esters, since it catalyzes the hydrolysis of these esters to free cholesterol. The activity of the enzyme is also associated with the rapid transport of cholesterol across intestinal mucosal membranes and the increase in the biosynthesis of cholesterol esters in the small intestine of the diabetic rat.16,17 The specific activity of pancreatic CЕase in terms of a protein was increased in rats with STZ-induced diabetes. Thus, high CЕase activity may contribute to the development of hypercholesterolemia via the increased absorption of dietary cholesterol in diabetic rats.

CЕase present in intestinal absorptive cells may be important for the esterification of free cholesterol.16,17 The intestinal CЕase is immunologically identical to that secreted from the pancreas.27,28 Pancreatic CЕase activity increases in rats with STZ-induced diabetes, as demonstrated in the present work. In contrast, a previous study has demonstrated that CЕase activity in intestinal cells was slightly elevated in diabetic rats, but the increase was not statistically significant.24 Three possibilities can be considered: (1) there is no increased secretion of CЕase into pancreatic juices from zymogen granules of the diabetic rat pancreas under physiological conditions; (2) little or no absorption of the enzyme by intestinal cells from the pancreatic juices occurs in diabetic rats; and (3) rapid proteolytic degradation destroys the absorbed enzyme. Further detailed analyses of CЕase in pancreatic juices are required to examine these possibilities.

It is well known that digestive enzymes are localized in the zymogen granules of the exocrine pancreas.29-32 Various mechanical treatments cause severe disruption of zymogen granules.33,34 In the present study, pancreas was homogenized at pH 7.4 in 0.35M sucrose. Although the intact zymogen granules in cell homogenate are precipitable by centrifugation at 2000 × g,33,34 most of the activity was recovered in the supernatant by centrifugation at 8000 × g after different centrifugation (Table III
and Fig. 2). This is an indication of the almost complete destruction ofzymogen granules. A difference in the distribution of CEnase activity in the cytosol fraction from the normal pancreas was observed when two different assay methods were used. Recovery of CEnase activity was low in the cytosolic fraction of the normal pancreas when the fluorometric assay was used, compared with the results when the isotopic assay was used. The reason for the difference in the two methods needs to be explained.

Pancreatic neutral CEnase hydrolyzes p-nitrophenyl esters, typical substrates for non-specific esterases. 21, 23 Although acidic lysosomal CEnase hydrolyzes umbelliferyl palmitate preferentially, 36, 37 there is no direct evidence for the hydrolysis of this compound by pancreatic neutral CEnase. Since pancreatic neutral CEnase is selectively activated by bile acids, 20 - 23 stimulation by bile acid is one of the criteria for the identification of neutral CEnase. To ensure the specific measurement of the activity of pancreatic neutral CEnase when umbelliferol palmitate was used as a substrate, the difference between activities measured with and without sodium taurocholate was used to quantify the enzymatic activity to exclude any possible contribution by non-specific esterases. The results obtained in this study under these conditions strongly suggest that the activity of pancreatic neutral CEnase was measurable even in the presence of lipase and non-specific esterases. We tried to measure neutral or alkaline CEnase activities in extracts from several tissues by the sensitive fluorometric method in the presence of cholate. Although non-specific esterase activities were detected in all of the extracts tested (liver, kidney, adrenal, testis and plasma) in the absence of cholate, no measurable CEnase activity was detected in extracts of those tissues from both normal and diabetic rats. Our observations confirm the previous report that pancreas alone of several tissues contains CEnase and lipase with an affinity for long-chain fatty acids in substrate molecules. 38

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