Effect of 2-Hydroxy-4-methoxy Benzoic Acid Isolated from *Hemidesmus indicus* on Erythrocyte Membrane Bound Enzymes and Antioxidant Status in Streptozotocin-induced Diabetic Rats

M. GAYATHRI* AND K. KANNABIRAN
Division of Industrial Biotechnology, School of Biosciences and Technology, Vellore-632 012, India

Gayathri and Kannabiran: Erythrocyte Membrane Bound Enzymes and Antioxidant Status by 2-hydroxy-4-methoxy Benzoic Acid

In the present study, the effect of 2-hydroxy-4-methoxy benzoic acid isolated from roots of *Hemisdesmus indicus* on the erythrocyte membrane bound enzymes and antioxidant status in streptozotocin-induced diabetic rats was investigated. The streptozotocin-induced diabetic rats were treated with 2-hydroxy-4-methoxy benzoic acid (500 μg/kg/day) for 7 weeks by oral intubation and compared with glibenclamide, a standard hypoglycemic agent (100 mg/kg). The erythrocyte membrane was isolated and the activity of Na^+/K^+-dependent ATPases, Ca^{2+}-ATPases, Mg^{2+}-ATPases were determined. Superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, vitamins C, vitamin E, plasma reduced glutathione and erythrocyte glutathione, reduced glutathione content in the tissues was also assayed. Administration of 2-hydroxy-4-methoxy benzoic acid to diabetic rats significantly (F>0.05 and P<0.001) elevated the activity of total ATPases, Na^+/k^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase to near normal level. The activities of catalase, superoxide dismutase and glutathione peroxidase and glutathione-S-transferase in erythrocytes were decreased significantly (F>0.05; P<0.001) in diabetic rats. Diabetic rats treated with 2-hydroxy-4-methoxy benzoic acid showed a significant (F>0.05; <0.001) increase in the enzymic antioxidants in erythrocytes. The elevated levels of vitamin E and low level of vitamin C and glutathione level in plasma and erythrocytes were observed in diabetic rats when compared to control rats and were restored significantly (F>0.05; P<0.001) after the administration of 2-hydroxy-4-methoxy benzoic acid. This study concludes administration of 2-hydroxy-4-methoxy benzoic acid supports the restoration of antioxidant defence, reduces the free radial production, lipid peroxidation and the glycosylation of haemoglobin in diabetic rats.

Key words: Antioxidants, diabetes, erythrocyte membrane bound enzymes, 2-hydroxy-4-methoxy benzoic acid

Diabetes mellitus is a metabolic disease as old as mankind and its incidence is considered to be high (4-5%) all over the world[1]. The number of cases of diabetes is currently 171 million and is predicted to reach 366 million by the year 2030. Diabetes mellitus is characterized by an increased concentration of blood glucose because of defective secretion of insulin and impaired carbohydrate metabolism. These metabolic disturbances result in acute and long-term diabetic complications[2]. Diabetic complications are associated with overproduction of free radicals and accumulation of byproducts of lipid peroxidation. Several studies have proposed the mechanism for the role of free radicals in the pathogenesis of various diseases. Arrays of enzymic and nonenzymic antioxidants are involved in the protection of free radicals induced oxidative damage. Erythrocytes earlier regarded as 'nontarget' cell for insulin but later it was reported that it possess specific receptors for insulin with characteristics similar to typical target cells. Membrane bound enzymes are the enzymes in a membrane and are responsible for the maintenance of cellular functions such as ion transport, secretion and uptake of a variety of substances, as well as cell to cell interactions. It has been hypothesized that the abnormality in dynamic properties of erythrocyte membrane in diabetes may play a major role in determining the alterations in enzymatic activities. The abnormalities in the membrane bound enzymes in erythrocytes is characterized by reduced contractibility, relaxation, cardiac work and diastolic complications which are linked to cardiovascular, ocular and neural manifestations of diabetic disease process[3]. Diabetes-induced hyperlipidemia; oxidative stress and protein glycation seem to be the major contributing

***Address for correspondence**
E-mail: gayathrigopinath@vit.ac.in

factors associated with abnormal membrane bound enzyme activities resulting in cardiac dysfunction. Normalization of membrane-bound enzymes was proposed to be one of the important mechanisms for the management of diabetes and to protect diabetic heart.

