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Efficacy of a growth hormone-releasing peptide mimetic in cardiac ischemia/reperfusion injury

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Abstract

The cardioprotective efficacy of the pyrazolinone–piperidine dipeptide growth hormone secretagogue (GHS) CP-424,391 was studied in an in vivo rabbit model of ischemia and reperfusion. CP-424,391 was administered at 25 mg/kg p.o. × 7 days. Ischemia was induced by left coronary artery occlusion for 30 min, after which the heart was reperfused for 2 h. At the end of reperfusion, animals were euthanized and the infarct size was determined. The area at risk of infarct was not different between the control (45.8 ± 3.7%, $n = 6$) and CP-424,391-treated groups (36.9 ± 4.3%, $n = 11$). The infarct size of the control animals was 49.5 ± 7.1% and was significantly ($P < 0.05$) lower in the CP-424,391-treated group (infarct size = 17.3 ± 3.0). There was a trend, albeit not significant, for the left ventricular function to recover to a greater extent in CP-424,391-treated rabbits. Thus, the treatment of rabbits for 7 days with CP-424,391 was cardioprotective against ischemia/reperfusion injury.

Keywords: Cardiac ischemia; Infarct; Growth hormone secretagogue; CP-424,391; Insulin-like growth factor-1 (IGF-1)

1. Introduction

The influence of growth hormone (GH), GH-releasing peptides (GHRPs) and insulin-like growth factor-1 (IGF-1) on the heart under physiological and pathophysiological conditions is of increasing academic and drug discovery interest. In experimental paradigms of heart failure (i.e. rapid pacing-induced heart failure in pigs and myocardial infarction-induced heart failure in rats), treatment with GH, GHRPs (i.e. hexarelin) or the GH secretagogue (GHS) CP-424,391 attenuated the development of ventricular failure and/or improved hemodynamic function (Cittadini et al., 1997; King et al., 2001; Houck et al., 1999; Isgaard et al., 1997; Tajima et al., 1999; Yang et al., 1995a; Tivesten et al., 2000). Clinically, recombinant human GH (rhGH) administered for 3 months to patients with idiopathic dilated cardiomyopathy or ischemic cardiomyopathy significantly improved hemodynamics, myocardial energy metabolism and exercise capacity/clinical function (Fazio et al., 1996; Genth-Zotz et al., 1999; Spallarossa et al., 1999), supporting a therapeutic role for pharmacologic manipulation of the GH–IGF-1 axis in heart failure. However, the GH treatment of patients with heart failure has not universally demonstrated efficacy, and indeed, two clinical trials have failed to demonstrate cardiac functional benefit after rhGH treatment (Isgaard et al., 1998; Osterziel et al., 1998). The clinical efficacy of GHRPs such as hexarelin, however, remains to be reported: thus, the treatment of heart failure with GH and/or GHRP may be considered as an evolving approach to the treatment of heart failure as specific treatment modalities and patient populations that may benefit are defined (Ng et al., 2000).

In addition to heart failure, GH and/or GHRPs have been reported to be cardioprotective in experimental paradigms of ischemic cardiac injury. Initial studies demonstrated that suppression of GH levels via administration of anti-GHRH serum to rats from postnatal days 20–40 exacerbated the ventricular functional deficit medicated by low flow ischemia in an isolated heart preparation (DeGennaro-Colonna et al., 1996). Other studies have shown that GH treatment for periods of 7–28 days prior to cardiac ischemia/reperfusion injury protected the heart: prophylactic GH treatment was associated with improved functional recovery in isolated Langendorff-perfused heart preparations (Rossoni et al.,

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1998, 2000), and reduced scar tissue formation was determined 4 weeks after experimental myocardial infarction (Laguens et al., 1998). It has also been shown in obese Zucker rats that hexarelin was functionally cardioprotective in response to ischemia/reperfusion injury (DeGennaro-Colonna et al., 2000). Furthermore, administration for 2 weeks of the GHRP, GHRP-2, but not rhGH was shown to attenuate the diastolic dysfunction associated with myocardial stunning in an isolated blood-perfused rabbit heart paradigm (Weekers et al., 2000). Lastly, it has been qualitatively stated that GH treatment reduced the extent of myocardial ischemia-induced necrosis (Castagnino, 1999), however, infarct size data were not reported.

