Differential effects elicited by neocuproine, a copper (1) chelator, on the bladder activity in mouse and rat

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Abstract

Background and Purpose: The aim of this study was to investigate the mechanism of a possible differential effect of Neocuproine (NC), selective Cu(1) chelator, in rat and mouse bladder tissues. Experimental Approach: Bladder function was evaluated by 1. in vitro isolated bladder strips, 2. in vitro preparations of whole bladders and 3. İn vivo cystometrogram (CMG) in rat and mouse. Selective Cu(I) and Cu(II) chelators and non-selective purinergic antagonists were examined on EFS-induced bladder contractions in isolated bladder strips. The spontaneous contraction activities of whole bladders were recorded to evaluate the amplitudes and frequencies. In CMGs experiments, the values of maximum bladder pressure during micturition and intercontraction intervals (ICI) were evaluated. Key Results: Whereas Neocuproine (NC) enhances the opposite effect on isolated bladder strips and whole bladder experiments, this opposite effect was not observed in in vivo CMG experiments in rat and mouse. NC caused a significant suppression on spontaneous contractions and baseline tonus in isolated mouse bladder tissues, whereas it caused facilitating effects in the rat. However, NC significantly decreased the ICI in both rat and mouse in CMGs. Conclusion and Implications: The effect of Neocuproine on rat and mouse bladder activity is likely to be the role of myogenic mechanisms. It is possible to trigger mechanisms associated with intracellular calcium (Ca2+) reduction and purinergic pathway, P2X purinoceptors besides P2Y purinoceptors, may have an important role in the inhibitory activity of NC on mouse bladder.

INTRODUCTION

ATP (adenosine 5-triphosphate) has been identified as nonadrenergic-noncholinergic (NANC) transmitters in the lower urinary tract (Hoyle et al., 1989; Burnstock, 2002). ATP regulates many cell functions by acting on special types of receptors, called P2 purinoceptors (Burnstock, 2003). According to the current classification, P2 purinoceptors are divided into 2 families: P2X (contractile) and P2Y (relaxant) receptors (Burnstock and Kennedy, 1985; Abbrachio and Burnstock, 1994; Fredholm et al., 1994).

It is well known that Neocuproine (NC) enhances bladder and vas deferens activity by facilitating purinergic excitatory responses (Gocmen et al., 2004, 2005). NC can also inhibit the relaxation of electrically stimulated mouse corpus cavernosum (Gocmen et al., 2000). In addition, other previous studies have shown that copper inhibits purinergic transmission in the bladder, vas deferens, and pregnant uterine tissues and the copper(I) chelator NC (2,9-dimethyl-1,10-phenanthroline) enhances bladder activity, by excitating purinergic excitatory responses in rat (Kumcu et al., 2009). In some experimental studies, species differences have been shown to cause different outcomes in the mechanism of drug action (Hou et al., 2005, De Wachter, 2011; Kawase et al., 2009). It has been shown that the purinergic transmission on the bladder tissue may be different between the cat and the rat (Birder et al., 2004). Studies have also been published indicating that

there may also be significant differences in purinergic activity in mouse and rat bladder (Chen & Gebhart, 2010).

Although there has been an increasing number of studies on the therapeutic value of copper and copper chelators in clinical use in recent years, there are no clinical studies demonstrating the effects of these agents on bladder functions other than experimental animal studies (Arnal et al., 2011; Ding et al., 2011). To demonstrate the contribution of copper-dependent mechanisms in the regulation of purinergic and nitrergic mechanisms in the rat bladder, we investigated the effects of a selective Cu(I) chelator, NC (Neocuproine; 2,9-dimethyl-1,10-phenanthroline) (De Man et al., 1999; Göçmen et al., 2000), and a selective Cu(II) chelator, cuprizone (De Man et al., 1999), on neurally evoked contractions of bladder strips and on voiding function in urethane-anaesthetized rats. It is important to investigate the mechanism of rat and mouse bladder whether these findings (De Man et al., 1999; Göçmen et al., 2000) are related to species diversity. Because "the binding of endogenous copper(1) in tissues enhances tissue activities by enhancing purinergic excitatory responses" hypothesis which we have asserted based on the results we have obtained in rats, may change according to the experimental animal species. This difference will draw attention to the consideration of species differences in scientific researches. Therefore, in this study, we aimed to investigate the effects of copper (1) chelator neocuproine in parallel experiments*in vitro* and *in vivo* on rat and mouse bladder, and to investigate the contributions of adrenergic, cholinergic, nitrergic and purinergic pathways in this mechanism of the possible different effects.

