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Effects of phenolic acids on gastrointestinal smooth muscle complications in streptozotocine-induced diabetic rats

Nurcan BEKTAŞ, Yusuf ÖZTÜRK

Aim: To identify the effects of antioxidants on gastrointestinal smooth muscle complications in 6-week streptozotocine (STZ)-induced diabetic rats.

Materials and methods: The following steps were carried out: 1. Three weeks after STZ injection, saline and 10 mg/kg p-OH benzoic, protocatechic, and gallic acids were separately administered to the rat groups every day for 3 weeks. 2. The responses of the fundus and ileum to agonists were recorded using isolated organ bath experiments.

Results: Diabetic smooth muscle complications developed in the diabetic rats. The responses of the ileum to acetylcholine increased whereas the responses of the fundus to serotonine decreased in the diabetic rats. The test drugs inhibited and promoted but did not affect the smooth muscle complications in the diabetic rats, and sometimes they affected the smooth muscle activity, which did not change with diabetes.

Conclusion: The antioxidants used elicited pro-oxidant activation and/or exhibited effects independent of diabetic complications of gastrointestinal smooth muscles in the experimental conditions applied.

Key words: Diabetes mellitus, gastrointestinal tract complication, p-OH benzoic acid, protocatechic acid, gallic acid

Fenolik asitlerin streptozotosin ile diyabet oluşturulmuş sıçanlarda gelişen gastrointestinal düz kas komplikasyonları üzerine etkileri

Amaç: Bu çalışma, antioksidanların streptozotosin (STZ) ile diyabet oluşturulmuş altı haftalık diyabetik sıçanlarda gelişen gastrointestinal düz kas komplikasyonları üzerine etkilerini incelemeyi amaçlamıştır.

Yöntem ve gereç: Sırasıyla şu işlemler uygulanmıştır: 1. STZ enjeksiyonundan 3 hafta sonra; saline, 10 mg/kg p-OH benzoik, protokateşik ve gallik asid, oluşturulan sıçan gruplarına üç hafta süresince ayrı ayrı uygulanmıştır. 2. İzole fundus ve ileumun agonistlere karşı verdikleri cevaplar izole organ banyosu deneyleri kullanılarak kaydedilmiştir.

Bulgular: Diyabetik sıçanlarda düz kas komplikasyonları oluşmuştur. Diyabetik sıçanlarda, ileumun asetilkoline karşı verdiği cevaplar artarken fundusun serotonine karşı verdiği cevaplar azalmıştır. Kullanılan fenoliklerin düz kaslarda oluşan bu değişiklikleri önlediği, değiştirmediği veya ilerlettiği, kimi zaman ise diyabet ile bozulmayan düz kas aktivitesinde değişikliklere neden oldukları belirlenmiştir.

Sonuç: Oluşturulan diyabetik koşullarda, kullanılan antioksidanların pro-oksidan aktivite gösterdiği ve/veya diyabetik gastrointestinal düz kas komplikasyonlarından bağımsız etkiler sergilediği sonucuna varılmıştır.

Anahtar sözcükler: Diabetes mellitus, gastrointestinal sistem komplikasyonları, p-OH benzoik asid, protokateşik asid, gallik asid

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Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Eskişehir - TURKEY

Correspondence: *Nurcan BEKTAŞ, Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Tepebaşı TR-26470 Eskişehir - TURKEY*
E-mail: nurcanbektas@anadolu.edu.tr

Introduction

Diabetes mellitus affects the functions of various smooth muscles as a long term complication and conflicting response to several agonists in the smooth muscles of experimentally diabetic animals, suggesting the defective contractile process of smooth muscle (1). One of the most important structural changes in smooth muscles due to experimental diabetes is autonomic neuropathy. As autonomic nerves are highly integrated with smooth muscle cells in various physiological systems, autonomic neuropathy due to experimental diabetes may cause consequent changes in smooth muscles (2,3). It has been reported that increasing oxidative stress during diabetes, largely due to hyperglycemia, has an important role in the development of neuropathy, and nerve damage may cause gastroenteropathy (4,5).

Phenolic acids are commonly found in plants and their antioxidant potentials are well known. It has been indicated that phenolic acids and the plants that contain these compounds have antidiabetic activity (6,7). The antioxidant activity of phenolic acids correlated positively with the number of hydroxyl groups bonded to the aromatic ring (8).

