Antiatherosclerotic and Renoprotective Effects of Ebselen in the Diabetic Apolipoprotein E/GPx1-Double Knockout Mouse

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OBJECTIVE—To investigate the effect of the GPx1-mimetic ebselen on diabetes-associated atherosclerosis and renal injury in a model of increased oxidative stress.

RESEARCH DESIGN AND METHODS—The study was performed using diabetic apolipoprotein E/GPx1 (*ApoE*$^{-/-}$*GPx1*$^{-/-}$)-double knockout (dKO) mice, a model combining hyperlipidemia and hyperglycemia with increased oxidative stress. Mice were randomized into two groups, one injected with streptozotocin, the other with vehicle, at 8 weeks of age. Groups were further randomized to receive either ebselen or no treatment for 20 weeks.

RESULTS—Ebselen reduced diabetes-associated atherosclerosis in most aortic regions, with the exception of the aortic sinus, and protected dKO mice from renal structural and functional injury. The protective effects of ebselen were associated with a reduction in oxidative stress (hydroperoxides in plasma, 8-isoprostane in urine, nitrotyrosine in the kidney, and 4-hydroxynonenal in the aorta) as well as a reduction in VEGF, CTGF, VCAM-1, MCP-1, and Nox2 after 10 weeks of diabetes in the dKO aorta. Ebselen also significantly reduced the expression of proteins implicated in fibrosis and inflammation in the kidney as well as reducing related key intracellular signaling pathways.

CONCLUSIONS—Ebselen has an antiatherosclerotic and renoprotective effect in a model of accelerated diabetic complications in the setting of enhanced oxidative stress. Our data suggest that ebselen effectively repletes the lack of GPx1, and indicate that ebselen may be an effective therapeutic for the treatment of diabetes-related atherosclerosis and nephropathy. Furthermore, this study highlights the feasibility of addressing two diabetic complications with one treatment regimen through the unifying approach of targeted antioxidant therapy. ***Diabetes*** **59:3198–3207, 2010**

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Chronic kidney disease is associated with enhanced morbidity and mortality, particularly caused by accelerated cardiovascular disease (1). Diabetes is emerging as an independent risk factor for both chronic kidney disease and atherosclerosis (2). Diabetic renal and cardiovascular complications are known to share common underlying pathogenic mechanisms, with oxidative stress and systemic inflammation contributing to both (3,4). Limiting oxidative stress through the removal of reactive oxygen species (ROS) is one strategy postulated to limit ROS-mediated chronic kidney disease and atherosclerosis in diabetic patients (5). However, antioxidant therapies such as vitamins E and C have shown limited benefits in clinical trials targeted at reducing cardiovascular outcomes (6,7). This has led to intensive efforts to define alternative antioxidant strategies for clinical applications (8).

Recent studies by our group have suggested that targeting antioxidant defenses, which are reduced in organs susceptible to injury in the diabetic setting (9), may be an appropriate strategy (10). Our studies have shown an involvement of the key antioxidant enzyme, glutathione peroxidase-1 (GPx1), in diabetes-associated proatherogenic pathways that included proinflammatory and profibrotic mediators, suggesting that this antioxidant holds promise as a therapeutic target (10). Our data are also supported by clinical findings where a reduction in GPx1 activity has been linked to an increased risk of cardiovascular disease, both within a diabetic setting and associated with coronary artery disease (11–13).

Modulation of GPx1 activity can be achieved through administration of selenium, the essential trace element found within the active site of GPx1, (14) although this holds less pharmacologic appeal because of selenium toxicity (15). In addition, lack of specificity caused by incorporation of selenium into several key enzymes makes this approach less attractive (14). Thus, compounds that mimic GPx1 activity offer an alternate way to increase GPx1-like activity (16).

Ebselen, a lipid-soluble low molecular weight selenoorganic compound and a known GPx1-mimetic, (17) has shown potential in reducing pathogenesis in various experimental models (18–20). Of significance in the Zucker diabetic fat rat, a model of type 2 diabetes associated with the metabolic syndrome, ebselen improved endothelial dysfunction and renal insufficiency (21,22). Furthermore, data from our laboratory have shown that ebselen reduces diabetes-associated lesions and proinflammatory media-

tors in the ApoE-deficient mouse (23). However, no study has investigated the effects of ebselen on atherosclerosis and nephropathy, two clinically relevant and often linked comorbidities, in one animal model. Such a study would strengthen the notion that oxidative stress underpins these diabetic complications, and would highlight the effectiveness of a targeted antioxidant approach against both conditions.

Consequently, in this study our objectives were as follows: First, our study investigated whether ebselen protects against diabetic nephropathy and diabetes-associated atherosclerosis in the diabetic ApoE/GPx1-deficient mouse. Second, we specifically chose the ApoE-deficient mouse additionally lacking Gpx1 to establish whether ebselen replenishes the lack of GPx1 activity. This has been performed to further clarify the mechanism of action of ebselen in an in vivo context, as well as strengthen the idea that replenishing GPx1-like activity may be of therapeutic benefit (11). Finally, our study also addressed potential pathways affected by ebselen in a rat kidney cell line.

RESEARCH DESIGN AND METHODS

Animal groups and experimental design. Eight-week-old male *ApoE*$^{-/-}$*GPx*$^{-/-}$ double knockout (dKO) mice (10) were rendered diabetic by two intraperitoneal injections of streptozotocin (Sigma, USA) on consecutive days, at 100 mg/kg/day (10). For comments on group selection, please see the supplementary appendix available online at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0195/DC1. Diabetic mice were divided into ebselen-gavaged and cellulose-gavaged groups. Ebselen (Sapphire Bioscience, Australia), dissolved in 5% CM-cellulose (Sigma, USA), was gavaged twice daily at 10 mg/kg starting at 10 weeks of age. Diabetes was allowed to progress for 10 or 20 weeks. Kidneys from nondiabetic and diabetic *ApoE*$^{-/-}$ mice rendered diabetic as detailed above, together with their ebselen-gavaged counterparts, were also assessed to facilitate comparisons with dKO kidneys in the development of diabetic renal complications.

Blood sampling, plasma biochemistry, and tissue collection. Plasma was analyzed for diamicron reactive oxygen metabolites (dROMs), a marker of hydroperoxides, using the FRAS-4 system as well as glucose, cholesterol, HDL, and triglycerides using commercial kits (see the supplementary online appendix for expanded METHODS). Glycated hemoglobin was measured in erythrocyte lysates by HPLC (Bio-Rad, USA). Urinary albumin excretion was measured using a mouse albumin ELISA kit (Bethyl Laboratories, USA). Plasma and urinary creatinine was measured by cation exchange chromatography (24).

One kidney was snap-frozen and stored at −70°C for gene expression and protein studies. The other was divided longitudinally, fixed in 10% neutral buffered formalin (NBF), and processed for immunohistochemical analysis, after which 3-μm paraffin-embedded cross-sections were cut.

Twenty weeks after initiation of diabetes, aortas from diabetic mice or sham-injected controls were fixed in 10%-NBF for en face assessment of atherosclerotic lesions. Aortas were stained with Sudan IV and dissected into arch, thoracic, and abdominal regions, after which photographic images were quantitated. Aortas from mice that were diabetic for 10 weeks, together with aged-matched controls, were either snap-frozen in liquid nitrogen and stored for gene expression studies, or fixed in 10%-NBF and processed for immunohistochemistry.

Immunohistochemistry. Aortic paraffin sections were stained for nitrotyrosine, 4-hydroxynonenal (4-HNE), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), NADPH oxidase-2 (Nox2), and vascular cell adhesion molecule (VCAM-1). Kidney sections were stained for nitrotyrosine, Nox2, collagen I, and fibronectin. Detailed methods and antibodies (supplementary Table 1) are described in the online appendix, including Mouse on Mouse immunodetection to localize antibodies raised in mouse (4-HNE, VCAM-1). For aortic analysis, three sections of each region were assessed per mouse, and 7 to 9 mice analyzed per group. Results were calculated as the percentages of positively-stained tissue and expressed relative to nondiabetic dKO mice. For glomerular assessments, 20 glomeruli were analyzed per mouse, and 6 to 8 mice assessed per group. For tubular staining, 4 to 6 fields were assessed per kidney and 6 to 8 mice were assessed per group.

