ANTIDIABETIC AND VASCULAR RESTORATION ACTIVITY OF VALPROATE

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Summary

Untreated type 2 diabetes may leads to various complications in diabetic subjects. We aimed to determine the effect of sodium valproate treatment in diabetes and associated vascular complications in diabetic rats. Diabetes was produced by high fat diet and low dose of streptozotocin (STZ). After 2 week of high fat fed rats were injected with STZ (35 mg/kg, i.p.) to develop type 2 diabetes. After six week of STZ administration diabetic rats were divided in various treatment groups according to fasting blood glucose. Sodium valproate (100, 200 and 300 mg/kg, p.o., once in a day) treatment was given for four weeks in diabetic rats. Various biochemical parameters (glucose, insulin, triglyceride and cholesterol) and tissue parameters (liver glycogen, SOD, Catalase and Lipid peroxidation of aorta) were measured before initiation and after completion of treatment in all groups. After the completion of treatment all rats were sacrificed under mild anesthesia and thoracic aorta was isolated. Contractile response of H_2O_2 (10^{-6} M to 10^{-3}M) and Angiotensin II (10^{-11} to 10^{-7} M) in control, diabetic and treated rat thoracic aorta was measured on multi channel data acquisition system Poworlab/SSP (AD Instruments. Australia). Treatment with sodium valproate significantly reduced the blood glucose, insulin, triglycerides and cholesterol in 300 mg/kg dose indicates the hypoglycemic and hypolipidemic activity. Four week sodium valproate treatment also showed significant increase in SOD, Catalase and decrease in MDA levels, indicate anti-oxidant activity. Contractile responses of H_2O_2 and Angiotensin II were increased in diabetic rats compared to normal control rats. While treatment with sodium valproate showed the decrease in % Emax in rat thoracic aorta in dose dependent manner. From this results it can be concluded that sodium valproate treatment reduces the diabetic state as well as diabetes induced vascular dysfunction.

Key words: Diabetes, Sodium valproate, H_2O_2, Angiotensin II and Vascular complications.

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and hyperinsulinemia. Prolonged untreated diabetes mellitus may leads to various chronic complications like nephropathy, retinopathy, neuropathy and vascular complications. Vascular complications associated with the diabetes are major cause for the increased morbidity and mortality in diabetic patients[1-4]. Diabetes is still not completely curable by the present anti diabetic agents. Insulin therapy is the only satisfactory approach in diabetic mellitus, even though it has several drawbacks like insulin resistance[5], anorexia, brain atrophy and fatty liver in chronic treatment[6].

Valproate is a broad spectrum anticonvulsant drug. Valproate has multiple mechanisms for its anticonvulsant action, mainly by inhibiting the degradation and increasing the release of inhibitory neurotransmitter GABA. Valproate also inhibits the Glycogen Synthase Kinase-3, an enzyme involved in the insulin signaling pathway and apoptosis. GSK-3 is a serine threonine kinase and has important role in the regulation of Glycogen Synthase and thereby regulates the synthesis of glycogen. So our main aim of the project was to elucidate the effect of sodium valproate treatment on diabetes and associated vascular complications.

Materials and methods:

Animals: Male *Sprague Dawley* rats of weighing 250-300g were procured from central animal facility of S. K. Patel College of Pharmaceutical Education and Research. The animals were maintained in controlled temperature as well as humidity. Animals were free to access water and feed. Experimental protocol was approved by IAEC.

Chemicals: Streptozotocin and Angiotensin II were purchased from the Sigma Chemicals Company (St. louis, MO, USA.), Sodium Valproate (provided as a gift sample from Sun Pharma. Ltd., Baroda) and H_2O_2 (Merck Ltd, India). Dilutions will be made with Kreb-Hansellet solution that is free of glucose. Adjust the pH 7.4 and it was done by addition of 0.1 N NaOH if required. Drug concentrations were expressed as final molar concentration in bath solution.

