SUPPLEMENTARY INFORMATION

Cytoprotective activated protein C averts Nlrp3 inflammasome induced ischemia reperfusion injury *via* mTORC1 inhibition

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Running headline: aPC restricts inflammasome activation

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Supplement material and methods

Reagents

Detailed ordering information of reagents are shown in supplementary table 1. The following antibodies were used in the current study: caspase-1, Nlrp3 and PAR4 (Santa Cruz, Heidelberg, Germany); caspase-3, caspase-7, BAX, raptor, hexokinase1, TSC1, p70S6K, and phospho-p70S6K (Cell Signaling Technology, Germany); IL-1β (Boster Immunoleader, CA, USA); EPCR and GAPDH (Sigma-Aldrich, Taufkirchen, Germany), and kidney injury molecule (KIM-1, Abcam, Germany). The following HRP-conjugated secondary antibodies were used for immunoblotting: rabbit IgG (Cell Signaling Technology, Germany) and mouse IgG (Abcam, Germany). The following secondary antibodies for immunofluorescence were used: Texas red-conjugated anti-mouse and FITC-conjugated anti-mouse and FITC-conjugated anti-goat (Vector Laboratories, CA, United States).

Other reagents were as follows: triphenyltetrazolium chloride (TTC), RPMI 1640, 2% Gelatin, tamoxifen, and pancreatin (Sigma-Aldrich, Taufkirchen, Germany); FLICA™ caspase-1 activity assay kit (Immunochemistry Technologies LLC.); DMEM, Trypsin-EDTA, penicillin, streptomycin, FCS, FBS, and HEPES (PAA laboratories, Pasching, Austria); protease inhibitor cocktail (Roche Diagnostics, Germany); BCA reagent (Perbio Science); Vectashield mounting medium (Vector Laboratories); PVDF membrane and immobilon^{TM}western chemiluminescent HRP substrate (Merck, Millipore, Massachusetts, United States); mouse IL-1β ELISA (R&D system); mouse IL-18 ELISA (Medical & biological laboratories co., Ltd); powdered milk, albumin fraction, and acrylamide (Carl ROTH, Karlsruhe, Germany) and collagenase II (Worthington, United States); formaldehyde 4% solution; medium 199, horse serum, and turbofect transfection reagents (Thermofisher Scientific, Germany); Trizol Reagent and PBS (Life Technologies; Germany); RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Germany); rompun 2% (Bayer, Leverkusen, Germany); ketamine 10% (beta-pharm, Vechta, Germany).

Myocardial ischemia reperfusion injury model

All mice were anesthetized with sodium ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg body weight, i.p.). In preliminary experiments we determined that equal dosing was required and sufficient in the different genotypes. After endotracheal intubation ventilation was performed using 0.3 L/min of oxygen and 1.5% isoflurane. Mice were placed on a 37°C thermostatically controlled operating platform. The surgery procedure did not differ among the groups. A left parasternal incision was made between the third and fourth ribs. The epicardium was removed to expose heart. The lateral anterior descending (LAD) artery was ligated using an 8-0 silk suture. Myocardial ischemia reperfusion injury (IRI) was induced by cautiously tightening the ligature around LAD. After LAD ligation the wound was temporally closed using a tape. After 90 min of ischemia the ligation was removed to restore blood flow through the LAD artery. Recovery of blood flow was visually ensured (as indicated by a reddish color). The chest was closed with continuous 4-0 polypropylene sutures. All animals received subcutaneous analgesic (buprenorphine 0.1 mg/kg) post-surgery. Mice were placed in a temperature controlled (~35°C) environment during the recovery phase and were regularly inspected. After full recovery animals were returned to their cages with free access to food and water. No differences in the recovery time between the different genotypes were noticed. Sham surgery consisted of an identical procedure without LAD ligation. After 24 hr of reperfusion animals were sacrificed. Blood samples were obtained from the inferior vena cava and the heart was isolated for further analyses. Different mice were used for infarct size determination and for protein expression.

Determination of infarct size

Isolated hearts were cut into 5 equally thick slices beginning from the lower tip of the heart up to the level where the ligature was set. Fresh heart slices were bathed in 1 % TTC solution (in PBS) at 37°C for 5 min. Tissue slices were then fixed in 10 % formalin solution for 10 min at room temperature (RT). Photos of heart tissue slices were taken using a digital camera (Nikon D750 KIT2) and the infarct area was determined using Image J software by two independent blinded investigators.

Isolation and culture of neonatal cardiomyocytes and cardiac fibroblasts

Primary neonatal cardiomyocytes and cardiac fibroblasts were isolated as described elsewhere.[1] Briefly, new born mice (age: 1 day) were decapitated and hearts were removed and placed in ice-cold 1 x ADS solution (6.8 g NaCl, 4.76 g HEPES, 0.12 g NaH_2PO_2, 1.0 g glucose, 0.4 g KCl, 0.1 g $MgSO_4$, pH was adjusted to 7.4 using 1 M NaOH) in a petri dish. After removing the atria heart ventricles were transferred to a tube containing 1 ml fresh 1 x ADS solution. Using sharp scissors heart ventricles were cut into small pieces, which were allowed to settle down. Then ADS solution was removed and 1.5 ml of freshly prepared enzyme solution was added (180 ml 1 x ADS with ~16800 U collagenase II (60mg) and 160 mg pancreatin). Tubes containing tissues pieces were placed on a shaker at 800 rpm at 37°C. After 6 min tissue were taken from shaker and homogenate were pipetted up and down, digested tissues samples were allowed to settle down, and enzyme solution was discarded. Fresh 1.5 ml enzyme solution was added again and the same procedure was repeated, except that the incubation time was increased from 6 to 10 min. After each digestion step the supernatant were collected and eventually pooled in “dark” medium containing 25 ml horse serum, 12.5 ml FCS and 212.5 ml of “light” medium (375 ml DMEM 4500 mg/l glucose, 125 ml Medium 199, 5 ml HEPES, 5 ml of Penicillin/Streptomycin). Tubes containing cell suspension were centrifuged at 1200 x g for 6 min at room temperature (RT). Following centrifugation supernatant was discarded and the pellet was re-suspended in 1 ml FBS and placed on ice. This final cell preparation was initially seeded into non-coated cultured dishes for 30 min and cardiac fibroblasts were allowed to adhere. These fibroblasts were used as neonatal cardiac fibroblasts for experiments. For cardiomyocytes, non-adherent cells were collected and centrifuged at 1200 x g for 10 min at RT. After resuspension of the pellet in 37°C “dark” medium cells were seeded onto plates pre-coated with gelatin (0.2% gelatin in PBS) and maintained at 37°C, in 5% CO_2. The purity of cells, which was routinely determined using the cardiomyocytes marker cTNT, was higher than 90% as measured by FACS analysis. These cells were used as cardiomyocytes for further experiments.

