PROTECTIVE EFFECT OF POLYMYXINE B AND NIFEDIPINE ON DIABETIC COMPLICATIONS IN RAT: ROLE OF PROTEIN KINASE C

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Abstract- Patients with diabetes mellitus (DM), experience significant morbidity and mortality from microvascular retinopathy, nephropathy and neuropathy. Hyperglycemia can induce diabetic complications through multiple pathways. Activation of protein kinase C (PKC) by hyperglycemia is one of the pathways which causes diabetic complications. Effect of nifedipine (a calcium channel blocker), and polymyxine B sulphate (a Protein kinase C inhibitor) was studied in adult male Sprague- dawley rats, who was made diabetic with streptozotocin. PKC activity was determined in tissues and serum enzymes and metabolite level was measured in all controls, diabetic and drug treated animals. The results showed that, levels of the, urea (two –fold), creatinine (60%), triglyceride (two-fold) and liver alanine transaminase (ALT) activity (two-fold), were significantly increased in diabetic group. In nifedipine, treated diabetic group, although urea and creatinine level was increased, but liver enzymes were not significantly different from those of control group. In diabetic group which was treated with polymyxine, all the measured metabolites and enzyme levels were the same as the control group, except glucose level which was increased and liver glycogen was decreased significantly. Protein kinase C activity in the cytoplasm of diabetic liver was increased comparing to its control group (5.73 ± 0.56 Vs, 4.00 ± 0.62). The enzyme activity in the plasma membranes of untreated and nifedipine treated diabetic groups was significantly increased (6.2 ± 0.42 and 3.66 ± 0.31 Vs 2.38 ± 0.36). These results show that polymyxine is more effective than nifedipine against protein kinase C activity in diabetic complications. In conclusion our results show that, liver and kidney damage in DM are related to PKC activation. The fact that polymyxine prevents diabetic related increase in PKC activity more than nifedipine, support the hypothesis that different PKC isozymes may play different roles in the development of diabetic complications.

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Key Words: Protein kinase C, nifedipine, polymyxine B sulphate, diabetic complications

INTRODUCTION

The increasing burden of diabetes mellitus (DM) and its complications is alarming. Each year, an estimated 130,000–150,000 people develop blindness, kidney failure and vascular disease from diabetes related complications (1). Patients with diabetes experience significant morbidity and mortality from microvascular retinopathy, nephropathy, neuropathy, and macrovascular heart attacks, stroke or peripheral vascular complications (2). Phospholipid- and calcium-dependent protein kinase (PKC) is involved in the regulation of physiological responses to many different stimuli and mediates a variety of events ranging from cellular proliferation, differentiation and exocytosis to the control of neural activity (3,4). PKC distribution between cytosolic and membrane fraction is dependent upon physiological state and tissue source. PKC has different isozymes in mammalian tissues, of which some are calcium dependent and some calcium independent. The activation of PKC is believed to occur upon distribution of the inactive cytoplasmic enzyme to the phospholipid environment of membrane (5). Hyperglycemia can induce vascular complications probably through multiple pathways. The mechanism of hyperglycemia's effect on the diacylglycerol and protein kinase C pathways is reviewed and its intracellular and *in vivo* effects on the vessels are described (6). The activation of PKC induced by hyperglycemia appears to be due to an increase in diacyglycerol levels, a physiological activator of PKC. Studies involving cultured cells, animal models of diabetes and patients have shown that inhibition of PKC by specific PKC inhibitors was able to reverse many of the vascular dysfunctions in the retina, kidney, and cardiovascular system, induced by either hyperglycemia or diabetes (7). Most studies

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have used immunological methods to detect PKC level, which does not correlate with enzyme activity (8). In various forms of liver and kidney damage, serum level of numerous enzymes and metabolites are increased. In this study diabetes was induced by streptozotocin in rat, a rodent model. The aim of this study was to use polymyxine B sulfate as a PKC inhibitor and nifedipine as a calcium channel blocker which reduces intracellular calcium concentration. PKC activity and serum enzymes and metabolite concentrations were investigated.

