BJP BRITISH JOURNAL OF PHARMACOLOGY

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BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00914.x
www.brjpharmacol.org

RESEARCH PAPER

# Differential effects of uridine adenosine tetraphosphateon purinoceptors in the rat isolated perfused kidney

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**Keywords**
purinoceptors; uridine adenosine tetraphosphate; P2 receptors; rat isolated perfused kidney; vasoconstriction; vasodilation

**Received**
31 July 2009
**Revised**
15 April 2010
**Accepted**
25 April 2010

This article is commented on by Burnstock, pp. 527–529 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2010.00937.x

## BACKGROUND AND PURPOSE

Purinergic signalling plays an important role in vascular tone regulation in humans. We have identified uridine adenosine tetraphosphate ($Up_4A$) as a novel and highly potent endothelial-derived contracting factor. $Up_4A$ induces strong vasoconstrictive effects in the renal vascular system mainly by $P2X_1$ receptor activation. However, other purinoceptors are also involved and were analysed here.

## EXPERIMENTAL APPROACH

The rat isolated perfused kidney was used to characterize vasoactive actions of $Up_4A$.

## KEY RESULTS

After desensitization of the $P2X_1$ receptor by α,β-methylene ATP (α,β-meATP), $Up_4A$ showed dose-dependent $P2Y_2$-mediated vasoconstriction. Continuous perfusion with $Up_4A$ evoked a biphasic vasoconstrictor effect: there was a strong and rapidly desensitizing vasoconstriction, inhibited by $P2X_1$ receptor desensitization. In addition, there is a long-lasting $P2Y_2$-mediated vasoconstriction. This vasoconstriction could be blocked by suramin, but not by PPADS or reactive blue 2. In preparations of the rat isolated perfused kidney model with an elevated vascular tone, bolus application of $Up_4A$ showed a dose-dependent vasoconstriction that was followed by a dose-dependent vasodilation. The vasoconstriction was in part sensitive to $P2X_1$ receptor desensitization by α,β-meATP, and the remaining $P2Y_2$-mediated vasoconstriction was only inhibited by suramin. The $Up_4A$-induced vasodilation depended on activation of nitric oxide synthases, and was mediated by $P2Y_1$ and $P2Y_2$ receptor activation.

## CONCLUSIONS AND IMPLICATIONS

$Up_4A$ activated $P2X_1$ and $P2Y_2$ receptors to act as a vasoconstrictor, whereas endothelium-dependent vasodilation was induced by $P2Y_{1/2}$ receptor activation. $Up_4A$ might be of relevance in the physiology and pathophysiology of vascular tone regulation.

## Abbreviations

α,β-meATP, α,β-methylene ATP; AngII, angiotensin II; $Ap_nA$, diadenosine-*n*-phophate (*n*: number of phosphates); $Ap_nG$, adenosine-guanosine-*n*-phosphate (*n*: number of phosphates); ApoE, apolipoprotein E; CI, confidence interval; DMSO, dimethyl sulphoxide; eNOS, endothelial NOS; $Gp_nG$, diguanosine-*n*-phosphate (*n*: number of phosphates); L-NAME, $N^G$-nitro-L-arginine methyl ester; MAP, mean arterial blood pressure; MCP-1, monocyte chemoattractant protein-1; MRS2179, 2′-deoxy-N6-methyladenosine 3′,5′-bisphosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2;4-disulphonic acid; RB2, reactive blue 2; $Up_4A$, uridine adenosine tetraphosphate

## Introduction

Over the past two decades, there has been an increase in evidence that the purinoceptor system is involved in vascular tone control (van der Giet *et al.*, 2002a; Buvinic *et al.*, 2006), and also potentially involved in the pathogenesis of hypertension (Jankowski *et al.*, 2005; Tolle *et al.*, 2008). In the mid-1990s, a new group of purinergic compounds, the so-called diadenosine polyphosphates, were identified as highly potent vasoactive substances (Schluter *et al.*, 1994; Gabriels *et al.*, 2002). In the following years, it became evident that these dinucleoside polyphosphates induce vasoconstriction in various vascular systems, mainly via $P2X_1$ receptor activation. However, some of the vasoactive effects observed with diadenosine pentaphosphate ($Ap_5A$) and the corresponding hexaphosphate ($Ap_6A$) were also mediated via activation of G-protein-coupled P2Y receptors (Gabriels *et al.*, 2002). In the following years, more dinucleoside polyphosphates containing either two adenosines, one adenosine and one guanosine, or two guanosines were identified. A complex family of purinergic dinucleoside polyphosphates with a variable phosphate chain of 2–7 phosphates (Jankowski *et al.*, 2009) has now been described. Adenosine-containing dinucleoside polyphosphates act more as vasoconstrictive agents, and guanosine-containing dinucleoside polyphosphates act as cell-proliferating agents. One of the guanosine-containing dinucleoside polyphosphates is of special interest. Diguanosine pentaphosphate ($Gp_5G$) is a potent activator of Rho-kinase and modulates the vasoactive responses of other known vasoactive substances such as angiotensin II (AngII) (Tolle *et al.*, 2006). $Gp_5G$ activates $P2Y_2$ receptors (receptor nomenclature follows Alexander *et al.*, 2009) to induce calcium sensitization, and such sensitization is believed to be an important mechanism in the control of vascular tone and blood pressure.

Recently, our group identified the first pyrimidine-containing dinucleoside polyphosphate as a highly potent, new endothelial-derived vasoconstrictive factor. The substance was characterized as uridine adenosine tetraphosphate ($Up_4A$) (Jankowski *et al.*, 2005). $Up_4A$ shares properties of both P2X receptor and P2Y receptor agonists. In the first study, we demonstrated that $Up_4A$ acts as a vasoconstrictive agent by $P2X_1$ receptor activation (Jankowski *et al.*, 2005). The response was only partially inhibited by the $P2X_1$ and $P2X_3$ receptor desensitizer α,β-methylene ATP (α,β-meATP), indicating the activation of other purinoceptors. It was proposed that P2Y receptors are responsible for the remaining vasoactive properties of $Up_4A$. These $Up_4A$-activated P2Y receptors have not yet been characterized in depth.

