Effects of Pelvic Nerve Neurectomy and Estrogen on the M₂ Muscarinic Receptor of the Urinary Bladder

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Background: Normal physiological voiding, as well as generation of abnormal bladder contractions in the diseased state, is critically dependent on acetylcholine-induced stimulation of contractile muscarinic receptors on the detrusor muscle of the urinary bladder. This study investigated the effect of pelvic nerve neurectomy and estrogen treatment on the M₂ muscarinic receptors of urinary bladder.

Methods: We studied the muscarinic cholinergic receptor density in the urinary bladder of castrated female rats following four weeks of estrogen treatment and/or pelvic nerve neurectomy. We used immunohistochemistry to detect the expression of M₂ receptors on the smooth muscle cells of urinary bladder.

Results: When compared to a sham-operated group, the M₂ receptors in the urinary bladders of rats that had undergone bilateral pelvic nerve neurectomy and estrogen treatment, showed no significant change. However, the M₂ receptors decreased significantly in the urinary bladders of rats that had undergone simultaneous estrogen treatment and bilateral pelvic nerve neurectomy, when compared to the other groups (p < 0.05).

Conclusion: Pelvic nerve denervation has a synergetic effect on the estrogen-induced down regulation of M₂ receptor immunoreactive cells in the urinary bladder.

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Key words: muscarinic receptor, urinary bladder, pelvic nerve, estrogen.
and a 60% increase in M2 receptor density but not M3, in the denervated bladder.\textsuperscript{(8)} They also found bilateral major pelvic ganglion electrocautery and spinal cord injury in rats might induce bladder hypertrophy and a change in muscarinic receptor subtype from M3 to M2.\textsuperscript{(9)} Hence, monitoring the fluctuation of M2 receptors may reflect the function of detrusor contractions in the parasympathetic denervated bladder.

In addition, sex steroid hormones can modulate neurotransmitter receptor density in the urinary bladder. Several published reports have shown that estrogen administration is associated with a change in the muscarinic receptor density in the urinary bladder.\textsuperscript{(10-12)} Levin et al demonstrated that administration of estrogen to immature female rabbits for four days resulted in not only an increased sensitivity of the bladder to a muscarinic agonist but also increased muscarinic receptor density.\textsuperscript{(10)} In contrast, Shapiro’s study showed that estrogen treatment for three weeks was found to cause a 45% decrease in the muscarinic receptor density in the adult female rabbit bladder and urethra.\textsuperscript{(11)} Batra and Andersson showed that after estrogen treatment for four weeks, the muscarinic receptor concentration was reduced to approximately 10% of the control value in the ovariectomized rabbit.\textsuperscript{(12)} However, the true mechanism of the response of muscarinic receptors to estrogen is still unclear, and the effect of estrogen treatment on the bladder’s muscarinic receptors deserves further study. In this study, we tried to identify the influence of parasympathetic bladder innervation in relation to estrogen treatment on the M2 muscarinic receptors in the castrated female rat.

METHODS

Twenty-five mature female Sprague-Dawley rats (age range: 10-12 weeks), obtained from the National Laboratory Animal Center and weighing between 200 and 250 g, were divided into five experimental groups. Group 1 consisted of sham-operated animals that received no hormonal manipulation (Sham). The remaining four groups of rats were castrated two weeks before any treatment. Group 2 underwent bilateral salpingo-oophorectomy (BSO) with no hormonal manipulation. Group 3 underwent BSO with no hormonal manipulation and then received bilateral pelvic nerve neurectomy four weeks after BSO (BSO + PN). Group 4 underwent BSO with intramuscular injection of estrogen every week (BSO + E). Group 5 underwent BSO with intramuscular injection of estrogen every week and then received bilateral pelvic nerve neurectomy four weeks after BSO (BSO + PN + E). Estrogen treatment was performed by intramuscular injection of 1 mg estradiol polyphosphate/kg body weight weekly. In our previous experiment (unpublished), we found that a single injection of estradiol polyphosphate could maintain a steady level of plasma estradiol in rats. All animals were sacrificed six weeks after BSO.

