Vol. 86, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1979 Pages 379-386

MEASUREMENT OF PROTEIN ACTIVATOR LEVELS IN EXPERIMENTAL DIABETIC RAT ADIPOSE TISSUE

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Received December 12, 1978

SUMMARY: Experimental diabetes induced by streptozotocin has been shown to decrease the level of cyclic AMP phosphodiesterase activity in rat adipose tissue. This reduced activity was restored with insulin. Protein activator, a small molecular weight substance, is essential for full activity of some component phosphodiesterases. Herein we demonstrate a significant decrease in protein activator level in the 13,000 X g boiled supernatant from streptozotocin-diabetic rat adipose tissue. However, although a decrease in protein activator level is consistent with diabetic inactivation of phosphodiesterase activity, additional studies presented here suggest that a defect in the diabetic phosphodiesterase enzyme itself also contributed to the decrease of total phosphodiesterase activity.

INTRODUCTION

Insulin was first shown to stimulate cyclic AMP phosphodiesterase (PDE, EC 3.1.4.c) activity in adipose tissue in 1970 (1). Subsequently, experimental streptozotocin-diabetes in rats has also been shown to be associated with decreased PDE activity (2). Cheung et al (3) and Kakiuchi et al (4) have demonstrated that a small molecular weight, calcium-dependent, heat stable protein activator is essential for full activity of the calcium-sensitive form of PDE. Furthermore, this material appears to play a more universal role in cellular metabolism (17, 18, 19). In light of this, we thought it important to measure and compare the levels of this protein activator in adipose tissue from streptozotocin-diabetic and normal rats.

MATERIAL AND METHODS

Holtzman rats were rendered diabetic by streptozotocin injections via the tail vein using standard doses of this drug (65mg/kg) (2). Control animals were treated similarly in a sham-operative procedure by injecting normal saline.

0006-291X/79/020379-08$01.00/0

Following injection the rats were maintained on standard rat chow, in metabolic cages, for three days during which time daily weights, urine volumes and the presence or absence of glucose and ketone bodies in the urine were recorded. At the time of sacrifice, plasma glucose and plasma immunoreactive insulin measurements were made (2,5).

The heat stable, calcium-sensitive protein activator was prepared from rat fat pads by homogenization, sonication, adjustment of pH to 5.9 and centrifugation at 13,000 x g. The resultant supernatant was boiled for 5 minutes, rapidly cooled and centrifuged as above. This procedure was repeated. The supernatant contained protein activator (60 μg protein/ml). Cyclic AMP phosphodiesterase was assayed by modification (6) of the method of Thompson and Appleman (7) as routinely done in our laboratories. The PDE activity in adipose tissue was measured in the 100,000 x g supernatant prepared from rat fat pads as described in detail elsewhere (2,6,7). PDE activity was measured at high and low cyclic AMP and cyclic GMP substrate concentrations (low, 5×10^{-7}M; high, 5×10^{-5}M). Protein activator was assayed by measuring the stimulation of protein activator-deficient PDE prepared by DEAE cellulose column chromatography of partially purified beef heart PDE (Sigma, St. Louis, MO, 3,9). Protein activator activity was assayed in the absence and presence of added calcium (1.0mM) with or without 0.1mM EGTA being present. Protein activator prepared from several tissues (rat brain, human RBC and rat and human fat) consistently produced a 4-5 fold tissue stimulation of activator-deficient beef heart PDE. Protein activator levels were measured at two concentrations (1:1, 60 μg/ml and 1:5, 12 μg/ml) to determine the optimal level of specific stimulation. The amount of protein activator was determined by its ability to activate this activator-deficient beef heart PDE (Table 2) and was expressed as relative activator activity/mg protein, diabetic compared to control. The total amount of protein activator present in the adipose tissue was also determined per wet weight of tissue.

