JAK/STAT Signaling Is Associated With Cardiac Dysfunction During Ischemia and Reperfusion

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Background—Activation of the heart renin-angiotensin system (RAS) under pathophysiological conditions has been correlated with the development of ischemic injury. The binding of angiotensin II to its receptors triggers induction of several, perhaps multifunctional, intracellular signaling pathways, notable among them the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. In this study, we investigated whether the JAK/STAT signaling is involved in the ischemia/reperfusion injury in adult rat myocardium.

Methods and Results—We report here that 2 components of the JAK/STAT signaling pathway, namely STAT 5A and STAT 6, are selectively activated in the rat heart subjected to ischemia/reperfusion. The activated STATs bind to a conserved nucleotide sequence (St domain) in the promoter of the angiotensinogen (ANG) gene and consequently upregulate the level of ANG mRNA. Treatment of the hearts with losartan (4.5 μmol/L), an AT1 blocker, or with tyrphostin AG490 (5 μmol/L), an inhibitor of JAK 2 phosphorylation, results in loss of the STAT/ANG promoter binding activity and an upregulated level of ANG mRNA. Hearts treated with the JAK 2 inhibitor tyrphostin AG490 showed a reduction in myocardial infarct size and in number of cardiomyocytes undergoing apoptosis. The treated hearts also showed a recovery in functional hemodynamics of the myocardium.

Conclusions—These findings suggest that activation of the JAK/STAT signaling pathway is a significant contributing factor to the pathogenesis of myocardial ischemia and that interference in activation of the pathway potentiates recovery in cardiac function. (Circulation. 2001;104:325-329.)

Key Words: ischemia ■ reperfusion ■ signal transduction ■ renin ■ angiotensin

It is well established that the tissue and systemic renin-angiotensin systems (RAS) play a major role in regulation of pathological cardiovascular functions, such as in hypertension, left ventricular hypertrophy, ischemic dilated cardiomyopathy, and heart failure. The binding of Ang II to its receptors (AT1 and/or AT2) triggers a variety of signal transduction pathways that are attributed to modulations in cardiovascular function. Several studies using isolated working heart as an in vivo model have documented the involvement of AT1 in the pathogenesis of ischemia/reperfusion injury and in the consequent myocardial dysfunctions. Administration of losartan, the AT1 receptor antagonist, causes functional recovery of the ischemic heart, suggesting that the signaling pathway mediated through the AT1 receptor is indeed involved in cardiovascular dysfunction. At the cellular level, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, among other second-messenger systems, is prominently associated with activation of the autocrine loop of the heart tissue–localized RAS. The JAK/STAT pathway, originally identified as a major signal transduction pathway of the cytokine superfamily, is known to be activated by several hypertrophic agonists, including Ang II. We have recently demonstrated that activation and translocation of 3 specific members of the STAT family, STAT 3, STAT 5A, and STAT 6, is mediated by Ang II, which results in activation of the autocrine RAS loop via transcriptional activation of the angiotensinogen (ANG) gene promoter in the hypertrophied myocardium. Whether JAK/STAT signaling is also involved in the onset of ischemic injury is not known at present.

We report here that when rat hearts are subjected to ischemia/reperfusion, there is a significant increase in the activated STAT 5A and STAT 6 binding to ANG promoter, with a concomitant increase in the ANG mRNA level. Treatment of the hearts with losartan resulted in loss of STAT protein interactions with ANG promoter DNA and consequently in loss of activated ANG mRNA levels. Likewise, inhibition of JAK 2, which phosphorylates STAT 5A and STAT 6, by tyrphostin AG490 caused reduction in STAT-ANG promoter interaction and in the level of ANG mRNA.
AG490 treatment also caused a marked reduction in the infarct size and in apoptotic cell death of cardiomyocytes, which is accompanied by an improvement in the hemodynamic performance of the heart. Taken together, these results provide the first evidence that JAK/STAT signaling plays a pivotal role in stimulation of the RAS in ischemia/reperfusion injury.

Methods

Isolated Perfused Heart Preparation
Hearts from adult male rats were randomly divided into 4 groups and subjected to ischemia/reperfusion as described earlier.18 In the ischemic group, hearts were perfused with Krebs-Henseleit buffer18 for 60 minutes, followed by 30 minutes of global ischemia. In the ischemic/reperfused group, hearts were perfused for 60 minutes, followed by 30 minutes of global ischemia and 120 minutes of reperfusion. Control group hearts were perfused for the same lengths of time. Losartan 4.5 μmol/L or tyrphostin AG490 in concentrations as indicated was administered during perfusion in Krebs-Henseleit buffer.

Primer Extension and RNase Protection Assay
A DNA primer spanning the complementary sequence of the rat ANG cDNA between nucleotides 302 and 279 (5'-AGGAGA-TGAAGGGGGTTGGAATGAT-3') was end-labeled and used to evaluate the expression of ANG mRNA in total RNA isolated from the rat heart. The primer extension protocol was according to instructions by the supplier (Promega). Rat GAPDH cDNA specific primer was used as control.

