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BASIC SCIENCE

Genetic deletion of the adaptor protein p66^{Shc} increases susceptibility to short-term ischaemic myocardial injury via intracellular salvage pathways

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Aims Several intracellular mediators have been implicated as new therapeutic targets against myocardial ischaemia and reperfusion injury. However, clinically effective salvage pathways remain undiscovered. Here, we focused on the potential role of the adaptor protein p66^{Shc} as a regulator of myocardial injury in a mouse model of cardiac ischaemia and reperfusion.

Methods and results Adult male p66^{Shc} deficient (*p66$^{Shc-/-}$*) and C57Bl/6 wild-type (WT) mice were exposed to 30, 45, or 60 min of ischaemia and reperfusion (5, 15 min, or 24 h). Infarct size, systemic and intracardiac inflammation and oxidants, as well as cytosolic and mitochondrial apoptotic pathways were investigated. Following 30, but not 45 or 60 min of ischaemia, genetic p66^{Shc} deficiency was associated with larger infarcts. In WT mice, *in vivo* p66^{Shc} knock down by siRNA with transient protein deficiency confirmed these findings. P66^{Shc} inhibition was not associated with any modification in post-infarction inflammation, oxidative burst nor cardiac vessel density or structure. However, in *p66$^{Shc-/-}$* mice activation of the protective and anti-apoptotic *Reperfusion Injury Salvage Kinases* and *Survivor Activating Factor Enhancement* pathways were blunted and mitochondrial swelling and cellular apoptosis via the caspase-3 pathway increased compared with WT.

Conclusions Genetic deletion of p66^{Shc} increased susceptibility to myocardial injury in response to short-term ischaemia and reperfusion in mice. Still, additional studies are needed for assessing the role of this pathway in acute coronary syndrome patients.

Keywords Acute myocardial infarction • Inflammation • Ischaemia • Reperfusion

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Translational perspective

Genetic deletion of p66^{Shc}, as shown in the present study, leads to increased myocardial infarction in response to short-term ischaemia and reperfusion. Therefore, heart-specific activation of p66^{Shc} protein may represent a promising novel strategy to prevent ischaemic and reperfusion myocardial injury. In particular, pharmacological modulation of apoptosis via myocardial salvage pathways involving p66^{Shc} might be a promising approach to limit short-term ischaemic injury, for instance in patients with acute coronary syndrome (ACS) from the time of symptom onset to percutaneous coronary intervention. However, the present study also adds complexity to the use of this pathway as a therapeutic target. Indeed, given the different effects of activation and silencing of p66^{Shc} in different cells, tissues and organs, tissue selective inhibition would be required. Indeed, while short-term activation might be protective in the context of an ACS, long-term inhibition may prevent endothelial dysfunction, atherosclerosis, and diabetic vascular disease. Obviously, this complexity also raises safety concerns for the potential use of p66^{Shc} in acute myocardial infarction that need to be clarified by additional research.

Introduction

Myocardial injury during short-term ischaemia and reperfusion has become clinically important with the use of primary percutaneous coronary angioplasty as a first-line strategy in patients with acute coronary syndrome (ACS). Indeed, time from symptom onset to reperfusion is a major determinant of outcome.[1] Thus, activation of protective pathways during this vulnerable period of ACS remains a clinical need. Knowledge of the mechanisms of myocardial ischaemia and reperfusion has been deepened with the use of animal models of human disease.[2,3] Several molecular and cellular targets involved in cardiac injury and repair have been identified that are activated to protect cardiomyocytes from ischaemic insults.[4–7] Indeed, a large number of protein kinases are activated at different levels and time points during ischaemia–reperfusion injury. Recently, three distinct cardioprotective pathways for those protein kinases have been proposed.[8] The first is activated during the initial and middle phase of acute ischaemic preconditioning and includes activation of phosphoinositide 3 kinase (PI3K)/Akt and in turn endothelial nitric oxide synthase (eNOS) and nitric oxide, followed by guanylate cyclase and protein kinase C activation leading to activation of the ATP-dependent mitochondrial potassium channels and generation of reactive oxygen species (ROS),[9,10] finally activating mitogen-activated protein kinase p38 and mitochondrial permeability pore (mPTP) opening.[11] The second becomes active during the early in reperfusion and results in the activation of the *Reperfusion Injury Salvage Kinases* (RISK) pathway, which comprises PI3K and extracellular signal-regulated mitogen-activated protein kinase (ERK1/2), and leads to the inhibition of mPTP opening. The third is recruited during ischaemic pre- and post-conditioning and includes the *Survivor Activating Factor Enhancement* (SAFE) pathway, which comprises signal transducer and activator of transcription (Stat) and results in rearrangements in the nucleus and mitochondria.[12] The complex interplay between these intracellular pathways remains largely unknown, but might represent a promising target to reduce cardiac damage during ischaemia and reperfusion.

In this context, cardiac ROS released following ischaemia-reperfusion are of interest as regulators of cardiac salvage pathways.[13] To investigate this, we focused on the adaptor protein p66^{Shc} which regulates cellular redox states, metabolism and life span and is a critical mediator of oxidative signal transduction.[14–17] p66^{Shc} acts not only in the cytosol but upon phosphorylation also as a specific redox enzyme in mitochondria, generating hydrogen peroxide.[18] As such, the adaptor protein p66^{Shc} might regulate ischaemia–reperfusion damage.[19,20] However, whether such effects occur *in vivo* remains unknown.

Thus, we designed an *in vivo* study to investigate the role of this protein in a mouse model of ischaemia and reperfusion taking advantage of genetic deletion of p66^{Shc} or transient *in vivo* knock down of the adaptor protein. In this setting, we investigated infarct size as well as activation of intracellular salvage or apoptotic pathways and their regulation by p66^{Shc}.

Materials and methods

Animals

P66$^{Shc-/-}$ knockout mice were originally obtained from the Centro Nationale di Oncologia, Milano, Italy.[15] The *p66^{Shc}* deficient mouse colony was maintained in the own animal facility by crossbreeding homozygous animals. All animal experiments were performed on 12- to 14-week-old *p66$^{Shc-/-}$* knockout and wild-type (WT) male mice. Both animal cohorts were maintained on identical C57Bl/6 genetic background. Genotyping was performed by PCR on ear punch biopsies using *p66^{Shc}*-specific primers (data not shown).

All mice were maintained at 24°C with 12 h light–dark cycle and free access to food and water. They were kept on a standard western type chow. All procedures were approved by local ethics committees (both in Zurich and Geneva) and Swiss authorities and conformed to the 'position of the American Heart Association on Research Animal Use' and ARRIVE guidelines.

In situ hybridization

P66^{Shc} *In situ* hybridization was performed using Leica BOND-MAX^{TM} (Leica Biosystems, Buffalo Grove, IL, USA). Briefly, isolated murine WT and *p66$^{Shc-/-}$* hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. Formalin-fixed paraffin-embedded tissue sections (5 μm) were deparaffinized in xylene, rehydrated in ascending alcohols, digested with Enzyme 2 (5 g/mL) for 15 min at ambient temperature. Samples (heart sections) were then denatured on a hot plate for 3 min at 90°C, and incubated for 1 h at 53°C in hybridization buffer (Exiqon, Woburn, MA, USA) containing 250 nM of double digoxigenin-labeled p66^{Shc} LNA mRNA detection probe (Exiqon). After incubation, the samples were washed twice in BOND washing buffer at ambient temperature for 5 min. The samples were incubated in blocking solution (AB-Blocking kit, Cell Marque, Rocklin, CA, USA), supplemented with 1 : 500 mouse anti-digoxigenin secondary Ab (Sigma) in BOND diluent buffer (Leica Biosystems) and detected with the BOND Polymer Refine Detection kit (Leica Biosystems).

