Elevated Ecto-5'-nucleotidase-Mediated Increased Renal Adenosine Signaling Via A2B Adenosine Receptor Contributes to Chronic Hypertension

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Rationale: Hypertension is the most prevalent life-threatening disease worldwide and is frequently associated with chronic kidney disease (CKD). However, the molecular basis underlying hypertensive CKD is not fully understood.

Objective: We sought to identify specific factors and signaling pathways that contribute to hypertensive CKD and thereby exacerbate disease progression.

Methods and Results: Using high-throughput quantitative reverse-transcription polymerase chain reaction profiling, we discovered that the expression level of 5'-ectonucleotidase (CD73), a key enzyme that produces extracellular adenosine, was significantly increased in the kidneys of angiotensin II–infused mice, an animal model of hypertensive nephropathy. Genetic and pharmacological studies in mice revealed that elevated CD73-mediated excess renal adenosine preferentially induced A2B adenosine receptor (ADORA2B) production and that enhanced kidney ADORA2B signaling contributes to angiotensin II–induced hypertension. Similarly, in humans, we found that CD73 and ADORA2B levels were significantly elevated in the kidneys of CKD patients compared with normal individuals and were further elevated in hypertensive CKD patients. These findings led us to further discover that elevated renal CD73 contributes to excess adenosine signaling via ADORA2B activation that directly stimulates endothelin-1 production in a hypoxia-inducible factor-α–dependent manner and underlies the pathogenesis of the disease. Finally, we revealed that hypoxia-inducible factor-α is an important factor responsible for angiotensin II–induced CD73 and ADORA2B expression at the transcriptional level.

Conclusions: Overall, our studies reveal that angiotensin II–induced renal CD73 promotes the production of renal adenosine that is a prominent driver of hypertensive CKD by enhanced ADORA2B signaling–mediated endothelin-1 induction in a hypoxia-inducible factor-α–dependent manner. The inhibition of excess adenosine-mediated ADORA2B signaling represents a novel therapeutic target for the disease. (Circ Res. 2013;112:1466-1478.)

Key Words: adenosine ■ hypertension ■ chronic renal disease

Hypertension is a leading cause of morbidity and mortality in the United States and worldwide. The condition affects 1 in 3 adults in the United States and 25% of the adult population worldwide. The estimated total number of adults with hypertension in 2000 was 972 million, and it is predicted to increase by ≈60% to a total of 1.56 billion by 2025. In the United States, hypertension accounts for 1 in 7 deaths, >$93.5 billion in medical costs per year, and immeasurable human suffering. Because of the increasing morbidity, mortality, and extensive medical costs associated with hypertension, novel therapeutic strategies are desperately needed to reduce hypertension and delay disease progression. Defining the molecular mechanisms underlying the disease is important for developing novel strategies for disease prevention and treatment.

Chronic kidney disease (CKD) is a devastating disease including kidney injury, progression to renal fibrosis, and...
Ang II can stimulate multiple signaling pathways, but renal fibrosis. In both humans and animals with hypertension, signaling pathways involved in hypertensive CKD, the renin–angiotensin system is considered as a key signaling cascade contributing to hypertension, CKD, and its progression to renal fibrosis. In both humans and animals with hypertensive nephropathy, circulating angiotensin II (Ang II), the end effector of the renin–angiotensin system, is elevated and therapies that inhibit this signaling cascade are effective. Ang II can stimulate multiple signaling pathways, but it is unclear which of these pathways drives hypertensive CKD and, when inhibited, which results in disease amelioration. Here, we sought to identify specific factors and signaling pathways that contribute to hypertension and CKD, and thereby exacerbate disease progression in both humans and mice.

Methods

Detailed Methods are available in the Online Data Supplement.

Human Subjects

Kidney biopsy specimens were collected from normal control individuals (n=12), those with CKD alone without hypertension (n=24), and hypertensive patients with CKD (n=32). CKD patients without hypertension and hypertensive CKD patients admitted to the First XiangYa Hospital were identified by nephrologists of the Central South University at Changsha, Hunan, China. Normal individuals were selected on the basis of having normal blood pressure and kidney function before acute kidney rupture resulting from trauma. CKD patients without hypertension were selected on the basis of displaying kidney damage (structural or functional abnormalities of the kidney) with glomerular filtration rate <60 mL/min per 1.73 m² for >3 months in the absence of hypertension. Individuals with hypertensive CKD were selected on the basis of presenting kidney damage (structural or functional abnormalities of the kidney) with glomerular filtration rate <60 mL/min per 1.73 m² for >3 months in the presence of hypertension (systolic blood pressure ≥140 mm Hg; diastolic blood pressure ≥90 mm Hg). The research protocol was approved by the Central South University Ethics Committee for the Protection of Human Subjects. Clinical data for normal individuals and patients with mild or severe CKD are listed in the Table.

Animals

Wild-type (WT) 8- to 10-week-old C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). Ecto-5′-nucleotidase (CD73)−/− deficient mice and A2B adenosine receptor (ADORA2B)−/− deficient mice congenic on a C57BL/6 background were generated and genotyped as described. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Houston Health Science Center. Six to 10 mice for each group were used.

Reverse-Transcription Polymerase Chain Reaction

Gene Expression Profiling of Kidneys of Mice With or Without Ang II Infusion

RNA was extracted using total RNA isolation reagent (Invitrogen, Carls-View, CA) from the kidneys after 14-day infusion with saline or Ang II. Genomic DNA contamination was eliminated by DNase treatment with RNeasy Micro Kit (Qiagen GmbH, Hidden, Germany). High-throughput mouse RT² profiler polymerase chain reaction (PCR) array and RT² real-timer SYBR Mix were purchased from SuperArray Bioscience Corporation (Frederick, MD). PCR was performed on an ABI Prism 7700 sequence Detector (Applied Biosystems). For data analysis, the ΔCt method was used. For each gene, the fold changes were calculated as difference in kidney gene expression between Ang II−infused and saline-infused mice. P<0.05 is considered significant.

