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Original Article

The effects of the urotensin-II receptor antagonist palosuran treatment on the corpora cavernosa of streptozotocin-induced diabetic rats

Murat Olukman [a], Cenk Can [a], Sibel Ülker [a], Yiğit Uyanikgil [b], Türker Çavuşoğlu [b,c], Neslihan Düzenli [a,d,*], Deniz Coşkunsever [a], Fatma G. Kozcu [a]

[a] *Department of Medical Pharmacology, Faculty of Medicine, Ege University, Izmir, Turkey*
[b] *Department of Histology and Embryology, Cord Blood, Cell-Tissue Application and Research Center, Faculty of Medicine, Ege University, Izmir, Turkey*
[c] *Department of Histology and Embryology, Faculty of Medicine, Izmir Bakırcay University, Izmir, Turkey*
[d] *Department of Medical Pharmacology, Faculty of Medicine, İzmir Democracy University, Izmir, Turkey*

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Abstract *Objective:* This study aimed to investigate the effects of treatment with palosuran, a urotensin receptor blocker, on molecular changes in the corpora cavernosa (CC) in diabetic rats.
Methods: Streptozotocin-induced diabetic rats were treated with palosuran 300 mg/kg per day for 6 weeks. Contraction of CC induced by potassium chloride, phenylephrine, and NG-nitro-*L*-arginine methyl ester and relaxation of CC induced by electrical field stimulation (EFS) and sodium nitroprusside (SNP) (endothelium-dependent and endothelium-independent stimuli, respectively), and Y-27632 (Rho-kinase inhibitor) were examined in organ baths. Direct contraction or relaxation induced by palosuran and urotensin-II (U-II) were also evaluated. The expression levels of nitric oxide synthetases (NOSs), RhoA, oxidative stress regulators, and U-II were analyzed by Western blotting or immunohistochemistry.
Results: Induction of diabetes in rats resulted in the decreased relaxant response to SNP, decreased pD_2 value of SNP, attenuated relaxant response to Y-27632 as well as the decreased RhoA expression in CC. Palosuran treatment of diabetic rats reversed all of these parameters; however, it further impaired the already weakened relaxation of diabetic CC in response to EFS. Although induction of diabetes did not change U-II expression in CC significantly,

* Corresponding author. Department of Medical Pharmacology, Faculty of Medicine, İzmir Democracy University, Izmir, Turkey.
E-mail address: drneslihanduzenli@gmail.com (N. Düzenli).
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palosuran treatment reduced U-II expression in diabetic CC. The expression level of nNOS was lowered in diabetic CC; however, palosuran treatment did not change the decreased the neuronal NOS expression. *In vitro* exposure of diabetic CC strips to palosuran produced a direct relaxant response.
Conclusion: Palosuran treatment did not affect the expression of NOSs or reduce nitrergic conduction induced by EFS stimulation in diabetic CC. However, while directly triggering a relaxant response, it did not induce a prominent contraction either by decreasing U-II expression, or increasing the sensitivity of CC to nitric oxide which suggested that palosuran has the potential to support erectile function. Further and comprehensive studies are required to clarify this issue.

1. Introduction

Diabetes mellitus (DM) may cause functional disorders by inducing structural and molecular changes in many systems, organs, and/or tissue. Erectile dysfunction (ED) is one of the important complications of DM associated with neuropathy and vasculopathy [1]. The prevalence of ED is 50%—75% in diabetic men and it is 3.5 times more frequently seen compared with non-diabetic men [2].

Under physiological conditions, the penis is kept in a flaccid state as a result of high cavernosal smooth muscle (SM) tone under the influence of local vasoconstrictors such as norepinephrine and endothelin-1. Contraction of cavernosal SM is mediated by the RhoA/Rho-related protein kinase (ROCK) signaling pathway, which facilitates chronic tonic contractions of the SM by increasing sensitivity to calcium [3]. This high relaxant muscle tone should decrease, and SM should relax to allow penile erection. This mechanism is mainly mediated by the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway, which reduces the activity of the RhoA/ROCK pathway. NO is synthesized by the neuronal NO synthase (nNOS) in cholinergic and nonadrenergic noncholinergic nerve fibers, and by the endothelial NO synthase (eNOS) in the endothelium. NO rapidly passes into the arterial and trabecular SM of the corpora cavernosa (CC), stimulating the synthesis of cGMP, which mediates SM relaxation by reducing intracellular calcium concentrations and/or sensitivity, resulting in penile erection. Following the degradation of cGMP by the phosphodiesterase type-5, the effects of local vasoconstrictor mediators become dominant again [4—6]. Disruption of the homeostatic mechanisms interacting between these pathways strongly potentiates the RhoA/ROCK signaling [3] and inhibition of this pathway leads to erection [6,7].

Defects in the NO/cGMP pathway are mainly responsible for the pathogenesis of diabetic ED [8,9]. Sildenafil, tadalafil, vardenafil, and avanafil are phosphodiesterase type-5 inhibitors that are frequently used in treating ED nowadays. However, since there is not enough bioactive NO in the cavernosal tissue of diabetics, these drugs may not provide sufficient erection for intercourse in patients with diabetic ED [1]. In addition, the potential interactions of these drugs with some antianginal and antihypertensive drugs used in treating cardiovascular diseases may cause life-threatening medical conditions. Other treatment options including intracavernosal injection of alprostadil or penile prosthesis are invasive procedures and reduce patient comfort. Therefore, studies on the development of safe and non-invasive treatments for diabetic ED retain their importance. These approaches require a better understanding of the mechanisms involved in the pathogenesis of diabetic ED.

