STAT signaling in ischemic heart: a role of STAT5A in ischemic preconditioning

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Submitted 15 January 2003; accepted in final form 4 April 2003

STAT signaling in the ischemic heart: a role of STAT5A in ischemic preconditioning. Am J Physiol Heart Circ Physiol 285: H476–H482, 2003; 10.1152/ajpheart.00079.2003.—We recently demonstrated that ischemic preconditioning (PC) induced by cyclic episodes of short duration of ischemia and reperfusion potentiates a signal transduction cascade involving Janus kinase (JAK) 2 and signal transducer and activator of transcription 3 (STAT3). A rapid activation of JAK and several STATs, including STAT3, STAT5A, and STAT6 also occurred during myocardial ischemia and reperfusion. This study sought to examine whether STAT5A and STAT6 were involved in PC. Two different animal models were used: isolated perfused working rat hearts and STAT5A and STAT6 knockout mouse hearts. The results of our study indicated phosphorylation of STAT 5A and STAT6 in the preconditioned myocardium. Tyrophostin AG490, a JAK2 inhibitor, or 4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo-3,4-d-pyrimidine (PPI), a Src kinase blocker, blocked STAT5A phosphorylation, whereas STAT6 phosphorylation was blocked only with tyrophostin. As expected, significant cardioprotection was achieved in the preconditioned heart as evidenced by reduced myocardial infarct size and decreased number of apoptotic cardiomyocytes. PC-mediated cardioprotection was partially abolished when hearts were pretreated with tyrophostin, PPI, or LY-294002, a phosphatidylinositol (PI)-3 kinase inhibitor. Studies with STAT5A and STAT6 knockout mouse hearts revealed that STAT6 knockout mouse hearts were resistant to myocardial ischemia-reperfusion injury. The hearts from STAT5A knockout mice could not be preconditioned, whereas those from STAT6 knockout mice were easily preconditioned. The results of the present study demonstrate that STAT5A, and not STAT6, plays a role in ischemic PC. For the first time, the results also indicated a role of Src kinase pathway in STAT5A PC and PI-3 kinase-Akt pathways appear to be the downstream regulator for STAT5A-STAT6 signaling pathway.

Janus kinase; apoptosis; ischemia-reperfusion; Src kinase; Akt; phosphatidylinositol 3-kinase

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 (STAT3) has recently been found to play an important role in cardioprotection induced by ischemic preconditioning (6, 24). Preconditioning can potentiate a Janus kinase (JAK)-STAT signaling by rapidly phosphorylating JAK and activating STAT3, resulting in a survival signal to the myocardium. Inhibition of JAK2 with tyrphostin AG 490 abolishes cardioprotective effects of preconditioning as evidenced by increased myocardial infarct size and cardiomyocyte apoptosis.

In another related study, we found a rapid activation of two components of JAK-STAT signaling pathway, STAT5A and STAT6, in the rat heart subjected to ischemia and reperfusion (10). These activated STATs bound to a conserved nucleotide sequence (ST domain) in the promoter of the angiotensinogen gene and upregulated the level of its mRNA. Treatment of the hearts with the AT1 blocker losartan resulted in loss of the STAT-angiotensinogen promoter binding activity and upregulated level of angiotensinogen mRNA. Because angiotensin has been shown to play a role in preconditioning (13, 18) and because these STATs are required for angiotensin II-mediated signaling in the heart (14), we hypothesized that STAT5A and STAT6 might also play a role in preconditioning.

There are two highly related STAT5, STAT5A, and STAT5B, of which the former was found to be activated in the heart in response to ischemia and reperfusion (10). The main function of STAT5A is prolactin signaling and, it is required for mammary gland development (26). STAT5B is also located downstream of growth hormone signaling, which has recently found to play a crucial role in angiogenic response during hypoxic or ischemic preconditioning (23, 27). STAT6 is an interleukin-4 (IL-4)-responsive transcriptional activator and is required for induction of IL-4-dependent gene expression (8). STAT6-deficient mice lack the physiological functions mediated by IL-4 (19).

