Pharmacological Reports
2007, 59, 339–348
ISSN 1734-1140

Influence of umbelliferone on membrane-bound ATPases in streptozotocin-induced diabetic rats

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Abstract:
The activities of membrane-bound ATPases are altered both in erythrocytes and tissues of streptozotocin (STZ)-induced diabetic rats and diabetic patients. Umbelliferone (UMB), a natural antioxidant, is a benzopyrone occurring in nature, and it is present in the fruits of golden apple (*Aegle marmelos Correa*) and bitter orange (*Citrus aurantium*). Earlier we evaluated and reported the effect of UMB on plasma insulin and glucose, and this study was designed to evaluate the effect of umbelliferone on membrane-bound ATPases in erythrocytes and tissues (liver, kidney and heart) of STZ-induced diabetic rats. Adult male albino rats of Wistar strain, weighing 180–200 g, were made diabetic by an intraperitonial administration of STZ (40 mg/kg). Normal and diabetic rats were treated with UMB dissolved in 10% dimethyl sulfoxide (DMSO) and diabetic rats were also treated with glibenclamide as drug control, for 45 days. In our study, diabetic rats had increased level of blood glucose and lipid peroxidation markers, and decreased level of plasma insulin and decreased activities of total ATPases, $(Na^+ + K^+)$-ATPase, low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocytes and tissues. Restoration of plasma insulin and glucose by UMB and glibenclamide seemed to have reversed insulin, glucose and lipid peroxidation markers, and diabetes-induced alterations in the activities of membrane-bound ATPases. Thus, our results show that the normalization of membrane-bound ATPases in various tissues, is due to improved glycemic control and antioxidant activity by UMB.

Key words:
diabetes, streptozotocin, umbelliferone, membrane-bound ATPases

Abbreviations: DMSO – dimethyl sufloxide, STZ – streptozotocin, UMB – umbelliferone

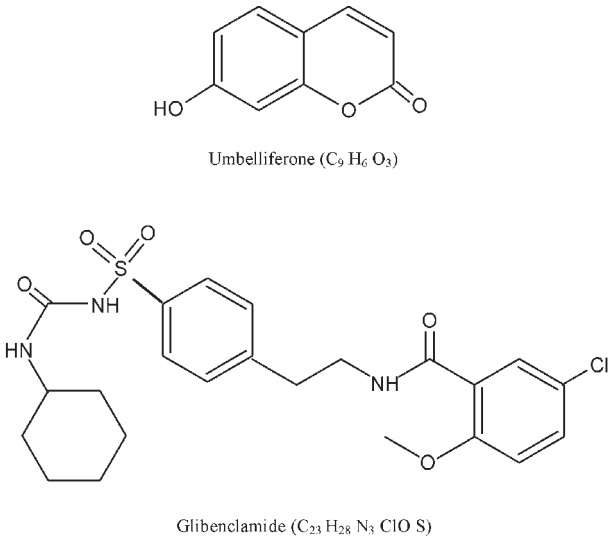
Introduction

The ubiquitous cellular enzyme $(Na^+ + K^+)$-adenosine triphosphatase (ATPase) is responsible for the maintenance of intracellular sodium and potassium concentrations [33, 50]. The function of this enzyme is to transport three ions of sodium from the intracellular space to the extracellular environment and, in return, to allow two ions of potassium to enter the cell. The high-affinity Ca^{2+}-ATPase is the major active calcium transport protein responsible for the maintenance of normal intracellular calcium levels in a variety of cell types. Maintenance of the cation gradient by high-affinity Ca^{2+}-ATPase is of fundamental importance in the control of hydration, volume, nutrient uptake and fluidity of cells, and is also essential for the contractility and excitability of muscles [29, 10]. Low-affinity Ca^{2+}-ATPase is considered to be responsible for the shape and deformability of the erythrocyte membranes [28].

