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The Role of Rac1 on Carbachol-induced Contractile Activity in Detrusor Smooth Muscle from Streptozotocin-induced Diabetic Rats

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Abstract: This study was designed to determine the role of the small GTPase Rac1 on carbachol-induced contractile activity in detrusor smooth muscle using small inhibitor NSC 23766 in diabetic rats. Rac1 expression in bladder tissue was also evaluated. In the streptozotocin (STZ)-induced diabetic rat model, three study groups were composed of control, diabetic and insulin-treated diabetic subjects. The detrusor muscle strips were suspended in organ baths at the end of 8–12 weeks after STZ injection. Carbachol (CCh) (10^{-9}–10^{-4} M) concentration–response curves were obtained both in the absence and in the presence of Rac1 inhibitor NSC 23766 (0.1, 1 and 10 μM). Diabetes-related histopathological changes and Rac1 expressions were assessed by haematoxylin and eosin staining and immunohistochemical staining, respectively. CCh caused dose-dependent contractile responses in all the study groups. Rac1 inhibitor NSC 23766 inhibited CCh-induced contractile responses in all groups, but this inhibition seen in both diabetes groups was greater than in the control group. Histological examination revealed an increased bladder wall thickness both in the diabetes and in the insulin-treated diabetes groups compared to the control group. In immunohistochemical staining, expression of Rac1 was observed to be increased in all layers of bladder in both diabetic groups compared to the control group. In the diabetic bladders, increased expression of Rac1 and considerable inhibition of CCh-induced responses in the presence of NSC 23766 compared to those of the control group may indicate a specific role of Rac1 in diabetes-related bladder dysfunction, especially associated with cholinergic mediated detrusor overactivity.

Diabetes mellitus (DM) is the most common endocrine disease encountered all over the world. In more than 50% of patients with diabetes, diabetic voiding dysfunction has been observed [1]. However, there is limited information regarding the development process of the disease and initiation of the urological complications. This lack of information is limiting the development of new and effective prevention and/or therapeutic options in patients with diabetic voiding dysfunction [2].

In patients with diabetes, the pathophysiology of bladder dysfunction is multi-factorial. Patients display neuronal dysfunction, smooth muscle dysfunction and a broad spectrum of signs and symptoms of urothelium [3]. In the early stage of diabetes, hyperglycaemia causes myogenic and neurogenic changes and also compensatory hypertrophy due to induced polyuria. In the late stage of diabetes, the accumulation of oxidative stress products leads to decompensation of bladder tissue and bladder functions [3].

Rac1 is a small GTPase which belongs to the Rho family. Rac1, a subunit of NADPH oxidase, has been shown to increase the generation of reactive oxygen species (ROS) and, depending on the increased free radicals within the cell, plays a significant role in many cellular events and affects smooth muscle contraction [4]. Rac1 may have a role in increased oxidative stress due to hyperglycaemia in diabetes. Very few studies are available on this issue, and particularly on its role in the development of diabetes-related complications. Even though one study has reported increased expressions of Pak1, which is known as the kinase activated by p21, the effector molecule of Rac1 in the bladder tissue of diabetic animals, there is no study on the expression of small GTPase Rac1 in diabetic mouse bladder [5].

Similar to its beneficial effects shown in patients with diabetes, insulin therapy has been reported to delay or prevent diabetes-related complications in diabetic animal models. However, very few studies are available on the effects of insulin therapy on diabetes-related bladder dysfunction. In diabetic mice, a long-term insulin therapy has been reported to restore most of the changes in bladder function (i.e. increase in bladder capacity and compliance, increase in urine volume and residual volume) [6]. Low-dose insulin infusion has been reported to have anti-inflammatory, antiatherosclerotic effects as well as reducing effects for free radicals, which were probably mediated by its hypoglycaemic effect [7]. However, a study investigating how insulin treatment would affect the Rac1 pathway in diabetes-related bladder dysfunction could not be found in the literature.

Although voiding dysfunction can be seen in the vast majority of the patients with diabetes, there is limited information on

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its development process and complications as well as effective prevention and/or treatment options. The aim of this study was to investigate the role of Rac1 on diabetes-related bladder dysfunction, especially associated with cholinergic mediated detrusor overactivity.

Materials and Methods

Animals. Adult, male Wistar rats weighing 180–220 g were used in this study. The study was approved by the Ethics Committee of the Research of Laboratory Animals, Dokuz Eylül University Medical School, and all procedures were performed according to the 'Principles of Laboratory animal care' by the 'National Institute of Health' publication. All animals were kept in standardized conditions of temperature (21–22°C) and illumination (12:12 light/dark) and cages with mesh bottoms providing free access to tap water and pelleted food.

Induction of diabetes mellitus. Freshly prepared streptozotocin (STZ) was dissolved in 0.02 M citrate buffer (pH 4.5) and administered intraperitoneally to the rats at 45 mg/kg. The age-matched control group received citrate buffer only. On the third or fourth day after STZ administration, serum glucose levels were measured using an Optium Xceed glucometer (Abbott Diabetes Care, Oxon, UK) with a drop of blood taken from the tail vein under diethyl ether anaesthesia. Rats with blood glucose levels higher than 300 mg/dL were considered diabetic. The treatment (insulin or saline) was started on the fourth or fifth day after STZ injection, this was considered as the 1st day of treatment, and the same protocol was continued for 8–12 weeks. All the animals could not be included in the experiment at the end of 8th week. To reduce bias, we managed the same number animals of each study group (C, D and D+I) at the same study week while taking into account differences of study period. However, all the animals in the insulin group received insulin treatment until they were included in the experiment (8 or 12 weeks).