There is some evidence that $Up_4A$ might have implications in the pathogenesis of human hypertension. $Up_4A$ plasma levels in young hypertensives are significantly increased compared to age-matched controls. The $Up_4A$ concentration is significantly correlated with the left ventricular mass and intima media wall thickness in these young hypertensives (Jankowski *et al.*, 2007).

The purpose of the current work was to identify all purinoceptors other than $P2X_1$ which are activated by $Up_4A$. It is necessary to know more about the activation of the P2 receptors other than $P2X_1$ receptors activated by $Up_4A$. We sought to understand the complex vasoregulatory properties of $Up_4A$ as a novel endothelium-derived vascoconstrictive factor. In this study, we focused on the P2Y receptor-mediated physiological vasoactive actions of $Up_4A$. To study the vasoactive effects of $Up_4A$, we used the model of the rat isolated perfused kidney. We demonstrated that $Up_4A$ exerted vasoconstriction not only via $P2X_1$ receptors, but also by activation of $P2Y_2$ receptors. The $Up_4A$-induced vasodilation was mediated via endothelial activation of $P2Y_1$ and $P2Y_2$ receptors.

## Methods

### *Animal experiments*

All animal care and experimental procedures were approved by the State Ethics committee Landesamt für Gesundheit, Ernährung und Technische Sicherheit Berlin.

### *Preparation of the rat isolated perfused kidney*

Adult male Wistar–Kyoto rats (4–6 months old) were anaesthetized with ketamine (50 $mg \cdot kg^{-1}$, intraperitoneally)/xylazine (10 $mg \cdot kg^{-1}$, intraperitoneally). The abdominal cavity was opened by a mid-ventral incision. The aorta and the left kidney were carefully isolated from adhesive tissue by blunt dissection. Ligatures were placed around the left renal artery and the infrarenal aorta. A polyethylene catheter (20-gauge) was placed in the distal aorta. Immediately after the insertion of the catheter, 500 U of heparin sodium was injected. Perfusion was then started. The catheter was gently advanced into the left renal artery without interruption of flow. The kidney was excised and immediately mounted in the perfusion system (Hugo Sachs Electronic, Freiburg, Germany).

### *Perfusion system*

The perfusion procedure generally followed the description given by Hofbauer *et al.* (1973). The

kidney was perfused at a constant flow rate using a peristaltic pump equilibrated to a perfusion pressure of about 70 mmHg Tyrode's solution of the following composition (in mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.1; $NaHCO_3$, 12; $NaH_2PO_2$, 0.42; and glucose, 5.6 gassed with 95% $O_2$–5% $CO_2$ and maintained at 37°C was used as perfusate. The pH was measured continuously by a pH sensor included in the perfusion system and was held between 7.35 and 7.45. Responses were measured as changes in perfusion pressure (mmHg) with a pressure transducer (Statham Transducer P23Gb, Siemens, Erlangen, Germany) on a side arm of the perfusion catheter, connected to a bridge amplifier (Hugo Sachs), and recorded digitally. Preparations were allowed to equilibrate for 30 min prior to experimentation.

### *Basal tone preparations*

Vasoconstrictor responses of preparations to doses of $Up_4A$, α,β-me-ATP, UTP, UDP or AngII were assessed at basal tone. For each substance, dose–response curves were constructed, with a minimum of 20 min being allowed to elapse between consecutive doses to avoid desensitization. This procedure allowed dose–response curves for several agonists to be constructed for the same preparation. A significant degree of cross-desensitization or auto-desensitization was not detected. The procedure has been described previously (van der Giet *et al.*, 1999). The non-selective P2 receptor antagonists suramin (100 μM), pyridoxal phosphate 6-azophenyl-2;4-disulphonic acid (PPADS; 10 μM), reactive blue 2 (RB2, 100 μM), selective $P2Y_1$ receptor antagonist MRS2179 (10 μM) or the $P2X_{1/3}$ receptor desensitizing agent α,β-meATP (10 μM) were added to the perfusate 30 min before challenge with $Up_4A$. In some experiments, NOS was inhibited by continuous perfusion with $N^G$-nitro-L-arginine methyl ester (L-NAME) (100 μM). In some experiments, we performed endothelial cell removal prior to the experiments. Endothelium removal was performed with Triton X-100. The endothelium was removed by perfusion of the isolated kidney for 5 s with 0.1% Triton X-100. The lack of response to acetylcholine was used to check endothelium removal. Unaffected contraction to $K^+$ (130 mM bolus) indicated an intact vascular smooth muscle cell function, which was tested before and after endothelium removal.

### *Continuous perfusion with $Up_4A$*

Vasoconstrictor responses to continuous perfusion with $Up_4A$ were assessed at basal tone. Dose–response curves were constructed for each substance, with 20 min being allowed to elapse between consecutive continuous perfusions. A significant cross-desensitization or auto-desensitization was not detected when substances were being given in intervals of at least 20 min. Desensitization was tested prior to experiments with all substances used (data not shown), and the results were compatible with previous observations with purinergic substances (van der Giet *et al.*, 1999).

### *Continuous perfusion with P2X receptor antagonists*

The non-selective P2 receptor antagonist suramin (100 μM) and the P2X receptor antagonist PPADS (10 μM) were added to the perfusate 30 min before challenge with $Up_4A$. In an additional experiment, the P2X receptor agonist α,β-meATP (10 μM) was also perfused before challenge with $Up_4A$.

### *Raised tone preparations*

Vasodilator responses to doses of $Up_4A$ and ACh were assessed in raised-tone preparations. Perfusion pressure was increased by continuous perfusion with AngII (200 nM). The resistance of vasodilator responses to desensitization and the reproducibility of responses with time-allowed dose–response curves for several agonists to be constructed for the same preparation. The P2 receptor antagonist suramin (100 μM), PPADS (10 μM, 100 μM), RB2 (100 μM), MRS 2179 (10 μM) and the antagonist of NOS L-NAME (100 μM) were added to the perfusate 30 min before challenge with $Up_4A$.

### *Assessment of oedema during perfusion experiments*

To assess the development of oedema during the perfusion experiments, rat kidneys were weighed before and after the experiments. After perfusion, the weight was 132 ± 16% of the initial weight, indicating that a slight oedema of the kidneys developed. The response to 10 nM AngII at the end of the experiments was 106 ± 9% of the initial response.

