Dual activation of STAT-3 and Akt is required during the trigger phase of ischaemic preconditioning

Naushaad Suleman, Sarin Somers, Robert Smith, Lionel H. Opie, and Sandrine C. Lecour*

Hatter Cardiovascular Research Institute, Department of Medicine, Faculty of Health Sciences, University of Cape Town, Private Bag 3, 7935 Observatory, South Africa

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Aims During preconditioning by tumour necrosis factor-α (TNFα), activation of the signal transducer and activator of transcription-3 (STAT-3) but not Akt, is essential, whereas ischaemic cardiac preconditioning (IPC) requires both STAT-3 and Akt at the time of reperfusion. However, it is not known whether the same signalling pattern occurs during the preconditioning stimulus (trigger phase) and whether links exist between STAT-3 and Akt. Hence, our hypothesis is that concomitant activation or co-interaction between these two key signals is required during the trigger phase for IPC. Conversely, we proposed that there would be no such interaction when preconditioning was induced by TNFα (TNF-PC).

Methods and results Cardiomyocytes, isolated from adult wild-type (WT) and cardiac-specific STAT-3 knockout (KO) mice, were exposed to simulated ischaemia (SI) reperfusion. Cells were preconditioned either by 30 min SI or by 30 min TNFα (0.5 ng/mL) in the presence or absence of AG490 (100 nM) or wortmannin (100 nM) to inhibit STAT-3 or Akt, respectively. Cell viability was evaluated by trypan blue, and phosphorylation levels of STAT-3 and Akt were measured by Western blot analysis. Similar experiments were conducted in isolated rat hearts subjected to an ischaemia-reperfusion insult. Both preconditioning stimuli failed to protect KO cardiomyocytes, and addition of AG490 abolished preconditioning in WT cardiomyocytes or isolated hearts. Wortmannin abolished the protection afforded by IPC, but did not affect TNF-PC in both models. Western blot analysis demonstrated that added wortmannin during IPC stimulus decreased STAT-3 phosphorylation while, conversely, AG490 reduced Akt phosphorylation.

Conclusion STAT-3 activation could be achieved independent of Akt during TNF-PC. In contrast, during an IPC stimulus, both prosurvival signalling molecule cascades acted in concert so that inhibiting activation of STAT-3 also inhibited that of Akt and vice versa.

1. Introduction

Phosphorylation of both signal transducer and activator of transcription-3 (STAT-3) and Akt (protein kinase B) occurs in response to ischaemia reperfusion to mediate protection via prosurvival signalling cascades or the inhibition of proapoptotic factors.1,2 STAT factors are a family of cytoplasmic transcription factors that mediate intracellular signalling initiated at cytokine cell surface receptors [for example, tumour necrosis factor-α (TNFα) receptors] and transmitted to the nucleus. STAT-3 is activated after phosphorylation of Janus kinases (JAKs) or by mitogen-activated protein kinases (MAPKs), which allow STAT-3 to dimerize and translocate to the nucleus (see reviews).3,4 Activation of the JAK/STAT-3 pathway during both the ischaemic preconditioning (IPC) stimulus5 and the early phase of reperfusion3 is crucial to achieve maximal protection in IPC. Such activation can confer protection via phosphorylation and inactivation of proapoptotic factors such as BAD5 and glycogen synthase kinase 3β (GSK-3β). Interestingly, pharmacological preconditioning with the cytokine TNFα also requires the activation of STAT-3 during the early phase of reperfusion,2 but whether its activation is required during the preconditioning stimulus has not yet been demonstrated.

Similarly, Akt phosphorylation is required both during the IPC stimulus2 and the early phase of reperfusion6 for IPC to confer cardioprotection. In addition, its activation is also required in many pharmacological preconditioning stimuli to inactivate several proapoptotic factors (BAD, Bax, Bim, and caspases) and to activate prosurvival factors such as endothelial nitric oxide synthase, p70s6kinase and protein kinase C (see review).1 To our knowledge, the only pharmacological preconditioning stimulus that can confer protection independent of the activation of Akt during the early phase of reperfusion is the cytokine TNFα. Of note, Akt and STAT-3 target some similar prosurvival pathways but it...
is not clear whether links exist between these two major protective signals in IPC.

