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A porcine model of ureteral contractile activity: influences of age, tissue orientation, region, urothelium, COX and NO

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Abstract

Introduction: We aimed to investigate factors contributing to ureteral responses and establish a reliable porcine model for studying ureteral contractility.

Methods: Isolated ureteral strips from young (6-month old) and older (3-year old) pigs were mounted in organ baths and subjected to phenylephrine, 5-HT, carbachol and histamine. Ureteral strips developed bursts of contractile activity which was measured as area under the curve (AUC) and frequency. Phenylephrine and 5-HT-induced responses of proximal and distal ureters were obtained, in the presence and absence of indomethacin (10μM) and L-NNA (100μM), and the influence of an intact mucosa was examined.

Results: Phenylephrine and 5-HT-induced contractile responses were greater than those to carbachol in the porcine ureter. In fact, responses to carbachol were only present in ureters from older animals. Ureters suspended longitudinally had increased phenylephrine-induced contractions compared to those suspended circularly ($p<0.05$). A greater amount of tissue strips developed spontaneous contractions from the proximal region compared to distal (83% vs 25%). There was an increase in maximum phenylephrine-induced responses in the distal ureter when compared to the proximal ureter ($p<0.05$). In the presence of indomethacin, only 5-HT-induced contractions in the young animals were depressed ($p<0.05$) while L-NNA did not affect any ureteral responses. The intact mucosa significantly decreased contractile responses to phenylephrine and 5-HT in the porcine ureter.

Discussion: The complexity of ureteral contractions depicting bursts of phasic activity requires AUC assessment. Porcine ureteral contractile properties, such as regional differences, influence of mucosa and lack of response to carbachol, are similar to those reported in the literature for human ureter.

Keywords:

Age; distal; isolated tissue; methods; nitric oxide; pharmacology; porcine; proximal; ureter; urothelium

Abbreviations:

5-HT (5-hydroxytryptamine), AUC (area under the curve), L-NNA (Nω-Nitro-L-arginine)

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1. Introduction

The incidence of urolithiasis, the formation of kidney stones, affects 1 in 10 individuals in western countries (Macneil & Bariol, 2011). While the pathophysiology of urolithiasis has not been clearly elucidated, this condition is frequently accompanied with ureteral colic due to constriction of the ureteral 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, Ali, Sahsah, Terra, & Kehinde, 2014).

Spontaneous ureteral contractility originates at the renal pelvis (Hammad, 2015). The most likely candidate for the renal pacemaker cells of the pyeloureteral complex are 'atypical' smooth muscle cells, while interstitial cells of Cajal-like (ICC-like) cells provide a compensatory pacemaker mechanism to maintain peristaltic waves in 'typical' smooth muscle cells (Lang & Hashitani, 2019). While normal ureteral contractility is dependent on peristaltic waves originating from 'atypical' smooth muscle cells, it also relies on effective contraction and relaxation of the 'typical' smooth muscle cells (in this article, referred to as smooth muscle cells) which constitute the bulk of the ureteral wall (Lang & Klemm, 2005). The mammalian ureter consists of two main cell layers: a multilayered water-tight transitional epithelium called the urothelium, and the smooth muscle cells (Woolf & Davies, 2013). There are two distinct layers of smooth muscle cells: an inner longitudinal layer and an outer circular layer, surrounded by fibrous tissues. The longitudinal smooth muscle is responsible for ureteral shortening and plays a role in movement of urine, while circular smooth muscle that coats the ureteral walls contracts to generate pressure (Vargiu, Perinu, Tintrup, Broccia, & Lisa, 2015).

While it is known that the myogenic properties of the pyeloureteral complex is sufficient for normal ureteral peristalsis, stimulation of sensory nerves by neurotransmitters released in the presence of stones or infections, can excite latent pacemakers present in the smooth muscle. The distal regions of the ureter have also been shown to be dependent on neurogenic mechanisms for the modulation of contractility (Santicioli & Maggi, 1998). The ureters have both efferent and afferent innervation including adrenergic, cholinergic and non-adrenergic non-cholinergic components, with the distal ureter receiving a greater density of innervation compared to the proximal regions (Edyvane, Trussell, Jonavicius, Henwood, & Marshall, 1992). The main motor innervation to the ureter is adrenergic and noradrenaline causes contraction of the human isolated ureter (Hernandez et al., 1992). Although the mRNA expression of $\alpha 1_D$-adrenoceptors is observed to be the greatest of the subtypes, a functional study performed on isolated human ureter suggested that the $\alpha 1_A$-adrenoceptor is the subtype that plays the major functional role in mediating contractions (Sasaki et al., 2011). This is

