Infarct size reduction by cyclosporine A at reperfusion involves inhibition of the mitochondrial permeability transition pore but does not improve mitochondrial respiration

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Abstract

Introduction: Ischemic postconditioning (PoCo) and cyclosporine A (CysA) given prior to reperfusion reduce myocardial infarct size after ischemia/reperfusion. Ischemic postconditioning's protection is characterized by better preservation of mitochondrial respiration and calcium retention capacity. Protection by CysA is not entirely clear. Cyclosporine A inhibits not only mitochondrial permeability transition pore (mPTP) opening but also the phosphatase calcineurin. We have investigated whether CysA mediates protection not only by mPTP inhibition but also through a more upstream inhibition of calcineurin with subsequently better preserved mitochondrial respiration.

Material and methods: Anesthetized pigs were subjected to 90 min ischemia and 10 min reperfusion initiated with either PoCo (6 × 20 s reperfusion/re-occlusion; $n = 9$), CysA infusion (5 mg/kg *i.v.*; 5 min before reperfusion; $n = 4$), or immediate full reperfusion (IFR; $n = 8$). Mitochondria were isolated from myocardial tissue for measurement of respiration and calcium retention capacity.

Results: In mitochondria from ischemic/reperfused myocardium, ADP-stimulated complex I respiration was similar between CysA (116 ±11 nmol O_2/min/mg protein) and IFR (117 ±8), but better preserved with PoCo (160 ±9; $p < 0.05$). Calcium retention capacity was greater with both PoCo and CysA (1096 ±45 and 1287 ±128 nmol Ca^{2+}/mg protein) than with IFR (756 ±103; $p < 0.05$).

Conclusions: Cyclosporine A's protection is not associated with improved mitochondrial respiration. Protection is unlikely related to an upstream calcineurin inhibition, but is indeed secondary to mPTP inhibition.

Key words: cyclosporine A, infarct size, ischemic postconditioning, mitochondrion, myocardial ischemia/reperfusion.

Introduction

Timely restoration of blood flow is mandatory to salvage ischemic myocardium from irreversible damage; however, reperfusion induces additional damage, i.e. reperfusion injury, and contributes to final infarct size [1–3].

Ischemic postconditioning (PoCo) – i.e. brief episodes of intermittent coronary re-occlusion during early reperfusion – and gentle reperfusion – i.e. slow restoration of coronary blood flow – protect myocardium from reperfusion injury [4, 5]. Attenuation of reperfusion injury and thus reduction of final infarct size can also be achieved pharmacologically

by cyclosporine A (CysA) given prior to reperfusion [6, 7].

Infarct size reduction by PoCo and by CysA treatment has been confirmed experimentally in all species tested so far [8–11] and the cardioprotective effects of both PoCo and CysA are also operative in humans. In patients with acute myocardial infarction, PoCo induced by intermittent re-inflation of the balloon-catheter used for coronary angioplasty decreased myocardial injury, as measured by the release of marker enzymes [12–14]. A protective effect of similar magnitude was also seen in patients with acute myocardial infarction who were treated with CysA prior to reperfusion [15]. Both types of cardioprotection may thus share a common step in their signal transduction. The underlying mechanisms of cardioprotection during early reperfusion are not completely understood, but mitochondria are potential end-effectors of cardioprotection. The preservation of mitochondrial function after ischemia/reperfusion is decisive for survival of cardiomyocytes and thus salvage of myocardium [16]. The mitochondrial permeability transition pore (mPTP) plays an important role in cell death. Opening of the mPTP results in collapse of the mitochondrial membrane potential, uncoupling of the respiratory chain, and efflux of cytochrome c and other pro-apoptotic factors which finally induce apoptosis and necrosis [17]. Thus, the inhibition of mPTP opening appears to be decisive for cardiomyocyte survival at early reperfusion [17–19], in particular when the duration of ischemia is longer than 30 min [20].

