Insulin Causes [Ca\(^{2+}\)]\(_i\)-Dependent and [Ca\(^{2+}\)]\(_i\)-Independent Positive Inotropic Effects in Failing Human Myocardium

Dirk von Lewinski, MD; Sebastian Bruns; Stefanie Walther; Harald Kögler, MD; Burkert Pieske, MD

Background—Insulin has been shown to exert positive inotropic effects in several in vitro and in vivo models, but signal transduction and substrate dependency remain unclear. We examined inotropic responses and signal transduction mechanisms of insulin in human myocardium.

Methods and Results—Experiments were performed in isolated trabeculae from end-stage failing hearts of 58 nondiabetic and 3 diabetic patients undergoing heart transplantation. The effect of insulin (0.3 and 3 IU/L) on isometric twitch force (37°C, 1 Hz) was tested in the presence of glucose or pyruvate as energetic substrate. Furthermore, intracellular Ca\(^{2+}\) transients (aequorin method), sarcoplasmic reticulum (SR) Ca\(^{2+}\) content (rapid cooling contractures), and myofilament Ca\(^{2+}\) sensitivity (semiskinned fibers) were assessed. In addition, potential signaling pathways were tested by blocking glycolysis, PI-3-kinase, protein kinase C, diacylglycerol kinase, insulin-like growth factor-1 receptors, or transsarcolemmal Ca\(^{2+}\) entry via the Na\(^+\)/Ca\(^{2+}\) exchanger. Insulin exerted concentration-dependent and partially substrate-dependent positive inotropic effects. The phosphatidylinositol-3-kinase inhibitor wortmannin and the Na\(^+\)/Ca\(^{2+}\) exchanger reverse-mode inhibitor KB-R7943 completely or partially prevented the functional effects of insulin. In contrast, insulin-like growth factor-1 receptor blockade, protein kinase C inhibition, and diacylglycerol kinase blockade were without effect. The inotropic response was associated with increases in intracellular Ca\(^{2+}\) transients, SR Ca\(^{2+}\) content, and increased myofilament Ca\(^{2+}\) sensitivity.

Conclusions—Insulin exerts Ca\(^{2+}\)-dependent and -independent positive inotropic effects through a phosphatidylinositol-3-kinase–dependent pathway in failing human myocardium. The increased [Ca\(^{2+}\)]\(_i\) originates at least in part from enhanced reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange and consequently increased SR-Ca\(^{2+}\) load. These nongenomic functional effects of insulin may be of clinical relevance, eg, during insulin-glucose-potassium infusions. (Circulation. 2005;111:2588-2595.)

Key Words: insulin ■ calcium ■ heart failure ■ myocardium ■ contractility

Patients with chronic heart disease often have insulin resistance, diabetes, or altered glucose metabolism.\(^1\) Insulin, a polypeptide of 51 amino acids, is known to regulate serum glucose levels, protein synthesis, and growth. Effects of insulin on myocardial function have been tested in several animal models under in vitro and in vivo conditions, and controversial results with both enhanced contractility and myocardial performance\(^2\)-\(^6\) or no functional effects\(^7\)-\(^8\) have been reported. Insulin infusions improved ventricular function in one study in humans\(^9\) but had no effect in another.\(^10\) In addition, the functional response to insulin may be different in diabetic versus nondiabetic animals\(^3\),\(^11\),\(^12\) and humans.\(^9\),\(^13\)

Recently, the onset of insulin resistance has been shown to coincide with progression from pressure-overload hypertrophy to dilatation.\(^14\) Whole-body insulin resistance is prevalent in congestive heart failure patients with either ischemic heart failure or idiopathic dilated cardiomyopathy.\(^15\),\(^16\) Therefore, insulin resistance may contribute to contractile dysfunction by genomic, metabolic, and direct functional effects. Despite the controversial reports on direct effects of insulin on myocardial function in mammalian myocardium, no data are available that directly test the inotropic response to insulin in human myocardium. In addition, the mechanisms of action of the functional effect of insulin in mammalian myocardium remains controversial.\(^5\) Under in vivo conditions, improved global ventricular function during insulin infusions\(^9\) might exclusively result from insulin-dependent arteriolar vasodilation with peripheral unloading\(^13\),\(^17\) and improved myocardial blood flow.\(^17\) Therefore, we directly assessed functional effects and mechanisms of action of insulin in isolated failing human myocardium. Our main findings were that insulin exerts both Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent positive inotropic effects. These functional effects were related to activation of phosphatidylinositol-3-kinase (PI-3-kinase) and, in part, reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange. These data help to improve our understanding of nongenomic hormonal effects in the human heart.
Methods

