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Hexarelin, But Not Growth Hormone, Protects Heart from Damage Induced In Vitro by Calcium Deprivation Replenishment

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The effects of hexarelin, a growth hormone (GH) secretagogue, and human GH on the mechanical and metabolic changes measured in isolated rat hearts submitted to 5 min of Ca^{2+} deprivation followed by reperfusion with Ca^{2+}-containing medium, the so-called calcium paradox phenomenon, were studied. Hexarelin (80 μg/kg bid, subcutaneously) administered for 7 d to male rats effectively antagonized the sudden increase in resting tension measured in vitro on Ca^{2+} repletion. Moreover, during Ca^{2+} repletion the release of creatine kinase activity (an index of cell damage) in the perfusate of these hearts was reduced up to 40% compared with controls. By contrast, administration of hexarelin for 3 d or GH (400 μg/kg bid, subcutaneously) for 7 d did not affect the mechanical and metabolic alterations induced by the calcium paradox. To assess its direct and acute cardiac effects, hexarelin (8 μg/mL) was perfused in vitro in recirculating conditions for 60 min through the hearts of normal rats. In this case, hexarelin did not stimulate heart contractility and failed to prevent ventricular contracture upon Ca^{2+} readmission, whereas diltiazem, a Ca^{2+}channel blocker, effectively antagonized the calcium paradox phenomenon. We conclude that short-term in vivo exposure to hexarelin, but not GH, enables cardiac myocytes to prevent cytoplasmatic electrolytic unbalance and to control intracellular Ca^{2+} gain, two functions largely impaired during the calcium paradox phenomenon. Moreover, because the effect of hexarelin is not acute but dependent on the length of in vivo treatment, we suggest that it requires modifications of myocardiocyte physiology.

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Introduction

We have recently demonstrated that hexarelin exhibits a significant protective activity against postischemic ventricular dysfunction in isolated hearts subjected to low-flow ischemia and reperfusion (*1–3*). To explain this effect, it was proposed at first that growth hormone (GH) release mediated hexarelin actions. Because hexarelin induced a clear-cut protection also in the hearts of hypophysectomized rats, we then suggested that its mechanism of action is largely independent of GH and that it is exerted directly on the heart (*4*). Supporting this view, specific binding sites for hexarelin have been recently demonstrated in cardiac membranes (*5*). In several studies, myocardial injury has been associated with accumulation of Ca^{2+} in cardiac cells (*6,7*). It is known that Ca^{2+} plays a pivotal role in the regulation of the energy metabolism of cardiac muscle, and disorders of intracellular Ca^{2+} distribution may affect the energetics of myocardial cells. The present study was designed to investigate the activity of a short-term in vivo treatment with hexarelin or GH on the performances of isolated hearts submitted to transient exposure to Ca^{2+}-free solution followed by Ca^{2+} repletion. In this setting, the sharp increase in resting tension is the mechanical manifestation of a phenomenon called calcium paradox (*8,9*). In addition, the direct acute effects of hexarelin have been studied by perfusing the compound through the heart in recirculating conditions for 60 min before the induction of calcium paradox.

Results

Treatment with hexarelin or GH did not apparently affect the somatotropic function of the rats. In fact, in all treatment groups insulin-like growth factor-1 (IGF-1) levels in

plasma and hearts were not significantly different from those measured in control rats (data not shown). Treated rats did not lose weight, nor did they show behavioral deviation from normality or significant signs of toxicity. The heart/body weight ratio remained similar in the four experimental groups, indicating that hexarelin and GH had not induced disproportional increases in the cardiac ventricular mass (data not shown).

Effects After In Vivo Treatments

When hearts obtained from control rats were exposed to Ca^{2+}-free medium, contractility declined rapidly (complete ventricular standstill was reached in <1 min) and left ventricular end diastolic pressure (LVEDP) (resting tension) increased moderately within 5 min (from 4.5 ± 0.5 to 23 ± 1.5 mmHg; $p < 0.01$). On Ca^{2+} readmission to the perfusing medium, LVEDP increased sharply, reaching a peak of 76 ± 7 mmHg.