Evaluation of kidney nitrotyrosine using ELISA and urinary 8-isoprostane. Nitrotyrosine was assessed in kidney mitochondrial fractions using an ELISA nitrotyrosine immunoassay kit (Oxis Research). Total protein was determined using a Pierce BCA kit. Urinary 8-isoprostanes were measured using a competitive assay per the manufacturer's recommendations (Cayman Chemical Company, Ann Arbor, MI).

Quantitative reverse-transcription polymerase chain reaction. RNA extraction and reverse transcription of aortic samples have been described previously (10). Total RNA was extracted from kidney cortex. Kidney gene expression of *Nox1*, *Nox2*, *Nox4*, fibronectin, *VEGF*, *TGF-β*, *CTGF*, *TNF-α*, *VCAM-1*, *MCP-1*, *α-SMA*, collagen I, collagen III, collagen IV, *GPx1*, *GPx3*, *GPx4*, and catalase were analyzed by quantitative RT-PCR as described previously (10) and in the supplementary online material. Probes and primers are shown in supplementary Table 2.

Glomerular injury. Three micron-thick kidney sections were stained with periodic-acid Schiff (PAS) to assess glomerular injury, which was determined in digital images using Image-Pro Plus 6.0 software (Media Cybernetics) and determined as the percentage of increased deposition of extracellular matrix within the glomerulus. The degree of injury was assessed in glomeruli showing a clearly identifiable vascular pole. ~20 glomeruli were analyzed per mouse, and 6 to 8 mice were assessed per group.

Cell culture and immunoblot analysis of normal rat kidney cells. Normal rat kidney interstitial fibroblasts were maintained in DMEM, and proteins extracted as described previously. Forty-five micrograms of protein was separated on 10% SDS polyacrylamide gels, transferred onto nitrocellulose membranes, and subjected to primary antibodies against phospho-IKKα^{ser180}/IKKβ^{ser181}, phospho-JNK(Thr183/Tyr185), and phospho-p38. Membranes were also probed with α-tubulin. Antibody binding was detected using the ECL Advance Western Blotting detection kit and quantified by densitometry. To determine whether the observed changes in phosphorylation were caused by changes in total protein, separate membranes were hybridized with total IKK, JNK, and p38, and standardized for loading with α-tubulin. Phosphorylated protein was then expressed per total protein.

Statistical analysis. Data were analyzed with one-way ANOVA using GraphPad Prism 5 software. Student-Newman-Keuls multiple comparison was used to compare group means. $P < 0.05$ was considered significant. Results are expressed as mean ± SEM unless otherwise stated.

RESULTS

Phenotypic assessment of ebselen-gavaged *ApoE*$^{-/-}$*GPx1*$^{-/-}$ mice and metabolic parameters after 20 weeks of diabetes. Supplementary Table 3 shows body weight and metabolic parameters in cellulose- and ebselen-gavaged nondiabetic and diabetic dKO mice. Diabetic dKO mice have significantly lower body weights compared with nondiabetic controls ($P < 0.001$). Ebselen-gavage caused a small yet significant reduction in body weight in nondiabetic dKO mice, although this was not observed in nondiabetic *ApoE*$^{-/-}$ mice gavaged with ebselen (supplementary Table 4). Diabetes was associated with a significant increase in glycated hemoglobin, plasma glucose, water intake, and urinary output in diabetic dKO mice compared with nondiabetic counterparts. Diabetes had no effect on lipids (cholesterol, triglycerides, HDL, and LDL), and these parameters as well as glucose and glycated hemoglobin were not affected by ebselen-treatment, emphasizing that ebselen has no effect on glucose or lipid pathways in diabetic dKO mice. The same parameters are shown for *ApoE*$^{-/-}$ mice in supplementary Table 4. Ebselen had no effect on any parameters measured in nondiabetic *ApoE*$^{-/-}$ mice. Diabetic *ApoE*$^{-/-}$ mice showed significantly increased glycated hemoglobin, plasma glucose, triglycerides, water intake, and urinary output. No significant differences were noted between untreated and ebselen-treated diabetic *ApoE*$^{-/-}$ mice, with the exception that ebselen-treated diabetic *ApoE*$^{-/-}$ mice lost less weight than their untreated diabetic counterparts.

Effect of ebselen on organic hydroperoxides in plasma and 8-isoprostane in urine. The dROMs test measures organic hydroperoxides present in plasma and is proportional to the free radicals from which they form (25). Our study (supplementary Table 3) shows a significant twofold increase in dROMs as a result of diabetes

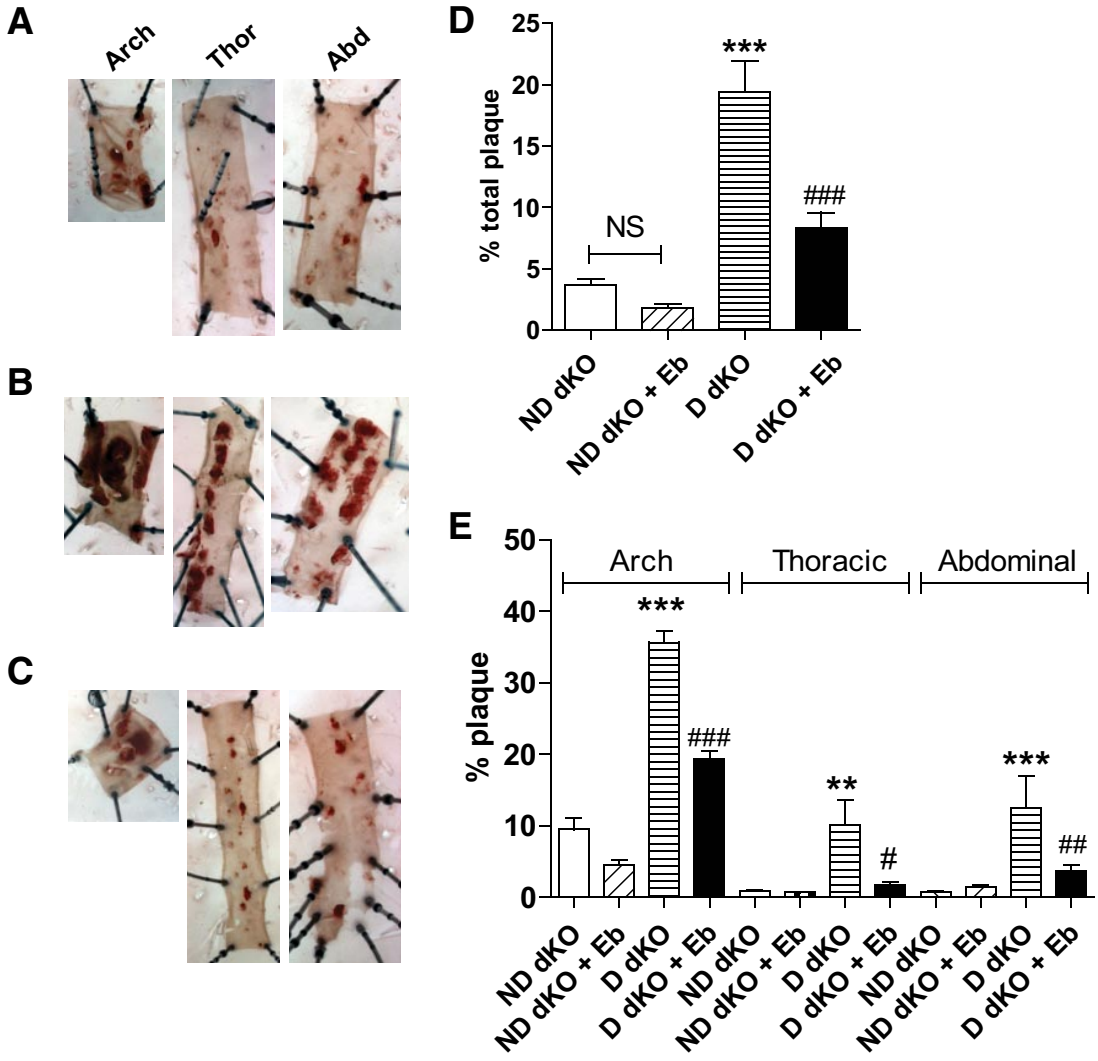

FIG. 1. Ebselen reduces diabetes-associated atherosclerosis in the $ApoE^{-/-}GPx1^{-/-}$ (dKO) aorta. Sudan IV-stained aortas from (*A*) nondiabetic, (*B*) diabetic, and (*C*) ebselen-gavaged diabetic dKO mice, 20 weeks after sham or streptozotocin-induced diabetes. Total and regional plaque is shown in *D* and *E*, respectively; Bars, mean ± SEM (n = 6–10 aortas/group). ***$P < 0.001$, **$P < 0.01$ vs. nondiabetic controls; ###$P < 0.001$, ##$P < 0.01$, #$P < 0.05$ vs. diabetic dKO aortas. Abd, abdominal; D, diabetic; Eb, ebselen; ND, nondiabetic; Thor, thoracic. (A high-quality digital representation of this figure is available in the online issue.)

