Deletion of soluble epoxide hydrolase gene improves renal endothelial function and reduces renal inflammation and injury in streptozotocin-induced type 1 diabetes

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Elmarakby AA, Faulkner J, Al-Shabrawey M, Wang MH, Maddipati KR, Imig JD. Deletion of soluble epoxide hydrolase gene improves renal endothelial function and reduces renal inflammation and injury in streptozotocin-induced type 1 diabetes. Am J Physiol Regul Integr Comp Physiol 301: R1307–R1317, 2011. First published August 10, 2011; doi:10.1152/ajpregu.00759.2010.—Studies suggest that soluble epoxide hydrolase (sEH) inhibition reduces end-organ damage in cardiovascular diseases. We hypothesize that sEH gene (Ephx2) knockout (KO) improves endothelial function and reduces renal injury in streptozotocin-induced diabetes. After 6 wk of diabetes, afferent arteriolar relaxation to acetylcholine was impaired in diabetic wild-type (WT) mice, as the maximum relaxation was 72% of baseline diameter in the WT but only 31% in the diabetic mice. Ephx2 KO improved afferent arteriolar relaxation to acetylcholine in diabetes as maximum relaxation was 58%. Urinary monocyte chemotactant protein-1 (MCP-1) excretion significantly increased in diabetic WT mice compared with control (868 ± 195 vs. 31.5 ± 7 pg/day), and this increase was attenuated in diabetic Ephx2 KO mice (420 ± 98 pg/day). The renal phospho-IKK-to-IKK ratio and nuclear factor-κB were significantly decreased, and hemeoxygenase-1 (HO-1) expression increased in diabetic Ephx2 KO compared with diabetic WT mice. Renal NADPH oxidase and urinary thiobarbituric acid reactive substances excretion were reduced in diabetic Ephx2 KO compared with diabetic WT mice. Albuminuria was also elevated in diabetic WT mice compared with control (170 ± 43 vs. 37 ± 13 μg/day), and Ephx2 KO reduced this elevation (50 ± 15 μg/day). Inhibition of sEH using trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (tAUCB) also reduced renal inflammation and injury in diabetic WT mice. Furthermore, inhibition of HO with stannous mesoporphyrin negated the renoprotective effects of tAUCB or Ephx2 KO during diabetes. These data demonstrate that Ephx2 KO improves endothelial function and reduces renal injury during diabetes. Additionally, our data also suggest that activation of HO-1 contributes to improved renal injury in diabetic Ephx2 KO mice.

Afferent arteriolar relaxation; albuminuria; NF-κB; HO-1; NADPH oxidase

DIABETIC RENAL INJURY IS CONSIDERED the leading cause of end-stage renal disease nowadays (11, 19, 24). The disease begins with proteinuria, which then progresses to renal inflammation and a decline in glomerular filtration rate (40, 46). The pathogenesis of diabetic renal injury could be linked to the impairment of endothelial function and increased vascular inflammation (4, 40). In diabetes, NADPH oxidase, the major source of superoxide production in the vasculature, has been shown to be activated, and its expression is augmented in the glomerulus and distal tubules (17, 51). Recent evidence demonstrates increased renal macrophage infiltration and overproduction of leukocyte adhesion molecules in experimental diabetic animal models (7, 16). Clinically, inflammatory processes in the kidney contribute to the progression of diabetic renal injury in patients with type 1 diabetes (35, 48). Oxidative stress has been shown to activate nuclear factor-κB (NF-κB), which then modulates the expressions of inflammatory genes in diabetes (25), suggesting that oxidative stress and inflammatory cytokines play a role in the pathogenesis of diabetic renal injury.

Cytochrome P-450 (CYP)-derived metabolites epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) are synthesized from arachidonic acid by CYP epoxygenase and ω-hydroxylases, respectively (45). Renal 20-HETE production is catalyzed primarily by CYP4A isoforms, whereas renal EETs production is catalyzed by CYP2C and CYP2J1 isoforms in rodents (45). Although 20-HETE is a potent vasoconstrictor and prohypertensive metabolite in the vasculature, it has been shown that 20-HETE inhibits sodium transport and promotes natriuresis at the renal tubule (45). Recent studies suggest that EETs could have potential impact in combating cardiovascular diseases as they are endothelium-derived hyperpolarizing factors (EDHF) with anti-inflammatory and antiplatelet aggregation properties (5). Overexpression of endothelial CYP epoxygenase reduces the elevation in blood pressure and renal injury in salt-sensitive angiotensin II hypertensive mice (26). However, EETs are quickly degraded by soluble epoxide hydrolase (sEH) to their less active or inactive dihydroxyeicosatrienoic acids (DHETEs) (20). We have previously shown that inhibition of TNF-α or chemo-kine receptor-2b (CCR2b) upregulates CYP2C23, the main enzyme responsible for the generation of EETs, and reduces renal injury in salt-sensitive angiotensin II hypertension (14, 15). Thus, a diminished EETs availability could be a potential mechanism for the progression of cardiovascular diseases, including diabetic renal injury.