Heart preparations showed difficulties in following the electrical pacing, and the recovery of contractility was markedly impaired. In fact, 30 min after Ca^{2+} readmission, LVEDP was still elevated (62 ± 5 mmHg) (**Fig. 1**). Treatment of the rats with hexarelin for 3 or 7 d induced a time-dependent reduction in these mechanical alterations. The protection afforded by the 7-d hexarelin treatment was particularly impressive, because on Ca^{2+} readmission the increase in LVEDP was 69% lower than in controls (23 ± 1.5 mmHg; $p < 0.01$). By contrast, GH treatment for 7 d did not protect the hearts from the calcium paradox, and these preparations behaved quite similarly to those of controls (**Fig. 1**). Thirty minutes after Ca^{2+} readmission, in the control preparations left ventricular developed pressure (LVDP) had recovered only 32% of basal values (**Fig. 2**). LVDP is an index of the strength of contractility of cardiac myocytes. The 7-d hexarelin treatment significantly increased the recovery of LVDP (up to 66% of basal values; $p < 0.01$ vs controls), whereas the GH and 3-d hexarelin treatments were ineffective.

During both stabilization and Ca^{2+}-free perfusion, the hearts released small amounts of creative kinase into the coronary effluent (30 ± 2 mU[min.g wt]). On Ca^{2+} restoration, creatine kinase release from control preparations increased significantly, reaching a peak (212 ± 19 mU/min/g wt) in 5–7 min. The 7-d hexarelin treatment significantly reduced peak creatine kinase release (40% lower than controls; $p < 0.01$), whereas GH and the 3-d hexarelin treatment were ineffective.

Effects of In Vitro Stimulations

Hexarelin (8 μg/mL) had no direct effects on heart mechanics, being the strength of contraction unaltered before and during hexarelin recirculation. On Ca^{2+} readmission, the extent of ventricular contracture was not statistically different from that measured in control preparations (LVEDP: peak at 66 ± 6 and 76 ± 6 mmHg for hexarelin

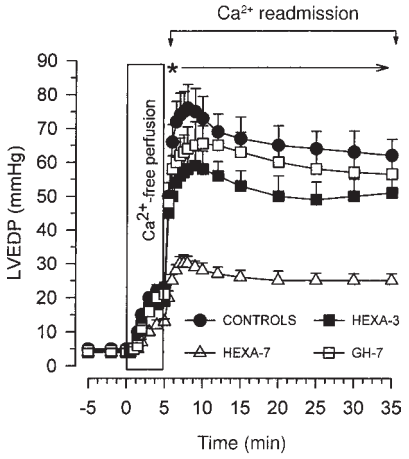

Fig. 1. LVEDP in isolated hearts subjected to calcium paradox. Rats were treated in vivo as described in Materials and Methods. After a period of equilibration, hearts were exposed for 5 min to a Ca^{2+}-free solution followed by Ca^{2+} readmission for 30 min. Each point is the mean ± SEM of six determinations. *$p < 0.01$ for HEXA-7 vs controls, HEXA-3, and GH at all time points from min 5 through 35.

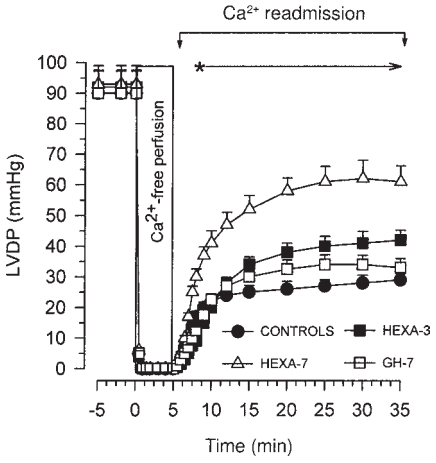

Fig. 2. LVDP. Treatments are as described in the legend of Fig.1. Each point is the mean ± SEM of six determinations. *$p < 0.01$ for HEXA-7 vs controls, HEXA-3, and GH at all time points from min 7 through 35.

and controls, respectively) (Fig. 3). Furthermore, there was a low recovery of contractility at the end of the experiments (LVDP: 36 and 28% for hexarelin and controls, respectively). By contrast, diltiazem (4 μg/mL for 60 min) significantly inhibited inotropism (21% reduction in contractility; $p < 0.05$). Afterward, on Ca^{2+} readmission, an almost complete inhibition of ventricular contracture occurred (peak LVEDP: 22 ± 2 mmHg) (Fig. 3), thus allowing consistent recovery in the force of contraction (LVDP:73% of the basal inotropism).

