Kidney

Alkaline Phosphatase Inhibitors Attenuate Renovascular Responses to Norepinephrine

Edwin K. Jackson, Yumeng Zhang, Dongmei Cheng

Abstract—Tissue nonspecific alkaline phosphatase (TNAP) contributes to the production of adenosine by the kidney, and A1-receptor activation enhances renovascular responses to norepinephrine. Therefore, we hypothesized that TNAP regulates renovascular responsiveness to norepinephrine. In isolated, perfused rat kidneys, the TNAP inhibitor l-p-bromotetramisole (0.1 mmol/L) decreased renal venous levels of 5′-AMP (adenosine precursor) and adenosine by 61% (P<0.0384) and 62% (P=0.0013), respectively, at 1 hour into treatment and caused a 10-fold rightward shift of the concentration–response relationship to exogenous norepinephrine (P<0.0001). Similarly, 2 other TNAP inhibitors, levamisole (1 mmol/L) and 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide (0.02 mmol/L), also right shifted the concentration–response relationship to norepinephrine. The ability of TNAP inhibition to blunt renovascular responses to norepinephrine was mostly prevented or reversed by restoring A1-adenosinergic tone with the A1-receptor agonist 2-chloro-N9-cyclopentyladenosine (100 mmol/L). All 3 TNAP inhibitors also attenuated renovascular responses to renal sympathetic nerve stimulation, suggesting that TNAP inhibition attenuates renovascular responses to endogenous norepinephrine. In control propranolol-pretreated rats, acute infusions of norepinephrine (10 μg/kg/min) increased mean arterial blood pressure from 95±5 mm Hg to a peak of 169±4 mm Hg and renovascular resistance from 12±2 mm Hg/mL/min to a peak of 55±12 mm Hg/mL/min; however, in rats also treated with intravenous l-p-bromotetramisole (30 mg/kg), the pressor and renovascular effects of norepinephrine were significantly attenuated (blood pressure: basal and peak, 93±7 and 146±6 mm Hg, respectively; renovascular resistance: basal and peak, 13±2 and 29±5 mm Hg/mL/min, respectively). TNAP inhibitors attenuate renovascular and blood pressure responses to norepinephrine, suggesting that TNAP participates in the regulation of renal function and blood pressure. (Hypertension. 2017;69:484-493. DOI: 10.1161/HYPERTENSIONAHA.116.08623.) • Online Data Supplement

Key Words: adenosine ■ adenosine receptors ■ alkaline phosphatase ■ kidney ■ norepinephrine ■ vasoconstriction

Previously, we discovered that activation of A1-receptors by endogenous adenosine modulates renovascular responses to renal sympathetic nerve stimulation (RSNS) and to exogenous norepinephrine.1,2 This conclusion is supported by our observations that in isolated, perfused rat kidneys selective A1-receptor antagonism reduces renovascular responses to RSNS1 and that in isolated, perfused mouse kidneys A1-receptor deletion suppresses renovascular responses to RSNS and exogenous norepinephrine.3 Mechanistically, there are 3 reasons A1-receptors contribute to RSNS-induced renal vasoconstriction: (1) RSNS triggers adenosine formation4–6; (2) preglomerular microvessels express high levels of vasoconstrictor A1-receptors; and (3) in the renal vasculature, the Gi signaling pathway (which adenosine acting via the A1-receptor engages) converges with the Gq signaling pathway (which norepinephrine acting via the α1-adrenoceptor engages) to trigger coincident signaling at phospholipase C, leading to augmentation by adenosine of the renovascular response to released norepinephrine.1 These facts may explain why most of the RSNS-induced increase in renovascular resistance is caused by contraction of the preglomerular microcirculation6 (where A1-receptors are highly expressed).

Because ATP is released from noradrenergic varicosities,7–10 as well as from vascular smooth muscle11,12 and endothelial cells,13–16 the main precursor of adenosine in the renal vasculature is most likely ATP. CD39 catalyzes the metabolism of ATP to ADP and ADP to 5′-AMP, and CD73 metabolizes 5′-AMP to adenosine; thus, these twin ectoenzymes acting in tandem are justifiably considered the most important mechanism for producing extracellular adenosine from ATP.17–20 Surprisingly, however, our experiments show that in isolated, perfused mouse kidneys, neither pharmacological inhibition nor genetic deletion of CD73 attenuates renovascular responses to RSNS.21 Moreover, our unpublished experiments show that in mouse kidneys even high concentrations (100 μmol/L) of the potent CD39 inhibitor ARL67156 have no effect on renovascular responses to RSNS.