### *Data analysis*

Responses were measured as changes in perfusion pressure (mmHg), and results presented as the means ± SEM and if necessary their 95% confidence interval (95% CI). Statistical analysis was performed using Friedman's test. To compare columns for statistical variance, we applied Dunn's correction where applicable. $P < 0.05$ were considered significant.

### *Materials*

All vasoactive substances were applied as 100 μL bolus into a valve proximal to the perfused kidney preparation. Drug dilutions were performed daily from stock solutions of 10 mM (concentrates stored frozen) in HPLC grade water or HPLC grade

dimethyl sulphoxide (DMSO) unless otherwise indicated. Heparin (sodium salt), suramin (hexasodium salt), α,β-meATP and ketamine/xylazine were purchased from Sigma Aldrich (Schnelldorf, Germany). Up$_4$A was purchased from Jena Bioscience (Jena, Germany). Prior to use, Up$_4$A was purified according to a procedure described by Heidenreich *et al.* (1995).

## Results

### *Vasoconstrictor responses in basal tone preparations*

The baseline perfusion pressure of the rat isolated perfused kidneys decreased by 3.0 ± 0.5 mmHg during the first, and by 2.0 ± 1.5 mmHg during the second hour of perfusion. Vascular reactivity to vasoactive agents did not diminish during this time. After the equilibration period, the baseline pressure was 68 ± 2 mmHg ($n$ = 73). The addition of suramin (100 μM) to the perfusate caused an increase of perfusion pressure of 7 ± 5 mmHg. After addition of L-NAME (100 μM), non-significant increases of perfusion pressure of 10 ± 4 mmHg and for RB2 (100 μM) of 6 ± 2 mmHg were observed. The addition of MRS2179 (10 μM), PPADS (10 μM) to the perfusate did not induce any change in baseline perfusion pressure (data not shown).

At basal tone, Up$_4$A caused a dose-dependent vasoconstriction ($EC_{50}$ [log mol] = –8.3 ± 0.1 and maximal change in perfusion pressure ($V_{max}$) of 107 ± 8 mmHg, $n$ = 7, Figure 1A,B). In the presence of the P2X$_1$ receptor desensitizer α,β-meATP (10 μM) in the perfusate, responses to bolus application of Up$_4$A were significantly decreased, but not completely abolished ($EC_{50}$ [log mol] = –8.6 ± 0.2, $V_{max}$ = 32 ± 3 mmHg, $n$ = 7, Figure 1A,B). This remaining vasoconstriction could be almost totally blocked by the non-selective P2 receptor antagonist suramin (100 μM), whereas the selective P2Y$_1$ receptor antagonist MRS2179 (10 μM), the non-selective P2 receptor antagonist PPADS (10 μM) or RB 2 (100 μM) had no effect (Figure 1C). UTP induced a dose-dependent vasoconstriction [$EC_{50}$ [log mol] = 7.9 ± 0.2 and maximum change in perfusion pressure ($V_{max}$) of 39.0 ± 1.9 mmHg, $n$ = 7, Figure 1D]. UDP only showed a very small change of perfusion pressure at high dosages. In the presence of suramin, there was a profound and significant ($P$ < 0.05) inhibition of UTP-induced vasoconstriction. PPADS also showed a significant ($P$ < 0.05) inhibitory effect. MRS2179 showed no significant effect. (Figure 1D).

### *Continuous perfusion with Up$_4$A*

Continuous perfusion with Up$_4$A led to a concentration-dependent increase of the perfusion pressure (Figure 2A). The perfusion pressure increase was divided into two phases. The first phase consists of a fast vasoconstrictive response with a rapid desensitization ($EC_{50}$ [log mol·L$^{-1}$] = –6.6 ± 0.1, $V_{max}$ = 96.2 ± 9.2 mmHg, $n$ = 8, Figure 2B). The second phase was characterized by a long-lasting, stable vasoconstriction ($EC_{50}$ [log mol·L$^{-1}$] = –6.6 ± 0.1, $V_{max}$ = 42 ± 2 mmHg, $n$ = 8, Figure 2C). Both phases showed a concentration-dependent vasoconstriction. The first, fast desensitization effect of Up$_4$A could be completely inhibited by parallel continuous perfusion with α,β-meATP, suramin and PPADS. MRS2179 and RB2 had no significant ($P$ < 0.05) inhibitory effect (Figure 2D).

The second, long-lasting vasoconstrictive effect of Up$_4$A was significantly ($P$ < 0.05) blocked by suramin, whereas PPADS, MRS2179 and RB2 showed no significant inhibition (Figure 2E).

