SUPPLEMENTAL MATERIALS

Calciprotein particles induce endothelial dysfunction by impairing endothelial nitric oxide metabolism

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Supplemental Methods

Transmission electron microscopy (TEM) CPP analysis

To confirm the morphology and crystallinity of the secondary CPPs, transmission electron microscopy (TEM) imaging was performed (Suppl. Fig. 1). CPPs were transferred on to a Formvar-coated copper grid. After overnight air-drying, high-resolution images were obtained using a JEOL JEM 1400 microscope (JEOL USA Inc., USA) with an accelerating voltage of 60 kV. Images were acquired using a Gatan Orius digital camera system (Gatan Inc., USA).

Energy dispersive X-ray (EDX) analysis of the CPPs

To determine the calcium and phosphate ratios of the secondary CPPs, energy dispersive X-ray (EDX) analysis was performed. Hereto, CPPs were airdried on copper tape. Thereafter, micro-elemental analysis was performed in combination with a GeminiSEM Sigma 300 electron microscope (Zeiss, Germany), using a Quantax 200 detector (Bruker, USA). Calcium to phosphate ratios were determined by dividing the atom percentage (%) of calcium by the atom percentage (%) of phosphate. Calcium and phosphate (atom) ratios of the CPPs are included in the legend of Supplemental Figure 1.

Validation of dihydroethidium (DHE)-based probes

To determine specificity of the dihydroethidium (DHE)- based probes used to measure CPP-induced superoxide production, HUVECs were seeded in 96-well plates up to 80% confluency. After 24 h, cells were incubated with 5µM dihydroethidium (#D11347, DHE, Thermo Fisher Scientific, USA) for 30 min 37 °C. Subsequently, cells were exposed to control (1x TBS solution in ECM) or 50 µg/mL of CPPs in the presence or absence of the superoxide inhibitor YCG063 (20µM, #557354, Calbiochem, Sigma-Aldrich, USA) for 30 min 37 °C. Fluorescent intensity was measured using 518/606 nm excitation/emission wavelength. Data is presented as relative fluorescence units (RFU) as percentage of the experimental control (Suppl. Fig. 5).

CD31 immunofluorescent staining

To demonstrate presence of ECs in the porcine coronary artery rings after CPP exposure, ring were stained for CD31 expression. Hereto, rings were fixed in formalin, and embedded in paraffin afterwards. Tissue sections were deparaffinized, followed by overnight antigen retrieval in 10 mmol/L Tris-HCl (pH = 9) at 80°C. Sections were blocked in 1% BSA-PBS and 5% serum-PBS for 30 min RT and incubated with the primary antibody CD31 (#SC-1506, 1:50, Santa Cruz Biotechnology, USA) in 1% BSA-PBS and 5% serum-PBS for 3 h RT. In between antibody incubation, sections were washed with PBS-Tween (0.05%). The primary antibody was detected with the secondary AlexaFluor-555 antibody (#A-21431, 1:250, Thermo Fisher Scientific, USA) in 10% BSA-PBS and DAPI (1 µg/mL) (30 min RT). Finally, sections were mounted in Citifluor and visualized with the TissueFAXS (TissueGnostics, Austria).

Supplemental Figures

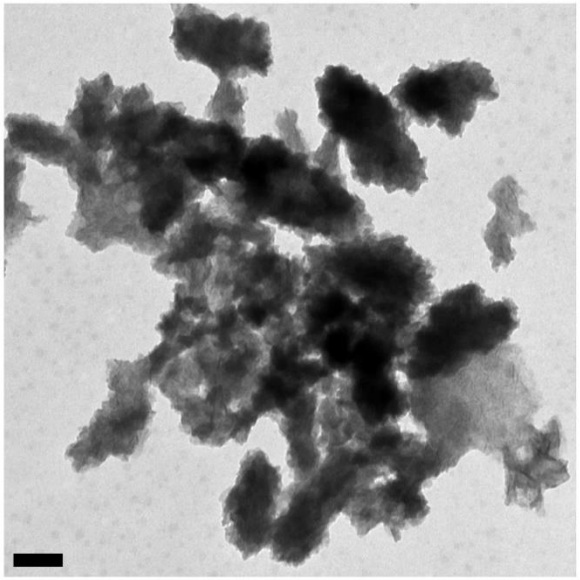

Figure S1: Transmission electron microscopy (TEM) analysis of secondary CPPs. TEM picture of secondary CPPs showing a spindle-shaped crystalline structure. Scale bar represents 100 nm. Energy dispersive X-ray (EDX) analysis indicated a calcium to phosphate (atom) ratio of the secondary CPPs of 2.061 ± 0.033 (mean ± SEM).

