Anti-atherosclerotic and renoprotective effects of Ebselen in the diabetic
Apolipoprotein E/GPx1-double knockout mouse.

Short title: Ebselen and diabetic complications

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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 randomised into two groups, one injected with streptozotocin, the other with vehicle at 8-weeks of age. Groups were further randomised 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-HNE 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 signalling pathways.

**Conclusions**—Ebselen has an anti-atherosclerotic 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.

Chronic kidney disease (CKD) is associated with enhanced morbidity and mortality, particularly due to accelerated cardiovascular disease.(1) Diabetes is emerging as an independent risk factor for both CKD 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 CKD 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 defences, 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 pro-atherogenic pathways that included pro-inflammatory 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.\textsuperscript{(11-13)}

Modulation of Gpx1 activity can be achieved through administration of selenium, the essential trace element found within the active site of GPx1,\textsuperscript{(14)} although this holds less pharmacological appeal due to selenium toxicity.\textsuperscript{(15)} In addition, lack of specificity due to incorporation of selenium into several key enzymes makes this approach less attractive.\textsuperscript{(14)} Thus, compounds that mimic GPx1 activity offer an alternate way to increase GPx1-like activity.\textsuperscript{(16)}

Ebselen, a lipid-soluble low molecular weight seleno-organic compound and a known GPx1-mimetic,\textsuperscript{(17)} has shown potential in reducing pathogenesis in various experimental models.\textsuperscript{(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 pro-inflammatory mediators in the ApoE-deficient mouse.\textsuperscript{(23)} However, no study has investigated the effects of ebselen on atherosclerosis and nephropathy, two clinically relevant and often linked co-morbidities, 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: Firstly, our study investigated whether ebselen protects against diabetic nephropathy and diabetes-associated atherosclerosis (DAA) in the diabetic ApoE/GPx1-deficient mouse. Secondly, we specifically chose the ApoE-deficient mouse additionally lacking Gpx1, to establish whether ebselen replenishes the lack of GPx1 activity. This has been performed in order to further clarify the mechanism of action of ebselen in an \textit{in vivo} context, as well as strengthen the idea that replenishing GPx1-like activity may be of therapeutic benefit.\textsuperscript{(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\textsuperscript{-/-}GPx1\textsuperscript{-/-} double knockout (dKO) mice\textsuperscript{(10)} were rendered diabetic by two intraperitoneal injections of streptozotocin (Sigma, USA) on consecutive days, at 100mg/kg/day.\textsuperscript{(10)} For comments on group selection, please see online appendix at \url{http://diabetes.diabetesjournals.org}. 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 10mg/kg starting at 10 weeks of age. Kidneys from non-diabetic and diabetic ApoE\textsuperscript{-/-} 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, high-density lipoprotein (HDL) and triglycerides using commercial kits (see online appendix for expanded methods). Glycated haemoglobin was measured in erythrocyte lysates by HPLC (Biorad, 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.\textsuperscript{(24)}

One kidney was snap frozen and stored at -70\degree 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.

20-week aortas 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. 10-week aortas 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 (NT), 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 NT, Nox2, collagen 1 and fibronectin. Detailed methods and antibodies (Table I) are described online, including Mouse-On-Mouse immunodetection to localize antibodies raised in mouse (4-HNE, VCAM-1). For aortic analysis, 3 sections of each region were assessed per mouse, and 7-9 mice analysed per group. Results were calculated as percentage positively stained tissue and expressed relative to non-diabetic dKO mice. For glomerular assessments, 20 glomeruli were analysed per mouse, and 6-8 mice assessed per group. For tubular staining, 4-6 fields were assessed per kidney and 6-8 mice 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 as per the manufacturer’s recommendations (Cayman EIA, USA).

**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 1, collagen 3, collagen 4, GPx1, GPx3, GPx4 and catalase were analyzed by qRT-PCR as described previously (10) and online. Probes and primers are shown online (Table II).

**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 increased deposition of extracellular matrix within the glomerulus. The degree of injury was assessed in glomeruli showing a clearly identifiable vascular pole. Approximately 20 glomeruli were analysed per mouse, and 6-8 mice were assessed per group.

**Cell Culture and Immunoblot analysis of NRK cells.** Normal rat kidney (NRK) 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 due to changes in total protein, separate membranes were hybridised with total IKK, JNK and p38 and standardised
Phosphorylated protein was then expressed per total protein.