The results of our study demonstrated that STAT5A, and not STAT6, is involved in preconditioning, although both are activated in the heart during ischemia and reperfusion. Interestingly, STAT5A potentiates the preconditioning stimulus via both Src kinase and JAK signaling pathways. Knockout mouse hearts de-
void of any copies of STAT5A lacked ability for preconditioning, whereas the hearts devoid of any copies of STAT6 did not affect cardioprotection afforded by preconditioning.

MATERIALS AND METHODS

Knockout Mice Devoid of STAT5A and STAT6

The knockout mice devoid of any copies of either STAT5A or STAT6 were obtained from the Jackson Laboratory (Bar Harbor, ME). The Western blot analysis demonstrates complete absence of STAT5A and STAT6 in the heart of these mice (data not shown). Corresponding wild-type mice (B6129SF2/J101045 for STAT5A and BALB/cJ000651 for STAT6) were also obtained from the same supplier.

Experimental Protocol

The study used two different animal models: 1) isolated working rat hearts subjected to ischemia-reperfusion and ischemic preconditioning protocol and 2) isolated working STAT5A and STAT6 genes knockout mouse hearts (Fig. 1). Isolated rat hearts were initially divided into three groups: 1) isolated hearts were perfused with Krebs-Henseleit bicarbonate (KHB) buffer for 3 h and 45 min (baseline control); 2) the hearts were subjected to 30 min of ischemia followed by 2 h reperfusion; and 3) the hearts were preconditioned to ischemic stress by four cyclic episodes of 5 min ischemia each followed by another 10 min reperfusion. The preconditioned hearts were further divided into three groups: hearts were preperfused for 15 min (before PC) with 1) tyrphostin AG 490, an inhibitor for JAK 2; 2) 4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo-3,4-u-pyrimidine (PPI), an inhibitor of Src kinase; and 3) LY-294002, an inhibitor of phosphatidylinositol (PI)-3 kinase. All hearts (after preconditioning) were subjected to 30 min of ischemia, followed by 2 h of reperfusion. In addition, three groups of hearts were perfused with 1) tyrphostin, 2) PPI, or 3) LY-294002 for 15 min followed by 2 h and 45 min perfusion with KHB buffer. To determine whether these inhibitors have any nonspecific effects, hearts were perfused with inhibitors only (not shown in Fig. 1).

For mouse hearts, STAT5A or STAT6 knockout mouse hearts and corresponding wild-type hearts were subjected to 30 min ischemia followed by 2 h reperfusion or preconditioning.

Isolated Working Rat and Mouse Heart Preparations

Male Sprague-Dawley rats and STAT5A and STAT6 knockout and wild-type mice were used for this study. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1985). The mice (25–34 g) and rats (250–300 g) were anesthetized with pentobarbital sodium (rats: 100 mg/kg body wt ip; mice: 80 mg/kg body wt ip) (Abbott, North Chicago, IL) and anticoagulated with heparin sodium (500 IU/kg body wt ip) (Elkin-Sinn, Cherry Hill, NJ) injection.

After sufficient depth of anesthesia was ensured, the hearts were excised and perfused in retrograde Langendorff mode against a constant perfusion pressure of 100 cmH2O (10 kPa) for standardization period in case of rats (18). For the mouse, a perfusion pressure of 50 cmH2O (5 kPa) was used (17). Any heart that showed any cardiac disturbance...
(ventricle arrhythmia and fibrillation) during the entire experiment was excluded from this study. All hearts were perfused by working mode according to the protocol described above. To ascertain the normal function of the heart, the heart rate, left ventricular developed pressure (the difference between the maximum systolic and diastolic pressure), left ventricular end-diastolic pressure, and the first derivative of developed pressure were recorded with a Gould P23 XL transducer (Gould Instrument System, Valley View, OH). The signal was amplified by using a Gould 6600 series signal conditioner (Gould Instrument System) and monitored on a Cordat II real-time acquisition system (Triton Technologies, San Diego, CA) (16, 17). The aortic flow was measured by flowmeter. The coronary flow was measured by time collection of the coronary effluent dripping from the heart.