Hyperglycemia results in the increased oxidative stress from excessive ROS production in the autoxidation of glucose and glycated proteins [2, 48]. The increased ROS activity initiates peroxidation of lipids and MDA accumulation, which in turn can stimulate glycation of proteins in diabetes [18]. As indicated before, plasma membrane and the membranes of intracellular organelles are crucial targets of ROS attack. Diabetes-induced hyperlipidemia and the alterations in membrane phospholipids and fatty acids have been shown to depress membrane-bound enzyme activities, which influence intracellular calcium metabolism resulting in cardiac dysfunction [27]. Another possibility involves the modification of enzyme molecules either by direct oxidation or by modification mediated by products of lipid peroxidation [48]. A decrease in ATPase activity in any diabetic tissue could be due to excessive nonenzymatic glycation of the enzyme itself and/or of calmodulin [11]. Glycosylation of proteins may alter their physiological properties, particularly their binding affinities [4, 56]. Insulin is one of the hormones that regulate the synthesis and activity of $(Na^+ + K^+)$-ATPase [54]. The amount and the activity of $(Na^+ + K^+)$-ATPase in the plasma membrane are reduced in diabetic animals and insulin administration partially restores normal conditions [13]. In the metabolic disorder theory, persistent hyperglycemia activates the polyol pathway, and a decrease in myoinositol associated with accumulation of sorbitol reduces $(Na^+ + K^+)$-ATPase activity. Several alterations in structural and dynamic properties of erythrocyte membrane have been reported in type 1 diabetes and type 2 diabetes, respectively [53]. Of particular interest has been the observation of an altered activity of erythrocyte membrane $(Na^+ + K^+)$-ATPase in type 1 diabetes and type 2 diabetes, [22, 46, 49]. The membrane bound $(Na^+ + K^+)$-ATPase or the sodium pump regulates a large number of basic and specialized cellular functions, which depend upon the intra- and extracellular Na^+ and K^+ concentrations. The ion gradient produced by $(Na^+ + K^+)$-ATPase influences cell volume and osmotic pressure, acts as a driving force for inward co-transport of amino acids and monosaccharides, and helps in the active co-transport of ions such as H^+, Ca^{2+} and K^+. Impaired $(Na^+ + K^+)$-ATPase is a feature of diabetes mellitus in many cell types and is believed to be a pivotal regulator of various cell functions. It is widely believed that an impairment in $(Na^+ + K^+)$-ATPase activity may play a major role at the cellular level in the pathophysiology of many late complications of diabetes mellitus: neuropathy, nephropathy and retinopathy [17, 12] and in the development of diabetic vascular complications [19].

Plant-derived phenolic coumarins might play a role as dietary antioxidants because of their consumption in the human diet in fruits and vegetables [16]. Umbelliferone (7-hydroxycoumarin), a natural antioxidant, is a benzopyrone occurring in nature, and it is present in the edible fruits such as golden apple (*Aegle marmelos Correa*) [36] and bitter orange (*Citrus aurantium*) [55]. The parent compound coumarin has been reported to reduce blood glucose level [31] and we have also reported that UMB has antihyperlipidemic, antidiabetic and antihyperglycemic properties [41, 42]. 4-Methylcoumarins, having one hydroxyl or two acetoxy groups in the benzoid ring at positions ortho to each other, have shown very strong antioxidant and radical scavenging properties better than those of α-tocopherol [38]. UMB has one hydroxyl and one acetoxy group in the benzoid ring, which may be responsible for the antioxidant and radical scavenging properties. Previous report has shown that UMB has alkylperoxy radical scavenging property [16], and we have also reported that UMB has antioxidant [40, 43, 44] properties and regulates glycoprotein components [45] in STZ-diabetic rats. Membrane lipid peroxidation [48] and glycoxidation would also be responsible for the inhibition of the activities of ATPases during diabetic hyperglycemia. Increased glycoprotein components are involved in increased glycation of membrane proteins [4], and diabetic hyperlipidemia [27], which may also be responsible for the alteration of the activities of ATPases. In light of the above, the present study was carried out to evaluate the influence of UMB on plasma insulin, glucose and lipid peroxidation markers, and membrane-bound ATPases such as total ATPases, $(Na^+ + K^+)$-ATPase, low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocytes and tissues (liver, kidney and heart) of control and experimental animals as there was no detailed study on these aspects.

