Regulation of CCAAT/Enhancer Binding Protein, Interleukin-6, Interleukin-6 Receptor, and gp130 Expression During Myocardial Ischemia/Reperfusion

Bysani Chandrasekar, PhD; Donald H. Mitchell, BS; James T. Colston, PhD; Gregory L. Freeman, MD

Background—Interleukin (IL)-6 is elevated in myocardium after ischemia and reperfusion. The IL-6 promoter/enhancer region contains response elements for nuclear factor-κB, AP-1, and CCAAT/enhancer binding protein (C/EBP). Expression and regulation of C/EBP in rat myocardium after ischemia and reperfusion has not been defined, nor has the behavior of the specific IL-6 receptor (IL-6R) or the signal transducer gp130.

Methods and Results—C/EBP DNA binding activity was not detected in shams or in previously ischemic tissue at 15 minutes of reperfusion; it was detected at 30 minutes of reperfusion, increased at 1 hour of reperfusion, and declined by 6 hours of reperfusion. A supershift was observed with anti–C/EBP-β but not with anti-α or anti-δ antibodies. mRNA and protein levels of IL-6 and gp130 were detected at low levels in controls, increased at 1 hour of reperfusion, and remained high until 6 hours of reperfusion. IL-6R mRNA and protein were not detected in controls, but its mRNA was induced at 1 hour of reperfusion and its protein at 2 hours of reperfusion. Although effects of reperfusion were rapid, in ischemic tissue not reperfused, low levels of C/EBP were detected at 4 hours, and at 24 hours the levels were slightly elevated. Significant upregulation in IL-6, IL-6R, and gp130 occurred only at 24 hours of sustained ischemia.

Conclusions—Reperfusion after a brief period of ischemia caused induction of myocardial C/EBP (β-subunit). The rapid and sustained production of IL-6 with concomitant expression of IL-6 receptor and gp130 suggest that these factors may participate in a local inflammatory cascade after myocardial ischemia and reperfusion. (Circulation. 1999;99:427-433.)

Key Words: myocardial ischemia ■ interleukins ■ receptors ■ reperfusion

Interleukin (IL)-6 is a pleiotropic cytokine with varying effects on cells of the immune system and other tissues. Recently, cardiac myocytes have been shown to produce IL-6. Yamauchi-Takihara et al found induction of IL-6 in isolated cardiomyocytes exposed to hypoxia followed by reoxygenation. Kukielka et al showed induction of IL-6 in ischemic myocardium of dogs, but not in adjacent nonischemic tissue. We have confirmed these observations on the myocardium, showing increased IL-6 mRNA and protein levels in tissues taken from rat hearts after in vivo ischemia and reperfusion. We found that IL-6 levels were upregulated 1 hour after reperfusion, and that unlike tumor necrosis factor (TNF)-α and IL-1β, which showed a return to low levels by 3 hours of reperfusion, the levels of IL-6 mRNA were elevated in a sustained manner for 6 hours after reperfusion.

IL-6 exerts its biological activities through binding to an 80-kDa ligand binding subunit, IL-6 receptor (IL-6R). On binding to IL-6R, IL-6 induces interaction and homodimerization of gp130. This leads to the activation of a cytoplasmic tyrosine kinase, phosphorylation of gp130, and subsequent transduction of intracellular signals. Given the upregulation of IL-6 in postischemic myocardium, the behavior of other aspects of its signaling cascade is of interest. No reports are available demonstrating expression and changes in IL-6R and gp130 during myocardial ischemia and reperfusion.

The promoter/enhancer region of IL-6 contains response elements for nuclear factor (NF)-κB, AP-1, and CCAAT/enhancer binding protein (C/EBP). We have reported induction and biphasic regulation of NF-κB and monophasic regulation of AP-1 during ischemia and reperfusion. However, patterns of expression and role of C/EBP in myocardium, especially during ischemia and reperfusion, have not been described. Given the observation that IL-6 mRNA and protein expression follow a pattern that differs from other proinflammatory cytokines after myocardial ischemia and reperfusion, its expression may be uniquely controlled. As...
such, alterations in the pattern of expression of C/EBP are of interest. In the present study, we analyzed myocardium for mRNA and protein levels of IL-6, IL-6R, and gp130 and for C/EBP and C/EBP subunit levels during reperfusion after a single episode of sublethal ischemia.