Urotensin-II (U-II) is the most potent endogen vasoconstrictor discovered so far [10]. Its vasoconstrictor effect is mediated by the RhoA/ROCK signalling pathway in the SM like other vasoconstrictor mediators [11,12]. However, it may also produce vasodilation by stimulating the NO/cGMP pathway in the intact endothelium [13—16]. Its net vasoactive effect is related to the localization of U-II receptors (UTRs) [14]. Although U-II is expressed widely but at low levels in many organs and tissue [17,18], its expression may be increased by endothelial dysfunction observed in various cardiovascular and metabolic diseases [15,19]. In this case, its endothelium-dependent vasodilator effect decreases, especially in peripheral small arteries such as cavernosal arteries, and its vasoconstrictor effect becomes more pronounced [20]. Additionally, U-II also has hypertrophic, mitogenic, proliferative, profibrotic, diabetogenic, proinflammatory, and oxidative stress-inducing effects [15,20—24], which all have important roles in DM-related multi-organ system involvement. Blockage of the urotensinergic system may be an important therapeutic target in treating or slowing the progression of DM-related complications, including ED.

Taken together, the present study aimed to investigate the changes in mechanisms related to erectile function in the CC tissue samples of streptozotocin (STZ)-induced diabetic rats, and effects of treatment with palosuran, a non-peptide UTR blocker, on these changes.

2. Materials and methods

The protocol of this trial was approved by Ege University Animal Experiments Local Ethics Committee (Approval No. 2009-133) and performed according to the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council Committee [25].

2.1. Animals and experimental groups

Male Wistar rats (250–300 g, 8–10 weeks old, $n=60$) were obtained from Kobay Experimental Animals Laboratory (Ankara, Turkey), and housed in cages in a temperature- and lighting-controlled room and fed ad libitum.

Rats were allocated into control (non-diabetic), diabetic, and palosuran-treated diabetic groups ($n=20$ per group [approximately $n=15$ for organ bath and Western blotting studies; $n=5$ for immunohistochemical study]). DM was induced by a single intraperitoneal injection of STZ (55 mg/kg, Sigma–Aldrich, St. Louis, MO, USA). Three days later, glycemic levels of rats were measured with a glucometer (Bayer HealthCare, Mishawaka, IN, USA) using blood samples taken from the tail veins and rats with glycemic levels above 250 mg/dL were considered diabetic. After confirmation of DM, palosuran-treated diabetic group received daily oral doses of 300 mg/kg per day palosuran (ACT-058362, Actelion Pharmaceuticals, Allschwil, Switzerland) administered via a gastric tube for 6 weeks. Studies by Clozel et al. [10,26] were taken as references in the selection of the doses used.

2.2. Isolated organ bath studies

Six weeks after administration of palosuran, rats were sacrificed under ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively, intraperitoneal) anesthesia. CC of the rats were excised, and dissected into 1 mm×1 mm×3 mm strips after clearing away all the surrounding fat tissue. Two strips were suspended vertically between two platinum wire electrodes in a 20 mL organ bath filled with Krebs–Henseleit buffer solution (NaCl, 118.1 mmol/L; KCl, 4.7 mmol/L; $MgSO_4$, 1.2 mmol/L; KH_2PO_4, 1.22 mmol/L; $CaCl_2$, 2.5 mmol/L; $NaHCO_3$, 25 mmol/L; glucose, 11 mmol/L), gassed with 95% oxygen and 5% carbon dioxide, at 37 °C and in pH 7.4. The strips were put to rest under 1 g resting tension for 1 h and rinsed every 15 min with Krebs–Henseleit buffer solution.

A single dose of KCl (120 mmol/L) and cumulatively increasing concentrations of phenylephrine (PE, $1x10^{-9}$–$3x10^{-4}$ mol/L) were applied to the organ baths to evaluate the contractility of the CC strips. Since cavernosal SM tone is increased in the flaccid penis under normal conditions, the relaxant responses were recorded in the strips precontracted with a submaximal concentration of PE (1×10^{-4} mol/L). The procedures applied on the strips were as follows:

i) Electrical field stimulation (EFS) in the presence of atropine and guanethidine (40 V; 0.5 ms pulse; 2 Hz, 4 Hz, 8 Hz, 16 Hz, 32 Hz, and 64 Hz, at 15 s intervals, consecutively) to evaluate nonadrenergic noncholinergic neurotransmission;
ii) Cumulatively increasing concentrations of acetylcholine ($1x10^{-9}$–$3x10^{-5}$ mol/L), carbachol ($1x10^{-9}$–$3x10^{-5}$ mol/L), bradykinin ($1x10^{-9}$–$3x10^{-7}$ mol/L) and Ca^{2+} ionophore A23187 ($1x10^{-9}$–$3x10^{-7}$ mol/L) to evaluate endothelium-dependent relaxation;
iii) Cumulatively increasing concentrations of sodium nitroprusside (SNP, $1x10^{-11}$–$1x10^{-5}$ mol/L) to evaluate endothelium-independent relaxation;
iv) Cumulatively increasing concentrations of ROCK inhibitor Y-27632 ($1x10^{-9}$–$3x10^{-5}$ mol/L);
v) Cumulatively increasing concentrations of palosuran ($3x10^{-9}$–$1x10^{-4}$ mol/L).