Isolation and culture of bone marrow derived macrophages

Bone marrow derived macrophages (BMDM) were isolated and cultured as described elsewhere.[2,3] Briefly, 10 to 12 weeks old C57BL/6J mice were sacrificed by cervical dislocation and bones were isolated from hind limbs (tibia, femur). Bones were kept in and flushed with RPMI-1640 complete medium containing 15% L929-cell conditioned medium (LCM) to isolate bone marrow cells. Bone marrow cells were further washed with 1 x PBS and resuspended in culture medium (RPMI-1640 (85%) – LCM (15%) medium. This procedure was repeated twice to remove dead cells. After the final washing step pelleted cells were resuspended in above culture medium. Cells were cultured for 7 to 10 days until ~80% confluence. The purity of cells was confirmed by CD11b staining and FACS analyses and was consistently found to be higher than 90%. These cells were used as BMDM for experiments.

Renal ischemia reperfusion injury model

Renal ischemia reperfusion injury was induced as described previously.[4] All mice were anesthetized with sodium ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg bodyweight, i.p.). Body temperature was maintained by placing the mice on a 37°C thermostatically controlled operating platform. The surgery procedure did not differ among the groups. Body fluid was maintained in all mice by subcutaneous administration of 300 µL 0.9% normal saline pre-operatively. A midline abdominal incision was made and both kidneys were exposed. The main renal arteries and veins were identified using a stereotactic microscope (Olympus, Germany), and great care was taken to identify all vascular branches. All renal arteries and veins were then bilaterally occluded for 30 min with non-traumatic microaneurysm clamps (F.S.T Instruments, Germany). To help maintain thermoregulation during ischemia time, the intestine was relocated and the abdomen was temporarily closed with few stitches. After 30 min of renal ischemia the abdomen was reopened and the clamps were removed. The kidneys were inspected for at least 1 min to ensure restoration of blood flow (as indicated by a pink color) and 0.5 ml of pre-warmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed with continuous 4-0 polypropylene sutures. All animals received subcutaneous analgesic (buprenorphine 0.1 mg/kg) at the end of surgery. Mice were routinely placed in a temperature controlled (~35°C) environment during the recovery phase and regularly inspected. No differences in the recovery time between the different genotypes were noticed. After full recovery animals were returned to their cages with free access to food and water. Sham surgery consisted of an identical procedure without application of the microaneurysm clamps. Animals were sacrificed 24 hr after renal ischemia reperfusion injury or sham surgery to obtain blood and tissue samples.

Preparation of activated protein C

Activated protein C was generated as previously described with slight modifications.[4,5] Briefly, prothrombin complex (Prothromplex NF600), containing all vitamin K dependent coagulation factors, was reconstituted with sterile water and supplemented with $CaCl_2$ at a final concentration of 20 mM. The column for purification of protein C was equilibrated at RT with 1 liter of washing buffer (0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide). The reconstituted prothrombin complex was gravity eluted on a column filled with Affigel-10 resin covalently linked to a calcium-dependent monoclonal antibody to PC (HPC4). The column was washed first with two column volumes of washing buffer and then two column volumes with a wash buffer rich in salt (0.5 M NaCl, 20 mM Tris, pH 7.5, mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide). Then the benzamidine was washed off the column with a buffer of 0.1 M NaCl, 20 mM Tris, pH 7.5, 2 mM Ca^{2+}, 0.02% sodium azide. To elute PC the column was gravity eluted with elution buffer (0.1 M NaCl, 20 mMTris, pH 7.5, 5 mM EDTA, 0.02% sodium azide, pH 7.5) and 3 ml fractions were collected. The peak fractions were identified by measuring absorbance at 280 nm. The peak fractions were pooled. The recovered PC was activated with human plasma thrombin (5% w/w, 3 hr at 37°C). To isolate activated protein C (aPC) ion exchange chromatography with FPLC (ÄKTAFPLC®, GE Healthcare Life Sciences) was used. First, thrombin was removed with a cation exchange column MonoS (GE Healthcare Life Sciences). Then a MonoQ anion exchange column (GE Healthcare Life Sciences) was equilibrated with 10% of a 20 mM Tris, pH 7.5, 1 M NaCl buffer. After applying the solution that contains aPC a 10-100% gradient of a 20 mM Tris, pH 7.5, 1 M NaCl buffer was run through the column to elute aPC at a flow of 1-2 ml/min under continuous monitoring of OD and conductivity. APC eluted at ~36 mS/cm by conductivity or at 40% of the buffer. Fractions of 0.5 ml were collected during the peak and pooled. Proteolytic activity of purified aPC was ascertained with

the chromogenic substrate SPECTROZYME® PCa. Human recombinant aPC variant lacking specifically anticoagulant function 3K3A-aPC was prepared as described elsewhere.[6]

Determination of plasma BUN and creatinine

Plasma BUN and creatinine were measure as described. [4,7] Mice were anesthetized 24hr after reperfusion with sodium ketamine (100 mg/kg bodyweight, i.p.) and xylazine (10 mg/kg body weight, i.p.) and sacrificed. Blood samples were obtained from the abdominal vena cava and collected into tubes pre-filled with sodium citrate (final concentration 0.38%). Plasma was obtained by centrifugation at 2000 x g for 10 min. Plasma BUN was measured using a kinetic test kit with urease (Roche Diagnostics, Cobas c501 module) and creatinine was determined by an enzymatic based kit (Roche Diagnostics, Cobas c501 module) at the Institute of Clinical Chemistry and Pathobiochemistry, Medical Faculty, Otto-von-Guericke University, Magdeburg, Germany.

Immunoblotting

Proteins were isolated and immunoblotting was performed as described.[4,7-12] Cell lysates were prepared in RIPA buffer (50 mM Tris at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF supplemented with protease inhibitor cocktail). Lysates were centrifuged (10,000 × g for 10 min at 4 °C) and insoluble debris was discarded. The protein concentration in supernatants was quantified using BCA reagent. Equal amounts of protein were electrophoretically separated on 7.5%, 10% (vol/vol) or 12.5% (vol/vol) SDS polyacrylamide gels, transferred to PVDF membranes, and probed with the desired primary antibodies overnight at 4°C. Membranes were then washed with PBS-tween (PBST) and incubated with anti-mouse (1:2,000), anti-rat IgG (1:2,000), or anti-rabbit IgG (1:2,000) horseradish peroxidase-conjugated antibodies, as indicated. Blots were developed with the enhanced chemiluminescence system. To compare and quantify levels of proteins, the density of each band was measured by using ImageJ software. Equal loading was confirmed by immuno-blotting with GAPDH antibody.

