Potent Long-Term Cardioprotective Effects of Single Low-Dose Insulin-Like Growth Factor-1 Treatment Postmyocardial Infarction

John F. O’Sullivan, MD; Anne-Laure Leblond, PhD; Geraldine Kelly, BSc; Arun H.S. Kumar, DVM, PhD; Pat Metharom, PhD; Chirlei K. Büneker, PhD; Niki Alizadeh-Vikali, MSc, BHSc; Ivvalna Hristova, BS; Brian G. Hynes, MD; Rosemary O’Connor, PhD; Noel M. Caplice, MD, PhD

Background—Insulin-like growth factor-1 (IGF-1) is recognized as an important regulator of cardiac structure and cardiomyocyte homeostasis. The prosurvival and antiapoptotic effects of IGF-1 have been investigated in vitro and in rodent models of myocardial infarction (MI). However, the clinical application of IGF-1 has been hampered by dose-dependent side effects both acutely and during chronic administration. We hypothesized that single, low-dose IGF-1 (LD-IGF-1) administered locally and early in the reperfusion phase after acute MI in a large animal model would avoid significant side effects but would have prosurvival effects that would manifest in long-term structural and functional improvement after MI treatment.

Methods and Results—Forty-four female Landrace pigs underwent intracoronary administration of LD-IGF-1 or saline 2 hours into the reperfusion phase of acute left anterior descending artery occlusion MI. In the area of infarction, IGF-1 receptor and signaling responses were activated at 30 minutes and cardiomyocyte cell death attenuated at 24 hours after LD-IGF-1 but not saline treatment. Hemodynamic and structural studies using pressure-volume loop, CT, and triphenyltetrazolium chloride analysis 2 months post-MI confirmed a marked reduction in infarct size, attenuation of wall thinning, and augmentation of wall motion in the LD-IGF-1-treated but not in the saline-treated animals. These regional structural benefits were associated with global reductions in left ventricular volumes and significant improvement in left ventricular systolic and diastolic function.

Conclusions—One-time LD-IGF-1 effects potent acute myocardial salvage in a preclinical model of left anterior descending artery occlusive MI, extending to long-term benefits in MI size, wall structure, and function and underscoring its potential as an adjunctive therapeutic agent. (Circ Cardiovasc Interv. 2011;4:327-335.)

Key Words: myocardial infarction ■ remodeling ■ myocardial contraction ■ myocytes ■ proteins

Insulin-like growth factor-1 (IGF-1) is recognized as an important regulator of cardiac structure and performs a key role in cardiomyocyte homeostasis, including inter alia promotion of cell growth, inhibition of apoptosis, and augmentation of calcium signaling.1,2 The most intriguing function of IGF-1 from a therapeutic perspective remains its prosurvival antiapoptotic effects that are mediated in large part through the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway.3,5 Moreover, recent evidence suggests that IGF-1 alters the mitochondrial calcium flux maintaining outer mitochondrial membrane potential, thus protecting against necrosis induced by changes in mitochondrial permeability transition pore (mPTP).4 Together, these signaling and membrane-stabilizing effects occur over minutes to hours and are distinct from the effects of chronic IGF-1 treatment in the heart, which includes potentially maladaptive promotion of cardiomyocyte hypertrophy.6,7 To date, the acute beneficial effects of IGF-1 have not been fully explored clinically because of perceived short- and long-term side effects of early IGF-1-related trials.

Editorial see p 311
Clinical Perspective on p 335

The first uncontrolled clinical trial to suggest positive IGF-1 effects in patients with heart failure involved administration of human growth hormone, the tissue effects of which are mediated through IGF-1.8 Subsequent randomized controlled trials, however, showed no sustained benefit of...
human growth hormone, and indeed, chronic administration was associated with a variety of side effects, including bone tenderness, arthralgias, edema, orthostatic hypotension, and tachycardia. Consequently, pharmaceutical interest in the use of IGF-1 in cardiac disease diminished. However, it is important to note that no clinical trial has ever used IGF-1 to target the cardiomyocyte dysfunction for which it is likely to be most therapeutic, that is, acute cardiomyocyte death in the context of myocardial infarction (MI).