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To reconcile our findings, we hypothesize that although CD39 and CD73, acting in tandem, provide the most important pathway of adenosine production in most biological contexts, this may not be true for all biological compartments. In this regard, it is important to note that tissue nonspecific alkaline phosphatase (TNAP) is in many ways similar to CD73. Both of these ectoenzymes are anchored to cell membranes by glycosylphosphatidylinositol (GPI) with the catalytic domains facing the extracellular space, contain metal ions (eg, Zn\(^{2+}\)), are glycosylated, have similar molecular weights, form homonomic dimers, are widely expressed, can be released as soluble forms, and can catalyze conversion of AMP to adenosine. However, unlike CD73, TNAP does not require CD39 to complete the ATP to adenosine pathway; that is, the entire biochemical pathway (ATP→ADP→5′-AMP→adenosine) can be accomplished by TNAP.

Because CD39 and CD73 do not seem to be involved in producing the adenosine that regulates renal sympathetic neurotransmission and because TNAP mRNA, protein, and activity are present in kidneys and TNAP contributes to the metabolism of 5′-AMP to adenosine in kidneys, TNAP may be involved in modulating renovascular responses to norepinephrine. We, therefore, hypothesized that TNAP inhibition would attenuate renovascular responses to exogenous norepinephrine presented to the luminal aspect of renal blood vessels and to endogenous norepinephrine presented to the abluminal side of renal blood vessels via RSNS.

**Methods**

**Materials**

Levamisole, 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, 0.02 mmol/L. Each inhibitor, 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, 0.02 mmol/L. Each inhibitor was obtained from Sigma-Aldrich (St Louis, MO). l-p-Bromotetramisole was obtained from Santa Cruz Biotechnology (Dallas, TX).

**Animals**

This study used male Sprague-Dawley rats (Charles River, Wilmington, MA) that were +16 weeks of age. The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Isolated, Perfused Rat Kidney**

Rats were anesthetized with thiobutabarbital (100 mg/kg, IP injection). Kidneys were isolated without interruption of perfusion, transferred to a Hugo Sachs Elektronik-Harvard Apparatus GmbH (March-Hugstetten, Germany) kidney perfusion system, and perfused at 5 mL/min (constant flow) with oxygenated (95% O\(_2\)/5% CO\(_2\)) Tyrode solution (contains in mmol/L: NaCl, 137.0; KCl, 2.7; CaCl\(_2\), 1.8; MgCl\(_2\), 1.1; NaHCO\(_3\), 12.0; NaH\(_2\)PO\(_4\), 0.42; and n (+)-glucose, 5.6). The kidney perfusion system included the following components: Model UP 100 Universal Perfusion System; Model ISM 834 Channel Reglo Digital Roller Pump; a glass double-walled perfusion reservoir maintained at 37°C and oxygenated; a R 120144 glass-oxygenator maintained at 37°C; mechanical integration of the oxygenator with the Universal Perfusion System UP 100; a Windkessel for absorption of pulsations; an inline holder for disc particle filters (80 μm); a temperature-controlled plexiglass kidney chamber integrated with the UP 100; and a thermostatic circulator. The plexiglass chamber contained a heat exchanger to maintain the temperature of the perfusate at 37°C at the point of entry into the tissue and also contained a device to extract bubbles from the perfusate just before the perfusate entered the kidney. Perfusion pressure was monitored with a pressure transducer and recorded on a polygraph.

**Analysis of 5′-AMP and Adenosine**

5′-AMP and adenosine were quantified using ultraperformance liquid chromatography–tandem mass spectrometry, using our most recently updated version of the assay.

**Protocol 1**

Rat kidneys were isolated and perfused as described above and allowed to stabilize for 30 minutes. Renal venous samples were collected at baseline and then at 15-minute intervals after treatment with either vehicle (Tyrode solution) or 1-p-bromotetramisole (0.1 mmol/L). The samples were immediately heat inactivated (ie, placed in a heating block at 100°C for 90 seconds) to denature any enzymes that might degrade 5′-AMP or adenosine. Samples were stored at −80°C until assayed for 5′-AMP and adenosine as described above.

