SUPPLEMENTARY METHODS

Cell culture. Bovine aortic endothelial cells (BAEC) (Cambrex) were grown to confluence in Dulbecco's MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged twice weekly by harvesting with 0.5% trypsin:EDTA. Experiments were conducted on cells from passages 4-10. Human coronary artery endothelial cells (Cambrex) were grown to confluence in EGM-MV media supplemented with the EGM-MV BulletKit (Cambrex). Experiments were performed on cells from passages 2-5. Cells were treated with aldosterone (10^{-9}–10^{-7} mol/l) (Steraloids) for 12, 24, 36, or 48 h. Aldosterone was dissolved in dimethylsulfoxide (10 nmol/l), which served as the vehicle control. In selected studies, cells were co-incubated with spironolactone (10 μmol/l) (Sigma-Aldrich) dissolved in 0.01% ethanol.

To increase G6pd expression, a recombinant adenovirus encoding rat *G6pd* cDNA under control of the CMV promoter, *AdG6pd*, or an empty viral vector, *Ad*, was utilized as previously described[7,17,40]. Briefly, an adenoviral G6pd expression vector encoding rat G6pd cDNA, *pAdG6PD*, was constructed and the sequence of *pAdG6pd* was confirmed by DNA restriction digestion and Western blotting of the expressed G6pd protein following transfection of HEK-293 cells. To create the recombinant virus, *pAdG6pd* was linearized with *Pac*I and transfected into HEK-293 cells using Lipofectamine (Invitrogen). The virus, *AdG6pd*, was harvested from the cells by 4 cycles of freezing and thawing in PBS containing 10% (vol/vol) glycerol. Viral amplification was performed by several rounds of infection of HEK-293 cells and the titer of the *AdG6pd* produced was determined by viral plaque assay.

To decrease Creb-1 expression BAEC were transfected with siRNA (5'-CAAGTCCAGACAGTTCAGATTTCAA-3'), or scrambled control (5'-CAAACCCAGTGAACTTAGTTGTCAA-3'), using Lipofectamine™ 2000 (Invitrogen) for 5 h in OptiMEM® I media. After this time, cells were placed in full growth media and experiments were performed after 48 h. To decrease Crem expression BAEC were transfected with siRNA (5'-ACAAACTGTTCAGGTAGCAACCATT-3') or a scrambled control (5'-ACACTGTTCAGGTAGCAACCAAATT-3') under similar conditions.

To isolate endothelial cells from aortas of G6PD-deficient hemizygous male mice, aortas were harvested from C3H wild-type mice and G6PD-deficient male hemizygous mice age 4-6 weeks. Aortas were rinsed free of red blood cells with ice-cold phosphate buffered saline, mechanically minced, and digested with collagenase (Worthington). Digests underwent a double selection process by incubation with monoclonal antibodies to CD31 (Santa Cruz Biotechnology) and VE-cadherin (Research Diagnostics) adhered to magnetic microbeads (Dynabeads, Dynal Biotech). Cells were grown to confluence and found to be > 98% pure by uptake of DiI-Ac-LDL (Molecular Probes)[41].

Immunoblotting and densitometry. Cells were harvested and centrifuged at 1000 x *g* at 4 °C for 10 min, after which the supernatant was discarded and the samples were frozen at –80 °C overnight. The pellet was homogenized and 25 μg cell protein per sample was added per lane. Proteins were size-fractionated electrophoretically using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes blocked with 5% milk solution. The membranes were incubated with 1:1000 dilution of antibodies to G6pd (Bethyl Laboratories), actin (Santa Cruz), eNos (Transduction Laboratories), mineralocorticoid receptor (Santa Cruz), PkA (Santa Cruz), CREB, phospho-CREB (Cell Signaling Technology), CREM (Santa Cruz), Sgk and phospho-SGK (Santa Cruz), Msk-1 (Sigma), Camk II (Cell Signaling Technology), phospho Camk II (Cell Signaling Technology), p90Rsk (Cell Signaling Technology), and p44/42 Mapk (Cell Signaling Technology) and visualized utilizing the ECL detection system (Amersham). Densitometry was performed on a minimum of 3 representative blots.

RNA isolation, cDNA generation, and northern hybridization. Total RNA was prepared from cells cultured in 100-mm tissue culture dishes by using the RNeasy mini kit (Qiagen) with the addition of DNase I step to remove residual DNA. For the RT-PCR reactions, 1 μg of RNA was used to generate cDNA using the SuperScript™ III One-Step RT-PCR System (Invitrogen). The cDNA was aliquoted and stored frozen at –80 °C. PCR conditions were as follows: an initial denaturation step at 94 °C for 2 min, followed by 40 PCR cycles of 94 °C for 15 s, 55°C for 30 s, and 68 °C for 1 min, with a final extension at 68 °C for 5 min. DIG-High Prime DNA Labeling and Detection Starter Kit

II (Roche) was used to label bovine G6PD, CREM, or GAPDH cDNA fragments with digoxigenin for nonradioactive detection of mRNA according to manufacturer's instructions**.** For Northern blot analysis, 5 µg total RNA from each sample was fractionated on 1.2% formaldehyde-agarose gels and transferred onto positively charged nylon membranes (Roche). Hybridization was performed for 24 hours at 65°C in Quickhyb solution, followed by 2 washes for 5 min each in 2X SSC, 0.1% SDS at room temperature, 2 washes for 15 min each in 0.1X SSC, 0.1% SDS at 55°C. Detection of mRNA was performed according to manufacturer's instructions. Membranes were stripped and rehybridized with the *Gapdh* probe. The quantitative analysis of hybridization signals was performed using a Versadoc imager. Analysis was performed with the QuantityOne software from Biorad.