Drugs. Streptozotocin (N-(Methylnitrosocarbamoyl)-α-D-glucosamine, cat. no. S0130, FW: 265.22) and Carbachol (2-Hydroxyethyl) trimethylammonium chloride carbamate, Carbachol, Carbamoylcholine chloride, Carbamylcholine chloride, FW: 182.65) were purchased from Sigma-Aldrich Chemie GmBH (Stenheim, Germany) and Sigma-Aldrich Corporation (St. Louis, MO, USA) respectively.

Rac1 inhibitor NSC23766 (N6-[2-[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride) was purchased from Tocris Bioscience, Bristol, UK (catalogue no: 2161, FW (molecular weight): 530.97). Humulin N suspension 10 mL 100 IU/mL [NPH human insulin (Lilly, USA)] was used in the insulin treatment group. Streptozotocin (STZ) was immediately dissolved in citrate buffer before usage. Carbachol (CCh) and Rac-1 inhibitor NSC-23766 were dissolved in distilled water.

Experimental study design. Twenty-eight rats were divided into three groups as follows: STZ-induced diabetic rats (D, n = 10), age-matched control rats (C, n = 8) and insulin-treated diabetic rats (D + I, n = 10). While the insulin-treated diabetic rats (D + I) were treated with 6 U/kg/day NPH insulin (which is accepted to be effective for 18–24 hr, Lilly, USA) subcutaneously (s.c.) for 8–12 weeks, control (C) and diabetic groups (D) were treated with saline s.c. for the same period [7]. The rats were killed by cervical dislocation, and the urinary bladder was removed immediately for organ bath experiments, histological examination and immunohistochemical assessment. The blood samples were obtained via cardiac puncture for measuring blood glucose level.

Organ bath experiments. The urinary bladders were removed and placed into a Petri dish containing ice-cold Krebs–Henseleit buffer. The middle region of the bladder was cut into three strips [total strip number: D or D+I (n = 30, each) and C group (n = 24)]. The detrusor muscle strips (2 × 10 mm) were mounted in a 10-mL organ bath containing Krebs–Henseleit solution (composition in mmol/L: NaCl 118.2, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11, pH adjusted to 7.4), gassed 95% O_2 and 5% CO_2 and maintained at 32°C for decreasing spontaneous muscle contractions. Tissues were allowed to equilibrate for 30 min. prior to the beginning of each set of experiments (washed every 10 min. with fresh buffer) with a resting tension of 2 g. Contractions were recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac System Inc, Goleta, CA 93117,USA).

After the equilibration period, 120 mM KCl was added into the bath to check the viability of the tissues and equilibrated for 20 min. before specific experimental processes were initiated. Contractile responses were determined using cumulative concentrations of CCh (10^{-9}–10^{-4} M).

To determine the effect of Rac1 inhibitor NSC 23766 on CCh-induced contractions, in some experiments, Rac1 inhibitor NSC 23766 (0.1, 1 and 10 μM) was added to the organ bath 30 min. before the addition of cumulative CCh [8]. Only one concentration of NSC 23766 was tested on each strip. Concentration–response curves to carbachol were carried out in the absence and in the presence of Rac1 inhibitor NSC 23766 (0.1, 1 and 10 μM). Three concentrations of Rac1 inhibitor NSC 23766, respectively, 0.1, 1 and 10 μM were added into three different organ baths including the detrusor strips obtained from the same animal. Thus, we could evaluate the effects of 3 different doses of Rac1 inhibitor NSC 23766 on CCh-induced contractile responses of the detrusor strips of the same animal. Thus, only one single dose of Rac1 inhibitor for each strip was tested in each organ bath. All further contraction data are expressed as percentage of the maximum carbachol effect within the same preparation before addition of Rac1 inhibitor.

Isolated urinary bladder strips from D, DI and age-matched C animals were always set up in parallel under the same experimental conditions, to allow for adequate comparison of the tissues' contractile responses to KCl and CCh (in the absence or presence of NSC23766).

At the end of each experiment, the contractile response to 120 mM KCl was remeasured to verify that the contractile mechanisms were intact.

Histological examination. Bladder tissues were fixed in 10% buffered formalin and embedded in paraffin wax. Five-micron-thick sections were stained with haematoxylin and eosin. Histomorphometric measurements were taken with the UTHSCA Image Tool (software version 3.0, University of Texas Health Science Center, San Antonio, Texas, USA). Histological findings were recorded in detail.

