Mitochondrial STAT3 Activation and Cardioprotection by Ischemic Postconditioning in Pigs With Regional Myocardial Ischemia/Reperfusion

Gerd Heusch, Judith Musiolik, Nilguen Gedik, Andreas Skyschally

Rationale: Timely restoration of coronary blood flow is the only way to salvage myocardium from infarction, but reperfusion per se brings on additional injury. Such reperfusion injury and the resulting size of myocardial infarction is attenuated by ischemic postconditioning, ie, the repeated brief interruption of coronary blood flow during early reperfusion. The signal transduction of ischemic postconditioning is under intense investigation, but no signaling step has yet been identified as causal for such protection in larger mammals in situ.

Objective: We have now in an in situ pig model of regional myocardial ischemia/reperfusion addressed the role of mitochondrial signal transducer and activator of transcription 3 (STAT3).

Methods and Results: We demonstrated reduction of infarct size by ischemic postconditioning (26 ±3% of area at risk versus 38±2% in controls with immediate full reperfusion) along with more markedly increased tyrosine705 phosphorylation of STAT3 in myocardial biopsies (at 10 minutes reperfusion: 9.2±3.0-fold from baseline versus 6.6±2.9-fold in controls with immediate full reperfusion). Increased tyrosine705 phosphorylation of STAT3 and better preservation of complex 1 respiration and calcium retention capacity were also present in isolated mitochondria from postconditioned myocardium in vitro. Prior janus kinase/STAT inhibition with AG490 in vivo abrogated the infarct size reduction and the better preservation of mitochondrial function, and the STAT3 inhibitor Statin in vitro also abrogated better preservation of mitochondrial function.

Conclusions: Our data support a causal role for mitochondrial STAT3 activation to mediate cardioprotection through better mitochondrial function. (Circ Res. 2011;109:1302-1308.)

Key Words: infarct size ■ mitochondrion ■ myocardial ischemia ■ postconditioning ■ reperfusion

Myocardial infarction continues to be a major cause of mortality and morbidity, and infarct size is the major determinant of patients’ prognosis. The only way to reduce infarct size is early reperfusion of the occluded coronary artery, but reperfusion not only salvages myocardium but also brings on additional “reperfusion injury.” Ischemic postconditioning, ie, repeated brief interruption of coronary blood flow during early reperfusion, attenuates such reperfusion injury and reduces ultimate infarct size. Ischemic postconditioning is operative in all species tested so far, including humans. The signaling of cardioprotection is still under intense investigation and involves 3 major intracellular pathways, ie, the nitric oxide synthase/protein kinase G program, the reperfusion injury salvage kinase program, and the survival activating factor enhancement program, all converging at the mitochondria as an integration point that is decisive for cardiomyocyte survival.

Signal transducer and activator of transcription 3 (STAT3) is a central element of cardioprotection, notably of the survival activating factor enhancement program, and it is activated by phosphorylation at tyrosine705 and serine727 during myocardial ischemia and even more during early reperfusion. Ischemic postconditioning increases STAT3 activation beyond that by reperfusion per se, and pharmacological inhibition of STAT3 activation or its genetic ablation abrogates cardioprotection. The exact role of STAT3 in cardioprotection is not clear; its established function as a transcription factor that regulates cardioprotective proteins is probably too slow to account for the immediate rescue from cell death during the early minutes of reperfusion. Recently, STAT3 has been identified in cardiomyocyte mitochondria, and its pharmacological inhibition or genetic ablation impaired complex 1 respiration and calcium retention capacity. Conversely, a mitochondrial-targeted STAT3 overexpression in mice preserved complex 1 respiration during simulated ex vivo ischemia and reduced the formation of reactive oxygen species. However, an improved mitochondrial function secondary to acute STAT3 activation by STAT3 inhibitors may provide a new strategy to reduce infarct size. © 2011 American Heart Association, Inc.
activation by a cardioprotective intervention in vivo has not been demonstrated so far. Of note, all available evidence for the involvement of STAT3 in cardioprotection has been derived from more reductionist rodent models so far, which largely differ from larger mammals in heart rate, temporal and spatial infarct development, and also in parts of their cardioprotective signaling.26 Translation rather than innovation, however, is the critical deficit in research on cardioprotection.27,28

We have therefore now taken advantage of an in situ pig model of protection from infarction by ischemic postconditioning29 and in an integrative approach measured reduction of infarct size, activation of mitochondrial STAT3 and better preservation of mitochondrial function, and ultimately by abrogating protection with pharmacological inhibition of STAT3 provided evidence for its causal involvement.