**Protocol 2**

In this and all protocols described below, the concentrations provided are final concentrations of the substance in the perfusate entering the kidney. Rat kidneys were isolated and perfused as described above and allowed to stabilize for 30 minutes. Next, norepinephrine was administered to the kidney at 0.03, 0.1, 0.3, 3, and 10 µmol/L for 5 minutes at each concentration, and the change in perfusion pressure from the initial baseline was recorded (ie, cumulative concentration–perfusion pressure response experiment; period 1). After a 30-minute washout, an inhibitor of TNAP was administered as follows: 1-p-bromotetramisole, 0.1 mmol/L; levamisole, 1 mmol/L; or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, 0.02 mmol/L. Each kidney received only a single TNAP inhibitor. The concentrations of TNAP inhibitors were selected on the basis of published concentrations of each necessary to inhibit TNAP. After 15 minutes, the cumulative concentration–perfusion pressure response experiment to norepinephrine was repeated in the presence of the TNAP inhibitor (period 2). For each TNAP inhibitor, a time-control group of kidneys was randomized into the protocol. In these groups, the experiment was identical to that described above, except that no inhibitors were administered during period 2.

**Protocol 3**

Rat kidneys were isolated and perfused as described above and allowed to stabilize for 30 minutes. After stabilization, baseline perfusion pressures were recorded, and then, norepinephrine was infused (0.5 µmol/L) for 5 minutes, and perfusion pressures were again recorded. After a 15-minute recovery period, kidneys were treated with the TNAP inhibitor 1-p-bromotetramisole (0.1 mmol/L), and this treatment was continued for the remainder of the experiment. After 15 minutes, perfusion pressures were again recorded before and 5 minutes into an infusion of norepinephrine (0.5 µmol/L). Next, kidneys were treated with the highly selective A₁-receptor agonist CCPA (100 nmol/L) and the CCPA treatment was continued for the remainder of the protocol. After 15 minutes, perfusion pressures were again recorded before and 5 minutes into an infusion of norepinephrine (0.5 µmol/L).

**Protocol 4**

Rat kidneys were isolated and perfused as described above and allowed to stabilize for 30 minutes. Next, kidneys were treated with the highly selective A₁-receptor agonist CCPA (100 nmol/L), and the CCPA treatment was continued for the remainder of the protocol. After 5 minutes, perfusion pressures were recorded before and 5 minutes into an infusion of norepinephrine (0.5 µmol/L). The norepinephrine treatment was stopped, and then, after 5 minutes, kidneys were treated with the TNAP inhibitor 1-p-bromotetramisole (0.1 mmol/L), and this treatment was continued for the remainder of the experiment. After 5 minutes, perfusion pressures were again recorded before and 5 minutes into an infusion of norepinephrine (0.5 µmol/L).
Procotol 5
Rat kidneys were isolated and perfused as described above. Immediately after initiating perfusion of the kidney, a platinum bipolar electrode was positioned around the renal artery close to the kidney for renal nerve stimulation, and the electrode was connected to a Grass stimulator (Model SD9E; Grass Instruments). After 30 minutes of stabilization, a response to RSNS was elicited at 5 Hz for 2 minutes. This response was used to normalize subsequent responses so as to reduce variability because of kidney-to-kidney differences in contact of the electrodes with periarterial sympathetic nerves.
Next, some kidneys were treated with an inhibitor of TNAP as follows: 1-p-bromotetramisole, 0.1 mmol/L; levamisole, 1 mmol/L; or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, 0.02 mmol/L. Each kidney received only a single TNAP inhibitor. Fifteen minutes after starting the TNAP inhibitor treatments, a frequency–perfusion pressure response relationship was generated by periarterial nerve stimulation at 2, 3, 4, 5, 6, 7, 8, 9, and 10 Hz for 2 minutes at 15-minute intervals. For each TNAP inhibitor group, a time-control group of kidneys was randomized into the protocol. In these groups, the experiment was identical to that described above, except that no inhibitors were administered.

Protocol 6
To determine whether TNAP inhibition alters renal levels of phosphate, perfused kidneys were treated with 1-p-bromotetramisole (0.1 mmol/L) and phosphate levels in kidney perfusate were measured before and during treatment with 1-p-bromotetramisole using the Abcam (Cambridge, MA) fluorometric phosphate assay kit (Cat no ab102508).

Protocol 7
To determine whether norepinephrine regulates the release of TNAP, TNAP activity (Abcam alkaline phosphatase assay Kit, Cat no ab83369) was measured in renal venous perfusate collected before and after treatment with norepinephrine (0.5 μmol/L for 5 minutes) in both naive kidneys and kidneys pretreated with CCPA (0.1 mmol/L; beginning 10 minutes before administering norepinephrine).

