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ROLE OF M2 AND M3 MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPES IN ACTIVATION OF BLADDER AFFERENT PATHWAYS IN SPINAL CORD INJURED RATS

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Abstract

Purpose—In this study, we evaluated the role of M2 and M3 mAChR subtypes in activation of bladder afferent pathways in rats with chronic spinal cord injury (SCI).

Materials and Methods—Adult female Sprague-Dawley rats were spinalized at the Th9 level. Continuous cystometry was performed under an awake condition 2 or 4 weeks after SCI. The effects of intravesical administration of an mAChR agonist (oxotremorine-M; Oxo-M), a non-selective antagonist (atropine), an M2-selective antagonist (methoctramine) and an M3-selective antagonist (darifenacin) were examined. After cystometry, the bladder was removed and separated into mucosa and detrusor, and M2 and M3 mAChR mRNA expression in the mucosa was determined with real time quantitative PCR.

Results—At 2 and 4 weeks after SCI, intravesical administration of a non-selective mAChR agonist (25μM Oxo-M) increased the area under the curve of non-voiding contractions (NVCs) although intercontraction interval (ICI) of voiding contractions and maximum voiding pressure (MVP) did not change. This effect was blocked by atropine and methoctramine (10μM), but not by darifenacin (50μM). However, mAChR antagonists alone (50μM) had no effect on cystometric parameters. M2 mAChR mRNA expression was increased in mucosa of SCI rats compared to normal rats.

Conclusions—Our results suggest that the M2 mAChR subtype plays an important role in bladder afferent activation that enhances detrusor overactivity in SCI rats. However, because mAChR antagonists alone did not affect any cystometric parameters, the muscarinic mechanism controlling bladder afferent activity may not be involved in the emergence of detrusor overactivity in SCI.

Keywords

rat; urinary bladder; spinal cord injury; afferent pathway; muscarinic receptor

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Introduction

Overactive bladder (OAB) is a most common functional bladder disorder, which is characterized by symptoms of urinary urgency with or without urge incontinence, usually with frequency and nocturia. OAB with a neurological etiology is often related to neurogenic detrusor overactivity (NDO). A frequently used animal model to study NDO is the spinal cord injured rat at the thoracic vertebral levels T9–T12. Spinal cord injury (SCI) rostral to the lumbosacral level causes initially are flexic bladder and urinary retention, followed by inefficient voiding due to detrusor-sphincter dyssynergia and NDO shown by non-voiding contractions during the storage phase of cystometry.¹ Intravesical or systemic administration of capsaicin or resiniferatoxin in SCI cats and rats reduced NDO, indicating that SCI-induced bladder dysfunction is triggered, at least in part, by capsaicin-sensitive C-fiber bladder afferents.^{1,2}

Recently, there is a great interest in the interaction between the neuronal system and the uroepithelium. The urothelium expresses various receptors including muscarinic, nicotinic, purinergic and transient receptor potential and also, releases various neurotransmitters (such as ACh; acetylcholine, ATP; adenosine 5'-triphosphate and NO; nitric oxide) in response to mechanical and/or chemical sensory stimuli, thereby modulating activity of bladder afferent pathways.³

The mainstay of pharmacological treatment used for OAB symptoms are antimuscarinics, which were initially thought to act on detrusor smooth muscle. However, as antimuscarinics work during the filling phase of the bladder when parasympathetic efferents are effectively silent; hence the muscle seems an unlikely target, particularly for urgency symptoms. Recent data including ours showed that activation of muscarinic acetylcholine receptors (mAChRs) in the urothelium and/or bladder afferent pathways by intravesical administration of mAChR agonist alters reflex bladder contractions.⁴ Accordingly, activation of mAChRs releases substances (ATP and NO) from the urothelium that can modulate afferent nerves,⁴ suggesting that activation of mAChRs in the urothelial and/or suburothelial layers affects bladder afferent activity in the control of the micturition reflex.