In vivo cardioprotective efficacy of GHRP treatment against acute myocardial ischemia/reperfusion injury arbitrated by infarct size measurement has not been reported. Therefore, the current study determined if treatment with a novel orally active GHS, CP-424,391, would affect infarct size in an in vivo rabbit model of ischemia/reperfusion injury. CP-424,391 is a member of a novel series of orally active pyrazolinone–piperidine dipeptide GH secretagogues (Fig. 1). This compound was discovered following the comparison of peptidomimetic GHS structures discovered by Merck with a series of active tetrahydroquinolines possessing potent activity but low oral bioavailability (Lefker et al., 2001). The intrinsic GH-releasing activity of these novel structures was determined in rat pituitary cell cultures with in vivo GHS activity determined in both rat and canine models. CP-424,391 is the most extensively characterized compound from this series and was selected for clinical evaluation based on its potent activity and excellent oral bioavailability in preclinical species (Pan et al., 2001).

We determined the effect of a 7-day treatment regimen with CP-424,391 on infarct size occurring as a result of regional myocardial ischemia and reperfusion. Efficacy was defined as a statistically significant reduction in myocardial infarct size. Our results show for the first time that GHS treatment produces a salutary effect on the survival of heart tissue subjected to a period of ischemia and reperfusion, suggesting that GHS may play a therapeutic role as prophylactic cardioprotective agents.

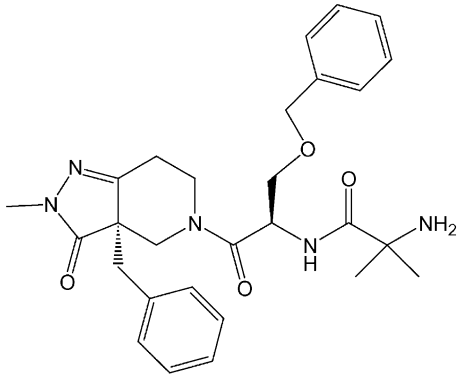

Fig. 1. The chemical structure of CP-424,391.

2. Materials and methods

2.1. Dosing protocol

Male New Zealand white rabbits (2.7–3.4 kg) were administered either CP-424,391 at a dose of 25 mg/kg or the equivalent volume of vehicle (water) once daily × 7 days by oral gavage. CP-424,391 or the vehicle was administered each day between 8:00 and 9:00 a.m. Rabbits were fasted for at least 2 h prior to dosing and for 1 h after dosing. On the seventh day of dosing, the in vivo ischemia/reperfusion cardioprotection experiment was conducted. During the dosing period and during the ischemia/reperfusion protocol, the plasma was collected and frozen for IGF-1 and GH concentration determinations. Body weight of rabbits in the control and CP-424,391-treated groups was recorded daily. See Fig. 2 for a schematic representation of the dosing schedule and experimental procedure used.

2.2. Determination of plasma GH and IGF-1 concentrations

Rabbit blood samples were drawn into Na–EDTA-containing tubes and the plasma was immediately isolated by centrifugation. The rabbit plasma was sub-aliquoted, immediately frozen and stored at −20 °C until assayed. Plasma growth hormone was quantified by a double antibody radioimmunoassay using purified hormone antigen and specific antisera obtained from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. IGF-1 concentrations were determined by radioimmunoassay (IGF-1 by extraction kit, Nichols Institute) following acid–ethanol extraction of plasma samples as recommended by the manufacturer.

2.3. Ischemia/reperfusion protocol

Rabbits were anesthetized by i.v. administration of sodium pentobarbital (30 mg/kg; marginal ear vein) followed by intubation and ventilation with room air using a positive pressure ventilator. A left thoracotomy was performed, the heart was exposed, and a snare (2–0 silk) was placed loosely around the main branch of the left coronary artery. Catheters were placed in the left external jugular vein and in the left common carotid artery for access to venous blood (e.g. for determining compound plasma concentration) and to monitor mean arterial pressure (heart rate is derived from the pressure pulse). Electrocardiographic leads were placed subcutaneously in a Lead II configuration to monitor the electrocardiogram. All hemodynamic and electrocardiogram data were recorded and analyzed continuously throughout the experiment using a Ponemah Data Acquisition and Archive System. Raw data were archived onto a CD.