### *Vasoactive responses in raised tone preparations*

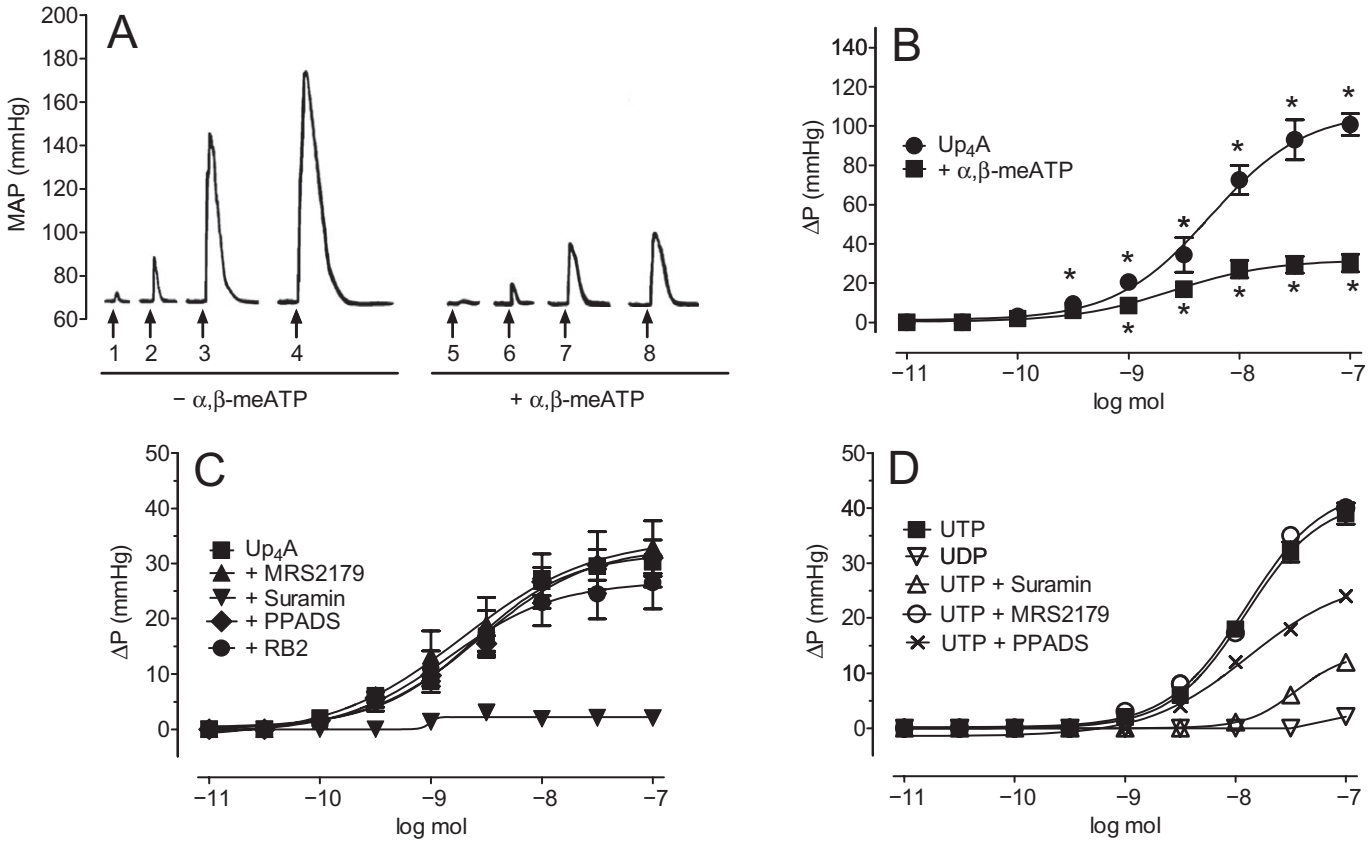
The basal pressure of the rat isolated perfused kidney was raised by continuous perfusion with AngII (200 nM) by 76.2 ± 4.2 mmHg. Under conditions of raised perfusion pressure, Up$_4$A induced a dose-dependent increase of the perfusion pressure ($EC_{50}$ [log M] = –8.7 ± 0.1, $V_{max}$ = 87.5 ± 3.7 mmHg, $n$ = 7, Figure 3A,B). This vasoconstriction was followed by a dose-dependent decline of perfusion pressure ($EC_{50}$ [log M] = –8.0 ± 0.1, $V_{max}$ = –65.7 ± 9.3 mmHg, $n$ = 7, Figure 3A,C).

As was the case under basal conditions, the initial increase of the perfusion pressure was significantly attenuated by α,β-meATP, suramin and PPADS, whereas MRS2179 and RB2 had no significant effect (Figure 4A). Under conditions of continuous perfusion with AngII (200 nM) and α,β-meATP (10 μM), a residual perfusion pressure increase by Up$_4$A could be detected. This remaining vasoconstriction was significantly blocked by suramin. PPADS, RB2 and MRS2179 had no significant effect on this remaining perfusion pressure increase (Figure 4B).

The Up$_4$A (10 pmol)-induced decrease of the perfusion pressure could be significantly diminished by continuous perfusion with the non-selective NOS antagonist L-NAME and after chemical removal of the endothelium with Triton X-100 (Figure 4C). The vasodilatation could also be significantly attenuated by the non-selective P2 receptor antagonists suramin, PPADS and RB2, and by the selective P2Y$_1$ receptor antagonist MRS2179 (Figure 4D).

## Discussion

Our results clearly demonstrated that Up$_4$A activated at least three different purinoceptor subtypes