Methods

Experimental Preparation and Protocols

Enflurane-anesthetized open-chest pigs were subjected to 90 minutes regional low-flow ischemia and 120 minutes reperfusion; infarct size was determined by triphenyl-tetrazolium chloride staining and expressed as percent of the area at risk.29 Ischemic postconditioning was induced by 6 cycles of 20 seconds reocclusion/reperfusion. Drill biopsies for Western blot analyses were taken at baseline, at 85 minutes ischemia, and at 5, 10, 30, and 120 minutes reperfusion. For isolation of mitochondria from both, the ischemic-reperfused and the remote non-ischemic control myocardium, experiments were terminated at 10 minutes reperfusion and myocardial tissue was cleaned, minced, homogenized, and subjected to stepwise centrifugation in ice-cold buffer.22,23 Experiments were performed in the absence and presence of the janus kinase (JAK)/STAT inhibitor AG490 (9 μg/kg/min i.c. starting 10 minutes before ischemia, with 1 mg/kg total dose16). The attempt to use Static as a different STAT3 inhibitor (AG490, 9 μg/kg/min i.c. starting 10 minutes before ischemia) was abandoned due to its toxicity (see Online Supplement available at http://circres.ahajournals.org).

Mitochondrial Respiration and Calcium Retention Capacity

Oxygen consumption of 50 μg mitochondrial proteins was measured with a Clark-type electrode at 37°C in 0.5 mL incubation buffer (in mmol/L: 125 KCl; 10 4-morpholine propane sulfonic acid; 5 MgCl2; 5KH2PO4; 0.02 ethylene glycol tetraacetic acid) using glutamate (5 mmol/L) and malate (5 mmol/L) as substrates for complex 1, or succinate (5 mmol/L) as substrate for complex 2 and inhibition of complex 1 by rotenone (2 μmol/L). Baseline and ADP (400 μmol/L)-stimulated respiration were measured. Complex 4 respiration was measured with 300 μmol/L TMPD (N,N,N'-tetramethyl-p-phenylenediamine) and 3 mmol/L ascorbate, and maximal oxygen uptake after uncoupling of the respiratory chain with 30 mmol/L FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone).

Calcium retention capacity of 100 μg mitochondrial proteins was measured in 1 mL incubation buffer (without ethylene glycol tetraacetic acid) at 37°C using 5 mmol/L glutamate and 5 mmol/L malate as substrates, with ADP (400 μmol/L)-stimulated respiration. Using calcium green-5N as indicator, extramitochondrial calcium was determined with a spectrophotometer while pulses of 5 nmol CaCl2 were added every minute until calcium was no longer taken up and a rapid calcium release occurred. As a control, cyclosporine A (10 μmol/L) was used to inhibit mitochondrial permeability transition pore opening.

Mitochondrial respiration and calcium retention capacity were measured in the absence and presence of the STAT3 inhibitor Static (100 μmol/L). The solvent DMSO (10 mmol/L, dimethyl sulfoxide) had no effect on respiration or calcium retention capacity.

Western Blot Analysis

Biopsies were homogenized and centrifuged; mitochondria were further purified by percoll-gradient ultracentrifugation. Protein aliquots of 20 μg (biopsies) or 100 μg (mitochondria) were electrophoretically separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk, membranes were incubated with antibodies directed against the phosphorylated forms of STAT1 (phosphorylation sites: tyrosine701 and serine727), STAT3 (tyrosine705 and serine727), and STAT5 (tyrosine694). After incubation with the respective secondary antirabbit antibodies, immunoreactive signals were detected by chemiluminescence and quantified. Immunoreactivities of phosphorylated proteins were normalized to those of the respective total proteins. Baseline values were not different between groups and therefore set as 100% to calculate the time courses of STAT phosphorylation within individual animals.

Statistics

Data are mean±SEM. Hemodynamics and time courses of STAT phosphorylation were analyzed by 2-way (group, time) ANOVA for repeated measures. When a significant difference was detected, individual mean values were compared by posthoc tests (least significant difference). Respiration and calcium retention capacity of mitochondria from the area at risk were compared between ischemic postconditioning and immediate full reperfusion by univariate ANOVA with the respective data from the intraindividual remote control area as covariates. Areas at risk and infarct sizes were analyzed by one-way ANOVA. Mitochondrial STAT3 phosphorylation was compared between ischemic postconditioning and immediate full reperfusion by Student t-test. Differences were considered significant at the level of P<0.05.