Induction of diabetes in rats: Healthy *S.D.* rats showing normal plasma glucose level in the range of 80-120 mg/dl were used. Animals were fed with high fat diet for two weeks prior to Streptozotocin (STZ) injection and were continued till the end of study. A single dose of streptozotocin (35mg/kg, i.p.) was administered for induction of diabetes. Plasma glucose level was measured after 72 hours of streptozotocin treatment. Those animals showing fasting blood glucose more than or equal to 250 mg/dl were considered as diabetic and was used for further studies. Diabetic animals were also fed with the high fat diet till the experiment termination. Plasma glucose was measured again at the end of every week to confirm consistent hyperglycemia.

Study design: Animals were grouped into normal control, diabetic control and Sodium Valproate (100 mg/kg, 200 mg/kg and 300 mg/kg) treated diabetic rats. Total duration of the study was 12 weeks. After the STZ administration diabetic animals were kept as such for six weeks without any treatment for the development of vascular complications. Development of vascular complications was confirmed by measuring the blood pressure using tail-cuff BP measurement with NIBP controller on Powerlab. Rats were acclimatized in rodent restrainer for

half an hour before recording. An average of three recordings will be made for each rat. After confirmation of vascular complications diabetic rats were treated with sodium valproate for four weeks.

Biochemical analysis: The blood samples (approximately 0.3 ml) were collected from rat tail vein under light anesthesia in heparinized centrifuge tubes. The plasma was separated by centrifugation (5000 rpm, 5 min at 4 °C) and analyzed for glucose (GOD-POD), triglycerides (GPO-POD) and total cholesterol (CHOD-POD) using commercially available spectrophotometric kits. The remaining plasma samples were stored at -20 °C till the insulin determination was made by ELISA kit using rat insulin as standard.

Glycogen Estimation from Liver: Glycogen estimation was done according to method described by Osterberg A. E., 1929[7]. It has been definitely established that 60 per cent potassium hydroxide at 100 ºC does not destroy glycogen, that glycogen is quantitatively precipitated from a 70 per cent solution of alcohol, and that the optimal condition for its conversion to glucose is in 2.2 per cent hydrochloric acid at 100 ºC. Following the conversion of glycogen to glucose, the Glucose can be estimated with the help of photometric method. Glycogen estimation was done in terms of glucose equivalent to glycogen in mg/gm of tissue unit.

In vivo antioxidants study:

Assay for SOD activity:
Isolated thoracic aorta was cleaned of surrounding fat and homogenized in 50 mM PBS buffer pH 7.0 using homogenizer. Homogenate was then centrifuged at 4 ºC; 15,000 rpm for 10 min. Supernatant was used for the estimation of SOD activity by adrenaline auto-oxidation method as described by Misra et al., 1972[8].

Assay for catalase activity:
Catalase activity was measured according to Grover et al., 2000[9]. Thoracic aorta was homogenized (20 mg of tissue/ml of PBS, pH 7.0) and centrifuged at 4 ºC (15,000 rpm for 10 min). The supernatant obtained was used for the assay. The degradation pattern of exogenously added H_2O_2 by catalase enzyme present in 200 µl of tissue supernatant was monitored at 240 nm in spectrophotometer at 15 s interval for 5 min and its activity calculated. Catalase activity is expressed as U/mg of protein. Protein was estimated by Lowry's method.

Lipid peroxidation assay:
The concentration of MDA [thiobarbituric acid reactive substance (TBARS)] was assayed using the method described by Beltowski et al., 2000[10]. 1ml of tissue supernatant of thoracic aorta was mixed with 1ml of 10% trichloroacetic acid and allowed to stand for 30 min at 37 ºC. Then 1ml of 0.67% (w/v) thiobarbituric acid and 20µl of 20% butylated hydroxytoluene (BHT) and the sample were heated at 95 ºC for 30 min in boiling water bath. After cooling to room temperature, 2ml of *n*-butanol was added and vortex immediately and centrifuged for 5 min at 5000 rpm. The organic layer was removed and its absorbance was measured at 532 nm. The concentration of MDA is expressed as nM of MDA/mg of tissue.

