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Modulatory Effect of N-Benzoyl-D-Phenylalanine on Cholinesterases in Rat Retinas of Neonatal Streptozotocin Diabetic Rats

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Abstract: The aim of the study was to investigate the protective effect of N-benzoyl-D-phenylalanine (NBDP), an antidiabetic compound, on the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in normal and streptozotocin-induced diabetic cataract in wistar rats. Cholinesterases activities were significantly decreased with significant increase in lipid peroxidation as reflected by Thiobarbituric Acids Reactive Substances (TBARS) in the diabetic rats. This, in turn, could lead to alterations associated with diabetes complications. Treatment with NBDP appears to have reversed this effect. The decreased activity of cholinesterases observed in diabetes may be due to lack of insulin, which causes specific alterations in neurotransmitter, thus causing retinal dysfunction. NBDP could protect against direct action of lipid peroxidation on retina AChE and BChE and in this way it might be useful in the prevention of cholinergic retinal dysfunction in diabetes.

Key words: N-benzoyl-D-phenylalanine · cholinesterase · rat retina · lipid peroxidation · diabetic complication · neonatal diabetes

INTRODUCTION

Cholinesterases are polymorphic enzymes, which hydrolyze acetyl choline and other choline esters. Two types of cholinesterases have been characterized in various tissues: acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) [1], encoded by separate gene and differ in substrate specificity and reactivity with selective inhibitors [2]. Previous studies have established that both AChE and BChE are found as a set of asymmetric (A_{12}, A_8 and $A_{4)}$ and globular forms (G_4, G_2 and $G_{1),}$ which are characterized by sedimentation propagation and cellular localization [3]. Globular monomer (G_1) and globular tetramer (G_4) are the two predominant forms in rat brain and retina [4]. The hydrophilic monomer is preferentially extracted in detergent-free medium and a second treatment in detergent-containing medium extracts the hydrophobic tetramer. The activity of detergent-soluble (DS) AChE is approximately 10-fold higher than the salt-soluble (SS) protein [5].

Abnormalities affecting AChE and BChE activities have been reported in various diseases including diabetes. Several studies have reported decreased activities of AChE and BChE during diabetes leads to accumulation of acetylcholine, cholinergic hyperactivity, convulsion and status epileptics [6-8]. Several studies have reported the inhibitory effect of diabetes on AchE activity in several rat tissues and the erythrocyte membrane [9, 10]. In diabetic rats, changes in free-radical levels and AChE and BChE activity have been reported in retina and brain [11, 12]. In experimental diabetic rats, decreased AchE activity in brain and heart can be reversed by insulin administration [9]. Decreased brain enzyme activity and changes in neuromuscular transmission are involved in the progression of human diabetes [9, 10, 13]. The decreased level of cholinesterase probably presents one of the indicators for neural diabetes complications although it is not clear to what extent it contributes directly.

In diabetic humans and animals, several lines of evidence have suggested that both the retina and brain

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are susceptible to damage by oxygen radicals [14-16]. Retinopathy risk correlates with a high concentration of oxidative stress markers. Conversely, in STZ-induced diabetes in rats, decreased antioxidant enzyme activities in the retina are play an important role in the development of retinopathy [17, 18].

Oral antidiabetics, mainly non-sulfonylurea drugs and biguanides, are used for the treatment of NIDDM patients. The most recently developed group of compounds, which are structurally related to sulfonylurea and biguanides, is the non-sulfonylurea of which N-benzoyl-D-phenylalanine (NBDP) is the member currently in use [19]. NBDP acts directly on the pancreatic β-cell to stimulate insulin secretion that is rapid and short duration and depend upon the ambient glucose level. Generally D-phenylalanine derivative controls hyperglycemia, resulting in improved overall glycemic control in patients with type 2 diabetes.