We previously reported in normal rats that stimulation of local mAChRs using intravesical application of mAChR agonists induces bladder overactivity via activation of M2 mAChR subtype.⁵ In this study, we therefore studied the effects of M2 and M3-selective mAChR antagonists on bladder overactivity induced by local mAChR stimulation in SCI rats as well as the changes of M2 and M3 mAChR subtype mRNA expression in the bladder mucosa.

Materials and methods

Animals

Adult female Sprague–Dawley rats weighing 250–300 g (Hilltop, Scottsdale, PA) were used. All experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Spinal cord transection

Rats were anesthetized with 2% isoflurane and Th9 laminectomy was performed. To produce SCI, the dura was opened, complete transection of the Th9–Th10 spinal cord was performed with scissors and a sterile Surgifoam™ sponge was placed between the cut ends of the spinal cord. The overlying muscle and skin were then sutured to close the wound. The animals were postoperatively treated with ampicillin (100 mg/kg intramuscularly) for 5 days. The bladder of spinalized rats was emptied by abdominal compression twice per day until reflex voiding recovered, usually 10 to 14 days after spinalization.

Cystometrograph

Two and four weeks after spinal cord transection, rats were anesthetized with 2% isoflurane. A midline abdominal incision was made and a transvesical catheter with a fire-flared tip (PE-50) was inserted into the dome of the bladder and secured with a silk thread for bladder filling and pressure recording. After the abdomen was closed, the rats were placed in a restrainer (Braintree Scientific, Braintree, Massachusetts) and allowed to recover from anesthesia for 1 to 2 hours. A 3-way stopcock was connected to the transvesical catheter to monitor bladder pressure. During cystometry, intercontraction interval (ICI) of voiding contractions, maximum voiding pressure (MVP), and non-voiding contraction (NVC) were measured by using a PowerLab unit and Chart software (AD Instruments, Colorado Springs, CO). NVC, which was defined as 4 cmH₂O or more increases of intravesical pressure above the baseline without voiding during the storage phase, was evaluated as area under the curve (AUC) of bladder contractions calculated by the integral of a 3-minute period prior to voiding contraction.

Drugs

We used oxotremorine methiodide (Oxo-M; Sigma, St. Louis, MO) as a non-selective mAChR agonist, atropine sulfate (Sigma) as a non-selective mAChR antagonist, methoctramine hemihydrates (Sigma) as an M2-selective mAChR antagonist and darifenacin hydrobromide (Kemprotec, Middlesbrough, UK) as an M3-selective antagonist. For intravesical administration, drugs were dissolved in saline to a final concentration and applied into the bladder through the intravesical catheter.

Experimental Protocols

Saline at a room temperature was continuously infused for at least 2 hours at a rate of 0.08 mL/min to record cystometrograms during a control period. After baseline cystometry was performed, vehicle (saline) or an antimuscarinic agent (10 μ M) was instilled intravesically for 30 minutes. Thereafter, 25 μ M Oxo-M was added to the infusate to examine whether intravesical application of Oxo-M with vehicle or an antimuscarinic agent induces bladder overactivity. We selected this dose of Oxo-M because 25 μ M Oxo-M elicited significant and consistent bladder overactivity as shown by an increase of NVC without apparent effects on the voiding detrusor contractions (MVP) in preliminary experiments.

PCR Quantification of M2 and M3 mAChR subtype mRNA

After cystometry, the bladder was rapidly removed, and the mucosa and detrusor layer were separated by microscissors in Kreb's solution (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 10). Total RNA was extracted from the bladder mucosa using Trizol® reagent. Three μ g RNA was reverse-transcribed into cDNA using SuperScript™ II. Primers for M2 and M3 mAChR subtypes were as follows;

M2: sense 5'-TTTGGGACCTGTAGTATGTGACC-3',

antisense 5'-GCTTAACTGGGTAGGTCAGAGGT-3',

M3: sense 5'-TATCCAGCACAGAGAATCCAGAC-3',

antisense 5'-GAGCTAACAGTGCAGGAGACAGT-3'.