BSO was performed through a lower abdominal midline incision using a sterile technique under inhalation anesthesia with 2% isoflurane in oxygen. Bilateral pelvic nerve neurectomy was performed in Groups BSO + PN and BSO + PN + E by making a lower abdominal midline incision and stripping the pelvic peritoneum from the iliac vessels.\textsuperscript{(3)} The fascial plane between the flexor caudae brevis and the abductor caudae internus muscles was located by following the internal pudendal and the superior gluteal arteries that transverse it. The fascial plane was split to expose the pelvic nerve, which branches from the internal pudendal nerve in one discrete bundle. The pelvic nerves were cut near the pudendal nerves and the distal portion was removed. Resection of both pelvic nerves abolished the micturition reflex with consequent distention of the bladder. For sham-operated animals, the nervous plexus was exposed but left intact. The subcutaneous tissue, muscle and skin were sutured.

After surgery, urine was drained out by using manual compression of the rats’ lower abdomen twice daily to prevent overdistention of the bladder. The animals were sacrificed by rapid exsanguination after intraabdominal pentobarbital administration, and the urinary bladder was removed, frozen in powdered dry ice, and stored at –70°C. The urinary bladder was cut at 10-µm thickness on a cryostat at –18°C and collected on silane-coated glass slides (Dako, Denmark).

Immunostaining of M2 muscarinic receptors was performed using the avidin-biotin-peroxidase complex (ABC) method with a commercially available kit (PK-6101, Vector Laboratories, Burlingame, CA, USA), according to our previously published method.\textsuperscript{(13)} First, the fresh-frozen sections were fixed
in ice-cold acetone for 10 minutes and air-dried. After blocking with 2% normal goat serum for one hour, the sections were incubated for 12 to 15 hours at 4°C with a rabbit polyclonal antibody against the M₂ subtype receptor (diluted 1/2,000, Chemicon International, Temecula, CA, USA). Endogenous peroxidase was blocked for 30 minutes with 0.1% H₂O₂ and 100% methanol. The sections were then washed and incubated for one hour with biotinylated goat anti-rabbit IgG, followed by incubation for 30 minutes with avidin-biotin-horseradish peroxidase complex. Staining was developed using DAB substrate kit (Vector Laboratories, Burlingame, CA, USA). The negative control studies consisted of: (1) omitting the primary M₂ receptor antibody, (2) using the polyclonal anti-M₂ receptor antibody preadsorbed to M₂ antigen (Chemicon International, Temecula, CA, USA) as the primary antibody, (3) replacing the primary antibody with the preimmune rabbit IgG (Vector Laboratories, Burlingame, CA, USA) (Fig. 1). Preadsorption was accomplished by incubating the polyclonal anti-M₂ receptor antibody with excessive amounts of purified M₂ antigen at 4°C for 24 hours; the supernatant taken from the mixture was used as the preadsorbed control.

For calculating the M₂ receptor density, every fifth urinary bladder section was collected, and a total of four sections from each animal were immunostained. The number of M₂ receptor immunoreactive cells in a smooth muscle area (number/mm² = M₂ receptor density) in each section was counted. The area of smooth muscle was mapped on a scale diagram and measured by a computerized graphic system (Image-Pro Plus, version 1.3, Media Cybernetics, Silver Springs, MD, USA). The measurements were conducted by an observer (LSR).

**Fig. 1** A: M₂ receptor immunoreactive cell on the smooth muscle cell of the urinary bladder. Negative control studies consist of (1) B: omitting the primary antibody, (2) C: using polyclonal anti-M₂ receptor antibody preadsorbed to M₂ antigen as the primary antibody, (3) D: replacing the primary antibody with the preimmune rabbit IgG. Bar in the top left indicates 100 µm.
who was blind to the experiment. The M2 receptor immunoreactive cell density of the four sections from each rat was summated and expressed as the mean ± standard deviation (SD), and the median value with maximum and minimum was also presented. Multiple comparisons between groups were made by 1-way ANOVA with the post-hoc Bonferroni test. A value of $p < 0.05$ was considered significant.