Human Myocardium

Experiments were performed in muscle strips obtained from 58 end-stage failing hearts (23 of ischemic origin and 35 of dilative origin; 50 males and 8 females, all without diabetes). Three additional hearts were obtained from patients with long-standing diabetes mellitus. The mean age of the patients was 51.8±2.3 years, and the mean ejection fraction before transplantation was 23.7±1.2%. The study protocol was approved by the local ethics committee, and all patients gave informed consent.

Muscle Strip Preparation

Small endocardial trabeculae were dissected from the left or right ventricle as described previously, connected to an isometric force transducer, and superfused with bicarbonate-containing Tyrode’s solution. Muscles were electrically stimulated at 1 Hz (37°C), and isometric contractions were recorded at optimum preload (Lmax). The resulting cooling contractures were elicited by a rapid decrease in the temperature of the muscle chamber from 37°C to 1°C by switching from a warm to a cold solution with solenoid pinch valves in the temperature of the muscle chamber from 37°C to 1°C by switching from a warm to a cold solution with solenoid pinch valves.

Rapid Cooling Contractures

Rapid cooling contractures (RCCs) were elicited by a rapid decrease in the temperature of the muscle chamber from 37°C to 1°C by switching from a warm to a cold solution with solenoid pinch valves at the bath inlet as previously described. The resulting cooling contracture is an index for sarcoplasmic reticulum (SR) Ca2+ content.

Aequorin Measurements

At steady state contractile function, the Ca2+-regulated bioluminescent photoprotein aequorin was microinjected into the quiescent muscle as described previously. Aequorin light emission was detected with a photomultiplier, which was vertically mounted with its cathode just above the glass cuvette that contained the muscle.

Semiskinned Fiber Preparations

Fibers were skinned by the technique and solutions described by Hambarchian et al. Saponin (50 μg/mL) was added for 45 minutes to functionally skin the trabeculae. This technique allows the control of intracellular ion concentrations but maintains sarcolemmal receptors and leaves subcellular signaling mechanisms intact.

Concentration-response curves for Ca2+ (tension-Ca2+ relationship) were obtained in each muscle, first without insulin and after complete reequilibration in the presence of 3 IU/L insulin.

Drugs

Insulin (Insumin Rapid 100 IU/mL, Hoechst Marion Russel) was used as supplied. Insulin-like growth factor-1 (IGF-1) receptor antibody (α-IR-3 clone; Oncogene Research Products) was dissolved as recommended by the manufacturer. KB-R 7943 (5 μmol/L; Tocris), diacylglycerol (DAG)-kinase inhibitor (1 μmol/L), cyclopiazonic acid (10 mmol/L), ryanodine (1 μmol/L), wortmannin (0.1 μmol/L), and iodoacetate (0.5 μmol/L; all from Sigma) were added to the organ bath 30 minutes before the experiment.

Statistical Analysis

Data are expressed as mean±SEM. Differences were compared by paired Student t test or 1-way repeated-measures ANOVA followed by Student-Newman-Keuls test when appropriate. Statistical significance was taken as P<0.05.

Results

Inotropic Effects of Insulin

Insulin exerted pronounced transient positive inotropic effects in isolated human cardiac muscle (Figure 1A) without changes in diastolic tension. These functional effects of insulin were concentration dependent in glucose-containing solution, with a maximum increase in developed force of 10.6±1.8% at low insulin concentrations (0.3 IU/L; n=8) and 28.1±5.7% (of basal twitch force, respectively) at high insulin concentrations (3 IU/L; n=8; Figure 1B). In pyruvate-containing solution, the relative increase in twitch force with insulin was smaller but independent of the insulin concentration used (10.5±3.2%, n=8 and 13±3.8%, n=9, respectively; Figure 1B). Developed force at baseline conditions was significantly higher in the pyruvate group (50.3±10.6
Influence of Insulin on Twitch Kinetics

