Insulin-like Growth Factor-1 but Not Growth Hormone Augments Mammalian Myocardial Contractility by Sensitizing the Myofilament to Ca\textsuperscript{2+} Through a Wortmannin-Sensitive Pathway
Studies in Rat and Ferret Isolated Muscles

Antonio Cittadini, Yoshiki Ishiguro, Hinrik Strömer, Matthias Spindler, Alan C. Moses, Ross Clark, Pamela S. Douglas, Joanne S. Ingwall, James P. Morgan

Abstract—A growing body of evidence has been accumulated recently suggesting that growth hormone (GH) and insulin-like growth factor-1 (IGF-1) affect cardiac function, but their mechanism(s) of action is unclear. In the present study, GH and IGF-1 were administered to isolated isovolumic aequorin-loaded rat whole hearts and ferret papillary muscles. Although GH had no effect on the indices of cardiac function, IGF-1 increased isovolumic developed pressure by 24% above baseline. The aequorin transients were abbreviated and demonstrated decreased amplitude. The positive inotropic effects of IGF-1 were not associated with increased intracellular Ca\textsuperscript{2+} availability to the contractile machinery but to a significant increase of myofilament Ca\textsuperscript{2+} sensitivity. Accordingly, the Ca\textsuperscript{2+}-force relationship obtained under steady-state conditions in tetanized muscle was shifted significantly to the left (EC\textsubscript{50}, 0.44±0.02 versus 0.52±0.03 \mu mol/L with and without IGF-1 in the perfusate, respectively; P<0.05); maximal Ca\textsuperscript{2+}-activated tetanic pressure was increased significantly by 12% (211±3 versus 235±2 mm Hg in controls and IGF-1–treated hearts, respectively; P<0.01). The positive inotropic actions of IGF-1 were not associated with changes in either pH\textsubscript{i} or high-energy phosphate content, as assessed by \textsuperscript{31}P nuclear magnetic resonance spectroscopy, and were blocked by the phosphatidylinositol 3-kinase inhibitor wortmannin. Concomitant administration of IGF binding protein-3 blocked IGF-1–positive inotropic action in ferret papillary muscles. In conclusion, IGF-1 is an endogenous peptide that through a wortmannin-sensitive pathway displays distinct positive inotropic properties by sensitizing the myofilaments to Ca\textsuperscript{2+} without increasing myocyte [Ca\textsuperscript{2+}].

Key Words: inotropy ■ insulin-like growth factor-1 ■ somatotropin ■ Ca\textsuperscript{2+} ■ aequorin ■ heart

The concept that GH and IGF-1, the mediator of many of the effects of GH on peripheral tissues, target the heart has recently emerged from a series of animal and human studies.\textsuperscript{1} Conditions of GH/IGF-1 deficiency in humans are associated with cardiac atrophy and impaired cardiac function.\textsuperscript{2–4} The hypertrophic response with enhanced cardiac performance observed in rats subjected to chronic GH/IGF-1 excess\textsuperscript{5–7} appears to be beneficial in the setting of experimental\textsuperscript{8–11} and, recently, human heart failure.\textsuperscript{12} Likewise, acute administration of IGF-1 has been shown to increase cardiac output in normal humans\textsuperscript{13} and, recently, in subjects with heart failure.\textsuperscript{14} These observations open new possibilities with regard to the potential therapeutic use of GH and IGF-1, which has been currently focused on growth failure, catabolic states, renal failure, and, recently, diabetes mellitus.\textsuperscript{15,16} Nonetheless, the mechanism(s) of action of GH and IGF-1 on heart function is largely obscure.

As to chronic conditions of GH and IGF-1 excess, recent evidence suggests that peripheral vasodilatation,\textsuperscript{17} cardiac growth,\textsuperscript{5,6} and increased response of the myofilament apparatus to Ca\textsuperscript{2+} may contribute to the positive inotropic action mediated by the GH/IGF-1 axis. Specific changes at the myofibrillar level occurring during GH-induced myocardial growth have been postulated to play a role in the observed increase of myofilament Ca\textsuperscript{2+} sensitivity.\textsuperscript{18,19} Data dealing with acute effects of GH and IGF-1 on isolated heart preparations are very limited, as these drugs are generally administered for their chronic effects. Such studies would enhance our understanding of GH and IGF-1 mechanisms of action because their growth-promoting and periph-

Received November 24, 1997; accepted April 17, 1998.

From the Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory Cardiovascular Division (A.C., Y.I., H.S., P.S.D., J.P.M.) and Endocrinology (A.C.M.), Beth Israel Deaconess Medical Center, Boston, Mass; the NMR Laboratory for Physiological Chemistry (M.S., J.S.I.), Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass; and Genentech Inc (R.C.), South San Francisco, Calif.


Correspondence to Antonio Cittadini, MD, Department of Internal Medicine (III Division), Federico II Medical School, Via S. Pansini, 5, 80131 Naples, Italy. E-mail cittadin@unina.it

© 1998 American Heart Association, Inc.
eral vasodilatory properties would be separated from possible direct effects on the contractile apparatus. In this regard, several lines of experimental evidence support the concept that IGF-1 may influence myocardial contractility directly. First, a preliminary study by Vetter et al. reveals that IGF-1 increases the contractility of isolated neonatal rat cardiomyocytes. IGF-1 receptors are expressed on neonatal and adult rat cardiomyocytes, and stimulation of these receptors with exogenous IGF-1 rapidly activates multiple signal transduction pathways and leads to the accumulation of inositol 1,4,5-trisphosphate. Contrary to the situation with IGF-1, it remains inconclusive whether GH exerts direct action on the heart. In fact, although Hjalmanson et al. demonstrated that GH increases the amino acid transport in the isolated heart of hypophysectomized rats, a recent study failed to observe any effect of GH administration on protein synthesis in isolated neonatal cardiomyocytes. However, the presence of GH receptors on the heart and the differential effects of GH and IGF-1 on cardiac structure raise the possibility that GH may affect cardiac function directly, independent of circulating or locally produced IGF-1.