In vivo knockdown of p66^{Shc}

In vivo knockdown of p66^{Shc} was performed by injecting a predesigned siRNA specifically targeting *p66^{Shc}* (5′-UGA GUC UCU GUC AUC GCU G dTdT-3′, Microsynth AG, Switzerland). A *scrambled* siRNA

was used as a negative control (5′-UAC ACA CUC UCG UCU CU dTdT-3′, Microsynth AG, Switzerland). Amount of $p66^{Shc}$ siRNA was selected based on dose optimization studies (data not shown). The siRNA mix at the final dose of 1.6 mg/kg was incubated with the *in vivo*-jetPEI delivery reagent (Polyplus-Transfection, Inc., New York, NY, USA) for 15 min at room temperature and intravenously injected in a final volume of 160 μL, as previously reported.[21] Successful knock-down of $p66^{Shc}$ was assessed by western blot in heart lysates.

In vivo pharmacological inhibition of Stat3

Some WT and $p66^{Shc-/-}$ knockout mice were pretreated with WP1066 (40 mg/kg, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or vehicle (10% DMSO in PBS) by intraperitoneal injection 5 min before ischaemia onset. Then, at 24 h of reperfusion, mice were euthanized and infarct size was histologically determined by triphenyltetrazolium chloride (TTC) staining.

Ischaemia and reperfusion *in vivo*

$P66^{Shc-/-}$ and WT (including those injected with siRNA) male mice (10–12 weeks of age) were initially anaesthetized with 4% isoflurane and intubated. After starting mechanical ventilation (tidal volume of 150 μL, 120 breaths/min) by supplementation with 100% oxygen, anaesthesia was maintained with 2% isoflurane. During surgery anaesthesia was monitored by careful visual and tactile control of mouse consciousness (i.e. breathing rate and volume, heart rate, sweating and tearing). A thoracotomy was performed in the left third intercostal space and the pericardium was removed. Ligature of the left anterior coronary artery at the inferior edge of the left atrium was performed using an 8-0 Prolene suture. A small piece of polyethylene tube was used to secure the ligature without damaging the artery.

After 30, 45, or 60 min of ischaemia, the occlusion of the left anterior coronary artery occlusion was released and reperfusion occurred. Analgesia was extended with s.c. injection of 0.05 mg/kg buprenorphine HCl every 12 h till sacrifice. Reperfusion was confirmed by visible restoration of colour to the ischaemic tissue. Then, chest was closed and the ventilator removed to restore normal respiration. After 24 h of reperfusion, animals were anaesthetized with 4% isoflurane and i.p. injection of ketamine–xylazine (4 mg/0.2%) and sacrificed for infarct size determination and immunohistochemical analyses.

Area at risk and myocardial infarct size (*I*) assessment

To assess area at risk (AAR) and infarct size (*I*) in *in vivo* myocardial protocols, mice were anaesthetized with ketamine–xylazine and sacrificed at 24 h of reperfusion, as described.[22] Left anterior coronary artery was re-occluded before the heart was taken out. Next, Evan's blue dye (2%; Sigma, St. Louis, MI, USA) was injected into the aortic cannula to delineate the *in vivo* AAR. The heart was rapidly excised and rinsed in NaCl 0.9%. Then, hearts were frozen and sectioned into 2-mm transverse sections from the apex to the base (5–6 slices/heart). The sections were incubated at 37°C with 1% TTC (Sigma) in phosphate buffer (pH 7.4) for 15 min, fixed in 10% formaldehyde solution and photographed with a digital camera (Nikon Coolpix) to distinguish continuously perfused tissue (blue), stained ischaemic viable tissue (red), and unstained necrotic tissue (white). The different zones were determined using MetaMorph software (version 6.0, Universal Imaging Corporation). Area at risk and left ventricular *I* were expressed as percentage of ventricle surface (AAR/V) and AAR (I/AAR), respectively.

Detection of cardiac troponin I and inflammatory mediators in mouse serum

Circulating cardiac troponin I (cTnI) levels were measured in serum after 24 h of reperfusion, using a high-sensitive ELISA kit (Life Diagnostics, Inc.). Serum levels of CXCL1 and CCL2 were measured by colorimetric enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA), following manufacturer's instructions. The limit of detection was 0.156 ng/mL for cTnI, 15.6 pg/mL for CXCL1, 7.8 pg/mL for CCL2. Mean intra- and interassay coefficients of variation were $<6\%$ for all mediators.

Immunostaining

Hearts from animals sacrificed both before and after 30 min of ischaemia and 24 h of reperfusion were frozen in optimal cutting temperature and serially cut from the occlusion locus to the apex in 7 μm sections. Immunostainings for neutrophils (anti-mouse Ly-6G Ab, dilution 1 : 100; BD Pharmingen™, San Jose, CA, USA), macrophages (anti-mouse CD68 Ab, dilution: 1 : 400; ABD Serotec, Dusseldorf, Germany), endothelial (anti-mouse CD31 Ab, dilution 1 : 500, Santa Cruz Biotechnology) or smooth muscle cells (anti-mouse smooth muscle actin Ab, dilution 1 : 20, Thermo Scientific, Inc., Waltham, MA, USA) were performed on five midventricular cardiac or brain sections per animal, and quantification performed with the MetaMorph software, as previously described.[22–24]

Oxidative stress determination in mouse infarcted hearts

Measurement of O_2^- in mouse hearts at 30 min of ischaemia and 24 h of reperfusion was performed using the O_2^--sensitive dye dihydroethidium (DHE, Molecular Probes, Life Technologies Corporation, Zug, Switzerland) as previously described.[22] Five frozen midventricular cardiac sections per animal were used, and nuclei were stained with 4′,6-diamidino-2-phenylindole. *In situ* fluorescence was assessed using fluorescence microscopy and quantification performed with MetaMorph software.

The production of other ROS was assessed by two histological mediators: the highly toxic product of lipid membrane peroxidation 4-hydroxy-2-nonenal (mouse anti-4-HNE monoclonal antibody at 1 μg/mL, Oxis International, Inc., Foster City, CA, USA) and the 3,5-dibromotyrosine (mouse anti-Di bromo tyrosine monoclonal antibody at 10 μg/mL, AMS biotechnology, LTD, Abingdon, UK).[22] To avoid any potential cross-reactivity with mouse heart antigens and to increase the specificity of the primary antibodies, the VECTOR M.O.M Immunodetection kit and the VECTOR VIP substrate kit for peroxidase (Vector Laboratories, Inc. Burlingame, CA, USA) were used, following the manufacturer's instructions. Quantification was performed with MetaMorph software. Results were expressed as percentages of stained area on total heart surface area.

Western blotting

Mouse hearts were isolated after 30 min of ischaemia followed by either 5 or 15 min of reperfusion and were homogenized in lysis buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1 mM PMSF, and 1 mM DTT. Total protein extracts were cleared by centrifugation and 40 μg of proteins separated by gel electrophoresis on 10% SDS–polyacrylamide gel followed by semi-dry transfer onto PVDF membranes. Membranes were incubated with primary anti-phospho-Akt(Thr308), anti-phospho-Akt(Ser473), anti-Akt, anti-phospho-p42/p44 MAPK, anti-p42/p44 MAPK, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-Stat3(Ser727), anti-phospho-Stat3(Tyr705), anti-Stat3, anti-phospho-SAPK/JNK(Thr183/Tyr185), and anti-SAPK/JNK antibody (all

from Cell Signaling, Danvers, MA, USA). Primary anti-GAPDH (Millipore Corporation, Billerica, MA, USA) antibodies were used as loading control. Protein expression was quantified using Scion Image™ and expressed as ratio to corresponding loading control.