($P < 0.001$ vs. nondiabetic dKO) in dKO plasma, which is significantly attenuated by ~30% after ebselen treatment ($P < 0.001$ vs. diabetic dKO). Similarly, 8-isoprostane levels, a marker of chronic oxidative stress (26), were increased in diabetic urines and reduced by ebselen ($P < 0.05$, diabetic plus ebselen vs. diabetic mice; supplementary Tables 3 and 4).

Effect of ebselen on atherosclerotic lesions. After 20 weeks of diabetes, total aortic plaque was significantly increased in dKO aortas compared with nondiabetic controls (Fig. 1*B* vs. Fig. 1*A* and *D*; $P < 0.001$). Regional plaque evaluation showed highly significant increases in the arch ($P < 0.001$), thoracic ($P < 0.01$), and abdominal ($P < 0.001$) regions in untreated diabetic mice compared with nondiabetic controls (Fig. 1*E*). Ebselen reduced total aortic plaque by ~57% in diabetic mice compared with untreated diabetic aortas (Fig. 1, *C* vs. *B* and *D*; $P < 0.001$), with significant regional reductions in the plaque area of ~45% in the arch ($P < 0.001$), ~83% in the thoracic ($P < 0.05$), and ~70% in the abdominal aorta ($P < 0.01$; Fig. 1*E*). The diabetes-associated increase in atherosclerosis within the aortic sinus was not reduced after 10 or 20 weeks of ebselen treatment (supplementary online Fig. 1).

Effect of ebselen on Nox2, oxidative stress markers and GPx isoforms −3 and −4 in dKO aortic tissue

Nox2. After 10 weeks of diabetes, aortic Nox2 mRNA levels increased ~14-fold compared with nondiabetic controls ($P < 0.001$; Fig. 2*A*). There was a marked increase in Nox2 immunostaining both in the intimal and medial layers in diabetic aortas of ~1.7-fold compared with nondiabetic counterparts ($P < 0.05$; supplementary online Figs. 2*A* and 3*A*). Ebselen reduced Nox2 mRNA and protein levels by ~60% ($P < 0.01$) and 42% ($P < 0.05$), respectively, in diabetic aortas compared with untreated diabetic aortas.

Oxidative stress markers: nitrotyrosine and 4-HNE. Nitrotyrosine staining of dKO aorta was increased approximately threefold compared with nondiabetic controls (Fig. 3*B* and supplementary online Fig. 2*B*), although this fell just outside of significance. Ebselen reduced nitrotyrosine levels by ~73% in diabetic dKO aortas compared with untreated diabetic aortas. Similarly, 4-HNE, a major aldehyde product of lipid peroxi-

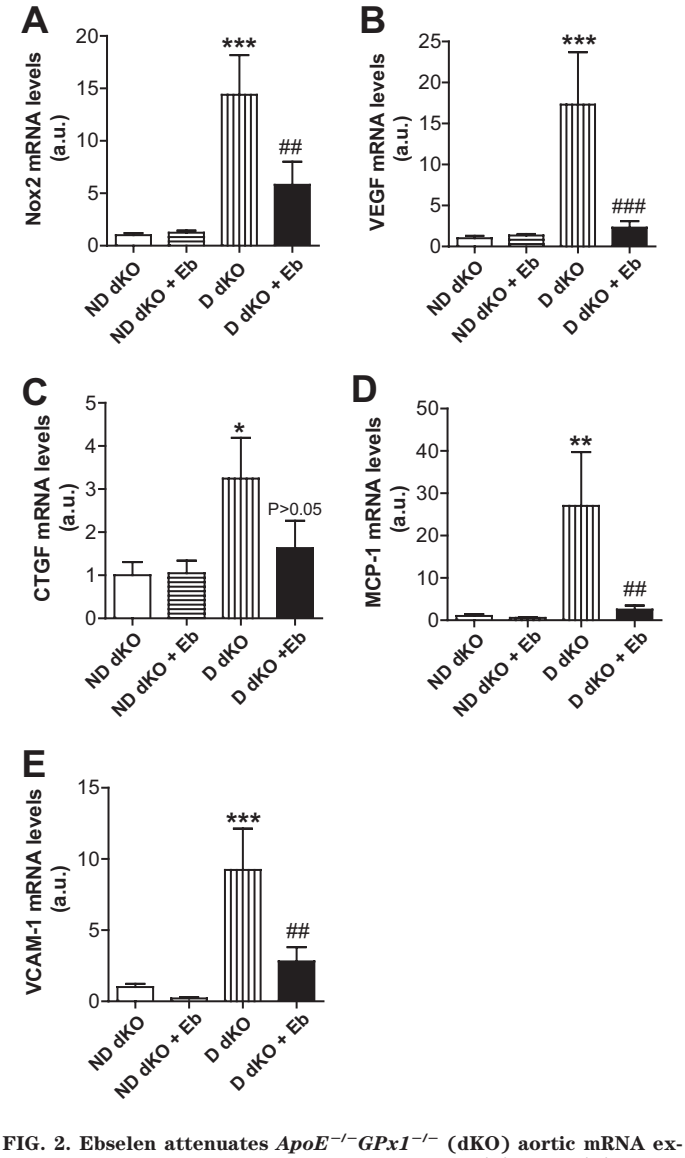

FIG. 2. Ebselen attenuates *ApoE*$^{-/-}$*GPx1*$^{-/-}$ (dKO) aortic mRNA expression after 10 weeks of STZ-induced diabetes. (*A*) *Nox2*, (*B*) *VEGF*, (*C*) *CTGF*, (*D*) *MCP-1*, and (*E*) *VCAM-1* quantitative RT-PCR levels were expressed relative to nondiabetic dKO levels, which were arbitrarily assigned a value of 1. Bars, mean ± SEM (*n* = 7–9 aortas/group). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. nondiabetic dKO aortas; ###$P < 0.001$, ##$P < 0.01$ vs. diabetic dKO aortas. D, diabetic; Eb, ebselen; ND, nondiabetic.