No study has systematically investigated and compared the acute effects of GH and IGF-1 on myocardial contractility and Ca2+ handling in isolated cardiac muscle preparations, independent of the confounding effects of cardiac growth and peripheral vasodilation found in vivo. Therefore, to address these issues, GH and IGF-1 were acutely administered in 2 in vitro preparations: the isovolumic buffer-perfused rat whole heart and the isometric ferret papillary muscle. In these preparations, GH produced no direct effects on contractility, whereas IGF-1 increased the force of contraction by 22% to 24% at a concentration of 10−7 mol/L. Thus, further experiments were performed with acute infusion of IGF-1 to investigate its mechanisms of action. Specifically, experiments were designed (1) to ascertain the changes in Ca2+ handling potentially responsible for the positive inotropic actions of IGF-1 by measuring [Ca2+]i, using the photoprotein aequorin during acute IGF-1 infusion and by evaluating the maximal response and the sensitivity of the myofilaments to Ca2+ responsiveness by plotting various [Ca2+]i levels and the corresponding force under steady-state conditions in tetanized muscles, (2) to assess pH and the possible linkage between mechanical changes and high-energy phosphate content using 31P-NMR spectroscopy, since intracellular alkalosis is a well-known sensitizer of myofilaments to Ca2+; (3) to determine the role of free IGF-1 in mediating these effects by investigating the role of the IGFBP-3, the major carrier of IGF-1 in the circulation, and (4) to test the hypothesis that the intracellular signaling cascade mediating IGF-1–induced positive inotropic effects would involve either the PI3-kinase or the protein kinase C pathways. The rationale for testing these intracellular pathways is provided by the following considerations: (1) PI3-kinase activity increases after IGF-1 application in isolated cardiomyocytes and is involved in the antiapoptotic properties of IGF-1, and (2) the phosphoinositidase second-messenger cascade is also activated by IGF-1 in cardiac tissue and its bifurcated end products, inositol phosphate production and, in particular, protein kinase C activation, may potentially increase cardiac contractility.

Materials and Methods

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) with body weights ranging from 340 to 570 g were used for the whole-heart experiments. Papillary muscles isolated from male ferrets aged 12 to 14 weeks with a body weight ranging from 1.0 to 1.4 kg were used for a second set of experiments. All the methods described are consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association and conform to the requirements of the American Heart Association. The studies were approved by the Animal Care Committee of Beth Israel Deaconess Medical Center. To allow a consistent interpretation of the data, we used similar conditions in all the experiments involving rat isolated isovolumic buffer-perfused whole-heart preparations in the Langendorff mode for both aequorin and NMR spectroscopy experiments. An additional set of experiments was performed using ferret papillary muscle for the following reasons: (1) to compensate for the limited availability of IGFBP-3 (in this experimental setting, the volume of the bathing medium is significantly smaller than that in the isolated whole heart), (2) to confirm the effects of IGF-1 in a different experimental setting and animal species, and (3) to exclude the possibility that coronary artery dilatation due to direct IGF-1 vasodilatory properties had mediated the positive inotropic effect observed.

Drugs

Recombinant human GH, IGF-1, and IGFBP-3 were kindly provided by Genentech (South San Francisco, Calif). After achieving steady-state conditions, hormones and chemicals were added to the bathing solution of the whole heart and/or of the papillary muscle starting at a concentration of 10−10 mol/L and increasing in a stepwise fashion until the maximal effect was reached. IGFBP-3 was added to the papillary muscle superfusion medium before exposure of the preparation to IGF-1 to test its effects on isometric tension. The protein kinase C inhibitor chelerythrine chloride (LC Laboratories) at a concentration of 10−6 mol/L and the PI3-kinase inhibitor wortmannin at a concentration of 10−7 mol/L (Sigma Chemical Co) were added to the superfusate alone and then before exposure of the preparation to IGF-1. In another subset of experiments, a concentration-response curve of the β-adrenergic agonist isoproterenol was obtained with and without IGFBP-3 (10−7 mol/L) and wortmannin (10−7 mol/L) in the superfusate.

Perfusion Technique

Rats were killed, and the isolated hearts were placed in a isovolumic buffer-perfused preparation according to the Langendorff technique, as previously described. Briefly, the rats were anesthetized with ether, followed by an injection of 200 IU heparin in the femoral vein. One minute later, the thorax was opened, and the heart was quickly removed and put into ice-cold Krebs-Henseleit solution (see below), weighed, and mounted on a cannula inserted into the ascending aorta. Retrograde aortic perfusion of the coronary arteries was performed within 30 seconds after the thoracotomy via a constant flow of 10 mL/min per gram heart weight. Pressure was monitored by a Statham...
IGF-1 and Myocardial Contractility

P23Db transducer. This flow rate was chosen since preliminary experiments with graded ischemia performed by Apstein et al.34 have demonstrated an aerobic pattern of lactate consumption. Cardiac temperature was set at 25°C, measured by a temperature probe inserted into the right ventricle. The composition of the perfusate was as follows (mmol/L): NaCl 118, KCl 4.7, KH2PO4 1.2, CaCl2 1.5, MgCl2 1.2, NaHCO3 23, and dextrose 5.5, saturated with a 95% O2/5% CO2 gas mixture to a pH of 7.4±0.2. LV pressure was measured using a fluid-filled latex balloon inserted into the LV via the mitral valve. After an equilibration period of 15 to 30 minutes at 25°C, the temperature was gradually increased to 30°C, and the hearts were paced at 3 Hz. Measurements of LV function were obtained when the preparation achieved a steady state after instrumentation (~15 minutes); the balloon volume was inflated to achieve a diastolic pressure of 2 to 4 mm Hg in all the animal groups.

