Activation of purinergic receptors (P2) in the renal medulla promotes endothelin-dependent natriuresis in male rats

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Abstract
Renal endothelin-1 (ET-1) and purinergic signaling systems regulate Na\(^+\) reabsorption in the renal medulla. A link between the renal (ET-1) and purinergic systems was demonstrated *in vitro*, however, the *in-vivo* interaction between these systems has not been defined. To test whether renal medullary activation of purinergic (P2) receptors promotes ET-dependent natriuresis, we determined the effect of increased medullary NaCl loading on Na\(^+\) excretion and inner medullary ET-1 mRNA expression in anesthetized adult male Sprague Dawley rats in the presence and absence of purinergic receptor antagonism. Isosmotic saline (NaCl; 284 mOsmol/kg H\(_2\)O) was infused into the medullary interstitium (500 μl/h) during a 30 min baseline urine collection period, followed by isosmotic or hyperosmotic saline (1800 mOsmol/kg H\(_2\)O) for two further 30 min urine collection periods. Na\(^+\) excretion was significantly increased during intramedullary infusion of hyperosmotic saline. Compared with isosmotic saline, hyperosmotic saline infused into the renal medulla caused significant increases in inner medullary ET-1 mRNA expression. Renal intramedullary infusion of the purinergic (P2) receptor antagonist, suramin, inhibited the increase in Na\(^+\) excretion and inner medullary ET-1 mRNA expression induced by NaCl loading in the renal medulla. Activation of medullary purinergic (P2Y\(_{2/4}\)) receptors by infusion of UTP increased urinary Na\(^+\) excretion. Combined ETA and ETB receptor blockade abolished the natriuretic response to intramedullary infusion of UTP. These data demonstrate that activation of medullary purinergic (P2) receptors promotes ET-dependent natriuresis in male rats, suggesting that the renal ET-1 and purinergic signaling systems interact to efficiently facilitate excretion of a NaCl load.

**Key Words.** Endothelin-1, purinergic receptors, natriuresis, kidney, inner medulla
Introduction

Endothelin-1 (ET-1) is an autocrine inhibitor of Na\textsuperscript{+} and water reabsorption by the kidney and plays a central role for the regulation of Na\textsuperscript{+} homeostasis and blood pressure control. Within the renal medulla, ET-1 is released in response to a high salt diet and inhibits tubular Na\textsuperscript{+} transport promoting natriuresis (14, 17). It appears that both ET\textsubscript{A} and ET\textsubscript{B} receptors are required for the full diuretic and natriuretic effects of ET-1 (7). However, the signaling mechanism by which NaCl loading to the renal medulla translates into an increase in ET-1 production and/or action is currently unknown.

Purinergic signaling has also emerged as another important system in the renal control of blood pressure and Na\textsuperscript{+} excretion (19, 31). In response to increased tubular flow, ATP is released from renal tubular cells inhibiting Na\textsuperscript{+} transport along the nephron (12, 19), mainly through P2Y\textsubscript{2} receptor activation (18, 23). Both P2Y\textsubscript{2} knockout mice and ET\textsubscript{B} deficient rats develop salt-sensitive hypertension (23, 30), supporting the fundamental roles for these two systems in blood pressure regulation by controlling Na\textsuperscript{+} homeostasis. However, the interaction between renal ET-1 and purinergic signaling is relatively unexplored. Recently, it has been demonstrated that renal P2Y\textsubscript{2} and P2X\textsubscript{7} receptor blockade inhibits ET-1 production in inner medullary collecting duct cells (22). However, the interaction between the endothelin and the purinergic signaling systems within the renal medulla has yet to be explored in vivo. Taken together, we uniquely designed the current study to elucidate the potential interplay between ET-1 and purinergic signaling systems on renal Na\textsuperscript{+} excretory function in male rats.

We hypothesize that the activation of purinergic (P2) receptors within the renal medulla promotes ET-dependent natriuresis. To test this hypothesis, we studied the effect of increased medullary NaCl loading on Na\textsuperscript{+} excretion and inner medullary ET-1 mRNA expression in adult male Sprague Dawley rats in the presence or absence of purinergic (P2) receptors.
antagonism. To determine the role of ET-1 system in response to increased NaCl loading to the renal medulla, we assessed the change in inner medullary ET-1 mRNA expression in response to infusion of hyperosmotic saline into the renal medulla. To assess whether inner medullary purinergic (P2) receptors are involved in ET-dependent control of Na⁺ excretion, we evaluated the effect of purinergic (P2) receptors blockade on natriuresis and ET-1 mRNA expression in inner medulla following increased medullary Na⁺ loading. Finally, to determine whether there is a crosstalk between medullary ET-1 and purinergic signaling systems, we studied the effect of intramedullary infusion of the purinergic (P2Y₂/₄) agonist, UTP, in the presence and absence of ETₐ and ETₐ receptors antagonists.