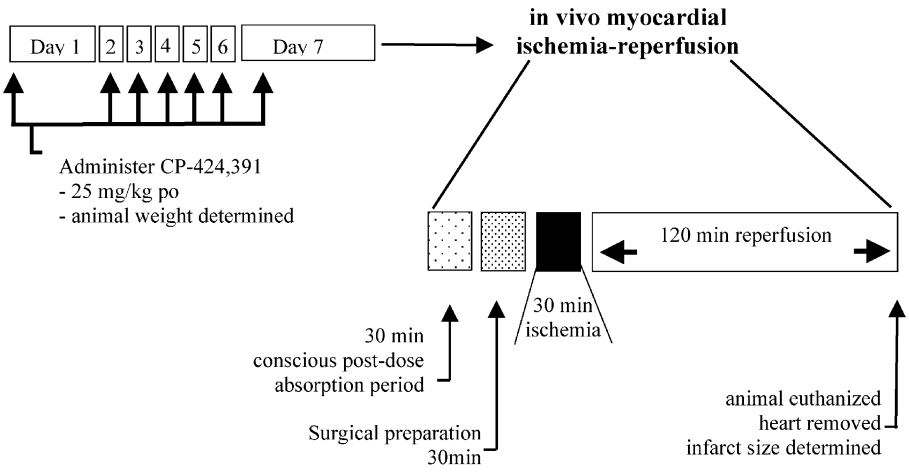

Fig. 2. Schematic representation of the protocol used to study the effect of the GHS CP-424,391 on cardiac ischemic/reperfusion injury in an anesthetized in vivo rabbit model. CP-424,391 or an equivalent volume of vehicle (water) was administered orally at a dose of 25 mg/kg/day for a period of 7 days. The cardioprotective efficacy was determined on the seventh day. After the last dose, a period of 30 min elapsed before the rabbits were anesthetized to permit the absorption of the compound.

After a stabilization period and 30 min of pre-ischemia hemodynamic recording, rabbits were subjected to 30 min of regional left ventricular ischemia (produced by occluding the main branch of the left coronary artery) followed by 120 min of reperfusion. At the end of the 120-min reperfusion period, rabbits were euthanized with an overdose of sodium pentobarbital, and the heart was rapidly removed. The heart was attached to a cannula via the aortic remnant and was perfused with saline to flush blood from the tissue. The silk suture that remained around the main branch of the left coronary artery was occluded, and the heart was perfused with a suspension of fluorescent zinc cadmium sulfate particles (0.5% w/v, 1–10 μm in diameter). These fluorescent particles delineated the area at risk for infarct development. The heart was removed from the saline/fluorescent zinc cadmium sulfate perfusion apparatus, blotted dry, wrapped in aluminum foil and stored overnight at −20 °C.

Infarct size was determined the following day. Frozen hearts were sliced into 2-mm transverse sections and incubated with 1% triphenyl tetrazolium chloride in phosphate-buffered saline for 20 min at 37 °C to further delineate non-infarcted (stained) from infarcted (non-stained) tissue. The viable cardiac tissue converted the yellow colored triphenyl tetrazolium chloride to a brick red formazan precipitate, while the dead/infarcted tissue did not convert the triphenyl tetrazolium chloride. Thus, viable and non-viable tissues were demarcated. The infarct area (yellow) and the area at risk (non-fluorescent) were calculated for each slice of the left ventricle using a precalibrated image analyzer, followed by the addition of the values for each tissue slice to obtain the total infarct area and total area at risk for each heart. To normalize the infarct area for differences in the area at risk between hearts, the infarct size was expressed as the ratio of infarct area vs. the area at risk (%infarct area/area at risk). The effect of CP-424,391 was compared against the vehicle-treated control rabbits subjected to the same dosing and experimental procedure.

2.4. Statistical analysis

For comparison of hemodynamic or infarct size variables between the control and CP-424,391-treated groups, the data were analyzed using a *t*-test (Excel 97, Microsoft, Seattle, WA). Mean data were considered different at the $P < 0.05$ level. All values are means ± standard error of the mean (S.E.M.).

3. Results

3.1. Dose selection

Prior to the cardioprotection study, an assessment of the effect of CP-424,391 on rabbit IGF-1 and GH levels was undertaken in a preliminary 3-day dosing protocol. These determinations established an appropriate dose of CP-424,391 to be used in the cardioprotection study. The aim was to determine a dose of CP-424,391 that produced an approximate twofold change in plasma IGF-1 levels. A dose of 25 mg/kg × 3 days CP-424,391 led to a consistent twofold change in plasma IGF-1 concentration relative to baseline (data not shown). Based on the robust change in plasma GH and IGF-1, the 25-mg/kg dose was chosen for the 7-day oral dosing regimen for the cardioprotection study.

