Research Article

Effect of phenolic acids on functions of rat aorta, vas deferens and on metabolic changes in streptozotocin-induced diabetes

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ABSTRACT

Objectives: This study aimed to investigate the effects of antioxidant treatment on streptozotocin (STZ)-induced diabetic metabolic and smooth muscle (SM) complications in rats.
Materials and Methods: Threeweeks after STZ injection (i.v.), vehicle, *p*-OH benzoic (p-OHBA), protocatechic (PA) and gallic acids (GA) were separately administered (10 mg/kg each, i.p.) to the rats everyday for 3 weeks. Metabolic functions were observedregularly. The rats in all groups were sacrificed andaorta and Vas deferens were dissected. Theresponses of isolated organs to agonists (acetylcholine and phenylephrine) were recorded.
Results: Protocatechic acid prevented increase in food consumption and feces output significantly. The responses of isolated organs to agonists increased in the STZ-diabetic rats. The test drugs either prevented, exacerbated or didnot affect the SMchanges in the STZ-diabetic rats.
Conclusions: It was concluded that *p*-OHBA, PA and GA may cause effects independently of their antioxidant effect and/or of diabeticcomplications. They may exhibit pro-oxidant activities in the experimental conditions applied.

KEY WORDS: Aorta, diabetic complications, oxidative stress, phenolic acids, Vas deferens

Introduction

Free radicals have an important role in the pathogenesis of diabetes mellitus (DM). Oxidative stress (OS) and/or decreased antioxidant defenses are believed to play a critical role in the contribution of smooth muscle(SM)complicationsin vascular and reproductive system in DM.[1,2] It has been reported that the increasing of OS during diabetes, largely due to hyperglycemia, causes neuropathy as a diabetic complication.[3] As autonomic nerves are highly integrated with SM cells in various physiological systems, autonomic neuropathy due to experimental diabetes may cause consequent changes in SMs.[4,5] Conflicting responses to several agonists in SMs of experimentally diabetic animals have been reported suggesting the defective contractile process of SMs.[4]

Anomalies of function and structure of autonomic nerves induced with augmented OS may cause erectile dysfunction, impaired ejaculation and decreased fertility in diabetic animals.[6] Complications of the reproductive system may be a distressing feature for the male patients with long-term DM. Cardiovascular complications are also common.[7]

Treatment with antioxidants may prevent or ameliorate abnormal function and biochemistry of nerve and protect nerves against to free radicals damage.[8] Consequently it is postulated that these agents can prevent diabetic complications.[9] Phenolic acids (PhA) are well-known antioxidants, and also their antidiabetic activities have been reported in many studies.[10,11] The antioxidant activity of PhA correlated positively with the number of hydroxyl groups bonded to aromatic ring.[12]

In this study, we attempted to identify the possible effects of 3-week treatment with 10 mg/kg *p*-OH benzoic acid (*p*-OHBA), protocatechic acid (PA) and gallic acid (GA), which have antioxidant activities with increasing potency order related to their hydroxyl number, on diabetic SMs complications developed in aorta and vas deferens. Experimental procedures were applied on the isolated organs which were isolated from 6-week streptozotocin (STZ)-diabetic rats.

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Materials and Methods

Animals

Male Wistar rats (250–300 g) were used in this study. The animals were maintained on 12 h light/12 h dark cycle and at

24 ± 1°C with standard pellet diet and tap water ad libitum. Animal care and research protocols were based on the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH publication No: 85-23, revised in 1985) and approved by theLocal Ethics Committee of Osmangazi University, Eskisehir.

Chemicals

The chemical used included streptozotocin (Sigma, St. Louis, USA), Citric acid (Merck, Darmstadt, Germany), Trisodiumcitrate (Merck), *p*-OHBA (Sigma), PA (Sigma), GA (Sigma), Phenylephrine.HCl (Sigma), Acetylcholine.HCl (Sigma), NaCl (Merck), KCl (Merck), $MgCl_2$ (Merck), KH_2PO_4 (Sigma), $NaHCO_3$ (Merck), Glucose.H_2O (Merck), $CaCl_2.2H_2O$ (Merck).

