EFFECT OF (-)EPICATECHIN IN MODULATING CALICUM-ATPASE ACTIVITY IN NORMAL AND DIABETIC HUMAN ERYTHROCYTES.

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ABSTRACT

Aqueous extract of the wood of *Pterocarpus marsupium*, commonly known as 'bijasar', is used in Indian Ayurveda system of medicine for treatment of diabetes. The active anti-diabetic principle in its aqueous extract has been found to be (-)epicatechin, a flavonoid. The present work was undertaken to study the effect of (-)epicatechin on erythrocyte membrane Ca^{++} -ATPase from type 2 diabetic patients. The activity of erythrocyte Ca^{++} -ATPase was significantly lower in type 2 diabetics. *In vitro* insulin treatment of erythrocyte ghosts, resulted in the increase of Ca^{++} -ATPase activity in diabetic patients. Treatment with (-)epicatechin (1mM) resulted in an increase in the activity of erythrocyte Ca^{++} -ATPase in both normal individuals and type 2 diabetic patients. The insulin like effect of (-)epicatechin on erythrocyte membrane Ca^{++} -ATPase in type 2 diabetics is an interesting finding. Further work is needed to elucidate the mechanism of action of (-)epicatechin on modulation of erythrocyte membrane bound enzymes.

KEY WORDS :

Erythrocyte, Ca^{++} -ATPase, diabetes mellitus, (-)epicatechin

INTRODUCTION

Erythrocytes earlier regarded as 'non target' cell for insulin, have been shown to possess specific receptors for insulin with characteristics similar to typical target cells (1). Several alterations in erythrocytes have been reported in type 1 and type 2 diabetes mellitus (2). An altered activity of erythrocyte membrane Ca^{++} -ATPase is seen in diabetic conditions (3, 4). Altered Ca^{++} regulation appears to play a major role in the development of cardiomyopathy as characterized by reduced contractibility, relaxation, cardiac work and diastolic complications (5) and may represent a common underlying abnormality linking the metabolic, cardiovascular, ocular, and neural manifestations of the diabetic disease process (6).

The bark of *Pterocarpus marsupium* is traditionally used in Indian Ayurveda system of medicine as an anti-diabetic drug. The compound responsible for

anti-diabetic effect has been identified to be (-)epicatechin (7), a member of a group of compounds collectively known as catechins belonging to the class of flavonoids. In earlier reports we have shown certain insulin like effects of (-)epicatechin on erythrocytes of type 2 diabetic patients (8-11), the present work has been undertaken to evaluate the effect of (-)epicatechin on erythrocyte membrane bound Ca^{++} -ATPase from normal and type 2 diabetic patients.

MATERIAL AND METHODS

Selection of subjects

The criterion for selection of type 2 diabetic patients was same as described (10, 11). Blood from 24 type 2 diabetic patients was taken for the study. None of the patients had hypertension or microalbuminuria. Hypertensive subjects were excluded to avoid any variation in results due to factors other than diabetes mellitus. The mean age of type 2 diabetic patients was 56 ± 8 years and their mean duration of diabetes was 10 ± 5 years. Fasting blood glucose values of these subjects was 206 ± 60 mg/100 ml. The control group consisted of 15 healthy volunteers, age group 22-50 years and

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fasting glucose level 78 ± 12 mg/100 ml. None of the controls were affected by hypertension.

Blood collection, isolation of erythrocytes and membrane preparation.

ATP (sodium salt), ouabain, and (-)-epicatechin were purchased from Sigma Chemical Company, St. Louis, USA. All other chemicals were of analytical grade and were purchased from HIMEDIA, LOBA or E. MERCK (India). Venous blood was collected from control and type 2 diabetic patients, after an overnight fast, with informed consent, using citric acid/sodium citrate/dextrose as anticoagulant. The blood sample was centrifuged at 4°C for 10 min. at 1000g to remove plasma and buffy coat, the isolated erythrocytes were washed 3 to 4 times with 0.154 M NaCl. Erythrocyte plasma membrane was prepared as described by Marchesi and Palade (12).

