Indapamide lowers blood pressure by increasing production of epoxyeicosatrienoic acids in the kidney

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Non standard abbreviations used:
20-HETE: 20-hydroxyeicosatetraenoic acid; AA: arachidonic acid; Ang II: angiotensin II; CYP: Cytochrome P450; DHET: dihydroxyeicosatrienoic acids; DMSO: dimethyl sulfoxide; EDHF: endothelium-derived hyperpolarizing factor; EET: epoxyeicosatrienoic acids; HCTZ: hydrochlorothiazide; HEETs: hydroxy-EETs; IDP: indapamide; IK\(\beta\alpha\): inhibitor of kappa\(\beta\alpha\); JNK: c-Jun N-terminal kinase; LVH: left ventricular hypertrophy; MAPK: mitogen-activated protein kinase; MS-PPOH: N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide; MDA: malondialdehyde; NF-\(\kappa\)B: nuclear factor \(\kappa\)B; PCR: polymerase chain reaction; PKA: protein kinase A; PPAR\(\alpha\): peroxisome proliferator-activated receptor \(\alpha\); ROS: reactive oxygen species; sEH: Soluble Epoxide Hydrolase; SHR: spontaneously hypertensive rat; siRNA: small interfering RNA; SOD: superoxide dismutase; TGF-\(\beta\)1: transforming growth factor-\(\beta\)1; WKY: Wistar-Kyoto rat
Abstract

Diuretics are widely used in the treatment of hypertension, although the precise mechanisms remain unknown. EETs (cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid) play critical roles in regulation of blood pressure. The present study was carried out to investigate whether EETs participate in the anti-hypertensive effect of thiazide diuretics (hydrochlorothiazide (HCTZ)) and thiazide-like diuretics (indapamide). Male spontaneously hypertensive rats (SHR) were treated with indapamide or HCTZ for eight weeks. Systolic blood pressure, measured via tail-cuff plethysmography and confirmed via intra-arterial measurements, was significantly decreased in indapamide- and HCTZ-treated SHR compared with saline-treated SHR. Indapamide increased kidney CYP2C23 expression, decreased soluble epoxide hydrolase expression, increased urinary and renovascular 11,12- and 14,15-EETs and decreased production of 11,12- and 14,15-DHETs in SHR. No effect on expression of CYP4A1 or CYP2J3, or on 20-HETE production, was observed, suggesting indapamide specifically targets CYP2C23-derived EETs. Treatment of SHR with HCTZ did not affect the levels of CYPs or their metabolites. Increased cAMP activity and PKA expression were observed in the renal microvessels of indapamide-treated SHR. Indapamide ameliorated oxidative stress and inflammation in renal cortices by down-regulating the expression of p47phox, NF-κB, TGF-β1 and phosphorylated MAPK. Furthermore, the p47phox-lowering effect of indapamide in angiotensin II-treated rat mesangial cells was partially blocked by the presence of MS-PPOH or CYP2C23 siRNA. Together, these results indicate that the hypotensive effects of indapamide are mediated, at least in part, by the CYP epoxygenase system in SHR, and provide novel insights into the blood pressure-lowering mechanisms of diuretics.
Introduction

The modern era of diuretic therapy for hypertension began in 1957 when Novello and Sprague synthesized the thiazide diuretic, chlorothiazide. Further modification of the benzothiadiazine core led to the synthesis of hydrochlorothiazide (HCTZ) and the thiazide-like diuretics: chlorthalidone (phthalimidine), metolazone (quinazolinone), and indapamide (indoline). Indapamide binds and inhibits the Na⁺-Cl⁻ cotransporter in the distal convoluted tubule and connecting tubule but does not contain the benzothiadiazine core (Reilly et al., 2010). Despite similarities to other members of the thiazide family, indapamide has unique features that render it a particularly efficacious and advantageous anti-hypertensive agent (Sassard et al., 2005).