Immunohistochemical assessment. All specimens were incubated with Rac1 (sc-217, Santa Cruz Biotechnology, 1/100 dilution) antibody, using a standard avidin–biotin complex method. After deparaffinization in xylene and rehydration with graded alcohols, sections were immersed in distilled water. They were then treated with trypsin (00-3008; Digest All 2A, Zymed, San Francisco, CA, USA) at 37°C for 15 min., to unmask antigens. Then, slides were rinsed three times for 2 min. each with deionized water. Sections were delineated using a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min. to inhibit endogenous peroxidase activity. They were then incubated with normal serum blocking solution for 30 min. and then incubated in a humidified chamber overnight at +4°C with primer antibody. Afterwards, they were washed three times for 5 min. each with PBS, followed by incubation with biotinylated secondary antibody and then

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with streptavidin–peroxidase conjugate (Histostain-Plus Broad Spectrum, 85-9043, Invitrogen, 542 Flynn Rd, Camarillo, CA9301, USA). After washing three times for 5 min. with PBS, sections were incubated for 5 min. with a DAB (3, 3′-diaminobenzidine) substrate containing diaminobenzidine (Cat No: 1718096, Roche Diagnostics Corporation, Indianapolis, USA) detect immunoreactivity and then with Mayer's haematoxylin. Sections were covered with Entellan mounting (Merck, Germany) and were analysed by light microscopy using a BH-2 microscope (Olympus, Tokyo, Japan). Negative control samples were processed in an identical manner but in the absence of primary antibody step. Immunolabelling intensity of Rac1 was graded (with semiquantitative assay) independently by two observers (G.E and S.C.M.) blind to the experimental conditions on the following scale: mild (+), moderate (+ +) and strong (+ + +) labelling [9].

Statistical analysis. All values were expressed as the mean ± standard error (mean ± S.E.M.). Statistical comparisons among the CRC of groups were analysed by two-way ANOVA and the post hoc Bonferroni test, using Prism statistical analysis packages (GraphPad Software, Prism 4.0, San Diego, CA, USA). The Kruskal–Wallis and Mann–Whitney *U*-test were used to compare Rac1 immunostaining intensity values between groups, using SPSS 15.0 software for Windows (Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

Results

Body and bladder weights and blood glucose values of animals.

Body-weights of the D group were significantly less than those of the C ($p < 0.05$) and DI ($p < 0.01$) groups. Bladder weights significantly increased in the D and DI groups compared to the C group ($p < 0.05$). Blood glucose levels were found to be increased in the D group compared to the C and DI groups ($p < 0.01$, table 1).

Investigation of changes in bladder detrusor muscle contractions.

Administration of KCl (120 mM) to bladder detrusor smooth muscle strips suspended in organ baths resulted in contractile responses in all the experimental groups. KCl-induced contractile responses significantly increased in the D (6707 ± 257 mg) and DI (6655 ± 254 mg) groups, compared to the C group (5647 ± 233 mg) ($p < 0.05$, fig. 1).

Administration of cumulative doses of carbachol (CCh, 10^{-9}–10^{-4} M) resulted in dose-dependent contraction responses in all groups. In the D and DI groups, CCh-induced contractile responses at high concentrations (10^{-5} and 10^{-4} M) were found significantly increased compared to the C group ($p < 0.001$, fig. 2). However, when CCh-induced contractions were evaluated as a percentage of KCl-induced (120 mM) contraction and compared, these two groups (diabetic groups *versus* control) did not differ significantly ($p > 0.05$, data not shown). Both CCh and KCl responses were increased in the diabetic groups (D and DI) but were less increased in the control group; as expected, CCh/KCl ratio remained unchanged among the study groups.

Rac1 inhibitor NSC 23766 (0.1, 1 and 10 μM) inhibited CCh-induced contractile responses in all groups (fig. 3A–C). In the control group, at 0.1 μM NSC 23766, the inhibition of CCh contractions was higher than that of 1 or 10 μM NSC 23766 ($p < 0.01$, fig. 3A,D). However, 1 and 10 μM NSC 23766 did not inhibit CCh responses significantly in the C group ($p > 0.05$, fig. 3A,D). In both diabetic groups, while each concentration of NSC 23766 significantly inhibited CCh-induced contractile responses ($p < 0.01$, fig. 3B,C), there was no significant difference in the Rac1 inhibitor-mediated inhibition of CCh responses among three different concentrations of NSC 23766. Also, in the presence of Rac1 inhibitor NSC23766, there were no differences between CCh contractile responses in the detrusor strips of the D and DI groups. While

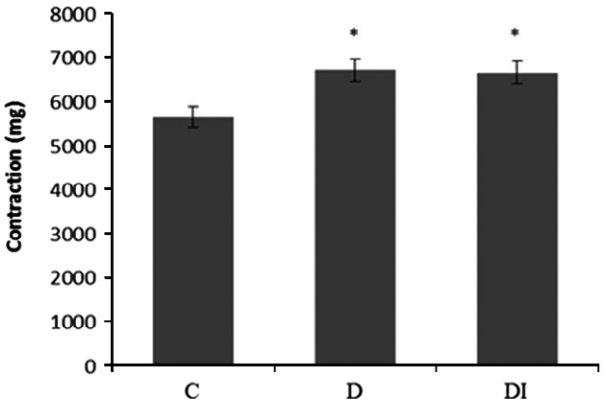


Fig. 1. Contraction response to KCl in all groups. *$p < 0.05$ compared to the C group.