The digital signal of the LV pressure tracing was further analyzed using customized software4 to obtain the following parameters: peak LV systolic pressure, LV end-diastolic pressure, LV developed pressure, time to peak pressure, time from peak systolic pressure to 90% of relaxation, time constant of exponential pressure decay using the variable asymptote method4 and maximum and minimum values of the first pressure derivative with respect to time.

Aequorin Loading

Aequorin loading was performed as described previously.4 Briefly, 3 to 5 μL of an aequorin-containing solution (1 μg/mL) was macro-injected into the interstitium of the inferior apical region of the LV. The heart was then positioned in an organ bath with the aequorin-loaded area of the LV directed toward the cathode of a photomultiplier (model 9635QA, Thorn-EMI, Gencom Inc) and submerged in Krebs-Henseleit solution. The organ bath was enclosed in a light-occlusive photographic bellows designed for studies with aequorin-loaded muscles by Blinks35 and modified for whole-heart studies by Kihara et al.36 Five to 10 minutes after loading, which was performed under Ca2+-free conditions, CaCl2 was gradually added to the coronary perfusate up to a total [Ca2+]i of 1 mmol/L. The temperature was increased to 30°C within 5 minutes, and the heart was paced at 3 Hz. The experimental protocol was started 5 to 10 minutes after a steady state of the mechanical parameters was reached.

Quantification of Intracellular Ca2+

Aequorin light signals were recorded on a 4-channel recorder in parallel with the LV pressure and coronary perfusion pressure tracings and were digitized as described above for the LV pressure tracings. At each step of perfusate Ca2+3.50 to 60 light transients were wave-averaged, and values were converted into [Ca2+]i using the method of fractional luminescence as described previously.6,33,35 At the end of each experiment, the heart was perfused with a solution containing 20 mmol/L Ca2+ and 5% Triton X-100 to lyse the aequorin-loaded cells and expose all of the remaining aequorin to the variable asymptote method. This resulted in an instantaneous burst of light, subsequently declining to baseline within 10 to 20 minutes. The area under the curve was integrated to obtain a value for Lmax. The ratio of the light versus Lmax is the fractional luminescence, which was converted into [Ca2+]i, by the use of a calibration curve derived in vitro. The wave-averaged signals were analyzed for peak systolic Ca2+, diastolic Ca2+, time to peak light, time from peak light to 50% of light decay, and time constant of exponential light decay, τ, using the variable asymptote method.

Analysis of the [Ca2+]i-Response Relationship

The response of the myofilament apparatus to Ca2+ consists of 2 terms: the myofilament Ca2+ sensitivity, which describes the affinity of the contractile apparatus for [Ca2+]i over the range of contractile responsiveness to [Ca2+]i, (the EC50 of the [Ca2+]i-response relationship), and the maximal Ca2+-activated force, which determines the amplitude of the contractile response.5,36 Both components were evaluated in the isolated whole heart by steady-state pressure-Ca2+ relations obtained by eliciting tetanus with rapid pacing after exposure to ryanodine, according to a previously described technique.35,36 Ryanodine at 10−7 mol/L (Calbiochem) was added to the perfusate to inhibit sarcoplasmic reticulum function. Typically, LV function declined gradually, reaching a new steady state after ~20 minutes. Tetanus was then elicited by 4 seconds of high-frequency electrical stimulation (15 Hz) with a pulse width of 50 milliseconds at 1.5 to 2.0 times the threshold. Pacing was discontinued in the intervals between tetanus, and the heart beat spontaneously at a rate of 60 to 90 bpm. Tetanic pressures peaked after ~2 seconds, and tetanic light signals reached a plateau after 2 to 3 seconds. Tetanic responses at 0.5, 1.0, 2.0, 4.0, and 6.0 mmol/L [Ca2+]i with repeated and averaged (3 to 5 tetani) measurements for peak tetanic pressure and light signals at each Ca2+ level were assessed. To avoid precipitation of Ca2+ salts at higher levels of [Ca2+]i, the perfusate was replaced by a phosphate-free solution. Preliminary data from our laboratory show that elevation of Ca2+ beyond 6 mmol/L generally fails to increase the twitch in rat myocardium, sometimes even diminishing the developed force. In a preliminary set of experiments, we also determined the [Ca2+]i-response relationships without eliciting tetanus. In twitching muscles, developed force at each [Ca2+]i was lower than under tetanic conditions, consistent with the hypothesis that tetanic force developed under high [Ca2+]i is indeed the maximal force of which the heart is capable. To obtain sensitivity (EC50) and maximal Ca2+-activated pressure of the [Ca2+]i-response relationship, peak tetanic pressure was plotted against peak systolic Ca2+ and fitted to the following function:

$$P(t) = \frac{\text{max } P_t}{1 + 10^{(\text{EC50}-z)}}$$

where P(t) is peak tetanic pressure, max P_t is maximum P_t, and Ca_s is peak systolic intracellular calcium, with EC50=a/b using nonlinear regression.

Since it was not possible to perform 2 separate [Ca2+]i-response curves under tetanic conditions in each heart before and after IGF-1 exposure, indices of myofilament Ca2+-responsiveness were compared between IGF-1–treated hearts (n=10) and isoproterenol (ie, running under identical experimental conditions during the same general period) control hearts (n=10).