In addition, basal NO-related relaxation was assessed by administrating a single dose (20 μL) of NG-nitro-*L*-arginine methyl ester (*L*-NAME, 100 μmol/L), a non-selective NO synthase (NOS) inhibitor into PE-precontracted strips, and additional contractile responses were recorded. Cumulative concentrations ($1x10^{-10}$–$3x10^{-7}$ mol/L) of U-II were injected into CC strips at resting tension and after PE precontraction, to determine whether U-II had a contractile or relaxant effect. Tension was recorded isometrically by a force displacement transducer (Commat Ltd., Ankara, Turkey), imaged on a Biopac acquisition system (Biopac System Inc., Goleta, CA, USA) and alterations in voltages were recorded. The baths were washed thoroughly between the protocols, and the CC strips were allowed to rest for 30–60 min in between. Contractile responses were expressed as changes in contraction in mg units and relaxant responses as percentages of PE-precontraction. At the end of each experimental period, strips were left to dry at room temperature for 24 h and then weighed. The pD_2 values (negative logarithm of molar concentrations producing the half maximum response [$-\log_{10} EC_{50}$]) were also calculated to determine the sensitivity of CC tissue to PE, SNP, and Y-27632.

2.3. Western blotting

CC strips were homogenized in 1:10 volume of ice-cold protein lysing buffer (Tris hydrochloric acid, 20 mmol/L; ethylene glycol tetraacetic acid, 2 mmol/L; ethylene diamine tetraacetic acid, 5 mmol/L; dithiothreitol, 2 mmol/L; phenylmethylsulfonyl fluoride, 0.5 mmol/L; aprotinin, 0.5 mg/mL; pepstatin, 0.001 mg/mL; leupeptin, 0.001 mg/mL; in pH 7.5) by gradually shredding in the mechanical homogenizer at 10000 r/min for 45 s on ice. The homogenates were centrifuged at 4 °C and 14000 r/min for 30 min and protein concentrations in the supernatants were measured spectrophotometrically at 750 nm wavelength using the Lowry method of protein estimation [27]. Sixty micrograms of extracted protein was loaded and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare, Amersham Biosciences, London, UK) in transfer buffer (Tris base, 25 mmol/L; glycine 192 mmol/L; 20% methanol) at 4 °C. Blots were blocked for 1 h at room temperature in blocking solutions (Tris base, 20 mmol/L; sodium chloride, 137 mmol/L, 0.1% Tween 20) containing 5% non-fat dried milk and then incubated overnight at 4 °C with antibodies against the inducible NOS (iNOS), nNOS, p65 (a subgroup of nuclear factor kappa-B [NF-κB]), RhoA, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), nitrotyrosine protein, and

β-actin. After extensive washing, blots were incubated with the secondary antibody for 1 h at room temperature. Detailed information on antibodies has been presented in Supplementary Table 1. The washed membranes were incubated with electrochemiluminescence solution (GE Healthcare UK Ltd., Amersham, UK) in the dark room and chemiluminescence films were obtained after consecutive exposures for 3 min, 5 min, 10 min, 15 min, and 20 min. Band images were scanned (UMAX PowerLook 1100, UMAX Technologies, Dallas, TX, USA) and transferred to the computer via the LabScan 5.0 software (GE Healthcare, Freiburg, Germany). The band densities were quantified using NIH ImageQuant TL v2005 (Amersham Biosciences, London, UK). The data were presented as the ratio of protein band density over β-actin band density.

2.4. Immunohistochemistry

At the end of the experiments, intracardiac perfusion with phosphate buffered saline (0.01 mol/L) followed by a 4% cold paraformaldehyde solution was carried out in rats under anesthesia. CC tissue was removed, post-fixated overnight in 4% paraformaldehyde solution, and passed through a series of decreasing concentrations of alcohol (100%–95%–80%) and dried. For the transparency process, the tissue samples exposed to xylol solvent were placed in melted paraffin in a 60 °C oven and kept overnight. The next day, the tissue samples taken away from the oven were embedded in paraffin solution and blocked. Then, 5 μm thick sections obtained using a cryostat device (LEICA 2145, Leica Microsystems Inc., Wetzlar, Germany) were placed on positively-charged microscope slides. Sections were washed in phosphate-buffered saline exposed to normal horse serum for 30 min and incubated with primary antibodies of the eNOS (rabbit polyclonal, 1: 100, Sigma–Aldrich Corp., St. Louis, MO, USA) and U-II (goat polyclonal, 1/1000, Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4 °C, and then blocked with 3% hydrogen peroxide for 10 min and incubated with secondary antibodies (mouse anti-rabbit immunoglobulin G conjugated with horseradish peroxidase, 1:100, and mouse anti-goat immunoglobulin G conjugated with horseradish peroxidase, 1:3000, respectively, Santa Cruz Biotechnology, Heidelberg, Germany) for 2 h at room temperature, and 3,3′-diaminobenzidine tetrahydrochloride (Histostain®-Plus kits, Invitrogen Laboratories, Inc., Ventura, CA, USA) was used as a chromogen. Finally, the sections were counter-stained with Mayer's hemotoxylin solution (Sigma-Aldrich Corp., St. Louis, MO, USA), dehydrated by a series of increasing concentrations of alcohol (80%–95%–100%), kept in xylol thrice for 2 min each time and coverslipped with 90% glycerol. The stained sections were photographed using a light microscope and a digital camera (Olympus BX-51 and C-5050, Tokyo, Japan, respectively). Protein expression levels were evaluated by the particle surface analysis using a computerized image-analyzer program ("Image J" program; National Institutes of Health, Bethesda, MD, USA). Surface areas of proteins, as well as sinusoids in each section, were analyzed on two different backgrounds. The immunoreactivities of proteins in the CC tissue were defined as the protein surface area (%) according to the following formula: protein surface area (%) = percentage of protein surface area/(100%–percentage of sinusoid surface area) × 100. These analyses were performed by two blinded researchers and then the mean of data obtained for each section was evaluated as a single value. Areas of artifacts that could cause incorrect evaluation were also measured and excluded from the data obtained.