Results

Ischemic postconditioning by 6 cycles of 20-second reocclusion/20-second reperfusion reduced infarct size (Figure 1), whereas hemodynamics (Table), residual blood flow,
Table. Systemic Hemodynamics With Ischemic Postconditioning (PoCo) vs Immediate Full Reperfusion (IFR) in the Absence or Presence of the JAK/STAT inhibitor AG490 (PoCo-AG490; IFR-AG490)

<table>
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<td>108±6</td>
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PoCo indicates ischemic postconditioning; IFR, immediate full reperfusion; Isch5/85/85, 5/85/85 min ischemia; R10/20/30/ 60/120, 10/20/30/60/120 min reperfusion; HR, heart rate; LVPmax, maximal left ventricular pressure; dP/dt max, maximum in the first derivative of LVP; CAPmean, mean coronary arterial pressure; CBFmean, mean coronary blood flow.

*P<0.05 vs Baseline.
†P<0.05 vs PoCo; 2-way ANOVA and Fisher’s LSD post-hoc tests.

and area at risk were not different from those with immediate full reperfusion; the protection was abrogated by prior JAK/STAT inhibition with AG490.

The phosphorylation of STAT1 at tyrosine701 and serine727 and of STAT3 at tyrosine705 and serine727 was increased during ischemia and even further increased during reperfusion; the phosphorylation of STAT5 at tyrosine694 was decreased during ischemia and transiently increased during reperfusion. The increment in STAT3 tyrosine705 phosphorylation was stronger with ischemic postconditioning than with immediate full reperfusion (Figure 2). Prior JAK/STAT inhibition with AG490 in vivo attenuated the increases in STAT3 tyrosine705 and serine727 phosphorylation and abolished the difference in STAT3 tyrosine705 phosphorylation between ischemic postconditioning and immediate full reperfusion (Figure 2). STAT3 phosphorylation at tyrosine705...
was also more pronounced in isolated mitochondria from myocardium that had undergone ischemic postconditioning than with immediate full reperfusion; this difference was again abolished by AG490 (Figure 3). Mitochondrial STAT3 protein content, normalized to ATP synthase as a marker of the inner mitochondrial membrane, was not different between ischemic postconditioning and immediate full reperfusion (0.51 ± 0.14 versus 0.33 ± 0.07 AU).

ADP-stimulated respiration at complex 1 ex vivo was reduced in myocardium that had undergone ischemia/reperfusion versus control remote myocardium, but it was better preserved with ischemic postconditioning than with immediate full reperfusion (Figure 4); complex 2 respiration (Figure 5) and maximal uncoupled oxygen uptake (Figure 6) were not different with ischemic postconditioning or immediate full reperfusion. Calcium retention capacity of isolated mitochondria ex vivo was reduced in myocardium that had undergone ischemia/reperfusion, but it was better preserved with ischemic postconditioning than with immediate full reperfusion (Figure 7). Both better preservation of ADP-stimulated complex 1 respiration and better preservation of calcium retention capacity were abrogated with prior JAK/STAT inhibition by AG490 in vivo and with STAT3 inhibition by Stattic in vitro.

Discussion

The present study is the first to demonstrate a causal role for mitochondrial STAT3 activation to mediate the cardioprotection by ischemic postconditioning: The reduction of infarct size by ischemic postconditioning in this large mammal in situ model was associated with increased STAT3 phosphorylation at tyrosine705 and both increased STAT3 phosphorylation at tyrosine705 and infarct size reduction were abrogated by prior JAK/STAT inhibition with AG490 in vivo. Previously, a role for STAT3 in cardioprotection has been suggested by abrogation of cardioprotection through its genetic ablation or pharmacological inhibition in rodents. However, the acute activation of mitochondrial STAT3 along with infarct size reduction by a mechanical cardioprotective maneuver such as ischemic postconditioning and their abrogation by STAT3 inhibition have not been shown before. The translation of findings on cardioprotection from rodent to larger mammal hearts is not trivial and cannot be taken for granted, and the importance of confirming data from rodent models in larger mammals such as the pig before translation to humans was recently emphasized by an NIH panel. Of note, the signal transduction of cardioprotection in larger mammals differs from that in more reductionist small rodent models, and STAT3 activation is the first signaling event identified to mediate ischemic postconditioning in pigs. The relatively modest further increase in STAT3 phosphorylation at tyrosine705 with ischemic postconditioning over that with immediate full reperfusion in myocardial biopsies was more pronounced at the level of mitochondria. In mitochondria, only STAT3 phosphorylation at tyrosine705 but not STAT3 protein content was increased, suggesting that STAT3 import into mitochondria played no role.

Figure 2. Myocardial STAT3tyr phosphorylation. Intraindividual time courses of STAT3tyr phosphorylation in samples from the area at risk (right panel in the presence of the JAK/STAT inhibitor AG490). Data are expressed as percent of baseline. Open symbols/dashed lines: ischemic postconditioning (PoCo), closed symbols/solid lines: immediate full reperfusion (IFR). Representative Western blots of the phosphorylated and total form of STAT3 at 86 kDa are on top. (BASE: baseline, ISCH: 85 minutes ischemia, R5/10/30/120: 5/10/30/120 minutes reperfusion).

