An essential role of the JAK-STAT pathway in ischemic preconditioning

Yu-Ting Xuan, Yiru Guo, Hui Han, Yanqing Zhu, and Roberto Bolli*

Experimental Research Laboratory, Division of Cardiology, University of Louisville and the Jewish Hospital Heart and Lung Institute, Louisville, KY 40292

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The goal of this study was to determine the role of the Janus tyrosine kinase (JAK)–signal transducers and activators of transcription (STAT) pathway in the late phase of ischemic preconditioning (PC). A total of 230 mice were used. At 5 min after ischemic PC (induced with six cycles of 4-min coronary occlusion/4-min reperfusion), immunoprecipitation with anti-phosphotyrosine (anti-pTyr) antibodies followed by immunoblotting with anti-JAK antibodies revealed increased tyrosine phosphorylation of JAK1 (+257 ± 53%) and JAK2 (+238 ± 35%), indicating rapid activation of these two kinases. Similar results were obtained by immunoblotting with anti-pTyr-JAK1 and anti-pTyr-JAK2 antibodies. Western analysis with anti-pTyr-STAT antibodies demonstrated a marked increase in nuclear pTyr-STAT1 (+301 ± 61%) and pTyr-STAT3 (+253 ± 60%) 30 min after ischemic PC, which was associated with redistribution of STAT1 and STAT3 from the cytosolic to the nuclear fraction and with an increase in STAT1 and STAT3 γ-IFN activation site DNA-binding activity (+606 ± 64%), indicating activation of STAT1 and STAT3. No nuclear translocation or tyrosine phosphorylation of STAT2, STAT4, STAT5A, STAT5B, or STAT6 was observed. Pretreatment with the JAK inhibitor AG-490 20 min before the six occlusion/reperfusion cycles blocked the enhanced tyrosine phosphorylation of JAK1 and JAK2 and the increased tyrosine phosphorylation, nuclear translocation, and enhanced DNA-binding activity of STAT1 and STAT3. The same dose of AG-490 abrogated the protection against myocardial infarction and the concomitant up-regulation of inducible NO synthase (iNOS) protein and activity observed 24 h after ischemic PC. Taken together, these results demonstrate that ischemic PC induces isoform-selective activation of JAK1, JAK2, STAT1, and STAT3, and that ablation of this response impedes the up-regulation of iNOS and the concurrent acquisition of ischemic tolerance. This study demonstrates that the JAK-STAT pathway plays an essential role in the development of late PC. The results reveal a signaling mechanism that underlies the transcriptional up-regulation of the cardiac *iNOS* gene and the adaptation of the heart to ischemic stress.

The late phase of ischemic preconditioning (PC) is a delayed response that renders the heart relatively resistant to ischemia/reperfusion injury 12–24 h after a brief ischemic stress (1–14). The protection afforded by late PC is sustained (≈72 h) and broad, involving both alleviation of myocardial stunning and limitation of infarct size (5, 7). As a result, considerable attention has focused on the pathophysiology and potential clinical implications of this defensive cardiac adaptation (5, 7). Recent evidence indicates that the beneficial actions of late PC are mediated in part by the inducible isoform of NO synthase (iNOS), which is synthesized in response to the initial ischemic stress (5, 6, 9–11, 13). Although protein kinase C (2, 3), Src protein tyrosine kinases (11, 12, 14), and NF-κB (8) have been implicated in the development of late PC, the precise signal transduction pathways whereby a sublethal ischemic stress results in up-regulation of iNOS and in delayed cardioprotection remain incompletely understood.

One mechanism that controls the expression of stress-responsive genes is the Janus tyrosine kinase (JAK)–signal transducers and activators of transcription (STAT) signaling pathway. It involves two families of proteins, JAKs and STATs, which transduce extracellular signals into the nucleus resulting in transcriptional activation of target genes (15–18). Four JAKs have been identified (JAK1, JAK2, JAK3, and TYK2), all of which are activated by tyrosine phosphorylation (15–17). The best-known substrate for JAKs is the family of STAT proteins, which includes seven isoforms (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) (15–17). Activation of STATs requires phosphorylation of tyrosine residues in the Src homology 2 domain (15–17). Once they are phosphorylated by JAKs, STAT proteins homodimerize or heterodimerize and translocate to the nucleus, where they transactivate STAT-responsive genes (15–17). The JAK-STAT pathway has been implicated in cardiac hypertrophy (18), apoptosis (19, 20), and inflammation (21, 22). Activation of JAK1 (23) and STAT3 (23, 24) has been reported in rat models of myocardial infarction produced by permanent coronary occlusion, where it has been suggested to limit apoptosis (24). STAT1 and STAT3 also have been found to exert proapoptotic and antiapoptotic effects, respectively, in cultured neonatal rat cardiac myocytes subjected to anoxia, metabolic inhibition, and acidosis (20). To date, however, the role of the JAK-STAT pathway in myocardial ischemia followed by reperfusion has not been evaluated and virtually nothing is known regarding whether JAK-STAT signaling contributes to the protective effects of ischemic PC.

The overall objective of the present study was to determine whether recruitment of JAK and STAT proteins is an important mechanism responsible for the up-regulation of iNOS that underlies late PC and for the concomitant tolerance to ischemia. The following specific questions were addressed: (*i*) which JAK and STAT isoforms are expressed in the adult myocardium, (*ii*) does an ischemic PC stimulus activate JAKs and/or STATs *in vivo*, (*iii*) if so, which specific JAK isoform(s) (among the four known isoforms) is/are activated, (*iv*) which specific STAT isoform(s) (among the seven known STATs) is/are activated, (*v*) does inhibition of the JAK-STAT pathway interfere with the up-regulation of iNOS protein expression and activity, and (*vi*) does inhibition of the JAK-STAT pathway interfere with the infarct-sparing effects of late PC? To address these issues, a systematic analysis of all known JAK and STAT proteins was performed at multiple time points in a murine model of late PC in which fundamental physiological variables that may impact on infarct size and cardiac response to stress (body temperature, arterial oxygenation, acid-base balance, heart rate, and arterial blood pressure) are carefully controlled and kept within normal limits (1, 13). The results demonstrate that activation of the JAK-STAT pathway by ischemic PC is an obligatory mechanism underlying the transcriptional activation of the *iNOS* gene and the protective effects of late PC.