2.5. Statistical analysis

Changes in body weights and mean blood glucose levels, KCl- and L-NAME-induced contractile responses, and data from immunohistochemistry were analyzed by the one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. The repeated measures ANOVA was used to analyze the concentration–response curves. Western blotting data were analyzed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test. Data were presented as mean with standard error of the mean (SEM). The level of statistical significance was accepted as $p<0.05$. Statistical analyses and graphs were performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Changes in body weights and blood glucose levels

STZ-induced diabetic rats had higher blood glucose levels and significant weight loss compared with control group ($p<0.001$), and palosuran treatment did not affect these changes (Table 1).

3.2. Assessment of contraction and relaxation responses of CC in the isolated organ bath

The contractile responses of CC strips to single concentration of KCl (120 mmol/L; $p<0.01$, compared to the control group, Fig. 1A) and cumulative concentrations of PE

Table 1 Blood glucose levels and changes in body weight of experimental rat groups.

Rat group	Blood glucose level, mg/dL	Change in body weight[a], %
Control	121.60±2.51	+25.66±2.26
DM	505.00±127.00***	−22.94±2.66[b]
DM+palosuran	473.00±41.00***	−26.82±2.35[b]

DM, diabetes mellitus; DM+palosuran, DM treated with palosuran.
Note: values are presented as mean±standard error of the mean.
[a] The change in body weight (%) of rats during the experimental period of 6 weeks was calculated using the following formula: change in body weight (%) = (last body weight [g]/basal body weight [g]) × 100.
[b] $p<0.001$ *vs.* control; $n=13–20$ for the change in body weight and $n=15–19$ for mean blood glucose levels.

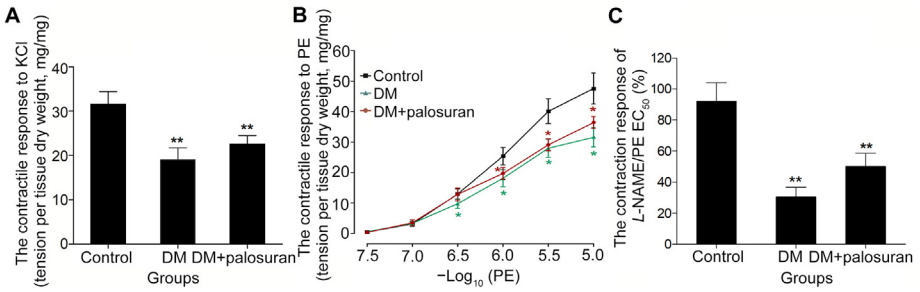

Figure 1 Contractile responses of rat corpora cavernosa in the isolated organ baths. (A) KCl (120 mmol/L)-induced contractile responses ($n=9-15$); (B) Response curves to cumulative concentrations of PE (doses of the PE have been presented as the negative logarithm [$-\log_{10}$] of its concentrations applied; $n=9-15$); (C) Additional contractile responses of PE-precontracted strips to 100 μmol/L L-NAME ($n=7-12$). DM, diabetes mellitus; DM+palosuran, DM treated with palosuran; L-NAME, NG-nitro-L-arginine methyl ester; PE, phenylephrine. * $p<0.05$ and ** $p<0.01$ *vs.* the control group.

($p<0.05$, Fig. 1B) were reduced in diabetic rats without a significant change in pD2 values of PE (means±SEMs: 5.469±0.034, 5.487±0.050, 5.403±0.063, in control, diabetic, and palosuran-treated diabetic groups, respectively). Contractile responses obtained by applying a single concentration of L-NAME (100 μmol/L) to PE-precontracted strips were also significantly weakened in diabetic rats ($p<0.01$, Fig. 1C). Palosuran treatment did not affect any of the weakened contractions in the diabetic group (Fig. 1).

When compared with the control group, relaxation responses induced by EFS in the presence of atropine and guanethidine were decreased significantly in palosuran-untreated ($p<0.05$) and palosuran-treated ($p<0.01$) diabetic rats (Fig. 2A). However, the reduction in EFS-induced relaxation was more prominent in the palosuran-treated diabetic rats compared to the untreated group ($p<0.05$, Fig. 2A).

Any relaxant responses could not be obtained by applying cumulative concentrations of acetylcholine, carbachol, bradykinin, or A23187, which were used to test endothelium-mediated relaxant responses in PE-precontracted strips. However, SNP, an endothelium-independent relaxant agent, induced relaxant responses in PE-precontracted strips which were significantly decreased in the diabetic group compared with the control group ($p<0.05$, Fig. 2B). The pD_2 values of SNP were significantly decreased in diabetic CC strips as well compared with the control group ($p<0.001$, Fig. 2C). On the other hand, the relaxant responses of Y-27632, a ROCK inhibitor ($p<0.05$, Fig. 2D) and the pD_2 values ($p<0.05$, Fig. 2E) were significantly increased in the diabetic group compared with the control group. Palosuran treatment prevented the changes in both SNP and Y-27632 induced relaxations in the CC strips of the untreated diabetic group (Fig. 2B–E).

These results indicate that both NO-mediated relaxation responses and ROCK-mediated contractions were reduced in diabetic rats. Although nitrergic conduction is further impaired by palosuran treatment, the recovery of relaxation and sensitivity to SNP, which is reduced in diabetes, suggests that palosuran treatment may have an effect on NO bioavailability in this tissue. Although the relaxant response to Y-27632 was reduced, the palosuran treatment did not reinforce other contractile responses.