supported by the efficacy of the most commonly used drug in medical expulsive therapy, tamsulosin, which has a high affinity for α_{1A}-adrenoceptors (Noble et al., 1997), to increase ureteral stone expulsion rate (Agrawal et al., 2009; Autorino et al., 2005; Dellabella, Milanese, & Muzzonigro, 2003; Yilmaz et al., 2005). Other neurotransmitters and mediators including acetylcholine, 5-hydroxytryptamine (5-HT), histamine, prostaglandins and nitric oxide have been shown to exert various responses in the isolated ureter from different species (Canda, Turna, Cinar, & Nazli, 2007). Nevertheless, the adrenergic system is widely accepted to play the dominant role of controlling ureteral motility and therefore, there is a greater focus on this receptor mechanism within the literature in comparison to others.

It is important to have a greater understanding of the pharmacological mechanisms controlling ureteral contraction, as this may reveal novel mechanisms and identify targets for development of more effective pharmacological agents for kidney stone expulsion and ureteral colic relief. The technique of *in vitro* isolated tissues is a useful and important method to investigate the physiology and pharmacology of animal and human tissues, including the ureter. Previous *in vitro* functional studies on the isolated ureter have utilised different methods of preparation where different regions, tissue orientations and age groups are utilised, preventing effective data comparison.

A previous study reported that different orientations of pig ureteral tissues, including open longitudinal (similar to the longitudinal preparation in the present study), closed longitudinal, closed ring and spiral cut segments all produced contractile responses that were similar in spontaneous contraction rates and maximum amplitude, in response to electrical field stimulation (EFS) and muscarinic stimulation with carbachol (Jerde, Saban, & Nakada, 1999). However, no studies have investigated the open circular segment.

Our previous findings suggest that contractile responses in ureters isolated from different ages vary with agonists, with ureteral contractions to α_1-adrenoceptor stimulation being greater in older animals, whilst contractions to 5-HT were smaller in younger animals (Lim, Chess-Williams, & Sellers, 2018a). However, the influence of age on responses mediated by carbachol and histamine, which have both been suggested to mediate ureteral contraction (Dodel, Hafner, & Borchard, 1996; Yalcin et al., 2013), have not been studied.

As it is difficult to obtain viable human ureteral tissues, porcine tissue can be used as a model reflecting mechanisms controlling ureteral contractility in humans. The difficulties that arise particularly, with the ureter is quantifying the complex phasic and tonic contractile responses with activity, which is also influenced by the mucosa. The aim of this study was to investigate

ureteral contractile response characteristics and to establish a reliable porcine ureteral model to promote effective investigation of isolated ureteral responses with optimised conditions.

2. Methods

2.1 Tissue specimen origin and preparation

Female pig urinary 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 Kreb's 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) and transported to the laboratory. The ureters were divided into proximal and distal segments, where proximal ureter was determined as the first 4cm of tube leaving the kidneys, and distal ureter as the 4cm just before entering the bladder. These were dissected into 4mm long tissue strip sections.

For consistency, we only examined strips from the distal ureter throughout the study, other than when comparing responses from distal and proximal ureter (Section 3.3). The mucosa was left intact with the smooth muscle strips in all experiments, except when investigating the modulatory effect of mucosa, where in denuded strips the urothelium together with the major part of the lamina propria was carefully removed with fine scissors (Section 3.5).

Tissue strips were mounted under approximately 1 g tension in 8 ml EZ-Bath organ baths (Global Towns Microtechnology, Sarasota, FL, USA) containing Krebs-bicarbonate solution, maintained at 37ºC and continuously gassed with 95% O_2 and 5% CO_2. All tissue strips were equilibrated for 1 hour, before addition of any drug. The tension developed by the tissues was recorded via a Powerlab recording system and Labchart software (ADInstruments, Castle Hill, NSW, Australia).

2.2 Data analysis

During the equilibration period, some tissue strips developed spontaneous contractions while others remained quiescent in the absence of agonists. In response to agonists, isolated ureteral strips developed bursts of phasic contractile activity (Fig 1). The amplitude of these phasic contractions was independent of agonist concentration, and there was no consistent pattern of increase or decrease of amplitude at various concentrations of agonists. Therefore, contractile responses of these tissues were expressed as area under the curve (AUC), by weight in grams seconds (gs g^{-1}) to account for both frequency and amplitude of the contractions and as frequency in Hertz (Hz), the number of phasic contractions in a second.

