



STAT3 as a common signal of ischemic conditioning: a lesson on “rigor and reproducibility” in preclinical studies on cardioprotection

Petra Kleinbongard¹ · Andreas Skyschally¹ · Sabine Gent¹ · Marion Pesch¹ · Gerd Heusch¹

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Abstract

Ischemic conditioning before (ischemic preconditioning, IPC) or after (ischemic postconditioning, POCO) sustained myocardial ischemia/reperfusion (I/R), induced locally or remotely from the heart (remote IPC, RIPC), reduces infarct size. However, none of the identified signaling steps of ischemic conditioning was robust across models and species to be successfully translated to humans. In prior separate studies in pigs, activation of signal transducer and activator of transcription 3 (STAT3) was causal for infarct size reduction by IPC, POCO, and RIPC but it remains unclear whether or not STAT3 is truly a common denominator of cardioprotective signaling. We therefore, now analyzed the phosphorylation of STAT3 and other signaling proteins in left ventricular biopsies from our prior studies on IPC, POCO and RIPC in one approach. We developed a strategy for the quantification of protein phosphorylation in multiple samples from many experiments on different gels/membranes by Western blot. Along with reduced infarct size, the ratio of STAT3_{tyr705} phosphorylation/total STAT3 protein at early reperfusion was significantly increased by IPC (IPC 2.0 ± 0.3 vs. I/R 1.2 ± 0.2 arbitrary units), but only trendwise by POCO and RIPC (1.3 ± 0.2 ; 1.4 ± 0.2 arbitrary units); storage time for IPC samples was shorter than for POCO and RIPC samples. No other signaling protein phosphorylation was associated with reduced infarct size. We confirmed STAT3 phosphorylation with IPC. For POCO and RIPC we could not reproduce the findings from our earlier more focused studies. At this point, we can not distinguish between lack of robustness of the biological signal and methodological issues of our retrospective approach.

Keywords Infarct size · Pigs · Reperfusion

Introduction

Infarct size is reduced by cycles of brief ischemia/reperfusion before (ischemic preconditioning, IPC) [29] or after (ischemic postconditioning, POCO) [38] sustained myocardial ischemia/reperfusion (I/R), induced either locally or

remotely from the heart (remote IPC, RIPC) [18]. These cardioprotective ischemic conditioning maneuvers are operative in all species tested so far, including humans [22]; however, their cardioprotective efficacy is attenuated in the presence of risk factors, co-morbidities and co-medications [7].

The underlying myocardial signal transduction of these ischemic conditioning maneuvers has been analyzed in many studies using different models and species [16]. Conceptually, three major cardioprotective signaling pathways were identified: the nitric oxide/protein kinase G (NO/PKG) pathway [6], the reperfusion injury salvage kinase (RISK) pathway [15], and the survival activating factor enhancement (SAFE) pathway [27]. In contrast to the ischemic conditioning procedures as such, none of the many identified signaling steps has emerged as robust enough to be translated successfully to humans, notably to patients suffering an acute myocardial infarction or undergoing cardiovascular surgery [2, 5, 10–12, 17, 19, 22, 28].

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✉ Gerd Heusch
gerd.heusch@uk-essen.de

¹ Institute for Pathophysiology, West German Heart and Vascular Center, Medical School, University of Essen, Hufelandstr. 55, 45122 Essen, Germany

In our previous studies in an established and clinically relevant pig model [23], the tyrosine₇₀₅ phosphorylation of signal transducer and activator of transcription 3 (STAT3), a decisive element of the SAFE pathway [27], was causally involved in cardioprotection by IPC [9], POCO [20], and RIPC [34]. Pharmacological blockade of STAT3 activation abrogated the cardioprotection induced by the different types of ischemic conditioning, suggesting it may be a common denominator of cardioprotective signaling. For POCO the cardioprotection was mediated through mitochondrial STAT3 activation, this activation was associated with improved mitochondrial function [20]. We never identified a signal step of the RISK pathway as important in our pig studies whereas others did [13]. However, we identified increased p38 mitogen-activated protein kinase (MAPK) β activity in IPC and abrogated IPC's protection by pharmacological blockade of p38 MAPK [32, 33]. None of the above signaling steps, but only the tyrosine_{694/699} phosphorylation of STAT5 at early reperfusion was identified in left ventricular biopsies in association with reduced troponin levels in patients undergoing cardiovascular surgery with prior RIPC [21].

To establish if STAT3 phosphorylation is indeed a common underlying mechanism of cardioprotection by ischemic conditioning, it is not sufficient to compare each IPC [9], POCO [20], and RIPC [34] in separate studies with I/R per se, but a comparison of all cardioprotective interventions with contemporary I/R controls in a single joint analysis is needed. Such joint analysis then requires the analysis of data from many experiments which can only be performed over a longer time frame and, for our specific target of phosphorylated signaling proteins; it requires Western blotting on multiple gels/membranes rather than under optimized conditions on a single gel/membrane. At this point, the purpose of our analysis runs into the problems of rigor and robustness of preclinical data which are of increasing concern [4, 24, 25] and pose a significant barrier for successful translation of cardioprotective strategies to the clinical situation [17]. We therefore developed a strategy to quantify and compare protein phosphorylation in multiple left ventricular biopsy samples from a large number of prior pig experiments. Using this approach, we analyzed again STAT3 tyrosine₇₀₅ phosphorylation [27, 35]. Both, STAT3 tyrosine₇₀₅ and serine₇₂₇ phosphorylation have been implicated in cardioprotection [3]. However, we focus on tyrosine₇₀₅ phosphorylation, because in our prior study on POCO the tyrosine₇₀₅ phosphorylation and not the serine₇₂₇ phosphorylation was involved in cardioprotection [20]. For comparison, we also analyzed representative signals of the other above pathways, i.e., on vasodilator-stimulated phosphoprotein (VASP) as a target of PKG [1],

on protein kinase B (AKT), extracellular signal-regulated kinase 1/2 (ERK1/2) and glycogen synthase kinase-3 β (GSK-3 β) as signals of the RISK pathway [14]. We also analyzed p38 MAPK as a potential signal step outside of the three above pathways, which we have found involved in IPC in our pig model [32, 33].

Methods

In vivo studies in pigs with sequential myocardial biopsies

The protocols of the present study were approved by the North Rhine Westphalia State Environment Agency (LANUV NRW, Germany) (G1240/11), and they conform with the Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, revised 1996.

The experimental preparation has been described in detail before [9, 34]. In brief, male Göttinger minipigs (20–40 kg) were anesthetized with isoflurane (2% *v/v*, Forene; abbvie, Ludwigshafen, Germany), and their hearts were exposed through a left lateral thoracotomy. A silk suture was placed around the left anterior descending coronary artery (LAD) distal to its second diagonal branch for later occlusion. Systemic hemodynamics were continuously recorded, and transmural myocardial blood flow was measured with colored microspheres [26]. All experiments, irrespective of the performed experimental protocols, were started in the morning.

Experimental protocols

Ischemia/reperfusion (I/R; n = 23; February 2012–September 2016) At baseline, systemic hemodynamics and regional myocardial blood flow were measured. Myocardial drill biopsies (2–4 mg) were taken from the designated area at risk, snap-frozen in liquid nitrogen and stored at -80°C for later Western blot analysis. After 5 min LAD occlusion, systemic hemodynamics and regional myocardial blood flow were measured again. Myocardial biopsies were taken again after 55 min LAD occlusion. Reperfusion was induced after 60 min LAD occlusion by quick release and removal of the suture and visually confirmed by the re-appearance of red color on the surface of the reperfused myocardium. Systemic hemodynamics were measured again at 30, 60 and 120 min reperfusion. Additional myocardial biopsies were taken at 10 and 120 min reperfusion. The experiment was terminated after 180 min reperfusion.

Ischemic preconditioning (IPC; n = 19; October 2012–March 2016) The experimental protocol was identical

to that of I/R, except that the LAD was occluded twice for 3 min, separated by 2 min reperfusion, 15 min before the 60 min LAD occlusion.

Ischemic postconditioning (POCO; n = 17; February 2012–April 2016) The experimental protocol was identical to that of I/R, except that at 1 min reperfusion the LAD was re-occluded 4 times for 1 min each, separated by 1 min reperfusion.

Remote ischemic preconditioning (RIPC; n = 23; March 2013–December 2014) RIPC was performed after induction of anesthesia by tightening a tourniquet around the left hindlimb; pale skin was taken to indicate leg ischemia. The tourniquet was released after 5 min, and the hindlimb was reperfused for 5 min; skin blush was taken to indicate reperfusion. Occlusion/reperfusion of the hindlimb was performed four times in total. The subsequent protocol was identical to that of I/R.

