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Decreased Citrate Synthesis by Lymphocytes from Alloxan-Diabetic Rat (39948)

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Introduction. The granulocytes and lymphocytes obtained from the peripheral blood of diabetic patients have been found to exhibit an altered glucose metabolism. Thus, glucose uptake, lactic acid production, and glycogen synthesis are lower in leukocytes from diabetic patients than in those obtained from normal individuals (1, 2).

It is claimed that lymphocytes from diabetic subjects exhibit a decreased binding with insulin (3). The circulating T lymphocytes are known to utilize glucose (4). Furthermore, in the presence of insulin, rat thymic lymphocytes exhibit an enhanced glucose metabolism (5). These observations suggest that lymphocytes from thymus may be added to the long list of cells known to be sensitive to insulin.

During the metabolism and oxidation of glucose one of the important steps is the synthesis of citrate. It is known that the citrate condensing enzyme is actively present in the lymphocytes of patients with chronic lymphocytic leukemia (CLL) (6). The decreased glucose uptake and oxidation by lymphocytes from diabetic patients may perhaps result in their decreased citrate content and citrate synthetic activity. We therefore felt that it would be of interest to determine whether the citrate synthesizing activity of lymphocytes from alloxan-diabetic rats is affected, as was found in other diabetic animal tissues (7, 8). In this communication we report that splenic lymphocytes from diabetic rats exhibited significantly decreased ability to synthesize citrate, and that this decreased enzyme activity was corrected following the administration of insulin.

Materials and methods. Young male rats of the Holtzman strain were administered alloxan (40 mg/kg body wt); the controls were injected with saline. The diabetic rats were divided into two groups: One group was injected for 10 days with a daily sc dose of 4 units of insulin (a 50:50 mixture of protamine zinc and lente insulin); the untreated group was injected with saline.

In vivo synthesis of labeled citrate from [1-^{14}C]acetate by rat spleen. The rats were weighed and injected iv with monofluoroacetic acid (MFA) (10 mg/kg body wt). Fifteen minutes later [1-^{14}C]sodium acetate (specific activity, 2.2 Ci/mole; purchased from New England Nuclear, Boston, Mass.) was injected iv as a tracer dose (5 μCi/100 g body wt). Thirty minutes later the animals were sacrificed by decapitation. Blood was collected in a heparinized beaker. One hundred-microliter aliquots were pipetted in triplicate samples in vials, and 1.0 ml of 9 *N* sulfuric acid containing 1 mg of citric acid as carrier was added. The labeled citrate in the blood was oxidized and determined as described earlier (9). Spleen was quickly excised, weighed, and transferred to a test tube containing 10 ml of 9 *N* sulfuric acid containing carrier citrate. The tissue was homogenized using Polytron, a high-frequency homogenizer (Brinkmann, Westbury, N.Y.). One milliliter of the splenic homogenate (in duplicate samples) was transferred to incubation vials for the determination of *de novo* synthesized labeled citrate. The principle of the method and the procedure for the quantitative determination of labeled citrate synthesized by rat tissues from the *in vivo* injected labeled acetate has been described in earlier publications (9, 10). The quantitative recovery of labeled citrate in the presence of labeled acetate was over 95%.

In vitro synthesis of citrate from [1-^{14}C]acetate by rat spleen. The spleens from diabetic, insulin-treated, and control rats were excised, weighed, placed in chilled

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beakers, and minced with scissors. Duplicate samples of 50 mg of minced splenic tissue from each rat were placed in incubation vials to which 0.25 μCi of [1-^{14}C]acetate and 2.0 ml of phosphate-buffered medium (pH 7.4), modified from that described by Dixon and Perkins (11) for the determination of "citrogenase" activity, were added. The final concentrations of the various constituents in the medium were 0.01 *M* sodium fluoroacetate, 0.01 *M* oxaloacetic acid, 0.1 *M* potassium chloride, 0.003 *M* magnesium sulfate, 0.01 *M* cysteine hydrochloride, 0.002 *M* adenosine triphosphate, and 0.01 *M* phosphate buffer (pH 7.4).