Vascular reactivity study: Ten weeks post-STZ administration, the rats were sacrificed and thoracic aorta was isolated from the heart to the diaphragm and cleaned of surrounding fat and connective tissues. Care was taken not to stretch the vessel. Helical strips of aorta of 2-3mm in width and 22 mm in length was cut with sharp iris scissors and placed in 10 ml organ bath containing Krebs–Henseleit buffer (NaCl 118 mM; KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $CaCl_2 \cdot 2H_2O$ 2.5mM, $NaHCO_3$ 25mM and glucose 5.5 mM) of pH 7.4 and osmolality (280–308 mOsmol). The solution was continuously aerated with 5% carbogen at 37 ºC. A resting tension of 2 g was applied to the strips and allowed to equilibrate for 2 h. After 2 h of equilibration, two wake up responses of KCl (80 mM) were recorded following which concentration response curves (CRC) of H_2O_2 (10^{-6} to 10^{-3} M) and Angiotensin II (10^{-11} to 10^{-7} M) were recorded in age matched normal and diabetic rat thoracic aortas. Changes in the isotonic contraction were recorded. The maximum vasoconstrictor response to the H_2O_2 or Angiotensin II in normal control aorta was considered as 100%. In case of sodium valproate treated rats, contractile responses of H_2O_2 and Angiotensin II were taken without incubation of any drug. These responses were compared with responses obtained from untreated diabetic rats.

Data and statistical analysis: Data were expressed as mean ± standard error of mean (SEM). Significances were determined using the Unpaired Student's t-test. Statistical comparisons between all groups were performed by using two tailed one way ANOVA followed by Dunnett test. P-values < 0.05 were considered statistically significant.

Results

Effect of sodium valproate on various biochemical parameters:
Treatment with sodium valproate in varying dosage for four weeks reduces the systolic blood pressure, fasting blood glucose, insulin, liver glycogen content and lipid profile dose dependent manner. The values are shown in table 3.1

Table 3.1 Effect of sodium valproate on body weight, blood pressure and biochemical parameters

Biochemical Parameteres	Normal	Diabetic	Sodium Valproate (100 mg/kg)	Sodium Valproate (200 mg/kg)	Sodium Valproate (300 mg/kg)
Body weight (gms)	288.56 ± 6.14	290.20 ± 8.15	299.16 ±4.48	302.94 ± 5.08	307.55 ± 6.19
Systolic blood pressure (mmHg)	116.25 ± 3.94	187 ± 3.33^{c}	184.3 ± 3.20	170.34 ± 4.62*	142.62 ± 4.53***
Glucose (mg/dl)	93 ± 7.83	432.28 ± 28.22^{c}	366.81 ± 19.32	301.83 ± 20.34**	267.15 ± 24.49**
Insulin (pmol/L)	168.31 ± 10.04	351.58 ± 20.44^{c}	319.31 ± 14.71	287.79 ± 12.57*	192.51 ± 21.19***
Triglycerides (mg/dl)	31.21 ± 5.93	280 ± 17.15^{c}	226.00 ± 13.61**	179.99 ± 18.45**	92.79 ± 9.91***
Cholesterol (mg/dl)	54.09 ± 6.21	210.34 ± 17.64^{c}	206.00 ± 13.12	169.55 ± 9.89*	141.58 ± 9.78**
Glycogen (mg/gm of tissue)	1.36 ± 0.04	1 ± 0.05^{c}	1.12 ± 0.04	1.17 ± 0.09***	1.18 ± 0.03***

Values are expressed as mean ± SEM. n = 6. *p<0.05, **p<0.01, ***p<0.001 compared with diabetic control. ^{C}p<0.001 compared with normal control.