Four pigs from the above protocols were excluded due to insufficient ischemia (transmural myocardial blood flow at 5 min ischemia > 0.06 ml/min/g), one from the IPC, one from the POCO and two from the RIPC protocols, respectively.

Infarct size

The LAD was re-occluded, and Patentblau V (Guerbet GmbH, Sulzbach, Germany) was injected into the left atrium to delineate the area at risk. Post mortem, the left ventricle was cut into five slices parallel to the atrioventricular groove. Infarcted tissue was demarcated by triphenyl tetrazolium chloride (TTC; Sigma-Aldrich Chemie GmbH, München, Germany) staining. The area at risk was expressed as a fraction of the left ventricle, and infarct size was expressed as fraction of the area at risk [9, 34].

Processing of myocardial biopsies

Myocardial biopsies were homogenized in 100 mmol/l tris(hydroxymethyl)-aminomethane (Tris, Sigma-Aldrich Chemie GmbH, München, Germany) with 2% sodium dodecyl sulfate (SDS; w/v; SERVA Electrophoresis GmbH, Heidelberg, Germany), heated to 70 °C for 5 min and centrifuged at 14,000 × g for 10 min. The proteins, lysed in the supernatant, were stored at –80 °C in aliquots to prevent freeze- and thaw-cycles. Protein lysates were separated by electrophoresis on precasted SDS–polyacrylamide gels (BioRad, Munich, Germany) and transferred to polyvinylidene fluoride membranes (BioRad, Munich, Germany). Membranes were stained with Ponceau S (SERVA, Heidelberg, Germany) as loading/transfer control. After blocking with fat-free milk (BioRad, Munich, Germany), membranes were sequentially incubated with antibodies directed against the phosphorylated forms of specific proteins over night at 4 °C (Table 1). Membranes were washed and then incubated with secondary antibodies for 45 min at room temperature. Immunoreactive signals were detected by enhanced chemiluminescence (ECL; Pierce Biotechnology, Rockford, USA) using a CCD camera (INTAS, Göttingen, Germany) and quantified with LabImage 1 D software (INTAS, Göttingen, Germany). Only images in which the signal intensity was at least twice the pixel intensity of the background were used for later analysis. After detection of the phosphorylated forms of each protein, membranes were stripped by incubation in stripping buffer (165 mmol/ltris(hydroxymethyl)-aminomethane, 250 mmol/l NaOH; Sigma-Aldrich Chemie GmbH, München, Germany) for 10 min at 55 °C. For each protein/primary and secondary antibody combination this stripping procedure was tested by incubation of the stripped membrane with the respective secondary antibodies and ECL reagent, and it resulted in no detectable chemiluminescence. After

Table 1 Antibodies

Protein	Phosphorylation site	Source	Antibody dilution	Order number	Manufacturer
Phospho AKT	ser ₄₇₃	Rabbit	1:500	#9271	Cell signaling
Total AKT		Rabbit	1:500	#9272	Cell signaling
Phospho ERK1	thr ₂₀₂ /tyr ₂₀₄	Rabbit	1:1000	#9101	Cell signaling
Phospho ERK2	thr ₁₈₅ /tyr ₁₈₇				
Total ERK1/2		Rabbit	1:1000	#9102	Cell signaling
Phospho GSK-3β	ser ₉	Rabbit	1:500	#9336	Cell signaling
Total GSK-3β		Mouse	1:500	#610202	BD biosciences
Phospho p38 MAPK	thr ₁₈₀ /tyr ₁₈₂	Rabbit	1:750	#9211	Cell signaling
Total p38 MAPK		Rabbit	1:1000	#9212	Cell signaling
Phospho STAT3	tyr ₇₀₅	Mouse	1:750	#9138	Cell signaling
Total STAT3		Rabbit	1:500	#12640	Cell signaling
VASP		Rabbit	1:300	#3112	Cell signaling

AKT protein kinase B, ERK1/2 extracellular signal-regulated kinase 1/2, GSK-3β glycogen synthase kinase-3β, p38 MAPK p38 mitogen-activated protein kinase, ser serine, STAT signal transducer and activator of transcription, thr threonine, tyr tyrosine, VASP vasodilator-stimulated phosphoprotein

Table 2 Amount of cardioprotective signaling proteins normalized to Ponceau S at baseline and at 10 min reperfusion

	Baseline				R10				<i>p</i>
	I/R (<i>n</i> = 23)	IPC (<i>n</i> = 18)	POCO (<i>n</i> = 16)	RIPC (<i>n</i> = 21)	IR (<i>n</i> = 23)	IPC (<i>n</i> = 18)	POCO (<i>n</i> = 16)	RIPC (<i>n</i> = 21)	
STAT3	1.06 ± 0.04	1.04 ± 0.05	1.09 ± 0.05	0.98 ± 0.03	1.02 ± 0.04	0.96 ± 0.03	1.02 ± 0.04	1.05 ± 0.03	0.36
AKT	1.03 ± 0.04	1.05 ± 0.04	1.09 ± 0.06	1.04 ± 0.06	0.98 ± 0.04	0.97 ± 0.04	1.01 ± 0.04	1.04 ± 0.03	0.58
ERK1/2	1.02 ± 0.03	1.05 ± 0.03	1.07 ± 0.05	0.98 ± 0.04	1.01 ± 0.03	1.01 ± 0.04	1.02 ± 0.03	1.0 ± 0.03	0.98
GSK-3β	1.08 ± 0.03	1.0 ± 0.05	0.98 ± 0.04	1.02 ± 0.06	0.96 ± 0.03	1.02 ± 0.04	1.05 ± 0.03	0.99 ± 0.3	0.26
VASP	0.96 ± 0.05	1.06 ± 0.07	1.02 ± 0.06	0.89 ± 0.06	1.13 ± 0.07	1.03 ± 0.07	0.90 ± 0.05	0.98 ± 0.08	0.16
p38 MAPK	1.02 ± 0.04	1.0 ± 0.05	0.97 ± 0.05	0.98 ± 0.06	0.96 ± 0.04	1.02 ± 0.04	1.04 ± 0.04	1.06 ± 0.04	0.28

Data are mean ± SEM normalized to Ponceau S
 I/R ischemia/reperfusion, IPC ischemic preconditioning, POCO ischemic postconditioning, RIPC remote ischemic preconditioning, AKT protein kinase B, ERK1/2 extracellular signal-regulated kinase 1/2, GSK-3β glycogen synthase kinase-3β, *p*-value using one-way ANOVA, p38 MAPK p38 mitogen-activated protein kinase, R10 10 min reperfusion, STAT3 signal transducer and activator of transcription 3, VASP vasodilator-stimulated phosphoprotein

Table 3 Amount of relative phosphorylation of cardioprotective signaling proteins at baseline and at 10 min reperfusion

Protein	Baseline				R10				<i>p</i>
	I/R (<i>n</i> = 23)	IPC (<i>n</i> = 18)	POCO (<i>n</i> = 16)	RIPC (<i>n</i> = 21)	I/R (<i>n</i> = 23)	IPC (<i>n</i> = 18)	POCO (<i>n</i> = 16)	RIPC (<i>n</i> = 21)	
p/t STAT3	1.11 ± 0.08	0.98 ± 0.11	0.92 ± 0.06	0.94 ± 0.07	1.0 ± 0.09	1.26 ± 0.07	1.0 ± 0.11	1.0 ± 0.09	0.14
p/t AKT	1.08 ± 0.09	0.84 ± 0.10	0.80 ± 0.08	0.93 ± 0.10	1.15 ± 0.08	0.92 ± 0.09	0.96 ± 0.1	1.05 ± 0.1	0.27
p/t ERK1/2	1.20 ± 0.13	0.98 ± 0.11	0.82 ± 0.08	0.91 ± 0.09	1.11 ± 0.09	1.11 ± 0.09	0.82 ± 0.08	1.02 ± 0.1	0.14
p/t GSK-3β	0.92 ± 0.06	0.97 ± 0.06	1.12 ± 0.13	0.94 ± 0.08	1.03 ± 0.06	0.96 ± 0.06	0.96 ± 0.09	1.12 ± 0.1	0.43
p/t VASP	1.20 ± 0.08	0.88 ± 0.07	1.17 ± 0.11	1.32 ± 0.15	0.98 ± 0.08	0.97 ± 0.1	1.14 ± 0.1	1.2 ± 0.1	0.21
p/t p38 MAPK	1.20 ± 0.15	0.75 ± 0.10	0.88 ± 0.13	0.91 ± 0.10	1.21 ± 0.13	1.07 ± 0.1	0.77 ± 0.09 ⁺	0.92 ± 0.09	0.04