Protocol 8
Rats were anesthetized with thiobutabarbital (100 mg/kg, IP injection) and pretreated with propranolol (3 mg/kg, SC injection) to prevent norepinephrine-induced activation of β-adrenoceptors. Body temperature was monitored with a rectal temperature probe and maintained with an isothermal pad and heat lamp. Polyethylene (PE) cannulas were inserted into the trachea (PE-240) to facilitate respiration and into the carotid artery (PE-50) for measurement of mean arterial blood pressure (MAPB) using a digital blood pressure analyzer (BPA 200; Micro-Med, Inc, Louisville, KY). Also, 2 PE-10 cannulas were inserted into the jugular vein (one for administration of norepinephrine and the other for administration of 1-p-bromotetramisole). An IV infusion of 0.9% saline (50 μL/min) was initiated to maintain volume status. Next, a transit-time flow probe (Model 1RB; Transonic Systems, Inc, Ithaca, NY) was placed on the left renal artery to monitor renal blood flow (RBf). After a 30-minute stabilization period, rats were treated with either 2 IV slow-bolus injections (separated by 15 minutes) of vehicle (saline) or 1-p-bromotetramisole (total dose, 30 mg/kg). Administering the 1-p-bromotetramisole as 2 slow injections minimized basal blood pressure perturbations. Fifteen minutes after the second bolus of 1-p-bromotetramisole, norepinephrine was infused at 10 μg/kg/min for 10 minutes.

Protocol 9
Preglomerular vascular smooth muscle cells and pregglomerular vascular endothelial cells (PGVECs) were isolated and placed in cell culture as previously described.30 Cell protein extracts were obtained, and TNAP expression in pregglomerular vascular smooth muscle cells and PGVECs was determined and compared using Western blotting as previously described.28 The primary antibody was Abcam’s anti-TNAP antibody (Cat no ab65834).

Statistics
Values are presented as mean±SEM. Statistical analysis was performed using appropriate models of ANOVA followed by Fisher least significant difference test if the overall effects or interactions in the ANOVA were significant. A paired Student t test was used for comparing 2 means in the same group of rats. P<0.05 was considered statistically significant.

Results
Protocol 1
To confirm that TNAP inhibition reduces adenosine production by rat kidneys, renal venous samples was collected at baseline and then at 15-minute intervals after treatment with either vehicle (Tyrode solution; n=6) or 1-p-bromotetramisole (0.1 mmol/L; n=6). Samples were analyzed for 5′-AMP (immediate adenosine precursor) and adenosine by ultraperformance liquid chromatography–tandem mass spectrometry.28 As shown in Figure 1, 1-p-bromotetramisole caused a significant and sustained reduction in the renal venous levels of 5′-AMP (Figure 1A; P<0.04) and adenosine (Figure 1B; P<0.002). In another 3 perfused kidneys, we observed that renal venous adenosine was 120±9 ng/mL before versus 47±7 ng/mL (P<0.02) 15 minutes after administration of an alternate TNAP inhibitor (levamisole; 1 mmol/L); similarly, in yet another 3 perfused kidneys, renal venous adenosine was 110±18 ng/mL before and 39±8 ng/mL (P<0.03) 15 minutes after administration of the TNAP inhibitor 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide (0.02 mmol/L).

Protocol 2
Figure 2A shows the concentration–response curve to norepinephrine (0.03, 0.1, 0.3, 3, and 10 μmol/L for 5 minutes at each concentration) before (period 1) and during (period 2) treatment with the TNAP inhibitor 1-p-bromotetramisole (0.1 mmol/L). As shown, there was an =30-fold shift in the norepinephrine concentration–response curve between period 1 and period 2. To assess whether the shift in the norepinephrine concentration–response curve between period 1 and period 2 was because of a time-related degradation of the experimental preparation, we randomized into the experimental series a group of kidneys that did not receive treatment either during period 1 or during period 2 (Figure 2B). In this group, there was slight shift that could be attributed to time-related changes in responsiveness to norepinephrine. Because there was a slight time-related shift in the concentration–response curve, we compared statistically the concentration–response curves to norepinephrine during period 2 of panels A and B. As shown in Figure 2C, even using this more conservative statistical approach, 1-p-bromotetramisole induced a significant (P<0.0001) shift in the norepinephrine concentration–response relationship. To further challenge our hypothesis, we repeated the experiment summarized in Figure 2 except rather than using 1-p-bromotetramisole to inhibit TNAP, we used either levamisole (1 mmol/L; Figure 3) or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide (0.02 mmol/L; Figure 4). New time controls were randomized into each of the TNAP inhibitor groups. As shown in Figure 3A and Figure 4A, treatment with either levamisole or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide markedly shifted the norepinephrine
concentration-response curve, whereas the shift because of time was much less (Figure 3B and 4B, respectively). Using the same statistical approach as with \( l \)-p-bromotetramisole, both alternative TNAP inhibitors significantly \((P<0.0001)\) right shifted the norepinephrine concentration–response relationship (Figure 3C and 4C for levamisole and 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide, respectively).