Methods

**General Methods.** Male Sprague-Dawley rats (16-18 weeks old, n=5-7 per group; Harlan Laboratories, Indianapolis, IN) were used in the current study. Rats were housed in a temperature and humidity-controlled room with a 12:12-h light-dark cycle, with free access to water. All protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, and were approved in advance by the UAB Institutional Animal Care and Use Committee.

**Acute Intramedullary Infusion.** Rats were anesthetized using thiobutabarbitone (Inactin, hydrate 100 mg/kg, ip, Sigma-Aldrich Co., St. Louis, MO), and surgically prepared for acute intramedullary infusion, as described in previous studies by our lab (6, 7). First, rats were placed on a heating surgical table to maintain body temperature at 37°C throughout the experiment, and a tracheotomy was performed to facilitate breathing. A catheter was inserted into the femoral vein for infusing 3% bovine serum albumin in phosphate-buffered saline at a rate of 1.2 ml/h to maintain euvolemia. Another catheter was inserted into the femoral artery, connected to a pressure transducer and a PowerLab data acquisition system to monitor blood pressure over the course of the experiment. A midline incision was then performed and a
catheter placed in the left ureter to allow urine collection. The left renal artery was isolated and
fitted with an ultrasonic perivascular flow probe (1PRB probe, Transonic Systems Inc., Ithaca,
NY) to measure the total renal blood flow continuously during each experiment. Finally, a
stretched PE10 catheter was inserted into the left kidney as deep as the outer/inner medullary
junction to allow infusion of isosmotic NaCl (0.9%) directly into the renal medulla (0.5 ml/h).

Following surgical preparation, a 60 min equilibration period and 30 min baseline urine
collection period was obtained during intramedullary infusion of isosmotic saline (284
mOsmol/kg H$_2$O, 0.9% NaCl, 154 mM NaCl). This was followed by isosmotic or hyperosmotic
saline (1800 mOsmol/kg H$_2$O, 5.7% NaCl, 976 mM NaCl) infusion for two further 30 min
periods. The experimental timeline used in this intramedullary infusion experiment is illustrated
in Fig. 1. It is important to highlight that we use the terms “isosmotic” and “hyperosmotic”
relative to plasma osmolality.

To determine the role of purinergic system in response to increasing intramedullary
NaCl loading, we continuously infused the non-selective purinergic (P2) receptors antagonist,
suramin (750 μg/kg/min, Sigma-Aldrich Co., St. Louis, MO), into the renal medullary
interstitium during the 30 min basal urine collection period. Suramin and hyperosmotic saline
were then co-infused for two subsequent 30 min urine collection periods. To our knowledge,
there is no available data on doses for intramedullary infusion of suramin. The suramin dose
used in the current study originates from pilot studies conducted in our lab using a range of
concentrations estimated based on prior intravenous studies (27).

To determine the role of ET-1 system in response to purinergic (P2) receptor activation,
rats received an IV bolus injection (0.5 ml/kg via femoral vein catheter) of vehicle or a
combination of the selective ET$_A$ receptor antagonist ABT-627 (5 mg/kg) plus the selective ET$_B$
receptor antagonist A-192621 (10 mg/kg, Abbott Laboratories) 30 min before the end of the
post-surgical equilibration period. These doses are known to maintain efficient blockade of
both receptor subtypes for the duration of our experimental protocol as previously
demonstrated (4, 7, 32). After the equilibration period and the 30 min baseline urine collection
period during which isosmotic saline was intramedullary infused, the purinergic (P2Y_{2/4})
receptor agonists (200 pmol/kg/min UTP, Sigma) was infused into the renal medullary
interstitium for two subsequent 30 min urine collection periods.

Blood pressure and renal blood flow data were monitored using a PowerLab data
acquisition system (ADInstruments). Renal blood flow was normalized to left kidney weight. At
the end of each experiment, the proper positioning of the catheter tip at the outer-inner
medullary junction was confirmed by dissection of the kidney and inner medulla was
harvested, snap frozen in liquid nitrogen and stored at -80°C until analysis.