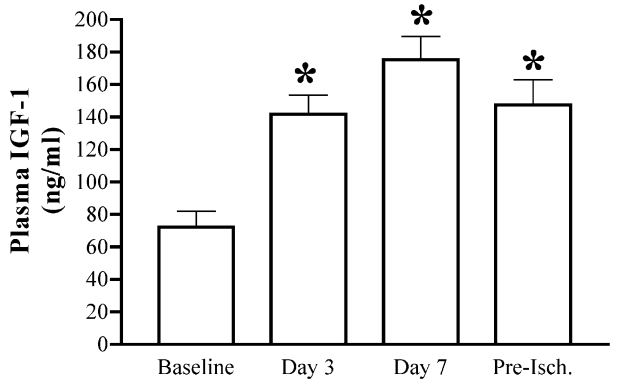

Fig. 3. The effect of CP-424,391 on rabbit plasma IGF-1 levels. Plasma IGF-1 levels were determined prior to the administration of CP-424,391 and thereafter before the compound was administered on days 3 and 7. As well, the effect of CP-424,391 on plasma IGF-1 levels was determined immediately before the onset of regional myocardial ischemia. Baseline $n=9$; day 3 $n=5$; day 7 $n=6$; pre-ischemia $n=9$. * $P<0.05$ vs. baseline.

3.2. Effects of CP-424,391 on plasma IGF-1 concentrations

As shown in Fig. 3, there was a robust and significant elevation in plasma IGF-1 concentrations in CP-424,391-treated rabbits. The IGF-1 levels in the CP-424,391-treated rabbits at days 3 and 7 represent the plasma IGF-1 observed in plasma isolated prior to the administration of the respective day's dose of CP-424,391 (i.e. 24 h after previous dose). There was a clear elevation of IGF-1 by CP-424,391 treatment. On day 7, the ischemia/reperfusion protocol was conducted, and plasma IGF-1 levels were determined immediately prior to the onset of regional myocardial ischemia. As shown in Fig. 2 (Pre-Isch), there was a significant elevation in plasma IGF-1 just prior to the onset of myocardial ischemia. These IGF-1 data indicate that the GH–IGF-1 axis was positively influenced by CP-424,391 treatment at 25 mg/kg/day.

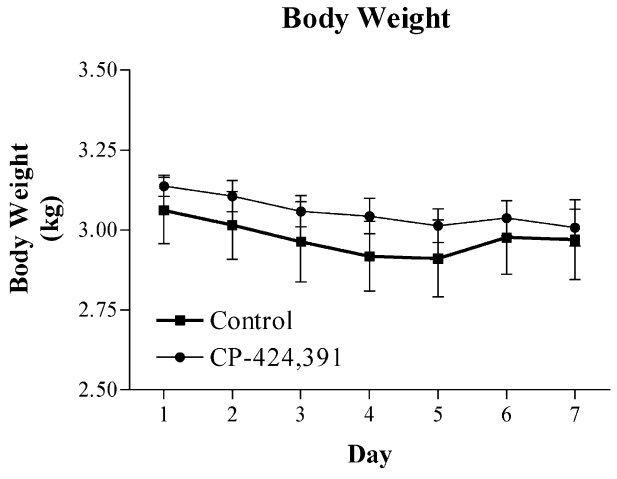

Fig. 4. The effect of CP-424,391 or vehicle (water) on body weight. There was no effect of daily oral administration of either water ($n=6$) or CP-424,391 ($n=9$) during the course of the study. There were no differences in body weight between the two groups studied.

3.3. Effect of CP-424,391 and vehicle on body weight

The vehicle and GHS CP-424,391 were administered to rabbits once daily for a period of 7 days by oral gavage. As shown in Fig. 4, there was no effect of either the vehicle (water) or CP-424,391 on body weight during the 7-day dosing period. There were no differences on the average body weights of rabbits in the two groups studied.

