Functional Significance of Hemodynamic Overload–Induced Expression of Leukemia-Inhibitory Factor in the Adult Mammalian Heart

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Background—Leukemia-inhibitory factor (LIF) is a member of the interleukin-6 family of cytokines that utilize gp130 as a common signaling component. In the present study, we examined the mechanisms that govern LIF expression and functional effects in the adult heart.

Methods and Results—LIF mRNA and protein biosynthesis were examined in the adult feline heart after hemodynamic overloading ex vivo. Both LIF mRNA and protein expression were detected within 60 to 90 minutes after hemodynamic overloading. Studies in isolated adult cardiac myocytes showed that these cells synthesized both LIF mRNA and protein. The functional effects of LIF in the heart were demonstrated by studies that showed that LIF stimulation led to a significant increase in general protein synthesis and an increase in sarcomeric protein synthesis. Pretreatment with LIF also protected the cells against hypoxia/reoxygenation-induced cardiac myocyte apoptosis and cellular injury. Finally, LIF had no effect on isolated cardiac myocyte cell motion.

Conclusions—Hemodynamic overload is a sufficient stimulus for LIF expression in the adult mammalian heart. Given that LIF confers both hypertrophic and cytoprotective responses in adult cardiac myocytes, this study suggests that the expression of LIF within the heart may play an important role in mediating homeostatic responses within the myocardium. (Circulation. 2001;103:1296-1302.)

Key Words: cytokines • hemodynamics • inhibitors • myocytes

Recent studies have suggested that the gp130 signaling complex plays an important role in regulating hypertrophic and cytoprotective responses in the adult heart. For example, double transgenic mice that overexpress ligand/receptor complexes that signal through gp130 develop a concentric hypertrophic phenotype, whereas mice that harbor a ventricular-restricted deletion of gp130 undergo accelerated cardiac myocyte apoptosis after hemodynamic pressure overloading.1,2 Relevant to this discussion is the recent observation that cytokines that signal through the gp130/leukemia-inhibitory factor receptor complex, such as cardiotrophin-1 and leukemia-inhibitory factor (LIF), confer hypertrophic and cytoprotective responses in cultured neonatal cardiac myocytes.3–5 Moreover, LIF mRNA expression has recently been detected in the adult human heart.6 Given that LIF is expressed by a number of resident cell types within the myocardium, including endothelial cells and fibroblasts, the expression of LIF within the myocardium might provide an important autocrine/paracrine mechanism that is responsible for integrating stress responses within the heart. However, the mechanisms that govern the expression and functional effects of LIF in the adult heart are not known. Accordingly, the purpose of the present study was 2-fold: first, to determine whether hemodynamic overloading was sufficient to provoke LIF expression in the adult heart, and second, to determine the functional effects of LIF in isolated adult cardiac myocytes.

Methods

Regulation of LIF Expression in the Adult Heart Ex Vivo

LIF mRNA Biosynthesis

Myocardial LIF mRNA production was assessed in freshly excised adult cat hearts with a modified Langendorff buffer (Krebs-Henseleit) perfusion apparatus.7 Hearts were either perfused at normal perfusion pressure (80 mm Hg) for 210 minutes or subjected to a brief period of hemodynamic overloading for 30 minutes (200 mm Hg), after which the perfusion pressure was returned to 80 mm Hg for an additional 180 minutes. For these studies, “time 0” refers to the time at which the elevated perfusion pressure was returned to normal (ie, 80 mm Hg). To determine the time course for...
LIF mRNA expression, a small sample (~500 mg) of myocardium was excised from the suspended heart starting at time 0 and for every 30 minutes thereafter for a total of 180 minutes (see online supplement for details of methodology).

**LIF Protein Biosynthesis**

Two approaches were taken to determine LIF production in the adult heart. First, we performed a Western blot analysis of LIF protein from myocardial extracts from hearts perfused at normal and elevated pressures; second, we examined LIF bioactivity in the superfusates from the buffer-perfused hearts (see online supplement for details).

**Cellular Source for Myocardial LIF Production**

To determine whether the cardiac myocyte was a potentially important source for LIF production, 2 different experiments were performed. First, we examined the relative production of LIF mRNA and protein by myocyte and nonmyocyte cell types isolated from pressure-overloaded hearts, as we have described previously. Second, we examined LIF expression in cultured cardiac myocytes that had been stimulated with either 200 U/mL tumor necrosis factor (TNF) or 125 μg/mL endotoxin (see online supplement for details).