Measurements of Infarct Size

After a global ischemic procedure, the heart was infused with 10% solution of the triphenyl tetrazolium (TTC) in phosphate buffer through the aortic cannula for 20 min (16). The left ventricle was removed and sliced into 1-mm thickness of cross-sectional pieces and weight. Each slice was scanned with computer-assisted scanner (Scanjet 5370C). The risk area of the whole myocardium was stained in red by TTC while the infarct zone remained unstained by TTC. These were measured with computerized software (Scion Image); areas were multiplied by the weight of each section and summed up to obtain the total of the risk zone and an infarct zone. The infarct size was expressed as the ratio of the infarct zone to the risk zone.

Western Blot Analysis

Left ventricles from the hearts were homogenized in a buffer containing (in mM) 25 Tris-HCl, 25 NaCl, 1 orthovanadate, 10 NaF, 10 pyrophosphate, 10 okadic acid, 0.5 EGTA, and 1 PMSF (11). Protein (100 μg) of each heart homogenate was incubated with 1 μg of antibody against STAT5A and STAT6 (Santa Cruz Biotechnology; Santa Cruz, CA) for 1 h at 4°C. The immune complexes were precipitated with protein A-Sepharose, and immunoprecipitates were separated by SDS-PAGE and immobilized on polyvinylidene difluoride membrane. The membrane was immunoblotted with phosphotyrosine (PY20) clone antibodies to evaluate the phosphorylation of STAT5A and STAT6. The membrane was stripped and rebotted with specific antibodies against STAT5A and STAT6. The resulting blots were digitized and subjected to densitometric scanning using a standard NIH image program.

TUNEL Assay for Assessment of Apoptotic Cell Death

Immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase end-labeling (TUNEL) assay.
ing (TUNEL) (12, 25). The sections were incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percentage of total myocyte population.

Statistical Analysis

The values for myocardial functional parameters, number of apoptotic cardiomyocytes, and infarct sizes were all expressed as the means ± SE. The statistical analysis was performed by one-way analysis of variance for any differences between the mean value of all groups. Differences between data were analyzed for significance by performing a Student’s t-test. The results were considered significant if \( P < 0.05 \).

RESULTS

Experiments with Isolated Rat Hearts

Phosphorylation of STAT5A and STAT6. We first examined the phosphorylation of STAT5A and STAT6 during preconditioning. As shown in Figs. 2 and 3, the Western blot analyses revealed that both STAT5A and STAT6 are extensively phosphorylated during preconditioning. The tyrphostin or PPI had no effect on the phosphorylation of either STATs (control), but they partially blocked the phosphorylation of STAT5A during preconditioning. Interestingly, PPI was quite effective in blocking the phosphorylation of STAT5A, but it had no effect on STAT6 phosphorylation, suggesting a role of Src kinase in STAT5A phosphorylation only. Tyrphostin reduced the phosphorylation of both the STATs, suggesting the involvement of JAK2 in STAT phosphorylation.

Effects of inhibition of phosphorylation of STAT5A and STAT6 on myocardial infarct size and cardiomyocyte apoptosis. Because necrosis and apoptosis independently contribute to myocardial infarct size and because ischemic stress adaptation reduces both necrosis and apoptosis, we determined the effects of tyrphostin and PPI on myocardial infarct size and cardiomyocyte apoptosis. As expected ischemia-reperfusion increased myocardial infarct size (Fig. 4) as well as apoptotic cell death (Fig. 5). Myocardial infarct size and cardiomyocyte apoptosis were significantly reduced in the preconditioned heart. Whereas none of these inhibitors including tyrphostin, PPI, and LY-294002 had any effect on the heart, they partially abolished the cardioprotection afforded by preconditioning by increasing the infarct size and the number of apoptotic cardiomyocytes (Figs. 4 and 5), suggesting a role of multiple signal transduction pathways in preconditioning.