Histology and immunohistochemistry

Sacrificed mice were perfused with ice-cold PBS and then with 4% buffered paraformaldehyde.[4,7-10,12] Tissues were further fixed in 4% buffered paraformaldehyde for 2 days at 4°C, embedded in paraffin and processed for sectioning. Kidney injury was evaluated using hematoxylin and eosin stained histological sections. Images of the outer third of the kidney sections were randomly chosen and captured using an Olympus Bx43 Microscope (Olympus, Hamburg, Germany). All tubuli within an image were individually scored on a scale of 0-4 based on the cellular damage as indicated by morphological signs of cell-swelling and tubular dilatation. The following scores were assigned: 0 – no cellular or tubular damage visible; 1 – damage visible, but less than 25% of the tubuli affected; 2 – 25% to 50% tubular damage; 3 – 50% to 75% tubular damage; and 4 – more than 75% damage. At least 5 random images per mouse and at least 6 mice per group were included in each group. All histological analyses were done by two independent blinded investigators. Images were obtained using an Olympus Bx43 Microscope (Olympus, Hamburg, Germany) at 20 x or 40 x magnification.

Caspase-1 activity assay

Caspase-1 activity was determined on frozen heart sections using the FLICA Casp-1 assay (Immunochemistry Technologies lcc.).[10] Frozen tissue sections (5µM thick) were prepared and allowed to air-dry. Then slides were fixed with acetone for 1 min, rehydrated by washing (twice for 5 min) in PBST and slides were blocked for 20 min with blocking solution containing 20% aqua-block in media

with 0.2% Tween. Blocking solution was decanted and then 50 µL of 3 x FLICA™ working solution (freshly prepared by diluting 150 x stock solution into 1:50 in PBS) was applied per section and incubated at RT for 2 hr, protected from light. Tissues were washed with PBST (twice for 5 min). Tissues were mounted with vactashield mounting medium containing DAPI and fluorescent images were captured with an Olympus Bx43-Microscope (Olympus, Hamburg, Germany). Images analyses were done by using Image J software.

Cell culture and *in vitro* hypoxia-reoxygenation

For hypoxia-reoxygenation (HR) injury neonatal cardiomyocytes cells were moved from routine culture conditions ("dark medium", 37°C, in 5% CO_2, 21% O_2) into hypoxic atmosphere (1% O_2, 94% N_2, and 5% CO_2) and serum- and glucose-depleted medium (HBSS) for 6 hr. For reoxygenation cells were returned to normal "dark" medium and 5% CO_2, 21% O_2.[4] Control cells were serum-starved and maintained in HBSS for 6 hr, but they were continuously exposed to 21% O_2. Cells were harvested after 12 hr of reoxygenation for protein isolation.

Reverse transcriptase polymerase chain reaction (RT-PCR)

To isolate RNA from cells, 3 ml TRIzol was added to 10 cm^2 dish. Cells lysed in TRIzol were incubated for 5 min at RT before adding 0.2 ml of chloroform per 1 ml of TRIzol. Following vigorous shaking the mixture was centrifuged at 12,000 x g for 15 min at 4°C to separate different phases. The aqueous phase containing the RNA was transferred to a fresh tube and RNA was precipitated by adding 0.5 ml of 2-propanol per 1 ml of TRIzol reagent. Following incubation at RT for 10 min samples were centrifuged at 12,000 x g for 10 min at 4°C. Supernatant was removed and RNA was washed by adding 1 ml 75 % ethanol per 1ml of TRIzol. Samples were vortexed and centrifuged at 7,500 x g for 5 min at 4°C. The RNA pellet was air-dried for 5 min, redissolved in 20 µl DEPC-water at 55°C for 10 min. RNA concentration was measured in a photometer and a 1.8 % agarose gel was run to verify the purity and integrity of RNA. cDNA was synthesized according to manufacture's protocol (SuperScript First-Strand Sythesis System for RT-PCR. Primers were custom synthesized by Thermo Fisher Scientific and PCR was performed using the Taq polymerase. PCR products were separated on a 1.5% (wt/vol) agarose gel and visualized by ethidium bromide staining. Reactions lacking reverse transcriptase served as negative controls. Primers used in the current study are shown in supplementary table S2.

IL-1β and IL-18 immunoassay

Mouse blood samples were obtained from the inferior vena cava of anticoagulated mice (sodium citrate). Plasma was obtained by centrifugation of blood samples for 10 min at 2000 g at RT. Plasma samples were stored at -80°C until analyses. We measured the concentrations of mouse IL-1β by ELISA and IL-18 by ELISA, according to manufacturer's instructions.

References

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Supplementary tables

Supplementary table S1:

REAGENTS	SOURCE	CATALOGUE #
Nlrp3	Santa Cruz Biotechnology	#sc-66846
Nlrp3	Cell Signalling Technology	#15101
Caspase-1	Santa Cruz Biotechnology	#sc-56036
Caspase-1	Merck Millipore	#AB1871
IL-1β	Boster Immunoleader	#PA1351
Caspas-3	Cell Signalling Technology	#9662S
Caspase-7	Cell Signalling Technology	#9492S
Bax	Cell Signalling Technology	#2772S
Raptor	Cell Signalling Technology	#2280
HK-1	Cell Signalling Technology	#2024
P70 S6	Cell Signalling Technology	#9202
Phospho-p70 S6	Cell Signalling Technology	#9234
TSC-1	Cell Signalling Technology	#4906
KIM-1	Abcam	#ab47635
GAPDH	Sigma-Aldrich	#G8795
Rabbit IgG	Cell Signalling Technology	#7074
Mouse IgG	Abcam	#ab6728
Caspase-11	Cell Signalling Technology	#14340
IRAK-1	Cell Signalling Technology	#4504
Rictor	Cell Signalling Technology	#2140
EPCR	R & D systems	#AF2749
ApoER2	Novus Biologicals	#NB100-2216
CD11b	Novus Biologicals	#NB110-89474SS
S1P1	Merck Millipore	#MABC94
Texas Red-conjugated anti-mouse	Vector Laboratories	#TI-1000
FITC-conjugated anti-mouse	Vector Laboratories	#FI-1000
Tamoxifen	Sigma-Aldrich	#T5648
LPS	Sigma-Aldrich	#LPS25
ATP	Sigma-Aldrich	#A2383
PAR-4 (Blocking antibody)	Santa Cruz Biotechnology	#SC-8461L
EPCR (mAb 1560; Blocking antibody)	Dr. Charles T. Esmon Laboratory of Coagulation Biology, Oklahoma Medical Research Foundation, Oklahoma City, United States.	
HAPC 1573	Dr. Charles T. Esmon Laboratory of Coagulation Biology, Oklahoma Medical Research Foundation, Oklahoma City, United States	
Parmodulin-2	Dr. Chris Dockendorff, Dept. of Chemistry, Marquette University, Milwaukee, WI, USA	
3K3A-aPC	Dr. John H. Griffin, Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA	
P1pal-12S	Genescript	#RSLSSSAVANRS

