Pharmacological Properties, Functional Alterations and Gene Expression of Muscarinic Receptors in Young and Old Type 2 Goto-Kakizaki Diabetic Rat Bladders

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Purpose: We investigated pharmacological properties, functional alterations and gene expression of the muscarinic receptor system in young and old Goto-Kakizaki rat bladders.

Materials and Methods: Male 12 and 70-week-old Goto-Kakizaki rats and age matched male Wistar rats were used in this study. Bladder function was estimated by voiding behavior, cystometric and functional studies using KCl, carbachol and various concentrations of subtype selective muscarinic antagonists, ie pirenzepine, methoctramine, 4-DAMP (Sigma®) and atropine (Wako Pure Chemical Industries, Osaka, Japan). The participation levels of M_2 and M_3 receptor mRNA in the bladder were investigated by real-time polymerase chain reaction.

Results: In voiding behavior studies there were no significant differences in urine output, although an age related decrease in micturition frequency and an age related increase in single voided volume were observed in Goto-Kakizaki and Wistar rats. In cystometric studies there were no significant differences in maximum detrusor pressure or bladder capacity, although residual urine volume was significantly increased in 70-week-old Goto-Kakizaki rats. In functional studies carbachol induced detrusor contractility was significantly increased in Goto-Kakizaki rats in each age group. Estimated pA_2 values for atropine, pirenzepine, methoctramine and 4-DAMP (Sigma) indicated that the carbachol induced contractile response was mediated through the M_3 receptor subtype in all groups. Furthermore, muscarinic M_2 and M_3 receptor mRNA was significantly up regulated in 70-week-old Goto-Kakizaki rat bladders.

Conclusions: Our data indicate that noninsulin dependent diabetes induces alterations in the muscarinic receptor system, which may contribute to the development of diabetic cystopathy.

Key Words: urinary bladder; diabetes mellitus; rats; receptors, muscarinic; gene expression

Diabetic cystopathy, which is a form of bladder dysfunction, is a major complication of diabetes, occurring in 25% to 83% of patients with diabetes mellitus.[1,2] Kaplan et al reported that classic diabetic cystopathy is not the most common urodynamic finding in patients with diabetes mellitus and voiding dysfunction, and in fact these patients present with variable pathophysiological findings.[3]

The STZ induced diabetic rat is the most commonly used and well investigated experimental model for type 1 diabetes.[4,5] Bladder function alterations are also seen in STZ induced diabetic rats.[4,5] For example, increased urine output, frequent voiding and an atonic bladder are observed after at least 2 weeks of diabetes induction in this animal model.[6] However, only limited information is available about type 2 diabetic rat cystopathy.

The GK rat represents a spontaneous, noninsulin dependent diabetes model. GK rats are produced from normal Wistar rats by selective breeding repetition and they are a widely accepted, genetically determined rodent model for human type 2 diabetes.[7,8] This genetic rat model is particularly relevant to human type 2 diabetes because defects in glucose stimulated insulin secretion, peripheral insulin resistance and hyperinsulinemia are seen as early as 4 weeks after birth, while abnormalities that occur later include insulin secretion and modest hyperglycemia.[9] Furthermore, some studies have shown age related and diabetes induced, duration related alterations in bladder function.[10,11]

To obtain more information on the detailed mechanisms of type 2 diabetes induced alteration of bladder smooth muscle we investigated bladder contraction using KCl and carbachol with subtype selective muscarinic antagonists. We also determined muscarinic M_2 and M_3 receptor mRNA expression levels by real-time PCR.

MATERIALS AND METHODS

Animal Model

All animal experiments were performed in accordance with the guidelines established by the Tottori University committee for animal experimentation. Six-week-old male GK and Wistar rats (SLC, Shizuoka, Japan) were maintained under identical conditions with free access to food and drinking

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Study received approval from the Tottori University committee for animal experimentation.