RESULTS

Table 1 presents the clinical data pertaining to normal and diabetic animals. These data allow us to assess the severity of experimental diabetes induced in these streptozotocin-treated animals. By the third day (72 hours), the diabetic animals had each lost 12 grams, whereas untreated rats had each gained 19 grams in body weight. In addition, the diabetic animals had increased their urinary output and were clinically diabetic with positive urinary glucose and acetone tests. Elevated plasma glucose and decreased immunoreactive insulin values ($p < .05$) in the diabetic rats further confirmed the differences between streptozotocin-treated and control rats.

Table 2 illustrates our standard protein activator assay. Beef heart PDE, rendered protein activator-deficient by passage through a DEAE cellulose column,

TABLE 1: Clinical Data Pertaining to Normal and Diabetic Animals

	N	Control Weight g	Weight on day #3 g	Δ Weight g	24-Hour urine volume on day #3 (ml)	Urine glucose on day #3	Urine ketones on day #3	Plasma glucose on day #3 (mg/100ml)	Immuno-reactive insulin (μU/ml**)
Control Rats	17	219	238	+19	4	0	0	106	20
Diabetic Rats	19	215	203	-12	56	2+	1+	517	8

All data shown in this table is expressed as the average value for the control or experimental animals.

* Streptozotocin-induced diabetes

** As μU/ml human insulin

TABLE 2
ASSAY OF PROTEIN ACTIVATOR IN RAT ADIPOSE TISSUE

Source of Adipose Tissue Activator		*Concentration of exogenous protein activator as µg protein/0.1 ml	CPM ^{3}H-Adenosine Release† PDE Alone	+Activator	Stimulation
A.	Rat	(Control) 0	312	312	1.0
		6	312	438	1.4
		18	312	735	2.4
		36	312	910	2.9
		72	312	1390	4.5

* 0.1 ml of boiled rat fat protein activator preparation contains 6 µg protein.

† These experiments were run in the presence of excess Ca^{2+} (1.0mM) in the assay of negate the effects of low endogenous Ca^{2+} or EGTA. The assay of cAMP PDE was conducted using 5 x 10^{-7}M cAMP. All values shown are the average of triplicate reaction mixtures with substraction of background counts. See Methods for further description of assay conditions using protein activator-deficient beef heart PDE (8 µg/0.1 ml).

was stimulated almost 5 fold by optimal concentrations of protein activator (72 µg/0.1 ml). Effects of Ca^{2+} and EGTA on these PDE and protein activator preparations are identical to other reported work and this data is not presented here again (3,4,9).

Figure 1 shows the level of protein activator obtained from the 13,000 x g boiled supernatant of both control and diabetic rat adipose tissue on the third day. This figure shows that protein activator activity in adipose tissue from normal and diabetic rats is markedly different, 60% lower in the diabetic ($p < .02$) when expressed per mg of adipose tissue protein. These conclusions were identical when the protein activator level was expressed either as "total activator activity in the boiled supernatant" or as protein activator activity per wet weight of tissue. All of these experiments comparing normal and diabetic tissues were statistically significant at $p < 0.05$ for the two concentrations (1:1 or 1:5 dilution) of the protein activator shown. Data shown in Table 3 illustrates the effect of exogenous rat fat protein activator on control or experimental streptozotocin-diabetic adipose tissue PDE for "low" and "high" Km cyclic AMP PDE activity. This table shows that in experimental