Mitochondrial swelling assay

Mitochondria (40 μg) from mouse hearts submitted to 30 min ischaemia and 24 h of reperfusion were isolated in swelling buffer (250 mM sucrose, 10 mM 3-(*n*-morpholino)propanesulfonic acid, 5 μM EGTA, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM pyruvate, and 5 mM malate) were incubated with 150 μM $CaCl_2$ in a final volume of 200 μL in 96-well plate for 20 min. Absorbance at 520 nm was read every 5 min.[25]

Caspase 3 assay

The caspase 3 assay was performed in total protein extracts from murine hearts after 30 min ischaemia and 5–15 min of reperfusion, using caspase 3 colorimetric assay kit (Sigma), according to manufacturer's recommendations.

Apoptotic cell measurement within infarcted hearts

Apoptosis was evaluated on cryosections of infarcted hearts after 30 min of ischaemia and 24 h of reperfusion. The staining was performed using the Dead End™ colorimetric terminal deoxinucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) system (Promega, Madison, WI, USA). Diaminobenzidine was used as the chromogenic substrate (according to the manufacturer's instructions). Results were expressed as percentages of stained area on total heart surface area.

Statistical analysis

The Mann–Whitney nonparametric test (the normality assumption of the variables' distribution in both groups was violated) was used for comparisons of continuous variables. Kruskal–Wallis one-way analysis of variance was used for multiple group comparison. All results are expressed as mean ± SEM. Values of $P < 0.05$ (two tailed) were considered significant. All analyses were done with GraphPad Prism software version 5.01.

Results

Expression pattern of $p66^{Shc}$ in cardiac tissue

To determine the expression of $p66^{Shc}$ within cardiac tissue, we performed *in situ* hybridization of paraffin-embedded cross sections of perfused hearts isolated from WT and $p66^{Shc-/-}$ mice using a $p66^{Shc}$-specific probe. In order to allocate $p66^{Shc}$ staining to cardiomyocytes, we performed H/E staining of adjacent sections (*Figure 1*). To visualize heart vessels and arterioles, we used smooth muscle

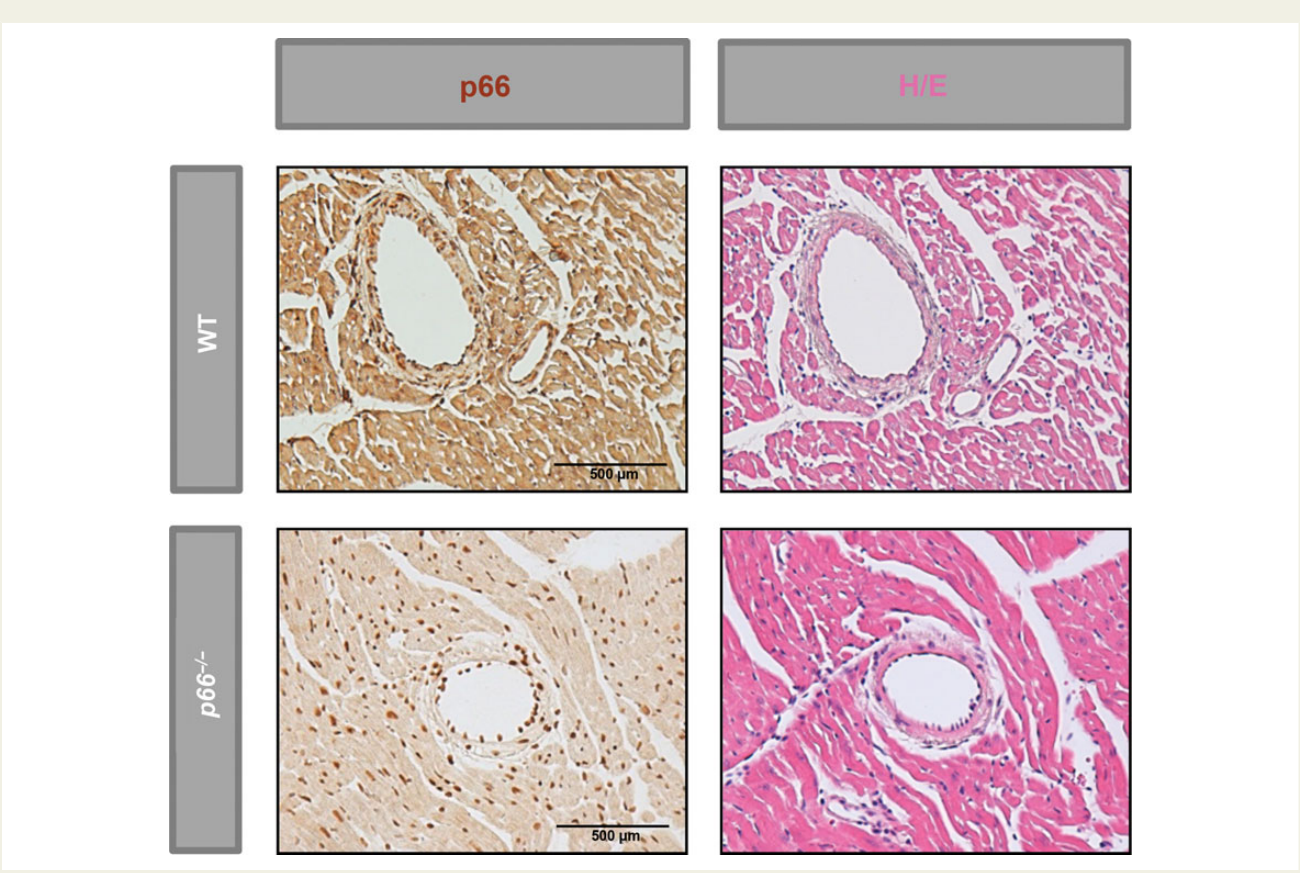

Figure 1 $P66^{Shc}$ expression pattern in mouse hearts. *In situ* hybridization with $p66^{Shc}$ mRNA LNA detection probe shows homogeneous expression of $p66^{Shc}$ in paraffin-embedded cross sections from wild-type (upper row) and $p66^{Shc-/-}$ hearts (lower row). Consecutive heart cross sections were stained with either $p66^{Shc}$ probe (left panels) or H/E (right panels).