Methods

The murine model of late PC has been described in detail (1, 13). This study was performed in 230 male ICR mice weighing 35.1 ± 0.4 g (age, 8.4 ± 0.2 wk).

Abbreviations: PC, preconditioning; GAS, γ-IFN activation site; JAK, Janus tyrosine kinase; STAT, signal transducer and activator of transcription; iNOS, inducible NO synthase; pTyr, phosphotyrosine; IP, immunoprecipitation; EMSA, electrophoretic mobility-shift assay.

*To whom reprint requests should be addressed. E-mail: rbolli@louisville.edu.

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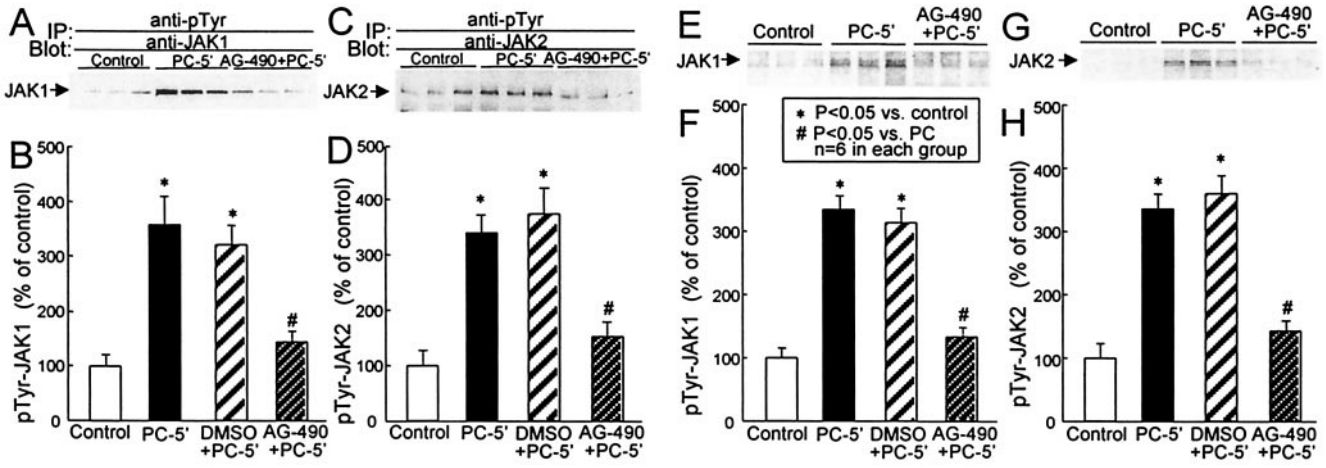

Fig. 1. Tyrosine phosphorylation of JAK1 and JAK2 by ischemic PC. Myocardial samples were obtained from mice that underwent a sham operation (control) and from the ischemic-reperfused region of preconditioned mice that received either no treatment (PC-5′), DMSO (DMSO + PC-5′), or AG-490 (AG-490 + PC-5′) 20 min before the ischemic PC protocol. All mice were euthanized 5 min after the sixth reperfusion. (*A–D*) The tyrosine-phosphorylated forms of JAK1 and JAK2 in homogenates were determined by IP of samples with anti-pTyr antibodies followed by Western blotting with anti-JAK1 or anti-JAK2 antibodies. (*E–H*) The tyrosine-phosphorylated forms of JAK1 and JAK2 in homogenates were directly determined by Western blotting with anti-pTyr(1022/1023)-JAK1 or anti-pTyr(1007/1008)-JAK2 antibodies. (*A*, *C*, *E*, and *G*) Western blots showing that the immunoreactivity of the tyrosine-phosphorylated forms of JAK1 and JAK2 increased markedly 5 min after ischemic PC, and that this increase was ablated by AG-490. (*B*, *D*, *F*, and *H*) Densitometric analysis of tyrosine-phosphorylated JAK1 and JAK2 signals. Data are means ± SEM.

Effect of Ischemic PC on JAKs and STATs. Mice were assigned to nine groups (groups I–IX) (see Fig. 8, which is published as supplemental data on the PNAS web site, www.pnas.org). Group I (control) underwent 1 h of open-chest state without coronary occlusion. Mice in groups II–IX underwent a sequence of six cycles of 4-min coronary occlusion/4-min reperfusion (a protocol that induces late PC; refs. 1 and 13) and were euthanized at 5 min, 30 min, 1 h, or 2 h after the last reperfusion. Myocardial samples were rapidly removed from the ischemic-reperfused region and frozen in liquid nitrogen. Groups IV and IX received the JAK inhibitor AG-490 (CalBiochem; 40 μg/g i.p. dissolved in 45% DMSO) 20 min before the first occlusion, whereas groups III (DMSO + PC-5′) and VIII (DMSO + PC-30′) received the same dose of vehicle (DMSO). Tissue samples were homogenized, and the cytosolic, membranous, and nuclear fractions were prepared as described (8, 10, 13). Western immunoblotting was performed by using standard techniques (3, 8, 13). Two independent methods were used to determine the tyrosine-phosphorylated (activated) forms of JAKs: (*i*) immunoprecipitation (IP) with an anti-phosphotyrosine (anti-pTyr) antibody (15, 25) followed by immunoblotting with anti-JAK antibodies; and (*ii*) immunoblotting with anti-pTyr(1022/1023)-JAK1 and anti-pTyr(1007/1008)-JAK2 antibodies. The sources of all antibodies used are indicated in Table 1, which is published as supplemental data on the PNAS web site. Electrophoretic mobility-shift assays (EMSAs), competition assays, and supershift assays were performed as described (8). A synthetic double-stranded probe with the sequence 5′-GATCAGCTTCAATTTCCCGTAAATCCCTA-3′ (GIBCO) was end-labeled by using [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Pharmacia) and T4 polynucleotide kinase, and purified with a G-25 Sephadex column (Amersham Pharmacia) (8). This oligonucleotide has the consensus sequence for γ-IFN activation site (GAS) elements, as indicated by underlines (26, 27).