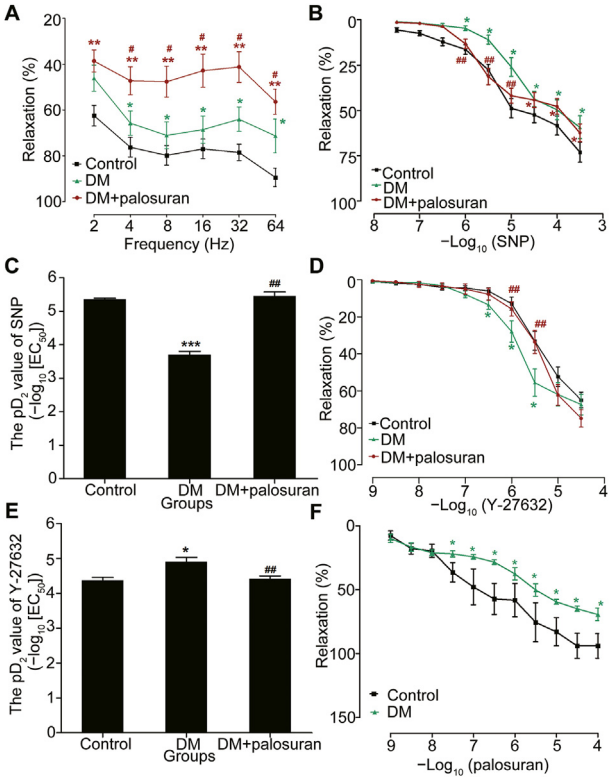

Figure 2 Relaxation of PE-precontracted rat cavernosal strips. (A) Frequency response curves with EFS, in the presence of atropine and guanethidine ($n=8-15$); (B) Response curves to cumulative concentrations of SNP ($n=11-16$); (C) The pD_2 values of SNP ($n=9-11$); (D) Response curves to cumulative concentrations of Y-27632 ($n=10-15$); (E) The pD_2 values of Y-27632 ($n=10-15$); (F) Response curves to cumulative concentrations of palosuran administered *in vitro* directly ($n=5-6$). Relaxation responses are presented as the percentage of the PE-precontraction in the A, B, D, and F. In the cumulative dose–response curves (B, D, and F), doses of agents have been presented as the negative logarithm ($-\log_{10}$) of their concentrations applied. PE, phenylephrine; EFS, electrical field stimulation; SNP, sodium nitroprusside; DM, diabetes mellitus; DM+palosuran, DM treated with palosuran. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* the control group; # $p<0.05$ and ## $p<0.01$ *vs.* the untreated diabetic group.

In the present study, incubation of U-II at increasing concentrations did not induce an effect in CC strips either at resting tension or after PE-precontraction. *In vitro* administration of palosuran at cumulative concentrations ($3x10^{-8}$–$3x10^{-4}$ mol/L) to PE-precontracted strips induced relaxation, but this effect was to a lesser extent in the untreated diabetic group compared with the control group ($p<0.05$; Fig. 2F). Although, *in vitro* administration of palosuran produced a relaxant response, endothelium-mediated relaxation could not be evaluated. These results indicate that palosuran exerts both direct and indirect effects on diabetic CC.

3.3. Assessment of the protein expression in CC by Western blotting

The expression levels of the nNOS and RhoA were considerably reduced in the untreated diabetic group compared with the control group ($p<0.05$ and $p<0.01$, Fig. 3A and B, respectively). These results are consistent with decreased nitrergic neurotransmission as well as decreased contractile responses in CC strips of diabetic rats. Palosuran treatment did not affect the decrease in nNOS expression in the untreated diabetic group (Fig. 3A), while it reversed the decrease in RhoA expression ($p<0.05$, Fig. 3B). There were no significant differences in the expression levels of the iNOS (Fig. 3C), antioxidant enzymes SOD and catalase, or oxidative stress indicators GPx and nitrotyrosine among all groups (Supplementary Figure 1). Expression of NF-κb did not change significantly in the untreated diabetic group compared to the control group, while palosuran treatment comparatively increased expression of NF-κb, which may mediate the formation of reactive oxygen species compared with other groups ($p<0.01$, Fig. 3D). However, lack of any other evidence of endothelial dysfunction concerning antioxidant enzymes and oxidative stress indicators, suggests that the palosuran-induced increase in NF-κb expression may not be functional.

3.4. Assessment of the protein expression in CC evaluated by immunohistochemistry

Representative images of the immunostaining of eNOS and U-II in the corporal tissue sections are presented in Fig. 4. Induction of diabetes did not change eNOS or U-II expression in CC strips significantly when compared to the control group (Fig. 4). However, palosuran treatment of diabetic rats resulted in a marked reduction in U-II expression ($p<0.05$ compared with the control group and untreated diabetic group, respectively, Fig. 4B).

4. Discussion

ED is an important precursor of systemic vascular disease in patients with DM [28,29]. Furthermore, since there are currently no non-invasive, safe, or adequately effective therapeutic options for the treatment of DM-related ED, the molecular and functional changes in the CC caused by DM need to be better defined.