Contractile responses expressed as AUC (gs g^{-1}) were normalised to individual tissue weights. Where responses are expressed as percentage, maximal response for each tissue strip was obtained by challenging tissues with high concentration KCl (80mM) at the end of the experiment.

GraphPad Prism software (GraphPad, San Diego, CA, USA) was used to perform statistical analysis and graphical representation. Following calculation of area under the curve (g^{-1} s) and frequency (Hz) for all contractile responses, paired or unpaired Student's *t*-tests were performed to identify statistically significant differences, where $p<0.05$ was considered statistically significant. All data were expressed as mean ± SEM of *n* experiments, where *n* is the number of animals. Non-linear regression analysis of the concentration-response curves was used to determine the potency of the agonists (pEC_{50}, the log of the concentration of a drug that produces half of the maximal response). Mean pEC_{50} values were calculated and compared using paired or unpaired Student's *t*-tests as appropriate.

2.3 Drugs and chemicals used

Chemicals were of analytical grade and purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). (R)-(-)-phenylephrine hydrochloride, carbamoylcholine chloride (carbachol), histamine dihydrochloride, indomethacin and Nω-Nitro-L-arginine (L-NNA) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) and 5-hydroxytryptamine hydrochloride (5-HT) was obtained from Abcam (Melbourne, VIC, Australia).

3. Results

All porcine ureteral strips were allowed to equilibrate to a passive tension of 1.05g ± 0.04g (n = 312). Spontaneous contractions in the absence of any pharmacological stimulus developed in 89 of 264 distal ureteral strips (34%), with no specific differences between any of the groups, except when comparing proximal vs distal ureter which is further discussed in Section 3.3. When quiescent tissues were subjected to increasing concentrations of agonist, they developed bursts of phasic contractions (Fig 1). Increasing concentrations of agonist induced increases in frequency of phasic activity in all porcine ureteral strips. However, the amplitude of phasic activity was not concentration-dependent. AUC was used as a measurement accounting for amplitude and frequency, to produce consistent and reproducible results in expression of concentration-related responses.

3.1 Influence of orientation on ureteral responses

The tissue strip orientations compared in the current study were open longitudinal and open circular preparations. Adjacent tissue strips from the same ureter were dissected and prepared as shown in Figs 2A and 2B. Considering the well-established role of the adrenergic system in ureteral contraction, cumulative concentration-response curves to α_1-adrenoceptor agonist phenylephrine were obtained in these tissues (Figs 2C and 2D).

The potency (pEC_{50}) of phenylephrine in the longitudinal preparations was significantly greater than in the circular preparations (for longitudinal, 5.08 ± 0.27 vs for circular, 4.54 ± 0.22, $p<0.05$, n=6). The maximum contractile responses expressed as both AUC and frequency were significantly greater in the longitudinal strips than circular strips ($p<0.05$, n=6, Figs 2C and 2D). Subsequently, all experiments within this study was performed using the longitudinal preparation.

3.2 Influence of age and agonist on ureteral responses

In the current study, we compared the contractile responses in tissues strips from young animals (6-month old) and older animals (3-year old) to two other mediators, carbachol and histamine, which have both been suggested to mediate ureteral contraction (Dodel et al., 1996; Yalcin et al., 2013). In the presence of increasing concentrations of carbachol, only isolated ureteral strips from the older age group produced contractile responses (Figs 3E and 3F). These carbachol-induced contractions were of a similar pattern to phenylephrine and 5-HT response as shown in the raw data trace in Fig 1. Increasing agonist concentrations also resulted in an increase of frequency in phasic activity. No ureteral strips responded to stimulation by histamine at concentrations up to 100mM (data not shown). Due to minimal contractile responses to carbachol and histamine, all subsequent studies were performed with phenylephrine and 5-HT in tissues from both age groups.

3.3 Influence of tissue region on responses

From the same ureter, the proximal region close to the kidneys and the distal region close to the bladder were isolated and separated. Contractile responses of these ureteral strips to phenylephrine and 5-HT were measured and compared. It was important to measure spontaneous contractions of ureter from different regions since it was expected that there would be more spontaneous contractions in the proximal regions, closer to the kidney. Spontaneous contractions developed during equilibration time (in the absence of any agonists) in 20 of 24 proximal ureteral strips (83%) and 6 of 24 distal ureteral strips (25%). The pEC_{50} of agonists were similar for tissues from the two different regions of the ureter, in both young and older animals (Table 1).