Cyclosporine A inhibits the opening of mPTP by binding to cyclophilin D at the inner mitochondrial membrane, and CysA's protection against myocardial infarction is usually attributed to this mechanism [21–23] (Figure 1; path A). However, CysA also binds to cyclophilin A, which inhibits the calcium-dependent serine-threonine phosphatase calcineurin [24]. The inhibition of de-phosphorylating properties of calcineurin could therefore increase/maintain the phosphorylation of proteins (Figure 1; path B). Cardioprotective signaling relies indeed on increased phosphorylation of cardioprotective proteins, and increased phosphorylation of one or more cardioprotective proteins results in better mitochondrial function, which becomes apparent as bet-

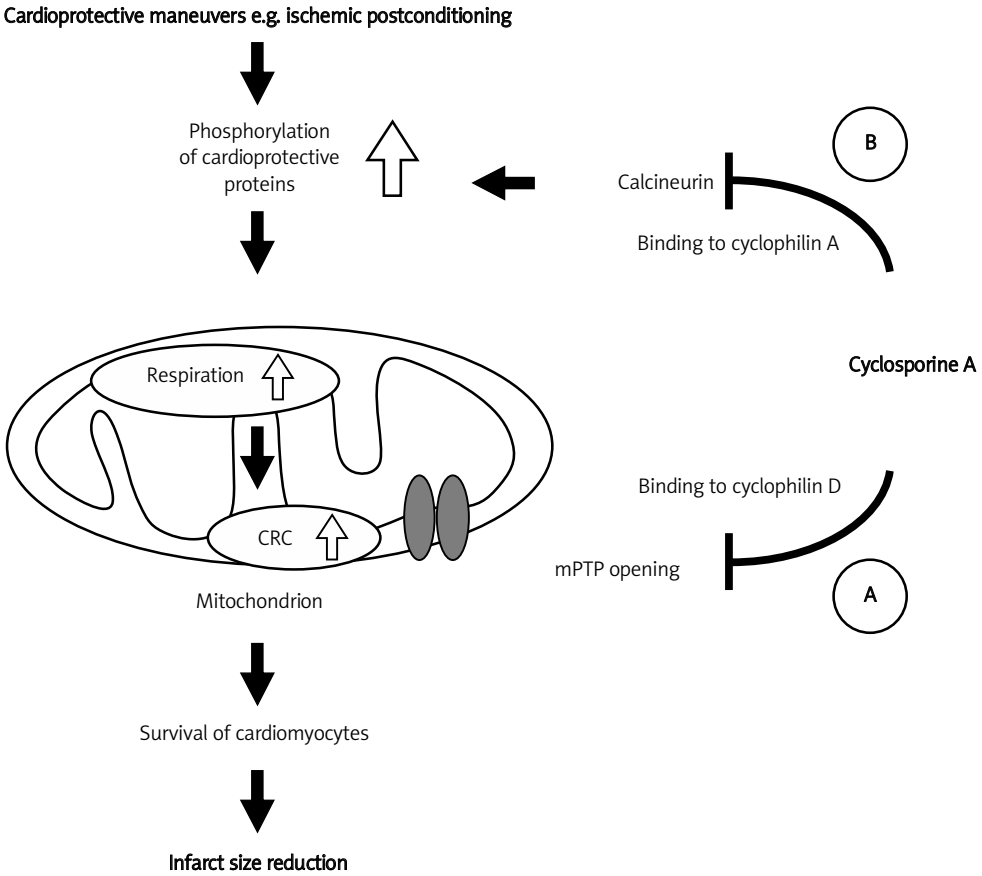

Figure 1. Potential mechanisms of protection by cyclosporine A (CysA) through mitochondrial permeability transition pore (mPTP) inhibition (path A) or calcineurin inhibition after ischemia/reperfusion (path B). Calcineurin inhibition can increase the phosphorylation of cardioprotective proteins. Such an increase in protein phosphorylation may contribute to better preserved mitochondrial respiration and calcium retention capacity (CRC) and thus ultimately to infarct size reduction

ter preserved mitochondrial respiration after ischemia/ reperfusion. Indeed, with cardioprotection by PoCo, the better preserved mitochondrial complex I respiration is causally linked to increased phosphorylation of signal transducer and activator of transcription 3 (STAT3) in pig cardiomyocyte mitochondria [25].

