Supplementary Data

Materials and Methods

The reagents used in the present study were purchased from Sigma Chemical except for U46619, which was from Cayman Chemical.

Western blot analysis

Protein was extracted from frozen tissue and its concentration measured spectrophotometrically with a colorimetric assay (Bio-Rad DC reagent). Thirty-five micrograms of total protein was separated by electrophoresis, transferred onto a nitrocellulose membrane, and incubated with specific antibody against glucose-6-phosphate dehydrogenase (G6PD, 1:500 dilution; Sigma Chemical) and protein kinase C (PKC) isoforms (1:100 dilution; BD Bioscience). After conjugation with the secondary antibody, the membranes were developed in a chemiluminescence substrate solution (Pierce Super-Signal Chemiluminescens Substrate), and re-probed for α-actin to verify the uniformity of protein loading. Bands were observed by autoradiography and quantified using commercially available software. Results are expressed as percentage of the density of a standard sample loaded on all membranes in triplicate.

Cell lines and culture conditions

Bovine coronary artery (CA) cells were purchased from Cell Application Inc., and maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum, 0.1 m*M* nonessential amino acids (GibcoBRL), 1 m*M* sodium pyruvate (GibcoBRL), and 100 U/ml penicillin and streptomycin (GibcoBRL). The human primary epithelial kidney 293T17 cells were purchased from ATCC and maintained in DMEM in the presence of 10% fetal bovine serum and 100 U/ml penicillin and streptomycin (GibcoBRL).

Detection of G6PD by immunohistochemistry and immunofluorescent microscopy

Cultured smooth muscle cells (SMCs) (1.2×10^5) were fixed in 100% methanol overnight at -20°C. Cells were washed twice with phosphate-buffered saline (PBS) containing 0.05% Triton X-100 and 0.5% bovine serum albumin, and incubated overnight at 4°C with a polyclonal anti-G6PD antibody (1:200) diluted in PBS containing 0.05% Triton X-100 and 0.5% bovine serum albumin. Cells were then incubated with Alexa-488-labeled anti-rabbit IgG for 1 h at room temperature. The coverslips were sealed onto the slides and images were captured with an MRC 1024 ES (Bio-Rad) confocal microscopy system with a black-and-white charge-coupled device camera, and then rendered in pseudocolor. Data were collected with an Olympus Plan x10/NA 0.25 Phi objective. All data within each experiment were collected at identical imaging settings.

CA rings were frozen in liquid nitrogen, and the frozen sections of 6- and 7-μm thickness were mounted on glass slides, air-dried for 30 min, and fixed in acetone for 15 min at room temperature. Each tissue section (up to five of each tissue) was washed twice with PBS containing 0.1% Triton X-100. After washing in PBS, preincubation was carried out with 20% goat serum in PBS followed by overnight incubation at 4°C with polyclonal anti-G6PD antibody (1:300; Santacruz Biotech) diluted in PBS containing 5% bovine serum albumin and 0.1% Tween 20. After three washings in PBS, sections were incubated with secondary goat IgG anti-rabbit conjugated with alkaline phosphatase for 2 h. Negative control in which tissue sections were not incubated with primary antibodies was performed for all three enzymes examined in this study. Staining was done by a protocol providedby Vector Laboratories. Counterstaining with hematoxylin solution and mounting in Aquamount (Vector Laboratories) completed the procedure. Data were collected with an Olympus Plan x10/NA 0.25 Phi objective. All data within each experiment were collected at identical imaging settings.