Metformin is an oral hypoglycemic agent, which belongs to the class known as the biguanides, chemically N-N-dimethylimidodicarbonimidicdiamide[20].Metformin is now widely used as one of the mainstays in the management of type 2 diabetes. Metformin reduces fasting plasma glucose concentration by reducing rate of hepatic glucose production via gluconeogenesis and glycogenolysis. Metformin improves glycemic control as monotherapy in combination with other oral antidiabetic agents, such as sulfonylureas and thiazolidine diones [21].

We previously reported the anti-hyperglycemic, anti-hyperlipidemic and anti-peroxidative effects of N-benzoyl-D-phenylalanine (NBDP) and metformin on neonatal streptozotocin (nSTZ) induced diabetic rats [22-25]. In the present study, we examine the relation between oxidative stress and STZ-induced changes in the activity of AChE and BChE in retinal tissue from diabetic rats and assess the protective influence of N-benzoyl-D-phenylalanine (NBDP) and metformin.

MATERIALS AND METHODS

Animals: Healthy Albino Wistar strain rats were kept for breeding in the Central Animal House, Rajah Muthiah Medical College, Annamalai University were used in the present study. The rats were fed on pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum*. The rats used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The ethical committee, Annamalai University, approved the use of rats in the present study (Approval. No; 99, 2002).

Drugs and chemicals: All the biochemicals and chemicals used in this experiment were purchased from Sigma chemical company Inc., St Louis, MO, USA. The chemicals were of analytical grade. N-benzoyl-D-phenylalanine was prepared by mixing equimolar amounts of D-phenylalanine with benzoyl chloride in the presence of appropriate solution and adopting the general procedure reported for benzoylation [26] and the structure was confirmed by IR and ^{13}C NMR spectral studies.

Experimental induction of type 2 diabetes (NIDDM) in rats: The model was developed according to the description of Bonner Weir *et al*. [27]. Wistar rats of either sex, aged 48±2 h, were injected intraperitoneally with streptozotocin in citrate buffer (pH 4.5) at a dose of 100 mg/kg body weight. After 12 weeks, only male rats weighing above 150g were selected for screening in the NIDDM model.

Experimental procedure: In the experiment, a total of 30 rats were used (24 diabetic surviving rats, 6 control rats). The rats were divided into five groups of six rats each.

Group 1. Control rats (vehicle-treated).
Group 2. Diabetic control rats.
Group 3. Diabetic rats given NBDP at 100 mg $kg^{-1}day^{-1}$ in 1 ml of 0.5 % methylcellulose suspension for 6 weeks.
Group 4. Diabetic rats given metformin at 500 mg $kg^{-1}day^{-1}$ in 1ml of saline for 6 weeks [28].
Group 5. Diabetic rats given NBDP (100 mg $kg^{-1}day^{-1}$ in 1 ml of 0.5 % methyl- cellulose suspension) and metformin (500 mg $kg^{-1}day^{-1}$ in 1 ml of saline) for 6 weeks.

At the end of experimental period, the rats were deprived of food overnight and blood was collected in a tube containing potassium oxalate and sodium fluoride for the estimation of blood glucose, hemoglobin and glycated hemoglobin levels. Plasma was separated for the estimation of insulin.

Eyes were rapidly removed and retinas gently peeled away using fine forceps. The Salt Soluble (SS) and detergent soluble (DS) fractions of AChE were extracted according to Sberna *et al*. [29]. Retinas were washed and homogenized (10% w/v) in Tris-saline buffer (1 M NaCl, 50 mM $MgCl_2$, 1 mM EDTA, 2 mM benzamidine, 1 mg mL^{-1} bacitracin, 0.1 mg mL^{-1} soybean trypsin inhibitor, 10 µg/mL pepstatin, 20 U/mL aprotinin, 10 mM Tris buffer, pH 7.0). After centrifugation for 1 h at 35,000 rpm, 4°C, in a 40Ti Beckman rotor, the salt-soluble fraction (S1) was recovered. The pellet was re-extracted with the Tris-saline buffer containing 1% (w/v) Triton X-100. After centrifugation, the detergent-soluble fraction (S2) and the pellet (P) were stored [10].