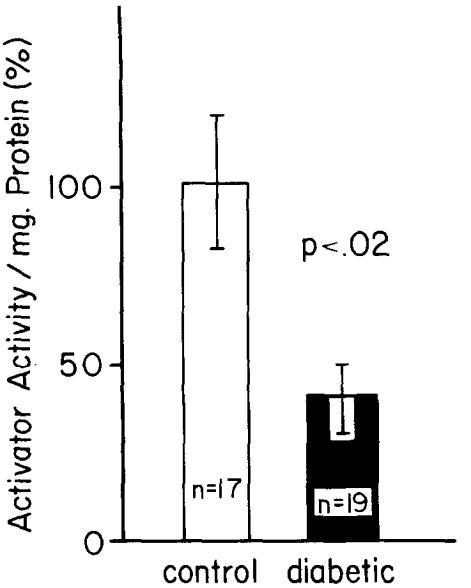

Figure 1 Measurement of protein activator (PA) /mg protein in adipose tissue from epididymal fat pads taken from normal and streptozotocin-diabetic animals. PA was measured in the 13,000 x g supernatant and was assayed by its ability to activate PDE at a c-AMP concentration of 5 X 10^{-7}M. Additional details of preparation and assay are reported under Methods. Control animal values were set at 100% ± S.E.M. Statistical comparisons are shown, with PA from diabetic lower than control, P < .02.

TABLE 3: Activity of cyclic AMP phosphodiesterase (PDE) from normal and streptozotocin-diabetic rat adipose tissue in the presence or absence of exogenous protein activator (PA).

	PDE Activity†			
	"Low" Km	P	"High" Km	P
Control Alone	2.4*	NS	34.9	NS
+PA	2.5		36.7	
Diabetic Alone	1.7*	NS	30.8	NS
+PA	1.5		38.0	

N = 9 experimental or control values.

† = as picomoles cAMP hydrolyzed 10 minutes per incubation vial. The concentrations of cAMP are 5 x 10^{-7}M (low) and 5 x 10^{-5}M (high). The PDE utilized is 0.1 ml of the 100,000 x g supernatant and PA concentration is 15 µg/0.1 ml. in a final volume of 0.4 ml.

* = Diabetic different from control for "low" Km PDE (P <.02).

diabetes the diabetic rats had a slightly decreased level of "low" Km PDE activity in their adipose tissue ($p < .02$, for all experiments). Furthermore, the addition of exogenous protein activator did not restimulate these values to normal levels of PDE activity (p=ns). Similar data was obtained when this experiment was done using 5×10^{-7}M and 5×10^{-5}M cyclic GMP as the substrate for the activatable PDE (data not shown).

DISCUSSION

Senft et al (10) originally reported the effect of insulin and experimental diabetes on cyclic AMP PDE activity. A number of investigators including Hepp and co-workers (11,12) observed little or no effect of insulin on PDE activity in adipose tissue. However, in 1970 Loten and Sneyd reported the specific stimulatory effect of insulin on the "low" Km membrane-bound PDE (1). This work has been substantiated by the work of Thompson, Little & Williams (13), Manganiello and Vaughan (14), Zinman and Hollenberg (15) and others. In 1975, Solomon and co-workers demonstrated that in experimental diabetes there was a reduced level of PDE activity in adipose tissue (2). This effect of experimental diabetes was specific in that there was a decrease only of "low" Km cAMP PDE activity and the effect of the diabetes on this PDE activity was reversed by incubation with insulin. The present work continues and extends these investigations elucidating the probable mechanisms involved in the insulin effect on PDE in diabetes. Herein we demonstrate a decrease in the levels of protein activator in adipose tissue from diabetic animals. This decrease was noted on the third day after the animals have been rendered diabetic through the use of streptozotocin. Expression of the data as activator activity per milligram of protein or as total activator activity assured us that this decrease was real and not related merely to a nonspecific decrease in the cellular

content of protein secondary to decreased protein synthesis that may occur in experimental diabetes (16). Whether or not these changes can be detected in naturally occurring animal models for diabetes or in humans with diabetes is presently under investigation.