**RESULTS**

The study of M2 receptor immunoreactive cell density (Figs. 2 and 3) demonstrated that group BSO, BSO + PN and BSO + E did not differ significantly when compared to the Sham Group: BSO cell density mean ± SD 32.72 ± 6.90, median 34.33 (min 40.84, max 24.72), BSO + PN 35.42 ± 3.72, 36.32 (38.75, 28.98), BSO + E 27.76 ± 4.76, 28.95 (32.73, 21.15) and Sham Group 31.65 ± 2.08, 31.60 (34.83, 28.98). However, Group BSO + PN + E, 12.10 ± 2.69, 11.27 (16.84, 10.42), showed a significant reduction in M2 receptor immunoreactive cell density when compared to the Sham Group ($p < 0.001$). Group BSO + PN + E had the most significant reduction of M2 cell density when compared to the other groups ($p < 0.05$).

**DISCUSSION**

Normal physiological voiding, as well as generation of abnormal bladder contraction in the diseased state, is critically dependent on acetylcholine-induced stimulation of contractile muscarinic receptors in the smooth muscle of the urinary bladder. Alm and Ekstrom reported that unilateral excision of the pelvic ganglion caused a loss of acetylcholinesterase positive nerve in the rat urinary bladder, both on the operated side and on the contralateral side, indicating a bilateral intramural distribution of cholinergic nerve derived from the pelvic nerve. The pelvic nerve is composed primarily of postganglionic sympathetic fiber, which terminates in the smooth muscle cells of the urinary bladder. Stimulation of the rat pelvic nerve results in muscarinic receptor-mediated contraction of the detrusor muscle. Denervation of the parasympathetic nerve may act like a chronic receptor blockade due to a muscarinic antagonist, and may be expected to

Fig. 2 The immunostaining of M2 receptors shows that there is a remarkable reduction of M2 receptor immunoreactive cells in Group 4 (G and H) and Group 5 (I and J) when compared to the Sham Group (A and B). However, there is no remarkable change of M2 receptor immunoreactive cells in Group 2 (C and D) and Group 3 (E and F) when compared to the Sham Group. Group 5 (I and J) has the most remarkable reduction of M2 receptor immunoreactive cells when compared to the other Groups. A and B: sham-operated rat (Sham); C and D: rat with bilateral salpingo-oophorectomy (BSO); E and F: rat with BSO + pelvic neurectomy (PN); G and H: rat with BSO + intramuscular injection of estrogen (E); I and J: rat with BSO + PN + E. Bars in A and B indicate 50 µm.
induce increased receptor density.\textsuperscript{(10)} Additionally, denervation of the pelvic nerve in the rat bladder results in supersensitivity to a muscarinic agonist, and bladder hypertrophy.\textsuperscript{(6,17-19)} Gunasena et al reported that postganglionic denervation caused a 37% increase in maximal binding and a twofold increase in the density of the muscarinic binding site in the smooth muscle of the bladder. This increased density could be the mechanism responsible for the increased contractile response to acetylcholine after bladder denervation,\textsuperscript{(15,17)} and in patients with bladder outlet obstruction.\textsuperscript{(20)}

Pharmacological, biochemical and molecular studies reveal that most tissues of the lower genitourinary tract, including the urinary bladder, are enriched with muscarinic receptors and express a mixture of subtypes.\textsuperscript{(6,19,21)} The binding and subtype selective immunoprecipitation studies demonstrate that the majority of muscarinic receptors in the urinary bladder are M\textsubscript{2} subtype and the rest are M\textsubscript{3} subtype.\textsuperscript{(6,19)} Maeda et al used the Northern blot method to identify mRNAs encoding the M\textsubscript{2} and M\textsubscript{3} muscarinic receptors in the rat urinary bladder.\textsuperscript{(21)} Wang et al investigated the distribution of muscarinic receptor subtypes in rats using a panel of M\textsubscript{1} to M\textsubscript{5} antisera, and found that more M\textsubscript{2} receptors were immunoprecipitated than M\textsubscript{3} receptors in bladder tissue.\textsuperscript{(6)} Braverman et al measured the density of total M\textsubscript{2} and M\textsubscript{3} receptors by subtype selective immunoprecipitation and found a 60% increase in M\textsubscript{2} receptor density but no change in M\textsubscript{3} receptor density three weeks after bladder denervation, when compared with normal or sham-operated controls.\textsuperscript{(6)} Muscarinic receptors can be detected in homogenates of bladder smooth muscle from the rabbit and the rat bladder using radioligand binding or autoradiographic study.\textsuperscript{(17)} However, radioligand binding study is non-selective and represents the total population of muscarinic receptors. In the present study, we used immunohistochemistry with polyclonal rabbit anti-