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water. The rats were divided randomly into 4 groups of 6 to 8 each. Groups 1 and 2 consisted of 12-week-old Wistar and GK rats, while groups 3 and 4 consisted of 70-week-old Wistar and GK rats, respectively. Upon attaining age 12 or 70 weeks the rats were sacrificed with an intraperitoneal overdose of pentobarbital (60 mg). Blood samples were collected from the vena cava and isolated bladders were used in tissue bath experiments or frozen at −80C for measurement of muscarinic M_2 and M_3 receptor mRNA.

Serum Glucose and Serum Insulin Measurement

The serum glucose concentration in experimental rats was measured by the hexokinase method (Glucose CII, Wako Pure Chemical, Osaka, Japan), which was done according to manufacturer instructions in all groups. In groups 3 and 4 the insulin concentration was also measured by enzyme-linked immunosorbent assay according to manufacturer instructions (Rat Insulin ELISA, Mercodia, Uppsala, Sweden).

Voiding Behavior Studies

Voiding behavior studies were performed according to methods used in our previous study[12] at age 12 or 70 weeks in all groups. All rats had free access to water from the time that they were initially placed in the cage. The parameters of the micturition reflex that were determined were micturition frequency, total urine output and single voided volume.

Cystometric Studies

Cystometric studies were performed according to methods used in our previous report[12] at age 12 or 70 weeks in all groups. According to our previous report the parameters evaluated were bladder capacity, Pdet, single voided volume and residual urine volume.

Measurement of Bladder Contractile Force

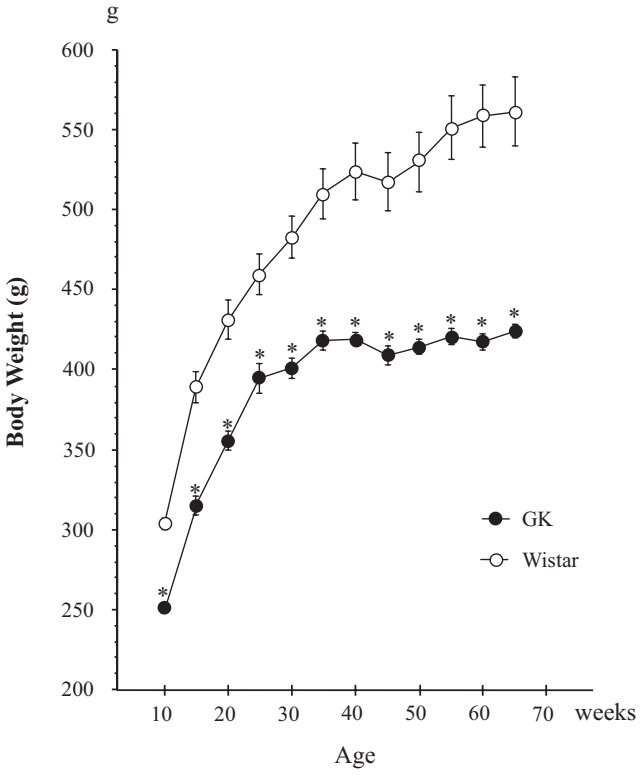
Functional studies were conducted according to methods used in our previous reports.[12,13] Razor blades were used to cut uniform longitudinal 1.5 × 5 mm strips of the posterior wall of the bladder dome. Changes in the tone of the strips were measured isometrically by force transducers and data were recorded on a Macintosh® G3 personal computer using Chart™, version 3.6.9 and a PowerLab®/16sp data acquisition system. Cumulative concentration-response curves to carbachol and KCl (100 mmol/l) were constructed. Carbachol induced contractile responses were measured cumulatively in the presence or absence of various concentrations of muscarinic receptor antagonists, including PRZ, MTR, 4-DAMP and ATR.[13] Antagonists were added 30 minutes before carbachol administration. After completing a concentration-response curve the tissue was washed until baseline force returned to the resting level and equilibrated for 30 minutes. The next consecutive concentration-response curve was then constructed.