Calcineurin inhibition might also be associated with protection of mitochondria. The regulatory subunit calcineurin B is present in mitochondria from rat kidneys [26], and calcineurin dephosphorylates the pro-apoptotic protein Bad [27] and enables its translocation into cardiomyocyte mitochondria [28]. Cell death induced by stimulation of rat cardiomyocytes with the β-adrenergic agonist isoproterenol is secondary to dephosphorylation of Bad and is inhibited by CysA or the selective calcineurin inhibitor FK506 [29]. In cardiomyocytes isolated from dogs with heart failure, CysA treatment improves mitochondrial respiration [30].

To elucidate whether an improvement of mitochondrial function by CysA is mediated not only by mPTP inhibition but also by a more upstream mechanism through calcineurin inhibition with subsequent better respiration, we have used our established and clinically relevant *in situ* model in anesthetized pigs [31].

In this experimental model infarct size is reduced by both PoCo and administration of CysA prior to reperfusion [10, 25]. In the present study we subjected pigs to the same experimental protocols as in those previous studies, but now measured mitochondrial respiration and calcium retention capacity of cardiomyocyte mitochondria harvested at 10 min reperfusion.

Material and methods

The experimental protocols were approved by the Bioethical Committee of the district of Düsseldorf, Germany, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by NIH Publication 85-23, revised 1996.

Experimental preparation and protocols

Göttinger minipigs (20–40 kg) of either sex were sedated using ketamine hydrochloride (1 g intramuscularly). Anesthesia was induced by thiopental (500 mg intravenously) and maintained using enflurane (1.5–2.0%) with an oxygen/nitrous oxide mixture (40 : 60%). We used an open-chest pig preparation with controlled hypoperfusion of the left anterior descending coronary artery (LAD) perfusion territory through an extracorporeal circuit [31]. Coronary inflow of the LAD was reduced to 10% of baseline flow and maintained constant at this level for 90 min. Reperfusion was initiated with a PoCo maneuver (6 cycles of 20 s re-occlusion/reperfusion; $n = 9$) or infusion of CysA (5 mg/kg *i.v.*; $n = 4$) 5 min before the onset of reperfusion. Due to the slightly higher viscosity of the CysA solution it had to be given slowly over 3–4 min. To ensure that the complete dosage of CysA had become evenly distributed in the circulation with the beginning of reperfusion, the CysA infusion was started 5 min before reperfusion. Pigs subjected to immediate full reperfusion ($n = 8$) served as controls. The experiments were terminated at 10 min reperfusion for tissue sampling (6–10 g) from the area at risk and a remote control zone as an intra-individual control. The time point of 10 min reperfusion was chosen in accordance with our previous studies on cardioprotection by PoCo [25, 32].

Infarct size

Unlike our previous study [10] in which we focused on infarct size reduction by PoCo or CysA, infarct size in the present study was not measurable due to tissue sampling at 10 min reperfusion for mitochondrial isolation. However, the same *in situ* pig model, the same protocols and the same drug administration scheme were used in the present study; thus, for infarct size reduction with CysA and PoCo we refer to our previous study [10].

Isolation of mitochondria

Samples were cleaned of adipose tissue and large vessels, minced and then homogenized (Ultra-Turrax, IKA, Staufen, Germany; 2 × 10 s at a shaft rotation rate of 6,500 rpm and 1 × 5 s at 9,500 rpm) in ice-cold isolation buffer (in mmol/l: sucrose 250; HEPES 10; EGTA 1, pH 7.4). Bovine serum albumin (BSA; 5 mg/ml) was added only during the mincing and homogenizing procedures. The homogenate was centrifuged at 700 g for 10 min. The supernatant was collected and centrifuged at 14,000 g for 10 min. The resulting pellet was resuspended in isolation buffer and centrifuged at 10,000 g for 5 min. This procedure was repeated, and the final mitochondrial pellet was resuspended in an appropriate volume of isolation buffer to get a stock suspension with a protein concentration of about 10 µg/µl. All procedures were performed on ice or at 4°C (centrifugation).