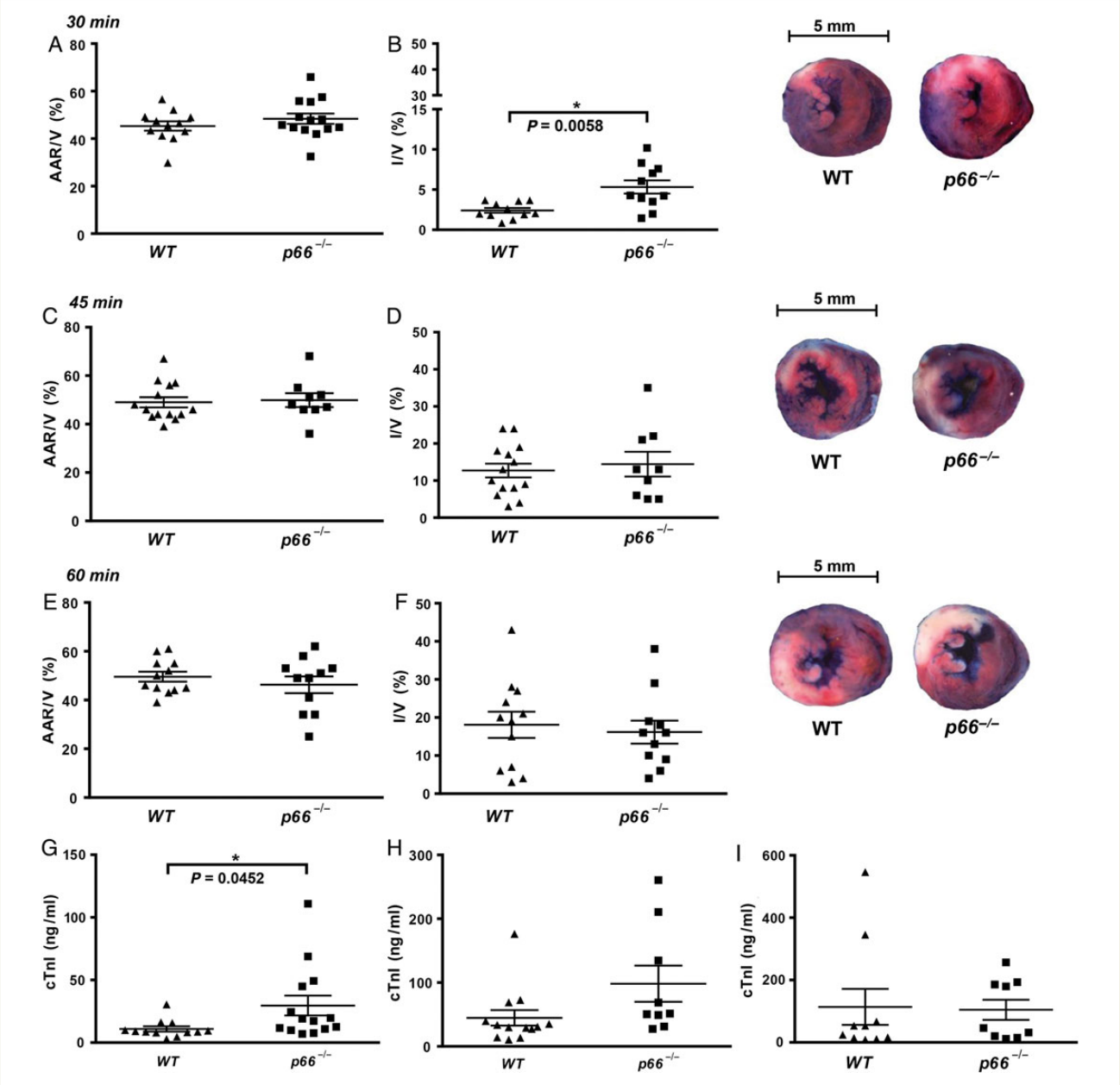

Figure 2 Genetic deletion of p66^{Shc} promotes infarct size after short-term ischaemia *in vivo*. (*A* and *B*) Thirty minute of ischaemia followed by 24 h of reperfusion. $n = 11$ for WT and $n = 14$ for $p66^{Shc-/-}$. (*A*) Quantification of area at risk per ventricle area ($P = 0.3127$). (*B*). Quantification of infarct size per ventricle area. Right panel: Representative images of 2-3-5-triphenyl tetrazolium chloride-stained middle heart sections of control wild-type and $p66^{Shc-/-}$ mice. (*C* and *D*) Forty-five minutes of ischaemia followed by 24 h of reperfusion. $n = 14$ for WT and $n = 9$ for $p66^{Shc-/-}$. (*C*) Quantification of area at risk per ventricle area ($P = 0.8014$). (*D*) Quantification of infarct size per ventricle area ($P = 0.6279$). Right panel: Representative images of 2-3-5-triphenyl tetrazolium chloride-stained-stained middle heart sections of wild type and $p66^{Shc-/-}$. (*E* and *F*) 60 min of ischaemia followed by 24 h of reperfusion. $n = 12$ for wild type and $n = 11$ for $p66^{Shc-/-}$. (*E*) Quantification of area at risk per ventricle area ($P = 0.4063$). (*F*) Quantification of infarct size per ventricle area ($P = 0.6838$). Right panel: Representative images of 2-3-5-triphenyl tetrazolium chloride-stained-stained middle heart sections of wild type and $p66^{Shc-/-}$. Serum cardiac troponin I levels of wild type or $p66^{Shc-/-}$ after 30 (*G*), 45 (*H*), or 60 (*I*) min of ischaemia followed by 24 h of reperfusion. $n = 10$ for both genotypes ($P = 0.0659$ for *H* and $P = 0.8915$ for *I*). Data are mean ± SEM; *$P < 0.05$ vs. wild type.

actin and CD31 immunostaining[26] of cardiac and brain tissues. Compared with WT no changes in either heart vessels (CD31+) or intramyocardial and coronary arterioles (SMA+) were noted in $p66^{Shc-/-}$ mice (see Supplementary material online, *Figures S1* and *S2*).

Infarct size after ischaemia and reperfusion in wild-type and $p66^{Shc-/-}$ mice

To investigate the impact of genetic deletion of p66^{Shc} on infarct size, we submitted mice to 30, 45, or 60 min of ischaemia followed by 24 h

of reperfusion. The AAR after 30 min of ischaemia were comparable for both genotypes indicating that coronary ligatures were placed at the same level of the LAD in both groups (*Figure 2A*), $p66^{Shc-/-}$ mice exhibited only increased infarct size after 30 min of ischaemia followed by 24 h of reperfusion as compared with WT (I/V, mean ± SEM: 5.3% ± 2.7 vs. 2.4% ± 1.0, *Figure 2B*). In contrast, after prolonged ischaemia of 45 or 60 min, despite comparable areas at risk (*Figure 2C* and *E*), infarct size was similar in both groups (*Figure 2D* and *F*). This indicates that $p66^{Shc-/-}$ mice display an increased susceptibility to ischaemia leading to larger infarcts at shorter, but not prolonged periods of ischaemia.

Serum cTnI levels (a biomarker of cardiac necrosis)[22,27] confirmed the histological results with significantly higher levels in $p66^{Shc-/-}$ mice only after 30 min of ischaemia (*Figure 2G–I*). Heart vessels (CD31+) or intramyocardial and coronary arterioles (SMA+) did not differ in $p66^{Shc-/-}$ mice after ischaemia and reperfusion compared with WT as assessed by smooth muscle actin and CD31 immunostaining, respectively (see Supplementary material online, *Figure S3*).

Finally, we found that expression of $p66^{Shc}$ protein in the heart from WT subjected to 30 min of ischaemia followed by either 12 or 24 h of reperfusion was increased compared with $p66^{Shc}$ protein expression without cardiac injury (see Supplementary material online, *Figure S4*).

