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Altered ureteral contractility with ageing: Role of the rho-kinase pathway

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ABSTRACT

This study investigated the role of calcium sensitisation in the regulation of ureteral contractility with ageing. Isolated ureteral strips from young (6-month old) and older (3-year old) pigs were mounted in Krebs bicarbonate solution and contractility induced by the α1-adrenoceptor agonist phenylephrine (30 and 300 μM) and 5-HT (10 and 100 μM), recorded in the absence and presence of the rho-kinase inhibitors Y-27632 (10 μM) and fasudil (30 μM). Ureteral strips developed bursts of contractile activity which was measured as area under the curve (AUC) and frequency. Maximum contraction to phenylephrine was significantly enhanced in tissues from older animals compared to younger animals ($p < 0.001$) while maximum contraction to 5-HT was greater in tissues from younger animals ($p < 0.001$). Both inhibitors significantly depressed AUC and frequency responses to both agonists in ureters from both age groups ($p < 0.05$). Inhibition by Y-27632 of phenylephrine (300 μM)- and 5-HT(100 μM)-induced contractions was greater in tissues from older animals than young ($p < 0.05$). Rho-kinase activity was also assayed in ureteral tissues, and basal activity was similar in ureters from both age groups. Neither phenylephrine nor 5-HT increased rho-kinase activity over basal levels. These data demonstrate the significant role rho-kinase plays in ureteral contractility and possible alterations with age.

1. Introduction

The incidence of urolithiasis, the formation of kidney stones, is high in western countries, affecting 1 in 10 individuals (Macneil and Bariol, 2011) and its prevalence is expected to escalate significantly in the coming decades (Soucie et al., 1994; Lee et al., 2002). While the pathophysiology of urolithiasis has not been clearly elucidated, this condition is frequently accompanied with ureteral colic, which is understood to be caused by constriction of the ureteric tube. The most common location for lodgement of kidney stones is the distal ureter approaching the ureterovesical junction where the ureter enters the bladder (El-Barky et al., 2014). Although normal ureteral contractility is dependent on peristaltic waves originating from pacemaker cells, it also relies on effective contraction and relaxation of the smooth muscle cells which constitute the bulk of the ureteral wall (Lang and Klemm, 2005). While it is well established that calcium is the intracellular trigger for smooth muscle contraction, recent research has demonstrated that smooth muscle contraction can occur even in the absence of large changes in calcium, a process currently defined as 'calcium sensitization' (Zhang and DiSanto, 2011; de Godoy and Rattan, 2011). A major enzyme involved in this pathway is rho-kinase, which is activated *via* G-protein coupled receptors by the monomeric GTP-binding protein RhoA (Christ and Andersson, 2007).

A limited number of studies have focused on the importance of rho-kinase in the ureter. In the rat ureter, it has been shown that rho-kinase inhibitors (Y-27632, fasudil and H-1152) significantly decrease phasic contractions (Shabir et al., 2004). This indicates that rho-kinase inhibition can modulate phasic contractions in the absence of calcium changes, suggesting a role for rho-kinase in calcium sensitization in ureteral smooth muscle. However, this was not evident in the guinea-pig ureter, indicating there are species differences (Shabir et al., 2004). In addition, another study on the sheep ureter demonstrated the presence of both isoforms of rho-kinase (rho-kinase-I and rho-kinase-II) and its mediation of agonist and electrical field stimulated (EFS) contractions, as well as spontaneous contractile activity (Levent and Buyukafsar, 2004). Immunohistochemistry and immunoblotting studies have demonstrated the presence of both rho-kinase isoforms in the human ureter, where spontaneous and EFS-induced contractile responses were depressed by the rho-kinase inhibitor Y-27632 (Hong et al., 2005).

It has been proposed that rho-kinase-mediated contractile mechanisms could be extensively altered in response to pathological insults and with ageing. Unilateral ureteral obstruction, as seen with ureteral stones, has been shown to enhance expression of rho-kinase-I and rho-kinase-II in the rabbit ureter and contractility was also enhanced in ureteral strips from rabbits with ureteral obstruction (Turna et al., 2007). Furthermore, rho-kinase inhibition with Y-27632 significantly suppressed contractility in both unilaterally obstructed and control

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ureters and reduced contractions in the obstructed ureters back to control levels (Turna et al., 2007).

Whilst no studies have investigated the effect of age on the rho-kinase pathway in the ureter, the calcium sensitization pathway has been shown to be altered with age in some tissues within the urinary tract. In the guinea pig bladder, contractions of detrusor smooth muscle strips were sensitive to blockade with Y-27632, although this inhibitor had a negligible effect on tissue strips from older animals, suggesting the possibility of alterations in rho-kinase expression and/or activity with age (Gomez-Pinilla et al., 2008). Furthermore, in the human bladder, there was a strong correlation between the amount of inhibition of contractility by Y-27632 with age, suggesting that rho-kinase-mediated contractions are age-dependent (Kirschstein et al., 2014).

The aim of this study was to investigate the effects of the rho-kinase inhibitors, Y-27632 and fasudil, on contractility of isolated distal ureter from young and older pigs, a species with similar urinary tract pharmacology and physiology to human.

2. Methods and materials

2.1. Tissue preparation

Fresh bladders, with ureters attached, from 6-month old (young) and 3-year old (older) female pigs were obtained from a local abattoir and immediately immersed in ice-cold Krebs-bicarbonate solution (4 °C) composed of NaCl (188.4 mM), $NaHCO_3$ (24.9 mM), glucose (11.7 mM), $CaCl_2$ (1.9 mM), $MgSO_4$ (1.2 mM) and KH_2PO_4 (1.2 mM). The ureters were detached from the bladders and the peri-ureteric fat was removed. The distal ureter was determined as being the region 5 cm from the entrance to the bladder, and this section was used to dissect 4 mm long tissue strips.