Glibenclamide (5-chloro-N-[2-[4-cyclohexylcarbamoylsulfamoyl)phenyl]ethyl]-2-methoxy-benzamide) is being used as a standard antidiabetic drug, and is used to stimulate insulin secretion in type 2 diabetic patients. Hence, we used it as a reference drug in this study. Both UMB and glibenclamide are phenolic compounds, but they are structurally different. The structures of UMB and glibenclamide are given on the next page (Scheme 1).



Scheme 1.

Materials and Methods

Animals

Male albino rats of Wistar strain with body weight ranging from 180–200 g, were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and were maintained in an air conditioned room (25 ± 1°C) with a 12 h light: 12 h dark cycle. Feed and water were provided *ad libitum*. Studies were carried out in accordance with Indian National Law on Animal Care and Use, and Ethical Clearance provided by The Committee for the Purpose of Control and Supervision of Experiments on Animals of Rajah Muthiah Medical College and Hospital (Reg. No. 160/1999/CPCSEA), Annamalai University, Annamalainagar, Tamil Nadu, India.

Chemicals

Streptozotocin was purchased from Sigma-Aldrich, St. Louis, USA. UMB (purity, > 98%) was procured from Carl Roth GmbH & Co, Germany. All other chemicals used were of analytical grade obtained from E. Merck or HIMEDIA, Mumbai, India.

Experimental induction of diabetes

The animals were made diabetic by a single intraperitoneal injection (*ip*) of STZ (40 mg/kg) in a freshly prepared citrate buffer (0.1 M, pH 4.5) and control animals were given citrate buffer alone after an overnight fast. STZ-injected animals were given 20% glucose solution for 24 h to prevent initial drug-induced hypoglycemic mortality. STZ injected animals exhibited massive glycosuria (determined by Benedict's qualitative test), and these animals were tested for hyperglycemia by measuring fasting plasma glucose (by glucose oxidase method), 96 h after the injection of STZ. The animals with the blood glucose levels more than 200 mg/dl were considered diabetic and used for the experiment.

Experimental design

The animals were randomly divided into 5 groups of six animals each and treated as given below. Dimethyl sulfoxide (DMSO) was used as a vehicle solution for the *ip* administration of UMB and glibenclamide.

Group I: Normal control received 10% DMSO
Group II: Normal + UMB (30 mg/kg) in 10% DMSO
Group III: Diabetic control received 10% DMSO
Group IV: Diabetic + UMB (30 mg/kg) in 10% DMSO
Group V: Diabetic + glibenclamide (600 μg/kg) in 10% DMSO

After 45 days of treatment, the 12 h fasted animals were anesthetized by giving an intramuscular injection of ketamine (24 mg/kg) and sacrificed by decapitation between 8 a.m and 9 a.m. Blood was collected in the tubes with EDTA and erythrocytes were separated by washing with 0.15 M sodium chloride solution. Erythrocytes and tissues (liver, kidney and heart) were collected for the measurement of membrane-bound ATPases such as total ATPases, $(Na^+ + K^+)$-ATPase, low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase. We have prepared Tris buffer using redistilled water and tissues were homogenized in Tris buffer and redistilled water was used throughout the experiment to avoid interference prior to phosphorous estimation in the assay of ATPases.

Biochemical analysis

Plasma glucose was measured by glucose oxidase method [51]. Plasma insulin was assayed with an ELISA kit by the method of Burgi et al. [5]. The estimation of TBARS, HP and CD was done by the methods of Niechaus and Samuelson [35]; Jiang et al. [20] and Klein [24], respectively. The activity of total ATPases was measured by the method of Evans [8]. The phosphate liberated was estimated by the method of Fiske and Subbarrow [9]. The activities of $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase were measured by the methods of Bonting [3]; Hjerten and Pan [14] and Ohnishi et al. [36], respectively.

Statistical analysis

All quantitative measurements were expressed as the means ± SD. The mean value was from six rats in each group. The data were analyzed using one-way analysis of variance (ANOVA) on SPSS/PC (Statistical Package for Social Sciences/Personal Computer) and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the p values were lesser than 0.05.