β -actin (Ambion, Austin, Texas) was used as an endogenous control to normalize M2 and M3 mRNA levels. The M2 and M3 subtype and β -actin mRNA level was quantified with an MX3000P real-time PCR system (Stratagene™) in a 25 μ L volume using SYBR® Green PCR Master Mix. Specificity for mAChR was confirmed by melting curve analysis. Sample quantification was achieved from the threshold cycle by interpolation from a standard curve

to calculate a copy number for mAChR and the ratio of mAChR to β -actin mRNA was compared.

Statistical Analysis

All data values are expressed as the mean $\pm$ standard error of the mean. $P < 0.05$ was considered significant. The overall comparisons among groups were performed using Prism software (GraphPad Software, San Diego, CA). For each cystometric parameter, 2 or 3 measurements were averaged. Student's t-test was used to compare baseline cystometric parameters of 2 and 4 weeks after SCI. Wilcoxon signed rank test was used to compare the cystometric parameters, before and after drug administration. Kruskal-Wallis test was used to compare changes in mAChR mRNA level among normal, 2 and 4 weeks after SCI.

Results

Cystometry

In baseline cystometry at 2 and 4 weeks after SCI, ICI of voiding contractions in 4 weeks after SCI was significantly smaller compared to that in 2 weeks (Table 1, 474.1 ± 64.00 vs 713.7 ± 63.31 seconds, $p = 0.0238$). There was no difference in NVC or MVP between 2-week and 4-week post-SCI groups.

Intravesical administration of Oxo-M (25 μ M) enhanced bladder overactivity (Fig. 1A, B) as evidenced by increased AUC of NVC both at 2 and 4 weeks after SCI (Table 2, 2wks; 1093 ± 321.5 to 1561 ± 340.3 cmH₂O*s, $p = 0.0313$, 4wks; 1025 ± 240.1 to 1424 ± 229.2 cmH₂O*s, $p = 0.0313$). ICI and MVP were not altered during Oxo-M instillation.

Intravesical administration of Oxo-M concomitant with a non-selective mAChR antagonist (atropine, 10 μ M) (Figure 1C, D) or an M2-selective antagonist (methoctramine, 10 μ M) (Figure 1E, F) suppressed Oxo-M-induced enhancement of NVC (Table 2). However, when Oxo-M was instilled with an M3-selective antagonist (darifenacin, 10 μ M), AUC of NVC significantly increased both at 2 and 4 weeks after SCI (data not shown). Therefore, we tried a high dose of darifenacin (50 μ M) to confirm the results. Despite its high dose, Oxo-M-induced increase of NVC was not prevented by darifenacin (Figure 1G, H, Table 2, 2wks; 745.2 ± 153.1 to 1060 ± 230.8 cmH₂O*s, $p = 0.0078$, 4wks; 1026 ± 269.4 to 1317 ± 230.7 cmH₂O*s, $p = 0.0313$).

However, intravesical administration of mAChR antagonists (atropine 10 μ M, methoctramine 10 μ M and darifenacin 10 & 50 μ M) alone prior to concomitant Oxo-M application did not affect any cystometric parameters such as NVC, MVP or ICI at 2 and 4 weeks after SCI.

Quantitative real-time PCR

In the bladder mucosa, the M2 mAChR subtype mRNA-to- β -actin mRNA ratio in 2 weeks after SCI rats was significantly greater than in normal rats (Figure 2A, 4.415 ± 1.604 vs 0.9467 ± 0.1485 , $p = 0.0420$). The M2 subtype mRNA-to- β -actin mRNA ratio in the mucosa at 4 weeks after SCI tended to be lower than that at 2 weeks after SCI and still higher than in normal rats, although the differences between 4-week and 2-week post-SCI groups or between 4-week post-SCI and normal groups were not significant. On the other hand, there was no significant difference in the M3 mAChR subtype mRNA-to- β -actin mRNA ratio in the mucosa among normal, 2-week and 4-week post-SCI groups (Figure 2B).