Infarct size after ischaemia and reperfusion during *in vivo* silencing of $p66^{Shc}$

In order to investigate whether transient silencing of $p66^{Shc}$ has similar effects on infarct size as in $p66^{Shc-/-}$ mice, we performed *in vivo* knockdown of $p66^{Shc}$ in WT by injecting small interference RNA specific for $p66^{Shc}$ or *scrambled* siRNA control into the tail vein. Four days after siRNA application, mice were submitted to 30 min of ischaemia followed by 24 h of reperfusion (*Figure 3A*). We first confirmed that $p66^{Shc}$ was significantly downregulated at the protein level upon *in vivo* siRNA treatment (*Figure 3B*) and that the AAR was similar in all treatment groups (*Figure 3C*). Similar to $p66^{Shc-/-}$ mice, $p66^{Shc}$-siRNA-mediated silencing was associated with a significant increase in infarct size as compared with controls at 30 min of ischaemia (14.9% ± 2.4 vs. 9.4% ± 2.9, *Figure 3D* and *E*). Again, there was no difference in arteriole or vessel density in WT mice injected

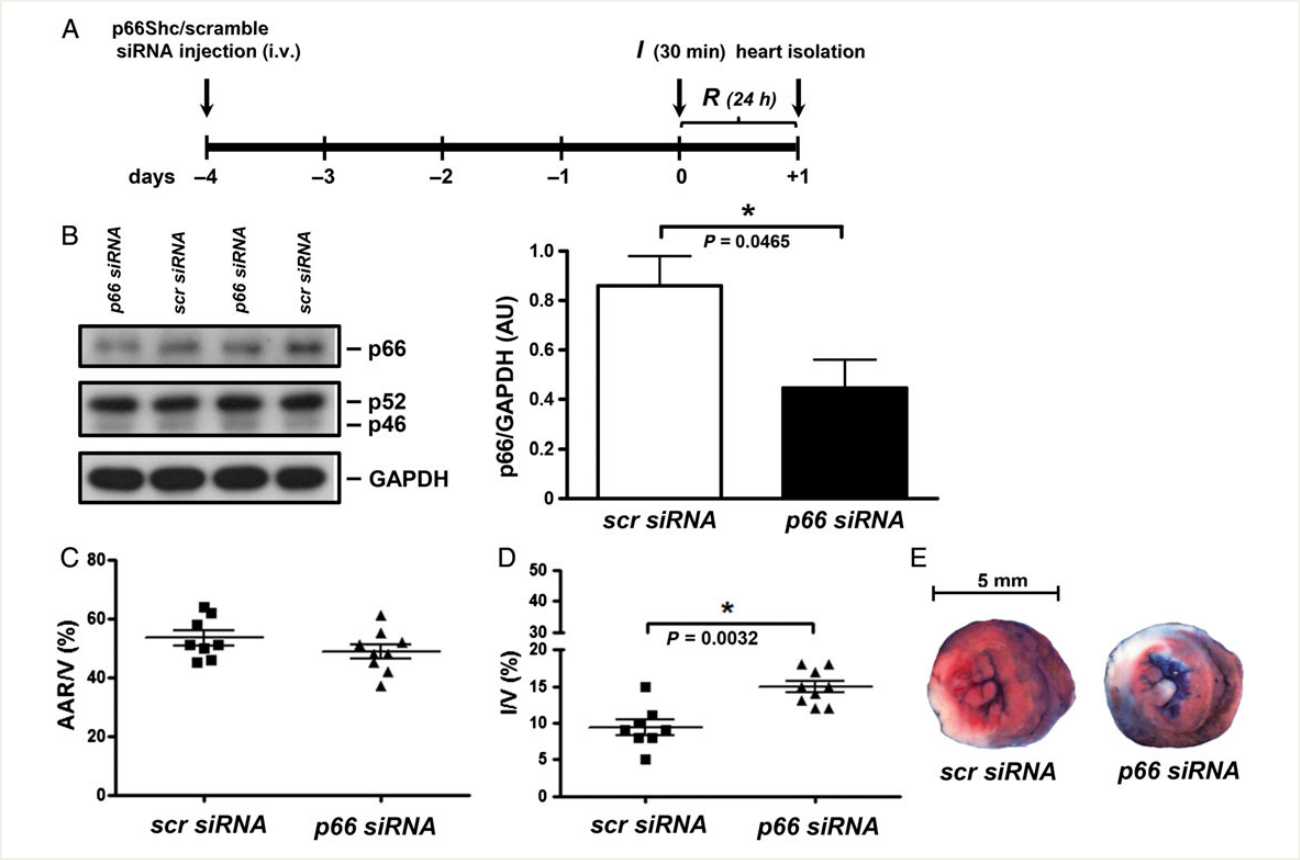

Figure 3 *In vivo* knockdown of $p66^{Shc}$ in wild-type mice increases infarct size after short-term ischaemia. (*A*) Scheme of the experiment set up. *Scrambled* (*scr* siRNA) or *p66Shc* siRNA was injected i.v. 4 days before *in vivo* ischaemia–reperfusion onset. Thirty minutes of ischaemia followed by 24 h of reperfusion were applied. (*B*) Cardiac silencing of p66Shc by siRNA was analysed by western blot. Representative immunoblots from four wild type injected with either *scr* siRNA or $p66^{Shc}$ siRNA. GAPDH immunoblot was used as a loading control. Bar graphs (left panels) represent densitometric quantification of immunoblots of $p66^{Shc}$ protein. Data are mean ± SEM; $n = 8$ for *scr* and $n = 11$ for $p66^{Shc}$ siRNA; $^*P < 0.05$ vs. *scr* siRNA. (*C*) Quantification of area at risk per ventricle area ($P = 0.2884$). (*D*) Quantification of infarct size per ventricle area. (*E*) Representative images of 2-3-5-triphenyl tetrazolium chloride-stained middle heart sections of wild type injected either with *scr* siRNA or $p66^{Shc}$ siRNA.

Table 1 Serum levels of CXCL1 (neutrophil chemoattractant) and CCL2 (monocyte/macrophage chemoattractant) after 30 min of ischaemia and 24 h reperfusion

Chemoattractant (pg/mL)	Genetic deletion		Transient knockdown	
	WT	$p66^{Shc-/-}$	*scr siRNA*	*p66 siRNA*
CXCL1	579.3 ± 635.7	476.6 ± 590.0	361.8 ± 234.6	515.2 ± 275.6
CCL2	28.2 ± 18.69	31.1 ± 17.35	54.7 ± 26.7	56.4 ± 32.34

Data are presented as mean ± SD; $n = 10–18$ per group.

with $p66^{Shc}$ siRNA after ischaemia and reperfusion compared with *scramble* injected WT (see Supplementary material online, *Figure S5*).

Genetic deletion or *in vivo* knockdown of $p66^{Shc}$ do not influence inflammatory processes during ischaemia and reperfusion

In order to investigate whether genetic deletion or silencing of $p66^{Shc}$ influences post-infarction inflammation (a critical mechanism of injury during reperfusion)[22,28] after short-term myocardial ischaemia, we investigated systemic levels of CC and CXC chemokines (attracting inflammatory cells),[22] cardiac leukocyte infiltration as well as cardiac oxidant content. No differences between $p66^{Shc-/-}$ and WT were noted in serum CXCL1 or CCL2 levels at 30 (*Table 1*) min of ischaemia followed by 24 h of reperfusion. Although some trend towards an increase for CXCL1 was observed in WT mice submitted to *in vivo* siRNA-mediated knockdown of $p66^{Shc}$, serum levels of both CXCL1 and CCL2 remained unchanged in $p66^{Shc}$ siRNA-treated animals when compared with *scr* siRNA controls after 30 min of ischaemia and 24 h of reperfusion (*Table 1*). As a consequence of these negligible differences between genotypes on post-infarction circulating levels of CC and CXC chemokines, $p66^{Shc-/-}$ mice and WT mice exhibited similar levels of infiltrating neutrophils (Ly-6G+ cells, Supplementary material online, *Figure S6A*), macrophages (see Supplementary material online, *Figure S6B*), and ROS (assessed by 4-hydroxy-2-nonenal [4-HNE], dibromotyrosine [DiBrY], superoxide [DHE] stainings, respectively) (see Supplementary material online, *Figure 6C–E*). Accordingly, *in vivo* $p66^{Shc}$ knockdown did not modify neutrophils (Ly-6G+ cells, Supplementary material online, *Figure S7A*), macrophages (see Supplementary material online, *Figure S7B*), or ROS (see Supplementary material online, *Figure 7C–E*) within infarcted hearts when compared with *scr* siRNA controls. Finally, no significant changes were observed in basal cardiac and brain ROS levels in both groups of mice as assessed by 4-HNE and DiBrY stainings (see Supplementary material online, *Figures S8* and *S9*).