Therefore, the hypothesis under test in this work was dual. First, we proposed using an isolated rat heart model (in conjunction with Akt and STAT-3 inhibitors) to demonstrate that TNFα, unlike IPC, can protect the heart via activation of STAT-3 but not of Akt during the preconditioning stimulus. Secondly, we used isolated cardiomyocytes from cardiac-specific STAT-3-deficient mice to test the hypothesis that concomitant activation or co-interaction between STAT-3 and Akt is required for IPC.

2. Methods

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [NIH Publication No. 85 (23), revised 1996], and all procedures were approved by the Faculty of Health Sciences Animal Ethics Committee University of Cape Town.

2.1 Isolated rat heart perfusion

Hearts from adult male Long-Evans rats (250–300 g) were excised rapidly and perfused retrogradely by the Langendorff technique as described previously. All hearts were subjected to 30 min of regional ischaemia by occlusion of the left coronary artery and 120 min of reperfusion (Figure 2). Hearts were preconditioned either with a low concentration of TNFα (0.5 ng/mL) that was given for 7 min followed by a 10 min washout period before standard ischaemia or with two cycles of 5 min of global I/R prior to standard ischaemia. Additional groups were perfused with AG490 (100 nM), a blocker of the JAK/STAT-3 pathway or wortmannin (100 nM), a blocker of the PI3 k/Akt pathway, for 15 min followed by 5 min washout prior to the ischaemia/reperfusion insult. AG490 or wortmannin was infused directly above the heart alone or in the presence of TNFα or an IPC stimulus. For infarct size measurements, the coronary artery was re-occluded at the end of the reperfusion period, and a solution of 2.5% Evans blue was perfused to delineate the area at risk. Hearts were then frozen overnight and sliced into five equal sections the following day. They were stained with a 1% TTC solution at 37°C for 5–7 min. The sections were then scanned.
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2.2 Isolated mouse cardiomyocytes

STAT-3-deficient mice were created by crossing homozygous floxed STAT-3 mice with heterozygous MLC2V-driven Cre-recombinase mice. Cardiomyocytes from wild-type and STAT-3 knockout (KO) mice (10-week-old males) were isolated using the modified method of Zhou et al.11 as described previously.10 Isolated mouse cardiomyocytes were cultured for 16–24 h at 37°C in a humidified 5% CO2 atmosphere prior to experimentation. They were exposed to simulated ischaemia (SI) in a modified Esumi buffer12 containing 118 mM NaCl, 3.58 mM KCl, 0.46 mM MgCl2, 0.9 mM CaCl2, 4 mM HEPES, and 20 mM 2-deoxy-D-glucose (2-DG) at pH 6.2 for 26 h, at 37°C, in a humidified environment containing 1% O2, 5% CO2, and the balance N2. Of note, the Esumi buffer is formulated to provide conditions similar to those found in ischaemic environment in vivo, with a decreased pH, a lack of metabolic substrates, and the presence of an inhibitor of glycolysis (2-DG). This was followed by 2 h of reoxygenation in insulin-free KLSMS (Kawasato, Sato, McClure, Sato)12 media under normoxic conditions (4% CO2 for protein extraction. Briefly, cytosolic proteins were extracted from isolated myocytes in 100 μL of lysis buffer containing 1% Nonidet P-40, 4 M NaCl, 1 M Hepes (pH 7.9), 500 mM EDTA and 400 μL of an EDTA-free protease inhibitor cocktail (Roche Systems, USA) per 1 000 000 myocytes. Samples were resuspended in lysis buffer and centrifuged at 3000 rpm for 30 s and the supernatant transferred to a new tube. This was then spun at 5000 rpm for 5 min to remove cellular debris. The resultant supernatant was used for Western blot analysis. Proteins were quantified using the Lowry Protein assay. Phosphorylated states of Akt (phospho-Akt; serine 473) and STAT-3 (phospho-STAT-3 tyrosine 705) as well as the total levels of Akt, STAT-3, and β-actin were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were transferred to PVDF membranes by electrophoretic transfer. Equal loading was verified by Ponceau staining or β-actin, and phosphorylated levels of proteins were normalized to their total protein levels in the same samples and under the same conditions but on a separate membrane. Relative peptide levels were measured using densitometric analysis with UVI band (UVI Tech, Cambridge, UK) software on a PC.

