

*Forum Minireview***Basic and Clinical Aspects of Non-neuronal Acetylcholine:
Expression of Non-neuronal Acetylcholine in Urothelium and Its Clinical
Significance**

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Abstract. Recently, several reports demonstrate that non-neuronal acetylcholine (ACh) release may contribute to various pathophysiological conditions. In this review, we presented our experiments designed to evaluate the non-neuronal cholinergic system in human bladder. After insertion of the microdialysis probe, human bladder strips were suspended in an organ bath filled with Krebs-Henseleit solution, and Ringer solution was perfused into the probe. ACh release was measured by microdialysis and HPLC. The contribution of urothelium and the effects of age and stretch of bladder strips on non-neuronal ACh release were evaluated. Choline acetyltransferase (ChAT) immunohistochemical staining of bladder was also performed. Immunohistochemistry showed marked ChAT-positive staining in the urothelium. There was tetrodotoxin-insensitive non-neuronal ACh release and this was significantly higher in strips with urothelium than in strips without urothelium. The non-neuronal ACh release was increased with age. Stretch of bladder strips caused increases in non-neuronal ACh release. The stretch-induced release of non-neuronal ACh was increased with age. Our data demonstrate that there is a non-neuronal cholinergic system in human bladder and that urothelium contributes to non-neuronal ACh release. There was significant age-related and stretch-induced increase in non-neuronal ACh release. It is suggested that the non-neuronal cholinergic system may contribute to the physiology and pathophysiology of human bladder. We also discussed the clinical significance of the non-neuronal cholinergic system in human bladder.

Keywords: non-neuronal cholinergic system, acetylcholine, human bladder, urothelium, overactive bladder

Introduction

The two main functions of the bladder, urine storage and bladder emptying, involve a complex pattern of efferent and afferent signaling in autonomic (parasympathetic, sympathetic) and somatic nerve (1). Disturbances of the normal control of the bladder reflexes may lead to an overactive bladder (OAB), clinically characterized by symptoms of urgency, with and without urge incontinence, usually with frequency and nocturia: OAB syndrome (2). It has been reported that bladder

function changes with age (3–7), and symptoms associated with OAB are increased with age (8).

The main neurotransmitter in bladder contraction is acetylcholine (ACh) released from cholinergic nerve endings. In OAB, it has been supposed that ACh released from cholinergic nerves causes stimulation of the muscarinic receptors on the detrusor smooth muscle, which results in involuntary detrusor contraction (9). Antimuscarinic drugs are usually used for treatment of OAB (10). Symptoms of OAB usually occur during the storage phase, and antimuscarinic drugs act mainly during this cycle, increasing bladder capacity and decreasing urge (11). However, during the storage phase, there is normally no activity in parasympathetic nerves (12). Thus, pathological mechanisms of symptoms

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of OAB still are not clearly elucidated.

Recently, it has been suggested that bladder epithelium and afferent neurons have an important role in the pathogenesis of detrusor overactivity (13). In addition, there are several reports suggesting the biological role of the non-neuronal cholinergic system in epithelial cells from several types of tissues (human airway, alimentary tract, and epidermis); endothelium; muscles; and immune cells (14–18). Additionally, it has been reported that this system contributes to both physiological and pathophysiological conditions (19–22). However, there is no available information about the non-neuronal cholinergic system in the epithelium of urinary tract (urothelium). Recently, we demonstrated the presence of a non-neuronal cholinergic system in human bladder urothelium. In this manuscript, we presented our experimental results and discussed the role of non-neuronal ACh on the pathogenesis of OAB and the action mechanisms of antimuscarinic drugs in OAB treatment.

Expression of choline-acetyltransferase (ChAT) in human bladder

Specimens of human urinary bladder were obtained from 15 patients (13 males and 2 females, mean age 65.4-year-old) who underwent total cystectomy due to malignant bladder tumor under permission for using human bladder tissue from the ethics committee at Graduate School of Medical Science, Kumamoto University.

ChAT-protein was demonstrated by immunohistochemistry using a goat polyclonal anti-ChAT antibody (Chemicon, Limburg, UK), as previously described (23). The normal part of human bladder tissue of all patients was immediately frozen in an OCT block by liquid nitrogen and then cut 10- μ m-thick with a cryostat.

The immunohistochemistry of ChAT showed that brown-stained positive immunoreactivity was observed in bladder urothelial cells and cells in the suburothelial space in all specimens. The immunoreactivity of the urothelial cells is relatively higher in the elderly patients, as compared with the young ones.