Indapamide is a relatively weak diuretic that has been shown to produce a significant and sustained reduction in blood pressure with a lower incidence of serious hypokalemia and hyperglycemia (Ambrosioni et al., 1998), and retains efficacy in patients with chronic kidney disease (Madkour et al., 1996). It has been demonstrated to reduce left ventricular hypertrophy (LVH) to a greater degree when compared with enalapril or atenolol monotherapy (Dahlof et al., 2005; de Luca et al., 2004; Gosse et al., 2000). It is also effective in reducing microalbuminuria in patients with diabetes and hypertension (Puig et al., 2007). Additional mechanisms by which indapamide may exert its anti-hypertensive effects have been proposed (Sassard et al., 2005). Uehara et al. showed that indapamide induces an increase in the levels of prostacyclin, a cyclooxygenase-derived metabolite of arachidonic acid (AA), in the vascular smooth muscle cells (Uehara et al., 1990). This raised the possibility that other AA metabolites may also play a role in the anti-hypertensive effects of indapamide.
In addition to the cyclooxygenases, AA can be metabolized by enzymes of the cytochrome P450 (CYP) superfamily. The CYP epoxygenases generate 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs), which are further metabolized to their corresponding less-active dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) (Fleming, 2001). The CYP ω-hydroxylases produce 20-hydroxyeicosatetraenoic acid (20-HETE) (Zhao and Imig, 2003). Both EETs and 20-HETE are involved in the regulation of vascular function (Zhao and Imig, 2003). In the renal microcirculation, 20-HETE promotes vasoconstriction by blocking large-conductance Ca\(^{2+}\)-activated K\(^+\) channels (Imig et al., 1996b) and stimulating L-type Ca\(^{2+}\) channels (Miyata and Roman, 2005), which contribute to an increase in blood pressure. On the other hand, EETs, which have been identified to be endothelium-derived hyperpolarizing factors (EDHF), promote vasodilation in the preglomerular arterioles via activation of renal smooth muscle cell Ca\(^{2+}\)-activated K\(^+\) channels, therefore leading to hyperpolarization of vascular smooth muscle cells and reduction in blood pressure. EETs also markedly enhance the production of atrial natriuretic peptide in the heart, which contributes to vasodilation and natriuretic effects (Xiao et al., 2010). The vasodilatory properties of EETs have been well characterized in many animal models.

The CYP2C subfamily enzymes are the major CYP epoxygenases in the kidney. In particular, CYP2C23 is the predominant enzyme expressed in the rat kidney and converts AA to 8,9-EET, 11,12-EET and 14,15-EET in a ratio of 1:2:1 (Imaoka et al., 1993). Furthermore, CYP2C23 can increase levels of hydroxy-EETs (HEETs) (Muller et al., 2004), which are endogenous activators of PPAR-α. PPAR-α activators are also highly expressed in kidney (Braissant et al., 1996) and exert
antioxidant and anti-inflammatory effects (Devchand et al., 1996; Diep et al., 2002; Kono et al., 2009). The production of CYP metabolites in the kidney is altered in rodent models of hypertension such as the spontaneously hypertensive rats (SHR) (Sacerdoti et al., 1989; Yu et al., 2000), and it is likely that changes in this system contribute to the abnormalities in renal function in these models.

In this study, we investigated the possibility that the beneficial effects of indapamide in SHR may be mediated through induction of CYP enzymes and alterations in levels of EETs or 20-HETE.
Materials and Methods

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Animal Research Committee of Tongji College. Eleven-week-old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rat (WKY) controls were obtained from the Experimental Animal Center of Beijing (Beijing, China). Rats were treated daily with indapamide (IDP, 1 mg/kg/day; Servier, France), hydrochlorothiazide (HCTZ, 20 mg/kg/day; Qingdao Huanghai Pharmaceutical Co., LTD, China) or saline (0.9% NaCl) via gastric gavage for 8 weeks.