MATERIALS AND METHODS

Chemicals and animals

All chemicals, drugs and coupling enzymes of the highest purity available were obtained from Boehringer (Mannheim, Germany) or Merck (Darmstadt, Germany). Male Sprague-dawley rats were purchased from Pasture Institute (Tehran, Iran)

Experimental design

Adult male Sprague-dawley rats were received at body weight ranging from 150-250 grams and maintained with *ad libitum* feeding for two weeks before assignment to one of the following experimental groups (6 animal in each group): group I, diabetic rats; group II, nondiabetic control rats: group III, diabetic rats treated with nifedipine; group IV, nondiabetic rats treated with nifedipine; group V, diabetic rats treated with polymyxine B sulfate; and group VI, non diabetic rats treated with polymyxine B sulfate. Diabetes was induced by injection of 60-65 mg/kg i.p. anhydrous streptozotocin and citric acid reconstituted with 0.9% saline (9). The control groups received citrate buffer alone. Rats were weighted twice weekly when tail vein blood was collected for the determination of fed plasma glucose concentration. Rats which have less than 200 mg/dl glucose were excluded from the experiments. Diabetic rats were maintained diabetic for 7days. After 7 days of hyperglycemia in diabetic rats; group III diabetic and group IV nondiabetic rats were treated daily with 40mg/kg oral nifedipine (a calcium channel blocker) (10), and group V diabetic and group VI non diabetic rats were injected 1 mg/kg i.p polymyxine B sulfate (PKC inhibitor) in phosphate buffered saline (PBS) daily for 4 months (11). During this time, the diabetic and control animals were housed in wired cage each containing only one animal. After 4 months of induction of diabetes, animals were killed by decapitation; blood was removed for metabolite determination and liver and kidneys were removed and immediately transferred to – 80°C for storage until use.

Metabolite and serum enzymes determination

Serum was prepared by centrifugation of the blood for 10 min at 3000 g, 4°C, and stored at –20°C. Glucose was determined with hexokinase, urea with diazine, creatinine with *Jaffe* reaction, phosphate with ammonium molibdate and calcium with ortho-cresolphthalein complexion, all standards methods (12). Plasma cholesterol and triglyceride were measured with coupling enzymes assay (13). Activity of plasma aspartate and alanine transaminases (AST and ALT respectively) was determined with coupling enzyme continuos-monitoring (14). Lactate dehydrogenase (LDH) was assayed by measurement of NADH consumption at 340 nm (15), and activity of alkaline phosphatase (ALP) was determined by using para nitrophenyl phosphate as the substrate (16). Liver glycogen was extracted and digested with amyloglucosidase according to Keppler and Decker (17), and released glucose was determined with hexokinase method. Preparation of enzyme extract and protein kinase C assay in liver and kidney cytoplasm and membrane fraction was done exactly as described previously (18,19).

Data analysis

Statistical analysis used were the Student's t-test or one-way analysis of variance (ANOVA) followed by the Dunnett's test.

RESULTS

Body weight: Table. 1 shows that at the time of study (4 months after induction of diabetes), the body weight of group I diabetic rats was significantly reduced, but in the other diabetic groups which received the drugs (groups III & V), the body weights were not significantly changed. In all of the non-diabetic control groups, the body weight was significantly increased during the experimental period.

Metabolite and serum enzymes

The serum glucose concentrations were significantly increased in all diabetics compared to their control groups (Fig. 1). The mean glucose con-

centration in all groups before the experiments was 121 ± 16 mg/dl postprandial, and was not significantly different among the groups. The blood urea nitrogen (BUN) concentration in group I diabetic rats was increased two-fold comparing to the group II control group (Table 2). In group III diabetics, which received the daily nifedipine, the serum urea concentration versus control non-diabetic (group IV) was increased, but in the group V diabetics which was treated with polymyxine, there were no significant differences with group VI control group. The serum creatinine concentration also followed the same pattern as urea concentration. The serum creatinine concentrations in group I and III diabetic groups were significantly increased compared to group II and IV control groups, but there was no significant change in serum creatinine of group V diabetics and group VI control groups, who received polymyxine (Table 2). Serum phosphate and calcium concentration was not significantly changed among all experimental groups (Table 2).