**Functional Effects of LIF in Adult Cardiac Myocytes**

To determine the functional consequences of LIF expression in the adult heart, we examined the effects of LIF on cardiac myocyte growth and contractility (see online supplement) and cell viability (see below).

**Cytoprotective Effects of LIF**

The potential cytoprotective effects of LIF were assessed by use of a previously described model of hypoxia/reoxygenation injury. The end points for these studies were lactate dehydrogenase (LDH) release and cardiac myocyte apoptosis. Adult cardiac myocyte cultures (day 1) were pretreated for 6 hours with diluent or with 10 ng/mL LIF. The myocyte cultures were then subjected to hypoxia/reoxygenation injury, as described previously. The hypoxic conditions were maintained for 12 hours, after which the cells were reoxygenated for 10 to 15 minutes, and the extent of cellular injury was assessed by measurement of LDH release or of the degree of cardiac myocyte apoptosis by a modification of the recently described in situ DNA ligation technique (see online supplement for details).

**Mechanism for the Cytoprotective Effect of LIF**

Previous studies have shown that ligands that signal through the LIF receptor/gp130 signaling complex confer cytoprotective responses in neonatal cardiac myocytes through activation of the mitogen-activated protein kinase (MAPK) (p44/p42), extracellular signal-regulated kinase [ERK]1/ERK2) pathway and/or the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. To determine whether the concentrations of LIF used in the present study were sufficient to activate ERK1/ERK2 and/or STATs in adult cardiac myocytes, we examined the degree of phosphorylation of ERK1/ERK2 and STAT3 (a prototypical STAT), respectively (see online supplement for details).

To determine whether MAPK inhibition was sufficient to block the functional effects of LIF in cardiac myocytes, 2 interleaved studies were conducted. First, to determine whether LIF-induced activation of ERK1/ERK2 mediated the cytoprotective effects in adult myocytes, we pretreated myocyte cultures for 30 minutes with PD98059 (1 to 10 μmol/L [Calbiochem]), a specific MAPK/ERK (MEK1) inhibitor, in the presence and absence of LIF. The myocyte cultures were then subjected to hypoxia/reoxygenation injury as described above. Second, to determine whether LIF-induced activation of ERK1/ERK2 was potentially important in terms of mediating the growth-stimulatory effects in adult myocytes, we pretreated myocyte cultures for 30 minutes with PD98059 (10 μmol/L) in the presence and absence of LIF. To confirm that the concentrations of PD98059 used were sufficient to block MAPK activation, we pretreated cardiac myocytes for 30 minutes with PD98059 (10 μmol/L), in the presence and absence of 10 nmol/L angiotensin II (Sigma) for 5 minutes, insofar as PD98059 has been shown to abrogate angiotensin II–induced ERK1/ERK2 expression in isolated adult cardiac myocytes.

**Statistical Analysis**

Data are expressed as mean±SEM. One-way ANOVA was used to test for mean differences in protein synthesis, LDH release, and the extent of cardiac myocyte apoptosis; post hoc testing (Dunnett’s or Tukey’s) was performed when appropriate. Two-way ANOVA was used to evaluate differences between and within groups of LIF bioassay and cell motion assay. The degree of net myosin heavy chain and actin synthesis was analyzed by nonpaired Student’s t tests.

**Results**

**Regulation of LIF Expression in the Heart**

**LIF mRNA Biosynthesis**

Figure 1A shows that LIF mRNA was not observed from 0 to 150 minutes and was only weakly detectable at 180 minutes in the hearts that were perfused at normal pressure. In contrast, Figure 1B shows that LIF mRNA expression was detectable 60 to 90 minutes after hemodynamic overloading.
and increased thereafter from 120 to 180 minutes. The group data summarized in Figure 1C show that LIF mRNA expression was maximal between 120 and 150 minutes and decreased slightly by 180 minutes.

**LIF Protein Biosynthesis**

Figure 2A shows that myocardial LIF was not detectable in hearts perfused at normal pressures (data not shown). In contrast, LIF protein levels were detectable within the myocardium as early as 60 to 90 minutes after hemodynamic overloading and increased 12-fold by 180 minutes (Figure 2A). Consistent with the findings in myocardial tissue, LIF bioactivity was not detectable in the superfusates from the control hearts (n=2), whereas LIF bioactivity increased in the superfusates from the hemodynamically overloaded hearts (n=5) within 90 minutes and continued to increase for up to 180 minutes after the cessation of hemodynamic overloading (Figure 2B). The specificity of the effects of LIF on M1 cell proliferation was confirmed by use of a neutralizing anti-LIF antibody, which completely blocked the effects of LIF on M1 cell proliferation (see inset of Figure 2B). One-way ANOVA indicated that there were significant differences in LIF protein levels in the hearts subjected to hemodynamic overloading (P<0.001); post hoc multiple comparison testing (Dunnett’s test) indicated that LIF protein levels were significantly different (P<0.05) from control at ≥120 minutes.