<table>
<thead>
<tr>
<th></th>
<th>TPT</th>
<th>RT₅₀</th>
<th>RT₉₀</th>
<th>+dT/dtmax</th>
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<tr>
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<td>137±9</td>
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<td>Insulin 3 IU/L</td>
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<td>140±9</td>
<td>363±32</td>
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<tr>
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<td>603±23</td>
<td>253±75</td>
<td>204±59</td>
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<tr>
<td>Insulin 3 IU/L</td>
<td>279±11</td>
<td>182±8</td>
<td>581±23</td>
<td>279±80</td>
<td>214±59</td>
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TPT indicates time to peak tension (ms); RT₅₀, 50% relaxation from peak tension (ms); RT₉₀, 90% relaxation from peak tension (ms); +dT/dtmax, maximum rate of tension increase (mN/s/mm²); and −dT/dtmax, maximum rate of tension decline (mN ⋅ s⁻¹ ⋅ mm⁻²).

versus 25.7±6.5 mN/mm² in the presence of glucose). Insulin did not significantly affect twitch kinetics (Table) but tended to prolong relaxation times.

To further assess the underlying mechanisms of the substrate dependence, additional experiments were performed after glycolysis was blocked with iodoacetate (0.5 μmol/L). In muscles preincubated with higher concentrations of iodoacetate (1 μmol/L), muscles developed contracture within the first minutes. The insulin-mediated positive inotropic effect was reduced by ~50% in the glucose group in the presence of 0.5 μmol/L iodoacetate and was comparable to the insulin-mediated effect in the pyruvate group under this experimental condition. These data show that at least some of the insulin-dependent positive inotropic effects are not related to increased glycolysis.

Additional experiments were performed in 9 trabeculae from 3 hearts obtained from patients with diabetes mellitus. Insulin exerted identical inotropic effects in both glucose- and pyruvate-containing solutions compared with nondiabetic myocardium (at 3 IU/L, 33±13% [n=6] and 15.8±11% [n=3], respectively).

Ca²⁺-Dependent and Ca²⁺-Independent Effects of Insulin

All further experiments designed to test the subcellular mechanisms of action of insulin were performed in glucose-containing Tyrode’s solution at an insulin concentration of 3 IU/L. First, experiments were performed in aequorin-loaded muscle strips. Figure 2A shows original tracings of the effects of insulin on twitch force and aequorin light signals. The inotropic effect of insulin (23.2±10%; P<0.05) was associated with a parallel, albeit smaller increase in aequorin light emission (15.8±4.2% of the basal value, P<0.05; Figure 2B). These data demonstrate that the functional effects of insulin are related to increased [Ca²⁺], at least in part.

We then compared these data to results from an intervention that increases force by an exclusively Ca²⁺-dependent mechanism, i.e., elevated [Ca²⁺]o. Increasing [Ca²⁺]o, from 2.5 to 3.2 mmol/L (Figure 2B) resulted in an increase in twitch force by 23.7±4.3% (n=8; P<0.05) and an increase in aequorin light emission by 20.5±7.2% (P<0.05). Although not significantly different between groups, for an almost identical increase in force, the increase in aequorin light was smaller with insulin than with [Ca²⁺]o, 3.2 mmol/L. This was quantified by calculating the relative increase in force (F) versus aequorin light emission (L). ΔF/ΔL was 0.98±0.05 with elevated [Ca²⁺]o, and 1.34±0.18 with insulin (P<0.05). These findings, in association with the tendency to prolonged relaxation kinetics, may point to an additional Ca²⁺-sensitizing effect of insulin. Interestingly, we have previously shown that IGF-1 does not affect Ca²⁺ sensitivity compared with 4 mmol/L [Ca²⁺]o, (ΔF/ΔL was 1.02±0.38 and 1.03±0.12, respectively), but β-adrenergic stimulation reduced the relative increase in ΔF/ΔL to 0.56±0.11, as an index for SR Ca²⁺ uptake.19 The rationale for these experiments is that during the rewarming period (in the unstimulated muscle), cytosolic Ca²⁺ is competitively removed from the cytosol by sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA2a) and Na⁺/Ca²⁺-exchange.
Insulin-mediated positive inotropic effects were not affected by DAG-kinase inhibition (n=6) and were blocked neither by the unspecific protein kinase C (PKC) inhibitor chelerythrine (n=4) nor GF109203X, which more specifically blocks typical (α,β1) and new (δ, ε) isoforms of PKC (the inotropic response to insulin was 26.8±6.4% in the presence of GF109203X and 22.2±5.4% for control experiments in muscles from the same hearts; n=9 for each group; P=NS; Figure 6).