**Tissue Analysis.** Renal inner medullary tissue was immediately harvested after the second 30
min urine collection period, and total RNA was isolated using Purelink Mini RNA extraction kit
(Ambion) according to manufacturer’s instructions. Then, the isolated RNA was reverse
transcribed using QuantiTect Reverse Transcription kit (Qiagen). Finally, the resulting cDNA
was used to quantify ET-1 mRNA by Real Time-PCR (CFX96 Real-Time System, BIORAD)
using TaqMan primer gene expression assays with ET-1 (catalog no. Rn01775763_g1), P2Y_{2}
receptor (catalog no. Rn02070661_s1), P2Y_{4} receptor (catalog no. Rn02133903_s1), P2Y_{6}
receptor (catalog no. Rn02134326_s1) and GAPDH (catalog no. Rn01775763_g1) primers.
We assessed the expression of these three purinergic (P2Y) receptors because UTP can
directly activate both P2Y_{2} and P2Y_{4} and may be converted to UDP that is an agonist for P2Y_{6}
receptors (11). ET-1 and P2Y receptor gene expression was quantified relative to GAPDH
using 2^{-ΔΔCt} method.

**Urine Analysis.** Urinary Na^{+} and K^{+} concentrations were measured with an atomic
absorption spectrometer in the flame photometry mode (model 3100; Perkin Elmer, Rodgau,
Osmolality of infused solutions and urine samples were determined by vapor pressure osmometer (VAPRO 5600, ELITechGroup Inc., Logan, UT).

Statistics. Data are presented as means ± SEM. Statistical comparison of 2 experimental groups was performed by unpaired Student’s t-test. When several groups were compared, we used one-way ANOVA followed by post hoc analysis with Bonferroni correction. A probability of p<0.05 was considered significant.

Results

During infusion of isosmotic saline into the renal medulla, urine flow, electrolyte excretion, and urine osmolality remained unchanged (Fig. 2). Both urine flow and Na⁺ excretion increased markedly within the first 30 min of commencing intramedullary infusion of hyperosmotic saline (1800 mOsmol/kg H₂O, Fig. 2A & B). These diuretic and natriuretic responses demonstrated during the first 30 min of medullary NaCl loading continued further during the second 30 min time interval (Fig. 2A & B). Increasing NaCl load into the renal medulla did not change urinary K⁺ excretion (Fig 2C). Urine osmolality was significantly increased during the first as well as the second 30 min of intramedullary infusion of hyperosmotic saline (Fig. 2D). Mean arterial pressure (Fig. 3A) and total renal blood flow (Fig. 3B) remained unchanged during intramedullary infusions of isosmotic or hyperosmotic saline.

Increased medullary NaCl loading enhances ET-1 mRNA expression in the inner medulla. To determine whether the natriuretic effect of increasing medullary NaCl loading was associated with stimulation of the ET-1 system, we assessed the expression of ET-1 mRNA within the inner medulla in response to increased medullary NaCl loading. Infusion of hyperosmotic saline into the renal medulla for 60 min increased the expression of inner medullary ET-1 mRNA compared to corresponding values from rats infused with isosmotic saline (Fig. 4). Additionally, we assessed the expression of inner medullary P2Y receptors in response to NaCl loading. Intramedullary infusion of hyperosmotic saline for 60 min had no
significant effect on the expression of P2Y$_2$, P2Y$_4$, or P2Y$_6$ receptors within the inner medulla (Fig. 5).

**Blockade of purinergic (P2) receptors attenuated natriuresis in response to increased medullary NaCl loading.** To determine the role of purinergic (P2) receptor activation in mediating the natriuretic effect of increasing the medullary NaCl delivery, we tested the effect of intramedullary infusion of hyperosmotic saline with or without the non-selective antagonist of purinergic (P2) receptors, suramin. Intramedullary infusion of suramin (750 μg/kg/min) significantly attenuated the diuretic as well as the natriuretic responses to increased medullary NaCl during the first as well as the second 30 min urine collection periods (Fig 5A & B). Increases in urine osmolality in response to intramedullary infusion of hyperosmotic saline were still evident in the presence of suramin, and even rising further during the second 30 min time interval (Fig. 5D). Urinary K$^+$ excretion, mean arterial pressure and total renal blood flow were not significantly altered by intramedullary infusion of hyperosmotic saline alone or when combined with the purinergic (P2) antagonist, suramin (Fig. 5C & 6).