**Cellular Source for Myocardial LIF Production**

Figure 3, A and B, shows the relative production of LIF mRNA and protein, respectively, by cardiac myocytes and nonmyocyte cell types in the heart after hemodynamic pressure overload. The supernatant from the cell isolation, which is composed predominantly of nonmyocyte cell types (>95%), expressed both LIF mRNA and protein, whereas the cell pellets, which are composed predominantly (>95%) of cardiac myocytes, expressed both LIF mRNA and protein. To further confirm that isolated adult cardiac myocytes synthesized LIF, we stimulated adult cardiac myocyte cultures with TNF (200 U/mL) and endotoxin (125 μg/mL). Figure 3C shows that the level of mRNA expression was minimal in diluent-treated myocyte cultures, whereas LIF mRNA expression increased 5.5- and 11-fold, respectively, after stimulation with TNF or endotoxin. Figure 3B shows that LIF protein levels were barely detectable in the diluent-treated cultures, whereas they were increased 3.8-fold (for both) after treatment with TNF or endotoxin. Each of the above experiments was confirmed in 2 additional experiments.

**Functional Effects of LIF in Adult Cardiac Myocytes**

We examined the effects of LIF on cardiac myocyte hypertrophy and cell motion. In brief, these studies showed that LIF increased general protein synthesis (Figure IA) and sarcomeric protein synthesis (Figure IB) in cultured adult cardiac myocytes, whereas LIF had no effect (Figure II) on...
isolated cardiac myocyte contractility (presented in full in the online supplement). The effects of LIF on cardiac myocyte viability after hypoxia/reoxygenation injury are presented below.

Cytoprotective Effects of LIF: LDH Release
Figure 4 shows 3 important findings with respect to the cytoprotective effects of LIF. First, there was no significant difference in LDH release in the diluent-treated (n=32 cultures) and LIF-stimulated (10 ng/mL; n=12 cultures) cultures studied under normoxic conditions. Second, hypoxia/reoxygenation resulted in a significant 2-fold increase in LDH release in diluent-treated normoxic cardiac myocyte cultures (n=27 cultures) compared with diluent-treated normoxic cardiac myocyte cultures, consistent with our earlier observations.9 Third, LIF (10 ng/mL) pretreatment significantly attenuated LDH release compared with diluent-treated hypoxic myocyte cultures (n=27 cultures). As shown in Figure 4, however, the degree of LDH release in LIF-treated hypoxic cells was still significantly greater than that observed in diluent-treated normoxic cells, suggesting that LIF did not completely protect the myocytes from hypoxia/reoxygenation injury (P<0.05). Importantly, the cytoprotective effects of LIF were abrogated completely by an anti-LIF antibody (n=9 cultures). One-way ANOVA indicated that there were significant overall differences between groups (P<0.001); post hoc ANOVA testing (Tukey) indicated that there were no significant differences in LDH release in normoxic cells in the presence and absence of LIF (P>0.05), whereas there was a significant increase in LDH release in normoxic compared with hypoxic cells (P<0.01) and a significant decrease in LDH release in LIF-treated hypoxic cells compared with diluent-treated hypoxic cells (P<0.01).