Papillary Muscle Studies

Papillary muscles (n=16; diameter, <1 mm) were isolated from the right ventricles of young adult male ferrets (aged 12 to 14 weeks, with a body weight ranging from 1 to 1.4 kg) and mounted vertically between a miniature clamp and an isometric force transducer with a 9-0 Tevdek thread.37 The muscles were stimulated with a voltage 10% above threshold, and 5-millisecond square-wave pulses at 0.33 Hz were applied through a platinum electrode located at the lower end of the muscle, just above the muscle clamp. The experiments were conducted in the presence of 6×10−7 mol/L (±)-propranolol to prevent the effects of any endogenous norepinephrine that might be liberated by the driving stimulus. The physiological salt solution was of the following composition (mmol/L): NaCl 120, KCl 5.9, glucose 11.5, NaHCO3 25, NaH2PO4·H2O 1.2, MgCl2 2.0·6H2O 1.2, and CaCl2 2.5. The solution was bubbled continuously with 95% O2/5% CO2 at 30°C and equilibrated to a pH of 7.4. The muscles were stretched to the length at which there was no further increase in peak active force. Each muscle was stabilized for ~1 hour before the protocols were begun. After the dose-response relationships to IGF-1 (n=8) and IGFBP-3 (n=4) were obtained, the effects of IGF-1 (10−7 mol/L) was tested in the presence of IGFBP-3 (10−7 mol/L, n=4), chelerythrine chloride (10−7 mol/L, n=4), and wortmannin (10−7 mol/L, n=4). Moreover, isoproterenol concentration-response curves were assessed both alone (n=5) and in the presence of IGFBP-3 (10−7 mol/L, n=4) and wortmannin (10−7 mol/L, n=4).

31P-NMR Spectroscopy

Isovolumic isolated heart preparation and instrumentation were the same as described for the aequorin measurements, except a phosphate-free buffer was used.31P-NMR spectra were obtained at...
TABLE 1. Isolated Whole-Heart Mechanical Data Before and After IGF-1

<table>
<thead>
<tr>
<th>Pressure</th>
<th>Baseline</th>
<th>After IGF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic, mm Hg</td>
<td>124±4</td>
<td>153±3*</td>
</tr>
<tr>
<td>Diastolic, mm Hg</td>
<td>3±0.7</td>
<td>3±0.6</td>
</tr>
<tr>
<td>Developed, mm Hg</td>
<td>121±4</td>
<td>150±3*</td>
</tr>
<tr>
<td>+dP/dt, mm Hg/s</td>
<td>2614±107</td>
<td>3068±111*</td>
</tr>
<tr>
<td>−dP/dt, mm Hg/s</td>
<td>1444±58</td>
<td>1663±60*</td>
</tr>
<tr>
<td>TP, ms</td>
<td>82±3</td>
<td>85±3*</td>
</tr>
<tr>
<td>T90, ms</td>
<td>116±3</td>
<td>127±3*</td>
</tr>
<tr>
<td>r, ms</td>
<td>44±2</td>
<td>46±2</td>
</tr>
<tr>
<td>CPP, mm Hg</td>
<td>75±2</td>
<td>74±2</td>
</tr>
</tbody>
</table>

TP indicates time to peak pressure; T90, time to 90% relaxation; r, time constant of exponential LV pressure decay; and CPP, coronary perfusion pressure. Values are mean±SEM (n=10).

*P<0.05 vs baseline values.

161.94 MHz on a GE-400 Omega spectrometer. The heart was placed in an NMR sample tube and inserted into a 1H/31P double-tuned probe, which was situated in a 20-mm bore, 9.4-T superconducting magnet. Temperature was maintained at 30°C using a variable temperature controller. Spectra were collected without proton decoupling at a pulse width of 6000 Hz and obtained by averaging the signals from 104 free induction decays in a 4-minute period. Spectra were subsequently analyzed using 20-Hz exponential multiplication and zero and first-order phase corrections. Each resonance peak was fitted to a lorentzian function, and the area under each peak was calculated using the commercially available program NMR1 (NMRi). By comparing the peak areas of fully relaxed spectra (recycle time, 10 seconds) and those of partially saturated spectra (recycle time, 2.14 seconds), the correction factors for saturation were calculated for [γ-P]MgATP (1.0), [PCr] (1.2), and [P] (1.15). The value of 10.8 mmol/L for [ATP] was used to calibrate the [γ-P]MgATP peak area of the NMR spectrum obtained during the initial equilibration period. Changes in [MgATP], [PCr], and [Pi] during the protocol were calculated by multiplying the ratio of their peak areas to the area of [γ-P]MgATP from the initial baseline spectrum by 10.8 mmol/L. pH was determined by comparing the chemical shift of P and PCr in each spectrum with values from a standard curve. P (10 mmol/L, KH2PO4), dissolved in a solution of high osmolarity, was titrated over the pH range of 5.95 to 7.6 to generate the standard curve. Under the experimental conditions used in the present study, 31P-NMR is accurate enough to detect changes of ±0.01 in pH units.

After being placed in the magnet, each heart underwent a stabilization period of ~20 minutes. Baseline data defining cardiac performance, pH, and high-energy phosphate content were then collected during the next 16 minutes. Then one group of hearts (n=5) was perfused with buffer containing IGF-1 (10−7 mol/L), and LV function and 31P-NMR spectra were obtained at 4-minute intervals for 20 minutes, providing 5 spectra for each heart. In 3 other hearts, the baseline data acquisition was followed by a 20-minute perfusion period with unchanged buffer.

Statistical Analysis

Data are reported as mean±SEM. Comparisons of the maximal Ca2+-activated pressure and the EC50 of the Ca2+-force relationship between IGF-1-treated and isochronic control hearts were performed using the unpaired Student t test. All the other variables related to the mechanical twitch and to the Ca2+ transient before and after IGF-1 exposure were compared by using the 2-tailed paired Student t test. A P<0.05 was considered significant.