Quantitative real-time PCR. For the RT-PCR reactions, 1 µg of RNA was used to generate cDNA with oligo(dT) primers using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed using 1 ng of first strand cDNA, TaqMan® Universal Master Mix, and 20X TaqMan® G6PD or GAPDH Gene Expression Assay. Samples were run on Applied Biosystems 7700. PCR conditions were as follows: an initial activation step at 50 °C for 2 min, a second activation step at 95 °C for 10 min, followed by 40 PCR cycles of 95 °C for 15 s and 60°C for 1 min. Quantitation of data was performed using the comparative C_T ($\Delta\Delta C_T$) method using GAPDH gene expression as an endogenous reference.

cAMP and cGMP levels. Confluent BAEC were pretreated with 0.5 mM isobutylmethylxanthine (IBMX) at 37 °C for 15 min. They were then stimulated with agonists in the presence of IBMX. The reaction was stopped by the addition of ice-cold 6% trichloroacetic acid. After three freeze-thaw cycles, the cells were scraped from the plate. The supernatant fractions of the cell lysates were extracted with water-saturated ether, dried with nitrogen gas at room temperature, and acetylated. Cyclic AMP or GMP levels were quantitated using an enzyme immunoassay kit (Cayman) and results standardized by protein levels. For studies performed in isolated aortas, aortas were

harvested, rinsed with PBS, and dissected free of adventitia. Cyclic GMP levels were determined in aortic homogenates and standardized to total protein.

Nuclear run-on assay. Nuclear preparations were isolated from cells using the BD™ TransFactor Nuclear Extraction Kit (Clontech) according to manufacturer's instructions. To perform the nuclear run-on transcription, 5×10^6 nuclei were incubated in a reaction buffer (5 mmol/l Tris·HCl, pH 8.0/2.5 mmol/l $MgCl_2$/150 mmol/l KCl/2.5 mmol/l each of ATP, GTP, CTP) and digoxigenin-11-UTP at 30°C for 45 min in a final volume of 60 µl. The reaction was stopped by the addition of 1,000 units of RNase-free DNase and incubated for 10 min at 37°C. The nuclei were then lysed by the addition of lysis buffer containing 10 mmol/liter Tris·HCl, 1% SDS, and 5 mmol/l EDTA. The reaction mixtures were treated with 20 µl of proteinase K (10 mg/ml). RNA was extracted with TRIzol reagent, ethanol-precipitated, and resuspended in 50 µl of RNase-free H_2O. Equal amounts (1 µg) of full-length bovine *G6pd*, *Gapdh*, and *pGEM-3z* cDNA were vacuum-transferred onto nitrocellulose membranes with a slot blot apparatus (BioRad). Hybridization of digoxigenin labeled transcripts was carried out at 45°C for 48 h. The membranes were then washed and developed using a Digoxigenin Luminescent Detection Kit (Roche).

Electrophoretic mobility shift assay. Nuclear preparations were isolated from cells using the BD™ TransFactor Nuclear Extraction Kit (Clontech) according to manufacturer's instructions. The oligonucleotide probe was biotin-labeled with Biotin 3' End DNA Labeling kit (Pierce) according to manufacturer's instructions and annealed at room temperature for 60 min to create a double stranded probe. The probe contained the CREB/CREM binding site (–889 - –881) from the G6PD promoter and was included using the oligonucleotides: forward 5'-CAGAAACAGTATGACGATAGGCAGAT-3' and reverse 5'-ATCTGCCTATCGTCATACTGTTTCTG-3'. Electrophoretic mobility shift assays were performed using the LightShift® Chemiluminescent EMSA kit (Pierce) according to manufacturer's instructions. Briefly, nuclear extract (5 µg) was incubated with 10X binding buffer, 1 µg/µl poly (dI•dC), and the biotin-labeled double stranded oligonucleotide probe for 20 min at room temperature. Antibodies to CREB (Santa Cruz),

CREM (Santa Cruz), p65 (Santa Cruz), or ATF-2 (Santa Cruz) were preincubated with the nuclear extract for the supershift reaction and preincubated overnight at 4°C prior to adding labeled probe. Similarly, cold competitor oligonucleotides (100-fold molar excess) were preincubated with the nuclear extract for 30 min prior to the addition of the labeled probe for the competition reactions. Complexes were separated on a 5% nondenaturing native gel, transferred to a nylon membrane, dried for 1 h, and exposed for autoradiography.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation assay was performed using the QuikChIP assay (Imgenex) according to manufacturer's instructions. Briefly native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 10 min. Equal aliquots of isolated chromatin were sheared and subjected to immunoprecipitation with various antibodies or human IgG (Sigma) as a negative control. The DNA associated with specific immunoprecipitates was utilized as a template for PCR to amplify the *G6PD* promoter sequence containing the CREB binding site. PCR conditions were: denature 94°C for 30 s, anneal 55°C for 30 s, extend 72°C for 60 s for 30 cycles. The primers used were: forward 5'-TGACCTCCGTGCTATTCCTC-3' and reverse 5'-ACCAAACTTGACTGCGCTCT-3'. As a specificity control, the *β-actin* promoter was similarly amplified using the primers: forward 5'-GTAGAGCCCACCTTCCTTCC-3' and reverse 5'-AGAGCGAGAGCGAGATTGAG-3'.

eNOS activity. eNOS activity was measured in intact cells without the addition of exogenous cofactors as previously described[44]. Briefly, cells were loaded with 0.75 μCi/ml [^{3}H]-L-arginine and incubated for 15 min. The reaction was terminated by the addition of 500 μl ice-cold 1N trichloroacetic acid to each plate. The cells were twice freeze-fractured in liquid nitrogen, ether-extracted, and neutralized with 1.5 ml of 25 mM HEPES, pH 8. Samples were applied to Dowex AG50WX-8 (Tris form) columns and eluted with 1 ml 40 mM HEPES buffer, pH 5.5 with 2mM EDTA and 2 mM EGTA. [^{3}H]-L-citrulline was quantified by liquid scintillation counting. In selected studies, exogenous NAPDH (20 mmol/L) was added to the assay.

Blood pressure. Baseline blood pressure (BP) and heart rate were determined using tail-cuff measurements. Mice were trained and acclimated to the BP apparatus (Visitech BP-2000, Visitech Systems), which consists of a heated platform, a holder that is open in the front, and a cuff that is placed around the mouse tail. Mice were trained for 2-3 d prior to the experimental protocols. Training involved two sessions of placing the mouse in the holder and inflating the tail cuff. Each session lasted 5 min after which the mouse was placed back in its cage for 15 min and then returned to the BP platform for an additional session. BP was then measured on a daily basis.

