SUPPLEMENTAL METHODS

Vascular function experiments

Septal coronary arteries from rats were prepared as previously described.[1] Briefly, vascular segments were mounted in a small vessel myograph (Danish Myo Technology), contracted with the thromboxane A_2 analog U46619, and tested for their ability to relax in response to progressive concentrations of acetylcholine. Other experiments were performed in coronary vessels before and after exposure to the arginase inhibitors, difluoromethyl ornithine (DFMO) or L-norvaline (50 μmol/L) for 1 and 2 hours, respectively. Control responses were obtained in vessels from the same animals. The vasorelaxant responses are expressed as percent decreases from U46619-induced contraction. The amount of contraction produced by 1 μmol/L U46619 in each ring from its initial resting tension was considered to be 100%.

Arginase activity

Arginase activity was assayed by measuring urea produced from L-arginine. Tissues were frozen in liquid nitrogen, pulverized, combined 1:4 (wt:vol) with ice-cold lysis buffer (50 mmol/L Tris-HCl, 0.1 mmol/L EDTA and EGTA, pH 7.5) containing protease inhibitors and homogenized on ice. The homogenate was centrifuged at 14,000 g for 20 minutes and the supernatant was removed for enzyme assay. BCEC were rinsed with ice-cold PBS, collected in lysis buffer and lysed by three freeze-thaw cycles. Arginase activity was assayed as previously described. [2]

Arginase protein expression

Rat tissues or BCEC were homogenized in lysis buffer containing protease inhibitors and centrifuged for 20 minutes at 14,000 *g*. Protein samples were electrophoresed, transferred to nitrocellulose membrane and reacted with anti-arginase I (BD Transduction Laboratories, 1:1000) or anti-arginase II antibodies (Santa Cruz Biotechnology, INC., 1:250), followed by sheep anti-mouse (Amersham, 1:4000) or donkey anti-rabbit (GE Healthcare, 1:4000) horseradish peroxidase-labeled secondary antibody, respectively, and enhanced chemiluminescence. Membranes were stripped and re-probed for α-actin to demonstrate equal loading and results were analyzed using densitometry.

Transfection of BCEC with arginase I siRNA

BCEC were transfected with arginase I siRNA (Smartpool, Dharmacon) or scrambled siRNA (non-targeting siRNA) using Lipofectamine2000 reagent (Invitrogen), according to the manufacturer's instructions. In brief, cells were transfected with 100 nmol/L of arginase I siRNA or scrambled siRNA for 5 hours. To evaluate the effects of high glucose (HG) on arginase activity and NO production, transfected cells were incubated in serum free media medium containing 25 mmol/L of D-glucose for 24 hrs. This glucose concentration corresponds to a blood glucose concentration of 500 mg/dL, which is commonly seen in diabetic rats or patients with uncontrolled diabetes.

Nitric oxide (NO) measurement

To measure NO, nitrite (NO^-_2) the stable breakdown product of NO in aqueous medium was analyzed using NO-specific chemiluminescence. In brief, samples containing NO_2^- were refluxed in glacial acetic acid containing sodium iodide. NO_2^- is quantitatively reduced to NO under these conditions, which can be quantified by a chemiluminescence detector after reaction with ozone in a NO analyzer (Sievers). The amount of NO generated is calculated as the difference in basal and NOS agonist - stimulated NO levels.

BCEC transfected with arginase I siRNA or scrambled siRNA were exposed to 5 mmol/L or 25 mmol/L D-glucose in serum free medium for 24 hours. Medium was then replaced with fresh serum free medium for 30 minutes and medium aliquots were collected for basal reading. Cells were then exposed to the calcium ionophore ionomycin (1 μmol/L) for 30 minutes and medium samples were collected.

Superoxide production

Superoxide production in rat coronary arteries was assayed by using the oxidative fluorescent dye dihydroethidium (DHE). DHE is oxidized on reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. Serial cryosections from fresh-frozen ventricular septum were first incubated in NAD(P)H (100 μmol/L) or NAD(P)H with PEG-SOD (400 U), or apocynin (30 μmol/L) or L-NAME (3 mmol/L) for 20 minutes followed by DHE with or without PEG-SOD (Sigma), apocynin or L-NAME (20 minutes, 37°C). Apocynin specifically blocks activity of NADPH oxidase by interfering with the assembly of the cytosolic NADPH oxidase components (p40phox, p47phox, p67phox) with the membranous components gp91phox and

p22phox. L-NAME is a non-specific inhibitor of NOS. DHE images from serial sections treated with or without inhibitors were obtained using a fluorescence microscope.