Erectile function is maintained by the balance between NO/cGMP and RhoA/ROCK-mediated pathways. The major

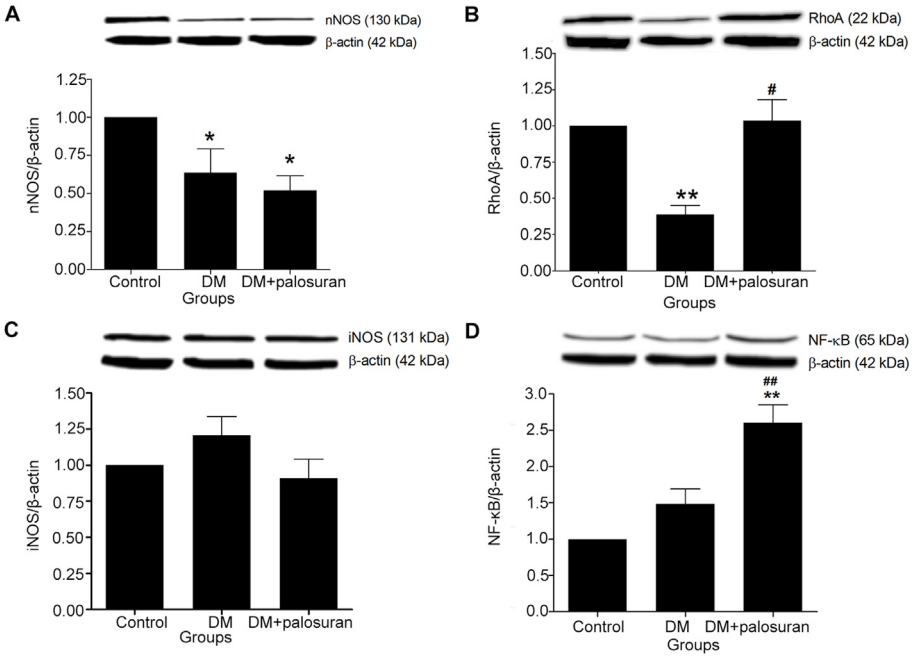

Figure 3 Representative band images and the expression levels obtained by Western blotting of proteins in rat corpora cavernosa. (A) nNOS; (B) RhoA; (C) iNOS; (D) p65 (a subgroup of NF-κb). $n=4$–6 for each parameter. DM, diabetes mellitus; DM+palosuran, DM treated with palosuran; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; nNOS, neuronal nitric oxide synthase. * $p<0.05$ and ** $p<0.01$ *vs.* the control group; # $p<0.05$ and ## $p<0.01$ *vs.* the untreated DM group. The results were normalized to β-actin which was considered as 1.00.

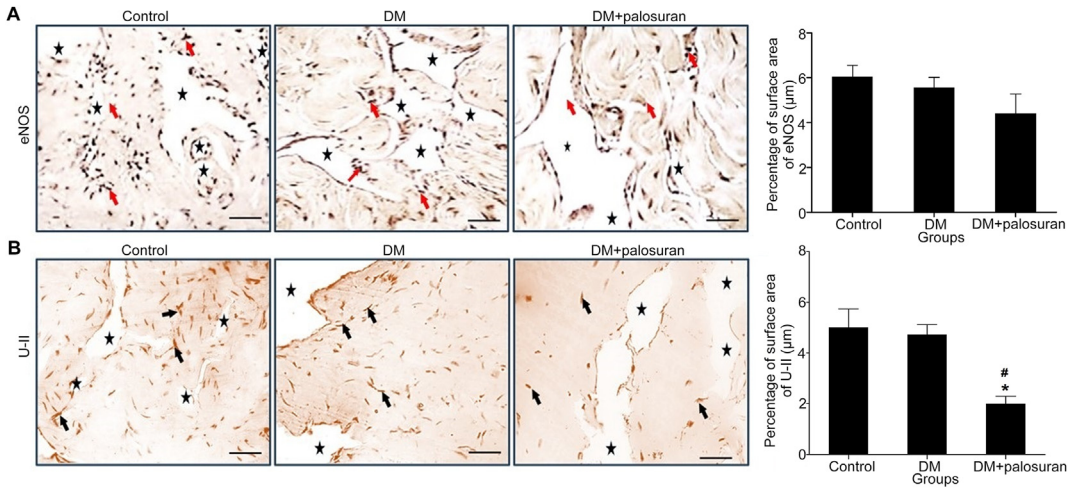

Figure 4 Representative images and expression levels in the rat corpora cavernosa obtained by immunohistochemistry. (A) eNOS; (B) U-II. DM, diabetes mellitus; DM+palosuran, DM treated with palosuran; eNOS, endothelial nitric oxide synthase; U-II, urotensin-II. Red and black arrows indicate eNOS and U-II proteins, respectively; stars indicate sinusoidal areas. Scale bar: 100 μm. $n=4-6$. * $p<0.05$ *vs.* the control group; # $p<0.05$ *vs.* the untreated diabetic group. To provide a more distinct representation, the background of the images has been made transparent and their clarity has been increased.

pelvic ganglia contain the cell bodies of autonomic motor neurons that innervate the urogenital organs in rats [30]. They are also surrounded by numerous small-diameter blood vessels, which are innervated by noradrenergic and nitrergic nerve fibers [31]. Insulin mediates vasodilation by increasing NO production or bioavailability. Deficiency of insulin or insulin resistance promotes vasoconstriction [32]. Blood flow to major pelvic ganglia decreases by nearly 50% in 1–4 weeks following induction of DM leading to hypoxia [28,33]. Both hypoxia and hyperglycemia predispose to the development of diabetic ED-associated autonomic neuropathy and endothelial dysfunction by decreasing NO production or bioavailability, triggering oxidative stress and inflammation, and increasing the activity of vasoconstrictor mediators [34,35]. Although these changes are initially reversible or tolerable, they may become permanent over time by advancing in the neurodegeneration [31], and by ongoing in the synthesis of advanced glycation end-products, which are produced by the covalent reaction between glucose and certain plasma proteins [29,35].