**Methods**

**Experimental Animals and Induction of Ischemia With or Without Reperfusion**

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85 to 23, revised 1985). Wistar-Kyoto rats weighing ~200 g were used for these studies (a total of 104 rats). Preparation of animals, induction of ischemia and reperfusion, and tissue collection were carried out as described earlier.³ Reperfusion was initiated after 15 minutes of ischemia for 15 minutes, 30 minutes, and 1, 2, 3, and 6 hours (n=8/group). Sham-operated animals euthanized at 15 minutes and 3 and 6 hours after surgery (n=8/group) were used as controls. Four animals selected at random were used for mRNA, protein analyses, and immunocytochemistry, and the other 4 were used for electrophoretic mobility shift assay (EMSA) and supershift assay.¹³

In a separate group of animals (n=32), ischemia alone (without reperfusion) was induced by ligating the left anterior descending coronary artery for 15 minutes and 1, 4, or 24 hours (n=8/group). After the ischemic period, the heart was excised and processed as described above. Four animals selected at random were used for mRNA and protein analyses, and the other 4 were used for EMSA.

**Electrophoretic Mobility Shift Assay**

All steps were carried out at 4°C unless otherwise noted. Nuclei from frozen myocardium were prepared as described by Muller et al.⁴ The ischemic and sham-operated portions of myocardium adjacent to the ischemic zone (4 hearts [~900 mg], pooled at each time period) were pulverized in the presence of liquid nitrogen. They were homogenized in lysis buffer (10 mmol/L HEPES, pH 7.4, 5 mmol/L KCl, 10 mmol/L MgCl₂, 5 mmol/L 2-mercaptoethanol) containing 0.32 mol/L sucrose and 0.1% Triton X-100. The homogenate was vacuum-filtered through a nylon filter (200 μm mesh) and centrifuged at 2000g for 10 minutes. Pellets were resuspended in lysis buffer containing 2.2 mol/L sucrose without detergent and centrifuged for 90 minutes at 100 000g. To verify and estimate the yield of nuclei, DNA content of the pellet was determined spectrophotometrically at 260 nm.

Proteins were extracted for 45 minutes from the nuclei as described by Dignam et al.¹⁰ Protein concentration was determined by BCA protein assay reagent (Pierce). The EMSA was performed as described by Dent and Latchman,¹¹ with some modifications.³ Consensus double-stranded oligonucleotides (5’-TGC AGA TTG CGC AAT CTG CA-3’, Santa Cruz Biotechnology, Inc) containing the binding site for C/EBP (TTGCGCAA) was added to the reaction mixture for 20 minutes, followed by the addition of labeled consensus probe. Binding reactions, separation of free DNA and DNA-protein complexes, autoradiography, and densitometry were carried out as described earlier.³

In the gel supershift assay, the nuclear protein extract (20 μg) was preincubated for 40 minutes on ice with either anti-α, anti-β, or anti-δ polyclonal antibodies (0.2 μg) before the addition of ³²P-labeled C/EBP consensus oligo. Subunit-specific rabbit anti-rat antibodies were from Santa Cruz Biotechnology, Inc.

**Total RNA Isolation and Northern Blot Analysis**

Total RNA isolation, Northern blotting, autoradiography, and densitometry were carried out as described earlier.¹²-¹³ The following probes were used: IL-6 (American Type Culture Collection, Rockville, Md; IL-6R, and gp130 (a kind gift from Dr G.M. Fuller, Professor, Department of Cell Biology and Anatomy, The University of Alabama at Birmingham). 28S rRNA probe (Oncogene Science, Inc, Uniondale, NY) was used as internal control.

**Protein Extraction and Western Blot Analysis**

Extraction of protein homogenates, detection of IL-6, IL-6R, and gp130, and densitometry were carried out as described.¹²,¹³ These antibodies were used: anti-rat (IL-6 [R-19]) or anti-mouse (IL-6R [M-20], gp130 [M-20]; cross-reactive with rat). Antibodies were from Santa Cruz Biotechnology, Inc (affinity-purified polyclonal antibodies).