Determination of erythrocyte membrane Ca^{++} -ATPase activity.

Ca^{++} -ATPase activity was assayed as described by Zaidi and Saleemuddin (13). 2.25 ml of the assay mixture contained: 80 mM NaCl, 15 mM KCl, 3 mM MgCl_2 , 18 mM Tris- HCl (pH 7.4), 0.1 mM ouabain, 0.1 mM EGTA, 0.2 ml of the membrane solution containing 0.4 to 1.5 mg membrane protein per ml and ± 0.2 mM CaCl_2 . The reaction was initiated by addition of 0.1 ml of 30 mM ATP. The incubation of the assay mixture was carried out at 37°C for 30 minutes. The reaction was stopped by adding 3.5 ml of solution containing 0.5 M H_2SO_4 , 0.5% ammonium molybdate and 2% SDS. Liberated phosphate was estimated by a modified method of Fiske and Subbarow (14) and Ca^{++} -ATPase activity is expressed as micro mole Pi liberated per mg membrane protein/hour.

In vitro experiments were carried out by adding bovine insulin (10^{-9}M) and/or (-)-epicatechin (1mM) to the enzyme assay medium and incubating at 37°C for 30 min. prior to enzyme assay. Appropriate controls were set up for each experiment. Blood glucose levels were measured using Ames Glucometer GX (Miles, India). Protein was estimated following the established method of Lowry et al. (15). Statistical analysis was carried out by employing students 't' test, a probability of 0.001 being used as a level of significance.

RESULT AND DISCUSSION

The erythrocyte membrane Ca^{++} -ATPase activity of

normal and type 2 diabetic patients is shown in table 1. The activity of enzyme was significantly lower in type 2 diabetic patients as compared to normal controls. ($p < 0.001$). Decrease in the activity of erythrocyte Ca^{++} -ATPase has also been reported by other workers in type 2 diabetic patients (4, 16). Reduced membrane Ca^{++} -ATPase activity as seen in type 2 diabetics may be responsible for increase in intracellular calcium and consequently, for elevated vascular resistance which is frequently associated with hypertension (17).

It has been hypothesized that the abnormal dynamic properties of the erythrocyte membrane in diabetic state may play a major role in determining the alterations in enzymatic activities (18). The decrease in the Ca^{++} -ATPase activity in the diabetic state may be due to altered membrane properties including functional and compositional changes (19-20).

Glycosylation is reported to cause changes in physical and biochemical properties of proteins and the rate of glycosylation depends on the proportion of sugar present in carbonyl form (21). A relationship was found between the blood glucose levels and erythrocyte membrane ATPases activity (22). It is reported that glycosylation of erythrocyte membrane proteins significantly inhibits Ca^{++} -ATPase activity (23). The decreased Ca^{++} -ATPase activity in diabetes may also be due to increased glycosylation of erythrocyte membrane protein.

In vitro insulin treatment of isolated erythrocyte membrane from type 2 diabetic patients resulted in the increase of Ca^{++} -ATPase activity, however, no significant effect was observed on Ca^{++} -ATPase from normal erythrocytes. Figure 1 shows the dose response of insulin (10^{-7} - 10^{-11}M) on the activity of erythrocyte membrane Ca^{++} -ATPase of normal individuals and diabetic patients. In diabetics maximum increase in Ca^{++} -ATPase activity is observed at 10^{-9}M hormone concentration, a lower inhibitory effect of insulin on the enzyme activity is observed at higher and lower insulin concentration resulting in a typical dose responsive curve. In contrast to its effect on diabetic erythrocyte Ca^{++} -ATPase, no significant effect of insulin was observed on erythrocyte Ca^{++} -ATPase of normal individuals. This observation is intriguing since it is difficult to explain why insulin does not affect Ca^{++} -ATPase of normal erythrocytes. Studies on $\text{Na}^{+}/\text{K}^{+}$ -ATPase has shown that insulin has a similar effect on the

enzyme from both normal and diabetic erythrocytes (9). Treatment with (-)epicatechin (1mM) resulted in significant increase in the activity of erythrocyte Ca^{++} -ATPase in both normal and type 2 diabetic patients. A similar increase in Ca^{++} -ATPase activity was observed at higher (-)epicatechin concentrations, with lesser or no increase in activity at lower concentration (Table 2). Incubation of erythrocyte membrane with both insulin (10^{-9}M) and (-)epicatechin (1mM) did not show any additive effect.