The serum triglyceride level among experimental groups is shown in fig. 2. The serum triglyceride in group I and group III versus group II and IV control groups was increased. This increase in group V was not significant compared group VI control group. The serum cholesterol level was increased in group 1 diabetic group but this increase was not significant in other experimental groups (Fig. 3).

Table 1. Animal body weight changes during experiments (4 months)

Experimental Group	Primary weight (gram)	Final Weight (gram)
Group I	236 ± 16	201 ± 14
Group II	214 ± 18	263 ± 21 *
Group III	211 ± 9	213 ± 26
Group IV	219 ± 7	299 ± 23 *
Group V	227 ± 19	243 ± 25
Group VI	228 ± 14	295 ± 25 *

* All data in tables and figures are meaning ± SEM. Numbers of animal in each group are 6.

* Significantly different from diabetic groups, $p<0.05$.

Table. 3 shows that the serum enzymes activity. ALT activity in group I increased almost two-fold, but there was no significant change in other groups. AST activity was not changed in all groups. LDH activity was increased in group I and III diabetic groups, but this change was not significant in group V diabetic group. Activity of ALP was increased in all diabetic groups, but this increase in group I was prominent. The liver glycogen level is shown in Fig. 4.

Table 2. Serum metabolite concentration in experimental groups

Groups	Urea(BUN) (mg/dl)	Creatinine (mg/dl)	Calcium (mg/dl)	Phosphorus (mg/dl)
Group I	31.1 ± 2.8 *	1.30 ± 0.02	9.0 ± 0.6	3.4 ± 0.3
Group II	16.5 ± 1.4	0.78 ± 0.03	7.6 ± 0.5	3.8 ± 0.1
Group III	28.5 ± 2.9 *	1.10 ± 0.08 *	8.2 ± 0.4	4.2 ± 0.2
Group IV	14.8 ± 2.6	0.79 ± 0.02	8.1 ± 0.2	4.1 ± 0.2
Group V	16.3 ± 1.7	0.81 ± 0.04	8.2 ± 0.3	3.9 ± 0.1
Group VI	13.4 ± 1.9	0.78 ± 0.01	8.0 ± 0.1	4.3 ± 0.3

* Significantly different from its control non-diabetic group, $p< 0.05$

Table 3. Serum enzymes activity in experimental groups

Groups	ALT (U/L)	AST (U/L)	LDH (U/L)	ALP (U/L)
Group I	12.7 ± 1.1 *	8.4 ± 1.1	347 ± 153*	496 ± 66 #
Group II	6.8 ± 1.6	7.7 ± 0.7	124 ± 40	241 ± 30
Group III	6.7 ± 1.9	7.2 ± 0.8	218 ± 61 *	339 ± 113
Group IV	6.2 ± 1.2	5.8 ± 0.6	112 ± 47	184 ± 25
Group V	6.6 ± 1.8	7.6 ± 1.2	103 ± 43	285 ± 44
Group VI	5.8 ± 1.3	4.9 ± 1.6	97 ± 45	181 ± 26

* Significantly different from its control non-diabetic group, $p< 0.01$

#. Significantly different from all experimental groups, except group III, $p< 0.05$

Table 4. Protein kinase C activity in kidney and liver of experimental groups

Group	Liver cytoplasm (nmol/gww[1])	Liver membrane (nmol/gww)	Kidney cytoplasm (nmol/gww)	Kidney membrane (nmol/gww)
Group I	5.73 ± 0.56 *	6.20 ± 0.42 #	10.68 ± 2.32	3.06 ± 0.52 *
Group II	4.00 ± 0.62	2.38 ± 0.36	9.68 ± 1.81	1.74 ± 0.16
Group III	4.89 ± 1.75	3.66 ± 0.31 *	9.88 ± 0.62	2.98 ± 0.54
Group IV	5.52 ± 1.37	2.25 ± 0.50	7.36 ± 1.28	2.34 ± 0.66
Group V	5.84 ± 1.38	2.92 ± 0.97	3.20 ± 0.56	2.10 ± 0.23
Group VI	5.04 ± 0.83	2.33 ± 0.77	3.42 ± 0.76 @	1.84 ± 0.20