**Protocol 3**

Figure 5A illustrates the ability of the highly selective \( \alpha_1 \)-receptor agonist CCPA (100 nmol/L) to reverse most of the inhibitory effect of \( l \)-p-bromotetramisole (0.1 mmol/L) on the renovascular response to norepinephrine (0.5 \( \mu \)mol/L). As expected, norepinephrine significantly increased perfusion pressure. Although \( l \)-p-bromotetramisole did not alter basal perfusion pressure, this TNAP inhibitor nearly abolished the response to norepinephrine. Importantly, administration of CCPA to kidneys receiving \( l \)-p-bromotetramisole reversed most of the suppression of norepinephrine responses induced by \( l \)-p-bromotetramisole.

**Protocoll 4**

Protocol 3 showed that CCPA could reverse most of the inhibitory effects of \( l \)-p-bromotetramisole on renovascular responses to norepinephrine. The purpose of protocol 4 was to determine whether pretreatment with CCPA could prevent most of the inhibitory effect of \( l \)-p-bromotetramisole on renovascular responses to norepinephrine. As shown in Figure 5B, in the presence of CCPA, norepinephrine significantly increased perfusion pressure. \( l \)-p-Bromotetramisole plus...
CCPA did not alter basal perfusion pressure. Moreover, in the presence of CCPA, L-p-bromotetramisole had little effect on norepinephrine-induced renovascular responses.

**Protocol 5**

In protocol 5, RSNS-induced responses were elicited in isolated, perfused rat kidneys. In this regard, at the beginning of each experiment, a response to RSNS was elicited at 5 Hz for 2 minutes, and this response was used to normalize subsequent responses so as to reduce variability because of kidney-to-kidney differences in contact of the electrodes with periarterial sympathetic nerves. Next, kidneys were treated with L-p-bromotetramisole (0.1 mmol/L), levamisole (1 mmol/L) or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide (0.02 mmol/L), and a frequency–response relationship (2, 3, 4, 5, 6, 7, 8, 9 and 10 Hz) was generated. With each TNAP inhibitor, a group of control kidneys not receiving any treatment received no treatment.

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**Figure 3.** Effects of levamisole on renovascular responses to norepinephrine. **A**, Levamisole (1 mmol/L) was administered between periods 1 and 2. **B**, No treatments were administered between periods 1 and 2. **C**, Period 2 of (A) is compared with period 2 of (B) by repeated-measures 2-factor ANOVA. *Significant difference (P<0.05; Fisher least significant difference [LSD] test) within a group between indicated concentration of norepinephrine versus lowest concentration of norepinephrine. **Figure 4.** Effects of 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide on renovascular responses to norepinephrine. **A**, 2,5-Dimethoxy-N-(quinolin-3-yl)benzenesulfonamide (0.02 mmol/L) was administered between periods 1 and 2. **B**, No treatments were administered between periods 1 and 2. **C**, Period 2 of (A) is compared with period 2 of (B) by repeated-measures 2-factor ANOVA. *Significant difference (P<0.05; Fisher least significant difference [LSD] test) within a group between indicated concentration of norepinephrine versus lowest concentration of norepinephrine. **Significant difference (P<0.05; Fisher LSD test) between groups at indicated concentration of norepinephrine. Values are means and SEMs.
was included (ie, 3 groups of control kidneys). Because the 3 groups of control kidneys responded similarly, for statistical purposes, we combined these into 1 control group of n=18. As shown in Figure 6, l-p-bromotetramisole (Figure 6A), levamisole (Figure 6B), and 2,5-dimethoxy-N-(quinolin-3-yl) benzenesulfonamide (Figure 6C) significantly *(P<0.006, P<0.0001, and P<0.0001, respectively) suppressed the RSNS-induced frequency–response relationship. Importantly, with all 3 inhibitors, the suppression of RSNS-induced renovascular responses did not occur at low frequencies of stimulation but only when frequencies were >4 Hz.

Protocol 6
To exclude any potential cofounding effect of changes in phosphate concentrations in the perfusate, we measured renal venous phosphate levels before and during administration of l-p-bromotetramisole (0.1 mmol/L). As shown in Figure S1 in the online-only Data Supplement, l-p-bromotetramisole did not alter renal venous perfusate levels of phosphate.