The observation of an increase in Ca^{++} -ATPase activity in response to (-)epicatechin pretreatment is an interesting finding. Significantly insulin also

causes elevation of Ca^{++} -ATPase activity in diabetic erythrocytes. Although (-)epicatechin does seem to mimic insulin in its effect on diabetic erythrocyte Ca^{++} -ATPase, it is difficult to predict its mechanism of action. Further work is needed to explain the effect of epicatechin on erythrocyte Ca^{++} -ATPase and also on other membrane bound enzymes (9,10).

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Table 1.

Erythrocyte membrane Ca^{++} -ATPase activity in normal human and type 2 diabetic patients and after *in vitro* treatment with insulin and (-)epicatechin.

Ca^{++} -ATPase activity [†]					
Condition	n	Control	+Insulin (1nM)	+Epicatechin (1mM)	+ Insulin (1nM) +Epicatechin(1mM)
Normal human	15	0.316±0.018	0.288±0.014	0.366±0.014*	0.354±0.023*
Type 2 diabetics	23	0.192±0.043**	0.266±0.022*	0.293±0.032*	0.302±0.029*

[†]Enzyme activity is expressed in terms of micro mole of P_i released /hr/mg membrane protein at 37°C. Each value is the mean of at least six independent experiments ± SD.

* $p < 0.001$ as compared to their respective control. ** $p < 0.001$ as compared to normal control.

Table 2.

Dose response of *in vitro* (-)epicatechin treatment on erythrocyte membrane Ca^{++} -ATPase activity in normal human and type 2 diabetic patients.

Condition	n	Ca^{++} -ATPase activity [†]				
		Control	(-)Epicatechin			
			(1nM)	(0.5mM)	(1mM)	(10mM)
Normal	15	0.316±0.018	0.322±0.026	0.358±0.019	0.366±0.014	0.362±0.020
Type 2 diabetics	23	0.192±0.043	0.202±0.034	0.286±0.022	0.293±0.032	0.290±0.028

[†]Enzyme activity is expressed in terms of μ mole of Pi released /hr/mg membrane protein at 37°C. Each value is the mean of at least six independent experiments $\pm$ SD.

Figure 1

Effect of different concentration of insulin (10^{-11} - 10^{-7} M) on erythrocyte membrane Ca^{++} -ATPase activity (each point is the mean $\pm$ SD of 4 to 5 independent experiments).

[†] Ca^{++} -ATPase activity is expressed in terms of μ mole of Pi released /hr/mg membrane protein at 37°C. Each value is the mean of at least six independent experiments $\pm$ SD.

* $p < 0.001$ § not significant as compared to respective control.