Results

Table 1 shows the plasma levels of glucose and insulin and body weight in control and diabetic rats. Diabetic rats had an elevated level of plasma glucose and decreased body weight and level of plasma insulin as compared with control rats, and treatment with umbelliferone (30 mg/kg/d) and glibenclamide (600 µg/kg/d) reversed glucose, body weight and insulin to near normalcy.

The levels of TBARS, HP and CD in the plasma of diabetic and control rats are presented in Table 2. Dia-

Tab. 1. Effect of umbelliferone on plasma glucose and insulin in control and diabetic rats

Name of the group	Glucose (mg/dl)		Insulin (µU/ml)
	0 day	45th day	
Normal control	79.60 ± 5.25	82.44 ± 2.68^{b}	18.04 ± 0.77^{ab}
Normal + umbelliferone (30 mg/kg/d)	82.14 ± 3.19	74.39 ± 4.17^{a}	18.73 ± 0.84^{a}
Diabetic control	240.47 ± 5.82	289.28 ± 3.18^{d}	5.38 ± 0.37^{c}
Diabetic + umbelliferone (30 mg/kg/d)	244.63 ± 6.29	114.28 ± 5.71^{c}	17.11 ± 0.66^{b}
Diabetic + glibenclamide (600 µg/kg/d)	242.85 ± 5.04	107.23 ± 7.23^{c}	17.49 ± 0.60^{b}

Values are given as the means ± SD from six rats in each group. Values not sharing a common superscript vertically differ significantly at $p < 0.05$. All five groups were compared with each other

Tab. 2. Effect of umbelliferone lipid peroxidation markers in the plasma of cntrol and diabetic rats

Name of the group	Thiobarbituric acid reactive substances (mmole/dl)	Lipid hydroperoxides (mmole/dl)	Conjugated dienes (ratio of absorbance at 240 and 214 nm)
Normal control	0.200 ± 0.03^{b}	9.51 ± 0.92^{b}	0.66 ± 0.02^{b}
Normal + umbelliferone (30 mg/kg/d)	0.173 ± 0.04^{a}	8.02 ± 0.82^{a}	0.50 ± 0.03^{a}
Diabetic control	0.337 ± 0.02^{d}	24.15 ± 1.8^{d}	0.93 ± .05^{e}
Diabetic + umbelliferone (30 mg/kg/d)	0.227 ± 0.01^{c}	11.60 ± 0.97^{c}	0.74 ± 0.06^{d}
Diabetic + glibenclamide (600 µg/kg/d)	0.212 ± 0.03^{bc}	10.41 ± 1.34^{bc}	0.69 ± 0.04^{c}

Values are given as the means ± SD from six rats in each group. Values not sharing a common superscript vertically differ significantly at $p < 0.05$. All five groups were compared with each other