Microdialysis procedure and measurement of non-neuronal ACh release

The functional experiments were performed as previously described (24). The human bladder strip with or without urothelium was suspended in an organ bath filled with Krebs-Henseleit solution. The microdialysis probe was inserted into each preparation; each preparation was connected to a force displacement transducer;

and the isometric force was recorded and monitored on an ink-writing recorder. The microdialysis procedure was performed as previously reported (25). In the strip with urothelium, the dialysis probe was inserted into the suburothelial space. In the strip without urothelium, it was inserted through the muscle layers. Ringer's solution containing 100 μ M physostigmine sulfate was continuously perfused. The internal standard, isopropylhomocholine, was fed into the perfusate tube. Dialysate was collected in a microtube every 10 min. We injected 10 mL of each sample into the ACh determination system. ACh determination was performed by a combination of HPLC, enzyme reaction, and electrical-chemical detection, as previously described (25). The detection limit of ACh was 0.005 pmol/injection.

Figure 1 demonstrates the typical recording of chromatograms for ACh release in human bladder strips with urothelium. A significant release of ACh occurred before EFS (basal release, Fig. 1A). The basal release was not affected by treatment with 1 μ M tetrodotoxin. EFS caused a significant elevation of the ACh peak (Fig. 1B), with a significant increase in the contractile response. Pretreatment with 1 μ M tetrodotoxin completely inhibited the EFS-induced contractile response. However, a small ACh release remained (Fig. 1C), and the remaining release was similar to the basal release. Thus, ACh release induced by EFS is considered of neuronal origin (neuronal ACh), and the basal release

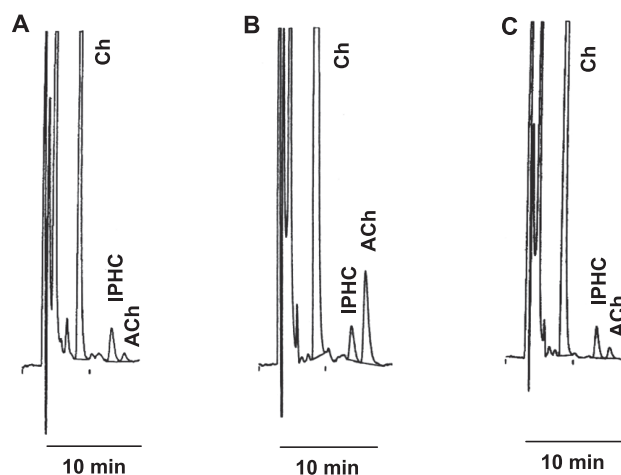


Fig. 1. Typical chromatograms of HPLC measurement of ACh releases from human bladder strip with urothelium. A: Before electrical field stimulation, there is small ACh release (basal release). The release was not inhibited by pretreatment with tetrodotoxin. B: Electrical field stimulation caused a significant increase in ACh release. After treatment with tetrodotoxin, electrical field stimulation-induced ACh release was significantly inhibited, but there was still only a small release (C). It is suggested that the tetrodotoxin-sensitive ACh release is of neuronal origin, while the tetrodotoxin-insensitive release is of non-neuronal origin. Ch: choline, IPHC: isopropylhomocholine, and ACh: acetylcholine

and remaining release after tetrodotoxin treatment in EFS are considered of non-neuronal origin.

Effects of urothelium and bladder stretch on non-neuronal ACh release

The amount of neuronal and non-neuronal ACh release was measured in human bladder strips with and without urothelium. The difference in neuronal ACh release between bladder strips with and without urothelium was not significant (3.62 ± 0.45 and 3.55 ± 0.42 pmol/g tissue, respectively; $n = 15$). However, the non-neuronal ACh release from strips with urothelium (0.056 ± 0.008 pmol/g tissue, $n = 15$) was significantly greater than that in strips without urothelium (0.018 ± 0.004 pmol/g tissue, $n = 15$). Non-neuronal ACh release was observed in strips with and without urothelium. However, the release was significantly greater in strips with urothelium than in those without urothelium. The data suggest that non-neuronal ACh is partly generated by the urothelium.

In the experiment on bladder-strip stretching, the resting tension was changed from 0–40 mN. In the strips with urothelium, significant stretch-induced increases in non-neuronal ACh release occurred. Stretch also caused significant ACh release from strips without urothelium. However, the increases did not show the tension-dependent changes and were significantly lower than the release from strips with urothelium.