Induction of Diabetes and Experimental Groups

The animals were injected with STZ in a single i.v. injection (50 mg/kg in 0.1 M citric acid buffer, pH 4.5, i.v.) to induce diabetes.[13] After 73 hours of injection, blood glucose levels were measured by Glukotrend® (Roche, Switzerland) and the ratswith a blood glucose level more than 300 mg/dl were selected. The other group was injected with citric acid buffer (i.v.) only to serve as non-diabetic control group. The diabetic rats were divided into 4 groups. The 1st group was used as STZ-diabetic control and injected with saline (i.p.). Three-weeks after induction of diabetes, the rats in the 2nd group were injected with (i.p.) 10 mg/kg *p*-OHBA, 3rd group was injected with 10 mg/kg PA and the last group was injected with 10 mg/kg GA everyday for 3-weeks. Blood glucose levels were measured at same hours in every week.

Metabolic-cages Measurements

The rats in all groups were individually housed in metabolic-cages (Ugo-basile, 41700, Italy) 3 weeks afterinduction of diabetes. Water and food intake, urine and feces excretion were monitored during 3 weeks. The weights of rats were also measured in every week.

Isolated Organ Bath Experiments

Both the diabetic and non-diabetic rats were sacrificed by cervical dislocation 6 weeks after induction of diabetes. Thoratic aorta and vas deferens were rapidly removed and placed in Kreibs-Henseleit solution (KHS) (g/L: NaCl-6.9544; KCl-0.3504; $MgCl_2$-0.0952; KH_2PO_4-0.1633; $NaHCO_3$-2.1002; Glucose.H_2O-2.20; $CaCl_2.2H_2O$-0.36) (pH=7.4). After cleaning of adhering fat and connective tissues, aorta ring was mounted with a resting tension of 1.0 g and vas deferens was mounted with a resting tension of 0.5 g in an isolatedorgan bath (Ugo-basile, 4050, Italy) containing 10 ml KHS, aerated with mixture of 95%O_2 and 5% CO_2 at 37°C.[14,15] The responses of isolated organs were recorded isometrically using a force-displacement transducer (Ugo-basile, 7003, Italy) connected to a pen recorder (Ugo-basile, 7070, Italy). After 1-h incubation period, concentration-response relationships of aorta were obtained with doses of 10^{-9}-10^{-3} M phenylephrine (PE). After resting period, aorta pre-contracted with PE (10^{-4} M) to relax inresponse to 10^{-9}-10^{-3} M acetylcholine (ACh). The vas deferens was firstly contracted with10^{-9}-10^{-3} MACh and then after resting period it was exposedto doses of 10^{-9}-10^{-3} M PE.

Statistical Analysis

All isolated organresponses were expressed as apparent affinity constant (pD_2) and percentage of corresponding maximal responses to each agonist (E_{max}). The statistical analyses were performed by one-way ANOVA, followed by Tukey's multiple comparison testsfor isolated-organ bath experiments, and Student '*t*' test for metabolic-cage measurements. The statistical analyses were carried out using GraphPad Prism version 5.0 and Microsoft Office Excel. The values were expressed asthe mean±S.E.M. to show variation in groups. $P \leq 0.05$ was considered statistically significant.

Results

Blood Glucose Levels

Blood glucose levels of all groups were observed to be significantly higher than the non-diabetic group and *p*-OHBA, PA and GA did not normalize hyperglycemia in STZ-induced diabetes [Figure 1].

Metabolic-Cage Measurements

p-OHBA, PA and GA did not change polyuria and polydipsia However, PA decreased the amounts of food intake and feces output significantly although *p*-OHBA and GA did not affect these parameters [Figure 2]. PA prevented weight loss when compared to *p*-OHBA and GA, but this effect was not significant [Figure 3].