2.3 Western blot analysis

Control or preconditioned rat hearts were subjected to the preconditioning protocol. Hearts were collected for Western blot analysis prior to the ischaemic insult, and Western blot analysis was performed as previously described.2

Control isolated myocytes were cultured for 16–24 h spun down and washed in ice-cold PBS before being pelleted and stored at −80°C for extraction. Myocytes were subjected to either the SI PC or TNFα-induced PC protocols which were stopped following 15 or 30 min of treatment and washed in ice-cold PBS before myocytes were pelleted and stored at −80°C for protein extraction. Briefly, cytosolic proteins were extracted from isolated myocytes in 100 μL of lysis buffer containing 10% Nonidet P-40, 4 M NaCl, 1 M Hepes (pH 7.9), 500 mM EDTA and 400 μL of an EDTA-free protease inhibitor cocktail (Roche Systems, USA) per 1 000 000 myocytes. Samples were resuspended in lysis buffer and centrifuged at 3000 rpm for 30 s and the supernatant transferred to a new tube. This was then spun at 5000 rpm for 5 min to remove cellular debris. The resultant supernatant was used for Western blot analysis. Proteins were quantified using the Lowry Protein assay. Phosphorylated states of Akt (phospho-Akt; serine 473) and STAT-3 (phospho-STAT-3 tyrosine 705) as well as the total levels of Akt, STAT-3, and β-actin were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were transferred to PVDF membranes by electrophoretic transfer. Equal loading was verified by Ponceau staining or β-actin, and phosphorylated levels of proteins were normalized to their total protein levels in the same samples and under the same conditions but on a separate membrane. Relative peptide levels were measured using densitometric analysis with UVI band (UVI Tech, Cambridge, UK) software on a PC.

2.4 Statistical analysis

Results are expressed as means ± SEM and were analysed by one-way ANOVA with Dunn’s post-test, using GraphPad InStat version 3.01 (Graphpad software, San Diego, CA, USA). Differences were considered significant at values of P < 0.05.

3. Results

3.1 Inhibition of STAT-3 activation during preconditioning abrogates the protection induced by both IPC and TNFα

3.1.1 Isolated rat hearts model

Our previous work has explored the role of STAT-3 and Akt as mediators in both IPC- and TNFα-induced preconditioning but their role as a trigger still remains to be confirmed in TNFα-induced preconditioning. To investigate the role of STAT-3 during the preconditioning stimulus, we measured infarct size as a percentage of the area at risk in preconditioned hearts perfused with AG490, an inhibitor of the JAK/STAT-3 pathway. The haemodynamic parameters and viability was quantified by exclusion of Trypan blue and rod-shaped morphology, and a total of 150 cells or more were counted per sample. The initial yield of the isolation procedure gave between 55 and 65% viable cells.
the area at risk between all groups were not statistically different (data not shown).

Ischaemic control (CTL) hearts had an infarct size of $32.7 \pm 3.0\%$ (calculated as a percentage of the risk zone) (Figure 2). Both IPC and pharmacological preconditioning with TNFα reduced infarct size compared to the CTL group (IPC $6.5 \pm 2.2\%$ and TNFα $9.4 \pm 2.9\%; P < 0.01$ vs. CTL), confirming previous data from our laboratory.\(^2\) When AG490 was given during the preconditioning stimulus, the protective effects of both IPC and TNFα were abrogated (IPC + AG490: $29.6 \pm 5.0\%$; TNFα + AG490: $41.64 \pm 4.6\%, n \geq 6$, ns vs. CTL). In addition, both preconditioning stimuli were associated with a large increase in STAT-3 phosphorylation ($P < 0.01$ vs. CTL).