Measurement of Blood Pressure

Systolic blood pressure was measured every two weeks at room temperature using tail-cuff plethysmography as described previously (Xiao et al., 2010). At 8 weeks after drug administration, the rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and a microtransducer catheter (SPR-838; Millar Instruments, Inc.) was inserted via the right carotid artery into the left ventricle according to a method described previously to measure blood pressure invasively (Xiao et al., 2010).

Cardiac functional study

Cardiac function was measured by echocardiograph (VIVID7, General Electric) equipped with a 15-MHz linear array ultrasound transducer. Parameters needed for
the calculation of cardiac function and dimensions were measured from a minimum of five systole-diastole cycles. Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured from LV M-mode tracing (with a sweep speed of 50mm/s) at the papillary muscle level: LV fractional shortening (FS) and ejection fraction (EF), measures of LV systolic function, were calculated from LV M-mode by the following equations:

\[
\text{FS\%} = \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100
\]

\[
\text{EF\%} = \left( \frac{\text{LVEDV} - \text{LVESV}}{\text{LVEDV}} \right) \times 100.
\]

**Isolation of Thoracic Aortic Rings and Determination of Vascular function**

Thoracic aortic rings were prepared as described previously (Xiao et al., 2010). We examined the responsiveness of aortic rings from rats treated with saline, indapamide (IDP) or hydrochlorothiazide (HCTZ) to norepinephrine (NE) and acetylcholine (ACh) with a multichannel physiologic recorder (ML-840 PowerLab; ADInstrument Pty Ltd.)

**Isolation of Renal Microvessels**

Renal microvessels were isolated according to a method described previously (Imig et al., 2001), collected, rapidly frozen in liquid nitrogen and stored at -80°C until use.

**Determination of 11,12- and 14,15-DHETs, 11,12- and 14,15-EETs and 20-HETE in Urine and Tissues**

An enzyme-linked immunosorbent assay (ELISA) kit (Detroit R&D Inc., Detroit, MI) was used to measure the concentrations of 11,12- and 14,15-EETs and their stable
metabolites, 11,12 and 14,15-DHETs, and 20-HETE in the urine and tissues, according to the manufacturer’s instructions. The amount of 11,12- and 14,15-EETs was quantitated by calculating the difference between total acidified 11,12- and 14,15-DHETs and non-acidified 11,12- and 14,15-DHETs, respectively, as described (Xiao et al., 2010).

**Western Blotting**

Western blot analysis was performed as described previously (Wang et al., 2003). Antibodies against CYP2C23, CYP2J2, CYP4A1 (Abcam), PKA, sEH, p47phox, p67phox, p-MAPK, MAPK, SOD1, SOD2, TGF-β1, p-IκBα, IKβα, NF-kB and β-actin (Santa Cruz) were used.

**Cyclic AMP, and MDA Assay**

Cyclic AMP (cAMP) levels in renal tissues were evaluated using the Cyclic AMP XP™ Assay Kit (Cell Signaling, MA), following the manufacturer’s instructions. Renal malondialdehyde (MDA) levels were measured as described previously (Li et al., 2010).

**Evaluation of Renal and Aortic Injury and Cardiac Hypertrophy**

Urinary microalbumin levels were measured using the Rat MALB ELISA kit according to the manufacturer’s instructions (Nanjing Jiancheng, Nanjing, China). Kidney sections (6 µm) were stained with Sirius red and hematoxylin-eosin, meanwhile, immunohistochemical detection of CD68 was performed as described previously (Xiao et al., 2010) using CD68 antibody (Santa Cruz Biotechnology, Inc.). Vessel wall collagen was assessed by Sirius red staining. Heart sections were stained with
hematoxylin-eosin. Cardiomyocyte diameter and the percentage of interstitial collagen content in the kidney were quantified using the HAIPS Pathological Imagic Analysis System (Tongji Qianping Image Company, Wuhan, China).

**Effects of Indapamide on CYP2C23 Production in HBZY-1 cells**

Rat renal mesangial (HBZY-1) cells were transfected with CYP2C23 siRNA (200 nmol/L) or treated with MS-PPOH (N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide, specific inhibitor of CYP epoxygenase, 10 µmol/L). Transfected or treated cells were incubated with/without indapamide (10 µmol/L) and angiotensin II (100 nmol/L) for 24 h, after which the cells were collected for western blot analysis.