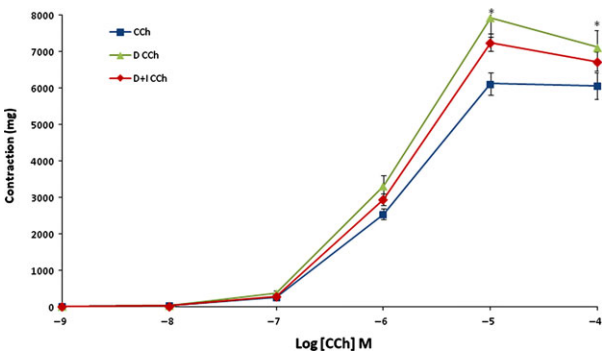


Fig. 2. Cumulative CCh (10^{-9}–10^{-4} M) concentration–response curves in all groups. *$p < 0.001$ compared to the C group.

Table 1.
Body-weight, bladder weight and blood glucose level in all groups.

	Body-weight (g)	Bladder weight (g)	Blood glucose level (mg/dL)[#]
C	231 ± 7	0.15 ± 0.02	210 ± 16
D	188 ± 11*	0.22 ± 0.01*	536 ± 35**
DI	234 ± 11***	0.21 ± 0.01*	290 ± 47***

C, control; D, diabetic and DI, insulin-treated diabetic group.
The values are expressed as mean ± S.E.M.
*$p < 0.05$, **$p < 0.01$ compared to the C group, ***$p < 0.01$ compared to the D group, [#]Non-fasting blood glucose level.

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the inhibition of CCh contractile responses by NSC23766 (1 and 10 μM) was greater in the D and DI groups, compared to the C group, there were no significant differences (fig. 3E,F).

CCh Emax values significantly increased in the D and DI groups compared to the C group ($p < 0.001$, table 2). CCh Emax values decreased significantly in the presence of Rac1 inhibitor NSC 23766 in all groups ($p < 0.001$, table 2). No significant difference was found among the CCh pD2 values of the groups in the absence of Rac1 inhibitor ($p = 0.101$, table 2). NSC 23766 caused a decrease in the CCh pD2 values in all groups, but the maximum decrease was observed in the presence of 10 μM NSC 23766 in all groups ($p < 0.001$, table 2).

Histological examination.

Histologically, all layers of the bladder sections demonstrated no difference in the C and DI group, while muscle fibres of the tunica muscularis layer were found to be irregular in the D group (fig. 4). Histomorphometric measurements demonstrated that bladder wall was 1.5 and 1.4 times thicker, respectively, in the D (1365.73 ± 42.71 mm) and DI groups (1280.34 ± 32.22 mm) than in the C group (937.68 ± 59.29 mm) ($p < 0.000$), while no difference was detected between the D and DI group in this regard ($p > 0.05$).

Assessment of Rac1 immunoreactivity.

Immunohistochemical examination indicated a statistically significant increase in the expression of Rac1 in all the layers in the D and DI groups rather than in the C group. Immunoreactivity of Rac1 in the lamina propria and tunica muscularis layers in the D group was higher than in the DI group ($p < 0.05$, the arrows show the immunopositive staining, table 3, fig. 4).

Discussion

In this study, we observed that the expressions of Rac1 increased in the bladder tissues of the diabetic rats. Organ bath studies demonstrated that, in the presence of Rac1 inhibitor NSC 23766 (1 and 10 μM), the inhibition of