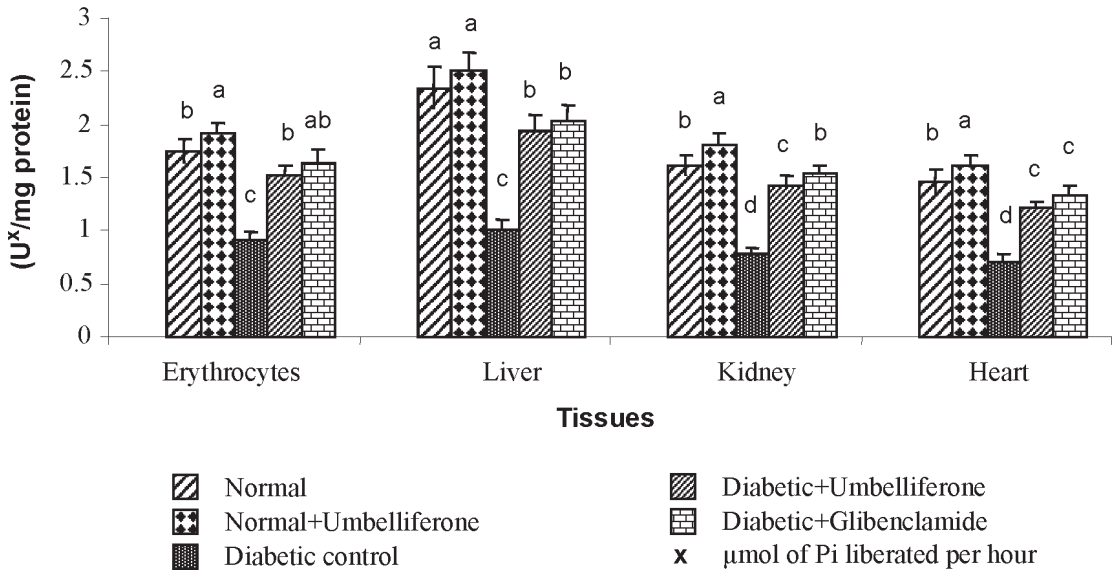


Fig. 1. Effect of UMB on total ATPases in erythrocytes and tissues of control and experimental rats. Data were given as the means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ as evaluated using Duncan's Multiple Range Test

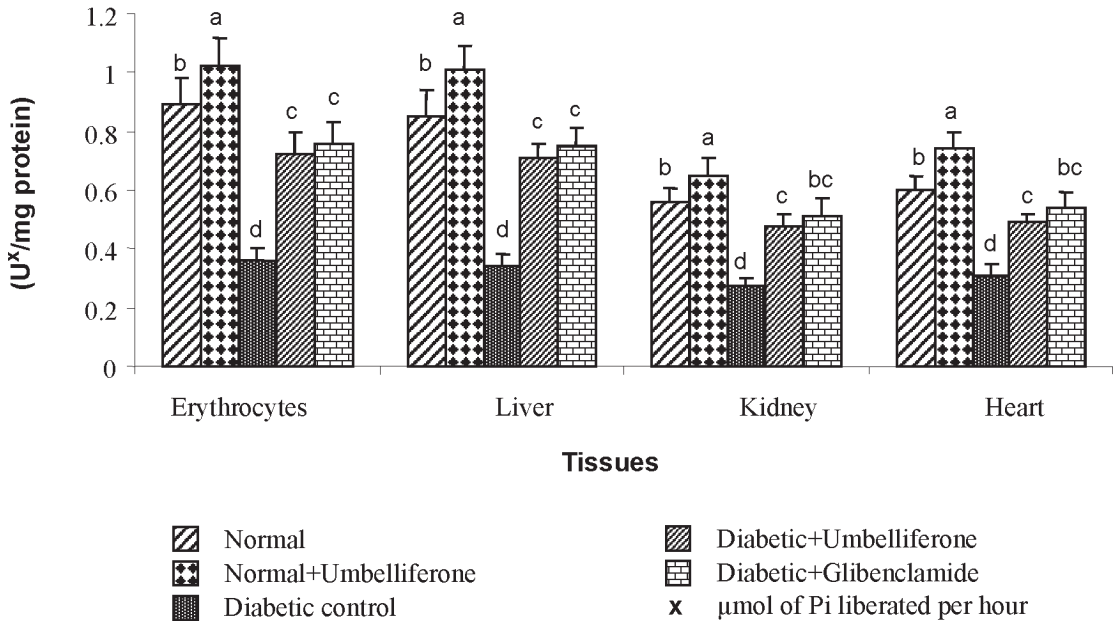


Fig. 2. Effect of UMB on $(Na^+ + K^+)$-ATPase in erythrocytes and tissues of control and experimental rats. Data were given as the means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ as evaluated using Duncan's Multiple Range Test

betic rats had elevated levels of TBARS, HP and CD in the plasma when compared with normal control rats. Treatment with umbelliferone and glibenclamide showed reversal of these parameters to near normalcy.