The present study aimed to evaluate 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 smooth muscle complications that developed in the gastrointestinal tract. Experimental procedures were applied to the ileum and fundus isolated from 6-week streptozotocin (STZ)-induced diabetic rats by using isolated organ baths.

Materials and methods

Experimental animals

Adult male Wistar rats (350-450 g) were used. The animals were housed in a well ventilated room, on 12-h light/12-h dark cycle, and at 24 ± 1 °C. All animals were allowed free access to standard pellet diet (Eysem A. Ş., Eskişehir, Turkey) and 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 the Local Ethics Committee of Osmangazi University, Eskişehir.

Chemicals

Streptozotocin (Sigma, St. Louis, MO, USA), citric acid (Merck, Darmstadt, Germany), trisodium citrate (Merck), *p*-OH benzoic acid (Sigma), protocatechic acid (Sigma), gallic acid (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), and $CaCl_2.2H_2O$ (Merck).

Diabetic model and experimental groups

Experimental diabetes was induced by a single i.v. injection of STZ (50 mg/kg body wt.) into the tail vein. STZ was dissolved in 0.1 mol/L citric acid buffer, adjusted to pH 4.50 (9). Seventy-two hours after the injection of STZ, blood glucose levels were measured by Glukotrend® (Roche, Switzerland). The rats with blood glucose levels above 300 mg/dL were considered diabetic and were selected for the experiments. The other rat group was injected with citric acid buffer (i.v.) only, as the non-diabetic control group (n = 6). Then STZ-diabetic rats were divided into 4 groups (n = 6 per group). Three weeks after, the rats in the 1st group were injected with (i.p.) 10 mg/kg *p*-OHBA, those in the 2nd group were injected with 10 mg/kg PA, and those in the 3rd group were injected with 10 mg/kg GA every day for 3 weeks. The last group, the STZ-diabetic control group, was injected with (i.p.) saline only. Blood glucose levels and the weights of rats in all groups were measured at the same hours every week.

Isolated organ bath experiments

The rats in all groups were sacrificed by cervical dislocation 6 weeks after the induction of diabetes. The gastric fundus and the longitudinal layer of the ileum were rapidly dissected and placed in Krebs-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

the adhering fat and connective tissues, the isolated organs were mounted with a resting tension of 1.0 g in an isolated organ bath (Ugo-basile, 4050, Italy) containing 10 mL of KHS, aerated with mixture of 95% O_2 and 5% CO_2 at 37 °C (10,11). 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). The gastric fundus was firstly contracted with 10^{-9}-10^{-3} mol/L acetylcholine (ACh) and then washed 3 or 4 times with KHS every 15 min, before exposure to doses of 10^{-9}-10^{-3} mol/L 5hydroxytryptamine (5-HT). Concentration-response relationships of the ileum were obtained with doses of 10^{-9}-10^{-3} mol/L ACh only.

Statistical analysis

The contraction responses were expressed as apparent affinity constant (pD_2) and percentage of the corresponding maximal responses to each agonist [E_{max} (intrinsic activity)]. pD_2 value is given by the negative logarithm of the molar agonist concentration that produces 50% of the maximal response produced by the agonist ($pD_2 = -\log EC_{50}$). The statistical analyses were performed by one-way ANOVA, followed by Tukey's multiple comparison tests. The statistical analyses were carried out using GraphPad Prism version 5.0 and Microsoft Office Excel. The results were expressed as the mean ± S.D. to show variation in groups. Differences were considered significant when $P \leq 0.05$.

Results

Blood glucose levels

STZ-diabetic rats exhibited characteristic symptoms of diabetes such as polyuria, polydipsia, polyphagia, and weight loss. Blood glucose concentrations in all experimental groups were significantly ($P < 0.001$) higher than those in the control group. The treatment with *p*-OHBA, PA, and GA did not normalize hyperglycemia that occurred through STZ-induced diabetes (Figure 1). Weight loss can be seen in Figure 2. There were no significances between the treated and STZ-diabetic groups.