The vials with the minced spleen tissue were incubated at 27° for 30 min, and the amount of labeled citrate synthesized from labeled acetate which was accumulated in the tissue was determined. Because of the presence of fluoroacetate in the medium, oxidation and utilization of the labeled citrate formed during the incubation was inhibited, resulting in its accumulation within the tissue as described earlier (9).

In vitro synthesis of citrate formed by condensation of [1-^{14}C]acetyl CoA and oxaloacetate by suspensions of lysed rat splenic lymphocytes. Spleens were excised from rats of the three different groups. For the isolation of lymphocytes the tissue was minced in chilled beakers and about 0.5 g of the tissue was incubated for 20 min at 37° in 25 ml of Hank's medium (pH 7.4) gassed with 95% O_2 + 5% CO_2 and containing 2750 units of collagenase (CLS Type I; Worthington Biochemical Corp., Freehold, N.J.). Following the incubation of the mixture, it was strained through a cheesecloth and centrifuged at 300*g* for 10 min. The supernatant was discarded and 10 ml of the Hank's medium was added to the pellets and mixed gently. The lymphocytes were separated using a Ficoll-Hypaque density gradient medium as described by Boyum (12). The density gradient method has been used successfully in these laboratories for the isolation of lymphocytes from the peripheral blood of patients with chronic lymphocytic leukemia (CLL) (6).

The isolated lymphocytes were washed (by centrifugation at 300*g* for 15 min), first with saline (containing 0.0001 *M* EDTA) and then with previously gassed Hank's medium (pH 7.4). The number of lymphocytes per unit volume was counted in a hemocytometer and diluted with 0.2 *M* phosphate buffer so as to contain 10^8 cells/ml of medium. A fifty-microliter aliquot containing 50×10^6 lymphocytes was diluted to 5 ml with phosphate buffer and the cells were lysed by repeated freezing and thawing in an acetone-dry ice mixture (13). Complete disruption of the cells was confirmed by staining a few drops of the medium with Wright stain and examining it under a light microscope. Aliquots from the lysed lymphocytes were pipetted into incubation vials containing 0.20 μCi of [1-^{14}C]acetyl CoA (sp act, 55 Ci/mole; New England Nuclear, Boston, Mass.), 0.53 μmoles of unlabeled acetyl CoA (purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio), 10 μmoles of oxaloacetate, 30 μmoles of phosphate buffer, and water to make a final volume of 1 ml (pH 7.4). The vials were incubated for 15 min at 27° as described earlier (6, 10). The reaction was stopped with 1 ml of 9 *N* sulfuric acid containing 1 mg/ml of carrier citric acid. Labeled citrate formed *de novo* by condensation of oxaloacetate with acetyl CoA by the enzymes released from the disrupted lymphocytes was determined quantitatively. This method of determining citrate synthetase has been found to correspond closely with a well-known colorimetric method (14), as reported earlier (10). The amount of citrate synthesized was calculated per milliliter volume of lymphocytes. Because the mean diameter of both the normal and diabetic rat lymphocytes was found to be 10 μm, it was estimated that 2×10^9 lymphocytes constituted a 1-ml volume.

Results. In vivo synthesis of labeled citrate from [1-^{14}C]acetate by rat spleen. Figure 1 depicts significant differences between the amount of citrate synthesized and accumulated by spleen obtained from untreated diabetic rats as compared to that from either insulin-treated or control rats. In contrast to the spleen, the amount of labeled citrate found in the blood of the diabetic rats was not significantly different from that in the normal or insulin-treated diabetic rats (data not shown).