Gelatin	Sigma-Aldrich	#G1393
Pancreatin	Sigma-Aldrich	#P3292
Collagenase Type II	Worthington	#LS004176
mEPCR shRNA	GE Dharmacon	#19124
mApoER2 shRNA	GE Dharmacon	#16975
CalPhos Mammalian Transfection Kit	Takara-Clonetech	#631312
cDNA synthesis Kit	Invitrogen	#18080051
S1P1 Antagonist	Tocris	#3602
NIF (CD11b) Antagonist	R&D systems	#5845-NF-050
FLICA™ Caspase-1 activity assay kit	ImmunoChemistry Technologies, LLC	#97
mouse IL-1beta ELISA kit	R&D Systems	#MLB00C
mouse IL-18 ELISA kit	Medical & biological laboratories co., Ltd	#7625
TTC	Sigma-Aldrich	#T8877
RPMI 1640	Sigma-Aldrich	#R0883
Trypsin-EDTA	Thermo Fisher Scientific	#2500-054
Penicillin/Streptomycin	Thermo Fisher Scientific	#15140-122
HEPES	Thermo Fisher Scientific	#15630-056
Pierce™ BCA protein assay kit	Perbio Science	#23227
Vector shield mounting medium with DAPI	Vector Laboratories	#CA94010
PVDF membrane	Merck Millipore	#IPVH00010
Immobilion™ chemiluminescent HRP substrate	Merck Millipore	#WBKLS0500
Powdered milk	CARL ROTH	#T145.2
Albumin fraction	CARL ROTH	#8076.2
Rotiphorese® Gel	CARL ROTH	#3029.1
Medium 199	Thermo Fisher Scientific	#3115002
Horse serum	Thermo Fisher Scientific	#26050088
Trizol Reagent	Life Technologies	#15596018
Phosphate Buffer Saline (PBS)	Thermo Fisher Scientific	#10-010-031
Rompun 2%	Bayer	770-081
Ketamine	Beta-pharm	798-744
Tween-20	CARL ROTH	#9127.1
2-Propanol	CARL ROTH	#CP41.1
HBSS	Thermo Fisher Scientific	#14025-050
DMEM	Sigma-Aldrich	#D6429
Paraformaldehyde	Thermo Fisher Scientific	#PI28908
Turbofect transfection kit	Thermo Fisher Scientific	#R0531
Protease Inhibitor Cocktail	Roche Diagnostics	#11 836 153 001

Supplementary table S1: List of reagents used in current study.

Supplemental table 2

Target	Primer Sequence
mPAR1	5'CCAGCCAGAATCAGAGAGGA3' 5'TCGGAGATGAAGGGAGGAG3'
mPAR2	5'CCAGGAAGAAGGCAAACATC3' 5’TGTCCCCCACCAATACCTC3'
mPAR3	5'CATCCTGCTGTTTGTGGTTG3' 5'TACCCAGTTGTTGCCATTGA3'
mPAR4	5'GCAGACCTTCCGATTAGCTG3' 5'CACTGCCGAGAACAGTACCA3'
mEPCR	5'CTACAACCGGACTCGGTATGAA3' 5'CCAGGACCAGTGATGTGTAAG A3'
mβ-actin	5'CTAGACTTCGAGCAGGAGATGG3' 5'GCTAGGAGCCAGAGCAGTAATC3'

Supplementary table S2: List of primers used in current study.