There are two documented approaches to experimentally increase EETs level in vivo. The first is to pharmacologically inhibit sEH. Pharmacological inhibition of sEH prevents EETs degradation and reduces renal injury and inflammation in salt-sensitive hypertension and in hypertensive Goto-Kakizaki type 2 diabetic rats (21, 38, 55). The second approach is to
utilize sEH null mice (Ephx2 KO). Ephx2 gene is responsible for sEH production, and Ephx2 KO mice are viable and have increased EETs availability and improved cardiac function after ischemic/reperfusion injury compared with wild-type (WT) mice (47). Manhiani et al. (33) have recently shown that Ephx2 gene deletion attenuates renal injury and inflammation in DOCA-salt hypertension. The role of sEH in the pathogenesis of diabetic renal injury has not been clearly explored. Accordingly, the present study will test the hypothesis that Ephx2 KO improves renal endothelial function and reduces renal injury and inflammation in streptozotocin-induced diabetes.

MATERIALS AND METHODS

The protocol for all animal studies was submitted to and approved by the Georgia Health Sciences University Animal Care and Use Committee. Wild-type (WT) C57BL/6J and homozygous male Ephx2 KO (Jackson Laboratory, Sacramento, CA) mice were used in this study at 14–16 wk of age. Diabetes was induced by overnight fasting followed by streptozotocin injection (Sigma, St Louis, MO) at a dose of 70 mg·kg⁻¹·day⁻¹ ip for three consecutive days. Urinary blood glucose was tested 3 days later to confirm glucosuria. Random blood glucose was also assessed at the end of the experiment, and any diabetic mice with blood glucose < 300 mg/dl were excluded. Four groups of mice were used (n = 6–8 per group): WT, diabetic WT, homozygous Ephx2 KO, and diabetic Ephx2 KO. Systolic blood pressure was recorded weekly using the tail cuff method for 6 wk, and mice were placed into metabolic cages for 24-h urine collection at the end of the 6-wk experiment. Mice were then anesthetized with pentobarbital sodium and kidneys were collected and immediately frozen in liquid nitrogen and then stored at −80°C. Liquid chromatography-mass spectrometry was used to measure the ratio of renal EETs/DHETEs as previously described (3).

Renal injury. Urinary albumin and nephrin excretions were measured as an index of renal injury using ELISA kit from Exocell (Philadelphia, PA). Kidney sections were embedded and frozen in optimal cutting temperature (Tissue-Tek, Hatfield, PA). Kidneys were then sliced into 5-μm sections and stained with Masson’s trichrome for collagen deposition according to the manufacturer’s recommended protocols (Richard Allan Scientific, Kalamazoo, MI). Ten images were taken per mouse at ×200 magnification power. Urinary collagen excretion was also determined using an ELISA kit from Exocell (Philadelphia, PA).

Renal inflammation. Renal p65-NF-kB activity and urinary monocyte chemoattractant protein-1 (MCP-1) excretion were measured as markers of renal inflammation. MCP-1 was determined using an ELISA kit from BD Bioscience (Minneapolis, MN) and renal NF-kB activity was assessed using NF-kB (p65) transcription factor assay from Cayman (Ann Arbor, MI).

Renal oxidative stress. NADPH oxidase activity was determined by the lucigenin chemiluminescence method using 35 μg protein in the presence of NADPH (100 μM) and lucigenin (5 μM) as previously described (30), and average sample counts (in cpm) were normalized to microgram of protein. Urinary thiobarbituric acid reactive substances (TBARS) were also assessed by using kits purchased from Cayman (Ann Arbor, MI). Renal hemeoxygenase-1 (HO-1) activity was determined using a kit from Assay Design (Ann Arbor, MI).

Western blot analysis. Fifty micrograms of homogenized kidney protein samples were separated by SDS-PAGE on a 10% Tris-glycine gel, and proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the blots overnight at 4°C in a Tris NaCl buffer (TBS) containing 5% nonfat dry milk and 0.1% Tween 20. The primary antibodies used were rabbit sEH, CYP2C44, CYP4A (Santa Cruz Biotechnology, Santa Cruz, CA), IKK, phospho-IKKα (ser180)/IKKβ (ser181) (Cell Signaling Technology, Danvers, MA), HO-1, and HO-2 (Assay Design, Ann Arbor, MI). The blots were then washed in TBS-0.1%...
Twin and incubated with the secondary antibody goat anti-rabbit (1:5,000) or goat anti-mouse (for β-actin) conjugated to horseradish peroxidase for 1 h. Detection was accomplished using enhanced chemiluminescence Western blot analysis, band intensity was measured densitometrically, and the values were normalized to β-actin.