3.4. Hemodynamic effects of CP-424,391

The hemodynamic parameters measured in the anesthetized rabbit preparation were the heart rate (HR), mean arterial pressure (MAP) rate of change of pressure during systole (contractility; $+dP/dt$) and the rate of change of

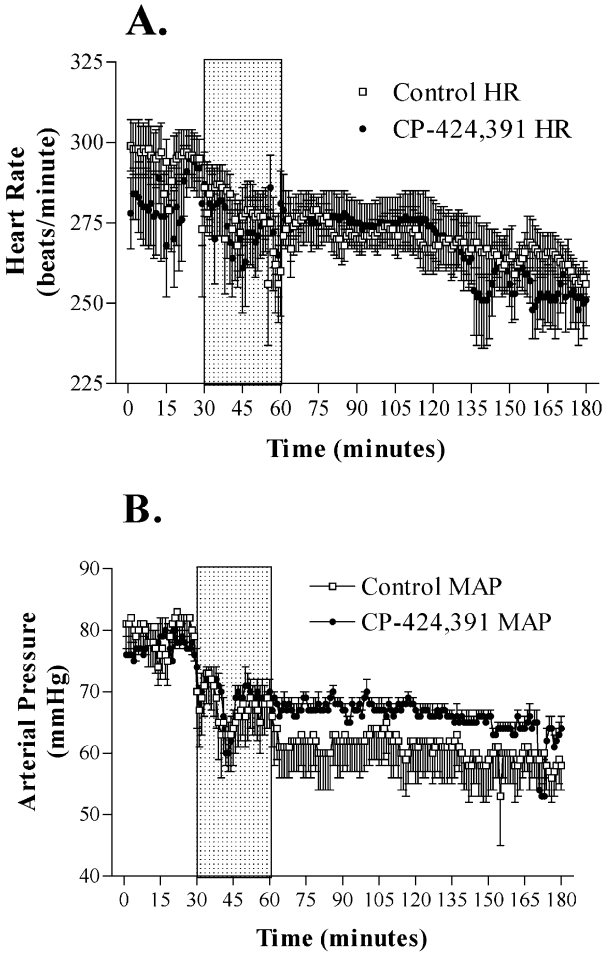

Fig. 5. The effect of CP-424,391 on heart rate and mean arterial pressure. The effect of 7-days vehicle or CP-424,391 administration on either heart rate (A) or mean arterial pressure (B) during the baseline, ischemic and reperfusion periods is shown. The data represent the means ± S.E.M. of each group. Control $n=6$ and CP-424,391 $n=9$.

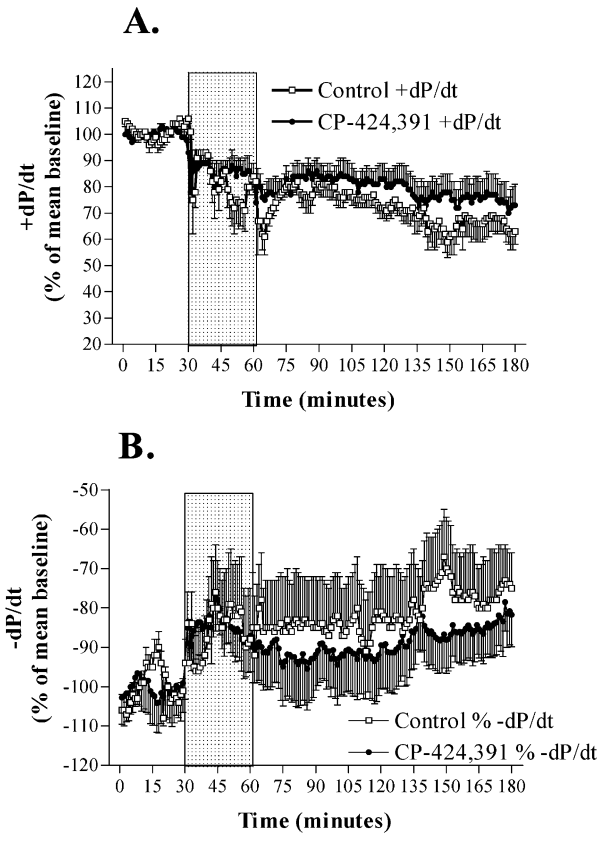

Fig. 6. The effect of CP-424,391 on the ventricular contractility and relaxation. The effect of 7-days vehicle or CP-424,391 administration on either (A) ventricular contractility, $+dP/dt$ (mm Hg/s) or (B) ventricular relaxation, $-dP/dt$ (mm Hg/s) during the baseline, ischemic and reperfusion periods is shown. The data represent the means ± S.E.M. of each group. Control $n=6$ and CP-424,391 $n=9$.

pressure during diastole (relaxation; $-dP/dt$). We qualitatively evaluated the Lead II ECG continuously during the study of each rabbit in the two groups studied. There were no differences in the incidence of ischemic or reperfusion-associated ventricular arrhythmias nor fatal ventricular arrhythmias, i.e. ventricular fibrillation, in either the control or CP-424,391-treated rabbits.