Cytoprotective Effects of LIF: Cardiac Myocyte Apoptosis
Figure 5, A and B, shows representative examples of DNA labeling in normoxic and hypoxic cardiac myocyte cultures, respectively. Figure 5C shows a pattern of diffuse nuclear staining that is observed with the in situ DNA ligation technique after DNase I treatment (positive control). The group data depicted in Figure 5D show that the extent of DNA labeling in diluent-treated normoxic cardiac myocyte cultures (n=6) was 3.3±0.5%. Hypoxia/reoxygenation injury led to a significant 2-fold increase in DNA labeling in the myocyte cultures (n=6), whereas pretreatment with LIF resulted in an ≈30% reduction in the number of apoptotic cardiac myocytes (n=6 cultures). It should be noted, however, that the frequency of apoptosis noted in the control cultures was in excess of that observed in normal myocardium, consistent with the findings reported by others in cultured adult myocytes.13 Accordingly, we cannot exclude the possibility that the degree of reduction of LIF-induced apoptosis in the cultured cells might be less in myocardial tissue. One-way ANOVA indicated that there were overall significant differences between groups (P<0.001); post hoc ANOVA testing (Tukey) indicated that there was a significant increase in the number of apoptotic myocytes in the diluent-treated hypoxic myocytes compared with diluent-treated normoxic myocytes (P>0.05) and that there was a significant decrease in myocyte apoptosis in the LIF-treated hypoxic cardiac myocytes compared with diluent-treated hypoxic myocytes (P<0.01); the level of apoptosis in the LIF-pretreated hypoxic/reoxygenation cells, however, was still significantly greater (P<0.05) than that observed in normoxic control cells.

Mechanism for the Cytoprotective Effect of LIF
In preliminary control experiments, we established that stimulation with LIF provoked a concentration-dependent increase in ERK1/ERK2 phosphorylation (Figure 6A) and STAT3 phosphorylation on serine 727 and tyrosine 705 (Figure 6B). To determine whether MEK1 inhibition was sufficient to block the effects of LIF on MAPK and/or STAT3 phosphorylation, we repeated the above assays after pretreatment with PD98059 (1 to 10 μmol/L). As shown in Figure 6A, PD98059 inhibited MAPK phosphorylation in a dose-dependent manner; at 1 μmol/L, we observed partial attenuation, whereas at 10 μmol/L, we observed complete attenuation of ERK1/ERK2 phosphorylation. As an additional control experiment, we showed that PD98059 (10 μmol/L) abrogated angiotensin II–induced ERK1/ERK2 activation (Figure 6A), as has been reported previously. Figure 6, B and C, shows that pretreatment with PD98059 resulted in a significant decrease in phosphorylation of STAT3 on serine 727, suggesting that there is cross-talk between the MAPK and JAK/STAT pathways. In contrast, PD98059 had no significant effect of the degree of phosphorylation on tyrosine 705. Finally, to determine whether inhibition of ERK1/ERK2 activity would attenuate the cytoprotective effects of LIF, we pretreated the cells with 10 μmol/L PD98059 before LIF stimulation. Table 1 shows that pretreatment with 10 μmol/L PD98059 resulted in a complete loss of the cytoprotective effects of LIF, both in terms of preventing LDH release (P<0.05) and cardiac myocyte apoptosis (P<0.05). Importantly, PD98059 (10 μmol/L) had no significant effect on LDH release or the rates of cardiac myocyte apoptosis in both
normoxic and hypoxic conditions, suggesting that the observed effects of PD98059 were not secondary to nonspecific cytotoxic effects of this compound. Table 2 further shows that pretreatment with PD98059 led to a significant decrease (P<0.05) in LIF-induced protein synthesis compared with cells that had been stimulated with LIF alone. The extent of protein synthesis in the LIF-stimulated cells that had been pretreated with PD98059, however, was still significantly greater than that in diluent-treated cells (P<0.05).

Discussion

The results of this study, in which we examined the expression and functional significance of leukemia-inhibitory factor (LIF) in the adult mammalian heart, permit several new and potentially important insights to be drawn with respect to the biological role that LIF plays in the adult heart. First, both LIF mRNA (Figure 1B) and protein (Figure 2, A and B) are rapidly synthesized within the adult heart in response to a superimposed environmental stress, such as hemodynamic overloading. The observation that LIF mRNA and protein were synthesized by adult cardiac myocytes and nonmyocytes (Figure 3) suggests that LIF may play an important autocrine/paracrine signaling role in the heart. Second, LIF-mediated signaling conferred both hypertrophic (Figure I) and cytoprotective (Figures 4 and 6D) responses in isolated adult cardiac myocytes that had been subjected to hypoxia/reoxygenation injury. Moreover, these hypertrophic and cytoprotective responses occurred in the absence of any discernable deleterious short-term effects on isolated cardiac myocyte cell motion (Figure II), as have been reported for interleukin-6.14 Thus, the pattern of expression of LIF in response to a superimposed environmental stress and the salutary functional effects of LIF in cardiac myocytes suggest that the stress-induced expression of LIF within the myocardium may play an important role in mediating homeostatic responses within this tissue.