Although our data suggest that bladder urothelium is the source of non-neuronal ACh, other sources could not be completely excluded. In the present experiment, in bladder strips without urothelium, a small amount of basal ACh release was observed, and stretching the strips also caused an increase in ACh release, although this was significantly lower than the stretch-induced

ACh release from strips with urothelium. It has been reported that smooth muscle cells have a non-neuronal cholinergic system (20). Furthermore, it is possible that removal of urothelium may remove some cellular components located in the suburothelial layer, such as myofibroblasts and interstitial pacemaker cells. Thus, the cells may be a source of non-neuronal ACh. In addition, it is speculated that nerves also leak ACh without nerve stimulation in various pathologic conditions of the bladder.

Effects of age on non-neuronal ACh release

Figure 2A shows the correlation between age and non-neuronal ACh release from bladder strips with urothelium under 0-mN resting tension. Non-neuronal ACh release increased with age and a significant positive correlation was found between age and release. Figure 2B shows the correlation between age and the stretch-induced maximal percentage of non-neuronal ACh release in strips with urothelium. In all strips, maximal release was obtained under 40-mN resting tension. The stretch-induced maximal percentage of release of non-neuronal ACh increased with age, and a significant positive correlation between age and release was observed. In addition to enhanced immunoreactivity of ChAT in the urothelium from the older patients compared to that from the younger patients, the data suggest that the aging process might partly contribute to enhancement of the non-neuronal cholinergic system in the human bladder.

The releasing mechanism and the targets of non-neuronal ACh

The releasing mechanisms of ACh from non neuronal

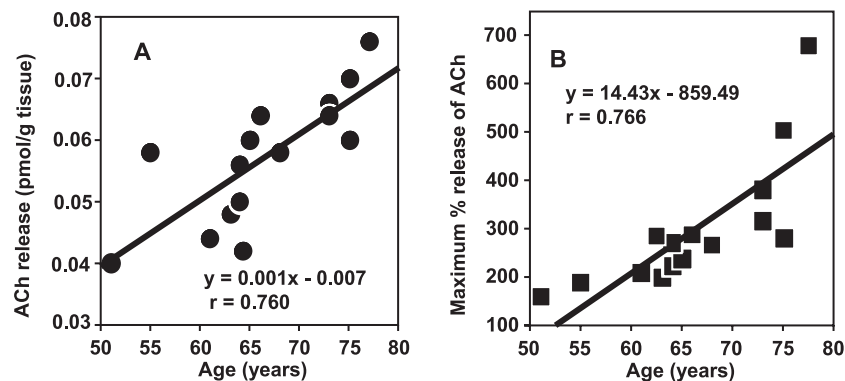


Fig. 2. Effect of age on non-neuronal ACh release and stretch-induced non-neuronal ACh release from human bladder strip with urothelium. A: Correlation between age and non-nerve-evoked ACh release. B: Correlation between age and stretch-induced maximal percentage of release of non-nerve-evoked ACh. For measurement of non-nerve-evoked ACh, the microdialysis procedure was performed in all bladder strips pretreated with tetrodotoxin ($1 \mu\text{M}$), under 0-mN resting tension.

origins have not been clearly elucidated. The finding that stretch of bladder strips caused an increase in the release implies that the shear stress of the urothelium may be one of the releasing mechanisms. It has been reported that other epithelial factors, such as nitric oxide (26), prostaglandins (27), and endothelin (28), are released by shear stress on the epithelium or endothelium.

Recently our preliminary experiments demonstrated that stretch-induced non-neuronal ACh releases from human bladder were significantly inhibited in Ca^{2+} -free medium. Further addition of a Ca^{2+} chelator resulted in a complete block of the release. Treatment with botulinum toxin (inhibitor of vesicular exocytosis or trafficking) caused significant reduction in ACh release. The data suggest that both intra- and extra-cellular Ca^{2+} contribute to stretch-induced non-neuronal ACh release in human bladder. In addition, Ca^{2+} -dependent vesicular exocytosis might be related to the release.

Although, there was positive immunoreactivity of ChAT in human bladder urothelium in our study, a recent report (29) demonstrated that there were negative RT-PCR results for ChAT. It has been suggested the presence of carnitine acetyltransferase (CarAT) mRNA shows that there is a potential for CarAT catalyzed synthesis of ACh. Thus, the authors concluded that CarAT might be the major ACh-synthesizing enzyme in human urothelium. In addition, with respect to the molecular components of the ACh release machinery, the urothelium markedly differs from cholinergic nerve terminals in that vesicular ACh transporter, the transporter shuffling ACh from the cytoplasm into synaptic vesicles in nerve endings (vesicular exocytosis), was detected neither at the mRNA level nor the protein level. The authors expected that polyspecific organic cation transporters (OCTs), particularly OCT isoform-1, represent a major ACh release mechanism from the bladder mucosa. Further study would be needed to clarify the releasing mechanism of non-neuronal ACh in human urothelium.