In vitro juxtamedullary nephron preparation. A separate set of mice were used for this experiment (n = 4–5). Mice were anesthetized with pentobarbital sodium, and the right kidney was isolated for study of renal microvasculature as previously described (12). Briefly, the right renal artery was cannulated through the superior mesenteric artery after a midline laparotomy, and the kidney was immediately perfused with a Tyrode’s solution containing 6% albumin and a mixture of l-amino acids. After a 20-min equilibration period, an afferent arteriole was chosen to assess baseline diameter. The afferent arteriole was constricted with phenylephrine and endothelium-dependent and -independent relaxations were assessed by using increasing concentrations of acetylcholine and sodium nitroprusside, respectively. To determine the relative contribution of EETs in endothelial-dependent relaxation, afferent arteriolar responses to acetylcholine were also assessed in the presence of the nitric oxide synthase (NOS) inhibitor Nω-nitro-l-arginine-methyl ester (l-NNAME) (100 μM) and cyclooxygenase inhibitor indomethacin (10 μM).

Inhibitor studies. To determine whether pharmacological inhibition of sEH provides the same degree of renal protection as Ephx2 gene deletion during diabetes and whether HO inhibition reduces diabetic protection of Ephx2 KO or sEH inhibition during diabetes, six additional groups of mice were studied as follows: 1) WT, 2) diabetic WT, 3) diabetic WT plus the sEH inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (tAUCA) at a dose of 10 mg/I for 6 wk, 4) diabetic WT plus tAUCA plus the HO inhibitor stannous mesoporphyrin (SnMP) at a dose of 10 mg/100 gm body wt ip weekly for 6 wk, 5) diabetic Ephx2, and 6) diabetic Ephx2 plus SnMP (n = 5–7 per group). Blood pressure and urinary excretion levels of albumin, nephrin, MCP-1, and TBARs were examined after 6 wk of diabetes.

**Statistical analysis.** All data are presented as means ± SE. Statistical significance was determined using a one-way ANOVA followed by a Tukey’s post hoc test to identify differences between WT and Ephx2 KO mice with or without diabetes. Student’s t-test was also used to compare between diabetic Ephx2 KO mice with or without SnMP treatment. A P value of < 0.05 was considered as statistically significant.

**RESULTS**

Initially, we verified that Ephx2 KO mice did not express sEH by using Western blot analysis for sEH protein in WT and Ephx2 KO renal homogenate. Renal sEH expression decreased in diabetic WT compared with control, although this change was not significant (P < 0.06). Ephx2 KO mice with or without diabetes did not express sEH protein (Fig. 1A). In addition, liquid chromatography-mass spectrometry analysis of the EETs-to-DHETEs ratio in renal homogenates from WT and Ephx2 KO mice showed a significant decrease in diabetic WT compared with control mice. The renal EETs-to-DHETEs ratio was significantly higher in Ephx2 KO mice compared with diabetic WT or control mice (P < 0.05). Although there was a mild decrease in the renal EETs-to-DHETEs ratio in diabetic Ephx2 KO compared with Ephx2 KO mice, the renal EETs-to-DHETEs ratio remained significantly higher in diabetic Ephx2 KO compared with diabetic WT mice (P < 0.05, Fig. 1A). We also assessed the expression of renal CYP2C44, the major CYP epoxide, and CYP4A, the major CYP ω-hydroxylase in mice kidney. Ephx2 KO mice tended to have a higher renal CYP2C44 expression than WT mice, although the change was not significant (Fig. 1B). Although induction of diabetes decreased renal CYP2C44 expression in WT mice, renal CYP2C44 expression level was significantly higher in diabetic Ephx2 KO than those in diabetic WT mice, and there was no difference in CYP2C44 expression between control and diabetic Ephx2 KO mice (Fig. 1B). There was no significant change in renal CYP4A expression between control WT and Ephx2 KO mice. Induction of diabetes significantly decreased renal CYP4A expression in both WT and Ephx2 KO mice, and there was no difference in CYP4A expression between diabetic WT and diabetic Ephx2 KO mice (Fig. 1C). Consistent with previous findings (33), there was no difference in basal blood pressure values between WT and Ephx2 KO mice (113 ± 4 in WT vs. 110 ± 6 mmHg in Ephx2 KO mice). Induction of diabetes with streptozotocin produced a modest increase in blood pressure in WT and Ephx2 KO mice (119 ± 7 in diabetic WT vs. 121 ± 7 mmHg in diabetic Ephx2 KO) after 6 wk; however, these changes were not significant.