Results

Elevated Renal CD73 Is a Novel Factor Underlying Chronic Increased Kidney Adenosine Production and Contributing to Hypertension and Kidney Injury in Ang II−Infused Mice

In an effort to identify specific renal factors contributing to the pathogenesis of CKD and hypertension, we used a high-throughput quantitative reverse-transcription PCR (RT-PCR) array to compare gene expression profiles in the kidneys of controls and Ang II−infused mice, an animal model of hypertensive CKD. Among all of the transcripts screened, CD73 mRNA levels were among the most highly induced in the kidneys of Ang II−infused mice compared with controls (Figure 1A). We further confirmed that CD73 mRNA and protein levels, as well as enzyme activity, were significantly elevated in the kidneys of Ang II−infused mice (Figure 1B and 1C). Intriguingly, CD73 mRNA levels were not significantly increased in the hearts of Ang II−infused mice (Online Figure I). Moreover, mRNA levels of CD38 and CD39, 2 other 5′-ectonucleotidases, were similar between mice with and without Ang II infusion (Online Figure II). In addition, adenosine deaminase (ADA) activity in the kidneys of Ang II−infused mice was reduced ≈15% (Online Figure III), which is much lower than CD73 activity induced by Ang II that increased ≈2-fold (Figure 1C). Thus, these studies revealed that CD73 is a key ectonucleotidase induced in the kidneys of Ang II−infused mice.

Because CD73 is responsible for production of extracellular adenosine, it is possible that the elevation in kidney CD73 resulting from Ang II infusion promotes the production of elevated renal adenosine. Consistent with this possibility, we found that adenosine levels increased significantly in the mouse kidney (Figure 1D) but not in the plasma (Figure 1E) of Ang II−infused mice, indicating that elevated renal CD73 induced chronic elevation of adenosine locally in the kidney, but not systemically. To determine whether CD73 contributes to elevation...
of adenosine and subsequent Ang II–induced hypertension and CKD in vivo, we took a genetic approach by infusion of Ang II into WT and CD73-deficient mice. As expected, we found that CD73 deficiency significantly reduced Ang II–mediated adenosine induction in the mouse kidneys (Figure 1D). These findings indicate that elevated CD73 leads to chronic elevation of renal adenosine production in Ang II–infused mice. Functionally, we found that infusion of Ang II into WT mice led to chronic hypertension and kidney injury characterized with proteinuria and decreased urine osmolality (Figure 1F; Online Figure IV A–IVC), features similar to those observed in hypertensive patients with CKD. In contrast, these features were significantly attenuated when Ang II infusion was performed in CD73-deficient mice (Figure 1F; Online Figure IV A–IVC).

To determine whether CD73 deficiency attenuated hypertension and whether renal damage is caused by effects on angiotensin receptor expression, we used quantitative RT-PCR to compare the abundance of transcripts encoding the 3 angiotensin receptors, angiotensin receptor type 1A, angiotensin receptor type 1B, and angiotensin receptor type 2 receptor, in kidneys of WT and CD73-deficient mice. The results (Online Figure VA) show no significant difference in the abundance of these receptor transcripts in the kidneys of WT mice and CD73-deficient mice. Thus, changes in angiotensin receptor expression do not account for the ability of CD73 deficiency to protect against chronic kidney damage and persistent hypertension in Ang II–infused mice.

Elevated Adenosine Contributes to Chronic Hypertension in Ang II–Infused Mice

To assess the direct effect of elevated renal adenosine in hypertension, we took a pharmacological approach of treating Ang II–infused mice with polyethyleneglycol-modified ADA to lower adenosine levels. Polyethyleneglycol itself showed no effects on blood pressure (Online Figure VI). However, polyethyleneglycol-modified ADA treatment significantly inhibited the increase in renal adenosine levels observed in Ang II–infused mice (Figure 1D). Likewise, Ang II–induced hypertension was significantly attenuated in mice chronically treated with polyethyleneglycol-modified ADA (Figure 1F; Online Figure VIA–VIC). These findings provide direct in vivo evidence that chronically elevated adenosine is a previously unrecognized detrimental mediator to drive Ang II–induced hypertension and kidney disease.

Excessive ADORA2B Adenosine Receptor Activation Underlies Hypertension in Ang II–Infused Mice

In an effort to determine the potential contribution of adenosine receptor signaling to Ang II–induced hypertensive CKD, we measured mRNA levels for the 4 adenosine receptors in WT and CD73-deficient mice with or without Ang II infusion. Unexpectedly, we found that only Adora2b mRNA levels were significantly elevated in the kidneys of Ang II–infused mice (Figure 2A–2D). Intriguingly, we found that CD73 deficiency significantly reduced Ang II–mediated elevation of Adora2b mRNA levels in the mouse kidneys (Figure 2A–2D), indicating that CD73-dependent elevated renal adenosine preferentially induces Adora2b gene expression in the kidneys of Ang II–infused mice.

Next, we used both genetic and pharmacological approaches to determine the role of ADORA2B in chronic hypertension.
Genetically, we found that ADORA2B deficiency significantly reduced Ang II–induced hypertension (Figure 2E and 2F). Consistently, we further demonstrated that selectively interfering with ADORA2B activation by an ADORA2B-specific antagonist, PSB1115 significantly attenuated Ang II–induced hypertension (Figure 2E and 2F). Altogether, we showed that elevated renal CD73 is associated with the chronic accumulation of renal adenosine and enhanced ADORA2B signaling, which underlies Ang II–induced hypertension.

**CD73 and ADORA2B Expression Levels Are Increased in the Kidneys of Mild CKD Patients Without Hypertension and Are Further Elevated in Severe CKD Patients With Hypertension**

To extend our mouse studies to humans, we first examined CD73 and ADORA2B protein levels in kidney biopsy specimens collected from normal controls (n=12), CKD patients without hypertension (n=24), and severe CKD patients with hypertension (n=32; Table shows clinical information of human subjects). Like the expression pattern seen in mice, immunostaining revealed that CD73 and ADORA2B were expressed in both glomeruli and tubules in normal control individuals. CD73 and ADORA2B levels were elevated in both glomeruli and tubules of kidneys isolated from CKD patients with or without hypertension (Figure 3A). Quantitative image analysis demonstrated that increased CD73 and ADORA2B staining in the kidneys of CKD patients was significantly higher than that in the controls, and that CD73 and ADORA2B levels were further elevated in severe CKD patients with hypertension compared with mild CKD patients without hypertension (Figure 3D and 3E). Intriguingly, the elevated CD73 and ADORA2B levels were significantly correlated to disease severity by clinical symptoms (Table), levels of kidney injury quantified by histological score based on hematoxylin and eosin staining (Figure 3A and 3C), and degrees of renal fibrosis by collagen score based on trichrome staining (Figure 3A and 3B). Thus, our human studies demonstrate, for the first time, that elevated CD73 and ADORA2B levels in the kidneys are associated with the severity of the disease.

**ADORA2B Signaling Contributes to Increased Endothelin-1 Production in the Kidneys of Ang II–infused Mice**

To identify signaling molecules functioning downstream of ADORA2B that contribute to hypertensive CKD, we re-examined the kidney gene expression profiles of control and
Ang II–infused mice, with a particular focus on hypertensive mediators. We found that prepro-endothelin-1 (prepro-ET-1), a precursor of ET-1, a potent vasoconstrictor, was elevated most in the kidneys of Ang II–infused mice (Online Table I; Figure 1A).