Over the past decade, regenerative medicine has ushered in newer, subacute approaches to MI repair after successful reperfusion in the context of thrombolysis or percutaneous intervention. A number of other studies have identified novel therapeutic targets that may acutely rescue ischemic cardiomyocytes: regulators of mPTP opening, such as glycogen synthase kinase-3 (GSK-3β) (9–14) and cyclophilin D (15); prosurvival pathways, such as the PI3K/Akt (3–5) and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) (16); and mediators of apoptosis, such as caspases. (17) We hypothesized that low-dose of IGF-1 (LD-IGF-1) administered 1 time early in the reperfusion phase after acute MI would have immediate beneficial anti-cell death effects without the negative side effects associated with higher dose regimens, such as hypotension, hypoglycemia, and tachycardia. Moreover, we hypothesized that acute salvage of cardiomyocytes undergoing cell death would have significant long-term beneficial effects in terms of chronic infarct size reduction and left ventricular (LV) remodeling. This latter approach would obviate the need for chronic IGF-1 administration with its attendant osteogenic and arthralgic side effects. To test this hypothesis we used a large animal (porcine) acute MI model where the size of the infarct, hemodynamic effects, nature of ischemia and reperfusion, and instrumentation used more closely approximate the clinical scenario.

Methods

Porcine Model of MI

Forty-four female Landrace pigs (25 to 30 kg) (of which 39 survived) fed on a normal diet were used in this study in accordance with the guidelines of the Experimental Animal Ethics Committee of University College Cork (Cork, Ireland). MI by balloon occlusion of the mid left anterior descending artery for 90 minutes followed by reperfusion for 2 hours was induced as described previously. (18–20)

LD-IGF-1 Intracoronary Administration

We derived the dose of IGF-1 from previous unpublished studies by our group in which we obtained potent paracrine antiapoptotic effects from EPC-conditioned medium. When we blocked IGF-1 in the conditioned medium with neutralizing antibody, the paracrine effect was completely abrogated. When the level of IGF-1 was measured in the conditioned medium, it was detected at 35 to 50 pg/mL. We used this concentration of IGF-1 as a guide to LD-IGF-1 dosing, and given that the original conditioned medium was administered as 12 mL (in aliquots of 4 mL) the LD-IGF-1 total dose used in the current study was 600 pg. At the end of reperfusion, a Voyager 3.0×12 mm over-the-wire coronary balloon (Abbot Laboratories; Abbot Park, IL) was positioned at the site of prior vessel occlusion, and LD-IGF-1 (recombinant human IGF-1, 50 pg/mL; Sigma-Aldrich; St Louis, MO) was delivered as 12 mL (in aliquots of 4 mL) the LD-IGF-1- (right) treated animals. Each BZ section includes 50% NZ and 50% IZ; junction represented by black dotted lines. Nuclei were stained in blue with DAPI. Apoptotic cells are TUNEL positive (green). B, Representative images (×60 magnification) of TUNEL (green), sarcomeric actin (red), and DAPI (blue) staining on BZ sections from saline- (left) and LD-IGF-1- (right) treated animals. Each BZ section includes 50% NZ and 50% IZ. Dotted white lines represent the junction between NZ and IZ. C, Quantification of apoptotic cells in the IZ by determining the percentage of apoptotic cells (DAPI and TUNEL positive) of total cells (DAPI positive). Five thousand nuclei were counted per animal. D, Quantification of caspase 9 activity within the BZ 24 hours after saline or LD-IGF-1 administration. Seven to 12×20 images per pig were analyzed; each sample was analyzed in duplicate (saline group, 4 to 5 pigs; LD-IGF-1 group, 5 pigs). Data are presented as mean±SEM. DAPI indicates 4’,6-diamidino-2-phenylindole; IZ, infarct zone; LD-IGF-1, low-dose insulin-like factor-1; NZ, normal zone; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling. *P<0.05.

Hemodynamic Parameters

Pressure-volume loops were recorded using a 5-F pig-tailed conductance catheter (Millar Instruments; Houston, TX) positioned in the LV with a sample frequency of 250 Hz using LabChart 5 Pro (AD
Euthanasia and Tissue Collection

Twenty-four hours (acute, 22 pigs used, 18 survived [saline group, 10 pigs; LD-IGF-1 group, 8 pigs]) or 2 months (chronic, 18 pigs used, 17 survived [saline group, 8 pigs; LD-IGF-1 group, 9 pigs]) after infarct generation, animals were euthanized by pentobarbitone overdose, and tissue was processed as previously described online-only Data Supplement Methods).

Preparation of Cellular Protein Extracts, Immunoprecipitation, and Western Blots

In a separate group of animals (8 pigs, 4 in each group, all survived), euthanasia was performed 30 minutes after normal saline or LD-IGF-1 infusion and the myocardium sectioned as just described. The remote (R), infarct zone (IZ), and border zone (BZ)-IZ samples were immediately snap frozen in liquid nitrogen and stored at -80°C. Tissue protein extracts were prepared as reported previously. For immunoprecipitation and proliferating cell nuclear antigen analysis, see the online-only Data Supplement Methods.