Analytical methods: Glucose level was estimated by O-toluidine method [30]. Plasma insulin was estimated by using Enzyme Linked Immunosorbant Assay (ELISA) kit (Boehringer Mannheim, Mannheim, Germany). Hemoglobin was estimated using the cyanmethemoglobin method described by Drabkin and Austin [31]. Glycated hemoglobin was estimated according to the method of Nayak and Pattabiraman [32] with modifications according to Bannon [33]. Lipid peroxidation marker in retina was estimated colorimetrically by the method of Niehius and Samuelsson [34]. AChE and BChE activities were assayed by the Ellman method [35].

Statistical analysis: All the data were expressed as mean±SD of number of experiments (n=12). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 7.5 (SPSS, Cary, NC, USA) and the individual comparison were obtained by Duncans' Multiple Range Test (DMRT). Values were considered statistically significant when $p< 0.05$ [36].

RESULTS

Table 1 shows the level of blood glucose, plasma insulin, total hemoglobin and glycated hemoglobin in control and experimental animals. The level of blood glucose and percentage of glycated hemoglobin was significantly increased whereas the plasma insulin and total hemoglobin was significantly decreased in nSTZ diabetic rats. The administration of NBDP significantly reversed the changes in dose-dependent manner. NBDP at a dose of 100 mg $kg^{-1}day^{-1}$ and Metformin at a dose of 500 mg $kg^{-1}day^{-1}$ showed a highly significant effect whereas combined administration of NBDP and metformin was more effective than either drug alone. Hence the effective dose was selected for further biochemical analysis.

The extent of lipid peroxidation in retina is presented in Fig. 1. TBARS levels were significantly ($p<0.05$)

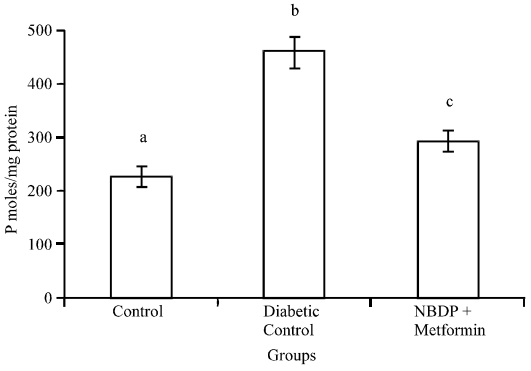

Fig. 1: Effect of N-benzoyl-D- phenylalanine (NBDP) and metformin on retina TBARS in control and experimental rats. Values are given as mean±SD from 12 rats in each group; ANOVA followed by DMRT. Values not sharing a common superscript letter differ significantly at $p<0.05$

Table 1: Changes in the level of blood glucose, plasma insulin, hemoglobin and glycated hemoglobin in control and experimental animals

	Blood glucose (mg dl^{-1})		Plasma insulin (µU dl^{-1})			
Groups	Initial	Final	Initial	Final	Hemoglobin (g dl^{-1})	Glycated hemoglobin (%)
Control	85.19±5.97 [a]	90.19±6.17 [a]	27.87±2.12 [a]	27.62±1.26 [a]	13.15±0.82 [a]	0.024
Diabetic control	196.50±10.38 [b]	235.16±10.71 [b]	21.92±1.34 [b]	21.10±0.75 [b]	6.11±0.39 [b]	0.089
NBDP (100 mg kg^{-1})	204.90±10.09 [b]	143.58±7.17 [d]	21.67±1.21 [b]	24.76±0.91 [d]	9.76±0.38 [d]	0.039
Metformin (500 mg kg^{-1})	207.18±8.57 [b]	154.85±8.74 [e]	20.99±1.26 [b]	23.88±0.43 [de]	9.06±0.43 [e]	0.046
NBDP (100 mg kg^{-1}) + metformin (500 mg kg^{-1})	194.66±8.87[b]	112.14±7.85 [f]	21.40±1.19 [b]	26.48±0.97 [f]	11.03±0.67 [f]	0.033