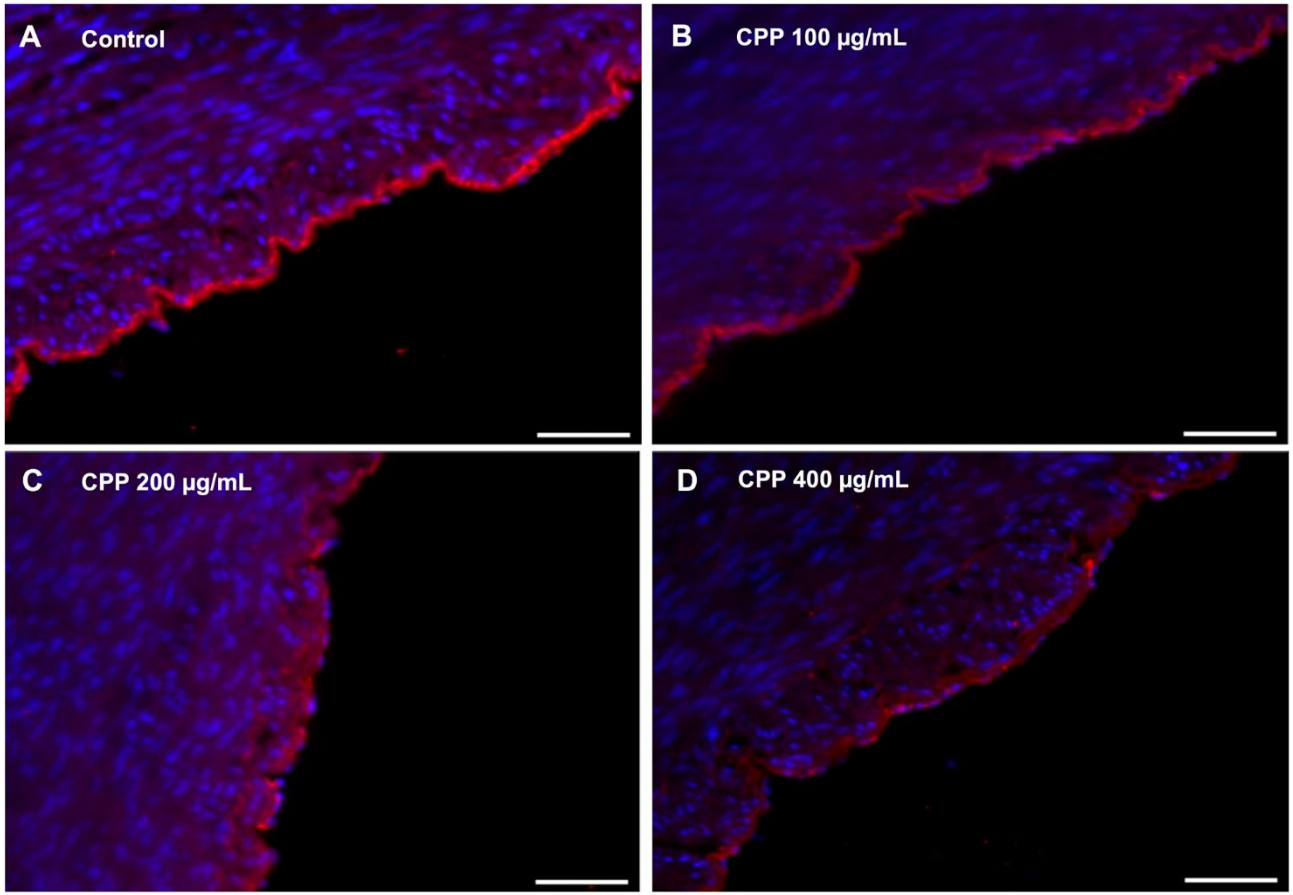

Figure S2: CD31 endothelial cell staining on coronary artery rings exposed to CPPs. Porcine coronary artery rings were stained for CD31 protein expression (endothelial cell marker) after overnight exposure to 0 (=control condition, Krebs buffer only), 100, 200 or 400 µg/mL CPPs (A-D respectively). Scale bar represents 50 µm.