Aorta ROS generation. Aortas were harvested at the completion of experimental protocols, irrigated the lumen with ice-cold phosphate-buffered saline until free of blood, dissected free the adventitia, and sectioned into rings. The rings were incubated with lucigenin (5 μmol/L) at 37°C in an oxygen-bubbled balanced salt solution, and lucigenin chemiluminescence was measured using a luminometer (TI-300, Turner Industries). Rings were then dried and results standardized to weight[47].

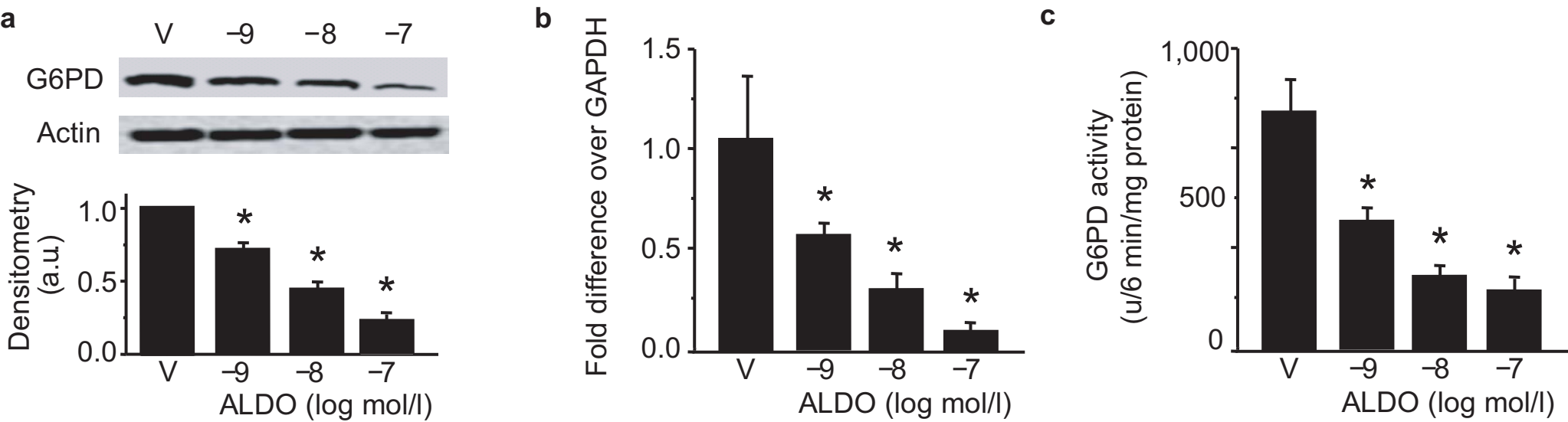
As a second measure of ROS, unfixed aortas were immediately frozen in OCT and cut into 10-μm-thick sections and placed on a glass slide. The sections were incubated with 20 μM dihydroethidine (Molecular Probes) in a dark incubator at 37°C for 30 min. Ethidium fluorescence (red signal) was detected with a 563-nm long-pass filter after excitation at 543 nm, and images were obtained with a Zeiss LSM 510 microscope equipped with a krypton/argon laser[48].

Immunofluorescence. For immunofluorescence of tissue samples embedded in paraffin, slides were first deparaffinized (xylene 2x, toluene 1x, 100% ethanol 2x, 95% ethanol, 70% ethanol, deionized H_2O, and phosphate-buffered saline (PBS) [7.4]) and incubated with 3% H_2O_2 for 5 minutes. After washing with PBS, the slides were incubated in 10% BSA for 1 h at 25°C, washed in PBS, and incubated with an antibody to eNos (Santa Cruz Biotechnology, Inc.) at 1:50 overnight at 4°C. After the slides were washed in PBS, the fluorescein secondary antibody (Vector Laboratories) was applied and incubated for 1 h at 25°C. Nuclei were counterstained with DAPI. The slides were mounted and sealed

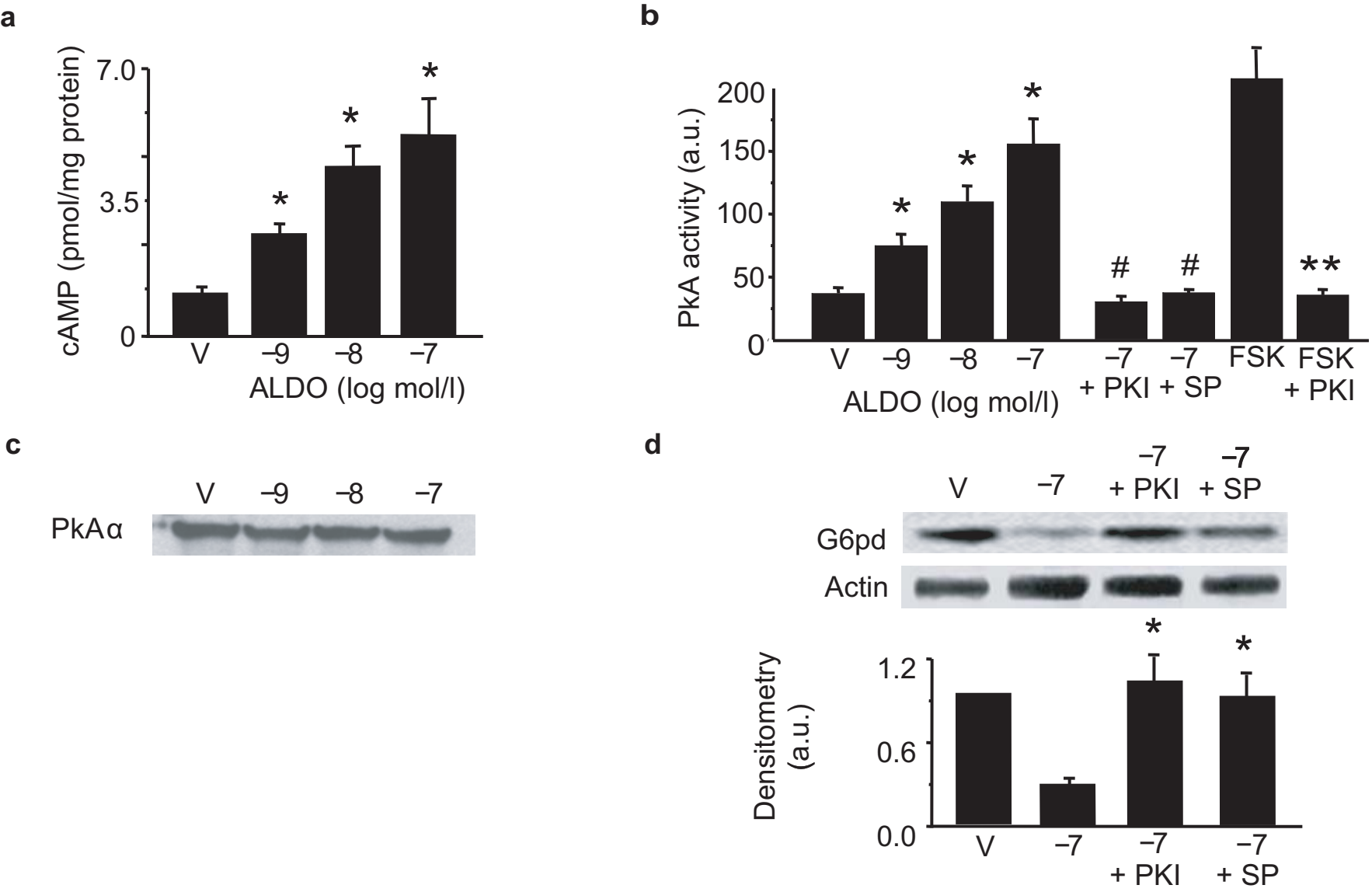
for microscopic visualization. Digital images were captured using a Nikon Eclipse TE300 inverted fluorescent microscope.