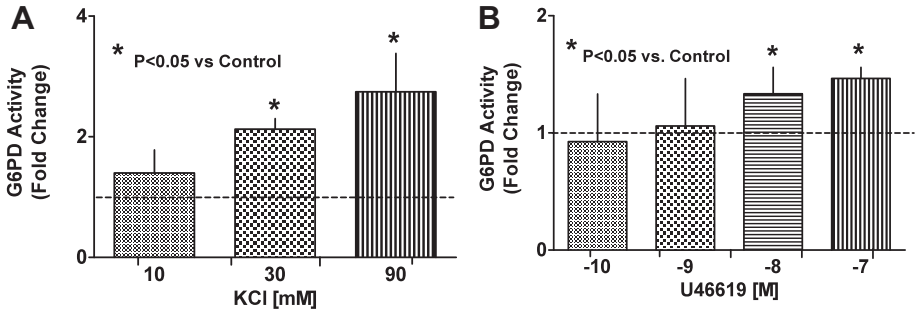
Silencing of PKCδ and G6PD in CA

Cultured CASMCs were grown in six-well plates at a concentration of 1×10^6 cells per well for 36 h in DMEM. Cells were transiently transfected with smartpool siRNA (100 n*M*) against G6PD (Dharmacon) using 2 μg of Fugene6 reagent (Roche) in serum free OPTI-MEM media (Invitrogen) for 72 h. Alternatively, CA rings (300–500 μm) harvested from bovine hearts were incubated in DMEM in a six-well plate overnight and then transiently transfected with smartpool siRNA (100 n*M*) against PKCδ (Dharmacon) or G6PD (Dharmacon) using 2 μg of Lipofectamine 2000 reagent (Invitrogen) in serum-free OPTI-MEM media (Invitrogen) for 67 h. Western blot analysis was performed as described previously to detect the extent of G6PD protein silencing in each cases.

NADPH levels

The levels of nicotinamide adenine dinucleotide phosphate reduced [NAD(P)H] in CA were determined by high-performance liquid chromatography using previously published methods (5). Briefly, CA were pretreated with and without contractile agents and immediately frozen in liquid nitrogen. The frozen tissues were crushed and homogenized in an extraction medium consisting of 0.02 N NaOH containing 0.5 m*M* cysteine at 0°C. The extracts were then heated at 60°C for 10 min and neutralized with 2 ml of 0.25 *M* glycylglycine buffer (pH 7.6).

Acidic extracts were prepared by homogenizing the tissues in hot 0.1 N HCl, followed by neutralization. NAD(P)H was eluted on a reverse-phase high-performance liquid chromatography column (4.6×250 mm, C18, Supelco; Sigma) at room temperature using a HP 1100 Series (Agilent Technologies)



SUPPLEMENTARY FIG. S1. Activation of G6PD by KCl and U46619. (A) G6PD activity is increased from baseline in CA by KCl (10–90 m*M*; $n = 5$–8) or U46619 (10^{-10} to 10^{-7} *M*; $n = 4$–7). CA, coronary artery; G6PD, glucose-6-phosphate dehydrogenase; KCl, potassium chloride. *Dotted line* indicates control levels.

and buffer system consisting of 100 m*M* potassium phosphate (pH 6.0; buffer A) and 100 m*M* potassium phosphate (pH 6.0) containing 5% methanol (buffer B). The column was eluted with 100% buffer A from 0 to 8.5 min, 80% buffer A plus 20% buffer B from 8.5 to 14.5 min, and 100% buffer B from 14.5 to 40 min. The flow rate was 1.0 ml/min, and the ultraviolet absorbance was monitored at 260 nm.

