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Modulating role of 'Saptarangi' (*Casearia esculenta*) on membrane bound ATPase in streptozotocin diabetic rats

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We have studied the activities of adenosine triphosphatase (Na^+/K^+ATPase, Mg^{2+}ATPase, Ca^{2+}ATPase and Total ATPase) in erythrocyte, liver, kidney and cardiac tissues of control and *Casearia esculenta* treated streptozotocin (STZ) diabetic rats. The activity of Na^+/K^+ATPase plays a central role in the regulation of intra and extra cellular homeostasis and alteration of this transport system is thought to be linked to several complications of diabetes mellitus. An Mg^{2+} dependent ATPase activity is responsible for controlling the energy requiring process in cells whereas Ca^{2+}ATPase is responsible for the signal transduction pathways and membrane fluidity. Activities of these enzymes were significantly altered ($p < 0.05$) in STZ diabetic rats. Oral administration of *C. esculenta* root extract for a period of 45 days resulted in significant ($p < 0.05$) reversal of these enzymes' activities to near normal. Thus the results suggest that *C. esculenta* protects the membrane integrity and functional status in STZ diabetic rats.

1. Introduction

Membrane bound ATPase is a ubiquitous enzyme essential for the maintenance of electrolyte balance and fundamentally involved in the maintenance of ion gradients that drives the co-transport of amino acids, sugars, and regulates cell volume and contributes to part of the membrane potential (Sweeney and Klip 1998). The activities of membrane bound enzymes may also be influenced by membrane environmental factors, such as lipid content. Alterations of membrane environment have been observed in various diseases. There is also evidence to suggest that membrane environment may have some effects on insulin sensitivity, insulin stimulated glucose uptake and ATPase activity (Gerbi et al. 1997).

In addition to being a major anabolic hormone regulating carbohydrate metabolism, insulin plays a pivotal role in Na^+ and K^+ homeostasis, since the entry of glucose into cells is mediated by specific transport ATPase in conjunction with the movement of Na^+ and K^+ ions across the cell membrane and the activities of these enzymes are largely influenced by the concentration of the hormone insulin in the blood. Diabetic state commands, the absence or lower level of insulin secretion, that leads to the increased level of glucose concentration, which in turn affects the membrane ATPase systems in organs (Sweeney and Klip 1998).

Concentration of Ca^{2+}ATPase activity in erythrocyte is maintained by several mechanisms. It has been reported that an abnormal plasma concentration of glucose will alter the erythrocyte membrane permeability to various cations such as Ca^{2+} ions (Ramana-Devi et al. 1997). Somer et al. (1979) have reported that decreased activity of Ca^{2+}ATPase represents the abnormal metabolism of Ca^{2+} in several conditions. Mg^{2+}ATPase activity is involved in other energy requiring processes in the cell. STZ diabetes was shown to result in decreased mitochondrial activity of Mg^{2+}ATPase in rat liver (Levy et al. 1986, 1994).

Casearia esculenta Roxb. (Flacourtiaceae, syn = *Casearia ovata)* an antidiabetic medicinal plant popularly known as "Kadala-Zhinjill", "Kottarkovai" in Tamil, "Wild cowrie fruit" in English and "Saptarangi" in Sanskrit is a shrub richly distributed in Konkan plateu, South India. In Indian traditional medicine, the plant has been a popular remedy for the treatment of diabetes mellitus (The Wealth of India 1992; Asolkar et al. 1992; Yoganarasimhan 2000) and our study drug is one of the major ingredients of D-400, a largest selling antidiabetic drug in India (Himalaya drug Co, Bangalore) (Mitra et al. 1995). In our previous communications we reported the glycemic control by *C. esculenta* – a short duration study in albino rats (Prakasam et al. 2003a) and the antihyperglycemic properties of *C. esculenta* root extract on normal and streptozotocin diabetic rats (Prakasam et al. 2002), blood glucose and plasma antioxidants status in streptozotocin diabetic rats (Prakasam et al. 2003b) and the hypolipidemic action of *C. esculenta* on STZ diabetic rats (Prakasam et al. 2003c). To our knowledge, no detailed investigations had been carried out to shed light on the role of *C. esculenta* on membrane bound enzymes in STZ diabetic rats. In the light of the above, the present study was carried out to evaluate the modulating role of *C. esculenta* root extract on membrane bound ATPases in control and experimental animals.