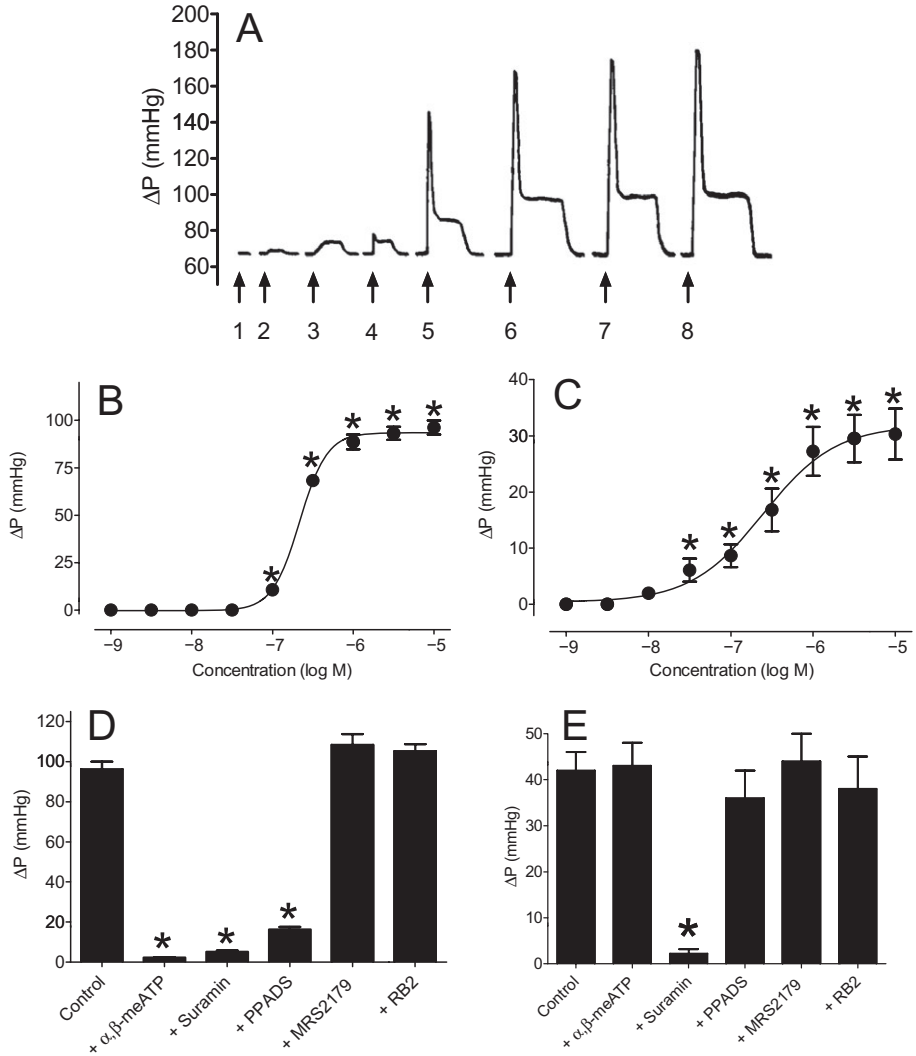



## Figure 1

(A) Original tracing of a representative experiment of the rat isolated perfused kidney showing the dose-dependent increase of the perfusion pressure (MAP) induced by $Up_4A$ in the absence (1–4) and the presence of α,β-meATP (1 μM; 5–8) (1:100 pmol; 2:1 nmol; 3:10 nmol; 4:100 nmol; 5:100 pmol; 6:1 nmol; 7:10 nmol; 8:100 nmol). (B) Dose–response curve of changes in perfusion pressure in the rat isolated perfused kidney induced by $Up_4A$ in the absence and presence of α,β-meATP. Each point is the mean of seven determinations, and vertical lines show SEM. For abbreviations, see text. Where error bars do not appear in figures, errors are within the symbol size. *<0.05 significant difference from baseline perfusion pressure. (C) Dose–response curve of changes in perfusion pressure in the rat isolated perfused kidney induced by $Up_4A$ in the presence of α,β-meATP (10 μM) and in the presence of the P2Y receptor antagonists suramin (50 μM), PPADS (10 μM), the specific $P2Y_1$ receptor antagonist MRS2179 (10 μM), and RB2 (100 μM). Each point is the mean of seven determinations and vertical lines show SEM. Where error bars do not appear in figures, errors are within the symbol size. *<0.05 significant difference from baseline perfusion pressure. (D) Dose–response curve of changes in perfusion pressure in the rat isolated perfused kidney induced by UDP and UTP in the presence of the P2 receptor antagonists suramin (100 μM), PPADS (10 μM) and the specific $P2Y_1$ receptor antagonist MRS2179 (10 μM). Each point is the mean of seven determinations, and vertical lines show SEM. Where error bars do not appear in figures, errors are within the symbol size. *<0.05 significant difference from baseline perfusion pressure.

in the kidney to induce a complex vasoactive response. Besides the $P2X_1$ receptor, which induces vasoconstriction (Jankowski *et al.*, 2005), we infer from our data that there was also a $P2Y_2$ receptor activation. The $P2Y_2$ receptor is responsible for additional, long-lasting vasoconstriction. Activation of the $P2Y_1$/$P2Y_2$ receptors results in an endothelium-dependent, NOS-mediated vasodilation.

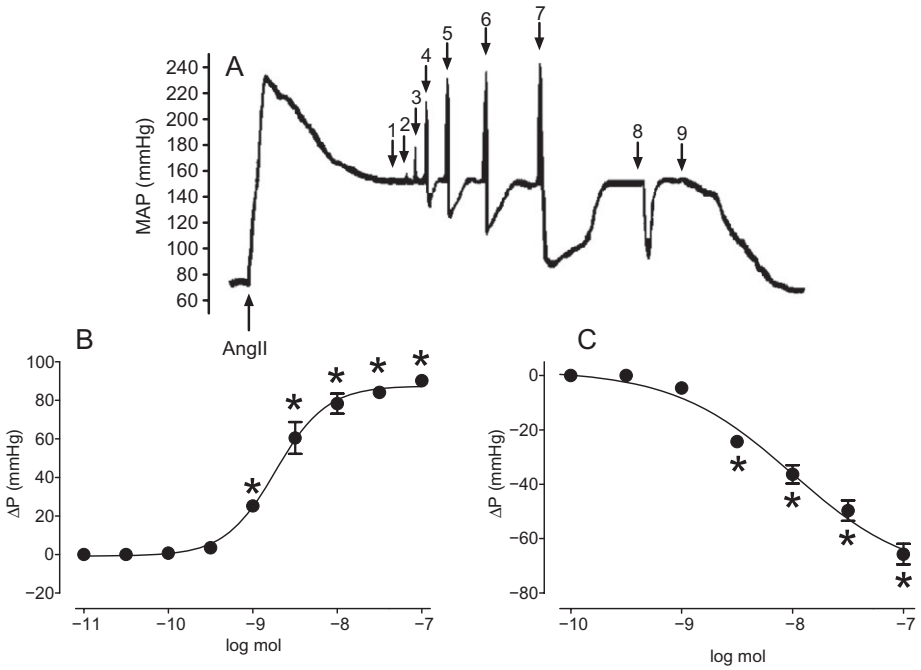
Studying the purinoceptor subtypes involved in the vasoconstriction and vasodilation observed in the current experiments is a complex undertaking. There are no specific pharmacological active agonists and antagonists available for purinoceptors. The purinoreceptor expression profile in kidney tissue has been extensively studied, but there are many open questions. Turner and coworkers demonstrated the expression of $P2X_1$, $P2X_2$ and $P2Y_1$ receptors in rat renal vascular smooth muscle cells (Turner *et al.*, 2003). Using a pharmacological approach, Inscho and colleagues identified a receptor-mediating vasoactive response in renal tissue that was activated by UTP (Inscho *et al.*, 1998). As UTP can activate $P2Y_{2/4}$ receptors, it is tempting to speculate that these receptors can activate response in renal tissue. Potentially, these receptor subtypes are expressed at a very low level and therefore they cannot be detected by immunohistochemistry (Turner *et al.*, 2003). In the present work, we demonstrated that UTP showed potent vasoactive actions, which were inhibited by