Western blotting was performed as previously reported.22 For a full description of specific Western blots, see the online-only Data Supplement Methods, Section l.

Cell Death in Infarct Area

Five-micrometer-thick cryosections from optimal cutting temperature (OCT)-embedded tissue were cut as infarct-representative slices, which were defined as having 50% normal myocardium and 50% infarcted myocardium. Detailed apoptosis analysis is provided in online-only Data Supplement Methods.

Chronic Remodeling, Cardiomyocyte Count, Collagen Staining, and Collagen and Transforming Growth Factor-β Quantitation in the IZ

For 13 pigs in the chronic group (6 in saline group, 7 in LD-IGF-1 group), the expansion index was calculated on triphenyltetrazolium chloride (TTC)-stained myocardium as the ratio between the endocardial length of the infarct segment and that of the noninfarcted segment. Thinning ratio was calculated as the ratio of the minimum wall thickness in the infarct-related segment to that of the noninfarcted segment.

Slides for cell counting and collagen staining were prepared as previously reported (9 in LD-IGF-1 group, 9 in saline group). A detailed methodology for cardiomyocyte counting, collagen staining, and collagen type 1 and transforming growth factor-β protein expression analysis by Western blotting is provided in online-only Data Supplement Methods.

Sixty-four-Slice CT Imaging

Cardiac CT imaging was performed (acute, 14 pigs [saline group, 8 pigs; LD-IGF-1 group, 6 pigs]; chronic, 13 pigs [saline group, 6 pigs; LD-IGF-1 group, 7 pigs]) using a 64-slice scanner (GE Discovery VCT RX). Iodixanol (Visipaque 320; Amersham Health) contrast agent was used.

Image Reconstruction and Data Analysis

All gated CT images were reconstructed at a 1.25-mm slice thickness, and phase data on all axial slices were reconstructed from 0% to 99% of the cardiac cycle in 9% increments for assessment of LV function parameters and ejection fraction (EF), as calculated on an online workstation (AW 4.4; GE Healthcare). Sixty-four-slice images were analyzed with CardIQ software (AW 4.4).

Statistics

Data are presented as mean ± SEM. Nonparametric tests were used to determine differences between groups (n < 30) as follows: Mann-Whitney test was used for 2-group comparison, and Kruskal-Wallis test was used for ≥3 groups, with subsequent pair-wise comparisons using Dunns test. For larger groups (n > 30), unpaired t tests and ANOVA were used to determine differences between groups (GraphPad Prism version 4; GraphPad Software, Inc; San Diego, CA). For the analysis of the defibrillation log and mortality outcomes, we constructed a...
Results

**LD-IGF-1 Treatment Reduced In Vivo Cardiomyocyte Death at 24 Hours Post-MI**

To determine the rate of apoptosis, TUNEL staining was performed on infarcted hearts, explanted at 24 hours post-MI (Figure 1A). The percentage of TUNEL-positive cells was significantly decreased within the IZ in the LD-IGF-1-treated group compared to the saline-treated group (P<0.001) (Figure 1A and 1C). In addition, 97.4±1.7% of the apoptotic nuclei in the saline group and 98.9±0.1% in the LD-IGF-1 group were confirmed to be cardiomyocytes (Figure 1B). Moreover, at 24 hours post-MI, there was a significant decrease in caspase 9 activity within the BZ in the LD-IGF-1-treated compared to the saline-treated group (P<0.001) (Figure 1D). At 24 hours, there was no significant change in inflammatory markers in serum or in the IZ between LD-IGF-1 and saline treatment (online-only Data Supplement Figure 1).

**LD-IGF-1 Induced Phosphorylation of IGF-1 Receptor, Akt, ERK, and GSK-3β But Not Insulin Receptor at 30-Minutes Posttherapy**

Tissues analyzed from the BZ-IZ but not remote zone 30 minutes after LD-IGF-1 therapy showed a 2-fold increase in phosphorylation of immunoprecipitated IGF-1 receptor (IGF-1R) compared to saline treatment (P<0.05) (Figure 2A and 2C). There was no significant change in phosphorylation of the insulin receptor after LD-IGF-1 treatment, indicating that the administered IGF-1 at this dose selectively targeted the IGF-1R (Figure 2B and 2D).