Isolated organ bath experiments

Isolated fundus

There was no significant difference between the groups in pD_2 values, which were calculated from the contractile response of the fundus to ACh. E_{max} values of the STZ-diabetic and GA-treated groups were lower when compared to the non-diabetic control group but this difference was not significant (Table 1). Moreover, statistically significant decreases were observed in E_{max} values of *p*-OHBA and PA-treated groups when compared to the non-diabetic and STZ-diabetic groups ($P < 0.001$).

pD_2 and E_{max} values calculated from contractions in the STZ-diabetic group in response to 5-HT contraction were found to be decreased when compared to those in the non-diabetic group ($P < 0.05$, $P < 0.01$) (Table 2). The injection of *p*-OHBA partially normalized the pD_2 value but the injections

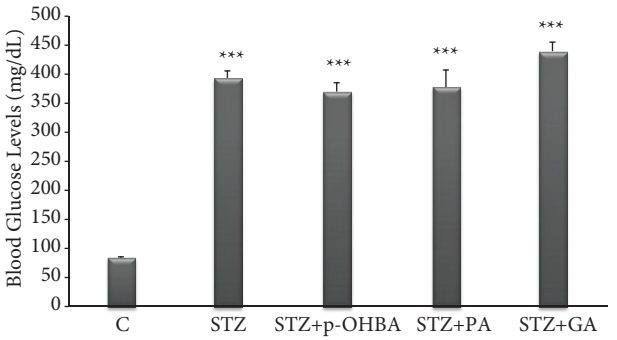

Figure 1. Blood glucose levels of non-diabetic control (C), STZ-diabetic control (STZ), *p*-OHBA-treated (STZ+*p*-OHBA), PA-treated (STZ+PA), and GA-treated (STZ+GA) rats, ***P < 0.001; significance relative to C (n = 6).

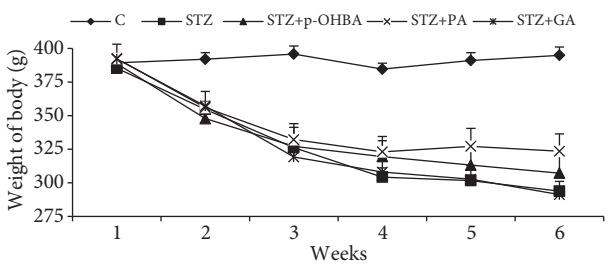

Figure 2. Weight loss of non-diabetic control (C), STZ-diabetic control (STZ), *p*-OHBA-treated (STZ+*p*-OHBA), PA-treated (STZ+PA), and GA-treated (STZ+GA) groups (n = 6).

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) groups' fundus response to Ach (n = 6).

	pD_2 ± S.D	**E_{max} ± S.D**
C	4.82 ± 0.40	100 ± 0.00
STZ	4.67 ± 0.19	97.41 ± 12.83
STZ+*p*-OHBA	4.93 ± 0.19	$^{***,\#\#\#}$68.03 ± 11.52
STZ+PA	4.56 ± 0.19	$^{***,\#\#\#}$71.27 ± 10.76
STZ+GA	5.00 ± 0.25	91.33 ± 15.68

*** P < 0.001; significance relative to C, $^{\#\#\#}$ P < 0.001; significance relative to STZ

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) groups' fundus response to 5-HT (n = 6).

	pD_2 ± S.D.	E_{max} ± S.D.
C	5.99 ± 0.46	100 ± 0.00
STZ	*5.36 ± 0.23	**79.51 ± 13.14
STZ+p-OHBA	5.66 ± 0.22	$^{***,\#\#\#}$55.01 ± 5.90
STZ+PA	*5.42 ± 0.18	***63.76 ± 15.41
STZ+GA	**5.33 ± 0.13	*77.76 ± 18.81

* P < 0.05, ** P < 0.01, *** P < 0.001; significance relative to C, $^{\#\#\#}$ P < 0.001; significance relative to STZ

of PA and GA did not affect the diabetes induced impairment. E_{max} values of the treatment groups were not significantly close to the E_{max} value of the control group even the E_{max} value of the *p*-OHBA-treated group (P < 0.001), whereas the PA- and GA-treated groups were partially decreased when compared to the STZ-diabetic group.

Isolated ileum

pD_2 and E_{max} values of the STZ-diabetic group were increased significantly compared to those of the non-diabetic group (P < 0.05, P < 0.001) (Table 3). The administrations of test drugs did not normalize the pD_2 values relative to the STZ-diabetic group. However, statistically significant decreases were observed in E_{max} values of all treatment groups (P < 0.001) even the E_{max} values of the *p*-OHBA and PA-treated groups were decreased when compared to the non-diabetic group.