**Blockade of purinergic (P2) receptors attenuated the increase in ET-1 mRNA expression in response to increased medullary NaCl.** To evaluate the possibility that medullary purinergic (P2) receptors stimulate the medullary ET-1 system following NaCl loading, we assessed the expression of ET-1 mRNA within the inner medulla after 60 min infusion of hyperosmotic saline alone or combined with suramin, the non-selective blocker of purinergic (P2) receptors. Interestingly, the increase in inner medullary ET-1 mRNA expression in response to increased medullary NaCl loading was completely abolished by suramin (Fig. 7).

**Dual blockade of ET$_A$ and ET$_B$ receptors inhibited the natriuretic response to purinergic receptor (P2Y$_{2/4}$) activation.** To determine a more direct interaction between the renal medullary ET-1 and purinergic systems, separate groups of male rats received intramedullary infusions of the purinergic (P2Y$_{2/4}$) receptor agonist, UTP (200 pmol/kg/min), with or without
combined ET$_A$ and ET$_B$ receptor blockade using the selective ET$_A$ antagonist (ABT-627) plus the selective ET$_B$ antagonist (A-192621). Intramedullary purinergic (P2Y$_{2/4}$) receptor activation with UTP enhanced the urine flow and Na$^+$ excretion significantly during the second 30 min period, compared to baseline as demonstrated in Fig. 8A & B. These diuretic and natriuretic responses to purinergic (P2Y$_{2/4}$) receptor activation were completely abolished by dual blockade of ET$_A$ and ET$_B$ receptors (Fig. 8A & B). Urinary K$^+$ excretion was unchanged by intramedullary P2Y$_{2/4}$ activation, but was significantly lower during the second 30 min urine collection in antagonist infused rats compared with those given UTP alone (Fig. 8C). Urine osmolality was not significantly changed during these experiments (Fig. 8D). Mean arterial pressure remained stable during intramedullary infusion of UTP, and was slightly, but significantly, decreased in rats receiving intravenous ET$_A$ and ET$_B$ receptor blockers during the second 30 min time period, compared to the corresponding time point in the UTP group (Fig. 9A). However, no statistically significant differences were detected between the second 30 min time point during dual blockade of ET receptors and corresponding baseline values. Total renal blood flow was not significantly different between these two groups (Fig. 9B).

**Discussion**

The current study shows that (1) increased NaCl load to the renal medulla of male rats results in a diuretic and a natriuretic response associated with an increase in inner medullary ET-1 mRNA expression, (2) blockade of purinergic (P2) receptors within the renal medulla inhibits the natriuresis and the increase in ET-1 mRNA expression following medullary NaCl loading, and (3) blockade of ET receptors inhibits to the diuretic and natriuretic response to UTP infusion. Together, these findings suggest an interaction between the ET-1 and the purinergic signaling systems within the renal medulla to efficiently excrete increased NaCl load.

Several earlier studies suggest that extracellular osmolality enhances ET-1 production
and release by freshly isolated or cultured renal tubular or collecting duct cells (9, 16, 22, 33). This \textit{in vitro} evidence was supported by \textit{in vivo} studies from our lab showing that infusion of hyperosmotic saline into the renal medulla resulted in diuresis, natriuresis and increases in urinary ET-1 excretion (6). The diuretic response following intramedullary infusion of hyperosmotic saline was attenuated by dual blockade of ET\textsubscript{A} and ET\textsubscript{B} receptors (7), suggesting that infusion of hyperosmotic saline to the renal medulla results in ET-dependent diuresis (7). This is in line with accumulating evidence highlighting a central role for renal medullary derived ET-1 in the control of renal excretory function (6, 7, 14, 15, 17, 21, 26).

In the current study, we demonstrate that increasing the medullary NaCl load enhances water and Na\textsuperscript{+} excretion in male rats. This diuretic and natriuretic effect is demonstrated during the first 30 min of infusing hyperosmotic saline and extended during the second 30 min urine collection period. Our lab has provided evidence that exposing the renal medulla to hyperosmotic saline stimulates ET-1 release in male rats over this time frame (6). Importantly, our current study further supported this evidence by showing significant increases in ET-1 mRNA expression within the renal inner medullary tissues in response to intramedullary infusion of hyperosmotic saline for 60 min.