Measurement of Myocardial Infarct Size
Infarct size measurements were done as described earlier.19 On termination of treatment, hearts were immersed in 1% triphenyl tetrazolium solution in phosphate buffer (Na2 HPO 4 88 mmol/L, NaH2 PO 4 1.8 mmol/L) for 10 minutes at 37°C and then stored at −70°C for processing. Frozen hearts (ventricular tissue) were sliced transversely in a plane perpendicular to the apicobasal axis into sections ≈0.5 mm thick, blotted dry, placed between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single-pass flatbed scanner. With the NIH 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Risk (equivalent to total left ventricular muscle mass) and infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels. The weight of each slice was then recorded to facilitate the expression of total and infarct masses of each slice in grams. The risk and infarct volumes of each slice was then recorded to facilitate the expression of total and infarct volumes for the whole slice thickness. The risk volumes and infarct volumes of each slice in cubic centimeters were then calculated on the basis of time. Losartan 4.5 μmol/L or tyrphostin AG490 in concentrations as indicated was administered during perfusion in Krebs-Henseleit buffer.

Evaluation of Apoptosis
Immunohistochemical detection of apoptotic cells was carried out by use of terminal dUTP nick end-labeling (TUNEL), in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase II as described earlier.19,20 The cells were incubated with a sheep polyclonal anti-digoxigenin antibody followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody as described by the manufacturer (ApopTag Plus, Oncor Inc). The sections were washed in PBS 3 times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing cardiac myosin heavy chain (Biogenesis Ltd) followed by staining with TRIRC-conjugated rabbit anti-mouse IgG (200:1 dilution, Dako Japan). The fluorescence staining was viewed with a confocal laser microscope (Olympus Co).

The apoptotic cells were counted and expressed as percentage of total myocyte population.

Preparation of nuclear extracts, electrophoretic gel mobility shift assay (GMSA), and tyrosine phosphorylation of JAK 2 protein during ischemia/reperfusion were performed as previously described.18 The Western blot was probed with polyclonal antibody against JAK 2 from Santa Cruz, diluted 1:1000, and developed according to the chemiluminescence protocol (Amersham).

Statistics
Data were analyzed by 1-way ANOVA to determine the statistical significance of multiple treatments as described earlier.20 Values shown are mean±SEM. Significant levels were established at levels of P<0.05 and P<0.01.

Results

Upregulation of Rat Heart ANG mRNA During Ischemia/Reperfusion
It was reported earlier19 that ANG mRNA is upregulated in the postinfarction rat heart; hence, the increase is thought to be detrimental to cardiac function. We therefore tested the possibility that stimulation of the RAS, as reflected by an increase in ANG mRNA, also occurs in ischemic injury when isolated rat hearts are subjected to ischemia/reperfusion. We examined the level of ANG mRNA by primer extension assay using gene-specific DNA probes. As shown in Figure 1A, there was a marked increase in the mRNA level after 30 minutes of ischemia and 120 minutes of reperfusion. The increase in mRNA was sensitive to blockage of the AT1 receptor, because pretreatment with losartan reduced it almost entirely to the level of the control sample. The levels of the ribosomal marker L32 mRNA used as control remained unchanged. The experiment was performed in quadruplicate. Data in the left panel are for 1 representative sample.

STAT Activation During Ischemia/Reperfusion
We have previously established10 that the sequence element St domain in the ANG promoter becomes the target for binding the Ang II–activated STAT proteins and thereby links STAT activation to stimulation of the RAS in the hypertrophied myocardium. To examine whether the STAT binding activity is also enhanced in hearts subjected to global ischemia, the heart nuclear extracts were examined in the GMSA by use of the chemically synthesized oligonucleotide sequence of the St domain.10 As shown in Figure 1B, there was a strong St-domain/STAT binding activity in the hearts subjected to 30-minute ischemia/120-minute reperfusion, which was almost entirely abolished in losartan-treated heart, suggesting that losartan treatment during perfusion resulted in loss of the activated STAT participation in complex formation. We10 and others21 have previously demonstrated that activation of STATs and the consequent binding to the St domain in the ANG promoter accounts for the increase in transcription of ANG mRNA. Thus, the loss of STAT/DNA interaction and the reduction in the ANG mRNA levels (see Figure 1A) due to losartan treatment appear to be correlative responses. To identify the STAT proteins that were activated in the ischemic hearts, nuclear extracts were preincubated with polyclonal antibodies against STAT 1, STAT 3, STAT 5A, and STAT 6 and examined by GMSA. The results in Figure 1C show that STAT 5A and STAT 6 DNA complexes
were prominently disrupted by antibodies against STAT 5A and STAT 6.