Figure 3. Mitochondrial STAT3tyr phosphorylation. Original Western blot of mitochondrial STAT3 phosphorylated at tyrosine705 and total STAT3 at 86 kDa extracted from the area at risk after ischemic postconditioning (PoCo) or immediate full reperfusion (IFR). The ratio pSTAT3tyr/STAT3 is presented numerically for each animal separately. Bar graphs represent statistics without and with JAK/STAT inhibition by AG490.
A facilitatory role of STAT3 for mitochondrial complex 1 respiration has been reported before in isolated mouse and rat cardiomyocyte mitochondria. The precise nature of this facilitatory role is not clear and probably not mediated through protein–protein interaction, given the low abundance of STAT3 as compared to complex 1 proteins; however, activated STAT3 can facilitate the docking of protein kinases to their targets and could thus affect respiration indirectly; in a recent study in isolated STAT3 overexpressing mouse mitochondria, not only complex 1 was better preserved but also reactive oxygen species formation reduced following simulated ischemia ex vivo. The present study is the first to demonstrate better preservation of mitochondrial complex 1 respiration with ischemic postconditioning and again implies a role for STAT3 activation in it, as the better preservation of complex 1 respiration was abrogated with JAK/STAT inhibition by AG490 in vivo and STAT3 inhibition by Stattic in vitro. The use of two structurally and functionally different inhibitors of the JAK/STAT pathway and the inhibition of both tyrosine705 and serine727 phosphorylation by AG490 provide solid evidence of STAT3 as the target molecule. The exact phosphorylation site that is important for the improvement in mitochondrial function by STAT3 is not clear but may vary with species: In mouse cardiomyocyte mitochondria it was the serine727 site, whereas it was both the serine727 and the tyrosine705 site in rat cardiomyocyte mitochondria and only the tyrosine705 site in the present study in pigs. The increased STAT3 phosphorylation at tyrosine705 was associated not only with better preservation of complex 1 respiration but also with improved calcium retention capacity as a measure of mitochondrial permeability transition pore inhibition. Very recently, improved mitochondrial respiration along with increased mitochondrial STAT3 phosphorylation at serine727 was also demonstrated in mice overexpressing heat shock protein 22, which in turn is important for cardioprotection in pigs.

Figure 4. Respiration with complex 1 substrates in mitochondria after ischemia/reperfusion and in remote non-ischemic control myocardium. Basal and ADP-stimulated mitochondrial complex 1 respiration of (A) mitochondria extracted from the area at risk after ischemic postconditioning (PoCo) or immediate full reperfusion (IFR) and (B) from the remote non-ischemic control myocardium, in the absence or presence of the JAK/STAT inhibitor AG490 in vivo or the STAT3 inhibitor Stattic in vitro.

Figure 5. Complex 2 respiration in mitochondria after ischemia/reperfusion. Basal and ADP-stimulated mitochondrial complex 2 respiration of mitochondria from the area at risk after ischemic postconditioning (PoCo) or immediate full reperfusion (IFR) with complex 2 substrates and rotenone. Complex 2 respiration was not affected by PoCo.

Figure 6. Complex 4 respiration and maximal uncoupled oxygen uptake in mitochondria after ischemia/reperfusion. Complex 4 respiration and maximal uncoupled oxygen uptake were similar between postconditioning (PoCo) or immediate full reperfusion (IFR).
In conclusion, our study in a clinically relevant in situ pig model of regional myocardial ischemia/reperfusion demonstrated activation of mitochondrial STAT3 along with better preservation of mitochondrial complex 1 respiration and calcium retention capacity and ultimately infarct size reduction by ischemic postconditioning.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What is Known?**

- Ischemic postconditioning, ie, the repeated interruption of coronary blood flow during the first minutes of reperfusion following a sustained period of myocardial ischemia, reduces infarct size in all species tested so far, including humans.
- The signal transduction of cardioprotection is complex, and many attempts of translation to the clinic have failed.
- Signal transducer and activator of transcription 3 (STAT3) is a transcription factor, but it is also involved in cardioprotective signaling in rodent hearts.
- STAT3 is present in cardiomyocyte mitochondria of rodent hearts and facilitates mitochondrial respiration.

**What New Information Does This Article Contribute?**

- Ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion relies on activation of STAT3 for reduction of infarct size.
- Ischemic postconditioning increases the phosphorylation of mitochondrial STAT3 at tyrosine705.
- The increased tyrosine705 phosphorylation of mitochondrial STAT3 improves respiration at complex 1 and inhibits permeability transition pore opening.