**Statistical Analysis**

Values of quantitative results were presented as mean ± S.E.M. The data were analyzed by one factor analysis of Variance (ANOVA) using GraphPad Prism software (GraphPad Software Inc.). Statistical significance was accepted if p < 0.05.
Results

Treatment with diuretics lowers blood pressure in SHR

Administration of indapamide or HCTZ for 8 weeks had no effect on the blood pressure of WKY control rats; however, treatment of SHR with either drug decreased the blood pressure by 16.9 and 15.4 mmHg, respectively, compared with saline-treated controls (Supplemental Figure 1A). Prior to sacrifice at the 8-week time point, the carotid intra-arterial pressure was measured, and the results were consistent with the noninvasive tail-cuff measurements (Supplemental Figure 1B). Moreover, analysis of cardiac hemodynamics showed that dp/dt\text{max} was increased in indapamide-treated SHR compared with saline-treated SHR (Supplemental Figure 1C). Measurement of cardiac function by echocardiograph showed that EF and FS were increased in indapamide-treated SHR compared with saline-treated SHR, but not in HCTZ-treated SHR (Supplemental Figure 1D-E).

Renal CYPC23 expression and 11,12- and 14,15-EET levels are elevated by indapamide in SHR

To investigate whether renal CYPs play a role in the anti-hypertensive effect of indapamide, we quantitatively analyzed the mRNA expression of two CYP epoxygenases, CYP2C23 and CYP2J3, and an ω-hydroxylase, CYP4A1, in the kidney by real-time PCR (Supplemental Table 1). As shown in Fig. 1A, CYP2C23 mRNA levels were upregulated by 2.3-fold in indapamide-treated SHR compared with saline-treated SHR, whereas levels of CYP2J3 and CYP4A1 remained unchanged. Treatment with HCTZ had no effect on the expression of CYPs in the rats. In addition,
the protein expression of CYP2C23 was increased in indapamide-treated SHR (Fig. 1B). Interestingly, both indapamide and HCTZ decreased the protein expression of sEH in SHR, although indapamide reduced it to a greater degree (Fig. 1B). To estimate CYP activity, levels of 11,12- and 14,15-EETs and 20-HETE were measured. Indapamide increased levels of 11,12- and 14,15-EETs (Fig. 1C) and decreased levels of 11,12- and 14,15-DHETs (Fig. 1D) in the urine of SHR compared with saline controls. As a result, the EET:DHET ratio was increased by 2.5-fold (Fig. 1E). No significant differences in 11,12- and 14,15-DHETs and 11,12- and 14,15-EETs, respectively, were observed in HCTZ-treated SHR and all WKY groups. Meanwhile, the levels of 20-HETE in the urine were markedly increased in SHR compared with WKY (Fig. 1F), but were unaffected by treatment with indapamide or HCTZ.

In addition, the expression of CYP enzymes and EET levels were assessed in renal microvessels. Indapamide increased CYP2C23 expression and decreased sEH expression in the SHR microvessels relative to saline control, while levels of CYP2J3 were not significantly different between the groups (Fig. 2A-D). Furthermore, treatment of SHR with indapamide increased and decreased levels of 11,12- and 14,15-EETs (Fig. 2E) and 11,12- and 14,15-DHETs, respectively (Fig. 2F), in the microvessels. No significant differences in 11,12- and 14,15-DHETs and 11,12- and 14,15-EETs were observed in HCTZ-treated SHR and all WKY groups.

These results suggest that indapamide, but not HCTZ, stimulates CYP2C23 to generate more EETs without affecting the levels of other CYP enzymes and their metabolites.

