Supplemental Material

Supplemental methods

Anesthesia

Mice were anesthetized with Pentobarbital (50mg/kg, i.p.).

Endothelial cell culture

Lung tissue, excluding central bronchi and vessels, was removed and minced with scalpels. The minced tissues were incubated for 1 hour at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 0.2% (w/v) collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) and 0.1% BSA. The tissue digest was passed through a 40μm strainer and centrifuged at 200 × *g*. The cells were seeded on dishes coated with 0.1% (w/v) collagen I (BioCoat, BD BioSciences, Franklin Lakes, NJ) and were grown in DMEM with 10% (v/v) horse serum, 50μg/ml endothelial cell growth substance (Biomedical Technologies, Stoughton, MA), and 100μg/ml heparin. After trypsin treatment during the first and second passage, cells were sorted with Dynabeads conjugated with sheep anti-rat IgG (Dynal/Invitrogen, Grand Island, NY) and complexed to ICAM-2 rat anti-mouse monoclonal antibody (BD Pharmingen, San Jose, CA) in DMEM containing 0.1% BSA for 30 minutes at 4°C. Cells were used at passages 3-6 after serum starvation for 16 hours in DMEM containing 0.1% BSA. The aorta endothelial cells were cultured as described above.

Glucose and insulin tolerance tests

Mice were fasted for 6 hours and then glucose (2mg/g body weight) or insulin (0.75mIU/g body weight) was administered to mice via intraperitoneal injection as described previously[1]. Blood was sampled from tail vein for glucose determination at 0, 30, 60,120 minutes after glucose or insulin injection.

Quantification of atherosclerotic lesion size in aorta

The mice were fed western diet (Harlan Laboratories, Madison, WI) which contained 0.15% cholesterol for 12 weeks. Then the aorta was harvested and stained for 5 minutes in 0.5% Sudan IV, and destained for 5 minutes in 70% ethanol. The stained aortas were placed on a glass slide and photographed with a QColor3 Digital Camera mounted on an Olympus stereo microscope. Lesion areas were measured by Photoshop and ImageJ as reported previously[1].

Immunohistochemistry

The abdominal aorta, from superior mesenteric artery to just below renal artery, was dissected and embedded in OCT medium and frozen on dry ice. The aorta containing plaque was sectioned with a 7μm thickness and 6-8 sections, each 70μm apart, were mounted on the slide. The slides were fixed in acetone in -20ºC for 10 minutes and then incubated with antibodies as indicated below.

Trichrome staining

Collagen and necrosis area in the plaque of abdominal aorta was stained using Trichrome Stain kit (Sigma-Aldrich Inc., St. Louis, MO).

Vascular reactivity studies

All reagents were obtained from Sigma-Aldrich and dissolved in distilled and double filtered water. Vessel reactivity studies were performed in a pressure myograph system (Danish Myo Technologies, Denmark) as described previously[2]. The left carotid arteries were isolated and mounted onto glass cannulas and maintained at 85mmHg pressure at 37°C in physiologic saline-HEPES buffer oxygenated with 95% O2 and 5% CO2. Vessels were constricted with 10^{-5} M phenylephrine followed by stepwise addition of ACh to generate vasodilator response curves. After basal NO production was blocked by

applying L-NAME (3×10^{-4} M, 30 minutes), vasodilator response curves were generated by stepwise addition of SNP.