Protocol 7
To determine whether norepinephrine regulates the release of TNAP, TNAP activity was measured in renal venous perfusate collected before and after treatment with norepinephrine (0.5 μmol/L for 5 minutes). This experiment was performed...
in both naive kidneys and kidneys pretreated with CCPA (0.1 μmol/L, beginning 10 minutes before administering norepinephrine). As shown in Figure S2, treatment with norepinephrine did not release TNAP activity from naive rat kidneys; yet in kidneys pretreated with CCPA, norepinephrine caused a 7-fold increase in the release of TNAP activity (P<0.03). CCPA per se had no effect on the release of TNAP activity. In preliminary experiments, we observed that RSNS (7 Hz) did not release TNAP activity either in the absence or presence of CCPA (data not shown).

Protocol 8
To determine whether TNAP inhibition attenuates norepinephrine-induced renal vasoconstriction in vivo, anesthetized rats were administered norepinephrine while monitoring MABP and renal blood flow. Rats were pretreated with the β-blocker propranolol to prevent norepinephrine-induced activation of β-adrenoceptors. This allowed an assessment of the effects of norepinephrine via α-adrenoceptors on MABP and renal blood flow without confounding by norepinephrine-induced renin release and tachycardia. Some rats were pretreated with l-p-bromotetramisole (30 mg/kg delivered IV as 2 slow boluses) and others were pretreated with the vehicle for l-p-bromotetramisole (ie, saline). Figure S3 demonstrates that the increases in MABP and renal vascular resistance (RVR=MABP/renal blood flow) induced by norepinephrine were significantly (P<0.002 and P<0.02, respectively) inhibited by l-p-bromotetramisole.

Protocol 9
To examine whether cell types within the renal microcirculation express TNAP, preglomerular vascular smooth muscle cells and PGVECs were isolated and placed in cell culture, and cell protein extracts were obtained for determination of TNAP expression by Western blotting. As shown in Figure S4, both preglomerular vascular smooth muscle cells and PGVECs expressed TNAP.

Discussion
Our previous published studies show that in the renal vasculature, the Gi signaling pathway (which A1-receptors engage) converges with the Gq signaling pathway (which α1-receptors engage) to trigger coincident signaling at phospholipase C, leading to augmentation by adenosine of renovascular responses to norepinephrine. This explains why activation of A1-receptors by endogenous adenosine affords full renovascular response to RSNS.1,2 However, this concept is at odds with our findings that neither inhibition of CD39 nor CD73 (ie, the most important set of enzymes for producing extracellular adenosine from ATP17–20) affect renovascular responses to RSNS.21

To reconcile the fact that A1-receptors are required to achieve a full renovascular response to RSNS with the fact that blocking CD39 or CD73 does not affect renal sympathetic neurotransmission, we hypothesized that the pool of adenosine that modulates renovascular responses to norepinephrine is produced by an alternative biochemical pathway. Because (1) TNAP mRNA, protein, and activity are present in kidneys,24 (2) TNAP can metabolize ATP to adenosine,23 and (3) TNAP contributes to the metabolism of 5′-AMP to adenosine in kidneys,24 we further hypothesized that TNAP mediates in part the alternative adenosine-producing pathway regulating renovascular responses to RSNS.

In this study, we observed that in isolated, perfused rat kidneys administration of TNAP inhibitors right shifted the concentration–response relationship to exogenous norepinephrine by ≈10-fold. Moreover, we found that the attenuation of renovascular responses to norepinephrine by TNAP inhibition was partially prevented or reversed by administration of the highly selective A1-receptor agonist, CCPA. That is to say, by restoring A1-receptor activation, the effects of TNAP inhibition were attenuated. These results are consistent with the concept that TNAP inhibition decreases the pool of adenosine that modulates renovascular responses to exogenous norepinephrine such that restoration or maintenance of adenosinergic tone partially rescues or prevents suppression of the norepinephrine response by TNAP inhibition. Indeed, measurement of renal venous levels of 5′-AMP (adenosine precursor) and adenosine per se showed that TNAP inhibition decreased the renal biosynthesis of adenosine. The present results, taken together with our previous findings that adenosine via the A1-receptor engages convergent signaling with norepinephrine at the level of phospholipase C, provides evidence supporting the conclusion that TNAP modulates renovascular responses to norepinephrine in part by maintaining a pool of adenosine that modulates the renovascular response to norepinephrine.

An important question is whether the ability of TNAP inhibition to attenuate the renovascular response to norepinephrine translates into attenuation of renovascular responses to norepinephrine released from sympathetic nerve varicosities. In the present investigation, we observed that all 3 TNAP inhibitors attenuated renovascular responses to all concentrations of norepinephrine. In contrast, although all 3 TNAP inhibitors attenuated renovascular responses to RSNS, this effect was observed only at frequencies >4 Hz. This unanticipated finding requires explanation.