Vascular reactivity. Vascular reactivity of mesenteric arterioles was assessed by intravital videomicroscopy as previously described[47]. After mice were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg), a segment of omentum was exteriorized through a small, midline abdominal incision. The mesenteric circulation was visualized with a Nikon Optip Hot-2 microscope and the image projected onto an HR-1000 monitor (Dage-MTI) using an Attofluor high-intensity camera (Atto Instruments Inc.). The final image was magnified 64,000X. A single mesenteric arteriole was identified and used for measurements throughout each experiment. The inner luminal diameter of the mesenteric arteriole was measured using a videocaliper apparatus. Arterioles were preconstricted with phenylephrine (10 μmol/L). Before each agonist dose, the omentum was washed with 0.9% saline and restoration of baseline vessel diameter confirmed. The response was expressed as percent change in vessel diameter compared with baseline.

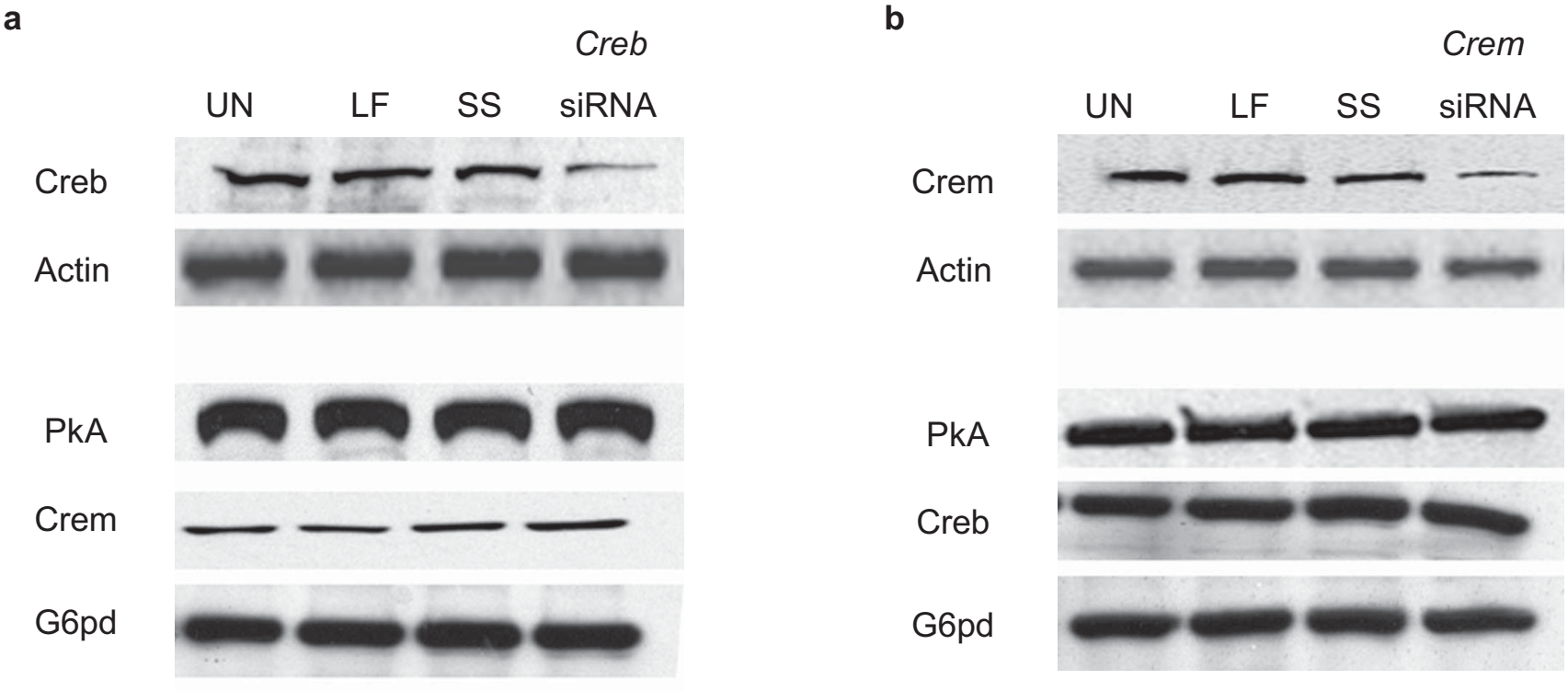
Accession numbers. *Bos taurus G6pd* – XM_583628; *Crem* – NM_001034710; *Gapdh* – NM_17540; *Homo sapiens G6PD* – NM_000402; *CREM* – NM_181571; *GAPDH* – NM_002046