Intracellular Ca\(^{2+}\) handling may be modulated by the Na\(^+/\)Ca\(^{2+}\) exchanger. This electrogenic ion transporter extrudes Ca\(^{2+}\) for Na\(^+\) influx in its forward mode but may also work in its reverse mode, resulting in Ca\(^{2+}\) influx during depolarization. Preincubation with the reverse-mode Na\(^+/\)Ca\(^{2+}\) exchanger inhibitor KB-R 7943 (5 μmol/L; n=8) reduced the positive inotropic effect of insulin by 62.1±6.3% (P<0.05; Figure 6). These data indicate that part of the inotropic response to insulin is related to reverse-mode Na\(^+/\)Ca\(^{2+}\) exchange activation.

Discussion

This is the first report on functional effects of insulin in isolated human myocardium. The results show that (1) insulin exerts a concentration-dependent positive inotropic effect that contains a Ca\(^{2+}\)-dependent and a Ca\(^{2+}\)-independent component; (2) the inotropic effect is partially substrate dependent; and (3) the positive inotropic effect involves PI-3-kinase and reverse-mode Na\(^+/\)Ca\(^{2+}\) exchange activation but is independent from DAG or PKC activation.
still be higher than those observed in previous studies in human end-stage failing myocardium. Therefore, higher concentrations of the inhibitor induced irreversible contracture of the muscles. Furthermore, basal developed force was significantly higher in the pyruvate group. This is in line with the described positive inotropic effect of pyruvate. With the higher basal twitch tension, an additional positive inotropic response may appear smaller if calculated as a percentage of basal force. In fact, the difference in the average increase in twitch force with 3 IU/L insulin was also less pronounced if absolute values were calculated: force increased by 4.3 mN/mm² in glucose-containing solution and by 3.3 mN/mm² in pyruvate-containing solution.

**Signal Transduction Pathways of Insulin in Human Myocardium**

In addition to binding to sarcolemmal insulin receptors, insulin is able to bind to both types of IGF receptors. We have previously shown that IGF-1 binding to the IGF-1

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**Short- and Long-Term Effects of Insulin in the Cardiovascular System**

Insulin exerts acute hemodynamic and long-term genomic effects in the cardiovascular system. Genomic effects include induction of endothelial dysfunction and antiapoptotic signaling. Acute application of insulin to human healthy volunteers or patients with diabetes increases left ventricular ejection fraction, but the mechanism underlying this observation remains unknown. Insulin also exerts direct vasodilatory effects in the human peripheral and coronary circulation. Therefore, whether improved ventricular performance exclusively results from peripheral unloading and enhanced myocardial blood flow or whether additional direct myocardial inotropic effects contribute remains to be determined.

**Direct Functional Effects of Insulin in Isolated Human Myocardium**

The inotropic response to insulin was tested at 0.3 and 3 IU/L in the present study. Although these concentrations are significantly lower than those used in previous studies in guinea pig and rat hearts, even the lower concentration used is up to 5 times higher than postprandial plasma insulin levels in healthy volunteers. However, local insulin concentrations at the receptor level are unknown and may be higher than plasma levels. In addition, higher plasma insulin concentrations may be reached under clinical conditions such as intravenous insulin application (eg, insulin-glucose-potassium infusions). We observed a concentration-dependent positive inotropic effect of insulin in isolated end-stage failing human myocardium that accounted to up to ≈25% increase in twitch force at 3 IU/L insulin. The inotropic effect was associated with minor prolongations of relaxation time and no change in diastolic tension. The magnitude of the inotropic responses to insulin in the present study is comparable to the effects reported in isolated guinea pig and rat hearts.

The acute functional effects of insulin in the present study were substantial and may be of functional relevance: maximal β-adrenergceptor stimulation in isolated failing human myocardium increases force by ≈100%, and therefore, the inotropic effect observed in this study with insulin accounts for up to one fourth of the maximal response to catecholamines in the same system. In addition, the inotropic response to insulin was identical in end-stage failing myocardium from patients with long-standing diabetes mellitus.