1. gww = gram wet weight
* Significantly different from its control non-diabetic group, p< 0.05
#. Significant Vs its control and other experimental groups, p< 0.01
@. Significant Vs all other experimental groups except its control group, p< 0.01

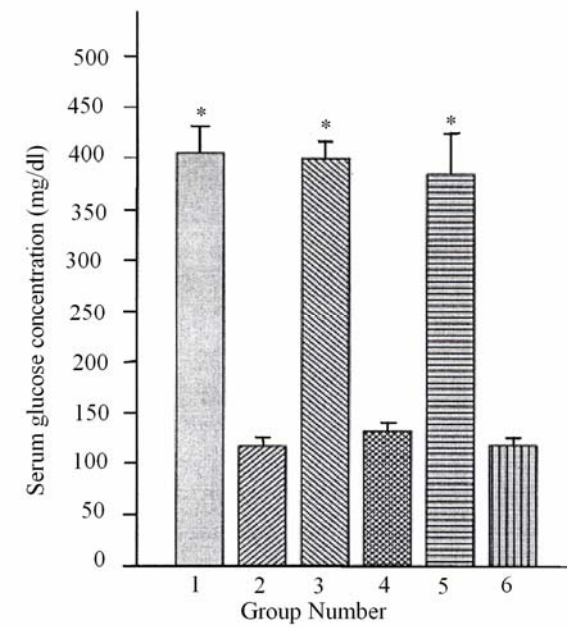

Fig. 1. Serum glucose concentration in experimental groups
* Significantly different from their control groups, p< 0.01

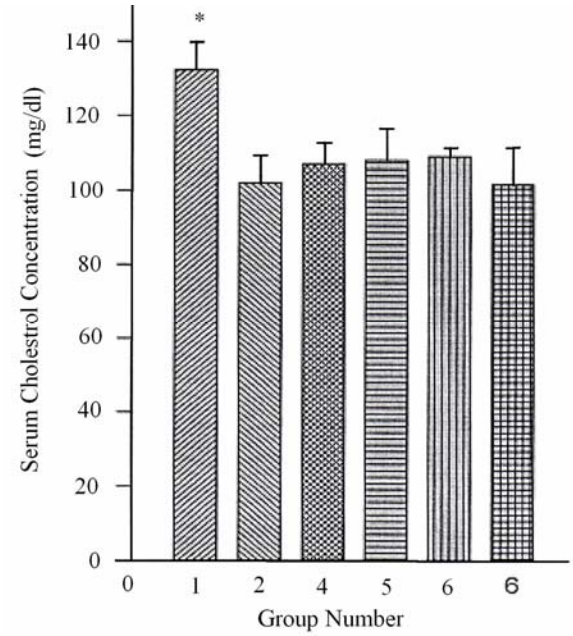

Fig. 3. Serum cholestrol level in experimental groups
*Significantly different from all other experimental groups, p< 0.05

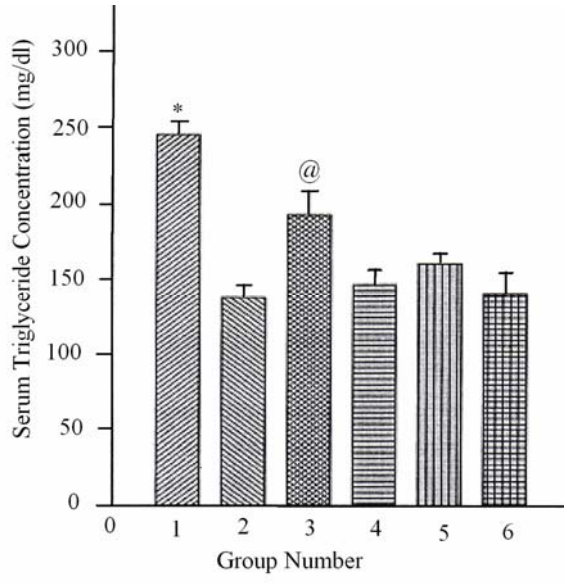

Fig. 2. Serum triglyceride level in experimental groups
*Significantly different from all other experimental groups, p< 0.01.
@ Significantly different from its control group, p< 0.05.