6-Phosphogluconate concentration

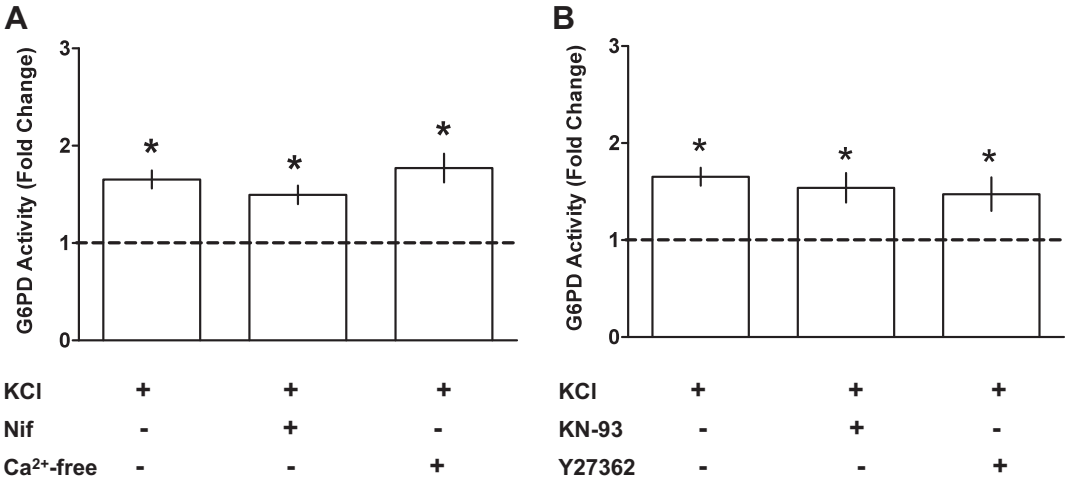
6-Phosphogluconate, a product of the oxidative branch of the pentose phosphate pathway, was measured in CA. The reduction of NADP+(0.02 m*M*) to NADPH was catalyzed by 6-phosphogluconate dehydrogenase (0.5 μg/ml) at 37°C for 20 min in Tris-HCl (0.05 *M*) buffer (pH 8.0) containing ethylene diaminetetraacetic acid (0.1 m*M*), dithiothreitol (0.1 m*M*), and ammonium acetate (0.03 *M*). NADPH concentration was then detected fluorometrically as previously described by us (6).

PKC activity

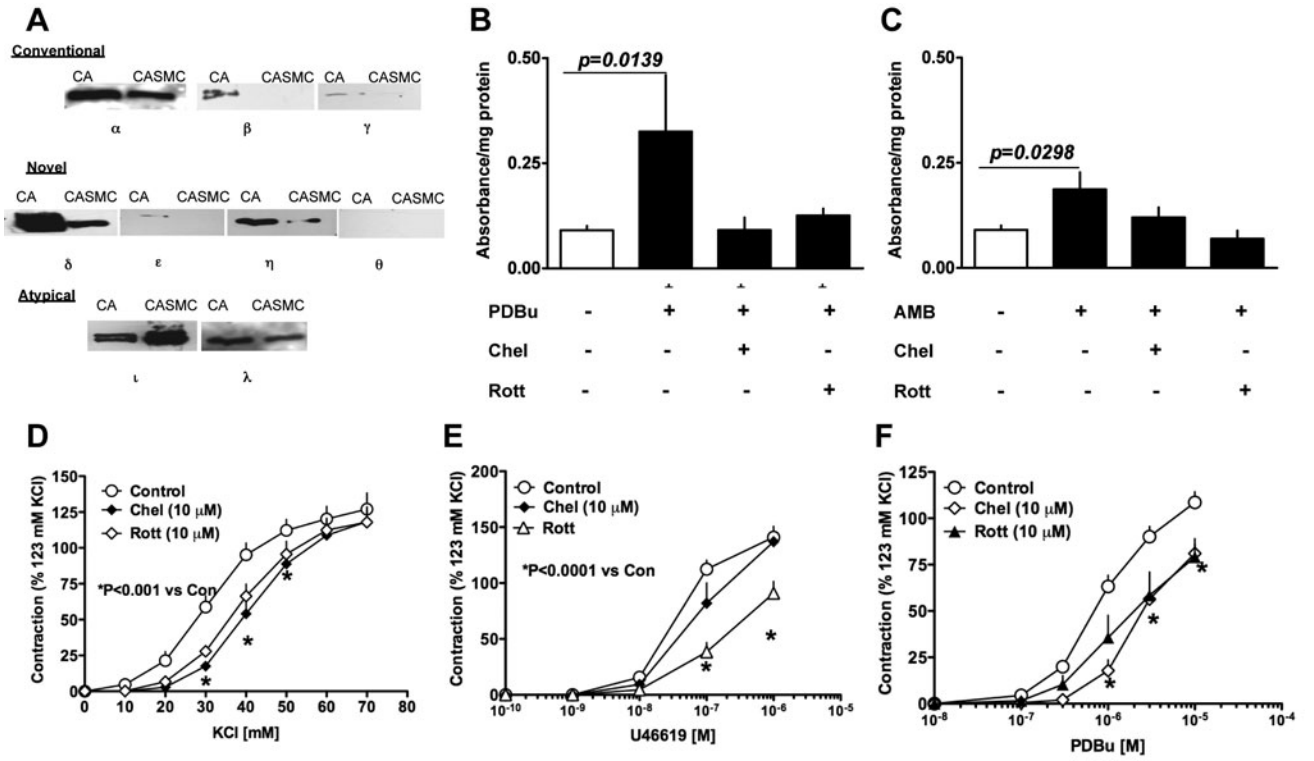
The activation of CA smooth muscle PKC by contractile agents was determined by nonradioactive assay kit purchased from StressXPress. Pretreated frozen samples (1 g) were homogenized in lysis buffer (5 ml) consisting of 20 m*M* 3-(N-morpholino) propane sulfonic acid, 50 m*M* β-glycerolphosphate, 50 m*M* sodium fluoride, 1 m*M* sodium vandate, 5 m*M* ethylene glycol tetraacetic acid, 2 m*M* ethylene diaminetetraacetic acid, 1% NP40, 1 m*M* dithiothreitol, 1 m*M* benzamidine, 1 m*M* phenylmethane-sulphonylfluoride, and 10 μg/ml leupeptin and aprotinin, pH 7.4. The ELISA was performed as per the manufacturer's protocol and activity was reported as absorbance/mg protein.