In the presence of atropine and guanethidine, EFS-induced NO-dependent relaxation [9] triggers nitrergic nerve conduction [36]. Since SNP directly releases NO, it is often used to test endothelium-independent relaxant responses in experimental DM studies [4]. L-NAME is a potent NOS inhibitor that prevents and reverses erection initiated by NO-mediated stimulation. Additional contraction obtained by incubation of L-NAME in PE-precontracted strips in the organ bath indirectly reflects the function of the NO/cGMP pathway. Our data obtained from the analysis of organ bath revealed that relaxations induced by EFS and SNP, the sensitivity of the erectile tissue to SNP (the pD_2 value of SNP), and the contractile response obtained by L-NAME incubation significantly decreased in diabetic rats. Furthermore, these changes were accompanied by a decrease in the nNOS expression. These findings indicate that nitrergic neurotransmission and the sensitivity of cavernosal SM to NO were reduced in diabetic rats.

Oxidative stress-induced activation of NF-κB, a transcription factor, is associated with neurovascular complications of DM [37]. Activated NF-κB stimulates the immune system [38] by mediating the synthesis of many cytokines, adhesion molecules, and inducible enzymes such as the iNOS, which are mainly found in macrophages [39]. U-II, a vasoactive peptide, is upregulated in endothelial dysfunction [40,41], and its proinflammatory and oxidative stress-inducing effects may worsen the ED [15,20–24]. The expression levels of almost all markers of endothelial dysfunction studied in the present study, *i.e.*, the eNOS, iNOS, NF-κb, U-II, GPx, or nitrotyrosine as oxidative stress markers; or SOD or catalase as antioxidant defence markers, did not change in the CC of STZ-induced diabetic rats. Despite our repeated attempts in isolated organ baths, we were unable to obtain any relaxant responses to the cumulative concentrations of acetylcholine, carbachol, bradykinin, or A23187. All these parameters were also used to assess endothelium-mediated relaxant responses. However, the above-mentioned results suggest that endothelial dysfunction might not have developed or progressed at this stage of diabetes in this study.

PE, an adrenergic receptor agonist, has a RhoA/ROCK pathway-mediated contractile effect, like endogenous local vasoconstrictors. Contractile responses induced via high concentration of KCl reflect the sensitivity of SM to calcium [42]. Although the contractile effect of KCl at the high concentration is associated with the opening of calcium channels, it has been suggested that activation of the RhoA/ROCK pathway also contributes to this effect [43]. Contrary to our expectations, the contractile response of diabetic CC strips to a high concentration of KCl significantly decreased and a similar decrease was also observed at cumulative concentration response curves of PE contraction. We unexpectedly detected that RhoA expression was also decreased in the diabetic CC tissue, which may explain the mechanisms underlying the decreased contractions of KCl and PE in diabetic CC strips. In addition,

concentration-dependent relaxation of Y-27632, a ROCK inhibitor, and sensitivity to Y-27632 augmented as well, suggesting that the RhoA/ROCK pathway has become more sensitive to suppression, despite decreased RhoA expression.

The RhoA/ROCK pathway may be also suppressed if the NO/cGMP signaling deteriorates due to various pathological conditions. Contrarily, under normal conditions, they interact to maintain penile hemostasis. Decreased RhoA/ROCK activity simultaneously with impaired NO/cGMP signaling may allow the erectile response to be achieved [3]. Thus, erectile function may be maintained at least for a while. Chronic NOS inhibition has been associated with decreased RhoA activity. Long-term inhibition of NOSs also increases the sensitivity to Y-2763245 [44] and knockout mice lacking nNOS expression still have adequate erectile function for coitus and reproduction [45]. Thus, it has been suggested that compensatory mechanisms may be activated to maintain erectile function in cases where only nNOS expression is decreased or blocked [46]. Measurement of intracavernosal pressure is a commonly used method to assess erectile function in rodents [47]. There was no evidence of impaired erectile function within 6 weeks in STZ-induced diabetic rats, although intracavernosal pressure began to decrease in the 8th week and became evident in the 10th–12th weeks [48,49]. The most important limitation of our study was the lack of intracavernosal pressure measurement. Therefore, we could not evaluate the clinical reflection of changes in relaxant and contractile responses of the diabetic CC. Our experimental period was 6 weeks. Taken together, we have concluded that the pathological changes leading to ED start at this stage of diabetes; however, a fully established clinical pathology may not have developed so far and the decrease in RhoA/ROCK-mediated contractile responses may be a compensatory mechanism against the impaired nitrergic neurotransmission.

Our results have shown that palosuran treatment did not affect the decreased nNOS expression. On the contrary, it further weakened the relaxant responses to EFS, and reversed the decrease in the relaxant response to the SNP and the pD_2 value of SNP. These results indicated that palosuran impairs nitrergic conduction but increases the sensitivity of trabecular SM to NO without affecting NOS synthesis. Although palosuran treatment reduced cavernosal U-II expression, it reversed the decreased RhoA expression and the increased relaxation response to Y-27632 and sensitivity to Y-27632 compared with untreated diabetic rats. However, it failed to reverse the decreased contractile responses to high concentration of KCl and cumulative concentrations of PE. This phenomenon indicates that the recurrent increases in expression levels of RhoA are partially suppressed by palosuran treatment. However, the effect of other RhoA effectors cannot be neglected.

It has been suggested that the activation of the urotensinergic system stimulates the NF-κB signaling by triggering the generation of reactive oxygen species [50], and UTR antagonism inhibits action of this mechanism [51]. However, in this study, U-II expression decreased while NF-κB expression increased with palosuran treatment. Since we have not revealed any evidence of oxidative stress, increased NF-κB expression may not be related to functional etiologic factors.

