Supplemental Section

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

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RESEARCH DESIGN AND METHODS (Additional comments)

Animal Groups and Experimental Design

Eight-week old *male* ApoE$^{-/-}$GPx1$^{-/-}$ double knockout (dKO) mice described by us previously(1; 2) were specifically chosen to investigate the effects of ebselen treatment since *female* mice do not achieve the same severity of diabetes as males. Indeed many female mice do not become diabetic as a result of the relative resistance of female β-islet cells to the diabetogenic effects of STZ.(3) Furthermore, previous work by us(1; 2; 4) and others(5) have generally used male mice since oestrogens have been reported to influence atherosclerosis in the setting of diabetes(6) which could act as a confounder in the interpretation of these studies.

Non diabetic and diabetic mice were divided into ebselen-gavaged and cellulose-gavaged groups. Ebselen (Sapphire Bioscience, NSW, Australia), dissolved in 5% CM-cellulose (Sigma, USA), was gavaged twice daily at 10mg/kg starting at 10 weeks of age. The chosen dosage was based on previously published data in mice(7) and our recent study in diabetic ApoE$^{-/-}$ mice.(2) Sham-injected male mice served as non-diabetic controls. Diabetes was allowed to progress for 10 or 20 weeks. There was no evidence of increased poor health after ebselen treatment in either diabetic ApoE-/- or diabetic dKO mice. This is consistent with our previous study(2) and that of others (7-9) that demonstrate no toxicity with this agent. Three days prior to termination, 24h urine was collected after housing in metabolic cages.

Blood Sampling, Plasma Biochemistry and Tissue Collection

Mice were fasted for 3-4hr prior to termination. Thereafter, mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (Sigma, USA). The thoracic cavity was opened and blood drawn by direct puncture of the right ventricle.

Kidneys were removed and one kidney was snap frozen and stored at -70^{o}C for gene expression and protein studies of the cortex. 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.

The heart with the aorta attached was carefully dissected from surrounding tissue. To release the heart and aorta from the mouse, the abdominal aorta was cut just proximal to the iliac bifurcation and the tissue immediately placed in cold saline. The aorta was then severed close to the heart with a sharp scalpel blade. 20-week aortas were fixed in 10%-NBF and stained with Sudan IV (Sigma, USA) for *en face* assessment of atherosclerotic lesions. Aortas were opened longitudinally and cut into the arch, thoracic and abdominal regions. To obtain the arch, a cut

was made distal to the left subclavian artery. Further cuts were made to remove the brachiocephalic and left common carotid artery as well as the left subclavian artery. To dissect the thoracic aorta from the abdominal aorta, a cut was made distal to the intercostal arteries. The aortic section is obtained after cutting the aorta just proximal to the iliac bifurcation. Care was taken to always dissect similar regions in all groups. The arch, thoracic and abdominal regions were then pinned onto dental wax.

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. Aortic cross-sections were prepared from arch, thoracic and abdominal areas.

Plasma was analyzed for diamicron reactive oxygen metabolites (dROMs) using the FRAS-4 system (see below), as well as glucose, cholesterol, high-density lipoprotein (HDL) and triglycerides using commercial kits. The Friedewald calculation was used to determine low-density lipoprotein (LDL). Glycated haemoglobin was measured in erythrocyte lysates by HPLC (Biorad, USA).

Urinary albumin excretion was measured by a mouse albumin ELISA kit, according to kit protocols (Bethyl Laboratories, USA). Plasma and urinary creatinine was measured by cation exchange chromatography, as previously described.(10) Creatinine clearance was estimated as the ratio of daily urinary creatinine excretion to plasma creatinine concentration, and expressed as $ml.min^{-1}.m^{-2}$ surface area. Urinary 8-isoprostane was measured in 24h urines at 1:40 dilution using a Cayman EIA Kit and following the manufacturers recommended protocols. 8-isoprostane concentration was determined by expressing 8-isoprostane (pg/ml) relative to the concentration of creatinine in the urine (ml/µmol). This gave the 8-isoprostane concentration in pg/µmol creatinine.

dROMs test to detect organic hydroperoxides using FRAS 4

The d-ROMs test allows for the determination of the blood concentration of reactive oxygen metabolites (ROMs) and, particularly, that of hydroperoxides, which are markers and amplifiers of free radical-induced oxidative damage (11). Briefly, 10µl of heparanized plasma is dissolved in acidic buffer as supplied by the manufacturer in the dROMs kit. The hydroperoxides present in the sample then react with transition metal ions liberated from the proteins by the acidic medium and are converted into alkoxy and peroxy radicals. These newly formed radicals oxidize N,N-diethyl-para-phenylendiamine to its corresponding radical cation. The concentration of this species is determed spectrophotometrically at 505nm using the Free Radical Analyser System 4 (FRAS 4) set at a k-factor of 18. dROMS are measured in Carratelli Units where one CARR Unit is equivalent to 0.8 mg/L hydrogen peroxide (11).

Evaluation of Atherosclerotic Lesions

Photographs were taken via a dissecting microscope (Olympus, Japan) equipped with a high-resolution camera (Zeiss Instruments, Germany). Images were analyzed for total and regional plaque area using image analysis software (Adobe Photoshop), and expressed as the percentage aortic surface occupied by red-stained plaque.

Immunohistochemistry

Four micron-thick aortic arch, thoracic and abdominal paraffin sections were stained for nitrotyrosine (NT), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), NADPH oxidase-2 (Nox2), vascular cell adhesion molecule (VCAM-1) and 4-

hydroxynonenal (4-HNE). Three micron-thick kidney sections were stained for NT, Nox2, collagen 1 and fibronectin. Primary and secondary antibodies and dilutions are shown in Table I below.

Immunohistochemical methods are briefly as follows. After hydration of sections, endogenous peroxidases were inactivated with 3% H_2O_2. Paraffin sections were incubated with protein blocking agent prior to application of the primary antibody. Primary antibody was left on overnight at 4^{o}C. This was followed by incubation with biotinylated secondary antibody for 10 min at room temperature. Sections were then incubated with Vectastain ABC reagent (Vector Laboratories). Peroxidase activity was identified by reaction with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). Sections were counterstained with hematoxylin.

Negative controls consisted of the addition of species-matched non-immune IgG subclass-specific antibody for the primary antibodies at equivalent dilutions to the specific primary antibody concentration.

Where antibodies had been raised in mouse (VCAM-1 and 4-HNE), an additional step was included prior to incubation with the primary antibody. This included using a M.O.M Immunodetection kit (Vector Lab, Burlingame, CA, USA). For 4-HNE detection, an antigen retrieval step was first performed as follows. Paraffin slides were incubated in 10mM citrate buffer (pH 6) and heated in a microwave for 5 min. Slides were then cooled for one hour before proceeding with the next step. For both VCAM-1 and 4-HNE, sections were incubated for 1 hour in M.O.M. Mouse Ig Blocking Reagent, followed by the M.O.M. diluent for 5 min. Sections were then incubated with primary antibody diluted in M.O.M. diluent for 30 min at RT. After washes with TBS, sections were incubated with M.O.M. biotinylated anti-mouse IgG reagent for 10 min. Section were then incubated with the Vectastain ABC reagent as previously described.

Immunohistochemical analysis of kidneys was performed as follows: NT primary antibody was applied at a dilution of 1:80 for staining of the glomeruli and at 1:150 for detection within the tubules. Nox2 primary antibody was applied at a dilution of 1:100 for staining of both the glomeruli and the tubules. The collagen I primary antibody was applied at a dilution of 1:100 for staining of the tubules. The fibronectin primary antibody was applied at a dilution of 1:100 for staining of both the glomeruli and the tubules.

Images were quantitated using Image Pro-Plus 6.0. Analysis was performed in a blinded fashion. Our procedure measures the intensity of the stain by choosing an intensity range which we then apply to the analysis of all sections stained with that antibody. The intensity range is chosen based on the level of staining of the diabetic dKO sections in most instances, since these stain most intensely due to increased levels induced by the diabetic condition. The results are calculated as a percentage positively stained tissue (i.e. the amount of brown stain per total area and is calculated based on the intensity range determined in pixels using our imaging software and expressed over the total area which is also calculated in pixels) and where aortic assessments were made, expressed relative to non-diabetic dKO mice that were arbitrarily assigned a value of 1. Measurements within the aorta included assessment of the endothelial cell-containing intimal layer as well as the smooth muscle cell-rich medial layer, but not the adventitial layer of the aorta. Immunohistochemical analysis of kidneys included ApoE-/- mice and in this instance,

results were normalised relative to the non-diabetic ApoE-/- values, which were arbitrarily assigned a value of 1.

Evaluation of Kidney Nitrotyrosine using ELISA

Mitochondrial fractions were prepared from 100mg of kidney cortex as follows. After homogenisation on ice, homogenates were centrifuged at 1500rpm at 4^{o}C to remove debris. Supernatant was then centrifuged for 15min at 10000rpm to obtain the mitochondrial pellet. Pellets were resuspended in 250µl of buffer (Krebs Ringer Phosphate Glucose Buffer, pH 7.35). Samples were then further diluted 1 in 2 with Working Dilution Buffer provided by the nitrotyrosine enzyme immunoassay kit (Oxis Research, Foster City, CA, USA). For the Elisa assay, 100ul of standard or sample was added to each well of a 96-well plate and the plate incubated for 1 hour at room temperature. The wells were then washed four times with Wash Buffer. 100ul of nitrotyrosine antibody was added to each well and incubated for 1 hour. This was followed again by 4 washes with Wash Buffer and a 1 hour incubation with 100ul of streptavidin peroxidase. After more washes with Wash Buffer, 100µl of TMB substrate was added and the incubation continued in the dark for 30min. 100µl of Stop Solution was added and the absorbance read at 450nm using a Victor3 plate reader. Total protein was determined using the Pierce BCA kit. A standard curve of known nitrotyrosine concentration, supplied with the kit, was used to determine the concentration of unknown samples and these values were standardized relative to their individual total protein levels.

Evaluation of Urinary 8-isoprostanes

50ul of mouse urine sample was added to each well of a 96-well plate together with the standards and controls provided by the kit (Cayman Chemical, USA). 50ul each of 8-isoprostane AChe Tracer and EIA Antiserum were added to each well and the plate incubated for 18 hours at 4^{o}C. The wells were then rinsed five times with Wash Buffer. 200ul of Ellman's reagent was added to each well and the plate incubated for 2 hours in the dark at RT. The plate was then read at 405nm using a Victor3 plate reader.