Results

Effects of GH and IGF-1 on Cardiac Function

GH had no effects on cardiac function. Isolated whole hearts responded to IGF-1 at a concentration of 5×10−8 mol/L, with the maximal response observed at 10−7 mol/L (Table 1, Figure 1). The onset of the response was fast, usually taking 60 seconds from the application, reaching a plateau after 5 to 10 minutes, and remained stable for the rest of the experiment. The average increase in developed pressure was 24% from baseline, which was solely due to an increase in systolic pressure. Ferret papillary muscles exhibited a similar concentration-effect relationship, with a maximal increase of developed tension of ~22% above baseline (Figure 1). The physiological effects of IGF-1 could not be washed out in these experiments in either papillary muscle or isolated whole-heart preparations. As for isovolumic developed pressure, isometric developed tension did not change significantly after application of GH (data not shown). To avoid significant aequorin consumption, we chose to use relative hypothermic conditions in our experiments. However, to test whether the

![Figure 1. Concentration-effect relationships of GH and IGF-1 in rat isolated whole hearts (n=10) (top) and of IGF-1 (n=8), IGFBP-3 alone (n=4), and IGFBP-3 plus IGF-1 (n=4) in isolated isometric ferret papillary muscles (bottom). Contractile response is displayed as percentage above baseline values. Each point with bars represents mean±SEM. *P<0.01 vs baseline values.](http://circres.ahajournals.org/DownloadedFrom)
Effects of IGF-1 were of similar magnitude under normothermia, 5 hearts were perfused in the Langendorff mode at 37°C. IGF-1 infusion at a concentration of $10^{-7}$ mol/L increased developed pressure from 109±9 to 137±10 mm Hg ($P<0.001$); the percentage increase (25%) was in the same range as observed at 30°C. Analysis of the time course of the twitch revealed that the time to peak pressure was longer following IGF-1 infusion, as observed with other Ca$^{2+}$ sensitizers. Time to 90% of relaxation were also significantly increased, whereas the $\tau$ value, a more accurate index of relaxation, did not differ significantly from baseline (Table 1). Peak positive and peak negative dP/dt were significantly higher than pretreatment values, suggesting enhanced contractility and relaxation. Coronary perfusion pressure in the whole heart did not change significantly. No significant differences were observed as to the time course and the amplitude of the response when either rat whole heart or ferret papillary muscle was used.

Effects of GH and IGF-1 on [Ca$^{2+}$], Transients

The subcellular mechanisms of the inotropic effects of IGF-1 were investigated in aequorin-loaded whole-heart preparations in which [Ca$^{2+}$], transients and the corresponding pressure tracings were simultaneously recorded. Figure 2 shows a typical [Ca$^{2+}$] transient and the corresponding LV pressure tracing at baseline perfusate Ca$^{2+}$. The first relevant finding was that the increase in developed pressure was not associated with an increase in the amplitude of the [Ca$^{2+}$], transient. On the contrary, there was a slight but consistent and significant decrease in peak systolic [Ca$^{2+}$], from 0.78±0.03 to 0.74±0.03 μmol/L, in the absence of changes of diastolic [Ca$^{2+}$], (Table 2). The time course of the transient was slightly but not significantly abbreviated after IGF-1 application. The $\tau$ value of the [Ca$^{2+}$], transient, which has been shown to reflect mostly the uptake of Ca$^{2+}$ by the sarcoplasmic reticulum, was significantly lower after IGF-1 application. Since activator Ca$^{2+}$ available to the myofilament was not increased but actually decreased, a major change in myofilament Ca$^{2+}$ responsiveness was expected. Indeed, this was the case. Maximal Ca$^{2+}$-activated pressure under tetanic conditions was significantly increased by 12%; the [Ca$^{2+}$]-response relationship was significantly shifted to the left, with $EC_{50}$ values decreased by $\approx14\%$, indicating an increase in Ca$^{2+}$ sensitivity (Figure 3). Figure 4 shows a representative tracing of intracellular Ca$^{2+}$ and of the corresponding pressure recorded under steady-state tetanic conditions in control and IGF-1–perfused hearts.

$^{31}$P-NMR Spectroscopy

In a separate group of hearts perfused in the same way as for the Ca$^{2+}$ measurements, IGF-1 perfusion increased the developed pressure from 116±5 to 139±8 mm Hg (21±2%, $P<0.001$). The increase was due solely to an increase in systolic pressure. pH, measured by $^{31}$P-NMR spectroscopy during the baseline perfusion was 7.15±0.01 and remained unchanged throughout the 20-minute period of IGF-1 perfusion. Analysis of the 3 control hearts showed that pH at baseline and during subsequent perfusion with buffer without IGF-1 also remained constant. The metabolites MgATP, P~o~ and PCr, also measured by $^{31}$P-NMR spectroscopy, remained unchanged during the protocol and were indistinguishable between control and IGF-1–perfused hearts (Table 3). Taken together, these results show no effect of acute IGF-1 infusion on either pH, or high-energy phosphate content in the isolated perfused rat heart.

IGFBP-3 Experiments

The role of the IGF binding proteins in modulating cardiac function is unknown, although a recent study has shown that IGFBP-3 was able to inhibit completely the hypertrophic response and the protein synthesis evoked by IGF-1 administration in neonatal cultured cardiomyocytes. IGFBP-3 alone administered to the ferret papillary muscle decreased isometric developed tension in a dose-dependent fashion, with a maximal reduction of 36% at $10^{-7}$ mol/L (Figure 1). Concomitant administration of IGFBP-3 and IGF-1 at equimolar concentrations ($10^{-7}$ mol/L) blocked almost completely IGF-1–positive inotropic effects, with a slight and not significant increase (5%) of isometric developed tension.
Moreover, the increase of developed tension induced by isoproterenol was similar with and without the presence of IGFBP-3 in the organ bath (Figure 5).

**Protein Kinase C and PI3-Kinase Inhibition**

Although chelerythrine chloride, a highly selective protein kinase C inhibitor (selectivity ratio, 260), at a concentration of $10^{-6}$ mol/L, did not affect the positive inotropic response elicited by IGF-1 application, wortmannin completely abolished the IGF-1–induced increase of developed tension (Figure 5). Furthermore, the isoproterenol concentration-response curve remained unchanged during concomitant wortmannin application to the superfusate, indicating that this signaling pathway was unaffected by wortmannin (Figure 5).