The IZ and BZ from these pigs also were used to measure phosphorylation of signaling pathways downstream of IGF-1R activation, including Akt, ERK, and GSK-3β (which is phosphorylated and inactivated by Akt and has been implicated in mPTP-associated pathway in initiation of necrosis).24 There was a significant 2-fold increase in phosphorylation of Akt (P<0.05) and ERK (P<0.05) in the BZ-IZ but not within the IZ or remote zone at 30 minutes after LD-IGF-1 treatment compared to saline treatment (Figure 3A and 3B). Consistent with increased Akt activity, a significant increase in phosphorylation of GSK-3β in the BZ-IZ but not in the IZ of the LD-IGF-1 group compared to the saline group. Four pigs were in each group; 97 samples were used in pAkt/Akt and pERK/ERK blots, and 20 samples were used in the pGSK-3β/GSK-3β experiments. Data are presented as mean±SEM. Akt indicates protein kinase B; BZ, border zone; ERK, extracellular signal-regulated kinase; IZ, infarct zone; LD-IGF-1, low-dose insulin-like factor-1; GSK-3β, glycogen synthase kinase-3β; p-, phosphorylated; R, remote zone. *P<0.05.

**LD-IGF-1 Treatment Reduced Infarct Size, Infarct Collagen Content, and Fibrotic Markers and Increased Cardiomyocyte Number in the IZ at 2 Months Posttherapy**

Gross pathological slices were analyzed using a digital camera and Image J software (National Institutes of Health; Bethesda, MD) (Figure 4A). LV infarct area (normalized to area at risk) and expansion index were
Cardiomyocytes/mm²) compared to the saline-treated group (2256.75 ± 3.7% and 44.99 ± 5.7%, respectively; *P < 0.05) (Figure 5C and 5D).

Western blot analysis of IZ tissue demonstrated decreased collagen type 1 (Figure 5E and 5F), and transforming growth factor-β expression (Figure 5G and 5H) in the LD-IGF-1 group compared to the control group. Together, these findings support a sustained long-term benefit from the acute prosurvival effects of single-dose LD-IGF-1. Infarct induction was of a similar size in both groups, as demonstrated by area at risk/LV (online-only Data Supplement Figure 3), suggesting that differences could not be accounted for by area-at-risk variance.

**LD-IGF-1 Improved Global LV Remodeling and Function at 2 Months Post-MI**

Further parameters of chronic remodeling after MI, global LV end-diastolic volume, LV end-systolic volume, and LVEF were determined by 64-slice CT (Figure 6). Representative images from LD-IGF-1-treated and saline-treated groups are seen in Figure 6A. LD-IGF-1 therapy significantly decreased LV end-diastolic volume and end-systolic volume compared to saline treatment (*P < 0.05) (Figure 6B and 6C). LVEF also was significantly increased at 2 months post-MI in LD-IGF-1-treated but not saline-treated animals, providing further evidence for sustained beneficial effects on LV remodeling after MI (*P < 0.01) (Figure 6D, online-only Data Supplement Video). To underscore these findings, LV function (±dP/dt) measured by conductance catheter at 2 months posttreatment showed that systolic and diastolic LV function were significantly improved in the LD-IGF-1-treated group compared to the saline-treated group (*P < 0.05) (Figure 6E).

**Regional Wall Motion and Thickening Are Improved 2 Months Post-MI After LD-IGF-1 Therapy**

Using multidetector CT and General Electric polar map software, we also investigated regional LV wall motion and thickening (Figure 7A and 7C). We focused on the infarct territory for quantitative analysis (Figure 7B and 7D). At 2 months post-MI, there was a significant increase in infarct-related wall motion in the LD-IGF-1-treated versus saline-treated group and a significant decrease in wall motion in the control group at 2 months compared to preinfarct values (*P < 0.05 and *P < 0.001, respectively) (Figure 7A and 7B). There was also a significant improvement in infarct-related wall thickening in the LD-IGF-1-treated group compared to the saline-treated group and a significant decrease in the saline-treated group at 2 months post-MI compared to preinfarct (*P < 0.001) (Figure 7C and 7D). Importantly, there was no significant difference between the LD-IGF-1 group and the saline group in terms of frequency of ventricular fibrillation and short-term or long-term mortality (Table).
Discussion

The major finding of this study is a hitherto unrecognized potent cardioprotective effect of 1-time intracoronary administration of LD-IGF-1 after acute MI. Our data extend established evidence that IGF-1 reduces cardiomyocyte apoptosis and promotes cardiomyocyte survival after infarction, to a dose regimen in a clinically relevant large animal model that is much lower than previously used, has no obvious side effects, and is clinically easy to use in the context of current percutaneous interventional approaches to acute infarct reperfusion. Finally, our study evinces salutary acute myocardial salvage effects of LD-IGF-1, which translate to long-term structural and functional improvement in the LV after MI.