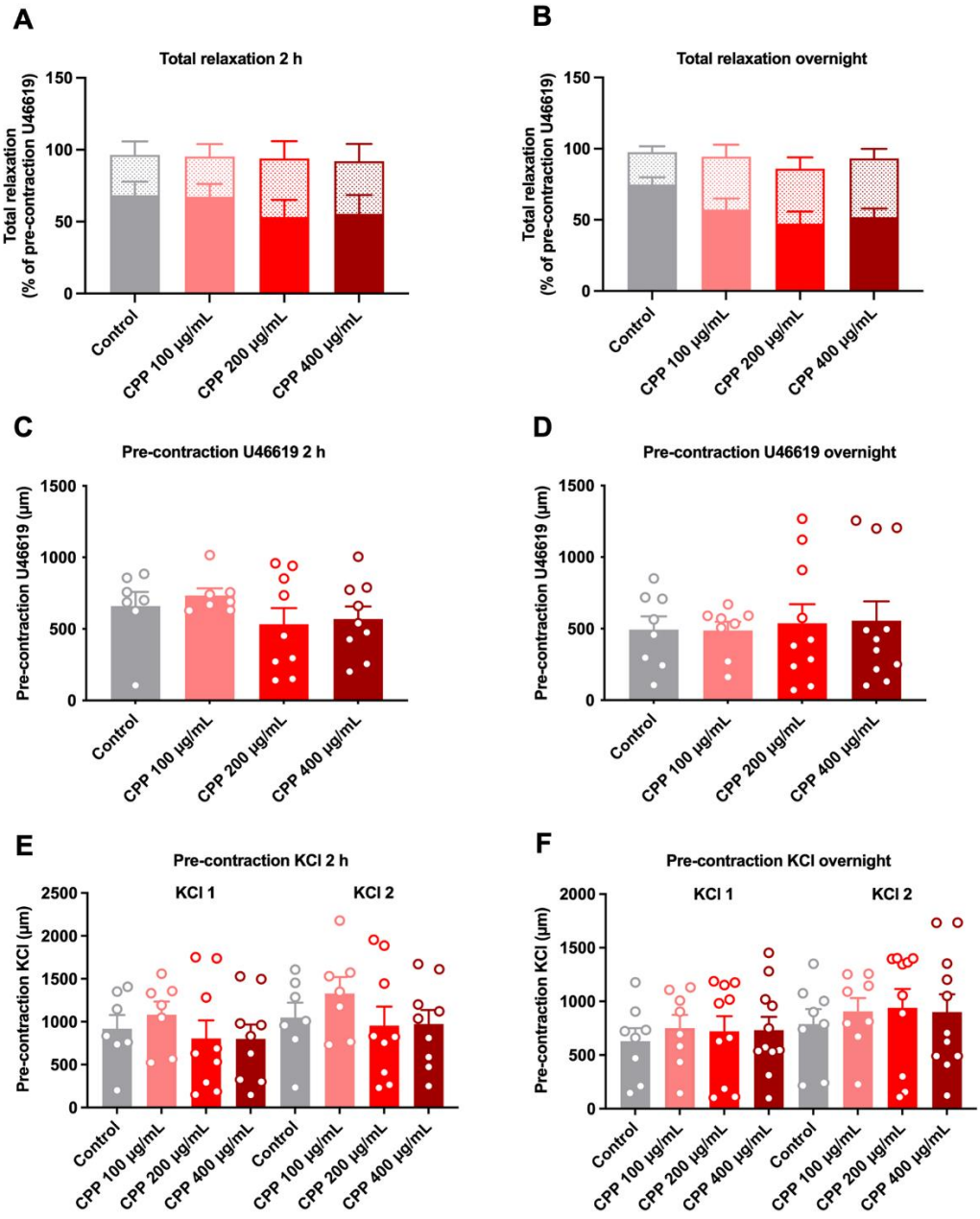

Figure S3: VSMC responses: total relaxation and pre-contraction of RCA-rings after secondary CPP exposure. A-B: Total relaxation of RCA-rings exposed for 2 h or overnight to secondary CPPs. The total relaxation is the sum of the endothelial-dependent relaxation to bradykinin (filled bars) + the endothelial-independent relaxation in response to SNP (dotted-pattern bars). C-D: Both acute and overnight exposure of the RCA-rings to secondary CPPs did not affect the VSMC pre-contraction to U46619. E-F: No differences of the RCA-rings on the KCl pre-contraction were observed. Data are shown as means ±SEM. Endothelial-independent relaxation is shown as % of pre-contraction to U46619. Vascular contractions are measured in µm. Rings were isolated from three individual porcine hearts and evenly distributed within groups (3-4 rings per heart per group; total 2h N = 7-9 rings per group, overnight N = 8-11 rings per group).