Coimmunoprecipitation

The interaction between G6PD and PKCδ was tested by immunopanning G6PD and PKCδ complex from CA extract. Briefly, two CA (3–4 mm) rings were homogenized in 20 m*M* Tris-HCl pH 7.4 buffer, containing 1% NP-40, 0.25% sodium deoxycholate, 100 m*M* NaCl, 1 m*M* ethylene glycol tetraacetic acid (EGTA), 1 m*M* phenylmethane sulfonylfluoride, 1 m*M* Na_3VO_4, 1 m*M* NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. After protein extraction (1–1.5 mg/ml), G6PD was immunoprecipitated using goat polyclonal antibodies (Sigma) bound to IgA and IgG beads (Santa Cruz Biotechnology), after which sodium dodecyl sulfate (SDS) gel electrophoresis was performed. Separated



SUPPLEMENTARY FIG. S2. G6PD is activated by KCl in Ca^{2+}-independent manner. (A) Activation of G6PD by KCl (30 m*M*; $n = 8$–10) was not blocked by nifedipine (1 μ*M*; $n = 8$–10) or by removal of extracellular calcium ($n = 10$). **(B)** KCl-induced activation of G6PD was unaffected by KN-93 (10 μ*M*; $n = 5$) or by Y27632 (10 μ*M*; $n = 5$). *$p < 0.05$ *versus* control represented by *dotted line*.



SUPPLEMENTARY FIG. S3. Activation of G6PD by depolarization is mediated by PKC. (A) Determination of PKC in CA by Western blot shows that classical (α and β), novel (δ and η), and atypical (ι and λ) isoforms are expressed in CA. **(B, C)** Activation of PKC in CA by PDBu (10 μM; $n = 5$) and AMP (50 μM; $n = 5$) is inhibited by nonspecific, Chel (10 μM), and PKCδ-specific, Rott (10 μM), antagonists. **(D–F)** KCl (30 mM), U46619 (100 nM), and PDBu (10 μM) evoked contraction was suppressed by Chel and Rott. AMP, amphotericin B; Chel, chelerythrine; PDBu, phorbol 12, 13-dibutyrate. PKC, protein kinase C; Rott, rottlerine. *$p < 0.05$ *versus* control.