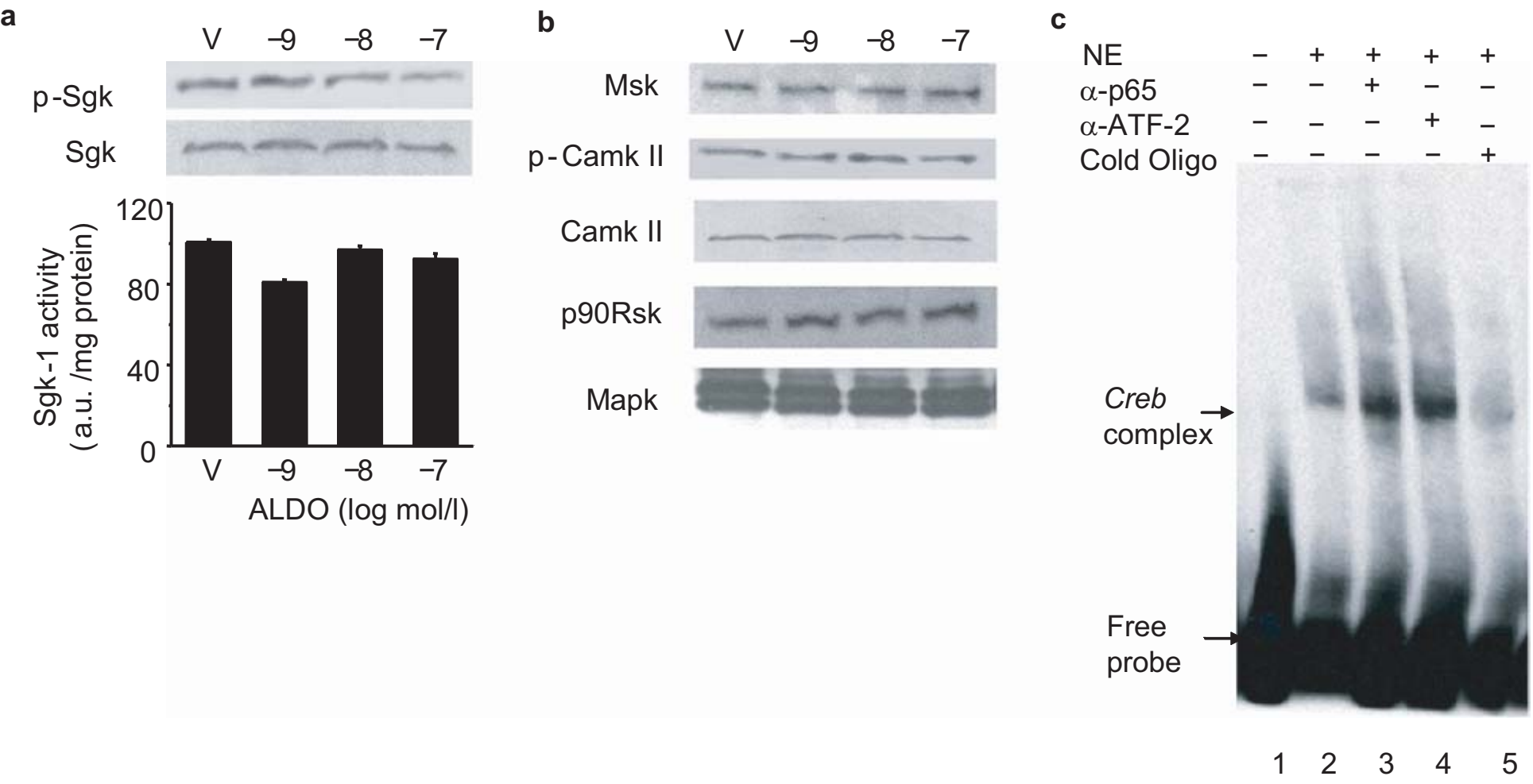
Supplementary Figure 1. Aldosterone decreases G6PD expression in human coronary artery endothelial cells. Human coronary artery endothelial cells were treated with aldosterone (ALDO) or vehicle (V) for 24 h and the influence of ALDO on (**a**) G6PD protein expression, (**b**) *G6PD* mRNA levels by quantitative real-time PCR (n = 4), and (**c**) G6PD activity were determined (n = 8). * P <0.01 vs. V. Densitometry was performed on a minimum of three blots and reported as arbitrary units (a.u.). Data represent mean ± SEM.



Supplementary Figure 2. Aldosterone decreases G6pd expression by protein kinase A activation. BAEC were treated with aldosterone (ALDO) or vehicle (V) for 24 h and (**a**) cAMP levels were determined (*P < 0.01 vs. V; n = 10), (**b**) protein kinase A (PkA) activity was determined in the presence of the PkA antagonist PKI (10 μmol/l) or the MR antagonist spironolactone (SP) (10 μmol/l). Forskolin (FSK) served as a positive control (*P < 0.01 vs. V; #P < 0.01 vs. –7; **P < 0.01 vs. FSK; n = 6). (**c**) PkA-α protein levels were assessed. (**d**) The influence of PKI or SP on G6pd expression was examined (*P < 0.01 vs. –7). Densitometry was performed on a minimum of three blots and reported as arbitrary units (a.u.). Data represent mean ± SEM.



Supplementary Figure 3. Inhibition of Creb or Crem expression by siRNA. BAEC were transfected with siRNA to *Creb* or *Crem*, or a scrambled control sequence (SS). After 48 h, (**a**) in cells transfected with *Creb* siRNA, Creb protein expression was examined in untreated (UN), Lipofectamine alone (LF), SS, and siRNA transfected cells. The influence of decreased Creb expression on PkA, Crem, and G6pd protein levels was also determined. (**b**) Crem protein expression was also examined in UN, LF, SS, and *Crem* siRNA transfected cells. The influence of decreased Crem expression on PkA, Creb, and G6pd protein levels was determined. Representative blots are shown.