**Indapamide increases cAMP levels and PKA expression in SHR renal**
microvessels

The vasodilatory effects of EETs in the renal vasculature have been associated with an increase in cyclic AMP (cAMP) levels and can be blocked by inhibitors of cAMP and PKA signaling (Carroll et al., 2006). To investigate whether these components were altered with indapamide treatment in SHR, renal microvessels were isolated. Interestingly, both cAMP levels (Fig. 3A) and PKA expression (Fig. 3B) were increased in indapamide-treated SHR compared with saline-treated SHR, suggesting that indapamide may increase vasodilation via a cAMP/PKA-dependent pathway. No significant differences in cAMP levels and PKA were observed in HCTZ-treated SHR and all WKY groups.

**Oxidative stress and inflammation in the renal cortex of SHR are attenuated with indapamide treatment**

In addition to increasing EET production, CYP2C23 is known to upregulate the expression of HEETs, endogenous PPAR-α activators that have both anti-oxidant and anti-inflammatory properties (Muller et al., 2004). To investigate whether indapamide affects oxidative stress and inflammation, both of which are commonly observed in SHR, renal cortices were isolated. Renal tissues from SHR displayed increased levels of malondialdehyde (MDA), a marker for oxidative stress (Fig. 4A), and elevated expression levels of two NADPH oxidase subunits (p47phox and p67phox), as compared to that from WKY (Fig. 4B). Treatment of SHR with indapamide or HCTZ significantly decreased levels of MDA (Fig. 4A) and prevented the increase in the expression of p47phox and p67phox (Fig. 4B and C). SOD1 and SOD2 were decreased in SHR compared with WKY but were markedly increased with HCTZ.
treatment, while SOD2 was subtly increased with indapamide treatment (Fig. 4B and D). In addition, indapamide, but not HCTZ, attenuated renal inflammatory responses by significantly decreasing the expression of CD68 and p65 NF-κB by 25 and 23%, respectively (Fig. 4E and F). Meanwhile, immunohistochemistry against CD68 showed that indapamide or HCTZ treatment decreased CD68-positive cells relative to the marked increase in saline-treated SHR (Supplemental Figure 2A). Furthermore, HCTZ treatment decreased phosphorylated IκBα, while indapamide or HCTZ treatment increased total IκBα expression (Fig. 4F). Protein levels of TGFβ1 and the phosphorylation of p38 MAPK and JNK were also enhanced in SHR; treatment with indapamide or HCTZ decreased these effects (Supplemental Figure 2B-D). No differences in pro-oxidant or inflammatory factors were observed among WKY groups.

Indapamide prevents renal and aortic damage and myocardial hypertrophy

Renal damage (Feld et al., 1990) and left ventricular hypertrophy are often observed in SHR. H&E staining of renal structures showed that increased solidified glomeruli (a glomerulopathy) in saline-treated SHR were decreased with indapamide or HCTZ treatment (Supplemental Figure 3A). Collagen staining of kidney sections revealed that indapamide or HCTZ significantly reduced the renal collagen content in SHR compared to saline controls (Supplemental Figure 3B-C). This was associated with a decrease in albuminuria suggesting that renal damage is attenuated by indapamide or HCTZ in these hypertensive rats (Supplemental Figure 3D). Furthermore, serum creatinine measured by Picric acid showed that the increased serum creatinine in saline-treated SHR was decreased by treatment with indapamide or HCTZ (Supplemental Figure 3E). Analysis of collagen in the aorta cell wall showed that
indapamide or HCTZ treatment decreased collagen deposition in the intima-media and ameliorated oxidative stress (Supplemental Figure 4A-B). Measurement of vascular function by artery rings showed that contraction in response to NE decreased and dilation in response to ACh increased in aortic rings from HCTZ- and indapamide-treated SHR compared with SHR control (Supplemental Figure 4C). We also evaluated the degree of myocardial hypertrophy in SHR by measuring cardiomyocyte diameter in H&E-stained heart sections and calculating the ratio of left ventricular weight:body weight (mg/g). A marked reduction in cardiomyocyte diameter (Supplemental Figure 5A-B) and the ratio of left ventricular weight:body weight (Supplemental Figure 5C) was observed in indapamide-treated SHR compared with saline-treated SHR, suggesting that indapamide also attenuates myocardial hypertrophy in hypertension.