The signaling mechanism by which a high medullary NaCl load is translated into an increase in ET-1 production/release \textit{in-vivo} is currently unclear. Our findings suggest that the purinergic receptors might be involved. This idea is originally based on a recent study revealing that purinergic receptors (P2) are required for flow-stimulated ET-1 release in inner medullary collecting duct cells (22). Our data showed that blockade of medullary purinergic receptors (P2) by suramin inhibited the diuretic and natriuretic response to increased medullary NaCl loading. More importantly, suramin completely abolished the increase in inner medullary ET-1 mRNA expression following intramedullary infusion of hyperosmotic saline.
These data provide strong *in-vivo* evidence that activation of the medullary P2 receptors is necessary for the ET-1 dependent natriuretic response in male rats. As suramin is a non-selective blocker of purinergic receptors (P2), it is unclear which P2 receptors could be involved in the regulation of ET-1 production in response to medullary NaCl loading. Pandit and colleagues’ findings suggest that P2X7 and P2Y2 receptors play a central role. Accordingly, the potential contribution of different subtypes of medullary P2Y and P2X receptors to ET-1 dependent natriuresis remains unclear and further studies are needed to address this particular point.

Our results showed that P2Y2, P2Y4 and P2Y6 receptor mRNA is expressed in renal inner medullary tissues. Previous studies have shown that P2Y2 and P2Y4 receptors are expressed in the proximal convoluted tubule and outer medullary collecting ducts (2). Additionally, Cha and colleagues found P2Y2 in the outer medullary collecting tubule (8). In the descending limb of Henle, P2Y2 mRNA was found, but P2Y4 was not expressed (2). Additionally, it has been shown that P2Y6 receptor mRNA is expressed in most segments of the rat nephron but basolateral expression of this protein is restricted to the proximal tubule (3). Our results showed that intramedullary NaCl loading did not significantly affect the expression of P2Y2, P2Y4 or P2Y6 receptors within the renal inner medulla.

It is important to comment on the effect of dual blockade of ET<sub>A</sub> and ET<sub>B</sub> receptors on the diuretic and natriuretic response to intramedullary infusion of UTP. As expected, intramedullary infusion of UTP for 60 minutes enhanced the urine flow and Na<sup>+</sup> excretion. This is in line with the well-established role for the purinergic signaling system in regulating Na<sup>+</sup> excretion (19, 24, 31). P2 receptors are expressed along the nephron, suppressing Na<sup>+</sup> transport at several sites (19). Our studies also revealed an inhibitory effect of combined blockade of ET<sub>A</sub> and ET<sub>B</sub> receptors on the diuretic and natriuretic effect of UTP. We used the
approach of blocking both receptor subtypes given evidence that both $\text{ET}_A$ and $\text{ET}_B$ receptors could participate in ET-1 dependent natriuresis even though the predominant action is via the $\text{ET}_B$ receptor (15, 29). Use of selective $\text{ET}_B$ receptor antagonists has the unfortunate consequence of increasing $\text{ET}_A$ receptor activity, which would make interpretation of such results problematic. Nonetheless, our findings further support the possibility of an interaction between the renal purinergic and ET-1 system within the renal medulla.

Collectively, our findings that pharmacologic blockade of P2 or both $\text{ET}_A$ and $\text{ET}_B$ receptors attenuated the diuretic and natriuretic response to intramedullary infusion of hyperosmotic saline or UTP, respectively, uncovers an important crosstalk between the medullary purinergic and ET-1 signaling systems in regulating $\text{Na}^+$ excretion in male rats. This is in line with recent findings demonstrating that blockade of P2Y$_2$ and P2X$_7$ receptors inhibits ET-1 production in inner medullary collecting duct cells (22). Furthermore, non-renal studies demonstrated that purinergic and ET-1 signaling interact in sensory neurons (5), blood vessels (1, 13), as well as myocardium (10). Barr et al. provided evidence that mechanical hypersensitivity caused by cutaneous ET-1 involves sensitization of P2X$_4$ receptors (5). In porcine pancreatic arteries, P2Y$_1$ receptor plays vasoconstrictive role that involves the release of ET-1 (1). Moreover, loss of mouse P2Y$_4$ receptors on cardiac microvascular endothelial cells protects against myocardial infarction through ET-1 downregulation (10).