In BCEC, superoxide production was determined by luminescence generated upon the addition of L-012 dye in a BMG Polar Star plate reader. Cells were seeded in 96 well luminescence plates and at ~80% confluence, cells were exposed to normal or high glucose for 24 hrs in the presence or absence of L-NAME, apocynin, the arginase inhibitor S–2-boronoethyl- L-cyteine (BEC), or L-citrulline.

Nitrotyrosine formation and lipid peroxidation (supplement)

Nitrotyrosine immunoreactivity, an indicator for $ONOO^-$ formation, was measured by slot blot analysis as described previously.[3] Lipid peroxide concentration was determined by measuring the amount of malondialdehyde (MDA) formed from thiobarbituric acid (TBA) during acid hydrolysis of lipid peroxides as described.[3]

Measurement of active RhoA

RhoA activation was determined using an affinity precipitation assay incorporating the Rho-binding domain (RBD) of rhotekin, which binds only the active GTP-Rho (Upstate). In brief, confluent BCEC were incubated in 25 mM D-glucose medium with or without simvastatin (10^{-7} M). Control cells were maintained in 5 mM D-glucose medium. After 24 hours, cells were scraped in lysis buffer (25 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 1% Igepal CA-630, 10 mmol/ l $MgCl_2$, 1 mmol/l EDTA, 10% glycerol, 10 µg/ml aprotinin, 10 µg/ml leupeptin) at 4 °C. Whole cell lysates were incubated with agarose conjugated rhotekin-RBD for 45 min at 4 °C and washed three times with lysis buffer. Agarose beads were boiled in Laemmli reducing sample buffer to release active Rho. Samples were resolved on a 12.5% polyacrylamide gel followed by immunoblotting with RhoA antibody.

Statistics

Data are given as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) with the Tukey post test. In some experiments, statistical differences were determined by the *Student T* test. Results were considered significant when $p < 0.05$.

SUPPLEMENTAL FIGURES

Supplemental Figure I. Western blot analysis of arginase II protein expression in aorta of Control, Diabetic Diab/Simv and, Diab/L-Cit rats at eight weeks.

Supplemental Figure II. Effect of the arginase inhibitor L-norvaline on ACh concentration-response curves of diabetic coronary arteries. In vitro treatment of diabetic coronary arteries (n =6 - 7 / group) for 2 hr with L-norvaline caused a significant increase in Emax to ACh compared with diabetic-untreated vessels. Values are expressed as means ± S.E.M.; *, $p < 0.05$ versus Diabetic-untreated.

Supplemental Figure III. Cardiac levels of lipid peroxides, as malondialdehyde (MDA) formation (A), and nitrotyrosine formation (B) in Control, Diabetic, simvastatin-treated diabetic (Diab/Simv) and, L-citrulline-supplemented diabetic (Diab/L-Cit) rats at 8 weeks (n = 8 / group). Values are expressed as means ± S.E.M.; *, $p < 0.05$ versus control, Diab/Simv and, Diab/L-Cit.

Supplemental Figure IV. Effect of high glucose (HG) on arginase I protein levels in BCEC. BCED were exposed to HG (25 mmol/L) or normal levels of glucose (control, 5 mmol/L) for 24 hours and arginase I levels were determined by Western blotting.