The active principle of *Hemidesmus indicus*, 2-hydroxy-4-methoxy benzoic acid (HMBA) was reported to possess hepatoprotective[4] and antidiabetic activity[5,6]. The present study was carried out to evaluate the effect of HMBA on enzymic and nonenzymic antioxidants in erythrocyte membrane of streptozotocin-induced (STZ-induced) diabetic rats.

The root of *Hemidesmus indicus* was identified and collected from the Morappur forest area, Dharmapuri District, Tamilnadu. The roots were identified and a voucher specimen was submitted to Vellore Institute of Technology, India. The roots were washed with distilled water, shade dried, powdered and stored in an air tight container until further use.

Male Wistar rats (150-200 g) were purchased from Tamil Nadu Veterinary Animal Science University, Madhawaram, Chennai, and housed under standard husbandry conditions (30±2°, 60-70% relative humidity, and 12:12 h day-night cycle) and allowed standard pellet rat feed and water *ad libitum*. The animal experiments were designed and conducted in accordance with the guidelines of the Institutional Animal Ethical Committee (IAEC), VIT University, India. Extraction, isolation and purification of the pure HMBA from the root powder of *Hemidesmus indicus* (100 g) was carried as per earlier reports[7].

The animals were fasted overnight and diabetes was induced by single intraperitoneal injection of freshly prepared solution of STZ (Sigma aldrich, USA) at a dose of 35 mg/kg, in 0.1 M cold citrate buffer, pH 4.5. STZ-injected animals were considered to be diabetic if the blood glucose values were above 250 mg/dl on the 3rd day after STZ injection. The control rats were injected with 0.1 M cold citrate buffer (pH 4.5) alone.

Animals were divided into six groups of six animals each. Group I served as a control; Group II had STZ-treated surviving diabetic rats; Group III served as a positive control and received a standard hypoglycaemic agent, glibenclamide (100 mg/kg body weight); group IV diabetic rats treated with HMBA (500 μg/kg/day) for 7 weeks by oral intubation.

The erythrocyte membrane was isolated[8] with a change in buffer[9]. Total ATPases activity[10] and the amount of liberated Pi[11] were estimated. The activity of Na^+/K^+-ATPases[12], Ca^{2+}-ATPases[13], Mg^{2+}-ATPases[14], superoxide dismutase (SOD)[15] catalase (CAT)[16], glutathione peroxidase (GPx)[17], glutathione S-transferase (GST)[18], vitamins C[19], vitamin E[20], plasma reduced glutathione (GSH) and erythrocyte GSH[21] were determined. The reduced GSH[22] content in the tissues were also assayed. The statistical analysis was performed using the SPSS software package, version 16.00. The values were analysed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). All the results were expressed as mean±SD for six rats in each group and $P<0.001$ was considered statistically significant.

The effect of HMBA on the activities of erythrocyte membrane bound enzymes total ATPase, Na^+/K^+-ATPase, Mg^{2+}-ATPase, Ca^{2+}-ATPase in diabetic rats were significantly ($F>0.05$; $P<0.001$) lowered, when compared to control rats (Table 1). Administration of HMBA to diabetic rats significantly ($F>0.05$ and $P<0.001$) elevated the activity of total ATPases, Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase to near normal level (Table 1).

The effect of HMBA on the activities of enzymic antioxidants like CAT, SOD and GPx and GST in erythrocytes were decreased significantly ($F>0.05$; $P<0.001$) in diabetic rats and the diabetic rats treated with HMBA showed a significant ($F>0.05$; $P<0.001$) increase in the enzymic antioxidants in erythrocytes (Table 2).

The elevated levels of vitamin E and low level of vitamin C and GSH in plasma and GSH level in erythrocytes were observed in diabetic rats when compared to control rats. The levels of these antioxidants were restored significantly ($F>0.05$; $P<0.001$) after the administration of HMBA (Table 3).