Signaling mechanisms involved in cardioprotection are complex, and translation of cardioprotective interventions from the laboratory to the clinic has not been successful. This is in part due to the fact that cardioprotective signaling in rodent hearts differs from that in larger mammalian hearts. Signal transducer and activator of transcription 3 (STAT3) is not only a transcription factor, but also involved in cardioprotective signaling in rodent hearts. Also in rodent hearts, genetic deletion or pharmacological inhibition of STAT3 impairs mitochondrial respiration. We have now identified that STAT3 plays a causal role in infarct size reduction by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. Ischemic postconditioning increased the phosphorylation of STAT3 at tyrosine705 within 10 minutes of reperfusion. Increased phosphorylation of STAT3 at tyrosine705 within 10 minutes of reperfusion by ischemic postconditioning was also identified in isolated mitochondria, and it improved respiration at complex 1 and inhibited the opening of the permeability transition pore. These data underscore the importance of mitochondrial STAT3 activation in cardioprotective signaling in larger mammals and could open new avenues for the development of strategies to induce cardioprotection in humans.
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Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion

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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

Göttinger minipigs (20–40 kg) of either sex were sedated using ketamine hydrochloride (1 g intramuscularly) and anesthetized with thiopental (500 mg intravenously). Through a cervical incision, the trachea was intubated and connected to a respirator (Dräger, Lübeck, Germany). Anesthesia was maintained using enflurane (1–1.5 %) with an oxygen/nitrous oxide mixture (40 %:60 %). The common carotid arteries were cannulated to measure arterial pressure and to supply blood to the extracorporeal circuit. The jugular veins were cannulated for volume replacement. A left lateral thoracotomy was performed and the pericardium opened. A micromanometer (P7, Konigsberg Instruments, Pasadena, CA, USA) was placed in the left ventricle through the apex together with a saline-filled polyethylene catheter (for calibration). The left anterior descending (LAD) coronary artery was dissected over a distance of 1.5 cm, ligated, cannulated, and perfused from an extracorporeal circuit. Pigs were anticoagulated with 20,000 IU sodium heparin and additional doses of 10,000 IU at 2 h intervals. The system included a roller pump, windkessel, and side-port for the injection of radiolabeled microspheres. Coronary arterial pressure was measured from the sidearm of a polyethylene T-connector by an external pressure transducer (pvb Medizintechnik, Kirchseon, Germany). Minimal coronary arterial pressure was held above 75 mmHg by adjusting the roller pump to avoid hypoperfusion prior to ischemia.

Infarct size

At the end of each study, the heart was sectioned from base to apex into 5 transverse slices in a plane parallel to the atroventricular groove. Slices were immersed in 90 mmol/L sodium phosphate buffer containing 1.0 % triphenyl tetrazolium chloride (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 8 % dextran for 20 min at 37 °C. The amount of infarcted tissue is expressed as percent of the area at risk, as defined by the microspheres technique. Radiolabeled microspheres (15 µm in diameter; ⁹⁵Nb, ¹⁰³Ru, ⁴⁶Sc; PerkinElmer, Waltham, MA, USA)
were injected into the coronary perfusion circuit to determine regional blood flow (Wizzard 2480, PerkinElmer, Waltham, MA, USA).

**Experimental protocols**

**Ischemic postconditioning (PoCo):** Following baseline measurements, coronary inflow was reduced to 10% of baseline and maintained constant for 90 min. At 5 min ischemia measurements of hemodynamics and regional blood flow were performed. At 20 s reperfusion 6 cycles of 20 s full occlusion of the coronary bypass/20 s reperfusion were performed. Then the myocardium was reperfused for 2 h before infarct size was determined.

**Immediate full reperfusion (IFR):** The protocol was identical to that with ischemic postconditioning, except that plain reperfusion was performed for 2 h.

Experiments with IFR and PoCo were performed in the absence and presence of the JAK/STAT inhibitor AG490 (9 μg/kg/min i.c. starting 10 min before ischemia, with 1 mg/kg total dose).

The attempt to use the STAT3 inhibitor Stattic (13 mg/kg i.v. over 30-40 min starting 1 h before ischemia, dissolved in DMSO and diluted in 10 ml saline) was discontinued after 5 experiments due to severely toxic effects: sinus tachycardia by 25-30 beats/min, sustained ventricular tachycardia and ventricular fibrillation during ischemia in 3 of 5 pigs, progressive and refractory decrease in ventricular pressure during reperfusion, increase in peak inspiratory pressure by 15-44 mm Hg and bloody secretion into the trachea.

**Tissue samples**

Myocardial biopsies (10-15 mg) from the area at risk were taken with a modified dental drill at baseline, 85 min ischemia, and at 5, 10, 30, and 120 min reperfusion and subjected to Western blot analysis. For isolation of mitochondria, experiments were terminated at 10 min reperfusion and larger tissue samples (6-10 g) from the area at risk and a remote control zone obtained for further processing.