Supplementary Figures
Figure S1

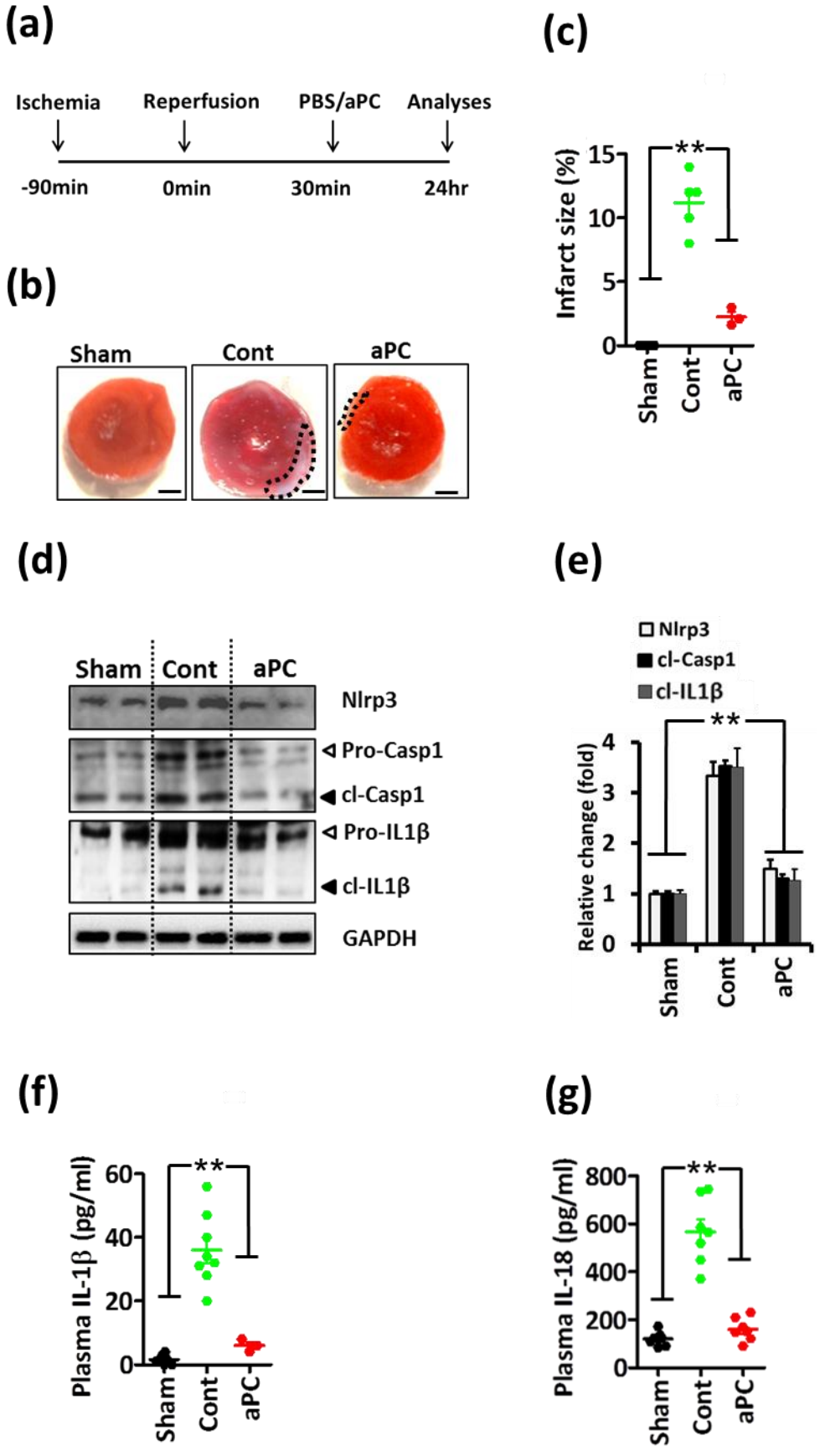

Figure S1: aPC post myocardial IRI treatment restricts Nlrp3 inflammasome activation

a: Experimental design.

b,c: aPC treatment 30 min after reperfusion reduces infarct size. Representative heart sections showing infarcted area detected by TTC staining (**b**, black doted encircled area, size bar: 20 µm) and dot-plot summarizing data (**c**).

d-g: aPC treatment 30 min after reperfusion significantly reduced cardiac Nlrp3 expression and cleaved caspase-1 (cl Casp1) and cleaved IL-1β (cl IL-1β). Representative immunoblots (**d**) and bar graph summarizing data (**e**); GAPDH: loading control. Arrowheads indicate inactive (white arrowheads) and active (black arrow heads) form of caspase-1 or IL-1β (**d**). The active form was quantified (**e**). Reduced plasma IL-1β (**f**) and IL-18 levels (**g**), dot blots summarizing data.

Sham operated mice (Sham) or mice with myocardial IRI without (Cont, PBS) or with aPC posttreatment (aPC). Data shown represent mean±SEM of at least 6 mice per group; **P<0.01 (**c, e, f, g**: ANOVA).

Figure S2

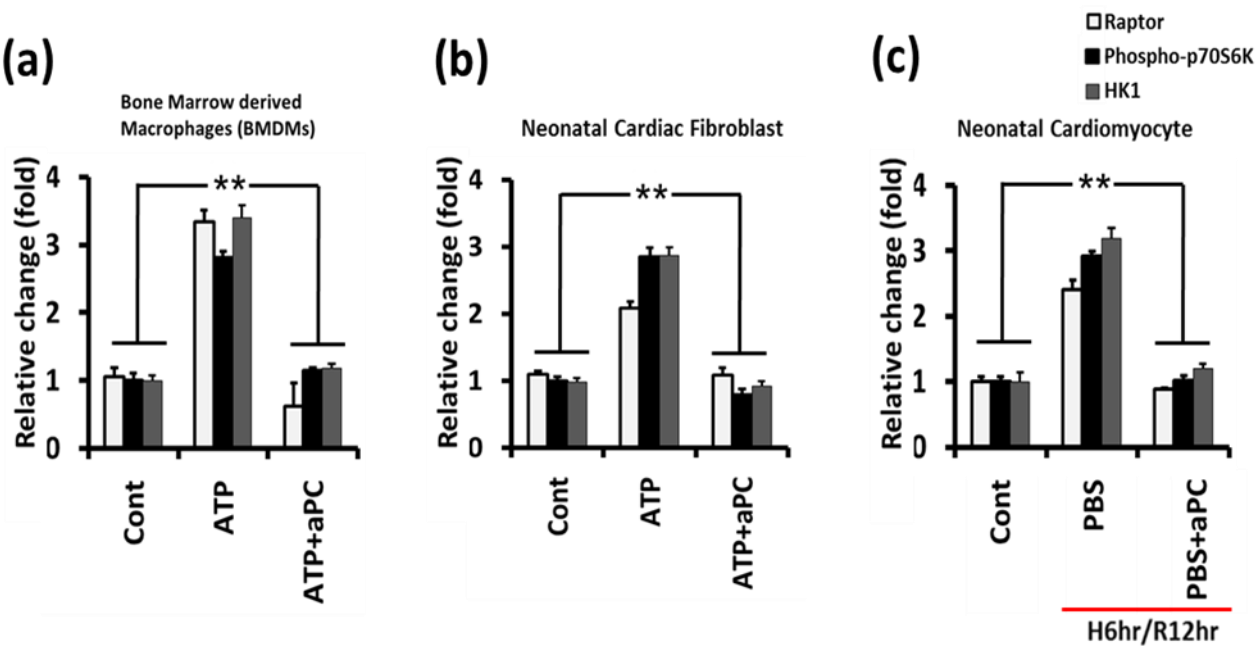

Figure S2: aPC restricts Nlrp3 inflammasome by suppressing mTORC1

a-c: Bar graphs corresponding to Fig. 4a-c. Bar graphs represent mean±SEM of Raptor (open bars) and HK1 (grey bars) expression and phosphorylation of ribosomal p70-S6 kinase (pS6K70, black bars) obtained from at least three independent experiments each with at least two technical replicates; **P<0.01 (**a, b, c**: ANOVA).

