ONLINE APPENDIX

RESEARCH DESIGN AND METHODS

BRP and BREC treatments. Eighty percent confluent BRECs and BRPs were treated with 100 nM Ang II (1; 2) (Sigma), for 24 hours in the presence of either candesartan (1 μM in DMSO) (3), or vehicle (diluted DMSO). Cells were incubated in 0.5% serum-containing media respectively during treatment. All treatments were in triplicate and the experiment repeated at least three-times.

Real-time PCR

Table S1: Real-time PCR primers - SYBR green.

Gene	Species	Accession number	SYBR green primers
GLO-I	rat	NM_207594	Forward: 5'TGAAGATGACGAGACGCAGAGT3' Reverse: 5'CCCAATGTGGCCAAATCC3'
TNF-α	rat	NM_012675	Forward: 5'TGATCGGTCCCAACAAGGA3' Reverse: 5'TGGGCTACGGGCTTGTCA3'
iNOS	rat	NM_012611	Forward: 5'CCTTCAGGTATGCGGTATTTGG3' Reverse: 5'TGTCATGAGCAAAGGCACAGA3'
18s	rat	XO3205	Forward: 5'CCGCAGCTAGGAATAATGGAAT3' Reverse: 5'CGGCGCAATACGAATGC3'
GLO-I	Bovine	XM_593291	Forward: 5'GGGCACGGAAGATGATGAGA3' Reverse: 5'CGGCGCAATACGAATGC3'
18s	Bovine	AF176811	Forward: 5'CGGCTACCACATCCAAGGAA3' Reverse: 5'CCTGTATTGTTATTTTTCGTCACTACCT3'

Real-time PCR primers and Taqman probes for rat ICAM-1, VEGFA and 18s were as previously described (4).

Retinal and cellular GLO-I activity. Flash-frozen whole retinae were homogenized in 250 μL ice-cold 10 mM Tris pH 7.4 containing protease cocktail inhibitors (Sigma) using a Polytron Homogenizer (HD Scientific, Vic, Australia). The homogenate was sonicated on ice at 40% amplitude in bursts for 20 seconds then centrifuged at 12,000x*g* for 20 minutes at 4°C. The supernatant (cytosolic fraction) was dialyzed overnight against 10 mM Tris pH 7.4 at 4°C. For BRP and BREC, after treatment, lysates were generated (5), and dialyzed overnight against 10 mM Tris pH 7.4 at 4°C.

$NO^{\bullet}$ assay in cells and retina. Cell lysates were generated as for the GLO-I assay except that the cells were not freeze-thawed, and homogenates were sonicated for 3x20 second bursts and not dialyzed. Both retina from each rat were pooled and homogenized in 300 uL of 10 mM sodium phosphate pH 7.4 and the lysate then centrifuged at 13,000 rpm for 10 min 4°C. Both rat and cell supernatants were first filtered through 100 kDa molecular weight-cut off (MWCO) and then 10 kDa MWCO filtration units (Amicon, Millipore, MA, USA). In cells, available nitrate in the 100 μL of filtrate was reduced as previously described (5), with some minor modifications. $NO^{\bullet}$ was detected by a fluorescence assay according to the method of Nussler et al (6), with

some modifications. Retinal filtrates were measured exactly as described in (6). The amount of nitrate in each sample was adjusted for the total protein concentration of the cell lysate by a Bradford protein assay (Biorad, CA, USA).

Acellular capillaries in retina. Eyes were immersed overnight in 2% Carson's fixative in 1.1 M sodium dihydrogen orthophosphate, 950 mM sodium hydroxide (BDH Laboratory Supplies, Poole, England), and 10% volume 40% formaldehyde (MERCK Pty. Ltd., Kilsyth, Victoria, Australia). Eyes were washed in 200 mM Tris buffer (Sigma-Aldrich Co., St. Louis, MO) for 24 hours. Retinas were dissected away from sclera and choroid and washed in Tris buffer for 4 days to loosen the vitreous from the retina. The neural retina was digested in a 1% trypsin solution (porcine pancreas; Sigma-Aldrich) at 37°C for 1 hour. After washing (45 min) in Triton X-Tris buffer solution (t-Octylphenoxypoly-ethoxyethanol; Sigma-Aldrich) only the retinal vascular network remained. Maltese-cross incisions were made and the retina mounted on silane-coated slides and stained with 1% periodic acid (CN Biomedicals Inc., Aurora, OH) and Schiff's reagent. Counterstaining with Mayer's Hematoxylin (Amber Scientific, Belmont, WA, Australia) was used to identify endothelial cells and pericytes. Acellular capillaries were counted in a double-masked manner in the central retina (0 to 1800±77 µm), mid retina (1801 to 3333±21 µm), and peripheral retina (3334 to 4917±60 µm) retina. Approximately 80 high-power (X40 objective) nonoverlapping photomicrographs were taken from each retina (15 central, 30 mid, 35 peripheral regions) with a Spot digital camera (SciTech Pty. Ltd., Preston, Victoria, Australia) attached to a Olympus BX51 microscope (Olympus, Tokyo, Japan). Results are expressed as acellular capillaries per field of central, mid or peripheral retina. Investigators were masked to the experimental groups.