A standard curve of known 8-isoprostane concentration, supplied with the kit, was used to determine the concentration of unknown samples and these values were standardized relative to their individual urine creatinine levels.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

RNA extraction, preparation of DNA-free RNA and RNA reverse transcription of aortic samples have been described by us previously.(1) Total RNA was extracted from kidney tissue after careful dissection of the cortex region. Kidney gene expression of Nox1, Nox2, Nox4, fibronectin, VEGF, TGF-β, CTGF, TNF-α, VCAM-1, MCP-1, α-SMA, collagen 1, collagen 3, and collagen 4 were analyzed by qRT-PCR as described previously.(1) Probes and primers are as shown in Table II below.

Briefly, total RNA was extracted after homogenisation of tissue (Polytron PT-MR2100; Kinematica AG) in TRIzol^{R} Reagent (Invitrogen Life Technologies). Contaminating DNA was removed after treatment with DNA-free^{TM} DNAse according to the manufacturer's specifications
(Ambion Inc, Austin, USA). DNA-free RNA was reverse transcribed into cDNA using the Superscript First Strand Synthesis System according to the manufacturer's specifications (Life

Technologies BRL, Grand Island, NY). Gene expression was analysed by real-time quantitative RT-PCR using the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA, USA). Fluorescence for each cycle was analyzed quantitatively by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems). Gene expression was normalised relative to the expression of the housekeeping gene 18S ribosomal RNA (18S rRNA Taqman Control Reagent kit; ABI Prism 7700) that was multiplexed together with the gene of interest. Probes and primers were designed using a Primer Express program (ABI Prism 7700) with care taken to ensure that primers spanned an intron. Amplifications were performed with the following time course: 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 50 cycles of 94^{o}C for 20s and 60^{o}C for 1min. Each sample was tested in duplicate and results expressed relative to non-diabetic control mice, which were assigned a value of 1.

Immunoblot analysis of Rat NRK cells.

Forty-five micrograms of protein from each sample was separated by electrophoresis on 10% SDS polyacrylamide gels at 150V. The proteins were then transferred onto nitrocellulose membranes using semi-dry transfer and incubated in blocking buffer [5% bovine serum albumin in Tris-buffered saline with 0.05% Tween (TBST)] for 2hr. Membranes were then subjected to overnight exposure to primary antibodies against phospho-IKKα^{ser180}/IKKβ^{ser181}, phospho-JNK (Thr183/Tyr185) and phospho-p38. Details of the primary and secondary antibodies used are as described in Supplementary Table I below. Membranes were also probed with α-tubulin. All primary antibodies were diluted 1 in 1000 in blocking buffer and incubations were at 4°C. The membranes were then washed and exposed to anti-rabbit horseradish peroxidase- conjugated secondary antibody (1 in 2000 in blocking buffer) for 1hr. After 30 min of washing, antibody binding was detected using the ECL Advance Western Blotting detection kit and quantified by densitometry using the program Quantity One. Please note that the two arrows in Fig. 8 indicate the two isoforms of P-JNK, namely p54 and p46. Both bands were quantitated to give total P-JNK and expressed relative to α-tubulin. To determine whether the observed changes in phosphorylation were due to changes in total protein, separate membranes were hybridised with total IKK, total JNK and total p38 and standardised for loading with α-tubulin. Levels of phosphorylated protein, which had been standardised for loading relative to α-tubulin, was then determined per standardised total protein.

ADDITIONAL DISCUSSION POINTS

Point 1: To further investigate the potential mechanism(s) whereby ebselen suppresses atherosclerosis and kidney disease, we investigated the expression levels of the phospholipid hydroperoxide GPx4 and the extracellular GPx3 isoforms of GPx in the kidney and aorta. We hypothesized that ebselen may mediate its protective effect by modulating other GPx isoforms. The best strategy to tease out the contributions of the other isoforms of GPx is by RT-PCR, since the GPx assay detects total GPx activity.(12) Our RT-PCR data showed that although diabetes was associated with an increase in GPx3 and GPx4 expression in both the kidney and aorta of the dKO mice, ebselen-gavage prevented this increase. In this instance it appears that the increase in the other isoforms of GPx occurs in response to the increased oxidative stress generated due to the lack of GPx1 in the diabetic milieu. Indeed, our data suggest that ebselen, in its capacity to act as a Gpx mimetic, lowers the oxidative stress and therefore reduces the need for the

compensatory increase in GPx3 and 4. This notion is also strengthened by our dROM data where we show that ebselen reduces reactive oxygen metabolites in the diabetic dKO plasma.

Point 2: Under physiological conditions, ebselen is known to upregulate GPx1 activity(13). Indeed, in a previous study where ebselen decreased atherosclerosis and atherogenic pathways, we showed an increase in GPx1 levels in these diabetic ApoE-/- aortas after ebselen treatment.(2) Interestingly, ebselen also increased catalase levels in these diabetic ApoE-/- aortas. Indeed, we proposed that in addition to its known antioxidant function in the removal of hydro- and lipid peroxides, ebselen further contributes to reductions in oxidative stress by increasing both GPx1 and catalase expression. In the current study, where GPx1 is ablated by homologous recombination, it is clear that ebselen reduces atherosclerosis and diabetic nephropathy independently of its ability to increase GPx1. Ebselen also appeared not to affect catalase levels in the diabetic dKO kidney. In this context, ebselen appears to act as an antioxidant in its own right in the lowering of peroxides, as was seen in the plasma where ebselen reduced reactive oxygen metabolites, thereby fulfilling its function as a glutathione peroxidase mimetic.(14)

Point 3: From the results of this study, it would appear that ebselen has a significant effect in lowering albuminuria in the diabetic dKO animals only and not in the diabetic ApoE-/- mice. This may have arisen due to the marked increase in albuminuria in the untreated diabetic dKO. Thus diabetic dKO mice may represent a model where one is more readily able to detect an anti albuminuric effect of ebselen since these animals may have a larger defect. It is therefore possible that ebselen is particularly effective when there is more of a deficiency in Gpx1, consistent with the underlying putative mode of action of this agent.

In addition, we have also shown that ebselen affords its greatest renoprotective influence on those genes and proteins significantly upregulated in the diabetic milieu, whether that be in the presence or absence of GPx1. For example, we showed that ebselen significantly reduces fibronectin expression (both at the mRNA level and at the protein level within the tubules) as well as the expression of Nox1, TGF-β, CTGF, TNF-α, VCAM-1, α-SMA, collagen I and collagen IV in the diabetic ApoE-/- kidney. However, where diabetes has not impacted on the oxidative stress marker, nitrotyrosine or Nox2 in the diabetic ApoE-/- kidney, administration of ebselen has no effect. In addition, these results suggest that that oxidative stress is not as perturbed in the diabetic ApoE-/- kidney compared with the diabetic dKO kidney. In summary, our data have shown that ebselen affords it greatest renoprotective influence in the absence of GPx1 but that it is still effective in reducing pro-fibrotic and pro-inflammatory mediators when GPx1 is present.

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TABLE I: Primary and secondary antibodies used in immunohistochemical (IHC) analysis and Western blots (WB).

Primary antibody	**Manufacturer/ Dilution/ Usage/Tissue**	**Secondary Antibody**	**Manufacturer and Dilution**
Polyclonal rabbit anti-mouse nitrotyrosine.	Chemicon; 1:200 IHC Aorta 1:80 IHC Glom 1:150 IHC Tub	Biotinylated anti-rabbit immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Polyclonal goat anti-mouse vascular endothelial growth factor (VEGF).	R&D System; 1:150 IHC Aorta	Biotinylated anti-goat immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Polyclonal goat anti-mouse connective tissue growth factor (CTGF)	Santa Cruz; 1:75 IHC Aorta	Biotinylated anti-goat immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Polyclonal mouse anti-rat vascular cell adhesion molecule CD106 (VCAM1)	Pharmingen; 1:100 IHC Aorta	M.O.M. Biotinylated anti- immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:250
Polyclonal goat anti-mouse Nox2 (gp91*phox*)	Santa Cruz; 1:300 IHC Aorta 1:100 IHC Glom, Tub	Biotinylated anti-goat immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Polyclonal goat anti-mouse collagen 1	Southern Biotech; 1:100 IHC Tub	Biotinylated anti-goat immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Polyclonal rabbit anti-human fibronectin	Dako; 1:100 IHC Tub & Glom	Biotinylated anti-rabbit immuno-globulin	Vector Lab, Burlingame, CA, USA; 1:500
Rabbit Polyclonal to human GPx1	Abcam Ltd, Cambridge, UK, 1:2000 WB	Polyclonal goat anti-rabbit IgG, peroxidase conjugated	Dako, Denmark, 1:2000
Rabbit Polyclonal to bovine catalase	Abcam Ltd, Cambridge, UK, 1:2000 WB	Polyclonal goat anti-rabbit IgG, peroxidase conjugated	Dako, Denmark, 1:2000
Rabbit Polyclonal to human SOD1	Abcam Ltd, Cambridge,UK,1:2000 WB	Polyclonal goat anti-rabbit IgG, peroxidase conjugated	Dako, Denmark, 1:2000
TABLE I CONTINUED: Primary antibody	**Manufacturer/ Dilution/ Usage/Tissue**	**Secondary Antibody**	**Manufacturer and Dilution**
Mouse monoclonal to β-actin	Abcam Ltd, Cambridge, UK, 1:10,000 WB	Polyclonal goat anti-mouse IgG, peroxidase conjugated	Dako, Denmark, 1:2000
Rabbit Polyclonal to human P-IKKαβ	Cell Signaling, Beverly, MA. 1: 1000 WB	Polyclonal donkey anti-rabbit IgG, peroxidase conjugated	GE Healthcare Uppsala, Sweden. 1: 2000
Rabbit Polyclonal to human	Cell Signaling, Beverly,	Polyclonal donkey	GE Healthcare

p38	MA. 1: 1000 WB	anti-rabbit IgG, peroxidase conjugated	Uppsala, Sweden. 1: 2000
Rabbit Polyclonal to human P-JNK	Cell Signaling, Beverly, MA. 1: 1000 WB	Polyclonal donkey anti-rabbit IgG, peroxidase conjugated	GE Healthcare Uppsala, Sweden. 1: 2000
Mouse Monoclonal to human α-tubulin	Sigma, St Louis, Missouri 1:10,000 WB	Polyclonal donkey anti-mouse IgG, peroxidase conjugated	Santa Cruz; 1:2000
Mouse Monoclonal to human 4-HNE	JaICA, Shizuoka, Japan 1:75	M.O.M. Biotinylated anti-mouse immuno-globulin	Vector Labs, 1:250
Normal Goat IgG Control	Alpha Diagnostic Int; Concentration equivalent to primary ab	M.O.M. Biotinylated anti-goat immuno-globulin	Vector Labs, 1:500
Normal Mouse IgG Control	Alpha Diagnostic Int; Concentration equivalent to primary ab	M.O.M. Biotinylated anti-mouse immuno-globulin	Vector Labs, 1:250
Normal Rabbit IgG Control	Alpha Diagnostic Int; Concentration equivalent to primary ab	M.O.M. Biotinylated anti-rabbit immuno-globulin	Vector Labs, 1:500