Endothelial function was assessed in WT and Ephx2 KO control with or without diabetes using in vitro juxtamedullary nephron preparation (Fig. 2). Afferent arteriolar relaxation to acetylcholine was impaired in diabetic WT mice compared with control WT as the maximum relaxation was 72% of baseline diameter in the WT mice but only 31% in the diabetic WT mice (P < 0.05, Fig. 2A). Although afferent arteriolar relaxation to acetylcholine was lesser in Ephx2 KO mice compared with WT, this change was not significant. Induction of diabetes did not significantly change afferent arteriolar relaxation to acetylcholine in Ephx2 KO (Fig. 2A). Afferent arteriolar relaxation to acetylcholine was also assessed after blocking both cyclooxygenase metabolites and NO dilatory

**Fig. 2.** Afferent arteriolar relaxation to acetylcholine in the absence (A) and presence (B) of indomethacin and Nω-nitro-l-arginine methyl ester (l-NNAME) in WT, diabetic WT, Ephx2 KO, and diabetic Ephx2 KO mice. A: induction of diabetes significantly decreased afferent arteriolar relaxation to acetylcholine in diabetic WT mice compared with control WT mice. Although afferent arteriolar relaxation to acetylcholine was lower in Ephx2 KO mice compared with WT mice, this change was not significant; however, induction of diabetes did not produce any further impairment in afferent arteriolar relaxation to acetylcholine in Ephx2 KO mice. B: blocking nitric oxide (NO) and cyclooxygenase metabolites using l-NNAME and indomethacin, respectively, reduced afferent arteriolar relaxation to acetylcholine in all the groups; however, there was no difference in afferent arteriolar relaxation to acetylcholine between control WT and Ephx2 KO. Induction of diabetes produced a further impairment in afferent arteriolar relaxation to acetylcholine in WT mice without a significant change in afferent arteriolar relaxation in Ephx2 KO mice (n = 4–5, *P < 0.05 vs. control WT mice).
Renal collagen deposition was assessed using Masson’s trichrome staining (Fig. 4A). The intensity of Masson’s trichrome stain increased in the diabetic WT mice, while it was lesser in the diabetic Ephx2 KO mice. The increase in collagen deposition in diabetic WT mice was also associated with the elevation in urinary collagen excretion in diabetic WT mice vs. control mice (Fig. 4B). Deletion of Ephx2 gene significantly reduced collagen excretion in Ephx2 KO mice compared with diabetic WT mice although collagen excretion remained significantly higher in diabetic Ephx2 KO compared with control WT or Ephx2 KO mice (Fig. 4B).

To determine the role of inflammation in the progression of renal injury in diabetic mice, renal phospho-IKK-to-IKK ratio, renal p65-NF-κB activity, and urinary MCP-1 excretion were determined in WT and Ephx2 KO mice with or without diabetes as shown in Fig. 5. Renal phospho-IKK-to-IKK ratio and p65-NF-κB activity significantly increased in diabetic WT mice compared with control mice (P < 0.05, Fig. 5). The renal phospho-IKK-to-IKK ratio was lower in control or diabetic Ephx2 KO mice compared with diabetic WT mice (P < 0.05). Renal p65-NF-κB activity was also lesser in control and diabetic Ephx2 KO mice compared with the diabetic WT group, although these changes were not significant (Fig. 5). Urinary MCP-1 excretion, another index of renal inflammation, was significantly elevated in diabetic WT mice. Control WT and Ephx2 KO mice displayed very low but delectable levels of urinary MCP-1, and diabetic Ephx2 KO mice excreted significantly lower levels of MCP-1 than diabetic WT mice (P < 0.05 vs. control WT, #P < 0.05 vs. diabetic WT, and †P < 0.05 vs. control Ephx2 KO).

Fig. 3. Indices of renal injury in streptozotocin-induced diabetic mice. There was no difference in albumin and nephrin excretions between WT and Ephx2 KO mice. Albumin and nephrin excretions increased significantly in diabetic WT compared with control WT. Diabetic Ephx2 KO mice displayed lesser albumin, and nephrin excretion levels compared with diabetic WT mice although nephrin excretion remained significantly higher in diabetic Ephx2 KO than control WT or Ephx2 KO mice (n = 6–8/group, *P < 0.05 vs. control WT, #P < 0.05 vs. diabetic WT, and †P < 0.05 vs. control Ephx2 KO).