We confirmed our screening results by showing that prepro-ET-1 mRNA levels were significantly elevated in the kidneys of Ang II–infused mice (Figure 4A).

Immunostaining and quantitative image analysis revealed that ET-1 protein levels were significantly elevated in the endothelial cells of glomeruli of Ang II–infused mice (Figure 4B and 4C). Previous studies showed that elevated ET-1 signaling plays an important role in hypertension, kidney dysfunction, and fibrosis in Ang II–infused mice.19–22 However, the role of elevated ADORA2B signaling in Ang II–mediated induction of ET-1 was not determined.

To test this possibility, we again used both genetic and pharmacological approaches. We found that Ang II–induced prepro-endothelin-1 mRNA and protein levels in the kidneys of Ang II–infused mice (Online Table I; Figure 1A). We confirmed our screening results by showing that prepro-ET-1 mRNA levels were significantly elevated in the kidneys of Ang II–infused mice (Figure 4A). Immunostaining and quantitative image analysis revealed that ET-1 protein levels were significantly elevated in the endothelial cells of glomeruli of Ang II–infused mice (Figure 4B and 4C). Previous studies showed that elevated ET-1 signaling plays an important role in hypertension, kidney dysfunction, and fibrosis in Ang II–infused mice.19–22 However, the role of elevated ADORA2B signaling in Ang II–mediated induction of ET-1 was not determined.

To test this possibility, we again used both genetic and pharmacological approaches. We found that Ang II–induced prepro-endothelin-1 mRNA production was significantly inhibited in the kidneys of ADORA2B-deficient mice and PSB1115-treated mice (Figure 4A). Similarly, immunostaining and quantitative image analysis further confirmed that ADORA2B deficiency and PSB1115 treatment significantly attenuated Ang II–induced ET-1 protein levels in endothelial cells of glomeruli of these mice (Figure 4B and 4C). Consistent with the findings of an important role for ADORA2B signaling in Ang II–induced production of ET-1 from kidneys, genetic deletion of CD73 also led to a significant reduction in prepro-ET-1 mRNA and protein levels in the kidneys of Ang II–infused mice (Figure 4A–4C). However, the endogenous ET system, including prepro-ET-1, ET receptor type 1A, ET receptor type 1B, and ET-converting enzyme, is intact in CD73-deficient mice (Online Figure VB). Taken together, these results revealed that CD73 activity and ADORA2B signaling contribute to the renal production of ET-1, a likely contributor to hypertension, CKD, and progression of fibrosis.

**ADORA2B Signaling Via Hypoxia-Inducible Factor-1α Underlies Ang II–induced ET-1 Production in Mouse Kidneys**

To determine what intracellular molecules functioning downstream of ADORA2B underlie Ang II–mediated ET-1 induction, we re-examined the kidney gene expression profiles. We found that hypoxia-inducible factor-1α (HIF-1α) was among the transcripts highly elevated in the kidneys of Ang II–infused mice (Online Table I; Figure 1A). Ang II–mediated induction of renal HIF-1α mRNA was confirmed by RT-PCR analysis (Figure 4D). Immunohistochemical analysis and image quantification studies demonstrated that HIF-1α protein levels were also significantly elevated in the glomeruli of kidneys of Ang II–infused mice (Figure 4E).
and 4F). Ang II–induced HIF-1α mRNA and protein levels in kidneys were significantly reduced in CD73-deficient mice, ADORA2B-deficient mice, and PSB1115-treated mice (Figure 4E and 4F). These studies provide in vivo evidence that elevated CD73-mediated adenosine induction and excess ADORA2B signaling are required for Ang II–mediated induction of HIF-1α.

Next, to assess the direct renal effect of excess adenosine signaling on HIF-1α and ET-1 induction, we isolated kidneys from WT mice, CD73-deficient mice, and ADORA2B-deficient mice to conduct experiments using kidney explant cultures.4,23 First, we treated kidney explants isolated from WT, CD73-deficient, and ADORA2B-deficient mice in the presence or absence of Ang II. Quantitative RT-PCR analysis showed that Ang II–induced prepro-ET-1 and HIF-1α mRNA levels were significantly reduced in the kidney explants of CD73−/− and Adora2b−/− mice compared with the WT mice (Figure 5A). These findings provide direct evidence for the importance of CD73 and ADORA2B in Ang II–induced HIF-1α and ET-1 production in the mouse kidneys.

Next, to determine whether adenosine signaling via ADORA2B activation directly induces HIF-1α and ET-1 production, we treated kidney explants isolated from both WT and Adora2b−/− mice with 5′-N-ethylcarboxamidoadenosine (NECA), a potent nonmetabolized adenosine analog.17 We found that NECA was capable of inducing both prepro-ET-1 and HIF-1α gene expression in cultured kidney explants from WT mice but not from Adora2b−/− mice (Figure 5B). Finally, to determine whether HIF-1α functioning downstream of ADORA2B is responsible for adenosine-induced ET-1
production, we treated kidney explants from WT mice with either chrysin (HIF-1α inhibitor) or dimethyloxalyl glycine (HIF-1α stabilizer). We found that chrysin significantly reduced NECA-induced prepro-ET-1 gene expression in WT mouse kidney explants (Figure 5C). In contrast, dimethyloxalyl glycine significantly enhanced NECA-induced prepro-ET-1 gene expression in WT mouse kidney explants (Figure 5C). Overall, our studies provide the direct evidence that ADORA2B-mediated HIF-1α induction contributes to Ang II-mediated induction of ET-1 in mouse kidneys.

**Figure 4.** A2B adenosine receptor (ADORA2B)–mediated induction of hypoxia-inducible factor-1α (HIF-1α) regulates endothelin-1 (ET-1) production in the kidneys of angiotensin II (Ang II)–infused mice. A, Quantitative reverse-transcription polymerase chain reaction (RT-PCR) measurement of prepro-ET-1 mRNA levels in wild-type (WT) mice, CD73−/− mice and Adora2b−/− mice with saline or Ang II infusion, and WT mice with PSB1115 treatment alone or with Ang II infusion. B, Immunohistochemical analysis of ET-1 expression in the kidneys of mice listed. C, Expression of ET-1 in the kidneys of mice listed in (B) was quantified using Image-Pro Plus image analysis software. D, HIF-1α mRNA levels were measured by quantitative RT-PCR in the kidneys of mice listed in A. E, Immunohistochemical analysis of HIF-1α expression in the kidneys of mice listed in (A). F, Expression of HIF-1α in the kidneys of mice listed in (E) was quantified using Image-Pro Plus image analysis software. Data are expressed as mean±SEM. *P<0.05 for Ang II–infused mice vs the control mice infused with saline; **P<0.05 for Adora2b−/−, CD73−/−, and WT mice with PSB1115 treatment infused with Ang II vs Ang II–infused WT mice (n=8–10 per group).