**Isolation of mitochondria**

To optimize the yield of intact mitochondria, procedures were carried out on ice and centrifugation steps performed at 4 °C. Tissues were rapidly cleaned from adipose tissue, and clotted blood and large vessels removed before being placed in ice-cold
isolation buffer (in mmol/L: sucrose 250; HEPES 10; EGTA 1, pH 7.4) with 5 mg/ml bovine serum albumin (BSA). Tissues were minced thoroughly using a pair of scissors and then homogenized with a tissue homogenizer (Ultra-Turrax, IKA, Staufen, Germany) using two 10 s treatments at a shaft rotation rate of 6,500 rpm each and one more at 9,500 rpm for 5 s. The homogenate was centrifuged at 700 g for 10 min. The supernatant was collected in 2 ml microfuge tubes and centrifuged at 14,000 g for 10 min. The resulting pellet was resuspended in isolation buffer by gentle pipetting without formation of foam and centrifuged at 10,000 g for 5 min. This procedure was repeated and the final pellet resuspended in an appropriate volume of isolation buffer.

The protein concentration of the isolated mitochondria was determined with a DC protein assay (Biorad, Hecules, CA, USA) with BSA as standard using the Lowry method ².

**Mitochondrial respiration**

Oxygen uptake of 50 µg mitochondrial proteins was measured with a Clark-type electrode (Strathkelvin, Glasgow, UK) at 37 °C during magnetic stirring in 0.5 ml incubation buffer (in mmol/L: 125 KCl; 10 MOPS; 5 MgCl₂; 5 KH₂PO₄; 0.02 EGTA) with glutamate (5 mmol/L) and malate (5 mmol/L) as substrates for complex 1 or succinate (5 mmol/L) as substrate for complex 2 and inhibition of complex 1 by rotenone (2 µmol/L). The oxygen electrode was calibrated using a solubility coefficient of 216 nmol O₂/ml at 37 °C. Basal oxygen consumption (state 2) was recorded for 3 min. Then 400 µmol/L ADP were added and stimulated respiration (state 3) measured for 3 min. With subsequent addition of 300 µmol/L TMPD (N,N,N,N’-tetramethyl-p-phenylenediamine) with 3 mmol/L ascorbate, which donates electrons to cytochrome oxidase via the reduction of cytochrome c, complex 4 respiration was determined. With 30 nmol/L FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), the maximal oxygen uptake of uncoupled mitochondria was measured. Of note, all mitochondrial function measurements were made at physiological temperature of 37 °C, and the lack of difference in respiration with TMPD/ascorbate and FCCP between the ischemic postconditioning and the immediate full reperfusion protocols in the anterior wall indicated equal loading with viable mitochondria.
Calcium retention capacity
The calcium uptake of 100 µg mitochondrial proteins was determined in 1 ml incubation buffer (without EGTA) at 37 °C using glutamate and malate as substrates, in the presence of ADP (400 µmol/L). Calcium green-5N (0.5 µmol/L, Invitrogen, Carlsbad, CA, USA) was used as indicator to detect extramitochondrial calcium with a spectrophotometer (Cary Eclipse, Varian, Mulgrave, Victoria, Australia) at excitation and emission wavelengths of 500 and 530 nm, respectively. Pulses of 5 nmol CaCl₂ were added every minute until calcium was no longer taken up and a rapid increase in calcium green fluorescence was detected. As a control, 10 µmol/L cyclosporine A was used to inhibit mitochondrial permeability transition pore opening. A relatively high concentration of cyclosporine A was chosen to inhibit permeability transition pore opening also at 37 °C and with stimulated complex 1 respiration.
Mitochondrial respiration and calcium retention capacity were measured in the absence and presence of the STAT3 inhibitor Stattic (100 µmol/L; 3, 4). The solvent DMSO (10 mmol/L, dimethyl sulfoxide) had no effect on respiration or calcium retention capacity.