METHODS

Animals

128 adult healthy male Wistar albino rats (n=48; 200–300 g) and Swiss albino mice (n=80; 25–35 g) were used in this study. The experimental procedures were approved by the animal care committee of the University of Cukurova (TIBDAM; protocol no: 2011/17-3) and the studies were carried out in accordance with the principles of laboratory animal care (National Institutes of Health guideline; publication No. 86-23, revised 1984). All animals were kept under standard laboratory conditions (12 h light/12 h dark). Three separate experimental methods were used in this study.

In vitro isolated organ bath studies

Sevoflurane anaesthetized rats (n=16) and mice (n=40) were sacrificed by decapitation. The bladders were isolated, and bladder strips (5- × 1-mm longitudinal sections) were prepared from the midportion of the bladder body. Strips (Strips of each rat: 4; strips of each mouse: 2) were subsequently mounted in 5-ml jacketed organ baths containing Krebs' solution (119 mM NaCl, 4.6 mM KCl, 1.25 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 24.8 mM NaHCO3, and 10 mM dextrose) maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2, pH 7.4. Tissues were allowed to equilibrate for 1 h, during which the preparation was washed with fresh Krebs' solution at 15-min intervals. The responses were recorded with isometric transducers (MAY COM, FDT 10-A). Data were recorded and stored using Biopac data acquisition software (Biopac MP35 Systems, Inc.). After the equilibrium period, neurally evoked isotonic contractions were induced using trains of electrical field stimulation (EFS; 10 Hz, 50 V, 0.5-ms duration, 10-s trains) delivered from a Grass S88 stimulator (Grass Instruments, Quincy, MA) at 2-min intervals through platinum electrodes positioned on the top and the bottom of the organ bath. In experiments in which EFS was used, atropine (2 μM) and guanethidine (2 μM) were always present in the bath with Krebs solution to block adrenergic and cholinergic transmission. In control experiments, EFS-evoked responses were recorded for a 3-h time period to examine the stability of the nerve-evoked responses. In the other experiments after the control responses to EFS were recorded for approximately 1-h, NC, a selective Cu(I) chelator, was applied at the concentrations of 100 and 200 μM to the same tissue consecutively at intervals ranging from 20 to 25 min. In some experiments, the effects of various agents including, prepared NC-copper(I) complex (Glutatione+NC+$CuCl_2$), a nitric oxide synthase inhibitor L-Nitroarginine (L-NOARG; 0.5 mM), a selective Cu(II) chelator cuprizone (5μM), or non-selective purinergic antagonist suramin (100 μM), P2X receptor antagonist PPADS (200 μM), P2X1 purinergic antagonist NF449 (20 μM), P2X2 purinergic antagonist A-

317491 (20 μM), P2X3 purinergic receptor antagonist NF110 (20 μM), P2Y1 purinergic antagonist MRS2179 (20 μM), P2Y2 purinergic receptor antagonist PSB1114 (20 μM) were examined on EFS-induced bladder contractions in the presence or absence of neocuproine. The neocuproine-copper(I) complex was prepared by reacting glutathione, neocuproine, and CuSO4 in molar ratios of 1:2:1, respectively (Dicks et al., 1996). In another series of experiments, we studied the effect of exogenous ATP on the basal tone of bladder strips. ATP (10, 50, or 100 μM) was applied four times to the same tissue consecutively at intervals ranging from 10 to 15 min. The exposure time of ATP was 60 s for each application. In some experiments, either ATP (10 or 100 μM) or neocuproine (5 or 10 μM) was applied to the bladder strips after prolonged (30-min) ATP treatment (10 or 100 μM) to examine the effects on the EFS-evoked contractions or basal tonus in preparations in which the purinergic receptors were desensitized (Driessen et al., 1993; Sahin et al., 2000).