Effect of sodium valproate on various antioxidant parameters:
Treatment with Sodium Valproate in varying dosage for four weeks significantly increases the level of antioxidant enzymes like SOD and Catalase and reduces the level of lipid peroxidation in terms of MDA content in dose dependent manner. The values are shown in table 3.2

Table 3.2 Effect of Sodium Valproate on various antioxidant parameters

Antioxidant Parameteres	Normal	Diabetic	Sodium Valproate (100 mg/kg)	Sodium Valproate (200 mg/kg)	Sodium Valproate (300 mg/kg)
SOD (U/mg protein)	25.84 ± 0.13	4.14 ± 0.09 ^{c}	12.32 ± 1.31***	14.32 ± 2.20***	18.68 ± 2.23***
Catalase (U/mg protein)	5.87 ± 0.19	3.32 ± 0.22 ^{c}	3.47 ± 0.19	5.01 ± 0.23**	5.33 ± 0.17**
MDA (nM MDA /mg protein)	0.20 ± 0.04	0.56 ± 0.01^{c}	0.48 ± 0.05	0.39 ± 0.03***	0.28 ± 0.04***

Values are expressed as mean ± SEM. n = 6. **p<0.01, ***p<0.001 compared with diabetic control.
^{C}p<0.001 compared with normal control.

Effect of Sodium Valproate treatment on H_2O_2 and Angiotensin II induced contraction:
The comparison of H_2O_2 and Angiotensin II induced contractions between normal rat thoracic aorta, diabetic rat thoracic aorta and thoracic aortas of Sodium Valproate treated groups shown in figure 3.3.1. While pD2 values of H_2O_2 and Ang II induced contraction in different treatment groups mentioned in table 3.3.1.

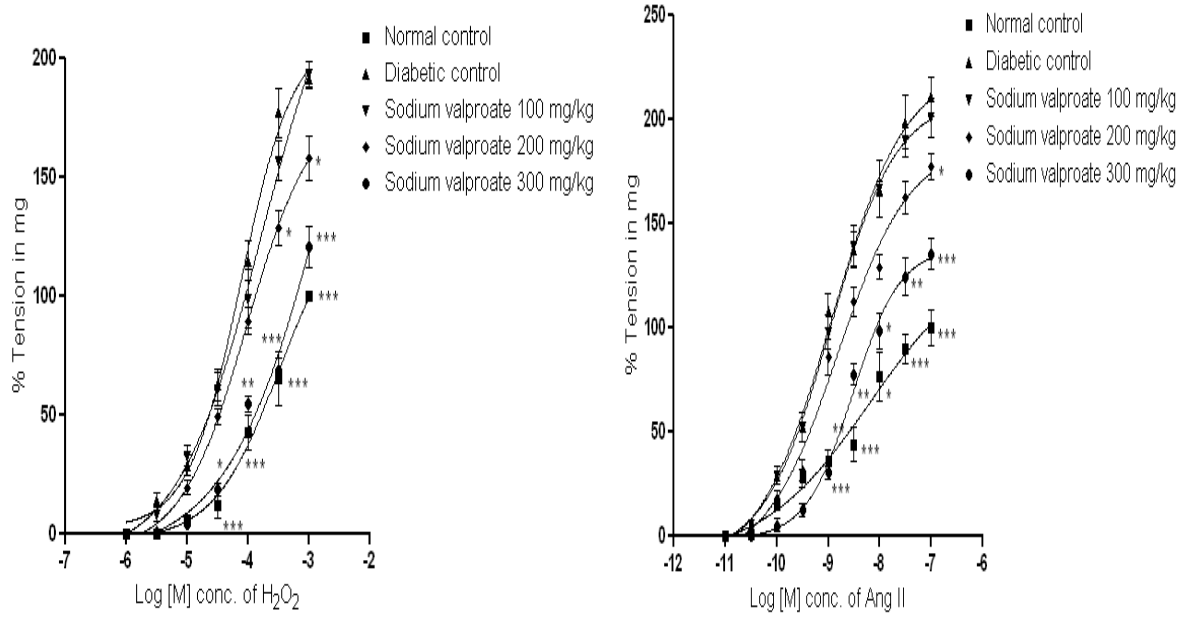

Figure 3.3.1 Cumulative concentration response curve of H_2O_2 and Angiotensin II on endothelium intact aortic spiral preparations obtained from untreated age matched normal rats (■), diabetic rats (▲), sodium valproate 100 mg/kg (▼), 200 mg/kg (♦) and 300 mg/kg (●) treated diabetic rats. Values are expressed as mean ± SEM. n = 6. *p<0.05, **p<0.01, ***p<0.001 Vs respective control group.