Effect of Ischemic PC on NOS. Mice were assigned to three groups (groups X–XII) (Fig. 9, which is published as supplemental data on the PNAS web site). Group X (control) underwent 1 h of open-chest state without coronary occlusion. Mice in group XI were preconditioned with six occlusion/reperfusion cycles with no treatment, whereas group XII received AG-490 (40 μg/g i.p.) 20 min before the six occlusion/reperfusion cycles. Both groups were euthanized 24 h later, and myocardial samples were harvested as described above. The expression of iNOS and endothelial NOS was assessed by Western immunoblotting (3, 8, 13). NOS activity was determined by measuring the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline (10, 13).

Effect of AG-490 on Infarct Size. Mice were assigned to five groups (groups XIII–XVII) (Fig. 9). Group XIII (control) underwent 30 min of coronary occlusion and 4 h of reperfusion without any prior manipulation. Groups XIV–XVII were subjected to the 30-min coronary occlusion on day 2 with or without undergoing PC (six occlusion/reperfusion cycles) on day 1 and with or without AG-490 pretreatment (Fig. 9). At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by post-mortem perfusion of the heart with phthalo blue dye and triphenyltetrazolium (1, 13). Infarct size was calculated by computerized videoplanimetry (1, 13).

Statistical Analysis. Data are reported as means ± SEM. Measurements were analyzed by ANOVA followed by unpaired Student's *t* tests with the Bonferroni correction. In all Western analyses, the content of the specific protein of interest was expressed as a percentage of the corresponding protein in the anterior left ventricular wall of control mice.

Results

Effect of Ischemic PC on JAKs. Using antibodies specific for JAK1, JAK2, JAK3, and TYK2 that recognize the kinases regardless of their phosphorylation state, three JAKs (JAK1, JAK2, and TYK2) were found to be expressed in the murine heart (Fig. 10, which is published as supplemental data on the PNAS web site), whereas JAK3 was not detected by using two different antibodies (Fig. 11*A*, which is published as supplemental data on the PNAS web site). Because all known JAKs are activated through tyrosine phosphorylation (15–17), we determined the tyrosine-phosphorylated (activated) forms of JAK1, JAK2, and TYK2 by IP of samples with a specific anti-pTyr antibody followed by immunoblotting with antibodies specific for JAK1, JAK2, or TYK2. As illustrated in Fig. 1, the sequence of six coronary occlusion/reperfusion cycles induced

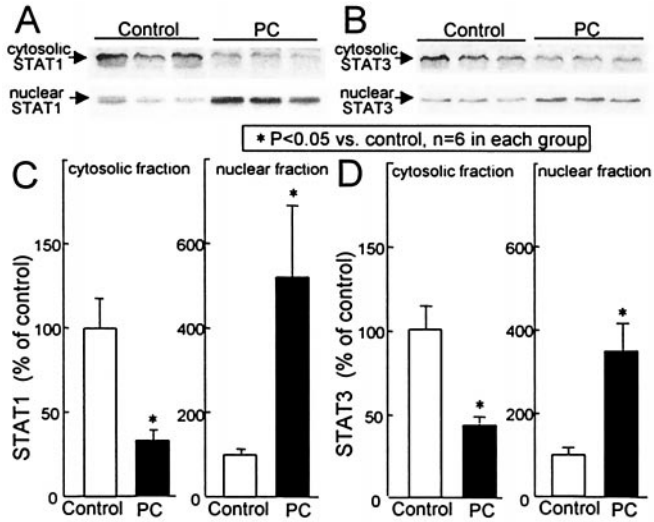

Fig. 2. Subcellular redistribution of STAT1 and STAT3 30 min after ischemic PC. Myocardial samples were obtained from mice that underwent a sham operation (control) and from the ischemic-reperfused region of preconditioned mice (PC). Total STAT1 and STAT3 expression in cytosolic and nuclear extracts was analyzed by Western blotting using anti-STAT1 or STAT3 antibodies that recognize STAT1 or STAT3 regardless of their phosphorylation state. (*A* and *B*) Western blots showing a marked decrease in STAT1 and STAT3 immunoreactivity in the cytosolic fraction and a commensurate increase in the nuclear fraction 30 min after ischemic PC. (*C* and *D*) Densitometric analysis of STAT1 and STAT3 signals. Data are means ± SEM.