Western blotting

Mouse lung endothelial cells were incubated with DMEM containing 0.1% BSA for 16 hours and then were stimulated with 100nM insulin for 5 minutes for detecting eNOS and Akt phosphorylation. The cells were incubated with 100nM insulin for 48 hours or 100nM PMA for 6 hours for detecting VCAM-1 protein. To observe the actions of insulin on eNOS phosphorylation in vivo, insulin (10mIU/g body weight) was administered to mice via intravenous injection and femoral arteries were harvested at 5 minutes after insulin injection. Protein extraction and immunoblot procedures were performed as reported previously. Protein samples were separated by electrophoresis in a 7% Tris-HCl polyacrylamide gel and transferred to a PVDF membrane, which was blocked with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20 and incubated with primary antibody in 4°C overnight. Detection was carried out using an ECL Plus Western Blotting Detection kit **(**Thermo Scientific, Rockford, IL**).** Quantitative densitometry was performed using ImageJ. Antibody directed against human S1177 phospho-eNOS and eNOS was obtained from BD Transduction Laboratories.

Real-time PCR analysis

1μg mRNA was used to generate cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). Gene expression level was normalized to the expression level of 36B4. PCR primers were: mouse PKCβ2 isoform, forward 5'-AGGGATTCCAGTGTCAAGTCTGCT-3', reverse 5'-GGACTGGAGTACGTGTGGAT CTT-3', mouse ET-1, forward 5'-GTGTCTACTTCTGCCACCTG-3', reverse 5'-CACTGACATCTAA CTGCCTGG-3', mouse VCAM-1, forward 5'- TCTGAACCCAAACAGAGGCAGAGT-3', reverse 5'-AGCTGGTATCCCATCACTTGAGCA-3', mouse 36B4, forward 5'- GCTCCAAGCAGATGCAGCA-3', reverse 5'- CCGGATGTGAGGCAGCAG-3'.

Leukocyte-endothelial cell adhesion

Mouse lung endothelial cells (10^5cell/well) were cultured for 48 hours and incubated with vehicle, PMA (100nM) and insulin (100nM) for 16 hours in DMEM (37°C, 5% CO2). After treatment, the medium was removed and endothelial cells were washed with PBS. Raw264.7 cells labeled with Leuko Tracker (Cell Biolabs Inc. San Diego, CA) were added using 10^6/cells per well and allowed to adhere for 30 minutes. Non-adherent cells were removed by washing with PBS. After lysing cells, fluorescence was measured by a fluorescence plate reader at 480 nm/520 nm.

Angiotensin II infusion

In mice 8 to 10 weeks of age, a catheter was placed in the right jugular vein. The animals were allowed to recover from surgery for 3 days. Angiotensin II was infused at 1000ng/kg body weight/min for 3 hours. PBS was infused in vehicle group. The aorta was isolated for measurement of ET-1 and Big ET-1 expression.

Measurement of aorta Big ET-1

Aortas were homogenized with RIPA buffer and Big ET-1 in the lysis was measured with an ELISA kit (Biotang Inc., Waltham, MA).

Measurement of ET-1

Mouse lung endothelial cells were cultured in DMEM containing 0.1% BSA for 24 hours and then the medium was collected for the measurement of ET-1 with an ELISA kit (Enzo Life Sciences, Farmingdale, NY). The value of ET-1 was normalized by the total protein extracted from the cells growing in the same well.

Cell death ELISA

Mouse lung endothelial cells were with or without withdrawing growth factors for 24 hours and DNA fragmentation was determined by using Cell Death ELISA kits (Roche, Indianapolis, IN).

References

(1) Rask-Madsen C, Li Q, Freund B, Feather D, Abramov R, Wu IH, Chen K, Yamamoto-Hiraoka J, Goldenbogen J, Sotiropoulos KB, Clermont A, Geraldes P, Dall'Osso C, Wagers AJ, Huang PL, Rekhter M, Scalia R, Kahn CR, King GL. Loss of insulin signaling in vascular endothelial cells accelerates atherosclerosis in apolipoprotein E null mice. *Cell Metab* 2010;11:379-89.

(2) Atochin DN, Wang A, Liu VW, Critchlow JD, Dantas AP, Looft-Wilson R, Murata T, Salomone S, Shin HK, Ayata C, Moskowitz MA, Michel T, Sessa WC, Huang PL. The phosphorylation state of eNOS modulates vascular reactivity and outcome of cerebral ischemia in vivo. *J Clin Invest* 2007;117:1961-7.