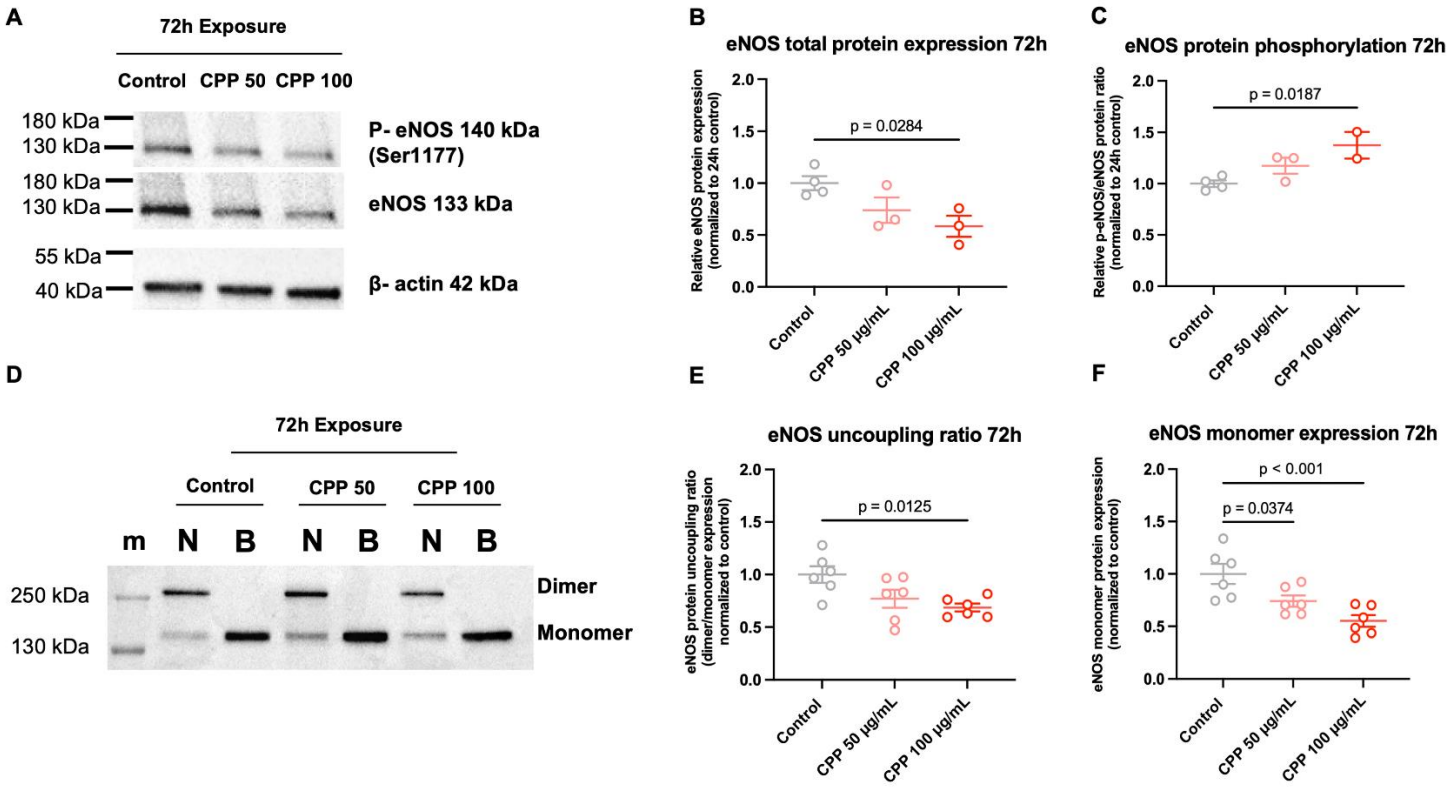

Figure S4: Exposure of secondary CPPs to HUVECs for 72 h reduces eNOS protein expression, increases Ser1177 eNOS protein phosphorylation and uncouples eNOS. A: Representative protein blots showing phospho-eNOS (Ser1177), total eNOS and β-actin protein expression. Molecular weight standards are indicated on the left side of the immunoblots in kilodaltons (kDa) based on the marker shown in Suppl. Fig. 8A&B. B: eNOS total protein expression was reduced after 72 h exposure to secondary CPPs (100 µg/mL). C: Secondary CPPs (100 µg/mL) increase the eNOS protein phosphorylation (Ser1177-activation side) within 72 h in HUVECs. D: Low temperature SDS-PAGE blots showing non-boiled/intact (indicated by symbol N) dimer/monomer eNOS expression and boiled/denatured (indicated by symbol B) eNOS total monomer expression. Molecular weight standards of the marker (m) are indicated on the left side of the immunoblots in kilodaltons (kDa). E-F: eNOS uncoupling ratios (dimer/monomer ratio) and total eNOS monomer expression were significantly decreased in HUVECs exposed to secondary CPPs. Data are presented as means (+/-SEM) with individual data points and normalized to the experimental control. Significant differences are indicated for *P*-values < 0.05.

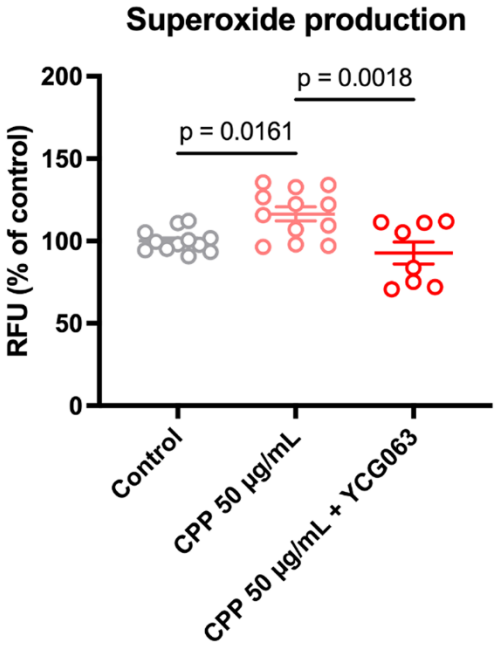

Figure S5: Superoxide production DHE including YCG063. Superoxide production was measured in HUVECs after CPP exposure (50 µg/mL) in the absence or presence of 20 µM ROS inhibitor YCG063 to validate specificity of the DHE-based superoxide probes. Data show an increase in superoxide production in ECs in response to CPP exposure, which can be inhibited by the addition of ROS inhibitor YCG063, indicating that the DHE readout is based mainly on mitochondrial-derived ROS. Graph shows mean +/- SEM and individual values. Relative fluorescence units (RFU) are shown as percentage of the experimental control. Significant differences are indicated for *P*-values < 0.05.