In vitro whole bladder preparation

Rats were anaesthetized with Sevoflurane and sacrificed by cervical dislocation. We used the previous techniques for whole bladder preparation (Ng et al., 2006). The bladder was exposed by a midline abdominal incision and removed from the abdomen by cutting at the bladder neck. A 26-gauge needle was inserted at the bladder neck and tied with 5–0 silk sutures. The needle was connected to an infusion pump and pressure transducer via polyethylene tubing and a 3-way stopcock. The needle and tubing were filled with Krebs solution (113 mM NaCl, 19.8 mM $NaHCO_3$, 11.1 mM dextrose, 1.2 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $MgCl_2$, 1.7 mM $CaCl_2$). The bladder was placed between two platinum stimulating electrodes inside an organ bath filled with 37 *C Krebs solution and bubbled with 95% O_2 and 5% CO_2. Bladder pressure was recorded by data acquisition software (BIOPAC MP30 Systems, Inc.). After a 30 min equilibration period, the bladder was filled slowly with Krebs solution in 50 μl increments during intermittent electrical field stimulation (50V, 1.5 ms, 10 Hz for 15–30 s) to determine the bladder volume necessary to produce maximal bladder contractions. Field stimulation was delivered by a Grass S88 stimulator (Grass Instruments, Quincy, MA). The distended bladder was washed three times with 15 ml of fresh Krebs, equilibrated for another 30 min and then drug treatment was started. We used the 5 min intervals within the 10 min observation period to calculate the mean amplitude and frequency of the spontaneous contractions, after a drug was administered. The peak amplitude of the spontaneous contractions was normalized as a percentage of the maximal K^+ evoked contraction amplitude. The K^+ evoked contraction was induced at the end of the experiments by bath solution containing 100 mM KCl. The frequency was determined by counting the number of contractions over a 5 min interval. In some experiments carbachol (1 M) was applied to elicit the cholinergic contractions.

Cystometrogram (CMG)

Animals were anaesthetized with subcutaneous injection of urethane (1.2 g/kg) 1 h before surgery. The rats and mice were placed in the supine position and the bladder was exposed via a midline abdominal incision and was catheterized. A PE-50 catheter, the bladder end of which was heated to create a collar, was inserted through a small incision in the bladder dome, and a suture was tightened around the collar. The other end was connected via a three-way stopcock to a pump for continuous infusion of physiological saline and to a pressure transducer to record bladder pressure as our previous study (Eser et al., 2012). Physiological saline was infused at room temperature into the bladder at a constant rate of 0.04 ml/min to elicit repeated voiding responses. In all experiments, control cystometrograms were recorded for about 3 h. The values of amplitude (maximum bladder pressure during micturition) and intercontraction interval (the time between two voiding cycles) were evaluated.

Drugs

Purinergic antagonists were obtained from Tocris; carbachol, atropine, guanethidine, L-Nitroarginine, cuprizone, neocuproine were obtained from Sigma-Aldrich, St. Louis, MO.

Statistical Analysis

The spontaneous contractile activity was quantified by calculating the maximal amplitude (cm-H_2O), the

frequency (contractions per min) and the area under the curve (AUC). Amplitude and developed tension were expressed as a percentage of KCl induced contraction at the end of each experiment. AUC was presented as "cm H_2O.min.". All data were expressed as mean ± S.E.M. All of the data were evaluated with the Bonferroni corrected *t*- test that was used in the analysis of variance (ANOVA). *P* values of less than 0.05 were considered significant. Statistical analysis was performed with GraphPad Prism software (San Diego, CA, USA).