Statistical Analysis. Data were analysed using one-way analysis of variance (ANOVA) using GraphPad Prism 5 Software. Student-Newman-Keuls Multiple Comparison compared 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. Online Table III shows bodyweight and metabolic parameters in cellulose- and ebselen-gavaged non-diabetic and diabetic dKO mice. Diabetic dKO mice have significantly lower bodyweights compared with non-diabetic controls (\( P<0.001 \)). Ebselen-gavage caused a small yet significant reduction in body-weight in non-diabetic dKO mice, although this was not observed in non-diabetic ApoE\(^{-/-}\) mice gavaged with ebselen (Online Table IV). Diabetes was associated with a significant increase in glycated haemoglobin, plasma glucose, water intake and urinary output in diabetic dKO mice compared with non-diabetic counterparts. Diabetes had no effect on lipids (cholesterol, triglycerides, HDL, LDL) and these parameters as well as glucose and glycated haemoglobin 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 Table IV. Ebselen had no effect on any parameters measured in non-diabetic ApoE\(^{-/-}\) mice. Diabetic ApoE\(^{-/-}\) mice showed significantly increased glycated haemoglobin, 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 (online Table III) shows a significant 2-fold increase in dROMs as a result of diabetes (\( P<0.001 \) vs ND dKO) in dKO plasma, which is significantly attenuated by \( \sim30\% \) 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; online Table III and IV).

Effect of Ebselen on Atherosclerotic Lesions. After 20 weeks of diabetes, total aortic plaque was significantly increased in dKO aortas compared with non-diabetic controls (Fig.1B vs Fig.1A and Fig.1D; \( P<0.001 \)). 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 non-diabetic controls (Fig.1E). Ebselen reduced total aortic plaque by \( \sim57\% \) in diabetic mice compared with untreated diabetic aortas (Fig.1C vs Fig.1B and Fig.1D; \( P<0.001 \)), with significant regional reductions in plaque area of \( \sim45\% \) in the arch (\( P<0.001 \), \( \sim83\% \) in the thoracic (\( P<0.05 \)) and \( \sim70\% \) in the abdominal aorta (\( P<0.01 \); Fig.1E). The diabetes-associated increase in atherosclerosis within the aortic sinus was not reduced after 10 or 20 weeks of ebselen treatment (online Fig.I).

Effect of Ebselen on Nox2, oxidative stress markers and GPx isoforms -3 and -4 in dKO Aortic tissue: a) Nox2 - After 10 weeks of diabetes, aortic Nox2 mRNA levels increased \( \sim14\)-fold compared with non-diabetic controls (\( P<0.001 \); Fig.2A). There was a marked increase in Nox2.
immunostaining both in the intimal and medial layers in diabetic aortas of ~1.7-fold compared with non-diabetic counterparts ($P<0.05$; online Fig.II, Part A and Figure 3A). 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.

b) Oxidative stress markers: Nitrotyrosine and 4-HNE - Nitrotyrosine staining of dKO aorta was increased ~3-fold compared with non-diabetic controls (Fig.3B and online Fig.II, Part 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 peroxidation,(27) is increased in diabetic dKO aorta and reduced by ebselen ($P<0.05$; Fig.3C and online Fig.II, Part C).

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), whilst ebselen prevented the diabetes-driven upregulation of GPx3 and GPx4. (online Fig. III).

Effect of Ebselen on Pro-atherogenic Markers. a) VEGF and CTGF - After 10 weeks of diabetes, dKO aortas showed ~17- and ~7-fold increase in VEGF mRNA ($P<0.001$; Fig.2B) and protein levels respectively ($P<0.05$; Fig.3D and online Fig.II, Part D), compared with non-diabetic 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 non-diabetic 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 a ~3- (Fig.2C) and ~4-fold (Fig.3E) increase in CTGF mRNA ($P<0.05$) and protein (online Fig.II, Part E) respectively, which were reduced to non-diabetic levels by ebselen.

b) MCP-1 and VCAM-1 - After 10 weeks of diabetes, dKO aortas showed ~27- ($P<0.01$; Fig.2D) and ~9-fold ($P<0.001$; Fig.2E), increase in MCP-1 and VCAM-1 mRNA levels respectively compared with non-diabetic 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. 10 weeks of diabetes caused a significant increase in VCAM-1 protein on immunohistochemical analysis (online Fig.II, Part F) of aortas from dKO mice (Fig.3F; $P<0.05$ vs non-diabetic dKO aortas), which ebselen significantly reduced by ~68% (Fig.3F; $P<0.05$ vs diabetic dKO aortas).