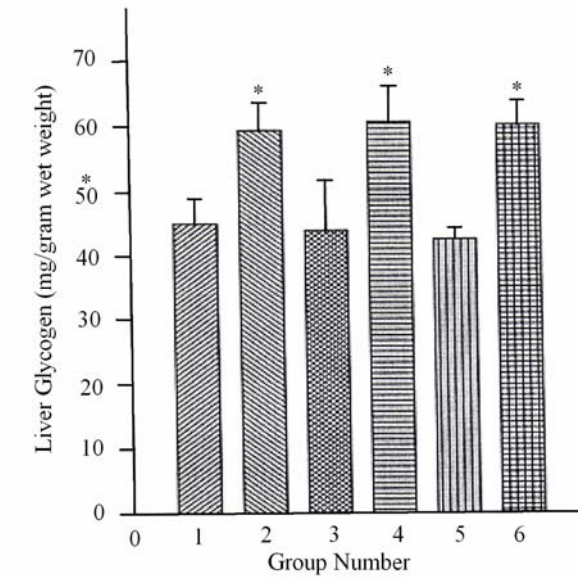

Fig. 4. Liver glycogen content in experimental groups
*Significantly different from the corresponding control groups P< 0.05

In all diabetic groups, the liver glycogen content comparing to control groups was significantly decreased. Table 4 shows the activity of PKC in livers and kidneys of diabetic Vs control rats. The liver cytoplasmic PKC activity in group I, was significantly higher than the other groups. The liver membrane fraction enzyme activity was higher in all diabetic groups. But these increases in groups I and III diabetic groups, were most significant. Kidney cytosolic PKC activity was significantly decreased in group V and IV (received polymyxine). The kidney membrane PKC activity was only increased in group I diabetics.

DISCUSSION

Diabetes mellitus is a chronic illness that requires continuing medical care and education to prevent and to reduce the risk of acute and long term complications. In this study, effects of nifedipine, a calcium channel blocker, and polymyxine, a PKC inhibitor, were investigated. Our results show that in spite of polyphagia, one of the prominent signs in diabetes is the body weight loss. The serum glucose was significantly increased reaching 350 mg/dl in diabetic animals, which agree with other studies (20). Increase in serum urea and creatinine is a sign of kidney damage in diabetic groups. Total and membrane bond PKC activity was also increased in diabetic animal kidneys, but this increase in animals receiving polymyxine B sulfate was not significant comparing to their control groups. Koya et. al have shown that, two weeks after induction of diabetes, total PKC levels were significantly increased in diabetic rat glomeruli (21). Nifedipine was not as effective as polymyxine in lowering PKC activity, urea and creatinine concentration. There may be different isozymes of PKC in the kidney, which are not calcium dependent (22). Increase in liver specific enzymes such as ALT and non-specific enzymes such as ALP and LDH activity and triglyceride level in serum show liver damage. ALT activity correlates with PKC activity and glycogen content in diabetic animal livers. Both nifedipine and polymyxine were effective in lowering ALT activity and serum triglyceride levels. Nawrocki et. al, also found that diabetes resulted in an almost two-fold increase in plasma fatty acids concentration (23). These data indicate that liver and kidney damage are related to PKC activity. While both drugs are known for their different mechanisms of actions, the fact that polymyxine prevents diabetes-related increase in PKC activity more than nifedipine, support the hypothesis that different PKC isozymes may play a role in the development of diabetic complications.

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