Figure S3

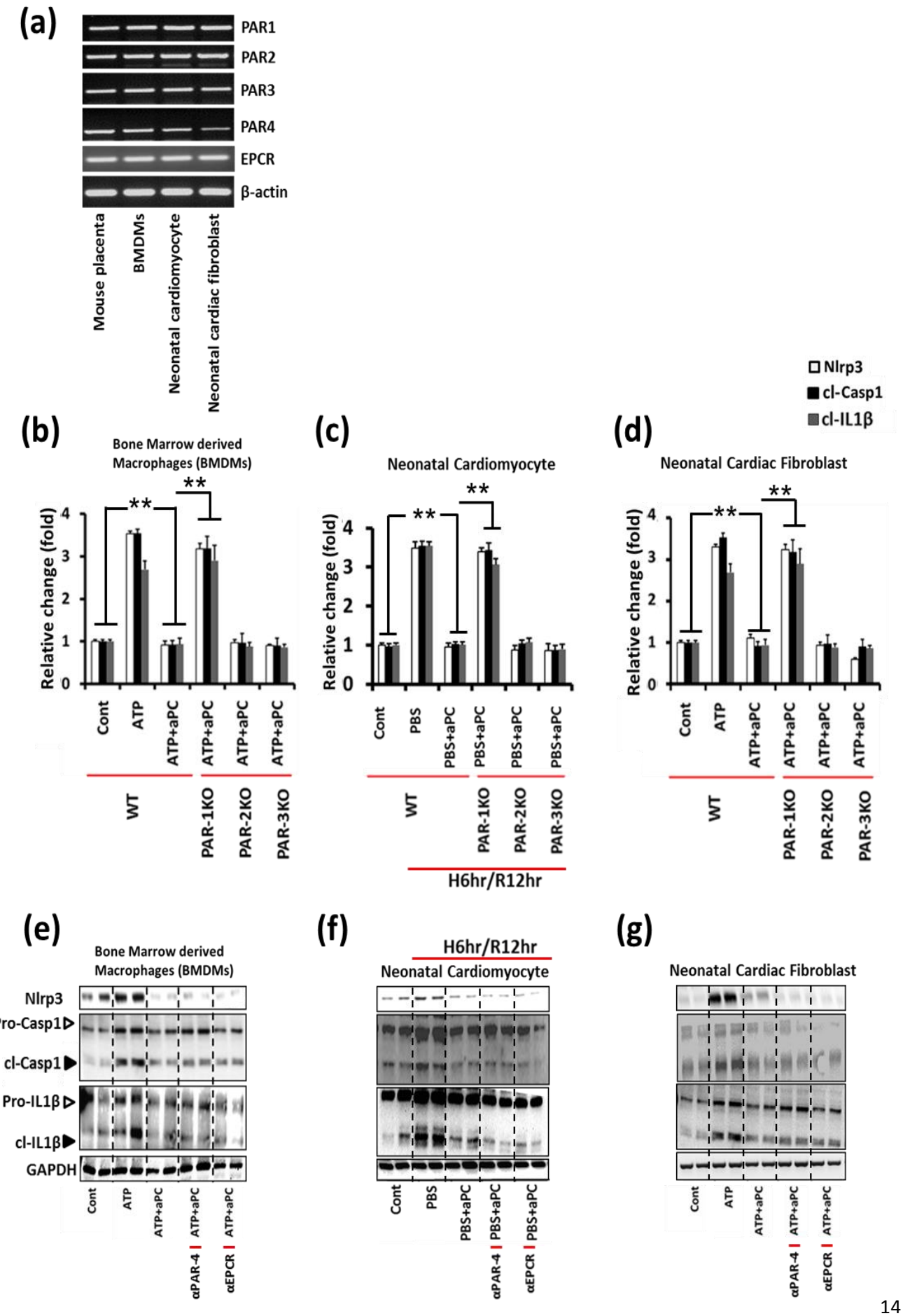
(a)
PAR1
PAR2
PAR3
PAR4
EPCR
β-actin
Mouse placenta
BMDMs
Neonatal cardiomyocyte
Neonatal cardiac fibroblast
Nlrp3
cl-Casp1
cl-IL1β
(b)
Bone Marrow derived Macrophages (BMDMs)
Relative change (fold)
Cont
ATP
ATP+aPC
WT
PAR-1KO
PAR-2KO
PAR-3KO
(c)
Neonatal Cardiomyocyte
PBS
PBS+aPC
H6hr/R12hr
(d)
Neonatal Cardiac Fibroblast
(e)
Bone Marrow derived Macrophages (BMDMs)
Nlrp3
Pro-Casp1
cl-Casp1
Pro-IL1β
cl-IL1β
GAPDH
αPAR-4
αEPCR
(f)
H6hr/R12hr
Neonatal Cardiomyocyte
(g)
Neonatal Cardiac Fibroblast

Figure S3: aPC restricts Nlrp3 inflammasome activation via PAR-1 in vitro

a: Mouse BMDMs, neonatal cardiomyocytes and neonatal fibroblasts express all PARs (PAR1-4) and EPCR; representative RT-PCR gel images; mouse placenta tissue was used as positive control and β-actin as loading control.

b-g: The effect of aPC on inflammasome activation was analysed following loss or inhibition of PARs or EPCR. BMDMs (**b**), neonatal cardiomyocytes (**c**), or neonatal fibroblasts (**d**) were isolated from wt, PAR1-/-, PAR2-/- or PAR3-/- mice (**b-d**) or PAR4 and EPCR function was blocked in wt cells using blocking antibodies (αPAR4, αEPCR; each 20 µg/ml; **e-g**). In all three cell types aPC fails to suppress LPS/ATP (**b,d**) or H/R (**c**) induced Nlrp3 expression (open bars) and levels of caspase-1 (cl Casp1, black bars) and cleaved IL-1β (cl IL-1β, grey bars) in the absence of PAR1, while loss or blocking of other receptors had no effect (**b-g**). Bar graphs summarizing results of PAR1-3 deficient BMDMs (**b**), neonatal cardiomyocytes (**c**) and neonatal cardiac fibroblasts (**d**, corresponding representative immunoblot images shown in Fig. 5a-c). Representative immunoblots of inflammasome activation in BMDMs (**e**), neonatal cardiomyocytes (**f**), and neonatal cardiac fibroblasts (**g**) following PAR4 and EPCR inhibition using blocking antibodies (αPAR4, αEPCR).

Arrowheads indicate inactive (white arrowheads) and active (black arrowheads) form of caspase-1 or IL-1β (e-g). The active form was quantified (**b-d**); GAPDH: loading control. Data shown represent mean±SEM of at least three independent experiments each with at least two technical replicates (**b-d**); **P<0.01 (**b, c, d**: ANOVA).