TABLE II: Primers and probes used in quantitative RT-PCR analysis

Gene	sense primer 5'-3'	anti-sense primer 5'-3'	probe 5'-3'
VEGF	GCA CTG GAC CCT GGC TTT ACT	ATG GGA CTT CTG CTC TCC TTC TG	6-FAM CTG TAC CTC CAC CAT GC
TGF-β	GCAGTGGCTGAAC CAAGGA	GCAGTGAGCGCTGA ATCGA	6- FAM AAAGCCCTGTATTCCGT
Nox1	GACCAATGTGGGA CAATGAGTTT	CCCCCACCGCAGAC TTG	6- FAM CTAGAATAGCTACTGCCC ACC
Nox2	CAA CTG GAC AGG AAC CT	AGT GCG TGT TGC TCG ACA AG	6-FAM CCA AGC TAC CAT CTT ATG GAA AGT G
Nox4	AAAAATATCACAC ACTGAATTCGAGA CT	TGGGTCCACAGCAG AAAACTC	6- FAM CATTTTGCTATTTCATCAA A
α-SMA	GAC GCT GAA GTA TCC GAT AGA ACA	GGC CAC ACG AAG CTC GTT AT	6- FAM TGC CAG ATC TTT TCC
CTGF	GCTGCCTACCGACT GGAAGA	CTTAGAACAGGCGC TCCACTCT	6- FAM ACTGCCTGGTCCAGAC
TNF-α	GGCTGCCCCGACTA CGT	TTTCTCCTGGTATGA GATAGCAAATC	6- FAM TCACCCACACCGTCAG
VCAM1	CTGCTCAAGTGATG GGATACCA	ATCGTCCCTTTTTGT AGACATGAAG	6-FAM CCAAAATCCTGTGGAGCA G
MCP-1	GTCTGTGCTGACCC CAAGAAG	TGGTTCCGATCCAG GTTTTTA	6- FAM AATGGGTCCAGACATAC
Coll 1	GACTGGAAGAGCG GAGAGTACTG	CCTTGATGGCGTCC AGGTT	6- FAM ATCGACCCTAACCAAG
Coll 3	GGGAATGGAGCAA GACAGTCTT	TGCGATATCTATGAT GGGTAGTCTCA	6- FAM AATATCAAACACGCAAGG C
Coll 4	GGCGGTACACAGT CAGACCAT	GGAATAGCCGATCC ACAGTGA	6- FAM CAGTGCCCTAACGGT
FN	ACATGGCTTTAGGC GGACAA	ACATTCGGCAGGTA TGGTCTTG	6- FAM CCCCGTCAGGCTTA
18S	TGT TCA CCA TGA GGC TGA GAT C	TGG TTG CCT GGG AAA ATC C	6-FAM TGC TGG CAC CAG ACC AGA CTT GCC CTC

TABLE III: Characteristics of dKO Mice at Conclusion of 20-week Study

Parameters	**ND (n=10/group)**	**ND+Ebselen (n=10/group)**	**Diabetic (n=8/group)**	**Diab+Ebselen (n=7/group)**
Body weight (g)	33.3 ± 1.0	29.7 ± 0.5**	23.9 ± 1.2***	24.2 ± 0.4***
Glycated haemoglobin (%)[1]	3.6 ± 0.1	2.9 ± 0.1	11.5 ± 1.0 ***	9.6 ± 1.2***
Plasma glucose (mmol/L)	9.5 ± 0.6	10.9 ± 0.6	27.6 ± 4.0**	29.2 ± 3.2**
Total cholesterol (mmol/L)	10.9 ± 0.8	11.7 ± 0.8	12.3 ± 1.8	12.0 ± 2.2
Triglycerides (mmol/L)	1.5 ± 0.2	0.9 ± 0.2	2.2 ± 0.4	2.8 ± 0.4
HDL (mmol/L)	2.1 ± 0.2	2.4 ± 0.2	1.9 ± 0.3	1.7 ± 0.4
LDL (mmol/L)	8.1 ± 0.5	8.9 ± 0.6	9.4 ± 1.5	9.0 ± 1.7
CCR ($ml/min/m^2$)	53.4 ± 4.5	57.4 ± 4.1	84.3 ± 4.3***	63.2 ± 4.0 †
H_2O intake (ml/24h)	4.1± 0.3	2.6 ± 0.5	22.5 ± 1.1***	18.4 ± 2.0*** †
Urinary output (ml/24h)	1.2 ± 0.2	1.0 ± 0.1	19.2 ± 1.7***	15.1 ± 2.0***
Plasma dROMS (U.Carr)	75.9 ± 4.8 (n=8)	74.1 ± 8.1 (n=7)	157.5 ± 7.8*** (n=10)	110.7 ± 6.9††† (n=11)
Urinary Isoprostanes (pg/μmol creatinine)	0.69 ± 0.11 (n=7)	0.52 ± 0.12 (n=4)	0.93 ± 0.09 (n=5)	0.56 ± 0.05† (n=9)

**P<0.01; ***P<0.001 vs non-diabetic dKO counterparts.
††† P<0.001, † P<0.05 vs diabetic group.
CCR=creatinine clearance rate.
[1] values after 10 weeks of treatment
Data shown as mean ± SEM; group sizes as indicated

TABLE IV: Characteristics of ApoE$^{-/-}$ Mice at Conclusion of 20-week Study

Parameters	**ND (n=10/group)**	**ND+Ebselen (n=10/group)**	**Diabetic (n=11/group)**	**Diab+Ebselen (n=10/group)**
Body weight (g)	30.2 ± 0.2	30.8 ± 0.5	19.9 ± 0.9***	23.3 ± 1.2*** †
Glycated haemoglobin (%)[1]	4.1 ± 0.1	4.7 ± 0.1	12.0 ± 0.5***	14.3 ± 1.1***
Plasma glucose (mmol/L)	11.4 ± 0.6	9.2 ± 0.6	25.8 ± 1.3***	30.5 ± 2.8 ***
Total cholesterol (mmol/L)	10.8 ± 0.3	9.2 ± 0.5	14.4 ± 2.3	13.3 ± 1.5
Triglycerides (mmol/L)	1.1 ± 0.1	2.0 ± 0.2	4.8 ± 1.1*	4.2 ± 0.6*
HDL (mmol/L)	2.4 ± 0.1	2.3 ± 0.1	1.7 ± 0.3	1.8 ± 0.4
LDL (mmol/L)	8.0 ± 0.2	6.1 ± 0.4	10.6 ± 1.7	9.6 ± 1.2
CCR (ml/min/m^2)	51.7 ± 3.9	55.4 ± 2.7	96.9 ± 4.0***	70.8 ± 6.7 †††
H_2O intake (ml/24h)	4.2 ± 0.3	3.9 ± 0.4	26.1 ± 1.6***	25.5 ± 2.1***
Urinary output (ml/24h)	1.2 ± 1.2	2.6 ± 0.2	24.1 ± 1.7***	23.0 ± 2.1***
Urinary Isoprostanes (pg/µmol creatinine)	0.49 ± 0.03 (n=5)	0.4 ± 0.03 (n=8)	1.88 ± 0.13*** (n=8)	1.32 ± 0.17† (n=7)

*** P<0.001; *P<0.05 vs non-diabetic ApoE$^{-/-}$ counterparts.
† P<0.05; ††† P< 0.001 vs diabetic group.
CCR= creatinine clearance rate.
[1] values after 10 weeks of treatment
Data shown as mean ± SEM; group sizes as indicated.

Figure I

Oil Red O-stained ApoE$^{-/-}$GPX1$^{-/-}$ (dKO) aortic sinuses, 10- (A-C) and 20-weeks (D-F) after STZ-induced diabetes. (A,D) ND dKO; (B,E) D dKO; (C,F) ebselen-gavaged D dKO aortas. Magnification: X40. G) Analysis of lesions, 10- and 20-weeks after STZ-induced diabetes. 10-w = 10 weeks of diabetes; 20-w = 20 weeks of diabetes; ND=non-diabetic; D=diabetic; Eb=ebselen; NS=not significant. Bars, mean ± S.E.M (n = 6-14 aortas/group). ***P*<0.01; **P*<0.05 vs ND counterparts; $^{+}$*P*<0.05; $^{+++}$*P*<0.001 vs 10-w ND counterparts.