**Immunohistochemistry**

At 6 hours of reperfusion, the left ventricle was divided into ischemic and nonischemic zones, embedded in OCT, snap-frozen in liquid nitrogen, and stored for not more than 3 days at −82°C. Cryosections of 6-μm thickness were prepared for immunostaining with the use of an immunoenzymatic staining kit (For IL-6, Universal DAKO PAP kit, K0 549; for IL-6R and gp130, DAKO LSAB2 kit, peroxidase for use on rat specimen, K0 609; DAKO).¹³,¹⁶ Omission of primary antibody, rabbit/goat preimmune serum in place of primary antibody, and primary antibody after neutralization with its peptide antigen served as controls. Immunohistochemistry staining was evaluated by light microscopy. Specific staining was graded on a semiquantitative scale from 0 to 3 (0=none, 1=weak, 2=intermediate, 3=strong).

**Statistical Analysis**

Comparisons between control (15 minutes sham-operated) and each of the 7 reperfusion time periods and between 15 minutes of ischemia and 1-, 4-, and 24-hour ischemic periods were performed for measures of mRNA and protein for IL-6, IL-6R, and gp130 by ANOVA with post hoc Dunnett’s t tests. F tests and Dunnett’s t tests with values of P<0.05 were considered statistically significant. Since IL-6R mRNA and protein values were undetectable for control and durations shorter than 1 hour of reperfusion for mRNA and 2 hours of reperfusion for protein, 1-sample Student’s t tests were performed. Similarly for immunohistochemistry, because no positive immunoreactivity was detected for IL-6 and IL-6R, significance was obtained by 1-sample Student’s t tests, and for gp130, ANOVA was used with post hoc Dunnett’s t tests.

**Results**

C/EBP and C/EBP Subunit Levels

Figure 1 shows C/EBP levels in the ischemic (no reperfusion), postischemic, and control (sham-operated) myocardium. At baseline, no specific C/EBP DNA binding activity was detected in the nuclear protein extracts from sham-operated hearts (at 15 minutes and 3 and 6 hours after surgery; Figure 1, lanes 22 to 24) or nonischemic regions adjacent to the ischemic zone (data not shown). In postischemic tissue, C/EBP was not detected at 15 minutes of reperfusion. It was, however, readily detected at 30 minutes of reperfusion. Its levels increased, being maximum at 2 hours of reperfusion, then declined by 6 hours of reperfusion (Figure 1, lanes 11 to 16). The specificity of the signal was verified by preincubating the nuclear protein extract with a 100-fold molar excess of cold consensus oligo (lane 5), which abolished the signal. There was also no interference in the signal obtained from labeled consensus oligo when the extract was preincubated with a 100-fold molar excess of cold mutant oligo (lane 4). Furthermore, no signal was obtained...
when the reaction mixture did not contain protein extract (lane 2) or when labeled mutant oligo was used in place of labeled consensus oligo (lane 3).

To define which subunit or subunits participated in the elevated C/EBP levels, we analyzed the nuclear protein extract at the time it peaked (2 hours of reperfusion) for C/EBP subunit levels by gel supershift assay with the use of subunit-specific polyclonal antibodies. As seen in Figure 1 (lanes 25 to 27), no further shift was observed when nuclear protein extracts were preincubated with anti-α and anti-δ antibodies before the addition of labeled consensus C/EBP oligo. However, supershift was observed when anti-β antibodies were used, indicating that the β-subunit was the moiety elevated at this time.

### IL-6, IL-6R, and gp130 Levels

Figure 2 shows total RNA from control and postischemic myocardium by Northern blotting. Confirming our earlier observations, low level expression of a single transcript (~1.4 kb) of IL-6 was detected in control myocardium at 15 minutes. It remained at this level in reperfused myocardium until 30 minutes of reperfusion; its level rose at 1 hour of reperfusion and remained high until 6 hours of reperfusion. Densitometric analysis of the autoradiographic bands (Figure 2) revealed that compared with sham-operated controls, the levels were significantly higher at 1 hour of reperfusion (1.66-fold; *P*<0.0001; Figure 3). The levels rose further by 2 hours of reperfusion (2.78-fold, *P*<0.005), increased further by 3 hours (3.19-fold; *P*<0.0001), and declined at 6 hours of...
reperfusion (3.01-fold; \( P < 0.0001 \)). IL-6R was not detected under steady-state conditions in control myocardium, nor was it present in postischemic myocardium until 30 minutes of reperfusion (Figures 2 and 3). However, it was readily detected at 1 hour of reperfusion (0.31 \pm 0.012; \( P < 0.01 \)), and its levels increased by 2 hours of reperfusion (0.46 \pm 0.026) and remained high until 6 hours of reperfusion (3 hours of reperfusion, 0.50 \pm 0.015; 6 hours, 0.53 \pm 0.23; all \( P < 0.0001 \)).