Erythrocytes are highly sensitive to peroxidative damage probably due to the high content of unsaturated fatty acid in their membrane. Therefore the activity of erythrocyte membrane of ATPases could serve as a marker for the assessment of intracellular damage[23]. The reduced activities of ATPase in

TABLE 1: EFFECT OF 2-HYDROXY-4-METHOXY BENZOIC ACID ON ERYTHROCYTE MEMBRANE BOUND ENZYMES IN DIABETIC RATS

Groups	Dose (mg/kg/day)	Total ATPase (micro mole Pi/mg of membrane protein/h)	Na^+/K^+-ATPase activity (micro mole Pi/mg of membrane protein/h)	Ca^{2+}-ATPase activity (micro mole Pi/mg of membrane protein/h)	Mg^{2+}-ATPase activity (micro mole Pi/mg of membrane protein/h)
Normal	-	1.69±0.02	0.89±0.01	0.36±0.01	0.39±0.02
Diabetic control	-	0.85±0.05#	0.35±0.04#	0.25±0.01#	0.28±0.05#
Diabetic+ΓA	100	1.40±0.03*	0.60±0.02*	0.31±0.01*	0.33±0.03*
Diabetic+HMBA	0.5	1.38±0.03	0.68±0.01	0.33±0.03	0.33±0.03
	0.5	1.52±0.02*	0.72±0.04*	0.35±0.04*	0.33±0.02*

ATPases - Adenosine tri Phosphatases. Values are mean±ΣD for six rats in each group (n=6).*Different from diabetic control, F>0.05 (ANOVA) and P<0.001 (DMRT). #Different from normal control, F>0.05 (ANOVA) and P<0.001 (DMRT). ND=Nondiabetic group, D=Diabetic group, GA=Glibenclamide, HMBA=2-hydroxy-4-methoxy benzoic acid

TABLE 2: EFFECT OF 2-HYDROXY-4-METHOXY BENZOIC ACID ON ERYTHROCYTE ENZYMIC ANTIOXIDANTS IN DIABETIC RATS

Groups	ND/D	Dose (mg/kg/day)	Catalase[a]	SOD[b]	Glutathione peroxidase[c]	Glutathione-S-transferase[d]
Normal	-	-	159±0.02	6.89±0.01	14.36±0.01	7.39±0.02
Diabetic control	-	-	85±0.05#	4.35±0.04#	10.25±0.01#	4.28±0.05#
Diabetic+GA	-	100	140±0.03*	5.60±0.02*	12.31±0.01*	8.33±0.03*
Diabetic+HMBA	ND	0.5	148±0.03	5.68±0.01	12.33±0.03	7.33±0.03
	D	0.5	152±0.02*	5.72±0.04*	12.35±0.04*	7.33±0.02*

Values are mean±SD for six rats in each group (n=6).*Different from diabetic control, F>0.05 (ANOVA) and P<0.001 (DMRT). #Different from normal control, F>0.05 (ANOVA) and P<0.001 (DMRT). ND=Nondiabetic group, SOD=Superoxide dismutase, D=Diabetic group, GA=Glibenclamide, HMBA-2-hydroxy-4-methoxy benzoic acid. [a]μmol of H_2O_2 consumed/min/mg Hb. [b]One unit of activity was taken as the enzyme reaction which gave 50% inhibition of NBT reaction in 1 min/mg Hb. [c]μg of GSH consumed/min/mg Hb. [d]μmol of CDNB-GSH/mg Hb

TABLE 3: EFFECT OF 2-HYDROXY-4-METHOXY BENZOIC ACID ON ERYTHROCYTE NONENZYMIC ANTIOXIDANTS IN DIABETIC RATS

Groups	ND/D	Dose (mg/kg/day)	Plasma			Erythrocytes reduced glutathione (μmol/g Hb)
			Vitamin C (mg/dl)	Vitamin E (mg/dl)	GSH (mg/dl)	
Normal	-	-	1.89±0.12	1.59±0.11	6.36±0.1	12.39±1.02
Diabetic control	-	-	0.85±0.15#	3.35±0.14#	4.29±0.1#	9.28±1.05#
Diabetic+GA	-	100	1.40±0.13*	2.60±0.12*	8.31±0.1*	11.33±1.03*
Diabetic+HMBA	ND	0.5	1.48±0.13	2.68±0.11	7.33±0.3	11.33±1.03
	D	0.5	1.59±0.12*	2.72±0.14*	7.45±0.4*	11.33±1.02*