**Indapamide-induced reduction of p47phox is CYP2C23-dependent in HBZY-1 cells**

To confirm the role of CYP2C23 in the effects of indapamide in the kidney, we evaluated the angiotensin II-induced increase in p47phox expression in rat mesangial (HBZY-1) cells that were either treated with MS-PPOH, a specific CYP epoxygenase inhibitor, or transfected with CYP2C23 siRNA. A 50% reduction in CYP2C23 protein was achieved in CYP2C23 siRNA-transfected cells. Treatment of control HBZY-1 cells with angiotensin II significantly increased the expression of p47phox and p67phox. Addition of indapamide to these cells decreased the angiotensin II-mediated induction in p47phox and p67phox expression. However, the p47phox or p67phox-lowering effect of indapamide was partially blocked in cells treated with MS-PPOH or transfected with CYP2C23 siRNA (Fig. 5A and B), which also exhibited...
significant decreases in CYP2C23 expression (Fig. 5C). These results further suggest that the anti-oxidant effects of indapamide are mediated via CYP2C23 in the kidney.
Discussion

This study was undertaken to investigate the effect of indapamide on blood pressure in hypertensive rats and the mechanisms involved. The results showed that indapamide reduced blood pressure in SHR and altered the expression of renal CYP2C23 and soluble epoxide hydrolase (sEH), leading to increases in EETs and decreases in DHETs. Indapamide did not have significant effects in WKY control rats. Interestingly, hydrochlorothiazide (HCTZ) decreased blood pressure in the SHR to a similar degree as indapamide, but failed to affect renal CYP expression and production of EETs/DHETs in both the urine and renal tissue. These results imply that CYP2C23-derived EETs may be involved in the anti-hypertensive effect of indapamide, but not of HCTZ.

CYP2C isoforms are considered to be the major arachidonic acid epoxygenases in the kidney. In particular, CYP2C23 is the major epoxygenase expressed in rat kidney and converts AA to 8,9-, 11,12- and 14,15-EET (Holla et al., 1999). Among these, 11,12-EET is the most active vasodilator in the preglomerular vasculature (Imig et al., 1996a) and a potent anti-inflammatory epoxide (Node et al., 1999). Induction of CYP2C23 not only increases the levels of EETs, but also stimulates the endogenous PPAR-α activator, HEET (Muller et al., 2004). PPAR-α is highly expressed in the kidney (Braissant et al., 1996) and exerts both antioxidant and anti-inflammatory effects (Devchand et al., 1996; Diep et al., 2002; Kono et al., 2009). These characteristics, combined with our observations, suggest that the hypotensive effects of indapamide may be due, at least in part, to increases in CYP2C23 expression and EET production.
EETs have been identified to be endothelium-derived hyperpolarizing factors (EDHF) (Campbell et al., 1996) and the predominant products generated by a rat CYP2C23 present in isolated renal microvessels (Imig et al., 2001). The current study shows that indapamide increased CYP2C23 expression and 14,15-EET levels in renal microvessels of SHR. Indapamide also decreased sEH expression, which may have a synergistic effect with CYP2C23 in increasing 14,15-EET production and decreasing the levels of DHETs, which are less active in the vasculature (Imig et al., 1996a). Previous studies have demonstrated that 11,12-EET analogs increase cAMP but not cGMP levels (Imig et al., 2008) and EETs dilate renal arteries by activating renal smooth muscle cell Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Zou et al., 1996), which are dependent on PKA activation (Imig et al., 1999). Our data showed that treatment of SHR with indapamide also increased cAMP levels and PKA expression in isolated renal microvessels. It is possible that these changes in cAMP and PKA may increase dilation in the renal vasculature, thus leading to a decrease in blood pressure.