We demonstrate specific biological activity of injected LD-IGF-1 in the infarct region within 30 minutes of administration, as manifested by phosphorylation of the IGF-1R but not the insulin receptor. Moreover, LD-IGF-1-treated animals exhibited concurrent activation signaling downstream of the IGF-1 receptor, with phosphorylation of PI3K/Akt, GSK-3β, and ERK, supporting prosurvival pathways affecting both membrane pore transition and caspase pathways.

It has been well described previously that inhibition of caspase activation occurs at least in part through phosphorylation of Akt and ERK, so it is likely that the reduction in infarct-related cardiomyocyte death seen in the LD-IGF-1-treated group was mediated through activation of these pathways. Similarly, phosphorylation and, thus, inhibition of GSK-3β in the infarct region 30 minutes after LD-IGF-1 may reduce activation of discrete signals essential for cardiomyocyte cell death and necrosis. Yang and colleagues recently proposed that activation of survival kinases (PI3K, Akt, and ERK) also may inhibit lethal mitochondrial membrane pore formation that normally uncouples mitochondria, leading to cardiomyocyte necrosis postreperfusion. Additionally, GSK-3β has emerged as an integration point for diverse pathways that play a central role in transferring signals downstream to targets that act at or in proximity to the mPTP. Thus, the consequences of LD-IGF-1 activation of a broad-based survival cascade involving inhibition of caspase and mPTP death pathways may be significant salvage of at-risk cardiomyocytes 24 hours posttreatment. In addition, IGF-1 is known to have positive effects on acute excitation-
contraction coupling events mediated through Ca\textsuperscript{2+} mobilization and Akt activation; attenuating decreases in Na-Ca exchanger, normalizing intracellular Ca\textsuperscript{2+} levels and transients; and, thus, preventing loss of mitochondrial membrane potential.\textsuperscript{28} Together, preservation of cardiomyocyte number and excitation-contraction mechanisms early after infarction may contribute to long-term preservation of LV mass and function during chronic remodeling after MI.

The current study showing potent acute prosurvival effects of a 1-time injection of IGF-1 protein after MI is consistent with other recent work by Kondo et al\textsuperscript{20} who demonstrated acute antiapoptotic, antiinflammatory, and antioxidative stress effects of single-dose adiponectin. These acute adiponectin effects manifested in infarct size reduction and improved cardiac performance. Important differences between this study and the current study include adiponectin administration 10 minutes into ischemia rather than LD-IGF-1 at 2 hours into reperfusion and the completion of analysis at 24 hours in the adiponectin study compared to additional long-term evaluation at 2 months post-MI in the current study. Moreover, the current study did not identify an antiinflammatory effect of LD-IGF-1 at 24 hours posttherapy (online-only Data Supplement Figure 1). Nevertheless, short-term findings in both studies suggest that it is feasible to effect significant cardiomyocyte survival in the early ischemia-reperfusion phase with 1-time drug dosing, and it is conceivable that correct timing of such cardioprotective agents may allow therapeutic application in the clinical setting.

An important aspect of any acute intervention after infarction is whether long-term benefits ensue. We demonstrate here that the acute prosurvival effects of LD-IGF-1 are associated with structural and functional benefits in the regional and global myocardium 2 months posttreatment. LD-IGF-1-treated animals exhibited marked reduction in infarct size and expansion index (by TTC analysis) at 2 months post-MI. Moreover, wall thinning ratio was reduced in the IZ after LD-IGF-1 treatment as measured by TTC and CT methods. Histological analysis of cardiomyocyte number in the IZ and extent of reduced collagen staining and profibrotic marker expression, such as collagen type 1 and TGF-β, underscored the cell preservation effects of LD-IGF-1 and its vitiating effects on long-term scar formation.

A major determinant of early and late survival in human subjects after a large MI is preservation of global LVEF, which reduces incidence of lethal ventricular arrhythmia and progression to heart failure.\textsuperscript{29} Reduction in LVEF frequently is accompanied by LV dilatation with increases in systolic and diastolic volumes. LD-IGF-1 in the current study markedly attenuated all of the features of maladaptive remodeling seen after large infarcts, reducing systolic and diastolic volumes and significantly improving LV structural parameters at 2 months posttreatment. This enhanced global contractile effect was contributed to by regional improvement in wall motion in the infarct-related area. It is important to note that both treatment groups (LD-IGF-1 and control) had similar infarct areas at risk before treatment, suggesting that the beneficial effects were mediated by the LD-IGF-1 therapy and not due to infarct size sampling bias (online-only Data Supplement Figure 3).