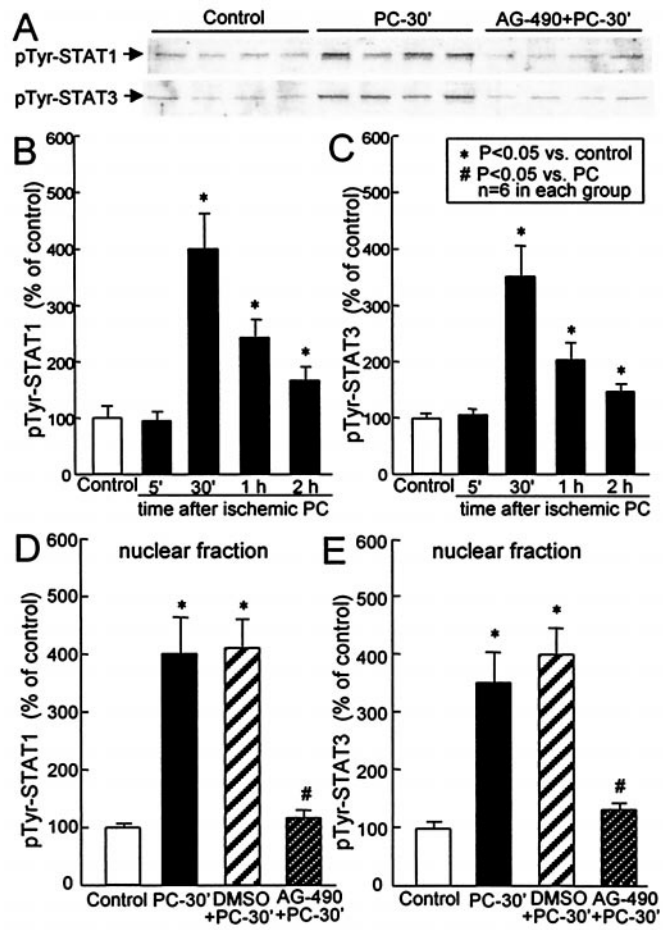

Fig. 3. Tyrosine phosphorylation of STAT1 and STAT3 by ischemic PC. Nuclear extracts were prepared from mice that underwent a sham operation (control), from the ischemic-reperfused region of preconditioned mice that were euthanized 30 min after the sixth reperfusion and received either no treatment (PC-30′), DMSO (DMSO + PC-30′), or AG-490 (AG-490 + PC-30′) 20 min before the ischemic PC protocol, and from the ischemic-reperfused region of untreated preconditioned mice that were euthanized 5 min, 1 h, or 2 h after the sixth reperfusion. Tyrosine-phosphorylated forms of STAT1 and STAT3 in nuclear extracts were analyzed by Western blotting with anti-pTyr(701)-STAT1 or anti-pTyr(704)-STAT3 antibodies. (*A*) Western blots showing that the immunoreactivity of tyrosine-phosphorylated STAT1 and STAT3 increased markedly 30 min after ischemic PC, and that this increase was ablated by AG-490. (*B* and *C*) Time course of tyrosine phosphorylation of STAT1 and STAT3. (*D* and *E*) Densitometric analysis of tyrosine-phosphorylated STAT1 and STAT3 at 30 min after PC. Data are means ± SEM.

a marked increase in the tyrosine-phosphorylated form of JAK1 (+257 ± 53% above control; Fig. 1 *A* and *B*) and JAK2 (+238 ± 35% above control; Fig. 1 *C* and *D*) 5 min after the sixth reperfusion, indicating rapid activation of these two kinases. In contrast, no tyrosine-phosphorylated TYK2 was detected either 5 min after ischemic PC or at subsequent time points (1 and 2 h after PC) (Fig. 11*B*). Control experiments for the IP studies are illustrated in Fig. 12, which is published as supplemental data on the PNAS web site. The augmented tyrosine phosphorylation of JAK1 and JAK2 was independently verified by a second method, i.e., by immunoblotting samples with specific anti-pTyr(1022/1023)-JAK1 and anti-pTyr(1007/1008)-JAK2 antibodies, respectively. Using this approach, a robust increase in both pTyr-JAK1 and pTyr-JAK2 (+219 ± 23% and + 235 ± 24% above control, respectively; Fig. 1 *E–H*) was observed 5 min after the sixth reperfusion, consistent with the results obtained by using IP followed by immunoblotting (Fig. 1 *A–D*). Pretreatment with AG-490 effectively inhibited the tyrosine phosphorylation of JAK1 and JAK2, whereas DMSO alone had no effect (Fig. 1). Analysis of mice subjected to one 4-min coronary occlusion with or without a subsequent 4-min reperfusion interval suggests that the activation of JAK1 and JAK2 occurs during reperfusion, not during ischemia (Fig. 13, which is published as supplemental data on the PNAS web site).

Effect of Ischemic PC on STATs. Using specific antibodies that recognize each STAT protein regardless of its phosphorylation state, all seven STAT isoforms were found to be expressed in the cytosolic fraction of the murine heart (Fig. 2 and Fig. 14, which is published as supplemental data on the PNAS web site). To thoroughly define the effects of ischemic PC on the STAT family of transcription factors, two indices of STAT activation (tyrosine phosphorylation and nuclear translocation) were systematically examined at serial time points (5 min, 30 min, 1 h, 2 h) after PC for all seven STAT isoforms. Western analysis of nuclear proteins with specific anti-pTyr(701)-STAT1 and anti-pTyr(704)-STAT3 antibodies demonstrated that the sequence of six occlusion/reperfusion cycles resulted in a marked increase in pTyr-STAT1 (+301 ± 61% above control; Fig. 3 *A* and *B*) and pTyr-STAT3 (+253 ± 60% above control; Fig. 3 *A* and *C*) at 30 min after the sixth reperfusion, indicating activation of these two transcription factors. Both of these changes were completely blocked by pretreatment with AG-490 (Fig. 3 *A*, *D*, and *E*), whereas DMSO alone had no effect (Fig. 3 *D* and *E*). The enhanced tyrosine phosphorylation of STAT1 and STAT3 30 min after the sixth reperfusion was associated with near-complete translocation of both transcription factors from the cytosol to the nucleus (Fig. 2). Interestingly, there was no increase in the nuclear content of pTyr-STAT1 and pTyr-STAT3 at 5 min after the sixth reperfusion (Fig. 3 *B* and *C*), a time when tyrosine phosphorylation of JAK1 and JAK2 was already evident (Fig. 1), indicating that the migration of pTyr-STAT1 and pTyr-STAT3 to the nucleus did not occur until after the activation of their upstream kinases, JAK1 and JAK2. The increase in tyrosine phosphorylation of nuclear STAT1 and STAT3 was transient, peaking at 30 min after