REFERENCES

1. Gambhir, K.K., Archer, J.A. and Carter, L. (1977) Insulin radio receptor assay for human erythrocytes. *Clin. Chem.*, 23, 1590-95.
2. Watala, C. (1993) Altered structural and dynamic properties of blood cell membranes in diabetes mellitus. *Diabetic Med.*, 10, 13-20.
3. Gronda, C., Rossi, J.P.F.C., Bermudaz, C.M., Cedola, N.V. and Gargliardino, J.J. (1986). Decreased Ca^{++} -ATPase activity in erythrocyte membranes from diabetic patients. *ICRS Med. Sci.*, 10, 479.
4. Gonzalez Flecha, F.L., Bermudaz, C.M., Cedola, N.V., Gargliardino, J.J. and Rossi, J.P.F.C. (1990) Decreased Ca^{2+} -ATPase activity after glycosylation of erythrocyte membranes *in vivo* and *in vitro*. *Diabetes*, 39, 707-711.
5. Allo, S.N., Lincon, T., Wilson, G.L., Green, F.J., Watanabe, A.M. and Schaffer, S.W. (1991). Non insulin dependent diabetes mellitus induced defect in cardiac calcium regulation. *Am. J. Physiol.*, 260, 1165-1171.
6. Levy, J., Gavin, J.R. and Showers, Y. (1994) Diabetes Mellitus: a disease of abnormal cellular calcium metabolism. *Am. J. Med.*, 96, 260-263.
7. Ahmad, F., Khalid, P., Khan, M.M., Rastogi, A.K. and Kidwai, J.R. (1989) Insulin like activity in (-)epicatechin. *Acta diabetol. Lat.* 26, 291-300.
8. Rizvi, S.I., Abu Zaid, M. and Suhail, M. (1995) Insulin mimetic effect of (-)epicatechin on osmotic fragility of human erythrocytes. *Indian J. Exp. Biol.* 33, 791-792.
9. Rizvi, S. I. and Abu Zaid, M. (1998) Modulation of erythrocyte membrane Na/K-ATPase activity by insulin in normal and type 2 diabetic patients. Evaluation of insulin-like role of (-)epicatechin. *Med. Sci. Res.* 26, 245-247.
10. Rizvi, S. I. and Abu Zaid, M. (2001) Insulin-like effect of (-)epicatechin on erythrocyte membrane acetylcholinesterase activity in type 2 diabetes mellitus. *Clin. Exp. Pharmacol. Physiol.* 28(9), 776-778.
11. Rizvi, S. I. and Abu Zaid, M. (2001) Intracellular reduced glutathione content in normal and type 2 diabetic erythrocytes: Effect of insulin and (-)epicatechin. *J. Physiol. Pharmacol.* 52(3), 483-488.
12. Marchesi, V.T. and Palade, V.E. (1967) The localization of Mg-Na-K-activated adenosine triphosphatase on red cell ghost membranes. *J. Cell Biol.*, 35, 385-404.
13. Zaidi, A. and Saleemuddin, M. (1993) Ca^{2+} -induced alterations in the activity of membrane (Ca^{2+} - Mg^{2+})-ATPase of human and rat erythrocytes. *Indian J. Biochem. Biophys.*, 30, 98-102.
14. Fiske, C. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.*, 66, 375-400.
15. Lowry, O. H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem.*, 193, 265-275.
16. Zemel, M.B., Bedford, B.A., Zemel, P.C., Marwah, O. and Sowers, J. R. (1998) Altered cation transport in non-insulin-dependent diabetic hypertension: effects of dietary calcium. *J. Hypertens.*, 6, S 228-230.
17. Zemel, M.B., Sowers, J.R., Shehin, S., Walsh, M.F. and Levy, J. (1990) Impaired calcium metabolism associated with hypertension in Zucker obese rats. *Metabolism*, 39, 704-708.
18. Testa, I., Rabini, R.A., Fumelli, P., Bertoli, E. and Mazzanti, L. (1988) Abnormal membrane fluidity and acetylcholinesterase activity in erythrocytes from insulin-dependent diabetic patients. *J. Clin. Endocrinol. Metab.*, 67, 1129-1133.

19. Greene, D.A. and Lattimer, S.A. (1986) Biochemical alterations and complications in diabetes. Clin. Chem., 32(10), B 42-47.
20. Winegrad, A.I. (1987) Does a common mechanism induce the diverse complications of diabetes. Diabetes, 36, 396-406.
21. Means, G.E. and Chang, M.R. (1982) Nonenzymatic glycosylation of proteins structural and functional changes. Diabetes, 31, 1-4.
22. Adamson, I., Okafor, C. and Abu-Bakre, A. (1986) Erythrocyte membrane ATPases in diabetes: effect of dikanut (*Irvingia gabonensis*). Enzyme, 36, 212-215.
23. Davis, F.B., Davis, P.J., Nat, G., Blas, S.D., MacGillivray, M., Gutman, S. and Feldman, M.J. (1985) The effect of *in vivo* glucose administration on human erythrocyte Ca^{++} -ATPase activity on enzyme responsiveness *in vitro* to thyroid hormone and calmodulin. Diabetes, 34, 693-646.