Similar to IL-6, gp130 mRNA (a single 5.1 kb transcript) was detected at low levels in control and postischemic myocardium. It remained at a low level until 30 minutes of reperfusion (Figures 2 and 3), rose by 1 hour of reperfusion (2.23-fold, \( P < 0.0001 \)), and remained elevated until 6 hours of reperfusion (2 hours of reperfusion, 1.92-fold; 3 hours, 1.99-fold; 6 hours, 2.17-fold; all \( P < 0.0001 \)).

As a separate control, we tested both sham-operated and postischemic myocardium for the presence of IL-1 \( \alpha \) mRNA. Results from these experiments confirmed our earlier observations that no signal was obtained in postischemic tissue, even after 8 days of autoradiographic exposure (data not shown).

Immunoblots for all 3 proteins are shown in Figure 4, with associated semiquantitation in Figure 5. Following the pattern of mRNA, both IL-6 and gp130 proteins were detected in low levels in controls. The levels remained low until 30 minutes of reperfusion, then increased gradually. IL-6 levels were significantly elevated at 1 hour of reperfusion (2.31-fold; \( P < 0.0001 \)) and remained elevated until 6 hours of reperfusion (2 hours of reperfusion, 1.92-fold; 3 hours, 1.99-fold; 6 hours, 2.17-fold; all \( P < 0.0001 \)).

Levels of gp130 were increased by 2.64-fold at 1 hour of reperfusion (\( P < 0.0001 \)), increased further at 2 hours of reperfusion (3.37-fold, \( P < 0.0001 \)), and remained elevated throughout the 6-hour study period (3 hours of reperfusion, 2.67-fold, \( P < 0.0005 \); 6 hours, 3.23-fold; \( P < 0.0001 \)). In contrast to IL-6 and gp130, IL-6R protein was not detected in controls at steady state nor in postischemic myocardium up to 1 hour of reperfusion. However, its levels were readily detected at 2 hours of reperfusion (169 \pm 11.2; \( P < 0.0001 \)) and progressively increased until 6 hours of reperfusion (3 hours of reperfusion, 192 \pm 7.5; 6 hours, 196 \pm 5.71; all \( P < 0.0001 \); Figures 4 and 5).

**Immunohistochemistry**

Immunohistochemical staining for IL-6, IL-6R, and gp130 was carried out in myocardium from sham-operated animals, in normal tissue adjacent to ischemic zone, and in postischemic myocardium at 6 hours of reperfusion (Figure 6). We chose this time period because high levels of IL-6, IL-6R, and gp130 protein were detected by Western blotting (Figure 4 and 5). No immunoreactivity for IL-6 was observed in shams (Figure 6A) or in normal tissue adjacent to the ischemic zone (Figure 6B). However, in postischemic myocardium, IL-6 immunoreactivity was readily detected, localized to the cytoplasm, and in a diffuse manner (control versus 6 hours of reperfusion; \( P < 0.0001 \); Figure 6C). Similar to IL-6, IL-6R immunoreactivity was detected neither in shams nor in normal tissue adjacent to ischemic zone (data not shown). However, IL-6R immunoreactivity was detected in a granular fashion in postischemic myocardium, localized to both membranous and cytoplasmic compartments (control versus 6

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**Figure 3.** Densitometric analysis of autoradiographic bands shown in Figure 2. Values are represented as a ratio of specific gene to that of 28S rRNA. *\( P < 0.05 \), †\( P < 0.0001 \) (vs control).