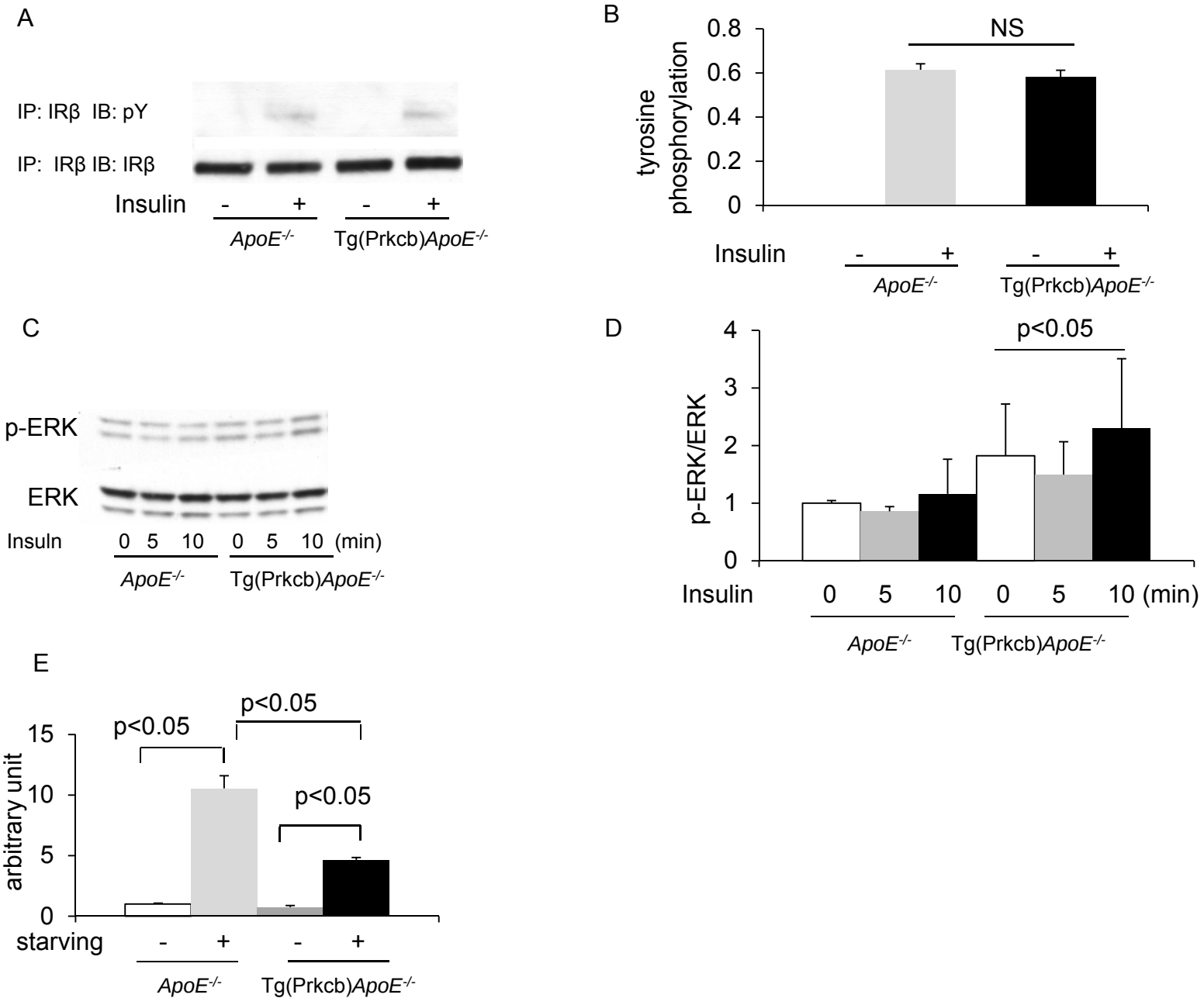
Supplemental legends

Supplemental Figure I. Insulin signaling and endothelial apoptosis.
(A-B), Tyrosine phosphorylation of insulin receptor β was stimulated by 100 nM insulin in MLECs from *ApoE*$^{-/-}$ or Tg(Prkcb)*ApoE*$^{-/-}$ mice. A. Representative western blots show tyrosine phosphorylation of insulin receptor β. B, mean values of tyrosine phosphorylation of insulin receptor β (n=3 for each group). (C-D), ERK phosphorylation was stimulated by 100nM insulin in MLECs from *ApoE*$^{-/-}$ or Tg(Prkcb)*ApoE*$^{-/-}$ mice. C. Representative western blots show ERK phosphorylation. D, mean values of phosphorylation of ERK (n=8 for each group). E, Apoptosis induced by withdrawing growth factors were determined by Cell Death ELISA (n=3 for each group).