The activities of total ATPase and $(Na^+ + K^+)$-ATPase in the erythrocytes and tissues (liver, kidney and heart) of control and experimental rats are given in Figures 1 and 2, respectively. Diabetic rats had de-

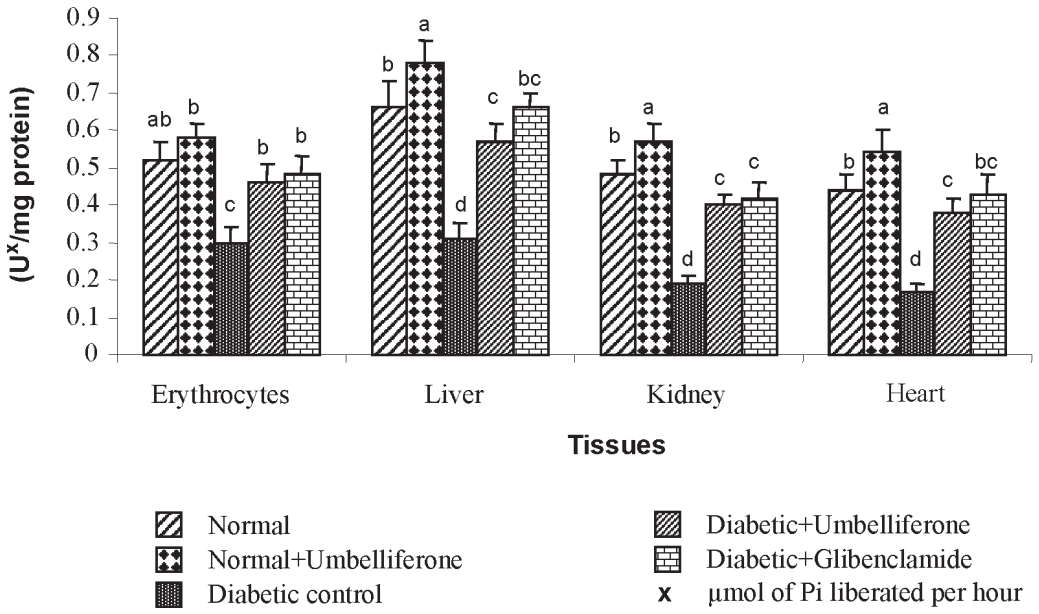


Fig. 3. Effect of UMB on low affinity Ca^{2+}-ATPase in erythrocytes and tissues of control and experimental rats. Data were given as the means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ as evaluated using Duncan's Multiple Range Test

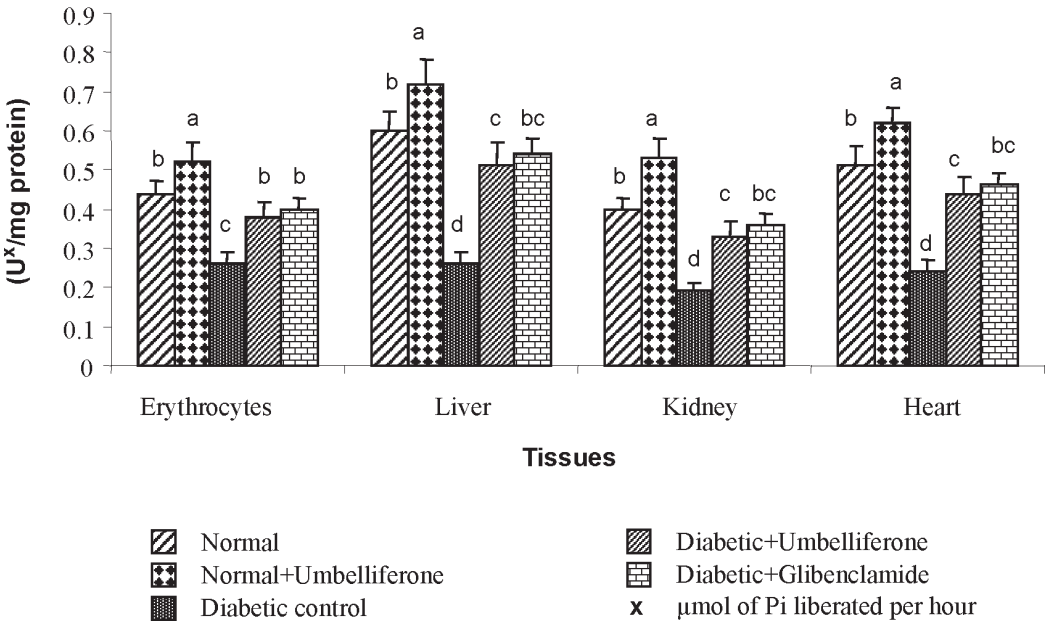


Fig. 4. Effect of UMB on Mg^{2+}-ATPase in erythrocytes and tissues of control and experimental rats. Data were given as the means ± SD from six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ as evaluated using Duncan's Multiple Range Test