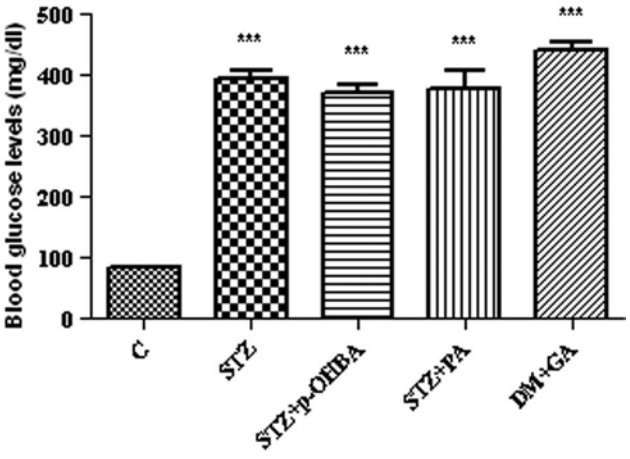
It was concluded that the positive effect of PA may be through different mechanisms other than diabeticmechanisms and/or antioxidant effect; because the other symptoms weren not affected by PAand the other test drugs did not change these parameters.

Isolated Organ Bath Experiments

Isolated aorta

Table 1 presents pD_2 and E_{max}values which were calculated from contractile responses of aorta to PE and relaxation responses to ACh. pD_2 of STZ-diabetic group was found to be unchanged. pD_2 of *p*-OHBA-treated group which was contracted with PE decreased according to non-diabetic control group; however, treatment with PA and GA didnot alter this value. Also in PE-contracted group, it was observed that E_{max} of STZ-diabetic group increased significantly when compared with the non-diabetic group. *p*-OHBA prevented this alteration partially while PA improved it. However, GA normalized this value even reduced it insignificantly under non-diabetic group' value.

Figure 1: Blood glucose levels of non-diabetic control (C), STZ-diabetic control (STZ), p-OHBA-treated (STZ+p-OHBA), PA-treated (STZ+PA) and GA-treated (STZ+GA) rats, ***P<0.001; significance relative to C, (n=6)



Diabetes, induced by STZ, and administration of *p*-OHBA and GA to STZ-diabetic rats aorta didnot cause alteration in relaxation responses of aorta. However, only pD_2 value decreased in PA-treated group.

Isolated vas deferens

Table 2 summarizes pD_2 and E_{max} values calculated from contractions of groups in response to PE and ACh. There was no significant difference between groups in pD_2 values which were calculated from contractile response of vas deferens to PE. However, E_{max} values demonstrated that STZ-diabetic group exhibited an increase in contraction of PE when compared with non-diabetic group. pD_2 values of STZ-diabetic and PA-treated groupswhich were contracted with AChwere found to be unchanged. Administration of *p*-OHBA and GA to the diabetic-rats increased the pD_2 values significantly when compared to non-diabetic and STZ-diabetic groups. Statistically significant increases were observed in the E_{max} of

Figure 2: Water and food intake amount, and urine and feces output of non-diabetic control (C), STZ-diabetic control (STZ), p-OHBA-treated (STZ+p-OHBA), PA-treated (STZ+PA) and GA-treated (STZ+GA) rats, (*n*=6)

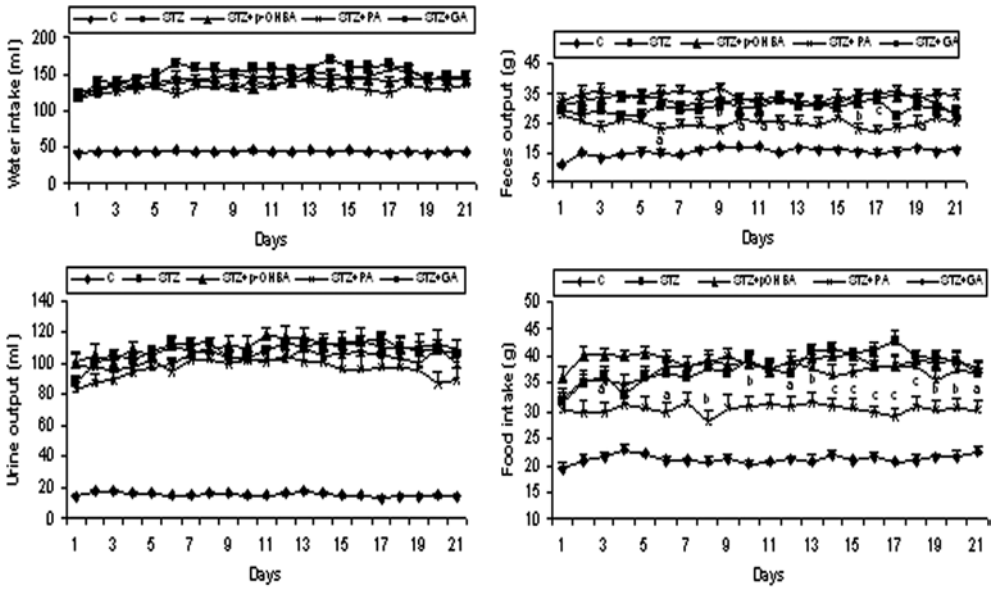


Table 1:

PD_2 and E_{max} values obtained from non-diabetic control (C), STZ-diabetic control (STZ), *p*-OHBA-treated (STZ+*p*-OHBA), PA-treated (STZ+PA) and GA-treated (STZ+GA) rats aorta response to PE and pre-contracted aorta response to ACh

	Aorta response to PE		*Pre-contracted aorta response to Ach*	
	pD_2±S.E.M.	*E_{max}±S.E.M.*	*pD_2±S.E.M.*	*E_{max}±S.E.M.*
C	7.48 ± 0.20	100 ± 0.00	6.69 ± 0.14	58.39 ± 6.65
STZ	7.31 ± 0.09	*121.60 ± 4.36	6.43 ± 0.15	58.02 ± 2.30
STZ+*p*-OHBA	*6.89 ± 0.10	109.60 ± 8.09	7.01 ± 0.27	68.85 ± 4.90
STZ+PA	7.23 ± 0.14	**129.40 ± 7.08	*6.07 ± 0.12	59.77 ± 2.87
STZ+GA	7.18 ± 0.20	##99.25 ± 6.38	6.59 ± 0.18	53.83 ± 6.44

*P<0.05, **P<0.01, significance relative to C, ##P<0.01, significance relative to STZ, (*n*=6)

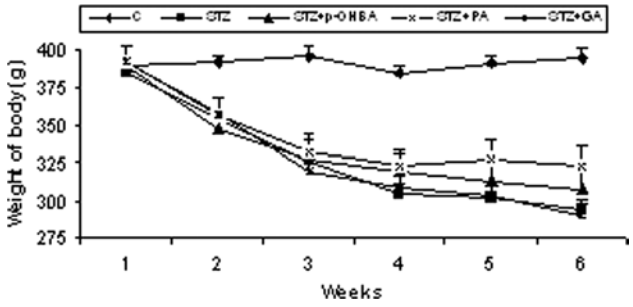
Table 2:

PD_2 and E_{max} values obtained from non-diabetic control (C), STZ-diabetic control (STZ), *p*-OHBA-treated (STZ+*p*-OHBA), PA-treated (STZ+PA) and GA-treated (STZ+GA) rats Vas deferens response to PE and ACh

	Vas deferens response to PE		*Vas deferens response to ACh*	
	pD_2±S.E.M.	*E_{max}±S.E.M.*	*pD_2±S.E.M.*	*E_{max}±S.E.M.*
C	5.63 ± 0.04	100 ± 0.00	3.84 ± 0,04	100 ± 0.00
STZ	5.76 ± 0.07	*122.60 ± 5.49	4.03 ± 0,02	***155.40 ± 6.36
STZ+*p*-OHBA	5.85 ± 0.07	111 ± 7.15	***,##4.32 ± 0.09	***,#136.80 ± 4.84
STZ+PA	5.58 ± 0.08	115.50 ± 5.98	3.97 ± 0.05	###112.70 ± 6,09
STZ+GA	5.52 ± 0.08	115.50 ± 5.10	***,##4.48 ± 0,14	###91.93 ± 5.01

*P<0.05, ***P<0.001, significance relative to C, #P<0.05, ##P<0.01, ###P<0.001, significance relative to STZ, (*n*=6)

Figure 3: Weight loss of non-diabetic control (C), STZ-diabetic control (STZ), p-OHBA-treated (STZ+p-OHBA), PA-treated (STZ+PA) and GA-treated (STZ+GA) rats, (*n*=6)



STZ-diabetic group when compared to non-diabetics. *p*-OHBA decreased E_{max}significantly as compared to STZ-diabetic group but couldnot normalize it. PA and GA normalized the value, theE_{max} of GA-treated group decreased when compared to non-diabetic group.