It has been reported that in isolated perfused rat pancreas, exposure to U-II reduced the hyperglycemia-induced insulin release [52] and long-term palosuran treatment reduced hyperglycemia by increasing insulin secretion and also caused weight loss in STZ-induced diabetic rats [10]. Therefore, it has been thought that U-II secreted from the pancreas may exert autocrine or paracrine effects on beta cells, while elevated plasma U-II concentrations acting as an endocrine factor may contribute to this effect [10]. Contrary to this assumption, in the present study, 6-week treatment with palosuran did not affect either hyperglycemia or weight loss in the STZ-induced diabetic rats. The main source of circulating U-II is believed to be vascular endothelial cells [53]. Since U-II is widely expressed in many tissue [17,18], the vascular bed covers a significant portion of the body and U-II can be released directly into the circulation. Plasma U-II levels are elevated in patients with atherosclerosis [41,53]. Atherosclerosis develops earlier in smaller arteries, such as the cavernous arteries, than in larger arteries, such as the coronary or femoral arteries [2,54]. Although available data related to cavernosal U-II expression are insufficient to comment on U-II levels in other organs or plasma, lack of evidence favoring the development of endothelial dysfunction predisposing to atherosclerosis suggests that plasma U-II expression does not probably increase. Although palosuran treatment can inhibit U-II expression, its lack of effect on hyperglycemia and weight loss may be related to the absence of U-II overexpression at the tissue or plasma level. STZ, a cytotoxic agent for pancreatic beta cells, is widely used in experimental DM studies. Its cytotoxic effect is dose-dependent and doses of 40–70 mg/kg [55] and 50 mg/kg or less [56] may be used to induce types 1 and 2 DM in rats, respectively [57]. In our study, the dose of STZ was 55 mg/kg. Another reason for the ineffectiveness of palosuran treatment on hyperglycemia may be the excessive decrease or depletion of insulin-secreting pancreatic beta cell reserve, depending on the STZ dose we administered.

Palosuran is a selective UTR antagonist with *in vitro* activity on human UTRs approximately 300-fold stronger compared with rats [58]. Indeed, we observed the relaxation responses when palosuran was directly administered *in vitro* to CC strips cumulatively, probably as a result of UTR receptor antagonism. There are strong indications that the urotensinergic system has an important role in DM and the beneficial effects of palosuran have been observed in rat models [10]. However, a clinical study conducted on patients with type 2 DM indicated that palosuran did not show any effect on insulin levels, glycemic regulation [58], or renal hemodynamic parameters [59]. It also should not be forgotten that animal studies may not always be sufficient to predict the human response. However, it has been suggested that this ineffectiveness has been largely due to the poor dosing regimen [60].

The vasoactive effect of U-II may vary significantly between animal species, vascular beds [11,61,62], and even vessels of the same type [19,62–66]. Additionally, exogenous U-II may also produce different vasoactive effects depending on its source (human, animal, or synthetic), the

method of administration (*in vivo* or *in vitro*) [67,68], and ambient conditions (physiological or pathological environment) [14,18]. We were unable to obtain any contraction or relaxation as a response to the incubation of CC strips with U-II at resting tension or after PE-precontraction. It has been suggested that unresponsiveness to U-II may result from the post-translational change related dysfunctional receptor production [69] or that UTR expression may be below the intensity required to elicit a response to the application of the U-II peptide [26,69]. However, multiple factors that may alter the effects of U-II make it difficult to perform comparisons between studies. On the other hand, cumulative concentrations of palosuran administered to PE-precontracted strips *in vitro* produced a relaxant response, although to a lesser extent in diabetic CC strips than in control. These responses correlated with the cavernosal RhoA expression. Palosuran may tend to acutely and directly block the vasoconstrictor effect rather than the vasodilatory effect of U-II, provided that U-II expression is unchanged. It may be postulated that palosuran may be effective in the treatment of ED by acting acutely and directly in case of overactivation of the RhoA/ROCK pathway.

5. Conclusion

Our results showed that both NO-mediated relaxation and ROCK-mediated contraction were reduced in the CC of diabetic rats. Palosuran treatment failed to reverse diabetes-related hyperglycemia and weight loss. In the diabetic CC, palosuran treatment did not affect NOS synthesis and worsened the nitrergic conduction; however, it increased the sensitivity of cavernosal SM to NO. Additionally, although palosuran decreased cavernosal U-II expression, it did not affect the ROCK-mediated attenuation in contraction. Some of the urotensinergic system-related effects of palosuran may have been overshadowed by the lack of overexpression of U-II and the short-lived experimental time. There may be hormonal and immunological factors outside the scope of this study contributing to the changes in the cavernosal tissue of diabetic rats, and this possibility cannot be excluded. Since U-II has a widespread distribution pattern and its overexpression plays an important role in diabetic complications and cardiovascular diseases, the development of treatments targeting U-II antagonism remains important.

Author contributions

Study concept and design: Murat Olukman.
Data acquisition: Yiğit Uyanıkgil, Türker Çavuşoğlu, Deniz Coşkunsever, Fatma G. Kozcu.
Data analysis: Murat Olukman, Neslihan Düzenli, Yiğit Uyanıkgil, Türker Çavuşoğlu.
Drafting of manuscript: Murat Olukman, Cenk Can, Sibel Ülker, Neslihan Düzenli.
Critical revision of the manuscript: Murat Olukman, Cenk Can, Sibel Ülker, Neslihan Düzenli.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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