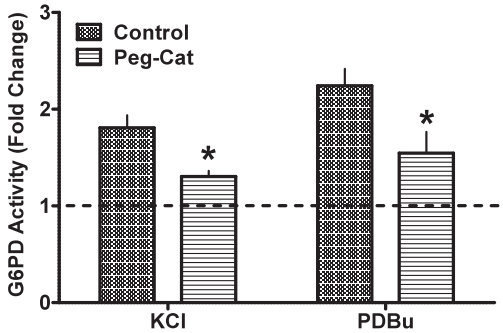
proteins were blotted onto 0.2 μm nitrocellulose (16 h, 30 V) and immunostained with anti-PKCδ antibody (BD Bioscience) followed by incubation with HRP-labeled appropriate secondary antibodies (Pierce). Specific proteins were detected by chemiluminescence (Pierce).

Contraction of CA

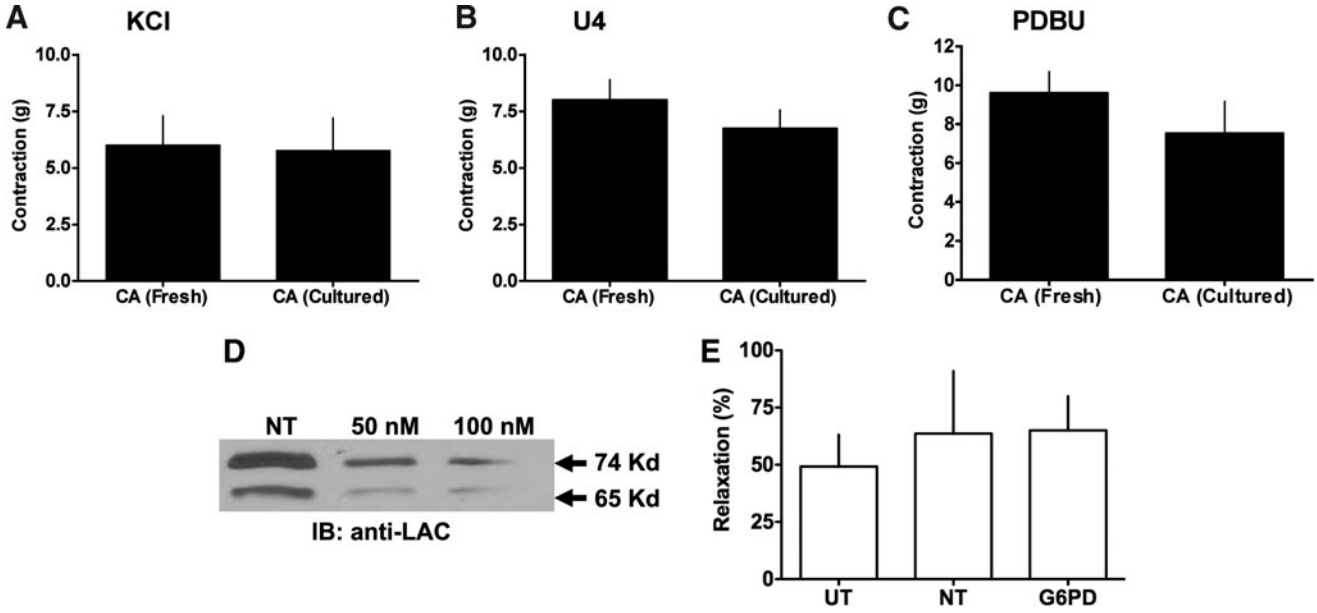
Isolated, endothelium-intact left anterior descending coronary arterial rings were prepared from slaughterhouse-derived bovine hearts and studied for changes in isometric force. The rings were incubated in individually thermostated (37°C) 10-ml baths (WPI Instruments) for 2 h at an optimal passive tension of 5 g in Krebs bicarbonate buffer (pH 7.4) containing the following (in mM): 118 NaCl, 4.7 potassium chloride [KCl], 1.5 $CaCl_2$, 25 $NaHCO_3$, 1.1 $MgSO_4$, 1.2 KH_2PO_4, and 5.6 glucose, gassed with 21% O_2–5% CO_2-balance N_2. After a 2-h equilibration and a brief depolarization with 123 mM KCl, CA was re-equilibrated in Krebs buffer for 30 min before the experiments were conducted. In studies examining the response of CA to contractile agents such as KCl, U46619, and phorbol 12, 13-dibutyrate (PDBu), drugs were added for at least 20–30 min.