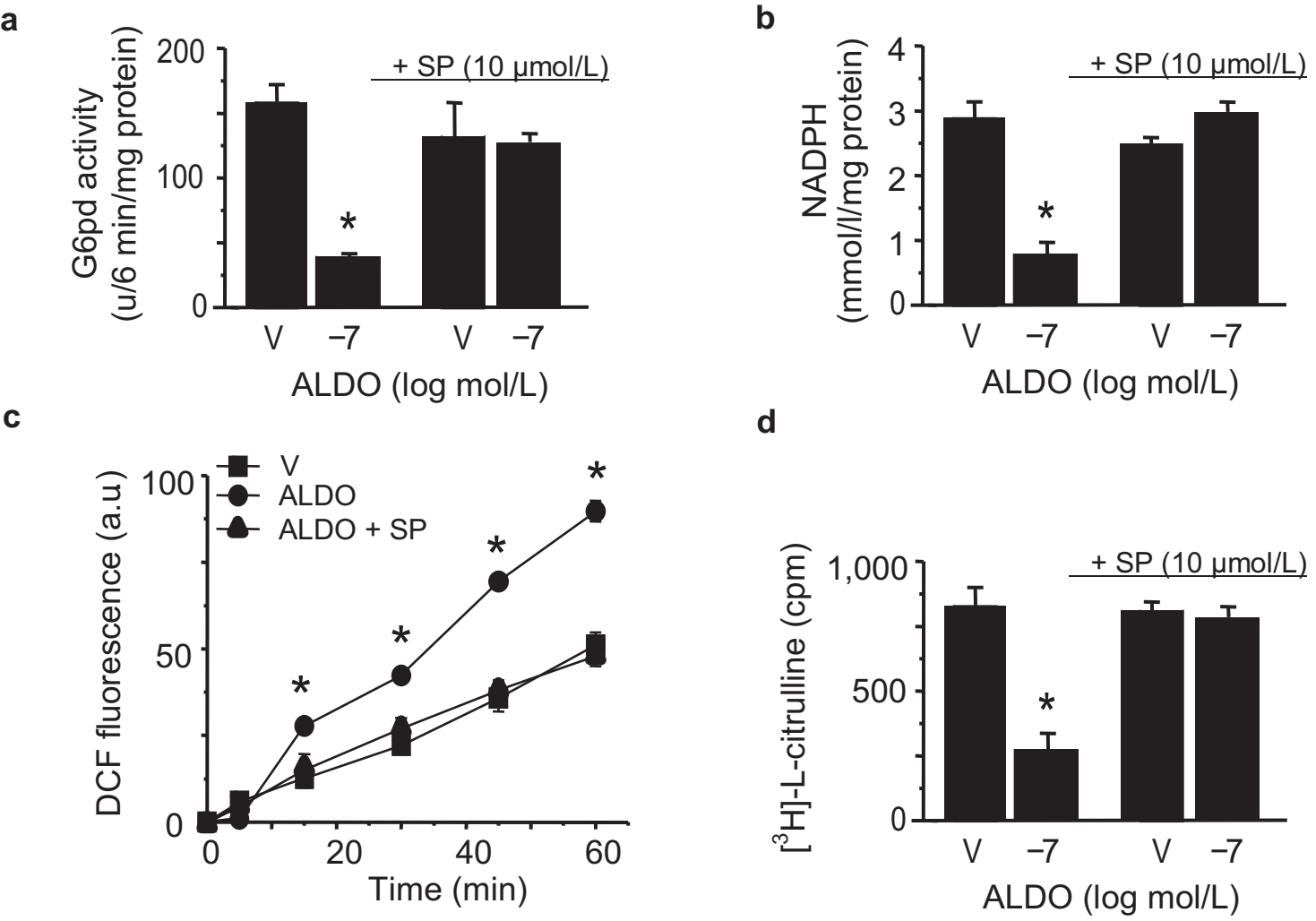


Supplementary Figure 4. Aldosterone and Creb activation: upstream signaling kinases and downstream transcription factors. BAEC were treated with aldosterone (ALDO) or vehicle (V) for 24 h and (**a**) serum-glucocorticoid kinase (Sgk) and phospho-Sgk expression were determined by Western blotting (*top*); and corresponding Sgk-1 activity was examined (*bottom*). (**b**) Expression of Msk, Ca^{2+}-calmodulin kinase (Camk), phospho-Camk, p90Rsk, and Mapk, all kinases known to activate Creb, was evaluated by Western blotting. (**c)** In human coronary artery endothelial cells, the influence of ALDO on DNA-binding to NF-κB and ATF was assessed by electrophoretic mobility shift assay using a biotin-labeled double stranded oligonucleotide containing the sequence of the CREB/CREM binding site from the *G6PD* promoter (5'-CAGAAACAGTATGACGATAGGCAGAT-3'). Nuclear extracts from ALDO-treated cells (lane 2) were incubated with the probe. Supershift assays were performed using antibodies to p65 (lanes 3) or ATF-2 (lanes 4). Competition assay (lane 5) was performed using 100-fold molar excess of cold oligonucleotide.