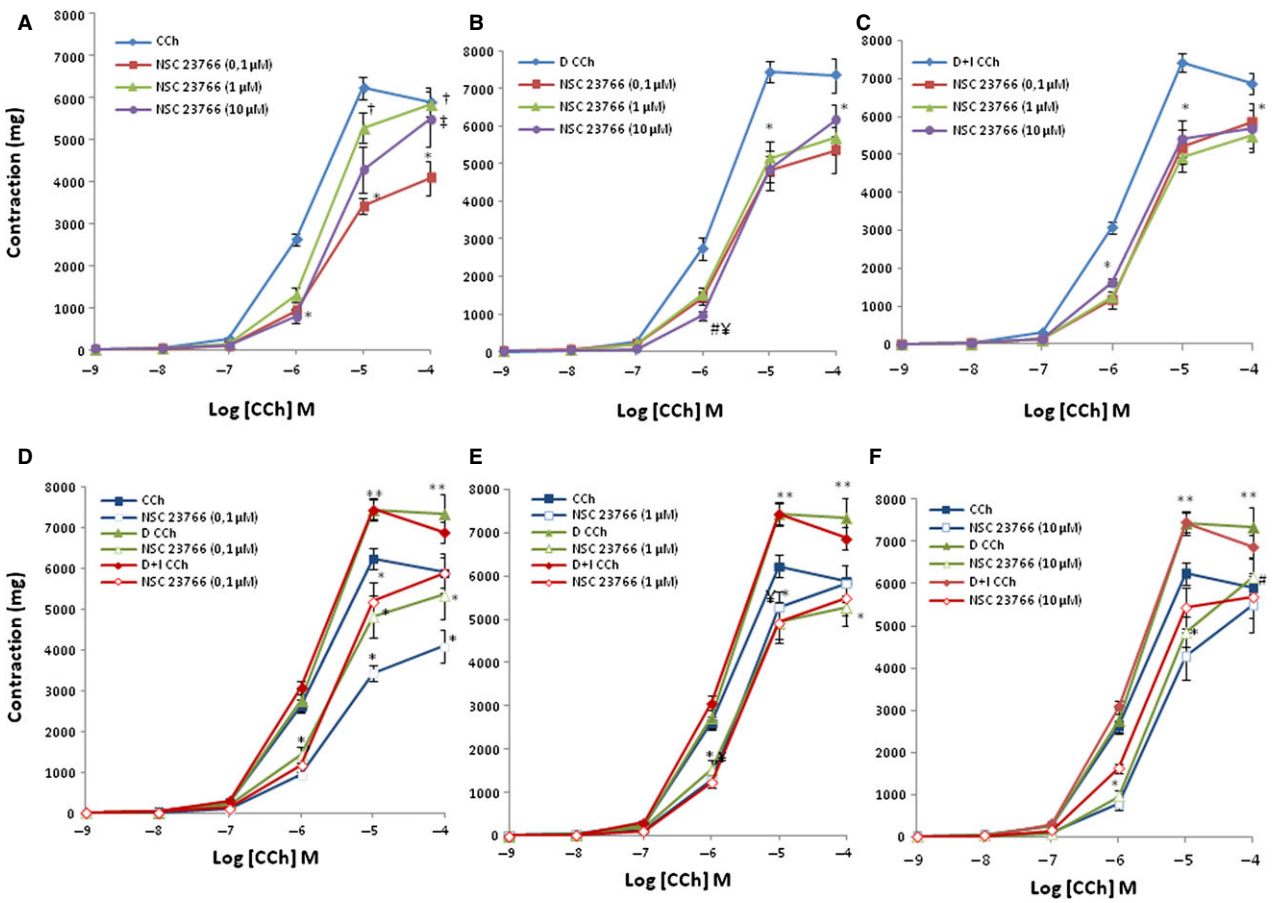


Fig. 3. The cumulative CCh concentration–response curves in the absence and presence of Rac1 inhibitor NSC 23766 (0.1, 1 and 10 μM) in control (A), diabetic (B) and insulin-treated diabetic groups (C). The cumulative CCh concentration–response curves in all study groups in the presence of NSC 23766 (0.1 μM) (D) and NSC 23766 (1 μM) (E) and NSC 23766 (10 μM) (F). $^{**}p < 0.001$ D and DI groups *versus* C for CCh responses in the absence of NSC23766 (Panel D, E and F). $^{*}p < 0.001$, $^{\#}p < 0.01$, $^{¥}p < 0.05$, CCh (in the absence of NSC23766) *versus* CCh in the presence of NSC23766 for intragroups (C *versus* C, D *versus* D, DI *versus* DI). $^{\dagger}p < 0.01$, CCh + (0.1 μM NSC23766) *versus* CCh+ (1 μM NSC23766) in the C group (panel A). $^{\ddagger}p < 0.01$, CCh + (0.1 μM NSC23766) *versus* CCh+ (10 μM NSC23766) in the C group (panel A).

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Table 2.
Maximum responses (Emax) and pD2 values of CCh in all groups.

	C		D		DI	
	Emax (mg)	pD2	Emax (mg)	pD2	Emax (mg)	pD2
CCh	6233 ± 259	5.90 ± 0.04	7437 ± 276†	5.80 ± 0.04	7445 ± 243†	5.91 ± 0.04
NSC 23766 (0.1 μM)	4087 ± 406*	5.55 ± 0.07*	5353 ± 616*	5.57 ± 0.10*,$^{\#}$	5875 ± 476*	5.62 ± 0.09*,$^{\#}$
NSC 23766 (1 μM)	5833 ± 421*	5.55 ± 0.08*	5679 ± 430*	5.62 ± 0.10*	5505 ± 423*	5.53 ± 0.06*
NSC 23766 (10 μM)	5485 ± 646*	5.37 ± 0.05*	6161 ± 417*	5.42 ± 0.05*	5679 ± 501*	5.39 ± 0.05*,$^{\#}$

$^{\dagger}p < 0.001$ *versus* C, *$p < 0.001$ *versus* CCh in all groups, $^{\#}p < 0.01$ *versus* C.

