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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Key Words: urinary bladder, Goto-Kakizaki (GK) rat, cystopathy, muscarinic M_2 receptor, muscarinic M_3 receptor, mRNA

Running head: Type 2 diabetic rat bladder

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Abstract

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

Materials and methods: Twelve- and 70-week-old male GK rats and age-matched male Wistar rats were used in this study. The bladder functions were estimated by voiding behavior studies, cystometric studies, and functional studies using KCl, carbachol, and various concentrations of subtype selective muscarinic antagonists, i.e., atropine, pirenzepine, methoctramine, and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP). The participation levels of M_2 and M_3 receptor mRNAs in the bladder were investigated by real-time PCR.

Results: In the voiding behavior studies, although there were no significant differences in urine output, an age-related decrease in micturition frequency and an age-related increase in single voided volume were observed in both GK and Wistar rats. In the cystometric studies, although there were no significant differences in maximum detrusor pressure or bladder capacity, residual urine volume was significantly increased in the 70-week-old GK rats. In the functional studies, carbachol-induced contractility of the detrusor was significantly increased in GK rats of both age groups. The estimated pA_2 values for atropine, pirenzepine, methoctramine, and 4-DAMP indicate that the carbachol-induced contractile response is mediated through the M_3 receptor subtype in all groups. Furthermore, muscarinic M_2 and M_3 receptor mRNAs were

significantly up regulated in the 70-week-old GK rat bladder.

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

Diabetic cystopathy, a form of urinary 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 classical 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 streptozotocin (STZ)-induced diabetic rat is the most commonly used and well-investigated experimental model for type 1 diabetes.[4, 5] Alterations of urinary bladder function are also seen in the STZ-induced diabetic rats.[4, 5] For example, increased urine output, frequent voiding, and atonic bladder are observed 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 Goto-Kakizaki (GK) rat represents a spontaneous non-insulin-dependent diabetes model. GK rats are produced from normal Wistar rats by repetition of selective breeding, and 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 four weeks after birth, and later-occurring abnormalities include insulin secretion and modest hyperglycemia.[9]

Furthermore, some reports have indicated age-related and diabetes-suffered duration-related alterations of bladder functions.[10, 11] To get more information on the detailed mechanisms of type 2 diabetes-induced alteration of bladder smooth muscles,

we investigated bladder contraction using KCl and carbachol with subtype-selective muscarinic antagonists as well as muscarinic M_2 and M_3 receptor mRNAs expression levels by real-time polymerase chain reaction (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 were purchased from SLC (Shizuoka, Japan). All rats were kept under identical conditions, and had access to food and drinking water ad libitum. The rats were divided randomly into four groups (n = 6-8). Groups A and B consisted of 12-week-old Wistar and GK rats, respectively, while Groups C and D consisted of 70-week-old Wistar and GK rats, respectively. Upon reaching either 12 or 70 weeks of age, the rats were sacrificed with an overdose of pentobarbital (60 mg i.p.). Blood samples were collected from the vena cava, and isolated bladders were used in tissue bath experiments or frozen at –80 ℃ for measurement of muscarinic M_2 and M_3 receptor mRNAs.

Serum glucose and serum insulin measurement

Serum glucose concentrations in the experimental rats were measured by the hexokinase method (Glucose CⅡ, Wako Pure Chemical, Osaka, Japan), which was carried out according to the kit manufacturer's instructions in all groups. In Groups C and D, the insulin concentrations were also measured by ELISA according to the manufacturer's instructions (Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden).

Voiding behavior studies

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

Cystometric studies

The cystometric studies were performed according to methods used in our previous report[12] at 12 or 70 weeks of age in all groups. The following parameters were evaluated according to our previous reports: bladder capacity, maximum detrusor pressure during voiding (Pdet), single-voided volume, and residual urine volume.

Measurement of contractile force in the bladder

Functional studies were conducted according to methods used in our previous reports.[12, 13] Razor blades were used to cut uniform longitudinal strips of the posterior wall of the bladder dome (1.5×5 mm). Changes in the tone of the strips were measured isometrically by means of force transducers, and the data were recorded on a personal computer (Macintosh G3, Apple Computer, Cupertino, CA, USA) with the use of the software program Chart version 3.6.9 and a PowerLab/16sp data acquisition system (AD Instruments, Castle Hill, NSW, Australia). 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: pirenzepine (PRZ), methoctramine (MTR), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), and atropine (ATR).[13] Antagonists were added 30 minutes prior to the administration of carbachol. After completion of a concentration-response curve, the tissue was washed until baseline force returned to the resting level, equilibrated for 30 minutes, and then the next consecutive concentration-response curve was constructed.