Data are mean ± SEM
 I/R ischemia/reperfusion, IPC ischemic preconditioning, POCO ischemic postconditioning, RIPC remote ischemic preconditioning, AKT protein kinase B, ERK1/2 extracellular signal-regulated kinase 1/2, GSK-3β glycogen synthase kinase-3β, *p*-value using one-way ANOVA, p38 MAPK p38 mitogen-activated protein kinase, *p*/t ratio of phosphorylated protein/total protein, R10 10 min reperfusion, STAT3 signal transducer and activator of transcription 3, VASP vasodilator-stimulated phosphoprotein
⁺ *p* < 0.05 vs. I/R

Table 4 Systemic hemodynamics, transmural blood flow, area at risk and infarct size

	HR (1/min)	LVP _{max} (mmHg)	dP/dt _{max} (mmHg/s)	TMBF (ml/min g)	Area at risk (% left ventricle)	Infarct size (% of area at risk)
I/R (n = 23)						
Baseline	116 ± 3	91 ± 2*	1767 ± 77*	0.82 ± 0.04*	23 ± 1	43 ± 2
I5	114 ± 2	80 ± 2*	1397 ± 53*	0.029 ± 0.003*		
I55	119 ± 3	82 ± 2*	1522 ± 36*			
R30	117 ± 3	81 ± 2*	1536 ± 61*			
R60	116 ± 3	81 ± 2*	1567 ± 54*			
R120	117 ± 3	77 ± 2*	1432 ± 67*			
IPC (n = 18)						
Baseline	116 ± 4*	88 ± 2*	1659 ± 67*	0.76 ± 0.09*	26 ± 1	21 ± 2 ⁺
I5	108 ± 4*	79 ± 2*	1394 ± 57*	0.023 ± 0.004*		
I55	110 ± 3*	79 ± 2*	1422 ± 64*			
R30	111 ± 3*	79 ± 1*	1441 ± 82*			
R60	111 ± 3*	79 ± 2*	1470 ± 89*			
R120	109 ± 3*	76 ± 2*	1381 ± 88*			
POCO (n = 16)						
Baseline	119 ± 3	88 ± 2*	1700 ± 94*	0.82 ± 0.05*	22 ± 2	26 ± 3 ⁺
I5	113 ± 4	79 ± 2*	1373 ± 65*	0.028 ± 0.005*		
I55	117 ± 3	79 ± 2*	1466 ± 108*			
R30	115 ± 3	75 ± 2*	1550 ± 139*			
R60	114 ± 3	74 ± 2*	1407 ± 91*			
R120	113 ± 4	73 ± 2*	1287 ± 83*			
RIPC (n = 21)						
Baseline	112 ± 2*	87 ± 2*	1657 ± 110	0.71 ± 0.04*	22 ± 1	21 ± 3 ⁺
I5	110 ± 3*	79 ± 2*	1335 ± 68*	0.029 ± 0.003*		
I55	113 ± 4*	79 ± 2*	1385 ± 65*			
R30	117 ± 4*	79 ± 2*	1465 ± 83*			
R60	119 ± 3*	79 ± 2*	1498 ± 89*			
R120	115 ± 3*	78 ± 2*	1380 ± 84*			

Data are mean ± SEM

I/R ischemia/reperfusion, IPC ischemic preconditioning, POCO ischemic postconditioning, RIPC remote ischemic preconditioning, I5/55 5/55 min ischemia, R30/60/120 30/60/120 min reperfusion, HR heart rate, LVP_{max} maximal left ventricular pressure, dP/dt_{max} maximal rate of rise of left ventricular pressure, TMBF transmural blood flow

**p* < 0.05 vs. baseline, ⁺*p* < 0.05 vs. I/R

the stripping procedure the membranes were re-probed with antibodies against their respective total form. For the detection of VASP, the antibody simultaneously targets the phosphorylated (50 kDa) and the total form (46 kDa) [1], and therefore both signals were detected on the same membrane. To correct for unequal loading, data for total and phosphorylated proteins were normalized to Ponceau S staining, and immunoreactivities of phosphorylated proteins were also normalized to their respective total forms (termed as “relative phosphorylation”).

We visually screened all membranes after Ponceau S staining. Degraded samples and samples with obviously different protein patterns were replaced by samples from

another myocardial biopsy taken at the same time from the same experiment. We assumed that different protein patterns reflect a largely different composition of cell types (i.e., cardiomyocytes, endothelial cells, erythrocytes, fibroblasts, leukocytes, neurons and vascular smooth muscle cells) in these particular samples. The number of excluded samples did not differ between I/R, IPC, POCO, and RIPC.

Quantitative characterization of data from multiple Western blots

Sequential samples from 78 different experiments precluded the comparison of samples on a single gel/membrane. We

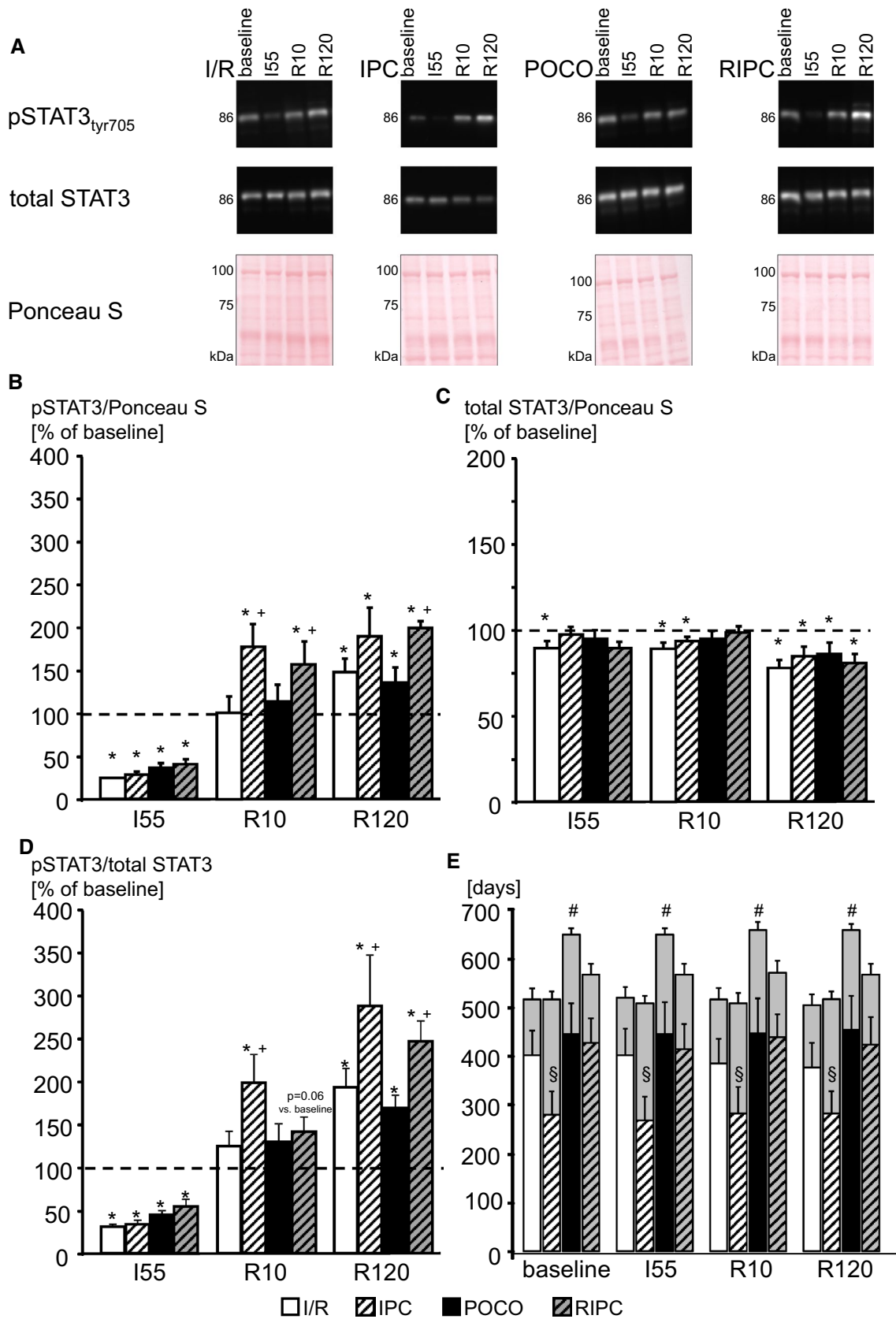


Fig. 1 The phosphorylation of signal transducer and activator of transcription 3 (STAT3) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic post-conditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated STAT3 at ty_{R705} and total STAT3 in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (a). Amount of phosphorylated STAT3 (b) and total STAT3 (c) normalized to Ponceau S staining, respectively, and relative phosphorylation of STAT3 normalized to the respective total form of STAT3 (d); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of STAT3 (e). Data are mean \pm SEM, two-way ANOVA for repeated measures (b–d) and two-way ANOVA (e), both with post hoc Fisher's LSD test, * $p < 0.05$ vs. baseline, [†] $p < 0.05$ vs. I/R, [§] $p < 0.05$ vs. myocardial biopsy storage time of all other groups, [#] $p < 0.05$ vs. total storage time of all other groups