Myosin light chain phosphorylation

Status of myosin light chain phosphorylation in CA treated with and without G6PD inhibitor in the absence and presence of contractile agents was determined by previously described protocols (15). Briefly, CA was collected in precooled (−80°C on dry ice) acetone containing 10% TCA and incubated at room temperature for 60 min, after which TCA was removed by washing the tissue in cold acetone three times, and air-dried for 24 h. The proteins were extracted from CA in 1% SDS, 20 mM dithiotheitol, and 10% glycerol (pH 7.2), and SDS-polyacrylamide gel electrophoresis was performed. Proteins were transferred onto a nitrocellulose membrane, and incubated with specific antibody against p-myosin light



SUPPLEMENTARY FIG. S4. Catalase inhibited activation of G6PD by KCl and PDBu. Peg-catalse (300 U/l) decreased KCl (30 mM; $n = 8$) and PDBu (10 μM) evoked G6PD activation. *$p < 0.05$ *versus* control. *Dotted line* indicates baseline activity level.



SUPPLEMENTARY FIG. S5. **(A)** Arterial rings (300 μm) were cultured in Dulbecco's modified Eagle's medium for 72 h. At the end of incubation period, rings were contracted with KCl (30 m*M*; **A**) or U46619 (100 n*M*; **B**), and PDBu (10 μM; **C**). CAs were transfected with NT (100 n*M*) and lamin A/C (100 n*M*) siRNA for 72 h and Western blot analysis was performed **(D)**. In parallel experiments, CAs rings were transfected with siRNA (100 n*M*) targeting G6PD and used for performing functional studies. **(E)** UT and NT controls, and G6PD-siRNA (G6PD)-treated CAs were contracted with phenylenephrine (1 μM) and relaxed with nitric oxide donor S-nitroso-N-acetyl penicillamine (1 μM; $n = 5$ in each group). NT, nontargeting; UT, untransfected.

chain (1:500 dilution, Santa Cruz) and p-myosin phosphatase target subunit 1 (1:100 dilution; Cell Signaling). After conjugation with the secondary antibody, the membranes were developed in a chemiluminescence substrate solution (Pierce SuperSignal Chemiluminescens Substrate), and re-probed for α-actin to verify the uniformity of protein loading. Bands were observed by autoradiography and quantified using commercially available software.

Measurement and imaging of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by dual-excitation ratiometric imaging using fura-PE3. CASMCs and rings were transferred to the experimental chamber and incubated in Ca^{2+}-free extracellular solution in (m*M*) 115 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 1 NaH_2PO_4, and 25 $NaHCO_3$ (pH 7.4 when bubbled with 5% CO_2) with 0.1 μM fura-PE3 and 0.8 μM pluronic acid for 20 min at room temperature. The cells/rings were washed with extracellular solution containing 2.0 m*M* Ca^{2+}, incubated at room temperature for 20 min, washed again, placed on the stage of an upright microscope, and perfused with a warmed extracellular solution (30°C). This loading method allows low concentrations of fura-PE3 to be quickly introduced in cells without affecting cell morphology. Changes in $[Ca^{2+}]_i$ were detected using a cooled charge-coupled device camera (Retiga EX; QImaging) and Northern Eclipse capture and analysis software (Empix, Inc.). Background fluorescence was subtracted before calculation of the 340-nm/380-nm ratio. Dissociation constants were calculated from *in vitro* calibration. Maximal and minimal ratio values were determined at the end of each experiment by first treating the cells with 1 μM ionomycin (maximal ratio) and then chelating all free Ca^{2+} with 10 m*M* EGTA (minimal ratio). Cells/rings not responding to ionomycin were disregarded, as will as cells/rings showing significant photobleaching. Peak increases in $[Ca^{2+}]_i$ were measured during each intervention, and data were reported as averaged peak values.