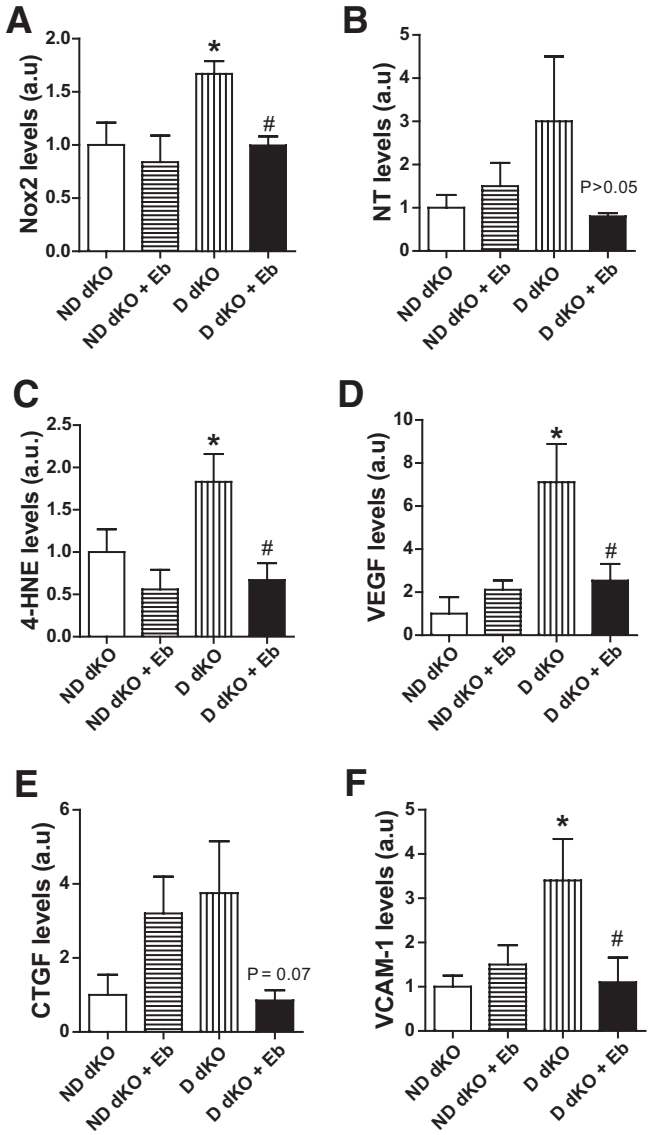

FIG. 3. Ebselen attenuates *ApoE*$^{-/-}$*GPx1*$^{-/-}$ (dKO) aortic protein levels after 10 weeks of STZ-induced diabetes. Representative immunhistochemical staining for each protein is shown in supplementary online Fig. 2*A–F*. Quantitation of immunohistochemical staining within aortic sections is shown for (*A*) Nox2, (*B*) nitrotyrosine (NT), (*C*) 4-HNE, (*D*) VEGF, (*E*) CTGF, and (*F*) VCAM-1. Values are expressed relative to the nondiabetic dKO group which is arbitrarily assigned a value of 1. Bars, mean ± SEM *n* = 5–7 aortas/group; *$P < 0.05$ vs. nondiabetic dKO group; #$P < 0.05$ vs. diabetic dKO group. a.u., arbitrary units; D, diabetic; Eb, ebselen; NT, nitrotyrosine; ND, nondiabetic.

dation (27). is increased in diabetic dKO aortas and reduced by ebselen ($P < 0.05$; Fig. 3*C* and supplementary online Fig. 2*C*).

Gpx3 and GPx4. Expression of the extracellular GPx3 and the phospholipid hydroperoxide GPx4 is significantly increased in diabetic dKO aorta ($P < 0.001$ and $P < 0.01$, respectively), while ebselen prevented the diabetes-driven upregulation of GPx3 and GPx4 (supplementary online Fig. 3).

Effect of ebselen on pro-atherogenic markers

VEGF and CTGF. After 10 weeks of diabetes, dKO aortas showed an ~17-fold and ~7-fold increase in VEGF mRNA ($P < 0.001$; Fig. 2*B*) and protein levels, respectively ($P < 0.05$; Fig. 3*D*, and supplementary online Fig. 2*D*), compared with nondiabetic controls. Ebselen significantly attenuated the diabetes-induced increase in VEGF at both the mRNA ($P < 0.001$) and protein level ($P < 0.05$) back to that seen in nondiabetic dKO mice. The increase in VEGF protein was observed in both intimal and medial layers of the diabetic aorta, and this was attenuated in both of these layers by ebselen. Analysis of CTGF showed similar trends with an approximately threefold (Fig. 2*C*) and approximately fourfold (Fig. 3*E*) increase in CTGF mRNA ($P < 0.05$) and protein (supplementary online Fig. 2*E*), respectively, which were reduced to nondiabetic levels by ebselen.

MCP-1 and VCAM-1. After 10 weeks of diabetes, dKO aortas showed ~27-fold ($P < 0.01$; Fig. 2*D*) and ~9-fold ($P < 0.001$; Fig. 2*E*) increases in MCP-1 and VCAM-1 mRNA levels, respectively, compared with nondiabetic dKO controls. Ebselen significantly reduced the increases in both MCP-1 and VCAM-1 mRNA back to basal levels ($P < 0.01$) in diabetic dKO mice. Ten weeks of diabetes

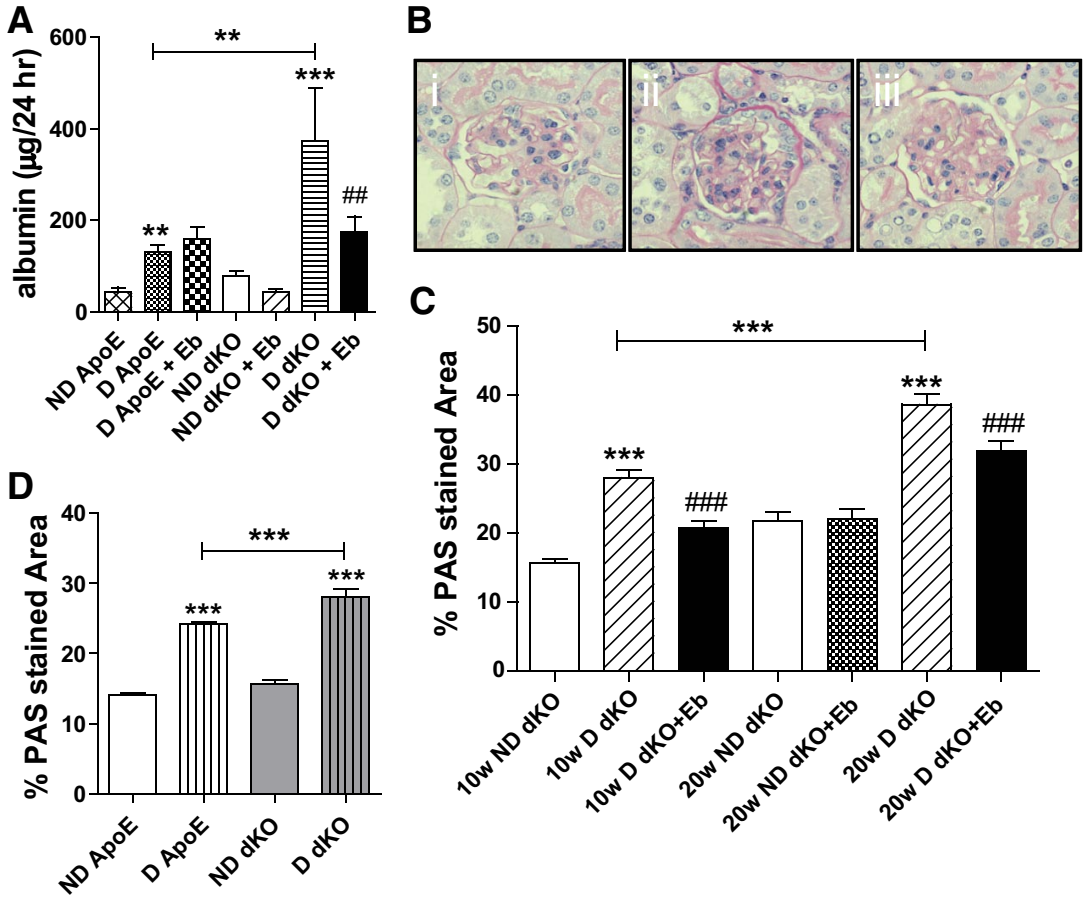

FIG. 4. Ebselen attenuates structural and functional markers of nephropathy in the diabetic *ApoE*$^{-/-}$*GPx1*$^{-/-}$ (dKO) kidney. *A*: Albuminuria is significantly reduced after 10 weeks of treatment in the diabetic dKO kidney, ##$P < 0.01$ vs. diabetic dKO, ***$P < 0.001$ and **$P < 0.01$ vs. nondiabetic counterparts, $n = 4–8$ urines/group, Bars, geometric mean ± error bars. *B*: Representative photomicrographs of PAS-stained glomeruli, (*i*) nondiabetic dKO, (*ii*) diabetic dKO, (*iii*) diabetic dKO+Eb. *C*: Ebselen significantly attenuated PAS staining after 10 and 20 weeks of treatment in diabetic dKO kidneys, ###$P < 0.001$ vs. diabetic dKO kidneys; ***$P < 0.001$ vs. nondiabetic age-matched dKO kidneys. *D*: PAS staining of kidneys showed significantly more damage in the diabetic dKO kidney versus diabetic *ApoE*$^{-/-}$ controls (*$P < 0.001$ vs. diabetic *ApoE*$^{-/-}$ kidneys), ***$P < 0.001$ vs. nondiabetic kidneys; $n = 6–9$ mice/group and at least 20 glomeruli/mouse. 10w, 10 weeks of diabetes; 20w, 20 weeks of diabetes; D, diabetic; Eb, ebselen; ND, nondiabetic. (A high-quality digital representation of this figure is available in the online issue.)**

caused a significant increase in VCAM-1 protein on immunohistochemical analysis (supplementary online Fig. 2*F*) of aortas from dKO mice (Fig. 3*F*; $P < 0.05$ vs. nondiabetic dKO aortas), which ebselen significantly reduced by ~68% (Fig. 3*F*; $P < 0.05$ vs. diabetic dKO aortas).