The protein concentration of the isolated mitochondria was determined with a DC protein assay (Biorad, Hercules, CA, USA) with BSA as standard using the Lowry method [33].

Mitochondrial respiration

The oxygen uptake of 50 µg of mitochondrial protein was measured with a Clark-type electrode (Strathkelvin, Glasgow, UK) at 37°C during magnetic stirring in 0.5 ml of incubation buffer (in mmol/l:

125 KCl; 10 MOPS; 5 $MgCl_2$; 5 KH_2PO_4; 0.02 EGTA) with glutamate (5 mmol/l) and malate (5 mmol/l) as substrates. In sequence, basal oxygen consumption and ADP (400 µmol/l)-stimulated respiration were recorded for 3 min each. After that, 300 µmol/l N,N,N,N`-tetramethyl-p-phenylenediamine (T) and 3 mmol/l ascorbate (A) were added to the respiration chamber to determine complex IV respiration. With subsequent addition of 30 nmol/l carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), the maximal oxygen uptake of uncoupled mitochondria was measured. The latter measurements were used as a quality check to prove equal loading of mitochondrial protein in the respiration chamber.

Calcium retention capacity

The calcium retention capacity of 100 µg of mitochondrial proteins was determined in 1 ml of incubation buffer (without EGTA) at 37°C using 5 mmol/l glutamate and 5 mmol/l malate as substrates in the presence of ADP (400 µmol/l). Calcium green-5N (0.5 µmol/l, Invitrogen, Carlsbad, CA, USA) was used to measure the extramitochondrial calcium concentration with a spectrophotometer (Cary Eclipse, Varian, Mulgrave, Victoria, Australia; excitation/emission wavelengths of 500/530 nm). Aliquots of 5 nmol $CaCl_2$ were added every minute until cessation of mitochondrial calcium uptake, and a rapid increase in calcium green fluorescence indicated mPTP opening. The small amount of EGTA which is transferred with mitochondria from stock suspension into the cuvette impacted only the first added calcium pulse (tested with mitochondrial free isolation buffer). This offset was similar with each measurement.

Protein phosphorylation

Myocardial samples from the area at risk taken at 10 min reperfusion were homogenized and centrifuged. Protein aliquots of 20 µg were electrophoretically separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk, membranes were incubated with antibodies directed against the phosphorylated forms of AKT, ERK1/2, GSK3β, and STAT3 (phosphorylation sites: tyr_{705} and ser_{727}). After incubation with the respective secondary anti-rabbit antibodies, immunoreactive signals were detected by chemiluminescence and quantified. Immunoreactivities of phosphorylated proteins were normalized to those of the respective total proteins.

Statistical analysis

Data are means ± SEM. Mitochondrial respiration and calcium retention capacity of mitochondria were compared between groups by one-way ANOVA (SigmaStat 3.5, SPSS inc.). Protein phosphorylation was compared by *t*-tests. Differences were considered significant at the level of $p < 0.05$.