NBDP-N-benzyl-D-phenylalanine. Values are Means±SD for 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$. Duncan's procedure: Range for the level 2.89, 3.03, 3.13, 3.20, 3.25

Table 2: Solubilization of Cholinesterase in control and experimental rat retinas

	Distribution (%)		
Fraction	AchE	BchE	protein
Control			
S_1	14±2	50±3	52±3
S_2	71±4	30±4	24±2
P	16±3	32±2	28±3
Diabetic			
S_1	10±2	43±3	51±2
S_2	61±3	24±2	26±3
P	20±2	25±3	24±2
NBDP+Metformin			
S_1	15±1	53±4	54±3
S_2	69±3	27±2	23±2
P	17±2	31±2	26±2

Cholinesterase and protein were determined in the supernatant obtained without (S_1) and with detergent (S_2) and in the pellet (P) as described in methods. The distribution is expressed as present of the total enzyme activity or protein recovered. Values are the mean±SD of 6 rats.

Table 3: Cholinesterases activities in fractions from control and experimental rat retinas

	AchE (U mg^{-1} protein)	BchE (U mg^{-1} protein)
Control		
H	3.67±0.230[a]	0.178±0.011[a]
S_1	0.93±0.048[a]	0.520±0.027[a]
S_2	3.89±0.240[a]	0.312±0.019[a]
P	0.78±0.049[a]	0.379±0.024[a]
Diabetic		
H	2.11±0.130[b]	0.085±0.005[b]
S_1	0.62±0.039[b]	0.228±0.014[b]
S_2	1.77±0.130[b]	0.165±0.012[b]
P	0.528±0.034[b]	0.180±0.013[b]
NBDP+Metformin		
H	3.19±0.170[c]	0.126±0.010[c]
S_1	0.78±0.043[c]	0.319±0.020[c]
S_2	3.12±0.180[c]	0.234±0.013[c]
P	0.60±0.037[c]	0.256±0.016[c]

Cholinesterases were determined in homogenate (H), solubilized enzyme without (S_1) and with detergent (S_2) and in the pellet (P) as described in methods. Cholinesterase activity is given in units (U- µmoles of substrate hydrolyzed per hour). Values are given as mean±SD from 6 rats in each group; ANOVA followed by DMRT. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

increased in nSTZ diabetic rats. Treatment with NBDP and metformin significantly ($p< 0.05$) brought down the levels of these lipid peroxide markers to near normal levels.

Table 2 shows the two-step procedure to the solubilization of cholinesterase from control and experimental rat retinas. The summed cholinesterase activity recovered in S_1+S_2+P was considered as 100%. The extent of enzyme solubilization in S_1+S_2 refers to this summed activity. Application of the two-step procedure to the solubilization of cholinesterase from control and diabetic rat retinas led to the release of approximately 33.0 and 54.49% of AChE and 48.57 and 47.11% of BChE. Similarly diabetic and NBDP and metformin treated group release of approximately 26.58 and 67.79% of AChE and 38.54 and 41.81% of BChE respectively. Table 3 represents the AChE and BChE activity in the S_1 and S_2 fraction of diabetic rat retina that showed a decrease with respect to control rats. The activity was significantly increased in NBDP and metformin treated rat retinas.

DISCUSSION

Diabetes related eye diseases are the major health problem in most countries where large proportions of the population is suffering from diabetes [37]. The combined administration of NBDP and metformin to decrease the elevated blood glucose level to normal glycemic level is an essential trigger for the liver to revert its normal homeostasis during experimental diabetes. The possible mechanism by which NBDP, belong to the group of hypoglycemic agents that induce insulin secretion through the interaction with sulfonylurea receptor in the plasma membrane of the pancreatic beta cell [38]. NBDP promote insulin secretion by closure of K^+-ATP channels, membrane depolarization and stimulation of Ca^{2+} influx, an initial key step in insulin secretion. Metformin reduces fasting plasma glucose level by reducing rates of hepatic glucose production [39, 40], its effect on the relative contributions of hepatic glycogenolysis [41, 42] and gluconeogenesis [43]. Combination treatment significantly decreases blood glucose and increases plasma insulin level compared with either drug alone.