Real-Time PCR to Quantify Muscarinic M_2 and M_3 receptor mRNA

To measure muscarinic receptor mRNA on the detrusor the urothelium in every rat was carefully peeled under illuminated magnifiers (Otsuka, Tokyo, Japan). Muscarinic M_2 and M_3 receptor mRNA in the experimental bladder dome was measured by real-time PCR according to our previous reports.[12,13] RNA was purified by an RNeasy® Fibrous Tissue Mini Kit according to manufacturer instructions. A reverse transcriptase mixture (28 μl) containing 2 μg total RNA was made and incubated at 37C for 60 minutes according to a previously reported method.[12,13] The mixture (5 μl) was used for real-time PCR, which was performed with a LightCycler® thermal cycler system with a LightCycler-FastStart DNA Master Hybridization Probe according to manufacturer instructions.[14] The primers and probe sequences specific to the genes of muscarinic M_2 (accession number NM 012527) and muscarinic M_3 (accession number NM 031016) receptors were used according to our previous reports.[12,13] The primer and probe of β-actin (accession number NM_031144) were used from the LightCycler-Primer/Probe Set (rat). A total of 15 μl solution were used for the sample. PCR products were subjected to 2% agarose gel electrophoresis. The β-actin gene served as the internal standard and analyzed by real-time PCR using the same RT mixture.

Data Analysis

EC_{50} and E_{max} values were obtained by a Macintosh G3 computer located with Chart version 3.6.9 software and a PowerLab/16sp data acquisition system. The dose ratio was obtained from the ratio of EC_{50} values for carbachol in the presence or absence of an antagonist. The pA2 values were obtained from Schild plots.[15] Schild plots were constructed by plotting the log of (dose ratio − 1) against the log of the molar concentration of the antagonist. EC_{50} values were calculated as geometric means, whereas E_{max} values were calculated as arithmetic means. The expression of muscarinic M_2 and M_3 receptor mRNA was quantified according to the expression of β-actin mRNA in experimental rat bladder



Mean ± SEM body weight in GK and Wistar rats. Data represent 6 to 8 determinations per group. Asterisk indicates significantly different at same age in Wistar rats. *g*, gm.

TABLE 1. *General features of experimental rats*

Group	Mean ± SEM Body Wt (gm) Age 12 Wks	Mean ± SEM Body Wt (gm) Age 70 Wks	Mean ± SEM Bladder Wt (gm)	Mean ± SEM Bladder/Body Wt ($\times 10^{-4}$)	Mean ± SEM Serum Glucose (mg/dl)	Mean ± SEM Serum Insulin (μg/l)
1	350.0 ± 4.9	—	0.095 ± 0.001	2.73 ± 0.05	153.4 ± 7.9	Not performed
2	298.3 ± 5.4*	—	0.084 ± 0.004*	2.82 ± 0.09	233.1 ± 12.3*	Not performed
3	—	561.3 ± 21.7†	0.128 ± 0.006†	2.34 ± 0.10†	132.2 ± 5.9	2.52 ± 0.27
4	—	424.3 ± 4.2*,†	0.127 ± 0.006†	3.21 ± 0.12*,†	212.1 ± 8.2*	0.43 ± 0.08*

Six to 8 separate determinations per group.
* Groups 1 vs 2 and 3 vs 4 significantly different.
† Groups 1 vs 3 and 2 vs 4 significantly different.

domes. A statistical comparison of differences between groups was performed using ANOVA and Fisher's multiple comparison test with $p < 0.05$ considered significant.

Drugs and Chemicals

Besides carbachol, 4-DAMP, PRZ. MTR and ATR, all other chemicals were available commercially and of reagent grade.

RESULTS

General Features of Experimental Animals

The figure and table 1 show data on the general features and serum concentration of insulin and glucose in the experimental animals. GK diabetic rats had a significantly small weight gain by age 10 weeks as well as a significantly small weight gain during the experimental period (see figure). There was no significant difference in bladder weight between groups 1 and 2 or 3 and 4. However, the bladder-to-body weight ratio in group 4 was markedly greater than that in groups 2 and 3. Significantly higher serum glucose levels were confirmed at age 12 weeks in GK diabetic rats. Significantly higher serum glucose and lower serum insulin levels than those in control rats were confirmed in 70-week-old GK diabetic rats.