In the older animals, maximum contractile responses to phenylephrine expressed as AUC were significantly greater in the distal region of the isolated ureter compared to the proximal region ($p<0.05$, n=6, Fig 4A) while maximum frequency was similar in both regions (Fig 4B). Although maximum AUC responses to phenylephrine were similar in tissues from the two regions in the young group (Fig 4C), maximum frequency was greater in the proximal strips compared to distal ($p<0.005$, n=6, Fig 4D). Maximum contractile responses to 5-HT expressed as AUC were smaller in the proximal region than in the distal region in both age groups ($p<0.001$, n=6), while maximum frequency of 5-HT-mediated phasic contractions was not different in the regions of the ureter (data not shown).

3.4 Influence of nitric oxide (NO) and prostaglandins on ureteral responses

Contractile responses to phenylephrine and 5-HT were compared in the presence and absence of the cyclo-oxygenase inhibitor indomethacin (10μM) and nitric oxide synthase inhibitor L-NNA (100μM). These were administered to the tissues in separate experiments and incubated with the tissue for 30 minutes before obtaining responses to agonists.

Indomethacin (10μM) had no significant effect on the pEC_{50} of phenylephrine or 5-HT in the isolated ureteral strips from either age group (Table 1). Maximum AUC and frequency responses to phenylephrine were similar in the presence and absence of indomethacin (10μM) in ureteral tissues from both age groups (data not shown). Maximum contractile activity to 5-HT stimulation expressed as AUC was decreased in the presence of indomethacin ($p<0.05$, n=6, Fig 5C) in tissues from the younger animals but not tissues from the older animals (Fig 5A). The maximum frequency of phasic contractions in response to 5-HT was not affected by indomethacin in any age group (Figs 5B and 5D).

The pEC_{50} values of the agonists were not altered in the presence of L-NNA (100μM) in the ureters from either age groups (Table 1). Additionally, maximum AUC and frequency responses to phenylephrine and 5-HT were also not affected in the presence of L-NNA (100μM) in both age groups (data not shown).

3.5 Influence of the mucosa on ureteral responses

Phenylephrine and 5-HT-induced contractions were compared in intact and mucosa-denuded ureteral strips. The pEC_{50} for either agonist was not significantly different in the presence or absence of mucosa (Table 1). In ureters from both age groups, maximum AUC and frequency responses to phenylephrine were significantly greater in denuded tissue strips compared to tissues with an intact mucosa ($p<0.05$, n=6, Fig 6). Similar results were also obtained for 5-HT-induced contractile responses ($p<0.05$, n=6, data not shown).

4. Discussion

The present study sought to optimise *in vitro* methodology for studying porcine isolated ureteral contractile responses. In order to achieve this, we investigated various factors that could affect contractile responses of the isolated ureter including tissue strip orientation, age of the animal, different agonists, regions of the ureter and the presence of an intact mucosa.

In a study investigating different methods of isolated ureteral suspension, Jerde and colleagues (1999) suggested that the contractile responses of the swine ureter are similar, regardless of tissue strip orientation. Subsequently, most studies on the isolated ureter have utilised the open longitudinal preparation (Fig 2B), as other suspension methods utilise a greater amount of tissue and are not ideal for studies involving limited availability of human tissues. However, Jerde and colleagues (1999) did not evaluate the open circular preparation (Fig 2A) that was investigated in the present study. In our comparison, the longitudinal preparations elicited significantly greater contractile responses when compared to the circular arrangement. In the light of these results and considering most studies on isolated ureter in the literature, longitudinal preparations would appear to be the optimal tissue orientation for studies and was therefore used throughout the rest of this present study.

