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STREPTOZOTOCIN-INDUCED DIABETES DECREASES THE CYCLIC AMP BINDING ACTIVITY OF THE REGULATORY SUBUNIT OF TYPE I cAMP-DEPENDENT PROTEIN KINASE FROM RAT LIVER[1]

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SUMMARY: Liver post-mitochondrial supernatant from diabetic rats showed a decrease in the [^{3}H] cAMP binding activity which was associated with a decrease in the number of cAMP binding sites. On the other hand, the cAMP binding activity of nuclear fractions from diabetic rat liver was not significantly different than that of control. The cAMP binding activity of post-mitochondrial supernatant was further analyzed by using 8-azido-[^{32}P] cAMP, a photoaffinity probe for cAMP binding sites. The diabetic supernatants showed a selective reduction in the photolabeling of a protein band representing the regulatory subunit of type I cAMP-dependent protein kinase without any appreciable change in the photolabeling of regulatory subunit of type II cAMP-dependent protein kinase.

cAMP has been shown to be a key regulator for many metabolic processes including glycogenolysis (1) and gluconeogenesis (2). Most, if not all, of the actions of cAMP are carried out by the activation of cAMP-dependent protein kinases (3, 4). At least

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Abbreviations used are: R, regulatory subunit of cAMP-dependent protein kinase; RI and RII denote the regulatory subunits of types I and II cAMP-dependent protein kinase respectively; C, catalytic subunit of cAMP-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis (α-aminoethylether)-N, N, N', N'-tetraacetic acid; SDS, sodium dodecyl sulfate.

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two soluble cAMP-dependent protein kinases have been identified in mammalian tissues and are called type I and II based on their order of elution from DEAE-cellulose by a salt gradient (5, 6). The cAMP dependent protein kinases exist as tetrameric holoenzymes in the non-activated state (6). In the presence of cAMP, the holoenzyme (R_2C_2) dissociates into the regulatory subunit dimer R_2 containing 4 mol of bound cAMP (7, 8) and two cAMP-independent, fully active catalytic (C) subunits. Type I and II protein kinases have similar C subunits (9) but their R subunits are different with respect to various physico-chemical (5, 6, 10) and immunological properties (11, 12) and in their ability to be phosphorylated by C (in case of RII) (13) or by cGMP-dependent protein kinase (in case of RI) (14). The respective physiological function for type I and II protein kinases has yet to be established. However, alterations in the proportions of two types of kinases have been observed during development (15, 16), different stages of cell cycle (17), in a variety of mutant cell lines (18-25), in solid tumors (26, 27) and in rat mammary carcinoma (28). Recently it has been shown that there is a decrease in the amount of RI in 8-bromo-cAMP resistant adrenal cell line (29) and in differentiating Friend erythroleukemia cells (30). These and other observations indicating that diabetic animals have increased hepatic cAMP levels (31), glycogenolysis (32, 33), gluconeogenesis (31, 34), and altered protein kinase activities (32, 33) prompted us to undertake the present studies to examine the effect of streptozotocin-induced diabetes on cAMP binding activities of rat liver.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (180-200g, body weight) were obtained from Charles-River, Canada. [2-8-^{3}H] cAMP (36Ci/mmol) was purchased from Amersham, Ontario, Canada and

8-azido-[^{32}P] cAMP (40Ci/mmol) was obtained from ICN, Irvine, California, USA. Streptozotocin, benzamidine-HCl, pepstatin A, 2-mercaptoethanol and histone IIA were purchased from Sigma Chemical Co. St-Louis, MO, USA. Leupeptin was obtained from Protein Research Foundation, Osaka, Japan. Electrophoresis Chemicals were obtained from Bio-Rad Laboratories, Missisauga, Ontario, Canada. Cronex lighting plus intensifying screens were from Dupont, Wilmington, DE, USA. All other chemicals were purchased from commercial sources.