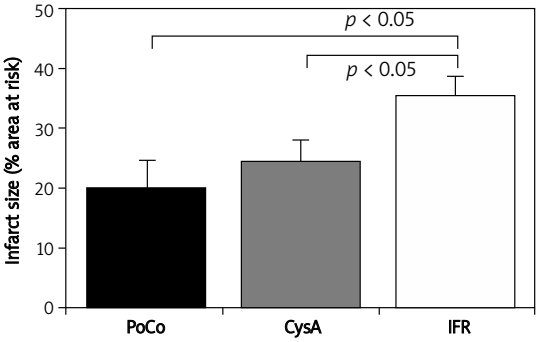

Figure 2. Infarct size after 90 min ischemia and 2 h reperfusion. Infarct size in pigs subjected to ischemic postconditioning (PoCo) and cyclosporine A (CysA) infusion prior to reperfusion was smaller than with immediate full reperfusion (IFR). From [10] with permission

Results

As reported previously, PoCo and CysA given prior to reperfusion reduced infarct size to a similar extent (Figure 2; from [10]).

In the present study, the basal oxygen uptake was not different between groups. Compared to mitochondria from the non-ischemic remote control zone, ischemia/reperfusion attenuated the ADP-stimulated respiration at complex I and maximal uncoupled respiration. With PoCo, the ADP-stimulated complex I respiration was better preserved after ischemia/reperfusion, whereas CysA infusion had no impact on complex I respiration, which was similar to that with immediate full reperfusion (Figure 3). Complex IV respiration as well as maximal uncoupled oxygen uptake was similar between the three groups, indicating even mitochondrial loading of the respiratory chamber (Figure 4).

Calcium retention capacity of mitochondria was reduced in myocardium which had undergone ischemia/reperfusion. Both PoCo and CysA better preserved calcium retention capacity, and an increased calcium retention capacity with CysA was also apparent in mitochondria from the non-ischemic remote control zone (Figure 5).

CysA treatment had no impact on the protein phosphorylation of AKT, ERK1/2, GSK3β, and STAT3 (tyr_{705} and ser_{727}) in myocardial samples taken from the area at risk at 10 min reperfusion (Figures 6 A–E).

Discussion

The notion that myocardial salvage by CysA could also be mediated by an upstream mechanism through calcineurin inhibition and subsequently bet-

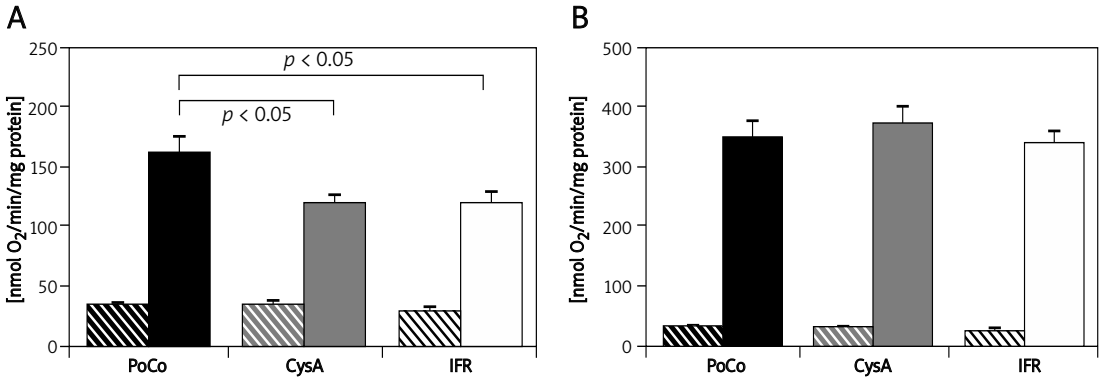

Figure 3. Respiration with complex I substrates in mitochondria from ischemic/reperfused myocardium and from remote non-ischemic control myocardium. Basal oxygen uptake (hatched bars) was not different between groups. ADP-stimulated complex I respiration of mitochondria extracted from the area at risk (**A**) was better preserved with ischemic postconditioning (PoCo; $n = 9$) than with cyclosporine A (CysA; $n = 4$) or with immediate full reperfusion (IFR; $n = 8$). In remote non-ischemic control myocardium (**B**) there were no differences between groups