Figure S4

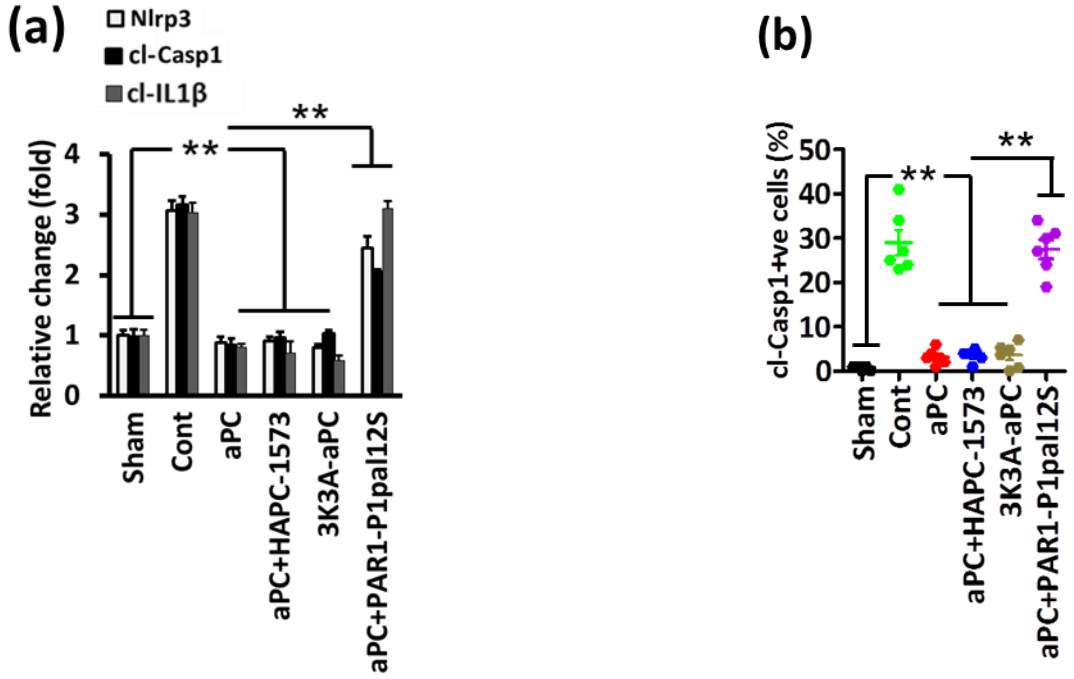

Figure S4: aPC restricts Nlrp3 inflammasome activation via PAR1 following myocardial IRI

a,b: Bar graph (**a**) and dot-plot (**b**) corresponding to Fig. 5g and h, respectively. Treatment of mice with aPC–HAPC1573 complex (aPC was preincubated before injection with HAPC1573 antibody at a 1:1 molar ratio for 10 min under gentle agitation to block its anticoagulant activity), or an aPC variant lacking specifically anticoagulant function (3K3A-aPC, 1mg/kg, i.p.) inhibits markers of inflammasome activation as efficient as aPC, while blocking PAR1 signaling (pepducin P1pal-12S, 2.5 mg/kg subcutaneously) abolishes aPC inhibitory effect. Bar graph corresponding to Fig. 5g, summarizing results of immunoblots; the active form of caspase-1 and IL-1β (cl Casp1 and cl IL-1β) was quantified (**a**). Dot-plot corresponding to Fig. 5h, summarizing data of caspase1-activity detected by caspase1-FLICA probes (**b**).

Sham operated mice (Sham) or mice with myocardial IRI with PBS (Cont), wild type aPC (aPC), aPC–HAPC1573 complex (aPC+HAPC1573), an aPC variant lacking specifically anticoagulant function (3K3A-aPC), or aPC plus P1pal-12S (aPC+P1pal-12S) pretreatment. Data shown represent mean±SEM of at least 6 mice per group; **P<0.01 (**a, b**: ANOVA).