Discussion

The present study demonstrated that, in spinal cord injured (SCI) rats, local application of a muscarinic receptor agonist (Oxo-M) enhanced bladder overactivity as evidenced by an increase of non-voiding contractions (NVCs) without affecting bladder function related to voiding (MVP or ICI). As previously reported, the NVCs in SCI rats are eliminated by C-fiber desensitization with capsaicin, indicating that these contractions are mediated predominantly by capsaicin-sensitive C-fiber afferents, while A δ -fiber afferents initiate voiding reflexes in both normal and in chronic spinal rats.⁶ In humans, NDO also appears to be mediated by C-fiber afferents, because intravesical capsaicin treatment is effective in suppressing bladder overactivity in patients with multiple sclerosis⁷ or SCI.⁸ Thus, it seems likely that local activation of muscarinic acetylcholine receptor (mAChR) increases C-fiber-mediated activity to enhance neurogenic detrusor overactivity (NDO) in SCI. In addition, the Oxo-M-induced increase of NVCs was prevented by intravesical co-application of atropine (non-selective mAChR antagonist) at the dose that did not affect voiding bladder contraction (MVP), indicating that atropine suppress locally expressed mAChR in the urothelial and/or suburothelial layers, but does not reach the detrusor layer to suppress detrusor muscle activity.

Furthermore, the M2-selective mAChR antagonist, but not the M3-selective antagonist, prevented Oxo-M-induced enhancement of NVCs, without affecting the baseline bladder activity by itself in SCI rats. Taken together, the results in the current study suggest that the M2 mAChR subtype plays a more important role than the M3 subtype in activation of C-fiber afferent pathways to enhance bladder overactivity in SCI rats. We also showed that expression of M2 mAChR subtype mRNA in bladder mucosa was increased in SCI rats compared to normal rats. Some *in vitro* studies also suggested the existence of excitatory mechanisms mediated by activation of the M2 mAChR subtype in other organs such as the respiratory and intestinal tracts.^{9–11} It has also been shown that activation of M1, M2 and M3 mAChR subtypes releases ATP from cultured rat urothelial cells, possibly through increases in intracellular Ca²⁺ levels.¹² In addition, there are many reports on altered M2-mediated responses in the neurogenic bladder. Despite its predominance in the bladder, M2 mAChR normally has just indirect effects on M3-mediated detrusor contraction.¹³ In decentralised rat bladders, an enhanced and direct contractile role for M2 has been reported.¹⁴ Similar results were reported in obstructed¹⁵ and neurogenic^{16,17} rat bladders. In humans, such altered M2 function might also develop in neurogenic detrusor overactivity, although there are conflicting reports on this topic.¹⁸ There is increasing evidence for the functional modulation by non-neuronal sources of transmitters in the bladder. For example, it has been shown that many factors including ACh¹⁹, ATP²⁰, NO²¹ can be released from the bladder urothelium. It is postulated that these non-neuronal sources of neurotransmitters interact with near-by tissues in the suburothelial layer to modulate afferent activity to control bladder function. Therefore, the urothelium is thought to play an important role in sensory transduction mechanisms that modulate micturition. We showed in this study that intravesical administration of non-selective mAChR agonist (Oxo-M) increased NVCs both at 2 weeks and 4 weeks after SCI. This increase of NVCs was inhibited by non-selective mAChR antagonist (atropine), which had no effects on detrusor contractility during voiding, indicating that local mAChR activation lead to bladder overactivity in SCI rats. One possible mechanism by which local mAChR activation increases NVCs is related with ATP. ATP levels and P2X receptor mediated responses are also increased in pathologic status. For example, urothelial ATP release is significantly enhanced in chronic SCI rats²² and in humans suffering from interstitial cystitis.²³ It has been suggested that P2X receptors found on bladder sensory nerve terminals²⁴ may be activated by enhanced ATP release from the urothelium in pathologies such as SCI.²⁵ Kullmann et al.⁴ also reported that intravesical administration of Oxo-M induced bladder overactivity through the release of ATP from the

urothelium in normal rats. Another possible mechanism is that Oxo-M directly activated mAChRs expressed on bladder afferent fibers since mAChRs are reportedly expressed on L6/S1 DRG cell bodies of bladder afferent neurons, suggesting that bladder afferent pathways might be under direct cholinergic control.²⁶