Voiding Behavior Studies

Table 2 shows the results of voiding behavior studies in experimental animals. These studies revealed no significant differences in urine production, micturition frequency or single voided volume between GK and control rats at either age. Although urine production was similar in all groups, age related alterations in micturition frequency and single voided volume were observed. Older rats (groups 3 and 4) showed significant decreases in micturition frequency and significant increases in single voided volume.

Cystometric Studies

Table 3 shows the results of cystometric studies. Although in these studies single voided volume in groups 3 and 4 tended to be larger, there were no significant differences in Pdet and single voided volume between any groups. However, residual urine volume in group 4 was markedly greater than in groups 2 and 3.

Measurement of Contractile Responses to Carbachol and 100 mM KCl

E_{max} values of the contractile responses of the longitudinal muscles to carbachol and KCl (100 mM) were determined (table 4). Diabetes induced detrusor hyperreactivity and age related hyporeactivity by carbachol was observed in these experimental rats when normalized to 100 mmol KCl. However, there were no significant differences in EC_{50} values with respect to carbachol between any groups. Table 5 lists pA_2 values and slopes of the Schild plots for these muscarinic receptor antagonists in rat detrusor. The pA_2 values calculated for series of muscarinic antagonists were similar in all groups. They were rank ordered as ATR > 4-DAMP > MTR > PRZ. The slopes of the Schild plots for these muscarinic receptor antagonists were similar between groups. These data suggest that in control bladder smooth muscle contractile responses induced by carbachol are mediated through the muscarinic M_3 receptor subtype and carbachol does not alter contractile systems according to age or the presence of diabetes.

Measurement of Muscarinic M_2 and M_3 Receptor mRNA in the Rat Bladder Dome

Table 6 shows the expression of muscarinic M_2 and M_3 receptor mRNA in the bladder dome. Although expression levels of muscarinic M_2 and M_3 receptor mRNA were similar in the younger groups, those in older GK rats were significantly higher than in age matched controls. Furthermore, control group 1 had higher expression of muscarinic M_3 than of muscarinic M_2 receptor mRNA. In all groups the expression level of muscarinic M_3 receptor mRNA was approximately 2 to 3 times higher than that of muscarinic M_2 receptor mRNA.

TABLE 2. *Voiding behavior studies in experimental rats*

Group	Mean ± SEM No. Voids/Day	Mean ± SEM Urine Production (ml/day)	Mean ± SEM Single Voided vol (ml)
1	12.3 ± 0.8	10.7 ± 1.1	0.87 ± 0.09
2	13.6 ± 0.9	12.8 ± 0.9	0.94 ± 0.08
3	9.1 ± 1.7*	12.8 ± 0.9	1.40 ± 0.22*
4	8.1 ± 1.2*	13.2 ± 1.3	1.62 ± 0.32*

Six to 8 determinations per group.
* Groups 1 vs 3 and 2 vs 4 significantly different.

TABLE 3. *Cystometrogram data in experimental rats*

Group	Mean ± SEM Pdet (cm H_2O)	Mean ± SEM Single Voided Vol (ml)	Mean ± SEM Residual Urine (ml)
1	42.4 ± 5.7	0.31 ± 0.03	0.054 ± 0.033
2	33.7 ± 2.9	0.34 ± 0.04	0.035 ± 0.010
3	35.3 ± 8.7	0.52 ± 0.13	0.044 ± 0.020
4	44.1 ± 4.3	0.51 ± 0.08	0.230 ± 0.057*

Six to 8 determinations per group.
* Groups 1 vs 2, 3 vs 4, 1 vs 3 and 2 vs 4 significantly different.

TABLE 4. *Functional studies in experimental rats*

Group	Mean ± SEM Carbachol Emax*	Mean ± SEM Carbachol ED_{50} ($\times 10^{-6}$ M)
1	1.57 ± 0.05	1.19 ± 0.12
2	1.88 ± 0.09†	2.88 ± 0.92
3	1.39 ± 0.05‡	2.01 ± 0.55
4	1.61 ± 0.05†,‡	2.22 ± 0.49

Six to 8 determinations per group.
* Contractile force to 100 mM KCl.
† Groups 1 vs 2 and 3 vs 4 significantly different.
‡ Groups 1 vs 3 and 2 vs 4 significantly different.