Assessment of diabetic renal injury in dKO mice

Albuminuria and creatinine clearance. Diabetes caused a significant increase in urinary albumin excretion rate (AER) in both the *ApoE*$^{-/-}$ (Fig. 4*A*, ~3-fold; $P < 0.01$) and dKO (~4.8-fold; $P < 0.001$) mice compared with nondiabetic counterparts after 10 weeks of diabetes. Furthermore, AER was significantly greater in diabetic dKO mice than in diabetic *ApoE*$^{-/-}$ mice (2.8-fold; $P < 0.01$). Ten weeks of ebselen gavage had no effect on AER in diabetic *ApoE*$^{-/-}$ mice, but significantly reduced albuminuria in diabetic dKO mice ($P < 0.01$). Similar results were obtained after 20 weeks of diabetes (supplementary online Fig. 4), where ebselen normalized the diabetes-driven increase in AER in diabetic dKO mice ($P < 0.05$ vs. diabetic dKO mice). Twenty weeks of ebselen-gavage significantly reduced the diabetes-induced increase in creatinine filtration in both *ApoE*$^{-/-}$ ($P < 0.001$) and dKO mice ($P < 0.05$) (supplementary Tables 3 and 4).

Glomerular injury. The percentage of PAS-positive material, indicative of mesangial expansion, was significantly increased after 10 and 20 weeks of diabetes in dKO glomeruli (Fig. 4*Bii* vs. *Bi* and *C*; $P < 0.001$ for both time points). The expansion was progressive, with an ~30% increase at 20 weeks compared with 10 weeks of diabetes (Fig. 4*C*; $P < 0.001$). Diabetic dKO glomeruli also displayed significantly more PAS staining compared with diabetic *ApoE*$^{-/-}$ glomeruli after 10 weeks of diabetes (Fig. 4*D*; $P < 0.001$). Ebselen reduced the percentage of PAS staining in diabetic dKO glomeruli after 10 and 20 weeks of diabetes (Fig. 4*C*; $P < 0.001$).

Effect of ebselen on nitrotyrosine, Nox, and GPx isoforms −3 and −4 in diabetic kidney

Nitrotyrosine. After 20 weeks of diabetes, dKO kidneys showed significantly increased levels of nitrotyrosine in both tubules and glomeruli compared with nondiabetic dKO kidneys (Fig. 5*B* vs. *A*, and *I* [tubules; $P < 0.001$]; Fig. 5*F* vs. Fig. 5*E* and *J* [glomeruli; $P < 0.001$]), as well as diabetic *ApoE*$^{-/-}$ kidneys (Fig. 5*I* and *J*; $P < 0.001$). Ebselen reduced dKO nitrotyrosine levels back to nondiabetic levels in both tubules (Fig. 5*C* vs. *B*; and Fig. 5*I*; $P < 0.01$) and glomeruli (Fig. 5*G* vs. *F*; and Fig. 5*J*; $P < 0.001$). These results were confirmed by ELISA when ebselen significantly reduced nitrotyrosine levels after 20 weeks of diabetes in the mitochondrial fraction of dKO kidneys (diabetic dKO: 5.04 ± 0.55 vs. diabetic dKO+Ebselen:

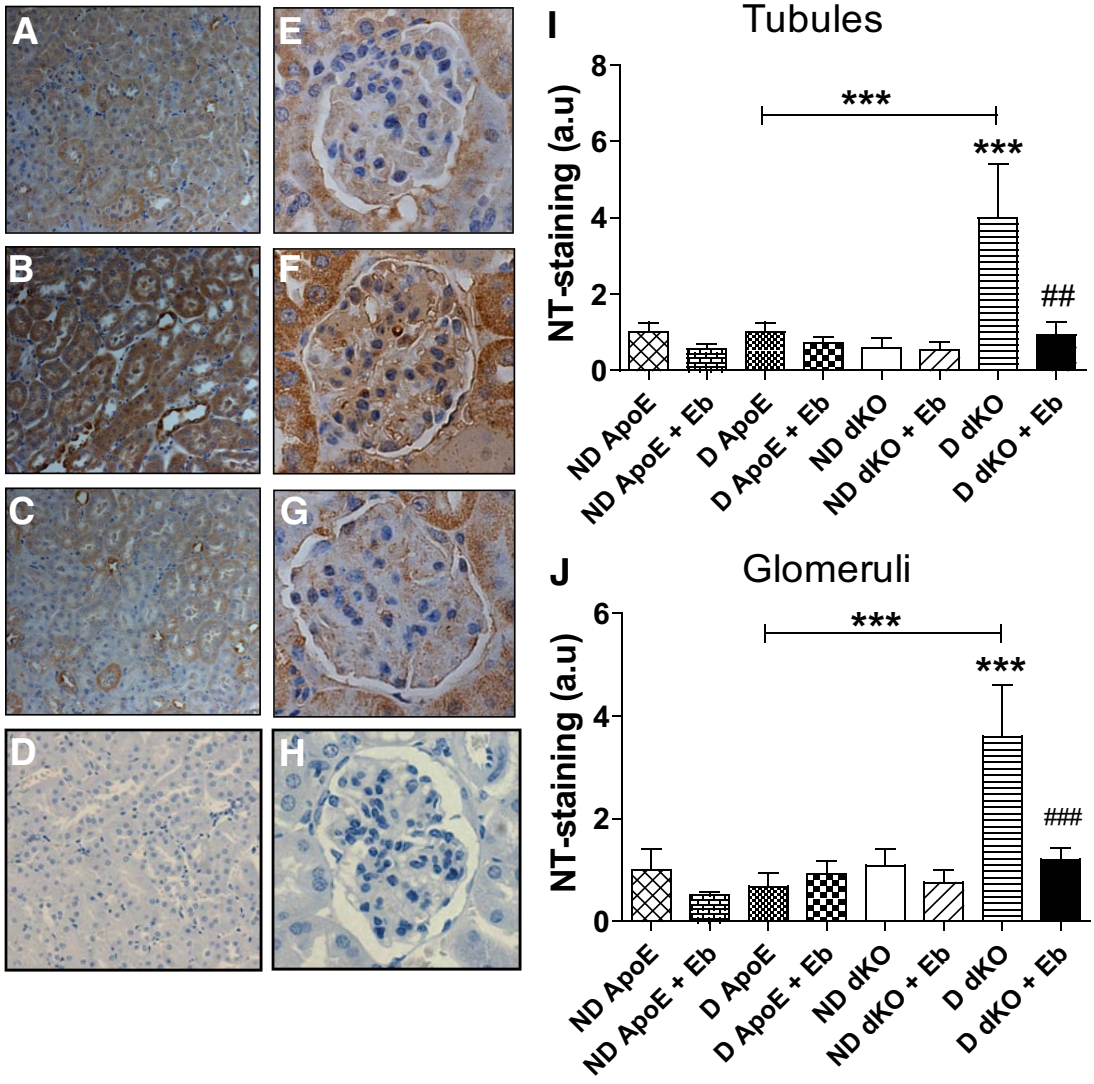

FIG. 5. The H_2O_2-mediated increase in nitrotyrosine (NT) is reduced by ebselen in the diabetic *ApoE*$^{-/-}$*GPx*$^{-/-}$ (dKO) kidney. *A–D*: Representative photomicrographs of kidney tubules after 20 weeks of diabetes. (*A*) nondiabetic dKO, (*B*) diabetic dKO, (*C*) diabetic dKO + Eb, and (*D*) negative control which consisted of species-matched nonimmune IgG in place of primary antibody. *E–H*: Representative photomicrographs of kidney glomeruli after 20 weeks of diabetes. Nondiabetic dKO (*E*); Diabetic dKO (*F*); diabetic dKO + Eb (*G*); and negative control which consisted of species-matched nonimmune IgG in place of primary antibody (*H*). Quantitation of nitrotyrosine-stained tubules and glomeruli is shown in *I* and *J*, respectively. ****P* < 0.001; diabetic dKO versus diabetic *ApoE*$^{-/-}$ kidneys. ##*P* < 0.01 and ###*P* < 0.001 vs. diabetic dKO kidneys. Bars, mean ± SEM; *n* = 6–8 kidneys/group. a.u., arbitrary units; D, diabetic; Eb, ebselen; ND, nondiabetic. (A high-quality digital representation of this figure is available in the online issue.)