**ADORA2B-Mediated Induction of HIF-1α Underlies Ang II–induced ET-1 in Cultured Human Microvascular Endothelial Cells**

HIF-1α and ET-1 were significantly elevated in the endothelial cells of the capillary lumens of kidneys of Ang II–infused mice (Figure 4B and 4E). These results suggest that microvascular endothelial cells are major cell types responsible for excessive adenosine-induced HIF-1α and ET-1 production. It is difficult to decipher the direct role of ADORA2B-mediated HIF-1α elevation in ET-1 induction in intact animals. Therefore, we extended our mouse studies to
human microvascular endothelial cells (HMECs). Although these cells are not derived from kidneys, they represent a clonally derived source of HMECs useful to examine the role of adenosine signaling in Ang II–mediated induction of HIF-1α and ET-1. First, we found that Adora2b transcripts are the predominant adenosine receptor transcript expressed in HMECs (Online Figure VIIA). Next, we found that Ang II–mediated induction of both HIF-1α and prepro-ET-1 gene expression in cultured HMECs was significantly attenuated by pretreatment with either α,β-methylene ADP (α,β-methylene ADP [APCP], a CD73 inhibitor) or MRS1706 (an ADORA2B antagonist; Figure 5D). These findings provide the direct evidence that CD73 and ADORA2B play an important role in Ang II–mediated induction of HIF-1α and ET-1 mRNA in HMECs.

Subsequently, we assessed the direct role of adenosine signaling in HMECs. We demonstrated that NECA was capable of inducing both HIF-1α and prepro-ET-1 gene expression in cultured HMECs in a time-dependent manner (Online Figure VIIA and VIIIB). In addition, we found that treatment of HMECs with either theophylline (a general AR antagonist) or MRS1706 (an ADORA2B-specific antagonist) significantly inhibited NECA-induced HIF-1α and prepro-ET-1 mRNA levels in human microvascular endothelial cells (HMECs). These data are expressed as mean±SEM. *P<0.05 vs untreated cells; **P<0.05 vs NECA-treated control cells (n=4–6).

Finally, to determine whether HIF-1α is required for adenosine-induced prepro-ET-1 gene expression, we generated HIF-1α-deficient HMECs by stably knocking down endogenous HIF-1α expression by means of a small interfering RNA strategy (HIF-1α-KD). First, we found that...
HIF-1α-specific siRNA significantly reduced HIF-1α protein levels in HIF-1α–KD HMEC cells (Figure 5F). More importantly, we found that NECA-induced prepro-ET-1 mRNA was significantly reduced in HIF-1α-KD cells compared with control cells (Figure 5F). Our findings show that ADORA2B activation can directly induce HIF-1α gene expression, and that HIF-1α is essential for adenosine-mediated ET-1 induction in human endothelial cells.

**HIF-1α Contributes to Ang II–induced CD73 and Adora2b Gene Expression in Both Cultured Mouse Kidneys and Human Endothelial Cells at Transcriptional Levels**

We have revealed that HIF-1α functioning downstream of ADORA2B is directly responsible for Ang II–induced ET-1 production by mouse kidneys and human endothelial cells. HIF-1α is known to be involved in increased CD73 and Adora2b gene expression under hypoxic conditions.24,25 However, whether Ang II is a hypoxia-independent mediator directly inducing HIF-1α levels and thereby responsible for Ang II-induced CD73 and Adora2b expression remains unknown. To test this possibility, we isolated kidneys from WT mice and conducted kidney organ culture as described.4,23 Quantitative RT-PCR analysis indicated that Ang II treatment directly induced both CD73 and Adora2b gene expression (Figure 6A). Moreover, we found that chrysin, a HIF-1α inhibitor, significantly reduced Ang II–induced CD73 and Adora2b gene expression in WT mouse kidney explants (Figure 6A). In contrast, dimethylxoxylglycine, a HIF-1α stabilizer, significantly enhanced Ang II–induced CD73 and Adora2b gene expression in WT mouse kidney explants (Figure 6A). Consistently, we found that Ang II significantly induced CD73 and Adora2b mRNA in the control HMEC cells (Figure 6B). However, in the HIF-1α knockdown cells (HMECs-HIF-1α KD), Ang II–induced CD73 and Adora2b mRNA levels were significantly reduced (Figure 6B). Overall, our studies demonstrate that Ang II is a previously unrecognized hypoxia-independent mediator directly inducing HIF-1α levels, and elevated HIF-1α is an important transcription factor responsible for Ang II–mediated induction of CD73 and Adora2b mRNA levels in cultured mouse kidneys and HMECs.

Next, we extended mouse studies to cultured HMECs. Specifically, we incubated both control and HIF-1α knockdown cells (HMECs-HIF-1α KD) with or without Ang II treatment. The results (Figure 6B) show that HIF-1α underlies Ang II–mediated induction of CD73 and Adora2b mRNA levels.

Both CD73 and Adora2b promoters contain a HIF-1α–responsive element (HRE). Thus, we hypothesize that HIF-1α underlies Ang II–induced CD73 and Adora2b gene expression at the transcriptional level. To test this hypothesis, we conducted transfection assays using luciferase reporter genes introduced into in HMECs in the presence or absence of Ang II. We found that Ang II–induced WT CD73 and Adora2b promoter activities compared with untreated controls (Figure 6C). However, deletion of HRE site in either CD73 or Adora2b promoter significantly reduced Ang II–induced CD73 and Adora2b promoter activity (Figure 6C). Thus, these results provide direct evidence that HREs in the promoters of both CD73 and Adora2b are essential for Ang II–induced transcriptional activation of these 2 genes.

**HIF-1α Is Responsible for NECA-induced CD73 and Adora2b Gene Expression in Cultured Human Endothelial Cells at Transcriptional Levels**

Finally, we determined whether adenosine-induced HIF-1α is responsible for activating CD73 and Adora2b gene expression at transcriptional levels. Similarly, we conducted transfection assays using luciferase reporter genes introduced into HMECs with or without NECA treatment. We found that NECA-induced WT CD73 and Adora2b promoter activities compared with untreated controls (Figure 6D–6F). However, deletion of the HRE in either the CD73 or the Adora2b promoter significantly reduced NECA-induced CD73 and Adora2b promoter activity (Figure 6D and 6E). Thus, these results provide direct evidence that the HRE in the CD73 and Adora2b promoters is essential for NECA-induced transcriptional activation of these 2 genes. Taken together, we revealed that adenosine–mediated HIF-1α induction underlies increased CD73 and Adora2b transcriptional activities and functions as a positive feedback to further promote Ang II–mediated elevation of adenosine and to amplify ADORA2B signaling (Figure 6F).