TABLE 6. *Muscarinic M_2 and M_3 receptor mRNA expression in bladder dome normalized to β-actin mRNA*

Group	Mean ± SEM M_2/β-Actin ($\times 10^{-3}$)	Mean ± SEM M_3/β-Actin ($\times 10^{-3}$)
1	2.57 ± 0.89	4.69 ± 0.88
2	2.72 ± 0.73	4.68 ± 0.17
3	1.50 ± 0.40	5.28 ± 0.11
4	4.48 ± 1.38*	8.26 ± 0.19*,†

Six to 8 determinations per group.
* Groups 1 vs 2 and group 3 vs 4 significantly different.
† Groups 1 vs 3 and 2 vs 4 significantly different.

DISCUSSION

Latifpour[4] and Fukomoto[5] et al reported significant up-regulation of muscarinic receptor expression and hypercontractility induced by the muscarinic receptor agonist carbachol in the bladder dome of rats with diabetes. Interestingly those series did not demonstrate significant alterations in EC_{50} values for carbachol or of K_i values of [^{3}H] quinuclydinyl benzylate for muscarinic receptor subtype selective antagonists. Those findings indicated that diabetes induced alterations in the bladder muscarinic receptor system are quantitative rather than qualitative. Tong[16] and Cheng[17] et al reported that STZ induced diabetes increases mRNA and protein expression of M_2 and M_3 muscarinic receptors in the urothelium as well as in the muscle layer. In our recent studies analysis of diabetes induced alterations in the bladder using pharmacological, biochemical and biological methods support their findings.[12,13] Recently Daneshgari et al noted that diabetic bladders may undergo a transition from a compensated to a decompensated state and this transition in the STZ rat model may begin 9 to 12 weeks after induction.[11] Thus, the STZ rat model of type 1 diabetes has been well investigated and characterized.

However, only limited information is available for bladder dysfunction in type 2 diabetic models. In 32-week-old female GK rats Miyamae et al observed that damage to the autonomic nervous system and peripheral nerves in the bladder causes a decrease in acetylcholine release during bladder contractions, which may be related to voiding dysfunctions in diabetes mellitus.[18] Yono et al reported age related alterations in the biochemical and functional properties of the bladder in type 2 diabetic GK rats.[19] In their study the maximum contractile responses to carbachol and adenosine triphosphate, and the release of acetylcholine induced by field stimulation were similar in bladders from GK and control rats until age 8 weeks. However, at ages 16 and 32 weeks GK rats had increased contractile responses to carbachol and levels of ATP along with decreased release of acetylcholine compared to controls.

Previously we reported that diabetes induces an increase in maximum detrusor pressure during voiding, which occurred by urethral dysfunction associated with diabetic neuropathy.[12] In this study we noted a similar tendency of Pdet in GK rats. Pdet in 70-week-old GK rats (group 4) tended to be higher than that in age matched Wistar rats (group 3). However, there was no significant difference between groups 3 and 4. This may be due to great variation of the data presented. In the current study we also observed the hypercontractility of detrusor smooth muscle to carbachol in GK rats at each age compared to that in age matched control rats. These data are similar to previously reported data on STZ induced diabetic rats.[12,13] We also noted that 70-week-old GK diabetic rats had significantly increased residual urine volume compared to age matched control rats and 12-week-old GK rats. These data are particularly interesting because early stage, STZ induced diabetic rats and late stage GK rats showed similar cystopathy patterns.

To clarify the mechanisms underlying type 2 diabetic cystopathy we performed pharmacological and biological examinations. To confirm these putative changes in the muscarinic receptor system we calculated pA_2 values and their slopes using 4 subtype nonselective and selective muscarinic alterations. There were no significant differences in pA_2 values and slopes between diabetic and nondiabetic rats for any of the muscarinic receptor antagonists in the current study. The results of pA_2 calculations for this series of muscarinic antagonists were similar in all groups with a rank order of values of ATR > 4-DAMP > MTR > PRZ. These findings indicate that alteration of the contractile response via the muscarinic M_3 receptor subtype is not due to changes in muscarinic receptor affinity in the diabetic rat detrusor. Rather, such changes appear to be the result of quantitative rather than qualitative changes in the muscarinic receptor system.