**Discussion**

The present study demonstrates that IGF-1 but not GH has a direct inotropic effect on the mammalian myocardium. The studies in isolated heart preparations and in papillary muscle demonstrate that the positive inotropic action of IGF-1 does not depend solely on its ability to induce cardiac hypertrophy with longer term administration in vivo. The effect of IGF-1 on cardiac contractility is blocked by IGFBP-3, which, per se,

### Table 2. Measurements of [Ca²⁺], Handling Before and After IGF-1

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After IGF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic, μmol/L</td>
<td>0.78±0.03</td>
<td>0.74±0.03*</td>
</tr>
<tr>
<td>Diastolic, μmol/L</td>
<td>0.35±0.001</td>
<td>0.35±0.001</td>
</tr>
<tr>
<td>TP&lt;sub&gt;Ca&lt;/sub&gt;, ms</td>
<td>47±2</td>
<td>44±2</td>
</tr>
<tr>
<td>T50&lt;sub&gt;Ca&lt;/sub&gt;, ms</td>
<td>42±1</td>
<td>41±4</td>
</tr>
<tr>
<td>TP&lt;sub&gt;Ca&lt;/sub&gt;+T50&lt;sub&gt;Ca&lt;/sub&gt;, ms</td>
<td>90±3</td>
<td>85±3</td>
</tr>
<tr>
<td>τ&lt;sub&gt;Ca&lt;/sub&gt;, ms</td>
<td>39±5</td>
<td>30±2*</td>
</tr>
</tbody>
</table>

TP indicates time to peak light; T50, time from peak light to 50% of light decay; and τ, time constant of exponential light decay. Values are mean±SEM (n=10).

**Figure 3.** Steady-state pressure-[Ca²⁺]<sub>i</sub> relations in IGF-1-perfused whole-heart preparations (dashed lines, n=10) and isochronic controls (solid lines, n=10). In the top panel, peak tetanic pressure is plotted against [Ca²⁺]<sub>i</sub> during the tetanic plateau phase of the light signal. In the bottom panel, peak tetanic pressure is normalized to the maximal response and fitted to the function described in the text to demonstrate a shift of EC<sub>50</sub>. Values of maximal Ca²⁺-activated tetanic pressure were (mean±SEM) 211±3 vs 235±2 mm Hg in control and IGF-1-perfused hearts, respectively; EC<sub>50</sub> was 0.52±0.03 vs 0.44±0.02 μmol/L in control and IGF-1-perfused hearts, respectively (P<0.01 for both).

**Figure 4.** Representative tracing of tetanic [Ca²⁺]<sub>i</sub> (top) and tetanic pressure (bottom) in IGF-1-perfused (dashed lines) and control (solid lines) whole hearts. Tetanus was triggered by 4 seconds of electrical stimulation at 15 Hz, with a pulse width of 50 milliseconds. [Ca²⁺]<sub>i</sub> in the perfusate was 6.0 mmol/L.
has a negative inotropic effect in the cardiac isolated muscle. This may in part reflect a role for endogenous IGF-1 production by cardiac myocytes in determining myocardial contractile response. The acute enhancement of contractility observed after IGF-1 administration does not depend on any increment of Ca\(^{2+}\) activator available to the contractile apparatus but is secondary to an augmented myofilaments responsiveness to Ca\(^{2+}\). The latter mechanism of IGF-1 action does not appear to be induced by intracellular alkalosis or by significant protein kinase C activation, whereas evidence supports the involvement of a wortmannin-sensitive pathway.

Cardiovascular Actions of GH/IGF-1

The absence of acute effects of GH on cardiac function on the one hand and the positive effects of IGF-1 on cardiac contractility on the other provide some insight into the complex interaction between the GH/IGF-1 axis and cardiovascular function. Although GH receptors have been demonstrated on the heart,\(^{26}\) their physiological role probably does not include acute modulation of myocardial contractility on the basis of the present observations. These receptors may mediate other functions, such as protein synthesis, or they may stimulate local IGF-1 production. On the other hand, circulating IGF-1 might also act as an endogenous regulator of myocardial contractility. The rapid onset, the long duration of effect, and the relatively modest magnitude of its action compared with other endogenous substances suggest that if IGF-1 has any acute cardioregulatory role, it may contribute to the modulation of the inotropic responsiveness of the myocardium over a time frame of minutes to hours, sensitizing the myofilaments to rises in [Ca\(^{2+}\)], induced by more potent but short-lived neurohumoral factors.

Whatever role IGF-1 may play in regulating myocardial contractility, the present study also demonstrates that the positive inotropic actions secondary to conditions of GH and IGF-1 excess are not solely due to the activation of cardiac growth or to peripheral vasodilatation. We\(^{5,6}\) and others\(^{8,18,19}\) have consistently demonstrated that conditions of GH and IGF-1 excess are associated with a hypertrophic response, enhanced cardiac function, and changes in Ca\(^{2+}\) handling. The increase in maximal active tension observed in these models points to an increased number of active crossbridges. This, in turn, has been proposed to depend on a distinct type of myocardial growth triggered by the activation of the GH/IGF-1 axis and capable to interfere with myofibrillar spatial organization, functional characteristics of the crossbridges (eg, unidentified alteration of the phenotype of proteins of the thin filament), or recruitment of previously “silent” myosin heads.\(^{18}\) Our findings of an increased contractility in an acute setting demonstrate that IGF-1–induced myofilament sensitization to intracellular Ca\(^{2+}\) may also occur independent of myocardial growth. The acute effects of IGF-1 suggest that these 2 mechanisms of enhanced cardiac function, ie, myocardial growth and myofilament sensitization to intracellular Ca\(^{2+}\), are independent, since protein synthesis occurs in a time frame far longer than the minutes needed for the acute effects observed in the present study.