While age-related changes in the biomechanical properties and composition of the human ureter have been reported (Sokolis, Petsepe, Papadodima, & Kourkoulis, 2017), this is the first study to investigate alterations with age in the pharmacological response of the ureter. We have previously reported that there are differences in contractions of the ureter in response to both α_1-adrenoceptor and 5-HT receptor stimulation (Lim, Chess-Williams, & Sellers, 2018b). The present results show that ureteral contraction in response to muscarinic receptor stimulation also differs in tissues from the two age groups. Ureters from the young animals did not respond to carbachol, and this is comparable to results reported for the human ureter (Roedel et al., 2018). In contrast, tissue strips from the older animals generated phasic contractile activity in a concentration-dependent manner to muscarinic receptor stimulation. A previous study demonstrated that there is an increase in cholinergic-induced contractile activity in the obstructed rabbit ureter (Yalcin et al., 2013). This suggests that the muscarinic contractile activity potentially develops under pathological conditions and ageing, and that the isolated ureters from older pigs could represent diseased conditions. It is evident that the adrenergic and 5-HT receptor systems play significantly greater roles in the ureter, as their stimulation resulted in far greater responses than muscarinic receptor stimulation. Therefore, all subsequent experiments were performed using phenylephrine and 5-HT as agonists. It was expected that α_1-adrenoceptor would be significantly involved in ureteral contractions, since α_1-adrenoceptor antagonists are currently used clinically to improve passage of kidney stones.

Interestingly, our findings suggest that the 5-HT receptor mechanism could also be considered as a target for treatment for the same purpose and warrants further investigations.

Our results also suggest that there are differences in contractile responses between the two regions of the ureter: proximal and distal. We confirmed that the isolated porcine distal ureter contractions to phenylephrine are significantly greater than those of the proximal ureter. Importantly, this is similar to previous findings with the human ureter (Sasaki et al., 2011). This regional difference is likely due to a greater α_1-adrenoceptor density in the distal ureter as was suggested in a receptor-binding study on the human ureter (Sigala et al., 2005). To our knowledge, our present study is the first to compare 5-HT-mediated responses in different regions of the ureter and we show that independent of age, these responses were significantly enhanced in the distal ureter. Further molecular studies are required to determine whether this is due to an increased density of 5-HT receptors in the distal region. Our findings of increased contractile response in the distal ureter support, and may explain, the clinical observation of an increased incidence of kidney stone lodgement within the distal ureter compared to other parts of the ureter (El-Barky et al., 2014). It was observed that the number of tissues strips that developed spontaneous contractions in the absence of agonist was greater in the proximal region compared to the distal. This may be explained by a greater number of pacemaker cells (known as atypical smooth muscle cells) responsible for initiating peristaltic waves at the renal pelvis, which is closer to the proximal ureter (Lang & Hashitani, 2019).

The non-selective cyclooxygenase inhibitor indomethacin had no effect on α_1-adrenoceptor-mediated contractile responses of porcine ureteral tissues, which again is similar to that reported for the isolated human ureter (Lee et al., 2010). Interestingly, indomethacin depressed 5-HT-mediated contractile responses in tissues from the younger animals, consistent with a previous study showing that both diclofenac and NS-398 caused a reduction in contractions to 5-HT in isolated ureter from pigs of a similar age range (3 – 6 months) to those utilised within the present study (Mastrangelo, Wisard, Rohner, Leisinger, & Iselin, 2000). The results suggest that ureteral cyclo-oxygenase activity is greater in tissues from young animals, but the effects of this on contractile activity is agonist-dependent.

The potent inhibitory effect of the mucosa is present throughout the lower urinary tract including in the bladder and urethra (Sellers, Chess-Williams, & Michel, 2018). In a recent report, phenylephrine-induced contractile responses of the human ureter were depressed in the presence of the urothelium (Roedel et al., 2018). Interestingly, a study on the rat ureter depicted the inhibitory effects of the mucosa on carbachol, angiotensin II and bradykinin-induced contractions but not on α_1-adrenoceptor stimulated response (Mastrangelo et al.,

2000). Here, we show that there is a similar effect in pig ureter to human, indicating that pig ureteral tissues can be used as a model for investigating mucosa inhibition.

5. Conclusion

It is difficult to compare and contrast findings reported in the scarce literature available on the pharmacology of ureteral motility, while taking into consideration the multiple factors that could contribute to ureteral contractions. To quantify the complex bursts of activity generated by the ureter, we propose that area under the curve assessment generates consistent and reproducible concentration-response curves. For optimal responses, longitudinally mounted strips, with distally located tissues, from younger animals best represent healthy human ureteral responses. To study ureteral smooth muscle responses directly, the mucosa should be removed and cyclooxygenase inhibited. Porcine tissues appear to be a good model for studying human ureter, with properties such as regional differences, influence of mucosa and lack of response to carbachol being similar to those reported in the literature for human ureter.

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