**Discussion**

In this study, we report that CD73, a critical enzyme in extracellular adenosine production, and ADORA2B, are elevated in kidneys of both mice and humans with hypertension and CKD. Using an Ang II infusion model of hypertensive renal disease in mice, we provide both genetic and pharmacological evidence that elevated renal CD73 contributes to hypertension, proteinuria, decreased tubular concentration ability, and progression of the disease by the excessive production of adenosine in the kidneys. Pharmacological inhibition or genetic disruption of the adenosine receptor ADORA2B significantly attenuates Ang II–induced hypertension. Mechanistically, we determined that HIF-1α is a key downstream mediator underlying excess renal adenosine–induced expression of ET-1, a potent vasoconstrictor. Finally, we revealed that HIF-1α is an important factor responsible for Ang II–induced CD73 and Adora2b expression at the transcriptional level. Without interference, elevated CD73-mediated increased renal adenosine functioning via amplified ADORA2B signaling further enhanced HIF-1α induction, resulting in additional elevation of renal ET-1 production. Thus, AngII–HIF-1α–CD73–ADORA2B functions as a malicious cycle to facilitate ET-1 production in the kidney and leads to hypertension (Figure 6F). Overall, our findings reveal a previously unrecognized detrimental role of chronically elevated renal CD73 levels and ADORA2B signaling in Ang II–induced hypertensive CKD and thereby identify novel and important therapeutic possibilities for the disease.26

Multiple factors are released from locally insulted renal tissue and responding cells. One of the best-known signaling molecules to be induced under hypoxic conditions is adenosine.7 Adenosine protects tissues like the brain,28,29...
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...and heart 

...intestinal... and heart... from acute ischemic damage, thereby exhibiting chemoprotective properties... However, in the setting of repeated or prolonged tissue injury, chronic elevation of adenosine becomes detrimental by promoting or exacerbating tissue injury and dysfunction in the lung, penis, and kidney... Of note, it has been speculated that increased adenosine levels under acute hypoxic conditions may be beneficial by increasing blood flow to ischemic or hypoxic tissues because of the potent vasodilatory effects in most organs. However, in the kidney, adenosine causes afferent arterial constriction and thereby decreases blood flow to the kidney, which is speculated to be a compensatory effect to reduce transport workload in the kidney under acute hypoxic conditions. Thus, persistent elevation of adenosine in the kidney may be detrimental because of prolonged vasoconstriction of afferent arterioles and subsequent severe ischemic renal injury. Notably, previous studies reported that Ang II infusion induces renal adenosine elevation, but not systemically, in rats... In addition, a previous report showed that intrarenal adenosine produces hypertension in the dog by activating the sympathetic nervous system... More recent studies showed that adenosine leads
to increased norepinephrine production from the renal sympathetic nervous system via the A1 adenosine receptor.\textsuperscript{31} However, specific renal factors causing chronic elevation of adenosine in Ang II–infused mice remained unidentified.

High-throughput quantitative RT-PCR array analysis allowed us to identify elevated renal CD73 accounting for increased kidney adenosine in Ang II–infused mice and revealed the detrimental consequences of enhanced ADORA2B signaling in this mouse model of hypertensive renal disease. Our studies have significantly enhanced our understanding of pathogenesis of hypertensive CKD and are strongly supported by previously studies. For example, previous reports have shown that Ang II infusion results in increased levels of renal interstitial ATP and adenosine.\textsuperscript{40,41} The adenosine is presumably derived from ATP by the consecutive action of CD39, an ectonucleotidase that converts ATP→ADP→AMP and CD73, which converts AMP to adenosine. Our genetic and pharmacological data showing the importance of CD73 in Ang II–induced accumulation of renal adenosine are in good agreement with previous studies by Franco et al indicating that the Ang II–induced elevation of renal adenosine was significantly inhibited by specific blockade of 5′-nucleotidase.\textsuperscript{42} It is especially noteworthy that Ang II–induced hypertension was not blocked by drugs that inhibit adenosine nucleotide–mediated (ie, ATP, ADP) P2 receptor signaling.\textsuperscript{43} Consistent with these findings, we present multiple lines of genetic and pharmacological evidence that Ang II–induced hypertension and CKD require adenosine signaling. We show that Ang II–induced hypertensive CKD is reduced in CD73-deficient mice, that Ang II–induced hypertensive nephropathy is reduced by treatment with polyethylene glycol-modified ADA to lower adenosine,\textsuperscript{40,41} raising a novel concept in which, in the short-term or acute setting, elevated adenosine may be beneficial to the kidney by inducing afferent arterial constriction to reduce workload via A1 adenosine receptor signaling\textsuperscript{51} and stimulating an anti-inflammatory response or maintaining endothelial barrier via A2A adenosine receptor signaling.\textsuperscript{27} However, in the setting of CKD, chronically elevated renal adenosine coupled with excessive ADORA2B signaling becomes detrimental by inducing renal production of ET-1 to promote hypertension and exacerbate kidney injury and disease progression (Figure 6F).

Ischemia and hypoxia have long been considered to be associated with CKD and are speculated to contribute to progression of the disease, including hypertension and renal fibrosis.\textsuperscript{52,53} Of note, HIF-1α is well-known to be induced under hypoxia and associated with hypertensive CKD.\textsuperscript{54} As with adenosine signaling, HIF-1α signaling can be tissue-protective, whereas chronic elevated HIF-1α also can be detrimental.\textsuperscript{27,55–57} The detrimental effects of prolonged elevation of HIF-1α in CKD has been revealed by studies showing that HIF-1α contributes to renal injury and progression of renal fibrosis in multiple animal models, including Ang II–infused rats.\textsuperscript{37} Although hypoxia is known to increase HIF-1α, a growing body of evidence indicates that hypoxia-independent mediators, including inflammatory cytokines and growth factors, also stimulate HIF-1α gene activity.\textsuperscript{56,59} Because baseline oxygen tension in the kidney is already low, Ang II infusion is unlikely to cause substantial additional hypoxia. Here, we show that Ang II treatment directly induces HIF-1α levels in the absence of hypoxia in cultured endothelial cells. Significantly, we further provide in vivo evidence that Ang II infusion leads to elevation of HIF-1α in endothelial cells of glomeruli in mice. Thus, both in vitro and in vivo studies indicate that Ang II is a novel hypoxia-independent mediator directly regulating HIF-1α and cytokine signaling.\textsuperscript{58,59} Consequently, inflammatory cytokines and growth factors are likely to contribute to ATII–mediated renal fibrosis, which could be attenuated by inhibiting HIF-1α signaling.