Values are mean±SD for six rats in each group (n=6).*Different from diabetic control, F>0.05 (ANOVA) and P<0.001 (DMRT). #Different from normal control, F>0.05 (ANOVA) and P<0.001 (DMRT). ND=Nondiabetic group, D=Diabetic group, GA=Glibenclamide, HMBA=2-hydroxy-4-methoxy benzoic acid, GSH=Glutathione

erythrocytes and other tissues of STZ-induced diabetic rats have been already reported[24]. STZ-induced diabetes is characterized by a severe rearrangement of sub cellular metabolism and structural alteration of cell membrane, which may in turn, play an important role in the development of diabetic vascular complications. The erythrocyte membrane Na^+/K^+-ATPase is concerned with the maintenance of low intracellular concentrations of Na^+. The decrease in the activity of Na^+/K^+-ATPase in diabetic condition can lead to a decrease in Na^+ efflux and thereby alter the membrane permeability. Insufficient Na^+/K^+-ATPase activity in diabetes consequently leads to an increase in the amount of Ca^{2+} accessible for contraction of the Na^+/K^+-ATPase in several tissues. These findings were accompanied by raise in intracellular Ca^{2+} concentrations, a fact that occurs when the Na^+/K^+-ATPase activity was reduced[25]. The intracellular concentration of calcium regulated the activities of Mg^{2+}-ATPase and Na^+/K^+-ATPase. The inhibition of these transport systems in the cell may result in a sustained increase in cytosolic Ca^{2+} concentrations producing over stimulation of cellular processes leading ultimately to cell death[26].

Multiple enzymatic and nonenzymatic antioxidant defence system present in cells inactivates those free radicals and reduces the amount of cellular oxidative damage. Endogenous antioxidant enzymes (SOD, CAT and GPx) are responsible for the detoxification and deleterious oxygen radicals[27]. The observed decrease in the activities of SOD, CAT and GPx in erythrocytes of diabetic rats, which could be due to increased utilization of these enzymes for

scavenging free radicals. HMBA treatment restored the activities of these enzymatic antioxidants, which could be a result of decrease in the levels of lipid peroxidation and thereby decreasing the utilization of these enzymes for scavenging free radicals.

Vitamins C and E are interrelated by recycling process. Recycling of tocopheroxyl radicals to tocopherol was achieved with vitamin C, which is a powerful watersoluble antioxidant present in the cytosolic compartment of the cell. Vitamin C serves as an electron donor for vitamin E radicals generated in the cell membrane during oxidative stress. Vitamin E neutralizes the free radicals by preventing the chain reaction that contributes to oxidative damage[28]. Vitamin E and C is one of the most important free radical scavenging chain-breaking antioxidant within biomembrane. Reduced GSH, a major endogenous antioxidant, plays a crucial role in the antioxidant defence. Vitamin C, a major extra cellular nonenzymatic antioxidant, has crucial role in scavenging several reactive oxygen species. Enzymatic antioxidants (SOD, CAT and GSHPx) form the first line of antioxidant defence mechanism against reactive oxygen species mediated oxidative damage. Several studies have demonstrated that decreased nonenzymatic antioxidant levels and enzymatic antioxidant activities in STZ-induced diabetic rats. GSH functions as free radical scavenger and helps in the repairment of free radical mediated biological damage. GSH is required for the recycling of vitamin C and acts as a substrate for GPx and GST that are involved in preventing the deleterious effect of oxygen radicals. GSH is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reactions. Indeed GSH depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects, for example, a decrease in the rate of gluconeogenesis or an increase in glycogenolysis[10]. Reduced level of GSH in the circulation during diabetes represents its increased utilization due to oxidative stress. In our study, the depleted levels of vitamin C and GSH and increased level of vitamin E in diabetic rats, which might be due to increased utilization of these reductants for scavenging free radicals produced in diabetic condition. HMBA treatment restored the altered levels of antioxidants, which could be due to reduction in lipid peroxidation levels in diabetic rats. Lipid peroxidation and glycosylation of proteins can cause reduction in the activities of enzymes and alteration in the structure and function of membranes[29]. A reduction in the lipid peroxidation and glycosylation of proteins can prevent the decrease in the activities of ATPases. Any reduction in ATPases activity can affect the intracellular concentrations of Na^+, K^+ and Ca^{2+}, alter the signal transduction pathway, and affect contractility, which in turn leads to cellular dysfunction beneficially.

Diabetic rats treated with HMBA showed significant elevation in the activities of total ATPases, Na^+/K^+-ATPases, and Mg^{2+}-ATPase in erythrocyte membrane. The reversal of erythrocyte membrane bound ATPases activity in diabetic rats by HMBA administration could be due to increase in metabolism of glucose, and thus the lowering of the glucose concentration in diabetic rats, that results in the restoration of antioxidant defence, reduction of free radial production, lipid peroxidation and the glycosylation of hemoglobin.

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