Fig. 4. A: Masson’s trichrome staining for collagen in kidney histological sections. Intensity of Masson’s trichrome stain was increased in diabetic WT compared with control WT and was reduced in control and diabetic Ephx2 KO mice (n = 4/group). These results were further confirmed by assessing urinary collagen excretion (B). Urinary collagen excretion significantly increased in diabetic WT compared with control WT or Ephx2 KO mice. Although collagen excretion was significantly reduced in diabetic Ephx2 KO compared with WT diabetic mice, its level remained significantly higher than control WT or Ephx2 KO mice (*P < 0.05 vs. control WT, †P < 0.05 vs. diabetic WT, and †P < 0.05 vs. control Ephx2 KO).
0.05), although MCP-1 excretion remained higher in diabetic Ephx2 KO mice than those in control WT or Ephx2 mice.

Because oxidative stress has been implicated in the pathogenesis of diabetic-induced renal injury (51), renal oxidative stress was assessed in WT and Ephx2 KO mice indirectly using urinary TBARs excretion. As shown in Fig. 7A, although there was no difference in urinary TBARs excretion in control WT and Ephx2 KO mice, induction of diabetes significantly increased TBARs excretion in both WT and Ephx2 KO mice compared with control WT or Ephx2 KO mice, respectively ($P < 0.05$). However, urinary TBARs excretion was significantly lower in diabetic Ephx2 KO compared with diabetic WT mice ($P < 0.05$). Because previous studies have shown that HO-1 expression is induced in response to oxidative insults under pathophysiological conditions (1), we also assessed renal HO-1 expression in control and diabetic WT and Ephx2 KO mice (Fig. 6A). There was no difference in basal HO-1 expression between control WT and Ephx2 KO mice. Induction of diabetes significantly increased renal HO-1 expression in diabetic Ephx2 KO compared with either control WT or Ephx2 KO ($P < 0.05$), and HO-1 expression was also higher in diabetic Ephx2 KO compared with diabetic WT mice ($P < 0.06$). Similarly, renal HO-1 activity was also higher in diabetic Ephx2 KO than other mice groups; however, this elevation was only significantly different compared with diabetic WT mice (Fig. 6B). There was no difference in renal HO-2 expression between groups (Fig. 6C). Finally, HO-1 induction has been demonstrated to inhibit NADPH oxidase-
derived superoxide production (10). Accordingly, renal NADPH oxidase activity was assessed in control and diabetic WT and Ephx2 KO mice (Fig. 7B). Renal NADPH oxidase activity was significantly increased in diabetic WT mice compared with control mice, and this increase was prevented in diabetic Ephx2 KO compared with diabetic WT mice.

To determine whether the effects of sEH inhibition on renal inflammation and injury are different than the effects of Ephx2 gene deletion, another set of diabetic WT mice were treated with tAUCB, a selective inhibitor to hydrolase domain of the sEH enzyme for 6 wk. Also, to further determine whether the inhibition of HO negates the reno-protective effects of Ephx2 gene deletion or sEH inhibition, a separate set of diabetic WT mice plus tAUCB and diabetic Ephx2 KO mice were treated with the HO inhibitor SnMP for 6 wk. Induction of diabetes did not significantly increase blood pressure in WT mice and tAUCB treatment had no significant effect on blood pressure in diabetic WT mice (data not shown). Pharmacological inhibitions of HO with SnMP tended to increase blood pressure in diabetic WT mice treated with tAUCB and in diabetic Ephx2 KO mice, although these changes were not statistically significant (data not shown). Inhibition of sEH with tAUCB decreased renal injury in diabetic WT mice as evidence by decreased albumin and nephrin excretion levels (Fig. 8). Inhibition of sEH also reduced urinary MCP-1 excretion as a marker of inflammation in diabetic WT mice; however, contrary to Ephx2 gene deletion, tAUCB treatment failed to lower urinary TBARs excretion as a marker of oxidative stress in diabetic WT mice (Fig. 9). Inhibition of HO with SnMP prevented the ability of sEH inhibitor tAUCB or Ephx2 gene deletion to reduce renal injury and inflammation during diabetes as SnMP treatment restored the decrease in albumin, nephrin, and MCP-1 excretion levels and increased urinary TBARs excretion in diabetic WT mice treated with tAUCB as well as in diabetic Ephx2 KO mice (Figs. 8 and 9).