2.5 ± 0.62 nmol/l/mg protein; $P < 0.05$, $n = 5$–6 kidneys per group).

Nox. We examined the gene expression of three isoforms of Nox (Nox 1, 2, 4) in the cortex of *ApoE*$^{-/-}$ and dKO mice (Fig. 6*A* and supplementary online Fig. 5). Expression was significantly increased in dKO kidneys ($P < 0.001$ for Nox2 and Nox4; and $P < 0.05$ for Nox1) compared with nondiabetic controls. Furthermore, diabetic dKO mRNA expression of Nox2 and Nox4 was significantly increased above that of diabetic *ApoE*$^{-/-}$ kidneys ($P < 0.001$). Nox2 protein was significantly increased in both the glomeruli and tubules (Fig. 6*B*) of diabetic dKO kidneys compared with nondiabetic controls ($P < 0.05$ for glomeruli; Fig. 6*C* and $P < 0.01$ for tubules; Fig. 6*D*), and was significantly increased compared with diabetic *ApoE*$^{-/-}$ kidneys ($P < 0.05$ for glomeruli and $P < 0.01$ for tubules). Ebselen significantly reduced the diabetes-driven increases in Nox2 ($P < 0.001$) and Nox4 ($P < 0.001$) expression in the diabetic dKO kidney, as well as Nox1 expression in the diabetic *ApoE*$^{-/-}$ kidney ($P < 0.001$). Furthermore, ebselen decreased Nox2 protein in the glomeruli and tubules ($P < 0.01$) of diabetic dKO kidneys.

Gpx3 and GPx4. GPx3 and GPx4 expression is significantly increased in diabetic dKO kidneys ($P < 0.01$ and $P < 0.001$, respectively) compared with diabetic ApoE$^{-/-}$ kidneys, while ebselen prevented this increase (see supplementary online Fig. 6).

Effect of ebselen on profibrotic and proinflammatory mediators and extracellular matrix proteins in diabetic kidneys. The mRNA expression of a range of profibrotic growth factors (TGF-β, CTGF), proinflammatory mediators (MCP-1, VCAM-1, and TNF-α), α-smooth muscle actin (α-SMA) and profibrotic markers (collagen I, III, IV, and fibronectin) were assessed in *ApoE*$^{-/-}$ and dKO nondiabetic and diabetic kidneys (supplementary online Figs. 7 and 8). TGF-β, collagen I, collagen III, MCP-1, VCAM-1 ($P < 0.001$) and fibronectin mRNA levels (Fig. 7*A*; $P < 0.001$) were significantly increased in diabetic dKO

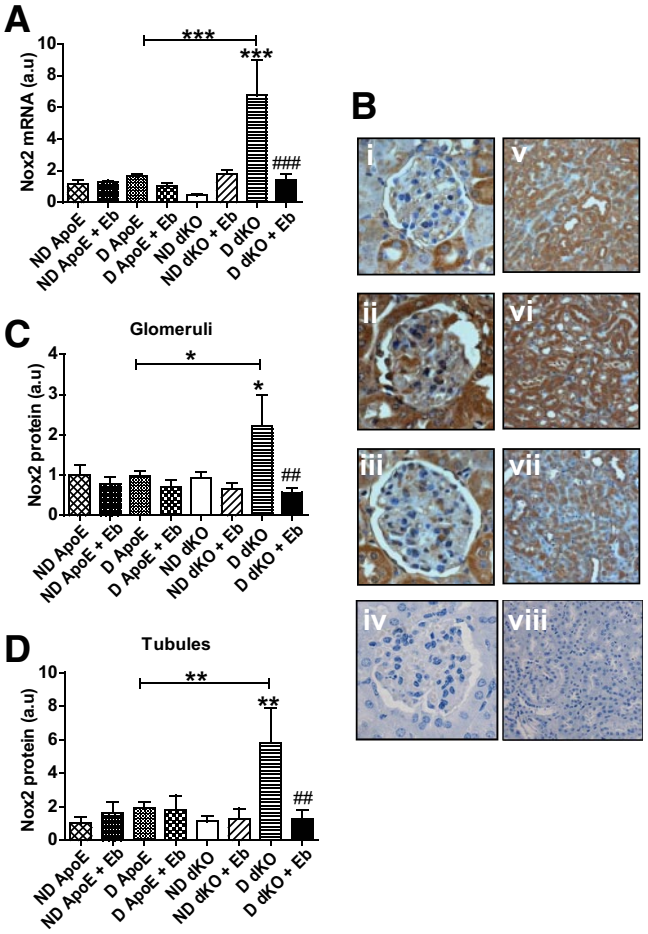

FIG. 6. The H_2O_2-mediated increase in Nox2 is reduced by ebselen in the diabetic $ApoE^{-/-}GPx1^{-/-}$ (dKO) kidney. *A*: Quantitative RT-PCR analysis of Nox2 in $ApoE^{-/-}$ and dKO kidneys after 20 weeks of diabetes; n = 8–10 kidneys/group. *B*: Representative photomicrographs of kidney glomeruli (*i–iv*) and tubules (*v–viii*); nondiabetic dKO (*i* and *v*); diabetic dKO (*ii* and *vi*); diabetic dKO+Eb (*iii* and *vii*); and negative control which consisted of species-matched nonimmune IgG in place of primary antibody (*iv* and *viii*). *C* and *D*: Quantitation of Nox2 protein within glomeruli and tubules, respectively. ***P < 0.001; **P < 0.01; *P < 0.05 diabetic dKO vs. diabetic $ApoE^{-/-}$ kidneys; and versus nondiabetic counterparts. ##P < 0.01 and ###P < 0.001 vs. diabetic dKO kidneys. Bars, mean ± SEM; n = 20 glomeruli/kidney and 4–6 kidneys/group and n = 6 tubular fields/kidney and at least 5 kidneys/group. a.u., arbitrary units; D, diabetic; Eb, ebselen; ND, nondiabetic. (A high-quality digital representation of this figure is available in the online issue.)