Real-time PCR (quantification of muscarinic M_2 and M_3 receptor messenger RNAs)

To measure muscarinic receptor mRNAs on the detrusor, urothelium was carefully peeled under illuminated magnifiers in every rat (Otsuka, Tokyo, Japan). Muscarinic M_2 and M_3 receptor mRNAs in the experimental bladder dome were measured by real-time PCR methods according to our previous reports.[12, 13] The RNA was purified by RNeasy® Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A reverse transcriptase (RT) mixture (28 μl) containing 2 μg of total RNA was made and incubated at 37 ℃ for 60 min according to a previously reported method.[12,13] Five microliters of the mixture was used for real-time PCR, which was carried out with the use of a LightCycler thermal cycler system with a LightCycler-FastStart DNA Master Hybridization Probe (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions.[14] The primers and probe sequences specific to the genes of muscarinicM_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 the β-actin (accession number: NM_031144) were used from the LightCycler-Primer/Probe Set (rat) (Roche Diagnostics). A total of 15 μl of solution was used for the sample. PCR products were subjected to 2% agarose gel electrophoresis. The β-actin gene was used as the internal standard and analyzed by real-time PCR by using the same RT mixture.

Data analysis

The EC_{50} and E_{max} values were obtained by a Macintosh computer (G3) 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 (the concentration of agonist that produces half-maximal contractile responses) 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. The EC_{50} values were calculated as geometric means, whereas E_{max} values were calculated as arithmetic means. The expressions of muscarinic M_2 and M_3 receptor mRNAs were quantified according to the expressions of β-actin mRNAs in the experimental rat bladder domes. A statistical comparison of differences between groups was performed using analysis of variance and Fisher's multiple comparison tests. $P<0.05$ was regarded as the level of significance.

Drugs and chemicals

Carbachol, 4-DAMP, pirenzepine, and methoctramine were purchased from Sigma

(St. Louis, MO, USA). Atropine was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were available commercially and of reagent grade.

RESULTS

General features of the experimental animals

The data obtained regarding the general features and serum concentrations of insulin and glucose in the experimental animals are shown in Table 1 and Figure 1. The GK diabetic rats showed significantly small weight gain by the age of 10 weeks, as well as significantly small weight gain during the experimental period (Figure 1). There was no significant difference in the bladder weight between Groups A and B, or Groups C and D. However, the bladder / body weight ratio in Group D was markedly greater than that in Groups B and C. Significantly higher serum glucose levels were confirmed at the age of 12 weeks in the GK diabetic rats. Significantly higher serum glucose and lower serum insulin levels than those of the control rats were confirmed in the 70-week-old GK diabetic rats.

Voiding behavior studies

Results of voiding behavior studies for the experimental animals are shown in Table 2. These studies revealed no significant differences in urine production, micturition frequency, or single voided volume between the 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. The older rats (Groups C and D) showed significant decreases in micturition frequency and significant increases in single voided volume.

Cystometric studies

The results of the cystometric studies are also shown in Table 3. In these studies, although single voided volume in Groups C and D tended to be larger, there were no significant differences in Pdet and single voided volume between any of the groups. However, the residual urine volume in Group D was markedly greater than those in Groups B and C.

Measurement of contractile responses to carbachol and 100 mM KCl

The E_{max} values for 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 by 100 mmol KCl. However, there were no significant differences in the EC_{50} values with respect to carbachol between any of the groups. The pA_2 values and slopes of the Schild plots for these muscarinic receptor antagonists in the rat detrusor are shown in Table 5. The pA_2 values calculated for series of muscarinic antagonists were similar in all groups and 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 that carbachol does not alter contractile systems according to age or the presence of diabetes.

Measurement of muscarinic M_2 and M_3 receptor mRNAs in the rat bladder dome

Table 6 shows the expressions of muscarinic M_2 and M_3 receptor mRNAs in the bladder dome. Although the expression levels of muscarinic M_2 and M_3 receptor mRNAs were similar in the younger groups, those of the older GK rats were significantly higher than those of age-matched controls. Furthermore, the control group (Group A) had higher expression levels of the muscarinic M_3 than the muscarinic M_2 receptor mRNAs; in all groups, the expression level of the muscarinic M_3 receptor mRNAs was approximately 2-3 times higher than that of the muscarinic M_2 receptor mRNAs.