**Figure 4.** Western blot analysis of protein homogenates isolated from control (sham-operated) and previously ischemic myocardium. Arrow indicates relative position of protein detected by the respective antibody.
hours of reperfusion; \( P<0.001 \). Though Western blotting revealed low levels of gp130 in sham-operated animals (Figure 4), immunohistochemistry revealed a very weak staining for gp130 (data not shown) in these tissues. However, gp130 immunoreactivity was detected in postischemic myocardium, localized to the cytoplasm as well as to the perinuclear region (4.6-fold; \( P<0.0001 \)), confirming previous results from a study in which we found a similar pattern of gp130 immunoreactivity in myocardium from animals infected with *Trypanosoma cruzi*.\(^{17}\) Omitting primary antibody and replacing it with respective preimmune sera abolished the signals (data not shown). Also, no immunoreactivity was detected when primary antibodies were neutralized with their respective peptide antigens. As an example, in Figure 4D, no IL-6 immunoreactivity was detected when anti–IL-6 antibodies were used after neutralization with their peptide antigen.

C/EBP, IL-6, IL-6R, and gp130 Levels After Sustained Ischemia Without Reperfusion

To determine the impact of ischemia alone on induction of C/EBP, IL-6, IL-6R, and gp130, we studied tissue after coronary ligation was sustained for 15 minutes, 1, 4, or 24 hours, and tissue was harvested without reperfusion. Figure 1 (lanes 6 to 9) shows C/EBP levels by EMSA in nuclear protein extracts taken from myocardium in the ischemic zone. C/EBP DNA binding activity was not detected at 15 minutes or 1 hour of ischemia. However, at 4 hours a weak signal was detected, and by 24 hours the levels increased modestly. These levels were considerably lower than the levels detected in previously ischemic tissue after reperfusion (Figure 1, lanes 12 to 16). Figures 7 and 8 show mRNA and protein levels of IL-6, IL-6R, and gp130 in ischemic myocardium. Northern blots revealed low levels of IL-6 mRNA at 15 minutes and 1 and 4 hours of ischemia, but significantly higher levels were detected at 24 hours (Figures 7 and 8; densitometry; 15 minutes versus 24 hours; IL-6, 3.79 fold, \( P<0.0001 \)). Its protein levels followed a similar trend (15 minutes versus 24 hours; 2.01-fold, \( P<0.0001 \)). Similar to IL-6, gp130 mRNA was detected at low levels at 15 minutes (0.19±0.011) and 1 hour (0.19±0.01) of ischemia, rose moderately at 4 hours (1.36-fold; \( P<0.005 \)), and increased further at 24 hours (2.95-fold; \( P<0.005 \)). However, its protein levels (Figures 7B and 8B) were found elevated only at 24 hours of ischemia (2.33-fold; \( P<0.0001 \)). In contrast to IL-6 and gp130, IL-6R mRNA and protein levels were undetectable at 15 minutes and 1 and 4 hours. They were detected only after 24 hours of ischemia (mRNA and protein, 15 minutes versus 24 hours, \( P<0.0001 \)).
Discussion

Our results show for the first time that in myocardial tissue, reperfusion after brief sublethal ischemia leads to increased nuclear C/EBP levels, with a predominant impact on the β-subunit. Although IL-6R was not present under baseline conditions, and only very low levels of gp130 were found, both were readily detected in the postischemic tissue. Furthermore, as we and others have previously shown, both mRNA and protein levels of IL-6 were upregulated in postischemic tissue. Thus, there appears to be coordinated expression of IL-6 and other factors that participate in its activity after ischemia and reperfusion in the myocardium.

A key observation in the present study is the detection of increased levels of C/EBP in the nuclear extracts. Specific C/EBP-DNA protein complexes were not detected under control conditions and were not found in reperfused myocardium until 30 minutes of reperfusion. Furthermore, a supershift was observed when the protein extracts were preincubated with anti-C/EBP-β but not with α- or δ-antibodies, suggesting that C/EBP-β may play an important role in postischemic myocardium. C/EBP is an inducible transcription factor, which is also known as IL-6 DNA binding protein (IL6DBP), liver-enriched transcriptional activator protein (LAP), and nuclear factor-IL-6. Whereas some C/EBP proteins are expressed constitutively and their levels are altered on cell differentiation, others, such as C/EBP-β and C/EBP-δ, are inducible in response to inflammatory mediators. The best-characterized inducible C/EBP protein is C/EBP-β. In cultured endothelial cells, during hypoxic conditions, induction of IL-6 was observed through activation of C/EBP-β. In addition to induction of IL-6, C/EBP has been shown to participate in the induction of other proinflammatory cytokines, including IL-1, as well as participating in TNF-α–induced IL-1β induction. This factor binds to the transcriptional regulatory regions in TNF-α, IL-8, and G-CSF, suggesting a role for C/EBP in inflammation and the acute phase response.