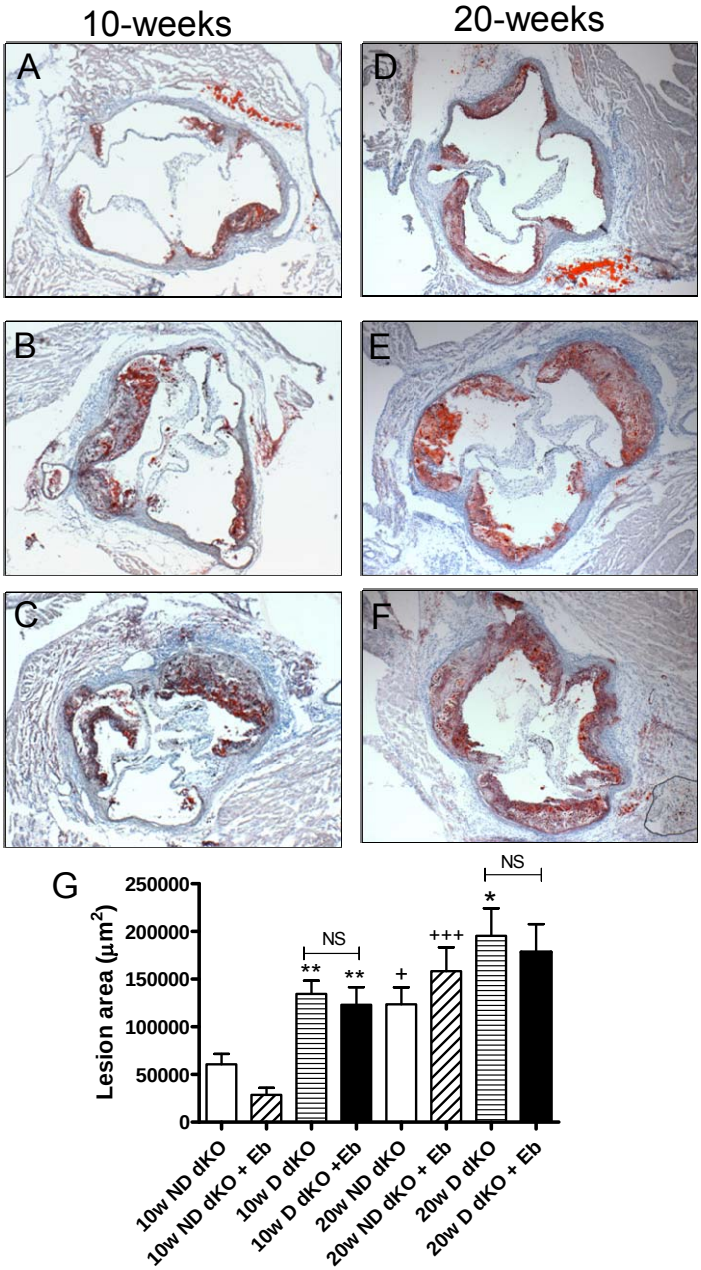
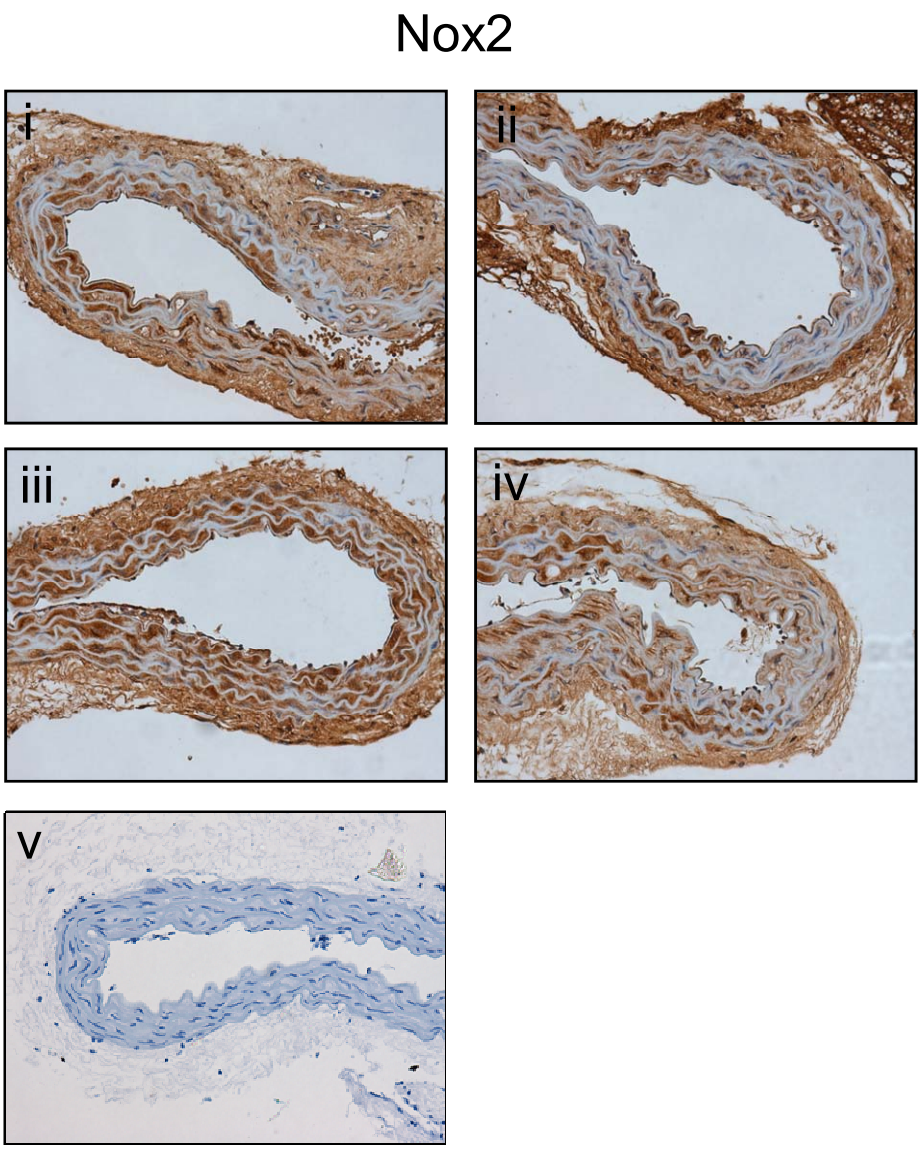


Figure II, Part A-F

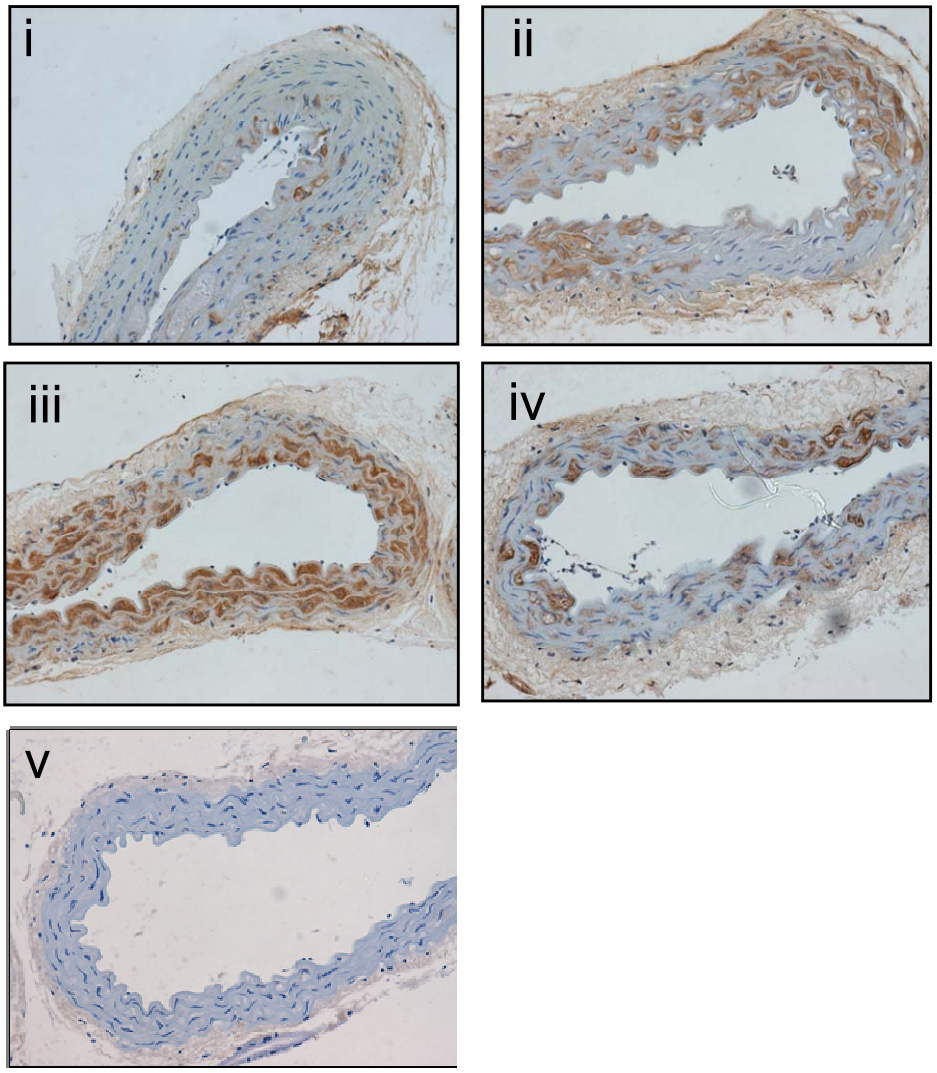
Part A=Nox2; Part B= Nitrotyrosine (NT); Part C= 4-Hydroxynonenal (4-HNE); Part D=Vascular Endothelial Growth Factor (VEGF); Part E=Connective Tissue Growth Factor (CTGF); and Part F= Vascular Cell Adhesion Molecule-1 (VCAM-1) (brown staining) in aorta from (i) non-diabetic $ApoE^{-/-}GPX1^{-/-}$ (dKO) mice ; (ii); non-diabetic dKO mice treated with ebselen; (iii) diabetic dKO mice; (iv) diabetic dKO mice treated with ebselen; and (v) negative control which consisted of species-matched non-immune IgG in place of primary antibody, 10-weeks after sham or STZ-induced diabetes. Magnification: X200.