Discussion

Our results demonstrate, for the first time, that hexarelin has cardioprotective effects that are not shared by GH. In

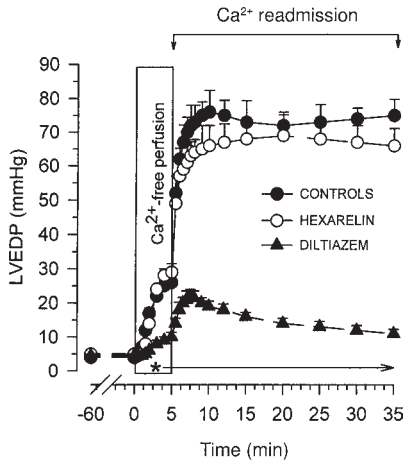

Fig. 3. LVEDP in isolated hearts subjected to calcium paradox. Hexarelin (8 μg/mL) and diltiazem (4 μg/mL) were perfused through the heart in recirculating conditions for 60 min before eliciting the calcium paradox event. Each point is the mean ± SEM of six determinations. *$p < 0.01$ for diltiazem vs controls and hexarelin at all time points from min 2 through 35.

fact, a 7-d in vivo administration of hexarelin produced in perfused hearts a clear-cut inhibition of the steep ventricular contracture that is the mechanical expression of calcium paradox. Although this phenomenon shares some aspects with the injury induced by postischemic reperfusion, e.g., a gain in tissue Ca^{2+}, there is no agreement on the possible routes for the Ca^{2+} influx during repletion (*10,11*). Many therapeutic strategies have been devised to protect the heart from metabolic and mechanical changes occurring during myocardial ischemia. A suitable way to achieve this goal is to prevent either intracellular Ca^{2+} overload or decline in cellular energy stores. Maintenance of the basic energy function is mandatory for the proper recovery of contractility at the end of ischemic insult. It has been proposed that Ca^{2+} depletion would cause weakening of intercalated disks of junction and, on Ca^{2+} repletion, Ca^{2+} entry through the Ca^{2+} channel would trigger contracture-mediated rupture of cell membrane and a massive secondary ion influx (*12*). In this context, hexarelin may have inhibited heart hypercontraction by reducing the oscillatory shifting of the Ca^{2+} ion between the sarcolemma and the sarcoplasmic reticulum of cardiac cells, preserving their integrity. In line with these observations, release of CK from the heart of hexarelin-treated rats was markedly reduced, indicating a better preservation of the integrity of myocardial cells.

In our study, 7-d administration of GH, at a dose that in previous experiments was protective in hearts from GH-deficient rats exposed to ischemia-reperfusion (*2*), did not prevent the rapid increase in resting tension of the myocardium on Ca^{2+} repletion. This would indicate that GH is unable to prevent changes in membrane permeability, namely Ca^{2+} movements, which occur in myocardial cells during the calcium paradox event. Thus, rather than by a GH-mediated action, the beneficial effects elicited by hexarelin on the ventricular contracture of ischemia-reperfusion model (*2*) or calcium paradox would be better explained by activation of specific cardiac receptors (*5*).

Moreover, the effect of hexarelin needs multiple exposures of the heart to the hexapeptide, since both the in vitro direct stimulation and the in vivo 3-d treatment were ineffective. In fact, in contrast to diltiazem, hexarelin perfused in recirculating conditions for 60 min failed to prevent the sudden rise in resting tension of the heart during Ca^{2+} repletion, leaving the ventricular function heavily depressed. Also, other data compound the interpretation of the action of hexarelin on the heart. Recently it has been reported that acute administration of hexarelin, but not GH, caused in healthy men an abrupt inotropic effect with significant increases in left ventricular ejection fraction and no changes in blood pressure and heart rate (*13*).

The investigators attributed the inotropic effect of hexarelin to activation of specific myocardial GH-releasing peptide binding sites (*5*). However, this effect is likely species-specific because it has never been reported in rats, and we have been unable to reproduce it either in vitro on isolated hearts (this and previous works) or in vivo in anesthetized rats (unpublished observations).