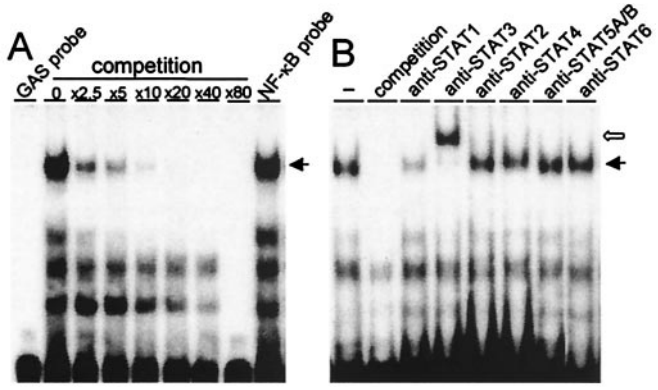

Fig. 4. Competition assay (*A*) and supershift assay (*B*) of STAT-GAS binding activity. Nuclear extracts were prepared 30 min after ischemic PC, and an EMSA was performed by using a ^{32}P-labeled GAS probe. (*A*) The competition assay was performed in the absence (lane 2) or presence of increasing amounts of unlabeled GAS consensus oligonucleotide (lane 3, 2.5-fold molar excess; lane 4, 5-fold; lane 5, 10-fold; lane 6, 20-fold; lane 7, 40-fold; lane 8, 80-fold), or a 100-fold molar excess of an unrelated NF-κB consensus oligonucleotide (lane 9). Lane 1 shows the reaction mixture alone without nuclear extracts. The specific band of the STAT-GAS complexes is indicated by the arrow. The GAS complex signal disappeared progressively with increasing folds of unlabeled GAS oligonucleotide (lanes 3–8) but was not affected by addition of the NF-κB oligonucleotide. (*B*) The supershift assay was performed in the presence of 1 μg of anti-STAT1 (lane 3), anti-STAT3 (lane 4), anti-STAT2 (lane 5), anti-STAT4 (lane 6), anti-STAT5A/5B (lane 7), or anti-STAT6 (lane 8) antibodies, in the presence of an 80-fold molar excess of unlabeled GAS oligonucleotide (lane 2), or in the absence of antibodies (lane 1). The specific STAT-GAS complex (solid arrow) was displaced or supershifted (open arrow) by anti-STAT1 and anti-STAT3 antibodies.

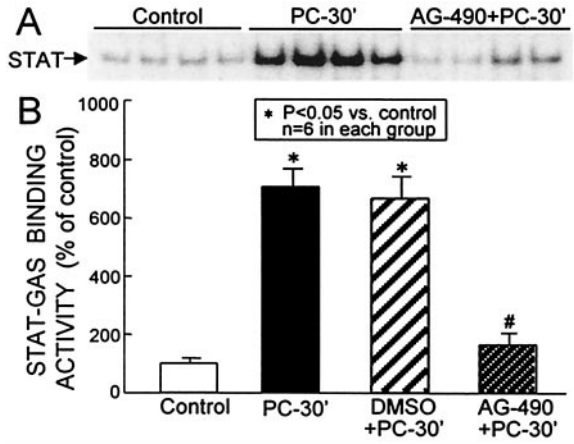

Fig. 5. Increased STAT-GAS binding activity 30 min after ischemic PC. Nuclear extracts were prepared as described in Fig. 3 and subjected to EMSA. (*A*) Representative EMSA showing that the shifted band corresponding to the STAT-GAS complex increased markedly 30 min after ischemic PC, and that this increase was ablated by AG-490 (the entire gel is shown in Fig. 16). (*B*) Densitometric analysis of the STAT-GAS complexes. Data are means ± SEM.

the sixth reperfusion and subsiding almost completely by 2 h (Fig. 3 *B* and *C*). Throughout this interval, the changes in tyrosine phosphorylation and subcellular distribution of STAT1 and STAT3 were of similar magnitude and exhibited a similar time course (Figs. 2 and 3). In contrast, no appreciable changes in either subcellular distribution or tyrosine phosphorylation of the five remaining STAT isoforms (STAT2, STAT4, STAT5A, STAT5B, STAT6) were observed at any time point from 5 min to 2 h after the sixth reperfusion (Figs. 14 and 15, which are published as supplemental data on the PNAS web site).

To determine whether the phosphorylated STAT1 and STAT3 bind to GAS motifs, tissue samples were analyzed by EMSA using a ^{32}P-labeled GAS oligonucleotide probe. As illustrated in Fig. 4*A*, a protein-GAS complex was observed in nuclear extracts from preconditioned tissue (lane 2) but not in the reaction medium alone (lane 1). Competition assays showed that the signal of the protein-GAS complex disappeared progressively when increasing fold (molar excess) (2.5–80 folds, lanes 2–8) of unlabeled GAS probe were added to the reaction mixture. In contrast, when a 100-fold molar excess of an unrelated oligonucleotide containing the consensus sequence for NF-κB was added to the reaction mixture, the protein-DNA complex signal was unaffected (lane 9). These results indicate that the signal is specific for the GAS motif. Supershift assays showed that the protein-GAS complex was diminished by the addition of anti-STAT1 antibodies and was supershifted by anti-STAT3 antibodies, but was not affected by anti-STAT2, STAT4, STAT5A, STAT5B, or STAT6 antibodies (Fig. 4*B*), indicating that both STAT1 and STAT3 (but not STAT2, STAT4, STAT5A, STAT5B, or STAT6) account for the augmented GAS-binding activity of nuclear extracts of preconditioned myocardium. As illustrated in Fig. 5, the STAT1/STAT3-GAS complex increased strikingly at 30 min after ischemic PC (+606 ± 64% above control); this change was suppressed by pretreatment with AG-490 but not by DMSO alone (the entire gel is shown in Fig. 16, which is published as supplemental data on the PNAS web site). Thus, the increased nuclear content of pTyr-STAT1 and pTyr-STAT3 in preconditioned myocardium (Fig. 3) was associated with a corresponding increase in STAT1 and STAT3 DNA-binding activity (Fig. 5).

Effect of AG-490 on NOS Protein Expression and Activity. The ischemic PC protocol resulted in augmented expression of iNOS protein 24 h later (+73 ± 15% above control in the cytosolic fraction; Fig. 6 *A* and *B*), whereas the expression of endothelial NOS was not altered (102 ± 5% of control in the entire homogenate). The increased iNOS protein content was associated with increased calcium-independent NOS (iNOS) activity (+377 ± 55% above control levels in the cytosolic fraction; Fig. 6*C*). cNOS (endothelial NOS and/or neuronal NOS) activity in the ischemic-

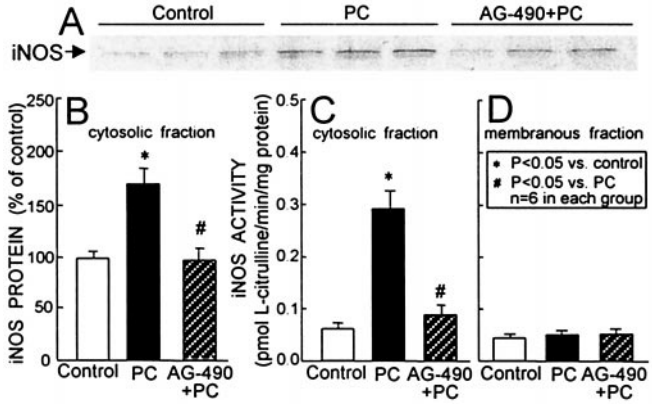

Fig. 6. Increased iNOS expression and activity after ischemic PC. Myocardial samples were obtained from mice that underwent a sham operation (control group) and from the ischemic-reperfused region of preconditioned mice that received either no treatment (PC group) or AG-490 20 min before ischemic PC (AG-490 + PC group). (*A*) Western blot showing that the immunoreactivity of iNOS in the cytosolic fraction increased 24 h after ischemic PC, and that this increase was ablated by AG-490. (*B*) Densitometric analysis of immunoreactive iNOS. (*C*) iNOS activity in the cytosolic fraction. (*D*) iNOS activity in the membranous fraction. iNOS protein expression in the AG-490 + PC group averaged 98 ± 12% of control in the cytosolic fraction (*B*) and 71 ± 16% of control in the membranous fraction. Data are means ± SEM.