Results

G6PD is activated by depolarization of membrane potential and by stimulation of G-coupled receptors in CA

It is apparent from our results that G6PD is activated in a dose-dependent manner by KCl (10–90 m*M*) and U46619 (0.1–100 n*M*)—a range of concentrations that is known to evoke contraction of CA (Supplementary Fig. S1A, B).

G6PD is activated in a Ca^{2+}-independent manner by KCl and U46619 in CA

As KCl and U46619 are known to increase $[Ca^{2+}]_i$ by activating L-type Ca^{2+} channels and release of Ca^{2+} from internal stores, we examined the role of Ca^{2+} in increasing G6PD activity. We blocked L-type Ca^{2+} channel with nitrendipine (1 μM) and estimated G6PD activity in CA stimulated by KCl. There was no difference in G6PD activity between nitrendipine-treated *versus* nitrendipine-untreated samples (Supplementary Fig. S2A). Second, we estimated G6PD activity in CA incubated with or without KCl in Ca^{2+}-free Krebs solution containing Ca^{2+} chelating agents, BAPTA-AM (200 μM) and EGTA (1 m*M*), and found that the increase in G6PD activity evoked by KCl was not different either in the presence or in the absence of Ca^{2+} (Supplementary Fig. S2A). These results indicate that changes in $[Ca^{2+}]_i$ did not activate

G6PD. In addition, Ca^{2+}-sensitive CaM kinase II and Rho kinase also did not play a role in KCl-evoked G6PD activation (Supplementary Fig. S2B).

Activation of G6PD by depolarization is modulated by PKC in CA

Six PKC isoforms (PKCα, PKCβ, PKCδ, PKCη, PKCι, and PKCλ) were found to be expressed in CA and CASMCs (Supplementary Fig. S3A), and pretreatment of CA with chelerythrine, a nonspecific inhibitor, and rottlerin (10 μM), a selective inhibitor of PKCδ, suppressed activation of PKC by PDBu (10 μM ; Supplementary Fig. S3B) and amphotericin B (50 μM ; Supplementary Fig. S3C). Additionally, we found that inhibition of PKC decreased KCl (30 m*M*; Supplementary Fig. S3D), U46619 (100 n*M*; Supplementary Fig. S3E), and PDBu (10 μM; Supplementary Fig. S3F)-elicited CA contraction.

G6PD activation is inhibited by catalase

To elucidate if G6PD activated by PKC through activating H_2O_2, we examined the effects of peg-catalase (300 U/ml) on KCl (30 m*M*)- and PDBu (10 μM)-induced activation of G6PD. As illustrated, peg-catalase partially reduced G6PD activity (Supplementary Fig. S4).

Contraction and relaxation of CA is not affected by organ culture

To determine if vasomotor function of CA is compromised by organ culture, we divided CA into two groups. One group was incubated of CA rings in the culture medium up to 72 h. At the end of incubation period, we compared the contraction responses to that of fresh CA rings (second uncultured group). The contractile responses of cultured CA rings were not different as compared to uncultured CA rings (Supplementary Fig. S5A–C). Further, we also assessed the effect of G6PD knockdown on nitric oxide (NO)-induced relaxation. To standardize transfection technique, CAs were first transfected with standard set of commercially available siRNAs such as nontargeting (scrambled; 100 n*M*; NT) and lamin A/C (50 & 100 n*M*)-targeting siRNA, and at the end of 72 h Western blot was performed to determine knockdown of lamin A/C. Western blot figure demonstrate that lamin A/C was silencing in a dose-dependent manner in CAs (Supplementary Fig. S5B). From these results we transfected a set of CAs with G6PD-siRNA (100 n*M*) for 67–72 h and confirmed G6PD knockdown by Western blot analysis; parallely, we also conducted tone studies to examine the effect of G6PD knockdown on NO-induced relaxation. Knocking down G6PD did not alter the response of CAs to NO-induced relaxation (Supplementary Fig. S5E).