RESULTS

In vitro isolated Bladder Strips

Isolated Bladder Activities in Rat and Mouse

In this group experiments, 2 μM atropine and 2 μM guanethidine (non-adrenergic-non-cholinergic; NANC) were always present in the organ bath with Krebs solution to block adrenergic and cholinergic transmission.

The baseline spontaneous contraction activities of isolated whole bladders of the rat and the mice were recorded for 4 hours. During this time, the amplitude and frequency of spontaneous contractions did not change significantly. The average amplitudes and frequencies recorded at the end of the 1-hour incubation was 0.37 ± 0.02 mg, 8 ± 1/min in the rat; 0.11 ± 0.01 mg and the frequency was 7 ± 1/min in mice, respectively.

The effect of Suramin on Neocuproine-induced inhibition and effects of Neocuproine on isolated rat and mice bladder strips

100 μM NC significantly increased the amplitude of basal spontaneous contractions on isolated rat bladder strips, while significant inhibition on isolated mouse bladder strips (Figure 1a,b). No significant difference was observed on the frequency of spontaneous contractions in both species (Control: 8.7 ± 0.6/min, NC: 7.0 ± 0.8/min in rat; Control: 9.7 ± 0.6/min, NC: 8.0 ± 0.5/min in mouse). There was an increase in baseline tonus (% 80.3 ± 16) in rat, while an inhibition on baseline tonus (%45.5 ± 0.08) in the mouse. It was observed that excitatory and inhibitor responses could be replicated with NC administration for the second time on the same tissues in rat and mouse. In the same way, it was observed that the effects of NC was eliminated and spontaneous contractions and basal tonus reversed to baseline values after washing with fresh Krebs solution.

Administration of nonspecific purinergic receptor antagonist 100 μM Suramin after applying of the first NC did not change statistically a significant effect on the amplitude of basal spontaneous contractions and baseline tonus in both species (Figure 1d,e). However, 100 μM NC was administered to the rat bladder strips in suramin, NC-induced potentialization on spontaneous contractions and baseline tonus were reversed significantly in the rat (Figure 1d). Likewise, 100 μM NC was administered to the mice bladder strips in suramin, NC-induced decrease on spontaneous contractions and baseline tonus were reversed significantly in mouse (Figure 1e). The frequency of spontaneous contractions did not change significantly in both species.

The effect of Neocuproine on neurogenic contractions EFS-induced and on spontaneous contractions stimulated with carbachol, 4-aminopyridine, substance P and 10 mM KCl on isolated mouse bladder strips

100 μM Neocuproine administered on neurogenic contractions EFS-induced (train stimulation; 10 Hz; 50 V; 0.5 ms pulse duration) on the rat and mouse bladder strips in NANC was statistically significant increased reversibly and recurrently (Figure 2a,b).

The spontaneous contractions were facilitated by applying the cholinergic receptor agonist carbachol (0.05 μM), 4-aminopyridine (100 μM), substance P (5 μM) and 10 mM KCl on isolated rat bladder tissues. 100 μM of neocuproine resulted in a significant inhibition (% percentages of inhibition: 31.1 ± 3.08 for carbachol, 40.6 ± 6.2 for substance P, 33.8 ± 6.2 for 4-aminopyridine and 44.1 ± 4.5 for KCl) on the spontaneous contractions.

Effect of Neocuproine-Cu (1) complex, Cuprizone and L-NOARG on neocuproine-induced inhibition on isolated mouse bladder strips

NC-Cu (1) complex which was administered after first neocuproine administration, caused in a partial but statistically insignificant decrease on basal spontaneous contractions (Figure 3a). NC-Cu (1) complex did not change the frequency of spontaneous contractions and baseline tonus.