For the targets of non-neuronal ACh from urothelium, it could be expected to enhance the muscarinic receptors-mediated myogenic contractile activity of the detrusor, which has been proposed to be increased in patients with detrusor overactivity (9). The increased myogenic activity may, in turn, increase firing in afferent nerves and contribute to symptoms of OAB. It is also possible that non-neuronal ACh acts on afferent nerves to initiate the micturition reflex. Currently, no evidence has shown that such receptors are present on the terminals of afferents that supply the bladder. Hawthorn et al. (30) reported 1.5 times greater numbers of muscarinic receptors in the urothelium than in the smooth muscle layer in the pig bladder. A recent report

(31) also demonstrated a high density of M_2 muscarinic receptors in the human bladder mucosa. Furthermore, Mukerji et al. (32) reported that M_2 - and M_3 -immunoreactive staining was present in human detrusor, myofibroblast-like cells, nerve fiber bundle, and dorsal root ganglion of small and medium sensory neurons in suburothelium. These muscarinic receptors might be targets of non-neuronal ACh from urothelium.

Recently, Beckel et al. (33) reported the expression of functional nicotinic acetylcholine receptors in rat urinary bladder urothelium. In addition, application of nicotine to cultured urothelial cells caused an increase in the Fura 2 signal, suggesting that the receptors were indeed functional. The urothelial nicotinic acetylcholine receptors should be considered to be the targets of non-neuronal ACh.

Clinical significance of non-neuronal ACh from bladder urothelium

Recently, it has been suggested that bladder urothelium and afferent neurons have important roles in the micturition reflex and pathogenesis of detrusor overactivity, and the concept of urothelial mechanosensitive transduction was proposed (13). Urothelium is not only a barrier for toxic substances in the urine but also is a metabolically highly active tissue that may take an active part in both storage and voiding phase. It has been reported that several mediators such as ATP (34), tachikins (35), and prostaglandins (36) may be released from urothelium in response to stretch, a process that was dependent on changes in the trans-epithelial potential. Each mediator could activate suburothelial sensory nerves or smooth muscles through each receptor and cause the initiation of bladder contraction. Non-neuronal ACh released from urothelium may be one of the mediators contributing to the urothelial mechanosensitive transduction.

The release of non-neuronal ACh was increased with age, and there was a significant positive correlation between age and the release, and the increased non-neuronal ACh release due to bladder strip stretch was significantly higher in the older patients than in the younger ones. In addition, the immunoreactivity of ChAT in bladder urothelium was higher in the elderly than in the young patients. Although, we did not evaluate the relationship between non-neurogenic ACh releases and symptoms of OAB, the data suggest that the increased level of non-neuronal ACh release in the elderly may contribute to the increased prevalence rate of overactive bladder (8).

In our study, non-neuronal ACh releases were gradually increased according to the elevation of the

resting tension by stretching the bladder strips with urothelium. It is supposed that the elevation of the resting tension of bladder strips is a condition similar to distension of bladder wall in the storage phase of the micturition cycle. Thus, it may be assumed that during the storage phase, there is an ongoing stimulation of detrusor tone by ACh released from non-neuronal sources, possibly the urothelium.

Muscarinic receptors are involved in both normal and disturbed contraction, and the most common drug treatment of OAB is antimuscarinic drugs. It is reported that antimuscarinic drugs act mainly during the storage phase, increasing bladder capacity and decreasing urge (11); and during the storage phase, there is normally no activity in parasympathetic nerves (12). However, if there is an increased non-neuronal ACh release during storage, which is proposed to be a contributing factor to OAB, it is possible that antimuscarinic drugs have some inhibitory effects on muscarinic receptors activated by the non-neuronal ACh. Thus, the present study may provide useful information about the action mechanism of antimuscarinic drugs during the storage phase.

Conclusions

There is a non-neuronal cholinergic system in human bladder, and the urothelium plays an important role in the release of non-neuronal ACh. There are significant age-related and stretch-induced increases in non-neuronal ACh release. These facts may contribute to the age-related increase in the prevalence of OAB and provide useful information about the pathogenesis of OAB in humans.

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