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possible that HIF-1α and interleukin-6 may work together to regulate Ang II–mediated ET-1 induction, an important issue for future studies. Besides ET-1, we further revealed that HIF-1α is important for Ang II–promoted expression of the genes encoding CD73 and ADORA2B at the transcriptional level and serves to perpetuate the malicious cycle of adenosine signaling, resulting in excessive ET-1 production (Figure 6F). Thus, the evidence provided by our human and mouse studies supports a novel working model that chronic elevated renal adenosine contributes to hypertensive CKD via HIF-1α–mediated adenosine regulation in the following 2 ways: (1) by inducing ET-1 production to promote hypertension and kidney injury; and (2) by inducing CD73 and Adora2b gene expression to further enhance adenosine production and preferentially amplifying HIF-1α signaling in a malicious cycle that promotes the progression of the disease (Figure 6F). Thus, interfering with this detrimental cycle is important for novel therapeutics in the treatment of hypertension and renal disease.

Sources of Funding

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Disclosures

None.

References


Novelty and Significance

What is Known?

- Hypertension is the most prevalent life-threatening disease worldwide and is frequently associated with chronic kidney disease (CKD).
- The renin–angiotensin system is a key signaling cascade contributing to hypertension, CKD, and progression to renal fibrosis.
- Elevated levels of angiotensin II (Ang II), the primary effector of the renin–angiotensin system, contribute to hypertension and CKD.

What New Information Does This Article Contribute?

- We used an Ang II infusion model of hypertensive renal disease in mice to identify new signaling pathways that contribute to disease pathogenesis.
- Our findings reveal important novel opportunities for therapeutic intervention for the treatment of hypertension and renal disease.

We sought to identify new factors and signaling pathways that contribute to hypertensive CKD in an effort to reveal novel therapeutic approaches for treatment. For our studies, we used the well-accepted Ang II infusion model of hypertension in mice. To identify specific factors and signaling pathways contributing to the pathogenesis of CKD and hypertension, we used a high-throughput analytic screening strategy to identify differences in patterns of gene expression in the kidneys of control mice and those infused with Ang II. Unexpectedly, our results revealed that a family of genes associated with a particular signaling pathway (adenosine signaling) was expressed at elevated levels in the Ang II–infused mice. A role for adenosine signaling in Ang II–induced hypertension previously has not been recognized. Our research findings revealed new therapeutic targets that we explored in the Ang II infusion model of hypertensive renal disease in mice. We tested a variety of pharmacological approaches to inhibit the pathophysiological consequences of excessive adenosine signaling and found that each approach resulted in reduced blood pressure and renal damage and reduced progression to renal fibrosis. Thus, our preclinical studies reveal potentially important novel opportunities for therapeutic intervention in the treatment of hypertension and renal disease.
Elevated Ecto-5'-nucleotidase-Mediated Increased Renal Adenosine Signaling Via A2B Adenosine Receptor Contributes to Chronic Hypertension
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Elevated CD73-mediated increased renal adenosine signaling via A2B adenosine receptor underlies chronic hypertension

By

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Running title: adenosine signaling in hypertensive nephropathy

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**Chronic Ang-II infusion into mice**
Mice were anesthetized with isoflurane (2%), and osmotic minipumps (Alzet model 2001; Alza, Palo Alto, CA) were implanted subcutaneously in the nape of the neck. Ang II (Sigma, MI, USA) was delivered at a rate of 425ng/kg/min into 12 week old mice for 14-days. Control mice were infused with saline.

**Real-time RT-PCR analysis**

Transcript levels were quantified using real-time quantitative RT-PCR. Syber green was used for analysis of CD73, CD38, CD39, ADORA1, ADORA2B, ADORA3, ADORA2B, HIF-1α, Prepro-ET-1 and β-actin using the following primers:

- **Mouse CD73:** Forward, 5'-CAG ATC CGC AAG GAA GAA CC-3' and reverse 5'-ATG GTG CCC TGG TAC TGG TC-3';
- **Mouse CD38:** Forward: 5'-CGA AGG AGC TTC CGG TGG TCT CC-3' and Reverse: 5'-TGG CAG GCC TGT TAT CC-3';
- **Mouse CD39:** Forward: 5'-TCT GGG TGG AAC ATG TCA AA-3' and 5'-ACC AGCCTC TGA GTC CTG AA-3';
- **Mouse ET1A:** Forward: 5'-caacacatagcagatg-3' and Reverse: 5'-caggaaagctatgcta-3';
- **Mouse ADORA1:** forward, 5'-GTTTGGCTGAAACAACCTGA-3' and reverse 5'-ACACTTGATCAGGGCTCC-3';
- **Mouse ADORA2A:** forward 5'-CCC TCT CAT CTA CGC CTA CAG-3' and reverse 5'-GTG GGT TCG GAT GAT CTT CC-3';
- **Mouse ADORA2B:** forward, 5'-GCG AGA GGG ATC ATT GCT G-3' and reverse, 5'-CAG GAA CGG AGT CAA TCC AA-3';
- **Mouse ADORA3:** forward 5'-ATA CCA GAT GTC GCA AA-3' and reverse 5'-GCA GGC GTA GAC AAT AGG GTT-3';
- **Mouse HIF-1α:** forward 5'-CCT TCA TCG GAA ACT CCA AA-3' and reverse 5'-TGG GGC ATG GTA AAA GAA AG-3';
- **Mouse Prepro-ET1:** forward 5'-CTG CAC TCC ATT CTC AGC TCC-3' and reverse 5'-TTC CCG TGA TCT TCT CTC TGC-3';
- **Mouse β-actin:** forward 5'-GGG AAT GGG TCA AAA CT-3' and reverse 5'-CAG CAC TAT GTC GTC CCA AA-3';
- **Mouse HIF-1α:** forward 5'-TGC TCA TCG GCC ACT CTG TA-3' and reverse 5'-TCC CAC GCA AAT AGG CTG-3';
- **Human ADORA2B:** forward 5'-TGT CCC GGT TCG GAT TAA AAG T-3' and reverse 5'-CCC AGG AAT GGA GTC AAT CCG-3';
- **Human CD73:** forward 5'-ACC ACG TAT CCA TGT GCA TTT-3' and reverse 5'-AAA GGG CAA TAC AGC AGC CAG-3';
- **human β-actin:** forward 5'-TGA CCG GTG CAC CCA CAC TGT GCC CAT CTA-3' and reverse 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