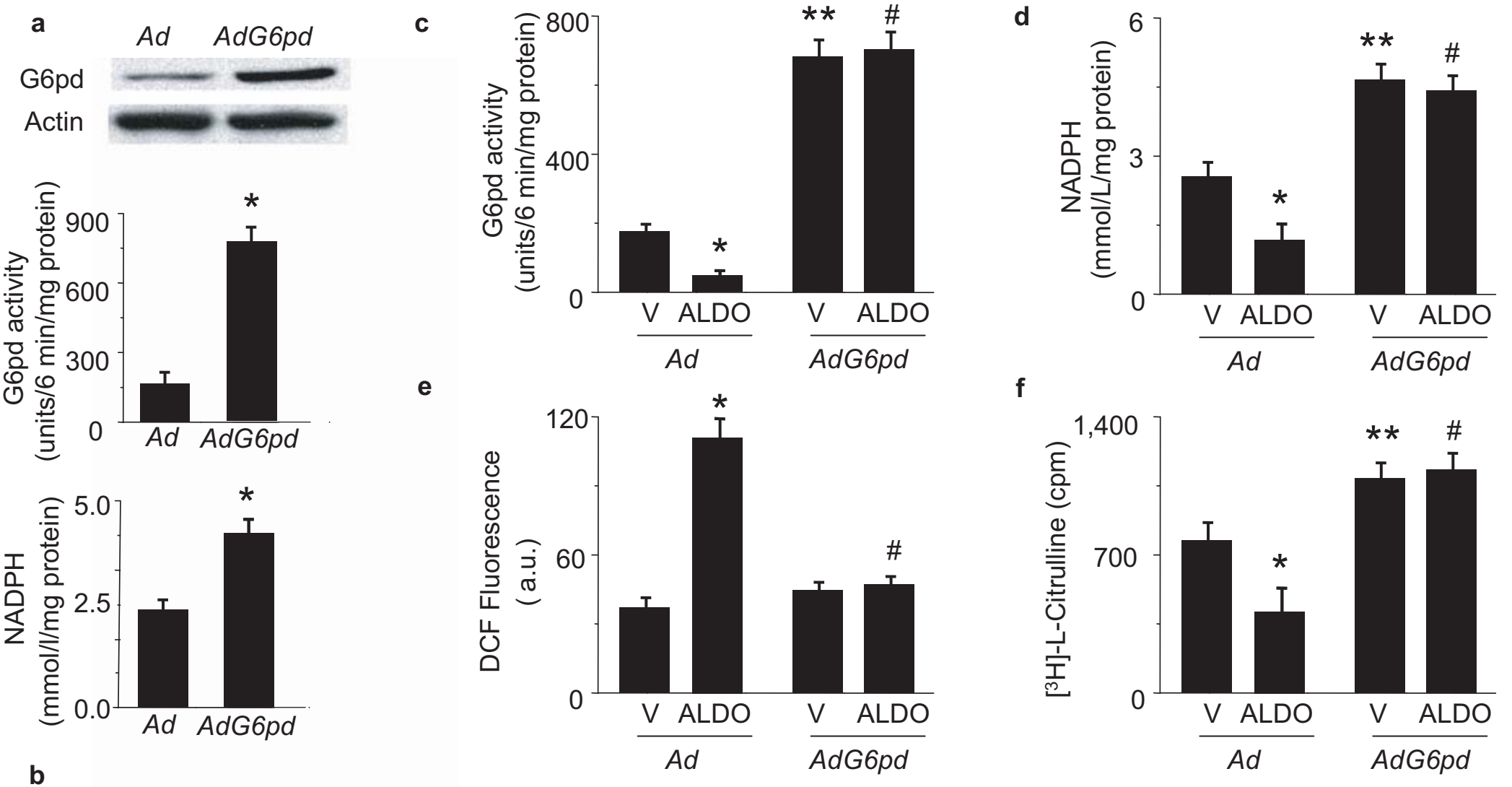
DCF Fluorescence (a.u.)

	V	ALDO–7
No addition	42.6 ± 4.6	95.8 ± 3.0*
L-NAME (1 mmol/l)	38.5 ± 5.1	52.5 ± 3.7#
Apocynin (30μmol/l)	31.2 ± 3.1	34.8 ± 4.4#
Indomethacin (10μmol/l)	44.8 ± 3.7	84.9 ± 4.5
Oxypurinol (100μmol/l)	45.6 ± 6.2	92.4 ± 6.7

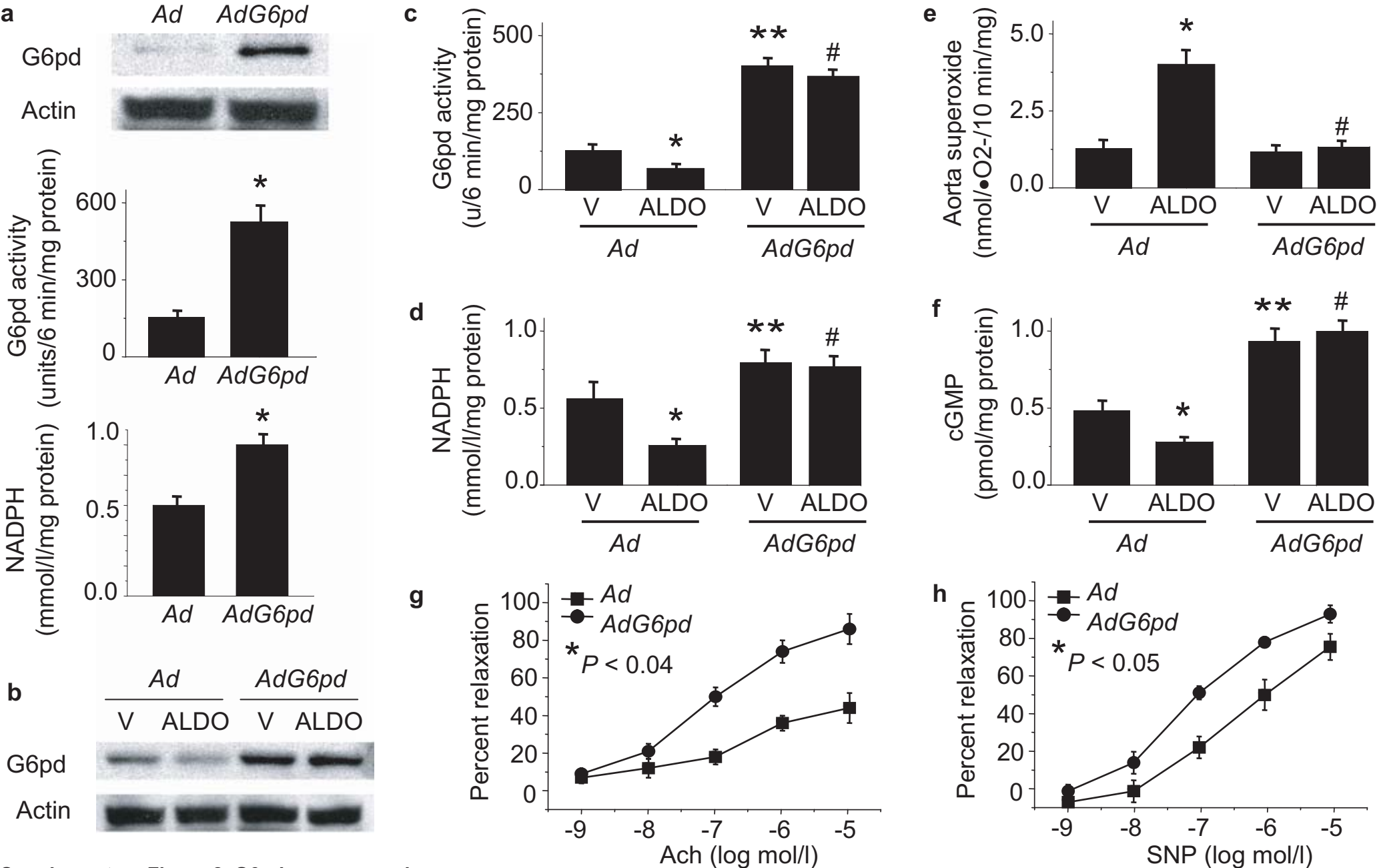
Supplementary Figure 5. Source of reactive oxygen species in aldosterone-treated cells. BAEC were treated with aldosterone (ALDO) or vehicle (V) for 24 h in the presence or absence of L-NAME (1 mmol/l), apocynin (30 μmol/l), indomethacin (10 μmol/l), or oxypurinol (100 μmol/l), inhibitors of eNos, Nadph oxidase, cyclooxygenase, and xanthine oxidase, respectively. Reactive oxygen species accumulation was measured by 6-carboxy-2'-7' dichlorodihydrofluorescein diacetate (DCF) fluorescence and reported as arbitrary units (a.u.). *P < 0.01 vs. V-No addition, #P < 0.01 vs. ALDO –7– No addition (n = 8). Data are presented as mean ± SEM.