Table 3. 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) groups' ileum response to ACh (n = 6).

	pD_2 ± S.D.	**E_{max} ± S.D.**
C	4.79 ± 0.53	100 ± 0.00
STZ	*5.66 ± 0.25	***151.40 ± 18.46
STZ+*p*-OHBA	*5.56 ± 0.58	$^{**,\#\#\#}$72.87 ± 12.42
STZ+PA	*5.48 ± 0.42	$^{*,\#\#\#}$74.69 ± 13.01
STZ+GA	**5.74 ± 0.38	$^{\#\#\#}$105.70 ± 17.29

* P < 0.05, ** P < 0.01, *** P < 0.001; significance relative to C, $^{\#\#\#}$ P < 0.001; significance relative to STZ

Discussion

Contraction and relaxation capacities of gastrointestinal smooth muscles change due to the impairment effect of diabetes (2). Experimental studies have indicated that adrenergic, cholinergic, and serotonergic stimulations of smooth muscles were damaged because of diabetes (12). However, the findings obtained from diabetic animal models seem to be conflicting (13). Increased, decreased, or unchanged contractions of smooth muscles have been observed in various studies (14-17). However, serotonin-induced contractions of isolated gastric fundus have been reported to be decreased significantly in both alloxan and STZ-diabetic rats as in our study (14). In addition, in our study, the cholinergic responsiveness did not change in the gastric fundus whereas it increased significantly in the ileum of 6-week STZ-diabetic rats. Our results are in general agreement with a group of studies, while conflicting with some other studies. The reasons for these controversies are not clear but are generally attributed to differences in the diabetic agents used, the duration of diabetes, the sex of the animals,

the animal strains, and the techniques applied for measuring and expressing contractile force. Since pD_2 and E_{max} values indicate the changes in receptor affinity and intrinsic activity, respectively (18), the changes in intestinal smooth muscle contractions were attributed to changes in the affinity, and either the number of receptors or the activity of the post-receptor mechanisms or both.

The impairments in the activities of protein kinase C, calcium channels, diacylglycerol dependent protein kinase, Na^+,K^+-ATP_{ase}, calcium influx, and sensitivity and/or affinity of calmodulin in intestinal smooth muscles may be responsible for these changed post-receptor mechanisms, since there are many studies that indicate that these mechanisms are affected by diabetes (14,15,19,20). It has also been reported that the structural alterations in diabetic ileum and G proteins in diabetic fundus may be involved in impaired functions of gastrointestinal smooth muscles in experimental diabetes (17,21). However, the underlying causes of impaired contractile responses of the gastric fundus to ACh and 5-HT and of the ileum to ACh require further investigation.

Since the relationship between DM and oxidative stress is known, it is thought that impairment of nerve conduction may be due to increased oxidative stress, and nerve damage may cause gastroenteropathy (22). Treatment with antioxidants may prevent or ameliorate abnormal functions and biochemistry of nerves, and also protects nerves from free radical damage (23). Thus antioxidants may have a potential to prevent gastroenteropathy. In contrast, in the present study, *p*-OHBA, PA, and GA generally showed impairing effects in STZ-diabetic fundus and ileum as explained in the results section. These effects were inversely correlated with –OH numbers in the molecules of antioxidants. Based on these results, it was concluded that the demonstrated preventive effects of the test drugs developed independently of antioxidant potential and diabetic complications. Moreover, since the damaging effects of the antioxidants used were observed, it was thought that these antioxidants may act like pro-oxidants in our experiment conditions. It has already been indicated that various phenolic acids such as GA may have pro-oxidant effects in different conditions (24,25). It is known that pro-oxidant activity of phenolics increases in the presence of redox active metal ions such as Fe^{++} and Cu^{++} and levels of these metal ions change in diabetic conditions (26,27). Polyphagia and metabolic changes in diabetic rats cause accumulation of metal ions in tissues (28). These metabolic changes were observed in our study. Conversely to accumulation of these metal ions, Zn^{++} ions, which have a role in preventing Fe^{++} and Cu^{++} -initiated lipid peroxidation, decrease in diabetes as a symptom of the disease (29,30). Thus, changed levels of all of these ions may have affected the test drugs' actions in our study.

Taking these findings into account, it seems possible that *p*-OHBA, PA, and GA showed pro-oxidant characteristics, since it is demonstrated that the levels of Fe^{++} and Cu^{++} increased and Zn^{++} decreased 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 used in this study.

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