Baseline values for each of the four hemodynamic parameters (HR, MAP, $+dP/dt$ and $-dP/dt$) were not different between the control and treated groups studied, indicating that at least in the anesthetized state, baseline hemodynamic function was not influenced by the 7-day CP-424,391 treatment protocol. As shown in Fig. 5A, there were no differences in HR between the groups during the experimental protocol: each group responded to the ischemic and reperfusion phases of the protocol to the same extent. In animals treated with CP-424,391, there was a trend towards the MAP recovering to a greater extent during the reperfusion period (Fig. 5B), however, the trend did not reach statistical significance. The data represented in Fig. 6A and B show the effect of ischemia and reperfusion on the left ventricular contractility (measured directly via an intraventricular catheter). As expected, there was a decline in both contractility ($+dP/dt$) and relaxation ($-dP/dt$) in response to the ischemia in both groups. There was a trend towards the improvements in contractility and relaxation in the CP-424,391-treated rabbits. The trends towards improved hemodynamic function were not statistically significant.

3.5. Effects of CP-424,391 on infarct size

The data of Fig. 7A and B show the area at risk of infarct and the infarct size, respectively, in the control and CP-424,391-treated groups after the 30-min period of ischemia and 120 min of reperfusion. The mean area of the left ventricle at risk of infarct (AAR) was not different between the two groups studied (Fig. 7A). Compared to the control and vehicle-treated groups, there was a significant reduction in the infarct size (expressed as a percentage of the area at risk of infarct) in the CP-424,391-treated rabbits (Fig. 7B). The infarct size was reduced by 65% in the CP-424,391-treated rabbits relative to the control group.

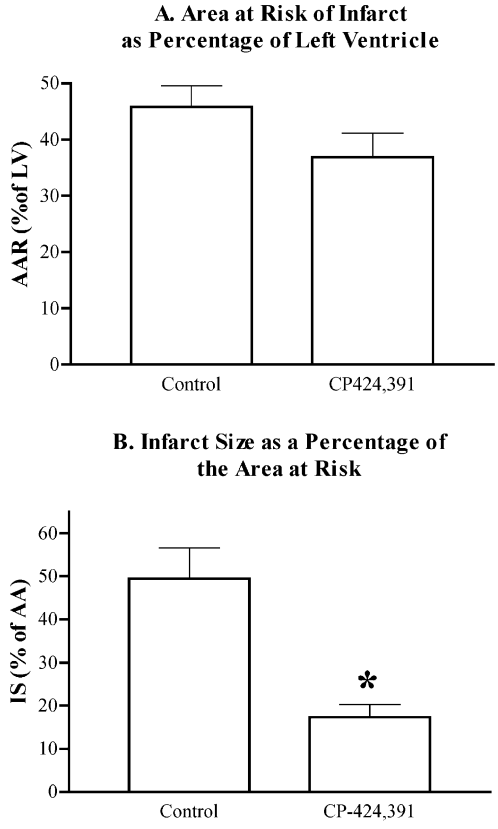

Fig. 7. The effect of vehicle or CP-424,391 treatment on left ventricular infarct size. The area at risk of infarct (A) was not different between the two groups studied. The infarct size (B) was measured after a period of 30 min of ischemia and 120 min of reperfusion using a dual staining procedure. The infarct size of the control (vehicle-treated; $n=6$) was significantly greater than that of the CP-424,391-treated ($n=9$) rabbits. The data represent the means ± S.E.M. of each group. * $P<0.05$ vs. control.