## Figure 2

(A) Original tracing of a representative experiment of the rat isolated perfused kidney showing the concentration-dependent increase of the perfusion pressure induced by the continuous perfusion with various concentrations of $Up_4A$ (1 = 1 nM; 2 = 10 nM; 3 = 50 nM; 4 = 100 nM, 5 = 500 nM; 6 = 1 μM; 7 = 5 μM; 8 = 10 μM). A biphasic vasoconstrictor response was observed; an initial transient effect and a subsequent sustained phase of vasoconstriction. (B) Concentration–response curve of the first short-acting part of the perfusion pressure change induced by continuous perfusion with $Up_4A$. Each point is the mean of eight experiments, and vertical lines show SEM. Significant difference ($^*P < 0.05$) from baseline perfusion pressure of the $Up_4A$ concentration (bolus application). Where error bars do not appear in figures, errors are within the symbol size. (C) Concentration–response curve of the sustained part of the perfusion pressure change induced by continuous perfusion with $Up_4A$. Each point is the mean of eight experiments, and vertical lines show SEM. Significant difference ($^*P < 0.05$) from baseline perfusion pressure of the $Up_4A$ concentration (bolus application). Where error bars do not appear in figures, errors are within the symbol size. (D) Influence of different P2 antagonists on the first short-acting part of the perfusion pressure increase induced by $Up_4A$ (5 μM). The $P2X_1$ and $P2X_3$ desensitizer α,β-meATP (10 μM), and the non-selective P2 receptor antagonist suramin (100 μM) and PPADS (10 μM) significantly inhibited ($^*P < 0.05$) the $Up_4A$-induced perfusion pressure increase, whereas the selective $P2Y_1$ receptor antagonist MRS2179 (10 μM) and the non-selective P2 receptor antagonist RB2 (100 μM) had no significant influence. (E) Influence of different P2 antagonists on the second sustained part of the perfusion pressure increase induced by $Up_4A$ (5 μM). In the presence of the $P2X_1$ and $P2X_3$ desensitizer α,β-meATP (10 μM), there was no significant effect on the $Up_4A$-induced sustained perfusion pressure. In the presence of the non-selective P2 receptor antagonist suramin (100 μM), the sustained perfusion pressure increase was significantly decreased ($^*P < 0.05$), whereas the selective inhibition of the $P2Y_1$ receptor by MRS2179 (10 μM) and the non-selective P2 antagonists PPADS (10 μM) and RB2 (100 μM) had no significant effect.

suramin, but not PPADS, indicating that the $P2Y_2$ receptor might be a receptor with vasoactive actions in the kidney. UDP, which is an agonist at $P2Y_6$ receptors, induced only a very mild vasoconstriction at high doses. In 2005, we identified $Up_4A$ as a potent, endothelium-derived vasoactive substance, and we identified the $P2X_1$ receptor as the main receptor mediating the observed vasoconstrictor

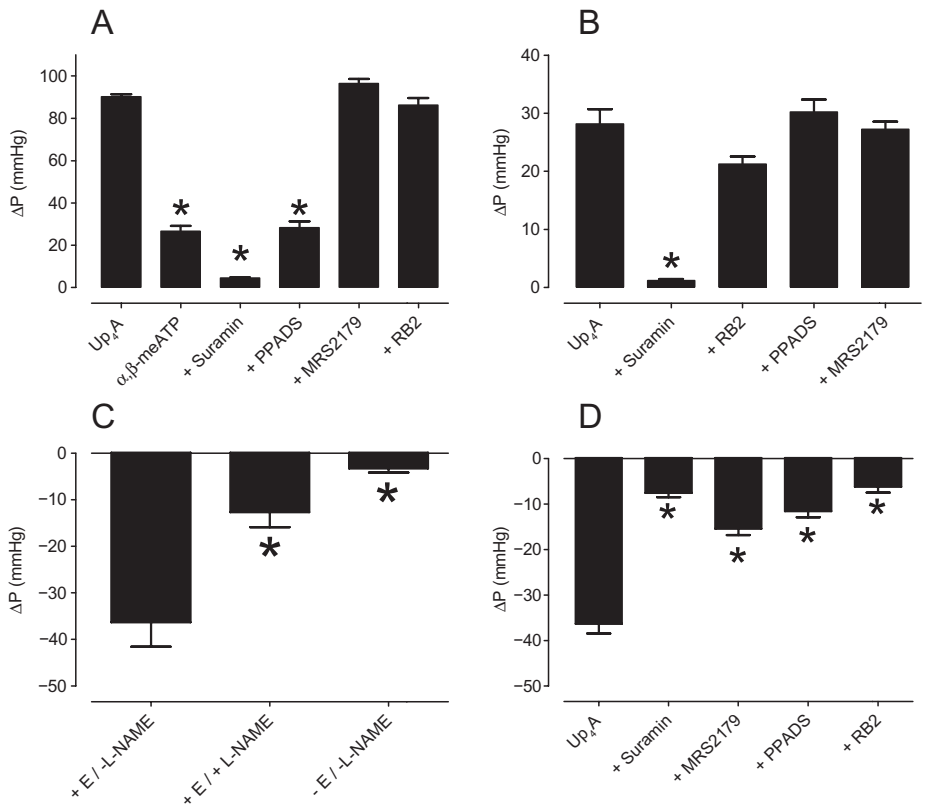



## Figure 3

(A) Original tracing of a representative experiment performed on the rat isolated perfused kidney showing the effect of bolus application of $Up_4A$ under raised tone with AngII (200 nM). The bolus application of $Up_4A$ induced a dose-dependent, rapid increase of the perfusion pressure with a subsequent dose-dependent vasodilatation. 1: $Up_4A$ 100 pmol; 2: $Up_4A$ 500 pmol; 3: $Up_4A$ 1 nmol; 4: $Up_4A$ 5 nmol; 5: $Up_4A$ 10 nmol; 6: $Up_4A$ 50 nmol; 7: $Up_4A$ 100 nmol; 8: ACh 1 nmol; 9: wash-out. (B) Dose–response curve of the first vasoconstrictor effect of $Up_4A$ under raised basal tone. Each point is the mean of seven determinations, and vertical lines show SEM. Significant difference (*$P < 0.05$) from increased perfusion pressure. For abbreviations, see text. Where error bars do not appear in figures, errors are within the symbol size. (C) Dose–response curve of the second vasodilator effect of $Up_4A$ under raised basal tone. Each point is the mean of six determinations, and vertical lines show SEM. Significant difference (*$P < 0.05$) from baseline perfusion. For abbreviations, see text. Where error bars do not appear in figures, errors are within the symbol size.