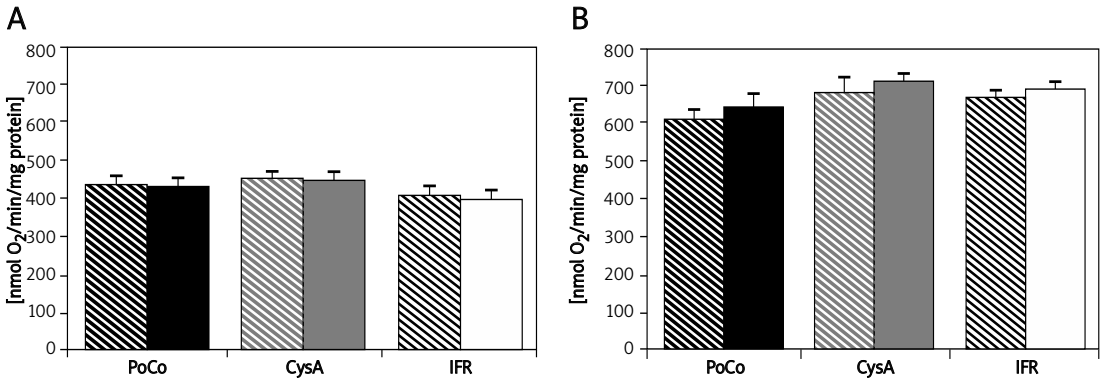

Figure 4. Mitochondrial complex IV respiration and maximal uncoupled oxygen uptake in mitochondria. Complex IV respiration (TMPD/Ascorbate; hatched bars) and maximal uncoupled oxygen uptake (FCCP; solid bars) were similar between postconditioning (PoCo; $n = 9$), cyclosporine A (CysA; $n = 4$), and immediate full reperfusion (IFR; $n = 8$) in (**A**) mitochondria from the area at risk and (**B**) remote non-ischemic control myocardium

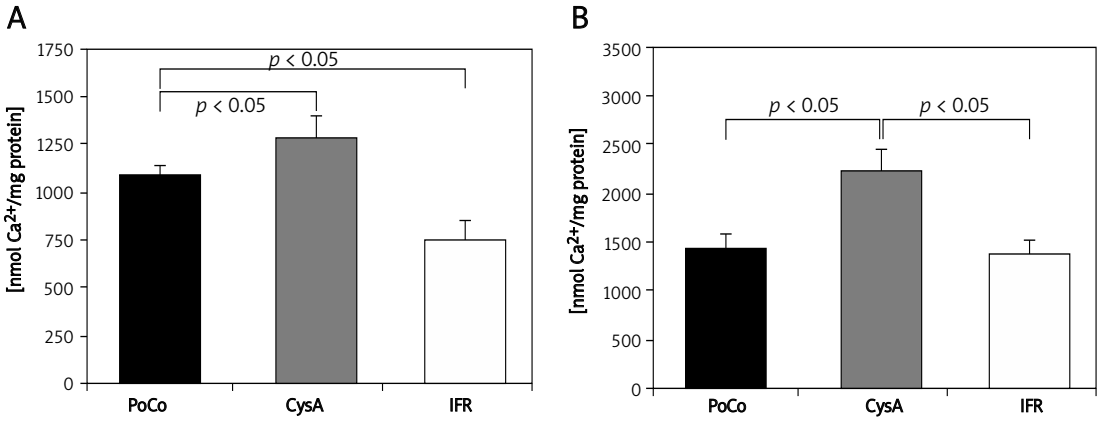

Figure 5. Calcium retention capacity with complex I substrates and ADP in mitochondria from ischemic/reperfused myocardium and from remote non-ischemic control myocardium. Calcium retention capacity of mitochondria extracted from the area at risk (**A**) was better preserved with ischemic postconditioning (PoCo; $n = 8$) and cyclosporine A (CysA; $n = 4$) than with immediate full reperfusion (IFR; $n = 8$). Calcium retention capacity of mitochondria from the remote non-ischemic control myocardium (**B**) was greater with CysA than with PoCo and IFR

ter preservation of mitochondrial respiration is not supported by our results. The general depression of respiration in mitochondria isolated from ischemic/reperfused myocardium may be related to the loss/release of cytochrome C during ischemia/reperfusion [34]. However, at 10 min reperfusion,