Administration of 50 μM Cu (2) chelators Cuprizone after applying of the first neocuproine did not change statistically a significant effect on the amplitude of basal spontaneous contractions, baseline tonus and frequency.

Administration of purinergic nitric oxide synthase inhibitor 100 μM L-NOARG after administrating of the first neocuproine did not change statistically a significant effect on basal spontaneous contractions and baseline tonus. Likewise, administrating L-NOARG to the mouse bladder strips did not affect neocuproine-induced the inhibition on spontaneous contractions and baseline tonus (Figure 3b).

The effect of PPADS, NF449, A-317491 and NF110 on neocuproine-induced inhibition on isolated mouse bladder strips

Administration of P_2X purinergic receptor antagonist 200 μM PPADS, P_2X_1 purinergic receptor antagonist 20 μM NF449, P_2X_2 purinergic receptor antagonist 20 μM A-317491 or P_2X_3 purinergic receptor antagonist 20 μM NF110 after administrating of the first NC did not elicit statistically a significant effect on the amplitude of basal spontaneous contractions and baseline tone (Figure 4a,b,c,d). However, applying PPADS and NF110 to the mouse bladder strips reversed significantly NC-induced the inhibition on baseline tone (Figure 4a,d), whereas administrating NF449 and A-317491 to the mouse bladder strips reversed partially but not significantly NC-induced the inhibition on baseline tone (Figure 4b,c) respectively. The frequency of spontaneous contractions did not change significantly.

The effect of MRS2179 on neocuproine-induced inhibition on isolated mouse bladder strips

Administration of P_2Y_1 purinergic receptor antagonist 20 μM MRS2179, P_2Y_2purinergic receptor antagonist 20 μM PSB1114, P_2Y_4 purinergic receptor antagonist 20 μM ATP and P_2Y_4 purinergic receptor antagonist 20 μM alpha-beta methylene ATP after administrating of the first NC did not change statistically a significant effect on the amplitude of basal spontaneous contractions and baseline tonus (Figure 5a,b,c,d). However, administered MRS2179 and ATP to the mouse bladder strips reversed significantly neocuproine-induced the inhibition on baseline tonus (Figure 5a,c), whereas administrating PSB1114 and alpha-beta methylene ATP to the mouse bladder strips reversed partially but not significantly neocuproine-induced the inhibition on baseline tonus (Figure 5b,d) respectively. The frequency of spontaneous contractions did not change significantly.

The effect of Calcium addition on neocuproine-induced inhibition on isolated mouse bladder strips

The inhibition, depending on the first application of NC on spontaneous contractions was observed. After the second NC-dependent inhibition on spontaneous contractions was stabilized and then 3 and 6 mM $CaCl_2$, administered to the mouse bladder strips at 5 min. intervals, significantly reversed the NC-induced dose-dependent inhibition on spontaneous contractions (Figure 6). Likewise, the addition of $CaCl_2$ completely reversed the inhibition on the baseline tonus.

In vitro isolated Whole Bladder

Isolated Rat and Mice Whole Bladder Activities

In this group experiments, 2 μM atropine and 2 μM guanethidine (non-adrenergic-non cholinergic; NANC) were always present in the organ bath with Krebs solution to block adrenergic and cholinergic transmission.

The basal spontaneous contraction activities of isolated whole bladders of the rats and the mice were recorded for 4 hours. During this time, the amplitude and the frequency of spontaneous contractions did not change

significantly. The average amplitudes and frequencies recorded after 1 hour incubation were 0.87 ±0.03 cmH2O, 2.8 ± 0.2 in rat; 0.72 ±0.09 cmH2O, 5.1 ± 0.3 in mouse, respectively.