Genetic deletion of $p66^{Shc}$ prevents activation of RISK and SAFE salvage pathways

Activation of survival protective pathways in response to short-term ischaemia early during reperfusion was then investigated.[12,29] We examined phosphorylation levels of Akt, ERK1/2 and Stat3, key members of RISK (Akt and ERK1/2) and SAFE (Stat3) intrinsic prosurvival signalling pathways known to limit reperfusion injury, during first 5 and 15 min of reperfusion. Significant abrogation in the phosphorylation of Akt (Thr308) and Stat3 (Ser727) was obvious at 5, but not at 15 min of reperfusion in $p66^{Shc-/-}$ mice when compared with WT, indicating a transient inhibition of these salvage pathways (*Figure 4A* and *C*). $p66^{Shc}$ deficiency was not associated with any modification in the phosphorylation of other amino acid residues of Akt (Ser473) or Stat3 (Tyr705) neither at 5 nor at 15 min of reperfusion (*Figure 4B* and *D*). On the other hand, no significant change in ERK1/2 phosphorylation was noted in $p66^{Shc-/-}$ animals when compared with WT (Supplementary material online, *Figure S10*). Finally, both WT and $p66^{Shc-/-}$ mice were treated with a single i.p. injection of the Stat3 inhibitor WP1066 (40 mg/kg) or vehicle 5 min before ischaemia (30 min) and 24 h of reperfusion (*Figure 4E*). Histological evaluation revealed an AAR of ~50–55% in all treatment groups (*Figure 4F*). The infarct size was significantly increased in both WT and $p66^{Shc-/-}$ WP1066-treated groups when compared with corresponding vehicle controls (*Figure 4G*).

Genetic deletion and *in vivo* knockdown of $p66^{Shc}$ promotes mitochondrial swelling and apoptosis in infarcted hearts

Phosphorylation of Stat3 on tyrosine 705 is known to drive $p66^{Shc}$ to the nucleus, whereas the phosphorylation on serine 727 drives the adaptor protein to the mitochondria where it regulates permeability transition pores (PTP).[30] To investigate the impact of Stat3 (Ser727) phosphorylation on mitochondrial function, we performed swelling assay using isolated mitochondria from WT and $p66^{Shc-/-}$ infarcted hearts induced by calcium chloride overload ($CaCl_2$). The rate of mitochondrial swelling was measured by light scattering at 520 nm. Stable absorbance at 520 nm was observed up to 20 min in extracts from WT mice. In contrast, mitochondria of $p66^{Shc-/-}$ hearts displayed significantly increased absorbance 15 and 20 min after $CaCl_2$ overload reflecting markedly increased swelling and thus mitochondrial disruption (*Figure 5A*). Accordingly, caspase-3 activity was increased in heart extracts from $p66^{-/-}$ mice after 15 min of reperfusion when compared with WT (*Figure 5B*).

We then assessed the effects of $p66^{Shc}$ deficiency on cardiac apoptosis after 30 min of ischaemia and 24 h reperfusion. In line with the activation of caspase-3, $p66^{Shc}$ deficiency was associated with an