Data shown in Table 3 indicated that the decrease in PDE activity detected in the 100,000 x g supernatant from diabetic rat adipose tissue was not obviously a direct result of decreased levels of endogenous protein activator. The additions of exogenous protein activator to the 100,000 x g supernatant of adipose tissue from diabetic rats did not restore the decreased PDE activity to near normal levels. Several explanations for this combination of decreased PDE activity and decreased protein activator levels in tissues from diabetic animals may be plausible. The protein activator may have become associated with another calcium-binding protein such as adenylate cyclase (17,18) or a calcium-magnesium dependent ATP-ase (19) or there was a decreased synthesis of protein activator. Alternatively, the protein activator may be avidly bound to a calcium-dependent PDE which itself has a decreased total level of activity. Protein activator may also be bound to specific subcellular components. Streptozotocin-induced diabetes may produce a shift of protein activator within the compartments of the cell, making it "less" available (20). Regardless of the mechanisms involved, one would have to postulate two defects, 1) a decrease in protein activator level and 2) a specific decrease in the activity of the PDE enzyme itself, to explain our data. A combination of both these factors might be essential for the decrease in PDE observed in experimental diabetes. Hence, despite the clear decrease in protein activator levels seen in diabetic rat adipose tissue, no totally unifying hypothesis can be drawn at this point. Additional experimental work will clearly have to be done to define the contribution of each component to the overall decrease in PDE activity.

ACKNOWLEDGEMENTS

The authors are indebted to Ms. J. McPherson, M.A. Bobal, and L. Holliday, for excellent technical assistance and to Ms. June Kimbrell for editoral and typing of the manuscript. The authors are especially indebted to Dr. L.E. King, Jr., for critical review of this manuscript prior to publication. This work supported in part by grants from the Medical Research Service of the Veterans Administration Hospital (077-30-9076) and the National Institutes of Health - AM18022.

REFERENCES

1. Loten, E.G., and Sneyd, J.G.T. (1970). Biochem. J. 120:187-193.
2. Solomon, S.S. (1975). Endocrinology 96:1366-1373.
3. Lin, Y.M., Liu, Y.P., and Cheung, W.Y. (1974) in Methods in Enzymology. Vol. 38 (C) pp. 262-273, Ed. by J.G. Hardman and B.W. O'Malley, Academic Press, NY.
4. Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K., and Miyamoto, E. (1975) in Advances in Cyclic Nucleotide Research (Vol 5) pp. 163-178, Ed. by G.I. Drummond, P. Greenwood, and G.A. Robison, Raven Press, NY.
5. Morgan, C.W., and Lazarow, A.M. (1963). Diabetes 12:115-126.
6. Solomon, S.S. (1972). J. Lab. Clin. Med. 79:598-610.
7. Thompson, W.J., and Appleman, M.M. (1971). Biochemistry 10:311-316.
8. Solomon, S.S., Palazzolo, M., and King, L.E., Jr. (1977). Diabetes 26:967-972
9. Cheung, W.Y. (1971). J. Biol. Chem. 246:2859-2869.
10. Senft, G., Schultz, G., Munske, K., and Hoffman, M. (1968). Diabetologia 4:322-329.
11. Menahan, L.A., Hepp, K.D., and Weiland, O. (1969). Eur. J. Biochem. 8:435-443.
12. Hepp, K.D., Menahan, L.A., and Wieland, O. (1969). Horm. Metab. Res. 1:93-94.
13. Thompson, W.J., Little, S.A., and Williams, R.H. (1973). Biochemistry 12:1889-1894.
14. Manganiello, V., and Vaughan, M. (1973). J. Biol. Chem. 248:7164-7170.
15. Zinman, B., and Hollenberg, C.N. (1974). J. Biol. Chem. 249:2182-2187.
16. Wool, I.G., and Cavicchi, P. (1967). Biochemistry 6:1231-1242.
17. Brostrom, C.O., Huang, Y.C., Brecken Ridge, B.McL., and Wolff, D.J. (1975). Proc. Nat. Acad. Sci. USA 72:64-68.
18. Lynch, T.J., Tallant, E.A., and Cheung, W.Y. (1977). Arch. Biochem. and Biophys. 182:124-133.
19. Luthra, M.G., Au, K.S., and Hanahan, D.J. (1977). Biochem. Biophys. Res. Commun. 77:678-687.
20. Donnelly, T.E., Jr. (1978). Biochem. Biophys. Res. Commun. 82:964-970.