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Cell density & Sham & BSO & BSO + PN & BSO + E & BSO + PN + E \\
Mean ± SD & 31.65 ± 2.08 & 32.72 ± 6.9 & 35.42 ± 3.72 & 27.76 ± 4.76 & 12.10 ± 2.69 \\
Median (maximum, minimum) & 31.60 (34.83, 28.98) & 34.33 (40.84, 24.72) & 36.32 (38.75, 28.98) & 28.95 (32.73, 21.15) & 11.27 (16.84, 10.42) \\
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\caption{Histogram shows differences in M\textsubscript{2} receptor immunoreactive cell density in the five groups. Results are expressed as the mean ± SD per mm\textsuperscript{2}. Group BSO + PN + E has a significant reduction of M\textsubscript{2} receptor cell density when compared to the Sham Group (\(p < 0.001\)). However, there is no significant difference in Group BSO, Group BSO + PN and Group BSO + E when compared to the Sham Group.}
\end{table}
body specific to the M2 receptor to determine the density of M2 receptors in the smooth muscle of rat bladders. Immunohistochemistry also allows a much higher degree of cellular resolution than autoradiographic methods.

Nilvebrant et al. pointed out that the muscarinic receptor density in the rat urinary bladder is, indeed, increased after bilateral parasympathetic denervation. Due to impaired bladder emptying, the denervated bladder becomes markedly hypertrophic and the major increase of receptors can be attributed to the increase in muscle mass. However, the receptor density decreases after urinary diversion, not only in innervated but also in denervated rat bladders. These results suggest that the rat bladder muscarinic receptor is influenced by functional state rather than by actual nerve supply and degree of receptor stimulation, i.e. muscarinic receptors would increase with the stretch in the denervated bladder wall after over-filling. In our present study, we found a tendency for an increase of muscarinic M2 receptors in the castrated female rat urinary bladder after bilateral pelvic neurectomy, although no significant difference was noted when compared with the control group. Bilateral ovariectomy may result in a not so significant increase in the density of muscarinic binding sites in the denervated rat, as previously reported.

Hence, we deduce the sex hormone (estrogen) from the rat ovary may play an essential role in the functional state of the bladder.