However, we also observed a partial substrate dependency of the inotropic effect. The maximal inotropic response to insulin was ≈50% smaller in pyruvate-containing Tyrode’s solution than in glucose-containing Tyrode’s solution at the higher insulin concentration. In addition, blocking glycolysis in muscles preincubated with glucose attenuated the inotropic response to insulin to the extent seen in the presence of pyruvate. We therefore suggest that the functional response to insulin may be related to 2 components: one that originates from improved glucose utilization and metabolism, and one that is independent from metabolic factors. However, we are not aware to what extent we have reduced glucose metabolism at the iodoacetate concentration used in these experiments, because higher concentrations of the inhibitor induced irreversible contracture of the muscles. Furthermore, basal developed force was significantly higher in the pyruvate group. This is in line with the described positive inotropic effect of pyruvate. With the higher basal twitch tension, an additional positive inotropic response may appear smaller if calculated as a percentage of basal force. In fact, the difference in the average increase in twitch force with 3 IU/L insulin was also less pronounced if absolute values were calculated: force increased by 4.3 mN/mm² in glucose-containing solution and by 3.3 mN/mm² in pyruvate-containing solution.
receptors results in a Ca\(^{2+}\)-dependent positive inotropic effect.\(^{22}\) Nevertheless, in the present study, insulin effects were not significantly blunted by preincubation with the selective IGF-1 receptor antibody a-IR-3. This demonstrates an IGF receptor–dependent signal transduction pathway for functional effects of insulin in human myocardium, most likely via direct activation of the insulin receptors. Both insulin receptors and IGF-1 receptors initiate signal transduction pathways that involve the insulin-receptor substrate (IRS). Tyrosine-phosphorylated IRS consecutively activates signaling molecules with SH2 domains, including PI-3-kinase.\(^{34,35}\)

The PI-3-kinase–dependent pathway mediates antiapoptotic signaling and trophic effects via activation of Akt. The role of PI-3-kinase activation in mediating metabolic actions of insulin is controversial.\(^{36–38}\) In the present study, preincubation of human ventricular muscle with the selective PI-3-kinase inhibitor wortmannin almost completely prevented the insulin-dependent inotropic effects. Similarly, we could previously demonstrate that PI-3-kinase also mediates IGF-1–dependent functional effects.\(^{22}\) These data suggest that PI-3-kinase activation is a key event in the signal transduction network activated by insulin or IGF-1 that ultimately results in functional effects. We also tested the involvement of other kinases, such as PKC, which may be activated downstream of PI-3-kinase;\(^{39}\) however, neither inhibition of PKC nor inhibition of DAG had any measurable effect on the inotropic response of failing human cardiac muscle to insulin. Therefore, the chain of events that couples insulin receptors and PI-3-kinase activation to functional effects deserves further investigation.

**Influence of Insulin on Intracellular Ca\(^{2+}\) Handling**

An increase in [Ca\(^{2+}\)], as the underlying mechanism for the inotropic effect of insulin was demonstrated for rat whole-heart preparations.\(^3\) Therefore, we assessed the effects of insulin on intracellular Ca\(^{2+}\) handling in human cardiac muscle. We could demonstrate that intracellular Ca\(^{2+}\) transients increased in parallel to twitch force in aequorin-loaded trabeculae. Increased Ca\(^{2+}\) transients may result from increased transsarcolemmal Ca\(^{2+}\) influx, increased SR Ca\(^{2+}\) release, or both. We therefore directly tested the effects of insulin on SR Ca\(^{2+}\) content and SR Ca\(^{2+}\) reuptake via SERCA2a (relative to transsarcolemmal Ca\(^{2+}\) elimination) using RCCs. We could demonstrate that the inotropic response to insulin was associated with but not dependent on an increase in SR Ca\(^{2+}\) content. In addition, data from the paired rapid cooling experiments revealed that insulin did not change the relative contribution of SERCA2a versus Na\(^+\)/Ca\(^{2+}\) exchange for cytosolic Ca\(^{2+}\) elimination. This supports the hypothesis that insulin increases transsarcolemmal Ca\(^{2+}\) influx without directly affecting SR Ca\(^{2+}\) handling properties. It also supports our notion that substantial parts of the inotropic effects of insulin are not related to metabolic changes, which might (by improved phosphorylation potential) increase the Ca\(^{2+}\)-reuptake capacity of SERCA2a.\(^{40}\)

PI-3-kinase–dependent activation of reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange recently has been shown by our group to be one source of Ca\(^{2+}\) entry in human myocardium.\(^{22}\) Therefore, we directly tested the effects of pharmacological reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange inhibition on the functional effects of insulin. In fact, this intervention partially prevented the inotropic response of human cardiac muscle to insulin. Therefore, a PI-3-kinase–dependent, reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange activation with consequent increased transsarcolemmal Ca\(^{2+}\) entry during the early phase of the action potential may be one of the mechanisms by which insulin increases Ca\(^{2+}\) transients, SR Ca\(^{2+}\) content, and twitch force. Nevertheless, the mechanisms that couple PI-3-kinase to Na\(^+\)/Ca\(^{2+}\) exchange remain to be determined.