Western Blot Analysis
Tissue samples were homogenized in 1x Cell lysis buffer (Cell Signaling, Danvers, MA, USA), supplemented with 1x Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After sonification and centrifugation at 16,000 g for 10 min at 4 °C the supernatants were collected. Mitochondria were further purified by percoll-gradient ultracentrifugation. For that, mitochondria were layered on top of a 30 % percoll solution in isolation buffer and centrifuged at 35,000 g for 30 min. The lower mitochondrial band was collected and washed twice in isolation buffer by centrifugation at 10,000 g for 5 min. The mitochondrial pellet was resuspended in an appropriate volume of 1x Cell lysis buffer (Cell Signaling, Danvers, MA, USA), supplemented with 1x Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After 1 h incubation at 4 °C and occasional mixing, the mitochondria were centrifuged at 16,000 g for 10 min. Supernatants were collected and the protein concentrations determined using the DC protein assay (Biorad, Hercules, CA, USA). Protein aliquots of 20 µg (biopsies) or 100 µg (mitochondria) 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
(Supplemental Table 1) directed against the phosphorylated forms of STAT1 (phosphorylation sites: tyrosine\textsubscript{701} and serine\textsubscript{727}), STAT3 (tyrosine\textsubscript{705} and serine\textsubscript{727}), and STAT5 (tyrosine\textsubscript{694}). The only commercially available antibody directed against STAT5 phosphorylated at serine\textsubscript{780} failed in our tissue samples. After incubation with the respective secondary anti-rabbit antibodies, immunoreactive signals were detected by chemiluminescence (LumiGLO Reagent and Peroxide, Cell Signaling, Danvers, MA, USA) and quantified with Scion Image software (Scion Corp, Frederick, MD, USA). Immunoreactivities of phosphorylated kinases were normalized to those of the respective total kinases. There were no differences in STAT phosphorylation at baseline between the samples from the ischemic postconditioning (PoCo) and the immediate full reperfusion (IFR) groups. Therefore, baseline values were set as 100% to calculate the time courses of STAT phosphorylation within individual animals. The signal from STAT1 phosphorylated at serine\textsubscript{727} was below the detection threshold. At 85 min ischemia signals were reliably detectable and not different between PoCo and IFR. Therefore, the time course for STAT1 phosphorylation at serine\textsubscript{727} was expressed as raw data.

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<tr>
<td>STAT1</td>
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<td>#9172</td>
<td>Rabbit</td>
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<tr>
<td>p-STAT3 tyrosine\textsubscript{705}</td>
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<td>#9131</td>
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<td>sodium/potassium (Na\textsuperscript{+}/K\textsuperscript{+})-ATPase</td>
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<td>sarcoplasmic calcium (SERCA2)-ATPase</td>
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<td>histone deacetylase 2 (HDAC2)</td>
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<td>glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH)</td>
<td>Hytest</td>
<td>5G4</td>
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<tr>
<td>manganese-superoxide-dismutase (MnSOD)</td>
<td>Millipore</td>
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<td>ATP-synthase (\alpha)</td>
<td>BD Transduction</td>
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**Supplemental Table I:** Detailed list of antibodies
The absence of sodium/potassium (Na\(^+\)/K\(^-\))-ATPase, sarcoplasmic calcium (SERCA2)-ATPase, histone deacetylase 2 (HDAC2), and glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) and the presence of manganese-superoxide-dismutase (MnSOD) confirmed the purity of mitochondrial preparations used in the Western blot analyses (Supplemental Figure I A). Glutaraldehyde-fixed samples of purified mitochondria were contrasted with OsO\(_4\), epon-embedded and cut at 60 nm. Sections were further contrasted with uranylacetate & lead citrate and investigated on a Zeiss EM902 transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) for mitochondrial integrity and the presence of residual cell fragments (Supplemental Figure I B). In a systematic comparison of remote control myocardium in 11 pigs, mitochondrial ADP-stimulated complex 1 respiration was similar after purification by percoll-gradient ultracentrifugation (346±18 nmol O\(_2\)/min/mg) as in a time control (344±19 nmol O\(_2\)/min/mg).
Supplemental Figure I: Purity of mitochondrial preparations after percoll-gradient ultracentrifugation was confirmed by Western blot (panel A; absence of sodium/potassium (Na⁺/K⁺)-ATPase, sarcoplasmic calcium (SERCA2)-ATPase, histone deacetylase 2 (HDAC2), and glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH); presence of manganese-superoxide-dismutase (MnSOD). Mitochondrial integrity was investigated by electron microscopy (panel B).
The total STAT1, 3 and 5 proteins were not different throughout the protocols and therefore the phosphorylated forms were normalized to their respective total proteins (Supplemental Figure II).

Supplemental Figure II: Time courses of STAT1, 3, and 5 protein concentrations in samples from the area at risk in the absence or presence of AG490. Data are expressed as raw data in arbitrary units (AU). Representative Western blots of the total form of each protein from animals subjected to ischemic postconditioning (PoCo) are on top. IFR: immediate full reperfusion, BASE: baseline, ISCH: 85 min ischemia, R10/30/120: 10/30/120 min reperfusion.