Previous studies revealed that sex steroid hormones might modulate neurotransmitter receptor density in various target tissues, including the urinary bladder. In the female rabbit, estrogen treatment increases tissue mass and the sensitivity of the smooth muscle to autonomic drugs in both the bladder and urethra. Ekstrom et al. treated the ovariecctomized adult rabbit for four to six months with either estrogen or progesterone, and found that estrogen resulted in bladder contraction in response to noradrenaline and phenylephrine, whereas these sympathomimetic agonists had no effect or evoked weak relaxation in castrated animals. Furthermore, both estrogen and progesterone treatments seemed to increase the sensitivity of bladder contraction to parasympathomimetics. Levin et al. demonstrated that the administration of estrogen for four days to immature female rabbits resulted in increased sensitivity of the bladder to a muscarinic cholinergic agonist and increased muscarinic receptor density. In contrast, Shapiro’s study showed that the muscarinic receptor density in the adult female rabbit bladder and urethra was decreased following three weeks of estrogen treatment. The conflicting results may be explained by the different intervals of estrogen administration. Batra and Andersson examined the effect of estrogen treatment on the mature ovariecctomized rabbit and found that estrogen treatment caused a marked reduction in the density of muscarinic receptors. There was already a significant reduction after just one week of estrogenization. After four weeks of estrogen treatment, the muscarinic receptor concentration was reduced to approximately 10% of the control value. However, the increase or decrease in muscarinic cholinergic response and receptor density is also intriguing from a clinical standpoint. Bladder function can be pharmacologically manipulated by neurotransmitter agonists and antagonists, or by agents that regulate the density of neurotransmitter receptors. Our data showed the castrated rat urinary bladder following estrogen treatment tended to decrease in the quantity of M2 receptors, although no significant difference was noted when compared with the Sham Group. Shapiro’s observations suggest that estrogen-induced down regulation of muscarinic receptors in the bladder may be another mechanism for the improved lower urinary tract dysfunction of frequency, urgency and urinary incontinence. Shapiro pointed out that the decreased muscarinic receptor density might result in the reduction of detrusor tone and an increase in bladder capacity. These changes may, in part, explain the improvement in lower urinary tract dysfunction of post-menopausal women with frequency, urgency and urinary incontinence following three to four weeks of estrogen therapy. However, in Batra and Andersson’s study, they found the effect on contraction caused by stimulation of the muscarinic receptors (carbachol-evoked contractions) seemed to be small. Up to now, the true mechanism of the response of muscarinic receptors to estrogen is still unclear but we have found that the pelvic nerve might play a role in muscarinic M2 receptor density. In the present study, after four weeks of estrogen administration, the muscarinic M2 receptor density in rat bladders decreased significantly in Group BSO + PN + E that underwent bilateral pelvic nerve neurectomy and castration, when com-
pared to the remaining groups. Further study is needed to determine if the down regulation of the muscarinic M2 receptor may result in a loss of contractile force.

In conclusion, our study demonstrated that the rat bladder muscarinic receptor is influenced by estrogen treatment and pelvic nerve stimulation. The estrogen treatment and pelvic nerve denervation may have a synergetic effect on the down regulation of M2 receptor immunoreactive cells in the bladder.

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骨盆神經截斷與雌激素治療對膀胱 M2 毒蕈鹼接受體的影響

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背 景：正常解尿與病變引起的不正業膀胱收縮一樣，都要靠乙酰膽鹼 (acetylcholine) 來刺激膀胱逼尿肌。毒蕈鹼接受體 (muscarinic receptor) 的作用。本研究的目的在探討骨盆神經截斷與雌激素治療對膀胱 M2 毒蕈鹼接受體的影響。

方 法：25 隻介於 200 至 250 公克成熟的雌性大白鼠被平均分為 5 組：組一為對照組，其餘四組的所有大白鼠則接受雙側卵巢切除。組二為單純的雙側卵巢切除的大白鼠；組三為雙側卵巢切除兩週後，加上雙側骨盆神經截斷的大白鼠；組四為雙側卵巢切除兩週後，加上每週給予雌激素肌肉注射一毫克 estradiol polyphosphate / 公斤的大白鼠；組五為雙側卵巢切除兩週後，加上雙側骨盆神經截斷，再加上每週給予雌激素肌肉注射一毫克 estradiol polyphosphate / 公斤的大白鼠。全部的大白鼠在雙側卵巢切除後六週犧牲，取出膀胱後冷凍並切片。利用免疫染色方法 (immunohistochemistry) 以得知位於膀胱平滑肌細胞 M2 毒蕈鹼接受體的表現。

結 果：免疫染色後，M2 毒蕈鹼呈現免疫反應 (immunoreactive) 的細胞在有給予雌激素肌肉注射與雙側骨盆神經截斷的組五的大白鼠膀胱平滑肌細胞，與對照組比較呈統計學上有意義的減少。雙側骨盆神經截斷加上接受雌激素肌肉注射的組五，其 M2 毒蕈鹼細胞濃度比其他四組呈統計學上有意義的減少。

結 論：骨盆神經是影響大白鼠膀胱平滑肌細胞 M2 毒蕈鹼細胞濃度重要的因素，一旦去勢雄鼠的雙側骨盆神經被截斷，會加大雌激素降低膀胱 M2 毒蕈鹼接受體的表現。

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關鍵字：毒蕈鹼接受體，膀胱，骨盆神經截斷，雌激素。