Discussion

STZ-induced diabetes results in changes in vascular reactivity to both vasoconstrictor and vasodilator agents. However, the results are conflicting, as increased, decreased or unchanged responses to same type of agonists have been observed in various studies.[4] It was reported that enhanced contractile responses of STZ-diabetic rat aorta to α-adrenergic agonists as noradrenaline are largely dependent on the presence of extracellular Ca^{++}, increased adrenergic stimulation, phosphoinositide metabolism, Ca^{++} channels sensitivity, deficiency of endothelial activity and/or decreased calmodulin levels.[16-18] In this study, by evaluation of increased response to PE of STZ-diabetic rat aorta, it was supposed that 6-week STZ-induced diabetes changed the α-adrenergic receptor number and/orthe mechanisms which were mentioned above without changing receptor affinity. However, muscarinic receptor affinity, number or post-receptor pathways remained unchanged.

Experimental diabetes also causes alterations in responses of Vas deferens to certain agonists.[4] It was reported that impaired responses to adrenergic nerve stimulation and hypersensitivity to ACh, noradrenaline and PE were observed in diabetic-rats. The enhanced α-adrenergic responsiveness of diabetic-rat Vas deferens was attributed to defective signal transduction mechanisms rather than an increase in the affinity or number of adrenoceptors.[19] In fact, altered metabolic product profile of arachidonic acid, hyperactivity of Na^+, K^+-ATP_{ase}, calcium channels and levels were reported as responsible mechanisms for the increased contractions in Vas deferens.[4] Another diabetic changes in the rat Vas deferens is the increased muscarinic responsiveness associated with the increase in muscarinic receptor density. Up regulation of M_2-muscarinic receptors was shown in STZ-diabetes.[20] As in many studies, in this study, α-adrenergic and muscarinic responsiveness in Vas deferens from 6-week STZ-diabetic rats increased significantly and it was attributed to increase in the number of receptors and activity of the post-receptor mechanisms as mentioned above since there was no changes in receptor affinity. Our results are compatible with some studies while conflicting with some other. The reasons for this controversy are not apparent but are generally attributed to differences in duration of diabetes, animal strains and techniques applied for the measuring and expressing contractile force. However, underlying causes of augmented contractile responses of Vas deferens to PE and ACh and aorta to PE require further investigations.

There has been a suggestion that antioxidants might be effective in preventing diabetic complications since free radicals have an important role in the pathogenesis of diabetes and its complications.[21] On the contrary, in this study, *p*-OHBA, PA and GA showed conflicting effects in STZ-diabetic aorta and Vas deferens. The test drugs either prevented or exacerbated or did not affect the SM changes in the STZ-diabetic rats, and sometimes, they affected the SM activity which didnot change through diabetes when compared to the non-diabetic rats. These effects of test drugs are not generally related with –OH number that is attributed to these PhA possesses different polarities so the rate of their migration into the cell is different. Based on these results, it was concluded that the demonstrated preventive effects of test drugs developed independently from antioxidant potential and restoration of diabetic complications. Moreover, since the impaired effects of used antioxidants were observed, it was thought that these antioxidants may act like pro-oxidant in our experiment conditions. Pro-oxidant effects of various PhA were demonstrated in different conditions.[22,23] It is known that pro-oxidant activity of phenolics increases in presence of redox active metal ions such as Fe^{++} and Cu^{++} and levels of Fe^{++}, Cu^{++}, and Zn^{++} ions change in diabetic conditions.[24,25] Polyphagia and metabolic changes in diabetic-rats cause accumulation of metal ions in tissues.[26] These metabolic changes were observed in our study and changed levels of all of these ions may have affected test drugs actions in this study.

On the basis of these findings and related studies, it seems possible that *p*-OHBA, PA and GA may exhibit improving or impairing effects independently from their antioxidant power and from mechanisms of diabetic complications. Furthermore, they may exhibit pro-oxidant activities in the experimental conditions applied, sincethe levels of Fe^{++} and Cu^{++} increase and Zn^{++} decrease in diabetic conditions. However, detailed investigations are needed to clarify the occurrence of the conditions when antioxidants act as pro-oxidants in the same experimental design in this study.

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