**ADA activity assay**
Lysis buffer contains 25ml distilled water, 12.5ml 0.1M K2PO4, 5ml 0.1M KH2PO4, 0.5% NP-40 and protease inhibitor (Sigma 1: 20) then adds water to 50ml. Small piece of kidney was lysed in 400µl lysis buffer followed by sonication. Then the samples were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant were transferred to a new centrifuge tube and further super-centrifuged at 45000 rpm for 20 minutes at 4°C. The supernatant were saved. Protein concentration were measured and adjusted to 1mg/ml. ADA activity was measured in the supernatants obtained from high-speed centrifugation under saturating substrate conditions using a spectrophotometric assay as described. Briefly, approximate 940µl assay buffer (lysis buffer without protease inhibitor and NP-40) was added to 50µl supernatant and 10µl of 10mM adenosine. Mixed immediately and the decrease in absorbance at 265 nm resulting from deamination of adenosine to inosine was continuously monitored in a Beckman DU-50 spectrophotometer and the rate of inosine production was calculated at linearity. Specific activities are presented as nanomoles of adenosine converted to inosine per min per mg of protein.
CD73 activity measurement
CD73 enzyme activity was measured by quantifying the conversion of etheno-AMP (E-AMP) to ethenoadenosine (E-ADO) as described previously. Briefly, membrane fraction proteins of frozen kidneys were extracted and used to measure CD73-specific activity. First, one μg of total protein was preincubated at room temperature with 200 nM deoxycoformycin in 0.1 M HEPES (pH 7.4), with 50 μM MgCl₂, with or without αβ-methylene ADP (APCP; Sigma-Aldrich). Next, samples were incubated at 37°C for 30 min in the presence of 100 μM AMP. AMP hydrolytic activity (AMPase) was measured by determining adenosine concentrations with reversed phase HPLC as described before. Absorbance was measured at 260 nm, and ultraviolet absorption spectra were obtained at chromatographic peaks. CD73 activity was expressed as percent E-AMP conversion in this time frame.

ADA enzyme therapy and PSB1115 treatment
Polyethyleneglycol-conjugated adenosine deaminase (PEG-ADA) was generated by the covalent modification of purified bovine ADA with activated PEG as described previously. PEG-ADA (2.5u/week) was given weekly by intraperitoneal injection after the minipump was implanted until mice were euthanized. (1 Unit is defined as the amount necessary to convert 1 μM of adenosine to inosine/min at 25°C). PSB1115, ADOA2B antagonist (Tocris Bioscience, St. Louis, MO), was injected intraperitoneally at 200 µg in PBS per day after the minipump was implanted.

Quantification of renal adenosine levels
Mice were anesthetized, and the kidneys were rapidly removed and frozen in liquid nitrogen. Adenosine was extracted from frozen kidney tissue using 0.4 N perchloric acid, separated and quantified using reversed phase HPLC as described previously.

Mouse blood pressure measurements
We used two methods to measure systolic blood pressure. First, systolic blood pressure was measured by a carotid catheter-calibrated tail-cuff system (CODA, Kent Scientific, Torrington, CT) before and after minipump implantation as described. Specifically, blood pressure was monitored on day 0 which was considered as baseline and continuously measured on the day 3rd, 7th, 10th, and 14th of the 2-week experimental period. After an initial acclimatization of the mice for five cycles, blood pressure was monitored over a period of 20 cycles and averaged for the final blood pressure measurement. To minimize the circadian effects, blood pressure measurement and urine collection were conducted at the same time each day. Besides the tail cuff system to measure blood pressure in live animals, we also measure the blood pressure in anesthetized animals. Specifically, on the final day of Ang II infusion the intracarotid mean arterial blood pressure was measured in the mice after anesthesia with isoflurane (2%). The carotid artery was isolated and cannulated with a PE-50 microtip catheter. The intracarotid mean artery blood pressure was measured with a pressure transducer connected to a Grass Model 7B chart recorder (AD Instrument Co, USA). Blood pressure was recorded and averaged over a 10-minute period.

Mouse urine collection and analysis
Twenty four hour urine was collected from mice with or without Ang II infusion using a metabolic cage on different days (Nalgene). Total microalbumin in the urine was determined by using an ELISA kit (Exocell). Creatinine was determined at the University of Texas, MD Anderson Cancer Center, Laboratory of Veterinary Medicine. We used the ratio of urinary albumin to urinary creatinine as an index of urinary protein as described. For urine osmolarity analysis, the collected 24 hour urine was diluted 10 times with distilled water and measured with vapor pressure osmometer (Wescor, Logan, UT).

Histological analysis and Semi-quantification of histological changes
Human kidneys were isolated and perfused with 5ml of PBS and then fixed in 10% PBS buffered formalin overnight at 4°C. Fixed kidneys were rinsed in PBS, dehydrated through graded ethanol washes and embedded in paraffin. Sections (5μm) were collected on slides and stained with haematoxylin and
eosin (H&E) or Masson’s trichrome, according to the manufacturer’s instructions (Shardon-Lipshaw) as described. The extent of renal damage was assessed by quantifying the glomeruli and tubules that showed characteristic features of damage in CKD: decreased Bowman’s space, occlusion of capillary loop spaces, sclerosis and necrosis in both glomeruli and tubules. To examine those features, the glomeruli and tubules were counted in 6-9 fields of randomized and blinded slides (×10 magnification), with each field having at least 16-22 glomeruli and tubules, respectively. The glomeruli and tubules in each field were given a score based on the amount of capillary space evident within the Bowman’s capsule and necrosis or sclerosis in glomeruli and tubules. A highest score of 5 was accorded to glomeruli and tubules with a normal amount of capillary space within Bowman’s capsule and normal structures of tubules. A score of 1 was assigned to the glomeruli that showed complete loss of capillary space and sclerosis or necrosis. An intermediate score of 3 was assigned to the glomeruli that displayed reduced, but not completely obliterated, capillary space and partial necrosis.

Morphometric analysis of the renal fibrosis in Masson’s trichrome stained sections
Ten consecutive non-overlapping fields of a mouse kidney stained with the Trichrome were analyzed. The fibrotic areas stained in light blue were picked up on the digital images using a computerized densitometry (ImagePro Plus, version 6.0, Media Cybernetics, Silver Spring, MD, USA) coupled to a microscope equipped with a digital camera as described. The percentage of the fibrotic area relative to the whole area of the field was calculated (percent fibrosis area). The average densities of ten areas per kidney were averaged and the SEM is indicated and n = 4-6 kidneys for each category.