Assessment of Diabetic Renal Injury in dKO mice: a) Albuminuria and Creatinine Clearance - Diabetes caused a significant increase in urinary albumin excretion rate (AER) in both the ApoE$^{-/-}$ (Fig.4A, ~3 fold; $P<0.01$) and dKO (~4.8 -fold; $P<0.001$) mice compared with non-diabetic counterparts after 10 weeks of diabetes. Furthermore AER was significantly greater in diabetic dKO mice than in diabetic ApoE$^{-/-}$ mice (2.8-fold; $P<0.01$). 10-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 (online Fig.IV), where ebselen normalised 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 ($P<0.05$) (online Table III and IV).

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

**Effect of Ebselen on Nitrotyrosine, Nox and GPx isoforms -3 and -4 in Diabetic Kidney**

a) **Nitrotyrosine** - After 20 weeks of diabetes, dKO kidneys showed significantly increased levels of nitrotyrosine in both tubules and glomeruli compared with non-diabetic dKO kidneys (Fig. 5ii vs Fig.5i, and Fig.5ix (tubules; \(P<0.001\)); Fig.5vi vs Fig.5v, and Fig.5x (glomeruli; \(P<0.001\))) as well as diabetic ApoE\(^{-/-}\) kidneys (Fig.5ix and Fig.5x; \(P<0.001\)). Ebselen reduced dKO nitrotyrosine levels back to non-diabetic levels in both tubules (Fig.5iii vs Fig.5ii; and Fig.5ix; \(P<0.01\)) and glomeruli (Fig.5vii vs Fig.5vi; and Fig.5x; \(P<0.001\)). These results were confirmed by ELISA where ebselen significantly reduced NT levels after 20 weeks of diabetes in the mitochondrial fraction of dKO kidneys (diabetic dKO: 5.04±0.55 vs diabetic dKO+Ebselen: 2.5±0.62 nM/mg protein; \(P<0.05\), n=5-6 kidneys/group).

b) **Nox** - We examined the gene expression of 3 isoforms of Nox (Nox 1, 2, 4) in the cortex of ApoE\(^{-/-}\) and dKO mice (Fig.6A and online Fig.V). Expression was significantly increased in dKO kidneys (\(P<0.001\) for Nox2 and Nox4; and \(P<0.05\) for Nox1) compared with non-diabetic 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.6B) of diabetic dKO kidneys compared with non-diabetic controls (\(P<0.05\) for glomeruli; Fig.6C and \(P<0.01\) for tubules; Fig.6D), 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 increase 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.

c) **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, whilst ebselen prevented this increase (see online Fig. VI).

**Effect of Ebselen on Profibrotic and Pro-inflammatory Mediators and Extracellular Matrix Proteins in Diabetic Kidneys.** The mRNA expression of a range of pro-fibrotic growth factors (TGF-\(\beta\), CTGF), pro-inflammatory mediators (MCP-1, VCAM-1 and TNF-\(\alpha\)), \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) and pro-fibrotic markers (collagen I, III, IV and fibronectin) were assessed in ApoE\(^{-/-}\) and dKO non-diabetic and diabetic kidneys (online Fig.VII and VIII). TGF-\(\beta\), collagen I, collagen III, MCP-1, VCAM-1 (\(P<0.001\)) and fibronectin mRNA levels (Fig.7A; \(P<0.001\)) were significantly increased in diabetic dKO 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 non-diabetic levels.

Two pro-fibrotic 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.7C-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. 7C; \( P < 0.05 \)) and tubules (Fig. 7D; \( P < 0.01 \)) to levels similar to that observed in non-diabetic kidneys. Collagen I was mainly detected in brush borders of proximal tubules (online Fig. IX), 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 (online Fig. VIII, Part B), collagen I protein was also significantly increased in diabetic dKO kidneys compared with diabetic ApoE \(^{-/-} \) kidneys (\( P < 0.001 \), online Fig. IX). Ebselen caused significant reductions in collagen I levels in diabetic dKO kidneys (online Fig. IX; \( P < 0.001 \)).