**Insulin and Myofilament Ca\(^{2+}\) Handling**

Inotropic interventions may be associated with increased, unchanged, or decreased responsiveness of the myofilaments for Ca\(^{2+}\).\(^{29}\) Typically, an intervention that results in an increased myofilament Ca\(^{2+}\) responsiveness prolongs twitch kinetics, whereas an intervention that reduces Ca\(^{2+}\) myofilament sensitivity abbreviates twitch kinetics. In the present study, the inotropic effects of insulin were associated with a slight, albeit nonsignificant, prolongation of twitch kinetics. Furthermore, Ca\(^{2+}\)-sensitizing inotropic interventions are characterized by underproportional increases in intracellular Ca\(^{2+}\) transients, whereas Ca\(^{2+}\)-desensitizing interventions show overproportional increases in intracellular Ca\(^{2+}\) transients.\(^{29,41}\) In the present study, insulin, compared with an increase in the extracellular Ca\(^{2+}\) concentration as a reference intervention without effects on myofilament sensitivity, showed a tendency toward an underproportional increase in Ca\(^{2+}\) transients relative to force increase. These 2 observations—a trend toward prolonged relaxation kinetics and underproportional Ca\(^{2+}\)-transient increases—point to the possibility that insulin may exert additional, direct Ca\(^{2+}\)-sensitizing effects in human cardiac muscle. This hypothesis was tested in semiskinned fiber preparations that allow the minute experimental control of ion homeostasis on the level of the myocytes but leave receptor-mediated signaling pathways operable. Indeed, in these experiments, insulin shifted the concentration-response curve for Ca\(^{2+}\) to the left, thus supporting the notion that the inotropic effect of insulin depends, at least in a minor part, on enhanced Ca\(^{2+}\) responsiveness of the myofilaments. To the best of our knowledge, there are no reports on direct effects of insulin on myofilament Ca\(^{2+}\) sensitivity, but a wortmannin-sensitive pathway that mediates increased myofilament sensitivity has been described for IGF-1 by Cittadini et al\(^42\) in rat whole hearts. Given the homology of the signal transduction pathways and the wortmannin sensitivity of the insulin-mediated inotropy, it is likely that a similar mechanism operates for both peptides. Nevertheless, the Ca\(^{2+}\)-sensitizing effect of IGF-1 was challenged by experiments in single myocytes of failing and nonfailing dogs,\(^{43}\) and hence, species and/or experimental approaches (ie, loaded versus unloaded preparations) may also play a role.

Taken together, we demonstrated for the first time a direct inotropic effect of insulin in failing human myocardium. Mechanistically, this functional response has 3 components, ie, a metabolic component, a Ca\(^{2+}\)-dependent component, and a Ca\(^{2+}\)-independent component. These interesting new as-
pects of insulin signaling in the human heart deserve further investigation.

Study Limitations
A limitation of the study is that aequorin light signals have not been converted to [Ca\textsuperscript{2+}]. This in itself is not a problem, but it reduces the validity of direct comparisons between different inotropic interventions.\textsuperscript{22} Although validated for human cardiac muscle,\textsuperscript{19} rapid cooling may not release all Ca\textsuperscript{2+} stored within the SR.\textsuperscript{44} In addition, RCCs are an indirect measure of SR Ca\textsuperscript{2+} content, and subcellular changes, such as the insulin-dependent increase in myofilament responsiveness to Ca\textsuperscript{2+}, may affect RCCs. KB-R7943 was used as an inhibitor of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger reverse mode; however, KB-R7943 is not an ideal agent because it can also affect other transport systems, such as K\textsuperscript{+}, Na\textsuperscript{+}, and Ca\textsuperscript{2+} channels, and it affects Ca\textsuperscript{2+} transients even in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger–knockout heart tubes.\textsuperscript{45} The apparent selectivity for outward versus inward Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger current is not well understood.\textsuperscript{46,47} Nevertheless, no better reverse-mode inhibitors are available presently.

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References
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