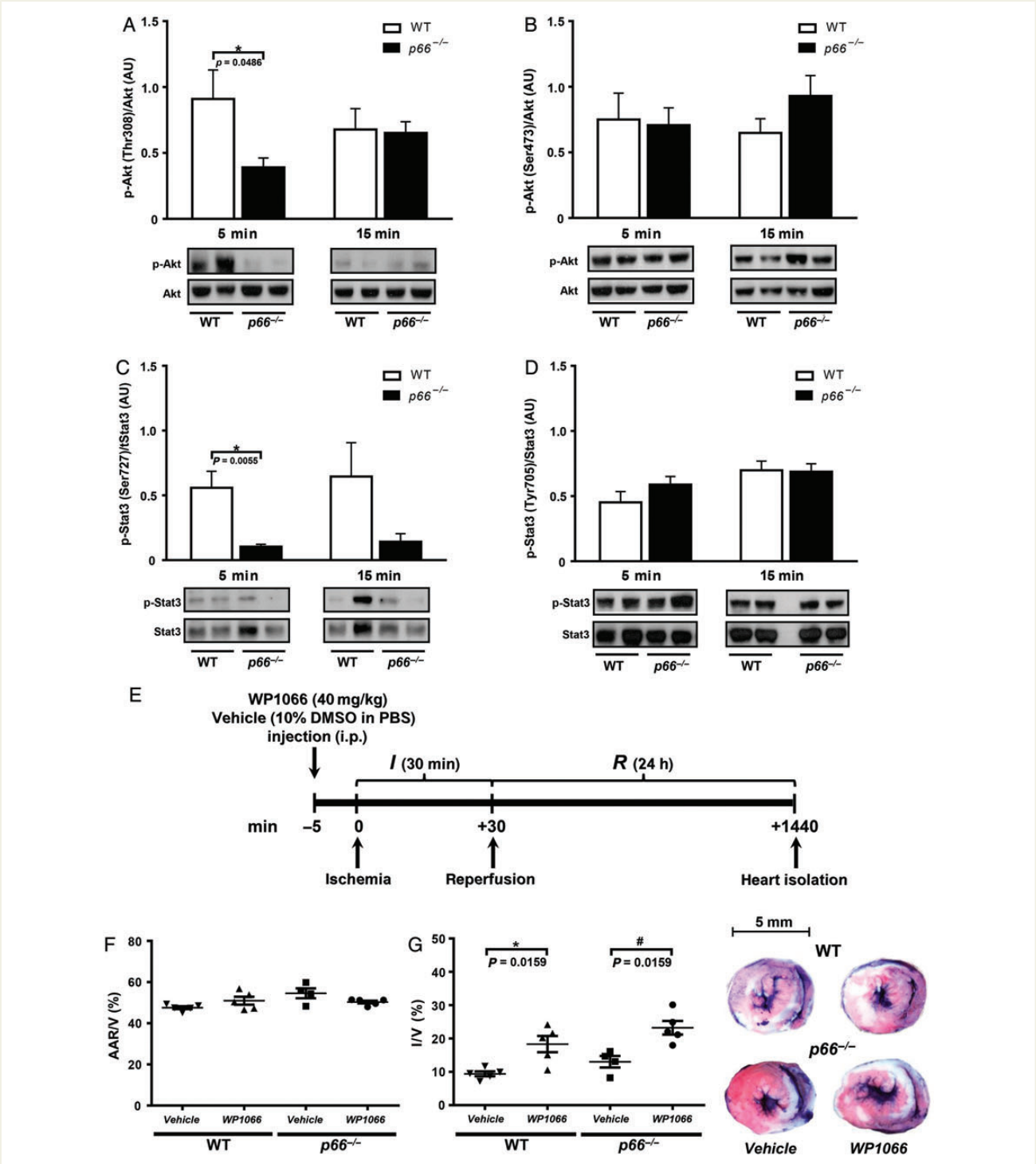

Figure 4 Genetic deletion of p66^{Shc} inhibits early activation of salvage signalling pathways in infarcted hearts. After 30 min ischaemia followed by 5–15 min of reperfusion, hearts were lysed and phosphorylation of intracellular kinases assessed by western blot. $n = 7$ for wild type and $n = 6$ for $p66^{Shc-/-}$. Data are mean ± SEM. *$P < 0.05$ vs. wild type. (*A*) Akt (Thr308) phosphorylation ($P = 0.8982$ for 15 min). (*B*) Akt (Ser473) phosphorylation ($P = 0.8610$ for 5 min and $P = 0.1544$ for 15 min). (*C*) Stat3 (Ser727) phosphorylation ($P = 0.1443$ for 15 min). (*D*) Stat3 (Tyr705) phosphorylation ($P = 0.2153$ for 5 min and $P = 0.9163$ for 15 min). (*E*) Scheme of the experiment set up. WP1066 or vehicle was injected i.v. 5 min before *in vivo* onset of 30 min ischaemia in wild type and $p66^{Shc-/-}$. At 24 h of reperfusion mice were euthanized and infarct size assessed. (*F*) Quantification of area at risk per ventricle area ($P = 0.1429$ for wild type and $P = 0.0971$ for $p66^{Shc-/-}$). (*G*) Quantification of infarct size per ventricle area. (*C*) Right panel: Representative images of 2-3-5-triphenyl tetrazolium chloride-stained middle heart sections of wild type and $p66^{Shc-/-}$.

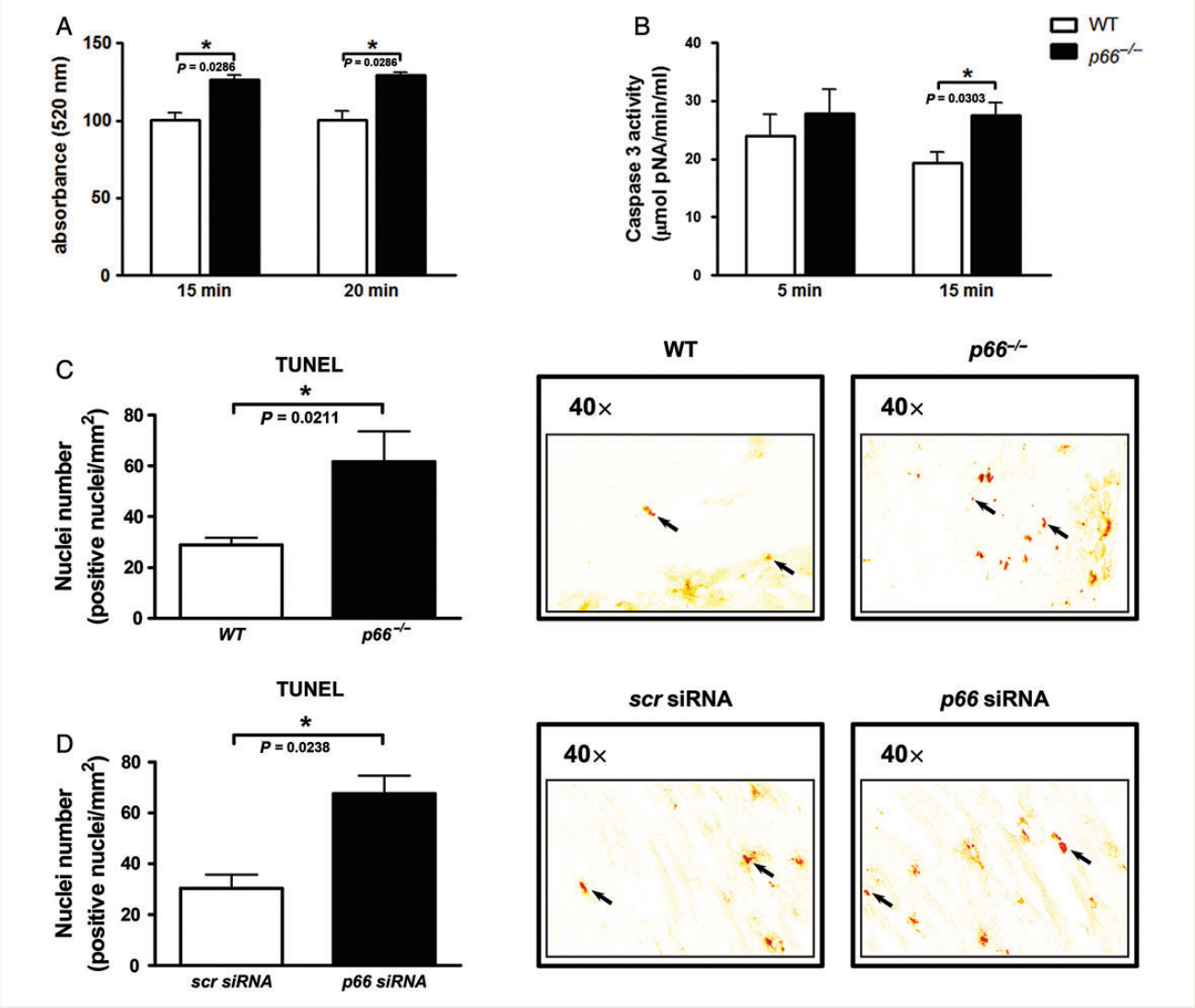

Figure 5 Genetic deletion of p66^{Shc} is associated with postinfarction mitochondrial dysfunction and increased apoptosis in infarcted hearts. (*A*) Light absorbance at 520 nm in wild type and *p66*$^{Shc-/-}$ infarcted hearts (30 min ischaemia and 24 h reperfusion) at 15 and 20 min of calcium overload (150 µM of $CaCl_2$). Values are shown as percentage of average absorbance of wild type extracts in the absence of calcium ($n = 4$/group). (*B*) Caspase-3 activity in infarcted heart extracts in wild type and *p66*$^{Shc-/-}$, submitted to 30 min ischaemia and 5 and 15 min of reperfusion ($n = 4$/group, $P = 0.2222$ for 5 min). (*C*) Quantification of Tunel-positive areas in frozen sections of infarcted hearts from wild type or *p66*$^{Shc-/-}$ submitted to 30 min of ischaemia followed by 24 h of reperfusion ($n = 8$ for wild type and $n = 6$ for *p66*$^{Shc-/-}$). Right panel: Representative images of Tunel staining are shown. (*D*) Quantification of Tunel-positive areas in frozen sections of infarcted hearts from wild type treated with scrambled (*scr* siRNA) or *p66*^{Shc} siRNA and submitted at 30 min of ischaemia followed by 24 h of reperfusion ($n = 7$ for *scr* and $n = 6$ for *p66*^{Shc} siRNA). Right panel: Representative images of Tunel stainings are shown. Data are mean ± SEM. *$P < 0.05$ vs. wild type.

increased Tunel-positive cell content when compared with WT (*Figure 5C*). A similar result was also seen in *p66*^{Shc} siRNA-treated mice when compared with *scr* siRNA controls (*Figure 5D*).

Discussion

This study for the first time demonstrates that, unlike in the brain,[19] p66^{Shc} protects the myocardium during short-term ischaemia and reperfusion from injury *in vivo* in mice. Indeed, we showed that (1) p66^{Shc} is expressed both in the myocardium and the coronary vasculature, (2) *p66*$^{Shc-/-}$ mice display increased infarct size when compared with WT controls when exposed to a short-term period of ischaemia only (i.e. 30 min), (3) similar effects can be obtained in WT mice by transient silencing of p66^{Shc} using siRNA, (4) p66^{Shc} regulates salvage pathways, and (4) prevents mitochondrial swelling and apoptosis via caspase-3.