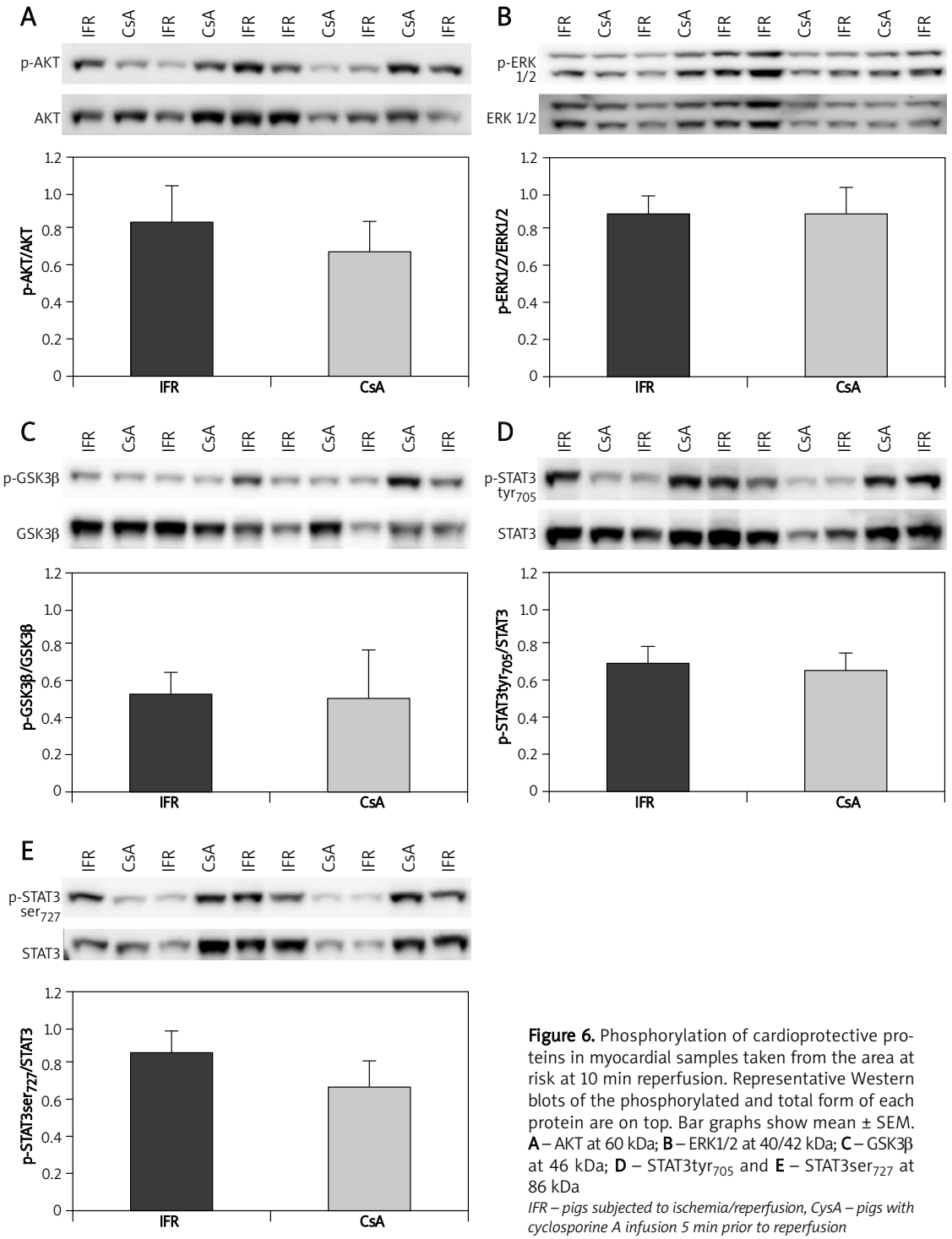

Figure 6. Phosphorylation of cardioprotective proteins in myocardial samples taken from the area at risk at 10 min reperfusion. Representative Western blots of the phosphorylated and total form of each protein are on top. Bar graphs show mean ± SEM. **A** – AKT at 60 kDa; **B** – ERK1/2 at 40/42 kDa; **C** – GSK3β at 46 kDa; **D** – STAT3tyr$_{705}$ and **E** – STAT3ser$_{727}$ at 86 kDa

IFR – pigs subjected to ischemia/reperfusion, CysA – pigs with cyclosporine A infusion 5 min prior to reperfusion

a time point at which we have already described changes in mitochondrial respiration, calcium retention capacity and protein phosphorylation related to the PoCo's cardioprotection [25, 32], mitochondrial respiration after ischemia/reperfusion was not improved by CysA, and phosphorylation of cardioprotective proteins was not different from that with ischemia/reperfusion alone (Figures 6 A–E). A few studies have related increased phosphorylation of cardioprotective proteins to calcineurin inhibition. In cardiomyocytes of rats, inhibition of calcineurin by CysA or the selective calcineurin inhibitor FK506 increased the phosphorylation of protein kinase B (AKT) [35]. Calcineurin inhibition by FK506 was also associated with increased phosphorylation of extracellular-signal-regulated kinases 1/2 (ERK1/2) after ischemia, and this interaction mediated the cardioprotection by δ-opioid receptor activation in rats

[36]. Treatment with Cys A or FK506 increased the phosphorylation of glycogen synthase kinase 3β (GSK3β) in human neuroblastoma cells [37] and increased phosphorylation of GSK3β in cardiomyocytes inhibits mPTP opening in rodents [38]. In isolated guinea pig hearts Cys A treatment prior to ischemia increased the phosphorylation of AKT and GSK3β [39]. Common to all these studies, however, was that they involved proteins of the reperfusion injury salvage kinase (RISK) pathway, which is not involved in cardioprotection at reperfusion in pigs [32].