have therefore established a method to facilitate the quantitative analysis of such a big sample number on Western blots, through correction for inevitable technical variations from use of multiple gels, with differences in the efficiency of the gel/membrane protein transfer and differences in the numerical readout resulting from a different light intensity of the ECL reaction per protein. Our approach preserved, however, the biological variations from interindividual differences and the impact of the different experimental protocols.

Prerequisite for such technical correction is a numerical readout of protein/antibody/ECL responses within the linear range. To test for this, 5, 10, 20, 30, and 50 μ g of a pooled lysate were electrophoretically separated and transferred to a membrane which was incubated with different dilutions for each antibody used in this study. Sample readouts were in the linear range when using 20 μ g protein lysate per lane in combination with the respective antibody at its specific dilution (Table 1), as optimized in this test. All analyses were then performed under these conditions.

To correct for technical variations but preserve biological variations when samples obtained under identical conditions are loaded on different gels and exposed to the same set of antibodies, the total numerical ECL signal intensity for each protein of interest must be set identical on each membrane. To set the signal intensity of a protein of interest when loaded on different gels and analyzed on different membranes as identical, the signal intensity of each protein of interest was normalized by multiplication with the reciprocal value of the respective total signal intensity of that protein on each membrane. Since total and phosphorylated proteins were normalized to Ponceau S staining and

phosphoproteins were normalized to their respective total forms (“relative phosphorylation”), the above normalization procedure was also done for the Ponceau S staining of the entire line of proteins on each membrane. To test this correction procedure we prepared six gels with protein lysates from baseline samples ($n = 3$ per group). After the above correction procedure, the variation coefficients for the Ponceau S staining from the same lysate were in the molecular weight range of AKT (50–70 kDa) $7 \pm 2\%$, ERK1/2 (35–50 kDa) $3 \pm 2\%$, and STAT3 (70–120 kDa) $7 \pm 2\%$, respectively. After probing these membranes with antibodies against the phosphorylated and total forms of AKT, ERK1/2, and STAT3, the variation coefficients for the same lysate, were for the amount of the total proteins AKT $7 \pm 1\%$, ERK1/2 $8 \pm 1\%$, and STAT3 $10 \pm 1\%$, and for the relative phosphorylation of AKT $8 \pm 1\%$, ERK1/2 $5 \pm 1\%$, and STAT3 $11 \pm 2\%$, respectively. We also increased the exposure time in the ECL detection system, resulting in a tenfold increase of the numerical readout. Again using the above correction procedure, the variation coefficients for the same lysate on one gel were for the amount of the total proteins AKT $0.8 \pm 0.1\%$, ERK1/2 $1.2 \pm 0.2\%$, and STAT3 $4.6 \pm 0.8\%$, and for the relative phosphorylation of AKT $1.1 \pm 0.1\%$, ERK1/2 $1.7 \pm 0.1\%$, and STAT3 $4.2 \pm 0.6\%$, respectively.

Having corrected for technical variations, we then assured that the total membrane signal intensity resulting from biological variations was also identical. For that, we loaded on each gel the same number of samples from all four experimental protocols (I/R, IPC, POCO, RIPC). To compensate for unequal n values in the experimental groups, some samples were used on multiple gels. Samples from different protocols were randomized on each gel. Seven baseline samples and eight samples taken at 10 min reperfusion were excluded due to protein degradation or obviously different protein patterns and replaced by alternate samples from the respective animal taken at the same time point. Excluded samples, however, were not excluded from the above correction procedure. The decisions to exclude samples were made by a person who was not involved in the present study and had no knowledge about the sample assignment, and it was made after visual inspection of all Ponceau S stainings.

Using the above correction procedure for technical variations, we compared baseline samples and samples at 10 min reperfusion from each protocol. Having assured, that baseline samples from the four protocols were not different (Tables 2, 3), we then normalized time courses of the expression of the total proteins (normalized to Ponceau S) and of the relative protein phosphorylation (normalized to the total protein) to their respective baseline. For the

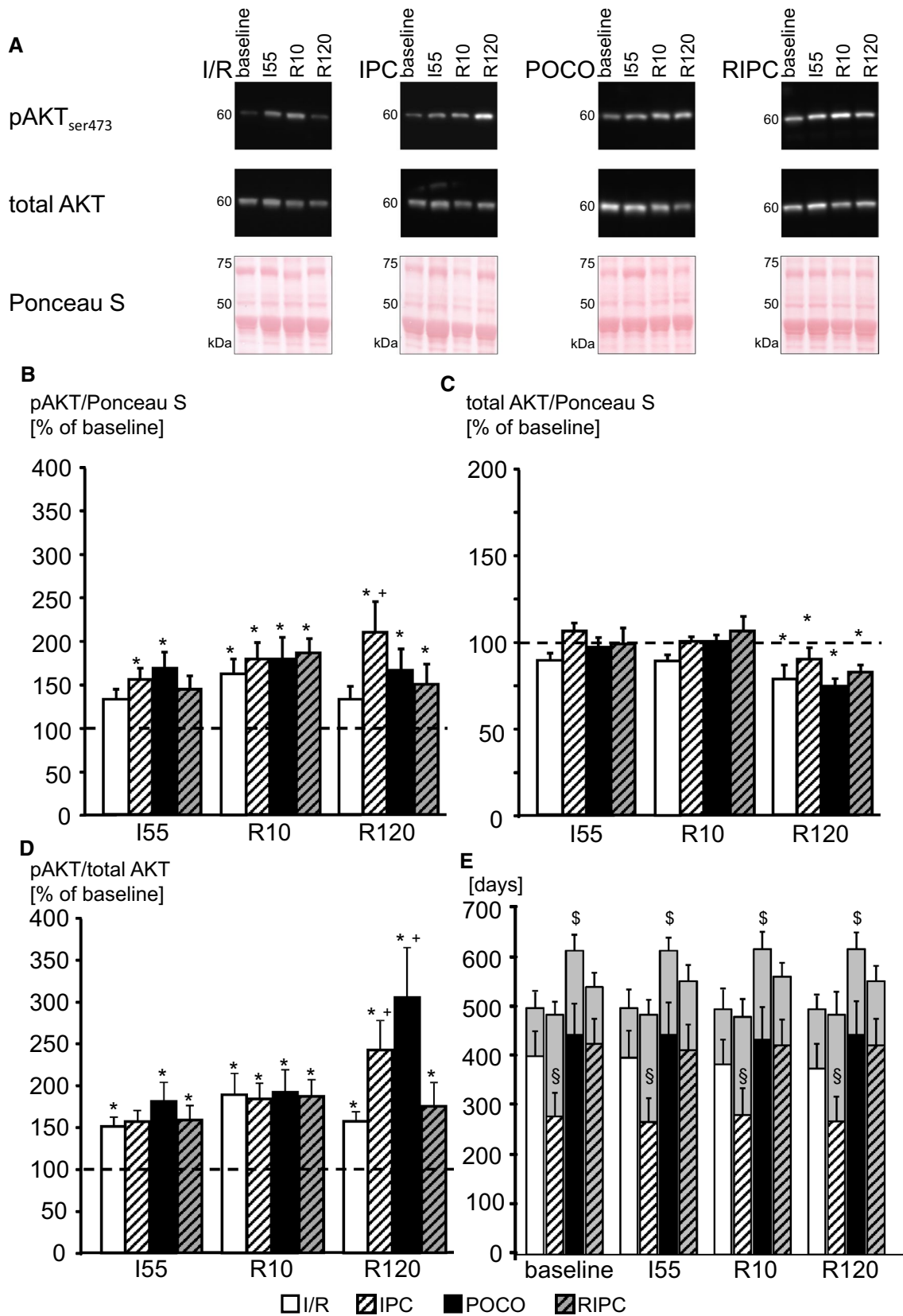


Fig. 2 Phosphorylation of protein kinase B (AKT) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic postconditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated AKT at ser₄₇₃ and total AKT in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (**a**). Amount of phosphorylated AKT (**b**) and total AKT (**c**) normalized to Ponceau S staining, respectively, and relative phosphorylation of AKT normalized to the respective total form of AKT (**d**); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of AKT (**e**). Data are mean \pm SEM, two-way ANOVA for repeated measures (**b–d**) and two-way ANOVA (**e**), both with post hoc Fisher's LSD test, * $p < 0.05$ vs. baseline, ⁺ $p < 0.05$ vs. I/R, [§] $p < 0.05$ vs. myocardial biopsy storage time of all other groups, [§] $p < 0.05$ vs. total storage time of I/R and IPC