Studies by Sedaa et al33 show that the vascular endothelium is the primary source of adenine nucleotides (including adenosine) in blood vessels, and the Western blot experiments in the present study demonstrate that PGVECs indeed express TNAP. Also, the combination of A1-receptor activation (in the present study with CCPA) plus α1-adrenoceptor stimulation with luminal (exogenous) norepinephrine causes release of TNAP activity into the renovascular circulation. Release of TNAP into the vascular lumen could amplify the luminal production of adenosine that could further augment the vascular effects of luminal norepinephrine. Thus, it would be expected that vascular smooth muscle cells positioned near the luminal side of the blood vessels would be constantly under the influence of endothelium-derived adenosine. Because administration of exogenous norepinephrine activates vascular smooth muscle cells underneath and adjacent to vascular endothelial cells, this may explain why TNAP inhibition attenuates renovascular responses to even low concentrations of norepinephrine.

Unlike exogenous norepinephrine, norepinephrine released from sympathetic nerve terminals activates abluminal vascular smooth muscle cells that are most likely under
the influence of adenine nucleotides coreleased from sympathetic varicosities. In this regard, measurable release of adenine nucleotides by sympathetic nerve stimulation seems to occur only at high frequencies of nerve stimulation. For example, Vonend et al. studied adenine nucleotide release from human kidney slices using field stimulation of 20 Hz; Ishii et al. investigated adenine nucleotide release from rabbit ear, femoral, renal, and pulmonary arteries using 16-Hz stimulation; and Gonçalves et al. used 7-Hz stimulation to examine adenine nucleotide release from guinea pig isolated vas deferens. We previously measured adenosine release from isolated, perfused rat kidneys and found that release of adenosine was difficult to detect at frequencies of nerve stimulation <5 Hz. Also, in the present study, we did not observe an interaction between A1-receptor activation with CCPA and abluminal (endogenous) norepinephrine with respect to the release of TNAP activity. It is likely then that the lack of effect of TNAP inhibition on responses to RSNS at low frequencies is related to the inadequate release of ATP and relatively low formation rate of adenosine at the abluminal aspect of renal blood vessels.

At present, we do not know how A1-receptor activation allows luminal norepinephrine to release TNAP into the renal circulation, but we do have a working hypothesis. TNAP is a GPI-anchored ectoenzyme, and in the secretory pathway, GPI-phospholipase D (GPI-PLD) cleaves the GPI anchor from and PKC accelerates the secretory pathway. Therefore, we are not confident that the observed effects of TNAP inhibitors on vascular responses to norepinephrine apply only to the kidneys or occur also in nonrenal vascular beds.

Because isolated, perfused kidneys are not in their natural environment, it is possible that TNAP inhibition reduces renovascular responses to norepinephrine in isolated kidneys but not in kidneys in vivo. In the present study, we tested this by infusing norepinephrine intravenously into rats treated or not with l-p-bromotetramisole. This experiment was conducted in rats pretreated with propranolol to block the ability of norepinephrine to affect MABP and RVR by activating the renin–angiotensin system and by increasing heart rate and cardiac output. Under these conditions, l-p-bromotetramisole attenuated norepinephrine-induced changes in MABP and RVR. These findings provide affirmation that our in vitro results can be extrapolated to the in vivo condition.

The size of the effect of TNAP inhibitors on renovascular responses to norepinephrine and RSNS exceeded our expectations. Therefore, we are not confident that the observed effects are only because of a reduction in adenosine levels and hypothesize that other yet-to-be-discovered mechanisms are involved that may or may not entail TNAP inhibition. However, some potential mechanisms can be excluded. For example, although it is conceivable that TNAP inhibitors cause direct α-adrenoceptor blockade, published studies are inconsistent with this hypothesis. Gulati et al. reported that in the guinea pig vas deferens levamisole actually enhanced responses to exogenous norepinephrine and field stimulation and concluded that levamisole does not block α-adrenoceptors but rather inhibits norepinephrine uptake. Joshi and Verma observed that levamisole caused contractions of the rat anococcygeus muscle that are blocked by phentolamine and concluded that levamisole is an agonist (not antagonist) of α-adrenoceptors. Vanhoutte et al. and Van Nueten did not observe α-adrenoceptor–blocking activity of levamisole in dog saphenous veins and rabbit spleens, respectively.