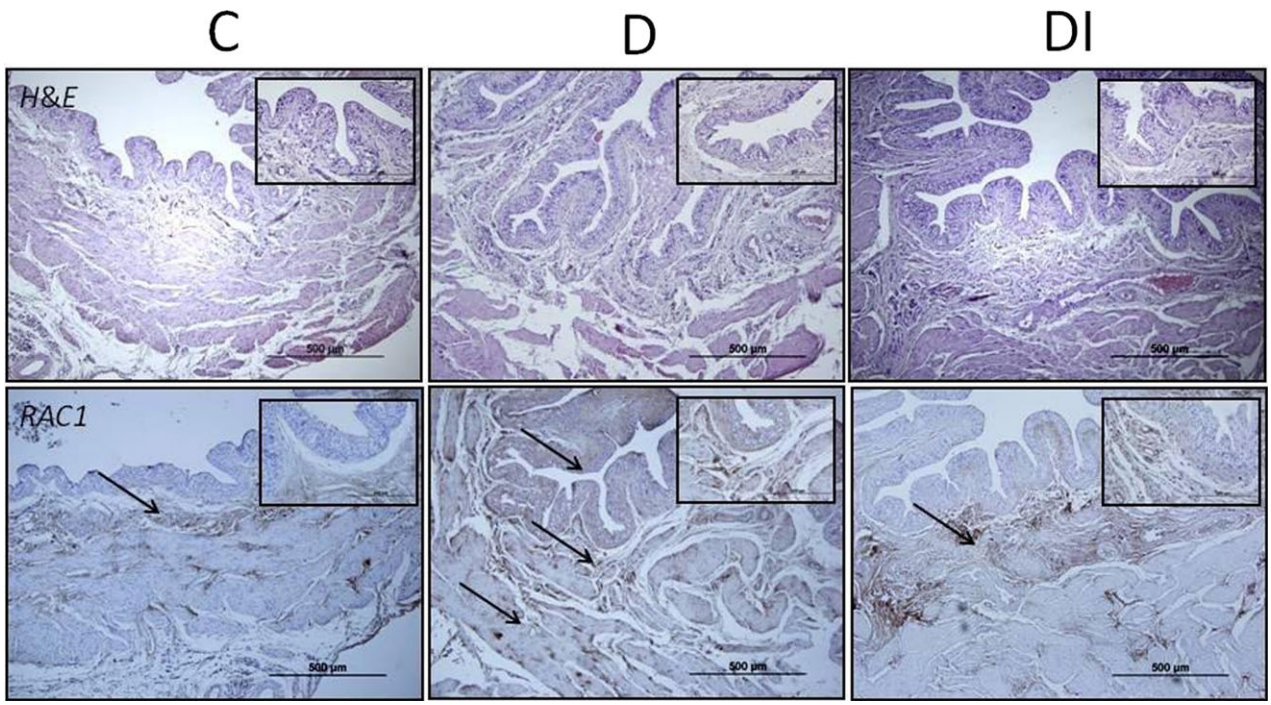


Fig. 4. Photomicrograph bladder sections from control (C), diabetic (D) and insulin-treated diabetic rats (DI). Haematoxylin–eosin (H&E; ×40 and ×100) stain and immunohistochemical staining for Rac1 (×40 and ×100). In H&E staining, all layers of the bladder sections demonstrated no difference in the C and DI groups, while muscle fibres of the tunica muscularis layer were found to be irregular in the D group. In immunohistochemical staining, expression of Rac1 was observed to be increased in all bladder layers in both the D and DI groups compared to the C group.

Table 3.
Rac1 immunoreactivity in urinary bladder in all groups.

Mean ± S.E.M.	Epithelium	Lamina Propria	Tunica Muscularis
C	0.16 ± 0.08	0.36 ± 0.10	0.24 ± 0.09
D	0.73 ± 0.11*	1.40 ± 0.12*	1.33 ± 0.11*
DI	0.57 ± 0.09*	0.70 ± 0.10*, $^{\#}$	0.77 ± 0.11*,$^{\#}$

*$p < 0.000$ compared to the C group, $^{\#}p < 0.01$ compared to the D group.

CCh-induced contractions of the detrusor muscle strips was more prominent for diabetic rats compared to the C group, but this was not of statistical significance. Increased expression of Rac1 in diabetic bladder, and also more prominent but statistically insignificant inhibition of CCh contractile responses with Rac1 inhibitor, indicates that Rac1 may be one of the reasons for the pathogenesis of diabetic bladder. As insulin therapy could not improve the decreased CCh contractile responses that were obtained in the presence of the Rac1 inhibitor, in addition to insulin, some other therapeutic choices may be required for the treatment of diabetes-related bladder dysfunction.

Consequently, further studies are required for promising therapies that target the small GTPase Rac1 in the bladder dysfunction in diabetes.

Hyperglycaemia is an independent risk factor causing complications in diabetes. One of the changes that hyperglycaemia may cause is bladder hypertrophy due to increased diuresis. Bladder hypertrophy was shown both in patients with diabetes and in diabetic animal models. Many studies reported significantly decreased body-weights, whereas there were significantly increased blood glucose levels and bladder weights for STZ-induced diabetic rats compared to the control group [6,7,10]. Consistent with the findings of previously reported studies, we observed increased blood glucose levels and bladder weights, and body-weights decreased in the diabetic rats.

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In the type I diabetic rat model, insulin therapy was shown to normalize blood glucose values, but did not improve bladder hypertrophy [11–14]. In accordance with these studies, we observed a decrease in the blood glucose levels and an improvement in the diabetes-related expected reduction in body-weight, while there was no increase in bladder weight in the diabetic group given insulin therapy.