**DISCUSSION**

Increased oxidative stress and inflammation play an important role in the pathogenesis of diabetic renal injury (4, 40). EETs are implicated in the regulation of vascular function and blood pressure control as they have anti-inflammatory properties (5). The major finding of the present study is that deletion of the Ephx2 gene improves renal endothelial function and reduces renal injury and inflammation in streptozotocin-induced diabetic mice. Renal injury markers were attenuated in diabetic Ephx2 KO mice as shown by reduced albumin, nephrin, and collagen excretion levels. Renal inflammation was also reduced in diabetic Ephx2 KO mice as seen by decreased renal NF-kB activation and MCP-1 excretion. The reduction in renal inflammation in diabetic Ephx2 KO mice was associated with increased renal HO-1 activation and decreased TBARs excretion and NADPH oxidase activity. Similar to Ephx2 gene deletion, inhibition of sEH reduced renal injury markers and decreased urinary MCP-1 excretion in diabetic WT mice. Additionally, inhibition of HO prevented the reno-protective effect of sEH inhibition or Ephx2 gene deletion during diabetes. The present study extends previous findings of the reno-protective effects of sEH inhibition or Ephx2 KO in salt-sensitive hypertension (33) as well as sEH inhibition in salt-sensitive hypertension and in hypertensive Goto-Kakizaki type 2 diabetic rats (21, 38, 55) and provides new evidence for the role of HO-1 induction in the reno-protective effects of Ephx2 KO in diabetic-induced renal injury.

Although previous studies suggest that the blood pressure lowering effect of sEH inhibition might be a contributing factor for decreased renal inflammation and injury and improved endothelial function (21, 55), the possibility of a pressure-dependent effect to reduce renal injury could be ruled out in the present study. Ephx2 KO mice have blood pressure similar to WT mice even after induction of diabetes. Likewise, inhibition of sEH with tAUCB also did not affect blood pressure in diabetic WT mice, suggesting that end-organ protection occurs independently of lowering blood pressure in renal disease models. This is also supported with previous findings that Ephx2 KO mice have decreased inflammation and end-organ damage independent of blood pressure lowering effect in DOCA-salt hypertension (33), although an initial study showed that Ephx2 KO mice had a lower blood pressure than WT mice (49). Olearczyk et al. (38) have also shown that inhibition of sEH improves renal injury without a blood pressure-lowering effect in salt-sensitive hypertensive diabetic rats. The Ephx2 gene is made up of a COOH-terminal hydrolase and NH2-modified tyrosine hydroxylase (53). The COOH-terminal hydrolase domain of sEH is highly conserved among mammals, whereas the NH2-terminal hydroxylase domain appears to be evolutionarily more divergent (53). Therefore, the COOH-terminal hydrolase domain may be responsible for the reno-protective effect of sEH inhibition in diabetic-induced renal injury.
terminal phosphatase domain. It becomes clearly evident that renal protection in Ephx2 KO is solely mediated to the inhibition of the hydrolase domain, as pharmacological inhibition of sEH using tAUCB as a selective inhibitor for the COOH-terminal hydrolase domain provides similar degree of renal protection as shown in Ephx2 KO mice in salt-sensitive hypertension (33) as well as in diabetic WT mice in the present study.

Interestingly, we observed a significant endothelial dysfunction after 6 wk of induction of diabetes only in WT mice. The relative contribution of EETs in endothelium-dependent relaxation to acetylcholine was also assessed by determining afferent arteriole relaxation to acetylcholine after blocking both cyclooxygenase metabolites and NO dilatory effects. Afferent arteriole relaxation to acetylcholine decreased in WT and Ephx2 KO after L-NAME and indomethacin treatment, suggesting the importance of Ephx2 gene deletion in attenuating reno-vascular responses during diabetes. However, the impairment in afferent arteriole dilation was exacerbated in diabetic WT mice and this impairment was lesser in diabetic Ephx2 KO mice, suggesting that cyclooxygenase and NO pathways could be upregulated in WT mice as a compensatory response during diabetes. Induction of diabetes with streptozotocin has been shown to lower renal and hepatic sEH expression levels and high glucose treatment also lowered sEH expression levels in cultured human hepatoma cell line (37). Consistent with these findings, renal sEH expression decreased in diabetic WT mice and was absent in Ephx2 KO mice. Renal CYP2C44 was also higher in Ephx2 KO mice and did not significantly decrease upon induction of diabetes, suggesting increased EETs bioavailability in control and diabetic Ephx2 KO mice. This was further supported by the elevation in the renal EETs-to-DHETEs ratio in control and diabetic Ephx2 KO mice. The increase in EETs levels in Ephx2 KO mice would also explain why impairment in endothelial function in diabetic Ephx2 KO mice was less compared with diabetic WT mice. Although there are conflicting data in the literature regarding renal sEH expression in diabetes (37, 41), our data are consistent with the previous finding of Luo et al. (31), which show a decrease in glomerular CYP4A, CYP2C23, CYP2C22, and CYP2J without a significant change in sEH expression in streptozotocin-induced type 1 diabetic rats. Together our data suggest that decreased EETs levels contribute to endothelial dysfunction in diabetic WT mice, whereas higher EETs levels function to improve incidence of endothelial dysfunction in diabetic Ephx2 KO mice.