Effect of JAK 2 Inhibition on STAT/DNA Binding and ANG mRNA

It is well established that both STAT 5A and STAT 6 are activated by JAK 2 via tyrosine phosphorylation. We therefore tested whether inhibition of the upstream JAK 2 by tyrphostin AG490, a potent and selective inhibitor of JAK 2 phosphorylation at 5 μmol/L, would interfere with activation of STATs and thereby with their binding to the St domain. First, we demonstrated that administration of AG490 in perfusion medium indeed was inhibitory at both 5 and 50 μmol/L for phosphorylation of JAK 2, which was activated readily in the ischemic heart in absence of the inhibitor (Figure 2A). When extracts from the same hearts were examined by GMSA for DNA binding as above, there was a total loss of STAT/DNA complex formation in the inhibitor-treated hearts (Figure 2B). Data in Figure 2C show that AG490 treatment also inhibited the stimulation of the ANG mRNA level that was observed in the ischemic tissues in absence of the inhibitor. These results therefore strongly suggest that activation of the JAK/STAT pathway, increases in the STAT/ANG promoter binding activity, and the upregulation of ANG mRNA all are causally related.

Cardioprotection by Inhibition of JAK 2

Finally, we examined whether inhibition of JAK 2 activity as above would afford protection against ischemia-induced changes in myocardial performance. Using spontaneously beating working hearts that were not paced, we observed that the absolute values and the first derivative of developed pressure were progressively decreased with reperfusion, as expected (Figure 3A). But the inhibitor at both concentrations was able to provide cardioprotection to approximately the same degree. This was particularly true during the first 60 minutes of reperfusion, when the dP/dt value was not lowered, and developed pressure was minimally lowered, in the treated groups. The baseline value for dP/dt increased slightly in high (50 μmol/L) concentrations of AG490. It also

Figure 1. Upregulation of ANG mRNA during ischemia/reperfusion (I/R) is mediated by STATs. A, Primer extension assay was performed with total RNA isolated from rat heart subjected to I/R as described in Methods. C indicates controls; I/R, 30 minutes ischemia and 120 minutes reperfusion; and L, with losartan. L32 mRNA levels determined by RNase protection assay. B, Nuclear extracts prepared from control tissues (C) and tissues subjected to I/R without losartan or with losartan (L) were subjected to GMSA using radiolabeled St-domain sequence of ANG promoter. Right, Averages of 4 independent experiments. Statistical analysis is shown as mean ± SEM (*P < 0.05 vs control). C, Nuclear extracts of hearts subjected to I/R as above were preincubated with polyclonal antibodies against STAT proteins followed by incubation with radiolabeled St-domain probe and examined by GMSA as above. Figure represents 1 of 3 independent experiments, all of which showed a comparable loss in STAT 5A and 6/DNA complex formation.

Figure 2. JAK 2 inhibition by tyrphostin AG490 during ischemia/reperfusion (I/R). A, Total extracts from hearts subjected to I/R in presence or absence of tyrphostin AG490 were immunoprecipitated with anti-phosphotyrosine antibodies (4G10) and tested against polyclonal anti-JAK 2 as described earlier. B, Nuclear extracts from hearts subjected to I/R in presence or absence of tyrphostin AG490 were analyzed by GMSA with radiolabeled St domain. Loss in DNA-protein complex is observed in nuclear extracts from I/R hearts pretreated with AG490. C, Primer extension was performed with an oligomer specific for ANG mRNA as described in Methods with total RNA isolated from rat hearts subjected to I/R as above. Right, Average of 4 experiments and statistical data analysis as in Figure 1 is shown as mean ± SEM.
ished in the ANG mRNA level. These effects were mimicked by AG490. In addition, the inhibitor AG490 at 5 \( \mu \)mol/L, a concentration at which AG490 is selective for JAK 2 inhibition, \( ^* \) caused recovery in cardiac performance, suggesting that JAK 2 activation is involved in ischemia-induced dysfunction of the heart.

Although the cardioprotective effect of losartan is well documented, \(^1,7\)–\(^9\) the precise mechanism by which JAK 2 activation influences myocardial performance is yet to be demonstrated. Activated RAS is known to facilitate apoptotic induction in cardiomyocytes. \(^22,23\) It appears, therefore, that the signaling pathways that include elements affecting cardiomyocyte cell death somehow converge on the JAK/STAT pathway programmed to activation of the RAS. It is thus likely that the JAK/STAT pathway plays a critical role in ischemia-induced apoptosis in cardiomyocytes. The cardioprotective effect of tyrphostin AG490 is perhaps achieved via modulation of intracellular Ang II mediated through JAK 2 signaling. Further studies are needed to delineate the precise mechanism(s) by which the STAT-mediated signaling interdigitates with the apoptotic pathway in ischemic heart.

References


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