In this study, neither Oxo-M nor antimuscarinics altered the amplitude of voiding contractions, indicating that the drugs at doses tested in this study did not affect the contractility of detrusor muscle or the activity of efferent pathways to the bladder. However, it is unclear to which layer of the bladder wall Oxo-M did penetrate. We can only suggest that Oxo-M, which was applied intravesically, did not penetrate sufficiently to the detrusor layer to affect detrusor activity. In this study intravesical administration of Oxo-M did not alter ICI of VCs. In SCI rats, VCs are thought to be mediated by A δ -fibers while NVCs are mediated by C-fibers.⁶ Most C-fibers end in the suburothelial layer immediately below the basal lamina of the bladder, and some nerve-endings penetrate the urothelium.²⁷ Thus, it may be because intravesically administered Oxo-M just affected C-fibers in urothelial and suburothelial layers, but not A δ -fibers in the detrusor, therefore pressure threshold of VC did not alter, leading to no changes in ICI.

In this study, the expression of M2 mAChR subtype mRNA in the bladder mucosa was increased in SCI rats compared to normal rats. We previously showed in normal rats that the activation of local M2 mAChR subtype leads to bladder overactivity although a high-dose (200 μ M) of Oxo-M was required to induce the reduction of ICI. In this study, a low dose (25 μ M) of Oxo-M increased NVCs in SCI rats. The increased expression of M2 mAChR subtype could be involved in the increased sensitivity for Oxo-M. Gevaert et al.²⁸ reported a significant increase of M2 subtype mRNA in the whole bladder at 3 weeks post-SCI rats and a significant decrease of M3 subtype mRNA at 24 hours post-SCI. An increased density of M2 protein and a decrease of M3 protein have been reported in decentralised¹⁴, obstructed¹⁵, and neurogenic¹⁷ rat bladders. Also, there is evidence that expression of M2 and M3 mAChR subtypes are altered on the urothelium and suburothelial interstitial cells²⁹ in different bladder pathologies. These changes may account for the increased sensitivity for mAChR agonists in SCI rats.

In our study, intravesical antimuscarinics alone did not affect any cystometric parameters even with high concentrations, suggesting that antimuscarinic agents did not penetrate to the detrusor muscle layer enough to affect bladder contractility and that local mAChRs are not involved in the emergence of bladder overactivity (NVC) in SCI rats. In normal animals, a considerable proportion of bladder C-fiber afferents are chemosensitive but insensitive to mechanical stimuli. However, following SCI, C-fiber afferents become mechanosensitive and can initiate bladder reflexes. It has been speculated that neurotrophic factors, such as the nerve growth factor or other chemicals released in the bladder, may be responsible for the afferent nerve plasticity after SCI while NVCs themselves are not under the endogenous muscarinic control at least in 4 weeks after SCI as shown in this study. However, if the urothelial release of ACh increases due to more dramatic pathophysiologic changes such as chronic inflammation induced by repeated infections, for example, in chronic SCI, the local enhancement of NDO may occur due to mAChR activation and antimuscarinics become effective to suppress SCI-associated NDO.

Conclusion

The results of this study suggest that the M2 mAChR subtype, which is expressed in the mucosal layer and increased after SCI, plays an important role in bladder afferent activation that enhances detrusor overactivity in SCI rats. However, mAChR antagonists alone did not affect any cystometric parameters, suggesting that the endogenous muscarinic mechanism

controlling bladder afferent activity may not contribute to the induction of detrusor overactivity in SCI. Antimuscarinics, which have antagonistic activity against M2 mAChR subtypes, could be more beneficial for the treatment of NDO in SCI patients if there is a condition that ACh release from the urothelium is increased to exacerbate bladder overactivity.

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Figure 1.

Representative cystometrograms. NVC was increased by intravesical administration of Oxo-M at 2 weeks (A) and 4 weeks (B) after SCI. The effect of Oxo-M was prevented by non-selective (C, D) or M2-selective mAChR antagonists (E, F). The M3-selective antagonist did not inhibit the effect of Oxo-M despite its high concentration (G, H). Atr: atropine, Met: methoctramine, Dar: darifenacin

Figure 2.