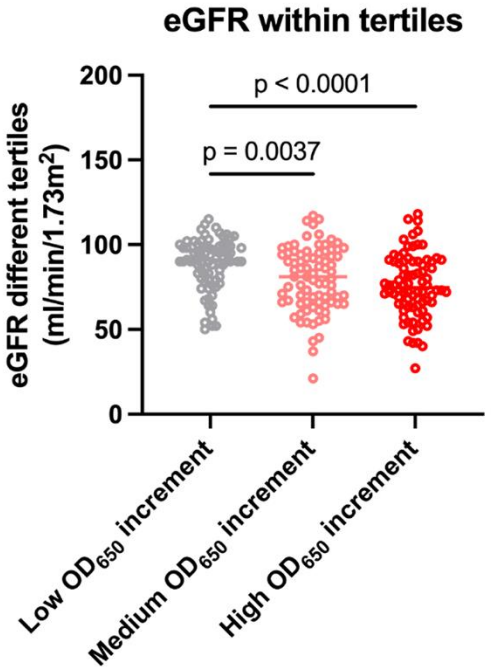

Figure S6: eGFR distribution within the OD_{650} tertiles. Individual eGFR data points are shown within the three (OD_{650} increment) tertiles. The horizontal line indicates the median. Significant differences are indicated for *P*-values < 0.05.