We next determined the contribution of the downstream signaling components of the survival pathway, PI-3 kinase and Akt. Inhibition of PI-3 kinase with LY-294002 completely abolished the cardioprotective

![Fig. 5. Effects of PPI, Tyr, and LY-294002 on the PC-mediated reduction of cardiomyocyte apoptosis. A: control, I/R, and PC values; B: PPI, Tyr, and LY-294002 values. PC lowered cardiomyocyte apoptosis by >75%. Apoptosis-lowering ability of PC was abrogated when the hearts were pretreated with any Tyr, PPI, or LY-294002, indicating that both Src kinase and JAK2 as well as PI-3 kinase are involved in PC. Data are expressed as means ± SE of 6 animals per group. *P < 0.05 vs. control; †P < 0.05 vs. PC.](http://ajpheart.physiology.org/)

![Fig. 6. Effects of PC on myocardial infarct size (A) and cardiomyocyte apoptosis (B) in STAT5A knockout mice. Stat5A wild-type mouse hearts were preconditioned (both infarct size and apoptotic cells are reduced), whereas PC was not achieved for STAT5A knockout mouse hearts. Data are expressed as means ± SE of 6 animals per group. *P < 0.05 vs. I/R hearts.](http://ajpheart.physiology.org/)
abilities of preconditioning, suggesting a crucial role of PI-3 kinase-Akt signaling in cardioprotection with STAT signaling (Figs. 4 and 5).

Experiments with Isolated Mouse Hearts

Recovery of myocardium contractile performance and infarct size after ischemia-reperfusion. Analysis of protein blots revealed complete absence of STAT5A and STAT6A in STAT5 knockout and STAT6 knockout mouse hearts, respectively. These hearts and the corresponding wild-type hearts were either subjected to ischemia-reperfusion or preconditioning protocol. The results of myocardial infarct size and cardiomyocyte apoptosis are shown in Figs. 6 and 7. Because of the high incidence of ventricular fibrillation and ventricular conduction disturbances, two wild-type and two knockout hearts from STAT6 and one wild-type and one knockout heart from STAT5A were excluded from this study. The results indicate that the hearts from STAT5A knockout mice could not be preconditioned because there were no differences in the infarct size and the number of apoptotic cardiomyocytes between the ischemia-reperfusion group and the preconditioned group. In contrast, preconditioning significantly decreased the myocardial infarct size and reduced the number of apoptotic cardiomyocytes in these hearts indicating that STAT6 knockout hearts could be preconditioned.

DISCUSSION

The results of the present study demonstrate that STAT5A, not STAT6, plays an important role in preconditioning of the heart, although both of these STATs are activated in the hearts after preconditioning. There are several salient features of the study including 1) both STAT5A and STAT6 are activated in the hearts after ischemia and reperfusion but only STAT5A plays a role in preconditioning; 2) STAT5A preconditioning is achieved with two different upstream signaling components, Src kinase and JAK, where Src kinase appears to play a predominant role; 3) Src kinase-STAT5A signaling is linked to PI-3 kinase Akt-mediated surviving signals leading to the reduction of cardiomyocyte apoptosis; and 4) STAT5A knockout hearts were resistant to preconditioning, whereas STAT6 knockout hearts are not affected by preconditioning stimulus. PC reduced myocardial infarct size as well as cardiomyocyte apoptosis, whereas these parameters were proportionately increased with the inhibitors of JAK2, Src kinase, or PI-3 kinase suggesting that both necrosis and apoptosis are independent contributors of myocardial infarction. The results of this study suggest a role of STAT5A, in addition to previously reported STAT3, in ischemic preconditioning of myocardium.

To date, seven members of the STAT family have been identified ranging from STAT1 to STAT6 with two members of STAT5, STAT5A and STAT5B. The most characterized feature of STAT proteins is that...

Fig. 7. Effects of PC on myocardial infarct size (A) and cardiomyocyte apoptosis (B) in STAT6 knockout mice. STAT6 wild-type and knockout mouse hearts were preconditioned (both infarct size and apoptotic cells are reduced). Data are expressed as means ± SE of 6 animals per group. *P < 0.05 vs. I/R hearts; †P < 0.05 vs. wild type.

Fig. 8. Proposed mechanism of STAT5A-mediated PC.
they contain an Src-homology 2 (SH2) phosphotyrosine binding domain that interacts with sites of tyrosine phosphorylation to recruit the STATs to the receptors (22). Dimerization between the SH2 domains and the carboxy-terminally localized phosphotyrosine-containing domain follows tyrosine phosphorylation of the STATs, which is an essential step for concomitant nuclear translocation of the dimer.