DISCUSSION

Latifpour and associates demonstrated that significant up-regulation of the expressions of muscarinic receptors and hyper-contractility induced by a muscarinic receptor agonist, carbachol, in diabetic rat bladder dome.[4, 5] Interestingly, those reports did not demonstrate significant alterations of EC_{50} values for carbachol and of K_i values of [^{3}H]QNB for muscarinic receptor subtype-selective antagonists. Those findings indicated that diabetes-induced alterations of the muscarinic receptor system in the bladder are quantitative rather than qualitative. Tong and associates reported that STZ-induced diabetes increases mRNA and protein expressions of M_2 and M_3 muscarinic receptors in the urothelium as well as in the muscle layer.[16, 17] In our recent studies, analyses of diabetes-induced alterations in the bladder using pharmacological, biochemical, and biological methods supported their findings.[12, 13] Recently, Daneshgari and associates reported that diabetic bladders may undergo a transition from a compensated to a decompensated state and that 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. Using 32-week-old female GK rats, Miyamae et al. reported 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. In their reports, the maximum

contractile responses to carbachol and ATP, and the release of acetylcholine induced by field stimulation, were similar in bladders from GK and control rats until 8 weeks of age; however, at the ages of 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.[19]

Previously, we reported that diabetes induces an increase in maximum detrusor pressure during voiding; this increase occurred by urethral dysfunction associated with diabetic neuropathy.[12] In this study, we demonstrated similar tendency of Pdet in GK rats; Pdet in 70-week-old GK rats (Group D) tended to be higher than that in age-matched Wistar rats (Group C). However, there was no significant difference between Groups C and D. It may be due to great variation of the data presented. In the present study, we also demonstrated the hypercontractility of detrusor smooth muscle to carbachol in GK rats at both ages compared to age-matched control rats. These data are similar to those for STZ-induced diabetic rats previously reported.[12, 13] We also demonstrated that 70-week-old GK diabetic rats had significantly increased residual urine volume compared to both 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 patterns of cystopathy. To clarify the mechanisms underlying type 2 diabetic cystopathy, we undertook pharmacological and biological examinations. To confirm these putative changes in the muscarinic receptor system, we calculated the pA_2 values and their slopes using four subtype nonselective and selective muscarinic alterations. There were no significant differences in the pA_2

values and slopes between diabetic and non-diabetic rats for any of the muscarinic receptor antagonists used in the present study. The results of the pA_2 calculations for this series of muscarinic antagonists were similar in all groups, and the rank order of the values was: 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 diabetic rat detrusor. Rather, such changes appear to be the result of quantitative rather than qualitative changes in the muscarinic receptor system. Mutoh and associates 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] and we also previously reported that the rank order of pA_2 values 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 both younger and older GK rats.

To elucidate the mechanisms underlying diabetes-induced hypercontractility to carbachol of the rat detrusor, we measured the expression levels of muscarinic M_2 and M_3 receptor mRNAs using real-time PCR. The results showed that the mRNA levels of both muscarinic M_2 and M_3 receptors were increased under diabetic conditions at 70 weeks, as compared to age-matched controls. Based on our previous and present data, it appears likely that the overexpressions of muscarinic M_2 and M_3 receptor mRNAs are related to the hypercontractility of detrusor in 70-week-old diabetic rats.[12, 13] The possible mechanisms of this up-regulation of muscarinic receptors are explained due to

decrease in cholinergic nerve density,[18] or due to defective neurotransmitter release mechanism.[21] Miyamae et al. reported decrease in cholinergic nerve density in GK rats, [18] while Tong et al. reported defective neurotransmitter release mechanism in two-week-old STZ induced diabetic rats.[21] The diabetes-associated neuropathy may inhibit the release of acetylcholine from cholinergic nerves, in turn inducing the overexpression of muscarinic receptors in the diabetic detrusor.[18, 19] Such overexpression may enhance signaling downstream of these receptors and may increase detrusor contraction, according to the results of the present organ bath study.

In conclusion, our data indicate that non-insulin-dependent diabetes induces alterations of the muscarinic receptor system that may contribute to the development of diabetic cystopathy.

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Figure legends

Figure 1. Body weight in GK and Wistar rats.