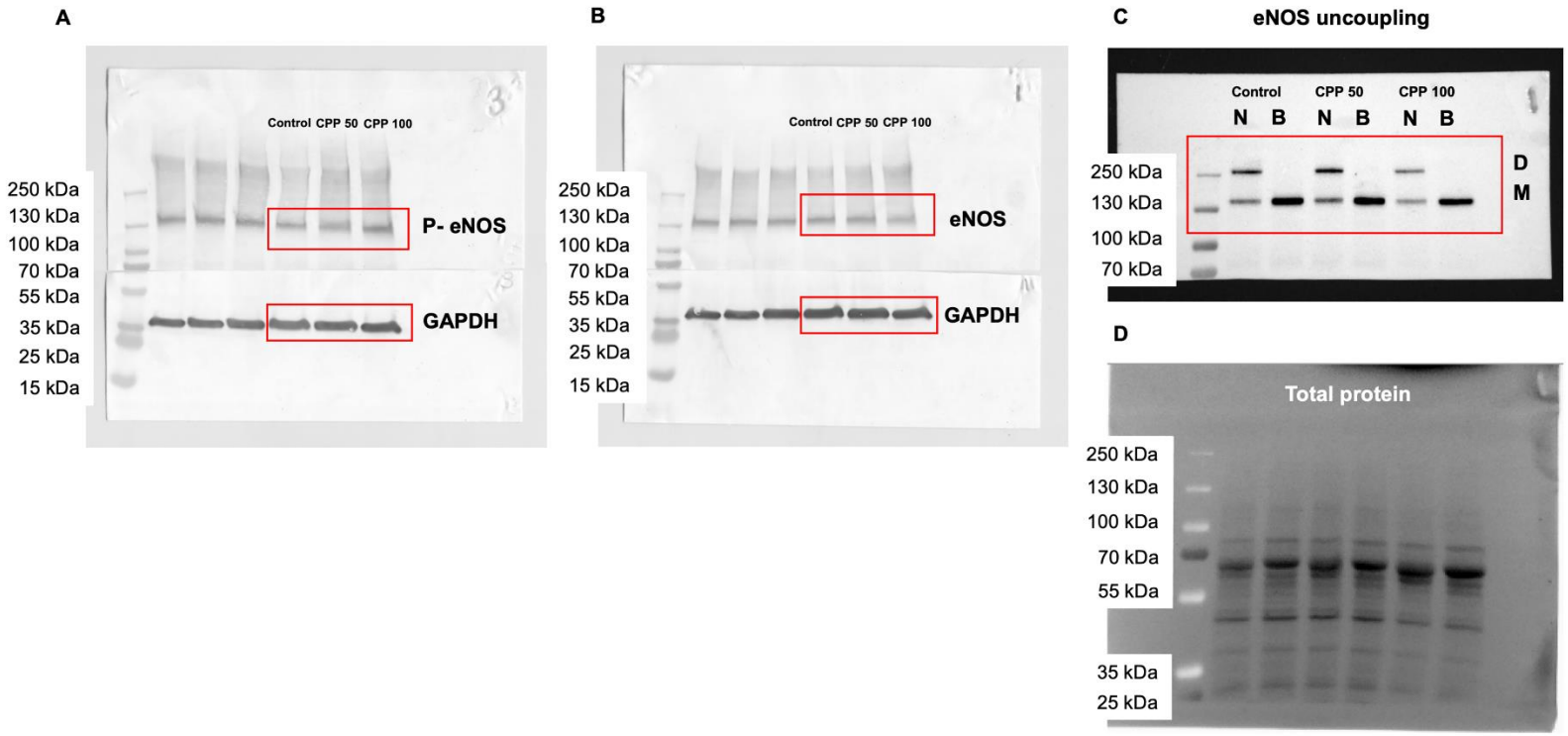

Figure S7: Full unedited blot of 24 h stimulation ECs with CPPs Figure 2. A: Uncropped blot of P-eNOS (Ser1177 phosphorylation) and GAPDH (input reference). B: Uncropped blot of eNOS and GAPDH (input reference). C: Low temperature blots with non-boiled/intact (indicated by symbol N) dimer/monomer eNOS expression (D/M) and boiled/denatured (indicated by symbol B) eNOS total monomer expression. Only upper section blot was used. D: Input correction total protein for eNOS uncoupling blot (Panel C). Molecular weight standards are indicated on the left side of the immunoblots in kilodaltons (kDa). Antibody names are indicated next to the corresponding band. Red squares show bands represented in the manuscript Figure 2, **24 h** exposure of ECs to CPPs.