P66^{Shc} is differentially expressed in different organs and tissues. Importantly, unlike in the brain (where p66^{Shc} is exclusively expressed in the endothelium of blood vessels),[19] in the heart the adaptor protein is expressed both in myocardial tissue and coronary blood

vessels. At baseline, *p66^{Shc}* knockout mice did not differ from WT in their vessel structure and density or metabolism of reactive oxidant species in both the heart and brain. Similarly, in infarcted hearts after 30 min of ischaemia and 24 h of reperfusion, no histological alterations of vessel density and structure or oxidant content were notable. This demonstrates that p66^{Shc} plays distinct roles at baseline and in response to an ischaemic insult in the heart and brain.

The surprising fact that both genetic deletion or transient silencing of p66^{Shc} led to increased infarct size after 30, but not after 45 and 60 min of ischaemia followed by 24 h reperfusion was supported by histological and biochemical data using troponin I as a marker. These solid observations are in contrast to previous *ex vivo* studies using isolated perfused hearts where p66^{Shc} deletion appeared to prevent from ischaemia–reperfusion injury.[20] Thus, as in other experimental situations, it is obvious that results obtained in isolated organs and tissues mostly perfused by artificial solutions and devoid of physiological filling pressure and neurohumoural regulation cannot be extrapolated to the *in vivo* situation. Indeed, *ex vivo* and *in vivo* models of ischaemic cardiac injury differ substantially (including different times of reperfusion and involvement of systemic inflammation).[22,28]

As in the brain where p66^{Shc} is only expressed in the vasculature, but not in neurons and in hepatic ischaemic injury p66^{Shc} contributes to reperfusion injury, we here demonstrate a differential role of p66^{Shc} in the myocardium. Indeed, in endothelial cells, p66^{Shc} inhibits the expression and activity of eNOS and increases O_2^- production.[31–34] However, the microcirculation of the heart (which is of importance during ischaemia) is mainly regulated by endothelium-derived hyperpolarizing factors rather than eNOS.[17,35,36] Thus, the protective effects of p66^{Shc} against short-term ischaemia and reperfusion must be related to a specific role of the adaptor protein in the myocardium. Indeed, in the heart, ROS may also play a protective role in response to ischaemia.[22,37] However, under our experimental conditions, p66^{Shc} silencing unlike in the murine aorta did not alter ROS production confirming a different regulatory role of p66^{Shc} in the heart compared with other organs.[38] Thus, the protective role of p66^{Shc} in the myocardium must involve other pathways than those described in endothelial cells of conduit arteries. Indeed, the fact that protein levels of p66^{Shc} in the infarcted hearts from WT mice increase after 30 min of ischaemia followed by 12 or 24 h of reperfusion support our initial hypothesis of a protective role of p66^{Shc} in the myocardium.

Further, p66^{Shc} deficiency was not associated with any modification in both systemic and cardiac inflammation postinfarction. This again was surprising, since inflammation is considered a critical mediator of myocardial injury after ischaemia and reperfusion.[5,6] However, our findings are in accordance with recent studies in animal models of acute myocardial infarction, in which the relevance of pro-inflammatory mediators (such as cytokines and chemokines) was questioned.[39] Furthermore, no anti-inflammatory interventions have been shown to be clinically effective.[40–42] It even has been suggested that inhibition of certain chemokines may favour scar formation, salvage of post-infarction remodelling and mouse survival.[27] On the other hand, others found that inhibiting CXC chemokines early after ischaemia may reduce infarct size, but ineffective in preventing post-infarction heart failure.[43,44] Be it as it may, p66^{Shc} does clearly not exert its protective effects via modulation of inflammatory responses or ROS activated during ischaemia and reperfusion.

Importantly, we found that p66^{Shc} is a critical regulator of cardiac protective pathways in mice. Indeed, p66^{Shc} deficiency was associated with a reduction in the phosphorylation levels of Akt at the Thr308 site and Stat3 at the Ser727 site after 5 min of reperfusion. Moreover, pharmacological inhibition of Stat3 activation through phosphorylation shortly before ischaemia onset further confirmed that Stat3 mediates the protective effects of p66^{Shc} during ischaemia–reperfusion. In line with that, activation of certain intracellular kinases, in particular those that are part of the RISK and SAFE pathways (i.e. Akt and Stat3), has been proposed as a preventive strategy against reperfusion injury.[12,45] Interestingly, to be protective against ischaemia, Akt must not be fully activated (i.e. phosphorylated at both Thr308 and Ser473).[46] This may explain why p66^{Shc}, although it selectively phosphorylates Akt only at the Thr308 residue, was able to activate the RISK pathway in the ischaemic myocardium. p66^{Shc}-mediated phosphorylation of Stat3 at Ser727 is of importance for mitochondrial respiration of cardiomyocytes during ischaemia as phosphorylation of Stat3 at Ser727 promotes the transcriptional activity and mitochondrial translocation of this kinase.[47] Data in mice and pigs confirmed the importance of Stat3 phosphorylation at Ser727 and Tyr705, respectively, for its mitochondrial localization and its effect on mitochondrial respiratory complex 1 and 2 as well as on mPTP.[48,49] Our data are in accordance with those data further extending the role of Ser727 phosphorylation on mitochondrial permeability transition process by regulating mitochondrial PTP status. Finally, considering that other potential intracellular pathways were only marginally affected by p66^{Shc} deficiency, p66^{Shc} appears to act selectively on these intracellular pathways during ischaemia and reperfusion.

In line with these findings, we further show for the first time that genetic deletion or transient silencing of p66^{Shc} is associated with increased apoptosis post-infarction in response to short-term ischaemia and reperfusion. In line with these morphological findings, caspase-3 activity was increased and marked mitochondrial swelling was noted in infarcted hearts obtained from *p66$^{Shc-/-}$* when compared with WT animals. Of note, post-infarction cardiac apoptosis is a key event in adverse cardiac remodelling and heart failure during follow-up.[50]

Study limitations

First, extrapolations from mouse models to the human situation are difficult. Thus, although p66^{Shc} is upregulated in peripheral cells of patients with infarction further experiments in human myocardial tissue should be considered. Second, we acknowledge that infarct size observed in the present study was rather small compared with the infarct size found in humans, and accordingly small was the potential for protection. Third, the use of conventional knockout mice does not exclude effects of p66^{Shc} deletion in the other cell types than cardiomyocytes. Fourth, it is not clear yet whether further activation of p66^{Shc} over the basal level would be beneficial. To sort this out, the development of mice overexpressing p66^{Shc} in the myocardium would be essential. Fifth, although we were able to identify p66^{Shc} as a regulator of protective pro-survival RISK and SAFE pathways within ischaemic hearts, we did not identify all intermediate molecules involved. Finally, the present study also adds complexity to the use of this salvage pathways as a potential therapeutic target. Indeed, given the different effects of activation and silencing of

p66^{Shc} in different cells, tissues, and organs,[17] tissue selective inhibition was shown to be effective in mice. Indeed, while short-term activation might be protective in the context of an acute myocardial infarction, long-term inhibition may prevent endothelial dysfunction,[34,38] atherosclerosis, and diabetic vascular disease in mouse models.[21,34] Obviously, this complexity also raises safety and bio-efficacy concerns for translating these results to human domain. Additional research is needed to further clarify the role of p66^{Shc} in other animal models of acute myocardial infarction, before speculating on human diseases.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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