**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 (online Fig. X) 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 increase in GPx1 protein in the diabetic ApoE \(^{-/-} \) kidneys compared with untreated non-diabetic 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 NRK cells.** Initial experiments established optimal treatment time and dosage with \( \text{H}_2\text{O}_2 \) (30min, at 1mM; data not shown) for increased expression of P-IKK, P-JNK and P-p38. Pre-treatment with 0.03μM ebselen for 30min, followed by treatment with 1mM \( \text{H}_2\text{O}_2 \) for 30min, significantly reduced \( \text{H}_2\text{O}_2 \)-mediated increases in phosphorylation of p38, IKK and JNK (Fig. 8; \( P < 0.05 \)).

**DISCUSSION**

This study has shown 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 pharmacological 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-isoprostanates. Furthermore, pro-inflammatory and pro-atherogenic 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 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 anti-atherosclerotic potential, the GPx1-mimetic ebselen prevents renal functional changes such as albuminuria and various renal structural changes and inflammatory responses associated with nephropathy in those diabetic mice that also had a deletion in GPx1. Our study strengthens the findings of Reddi and Bollineni, (33) where a deficiency in selenium lead to increased oxidative stress and accelerated nephropathy in diabetic rats, and 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. Firstly, ebselen lowered plasma levels of reactive oxygen metabolites, implying that ebselen acts as an antioxidant in the removal of ROS such as hydroperoxides. Secondly, 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 NT within the mitochondrial fraction of the diabetic kidney adds further information on 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 diabetic 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 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 (please also see online appendix Point 3). Furthermore, ebselen was associated with variable effects on body weight albeit that 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 non-diabetic 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 pathological 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 DN,(38) are unaltered in the diabetic C57Bl/J6 model. Indeed, hyperlipidemia is now recognised 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 DN against a background of elevated lipids in diabetic ApoE/GPx1-dKO mice. We now show increased albuminuria which is associated with pathological changes that include mesangial expansion and up-regulation of pro-fibrotic (collagen I and III, fibronectin, TGF-β) and pro-inflammatory 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. Type I and III collagens are mainly expressed 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 DAA and DN, since it is becoming increasingly appreciated that accelerated cardiovascular disease is a common phenomenon in subjects with DN.(43) Together with our previous study where we showed accelerated DAA 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 NRK 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 pro-inflammatory 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 H2O2-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 H2O2-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 pro-inflammatory pathways, not only in endothelial cells, but also within kidney cells to limit pro-inflammatory processes.

In conclusion, this study has shown 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.

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

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**Statement of Responsibility.** The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**FIGURE LEGENDS**

**Figure 1:** Ebselen reduces diabetes-associated atherosclerosis in the ApoE/−GPx1/− (dKO) aorta. Sudan IV-stained aortas from (A) non-diabetic, (B) diabetic and (C) ebselen-gavaged diabetic dKO mice, 20-weeks after sham or STZ-induced diabetes. Total and regional plaque is shown in D) and E) respectively; Bars, mean±S.E.M (n=6-10 aortas/group); ND=non-diabetic; D=diabetic; Eb=ebselen.; Thor=thoracic; Abd=abdominal. ***P<0.001, **P<0.01 vs ND controls; #P<0.001, ##P<0.01, #P<0.05 vs diabetic dKO aortas.

**Figure 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 qRT-PCR levels were expressed relative to ND dKO levels, which were arbitrarily assigned a value of 1. ND=non-diabetic; D=diabetic; Eb=ebselen. Bars, mean±S.E.M (n=7-9 aortas/group). ***P<0.001, **P<0.01, *P<0.05 vs ND dKO aortas; ###P<0.001, ##P<0.01 vs diabetic dKO aortas. ND=non-diabetic; D=diabetic; Eb=ebselen.

**Figure 3:** Ebselen attenuates ApoE/−GPx1/− (dKO) aortic protein levels after 10 weeks of STZ-induced diabetes. Representative immunohistochemical staining for each protein is shown online in Fig II, Part 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 non-diabetic dKO group which is arbitrarily assigned a value of 1. Bars, mean±S.E.M. n=5-7 aortas/group; a.u.=arbitrary units; *P<0.05 vs ND dKO group; #P<0.05 vs diabetic dKO group. ND=non-diabetic; D=diabetic; Eb=ebselen.

**Figure 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 D dKO, ***P<0.001 and **P<0.01 vs ND counterparts, n=4-8 urines/group, Bars, geometric mean±Error Bars; (B) Representative photomicrographs of PAS stained glomeruli, (i) ND dKO, (ii) D dKO, (iii) D dKO+Eb; (C) Ebselen significantly attenuated PAS staining after 10 and 20 weeks of treatment in diabetic dKO kidneys; (D) Periodic Acid Schiff staining (PAS) of kidneys showed significantly more damage in the diabetic dKO kidney vs diabetic ApoE/− controls (**P<0.001 vs diabetic ApoE/− kidney). ND=non-diabetic; D=diabetic; Eb=ebselen.