Part A



Part B

Nitrotyrosine



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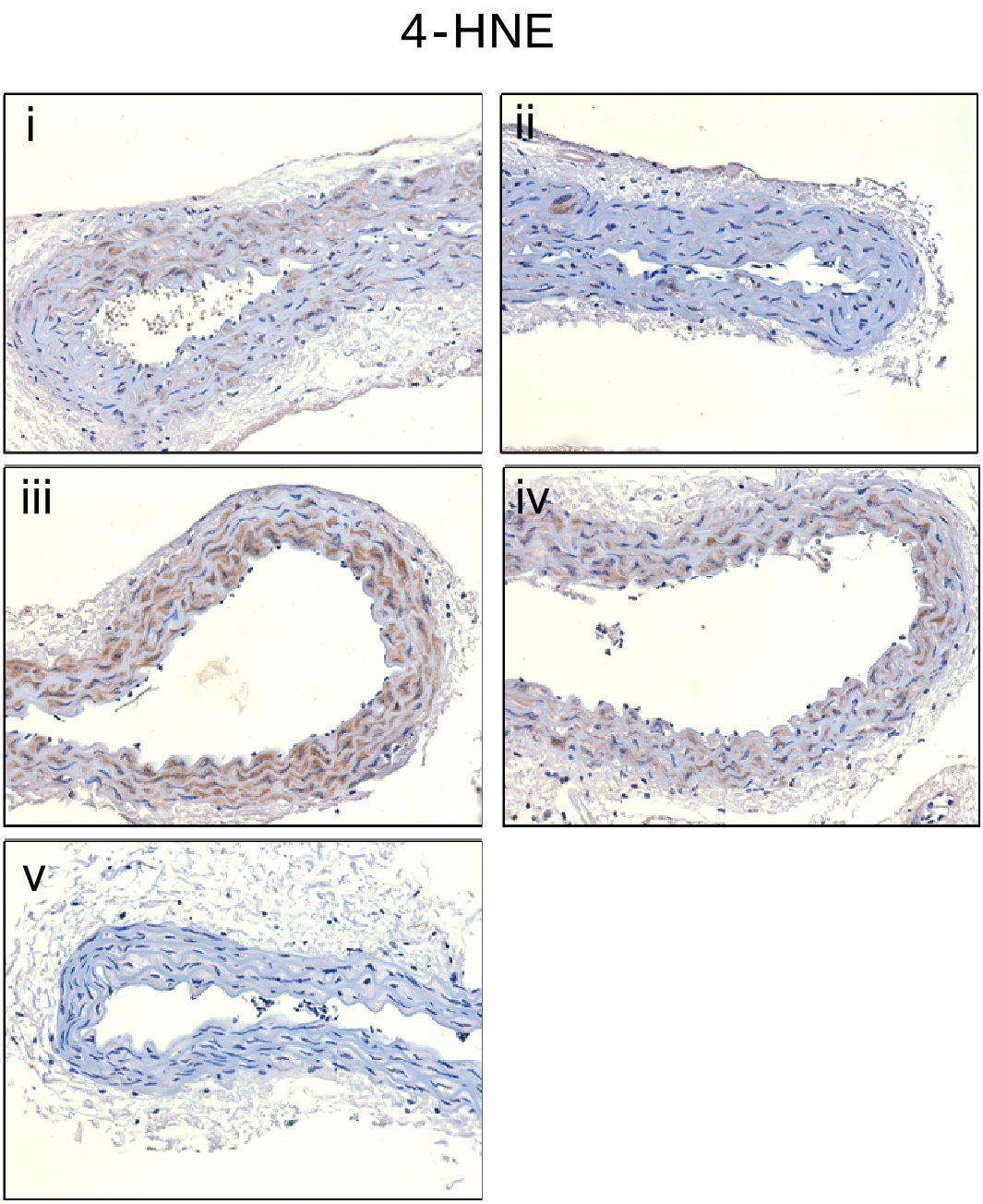

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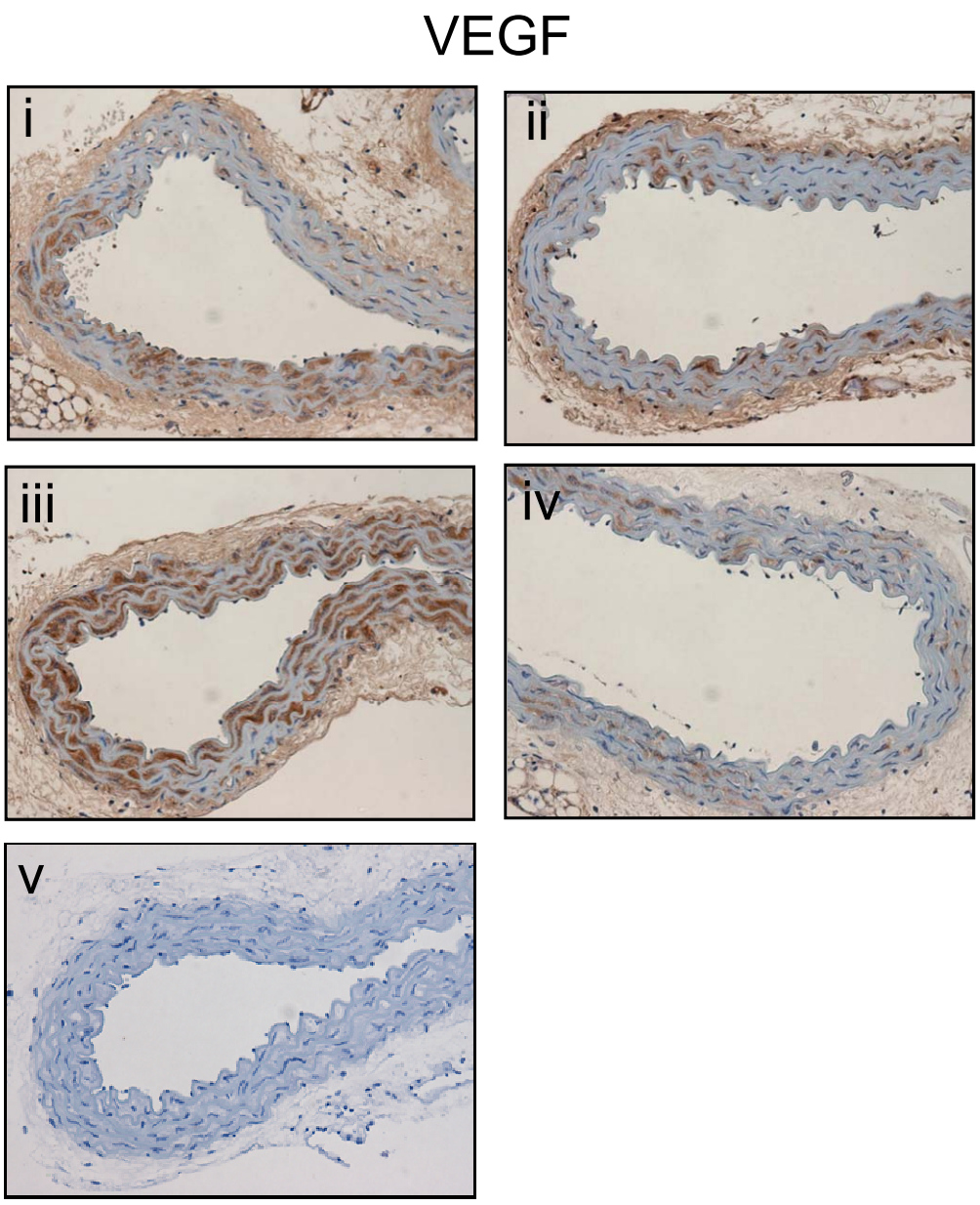

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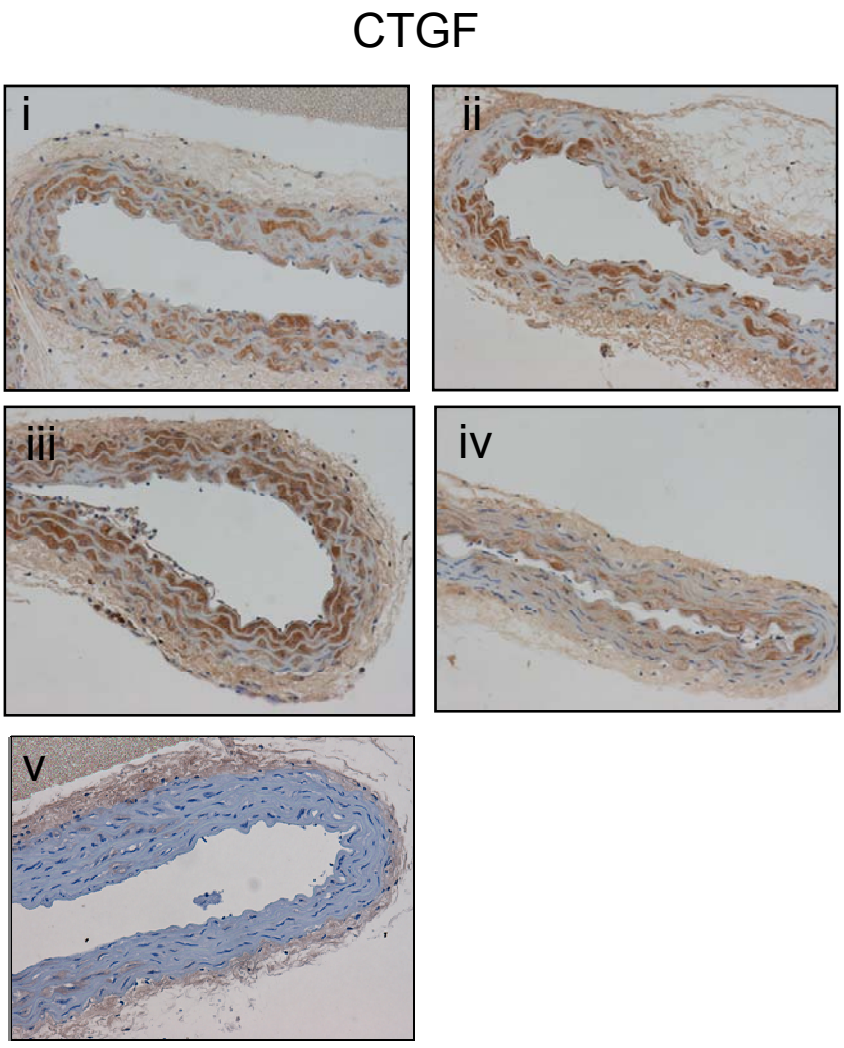