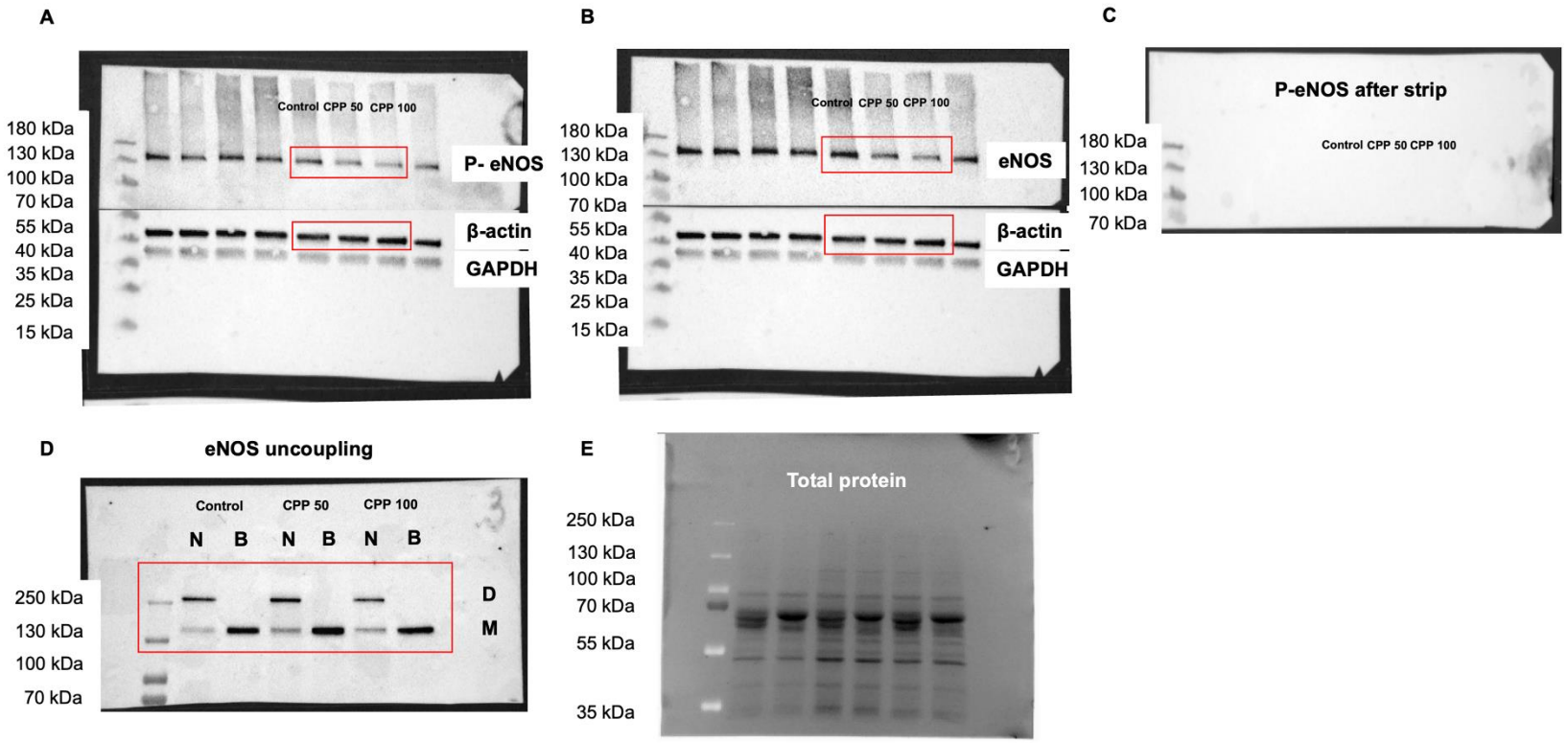

Figure S8: Full unedited blot of 72h stimulation ECs with CPPs Supplemental Figure 4. A: Uncropped blot of P- eNOS (Ser1177 phosphorylation) and corresponding β-actin and GAPDH expression. B: Uncropped blot of eNOS and corresponding β-actin and GAPDH expression. Both phosphorylated eNOS and total eNOS were measured on the same membrane. β-actin (input reference) is identical for both blots. C: P-eNOS blot after stripping blot, and before incubation with the total eNOS antibody. D: Low temperature blots with non-boiled/intact (indicated by symbol N) dimer/monomer eNOS expression (D/M) and boiled/denatured (indicated by symbol B) eNOS total monomer expression. Only upper section blot was used. Input correction was performed with total protein (Panel E). Molecular weight standards are indicated on the left side of the immunoblots in kilodaltons (kDa). Antibody names are indicated next to the corresponding band. Red squares indicate bands which are represented in the Supplemental Figure 4, **72 h** exposure of ECs to CPPs.

Supplemental Tables

Table S1: Half-maximum inhibitor concentration (IC_{50}) of RCA-rings after secondary CPP exposure.

Exposure time	Experimental group	IC_{50} (M)	
		Mean	***SEM***
2 h	Control	$2.335\ 10^{-8}$	$1.426\ 10^{-8}$
	CPP 100 µg/mL	$1.459\ 10^{-8}$	$1.657\ 10^{-9}$
	CPP 200 µg/mL	$1.741\ 10^{-8}$	$4.208\ 10^{-9}$
	CPP 400 µg/mL	$2.505\ 10^{-8}$	$1.070\ 10^{-8}$
Overnight (20 h)	Control	$1.266\ 10^{-8}$	$3.781\ 10^{-9}$
	CPP 100 µg/mL	$1.538\ 10^{-8}$	$4.247\ 10^{-9}$
	CPP 200 µg/mL	$3.114\ 10^{-8}$	$6.777\ 10^{-9}$
	CPP 400 µg/mL	$1.709\ 10^{-7}$	$1.188\ 10^{-7}$

Both acute (2 h) and long-term (overnight) exposure of RCA-rings to secondary CPPs (control, 100, 200 and 400 µg/mL) did not affect the IC_{50} of the endothelial-dependent relaxation curves. Rings were isolated from three individual porcine hearts and evenly distributed within groups (3-4 rings per heart per group; total 2h N = 7-9 rings per group, overnight N = 8-11 rings per group). Data are presented as means ±SEM (M).

Table S2: Multivariable linear regression analysis demonstrating associations between calcification propensity (OD_{650}) and NO_x levels as independent variables and eGFR as outcome variable.

	OD_{650}				NO_x		
	B	**95% CI**	***P*-value**		**B**	**95% CI**	***P*-value**
Model 1	-24.9	-31.1;-16.3	0.001	**Model 1**	34.9	29.8;39.2	<0.001
Model 2	-20.1	-26.5;-10.5	0.007	**Model 2**	29.9	24.5;34.5	<0.001
Model 3	-18.4	-25.2;-6.7	0.024	**Model 3**	26.3	19.8;31.5	<0.001
Model 4†	-17.8	-24.9;-3.9	0.040	**Model 4**‡	25.2	18.3;30.6	<0.001

Data are presented as unstandardized regression coefficients (B) with 95% confidence intervals (CI) and corresponding *P*-values. eGFR was square-transformed to achieve normally distributed distributions and homoscedasticity of residuals. Coefficients are presented after back-transformation, and are associated with a 1-SD increment or decrement in OD_{650} and NO_x since these variables were standardized using Z-scores. †Final model characteristics: $R^2 = 0.39$, df = 10, $p < 0.001$. ‡Final model characteristics: $R^2 = 0.49$, df = 10, $p < 0.001$. Model 1: crude model. Model 2, model 1 with adjustment for age and sex. Model 3, model 2 with adjustment for smoking, BMI, hypertension and chronic heart failure. Model 4, model 3 with adjustment for the use of statins, beta-blockers and nitrates.