The Effects of NC on Isolated Rat and Mouse Whole Bladder and The Effects of Suramin on NC-induced Inhibition of Isolated Mouse Whole Bladder Activities

100 μM NC significantly increased the amplitude of basal spontaneous contractions on isolated rat bladder activity, while caused a significant inhibition on isolated mice bladder activity (Figure 7a,b). A nonsignificant decrease was observed on basal tonus in the rat (%18.4 ± 9.4), while the non-significant increase was observed on basal tonus in mouse (%12.6 ± 5.4). There was no significant difference in the frequency of spontaneous contractions in rat and mouse. It shows that the second application of NC has a reducing and enhancing effect in mouse and rat, respectively (Figure 7a,b). In the same way, it was observed that the effects of NC were eliminated and spontaneous contractions and basal tonus returned to baseline values after washing with fresh Krebs solution. Nonspecific purinergic receptor antagonist 100 μM suramin administered alone after the first NC administration did not significantly affect basal spontaneous contractions and basal tonus. However, when 100 μM NC was administered to the tissues in the presence of suramin, suramin significantly reversed the inhibition depending on NC on spontaneous contractions (Figure 7c).

In vivo Cystometrogram (CMG)

The bladder pressures of control rats (n = 16) and mice (n = 20) under urethane anaesthesia were monitored for 4 hours. It was observed that inter contraction interval (ICI) and the bladder pressures during the basal and voiding stabilized at the end of 1 hour incubation period in the findings of rat and mouse cystometry.

NC (100-200 μM) was administered into the bladder by an infusion pump (0.04 ml/min) after bladder pressures were recorded for 2 hours in rat (n = 16) and mouse (n = 20) under urethane anaesthesia. NC significantly reduced ICI periods (Figure 8a,b,c,d). NC did not affect baseline of bladder pressures in both species. The amplitudes of contractions during voiding did not change significantly in both species. 100 μM Suramin, a non-specific purinergic receptor antagonist, reversed the reduction in neocuproine-induced ICI in a partial but statistically significant manner (Figure 8c) in rat, while 100 μM Suramin, a non-specific purinergic receptor antagonist, partial reversed the reduction in NC-induced ICI in mouse (Figure 8d).

DISCUSSION

In this study, copper(I) chelator neocuproine, carried out *in vitro* isolated bladder strips and isolated whole bladder experiments on rat and mouse species, generate an opposite effect on bladder activities, however, the opposite effect was not observed on *in vivo* cystometrogram studies. In parallel with some studies in several animal models showed that species differences may cause different mechanism of drug action (Hou, et al., 2005; De Wachter, 2011; Kawase, et al., 2009). Our pharmacological results reveal that NC caused significant potentialization on basal tone and spontaneous contractions on rat isolated bladder strips, while significant inhibition on baseline tonus and spontaneous contractions on mouse isolated bladder strips. Nevertheless, NC significantly reduced the intercontraction intervals (ICI) on *in vivo* bladder pressure measurements in both species.

In the present study, NC caused a recurrent and reversible increase on spontaneous contractions and basal tone on *in vitro* isolated rat bladder strips and whole bladder tissue. The excitatory effect was significantly inhibited by suramin, a purinergic receptor antagonist. These results are consistent with the previous studies in rat bladder and vas deferens tissues and the results have been suggested that NC-induced excitatory effects may be owing to the activation of the purinergic excitatory responses (Gocmen et al., 2004, 2005). In contrast to the response of rat bladder, NC caused recurrent and reversible inhibition on spontaneous contractions and basal tonus on *in vitro* isolated mouse bladder strips and whole bladder tissue. Cu(II) chelator, Cuprizone did not significantly affect the NC-induced inhibition. The inhibitor effect did not occur in the presence of pre-prepared NC-Cu(I) complex. The results indicate that a copper (I)-sensitive mechanism may play a role in the inhibition effect of NC on purinergic responses of mouse bladder tissue, and previous studies of Gocmen et al., 2004 and 2005 support that the copper(1)-sensitive mechanism may

play a role by facilitating purinergic excitatory response (Gocmen et al., 2004, 2005). In addition, it has been suggested that NO releases with S-nitrosothiols, a sulfhydryl-containing compound, and NC affect the responses of S-nitrosothiols by its ability to chelate copper in the bladder (De Man et al., 1999), however, it was exhibited that it is different from L-NOARG mechanism, because of that NOS inhibitor, L-NOARG did not change the effect of NC responses (Gocmen et al., 2004). In parallel with these studies, in the present study, the ineffectiveness of the nitric oxide synthase inhibitor, L-NOARG on NC-induced inhibition demonstrates that the L-arginine-NO pathway has any role in this inhibitor activity.