Treatment of animals. Diabetes was induced in rats by intraperitoneal injection of streptozotocin (70mg/kg body weight) in 0.15M NaCl following an overnight fast. Control rats received only 0.15M NaCl. The rats were fed ad libitum on purina lab chow. Blood glucose was monitored from the tail vein by using dextrostix and a dextrometer (Ames). Streptozotocin injected rats with blood glucose concentrations in excess of 350mg/dl were considered diabetic and used 5 days after the injection. Rats were sacrificed by decapitation and livers were quickly excised and frozen using Woolenberger clamps cooled in liquid nitrogen. Frozen livers were subsequently pulverized to a fine powder with a percussion mortar cooled in liquid nitrogen and stored at -70^{o}C until assayed.

Preparation of extracts. Approximately 200mg of frozen liver powder were suspended at 4^{o}C in 4 times their weight of a medium containing 10mM potassium phosphate (pH 6.8), 250 mM sucrose, 10% (v/v) glycerol, 5mM EDTA, 1mM EGTA, 10mM benzamidine-HCl, 2µM pepstatin A and 2µM leupeptin. Homogenization was done at medium speed for 60 seconds with a motor driven teflon pestle in a glass tube homogenizer. The homogenate was centrifuged in a Beckman J-21 centrifuge at 29,000 X g for 15 minutes at 4^{o}. The supernatant was clarified of floating lipid material by filtering through glass wool.

Preparation of nuclear fraction. The nuclear fraction from the above homogenate was isolated, purified and extracted essentially as described by Cho-Chung et al (44).

cAMP binding assay. Before the binding assay the supernatant was preincubated with 0.5mM ATP and 5mM magnesium chloride at 25^{o}C for 10 minutes to promote the reassociation of R and C subunits. Supernatant was then diluted 10 fold in the homogenization buffer containing 1mg/ml BSA and used for the binding assay. The binding was determined by millipore filtration method (35) as described previously (36). The assay was performed in a total volume of 100µl which contained 50mM potassium phosphate buffer pH 6.8, 1mM IBMX, 0.5mg/ml histone type II A, 1mM EDTA, 400nM [^{3}H] cAMP and 50µl of suitably diluted supernatant. The incubation was performed at 4^{o}C for 60 minutes. At the end of incubation the entire mixture was filtered through a millipore filter (HA 0.45µM). The filter was rinsed twice with 10ml of 20mM potassium phosphate (pH 6.8) and dried in an oven at 150^{o}. The radioactivity retained by the filter was determined by counting in 10ml of Econofluor in a liquid scintillation counter.

Photoaffinity labeling. The covalent labeling of cAMP binding sites by photoaffinity probe 8-azido-[^{32}P] cAMP was performed as described previously (36). The conditions employed were similar to those used for cAMP binding except that 8-azido-[^{32}P] cAMP was substituted for [^{3}H] cAMP. The reaction

mixture was incubated on ice in a cold room in the dark for 60 minutes and then irradiated with a mineralite short wavelength (254nm) hand lamp at a distance of 8cm for 10 minutes. After irradiation, the reaction was stopped by transferring 50µl of reaction mixture to 20µl of 7% SDS and 33% 2-mercaptoethanol, followed by heating in a boiling water bath for 5 minutes.

SDS-polyacrylamide gel electrophoresis and auto radiography. SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (37) with a 7.5% separating gel and 4% stacking gel. After electrophoresis, gels were fixed, stained and destained according to Segrest and Jackson (38). Autoradiography of dried gels was done by exposing to Kodak XAR-5 film in the presence of an intensifying screen (39).

Protein was determined according to Bradford (40) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

cAMP binding activities in control and diabetic rat livers. Several studies (32, 41) have indicated decreased cAMP-independent or dependent histone kinase activities in the livers of streptozotocin-diabetic rats. To examine whether the decreased activities of cAMP-dependent kinases observed in those studies were associated with decreased abilities to bind cAMP, the cAMP binding activities in the post-mitochondrial supernatant from control and streptozotocin-diabetic rat livers were determined. Total cAMP binding activity will be underestimated in standard cAMP-binding assays if significant amounts of dissociated R-cAMP complexes are present in the supernatants, therefore, prior to cAMP-binding assays the supernatants were preincubated with Mg-ATP to facilitate the reassociation of R and C subunits and dissociation of bound cAMP.