STAT1 phosphorylation at serine727 was not detectable at baseline and therefore data are presented as raw values. The phosphorylation of STAT1 at tyrosine701, STAT3 at tyrosine705 and serine727 and STAT5 at tyrosine694 at baseline were not different between ischemic postconditioning and immediate full reperfusion and time courses are therefore normalized to baseline.
<table>
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<td>mean ± SEM</td>
<td>mean ± SEM</td>
<td></td>
<td></td>
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<tr>
<td>STAT1&lt;sub&gt;ser&lt;/sub&gt;*</td>
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<td>0.61 ± 0.20</td>
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<td>1.03 ± 0.27</td>
<td>1.02 ± 0.17</td>
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<table>
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<tr>
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<th>IFR+AG490</th>
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<th>n</th>
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<td>mean ± SEM</td>
<td>mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT1&lt;sub&gt;ser&lt;/sub&gt;*</td>
<td>0.75 ± 0.40</td>
<td>0.68 ± 0.09</td>
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<tr>
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<td>0.50 ± 0.23</td>
<td>0.27</td>
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<tr>
<td>STAT3&lt;sub&gt;ser&lt;/sub&gt;</td>
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<td>0.86 ± 0.21</td>
<td>0.53</td>
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<td>STAT3&lt;sub&gt;tyr&lt;/sub&gt;</td>
<td>0.77 ± 0.23</td>
<td>1.00 ± 0.39</td>
<td>0.64</td>
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**Supplemental Table II:** STAT phosphorylation in samples taken at baseline from pigs subjected to ischemic postconditioning (PoCo) or immediate full reperfusion (IFR) in the absence or presence of the JAK/STAT inhibitor AG490. For group comparisons, samples were analyzed on the same gel. Phosphorylated STAT was normalized to the respective total STAT. *STAT1<sub>ser</sub>: signals of phosphorylated STAT1<sub>ser</sub> were below detection threshold; p: p-value of two-sided t-test PoCo vs. IFR; n: number of samples in each group.

STAT1 phosphorylation at tyrosine<sub>701</sub> and serine<sub>727</sub> and STAT3 phosphorylation at serine<sub>727</sub> were increased during myocardial ischemia and even further during reperfusion, without a difference between ischemic postconditioning and immediate full reperfusion. STAT5 phosphorylation at tyrosine<sub>694</sub> was decreased during myocardial ischemia and transiently increased during reperfusion. AG490 inhibited only the increased phosphorylation of STAT3 (Supplemental Figures IIIa-d).
Supplemental Figure IIIa: Time courses of STAT1\textsubscript{tyr} phosphorylation in samples from the area at risk (right panel in the presence of the JAK/STAT inhibitor AG490). Data are expressed as percent of baseline. Open symbols/dashed lines: Ischemic postconditioning, closed symbols/solid lines: immediate full reperfusion. Representative Western blots of the phosphorylated and total form of each protein at 91 kDa are on top. (BASE: baseline, ISCH: 85 min ischemia, R5/10/30/120: 5/10/30/120 min reperfusion).
**Supplemental Figure IIIb:** Time courses of $\text{STAT1}_{\text{ser}}$ phosphorylation in samples from the area at risk (right panel in the presence of the JAK/STAT inhibitor AG490). Data are expressed as percent of baseline. Open symbols/dashed lines: ischemic postconditioning, closed symbols/solid lines: immediate full reperfusion. Representative Western blots of the phosphorylated and total form of each protein at 91 kDa are on top. (ISCH: 85 min ischemia, R5/10/30/120: 5/10/30/120 min reperfusion).
Supplemental Figure IIIc: Time courses of STAT3_{ser} phosphorylation in samples from the area at risk (right panel in the presence of the JAK/STAT inhibitor AG490). Data are expressed as percent of baseline. Open symbols/dashed lines: ischemic postconditioning (PoCo), closed symbols/solid lines: immediate full reperfusion (IFR). Representative Western blots of the phosphorylated and total form of each protein at 86 kDa are on top. (BASE: baseline, ISCH: 85 min ischemia, R5/10/30/120: 5/10/30/120 min reperfusion).
**Supplemental Figure IIIId:** Time courses of STAT5\(_{\text{tyr}}\) phosphorylation in samples from the area at risk (right panel in the presence of the JAK/STAT inhibitor AG490). Data are expressed as percent of baseline. Open symbols/dashed lines: ischemic postconditioning; closed symbols/solid lines: immediate full reperfusion. Representative Western blots of the phosphorylated and total form of each protein at 90 kDa are on top. (BASE: baseline, ISCH: 85 min ischemia, R5/10/30/120: 5/10/30/120 min reperfusion).
Supplemental Figure IV: Maximal calcium retention capacity of mitochondria from the area at risk was increased by addition of cyclosporine A (CsA; 10 µmol/L). Albeit significant, there was only a modest increase in calcium retention capacity by cyclosporine A over that with ischemic postconditioning (PoCo), suggesting a ceiling effect of maximal protection.

References