CD73, ADORA2B, HIF-1α and ET-1 immunohistochemistry in the kidneys and quantification.
Immunohistochemistry for CD73, ADORA2B, HIF-1α and ET-1 was also carried out with the formalin fixed tissues. Sections of 4µm were cut and mounted on glass slides, deparaffinized through serial baths in xylene and rehydrated in a graded series of alcohol and distilled water. Endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) at 95°C for 15 min. Next, endogenous avidin and biotin blocking was performed with a Biotin Blocking System (Dako). The slides were then incubated with rabbit anti-CD73 antibody (1:100 dilution), rabbit anti-ADORA2B (1:200), rabbit anti-HIF-1α antibody (Lifespan, 1:200 dilution) and rabbit anti-ET-1 (Sigma, 1:100 dilution) in a humidified chamber at 4°C overnight. After the primary antibody incubation, anti-rabbit IgG ABC staining system (Santa Cruz) was used. Slides were stained with DAB (BD, USA) after being washed in PBS for 5 min, washed in PBS and DAB (BIOS) for 5 min and counterstained with hematoxylin. For negative controls, the primary antibody was replaced with the corresponding affinity-purified preimmune IgG. Quantification of the immunohistochemical staining was performed using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The density of the brown staining was measured. The average densities of 25 areas per kidneys were determined and the SEM is indicated. n=6 for each group.

Isolation of kidney and organ culture
Kidneys were surgically isolated from WT mice, CD73- and ADORA2B-deficient mice. The isolated kidneys were washed in PBS, minced into 2-3 mm³ pieces and suspended in Dulbecco’s modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and cultured in a humidified atmosphere of 5% CO2 at 37°C. After 24 hours in culture, tissues were serum-starved in DMEM without FBS and treated with various reagents including Ang-II (100nM) (Sigma), NECA (10µM), Chrysine (10µM) (Enzon) or DOMG (10µM). After 24-hour treatment, total RNA was isolated and quantitative RT-PCR was conducted as described above.

Cell culture
Human microvascular endothelial cells (HMEC-1) were derived from dermal tissues and were harvested and cultured by a modification of methods described previously. HMEC-1 cells were passaged in 25
cm² culture flasks in 5% CO₂ humidified air at 37°C in MCDB131 medium supplemented with 10% heat-
inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, 10ng/ml epidermal
growth factor, 1µg/ml hydrocortisone and 2 mM L-glutamine (Invitrogen, USA). Cells at Passages 10-15
were used for all experiments. Before experimental intervention, cultured cells at 85% confluency were
switched to serum free medium and subsequently treated without or with NECA at 1µM. Some of cells
were treated with theophylline (10µM), MRS1706 (10µM) or SCH442416 (10µM). HMEC-1 cells were
stably transected with siRNA for HIF-1α to knock down HIF-1α (HIF-1α-KD cells). These cells were
also treated with NECA at 10µM. At different time points, total RNA was isolated and quantitative RT-
PCR was conducted as described above.

Transfection and Luciferase assay
About 1.5x10⁵ HMEC cells were placed to each well of a 24-well plate. After 24 hours, when the cells
were about 90% confluent, the cells were transected with various plasmids including wild type 0.57NT
(CD73-Luc), 0.57HRE (CD73-HRE-Mut), wild type ADORA2B-Luc or ADORA2B-HRE-Mut,
respectively (0.4µg DNA for each well) using lipofectamine 2000 (Invitrogen). The wild type and HRE
mutants were generated and provided by Dr. Sean Colgan as described before. Briefly, CD73-HRE-
Mut was generated by site-directed mutagenesis by mutating HRE consensus sequence from 5’-CGTG-
3’ to 5’-C4TGG-3’. Similarly, ADORA2B-HRE mutant was generated by mutating HRE consensus
sequence from 5’-ACGTG-3’ to 5’- A4TGC-3’. The empty plasmid pGL2 was used as a control for
normalization of fold change. Four hour after transfection, the cells were washed by 1XPBS and switched
to serum-free medium. Then, the cells were treated with or without 200nM angiotensin II or 10µM
NECA for 24 hours. The cells were rinsed by PBS and lysed by 1X reporter lysis buffer for luciferase
activity assay (Promega).

Western blot Analysis
Western blots were performed as described. Blots were incubated with polyclonal rabbit anti-HIF-1α
antibody (1:1000 dilution) or rabbit anti-β-actin antibody (1:1000 dilution) and secondary anti-rabbit
antibody (1:5000 dilution) for 1 hour each. The signals were visualized using ECL chemiluminescent
western blotting detection.

Statistical analyses
All data were expressed as the mean ± SEM. Student’s t tests (paired or unpaired as appropriate) were
applied in two-group analysis. Differences between the means of multiple groups were compared by the
one-way analysis of variance (ANOVA), followed by a Tukey’s multiple comparisons test. A value of P
< 0.05 was considered significant. Statistical programs were run using GraphPad Prism 4 software
(GraphPad Software, San Diego, CA)

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2006;176:4449-4458


# Online Table I- RT-PCR gene expression profiling of kidneys of mice with or without Ang II infusion

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For each gene, the fold changes were calculated as difference in kidney gene expression between Ang II infused and saline infused mice. P<0.05 is considered significant.
Online Figure I. Quantitative RT-PCR measurement of CD73 mRNA in the heart of wild type mice with or without Ang II infusion.
Online Figure II. Quantitative RT-PCR measurement of CD38 (A) and CD39 (B) in the kidney of wild type mice with or without Ang II infusion.
Online Figure III. ADA activity in the kidney of wild type mice with or without Ang II infusion. n=8
Online Figure IV. (A) Intracarotid mean arterial blood pressure (MAP) was measured on day 14 as indicated. (n=8-12/group). (B) Ratio of albumin to creatinine in 24 hour collected urine was measured on day 14 (n=8-12/group). (C) Osmolarity of 24 hour collected urine was measured on day 14 (n=8-12/group).
Online Figure V. mRNA levels of Ang II receptors (AT), endothelin-1 (ET-1), its receptors (ET1a and ET1b) and endothelin converting enzyme (ECE) in wild type mice and CD73-deficient mice.
Online Figure VI. Effects of polyethyleneglycol (PEG) on blood pressure in wild type mice. Systolic blood pressure was measured at daily intervals by the tailcuff method until mice were sacrificed. (n=4).
Online Figure VII. (A) Quantitative RT-PCR assay of expression profiles of adenosine receptors in HMECs. (B-C) NECA induces HIF-1α and Prepro-ET mRNA levels in HMECs in a time-dependent manner. Data are expressed as mean ± SEM. *P<0.05, versus the control without NECA treatment.