**Figure 5:** The H2O2-mediated increase in nitrotyrosine (NT) is reduced by ebselen in the diabetic ApoE/−GPX1/− (dKO) kidney. (i-iv) Representative photomicrographs of kidney tubules after 20 weeks of diabetes. i = ND dKO; ii = D dKO; iii = D dKO + Eb; iv = negative control which consisted of species-matched non-immune IgG in place of primary antibody. (v-viii) Representative photomicrographs of kidney glomeruli after 20 weeks of diabetes. v = ND dKO; vi = Diab dKO; vii = D dKO + Eb; viii = negative control which consisted of species-matched non-immune IgG in place of primary antibody. Quantitation of NT-stained tubules and
glomeruli is shown in ix and x respectively. ***P<0.001; D dKO vs D ApoE−/− kidneys. ##P<0.01 and ###P<0.001 vs D dKO kidneys. a.u.=arbitrary units. Bars, mean±S.E.M; n= 6-8 kidneys/group. ND=non-diabetic; D=diabetic; Eb=ebselen. 

**Figure 6**: The H2O2-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); i & v =ND dKO; ii & vi = D dKO; iii & vii = D dKO+Eb; iv & viii = negative control which consisted of species-matched non-immune IgG in place of primary antibody. (C & D) Quantitation of Nox2 protein within glomeruli and tubules respectively. ***P<0.001; **P<0.01; *P<0.05 D dKO vs D ApoE−/− kidneys; and vs ND counterparts. ##P<0.01 and ###P<0.001 vs D dKO kidneys. a.u.=arbitrary units. Bars, mean±S.E.M; n= 20 glomeruli/kidney and 4-6 kidneys/group and n= 6 tubular fields/kidney and at least 5 kidneys/group. ND=non-diabetic; D=diabetic; Eb=ebselen.

**Figure 7**: The H2O2-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); i & v =ND dKO; ii & vi = D dKO; iii & vii = D dKO+Eb; iv & viii = negative control which consisted of species-matched non-immune IgG in place of primary antibody. (C & 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. a.u.=arbitrary units. Bars, mean±S.E.M; n= 20 glomeruli/kidney and 4-6 kidneys/group and n= 6 tubular fields/kidney and at least 5 kidneys/group. ND=non-diabetic; D=diabetic; Eb=ebselen.

**Figure 8**: Ebselen abrogates H2O2-mediated increases in (A, D) P-p38; (B, E), P-IKK and (C, F) P-JNK protein in NRK 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 hr; lane 3=DMSO-treated cells; lane 4=DMSO + 1mM H2O2 treated cells; lane 5=1mM H2O2 treated cells; lane 6=0.03μM ebselen treated cells; and lane 7 = 1mM H2O2+0.03μM 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 Suppl. Fig.XI) for each gene. *P<0.05 and **P<0.01 vs control cells, DMSO and serum starved cells. #P<0.05 vs H2O2-treated cells. Bars, mean ± S.E.M; n= 4 replicates/group.

**REFERENCES**


Figure 1

A

Arch  Thor  Abd

B

C

D

% total plaque

ND dKO  ND dKO + Eb  D dKO  D dKO + Eb

E

% plaque

Arch  Thoracic  Abdominal

ND dKO  ND dKO + Eb  D dKO  D dKO + Eb  ND dKO  ND dKO + Eb  D dKO  D dKO + Eb  ND dKO  ND dKO + Eb  D dKO  D dKO + Eb
Figure 2

Panel A: Nox2 mRNA levels (a.u.)

Panel B: VEGF mRNA levels (a.u.)

Panel C: CTGF mRNA levels (a.u.)

Panel D: MCP-1 mRNA levels (a.u.)

Panel E: VCAM-1 mRNA levels (a.u.)

ND dKO, ND dKO + Eb, D dKO, D dKO + Eb
Figure 3

A

B

C

D

E

F

Ebselen and diabetic complications
Figure 4

A

**

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###

B

C

***

****

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Figure 6

A

Nox2 mRNA (a.u.)

B

Nox2 protein (a.u.)

C

Glomeruli

D

Tubules

Ebselen and diabetic complications
**Figure 8**

### D

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