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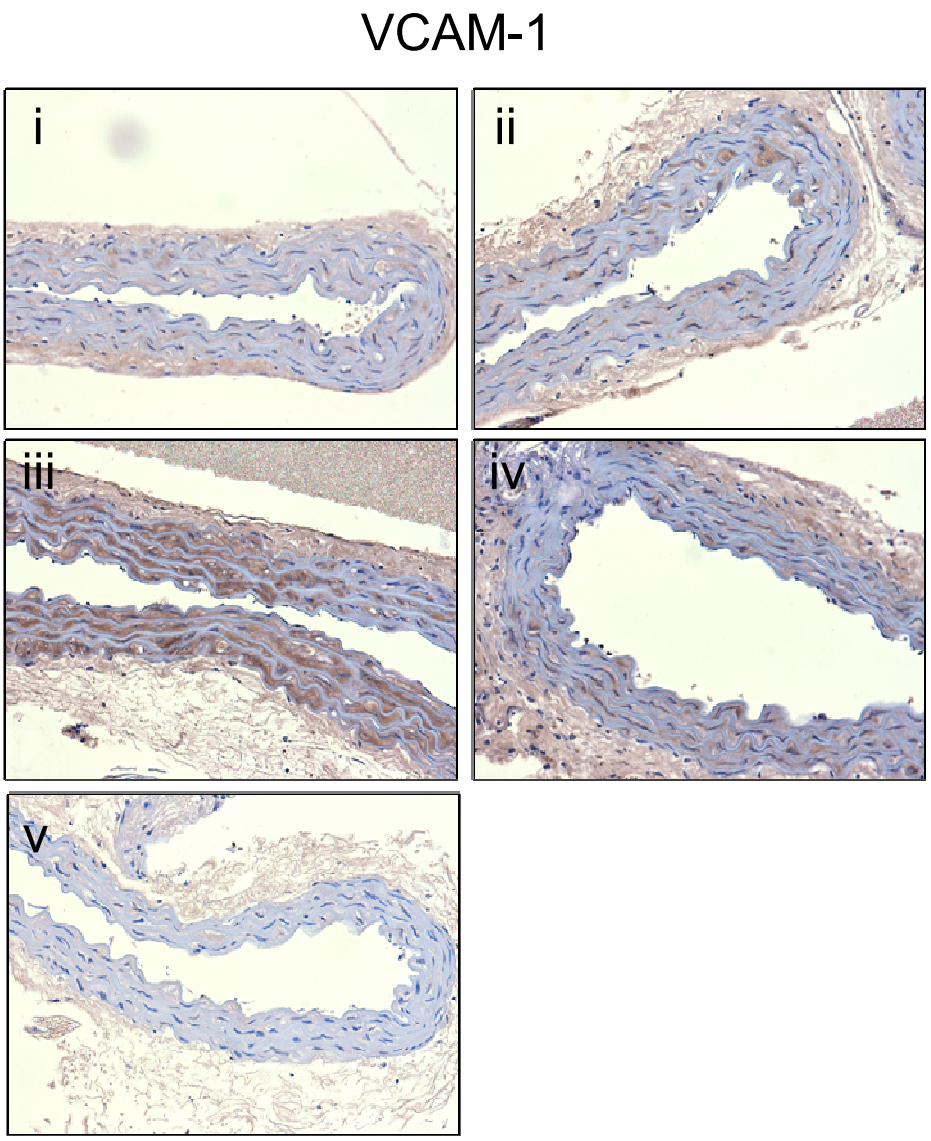

Figure III

Quantitative RT-PCR analysis of A) GPx3 and B) GPx4 in ApoE$^{-/-}$GPX1$^{-/-}$ (dKO) aortas after 10 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO= ApoE$^{-/-}$ GPX1$^{-/-}$. ***P<0.001; **P<0.01 vs ND counterparts; $^{###}P$<0.001; $^{##}P$<0.01 vs diabetic counterparts. a.u.= arbitrary units. Bars, mean ± S.E.M; n= 7-9 aortas/group.

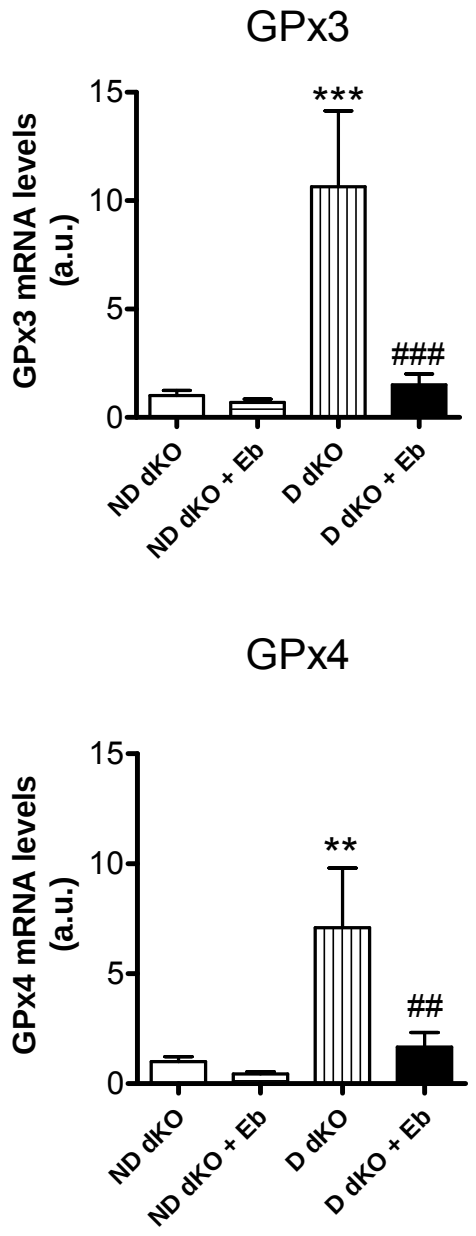

Figure IV
Albuminuria is significantly reduced after 20 weeks of ebselen treatment in the diabetic dKO kidney. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO= $ApoE^{-/-}GPX1^{-/-}$.
$^{\#}P<0.05$ vs D dKO; $^{**}P<0.01$ vs ND dKO counterparts; $^{*}P<0.05$ D dKO vs D $ApoE^{-/-}$; n = 4-10 urines/group, Bars, geometric mean ± Error Bars.

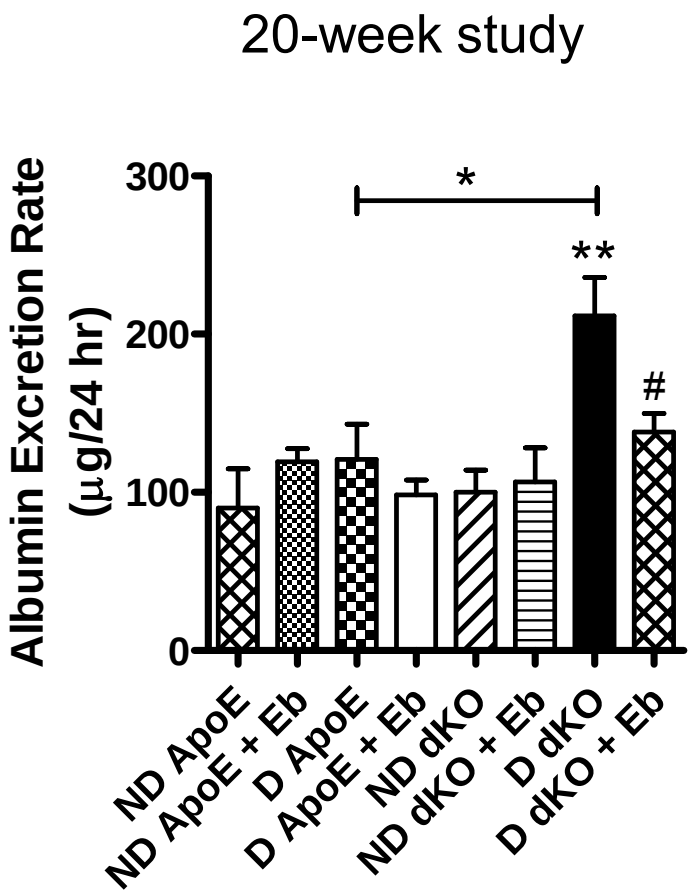

Figure V

Quantitative RT-PCR analysis of the Nox isoforms -1 and -4 in ApoE$^{-/-}$ and dKO kidneys after 20 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO=ApoE$^{-/-}$GPX1$^{-/-}$ kidneys. ***P<0.001; *P<0.05 vs ND counterparts; $^{###}P$<0.001 vs diabetic counterparts. a.u.=arbitrary units. Bars, mean ± S.E.M; n = 7-11 kidneys/group.

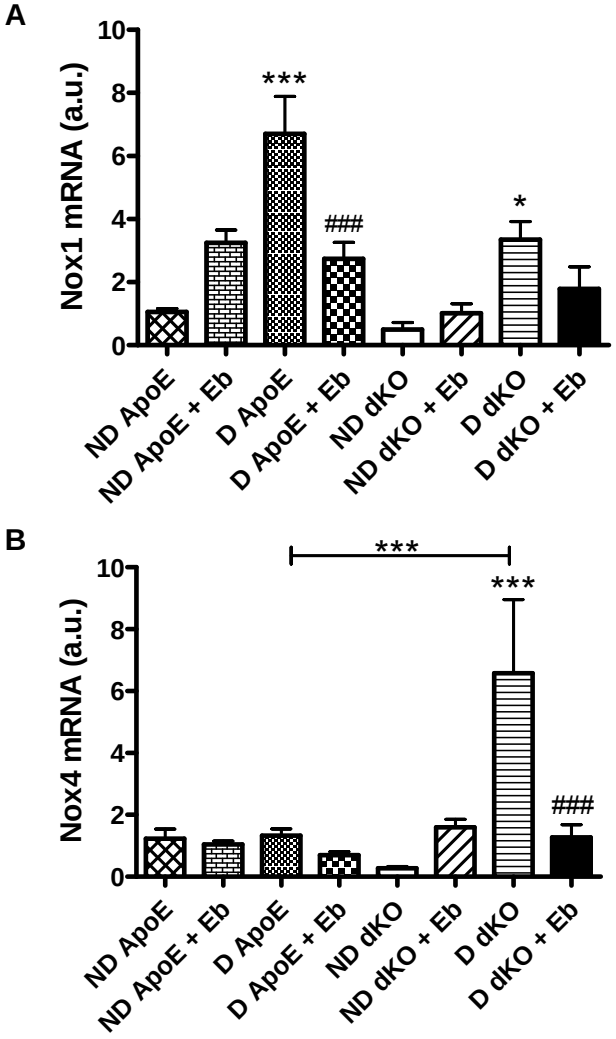

Figure VI

Quantitative RT-PCR analysis of A) GPx3 and B) GPx4 in ApoE$^{-/-}$ and dKO kidneys after 20 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO= ApoE$^{-/-}$ GPX1$^{-/-}$. ***P<0.001 vs ND counterparts; $^{###}P$<0.001; $^{##}P$<0.01 vs diabetic counterparts. a.u.= arbitrary units. Bars, mean ± S.E.M; n= 7-11 kidneys/group.