Biological Effects of LIF in Cardiac Myocytes

Although the functional significance of LIF-mediated signalling in the adult myocyte has not been studied heretofore, previous studies in neonatal cardiac myocytes suggest that LIF stimulation increases the rate of general protein synthesis.4,5,15 Thus, the findings in the present study both confirm and expand on these observations in neonatal cells by demonstrating that stimulation with LIF provokes an increase in sarcomeric protein synthesis (Figure IB). Moreover, the results of the present study suggest that the LIF-mediated increase in protein synthesis is partially sensitive to PD98059, a MEK1 inhibitor (Table 2). That is, there was a significant decrease in protein synthesis in the LIF-stimulated cells that had been pretreated with PD98059 (Table 2). It is not possible, however, to determine from the present studies whether the MAPK and/or JAK/STAT pathway was responsible for the hypertrophic effects of LIF, insofar as PD98059 significantly inhibited the phosphorylation of both MAPK and STAT3 phosphoproteins.

The findings in the present study that LIF protects isolated cardiac myocytes against hypoxia/reoxygenation-induced injury (Figures 4 and 6 and Table 1) are consistent with previous reports that showed that LIF confers cytoprotective responses in isolated myocytes and intact myocardial tissue.10,16 For example, a previous study in neonatal cells suggested that the cytoprotective/antiapoptotic effects of LIF are mediated through JAK/STAT-induced upregulation of
Bcl-xL. Although we cannot formally exclude a potential role for LIF-induced upregulation of Bcl-xL in adult myocytes, our results suggest that the cytoprotective effects of LIF are mediated through activation of the MAPK pathway and are thus consistent with a previous study that showed that activation of the MAPK pathway was required for the cytoprotective effects of cardiotrophin-1. Nonetheless, given that treatment with PD98059 resulted in decreased phosphorylation of STAT3, we cannot formally exclude the interesting possibility that cross-talk between the MAPK and JAK/STAT pathways may be responsible, at least in part, for the cytoprotective effects of LIF.

**Conclusions**

The observation that myocyte and nonmyocyte cell types synthesize a variety of “homeostatic proteins” in response to a superimposed environmental stress is certainly not new and was first suggested by a series of insightful experimental studies nearly 20 years ago. Recent studies from a number of different laboratories have also shown that an ensemble of stress-activated cytokines, including TNF, interleukin-1 and -6, and cardiotrophin-1, are expressed within the myocardium after hemodynamic overloading and/or ischemic injury. Although the precise role that these stress-activated cytokines play in the myocardium is unclear, there is now increasing evidence that suggests that the short-term expression of these molecules may play a critical role in initiating and integrating the myocardial response to a superimposed environmental stress. The findings in the present study are entirely consistent with this point of view and suggest that LIF may play an important autocrine/paracrine role in mediating both short-term and longer-term myocardial responses to environmental stress, both by preventing cardiac myocyte apoptosis and by stimulating hypertrophic cardiac myocyte growth. Accordingly, it will be important in future studies to determine whether selective activation of this pathway will confer adaptive myocardial responses in vivo.

**Acknowledgments**

This research was supported by research funds from the NIH (P50-HL-066, RO1-HL-58081-01, RO1-HL-61543-01, and HL-42250-10/10). The authors gratefully acknowledge Dr Colin L. Stewart, who kindly provided us with the human LIF cDNA, and the technical assistance of Dorelyn Lee-Jackson and Stacey Walker. We would also like to thank Dr Andrew I. Schafer for his past and present support and guidance.

**References**


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**Table 1. Effect of PD98059 on LIF-Induced Cytoprotection**

<table>
<thead>
<tr>
<th>PD98059 (−)</th>
<th>PD98059 (+)</th>
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</thead>
<tbody>
<tr>
<td><strong>Hypoxia</strong></td>
<td><strong>LIF + Hypoxia</strong></td>
</tr>
<tr>
<td>LDH release (treated/control)</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Apoptotic cells, % (rod- and ball-shaped)</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Apoptotic cells, % (rod-shaped)</td>
<td>6.0 ± 0.7</td>
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The extent of LDH release was expressed as a ratio of experimental to control values obtained in diluent-treated normoxic controls. Cardiac myocyte apoptosis was determined as the percentage of rod- and/or ball-shaped cardiac myocytes with positive DNA labeling.

*P < 0.05 vs respective diluent-treated cells.
†P = NS vs diluent-treated cells in the presence and absence of PD98059.
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Circulation. 2001;103:1296-1302
doi: 10.1161/01.CIR.103.9.1296

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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