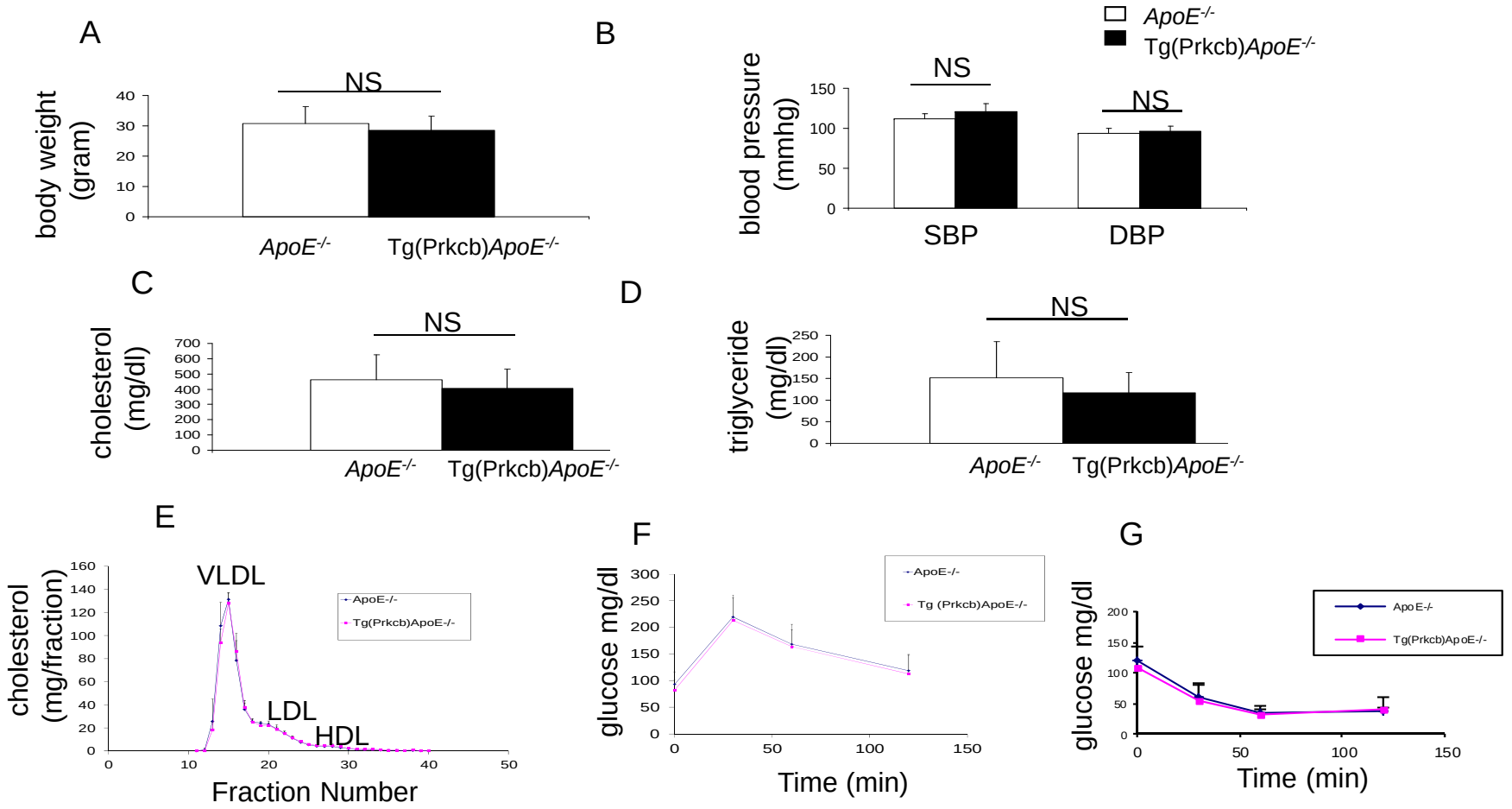
Supplemental figure II, Characteristics of Tg(Prkcb)*ApoE*$^{-/-}$ mice.
A. The body weight of *ApoE*$^{-/-}$ mice and Tg(Prkcb)*ApoE*$^{-/-}$ mice after 12 weeks high fat feeding (*ApoE*$^{-/-}$ n=17, Tg(Prkcb)*ApoE*$^{-/-}$ n=16). B, Systolic blood pressure (SBP) and diastolic blood pressure (DBP) of *ApoE*$^{-/-}$ mice and Tg(Prkcb)*ApoE*$^{-/-}$ mice after high fat feeding (*ApoE*$^{-/-}$ n=6, Tg(Prkcb)*ApoE*$^{-/-}$ n=7). (C-E). Plasma lipids were measured in *ApoE*$^{-/-}$ mice and Tg(Prkcb)*ApoE*$^{-/-}$ mice after high fat feeding. C Total cholesterol in plasma (*ApoE*$^{-/-}$ n=17, Tg(Prkcb)*ApoE*$^{-/-}$ n=13). D, Total triglyceride in plasma (*ApoE*$^{-/-}$ n=17, Tg(Prkcb)*ApoE*$^{-/-}$ n=13). E, Cholesterol concentration in FPLC fractions of plasma (*ApoE*$^{-/-}$ n=4, Tg(Prkcb)*ApoE*$^{-/-}$ n=4). (F-G) Glucose and insulin tolerance test in *ApoE*$^{-/-}$ mice and Tg(Prkcb)*ApoE*$^{-/-}$ mice after high fat feeding. F glucose tolerance test (*ApoE*$^{-/-}$ n=8, Tg(Prkcb)*ApoE*$^{-/-}$ n=7). G, insulin tolerance test (*ApoE*$^{-/-}$ n=9, Tg(Prkcb)*ApoE*$^{-/-}$ n=8).

Supplemental Figure III. Insulin-induced eNOS phosphorylation and VCAM-1 expression in aorta endothelial cells.
(A-B) aorta endothelial cells were stimulated with 100nM insulin and eNOS phosphorylation at Ser1176 was determined by western blotting *(ApoE*$^{-/-}$ unstimulated n=8, *ApoE*$^{-/-}$ insulin-stimulated n=11, Tg(Prkcb)*ApoE*$^{-/-}$ unstimulated n=5, Tg(Prkcb)*ApoE*$^{-/-}$ insulin stimulated n=9). (C-D), VCAM-1 expression in aorta endothelial cells. VCAM-1 expression in aorta endothelial cells was determined by western blotting. C, Representative western blots show VCAM-1 and actin expression. D, mean values of the ratio of VCAM-1 to actin (n=3 for each group).

Supplemental Figure I



Supplemental Figure II



Supplemental Figure III