### 3.1.2 Isolated cardiomyocytes

Similar results were obtained in isolated mouse cardiomyocytes. Myocyte cell viability following an SI/reperfusion insult was $55.9 \pm 1.9\%$ (Figure 3). Both ischaemia- and

![Figure 3](image3.png)

Figure 3 Inhibiting the activation of STAT-3 (AG490, AG) or Akt (Wortmannin, w) during the preconditioning stimulus abrogates the increase of viable cells induced by simulated ischaemic preconditioning in isolated wild-type mouse cardiomyocytes. In contrast, only AG490 could abrogate the increase of cardiomyocytes viability afforded by the pharmacological preconditioning stimulus TNFα. *P < 0.05 vs. control.

![Figure 4](image4.png)

Figure 4 Western blot analysis performed in isolated cardiomyocytes show that inhibition of STAT-3 with AG490 reduced activated STAT-3 in both simulated ischaemic (A) and pharmacological preconditioning (B). *P < 0.05 vs. 30’ SI (A) or 30’ TNFα (B). SI, simulated ischaemia.
TNFα-induced preconditioning improved myocytes viability to 88.5 ± 3.6 and 83.0 ± 3.6%, respectively (*P < 0.001 vs. SI for both preconditioning stimuli; n ≥ 6) (Figure 3). Administration of AG490 inhibited the cardioprotective effects of both ischaemia- and TNFα-induced preconditioning in isolated cardiomyocytes (myocyte viability in IPC + AG490: 62.9 ± 2.8% and TNFα + AG490: 47.6 ± 4.4%, ns vs. control, n ≥ 6), in association with a significant reduction of phosphorylated levels of STAT-3 (Figure 3).

3.2 Inhibition of Akt activation during preconditioning abrogates the protection induced by IPC but not by TNFα

A simulated ischaemic stimulus was associated with a time-dependent increase of Akt phosphorylation in both isolated hearts (Figure 2B) and WT cardiomyocytes (Figure 5A). In contrast, the preconditioning stimulus with TNFα stimulus did not change the levels of phosphorylated Akt (Figures 2B and 5B). To investigate the role of PI3K/Akt during the preconditioning stimulus, we administered an inhibitor of the pathway, wortmannin.

3.2.1 Isolated rat hearts

Treatment with wortmannin during the preconditioning stimulus attenuated the protective effect of IPC in the isolated rat heart model (IPC + wortmannin 16.9 ± 3.5%, ns vs. CTL) (Figure 2A), thus confirming data previously described in the literature. In contrast, the inhibitor did not alter the protection afforded by TNFα preconditioning (TNFα + wortmannin 8.8 ± 2.9%; *P < 0.01 vs. CTL, n ≥ 6).

3.2.2 Isolated cardiomyocytes

Interestingly, similar data were obtained in isolated cardiomyocytes. Wortmannin, given during the ischaemic preconditioning stimulus, abolished the protective effect afforded by preconditioning (myocytes viability 55.2 ± 4.0 and 53.2 ± 4.1%, respectively, ns vs. SI; n > 6) (Figure 3). In contrast, this inhibitor failed to reverse the protection afforded by TNFα (85.0 ± 5.9 and 93.4 ± 2.3%, respectively; *P < 0.05 vs. SI, n > 6) (Figure 3).