This study has limitations. A single dose of LD-IGF-1 was given 2 hours after reperfusion, and it is unknown whether delayed administration beyond this time point would provide similar acute and chronic benefits and whether there is a cutoff time for LD-IGF-1 efficacy. Although a single dose of LD-IGF-1 was effective in this study, a full-dose response for IGF-1 up to and including concentrations that are associated with side effects in human subjects was not studied.
In conclusion, these data suggest that the acute gains obtained from early, single-dose, LD-IGF-1 in the postreperfusion phase of a large MI translate into long-term preservation of myocardial cell structure and function. One-time LD-IGF-1 administration in the hours after reestablishing complete reperfusion may thus offer a novel adjunctive myocardial salvage approach to current percutaneous coronary interventional and pharmacological strategies after MI.

Table. The Incidence of Ventricular Fibrillation, Mortality, and Outcome

<table>
<thead>
<tr>
<th></th>
<th>Control (n=23)</th>
<th>LD-IGF-1 (n=21)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricular fibrillation</td>
<td>9 (23)</td>
<td>7 (21)</td>
<td>0.76</td>
</tr>
<tr>
<td>during procedure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality 30-min study</td>
<td>0 (4)</td>
<td>0 (4)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mortality 24-h study</td>
<td>2 (10)</td>
<td>2 (8)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mortality after 2 mo</td>
<td>1 (9)</td>
<td>0 (9)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Data are presented as incidence (number of animals in group). LD-IGF-1 indicates low-dose insulin-like growth factor-1.

Acknowledgments
We thank Janet Choi for technical assistance.

Sources of Funding
This study was funded by Molecular Medicine Ireland (R12699-JOS), Science Foundation Ireland (R11482-NMC, RFP06-NMC, and RFP07-ROC), and Health Research Board (R11831-NMC), Dublin, Ireland. This work also was supported through the National Biophotonics and Imaging Platform, Ireland, and funded by the Irish Government Programme for Research in Third Level Institutions, Cycle 4, Ireland EU Structural Funds Programmes 2007 to 2013.

Disclosures
None.

References
2. Laustsen PG, Russell SJ, Cui L, Entingh-Pearsall A, Holzenberger M, Liao R, Kahn CR. Essential role of insulin and insulin-like growth factor...


CLINICAL PERSPECTIVE

Chronic heart failure after ST-segment elevation myocardial infarction (MI) remains a public health burden despite current percutaneous interventional and pharmaceutical treatment. Strategies to reduce infarct size are needed but remain a therapeutic challenge. Insulin-like growth factor-1 (IGF-1) is a known central regulator of cardiac function with prosurvival, proliferative, and differentiation effects in the heart. However, clinical trials of IGF-1-related compounds in heart failure were abandoned >1 decade ago because of the side effect profile of chronically administered drug and presumed therapeutic inefficacy. Importantly, IGF-1 has never been tested clinically for its acute prosurvival effects in the context of MI. The current study examined the potency of IGF-1 at a lower dose and as a single injection that would not be expected to cause the side effects manifested in previous IGF-1-related studies. Moreover, we treated large MIs in pigs in the reperfusion phase to more closely model the scale, hemodynamic, and functional parameters seen in the clinical ST-segment elevation MI setting. We show here that a 1-time bolus of low-dose IGF-1 has potent acute cardioprotective effects on cardiomyocytes within the infarct zone associated with activation of classic prosurvival signaling pathways and that acute cell salvage translates into long-term preservation of cardiac structure and function without the significant side effects seen in previous trials. These data suggest that low-dose IGF-1 may be a useful adjunctive therapy for acute MI. Further study is warranted to investigate whether low-dose IGF-1 is a safe and effective treatment, particularly in patients with large MIs at risk of developing long-term heart failure.
Potent Long-Term Cardioprotective Effects of Single Low-Dose Insulin-Like Growth Factor-1 Treatment Postmyocardial Infarction


Circ Cardiovasc Interv. 2011;4:327-335; originally published online June 28, 2011; doi: 10.1161/CIRCINTERVENTIONS.110.960765

Circulation: Cardiovascular Interventions is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-7640. Online ISSN: 1941-7632

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circinterventions.ahajournals.org/content/4/4/327

Data Supplement (unedited) at:
http://circinterventions.ahajournals.org/content/suppl/2011/06/28/CIRCINTERVENTIONS.110.960765.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Cardiovascular Interventions can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Cardiovascular Interventions is online at:
http://circinterventions.ahajournals.org//subscriptions/
Supplemental Material