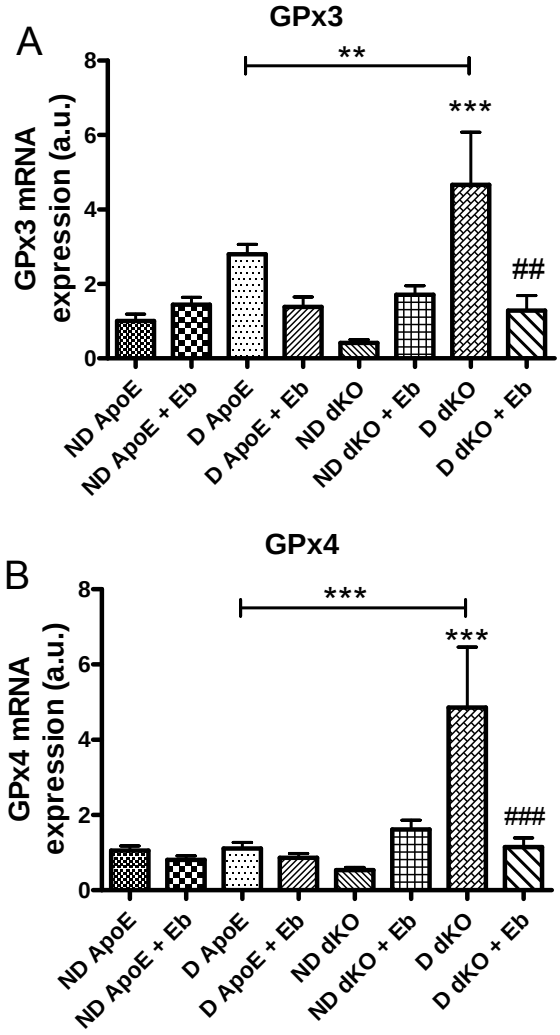

Figure VII

Quantitative RT-PCR analysis of A) TGF-β; B) CTGF; C) TNF-α; D) VCAM-1; and E) MCP-1 in ApoE$^{-/-}$ and dKO kidneys after 20 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO= ApoE$^{-/-}$ GPX1$^{-/-}$. ***P<0.001 vs ND counterparts; $^{###}P$<0.001; $^{##}P$<0.01 $^{#}P$<0.05 vs diabetic counterparts. a.u.= arbitrary units. Bars, mean ± S.E.M; n= 7-11 kidneys/group.

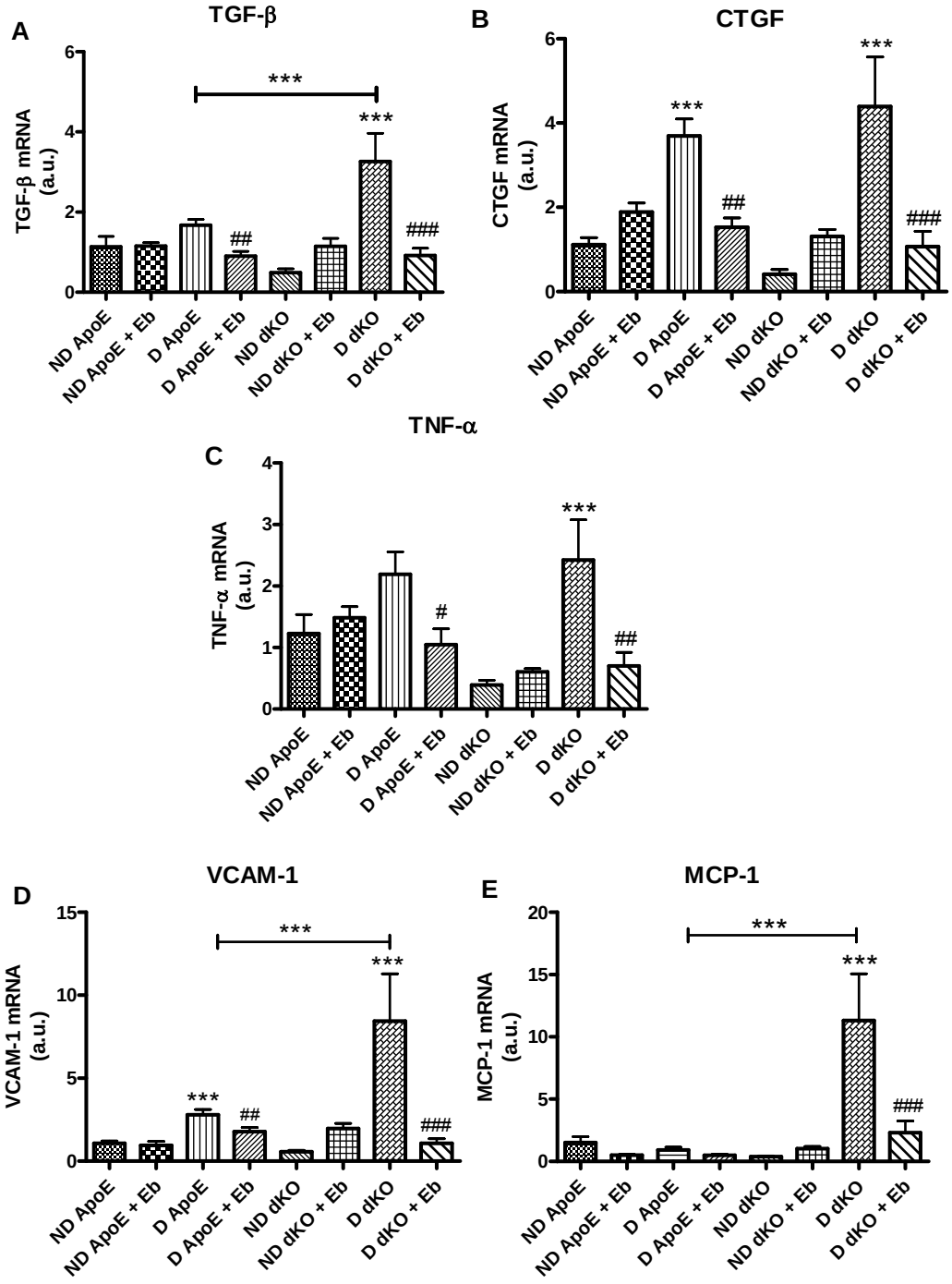

Figure VIII

Quantitative RT-PCR analysis of A) α-SMA; B) collagen I; C) collagen III; and D) collagen IV; in ApoE$^{-/-}$ and dKO kidneys after 20 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen; dKO= ApoE$^{-/-}$ GPX1$^{-/-}$. ***P<0.001; **P<0.01 vs ND counterparts; $^{###}P$<0.001; $^{##}P$<0.01 $^{#}P$<0.05 vs diabetic counterparts. a.u.= arbitrary units. Bars, mean ± S.E.M; n= 7-11 kidneys/group.

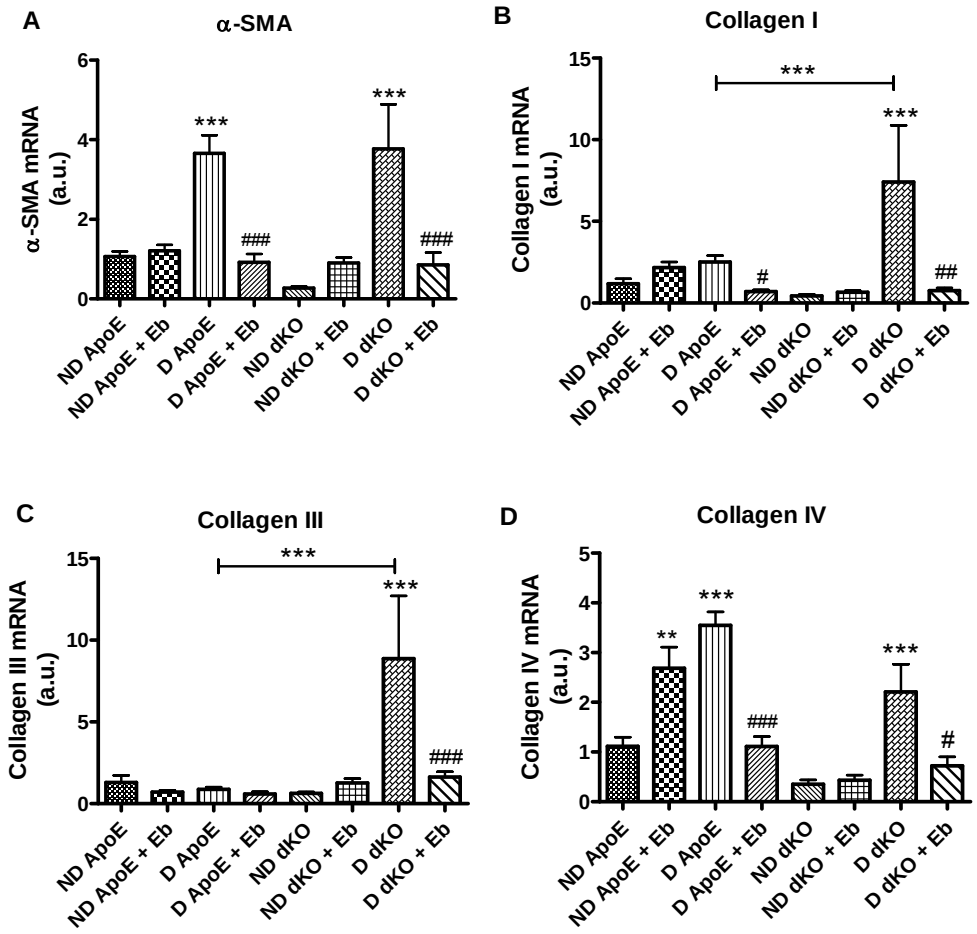

Figure IX

Immunohistochemical analysis of collagen I protein in ApoE$^{-/-}$ and ApoE$^{-/-}$GPx1$^{-/-}$ (dKO) kidneys after 20 weeks of diabetes. A-D) ApoE$^{-/-}$ kidneys; E-H) dKO kidneys. Treatments are as indicated above and to right of photomicrographs; I) negative control which consisted of species-matched non-immune IgG in place of primary antibody on a diabetic dKO section; Magnification=100X; J) Quantitative analysis of collagen I protein levels in ApoE$^{-/-}$ and dKO kidneys after 20 weeks of diabetes. ND=non-diabetic; D=diabetic; Eb=ebselen. $^{**}P<0.01$ vs ND counterparts; $^{\#\#\#}P<0.001$ vs diabetic counterparts. a.u.= arbitrary units. Bars, mean ± S.E.M; n= at least 5 mice/group.