Leukocyte adherence in retina. This technique is based on a modification of previously published methods (4; 7). Animals were perfused via the right atrium via a 21 gauge needle with 100 mM phosphate buffered saline at pH 7.4 (PBS, 100 mL) over 5 minutes to clear circulating blood cells and non-adherent leukocytes from the retina. All subsequent procedures were post-mortem. Animals were perfused with rhodamine-coupled Concanavalin A lectin (25mg/kg in PBS, 50 mL, Vector Laboratories, Inc., CA, USA) to stain adherent leukocytes and the vascular endothelium. Animals were then perfused with another 50 mL of PBS to clear any remaining Concanavalin A lectin. Eyes were then fixed in 4% paraformaldehyde in PBS for 30 minutes and retina flat-mounted. Adherent leukocytes were observed using an Olympus BX51 fluorescent microscope fitted with a rhodamine filter and Olympus U-RFL-T light source (Olympus. Tokyo, Japan). Adherent leukocytes were counted in all blood vessels including capillaries over the entire retina. Results are expressed as the number of adherent leukocytes per retina. No attempt was made to discriminate between occluded and non-occluded blood vessels. The investigator was blinded to the experimental group.

Retinal MGO-AGE and argpyrimidine levels. Five hundred ng of lysate was used to coat each well of a 96 well plate overnight at 4°C (in 100 mM Sodium Bicarbonate, pH 9.6). Plates were washed 3 times with 1xPBS/0.05%Tween-20 (PBS/T) and blocked with 1% BSA in PBS for 1 hour at 37°C. After incubation, plates were washed with PBS/T and 100 µL of either mouse anti-argpyrimidine (1:10,000) or rabbit anti-MGO-AGE (1:2,000) in 1% BSA/PBST added. Plates were incubated for 1 hour at 37°C, washed with PBS/T, and secondary applied for 1 hour at 37°C. For argpyrimidine, 1:10,000 goat anti-mouse IgG (GE Healthcare, NSW, Australia) and

for MGO-AGE, 1:3,000 goat anti-rabbit IgG (Cell Signalling) in 1% BSA/PBST were used. After incubation, plates were washed 3 times with PBS/T, and developed by addition of 100 µL of 3,3',5,5'-tetramethybenzidine Horseradish Peroxidase Substrate (Millipore, NSW, Australia). The reaction was quenched with 100 µL 1M HCl and absorbance recorded in a microplate reader (Bio Rad Benchmark Plus, Bio Rad, NSW, Australia) at 450nm.

MGO-AGE and total AGE levels in plasma by competitive ELISA.
Rat serum (diluted 1:8,000) or MGO-BSA (500ng/ml) was coated onto Maxisorb 96 well ELISA plates (Nunc) overnight at 4°C (in 1x PBS). Plates were washed 3 times with 1xPBS/0.05% Tween 20 (PBS/T) and blocked with 4% non-fat dry milk in PBS/T (mPBS/T) for 2 hours at room temperature. The plates were washed once before the addition of an in-house (Baker IDI) rabbit anti-AGE-BSA polyclonal antibody (for 2 hours at a 1/1000 dilution in 0.4% mPBS/T) that had been pre-incubated with either 50ug/ml fraction V BSA (Sigma) (for total AGE quantitation) or MGO-BSA (non-MGO-AGE quantitation) for 1hr at room temperature in PBS. After washing 3x with PBS/T the antibody bound to the plate was detected using a goat anti-rabbit HRP conjugate (Sigma) (at 1:2000 dilution in 0.4%mPBS/T). Following 3 more washes with PBS/T, 50µl of 3,3',5,5'tetramethylbenzidine liquid substrate was added to each well (TMB) (Sigma) followed by 50 µl of 2N H_2SO_4 to stop the reaction. The O.D. was scanned at 450-570nm using an ELISA plate reader (Bio Rad Benchmark Plus). BCA protein assay kit (Pierce, Thermo Scientific) was performed on serum samples diluted to 1/100 in PBS as per manufacturer's instructions to adjust the ELISA results for differences in total protein concentration. MGO-AGE content is calculated by subtraction of total AGE values from non-MGO-AGE values.