Table 1: Activities of membrane bound enzymes in the erythrocyte of control and experimental animals

Group	Treatment (dose/kg body wt.)	Total ATPase U[a]/mg protein	Na^+/K^+ATPase U[a]/mg protein	Mg^{2+}ATPase U[a]/mg protein	Ca^{2+}ATPase U[a]/mg protein
I	Control (2% gum acacia)	1.66 ± 0.05^a	0.75 ± 0.047^a	0.35 ± 0.01^a	0.36 ± 0.007^a
II	Diabetic control	0.82 ± 0.05^b	0.34 ± 0.02^b	0.33 ± 0.01^a	0.21 ± 0.02^b
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	1.34 ± 0.11^c	0.49 ± 0.03^c	0.34 ± 0.03^a	0.30 ± 0.01^c
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	1.54 ± 0.06^a	0.65 ± 0.01^a	0.33 ± 0.009^a	0.33 ± 0.01^a
V	Diabetic + glibenclamide (600 µg/kg body wt.)	1.66 ± 0.09^a	0.72 ± 0.04^a	0.35 ± 0.01^a	0.36 ± 0.02^a

Values are given as mean ± SD for six animals in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ Duncan's Multiple Range Test (DMRT)
a = µmoles of Pi liberated/h/mg protein

Table 2: Activities of membrane bound enzymes in the liver of control and experimental animals

Group	Treatment (dose/kg body wt.)	Total ATPase U[a]/mg protein	Na^+/K^+ATPase U[a]/mg protein	Mg^{2+}ATPase U[a]/mg protein	Ca^{2+}ATPase U[a]/mg protein
I	Control (2% gum acacia)	2.29 ± 0.26^a	0.64 ± 0.07^a	0.36 ± 0.04^a	0.60 ± 0.04
II	Diabetic control	1.25 ± 0.18^b	0.48 ± 0.02^b	0.27 ± 0.07^b	0.60 ± 0.06
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	1.52 ± 0.22^c	0.51 ± 0.08^b	0.33 ± 0.02^c	0.62 ± 0.05
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	1.76 ± 0.11^d	0.55 ± 0.10^c	0.36 ± 0.05^a	0.61 ± 0.04
V	Diabetic + glibenclamide (600 µg/kg body wt.)	2.05 ± 0.11^e	0.61 ± 0.15^c	0.35 ± 0.04^a	0.59 ± 0.05

Values are given as mean ± SD for six animals in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ Duncan's Multiple Range Test (DMRT)
a = µmoles of Pi liberated/h/mg protein

2. Investigations and results

The activities of erythrocyte membrane bound total ATPases, Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are depicted in Table 1. In diabetic animals total ATPases, Na^+/K^+ATPase and Ca^{2+}ATPase activities are markedly lowered ($p < 0.05$). *C. esculenta* treatment for 45 days in STZ diabetic rats restores the decreased activities of these enzymes to near normal.

Table 2 represents the activities of total ATPases, Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in the liver of control and experimental animals. The activities of total ATPases, Na^+/K^+ATPase and Mg^{2+}ATPase activities are significantly lowered ($p < 0.05$) in livers of STZ diabetic rats when compared to diabetic rats. Oral administration of *C. esculenta* significantly increases the activities after 45 days. No significant changes in Ca^{2+}ATPase of control and experimental animals.

Activities of total ATPases, Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in the kidney of control and experimental animals are shown in Table 3. The activities of total ATPases, Na^+/K^+ATPase and Mg^{2+}ATPase are significantly increased ($p < 0.05$) in the kidney of diabetic animals when compared with control rats. Oral administration of *C. esculenta* for 45 days to diabetic rats restores the altered levels of these enzymes to near normal. No significant changes in Ca^{2+}ATPase of control and experimental animals.

Table 4 represents the activities of total ATPases, Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in the cardiac tissue of control and experimental animals. The activities

Table 3: Activities of membrane bound enzymes in the kidney of control and experimental animals

Group	Treatment (dose/kg body wt.)	Total ATPase U[a]/mg protein	Na^+/K^+ATPase U[a]/mg protein	Mg^{2+}ATPase U[a]/mg protein	Ca^{2+}ATPase U[a]/mg protein
I	Control (2% gum acacia)	1.47 ± 0.19^a	0.54 ± 0.05^a	0.36 ± 0.04^a	0.35 ± 0.04^a
II	Diabetic control	2.41 ± 0.15^b	1.31 ± 0.10^b	0.67 ± 0.06^b	0.37 ± 0.03^b
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	2.03 ± 0.10^c	1.10 ± 0.07^b	0.47 ± 0.08^a	0.37 ± 0.10^b
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	1.94 ± 0.16^d	0.85 ± 0.22^a	0.35 ± 0.01^a	0.36 ± 0.04^a
V	Diabetic + glibenclamide (600 µg/kg body wt.)	1.71 ± 0.07^d	0.56 ± 0.09^a	0.32 ± 0.01^a	0.35 ± 0.01^a