In vitro synthesis of labeled citrate from

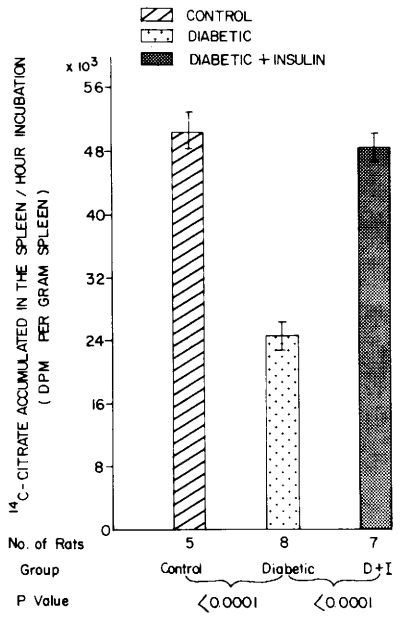

FIG. 1. Labeled citrate synthesized and accumulated *in vivo* by spleens of control rats, and untreated and insulin-treated diabetic rats. The vertical line on the top of each bar denotes the standard error of the mean (SEM).

[1-^{14}C]acetate by splenic tissue from diabetic and normal rats. The amount of labeled citrate synthesized by the rat splenic tissue was found again to depend upon the condition of the animal. Thus, spleen from untreated diabetic animals synthesized significantly less citrate than corresponding tissue obtained from normal ($P < 0.001$) or insulin-treated diabetic rats ($P < 0.0001$) (Fig. 2).

In vitro synthesis of labeled citrate from [1-^{14}C]acetyl CoA by isolated rat splenic lymphocytes. Figure 3 compares the citrate synthetase activity of lymphocytes obtained from the insulin-treated and untreated diabetic rats. The enzymes contained in the lymphocytes from untreated diabetic rats synthesized considerably less citrate per hour as compared to the splenic cells from the normal rat. Following 10 days of insulin treatment to the diabetic rats, the ability of splenic lymphocytes to synthesize citrate was significantly enhanced ($P < 0.0001$).

Discussion. It is well recognized that monofluoroacetate (MFA) injected into an animal blocks the oxidation of citrate. It is believed that MFA is incorporated to form monofluorocitrate which then competitively inhibits the enzyme aconitase, thus resulting in the accumulation of citrate in the tissues (15). From the *in vivo* studies it is evident that when the untreated diabetic animals were injected with MFA and labeled acetate, within half an hour the spleens accumulated considerably less labeled citrate than the corresponding tissues of insulin-treated and normal rats (Fig. 1). The accumulation of labeled citrate in the blood of the diabetic rats within half an hour was not appreciably different from that of normal or insulin-treated rats.

From Figure 2 it is also evident that splenic tissue from diabetic rats exhibited a decreased ability to synthesize citrate from acetate *in vitro*, as compared to the tissue of normal or insulin-treated diabetic rats. When isolated diabetic rat lymphocytes were lysed and then incubated with acetyl CoA and oxaloacetate, the amount of citrate synthesized was considerably lower than that synthesized by lymphocytes isolated from normal or insulin-treated diabetic rats (Fig. 3). In these experiments

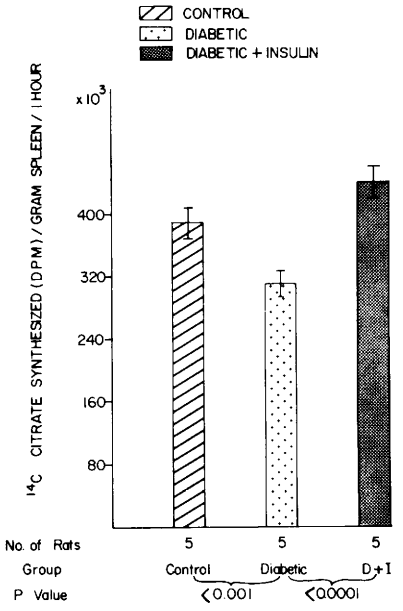

FIG. 2. *In vitro* synthesis of labeled citrate from [1-^{14}C]acetate by the fresh weight samples of splenic tissue of normal rats, and untreated and insulin-treated diabetic rats. The vertical line on the top of each bar denotes the standard error of the mean (1000 dpm = 57.5 nmoles of acetate utilized for citrate synthesis).