time course analysis, (phospho-)proteins for each animal were loaded on the same gel and analyzed on the same membrane. Again, some samples were excluded due to protein degradation or obviously different protein patterns (8 samples taken at 55 min ischemia and 6 samples taken at 120 min reperfusion). These samples were replaced by alternate samples from the respective animal taken at the same time point, and the complete individual time course was re-analyzed on a new gel/membrane. Storage time of myocardial biopsies and of their respective protein lysates, which were included in the final time course analysis, was determined.

Statistics

Statistical outliers were identified by the Dean-Dixon test and excluded. No more than one outlier per membrane was identified. Data are given as mean \pm SEM. Area at risk, infarct size and the amount of signaling proteins, separately for total proteins normalized to Ponceau S, phosphorylated proteins normalized to Ponceau S, and phosphorylated proteins normalized to total proteins ("relative phosphorylation"), at baseline and at 10 min reperfusion were compared between different protocols by one-way ANOVA. Hemodynamics, transmural myocardial blood flow and time courses of total as well as of signaling proteins were analyzed by two-way repeated measures ANOVA. Storage time of myocardial biopsies and that of their respective protein lysates were analyzed by two-way ANOVA. When a significant difference was detected, individual mean values were compared by Fisher's LSD post hoc test. Differences were considered significant at the level of $p < 0.05$. Pearson's coefficient of correlation was used to

characterize the relation between relative phosphorylation of STAT3 at 10 min reperfusion and infarct size and the relation between storage time and the total protein amount as well as protein phosphorylation, respectively. All statistical analyses were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, USA).

Results

Systemic hemodynamics, area at risk, transmural myocardial blood flow and infarct size in pigs

Heart rate was not different between the experimental groups and remained unchanged throughout the protocols. Maximal left ventricular pressure and maximal rate of rise of left ventricular pressure decreased with the onset of ischemia and remained below baseline up to the end of the protocols; these changes were comparable between groups. Area at risk was comparable between groups, and the transmural myocardial blood flow in the area at risk was similar between groups at baseline and reduced to the same extent during ischemia. The reduction of infarct size by IPC, POCO, RIPC versus I/R was of comparable magnitude (Table 4).

Comparison of total and phosphorylated proteins across protocols at baseline and at 10 min reperfusion

At baseline, the amounts of total protein (Table 2) and relative protein phosphorylation (Table 3) of STAT3, AKT, ERK1/2, GSK-3 β , VASP and p38 MAPK did not differ between groups. At 10 min reperfusion, the amount of all total proteins (Table 2) was also not different. The relative phosphorylation of STAT3 at 10 min reperfusion was higher with IPC than with I/R by trend, but not with POCO and RIPC (Table 3). The relative phosphorylation of p38 MAPK at 10 min reperfusion was lower with POCO than in the other groups (Table 3).

Time course of total and phosphorylated proteins in the different protocols

The amount of total STAT3, AKT, ERK1/2, GSK-3 β and p38 MAPK decreased slightly over time, most likely due to unspecific proteolysis during ischemia and reperfusion, but without differences between groups (Figs. 1c, 2c, 3c, 4c, 6c, respectively). The amount of total VASP, however, was stable over time (Fig. 5c).

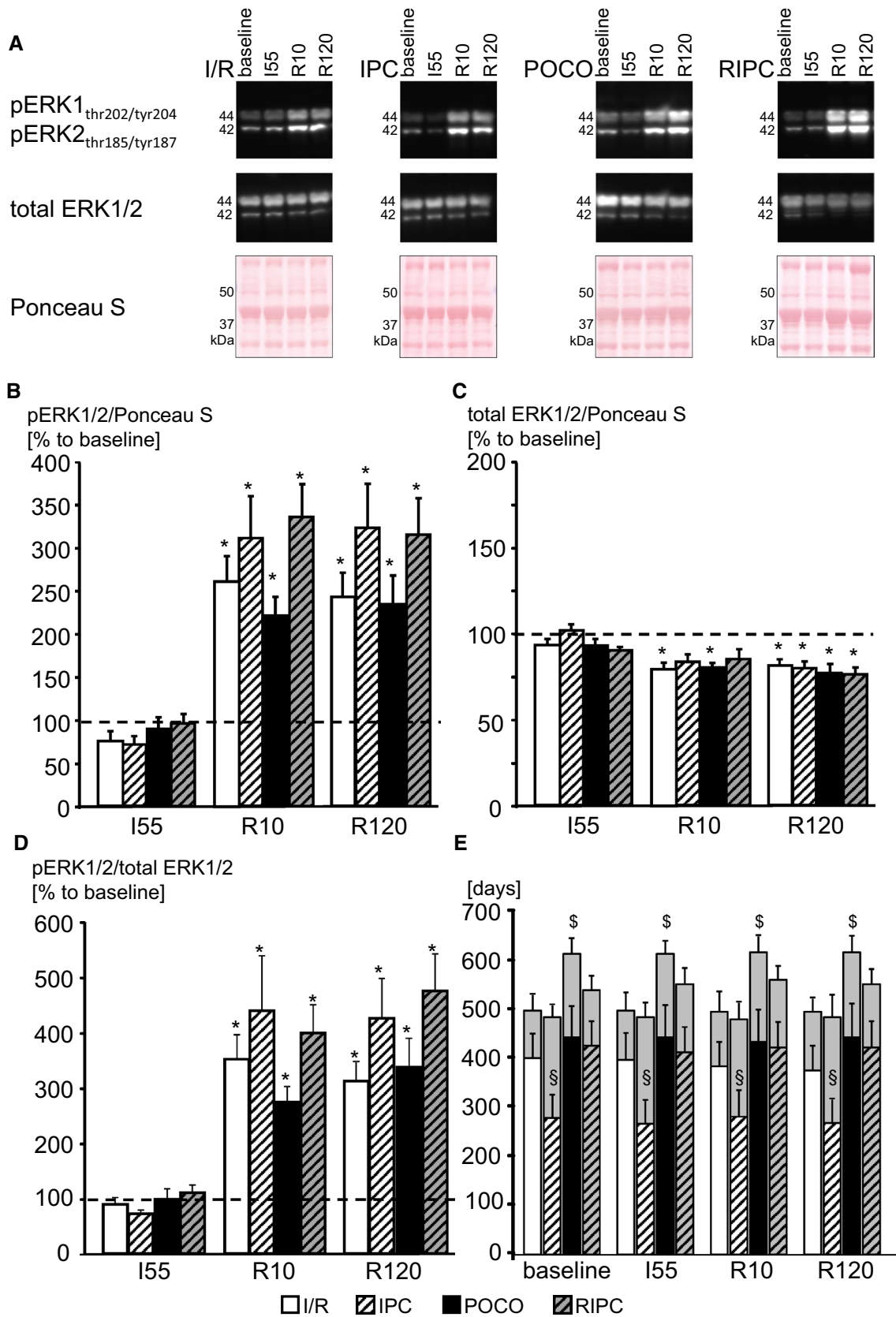


Fig. 3 Phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic postconditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated ERK1 at thr₂₀₂/tyr₂₀₄ and ERK2 at thr₁₈₅/tyr₁₈₇ and total ERK1/2 in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (a). Amount of phosphorylated ERK1/2 (b) and total ERK1/2 (c) normalized to Ponceau S staining, respectively, and relative phosphorylation of ERK1/2 normalized to the respective total form of ERK1/2 (d); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of ERK1/2 (e). Data are mean \pm SEM, two-way ANOVA for repeated measures (b–d) and two-way ANOVA (e), both with post hoc Fisher's LSD test, * $p < 0.05$ vs. baseline, [†] $p < 0.05$ vs. I/R, [§] $p < 0.05$ vs. myocardial biopsy storage time of all other groups, [§] $p < 0.05$ vs. total storage time of I/R and IPC