In addition to C/EBP, the IL-6 promoter region also contains binding sites for AP-1 and NF-κB, transcription factors regulated by the redox status of the cell. All of these sites have been shown to be essential for the induction of IL-6. We have previously shown increased NF-κB and AP-1 levels in postischemic reperfused myocardium. We observed low and consistent levels of NF-κB p50 and increased levels of p65 in reperfused myocardium. We speculate that the mechanism underlying the fact that the appearance of IL-6 follows a different pattern of expression than either TNF-α or IL-1β may be the more prominent role of C/EBP in transcriptional regulation of IL-6.

Another important observation in the present study is the induction of IL-6R and upregulation of gp130 in reperfused myocardium. Whereas IL-6R was not detected under basal conditions, it was induced at 1 hour of reperfusion and remained at significantly
higher levels throughout the 6-hour study period. On the other hand, both mRNA and protein levels of gp130 were detected at low levels under basal conditions, were significantly elevated at 1 hour of reperfusion, and remained elevated up to 6 hours of reperfusion. Whereas IL-6 exerts its biological effects through binding to IL-6R, IL-6Rb by itself cannot transduce signals intracellularly. This requires dimerization of gp130, which activates a cytoplasmic tyrosine kinase5,20 and subsequent transduction of intracellular signals. Proinflammatory cytokines such as IL-1ß and TNF-α have also been shown to upregulate gp130 in amnion (UAC) and hepatoma (Hep3B) cell lines.21 Our data show a concurrent expression of IL-6 and its receptor system, which probably are primary events not induced by cytokines but rather a direct result of the ischemia and reperfusion process.

To evaluate the importance of reperfusion on the patterns of gene expression, we studied groups of animals with persistent ischemia and no reperfusion. Prior studies on how sustained ischemia affects expression of IL-6 have been inconsistent. Herskowitz et al22 showed that IL-6 mRNA levels were increased after 24 hours of ischemia and reperfusion, whereas IL-6 was not expressed by cardiac myocytes during ischemia and no reperfusion. Prior studies on how sustained ischemia affects expression of IL-6 have been inconsistent. Herskowitz et al22 showed that IL-6 mRNA levels were increased in hearts of rats after 1 and 3 hours of ischemia but not after 24 hours. On the other hand, Kukielka et al2 that IL-6 is not expressed by tissue rendered ischemic in the absence of reperfusion at early time points, appearing only after 24 hours. In addition to IL-6, we also studied changes in C/EBP levels and expression of IL-6R and gp130 in ischemic tissue. C/EBP DNA binding activity was not detected either at 15 minutes or 1 hour of ischemia, but it was present if ischemia persisted for 24 hours. Our results support the observation of Kukielka et al2 that IL-6 is not expressed by tissue rendered ischemic in the absence of reperfusion at early time points, appearing only after 24 hours. Furthermore, both mRNA and protein levels of IL-6R and gp130 were only increased after 24 hours of ischemia. The levels were low compared with their expression in reperfused postischemic tissue, and overall our results suggest that changes in IL-6 and its receptors can occur very rapidly and that they are more pronounced after reperfusion than after ischemia alone. Although we did not study reperfusion after periods of ischemia longer than 15 minutes, both Herskowitz et al21 and Kukielka et al22 showed that after ischemic durations sufficient to cause cell death (35 minutes or 1 hour, respectively), IL-6 was expressed in reperfused myocardium. Under those conditions, the picture is more complex because cell death by necrosis or apoptosis is taking place. Whether cytokine expression is mediated by myocardium or by inflammatory cells, either in the early stages or at 24 hours, is not known and will require further study.

In conclusion, the present study demonstrates for the first time that reperfusion after a brief period of myocardial ischemia leads to the upregulation of C/EBP as well as IL-6, IL-6R, and gp130. This is a unique and temporally synchronized tissue response, consistent with the expression of other proinflammatory cytokines under these conditions. The fact that all of the components of the signaling cascade are induced in a coordinated fashion strongly suggests that there is a defined role for this protein in the postischemic heart. Full delineation of this role will require further studies.

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