Thus, there is fairly clear evidence to suggest that ET-1 and purinergic receptors are important in controlling renal tubular reabsorption of $\text{Na}^+$ and water. The tubular segment contributing to the interaction between ET-1 and purinergic receptors in controlling $\text{Na}^+$ excretion is not specifically known, however, the inner medullary collecting ducts are potential contributors based on recent data (22). Roles for proximal tubule and thick ascending limb are also postulated.
Finally, it is imperative to pinpoint that increasing the medullary NaCl load by intramedullary infusion of hyperosmotic saline is not used in the current study to specifically mimic the effect of high salt diet, but rather, it is used as an established stimulus for ET-1 production. Changes in the osmolality of the renal medulla resulting from water loading (20), diuretics (28), dehydration (25), and drinking salty water (9) have been reported in the literature. Drinking 1% NaCl in water for 7 days has been reported to increase the medullary osmolality in rats (9), however, the effect of high salt diet on medullary Na⁺ concentration is not yet totally clear.

It is important to note that our study used suramin as an antagonist for all purinergic (P2) receptors and so a limitation of our study is that we cannot specifically assign a role for subtypes of P2 receptors, P2Y or P2X, or furthermore, subtypes of these two major classes of purinergic receptor. Additionally, we used UTP that can activate P2Y₂ as well as P2Y₄ receptors. UTP may be also converted to UDP that can activate P2Y₆ receptors (11). Future studies using more specific P2 agonists and antagonists will help clarify the exact receptor subtypes involved in the interaction between ET-1 and purinergic signaling in the renal medulla.

In summary, our current findings suggest that purinergic (P2) receptors activate the ET-1-dependent natriuresis in male rats (Fig. 11). Consistently, ET-1 appears to contribute to the diuretic and natriuretic response to intramedullary infusion of UTP. Together, these observations provide strong in vivo evidence for possible renal medullary interplay between ET-1 and purinergic systems in controlling Na⁺ and water homeostasis in male rats.

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Conflict of Interest

The authors declare that there are no conflicts of interest

Literature Cited


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**Figure Legends**

**Fig. 1.** Experimental timeline.

**Fig. 2.** Effect of renal medullary interstitial infusions of isosmotic (284 mOsmol/kg H2O) or hyperosmotic saline (1800 mOsmol/kg H2O) on urine flow (A), urinary excretion rate of Na⁺ (U_{Na}V, B), K⁺ (U_{K}V, C) and urine osmolality (D) in anaesthetized male rats. Data are means ± SE of 5-7 samples in each group. Error bars for few time points are not apparent due to very low SE. *P<0.05 vs. corresponding isosmotic saline values.

**Fig. 3.** Effect of renal medullary interstitial infusions of isosmotic (284 mOsmol/kg H2O) or hyperosmotic saline (1800 mOsmol/kg H2O) on mean arterial blood pressure (MAP, A) and total renal blood flow (B) in anaesthetized male rats. Data are means ± SE of 5-7 samples in each group. Error bars for few time points are not apparent due to very low SE.

**Fig. 4.** Effect of renal medullary interstitial infusions of isosmotic (284 mOsmol/kg H2O) or
hyperosmotic saline (1800 mOsmol/kg H₂O) on the relative inner medullary mRNA expression of ET-1 in anaesthetized male rats. Values represent fold change from corresponding isosmotic saline levels. Data are means ± SE of 5-7 samples in each group. *P<0.05 vs. corresponding isosmotic saline values.

**Fig. 5.** Effect of renal medullary interstitial infusions of hyperosmotic saline (1800 mOsmol/kg H₂O) on relative inner medullary mRNA expression of purinergic receptors (P₂Y₂ R, P₂Y₄ R, and P₂Y₆ R) in anaesthetized male rats. Values represent fold change from corresponding control (contralateral kidney) levels. Data are means ± SE of 4-5 samples in each group.

**Fig. 6.** Effect of renal medullary interstitial infusions of hyperosmotic saline (1800 mOsmol/kg H₂O) with or without the non-selective antagonist of purinergic (P₂) receptors, suramin, on urine flow (A), urinary excretion rate of Na⁺ (UₙaV, B), K⁺ (UₖV, C) and urine osmolality (D) in anaesthetized male rats. Data are means ± SE of 5-6 samples in each group. Error bars for few time points are not apparent due to very low SE. *P<0.05 vs. corresponding baseline values. †P<0.05 vs. corresponding hyperosmotic saline values.