Circulating IGF-1, mainly synthesized by the liver under GH control, is bound to several specific carrier proteins, of which IGFBP-3 is the most abundant in serum. Although the physiological role of the IGFBPs is complex and poorly understood, most data support the notion that IGFBP-3 restrains IGF-1 action in vitro and in vivo and blocks the interaction of IGF-1 with its receptors. Despite a large body of literature concerning the endocrine role of IGF carrier proteins,\(^{39}\) their relevance for the cardiovascular system is unknown. In the present study, we extend observations of the effects of IGFBP-3 on other systems to the heart by demonstrating that this binding protein almost completely blocks the effects of IGF on contractility. This inhibition appears specific for IGF-1, since the isoproterenol-stimulated actions are

### TABLE 3. \(^{31}\)P-NMR Data

<table>
<thead>
<tr>
<th></th>
<th>P, mmol/L</th>
<th>PCr, mmol/L</th>
<th>ATP, mmol/L</th>
<th>pH i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.6±0.7</td>
<td>18.1±2.8</td>
<td>10.3±0.2</td>
<td>7.15±0.01</td>
</tr>
<tr>
<td>After IGF-1</td>
<td>4.4±0.6</td>
<td>17.2±2.7</td>
<td>10.4±0.2</td>
<td>7.15±0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n = 5).

**Figure 5.** Top, Concentration-effect relationships of IGFBP-3 (n = 4), IGFBP-3 plus isoproterenol (n = 3), and isoproterenol alone (n = 4) in isolated isometric ferret papillary muscles. Bottom, Concentration-effect relationships of IGF-1 plus chelerythrine (n = 4), IGF-1 plus wortmannin (n = 4), isoproterenol alone (n = 4), and isoproterenol plus wortmannin (n = 4). Contractile response is displayed as percentage above baseline values. Each point with bars represents mean±SEM. *P<0.01 vs baseline values.
not influenced by IGFBP-3. The observation that IGFBP-3 alone elicits a negative inotropic response in a concentration-dependent fashion was novel and unexpected. Binding and consequent inactivation of locally produced (paracrine/autocrine) IGF-1 by exogenous IGFBP-3 is one possible explanation for this finding. However, we cannot exclude the possibility that the IGFBP-3–induced decrease of developed tension may be in part independent of IGF-1 inhibition. In this regard, it has been shown that IGFBP-3 may directly inhibit cell growth, independent of IGF-1 binding, possibly through specific cell surface receptors. A generalized IGFBP-3–induced toxic effect, particularly at high concentrations, may also explain the observed negative inotropic action. Future studies are needed to elucidate this novel finding.

**Ca^{2+}-Handling Alterations Induced by IGF-1**

The contractile state of the muscle can be altered by the following mechanisms: “upstream” mechanisms that alter the amplitude or time course of the [Ca^{2+}] transient and/or alter the affinity of troponin C for Ca^{2+} or “downstream” mechanisms that alter the response of the myofilaments to a given level of occupancy of the Ca^{2+} binding sites on troponin C. Viewed from this perspective, the changes of the Ca^{2+} transients observed in the present study after the application of IGF-1 exclude upstream mechanisms, since the amplitude of the transient was significantly decreased compared with baseline. Therefore, the most likely mechanism is a combination of an increased affinity of troponin C for Ca^{2+} and mechanisms downstream from the troponin C complex. The first is suggested by the decreased amplitude of the Ca^{2+} transients combined with a tendency toward a shorter transient and a prolonged corresponding twitch; the shift of the Ca^{2+}-force relationship to the left is consistent with this hypothesis. On the other hand, the increase in maximal Ca^{2+}-activated force observed after IGF-1 application to the perfusate speaks in favor of downstream mechanisms, i.e., changes of myofilament kinetics after Ca^{2+} binding to troponin C. It is worth noting that few drugs act only by increasing myofilament Ca^{2+} sensitivity and maximal Ca^{2+}-activated force, without increasing but instead slightly reducing [Ca^{2+}].

Another distinctive feature of IGF-1 inotropic action is that the increase in the amplitude of the mechanical twitch is accomplished without significant impairment of the myocardial relaxation, as shown by the similar values of the mechanical twitch and after IGF-1 application. This is further supported by the observation that intracellular Ca^{2+} decline is abbreviated, as shown by lower values of the Ca^{2+} transients after IGF-1 application. On the other hand, the value of the Ca^{2+} transient has been proposed to depend primarily on sarcoplasmic reticulum Ca^{2+}-ATPase, the sarcolemma Na^{+}-Ca^{2+} exchanger, and buffering of Ca^{2+} by intracellular proteins, and its prolongation often precedes and causes abnormal myocardial relaxation. These findings are at variance with other known myofilament Ca^{2+} sensitizers, whose action is almost invariably associated with variable degrees of myocardial relaxation impairment and slowed intracellular Ca^{2+} decline.

These results differ from those we described recently after chronic GH and IGF-1 treatment in normal rats, in which there was an increase in maximal Ca^{2+}-activated force of the myofilaments, but the sensitivity was slightly reduced, as shown by higher EC_{50} values in GH and IGF-1–treated animals. There are several possible explanations for this apparent discrepancy. First, the model system used is different: the present study describes an acute in vitro system, whereas we had previously infused GH and IGF-1 chronically in vivo, with attendant changes of loading conditions and the activation of somatic and cardiac growth. Second, an accurate assessment of myofilament Ca^{2+} responsiveness was hampered in our previous study by the absence of steady-state Ca^{2+}-force relationships. In this respect, it has been shown that in twitching muscle the peak force–peak Ca^{2+} relationship does not accurately reflect changes at the level of the myofilament, particularly in the presence of differences in the time course of the Ca^{2+} transients. However, it is important to stress that although both studies suggest an increase in Ca^{2+} responsiveness, the explanations in the present study for the mechanisms of the acute effects of IGF-1 and GH may not apply with chronic use, because the actions on cardiac myofibrillar growth are lacking.