SUPPLEMENTAL METHODS

Western Blotting

All protein samples for Western blot analysis were resolved by SDS-PAGE on 10 %, 12 % or 15 % SDS PAGE gels (100 μg per sample). All primary antibody incubations were performed overnight at 4°C. Primary antibodies used were anti-IGF-1R, anti-IR, anti-ERK, and anti-phospho-ERK (all Santa Cruz Biotechnology), anti-Akt, anti-phospho-Akt, anti-GSK-3β, anti-phospho-GSK-3β (all Cell Signaling, Boston MA, USA), anti-Collagen Type 1 (Sigma Aldrich, MO, USA), and anti-TGFβ (R&D Systems, Abingdon, UK). For cell proliferation, the primary antibodies used were PCNA (clone PC10, Chemicon International), and Beta-Actin (Sigma Aldrich, MO, USA). For directly conjugated secondary antibodies (Alexa Fluor 680- and 800-coupled anti-rabbit and anti-mouse antibodies [LI-COR Biosciences Cambridge, UK]) detection was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Cambridge, UK). For HRP-conjugated secondary antibodies (anti-rabbit and -mouse, Jackson Laboratory), detection was performed using Chemiluminescent substrate (Supersignal West Pico, Thermo Scientific) and UVP Biospectrum Multispectral Imaging System. At least 10 tissue samples were used per pig per antibody, for each Western Blot.

Sacrifice and tissue collection

LAD coronary artery was re-occluded and the aorta clamped followed by administration of 20 mls of 0.5 % methylene blue (Sigma Aldrich, MO, USA) through the left ventricular apex, to determine the area at risk (AAR). The hearts were explanted, weighed and sectioned in 5mm transverse slices from apex to base (6 to 8 slices/heart), and were incubated in 2 % triphenyltetrazolium chloride (TTC) (Sigma Aldrich, MO, USA) for 15 minutes in the dark to allow the staining of the infarct area. Images of the sections were captured using a digital camera, and planimetry of images was performed using Image J software (U.S. National Institutes of Health, Maryland, USA)\(^1,2\). Samples from infarct-zone
IZ), border-zone (BZ) and region spanning both BZ and IZ (BZ-IZ) and remote-zone (R) myocardial areas were embedded or cryopreserved in liquid nitrogen for further analysis. All analyses were performed by at least two blinded and independent observers.

**Immunoprecipitation and PCNA analysis**

For immunoprecipitation of IGF-1 receptor (IGF-1R), lysates (1 mg of protein per sample) were incubated with 1 μg of mouse anti-phosphotyrosine antibody, clone 4G10 (Millipore, Molsheim, Germany), and for immunoprecipitation of insulin receptor (IR), lysates (1 mg of protein per sample) were incubated with 1 μg of mouse anti-phosphotyrosine antibody, clone 20 (Millipore, Molsheim, Germany), both overnight at 4 °C, followed by addition of 15 μl of protein G-agarose beads for 3 hrs at 4 °C. For animals sacrificed at 24 hrs, “snap” frozen BZ-IZ tissue was used to calculate cell proliferation using PCNA antibody.

**Apoptosis analysis**

Apoptotic cells within IZ myocardium were detected using terminal deoxynucleotidyl-mediated dUTP nick-end labeling (TUNEL) method (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics). Nuclei were stained with DAPI (Molecular Probes). The percentage of apoptotic cells was determined by counting the total number nuclei and the total number of apoptotic cells within the IZ. These sections were also dual-stained with TUNEL and Sarcomeric Actin (Sigma Aldrich, MO, USA) to confirm that the apoptotic nuclei were of cardiomyocyte origin. Over 5,000 nuclei were counted.

Caspase 9 activity in the BZ was determined using a Caspase-9 colorimetric activity assay commercial kit (Chemicon International). Each sample was analysed in duplicate. Protein content was measured with a standard Bradford protein assay (BioRad).

**Measurement of inflammatory mediators in the serum and infarct zone**
Using 24 hr serum samples and ELISA kits (R&D Systems, Abingdon, UK), we measured levels of IL-6 (10 pigs saline, 7 pigs LD-IGF-1) and TNF alpha (9 pigs saline, 7 pigs LD-IGF-1) in the serum. Using infarct zone tissue from hearts explanted at 24 hrs, we performed myeloperoxidase activity (9 saline pigs, 8 LD-IGF-1 pigs) using ELISA kit (R&D Systems, Abingdon, UK), and neutrophil staining (9 saline pigs, 7 LD-IGF-1 pigs) using anti-pig granulocyte (neutrophil) antibody (AbDSerotec, Oxford, UK).