M2 (A) and M3 (B) mAChR mRNA expression in the bladder mucosa. The M2 mAChR subtype mRNA-to-β-actin mRNA ratio was significantly increased in 2 weeks after SCI. The M2 subtype mRNA-to-β-actin mRNA ratio at 4 weeks after SCI tended to be lower than that at 2 weeks after SCI and still higher than in normal; however, the differences were not significant (A). On the other hand, there was not significant difference in the M3 mAChR subtype mRNA-to-β-actin mRNA ratio in the mucosa among normal, 2 weeks and 4 weeks after SCI (B) (n=6). * p=0.042

Table 1

Baseline cystometrogram in 2 and 4 weeks after SCI (n=24). NVC (non-voiding contraction) is expressed as AUC (area under the curve) of bladder contractions calculated by the integral of a 3-minute period prior to voiding contraction.

	NVC (AUC, cmH ₂ O* s)	MVP (cmH ₂ O)	ICI (s)
SCI 2wks	868.1 ± 122.5	63.57 ± 6.297	713.7 ± 63.31
SCI 4wks	1173 ± 147.6	58.35 ± 10.97	474.1 ± 64.00 *

MVP: maximum voiding pressure. ICI: intercontraction interval of voiding contraction.

*
p=0.0238

Table 2

Effects of intravesical administration of Oxo-M with or without antimuscarinic agents on cystometric parameters (n=6).

	NVC (AUC, cmH ₂ O* s)	MVP (cmH ₂ O)	ICI (s)
SCI 2w			
saline	1093 ± 321.5	63.57 ± 6.297	713.7 ± 63.31
Oxo-M	1561 ± 340.3 *	71.76 ± 6.786	785.0 ± 27.55
SCI 4w			
saline	1025 ± 240.1	58.35 ± 10.97	474.1 ± 64.0
Oxo-M	1424 ± 229.2 *	63.41 ± 8.412	444.7 ± 47.89
SCI 2w			
saline	926.4 ± 234.7	63.57 ± 6.297	713.7 ± 63.31
atropine	914.1 ± 224.7	64.31 ± 7.402	755.4 ± 238.7
Oxo-M+atr.	929.2 ± 200.1	64.03 ± 5.891	845.3 ± 172.7
SCI 4w			
saline	919.5 ± 212.8	56.21 ± 7.400	776.7 ± 191.8
atropine	866.0 ± 217.1	61.28 ± 9.806	663.6 ± 113.8
Oxo-M+atr.	1060 ± 294.1	67.46 ± 9.043	684.1 ± 93.71
SCI 2w			
saline	807.1 ± 167.2	64.21 ± 7.211	826.8 ± 279.0
metho.	843.5 ± 214.8	67.79 ± 6.613	710.2 ± 219.5
Oxo-M+metho.	867.4 ± 182.5	68.44 ± 5.643	708.2 ± 281.9
SCI 4w			
saline	1542 ± 223.4	63.33 ± 10.76	628.5 ± 79.76
metho.	1445 ± 274.7	61.37 ± 11.64	604.4 ± 80.41
Oxo-M+metho.	1310 ± 78.97	65.53 ± 14.01	553.0 ± 81.23
SCI 2w			
saline	745.2 ± 153.1	54.29 ± 7.585	789.4 ± 139.8
darifenacin	793.1 ± 174.8	51.55 ± 6.567	788.8 ± 153.3
Oxo-M+dar.	1060 ± 230.8 *	56.29 ± 8.060	773.4 ± 165.1
SCI 4w			
saline	1026 ± 269.4	62.06 ± 7.659	602.8 ± 102.2
darifenacin	965.0 ± 194.0	59.76 ± 6.957	548.4 ± 49.0
Oxo-M+dar.	1317 ± 230.7 *	69.22 ± 5.035	584.6 ± 77.96

atr: atropine, metho: methoctramine, dar: darifenacin.

*
p<0.05