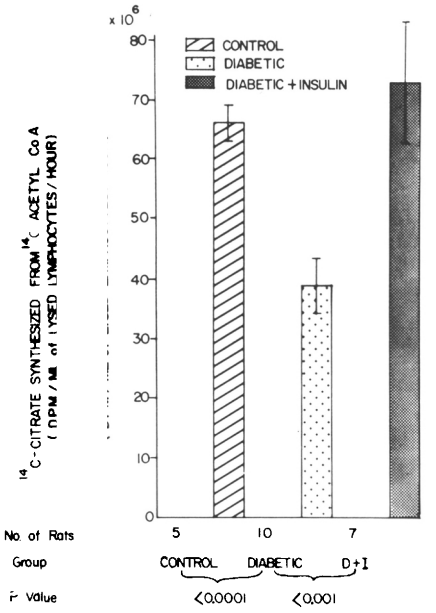

FIG. 3. *In vitro* synthesis of labeled citrate by citrate condensing enzyme from lysed splenic lymphocytes obtained from normal rats and untreated and insulin-treated diabetic rats on incubation with oxaloacetate and [1-^{14}C]acetyl CoA. The vertical line on the top of each bar denotes the standard error of the mean (1000 dpm = 1.75 nmoles of acetyl CoA utilized for citrate synthesis).

using acetyl CoA as the substrate, no MFA was added to preclude the further oxidation of the citrate that was synthesized *de novo* because pilot studies showed that the addition of MFA did not enhance the recovery of citrate that was synthesized.

It has been reported that there was a decreased blastogenesis of lymphocytes obtained from diabetic rat lymph nodes in response to mitogens (16). It is difficult to state whether this depressed cellular immune response of lymphocytes in the diabetic state could be associated with a decrease in citrate synthesis.

Hypercitremia has been reported in alloxan-diabetic rats by DeVilliers and coworkers (17). However, liver (6) and muscle tissue (18) of diabetic animals exhibited decreased citrate synthesis as compared to the corresponding normal controls. From the evidence presented in this communication, it would seem that the lymphocytes of splenic tissue from diabetic animals also exhibit impaired citrate synthesis. To our knowledge, this is the first report implicating the impairment of an important enzyme of the Krebs cycle in the lymphocytes of diabetic animals, and its subsequent correction by insulin treatment. Because the citrate condensing enzyme (EC 4.1.3.7) is located primarily in the mitochondria (19), the impaired enzyme activity in diabetes could be attributed to (a) a decrease in the number of mitochondria in the lymphocytes of diabetic animals, (b) decreased enzyme content in the mitochondria, (c) decreased affinity of the enzyme for the substrates, or (d) an impairment in the penetration of substrates into the intact mitochondria of the lymphocytes of diabetic animals, as was reported in the liver and muscle mitochondria of diabetic rats (18). Further work is necessary to determine whether similar differences could be shown in the circulating lymphocytes of diabetic animals, and whether this method could be of diagnostic use in the assessment of prediabetes.

Summary. The effect of alloxan-diabetes on the ability of rat splenic lymphocytes to synthesize citrate has been studied. Untreated and insulin-treated diabetic as well as normal rats were used for these studies. For the *in vivo* studies, [1-^{14}C]labeled acetate was injected into rats intravenously, 15 min after an intravenous injection of monofluoroacetate. The animals were sacrificed 30 min later and the amount of labeled citrate in the blood and spleen of the rats was determined. The splenic tissue of untreated diabetic rats contained significantly decreased amounts of labeled citrate as compared to insulin-treated and control rat spleens. No such differences were observed in the bloods of the three groups.

In another study, the splenic tissue of diabetic rats was incubated *in vitro* with tracer quantities of labeled acetate and substrates. The diabetic rat spleen was found to produce significantly decreased amounts of citrate in contrast to the spleen of normal or insulin-treated rats.

A method to isolate splenic lymphocytes has been described. The isolated splenic lymphocytes from the various groups of rats were lysed and the cell suspensions were incubated with labeled and unlabeled acetyl CoA and oxaloacetate. The labeled citrate synthesized by the diabetic rat lymphocyte

suspensions was found to be significantly lower than that synthesized by suspensions from normal or insulin-treated rats. These studies indicate that splenic lymphocytes from untreated diabetic rats exhibit decreased ability to synthesize citrate.

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