The amount of phosphorylated STAT3 and the relative phosphorylation of STAT3 decreased during ischemia in all groups (Fig. 1b, d). At 10 min reperfusion, the amount of phosphorylated STAT3 was significantly increased with IPC and RIPC over baseline and that with I/R (Fig. 1b). The relative phosphorylation of STAT3 was only significantly increased with IPC and trendwise with RIPC (Fig. 1d). The relative phosphorylation STAT3 at 10 min reperfusion did not correlate with infarct size in all groups, respectively (I/R: $r = 0.163$, IPC: $r = 0.284$, POCO: $r = 0.291$, and RIPC: $r = 0.1847$). The amount of phosphorylated STAT3 and the relative phosphorylation was further increased at 120 min reperfusion in all groups, but was higher with IPC and RIPC than I/R and POCO (Fig. 1b, d). For STAT3 analysis, the storage time for myocardial biopsy samples and their total storage time, including that for their protein lysates, varied for the different protocols (Fig. 1e), and it was clearly shortest for IPC where, in turn, STAT3 phosphorylation was most pronounced. The biopsy storage time and the total storage time ranged from 1 to 894 days and 64 to 1459 days, respectively. There was no correlation of biopsy storage time and total storage time to total STAT3 protein, but a weak correlation to relative STAT3 phosphorylation (biopsy storage time STAT3: $r = 0.052$, pSTAT3: $r = 0.325$; total storage time STAT3: $r = 0.023$, pSTAT3: $r = 0.384$, respectively) at baseline where all samples were obtained under identical conditions.

The amount of phosphorylated AKT and the relative phosphorylation of AKT was increased during ischemia and did not change until 10 min reperfusion in all groups.

At 120 min reperfusion, the amount of phosphorylated AKT was increased with IPC, the relative phosphorylation of AKT was increased with IPC and POCO (Fig. 2b, d).

The amount of phosphorylated ERK1/2 and the relative phosphorylation of ERK1/2 did not change during ischemia, but was increased at 10 min reperfusion and then did not change further until 120 min reperfusion (Fig. 3b, d). The increase in ERK1/2 phosphorylation at reperfusion was higher by trend with IPC and RIPC (Fig. 3b, d).

The amount of phosphorylated GSK-3 β was increased by trend (Fig. 4c), and the relative phosphorylation of GSK-3 β was increased significantly during ischemia in all groups (Fig. 4d). Both, the amount of phosphorylated GSK-3 β and the relative phosphorylation of GSK-3 β then decreased until 120 min reperfusion below baseline in all groups (Fig. 4b, d).

The amount of phosphorylated VASP and the relative phosphorylation of VASP did not really change in all groups (Fig. 5b, d).

The amount of phosphorylated p38 MAPK and the relative phosphorylation of p38 MAPK increased during ischemia and then decreased during reperfusion in all groups (Fig. 6b, d).

As for the myocardial biopsy samples used for STAT3, the storage time of samples used for AKT, ERK1/2, GSK-3 β VASP, and p38 MAPK varied for the different protocols (Figs. 2e, 3e, 4e, 5e, 6e, respectively). However, for none of these proteins was there a correlation between biopsy and total storage time and total protein or the protein phosphorylation (biopsy storage time AKT: $r = 0.046$, pAKT: $r = 0.061$, ERK1/2: $r = 0.112$, pERK1/2: $r = 0.099$, GSK-3 β : $r = 0.059$, pGSK-3 β : $r = 0.103$, VASP: $r = 0.165$, pVASP: $r = 0.036$, and p38 MAP: $r = 0.067$, p-p38 MAP: $r = 0.289$, and for total storage time AKT: $r = 0.091$, pAKT: $r = 0.135$, ERK1/2: $r = 0.149$, pERK1/2: $r = 0.236$, GSK-3 β : $r = 0.029$, pGSK-3 β : $r = 0.044$, VASP: $r = 0.102$, pVASP: $r = 0.052$, and p38 MAP: $r = 0.148$, p-p38 MAP: $r = 0.01$, respectively).

Discussion

In the present study, infarct size was reduced to more or less the same extent by IPC, POCO and RIPC as compared to I/R per se, thus confirming our prior reports about the cardioprotection by the different types of ischemic conditioning and permitting the quantitative comparison of potentially causally involved cardioprotective signals. AKT, ERK1/2, GSK-3 β p38 MAPK and

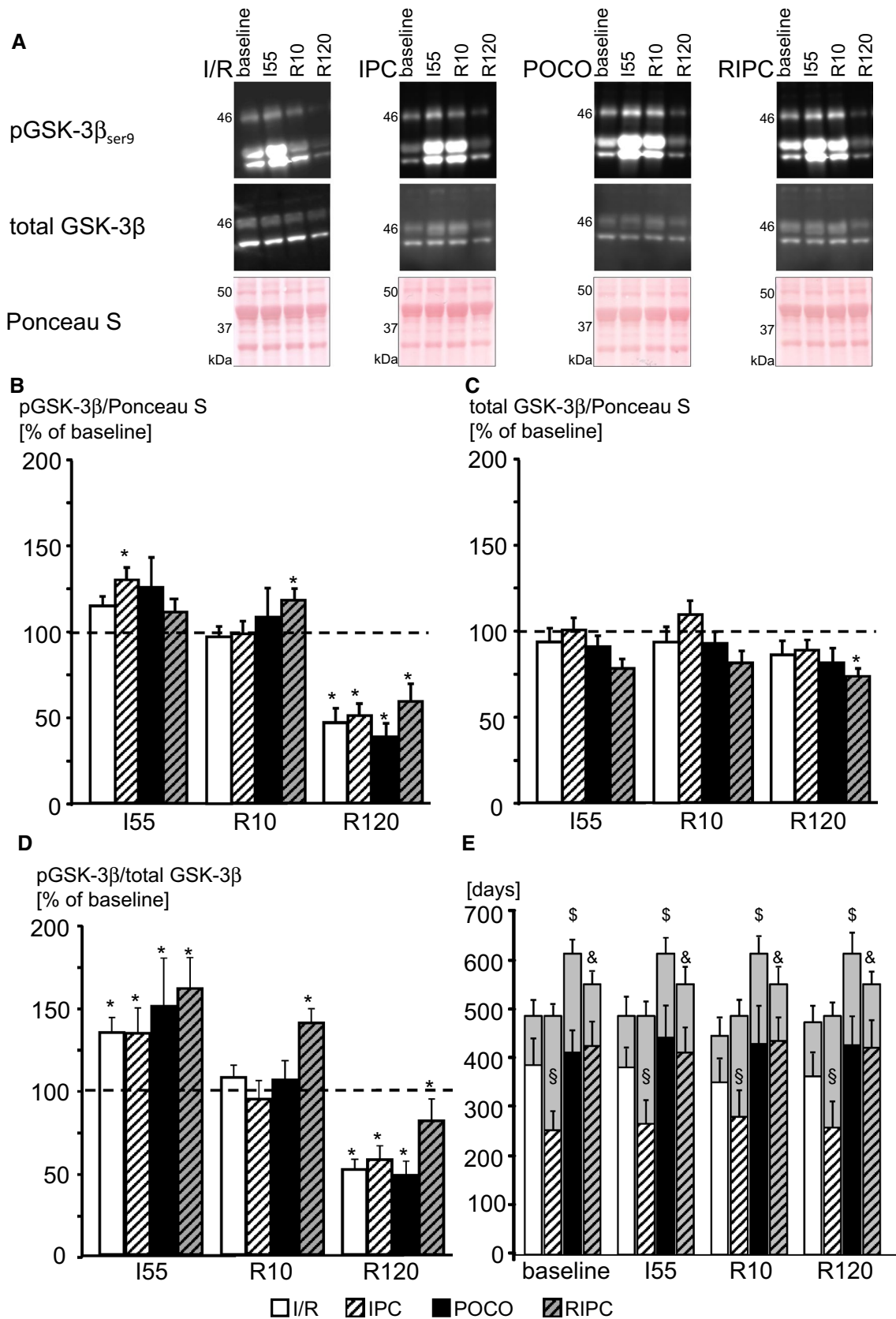


Fig. 4 Phosphorylation of glycogen synthase kinase-3 β (GSK-3 β) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic postconditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated GSK-3 β at ser₉ and total GSK-3 β in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (a). Amount of phosphorylated GSK-3 β (b) and total GSK-3 β (c) normalized to Ponceau S staining, respectively, and relative phosphorylation of GSK-3 β normalized to the respective total form of GSK-3 β (d); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of GSK-3 β (e). Data are mean \pm SEM, two-way ANOVA for repeated measures (b–d) and two-way ANOVA (e), both with post hoc Fisher's LSD test, * p < 0.05 vs. baseline, [†] p < 0.05 vs. I/R, [§] p < 0.05 vs. myocardial biopsy storage time of all other groups, [§] p < 0.05 vs. total storage time of I/R and IPC, [&] p < 0.05 vs. total storage time of I/R