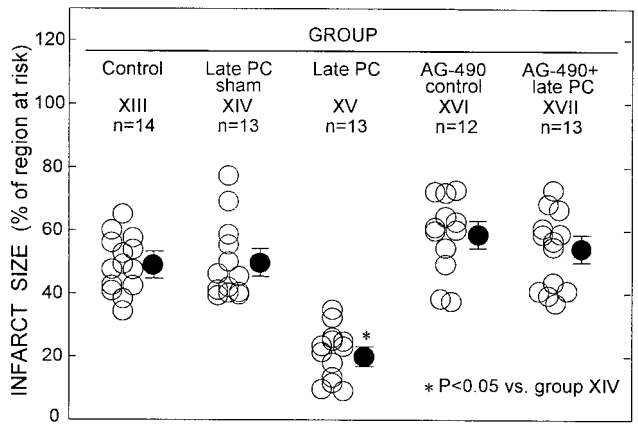

Fig. 7. Effect of AG-490 on myocardial infarct size. Infarct size is expressed as a percentage of the region at risk of infarction. ○ represent individual mice, whereas ● represent means ± SEM.

reperfused region did not change appreciably 24 h after ischemic PC, either in the cytosolic fraction (0.122 ± 0.009 pmol L-citrulline/min per mg protein in the control group and 0.131 ± 0.014 in the preconditioned group) or in the membranous fraction (0.239 ± 0.016 and 0.264 ± 0.026 pmol L-citrulline/min per mg protein, respectively). When mice were given AG-490 before the ischemic PC protocol on day 1, the increase in iNOS protein and activity on day 2 was completely abrogated (Fig. 6), indicating that activation of the JAK-STAT pathway by ischemic PC on day 1 is necessary for the up-regulation of iNOS 24 h later.

Effect of AG-490 on Infarct Size. Heart rate and rectal temperature were similar among groups XIII–XVII and within physiological limits (Table 2, which is published as supplemental data on the PNAS web site). There were no significant differences among groups with respect to body weight, left ventricular weight, or weight of the region at risk (Table 3, which is published as supplemental data on the PNAS web site). In control mice, infarct size averaged 49.1 ± 2.2% of the region at risk (Fig. 7). Infarct size was similar (49.4 ± 3.4%) in the late PC sham group (Fig. 7), indicating that a sternotomy with a 60-min period of open-chest state without coronary occlusion did not affect the extent of cell death induced by a 30-min coronary occlusion 24 h later. When mice were preconditioned with six coronary occlusion/reperfusion cycles on day 1 (late PC group), the size of the infarct induced by a 30-min coronary occlusion 24 h later (day 2) was reduced to 20.7 ± 2.3% of the risk region, indicating the development of a late PC effect. However, when preconditioned mice were given AG-490 20 min before the six occlusion/reperfusion cycles on day 1 (AG-490 + late PC group), infarct size was not reduced (56.2 ± 3.7% of the risk region; Fig. 7), indicating that the protective effect of late PC was completely abolished. AG-490 pretreatment had no effect in the absence of ischemic PC (AG-490 control group) (Fig. 7).

Discussion

The salient findings of this study can be summarized as follows: (*i*) in the intact mouse, brief episodes of myocardial ischemia (six cycles of 4-min occlusion/4-min reperfusion), which elicit late PC, induce rapid and isoform-selective activation of JAK1 and JAK2 (but not of JAK3 or TYK2) through tyrosine phosphorylation; (*ii*) JAK1 and JAK2 activation is followed by selective tyrosine phosphorylation of STAT1 and STAT3 (but not of STAT2, STAT4, STAT5A, STAT5B, or STAT6) and by their translocation into the nuclear compartment leading to increased STAT1 and STAT3 DNA-binding activity; (*iii*) pretreatment with the JAK inhibitor AG-490 before the six occlusion/reperfusion cycles blocks the enhanced tyrosine phosphorylation of JAK1 and JAK2 and the increased tyrosine phosphorylation and DNA-binding activity of STAT1 and STAT3 evoked by ischemic PC; and (*iv*) pretreatment with the same dose of AG-490 inhibits the ischemic PC-induced up-regulation of iNOS 24 h later and, at the same time, abrogates the protective effects of late PC against myocardial infarction. Taken together, these findings demonstrate that the JAK-STAT pathway plays an essential role in the development of late PC. The results reveal a signaling mechanism that underlies the transcriptional up-regulation of the *iNOS* gene and the attending protection 24 h after exposure of the heart to a sublethal ischemic stress.