The data presented in Table 1 indicate that in diabetic rat livers the total cAMP binding activity of post-mitochondrial supernatant expressed either as per mg protein or on a tissue weight basis was decreased by about 40-50% as compared to control rats. Analysis of cAMP binding activity of the post-mitochondrial supernatants by Scatchard plots (36) (Figure 1) generated linear curves for both control and diabetics. The

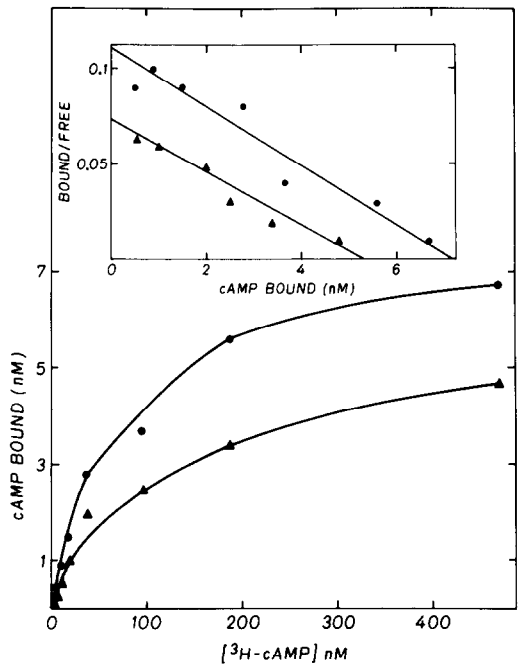

Figure 1: Saturation binding curve of [^{3}H-cAMP binding to post-mitochondrial supernatants from control (●) and diabetic (▲) rat livers. The amount of [^{3}H]-cAMP bound was measured at [^{3}H]-cAMP concentrations ranging from 5 to 500nM as described in the "Materials and Methods". The inset shows the Scatchard analysis of the binding data. Values are the means of triplicate determinations from one of three experiments.

apparent dissociation constant of diabetic liver (74 nM) was not significantly different from that of control (65 nM), whereas, about 30% decrease in the number of cAMP binding sites was observed in diabetics. Furthermore, the decreased cAMP binding activity in diabetic rats did not appear to be due to the translocation of type I cAMP-dependent protein kinase from cytosol to nucleus, because the cAMP binding activity of the nuclear fraction from diabetic liver was not significantly different than that of control as shown in Table I.

Photoaffinity labeling of post-mitochondrial supernatant from control and diabetic rat livers. Since rat liver has been shown to contain both type I and II

TABLE I
cAMP BINDING ACTIVITY IN POST-MITOCHONDRIAL SUPERNATANTS AND NUCLEAR FRACTIONS FROM CONTROL AND STREPTOZOTOCIN-DIABETIC RAT LIVER

	cAMP binding activity			
	pmole cAMP bound/g liver		pmole cAMP bound/mg protein	
	A	B	A	B
Control	392 ± 17	10.7 ± 1.2	4.1 ± 0.3	6.7 ± 0.9
Diabetic	227 ± 21 *	10.3 ± 1.0 †	2.1 ± 0.2*	6.3 ± 0.8 †

cAMP binding activity in post-mitochondrial supernatants (A) and nuclear fractions (B) from control and streptozotocin-diabetic rat liver was determined as described in "Materials and Methods". Values are the means ± S.E.M. of 8 animals (A) and 4 animals (B) in each group. p values relative to the control values were obtained by student's "t" test.

* $p < 0.0005$

† not significantly different from control

cAMP-dependent protein kinases (36) the changes in cAMP binding activity observed above could be due to alterations in the binding to one or both type of protein kinase R (RI, RII) subunits. The photoaffinity probe 8-azido-[^{32}P] cAMP which has been shown to label covalently and specifically the regulatory subunits of cAMP-dependent protein kinases (36) was used to differentiate the cAMP binding to RI and RII subunits.