Supplementary Figure 6. Spironolactone increases G6pd activity. BAEC were treated with aldosterone (ALDO) or vehicle (V) for 24 h in the presence or absence of spironolactone (10 µmol/L) (SP). (**a**) G6pd activity (n = 8) and corresponding (**b**) Nadph levels (n = 4) were measured. *$P < 0.001$ vs. V, –7 + SP. (**c**) Reactive oxygen species accumulation (n = 8) was assessed by 6-carboxy-2'-7' dichlorodihydrofluorescein diacetate (DCF) fluorescence and reported as arbitrary units (a.u.). *$P < 0.01$ vs. V, –7 + SP; and (**d**) eNos activity was measured in intact cells, *$P < 0.01$ vs. V, –7 + SP, n = 6. Data are presented as mean ± SEM.



Supplementary Figure 7. G6pd overexpression preserves G6pd activity *in vitro*. BAEC were infected with an adenoviral vector encoding *G6pd* (*AdG6pd*) at an MOI = 10 pfu/cell or with an empty vector (*Ad*) as a control. (**a**) G6pd expression (*top*), activity (n = 8) (*middle*), and Nadph levels (n = 4) (*bottom*) were determined (*$P < 0.01$ vs. *Ad*). G6pd overexpressing cells, or cells infected with the control vector, were then treated with aldosterone (ALDO) (10^{-7} mol/L) or vehicle (V) for 24 h and (**b**) G6pd expression, (**c**) activity (n = 8), and (**d**) Nadph levels (n=4) were reexamined (*$P < 0.05$ vs. V-*Ad*; **$P < 0.01$ vs. V-*Ad*, $^{\#}P < 0.005$ vs. ALDO-*AdG6PD*).(**e**) Reactive oxygen species accumulation (n = 8) was measured by 6-carboxy-2'-7' dichlorodihydrofluorescein (DCF) fluorescence and reported as arbitrary units (a.u.), *$P < 0.01$ vs. V-*Ad*, $^{\#}P < 0.01$ vs. ALDO-*Ad*), and (**f**) eNOS activity (n = 4) was determined in intact cells (in the absence of exogenous cofactors) (*$P < 0.05$ vs. V-*Ad*, **$P < 0.05$ vs. V-*Ad*, $^{\#}P < 0.01$ vs. ALDO-*Ad*). Representative blots are shown. Data represent mean ± SEM.



Supplementary Figure 8. G6pd overexpression preserves G6pd activity *in vivo*. C3H wild-type mice (n = 24) were injected by tail vein with 5 x 109 plaque forming units of *AdG6pd* or an empty control vector *Ad* (n = 24). (**a**) After 5 d, aorta G6PD protein expression (*top*), activity (*middle*), and Nadph levels (*bottom*) were examined (*$P < 0.01$ vs. *Ad*; n=4). At day 5 after infection, mice were infused with aldosterone (50 µg/kg/day) (ALDO) or vehicle (V) via Alzet pump for 14 days and aorta (**b**) G6PD expression, (**c**) activity, and (**d**) Nadph levels were measured (*$P < 0.05$ vs. V-*Ad*, **$P < 0.01$ vs. V-*Ad*, #$P < 0.01$ vs. ALDO-*Ad*; n = 8). (**e**) Aorta superoxide levels were determined by lucigenin (5 µmol/L) chemiluminescence (*$P < 0.001$ vs. V-*Ad*, #$P < 0.001$ vs. ALDO-*Ad* n = 6) and (**f**) cGMP levels were measured (*$P < 0.05$ vs. V-*Ad*, #$P < 0.01$ vs. ALDO-*Ad*, n=6). Vascular reactivity was assessed by intravital videomicroscopy to increasing concentrations of (**g**) acetylcholine (Ach) (*$P < 0.04$ by ANOVA, n = 5) or (**h**) sodium nitroprusside (SNP) (**$P < 0.05$ by ANOVA, n = 5). Representative blots are shown. Data are presented as mean ± SEM.