Data are shown as mean ± SEM of six to eight separated determinations in each group.

*)significantly different from the same age of Wistar rats.

Table 1. General features of the experimental rats

	Body Weight (g)		Bladder weight	Bladder /Body weight	Serum glucose	Serum insulin
	12 weeks old	70 weeks old	(g)	ratio ($x10^{-4}$)	(mg/dl)	(μg/l)
A	350.0 ± 4.9	-----	0.095 ± 0.001	2.73 ± 0.05	153.4 ± 7.9	n.p.
B	298.3 ± 5.4*	-----	0.084 ± 0.004*	2.82 ± 0.09	233.1 ± 12.3 *	n.p.
C	-----	561.3 ± 21.7**	0.128 ± 0.006**	2.34 ± 0.10**	132.2 ± 5.9	2.52 ± 0.27
D	-----	424.3 ± 4.2* **	0.127 ± 0.006**	3.21 ± 0.12* **	212.1 ± 8.2 *	0.43 ± 0.08*

A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group. n.p.: not performed *) significantly different from age-matched Wistar group (group A VS group B, and group C VS group D). **)significantly different from younger group (group A VS group C, and group B VS group D).

Table 2. Voiding behavior studies in the experimental rats

	micturion frequency (/day)	urine production (ml/day)	single voided volume (ml)
A	12.3 ± 0.8	10.7 ± 1.1	0.87 ± 0.09
B	13.6 ± 0.9	12.8 ± 0.9	0.94 ± 0.08
C	9.1 ±1.7 *	12.8 ± 0.9	1.40 ± 0.22*
D	8.1 ± 1.2 *	13.2 ± 1.3	1.62 ± 0.32*

A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group. *)significantly different from younger group (group A VS group C, and group B VS group D).

Table 3. Cystometrogram data in the experimental rats

	Pdet (cmH_2O)	single voided volume (ml)	residual urine (ml)
A	42.4 ± 5.7	0.31 ± 0.03	0.054 ± 0.033
B	33.7 ± 2.9	0.34 ± 0.04	0.035 ± 0.010
C	35.3 ± 8.7	0.52 ± 0.13	0.044 ± 0.020
D	44.1 ± 4.3	0.51 ± 0.08	0.230 ± 0.057* **

A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group. Pdet: maximum detrusor pressure during voiding *) significantly different from age-matched Wistar group (group A VS group B, and group C VS group D). **)significantly different from younger group (group A VS group C, and group B VS group D).

Table 4. Functional studies in the experimental rats

	Emax / KCl	ED_{50} (10^{-6} M)
A	1.57 ± 0.05	1.19 ± 0.12
B	1.88 ± 0.09*	2.88 ± 0.92
C	1.39 ± 0.05**	2.01 ± 0.55
D	1.61 ± 0.05* **	2.22 ± 0.49

A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group. E_{max} values and ED_{50} values are for carbachol. KCl means contractile force to 100 mM KCl. *) significantly different from age-matched Wistar group (group A VS group B, and group C VS group D). **)significantly different from younger group (group A VS group C, and group B VS group D).

Table 5. pA_2 values of atropine, pirenzepine, methoctramine, and 4-DAMP versus carbachol and regression slopes obtained from Schild prots.