PKG, as reflected by its target VASP, and their phosphorylation were clearly not associated with cardioprotection. STAT3 phosphorylation and relative STAT3 phosphorylation at 10 min reperfusion, which we have previously identified as a causal signaling step in separate comparisons of I/R versus IPC [9], POCO [20], and RIPC [34] by both Western blotting of lysates from myocardial biopsies and pharmacological blockade, was confirmed for IPC in the present, more comprehensive comparison against a broader and more diverse background. STAT3 phosphorylation at early reperfusion was also increased with RIPC, but this was only seen trendwise in relative STAT3 phosphorylation. For POCO, STAT3 phosphorylation was not significantly increased at early reperfusion. Therefore, we could not reproduce our earlier findings on relative STAT3 phosphorylation as a causal signal for POCO and RIPC. When looking at protein kinase phosphorylation, it is usually normalized to the respective total protein kinase content. However, to estimate the activity of a protein kinase, its total phosphorylated concentration may be more relevant than its relative concentration. In any event, we believe that we do not have to revoke the conclusions of our earlier studies at this point, because we simply cannot distinguish between biological issues (robustness of STAT3 phosphorylation in animals with a diverse background of different conditioning strategies in comparison to placebo ischemia reperfusion) and methodological issues, notably storage time. A truly causal role of storage time for the different STAT3 phosphorylation patterns is also only interferential at this point. Therefore, we can

simply not answer our original question on STAT 3 phosphorylation (relative/normalized or non-normalized) at early reperfusion as a common signal of cardioprotection by ischemic conditioning, but “absence of evidence is not evidence of absence”.

In fact, there were also some differences in surgical protocol and Western blot analysis in the prior studies on POCO [20] and RIPC [34]. These prior studies were performed under optimized conditions, notably over a short time frame with Western blot analysis on the same gel/membrane. In comparison to our prior studies, the POCO protocol was changed from the previous hypoperfusion of a cannulated coronary artery for 90 min [20] to 60 min coronary occlusion, but thus more consistent with the I/R, IPC and RIPC protocols in the present analysis. The time intervals from the ischemic conditioning stimulus to the critical biopsy sampling at 10 min reperfusion in the different protocols varied widely from a few minutes (POCO) to 90 min (IPC) or more (RIPC). Still, no matter when initiated by the ischemic conditioning stimulus, cardioprotection must be exerted during early reperfusion and any causal signal must be present then. However, we analyzed only three time points, and since the biokinetic properties of the different proteins may be different and also differ among the different conditioning strategies, we may have missed a transient activation of any of the analyzed signals that may have subsequently activated another signal that was present at 10 min reperfusion but not analyzed in our study. The protein lysate preparation differed between our prior study on POCO [20], where proteins were lysed in cell lysis buffer supplemented with protease inhibitor cocktail, and the latter studies [9, 34] including also the current study, where tris-SDS buffer was used to lyse the proteins and prevent protein degradation during long-term storage. The lysis buffer, however, largely determines the composition of extracted proteins [8]. Our experiments were performed within a time frame of several years, and variations in the animals over subsequent generations, changes in the investigator team and their surgical procedures, seasonal variations in temperature and humidity, batch variations in drugs and chemicals etc. may also have contributed to the lack of robustness of the STAT3 phosphorylation data. The sample storage time differed for all proteins because the present study was retrospective in nature and the individual protocols were not performed at random. Whereas the storage time did not affect the amount of total STAT3 protein, the shortest total storage time was associated with the highest STAT3 phosphorylation, i.e., with IPC. For AKT, we

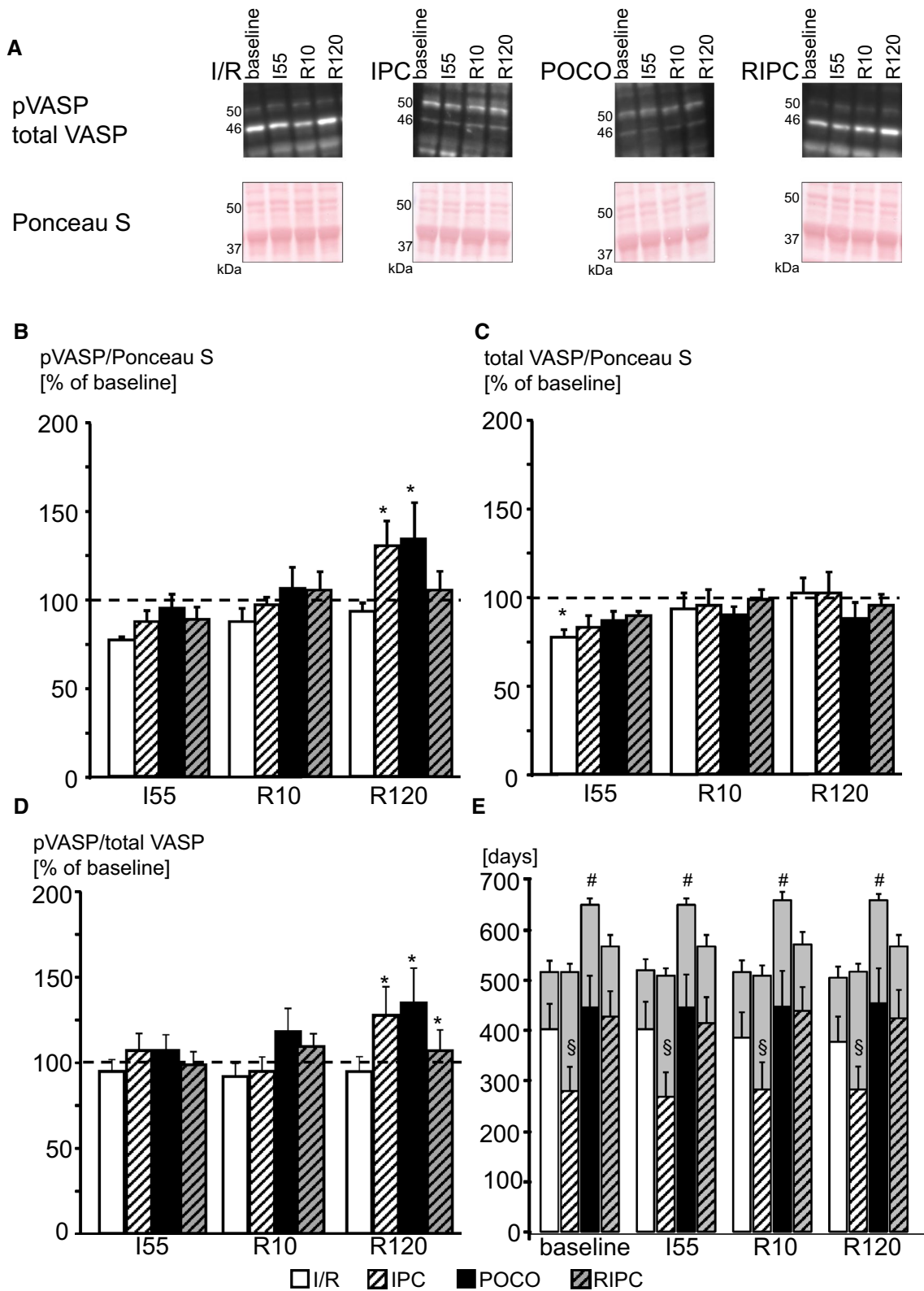


Fig. 5 Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic postconditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated VASP and total VASP in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (**a**). Amount of phosphorylated VASP (**b**) and total VASP (**c**) normalized to Ponceau S staining, respectively, and relative phosphorylation of VASP normalized to the respective total form of VASP (**d**); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of VASP (**e**). Data are mean \pm SEM, two-way ANOVA for repeated measures (**b–d**) and two-way ANOVA (**e**), both with post hoc Fisher's LSD test, * $p < 0.05$ vs. baseline, ⁺ $p < 0.05$ vs. I/R, [§] $p < 0.05$ vs. myocardial biopsy storage time of all other groups, [#] $p < 0.05$ vs. total storage time of all other groups

confirm the results of our prior separate studies. The AKT phosphorylation was increased by ischemia/reperfusion per se to the same extent as by the different ischemic conditioning procedures [9, 20, 34]. Thus, the phosphorylation of AKT was obviously not affected by storage time. The pattern of ERK1/2 phosphorylation was comparable to that of STAT3 phosphorylation, however, did not contradict our earlier studies, as the phosphorylation of ERK1/2 by ischemia/reperfusion per se did not significantly differ from that by IPC and RIPC [9, 34]. For GSK-3 β p38 MAPK, and VASP we have no prior data for comparison. Apparently, the impact of storage time on protein phosphorylation varies for different proteins.