Previous studies suggest that the impairment in endothelial function precedes the incidence of renal inflammation and injury in cardiovascular diseases. In diabetes, Luo et al. (32) recently reported a decrease in the production of glomerular 20-HETE and EETs in diabetic rats together with increased proteinuria and histological damage. Decreased 20-HETE and EETs productions have also been shown to increase glomerular TGF-β, which could lead to glomerular damage in diabetic and hypertension (8, 56). Because the degree of renal injury is in direct proportion to the increase in albumin excretion and increased nephrin excretion levels have been used recently as indication of renal injury (44), we assessed albumin and nephrin excretion levels as markers of renal injury. Urinary albumin and nephrin levels were elevated in diabetic WT mice and were significantly decreased in diabetic Ephx2 KO mice. This was also supported by increased intensity of Masson’s trichrome staining for collagen in kidney section from diabetic WT mice together with increased urinary collagen excretion.
These changes were also reduced in diabetic Ephx2 KO mice. These data suggest that Ephx2 gene deletion could slow but not prevent the progression of diabetic renal injury. These findings were supported by the previous findings of Manhiani et al. (33) as they showed decreased renal nephrin expression and increased albuminuria in DOCA-salt hypertensive mice where these changes were reversed in Ephx2 KO DOCA salt mice. Manhiani et al. (33) also showed that inhibition of sEH with tAUCB provides the same degree of renal protection as Ephx2 gene deletion, which is consistent with our data in the present study. Inhibition of sEH also reduced glomerular and tubular collagen excretion, vascular hypertrophy, and albuminuria in salt-sensitive hypertension and diabetic hypertensive rats (38). Therefore, the present study demonstrates that decreased renal EETs availability is associated with increased renal injury in diabetes and increased EETs levels via Ephx2 gene deletion is a novel approach to protect kidney from diabetic-induced renal injury.

There is growing evidence that diabetes is an inflammatory disease (9–13). Inflammatory cytokines are important mediators in the pathogenesis of streptozocin-induced diabetic injury (9, 23). Oxidative stress and NF-κB signaling contribute to the incidence of endothelial dysfunction and renal injury and inflammation in diabetes (4, 25, 51). Past studies suggest that EETs are anti-inflammatory, at least in part via inhibition of NF-κB inflammatory signaling activation (34, 36). Although EETs have been shown to inhibit NF-κB activation in vitro (36), there is no clear evidence in vivo about the mechanisms of EETs-inhibition of NF-κB inflammation. NF-κB proteins are composed of the two subunits p50 and p65, which are present in the cytoplasm as inactive heterodimers bound to the inhibitory protein IκB (18). The key step in NF-κB activation is the phosphorylation of IκB by the active phospho-IKK. Once phosphorylated, IκB is proteolytically degraded and NF-κB translocates into the nucleus to activate cell adhesion molecules and MCP-1, thereby inducing vascular injury (18). In our study, the renal P-IKK-to-IKK ratio, P65-NF-κB activity, and urinary MCP-1 excretion increased in diabetic WT mice, and these changes were reduced in diabetic Ephx2 KO mice. Pharmacological inhibition of sEH with tAUCB also decreased MCP-1 excretion in diabetic WT mice. Thus, Ephx2 gene deletion or sEH inhibition could reduce renal injury in diabetes via EETs inhibition of phospho-IKK-derived NF-κB activation. However, EETs inhibition of NF-κB-induced inflammation could be also attributed to the activation of the anti-inflammatory nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) as previously shown (29). Our data are consistent with the previous findings that renal inflammation, macrophage infiltration, and NF-κB activation increased in DOCA-salt hypertensive mice, and these changes were blunted in Ephx2 KO DOCA-salt hypertensive mice (33). Inhibition of sEH also reduced lipopolysaccharide-induced acute inflammation in mice (22, 28). In mice with pressure overload-induced myocardial hypertrophy, inhibition of sEH reversed left ventricular hypertrophy via the blockade of NF-κB activation (53). Inhibition of sEH also reduced macrophage infiltration, renal NF-κB activity, and MCP-1 excretion in salt-sensitive hypertensive diabetic rats (38). Although the alteration of glomerular hydrostatic pressure could be the driving force to increase albuminuria, we were not able to...
assess this parameter in the present study. Based on the results in the literature, we think that decreased renal inflammation and oxidative stress could drive the decrease in albuminuria in diabetic mice. We have previously shown that inhibition of TNF-α reduced albuminuria and renal NF-κB inflammatory signaling activation in DOCA-salt hypertension independent on blood pressure effects (13). Accordingly, we could postulate that increased renal EETs availability in diabetic Ephx2 mice decreased albuminuria via inhibition of IKK-induced NF-κB inflammatory signaling activation.