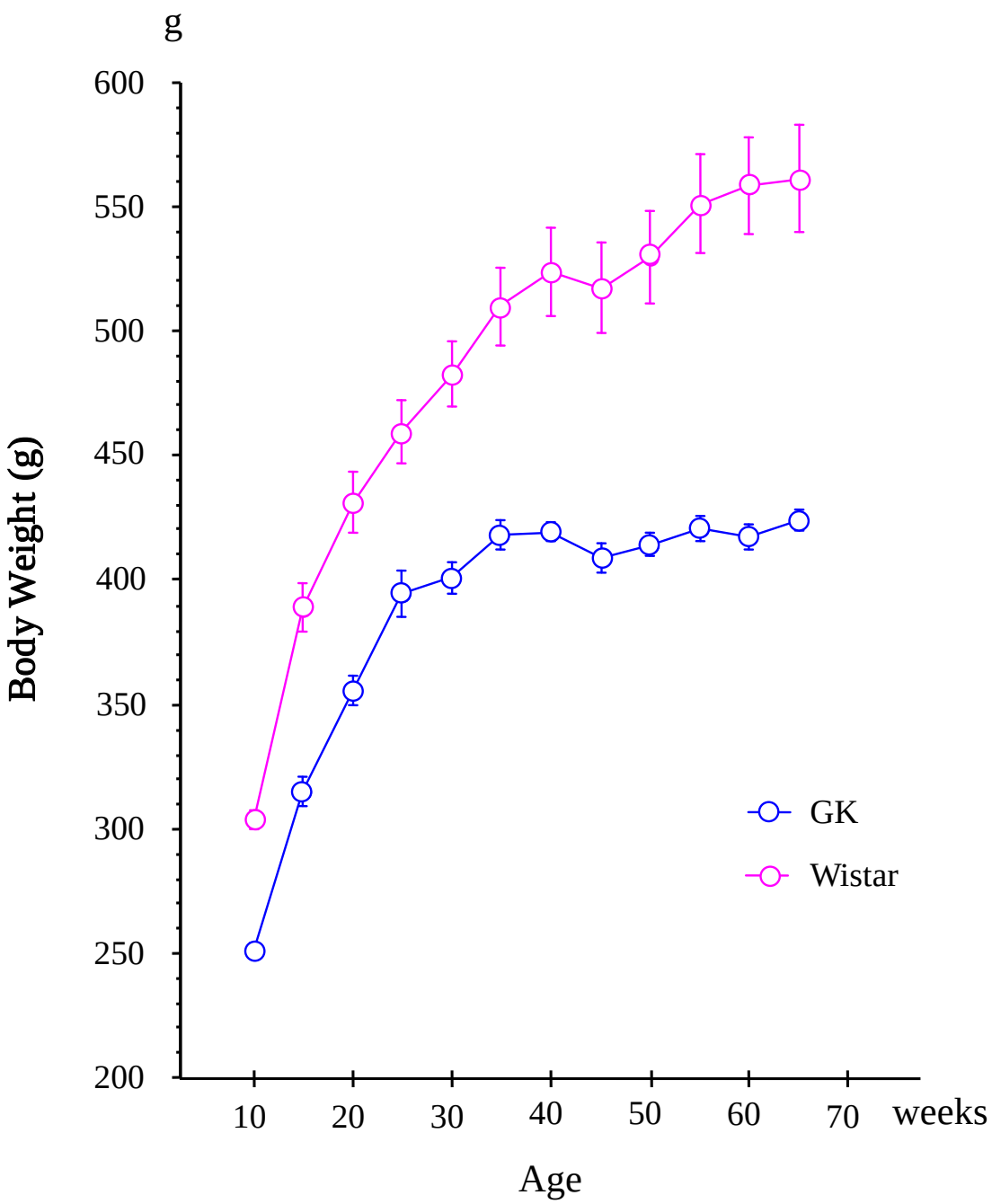
	atropine		pirenzepine		methoctramine		4-DAMP	
	pA_2	Slope	pA_2	Slope	pA_2	Slope	pA_2	Slope
A	9.48 (9.37 – 9.62)	1.07 ± 0.09	6.59 (6.45 – 6.82)	0.71 ± 0.08	7.57 (7.28 – 8.94)	0.80 ± 0.08	8.84 (8.74 – 8.96)	1.15 ± 0.08
B	9.58 (9.49– 9.71)	0.91 ± 0.11	7.27 (7.08– 7.61)	0.86 ± 0.10	8.03 (7.79– 8.60)	0.79 ± 0.16	9.17 (9.03– 9.25)	1.00 ± 0.07
C	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
D	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

A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group.

Table 6. Expression of muscarinic M_2 and M_3 receptor mRNAs in the bladder dome

	M_2 /β-actin (x 10^{-3})	M_3 /β-actin (x 10^{-3})
A	2.57 ± 0.89	4.69 ± 0.88
B	2.72 ± 0.73	4.68 ± 0.17
C	1.50 ± 0.40	5.28 ± 0.11
D	4.48 ± 1.38*	8.26 ± 0.19* **

Expression of muscarinic M_2 and M_3 receptor mRNAs were normalized with that of □-actin mRNAs. A: twelve-week-old Wistar rats, B: twelve-week-old GK rats, C: seventy-week-old Wistar rats, and D: seventy-week-old GK rats. Data are shown as mean ± SEM of six to eight separated determinations in each group. n.p.: not performed *) significantly different from age-matched Wistar group (group A VS group B, and group C VS group D). **)significantly different from younger group (group A VS group C, and group B VS group D).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