Taken together, it appears that the inotropic mechanism of action of IGF-1 is unique, since it qualitatively differs from the many other positive inotropic endogenous factors, such as α- or β-adrenergic agonists or endothelin, or drugs, such as digitalis or caffeine, which variably increase [Ca^{2+}], in addition to inducing changes in myofilament response to Ca^{2+}.

**pH and High-Energy Phosphate Content, Protein Kinase C, and PI3-Kinase Pathways**

The myofilament-sensitizing effect observed after IGF-1 exposure may represent the final result of several intracellular signaling pathways activated by the IGF-1–receptor complex. Although these pathways are largely unknown, experimental evidence has identified several major substrate proteins activated by IGF-1 binding to its cognate receptor: (1) IRS-1, (2) the Shc proteins, and (3) Crk, a cellular homologue of v-crk. IRS-1, in turn, binds the p85 β-subunit domain of PI3-kinase. In this regard, it has been recently demonstrated that the PI3-kinase pathway is activated by IGF-1 in rat cardiomyocytes and is involved in IGF-1–induced prevention of apoptosis in differentiated PC12 cells. In addition to the activation of IRS-1, Shc, and Crk, other second messengers may be generated by IGF-1 receptor activation, including the phospholipase C pathway. In fact, IGF-1 stimulation leads to a rapid accumulation of inositol phosphates and corresponding increases in cytoplasmic Ca^{2+} in thyroid cells. Activation of phospholipase C and rapid elevation of intracellular inositol phosphate levels have been reported in cardiac cells. Linkage of this signaling cascade to the modulation of cardiac contractility is provided by the observation that intracellular alkalosis, which sensitizes the myofilaments to Ca^{2+}–inducing changes of Ca^{2+} transients similar to those observed in the present study, occurs through a parallel bifurcated pathway involving diacylglycerol and protein kinase C. Moreover, this latter pathway has been recently shown to mediate at least a
component of the positive inotropy associated with agents that stimulate phospholipid turnover.20

Despite this rationale, at least 2 lines of evidence appear to exclude a significant role of phospholipase C activation in mediating the enhanced response of the myofilament apparatus to Ca2+—(1) Significant intracellular alkalosis, which is known to follow protein kinase C activation and partly mediate the inotropic responses of other hormones such as endothelin,46 does not occur after exposure to IGF-1 as measured by 31P-NMR. (2) Protein kinase C inhibition with chelerythrine did not affect the IGF-1–induced increase of contractility.

On the other hand, the results of the present study strongly suggest the involvement of the PI3-kinase signaling pathway or of wortmannin-sensitive molecules in the mediation of the positive inotropy elicited by IGF-1. The recent demonstration that PI3-kinase activity is increased 2.5-fold after 5 minutes of incubation with 30 nmol/L IGF-1 in isolated cardiomyocytes22 supports the hypothesis that this signal transduction pathway, which plays a relevant role in the response of many hormones, may also mediate, at least in part, inotropic responses in the mammalian myocardium, possibly by interacting with the excitation-contraction coupling process.

Considering the complexity of the growth factor signaling cascade, with >500 genes regulated by numerous diverging and cross-talking pathways, future research is needed to better define the role of the PI3-kinase transduction pathway in the regulation of IGF-1 changes in inotropy and, in general, of all the events downstream from the IGF-1–receptor ligand.

Interestingly, high-energy phosphate content did not change in parallel with the change in cardiac work induced by IGF-1. This observation parallels the finding obtained in normal humans subjected to acute IGF-1 infusions, in whom peak oxygen consumption was not increased although cardiac output increased by 18%.13

Clinical Implications
The acute positive inotropic effects achieved without increasing [Ca2+], but by sensitizing the myofilaments to Ca2+ opens new scenarios for the use of recombinant human IGF-1 as a pharmacological agent in patients with heart disease. Most of the inotropic drugs currently available act to variably increase [Ca2+]27,28 which, in turn, may predispose the patients to develop arrhythmias, particularly under conditions of heart failure or ischemia. Therefore, although the use of IGF-1 in heart failure may be promising, other potential results could involve myocardial stunning, characterized by decreased Ca2+ responsiveness,29 or states of acute hemodynamic impairment, in which the combined vasodilatory and positive inotropic properties of IGF-1 obtained without increases in [Ca2+], might ameliorate overall cardiac function. To support this view, 2 recent studies have demonstrated that IGF-1 in normal humans and in patients with heart failure increases cardiac performance.13,14 In accordance with the present results, part of these positive inotropic effects could be due to a direct enhancement of the inotropism. However, future research is needed to test these working hypotheses.

Acknowledgments
This study was supported in part by National Institutes of Health grants HL-31117 and HL-511307-01 (Dr Morgan) and HL-52320 and AG-10829 (Dr Ingwall). Dr Spindler was the recipient of a research fellowship of the Deutsche Forschungsgemeinschaft.

References


41. Herbert JM, Augereau JM, Gleye J, Maffrand JP. Cherythrythrine is a potent and specific inhibitor of protein kinase C. Biochim Biophys Res Commun. 1990;172:993–999.


Insulin-like Growth Factor-1 but Not Growth Hormone Augments Mammalian Myocardial Contractility by Sensitizing the Myofilament to Ca$^{2+}$ Through a Wortmannin-Sensitive Pathway: Studies in Rat and Ferret Isolated Muscles

Antonio Cittadini, Yoshiki Ishiguro, Hinrik Strömer, Matthias Spindler, Alan C. Moses, Ross Clark, Pamela S. Douglas, Joanne S. Ingwall and James P. Morgan

_Circ Res._ 1998;83:50-59
doi: 10.1161/01.RES.83.1.50

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/83/1/50

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ 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 Research_ is online at:
http://circres.ahajournals.org//subscriptions/