The pathogenesis of diabetes-induced bladder dysfunction is multi-factorial. One of these factors is the functional changes of the detrusor smooth muscle. Kaplan *et al.* [15] reported that detrusor overactivity was the most common urodynamic finding (48%), followed by decreased detrusor contractility (30%) and impaired compliance (15%). In our study, contractile responses of bladder detrusor muscle strips suspended in organ bath to KCl were detected significantly increased in the diabetes and insulin-treated diabetes groups compared to the control group, which was consistent with some studies yet incompatible with others. When we analysed the incompatible studies, we found that either the KCl concentration was too low (30 mM) [10] or the duration of diabetes was shorter (4 weeks) [16] or that a different experimental animal, such as a rabbit, or a different diabetes model, such as alloxan-induced diabetes, was used [17–19]. The contractile responses obtained to KCl in the diabetic group were compatible with those reported in the STZ-induced rat models.

Enhanced contraction responses to K^+ may be due to smooth muscle hypertrophy. However, in many different studies, increased extracellular K^+ has been shown in non-diabetic animals as well as diabetic animals to cause smooth muscle (included detrusor smooth muscle) contractions by depolarizing cell membrane, either via increasing intracellular levels or increasing sensitivity to Ca^{2+} [3,20]. In addition, Bezuijen *et al.* reported in their study, in which urinary bladders of diabetic animals were grouped as small (under 265 mg) and large (over 265 mg), that KCl responses were higher in the small group and lower in the large group. This was associated with the stage of diabetes and interpreted in the way that animals with large bladder may be in a decompensated stage of diabetes and respond less because of the development of autonomic neuropathy [21]. Therefore, the detrusor muscle hypertrophy alone should be taken into account when evaluating the response to contractile agents, but this is not a factor that is satisfactory alone.

In diabetes, an increase in intracellular Ca^{2+} levels due to increased M3 muscarinic receptor density in smooth muscle cells or increased Ca^{2+} sensitization due to Rho kinase system activation might have led to the increased contractile response [3,22]. There are numerous studies indicating that detrusor smooth muscle overactivity may be a result of an increase in muscarinic receptor density in smooth muscle [20–24]. In the STZ-induced diabetic rat model, bladder sensitivity and maximum contractile responses have been reported to increase with muscarinic stimulation. In our study, the cumulative doses of CCh were determined to induce dose-dependent contractile responses in all groups; however, CCh-induced contractile responses and Emax values were found significantly increased in the diabetes and insulin-treated diabetes groups, compared to the control group. Our study data were compatible with several other studies, showing that contractile responses to muscarinic agonists increased in diabetic rats compared to the control rats [23–25]. Latifpour *et al.* [26] have observed in 8-week STZ-induced rats that contractile response of detrusor muscle to muscarinic agonists (CCh, ACh and bethanechol) increased more than controls, but EC50 values were similar to controls and diabetic rats. In our study, CCh Emax values increased in the diabetic group, whereas CCh pD2 values were found close to each other in all groups. The lack of any difference in terms of potency among the groups was consistent with previous studies [26,27].

One of the factors that may lead to diabetic complications is the increase in oxidative stress. Rac1, a subunit of NADPH oxidase enzyme, increases ROS generation and, due to increased intracellular free radicals, plays a role in many cellular events such as cell growth, migration, transformation and apoptosis [28,29]. Rac1 is also a signal transduction molecule having a role in smooth muscle contractions [30–32]. Although it depends on the tissue types, Rac1 may induce either contracting or relaxing effect on the tissues [8,30–32]. More recently, Rahman *et al.* [33] have shown the role of Rac1 for smooth muscle contractions (including urinary bladder) using small-molecule inhibitors (EHT1864 and NSC23766) in the Rac1-knockout non-diabetic mouse strain. While Rac1 was shown to be associated with smooth muscle contractions, including urinary bladder in non-diabetic animals, there is no functional study carried out with diabetic bladder. There is only study reporting the increase of oxidative stress and expression of Pak1 (p21-Rac), which is the effector molecule of Rac1 in the bladder tissue of the diabetic mouse model [5]. The present study is the first to demonstrate that the role of the small GTP-binding protein Rac1 in detrusor smooth muscle contraction induced by CCh was examined using small-molecule inhibitor NSC23766, and also the expression of Rac1 was shown using immunohistochemical analysis in the urinary bladder of a STZ-induced diabetic rat model.

In the present study, Rac1 inhibitor NSC 23766 significantly inhibited CCh-induced contractile responses of bladder detrusor strips in all study groups. However, the inhibition of CCh contractile responses by NSC23766 (1 and 10 μM) was greater in the diabetic groups, compared to the C group, but there were no significant differences between the two groups. These results suggest that Rac1 may play a significant role in detrusor smooth muscle functions mediated by cholinergic stimulation in all study groups including diabetic rats. In this respect and to our knowledge, this is the first study to show the role of Rac1 on cholinergic activity in the diabetic rat bladder. Our results are consistent with the study by Levay *et al.* [34] reporting that NSC 23766 inhibits CCh-induced inotropic response mediated through M2 muscarinic acetylcholine receptor (M2 mAChR) in neonatal rat cardiac myocytes in organ bath and suggests that Rac1 inhibitor NSC 23766 is not only an inhibitor of Rac1 activation but also a competitive antagonist at mAChRs. Our experimental protocol does not allow us to determine whether NSC23766-induced inhibition

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of CCh responses occurs through selective Rac1 inhibition or non-selective cholinergic inhibition. In any case, Rac1 inhibitor NSC23766 inhibited CCh-induced contractions in diabetic bladder strips one way or another. To our knowledge, our study is the first to examine the effects of Rac1 inhibitor NSC23766 on diabetic urinary bladder strips *in vitro*.