It is well known that renal oxidative stress, inflammation and hypertension are highly interrelated (Rodriguez-Iturbe et al., 2001; Touyz, 2005; Vaziri, 2004); modulating any one of them could affect the status of the other two (Nava et al., 2003; Rodriguez-Iturbe et al., 2003). Meanwhile, antioxidants are known to reduce blood pressure in SHR (Nava et al., 2003; Rodriguez-Iturbe et al., 2003) and NF-κB blockade reduces oxidative stress and blood pressure in SHR (Elks et al., 2009). A recent study showed that expression of NADPH oxidase subunits, p47phox and p67phox, are upregulated in the kidney of SHR (Chabrashvili et al., 2002). In addition, studies have shown that superoxide dismutase (SOD) activity is suppressed in SHR (Ito et al., 1995; Ushiyama et al., 2004). The SODs act as the first line of defense
against ROS-mediated damage by catalyzing the dismutation of unstable superoxide anions to H$_2$O$_2$. Thus, treatment with SOD mimetics decreases superoxide anion production and attenuates the development of hypertension in SHR (Schnackenberg et al., 1998). The data presented in this study indicate that indapamide ameliorated oxidative stress in the renal cortex of SHR, potentially by decreasing p47phox and p67phox expression, increasing SOD expression, and attenuating renal inflammatory responses by decreasing p65NF-κB expression, which may be associated with the anti-inflammatory effects of 11,12-EET or HEETs.

Oxidative stress can trigger the activation of redox-sensitive signal transduction pathways such as those that include NF-κB, which in turn intensifies oxidative stress (Vaziri and Rodriguez-Iturbe, 2006) and upregulates c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways (Hehner et al., 2000). Moreover, JNK and p38 MAPK play important roles in renal fibrosis, acting downstream of TGF-β1. Previous studies showed that blockade of JNK abrogates the pathogenesis of interstitial fibrosis (Ma et al., 2007) and a p38 MAPK inhibitor reduces extracellular matrix production in the rat kidney (Stambe et al., 2004). The role of TGF-β1 in renal fibrosis is widely accepted (Schnaper et al., 2002). Enhanced expression of TGF-β1 has been shown to contribute to the development of renal fibrosis in hypertensive rats (Gallego et al., 2001). The present study revealed that indapamide treatment reduced renal collagen deposition, decreased levels of TGF-β1 and inhibited the activation of p38 and JNK in the renal cortex of SHR, suggesting that indapamide may reduce renal inflammation and fibrosis by decreasing oxidative stress and MAPK activation. Furthermore, indapamide attenuated oxidative stress by increasing CYP2C23 expression \textit{in vitro} in HBZY-1 cells, which was partially
abrogated by addition of MS-PPOH, a specific P450 epoxygenase inhibitor, or transfection with CYP2C23 siRNA.

In summary, the present study provides evidence that activation of the CYP2C23 epoxygenase pathway may be involved in the anti-hypertensive effect of indapamide. We suggest that indapamide increases EET production via the induction of CYP2C23 and the inhibition of sEH, which ameliorates the hypertension observed in SHR through increasing cAMP and PKA expression in the renal microvessels and decreasing the expression of NADPH oxidase subunits, p47phox and p67phox, NF-κB and TGF-β1 in the renal cortex. However, HCTZ decreased blood pressure and ameliorated the oxidative stress and inflammation in the renal cortex without activating the CYP epoxygenase pathway, which will be investigated in the future.
Authorship Contributions

Participated in research design: Ma, Lin, Y. Wang, Chen and D. W. Wang

Conducted experiments: Ma and Lin

Performed data analysis: Ma, Lin, Y. Wang and D. W. Wang

Wrote or contributed to the writing of the manuscript: Ma, Lin, D. W. Wang, Cheng and Zeldin
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Footnotes

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Fei Ma and Fan Lin contributed equally to this work.
Figure legends