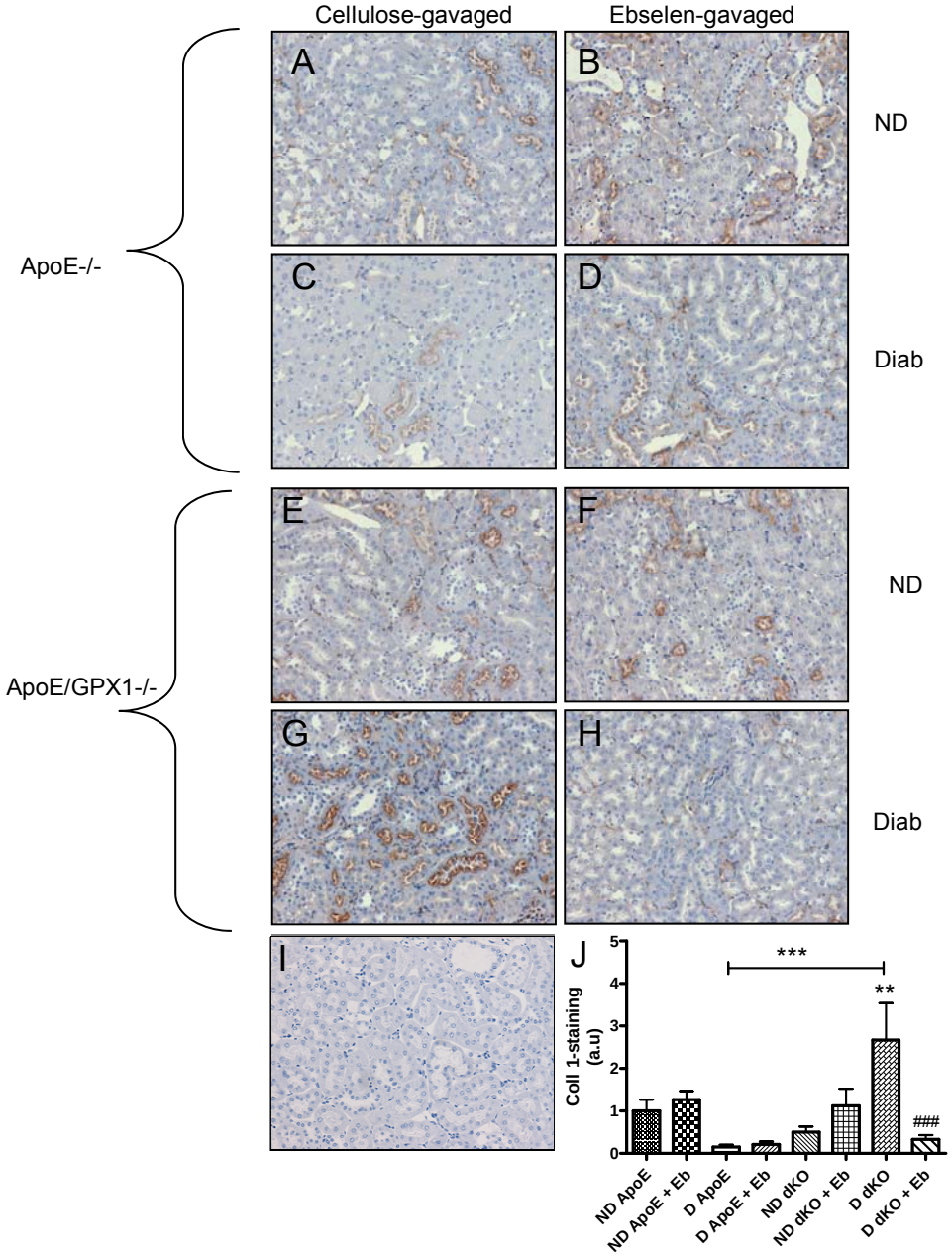

Figure X
Western blot analysis of antioxidant levels after 20 weeks of diabetes in non-diabetic and diabetic ApoE-/- and ApoE$^{-/-}$GPx1$^{-/-}$ (dKO) kidneys with and without ebselen treatment. A) Glutathione Peroxidase-1 (GPx1) levels; B) catalase; and C) Superoxide dismutase-1 (Sod1). a.u.=arbitrary units. Bars, mean ± S.E.M; n= 4 kidneys/group. *P<0.05 vs ND ApoE$^{-/-}$ kidney.

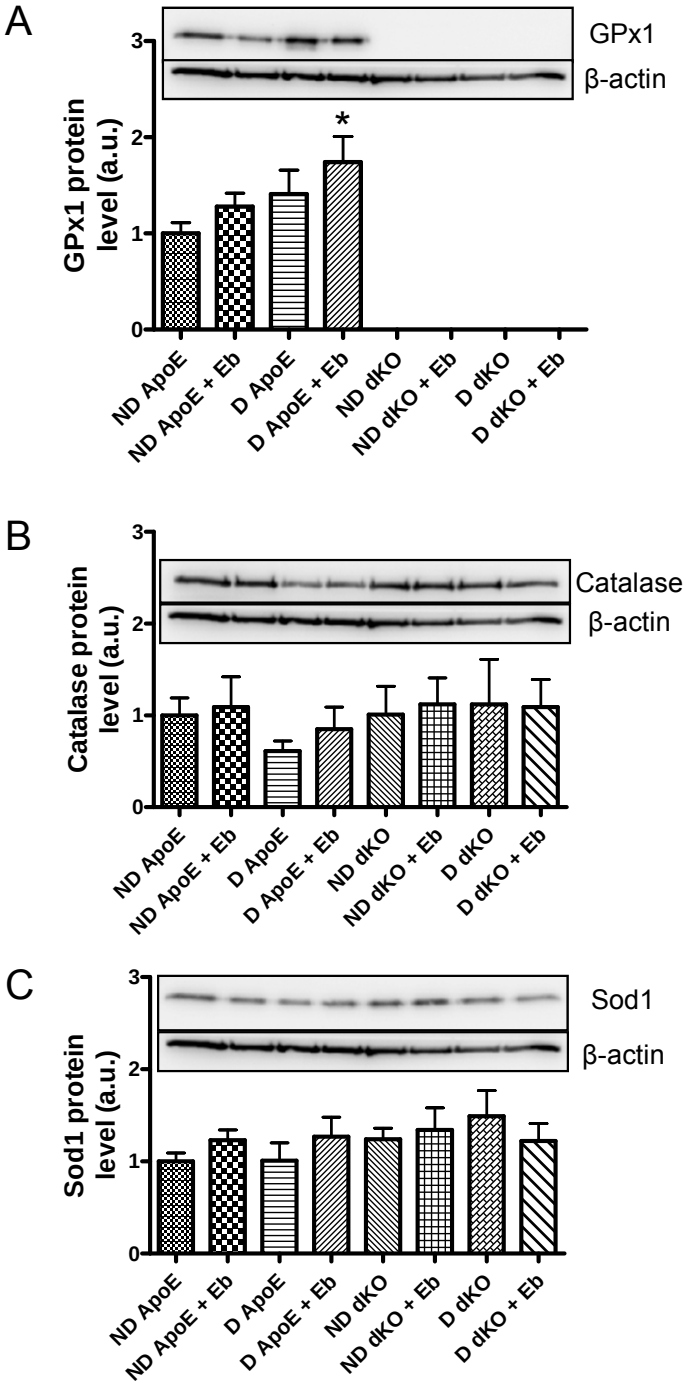
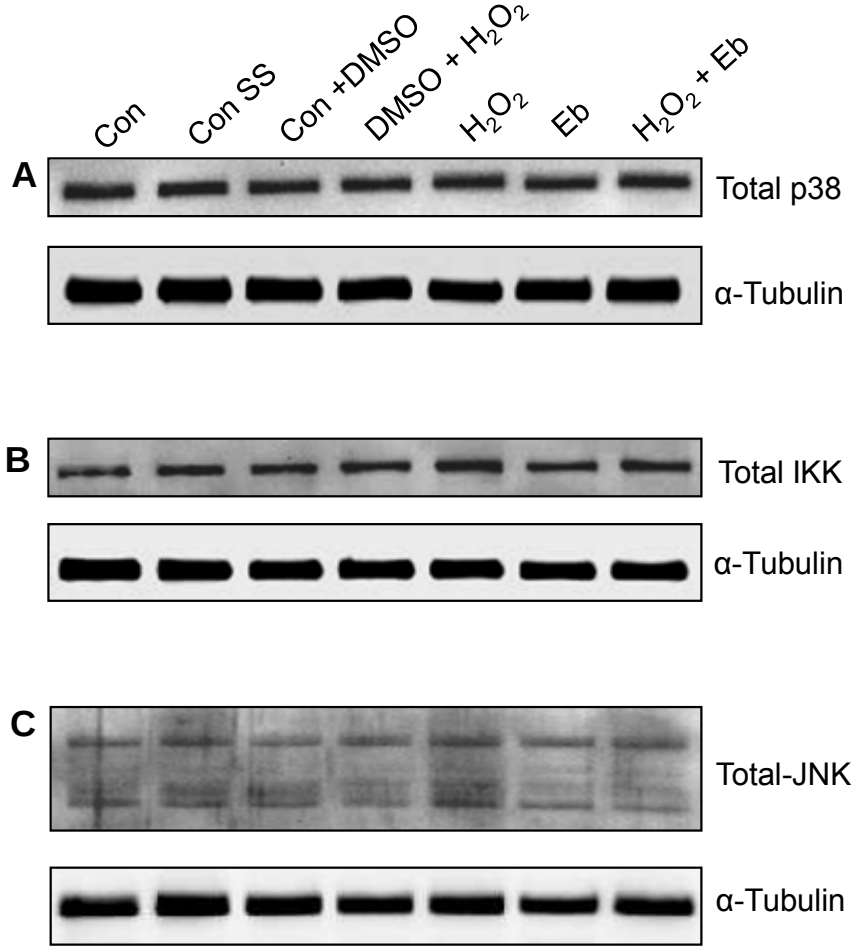
Figure XI
Immunoblots of total p38, total IKK and total JNK. Forty-five micrograms of protein from each sample was separated by electrophoresis on 10% SDS polyacrylamide gels. Blots were probed with total p38, total IKK and total JNK as well as α-tubulin to control for protein loading. Quantitation of bands and expression relative to α-tubulin confirmed that none of the treatments affected total p38, total IKK or total JNK protein levels. SS=serum starved; DMSO=dimethylsulphoxide (diluent for ebselen).