A recent study showed that that overexpression of CYP2J3 epoxygenase improved insulin resistance in diabetic db/db mice and in fructose-induced insulin resistant rats (54). Luo et al. demonstrated that eNOS inhibition or Ephx2 gene deletion prevented hyperglycemia in streptozotocin-induced diabetic mice via the increase in glucose-stimulated insulin secretion and inhibition of cytokines induced-β cell apoptosis. In our present study, there was no difference in random blood glucose levels between control WT and Ephx2 KO mice, and induction of diabetes increased blood glucose in both strains; however, diabetic Ephx2 mice had lower blood glucose levels than diabetic WT (485 ± 23 vs. 508 ± 22 mg/dl, respectively). Ephx2 gene deletion did not affect insulin levels during diabetes compared with WT mice (data not shown). The differences between our data and the data of Luo et al. could be attributed to the use of 14- to 15-wk-old mice in our study compared with younger age (6-wk-old) mice in their study besides the dose and number of injection of streptozotocin as well as the difference in glucose homeostasis and randomly assessing blood glucose in our study, rather than fasting mice. However, the present study supports the hypothesis that Ephx2 gene deletion could improve diabetic renal injury independent on hypoglycemic effects.

HO is the primary pathway for the catabolism of heme-generating biliverdin, iron, and CO and biliverdin, which is further metabolized to bilirubin (1, 2). There are two isoenzymes of HO: HO-1 and HO-2. HO-1 is inducible and is upregulated in response to hypoxia, oxidative stress, ischemia, and inflammatory cytokines (1, 2). HO-2 is the constitutive isoenzyme, which accounts for most HO activity in a normal healthy state. HO-1 is induced to yield a protective effect via the dissipation of the prooxidant heme and the generation of the antioxidant metabolites (1, 2). For example, CO can induce vasodilation in addition to its anti-inflammatory and antiapoptotic effects (52). Bilirubin is also a potent antioxidant by decreasing NADPH oxidase-derived superoxide production (27). Overexpression of HO-1 improves endothelial function and inhibits endothelial cell sloughing in diabetic rats (39). Because hyperglycemia increases renal oxidative stress and vascular inflammation (25, 40), HO-1 induction could protect diabetic kidney via antioxidant and anti-inflammatory effects. Although EETs and HO share overlapping anti-inflammatory biological activities, a possible link between them has only been examined in vitro. The 11,12-EET stimulates HO-1 activity and expression in cultured endothelial cells (43). Rat mesenteric vasoconstrictor activation of 11,12-EET has been also shown to be mediated via an increase in HO activity (42). Consistent with previous findings, renal HO-1 expression and activity increased in diabetic Ephx2 KO mice with no change in HO-2 expression. Renal NADPH oxidase activity and urinary TBARS excretion increased in diabetic WT mice and these increases were attenuated in diabetic Ephx2 mice. These data are consistent with the recent findings of Sodhi et al. (50) who showed that HO-2 null mice exhibit a marked vascular inflammation. Treatment of HO-2-null mice with a dual-activity EETs agonist/sEH inhibitor increased renal and vascular EETs levels and renal HO-1 expression and these changes were associated with a reduction in serum TNF-α and MCP-1 and restoration of acetylcholine relaxation (50). Inhibition of HO with SnMP in the present study negated the reno-protective effects of Ephx2 gene deletion or sEH inhibition during diabetes as it increased inflammatory and renal injury markers. These data suggest that HO-1 induction in Ephx2 KO mice upon diabetic insult could be attributed to the increase in EETs levels, and induction of HO-1 in Ephx2 KO mice could provide renal protection via the reduction in NADPH-derived oxidative stress and inflammation during diabetes.

Perspectives and Significance

Recently published data show that the selective sEH inhibitor AR9281 is now in clinical trial, targeting hypertension and type 2 diabetes (6), which is consistent with our findings that the Ephx2 gene plays a crucial role in the development of cardiovascular disease and sEH inhibition could be a new avenue in halting the progression of end-organ damage. Our study demonstrates that diabetic-induced renal injury and inflammation can be ameliorated by preventing EETs degradation. The reno-protective effects of Ephx2 gene could be correlated to a significant reduction in NF-κB-induced inflammation and activation of HO-1 antioxidant mechanisms and were not dependent on the prevention of hyperglycemia or lowering blood pressure. Therefore, the potential therapeutic benefits of using sEH inhibitors in diabetic renal injury include a reduction in inflammation and enhancement of antioxidant defense mechanisms, which could prevent the disease progression to end-stage renal diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

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