effects. There were hints that further P2 receptors are involved in the $Up_4A$-induced vasoconstriction (Jankowski *et al.*, 2005). Here, we could show that the $Up_4A$-induced vasoconstriction depends not only on the activation of the $P2X_1$ receptor (Jankowski *et al.*, 2005), but also on the activation of the $P2Y_2$ receptor. Under basal conditions, the bolus application of $Up_4A$ induced a dose-dependent vasoconstriction in the rat isolated perfused kidney. The $Up_4A$-induced vasoconstriction was blocked by the $P2X_1$ receptor desensitizer α,β-meATP, the non-selective P2 receptor antagonist suramin and PPADS. After α,β-meATP-induced desensitization of the $P2X_1$ receptor, the bolus application of $Up_4A$ induced a dose-dependent vasoconstriction. This vasoconstriction was completely blocked by the non-selective P2 receptor antagonist suramin. Suramin mainly inhibits activation of $P2Y_1$ and $P2Y_2$ receptors, and shows only weak low affinity at the $P2Y_6$ receptor (von Kugelgen, 2006). There was no significant inhibition of $Up_4A$-induced vasoconstriction by the non-selective P2 receptor antagonist RB2. RB2 inhibits activation of the $P2Y_1$, $P2Y_4$ and $P2Y_6$ receptors. There is also a very low inhibitory affinity at the $P2Y_2$ receptor (von Kugelgen, 2006). PPADS is a potent antagonist of the $P2Y_1$ and $P2Y_4$ receptors (von Kugelgen, 2006), whereas MRS2179 is the only tested selective antagonist at the $P2Y_1$ receptor (von Kugelgen, 2006). PPADS and MRS2179 showed no significant antagonistic effects on $Up_4A$-induced vasoconstriction. Taken together, these observations suggest that the $P2Y_2$ receptor is responsible for the residual vasoconstriction induced by $Up_4A$. The observations are in line with recent findings showing that the $P2Y_2$ receptor mediates the UTP-induced vasoconstriction in porcine isolated arteries. The $P2Y_4$ and $P2Y_6$ receptors were not involved (Rayment *et al.*, 2007). In control experiments, we can show that the UTP-induced vasoconstriction in the rat isolated perfused kidney was inhibited by suramin and not by PPADS.

Continuous perfusion of the rat isolated perfused kidney with $Up_4A$ induced a fast, concentration-dependent perfusion pressure increase with a subsequent desensitization. In addition, continuous perfusion with $Up_4A$ induced a sustained increase in perfusion pressure without signs of desensitization. The first part of the $Up_4A$-induced vasoconstriction




## Figure 4

(A) Influence of different P2 antagonists on the first short-acting part of the perfusion pressure increase induced by $Up_4A$ (5 μM). The $P2X_1$ and $P2X_3$ receptor desensitizer α,β-meATP (10 μM), and the non-selective P2 receptor antagonist suramin (100 μM) and PPADS (10 μM) significantly inhibited (*$P < 0.05$) the $Up_4A$-induced perfusion pressure increase, whereas the selective $P2Y_1$ receptor antagonist MRS2179 (10 μM) and the non-selective P2 receptor antagonist RB2 (100 μM) had no significant influence. (B) Influence of different P2 antagonists on the second sustained part of the perfusion pressure increase induced by $Up_4A$ (5 μM). In the presence of the $P2X_1$ and $P2X_3$ receptor desensitizer α,β-meATP (10 μM), the $Up_4A$-induced sustained perfusion pressure increase was significantly enhanced. In the presence of the non-selective P2 receptor antagonist suramin (100 μM), the sustained perfusion pressure increase was significantly decreased (*$P < 0.05$), whereas the selective inhibition of the $P2Y_1$ receptor by MRS2179 (10 μM) and the non-selective P2 antagonists PPADS (10 μM) and RB2 (100 μM) had no significant effect. (C) In the presence of an intact endothelium, the eNOS antagonist L-NAME (300 $\mu mol \cdot L^{-1}$) significantly reduced (*$P < 0.05$) the $Up_4A$- (10 nmol) induced decrease of the perfusion pressure. After removal of the endothelium, $Up_4A$ (10 nmol) could not induce any significant decrease of perfusion pressure. (D) The $Up_4A$-induced perfusion pressure decrease could be significantly inhibited (*$P < 0.05$) by the non-selective P2 receptor antagonist suramin (100 μM), the selective $P2Y_1$ receptor antagonist MRS2179 (10 μM), the non-selective P2 receptor antagonist PPADS (10 μM) and RB2 (10 μM).

was attenuated by α,β-meATP, suramin and PPADS, whereas RB2 and MRS2179 again had no significant effect. Thus, this part of the vasoconstriction is due to an activation of the $P2X_1$ receptor. The second part of the $Up_4A$-induced vasoconstriction was significantly attenuated by suramin, whereas α,β-meATP, PPADS, RB2 and MRS2179 had no effect. The continuous activation of the $P2Y_2$ receptor was responsible for this sustained $Up_4A$-induced vasoconstriction. Recently, it was shown in young hypertensive patients that the $Up_4A$ concentration correlates with blood pressure, left ventricular mass and intima media thickness (Jankowski *et al.*, 2007). It is possible that $P2Y_2$ receptor activation is involved in the physiology and pathophysiology of blood pressure regulation. After inter-arterial application of $Up_4A$ in rats, there is a potent transient increase in the mean arterial blood pressure (MAP) with signs of desensitization (Jankowski *et al.*, 2005). After the initial increase in blood pressure, there is a sustained increase in MAP. This is further evidence that $Up_4A$ is a compound that regulates and increases blood pressure (Jankowski *et al.*, 2005).

In the experiments with AngII-induced raised perfusion pressure in the rat isolated perfused kidney, we observed a biphasic, vasoactive response to bolus application of $Up_4A$. The first response was a dose-dependent increase in vascular tone that was followed by a sustained decrease of perfusion pressure. As was the case under basal conditions, the perfusion pressure increase could be significantly