Mutoh et al previously reported that the rank order of pA_2 values for these muscarinic antagonists was ATR > 4-DAMP > PRZ > MTR in the rabbit bladder dome.[20] We also previously reported that the rank order of pA_2 values

TABLE 5. *ATR, PRZ, MTR and 4-DAMP pA_2 carbachol and regression slopes from Schild plots*

Group	ATR Median pA_2 (range)	ATR Mean ± SEM Slope	PRZ Median pA_2 (range)	PRZ Mean ± SEM Slope	MTR Median pA_2 (range)	MTR Mean ± SEM Slope	4-DAMP Median pA_2 (range)	4-DAMP Mean ± SEM Slope
1	9.48 (9.37–9.62)	1.07 ± 0.09	6.59 (6.45–6.82)	0.71 ± 0.08	7.57 (7.28–8.94)	0.80 ± 0.08	8.84 (8.74–8.96)	1.15 ± 0.08
2	9.58 (9.49–9.71)	0.91 ± 0.11	7.27 (7.08–7.61)	0.86 ± 0.10	8.03 (7.79–8.60)	0.79 ± 0.16	9.17 (9.03–9.25)	1.00 ± 0.07
3	9.96 (9.83–10.14)	0.83 ± 0.09	7.59 (7.41–7.92)	0.76 ± 0.14	8.13 (7.98–8.45)	0.89 ± 0.21	9.16 (9.01–9.40)	1.01 ± 0.08
4	9.80 (9.68–9.98)	1.18 ± 0.13	6.84 (6.62–7.29)	0.75 ± 0.11	7.62 (7.47–7.85)	0.99 ± 0.25	9.16 (9.09–9.26)	1.06 ± 0.08

Six to 8 determinations per group.

for these muscarinic antagonists in the rat bladder smooth muscle was ATR > 4-DAMP > MTR > PRZ.[13] Regarding the role of muscarinic receptors these data suggest that the rat detrusor undergoes contractions via the muscarinic M_3 receptor subtype in younger and older GK rats.

To elucidate the mechanisms underlying diabetes induced hypercontractility to carbachol in the rat detrusor we measured the expression levels of muscarinic M_2 and M_3 receptor mRNA using real-time PCR. Results showed that the mRNA levels of muscarinic M_2 and M_3 receptors were increased under diabetic conditions at 70 weeks compared to those in age matched controls. Based on our previous and current data it appears likely that the over expression of muscarinic M_2 and M_3 receptor mRNA is related to detrusor hypercontractility in 70-week-old diabetic rats.[12,13]

The possible mechanisms of this up-regulation of muscarinic receptors are explained by a decrease in cholinergic nerve density[18] or by a defective neurotransmitter release mechanism.[21] Miyamae et al reported a decrease in cholinergic nerve density in GK rats,[18] while Tong et al reported defective neurotransmitter release mechanism in 2-week-old STZ induced diabetic rats.[21] Diabetes associated neuropathy may inhibit the release of acetylcholine from cholinergic nerves, in turn inducing the over expression of muscarinic receptors in the diabetic detrusor.[18,19] Such over expression may enhance signaling downstream of these receptors and increase detrusor contraction according to the results of the current organ bath study. In conclusion, our data indicate that noninsulin dependent diabetes induces alterations in the muscarinic receptor system that may contribute to the development of diabetic cystopathy.

Abbreviations and Acronyms

4-DAMP	=	4-diphenylacetoxy-N-methylpiperidine methiodide
ATR	=	atropine
EC_{50}	=	concentration producing half-maximal contractile response
E_{max}	=	maximum response
GK	=	Goto-Kakizaki
MTR	=	methoctramine
PCR	=	polymerase chain reaction
Pdet	=	maximum detrusor pressure during voiding
PRZ	=	pirenzepine
STZ	=	streptozotocin

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