3.3 Inhibiting STAT-3 in isolated cardiomyocytes decreases phosphorylation of Akt and vice versa

To determine whether an interaction between Akt and STAT-3 activation might exist, we determined STAT-3 phosphorylation in the presence of the PI3K/Akt inhibitor (wortmannin) and the phosphorylation of Akt in the presence of the JAK2/STAT-3 inhibitor (AG490). Akt was not phosphorylated in WT cardiomyocytes preconditioned in the presence of AG490 (156 ± 20 AU for IPC vs. 64 ± 12 AU for IPC + AG490, *P < 0.01) (Figure 6A). Conversely, STAT-3 was not phosphorylated in WT cardiomyocytes preconditioned in the presence of wortmannin (265 ± 45 AU for IPC and 120 ± 28 AU for IPC + wortmannin, *P < 0.05, n ≥ 6) (Figure 6B).

3.4 Akt is not activated in STAT-3-deficient cardiomyocytes subjected to a preconditioning stimulus

We have previously reported that STAT-3 cardiac-depleted mice failed to be preconditioned with ischaemia or with a pharmacological stimulus (including TNFα). Here, we demonstrate that isolated cardiomyocytes from cardiac-deficient mice failed to phosphorylate Akt when subjected to an ischaemic preconditioning stimulus (100 ± 0 AU in controls vs. 101 ± 6 AU in preconditioned hearts, ns, n ≥ 4) (data not shown).

4. Discussion

Using a combination of two different models, our data demonstrate, for the first time during the trigger phase of preconditioning, the co-existence of two distinct signalling protective cascades, one involving Akt and the other involving STAT-3. TNFα-induced preconditioning requires only the activation of STAT-3, but the two distinct cascades act in concert in IPC to promote maximal protection. In addition, we demonstrate the existence of a close interaction between these two IPC cascades such that inhibiting one will inhibit the other one and vice versa.
agents protect against an ischaemic insult by activation of the reperfusion injury salvage kinase (RISK) pathway that involves activation of both Akt and extracellular signal-regulated kinase (ERK) during the early phase of reperfusion.\textsuperscript{1} Our recent work has demonstrated for the first time that a preconditioning stimulus (TNF\textsubscript{a}) could protect against ischaemic insult independent of activation of the RISK pathway,\textsuperscript{2} therefore suggesting the concept of multiple protective pathways in IPC, namely the RISK pathway and the RISK-independent pathway (that involves TNF\textsubscript{a} and requires phosphorylation of STAT-3).

Other studies have also reported the possibility of an interaction between PI3K/Akt and JAK/STAT-3. The p85 regulatory subunit of PI3K may control serine phosphorylation of STAT-3, a critical step for the formation of stable STAT-3 homodimers.\textsuperscript{15} On the other hand, JAK can enhance the phosphorylation of Akt after binding to the p85-regulatory subunit of PI3K.\textsuperscript{22} There is also evidence that STAT-3 activation depends on the PI3K/Akt pathway, at least in pulmonary artery endothelial cells subjected to carbon monoxide exposure.\textsuperscript{23} More recently, Gross et al.\textsuperscript{23} studied ischaemia reperfusion in the in vivo rat and in a cell culture model to demonstrate opioid-induced cardioprotection via activation of both JAK/STAT-3 and PI3K/Akt pathways. Of note, STAT-3 phosphorylation was dependent on PI3K activation.

With the aim of delineating the existence of a possible interaction between these two protective pathways in the context of IPC, we used cardiac-specific STAT-3-deficient mice. When subjected to an IPC insult, these mice failed to increase phosphorylation of Akt, therefore suggesting that JAK/STAT-3 can regulate Akt phosphorylation. On the other hand, STAT-3 could not be phosphorylated in WT mice subjected to IPC in the presence of a PI3K inhibitor, therefore suggesting that PI3K can regulate STAT-3 phosphorylation. Similarly, 15 min of ischaemia did not phosphorylate STAT-3, whereas it caused an Akt phosphorylation which almost reached the levels of 30 min of ischaemia, therefore suggesting that Akt phosphorylation may precede STAT-3 phosphorylation. However, our experiments conducted with TNF\textsubscript{a} as a preconditioning stimulus clearly demonstrated that STAT-3 phosphorylation can be activated totally independent of PI3K.