Nothing is known about the local anesthetic actions of l-p-bromotetramisole or 2,5-dimethoxy-N-(quinolin-3-yl) benzenesulfonamide. However, Onuaguluchi and Igbo reported that levamisole caused surface anesthesia in the rabbit cornea with a half-maximal effective concentration of 300 mmol/L and infiltration local anesthesia in the guinea pig intradermal model with a half-maximal effective concentration of 0.8 mmol/L. Obviously, local anesthetics do not block α-adrenoceptor–induced vasoconstriction because epinephrine is commonly coadministered with lidocaine to cause vasoconstriction and limit the local absorption of lidocaine into the systemic circulation. Indeed, the local anesthetic dibucaine does not inhibit vasoconstriction by exogenous norepinephrine in the rat mesentery. Therefore, any local anesthetic action could not explain the ability of TNAP inhibitors to reduce the responsiveness of the renal vasculature to exogenous norepinephrine. Although a membrane-stabilizing effect of TNAP inhibitors could partially explain the effects of these agents on responses to RSNS, one would anticipate inhibition of responses to RSNS at all frequencies rather than just at frequencies >4 Hz. For example, dibucaine inhibits noradrenergic neurotransmission in the rat mesentry at 3 Hz nerve stimulation. Nonetheless, it remains conceivable that a local anesthetic action explains in part the effects of TNAP inhibitors on RSNS responses but not exogenous norepinephrine.

Another potential mechanism is that somehow changes in phosphate levels alter renovascular responsiveness to norepinephrine. However, we could not detect changes in phosphate levels exiting the renal vein. Additional studies, for example, in TNAP knockout animals, are warranted to confirm that TNAP inhibitors attenuate renovascular responses to norepinephrine by reducing TNAP activity and to elucidate the full mechanistic details by which TNAP inhibitors attenuate norepinephrine-induced increases in RVR. Also, it will be of interest to examine the effects of TNAP inhibitors on responses to norepinephrine in alternative vascular beds to determine whether the observed effects of TNAP inhibitors on vascular responses to norepinephrine apply only to the kidneys or occur also in nonrenal vascular beds.

**Perspectives**

The most important aspect of this research is that it identifies the ability of TNAP inhibitors to attenuate renovascular responses to both luminal and abluminal norepinephrine. This effect seems to be mediated at least partially by inhibition of adenosine production in the kidney that is normally maintained
by TNAP activity. We also find that TNAP inhibitors attenuate the effects of noradrenaline on MABP and RVR in vivo, suggesting a new modality of treating cardiovascular diseases and hypertension.

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Disclosures
None.

References

**Novelty and Significance**

**What Is New?**
- This study establishes that tissue nonspecific alkaline phosphatase (TNAP) inhibitors attenuate renovascular responses to both exogenous and endogenous norepinephrine and reduce the pressor effects of norepinephrine.
- This study also demonstrates that TNAP inhibitors reduce the production of adenosine by the kidneys and that likely this contributes, at least in part, to the attenuation by TNAP inhibitors of norepinephrine-induced renal vasconstriction.

**What Is Relevant?**
- TNAP inhibitors attenuate norepinephrine-induced renal vasoconstriction.
- These studies identify a new mechanism for blocking the effects of catecholamines on the renal vasculature.

- These studies also identify TNAP as a potential target for the treatment of hypertension and cardiovascular and renal diseases.

**Summary**

Here, we report that 3 TNAP inhibitors attenuate renovascular responses to exogenous norepinephrine and endogenous norepinephrine released by renal sympathetic nerve stimulation. These findings corroborate the hypothesis that TNAP inhibition attenuates renovascular response to both luminal and abluminal norepinephrine and suggest the prospect that TNAP inhibition could attenuate the effects of the sympathoadrenal axis on renal function and blood pressure.
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Running title: *TNP Inhibitors Reduce NE-Induced Vasoconstriction*

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Figure S1. L-p-bromotetramisole did not alter renal venous levels of phosphate.
Figure S2. (A) Norepinephrine (NE; 0.5 μmol/L) did not affect renal venous levels of TNAP activity in control kidneys. (B) In CCPA (A1-receptor antagonist; 0.5 μmol/L) pretreated kidneys, NE increased the renal venous levels of TNAP activity by 7-fold.
Figure S3. Intravenous administration of the TNAP inhibitor L-p-bromotetramisole (30 mg/kg) attenuated norepinephrine (NE; 10 \( \mu \text{g/kg/min} \)) induced increases in mean arterial blood pressure (MABP; panel A) and renal vascular resistance (RVR; panel B). P-values are from repeated measures 2-factor ANOVA. "a" indicates P<0.05 compared to corresponding baseline (time 0) and "b" indicates P<0.05 compared to vehicle group at corresponding time.
Figure S4. Western blot analysis detected the expression of TNAP in both preglomerular vascular endothelial cells and preglomerular vascular smooth muscle cells.