Several recent studies demonstrated rapid activation of several STATs, including STAT3, STAT5A, and STAT6 in the heart in response to ischemia and reperfusion (14). Among these STATs, STAT3 was found to be involved in preconditioning (2). STAT activation occurred through the upstream signaling component JAK2, and JAK2-STAT3 signaling was instrumental for changing the ischemia-reperfusion-mediated death signal into preconditioning-mediated survival signal. This was accompanied by an upregulation of the anti-death gene bcl-2, suggesting that bcl-2 may play a crucial role in the survival signal transmitted by JAK2-STAT3 signaling. The present study demonstrates that in addition to JAK2, Src tyrosine kinase is also involved in STAT5A signaling because the specific Src kinase inhibitor PPI mostly abolished the cardioprotective activities of STAT5A signaling. PPI is known to inhibit the Src family of tyrosine kinase at submicromolar concentrations without affecting other protein tyrosine kinase and receptor tyrosine kinase activities (1). We previously used PPI to successfully block Src kinase signaling in the ischemically preconditioned myocardium (3).

A growing body of evidence indicates a crucial role of Src kinase in preconditioning. A recent study indicated that Src kinase activation mediates ischemic injury but serves as the trigger for preconditioning in the position either upstream of or parallel to membrane-associated protein kinase C-ε (7). In conscious rabbits, protein kinase C-dependent activation of Src and Lck tyrosine kinases occurs during preconditioning (15). Thus, although activation of the Src family of tyrosine kinases during sustained ischemia is deleterious, it is not harmful when induced by a brief period of ischemia and is capable of generating signals that eventually confer myocardial protection.

A role of tyrosine kinases in preconditioning is widely recognized, and as mentioned above, Src kinase appears to play an essential role in transmitting preconditioning-mediated survival signal. However, there is some evidence indicating that both Src kinase-dependent and Src kinase-independent signal transduction pathways exist based on the observation that protein tyrosine kinase is not involved in the infarct size-limiting effect of early preconditioning in the canine heart (9). Consistent with this report, we found activation of STAT3 via JAK could also lower the myocardial infarct size induced by preconditioning (21). Thus it appears that both JAK and Src are equally important tyrosine kinases that can contribute to preconditioning. In the present study, we demonstrate that JAK2 and Src kinase equally contribute in transmitting survival signal in the preconditioned myocardium. Inhibition of either JAK2 with tyrphostin or Src kinase with PPI blocks the activation of STAT5A and consequently blocks cardioprotective abilities of STAT5A preconditioning.

Interestingly, Src kinase-STAT5A signaling appears to be linked with PI-3 kinase-Akt-mediated survival signals. Inhibition of Src kinase with PPI, but not inhibition of JAK2 with tyrphostin blocked Akt phosphorylation, suggesting that STAT5A activation via JAK2 does not contribute to PI-3 kinase-Akt-mediated survival signal. The importance of PI-3 kinase-Akt signaling has recently been recognized from the studies that inhibition of PI-3 kinase can abolish the cardioprotective abilities of preconditioning (5). Consistent with these reports, the present results demonstrate that inhibition of PI-3 kinase with LY-294002 blocked the phosphorylation of Akt and abolished the antiapoptotic abilities of preconditioning. Akt, a serine-threonine kinase, is a key effector of PI-3 kinase in the survival pathway against apoptosis (4). Akt can phosphorylate the proapoptotic protein Bad thereby inhibiting its proapoptotic function, which may account for the antiapoptotic effect of Akt (4).

There is evidence to indicate that Src kinase and PI-3 kinase signaling pathways converge to activate Rac1 and Jnk (20). Our results further demonstrated that inhibition of Src kinase with PPI was accompanied by inhibition of preconditioning-mediated activation of STAT5A and Akt signaling in the preconditioned myocardium (Fig. 8).

In summary, the results of the present study demonstrate a role of STAT5A in preconditioning-mediated cardioprotection through two different signaling pathways. Preconditioning activates STAT5A via Src kinase and JAK2. The Src kinase activation of STAT5 preconditions the heart through the PI-3 kinase-Akt survival pathway, whereas JAK2/STAT5A signaling occurs by an as yet unknown pathway.

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DISCLOSURES

This study was partially supported by National Heart, Lung, and Blood Institute Grants HL-34360, HL-22559, HL-33889, and HL-56803.

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