The balance between production and catabolism of Reactive Oxygen Species (ROS) by cells is critical for maintaining biological integrity. Ocular tissues contain antioxidants that prevent damage from excessive reactive oxygen metabolites either by decomposing or trapping ROS [12].

Our results show that in hyperglycemic rats the activities of AChE and BChE were significantly decreased. STZ, when injected intraperitonially in a diabetogenic dose to rats, has been found to cause prolonged impairment of glucose and energy metabolism [44]. Lipid peroxidation as reflected by increased TBARS may have induced the observed decrease in AChE and BChE. It has also been shown that increased concentration of free

radical can produce lipid peroxidation and decrease membrane fluidity. Therefore, decrease in AChE and BChE activities in diabetic rats reflect a decrease in membrane fluidity due to lipid peroxidation (TBARS), which may influence the enzyme activities through lipid-protein interaction. AChE and BChE activities measured in retina is supposedly related to cholinergic neurotransmission. The decreased activities were in accordance with other studies. The changes in cholinesterase activities might reflect impairment in biosynthetic, degradation or insertion into the plasma membrane. In this regard the diabetic state is known to cause membrane alteration that can affect the kinetic properties of the membrane bound cholinesterases [10].

The AChE and BChE specific activities were reduced considerably in S_1 and S_2 extract from diabetic rat retina. Cellular and secreted enzymes differ in the composition of molecular forms. The result suggests that diabetes produces specific changes in cholinesterases that could reflect impairment in the membrane. In agreement with those results, activities of all AChE and BChE molecular forms S_1 were reduced. Impairment in the activities has been reported in muscular dystrophies and Alzheimer's diseases [45-47]. Therefore the decrease in the activities of cholinesterases molecular forms may alter the retinal environment and this may precede the abnormalities observed in diabetic retinopathy.

The decreased AChE and BChE activities by the lipid peroxidation were reversed when diabetic rats were treated with NBDP and metformin. The observed stimulation of AChE and BChE in diabetic rats treated with NBDP may possibly due to increase in membrane fluidity. In a recent report, it has been suggested that Phenylalanine acts directly on AChE and BChE [48]. Therefore, phenylalanine seems to protect the enzyme against the direct actions of free radicals. This action could be exerted on cysteine, methionine, histidine and/or tyrosine residue to the AChE and BChE molecule [49-51]. It has been reported [52, 53] that phenylalanine reacts with OH radicals to form the aromatic hydroxylated production of o, m, p- tyrosine. Therefore NBDP also protect against the action of free radical on brain AChE and BChE activities.

Taken together our results suggest that decrease in cholinesterase activities may lead to impairment in retinal function. In addition to their role in cholergic neurotransmission several activities have suggested that cholinesterases are involved in non-synaptic functions such as morphogenesis, recognition ands cell adhesion mechanisms [11]. Changes we observed might lead to alteration associated to diabetic complications. We have shown that low cholinesterase activities in the rat retina at long periods after the induction of diabetes. It remains to be studies whether cholinesterases isoforms are regulated by insulin or hyperglycemia affects specific subset of cells expressing these isoforms. The role of insulin in nervous tissue is unclear, but the presence of insulin ands insulin growth factor has been reported in retina [54]. This growth factor may act as endogenous regulator of neural metabolism thus promoting cell survival.

Our data suggest that NBDP increased AChE and BChE activities increasing membrane fluidity and decreasing lipid peroxidation. This might be of special relevance for preserving the cells which constitute the retina and for maintaining the mutual action of retina. NBDP along with metformin might ameliorate the diabetes induced changes in the cholinesterases activities.

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