The significance of the JAK-STAT pathway in myocardial ischemia remains obscure. A major obstacle in addressing this issue is the fact that the complete expression profile of these families of tyrosine kinases and transcription factors in the heart has not been characterized. Although JAK1, JAK2, STAT1, and STAT3 have been described in neonatal cardiomyocytes (18–22) and in adult myocardium (23, 24), it is not known whether the other isoforms (JAK3, TKY2, STAT2, STAT4, STAT5A, STAT5B, and STAT6) are also expressed in the heart. Moreover, no information is available regarding (*i*) whether these proteins are activated by ischemic PC and if so, which specific isoform(s), and (*ii*) whether their activation is necessary for the protection of late PC to occur. The present study demonstrates that the adult murine heart constitutively expresses three of the four known members of the JAK family (JAK1, JAK2, and TYK2; Figs. 10 and 11*B*) and all of the seven known members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6; Figs. 2 and 14). These findings lay the indispensable groundwork for future investigations of the role of JAK-STAT signaling in myocardial ischemia and also in cardiac pathophysiology in general. Importantly, the present study documents that brief episodes of ischemia/reperfusion produce rapid activation of the JAK-STAT pathway and identifies two members of the JAK family (JAK1 and JAK2; Fig. 1) and two members of the STAT family (STAT1 and STAT3; Figs. 2–4) as the specific isoforms that are recruited by ischemic PC *in vivo*. The high degree of selectivity in the activation of JAK1, JAK2, STAT1, and STAT3 implies a distinctive role of these specific proteins in the response of the heart to ischemic stress. To exclude the possibility that a transient activation of other JAKs and STATs may have been missed because of the choice of one arbitrary time point, we systematically examined the tyrosine phosphorylation and/or nuclear translocation of all JAK and STAT proteins (except JAK3, which is not expressed; Fig. 11*A*) at multiple time points. The results of this analysis confirmed that ischemia mobilizes only JAK1/2 and STAT1/3 in an isoform-selective manner (Figs. 1–3, 11*B*, 14, and 15). STAT1 and STAT3 are known to be closely related, i.e., they form heterodimers, are phosphorylated by similar stimuli, share a tyrosine phosphorylation site for JAKs, and exhibit similar DNA-binding specificities (26, 28, 29). Our finding that the magnitude and time course of STAT1 and STAT3 activation in preconditioned myocardium are virtually superimposable (Figs. 2 and 3) and that both factors contribute to the enhanced GAS-binding activity (Fig. 4) is consistent with these previous observations.

Having found that JAK1/JAK2 and STAT1/STAT3 are activated by ischemic PC, we next sought to identify the downstream targets of the JAK-STAT axis that underlie late PC. We focused on iNOS because the cardioprotective effects of late PC are known to be mediated in part by this protein (6, 9, 10, 13). The promoter of the mouse *iNOS* gene contains three copies of the GAS consensus sequence for the binding of STAT1 and STAT3 (28–30), and the interaction of STAT1 with GAS elements has been shown to be necessary for the expression of iNOS after exposure to cytokines or lipopolysaccharide in murine macrophages (31, 32). Accordingly, we hypothesized that STAT1 and/or STAT3 participate in late PC by modulating *iNOS* gene transcription. We found that inhibition of

JAK1 and JAK2 with AG-490 (Fig. 1) blocked not only the activation of STAT1 and STAT3 (Figs. 3 and 5) but also the subsequent up-regulation of iNOS protein expression and activity (Fig. 6) and the attending cardioprotection (Fig. 7). Because AG-490 is a general JAK inhibitor (22, 25, 33, 34), the present data do not enable us to discern the relative roles of JAK1 vs. JAK2 and STAT1 vs. STAT3. Nevertheless, these data identify the JAK-STAT pathway as an important signaling mechanism responsible for the transcriptional activation of the cardiac *iNOS* gene by ischemia. Because NF-κB also plays an essential role in iNOS induction during late PC (8), the present results imply that the expression of the *iNOS* gene in this setting requires the simultaneous combinatorial action of two families of transcription factors, NF-κB and STATs, a concept that is consonant with studies of *iNOS* promoter regulation in other systems (24, 30). Recruitment of NF-κB after ischemic PC results from protein kinase Cε–Src/Lck tyrosine kinase signaling (5, 8). Thus, we propose that a sublethal ischemic stress activates two parallel signaling pathways (protein kinase Cε–Srk/Lck–NF-κB and JAK1/2–STAT1/3), and that both of these pathways are necessary for the transcriptional activation of *iNOS* and the development of late PC.

The functional significance of the observed recruitment of the JAK-STAT pathway by ischemic PC was interrogated with the use of AG-490. AG-490 is a specific JAK inhibitor with an $IC_{50} = 10$ μM and a maximal effect at 50–100 μM (22, 25, 33, 34). The dose used in this study (40 μg/g) was selected because it is calculated to give tissue levels of ≈100 μM (25). Although AG-490 inhibits all JAK isoforms, it does not inhibit other tyrosine kinases including Lck, Lyn, Btk, Syk, and Src (25). Previous investigations have documented that AG-490 inhibits tyrosine phosphorylation-dependent activation of JAKs and subsequent phosphorylation of STAT proteins in leukemic cells, vascular smooth muscle cells, T cells, epithelial cells, and cardiac myocytes (22, 24, 25, 33, 34). Our results demonstrate that this dose of AG-490 effectively blocked the activation of the JAK-STAT axis by ischemic PC, as evidenced by the fact that pretreatment with AG-490 suppressed not only the tyrosine phosphorylation of JAK1 and JAK2 (Fig. 1) but also the tyrosine phosphorylation and nuclear translocation of STAT1 and STAT3 (Fig. 3) as well as their binding to the GAS motif (Fig. 5). Importantly, the same dose of AG-490 abrogated the infarct-sparing effects 24 h later (Fig. 7), demonstrating that the mobilization of the JAK-STAT pathway plays an essential role in the cardioprotection afforded by late PC.

No previous study has examined the JAK-STAT pathway in the setting of myocardial ischemia followed by reperfusion. Two recent investigations have reported activation of JAK1 (but not JAK2) (23) and STAT3 (but not STAT1) (23, 24) in rat models of infarction produced by permanent coronary ligation. Because of the fundamental differences between cell death resulting from prolonged ischemia without reperfusion and reversible injury resulting from brief transient coronary occlusions, a comparison of these results with the present data is not possible.

In conclusion, the present investigation identifies a signaling mechanism whereby the heart adapts to stress *in vivo*. The results reported herein demonstrate that ischemic PC induces highly selective activation of JAK1, JAK2, STAT1, and STAT3, and that ablation of this response impedes the up-regulation of iNOS and the acquisition of ischemic tolerance. Although the JAK-STAT axis was described several years ago (15–17), its function in cardiovascular homeostasis remains poorly understood. The notion that JAK1/2 and STAT1/3 play an obligatory role in the shift of the heart to a defensive (preconditioned) phenotype identifies an important task of this signaling pathway and has broad implications for many other pathophysiological conditions besides ischemia in which JAK and STAT proteins are known to be activated.