Figure 1. Expression of CYP enzymes in the kidney and urinary levels of 11,12- and 14,15-EETs, 11,12- and 14,15-DHETs and 20-HETE measured using ELISA kits. Kidneys and urine were collected from SHR and WKY rats treated with saline, indapamide (IDP) or hydrochlorothiazide (HCTZ). (A) mRNA levels of CYP2C23, CYP2J3 and CYP4A1 was determined by real-time PCR and normalized to GAPDH. N=5, *p<0.05 vs. saline-treated WKY, #p<0.05 vs. saline-treated SHR. (B) Representative western blot depicting the protein expression of CYP2C23, sEH, CYP2J3 and CYP4A1. N=3, duplicated three times. (C) 11,12- and 14,15-EETs, (D) 11,12- and 14,15-DHETs, (E) EETs:DHETs ratios, and (F) 20-HETE levels. N=5, *p<0.05 vs. saline-treated WKY, #p<0.05 vs. saline-treated SHR.

Figure 2. Levels of CYP enzymes and their metabolites in the renal microvessels. Renal microvessels were isolated from WKY and SHR treated with saline, indapamide (IDP) or hydrochlorothiazide (HCTZ). (A-B) Representative western blots and densitometry analyses of CYP2C23, sEH and CYP2J3. N=3, duplicated three times, *p<0.05 vs. saline-treated WKY, **, #p<0.05 vs. saline-treated SHR. (C) 11,12- and 14,15-EETs, 11,12- and 14,15-DHETs levels and ratios of EETs/DHETs were determined via an ELISA kit and normalized to the amount of protein in the tissues. N=6, *p<0.05 vs. WKY, #p<0.05 vs. saline-treated SHR.

Figure 3. Renal vascular cAMP levels and PKA expression in rats. Renal microvessels were isolated from SHR and WKY rats treated with saline, indapamide (IDP) or hydrochlorothiazide (HCTZ). (A) cAMP levels in renal microvessels were determined using the cAMP assay kit. N=8, *p<0.05 vs. saline-treated WKY, #p<0.05
vs. saline-treated SHR. (B) Representative western blot and densitometry analysis for PKA. N=3, duplicated three times , *p< 0.05 vs. WKY, #p<0.05 vs. saline-treated SHR.

**Figure 4.** The effects of indapamide or HCTZ on oxidative stress and inflammation in the renal cortex. Renal cortices were isolated from SHR and WKY rats treated with saline, indapamide (IDP) or hydrochlorothiazide (HCTZ). (A) Malondialdehyde (MDA) levels were measured as an indicator of oxidative stress, using a commercial assay kit. N=5, *p<0.05 vs. saline-treated WKY, **, #p<0.05 vs. saline-treated SHR. (B) Representative western blots and corresponding densitometry analyses showing the expression of (C) p47phox, p67phox, (D) SOD1, SOD2, (E) CD68, (F) p65-NF-κB, p-IκBα , IκBα and β-actin was used as the loading control. N=3, duplicated three times , *p<0.05 vs. saline-treated WKY, ** , #p<0.05 vs. saline-treated SHR.

**Figure 5.** The effects of CYP inhibition and indapamide on the angiotensin II-induced increase in p47phox expression in rat renal mesangial (HBZY-1) cells. Cells were transfected with CYP2C23 siRNA (200 nmol/L) or treated with MS-PPOH (10μmol/L) in the presence/absence of indapamide (IDP; 10 μmol/L) and angiotensin II (100 nmol/L) for 24 h. (A-B) Representative western blots and corresponding densitometry analyses of p47phox and p67phox in MS-PPOH-treated and CYP2C23 siRNA-transfected cells. N=3, duplicated three times , *p<0.05 vs. DMSO, #p<0.05 vs. Ang II, **p<0.05 vs. Ang II + IDP. (C) Densitometry analyses of CYP2C23 in MS-PPOH-treated and CYP2C23 siRNA-transfected cells. N=3, duplicated three times , *p<0.05 vs. Ang II, #p<0.05 vs. Ang II + IDP.
Figure 2

A

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<td>CYP2J3</td>
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B

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<th>11,12-DHET (pg/mg tissue)</th>
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