In conclusion, our present data demonstrate that a 7-d treatment with hexarelin is mandatory in the rat to antagonize the hypercontracture induced by the calcium paradox. The exact mechanism of action of hexarelin remains largely unknown, but it clearly does not depress heart contractility, an energy-sparing effect that is distinctive for Ca^{2+} channel blockers. We suggest that hexarelin treatment makes myocardial cells more resistant to mechanical uncoupling and capable of better maintenance of electrolyte balance. This would reduce Ca^{2+} influx and preserve myocardial cell integrity.

Materials and Methods

Treatment of Animals

Male Sprague-Dawley rats weighing 200 ± 2 g (Charles River, Calco, LC, Italy) were treated subcutaneously twice daily with physiologic saline (1 mL/kg) for 7 d (controls), hexarelin (80 μg/kg) (Pharmacia & Upjohn, Stockholm, Sweden) for 3 d (HEXA-3), hexarelin (80 μg/kg) for 7 d (HEXA-7), and recombinant human GH (400 μg/kg) (Pharmacia & Upjohn) for 7 d (GH-7). Rats were killed by cervical dislocation 14 h after the last injection. All experimental protocols were approved by the Review Committee of the Department of Pharmacology and met the Italian Guidelines for laboratory animals, which conform with the European Union Directive of November 1986 (86/609/EEC).

IGF-1 Assay

Plasma samples, separated from blood, were cryoprecipitated in 87.5% ethanol and 2 *N* 12.5% HCl as previously described by Breier et al. (*14*). Hearts were weighed and frozen in liquid nitrogen. Single hearts were subsequently pulverized using a tissue pulverizer, and IGF-1 was

extracted using 1 mol/L of ice-cold acetic acid (5 mL/g of tissue) as previously described by D'Ercole et al. (*15*). After centrifugation at 600x*g* for 10 min, the supernatants were frozen at –20°C, lyophilized, and reconstituted with assay buffer. Total plasma IGF-1 levels and heart IGF-1 concentrations were determined using a commercially available kit (Amersham, Milan, Italy). The sensitivity of the assay was 50 pg/mL; intraassay variability was <10%. To avoid possible interassay variations, all samples were assayed in a single radioimmunoassay.

Hearts Preparation

Hearts were quickly dissected and perfused retrogradely at 37ºC through the aorta as previously described (*3*). The perfusion medium contained 118 m*M* NaCl, 2.8 m*M* KCl, 1.2 m*M* KH_2PO_4 2.5 m*M* $CaCl_2$ 1.2 m*M* $MgSO_4$, 25 m*M* $NaHCO_3$, and 5.5 m*M* glucose. After a period of equilibration with a gas mixture containing 5% CO_2 and 95% O_2, the pH of the perfusate was 7.4 and the perfusion flow was maintained at 15 mL/min. Left ventricular pressure (LVP) was recorded using a pressure transducer (HP-1280C; Hewlett-Packard, Waltham, MA). LVP was obtained by inserting a small latex balloon filled with saline through the left atrium. LVEDP was stabilized to 4 to 5 mmHg. The hearts were electrically paced at a frequency of 300 beats/min with rectangular impulses (1-ms duration, voltage 10% above threshold). According to Cavero et al. (*16*), at the end of the equilibration period (20 min), all the hearts were exposed for 5 min to a Ca^{2+}-free solution followed by Ca^{2+} readmission for 30 min. When Ca^{2+} is readmitted to the perfusing solution, the resting tension (LVEDP) of the heart rapidly increases. The magnitude of this effect is the mechanical expression of the calcium paradox. Changes in LVDP, (calculated as the difference between peak LVP minus LVEDP) were also evaluated.

In a separate experiment, the Ca^{2+} channel antagonist diltiazem (4 μg/mL) (Sigma, St. Louis, MO) and hexarelin (8 μg/mL) were perfused directly through the hearts of control rats in recirculating conditions (reservoir: 25 mL; flow rate: 15 mL/min) for 60 min before the induction of calcium paradox.

Assay of Creatine Kinase Activity

During the equilibration period, two samples of perfusate were collected over 150 s to evaluate the basal activity of creatine kinase. Similarly, samples were collected every 150 s during the period of Ca^{2+} readmission. Creatine kinase activity was determined as previously described *(17)* and expressed as milliunits/(minute . g) of wet tissue.

Statistical Analysis

Statistical analysis of the means was performed by one-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons. A value of $p < 0.05$ was considered significant.

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