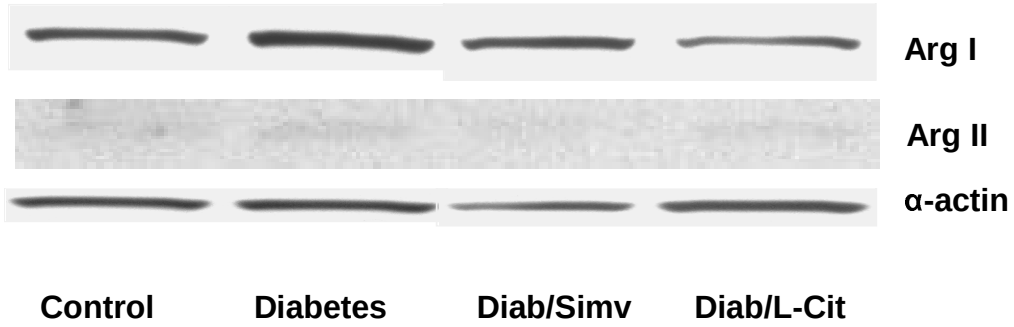
SUPPLEMENTAL REFERENCES

1. Tawfik HE, El-Remessy AB, Matragoon S, Ma G, Caldwell RB, Caldwell RW. Simvastatin improves diabetes-induced coronary endothelial dysfunction. *J Pharmacol Exp Ther*. 2006;319:386-95.
2. Corraliza IM, Campo ML, Soler G, Modolell M. Determination of arginase activity in macrophages: a micromethod. *J Immunol Methods*. 1994;174:231-5.
3. El-Remessy AB, Behzadian MA, Abou-Mohamed G, Franklin T, Caldwell RW, Caldwell RB. Experimental diabetes causes breakdown of the blood-retina barrier by a mechanism involving tyrosine nitration and increases in expression of vascular endothelial growth factor and urokinase plasminogen activator receptor. *Am J Pathol*. 2003;162:1995-2004.

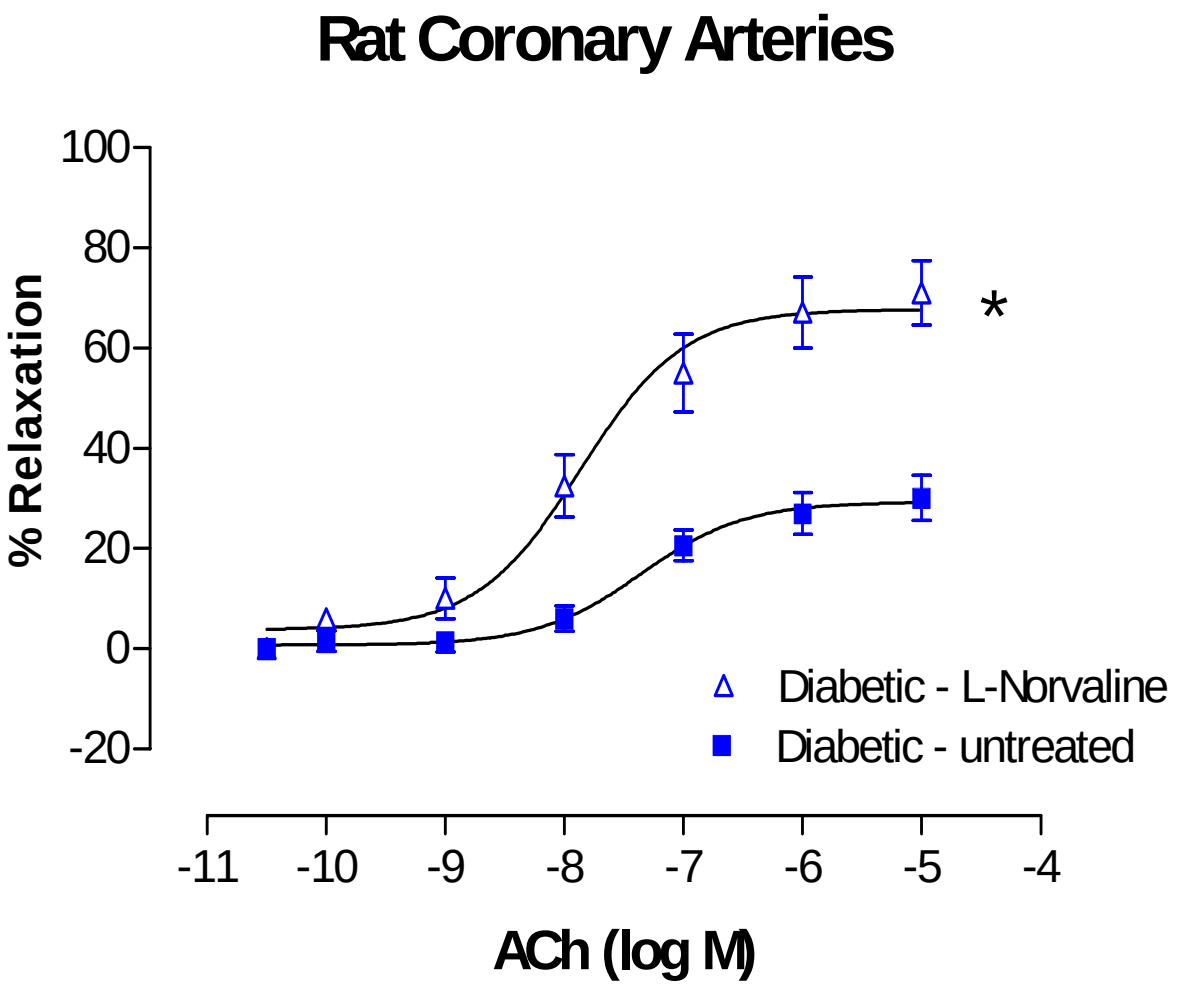
Supplemental Table I. Maximum vasorelaxant response after L-NAME treatment