Figure S5

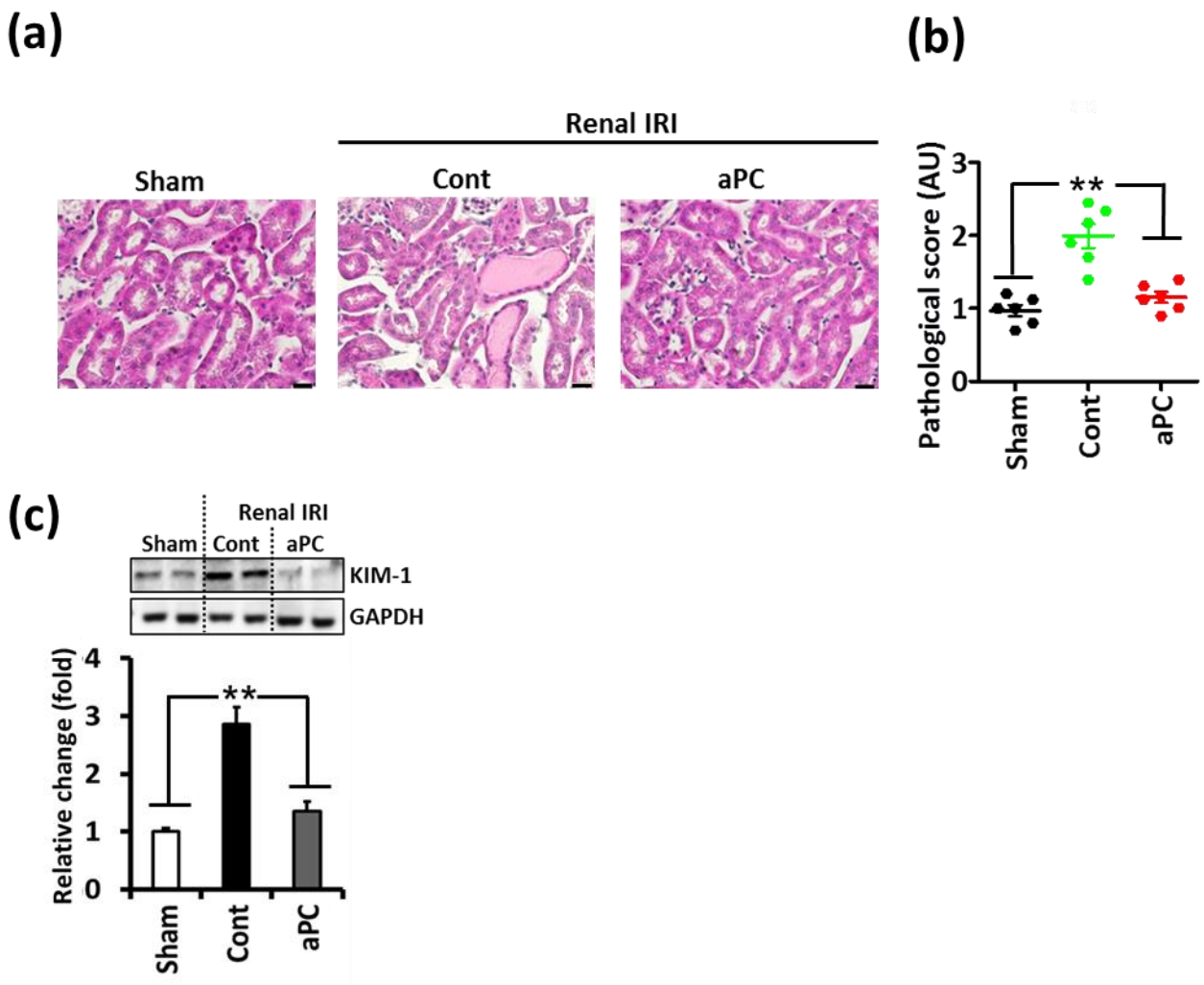

Figure S5: aPC protects against renal ischemia reperfusion injury by limiting Nlrp3 inflammasome

a-c: aPC protects mice from acute renal injury following IRI. Exemplary images of H&E-stained kidney section from wild-type sham operated mice and IRI mice without (Cont) or with aPC treatment (**a**) and dot-plot summarizing results of pathologic scores (**b**). aPC treatment inhibits expression of the kidney injury marker (KIM-1) in renal IRI. Representative immunoblots (GAPDH: loading control) and bar graph summarizing results (**c**).

Sham operated mice (Sham) or mice with renal IRI with PBS (Cont) or aPC treatment. Data shown represent mean±SEM of at least 6 mice per group; **P<0.01 (**b, c**: ANOVA).

Figure S6

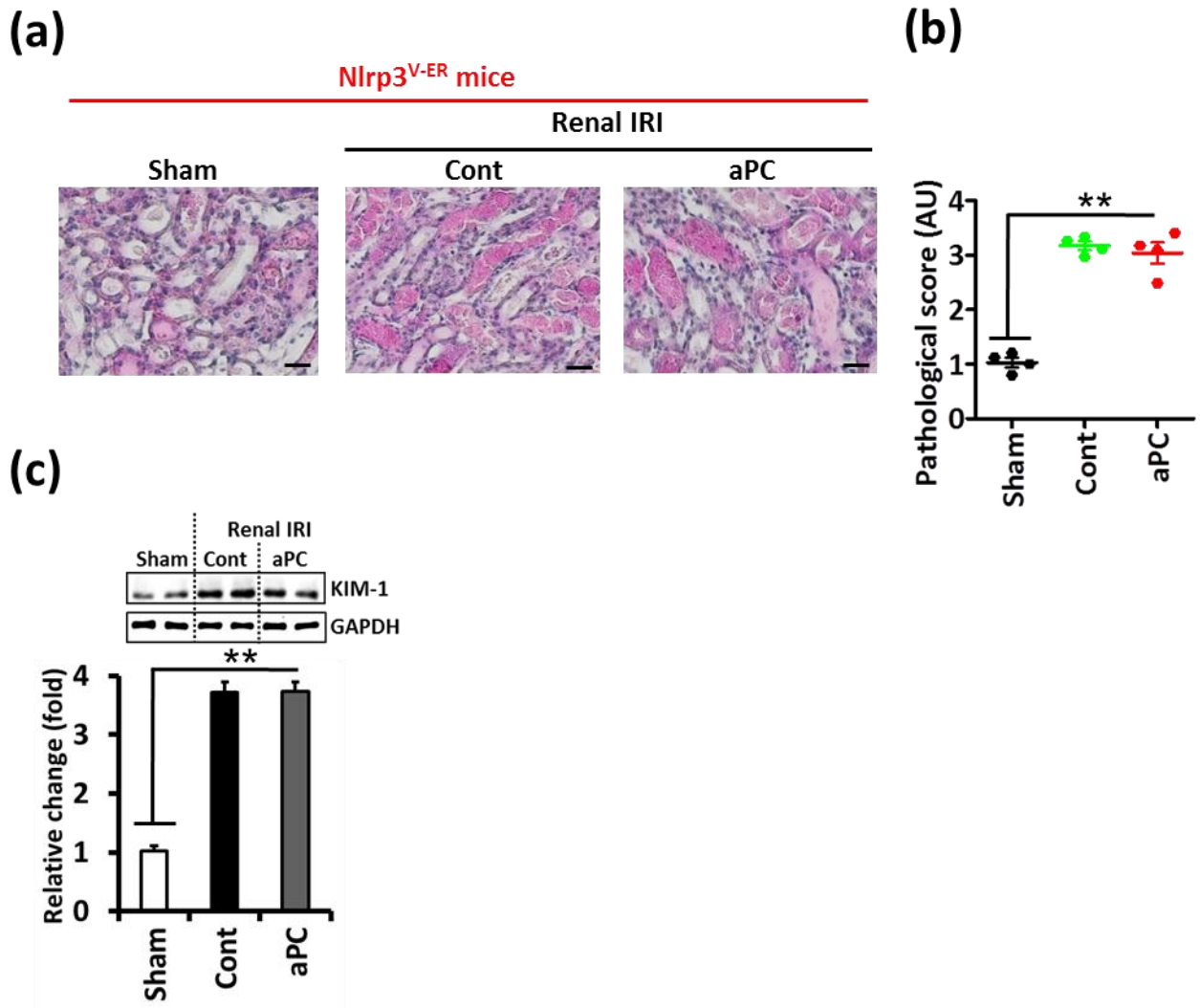

Figure S6: aPC fails to protect against renal IRI in Nlrp3$^{V\text{-}ER}$ mice.

(**a-c**): aPC treatment fails to protect against renal IRI in Nlrp3$^{V\text{-}ER}$ mice. Exemplary images of H&E-stained kidney section from Nlrp3$^{V\text{-}ER}$ mice (**a**) and dot-plot summarizing results of pathologic scores (**b**). aPC treatment failed to reduce expression of the kidney injury marker (KIM-1) in Nlrp3$^{V\text{-}ER}$ mice in renal IRI. Representative immunoblots (GAPDH: loading control) and bar graph summarizing results (**c**).

Sham operated mice (Sham) or mice with renal IRI with PBS (Cont) or aPC treatment. Data shown represent mean±SEM of at least 6 mice per group; **P<0.01 (**b, c**: ANOVA).