The nonspecific P2X purinergic receptor antagonist, suramin significantly reversed the NC-induced inhibitory effect on isolated mouse bladder strips and whole bladder tissue. These findings suggest that the NC-dependent inhibition is associated with the purinergic mechanism as reported in another study in the guinea pig urinary bladder (Hoyle et al., 1990). On the other hand, NC-induced inhibitory effect partially prevented by P_2X receptor blocker PPADS, P_2X_1 purinergic receptor antagonist NF449, P_2X_2 purinergic receptor antagonist A-317491 and P_2X_3 purinergic receptor antagonist NF110. This preventive effect indicates that P_2X subtype receptors are also involved in the NC mechanism. In the absence of P2X3 receptors in mouse knockouts, the bladder is hyperactive (Cockayne et al., 2000; Vlaskovska et al., 2001). The more recently developed P2X3 and P2X2/3 antagonist AF-219, the aryloxy-pyrimidinediamine, which is orally bioavailable and metabolically stable, is being explored as a therapeutic agent for urinary tract dysfunction. One of the main prediction to initiate this project is the argument that P_2Y receptor subtypes may play a major role in the inhibitory mechanism of neocuproine. In contrast with P2X purinoceptors, reversing the inhibitor effects, partly but significantly, also specific P_2Y purinergic receptor antagonists such as P_2Y_1 purinergic receptor antagonist MRS2179 and P_2Y_2purinergic receptor antagonist PSB1114 indicates that these receptor subtypes were also involved in the NC-induced inhibitory mechanism. It is known that P_2Y receptors have inhibitory mechanisms (Burnstock, 2016, 2017). In our study, selective P_2Y receptor antagonists reversed the inhibitor activity, significantly but not completely, corroborate the argument in that involved in NC-induced inhibitory mechanism. The presence of purinergic receptors, mediated relaxation such as P_2Y has been demonstrated on the bladder of many species (McMurray et al., 1998; Bolego et al., 1995; Boland et al., 1993). However, selective P_2X receptor antagonists also reversed the inhibitor activity, significantly but not completely, making it difficult to fully understand the inhibitory effect of NC mechanism.

NC-induced inhibition on spontaneous contractions were dose-dependent reversed significantly by administrating of 3 and 6 mM CaCl2 to the mouse bladder strips at 5 minutes intervals. This finding shows that extracellular calcium plays a crucial role in NC-dependent inhibitory mechanism. In a previous study on rat bladder, the prevention the increase on NC-induced baseline tonus by calcium channel blockers, or the absence of this increment in calcium-free medium supports the argument that calcium may also play a role in this inhibitory mechanism on mice (Gocmen et al., 2004).

As well as purinergic mechanism, peptidergic transmission involving substance P or other neurokinins are thought to be involved in NANC transmission in the bladder and promote to the constant component of the EFS-induced contraction (Benko et al., 2003). In parallel, in the present study, it was observed that inhibitor activity of NC on basal tone and spontaneous contraction activity of mouse bladder induced 10 mM KCl, cholinergic receptor agonist CCh, K+ channel blocker 4-AP, an inflammatory mediator neurokinin receptor agonist substance P. The results demonstrate that the NC-dependent inhibitor effect is not only related to the basal activity, peptidergic transmission were also affected in the mouse bladder.