**Fig. 7.** Effect of renal medullary interstitial infusions of hyperosmotic saline (1800 mOsmol/kg H₂O) with or without the non-selective antagonist of purinergic (P₂) receptors, suramin, on mean arterial blood pressure (MAP, A) and total renal blood flow (B) in anaesthetized male rats. Data are means ± SE of 5-6 samples in each group. Error bars for few time points are not apparent due to very low SE.

**Fig. 8.** Effect of renal medullary interstitial infusions of isosmotic (284 mOsmol/kg H₂O), hyperosmotic saline (1800 mOsmol/kg H₂O) with or without the non-selective antagonist of purinergic (P₂) receptors, suramin, on the relative inner medullary mRNA expression of ET-1 in anaesthetized male female rats. Values represent fold change from corresponding isosmotic saline levels. Data are means ± SE of 5-7 samples in each group. *P<0.05 vs. corresponding isosmotic saline values. †P<0.05 vs. corresponding hyperosmotic saline values.
**Fig. 9.** Effect of renal medullary interstitial infusions of the purinergic (P2Y$_{2/4}$) receptor agonist, UTP, with or without the selective ET$_A$ receptor blocker (ABT-627) plus the selective ET$_B$ receptor blocker (A-192621) on urine flow (A), urinary excretion rate of Na$^+$ (U$_{Na}V$, B), K$^+$ (U$_{K}V$, C) and urine osmolality (D) in anaesthetized male rats. Data are means ± SE of 5-7 samples in each group. *P<0.05 vs. corresponding baseline values. †P<0.05 vs. corresponding UTP values.

**Fig. 10.** Effect of renal medullary interstitial infusions of the purinergic (P2Y$_{2/4}$) receptor agonist, UTP, with or without the selective ET$_A$ receptor blocker (ABT-627) plus the selective ET$_B$ receptor blocker (A-192621) on mean arterial blood pressure (MAP, A) and total renal blood flow (B) in anaesthetized male and female rats. Data are means ± SE of 5-7 samples in each group. †P<0.05 vs. corresponding UTP values.

**Fig. 11.** Hypothetical sequence of the interaction between the purinergic and ET-1 signaling.
♂ Sprague Dawley Rats

Surgery

60 min
Equilibration

30 min
Baseline urine collection period

30 min
30 min
urine collection period

intramedullary infusion of isosmotic saline

intramedullary infusion of isosmotic, hyperosmotic saline or UTP

ET<sub>A/B</sub> receptors antagonists (IV)

inner medulla collection
**Urine Flow (μl/min)**

- **A** Isosmotic Saline
- **B** Hyperosmotic Saline

**U_{Na} V (μmol/min)**

**U_{K} V (μmol/min)**

**Urine Osmolality (mOsmol/kg H\textsubscript{2}O)**
MAP (mmHg)

Baseline
0-30 min
30-60 min

Isosmotic Saline
Hyperosmotic Saline

Renal Blood Flow (ml/min/g tissue)

Baseline
0-30 min
30-60 min
Relative ET-1 mRNA (Fold Change)

Osmolality of infused NaCl solution (mOsmol/kg H₂O)
Osmolality of infused NaCl solution (mOsmol/kg H₂O)
A. **Urine Flow (μl/min)**

- **Hyperosmotic Saline**
- **Suramin + Hyperosmotic Saline**

B. **U \text{Na}** (μmol/min)

C. **U \text{K} V** (μmol/min)

D. **Urine Osmolality (mOsmol/kg H_2O)**

* Baseline 0-30 min 30-60 min

* Significantly different from baseline

† Significantly different from other group
MAP (mmHg)

Baseline 0-30 min 30-60 min

Hyperosmotic Saline
Hyperosmotic Saline + Suramin

Renal Blood Flow (ml/min/g tissue)

Baseline 0-30 min 30-60 min
Relative ET-1 mRNA
(Fold Change)

Osmolality of infused NaCl solution
(mOsmol/kg H2O)
Urine Flow (μl/min)

A. ▼ UTP
  ▲ UTP+ABT-627+A-192621

Baseline 0-30 min 30-60 min

UTP+ABT-627+A-192621

Urine NaV (μmol/min)

B. *

Baseline 0-30 min 30-60 min

Urine Osmolality (mOsmol/kg H₂O)

C. †

Baseline 0-30 min 30-60 min

U NaV (μmol/min)

Baseline 0-30 min 30-60 min

D. †