**Measurement of long-term fibrotic markers: cardiomyocyte counting, collagen staining, and collagen type 1 and TGFβ protein expression on Western blot**

Using 2 months myocardial tissue samples, the total number of nuclei and the number of cardiomyocytes positive for sarcomeric actin and laminin staining (Sigma Aldrich, MO, USA) were counted in 10 HPF (magnification: 40x) per pig. The cardiomyocyte count was expressed as a percentage of total nuclei per HPF per infarct zone. At least 1,000 nuclei were counted per pig.

Slides were also stained with Masson’s Trichome (CellPath) for analysis of collagen content (8 pigs each group). In each section stained with Masson’s Trichrome staining, 10 to 15 fields were acquired (magnification: 20x), and analysed using NIS-Elements BR 3.0 image analysis software.

2 month infarct zone “snap frozen” tissue was also used for Western blotting for collagen type 1 (Sigma Aldrich, MO, USA; 4 pigs in each group), and TGFβ (R&D Systems, Abingdon, UK; 5 pigs in each group).
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Acute inflammatory data (24 hours). Serum samples were assessed for levels of IL-6 (A) and TNFα (B) at 24 hrs. There was a trend towards reduction but no significant difference comparing the LD-IGF-1 group to saline. Similarly, there was a non-significant reduction in MPO activity (C) and neutrophil count (D) in the infarct zone at 24 hours. (A) n=10 pigs in saline group, n=7 pigs in the LD-IGF-1 group. (B) n=9 pigs in saline group, and n=7 pigs in LD-IGF-1 group. (C) n=4 pigs in the saline group, n=4 pigs in the LD-IGF-1 group. (D) n=9 pigs in the saline group, n=7 pigs in the LD-IGF-1 group.

Supplemental Figure 2. Cell Proliferation in the BZ-IZ. Upper panel: representative immunoblot for PCNA expression at 24hrs in BZ-IZ from saline or LD-IGF-1 treated groups. Lower panel: quantification of PCNA expression relative to total protein (beta actin). n=12 samples per group, n=9 pigs per group, data are expressed as mean ± SEM. NS: non-significant.

Supplemental Figure 3. LD-IGF-1- and normal saline-treated group have similar area-at-risk (AAR) expressed as a percentage of left ventricular area. The average of at least five transverse slices was used for each animal. n=6 pigs for the saline group, n=7 pigs for the LD-IGF-1 group. Data are expressed as mean ± SEM. NS: non-significant.

Supplemental Figure 4. Hemodynamic Data. Average systolic (A) and diastolic (B) blood pressure (BP) for both treatment groups pre and 24 hours post MI; n= 10 pigs saline, n=8 pigs LD-IGF-1. Average systolic (C) and diastolic (D) BP for both treatment groups pre and 2 months post MI. There was a significant difference only between treatment groups for systolic BP at 2 months; n=8 pigs saline, n= 9 pigs LD-IGF-1. Acute and chronic heart rates for both groups pre and 24 hours post (E), and pre and 2 months post (F) myocardial infarction. N=10 pigs in 24 hour saline group, 8 pigs in 2 months saline group; n=8 pigs in 24 hour LD-IGF-1 group, 9 in 2 months LD-IGF-1 group.

***p<0.001.
Supplemental Figure 1

A) Serum IL-6 at 24 Hours

B) Serum TNFα at 24 Hours

C) MPO Peroxidation Activity
   Infarct at 24 Hours

D) % Neutrophil Count Infarct Zone
   at 24 Hours

[Graphs and images showing data for each condition: Saline and LD-IGF-1 for each parameter.]
Supplemental Figure 2

Relative Expression of PCNA in BZ-IZ at 24 Hours

Saline | LD-IGF-1
---|---
36 kDa | PCNA
42 kDa | Beta Actin

Rel Exp PCNA

Saline | LD-IGF-1
---|---
0 | 1.0

NS
Supplemental Figure 3

% Area at Risk/Left Ventricular Area

NS

% AAR/LV

Saline  LD-IGF-1
Supplemental Figure 4

A) Acute systolic BP

B) Acute diastolic BP

C) Chronic systolic BP

D) Chronic diastolic BP

E) Acute Heart Rate

F) Chronic Heart Rate

○ Saline
● LD-IGF-1

mmHg

BPM

Pre | Post 24 hours
Pre | Post 2 Months
Pre | Post 2 Months
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL VIDEO FILES

See attached video files. These dynamically illustrate LV function at 2 months post treatment.

Videos:

Video 1: Sagittal view of LV function at 2 months post MI in saline control group

Video 2: Sagittal view of LV function at 2 months post MI in LD-IGF-1 group

Video 3: 3D view of LV function at 2 months post MI in saline control group

Video 4: 3D view of LV function at 2 months post MI in LD-IGF-1 group