creased activity of total ATPase and $(Na^+ + K^+)$-ATPase in the erythrocytes and tissues. Treatment with UMB and glibenclamide restored the activities of total ATPase and $(Na^+ + K^+)$-ATPase to near normalcy.

Figures 3 and 4 represent the activities of low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase in the erythrocytes and tissues (liver, kidney and heart) of control and experimental rats, respectively. The activities of

Mg^{2+}-ATPase and low affinity Ca^{2+}-ATPase decreased in the erythrocytes and tissues (liver, kidney and heart) of diabetic rats, while treatment with UMB and glibenclamide brought back Mg^{2+}-ATPase and low affinity Ca^{2+}-ATPase to near normalcy.

Discussion

Diabetes affects about 5% of the global population [6] and management of diabetes without any side effects is still a challenge to the medical system [21]. Poorly controlled or undiagnosed disease may be associated with so-called late complications of diabetes such as accelerated atherosclerosis, blindness, renal insufficiency, stroke, and amputation of extremities. Diabetes is also associated with a decrease in life expectancy. These facts make diabetes a major health problem [44]. In our study, diabetic rats had elevated level of blood glucose and treatment with UMB and glibenclamide reversed blood glucose, which could be associated with increased secretion of insulin. Diabetes mellitus has been reported to generate reactive oxygen species (ROS). ROS such as free hydroxyl radicals ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) can cause lipid peroxidation [32]. Elevated levels of lipid peroxidation markers in our study are consistent with the previous report. Treatment with UMB and glibenclamide reversed these changes to near normalcy, which could be associated with improved glycemic control.

Total ATPases consists of $(Na^+ + K^+)$-ATPase, low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase. Insulin and catecholamines are the principal mediators of acute hormonal control of Na'/K+-ATPase [7]. In our study, diabetic rats had decreased level of $(Na^+ + K^+)$-ATPase in the tissues, which resembles the previous report [23]. This might be associated with the deficiency of insulin as insulin administration partially restored $(Na^+ + K^+)$-ATPase [13]. The oxidative damage of tissue lipids and proteins might have caused $(Na^+ + K^+)$-ATPase inactivation. $(Na^+ + K^+)$-ATPase is rich in thiol groups and oxidation of thiol groups has been reported to inhibit enzyme activity [52]. Treatment with UMB and glibenclamide restored $(Na^+ + K^+)$-ATPase. This may be due to insulin secretory effect along with decreasing peroxidative damage to membrane lipids as reported earlier [40, 43, 44].

Hyperglycemia can cause glycosylation of proteins and cellular lipid peroxidation, which, in turn, can cause inhibition/reduction in the activities of $(Na^+ + K^+)$- and Ca^{2+}-ATPases. This result can, in turn, affect the intracellular concentrations of Na^+, K^+, and Ca^{2+}, alter the signal transduction pathways, and affect contractility and excitability and cellular dysfunctions [17]. Diabetic rats had decreased activity of low affinity Ca^{2+}-ATPase as a consequence of interaction of glucose with these enzymes [39]. Treatment with UMB and glibenclamide restored low affinity Ca^{2+}-ATPase along with decreasing lipid peroxidation and protein glycosylation or improving glycemic control.

Insulin directly regulates the membrane bound $(Ca^{2+} + Mg^{2+})$-ATPase [15]. Low-affinity Ca^{2+}-ATPase is considered to be responsible for the shape and deformability of the erythrocyte membranes [28]. In our study, diabetic rats showed decreased activity of low affinity Ca^{2+}-ATPase. This could be due to insulin deficiency as insulin is the regulator of the enzyme. Treatment with UMB and glibenclamide restored low affinity Ca^{2+}-ATPase, which might be associated with insulin secretory effect.