Group	Control	Diabetic	Diab/Simv	Diab/L-Cit
E_{max} [#]	22 +/- 3.2	20 +/- 2.5	24 +/- 1.8	25 +/- 2.8

[#] Maximum vasorelaxant response to acetylcholine (10 μmol/L) after treatment of vessels with L-NAME (3 mmol/L). n = 5 –6.



Supplemental Fig. I



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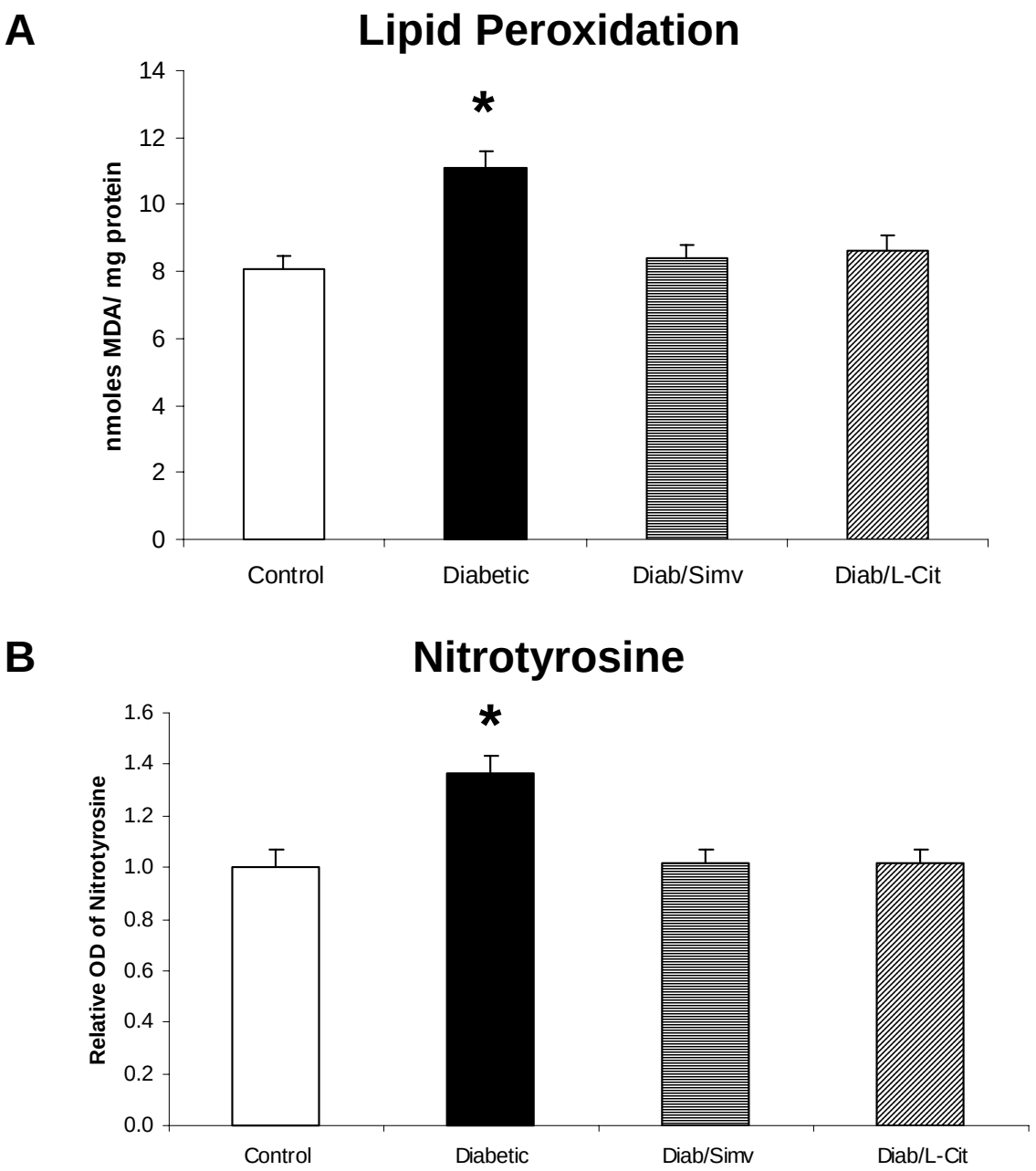
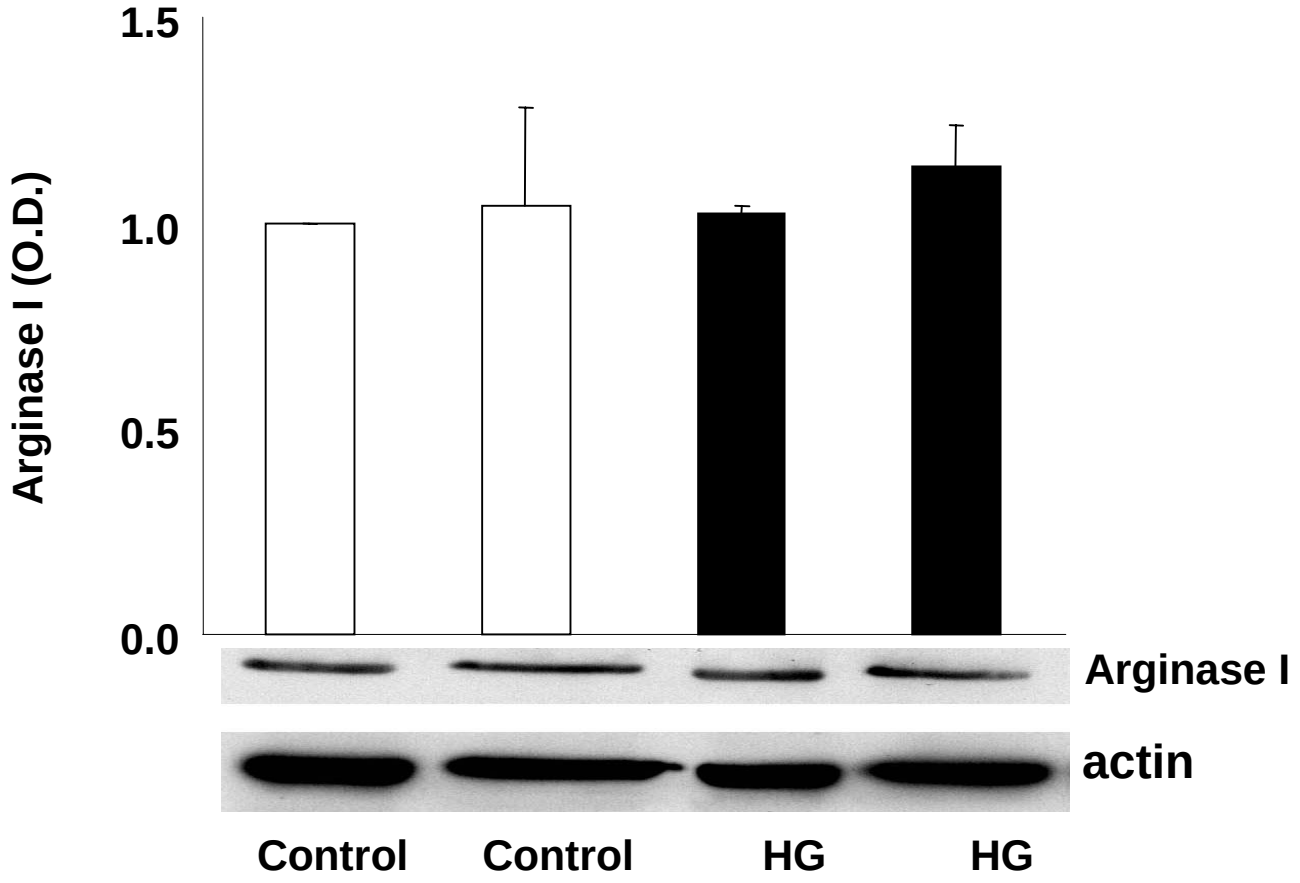


Supplemental Fig. III



Supplemental Fig. IV