Could we have done better to answer our original question? Using a prospective analysis, we could have better balanced storage times among the different protocols. Still, we could not have avoided loss of STAT3 phosphorylation with prolonged storage times and thus may have diluted the original biological effect for all conditioning protocols. Only the combination of a prospective approach with many contemporary experiments within a short time frame could have avoided the potential problems which we experienced in the present study. Such prospective large-scale studies, however, would not be feasible in our laboratory for logistic, financial and

legal reasons, and we doubt that it is possible in other laboratories.

Our inability to identify a common signal of cardioprotection by ischemic conditioning using Western blotting in myocardial tissue lysates corresponds to methodological problems using other methods. A protein phosphorylation analysis in different subcellular compartments, as we have done before for STAT3 in mitochondria under a POCO protocol, may be more sensitive [20]. Using a proteomic approach, changes of the myocardial proteome/phosphoproteome in response to myocardial I/R damage per se may mask changes of low abundance proteins and their phosphorylation [30], as was true for STAT3 in our own study on RIPC [8]. Indeed, when the IPC stimulus without subsequent myocardial I/R injury was analyzed in pigs by next generation sequencing, STAT3 was identified as one key regulator of IPC [30]. Pharmacological antagonists almost inevitably lack specificity, and transgenic approaches are often confounded by compensatory changes in other signaling proteins [17]. Pigs are a clinically relevant model for myocardial infarction and infarct size reduction by ischemic conditioning interventions, but may not be so for the underlying signaling. Indeed data in humans point to STAT5 activation as a potential cardioprotective signal in remote ischemic conditioning [21, 37], but STAT5 is not involved in RIPC in pigs [34].

The above problems which we experienced in the translation of our prior studies under optimized conditions to the current retrospective analysis with limited robustness highlight typical problems in preclinical data as previously demonstrated in the CAESAR trial [25]. Limited robustness of preclinical data is only one problem in the translation of experimental mechanistic studies to use in a clinical situation, and many of the problems which we have experienced here—data collection over a long time frame, sample storage times, different investigators etc.—also confound clinical studies and are in the way of successful translation [17]. Robust preclinical data from systematic methodological approaches, including multi-omic analysis [31], are an absolute prerequisite for development of pharmacological targets for eventual

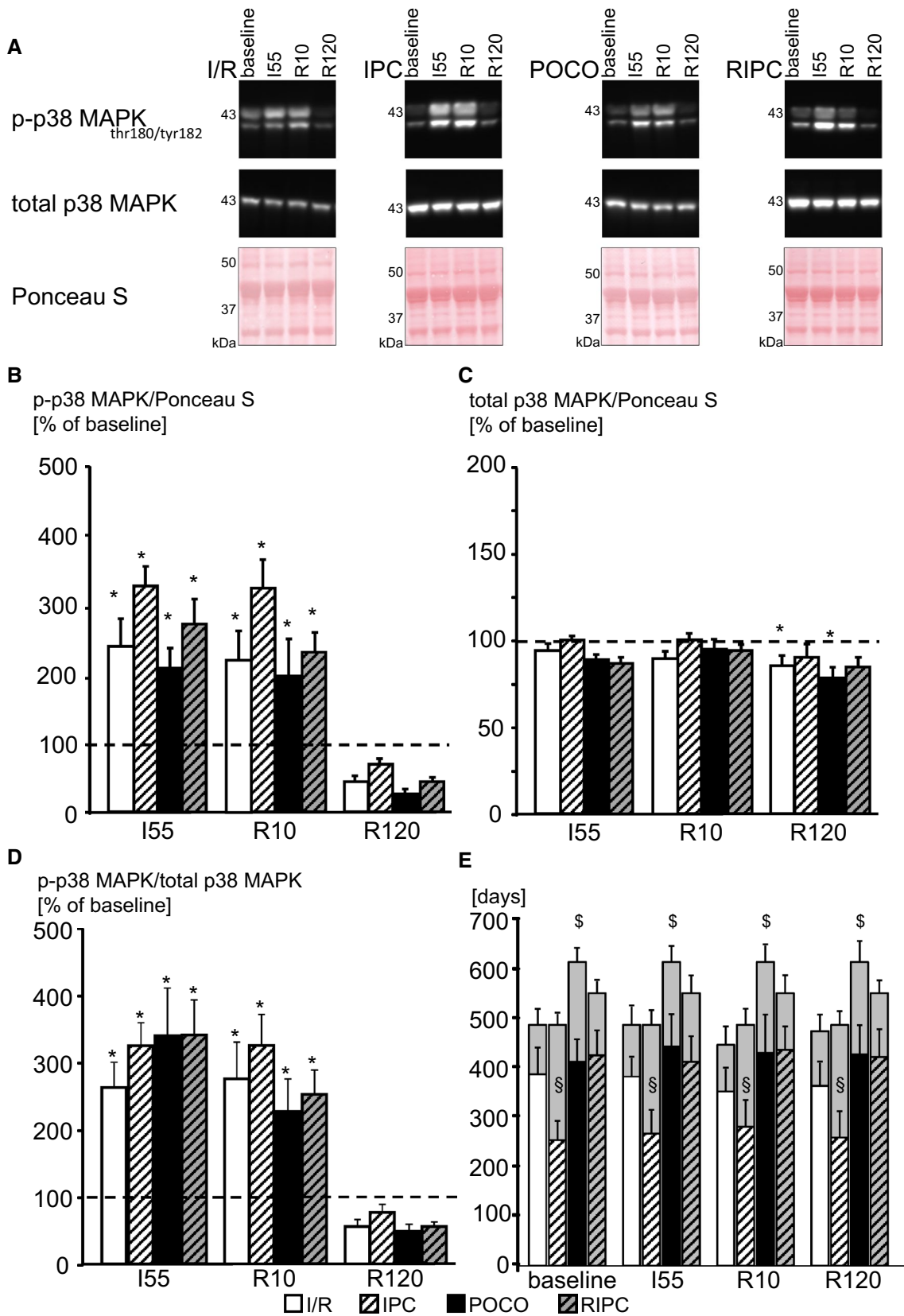


Fig. 6 Phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) during ischemia (I) and reperfusion (R) and cardioprotection by ischemic preconditioning (IPC), ischemic postconditioning (POCO), and remote ischemic preconditioning (RIPC). Representative immunoreactivities of the phosphorylated p38 MAPK at thr₁₈₀/tyr₁₈₂ and total p38 MAPK in biopsies from the area at risk taken at baseline, 55 min ischemia (I55) and 10 and 120 min reperfusion (R10/120) from pigs undergoing I/R, IPC, POCO, and RIPC (a). Amount of phosphorylated p38 MAPK (b) and total p38 MAPK (c) normalized to Ponceau S staining, respectively, and relative phosphorylation of p38 MAPK normalized to the respective total form of p38 MAPK (d); I/R (white bars), IPC (light grey bars with black stripes), POCO (black bars), and RIPC (dark grey bars with black stripes). Storage time of myocardial biopsies (bars as above) and that of their respective protein lysates (stacked grey bars) used for the analysis of p38 MAPK (e). Data are mean ± SEM, two-way ANOVA for repeated measures (b–d) and two-way ANOVA (e), both with post hoc Fisher's LSD test, **p* < 0.05 vs. baseline, +*p* < 0.05 vs. I/R, §*p* < 0.05 vs. myocardial biopsy storage time of all other groups, §*p* < 0.05 vs. total storage time of I/R and IPC

use in patients and for the translation of cardioprotective interventions to the clinic [17, 25, 36].

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Compliance with ethical standards

Conflict of interest All authors declared that they have no potential conflict of interest.

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