Erythrocyte $(Na^+ + K^+)$-ATPase plays a central role in the regulation of intra- and extracellular cation homeostasis. Alteration of this transport system was thought to be linked to several complications of diabetes [26]. As $(Na^+ + K^+)$-ATPase is an integral protein in the red cell membrane, its catalytic activity can be dictated by the vicinal activation of phospholipids. A direct interaction between enzyme and phospholipids was shown by Muczynsky and Stahl [34]. Lipid alteration in the erythrocyte membrane was reportedly related to the reduced $(Na^+ + K^+)$-ATPase activity in type 1 and type 2 diabetic patients [1]; this may be a possible factor in the present reduction of the enzyme activity. Treatment with UMB and glibenclamide restored $(Na^+ + K^+)$-ATPase along with decreasing peroxidative damage to membrane phospholipids.

The activities of low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase were found to decrease in the erythrocytes of diabetic rats. Increased lipid peroxidation can, in turn, diminish the activities of low affinity Ca^{2+}-ATPase and Mg^{2+}-ATPase in erythrocyte membrane when exposed to a higher glucose concentration-containing medium [17]. Diabetic rats treated with UMB and glibenclamide showed the reversal of low affinity Ca^{2+}- and Mg^{2+}-ATPases to near normalcy which might be associated with decreased per-

oxidative damage to membrane phospholipids along with improving glycemic control.

Several alterations in structural and dynamic properties of erythrocytic membrane have been reported in type 1 diabetes and type 2 diabetes [53]. Altered activity of erythrocyte membrane (Na^++K^+)-ATPase has been observed in type 1 diabetes and type 2 diabetes [46]. An impairment in (Na^++K^+)-ATPase activity may play a major role at the cellular level in the pathophysiology of many late complications of diabetes mellitus: neuropathy, nephropathy and retinopathy [12, 17] and in the development of diabetic vascular complications [19]. The normalization of membrane bound ATPases by UMB-treatment may protect erythrocytes from the risk of deformability and preserve specific properties of muscle and nerve tissue such as contractility and excitability, and prevent diabetic neuropathy.

Many of the well-characterized membrane ATPases (P type ATPases – Na^+, K^+- and Ca^{2+}-ATPases; V type ATPases – lysosomes;) are transport proteins and use intracellular ATP to drive active ion transport whereas F-type ATPases are involved in the formation of ATP in mitochondria. In contrast, the cell surfaces ATPases, which are also ubiquitous, hydrolyze extracellular ATP (and UTP, ADP) [25]. Ecto ATPases (extracellular ATPases) are cell surface ATPases that hydrolyze extracellular ATPs. E-type ATPase activity was first designated as Mg^{2+}-ATPase [47]. Mg^{2+}-ATPases are all cell surface ATPases, which hydrolyze extracellular ATP. Comparison between total ATPases and sum of individual ATPases (i.e. Na^+, K^+-ATPase and low affinity Ca^{2+}-ATPases and Mg^{2+}-ATPase) show almost no difference in our study, since we used tissue homogenates, which includes all these individual ATPases.

Membrane lipid peroxidation [48] and glycoxidation would also be responsible for the inhibition of the activities of ATPases during diabetic hyperglycemia. Increased glycoprotein components [45] are related with increased glycation of membrane proteins [4] and diabetic hyperlipidemia [27], which may also be responsible for the inhibition of the activities of ATPases. Glycation of ATPases is also possible during hyperglycemia. Hence improved glycemic control [41, 42] and redox status [40, 43, 44] by UMB and glibenclamide could be responsible for the restoration of enzyme activities along with increased insulin secretion as insulin is the regulator of ATPases. The effect of UMB is comparable with that of glibenclamide in all the parameters.

Acknowledgment:
The financial support to Dr. B. Ramesh as Senior Research Fellowship from Indian Council of Medical Research, New Delhi, is gratefully acknowledged.

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Received:
July 27, 2006; in revised form: June 25, 2007.