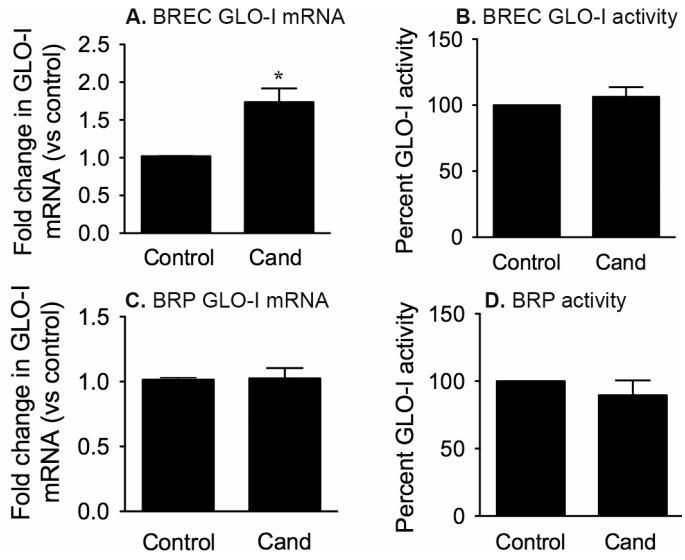
RESULTS
Body weight, blood glucose and systolic blood pressure. As expected, in both Ren-2 and Sprague Dawley rats, diabetes was associated with a reduction in body weight gain compared to non-diabetic controls (Table S2). Candesartan had no effect on body weight gain in Ren-2 rats (Table S2). Systolic blood pressure was similar in non-diabetic and diabetic Ren-2 rats, and higher than Sprague Dawley controls (Table S2). Candesartan reduced systolic blood pressure in Ren-2 rats after 4 weeks of diabetes, and by 20 weeks of diabetes had reduced systolic blood pressure to the level of Sprague Dawley controls (Table S2). Blood glucose was increased in diabetic rats compared to non-diabetic controls, and in diabetic Ren-2 rats there was no effect with candesartan (Table S2).

Table S2: Body weights, systolic blood pressure (SBP) and blood glucose in diabetic Ren-2 and Sprague Dawley (SD) rats.

Study group	Body weight (g)	SBP (mmHg)	Blood glucose (mmol/L)
4 weeks			
Non-diabetic Ren-2	231.5±3.3	235.9±6.2	7.0±0.4
Diabetic Ren-2	216.8±3.6*	238.5±14.7	30.1±0.6*
Diabetic Ren-2 + candesartan	219.5±3.2*	142.9±3.7‡	31.2±0.5*
Non-diabetic SD	245.6±7.9	117.6±2.3‡,§	7.2±0.3
Diabetic SD	224.6±3.3*	118.9±3.1‡,§	30.0±0.7*
20 weeks			
Non-diabetic Ren-2	305.7±5.6	230.3±2.4	7.2±0.1
Diabetic Ren-2	262.3±4.4*	235.4±3.6	29.4±0.5*
Diabetic Ren-2 + candesartan	260.7±2.6*	123.1±3.6‡	29.8±0.4*
Non-diabetic SD	323.9±8.9	123.8±3.6‡	7.2±0.2
Diabetic SD	287.8±3.2*	125.5±1.5‡	28.7±0. 9*

Data are mean±sem with N=9-15 animals/group. *P<0.01 to age- and strain-matched non-diabetic controls. ‡P<0.001 to Ren-2 diabetic and non-diabetic controls. §P<0.005 to 4 week diabetic Ren-2 + candesartan. Data were analyzed by Kruskal-Wallis tests, followed by Mann Whitney U tests.

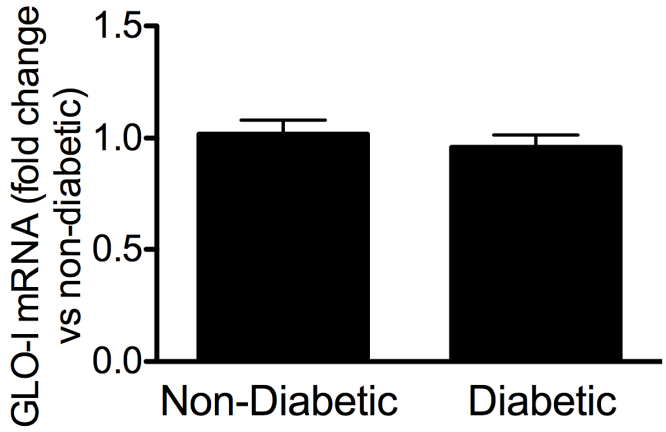
GLO-I mRNA and activity with candesartan alone. We investigated the possibility that candesartan in the absence of Ang II regulates GLO-I. Candesartan was found to increase GLO-I mRNA in BREC, but not to influence GLO-I activity (Supplemental Figures 1A and B). Candesartan did not influence GLO-I mRNA or activity in BRP (Supplemental Figures 1C and D).



Supplemental Figure 1: GLO-I mRNA and activity levels in BREC and BRP following treatment with candesartan (Cand, 1 μM) for 24 hours. In BREC, candesartan increased GLO-I mRNA compared to control (**A**), but had no effect on GLO-I activity (**B**). In BRP, candesartan had no effect on either GLO-I mRNA (**C**) or GLO-I activity (**D**) compared to control. *P=0.02 vs control. N=3-4 independent experiments. Values are mean±sem. Data were analyzed using unpaired t-tests.

GLO-I mRNA is unchanged after 4 weeks of diabetes

We investigated whether GLO-I mRNA levels were changed at in the retina after 4 weeks of diabetes. Although activity is decreased (Figure 7, manuscript results), mRNA is unchanged (Supplemental Figure 2).



Supplemental Figure 2: GLO-I mRNA is unchanged after 4 weeks of diabetes in Ren-2 rats compared to the non-diabetic Ren-2 controls.

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