Table 3.3.1 pD_2 values and % Emax of H_2O_2 and Ang II induced contraction in different treatment groups

Groups	H_2O_2 induced contraction		Ang II induced contraction	
	pD_2 Value	% Emax	pD_2 Value	% Emax
Normal	3.76 ± 0.08	100. 06 ± 3.45	8.63 ± 0.14	99.98 ± 4.37
Diabetic	4.18 ± 0.13 #	191.42 ± 8.56 ###	8.91 ± 0.12	210. 75 ± 10.99 ###
Sodium Valproate (100 mg/kg)	4.19 ± 0.23	193.32 ± 10.35	8.91 ± 0.11	200.08 ± 13.64
Sodium Valproate (200 mg/kg)	4.11 ± 0.14	157.46 ± 8.57*	8.79 ± 0.16	170.73 ± 12.22*
Sodium Valproate (300 mg/kg)	3.72 ± 0.19	120.61 ± 8.57***	8.53 ± 0.17	135.48 ± 7.34***

Values are expressed as mean ± SEM. n = 6. #p<0.05, # # #p<0.001 compared with normal control. *p<0.05, ***p<0.001 compared with diabetic control

Discussion

This study showing that in diabetic rats, treatment with different dosage of sodium valproate results an improvement in systolic blood pressure, fasting blood glucose, insulin sensitivity, lipid profile and vascular oxidative stress. In addition, there was an improvement in hydrogen peroxide and Angiotensin II induced contractile responses in thoracic aortas of sodium valproate treated diabetic rats. However there is no significant decrease in pD_2 values of H_2O_2 and Angiotensin II in thoracic aortas of sodium valproate treated diabetic rats compared to diabetic control rats.

High fat fed and low dose of STZ treated rats is one of the best models for screening the drugs effective in type 2 diabetes and associated complications[11]. After the STZ administration rats were kept for six weeks on same high fat diet without any treatment for development of insulin resistance and vascular complications. Our finding shows that six weeks were sufficient to significant increase in fasting blood glucose, insulin level and systolic blood pressure. As we know Insulin resistance plays a primary role in the development of type-2 diabetes[12] and is a characteristic feature of other health disorders including obesity, hypertension and cardiovascular disease[13]. GSK-3 activity is increased in skeletal muscle and adipose tissues of obese rodents and in skeletal muscle of obese humans with type 2 diabetes[14], and this elevated GSK-3 activity is associated with decreased insulin sensitivity[15]. Sodium valproate treatment in dose dependent manner decreases hyperglycemia, plasma insulin level, lipid profile and systolic blood pressure. While the treatment of sodium valproate also increased the glycogen synthesis in diabetic rats, this increase in glycogen synthesis can be correlated with GSK-3 inhibition by sodium valproate.

Oxidative stress implies an imbalance between the production of reactive oxygen species and the antioxidant defense system. Markers of oxidative stress are increased in individuals with diabetes and insulin resistance[16]. Lipid peroxidation products such as MDA are generated under high levels of un-scavenged free radicals[17]. These products may be important in the pathogenesis of vascular complication in diabetes mellitus[18]. Our findings demonstrated that sodium valproate has antioxidant activity which reduces development of free radicals inside the vasculature and it may important for prevention of vascular complications.

Our findings also demonstrated that H_2O_2 and Angiotensin II induced enhanced contractile responses can be reduced by sodium valproate treatment. However, treatment did not show significant decrease in pD_2 values of H_2O_2 and Angiotensin II compared to diabetic rat thoracic aortas. From the above findings we can reveal that sodium valproate can be used as

hypoglycemic agent in diabetes and also in epileptic patients with diabetes and associated diabetic vascular complications.

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