Tissue strips were mounted longitudinally under 1 g tension in 8 ml organ baths (EZ-baths, Global Towns, CA) containing Krebs-bicarbonate solution, maintained at 37 °C and continuously gassed with 95% O_2 and 5% CO_2. Isometric tension developed by the tissues was recorded *via* a Powerlab recording system and Labchart software (ADInstruments, Castle Hill, Australia).

2.2. The effect of rho-kinase inhibition on contractility of ureters

Ureteral tissue strips were isolated and cut in half: one strip acting as a control while the other was incubated for 30 min with one of the rho-kinase inhibitors, Y-27632 (10 μM) or fasudil (30 μM), before addition of an EC_{50} concentration (termed "low dose") of the α1-adrenoceptor agonist phenylephrine (30 μM) or 5-HT (10 μM). The responses to the agonists were recorded, and then after washout and return to baseline, a maximally effective concentration (termed "high dose") of the same agonist (phenylephrine 300 μM or 5-HT 100 μM) was added. The rho-kinase inhibitor was present throughout the experiment. The agonist concentrations were determined based on preliminary concentration-response curves (Fig. 1). Each ureteral tissue strip was exposed to only one inhibitor and one agonist. The contractile responses were measured as area under the curve (g s) and frequency (Hz).

2.3. Measurement of rho-kinase activity in ureteral smooth muscle

A rho-kinase activity assay (Cell BioLabs, Inc) was performed as per manufacturer's instructions on isolated distal ureteral tissue strips freshly homogenised with a RIPA lysis buffer system (Santa Cruz Biotechnology, Inc). This enzyme immunoassay detects the specific phosphorylation of MYPT1 at Thr^{696} by rho-kinase. The assays were performed in duplicate on tissue lysates of isolated ureteral strips incubated in the absence and presence of low and high concentrations of phenylephrine and 5-HT, as used in the functional contraction experiments, and in ureters from both age groups.

2.4. Materials

All components of Krebs bicarbonate solution were purchased from Sigma-Aldrich, New South Wales, Australia. The following drugs were used: 5-hydroxytryptamine hydrochloride (Abcam, Cambridge, U.S.A.), (R)-(-)-phenylephrine hydrochloride (Sigma-Aldrich, New South Wales, Australia), Y-27632 dihydrochloride (Tocris, Victoria, Australia) and fasudil hydrochloride (Tocris, Victoria, Australia). All drugs were dissolved in distilled water and dilutions performed using Krebs bicarbonate solution.

2.5. Data analysis

Paired Student's *t*-tests were used to compare data from two groups, while two-way ANOVA followed by Dunnett's post hoc test was used to compare data from more than two groups.

3. Results

Porcine ureteral strips were allowed to equilibrate to a passive tension of 1.19 ± 0.06 g (n = 96). Spontaneous contractions developed during the equilibration period in 16 of 96 ureteral strips (11.7%). When subjected to the agonists phenylephrine and 5-HT, all ureteral strips, from both age groups, demonstrated triggered bursts of phasic contractions, the frequency of which was concentration-dependent, with greater rates of phasic contractions at the higher concentrations of agonist (Fig. 1).

3.1. Effect of age on phenylephrine and 5-HT-induced contractility of ureters

The potency (pEC50) of both agonists in producing contraction of ureteral strips was similar for tissues from both age groups (young *vs* older: 4.42 ± 0.18 *vs* 4.83 ± 1.43 for phenylephrine, 5.16 ± 0.09 *vs* 5.43 ± 0.16 for 5-HT). However, the maximum contractile responses expressed as AUC, to phenylephrine were significantly enhanced in ureteral strips from the older animals (Fig. 1A, unpaired *t*-test, $p < 0.001$). Phenylephrine-induced frequency of contractions was also greater in ureteral strips from older animals (Fig. 1C, unpaired *t*-test, $p < 0.05$). In contrast, 5-HT maximum contractile responses (AUC) were depressed in tissues from older animals (Fig. 1B, unpaired *t*-test, $p < 0.001$), whilst the maximum frequency response to 5-HT was similar in tissue strips from both age groups (Fig. 1D).

3.2. Effect of the rho kinase inhibitor Y-27632 on phenylephrine-induced contractile responses

Incubation of ureteral strips with the rho-kinase inhibitor Y-27632 (10 μM) significantly depressed phenylephrine-induced contractions in tissues from both young and older animals, when responses were expressed as AUC (Fig. 2A and C, paired *t*-test, $p < 0.005$). Y-27632 produced a greater inhibition of contractions induced by the high dose of phenylephrine in tissues from older animals compared to those from young animals (Table 1, unpaired *t*-test, $p < 0.05$), but this was not observed when tissues were stimulated with the low dose of phenylephrine.

In ureteral strips from both age-groups, the frequency of contractions induced by the high dose phenylephrine was significantly inhibited by Y-27632, but not those induced by low dose of phenylephrine (Fig. 2A and C, paired *t*-test, $p < 0.005$). Similar to AUC data, the frequency of phenylephrine-induced contractions was also inhibited by Y-27632 to a greater extent in tissues from older animals, at the high dose phenylephrine (Table 1, unpaired *t*-test, $p < 0.05$).