The data in Figure 2 show that in both, control (lanes 1-3) and diabetic (lanes 7-9) rat livers, 8-azido-[^{32}P] cAMP labeled one major band with an apparent molecular weight of 48,000. Electrophoretic mobility of this band was identical to the highly purified rabbit skeletal muscle RI (lane 6) (7) and hence was identified as RI. One minor doublet with apparent molecular weights of 56,000 and 54,000 and an electrophoretic mobility similar to beef heart RII (lane 4) was also labeled; these bands represent the phospho- and dephospho-forms of RII (42). However, the supernatants from diabetic rat liver extracts (lanes 7-9)

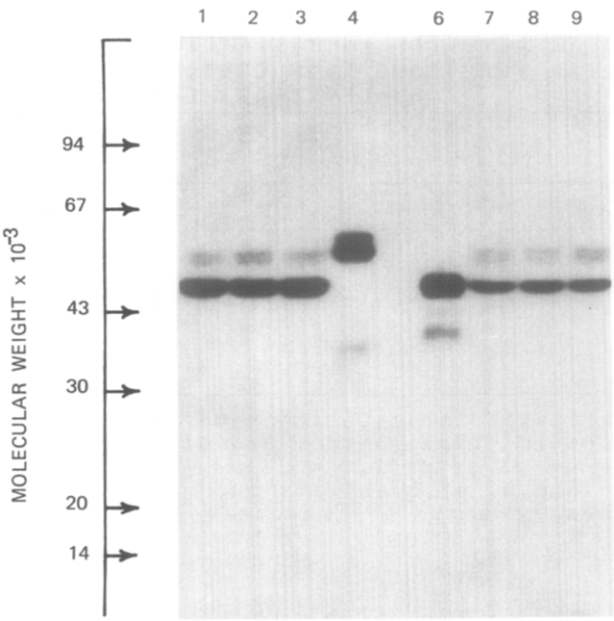

Figure 2: Autoradiograph showing the photoaffinity labeling of post-mitochondrial supernatants from control and diabetic rat livers. Supernatants were labeled with 400nM 8-azido-[^{32}P] cAMP and subjected to SDS-polycryamide gel electrophoresis followed by autoradiography as described in "materials and methods". Gels were calibrated using molecular weight standards (Pharmacia): Phosphorylase b (Mr=94,000), Albumin (Mr=67,000), Ovalbumin (Mr=43,000), Carbonic anhydrase (Mr=30,000), Trypsin inhibitor (Mr=20,100), β- lactalbumin (Mr=14,400). Highly purified beef heart and rabbit skeletal muscle regulatory subunits prepared as described (7) were used as standards for RII (lane 4) and RI (lane 6) respectively. Lanes 1-3 represent the labeling of 3 different normal liver supernatants and lanes 7-9 represent the labeling of 3 different diabetic liver supernatants. The amount of supernatant protein applied in each lane was between 86-90μg.

when compared with the controls (lanes 1-3) showed a selective reduction in the amount of RI photolabeled with the photoaffinity probe without any appreciable change in the photolabeling of RII. An alteration in the amount RI subunits have also been reported in Friend erythroleukemic cells (29) and in mutant cell lines of adrenocortical tumors (30).

The decreased cAMP binding to RI may have serious physiological consequences because it may lead to an alteration in the reassociation dissociation properties of R and C subunits and thereby defective substrate protein phosphorylation.

However, the mechanism(s) by which this decrease occurs in diabetes remains to be elucidated. At present, we can only speculate on some possible mechanism(s) to explain this phenomenon: (a) since the *in vitro* phosphorylation of RI by cGMP-dependent protein kinase has been shown to result in loss of one of its cAMP binding sites (43), it may be possible that in diabetic liver the RI is highly phosphorylated and shows a decreased cAMP binding activity; (b) a decreased *de novo* synthesis of type I cAMP-dependent protein kinase, which would explain not only the decreased RI labeling but also the decreased cAMP-independent and -dependent histone kinase activities observed in diabetics (32, 34).

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