On the other hand, EFS-induced neurogenic contractions on rat and mouse bladder strips were excitated with NC in both species. This finding may indicate that the intramural nerves do not have any role in the NC-induced inhibitor mechanism. In addition, the excitation of neurogenic contractions in both species supports to the aspect that the species difference on NC-induced effects on baseline spontaneous contractions and baseline tonus is myogenic activity.

In our study, intravesical administration of NC significant decrease the ICI in both rat and mouse of *in vivo* bladder pressures. In the study of Gocmen et al., 2004 showed that the NC-dependent inhibition on ICI response in rat (Gocmen et al., 2004). 100 μM Suramin, a non-specific purinergic receptor antagonist,

reversed the reduction in neocuproine-induced significantly ICI in rat, while 100 μM Suramin partial reversed the reduction in NC-induced ICI in Mouse. These results indicate that the neurogenic mechanism does not play a role in the species difference of the NC-dependent mechanism.

In conclusion, It is possible that myogenic mechanisms play a role in the differential effects of NC on rat and mouse bladder activity. It is possible that the mechanisms associated with decreased intracellular calcium in the inhibitor activity of NC in mouse bladder may be triggered. In the effect of NC-induced inhibition may also have an important role in the purinergic pathway. However, the effect of NC on mouse bladder activity appears to be partly attributed to P2X receptors as well as P2Y receptors, known to have an inhibitor activity.

AUTHOR CONTRIBUTIONS

N.E., and C.G. performed the research, designed the experiments and wrote up the manuscript. N.E., H.S.B., E.K., and C.G. helped out in*in vivo* and *in vitro* experiments and data analysis. The manuscript has been reviewed and approved by all authors.

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FIGURE LEGENDS

FIGURE 1 The effects of neocuproine on isolated (a) rat and (b) mouse bladder strips. The effects of 100 μM neocuproine (NC) on spontaneous contraction amplitudes of isolated rat (b) and Mouse (c) bladder strips. The effect of Suramin (SUR; 100 μM) on 100 μM NC-induced inhibition on spontaneous contractions of isolated rat (d) and mouse (e) bladder tissue (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 2 The effects of 100 μM NC on neurogenic contractions EFS-induced on isolated (a) rat and (b) mouse bladder tissues. (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 3 The effect of (a) neocuproine-copper (1) complex [NC-Cu (1)] and (b) 100 μM L-NOARG on 100 μM NC-induced inhibition on spontaneous contractions of isolated mouse bladder tissue (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 4 The effect of (a) 200 μM PPADS, (b) 20 μM NF449, (c) 20 μM A-317491 and (d) 20 μM NF110 on 100 μM NC induced inhibition on spontaneous contractions of isolated mouse bladder tissue (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 5 The effect of (a) 20 μM MRS2179, (b) 20 μM PSB1114, (c) 20 μM ATP and (d) 20 μM alpha-beta methylene ATP on 100 μM NC induced inhibition on spontaneous contractions of isolated mouse bladder tissue (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 6 The effect of 3 and 6 mM $CaCl_2$addition on 100 μM NC-induced inhibition on spontaneous contractions of isolated mouse bladder tissues. (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 7 The effects of 100 μM neocuproine (NC) on spontaneous contraction amplitudes of (a) rat and (b) mouse whole bladder tissues. (c) shows that the effect of Suramin (SUR; 100 μM) on 100 μM NC-dependent inhibition on spontaneous contractions of isolated mouse whole bladder tissue (*) indicates statistical significance compared to control ($p<0.05$).

FIGURE 8 . The trace of control and 200 μM NC-administration on the activity of (a) rat and (b) mouse bladder pressure under the urethane anaesthesia. (A) Control and (B) NC administration. The effect of 200 μM neocuproine (NC) and 100 μM Suramin on the activity of intercontraction intervals (ICI) in (c) rat and (d) mouse under the urethane anaesthesia. (*) indicates statistical significance compared to control ($p<0.05$).

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