attenuated by suramin, α,β-meATP and PPADS, whereas MRS2179 and RB2 showed no effect. In the presence of α,β-meATP, a perfusion pressure increase could be seen, which was blocked by suramin, whereas PPADS, RB2 and MRS2179 had no effect. Therefore, this remaining perfusion pressure increase was due to the activation of the $P2Y_2$ receptor. The $Up_4A$-induced perfusion pressure decrease was not present after de-endothelialization or after inhibition of NOS by L-NAME. $Up_4A$ activates NOS by stimulating $P2Y_1$ and $P2Y_2$ receptors on endothelial cells. The $Up_4A$-induced perfusion pressure decrease was partially inhibited by the $P2Y_1$ receptor antagonist MRS2179. In addition, the non-selective P2Y receptor antagonist RB2, which is a potent antagonist of the $P2Y_1$ and $P2Y_6$ receptors, and a weak antagonist of the $P2Y_2$ and $P2Y_4$ receptors, and the non-selective P2Y receptor antagonist PPADS, a potent antagonist of the $P2Y_1$ receptor, and a weak antagonist of the $P2Y_4$ and $P2Y_6$ receptors inhibited the $Up_4A$-induced vasodilation. A nearly complete inhibition of the $Up_4A$-induced reduction of perfusion pressure could be observed using suramin, a potent antagonist of the $P2Y_1$ and $P2Y_2$ receptors, and a weak antagonist of the $P2Y_6$ receptor. Thus, $Up_4A$ appears to activate NOS via stimulation of the $P2Y_1$ and $P2Y_2$ receptors. $Up_4A$-induced vasodilation is inhibited by the $P2Y_1$ receptor antagonist MRS2179 and even more pronounced inhibition by suramin. Suramin is not an antagonist of the $P2Y_4$, but a potent antagonist of the $P2Y_2$ receptor. These findings are in accordance with recent findings showing the activation of eNOS by stimulation of the $P2Y_1$, $P2Y_2$ and possibly the $P2Y_4$ receptors (da Silva *et al.*, 2009). Currently, it is not possible to study single P2Y receptor subtypes due to the lack of selective antagonists. From our experiments, we cannot exclude the possibility that the $P2Y_4$ receptor might play a role in NOS activation, but the expression of this receptor is low in the rat kidney.

We and others were able to demonstrate previously that diadenosine polyphosphates such as $Ap_4A$ activate various purinoceptor subtypes to induce vasoconstriction and vasodilation in isolated arterial vessels or in the rat isolated perfused kidney. $Ap_4A$ mainly induces vasoconstriction in the rat isolated perfused kidney and in the mesenteric bed via activation of $P2X_1$ receptors (van der Giet *et al.*, 1998; Gabriels *et al.*, 2002). In addition, $Ap_4A$ could also induce an endothelium-dependent vasodilation in isolated mesenteric arteries which was mainly attributable to the activation of endothelial expressed P2Y receptors (Busse *et al.*, 1988). However, those diadenosine polyphosphates with more than four phosphate groups mainly activate P2X receptors to induce vasoconstriction in most vascular models tested (Gabriels *et al.*, 2002). Using the rat isolated perfused kidney model, no vasodilation was observed in response to $Ap_5A$ or $Ap_6A$ (van der Giet *et al.*, 1997). However Gabriels and coworkers reported a possible vasodilation induced by $Ap_5A$ or $Ap_6A$ in the renal microcirculation (Gabriels *et al.*, 2000), which might potentially be attributable to the degradation products of $Ap_5A$ and $Ap_6A$. $Ap_4G$, $Ap_5G$ and $Ap_6G$ are also potent vasoconstrictors activating $P2X_{1/3}$ receptors in the rat isolated perfused kidney (Cinkilic *et al.*, 2001; van der Giet *et al.*, 2001). There was no $Ap_5G$- or $Ap_6G$-induced vasodilation observed in the rat isolated perfused kidney, whereas in coronary arteries we were able to show that $Ap_5A$, $Ap_5G$, $Ap_6A$ and $Ap_6G$ can activate $P2Y_1$ receptors with consequent activation of eNOS (van der Giet *et al.*, 2002b). The adenosine- and guanosine-containing dinucleoside polyphosphates mainly activate $P2X_1$ receptors to induce vasoconstriction in vascular models, and P2Y receptor-mediated vasoactive effects are rarely reported for these substances (Gabriels *et al.*, 2002). In the present work, we showed that the uridine-containing dinucleoside $Up_4A$ robustly activated $P2Y_{1/2}$ receptors, as well as $P2X_1$ receptors. This is of interest as there are some reports that activation of the $P2Y_2$ receptor is involved in the regulation of blood pressure. It was shown that mice lacking $P2Y_2$ receptor have salt-resistant hypertension and facilitated renal $Na^+$ and water reabsorption (Rieg *et al.*, 2007). In addition, there is paracrine regulation of the epithelial $Na^+$ channel in the mammalian collecting duct by purinergic $P2Y_2$ receptors (Pochynyuk *et al.*, 2008). All these mechanisms are believed to be relevant in blood pressure regulation. In addition, there are reports that activation of $P2Y_2$ receptor signalling might be potentially relevant in pro-inflammatory vascular disease conditions like atherosclerosis. Activation of $P2Y_2$ receptors in peritoneal macrophages can lead to the production of the chemokine CCL2 (monocyte chemoattractant protein 1; MCP-1) (Stokes and Surprenant, 2007), and this is a key chemokine in the initiation and progression of atherosclerotic disease (Viedt *et al.*, 2002). Activation of the $P2Y_1$ receptor also contributes to the pathogenesis of atherosclerosis, as elegantly demonstrated with $P2Y_1$/ApoE double knock-out mice (Hechler *et al.*, 2008). Unfortunately, the precise mechanism is not fully understood to date.

In summary, we were able to demonstrate that $Up_4A$ induced renal vasoconstriction in perfused kidneys via activation of the $P2X_1$ receptor, which shows fast desensitization, and via activation of the $P2Y_2$ receptor, which induces sustained vasoconstriction. $Up_4A$ activated NOS via stimulation of the

endothelial $P2Y_1$ and $P2Y_2$ receptors. Elevated serum concentrations of $Up_4A$ can potentially induce a significant elevation of the blood pressure. P2Y receptor signalling initiated by $Up_4A$ might be relevant in blood pressure control, in the pathogenesis of human hypertension and in the progression of vascular inflammatory disease.

## Acknowledgements

The study was supported by a grant from the Deutsche Nierenstiftung (M.T.), Deutsche Forschungsgemeinschaft (JA972/11-1, M.vdG. and J.J.), Sonnenfeld-Stiftung (M.vdG. and M.T.), BMBF (0313920D), Deutsche Hochdruckliga (M.T.), Dr Robert-Pfleger-Stiftung (M.T. and M.vdG.), Manchot-Stiftung (M.T.) and the Dr Werner Jackstädt-Stiftung (M.vdG. and M.T.).

## Conflict of interest

None.

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