Values are given as mean ± SD for six animals in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ Duncan's Multiple Range Test (DMRT)
a = µmoles of Pi liberated/h/mg protein

Table 4: Activities of membrane bound enzymes in the cardiac of control and experimental animals

Group	Treatment (dose/kg body wt.)	Total ATPase U[a]/mg protein	Na^+/K^+ATPase U[a]/mg protein	Mg^{2+}ATPase U[a]/mg protein	Ca^{2+}ATPase U[a]/mg protein
I	Control (2% gum acacia)	1.36 ± 0.06^a	0.54 ± 0.02^a	0.29 ± 0.01^a	0.42 ± 0.6^a
II	Diabetic control	2.75 ± 0.32^b	0.78 ± 0.09^b	0.69 ± 0.08^b	0.69 ± 0.07^b
III	Diabetic + *C. esculenta* (200 mg/kg body wt.)	1.96 ± 0.13^c	0.72 ± 0.14^b	0.56 ± 0.03^c	0.60 ± 0.03^c
IV	Diabetic + *C. esculenta* (300 mg/kg body wt.)	$1.62 \pm 0.09^{a,c}$	$0.62 \pm 0.03^{a,b}$	$0.45 \pm 0.02^{a,c}$	0.61 ± 0.04^b
V	Diabetic + glibenclamide (600 µg/kg body wt.)	$1.39 \pm 0.06^{a,c}$	0.55 ± 0.02^a	$0.38 \pm 0.01^{a,c}$	$0.52 \pm 0.02^{a,b}$

Values are given as mean ± SD for six animals in each group.
Values not sharing a common superscript differ significantly at $p < 0.05$ Duncan's Multiple Range Test (DMRT)
a = µmoles of Pi liberated/h/mg protein

of total ATPases, Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are significantly increased ($p < 0.05$) in STZ diabetic rats when compared to control rats. Oral administration of *C. esculenta* significantly decreased the activities after 45 days of *C. esculenta* treatment.

3. Discussion

Activities of the membrane bound ATPases (Na^+K^+ATPase and Ca^{2+}ATPase) were significantly altered in STZ diabetic rats as compared with control rats. STZ induced diabetes is characterized by a severe rearrangement of subcellular metabolism and structural alterations of cell membrane (Brasitus et al. 1985). Several factors are known to alter levels of ATPase, especially lipid peroxidation (LPO), membrane fluidity, extracellular calcium and oxidation of thiol groups by LPO. It could be due to oxygen radical mediated inhibition of Ca^{2+}ATPase activity in sarcoplasmic reticulum, hepatocyte plasma membrane and intestinal brush border enzymes (Ohta et al. 1989). Membrane of erythrocytes are highly sensitive to oxidative damage probably due to their content of unsaturated fatty acids. Therefore, the fair degree of ATPase activities in erythrocyte membrane could serve as a simple, safe and useful marker of liver damage (Ohta et al. 1989).
The plasma membrane Na^+K^+ATPase is concerned with the maintenance of a low concentration of Na^+ and in consequence of cellular water content. Altered activity of Na^+K^+ATPase can lead to a decrease in sodium efflux and thereby altering the membrane permeability (Finotti and Palatini 1986). A decrease in the activity of Na^+K^+ATPase in diabetes would also be expected in the light of the many reports that insulin stimulates this enzyme and sodium pump activity *in vivo* and *in vitro* in a variety of tissues (Takahashi et al. 1998).
Ca^{2+}ATPase regulates the calcium pump activity. Decrease in the activity of Ca^{2+}ATPase can increase intracellular concentration of free Ca^{2+} and alter the signal transduction pathways and cellular fluidity (Levy et al. 1986). Mg^{2+}ATPase activity is involved in other energy requiring processes in the cell. STZ diabetes was shown to result in decreased mitochondrial activity of Mg^{2+}ATPase in rat liver (Levy et al. 1986, 1994). In contrast to the present study Tahahashi et al. (1998) reported an increase in the activities of Ca^{2+}, Mg^{2+}ATPase in STZ diabetic rats.
The intracellular concentration of calcium regulates the activities of Mg^{2+} and Na^+K^+ATPases, therefore Ca^{2+} may play a role in the regulation of sodium reabsorption. The steady-state Ca^{2+} concentration is postulated to be regulated by calcium uptake by this specific Ca^{2+} activated ATPase (Levy et al. 1986, 1994). Jain and Lim (2000) have reported that lipid peroxidation can decrease the functions of Ca^{2+} and Mg^{2+}ATPases and the activity of membrane bound Ca^{2+} translocase. High glucose concentrations can result in increased oxidative stress from excessive oxygen radical production from the autoxidation of glucose (Wolff et al. 1991). Increased lipid peroxidation can in turn diminish the activities of Na^+K^+, Ca^{2+} and Mg^{2+}ATPases in erythrocyte membrane when exposed to higher glucose concentration medium (Jain et al. 2000). STZ diabetic rats are well known to have high blood glucose concentration. The increased blood glucose stimulating the lipid peroxidation may be a reason to decrease the activities of membrane ATPases observed in the present study.
In the erythrocyte membrane of STZ diabetic rats a decreased activity of Ca^{2+}ATPase is observed, which results in increased erythrocyte total Ca^{2+} levels, as a result of the permeability alterations. The decreased Ca^{2+}ATPase also decreases the membrane bound Ca^{2+} concentration. This also indicates the alteration in erythrocyte permeability (Ramana-Devi et al. 1997).
Loss of Ca^{2+} or Mg^{2+}ATPase activity in membranes is accompanied by an increase in the formation of MDA and 4 HNE (a byproduct of LPO) (Hoschstein and Jain 1981). The Ca^{2+}ATPase molecule has several lysine residues and some of them are closely related to the active site. Binding of LPO by-products on lysine residues, near or at the active site, probably account for the STZ-induced Ca^{2+}ATPase inhibition (Filoteo et al. 1987).
In the present study, oral administration of *C. esculenta* root extract for 45 days significantly reversed the diabetes induced alterations in the activities of membrane bound ATPases, possibly by decreasing the lipid peroxidative damage to the cell membrane or by its insulin secretory effects.