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1. Guo, Y., Wu, W. J., Qiu, Y., Tang, X. L., Yang, Z. Q. & Bolli, R. (1998) *Am. J. Physiol.* **257,** H1375–H1387.
2. Baxter, G. F., Goma, F. M. & Yellon, D. M. (1995) *Br. J. Pharmacol.* **115,** 222–224.
3. Ping, P., Zhang, J., Qiu, Y., Tang, X. L., Manchikalapudi, S., Cao, X. & Bolli, R. (1997) *Circ. Res.* **81,** 404–414.
4. Bolli, R., Bhatti, Z. A., Tang, X. L., Qiu, Y., Zhang, Q., Guo, Y. & Jadoon, A. R. (1997) *Circ. Res.* **81,** 42–52.
5. Bolli, R. (2000) *Circ. Res.* **87,** 972–983.
6. Bolli, R., Manchikalapudi, S., Tang, X. L., Takano, H., Qiu, Y., Guo, Y., Zhang, Q. & Jadoon, A. R. (1997) *Circ. Res.* **81,** 1094–1107.
7. Marber, M. S. & Yellon, D. M. (1996) *Ann. N.Y. Acad. Sci.* **793,** 123–141.
8. Xuan, Y. T., Tang, X. L., Banerjee, S., Takano, H., Li, R. C. X., Han, H., Qiu, Y. & Bolli, R. (1999) *Circ. Res.* **84,** 1095–1109.
9. Takano, H., Manchikalapudi, S., Tang, X. L., Qiu, Y., Rizvi, A., Jadoon, A. K., Zhang, Q. & Bolli, R. (1998) *Circulation* **98,** 441–449.
10. Xuan, Y. T., Tang, X. L., Qiu, Y., Banerjee, S., Takano, H., Han, H. & Bolli, R. (2000) *Am. J. Physiol.* **279,** H2360–H2371.
11. Dawn, B., Xuan, Y. T., Qiu, Y., Takano, H., Tang, X. L., Ping, P., Banerjee, S., Hill, M. & Bolli, R. (1999) *Circ. Res.* **85,** 1154–1163.
12. Imagawa, J., Baxter, G. F. & Yellon, D. M. (1997) *J. Mol. Cell. Cardiol.* **29,** 1885–1893.
13. Guo, Y., Jones, W. K., Xuan, Y. T., Tang, X. L., Bao, W., Wu, W. J., Han, H., Laubach. V. E., Ping, P., Yang, Z., *et al*. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11507–11512.
14. Ping, P., Zhang, J., Li, R. C. X., Dawn, B., Tang, X. L., Takano, H., Balafanova, Z. & Bolli, R. (1999) *Circ. Res.* **85,** 542–550.
15. Igarashi, K., Garotta, G., Ozmen, L., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Larner, A. C. & Finbloom, D. S. (1994) *J. Biol. Chem.* **269,** 14333–14336.
16. Darnell, J. E., Jr. (1997) *Science* **277,** 1630–1635.
17. Heim, M. H. (1999) *J. Recept. Signal Transduction Res.* **19,** 75–120.
18. Kunisada, K., Hirota, H., Fujio, Y., Matsui, H., Tani, Y., Yamauchi-Takihara, Y. & Kishimoto, T. (1996) *Circulation* **94,** 2626–2632.
19. Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J. H. & Chien, K. R. (1997) *J. Biol. Chem.* **272,** 5783–5791.
20. Stephanou, A., Brar, B. K., Scarabelli, T. M., Jonassen, A. K., Yellon, D. M., Marber, M. S., Knight, R. A. & Latchman, D. S. (2000) *J. Biol. Chem.* **275,** 10002–10008.
21. Fujio, Y., Kunisada. K., Hirota, H., Yamauchi-Takihara, K. & Kishimoto, T. (1997) *J. Clin. Invest.* **99,** 2898–2905.
22. McWhinney, C. D., Hunt, R. A., Conrad, K. M., Dostal, D. E. & Baker, K. M. (1997) *J. Mol. Cell. Cardiol.* **29,** 2513–2524.
23. Omura, T., Yoshiyama, M., Ishikura, F., Kobayashi, H., Takeuchi, K., Beppu, S. & Yoshikawa, J. (2001) *J Mol. Cell. Cardiol.* **33,** 307–316.
24. Negoro, S., Kunisada, K., Tone, E., Funamoto, M., Oh, H., Kishimoto, T. & Yamauchi-Takihara, K. (2000) *Cardiovasc. Res.* **47,** 797–805.
25. Meydan, N., Grunberger, T., Dadi, H., Shahar, M., Arpaia, E., Lapidot, Z., Leeder, J. S., Freedman, M., Gazit, A., Levitzkl, A. & Roifman, C. M. (1996) *Nature (London)* **379,** 645–648.
26. Decker, T.,Kovarik, P. & Meinke, A. (1997) *J. Interferon Cytokine Res.* **17,** 121–134.
27. Singh, K., Balligand, J., Fischer, T. A., Smith, T. W. & Kelly, R. A. (1996) *J. Biol. Chem.* **271,** 1111–1117.
28. Seidel, H. M., Milocco, L. H., Lamb, P., Darnell, J. E., Stein, R. B. & Rosen, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3041–3045.
29. Horvath, C. M., Wen, Z. & Darnell, J. E. (1995) *Genes Dev.* **9,** 984–994.
30. Xie, Q., Whisnant, R. & Nathan, C. (1993) *J. Exp. Med.* **177,** 1779–1784.
31. Gao, J., Morrison, D. C., Parmely, T. J., Russell, S. W. & Murphy, W. J. (1997) *J. Biol. Chem.* **272,** 1226–1230.
32. Meraz, M. A., White, J. M., Sheehan, K. C. F., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., *et al*. (1996) *Cell* **84,** 431–442.
33. Nielsen, M., Kaltoft, K., Nordahl, M., Ropke, C., Geisler, C., Mustelin, T., Dobson, P., Svejgaard, A. & Odum, N. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6764–6769.
34. Cavicchi, M. & Whittle, B. J. R. (1999) *Br. J. Pharmacol.* **128,** 705–715.