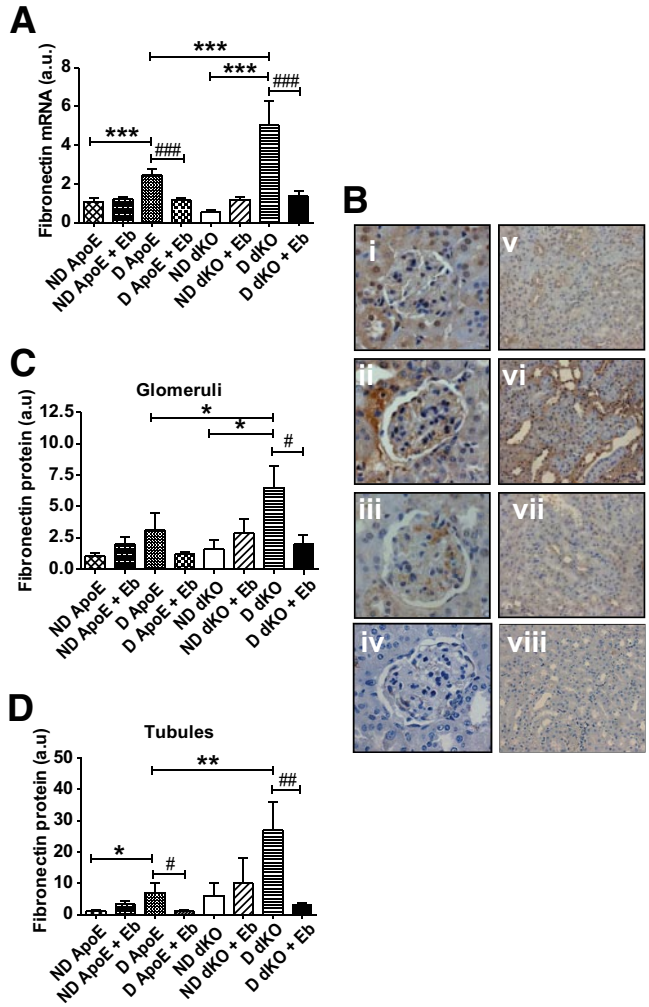

FIG. 7. The H_2O_2-mediated increase in fibronectin is reduced by ebselen in the diabetic $ApoE^{-/-}GPx1^{-/-}$ (dKO) kidney. *A*: Quantitative RT-PCR analysis of fibronectin in $ApoE^{-/-}$ and dKO kidneys after 20 weeks of diabetes; n = 7–10 kidneys/group. *B*: Representative photomicrographs of kidney glomeruli (*i–iv*) and tubules (*v–viii*); nondiabetic dKO (*i* and *v*); diabetic dKO (*ii* and *vi*); diabetic dKO+EB (*iii* and *vii*); and negative control which consisted of species-matched nonimmune IgG in place of primary antibody (*iv* and *viii*). *C* and *D*: Quantitation of fibronectin protein within glomeruli and tubules, respectively. ***P < 0.001; **P < 0.01; *P < 0.05 and ###P < 0.001; ##P < 0.01; #P < 0.05 as indicated by horizontal bars. Bars, mean ± SEM; n = 20 glomeruli/kidney and 4–6 kidneys/group and n = 6 tubular fields/kidney and at least 5 kidneys/group. a.u., arbitrary units; D, diabetic; Eb, ebselen; ND, nondiabetic. (A high-quality digital representation of this figure is available in the online issue.)

kidneys compared with diabetic $ApoE^{-/-}$ kidneys. Ebselen significantly reduced the expression of all genes investigated in the diabetic dKO and $ApoE^{-/-}$ kidneys, with the exception of collagen III and MCP-1 in the diabetic $ApoE^{-/-}$ kidney where expression was not different from nondiabetic levels.

Two profibrotic genes were chosen to verify these changes at the protein level. Fibronectin, both within the glomeruli and tubules, was significantly increased in diabetic dKO kidneys (Fig. 7*C* and *D*) compared with diabetic $ApoE^{-/-}$ kidneys ($P < 0.05$ and $P < 0.01$ for glomeruli and tubules, respectively). Ebselen significantly attenuated the diabetes-related increase in fibronectin in the glomeruli (Fig. 7*C*; $P < 0.05$) and tubules (Fig. 7*D*; $P < 0.01$) to levels similar to that observed in nondiabetic kidneys. Collagen I was detected mainly in brush borders of proximal tubules (supplementary online Fig. 9), but also in surrounding glomeruli and tubules as previously reported (28). Collagen I was upregulated in the tubular compartment in diabetic dKO kidneys, particularly within the brush borders of the tubules. In agreement with our RT-PCR data (supplementary online Fig. 8*B*), collagen I protein was also significantly increased in diabetic dKO kidneys compared with diabetic $ApoE^{-/-}$ kidneys ($P < 0.001$, supplementary online Fig. 9). Ebselen caused significant reductions in collagen I levels in diabetic dKO kidneys (supplementary online Fig. 9; $P < 0.001$).

Effect of ebselen on antioxidants in the diabetic dKO kidney. We investigated the expression of other antioxidants in addition to GPx1 that are part of the antioxidant pathway (29). Protein analysis by Western blots (supplementary online Fig. 10) confirmed the lack of GPx1 in the dKO kidney, in agreement with our previous studies in this model (30,31). Ebselen caused a small yet significant

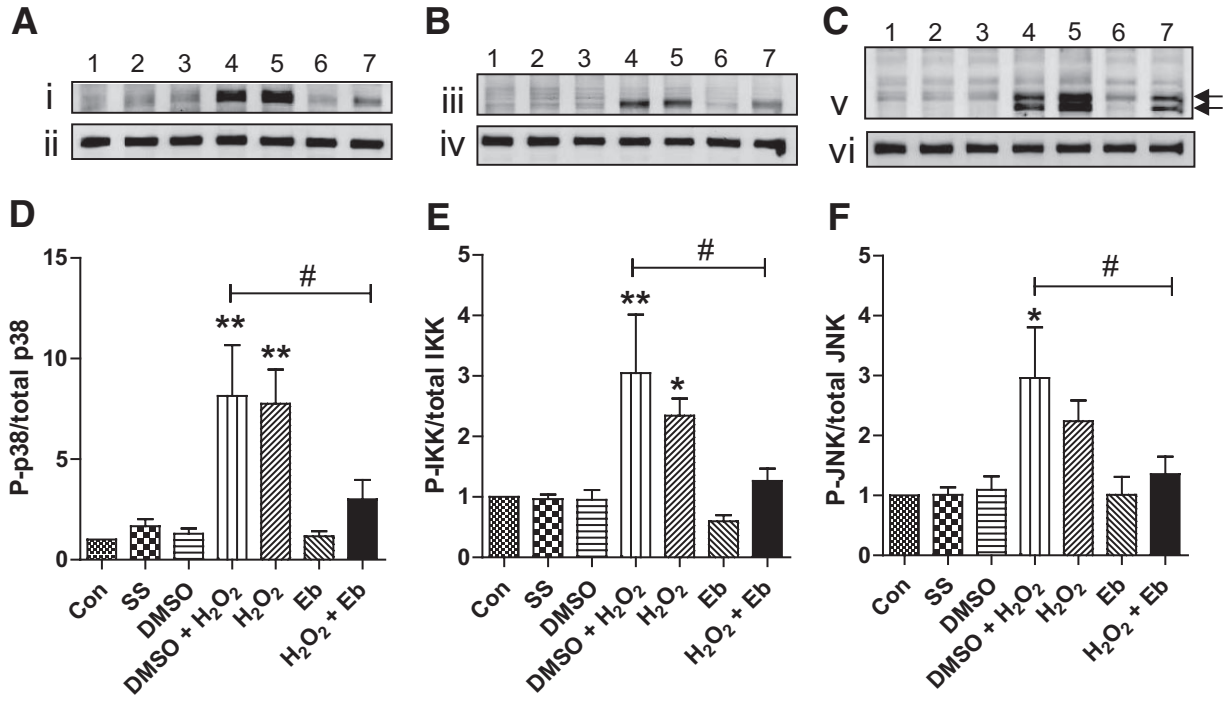

FIG. 8. Ebselen abrogates H_2O_2-mediated increases in (*A* and *D*) P-p38; (*B* and *E*) P-IKK, and (*C* and *F*) P-JNK protein in normal rat kidney cells. A representative gel with its internal α-tubulin control (*Aii*, *Biv*, and *Cvi*) is shown above the quantitation for each protein. Lane 1 = untreated cells; lane 2 = serum-starved (SS) cells for 4 h; lane 3 = DMSO-treated cells; lane 4 = DMSO + 1 mmol/l H_2O_2-treated cells; lane 5 = 1 mmol/l H_2O_2 treated cells; lane 6 = 0.03 μmol/l ebselen-treated cells; and lane 7 = 1 mmol/l H_2O_2+0.03 μmol/l ebselen-treated cells. *Arrows* point to the two isoforms of P-JNK. Phosphorylated protein was quantitated relative to total protein (total protein levels are shown in supplementary online Fig. 11) for each gene. *$P < 0.05$ and **$P < 0.01$ vs. control cells, DMSO, and serum-starved cells. #$P < 0.05$ Ebselen plus H_2O_2 versus H_2O_2-treated cells. Bars, mean ± SEM; $n = 4$ replicates/group.

increase in GPx1 protein in the diabetic $ApoE^{-/-}$ kidneys compared with untreated nondiabetic controls ($P < 0.05$). No significant differences in Sod1 or catalase levels were detected in any of the groups on Western blot analysis.

In vitro analysis of ebselen in normal rat kidney cells. Initial experiments established optimal treatment time and dosage with H_2O_2 (30 min, at 1 mmol/l; data not shown) for increased expression of P-IKK, P-JNK, and P-p38. Pretreatment with 0.03 μmol/l ebselen for 30 min, followed by treatment with 1 mmol/l H_2O_2 for 30 min, significantly reduced H_2O_2-mediated increases in phosphorylation of p38, IKK, and JNK (Fig. 8; $P < 0.05$).