4. Experimental

4.1. Plant material

Root of *Casearia esculenta* was collected from Western ghats of Tamil Nadu and the plant was botanically authenticated by Dr. C. Chelladurai, Research Officer, Survey Medicinal Plant Unit (S.M.P.U.), Central Council for Research in Siddha and Ayurvedic, Siddha Medical College, Palayamkottai, Tamilnadu. A voucher specimen was deposited in the (AU2145) Department of Botany, Annamalai University, Annamalainagar, Tamilnadu. The plant root was air dried at 25 °C and the dried root was ground with an auto-mix blender and the powdered part was kept in a deep freezer until the time of use.

4.2. Preparation of aqueous extract

(100 g) Dry fine powder was suspended in 250 ml water for 2 h and then boiled at 60–65 °C for 30 min (since a boiled decoction of the root of this plant has been used as remedy for diabetes).
The extract was preserved and the processes were repeated for three times with the residual powder, each time collecting the extract. The collected extract was pooled and passed through a fine cotton cloth. The filtrate upon evaporation at 40 °C yielded 12% semi-solid extract.

4.3. Drugs and chemicals

Streptozotocin (STZ) was obtained form Sigma chemical company. All other chemicals used were of analytical grade.

4.4. Animals

Male Wistar albino rats (weighting 140–160 g) were procured from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar. Animals were maintained at Central Animal House and the animals were fed with standard diet (Hindustan Lever, Bangalore) and water *ad libitum.* All studies were conducted in accordance with the National Institute Health "Guide for the Care and Use of Laboratory Animals" (National Institute of Health 1985) and the Ethical Committee of Rajah Muthaih Medical College and Hospital, Annamalai University, Annamalainagar approved the study.

4.5. Experimental induction of diabetes

Adult (9 weeks old) male *Wistar* rats were made diabetic with an intraperitoneal injection of streptozotocin (STZ, 50 mg/kg body weight) dissolved in citrate buffer (0.1 M, pH 4.5). Streptozotocin injected animals exhibited massive glycosuria and hyperglycemia within a few days. Diabetes was confirmed in STZ rats by measuring the fasting blood glucose concentration, 96 h after injection with STZ. Albino rats with a blood glucose level above 240 mg/dl were considered to be diabetic and were used in the experiment. Six rats were injected with 2% gum acacia alone that served as control.