A similar interactive phenomenon has been observed in IPC between Akt and ERK.\textsuperscript{8} Using the isolated rat heart model with specific inhibitors of PI3K/Akt and MEK/ERK, Hausenloy et al.\textsuperscript{8} suggest that the cross-talk between these two kinases is not balanced and that, in the scenario of cellular survival, the PI3K/Akt cascade is the more dominant cascade, with MEK/ERK playing a more significant role in mediating growth and hypertrophy. Our TNF\textsubscript{a} study suggests that STAT-3 may be more dominant in the context of preconditioning but we cannot exclude the possibility that an untested preconditioning agent may protect via activation of Akt and independent of activation of STAT-3.

On the basis of our data, we hypothesize that: (i) these two cascades can be activated independently (as demonstrated with TNF\textsubscript{a} PC); (ii) activating PI3K will further enhance JAK2 activity and vice versa; and (iii) activating JAK2 will facilitate Akt phosphorylation from PI3K. Therefore, we propose that the interaction occurs between PI3K and JAK2 and that the activation of PI3K by JAK2 is further enhanced once JAK2 has phosphorylated STAT-3. The exact

Figure 6 Inhibiting STAT-3 activation with AG490 results in inactivation of Akt in isolated wild-type mouse cardiomyocytes subjected to a simulated ischaemic preconditioning stimulus (A). Similarly, inhibiting Akt activation with wortmannin results in inactivation of STAT-3 (B) \((n \geq 4 \text{ per group})\); \(P < 0.05\) vs. 30’ simulated ischaemia.

To our knowledge, all major pharmacological preconditioning agents require the activation of Akt during the trigger phase (preconditioning stimulus) (see review).\textsuperscript{14} Our present work clearly demonstrates that TNF\textsubscript{a} can confer protection independent of Akt activation during the preconditioning stimulus period, therefore strongly suggesting the existence of diverse protective pathways during the trigger phase of IPC, one being Akt-dependent and another one being TNF\textsubscript{a}/STAT-3 dependent. Exogenous TNF\textsubscript{a} can, in a dose- and time-dependent manner, mimic IPC.\textsuperscript{9} Furthermore, activation of this cytokine is also required during IPC to confer cardioprotection.\textsuperscript{15–17} Similar to IPC, TNF\textsubscript{a} confers this protective effect via activation of reactive oxygen species, mitochondrial potassium ATP-dependent channel, and protein kinase C.\textsuperscript{9,18,19} Interestingly, sphingolipids are involved in TNF\textsubscript{a}-induced preconditioning but not necessarily in IPC.\textsuperscript{21} Most importantly, IPC and many pharmacological conditions.
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molecular mechanisms by which PI3K and JAK2 interact is unknown and further studies by immunoprecipitation may give more insights about the sequence of events that occurs in this interaction. Although both STAT-3 and Akt can invoke a cardioprotective effect from their nuclear localization, it is not clear whether this interaction occurs only in the nucleus. It is possible that this apparent signalling cross-talk could reflect the need for interaction between different subcellular compartments, rather than the same compartment. Interestingly, both signalling pathways seem to converge to common targets such as BAD and GSK-3beta but whether this convergence occurs directly or indirectly (ie, via p70S6 kinase) is unknown. Further work will be necessary to delineate the exact downstream cascade of these pathways.

In conclusion, we report, for the first time, the co-existence of two distinct signalling protective cascades during the trigger phase of IPC, the one involving PI3K/Akt phosphorylation and the other one involving the TNF\(\alpha/JAK/STAT-3\) pathway. These two cascades act in concert to promote maximal protection. Inhibiting the TNF\(\alpha/JAK/STAT-3\) pathway affects the activation of the PI3K/Akt and vice versa. These insights, combined with our previously published data, will hopefully open new therapeutic opportunities against ischaemic heart disease.

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