Epidemiological studies have proved the benefits of insulin therapy in improving glycaemic control and also in reducing the risk of long-term complications of both type I and type II diabetes [35]. In diabetic animal models, insulin therapy was shown to prevent or delay diabetes-related complications [7,12,14]. Low-dose insulin infusion therapy exhibits anti-inflammatory, antiatherosclerotic and ROS-reducing effects, most likely through its own hypoglycaemic activity [7,36]. Insulin therapy was reported to improve ACh-induced vasorelaxation in diabetic rats by increasing endothelial NO production [7,37]. In STZ-induced diabetic animal models, chronic insulin therapy was shown to prevent endothelial relaxation in mesenteric arteries and aortic rings [7,38,39], and also reduction of agonist-induced contractile responses in rat aorta [40]. Paskaloglu *et al.* [41] examined diabetic aorta and corpus cavernosum tissues in their study and reported that insulin therapy completely improved the reduction in phenylephrine-induced contractile responses, but not in ACh-induced relaxation responses.

There are very few studies conducted on the effects of insulin therapy on diabetes-related bladder dysfunction in patients with diabetes and diabetic animal models [6,11,35,40,42]. The first approach for the treatment of bladder dysfunction in patients with diabetes is metabolic control, and subsequently, other treatment modalities are recommended [35]. It was reported that diabetes-related muscarinic receptor density increase detected in the bladder of insulin-treated diabetic rats may be inhibited or improved with insulin treatment [11,42]. In diabetic mice, long-term insulin therapy was reported to reverse most of the changes regarding bladder functions (such as increase in bladder capacity and compliance, and increase in urine volume and residual volume) [6]. In our study, KCl and CCh responses of the DI group were much more similar to those of the D group. Although our data may seem contradictory with the studies reporting that insulin can inhibit the increase in the density of muscarinic receptors, Kobayashi *et al.* [40] demonstrated that low-dose insulin therapy (10 U/kg) did not change contractile responses of diabetic aorta to KCl, but nevertheless, high-dose insulin (50 U/kg) did this. The low dose of insulin (6 U/kg) in our study may be the reason for the lack of any improving or therapeutic effects on the contractile responses in diabetic urinary bladder.

When analysing diabetes-related changes in the bladder histologically, histomorphometric measurements of bladder wall thickness displayed 1.5 and 1.4 times higher values, respectively, for the D and DI groups, compared to the C group, which is compatible with previous studies [43,44]. We detected no difference between the bladder wall thickness measurements of D and DI groups. In some studies, hyperaemia and oedema were observed in the lamina propria of bladder mucosa of diabetic rats, and moreover, smooth muscle fibres were reported to be irregular [45]. In our study, we did not observe any difference in the bladder epithelium and lamina propria layers among the groups, whereas fibres of the tunica muscularis showed some irregularities in the D group compared to the C group. Tunica muscularis layer of DI group was similar to that of the D group. Analysing Rac1 expressions with immunohistochemical staining demonstrated that Rac1 expressions significantly increased in all the bladder layers in the D and DI groups compared to the C group. To our knowledge, no study has investigated Rac1 expression in diabetic bladder so far; thus, we believe our results are important as being the first to examine this issue.

Immunoreactivity of Rac1 in the lamina propria and tunica muscularis layers in the insulin-treated group was less than those of the untreated diabetic groups. While insulin treatment significantly decreased Rac1 overexpression in diabetic bladders, there were no modified detrusor contractile responses in the presence of Rac1 inhibitor. The exact reason for this is not known, and further detailed studies are needed. Perhaps, our insulin dose was sufficient to reduce Rac1 overexpression or insufficient to regulate smooth muscle functions. This may suggest that Rac1 overexpression is not a primary reason but does not rule out to be one of the reasons. Insulin therapy reduces blood glucose level but not the weight of the bladder, and hence, the smooth muscle hypertrophy. Therefore, the increase in hypertrophy-induced smooth muscle contractions may be an obstacle for insulin to show its optimal action. Furthermore, in the case of an extreme increase in diabetes-induced Rac1 activation (e.g. constitutive activation), even the expression is decreased; increased activation in the rest of the Rac1 protein may help to maintain its effect. This explanation is our untested hypothesis. As we have examined only the expression level and not the Rac1 activation in our study, we could not demonstrate this. Therefore, further analysis such as evaluation of Rac1 activation is definitely required. This is another limitation of our study.

In the light of the findings of this study, the increase in the CCh contractile responses and in the inhibitory effects of NSC 23766, a Rac1 inhibitor on the contractile responses of CCh in diabetes, may be explained by the increased expression and/or activity of the small GTPase Rac1 in diabetes. The mechanisms of this inhibition of CCh-induced contractions by NSC23766 are not clear and therefore worthy of further investigation. Although therapeutic or preventive effects of insulin therapy on other complications related to diabetes has been reported, but bladder dysfunction in diabetes can not be cured effectively with insulin alone, in our study.

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Declaration of Interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of this article.

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