We have recently shown in our experimental model that PoCo increased the phosphorylation of mitochondrial STAT3. which in turn resulted in better preserved mitochondrial ADP-stimulated complex I respiration after ischemia/reperfusion. The pharmacological inhibition of STAT3 phosphorylation during ischemia/reperfusion *in vivo* abolished this better preservation of mitochondrial function with PoCo and also abrogated the infarct size reduction [25]. Consequently, the lack of impact of CysA on mitochondrial respiration in the present study supports the notion that CysA's protection is indeed not mediated by increased protein phosphorylation.

Whether or not mitochondrial respiration is improved by CysA or the selective calcineurin inhibitor FK506 is currently controversial. The statin-induced impairment of mitochondrial ADP-stimulated complex I respiration of prostate cancer cells was improved with CysA and FK506, but in this study there was also an increase in the maximal uncoupled oxygen uptake with FCCP [40], possibly reflecting unequal loading of the respiratory chamber. Improvement of mitochondrial respiration in isolated rat hearts subjected to 30/60 min ischemia was observed with CysA, but not with FK506, which does not act on calcineurin [41]. Also, FK506 does not protect against infarction after ischemia/reperfusion in rat [6] or rabbits hearts [42].

In our *in situ* pig model, infarct size was reduced by CysA infusion to a similar extent as with PoCo [10], supporting the notion that cardioprotection by CysA is indeed induced by inhibition of mPTP opening. The calcium retention capacity was even better preserved than with PoCo, and increased calcium retention capacity was also seen in mitochondria harvested from the remote non-ischemic myocardium.

In conclusion, the lack of improvement in mitochondrial respiration in ischemic/reperfused myocardium with CysA treatment at reperfusion does not support a potential upstream mechanism through calcineurin inhibition.

Acknowledgments

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