DISCUSSION

This study shows that the synthetic antioxidant, ebselen, reduces atherosclerosis and renal injury in diabetic ApoE/GPx1-deficient mice. Atherosclerosis was attenuated in the major regions of the aorta. However, one region unaffected by ebselen was the sinus, in agreement with a growing list of studies showing the resistant nature of this region to pharmacologic intervention (23,32). The reduction in atherosclerosis was accompanied by significant reductions in vascular oxidative stress as reflected by reduced staining for two established oxidative stress markers, 4-HNE and nitrotyrosine, a reduction in the Nox2 subunit of NADPH oxidase (Nox), an enzyme implicated in the generation of vascular ROS, and a reduction in plasma hydroperoxides and urinary 8-isoprostanes. Furthermore, proinflammatory and proatherogenic mediators associated with the atherosclerotic phenotype (VCAM-1, MCP-1, CTGF, and VEGF) were reduced by ebselen in the diabetic ApoE/GPx1-deficient aorta. These data support the notion of Blankenberg et al. (11) that bolstering GPx-like activity reduces atherosclerosis, and it is in agreement with our previous study where lack of GPx1 greatly accelerated plaque deposition in the aorta of diabetic $ApoE^{-/-}GPx1^{-/-}$ mice (10).

This study has also shown that in addition to its antiatherosclerotic potential, the GPx1-mimetic ebselen prevents renal functional changes such as albuminuria and various renal structural changes and inflammatory responses associated with nephropathy in diabetic mice that also had a deletion in GPx1. Our study strengthens the findings of Reddi and Bollineni (33), where a deficiency in selenium led to increased oxidative stress and accelerated nephropathy in diabetic rats, and it is in agreement with Chander et al. (22) where ebselen improved renal outcomes in the Zucker diabetic fat rat. Importantly, by improving both renal and atherogenic outcomes in the diabetic ApoE/GPx1-deficient model, our study suggests that ebselen is effective as a monotherapy against both diabetes-associated conditions. Furthermore, this study has provided in vivo evidence that ebselen, in its ability to act as an antioxidant, functionally replenishes the lack of GPx1 in this murine model. Two pieces of evidence support this notion. First, ebselen lowered plasma levels of reactive oxygen metabolites, implying that ebselen acts as an antioxidant in the removal of ROS such as hydroperoxides. Second, ebselen reduced nitrotyrosine levels, a marker of peroxynitrite-mediated protein damage and a function mostly attributed to GPx1 in its capacity to act as peroxynitrite reductase (34,35). In addition, our data showing an ebselen-mediated reduction in nitrotyrosine within the mitochondrial fraction of the diabetic kidney adds further information about the mechanism of ebselen action. It is well accepted that mitochondrial ROS play a significant role in diabetes-associated injury (36), particularly that seen in dia-

betic nephropathy, hence a reduction by ebselen in peroxynitrite within this compartment may have contributed to the protective effects observed in this study. Our data also suggest that the protective effects of ebselen in diabetic dKO mice occur independently of any compensatory effects on the major antioxidants, Sod1, catalase, or the isoforms of GPx (for more detailed discussion please see supplementary online appendix, points 1 and 2).

Our study has also shown some differential effects of ebselen in the presence of GPx1, since ebselen had no effect in reducing diabetes-associated proteinuria nor various markers of oxidative stress such as nitrotyrosine or Nox2 in diabetic *ApoE*$^{-/-}$ kidneys. It remains to be ascertained if the degree of GPx1-depletion often seen in the diabetic milieu is an important predisposing factor for responsiveness to ebselen (also see the supplementary online appendix, point 3). Furthermore, ebselen was associated with variable effects on body weight, albeit these changes were modest. In addition, this treatment was associated with a reduction in creatinine clearance in both diabetic *ApoE*$^{-/-}$ and dKO mice, a phenomenon not observed in nondiabetic animals. It is likely that this represents a protective effect of ebselen on renal hyperfiltration rather than a deleterious effect on renal function, since after ebselen treatment, creatinine clearance was still within the normal range. The underlying explanation for these renal hemodynamic effects of ebselen remain to be elucidated.

This study has also demonstrated that lack of GPx1 on an ApoE-deficient background accelerates pathologic changes associated with diabetic nephropathy. Interestingly, our previous study failed to show a role for GPx1 in reducing diabetic nephropathy in C57Bl/J6 diabetic mice (30). This was an unexpected finding since the high glucose environment within the diabetic kidney is known to be a strong inducer of ROS (37), and furthermore, GPx1 is the major antioxidant in the removal of hydrogen and lipid peroxides within the kidney (31). However, the significance of a lack of GPx1 may not have been properly revealed since lipids, known to be important in the development of diabetic nephropathy (38), are unaltered in the diabetic C57Bl/J6 model. Indeed, hyperlipidemia is now recognized as a major phenotypic feature in people who are prone to nephropathy (39). In the present study we reassessed the role of GPx1 in the development of diabetic nephropathy against a background of elevated lipids in diabetic ApoE/GPx1-dKO mice. We now show increased albuminuria which is associated with pathologic changes that include mesangial expansion and upregulation of profibrotic (collagen I and III, fibronectin, and TGF-β) and proinflammatory mediators (VCAM-1, MCP-1) in diabetic ApoE/GPx1-dKO kidneys compared with diabetic *ApoE*$^{-/-}$ controls. Thus, we have confirmed a role for GPx1 in limiting and/or preventing diabetic nephropathy in the clinically relevant milieu of increased lipids known to accompany diabetes (38,39). It is also noteworthy that lack of GPx1 caused a further upregulation of the predominantly tubular collagens, collagen I and III, but not the major basement membrane type IV collagen, in the diabetic kidney. Types I and III collagens are expressed mainly in the interstitial regions, whereas type IV collagen plays a significant role in glomerular pathology (40,41). It is therefore possible that GPx1 plays a role in the protection of the interstitium against extracellular matrix deposition and influences tubulointerstitial injury.

A major deficiency in animal models of diabetic nephropathy has been the absence of a demonstrated concurrent increase in cardiovascular disease (42). This is particularly important in the design of effective treatments against both diabetes-associated atherosclerosis and diabetic nephropathy, since it is becoming increasingly appreciated that accelerated cardiovascular disease is a common phenomenon in subjects with diabetic nephropathy (43). Together with our previous study in which we showed accelerated diabetes-associated atherosclerosis in aortas of diabetic *ApoE*$^{-/-}$*GPx1*$^{-/-}$ mice (10) and the current study showing increased renal injury in these mice, we believe that diabetic *ApoE*$^{-/-}$*GPx1*$^{-/-}$ mice are a useful tool in which to test GPx1-mimetic antioxidant therapies that may be effective against both complications.

Finally, our in vitro studies in rat normal rat kidney cells suggest that ebselen mediates its renoprotective effects via pathways that include the modulation of p38, JNK, and IKK. By preventing or limiting phosphorylation of the proinflammatory mediator IκB-kinase (IKK), which in turn regulates the transcription factor NF-κB, ebselen plays a pivotal role in limiting the activation of proinflammatory genes. Likewise, by reducing the H_2O_2-mediated phosphorylation of the stress-activated kinase JNK, ebselen modulates a kinase that is involved in the activation of the transcription factor AP-1. The strong inhibition by ebselen of the phosphorylation of the MAP-kinase family member p38 suggests that ebselen affects H_2O_2-mediated signal transduction and is in agreement with previous studies in HUVECs (44). Together with our previous data in human aortic endothelial cells (HAECs), these new data strengthen the notion that ebselen impacts positively on proinflammatory pathways, not only in endothelial cells, but also within kidney cells to limit proinflammatory processes.

In conclusion, this study shows that ebselen limits the development of two major diabetic vascular complications, namely diabetes-associated atherosclerosis and diabetic nephropathy in ApoE/GPx1-deficient mice. This approach of GPx1 repletion via mimetics of GPx1 offers an alternative therapeutic antioxidant strategy that is worthy of further investigation.

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P.C. and M.T.C. researched and evaluated data. D.Y.C.Y., N.S., and J.P. researched data. K.A.J.-D. evaluated data and reviewed/edited the manuscript. M.C.T. evaluated data. F.R. and M.E.C. reviewed/edited the manuscript. J.B.d.H. conceived and designed experiments, evaluated data, wrote the manuscript.

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