Major Resources Table

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
eNOS (NOS3)	BD Biosciences	#610296	0.25 µg/mL (1:1000)	-	Purified Mouse Anti-eNOS/NOS Type III (bdbiosciences.com)
Phosphorylated eNOS (Ser1177)	BD Biosciences	#612393	0.50 µg/mL (1:500)	-	Purified Mouse Anti-eNOS (pS1177) (bdbiosciences.com)
GAPDH	Cell signaling	#5174S	34 ng/mL (1:1000)	-	GAPDH (D16H11) XP® Rabbit mAb | Cell Signaling Technology
β-actin	Cell signaling	#4967L	30 ng/mL (1:1000)	-	β-Actin Antibody | Cell Signaling Technology
3NT anti-nitrotyrosine	Santa Cruz	#39B6	4 µg/mL (1:50)	-	Anti-Nitrotyrosine Antibody (39B6) | SCBT - Santa Cruz Biotechnology
CD31	Santa Cruz	#SC-1506	4 µg/mL (1:50)	-	PECAM-1 Antibody (M-20) | SCBT - Santa Cruz Biotechnology
Isotype IgG_{2a} goat anti-mouse biotin	Southern Biotech	#1081-08	5 µg/mL (1:100)	-	Biotin Goat Anti-Mouse IgG2a | SouthernBiotech
AP-conjugated goat anti-mouse	DAKO	#D0486	0.67 mg/L (1:1500)	-	-
AP-conjugated goat anti-rabbit	DAKO	#D0487	0.64 mg/L (1:1000)	-	-
HRP-conjugated rabbit anti-mouse	DAKO	#P0260	1.3 mg/L (1:1000)	-	Rabbit Anti-Mouse Ig/HRP Antibody (solid-phase absorbed) | Agilent
HRP-conjugated goat anti-rabbit	DAKO	#P0448	0.3 mg/mL (1:1000)	-	P044801-2 | Agilent
Alexa Fluor 647 donkey anti- mouse	Invitrogen	#A-31571	8 µg/mL (1:250)	-	Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 647 (A-31571) (thermofisher.com)
Alexa Fluor 555 rabbit anti-goat	Thermo Fisher Scientific	#A-21431	8 µg/mL (1:250)	-	Rabbit anti-Goat IgG (H+L) Cross-Adsorbed, Alexa Fluor™ 555 (A-21431) (thermofisher.com)

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Human umbilical vein endothelial cells (HUVEC)	#CC-2519 Lonza	Mixed F & M	Pooled Human Umbilical Vein Endothelial Cells in EGM | Lonza

Other

Description	Source / Repository	Persistent ID / URL
Calcium colorimetric assay kit	#MAK022 Sigma-Aldrich	Calcium Colorimetric Assay sufficient for 250 colorimetric tests | Sigma-Aldrich (sigmaaldrich.com)
Measure-iT™ High-sensitivity Nitrite assay kit	#M36051 Thermo Fisher Scientific	Measure-IT™ High-Sensitivity Nitrite Assay Kit (thermofisher.com)
cDNA reverse transcriptase kit	#4368813 Thermo Fisher Scientific	High-Capacity cDNA Reverse Transcription Kit (thermofisher.com)
Protein Colorimetric Assay Kit	#5000111 Biorad	DC™ Protein Assay Kit I #5000111 | Bio-Rad
4-15% Mini-PROTEAN TGX Precast Protein gels	#4561084 Biorad	4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 50 µl #4561084 | Bio-Rad
Trans-Blot Turbo RTA Mini Nitrocellulose Transfer kit	#1704270 Biorad	Trans-Blot Turbo RTA Mini 0.2 µm Nitrocellulose Transfer Kit, for 40 blots #1704270 | Bio-Rad
MitoSOX Red reagent	#M36008 Thermo Fisher Scientific	MitoSOX™ Red Mitochondrial Superoxide Indicator, for live-cell imaging (thermofisher.com)
Avidin-Biotin kit	#SP-2001 Vector Laboratories	Avidin/Biotin Blocking Kit (vectorlabs.com)
Streptavidin-HRP conjugate	# P039701-2 DAKO	P039701-2 | Agilent
Lycopersicon esculentum Lectin (LEA)	#FL-1171 Vector Laboratories	Lectins: Lycopersicon Esculentum (Tomato) Lectin (LEL, TL), Fluorescein labeled (vectorlabs.com)
Fetuin-A ELISA kit	#RD191037100 BioVendor	Fetuin-A (AHSG) Human ELISA | BioVendor R&D
Nitrate and Nitrite (NO_x) kit	#ab65328 Abcam	Nitric Oxide Assay Kit (Colorimetric) (ab65328) | Abcam
Osteosense680EX dye	#NEV10020EX PerkinElmer	IVISense Osteo 680 Fluorescent Probe (OsteoSense) | PerkinElmer
PKH67 dye	#MINI67 Sigma-Aldrich	PKH67 Green Fluorescent Cell Membrane Label Sigma-Aldrich (sigmaaldrich.com)
Dihydroethidium (DHE) probe	#D11347 Thermo Fisher Scientific	Dihydroethidium (Hydroethidine) (thermofisher.com)
ROS inhibitor YCG063 (Calbiochem)	#557354 Sigma-Aldrich	https://www.sigmaaldrich.com/NL/en/product/mm/557354