4.6. Animals

After the induction of diabetes the rats were divided into five groups of six animals each.
Group I – Control rats received vehicle solution (2% gum acacia); group II – Diabetic control; group III – Diabetic rats received *C. esculenta* root extract (200 mg/kg body weight) in 2% gum acacia using an intragastric tube daily for 45 days; group IV – Diabetic rats given *C. esculenta* root extract (300 mg/kg body weight) in 2% gum acacia using an intragastric

tube daily for 45 days; group V – Diabetic rats received glibenclamide orally (600 μg/kg body weight) as aqueous solution using an intragastric tube daily for 45 days.

After 45 days of treatment, the animals were sacrificed by cervical decapitation. The blood was collected in heparinised centrifuge tubes and the plasma was collected and the blood was processed for the separation of intact RBC by the method of (Dodge et al. 1968) as modified by the method of Quist (1980). The liver, kidney and cardiac tissues were dissected out immediately and washed with ice-cold saline. A portion of these tissues was weighed using an electronic balancer and homogenized using a potter Elvejham homogenizer and centrifuged at 3000 × g. The supernatant were further used for the assay of ATPases. Tissue total protein (Lowry et al. 1951) and haemoglobin (Drakbin and Austin 1932) were estimated in control and drug treated animals.

4.7. Biochemical analysis

4.7.1. Assay of total ATPase (E.C. 3.6.1.2)

The activity of the enzyme was assayed according to the method of Evans (1969). The assay mixture consisted of 1.5 mL of Tris-HCl buffer (0.1 M, pH 7.0), sodium chloride (0.1 M), potassium chloride (0.1 M), adenosine triphosphaste (0.01 M) and suitable aliquot of the enzyme. A control was also run simultaneously without the enzyme. The tubes were incubated at 37 °C for 15 min. The reaction was arrested by trichloroacetic acid (10% w/v). The enzyme was then added to the control. The tubes were centrifuged at 3000 × g for 10 min. The inorganic phosphorus liberated was estimated by the method of Fiske and Subbarow (1925). The total ATPase activity is expressed as μmol of Pi liberated/h/mg protein.

4.7.2. Na^+/K^+ATPase (E.C. 3.6.1.3)

Sodium and potassium dependent ATPase activities were assayed by the method of Bonting (1970). The incubation volume of the test consisted of 1 mL of Tris-HCl buffer (184 mM, pH 7.5), 0.2 mL each of magnesium sulphate (50 mM), potassium chloride (50 mM), sodium chloride (160 mM), EDTA (1 mM), ATP (4 mM) and suitable aliquot of the enzyme. Control without enzyme was also treated similarly. The tubes were incubated at 37 °C for 15 min. The reaction was arrested by trichloroacetic acid (10% w/v) followed by the addition of the enzyme to the control. The tubes were centrifuged at 3000 × g for 10 min. The inorganic phosphorus was estimated by the method of Fiske and Subbarow (1925) and the enzyme activity is expressed as μmol of Pi liberated/h/mg protein.

4.7.3. Mg^{2+}ATPase (E.C. 3.6.1.4)

The activity of the enzyme was estimated by the method of Ohnishi et al. (1982). The incubation of volume of the test consisted of 0.1 mL each of Tris-HCl buffer (125 mM pH 7.6), magnesium chloride (25 mM), ATP (0.01 M), distilled water and enzyme preparation. Control without enzyme was run simultaneously. The tubes were incubated at 37 °C for 15 min. The reaction was then arrested by trichloracetaic acid (10% w/v) followed by 0.1 mL of the enzyme of the control. The tubes were incubated at 37 °C for 15 min and centrifuged at 3000 × g for 10 min. Fiske and Subbarow method estimated the inorganic phosphorus liberated and the enzyme activity is expressed as μmol of Pi liberated/h/mg protein.

4.7.4. Ca^{2+}ATPase (E.C. 3.6.1.5)

The activity of Ca^{2+}ATPase activity was measured in tissue homogenate and RBC membrane according to the method of Hjerten and Pan (1983). The incubation mixture contained 0.1 mL of buffer (125 mM, pH 8.0), 0.1 of calcium chloride (50 mM), 0.1 mL of ATP (10 mM), 0.1 mL of water and 0.1 of enzyme (tissue homogenate or RBC membrane). The contents were incubated at 37 °C for 15 min. The reaction was then arrested by the addition of 0.5 mL of ice-cold TCA (10% w/v). The amount of phosphorus liberated was estimated by the method of Fiske and Subbarow and the enzyme activity is expressed as μmol of Pi liberated/h/mg protein.

4.8. Statistical analysis

Values were represented as mean ± S.D., Data were analyzed using Analysis of Variance (ANOVA) and group mean were compared with Duncan's multiple range test (DMRT).

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