4. Discussion

Previous studies of GH or GHRP (i.e. hexarelin or GHRP-2) in models of cardiac ischemia/reperfusion injury have utilized ex vivo paradigms to determine the cardioprotective potential of these compounds. Results of these studies supported a role for GH or GHS/GHRP treatment as a means to provide cardioprotection to the ischemic/reperfused heart: left ventricular functional recovery was improved by GH/GHS treatment (Rossoni et al., 1998). The current results extend the observations that pharmacologic influence over the GH–IGF-1 axis is cardioprotective by demonstrating for the first time that 7 days of orally administered treatment with the GHS CP-424,391 protected the heart in vivo from ischemic/reperfusion injury by reducing the infarct size. Compared to control and vehicle-treated rabbits, the infarct size was significantly reduced by 65% in CP-424,391-treated rabbits. These results represent the first description of direct myocardial infarct size reduction by prophylactic oral treatment with GHS.

In addition to a significant reduction in infarct size, there was a trend towards an improvement in the left ventricular functional recovery in CP-424,391-treated rabbits. The lack of a statistically significant effect of CP-424,391 treatment on the left ventricular contractility or relaxation in the current study is in contrast to the previous studies of the effects of GH or the hexapeptide GHS hexarelin in both acute cardiac injury and post-myocardial infarction cardiac dysfunction models. This difference may relate to the paradigms used since previous studies of the left ventricular functional recovery utilized isolated Langendorff-perfused hearts. A direct benefit of GHS treatment on cardiac functional recovery in vivo may have been attenuated or masked by the more complex physiologic milieu of the injury occurring in the reperfusion phase of the in vivo paradigm. Furthermore, a previous study (Rossoni et al., 1998) utilized a prophylactic hexarelin treatment period of 21 days in contrast to the 7-day treatment period with CP-424,391 used it the current study. An additional consideration is that ex vivo cardioprotection demonstrated by hexarelin may be affected by cardiac receptors that distinguish between subsets of GHS ligands. A class of hexarelin-binding sites that are not competed by MK-0677 has been characterized in rat heart (Bodart et al., 1999). However, a direct coronary vasoconstrictive action of hexarelin, as described by Bodart et al. (1999), in rat heart is somewhat incongruous if considered within the context of a cardioprotective mechanism. Acute administration of hexarelin to GH-deficient patients and GH-replete control subjects showed a transient increase in the left ventricular ejection fraction without changing plasma catecholamine levels, mean blood pressure or cardiac output in either group although the GH response was absent in the GH-deficient group (Bisi et al., 1999). It remains to be determined in follow-up studies if a longer CP-424,391 treatment period (i.e. 21 days) will confer not only to a greater degree of cardioprotection via infarct size reduction but also by improvement in left ventricular functional recovery.

The pharmacologic effect of GH or GHS treatment on left ventricular function post-myocardial infarction in either the acute or chronic period after myocardial infarction is complex. Both no effect (Shen et al., 1996, 1998) and positive responses (Cittadini et al., 1997; Jin et al., 1995; Yang et al., 1995b) have been reported in this regard. While the variation in heart failure paradigms (i.e. species, mechanism of failure induction, etc.) cannot be discounted to have an influence on GH/GHS efficacy, neither can the technology used to assess cardiac function. In this respect, studies in which GH/GHS treatment has been shown to improve left ventricular function have utilized the non-invasive technique of transthoracic echocardiography to delineate functional improvements. In heart failure paradigms, left ventricular dilation was reduced in GH-treated vs. control animals (Cittadini et al., 1997), and left ventricular fractional shortening was higher and left ventricular wall stress was lower in GH-treated pigs with heart failure (Houck et al., 1999). Thus, the method used to measure the potential improvements in the left ventricular function may impact the conclusions drawn.

The molecular mechanism by which cardioprotection was afforded by CP-424,391 in the current study remains to be defined. However, each of the principal components of the GH–IGF-1 axis is present in the heart, including the GH receptor mRNA, GH, GHRP and IGF-1 receptors (Tiong et al., 1989; Bodart et al., 1999; Isgaard et al., 1994; Ong et al., 1998; Toyozaki et al., 1993; Haro et al., 1999). The direct interaction of GH with specific receptors in the cardiac tissue may mediate a component of the cardiac response in rabbits to the GHS CP-424,391. Since rabbit cardiac GH receptors have been shown to be pharmacologically distinct from GH receptors in other tissues, the possibility of tissue-specific effects in the heart remains a possibility (Haro et al., 1999). However, with respect to the cardioprotective effects of GHRP, the more relevant effects of GH may be considered to be mediated by its stimulation of the expression of IGF-1. In our study, we clearly demonstrated that a dose of 25 mg/kg p.o. of CP-424,391, albeit a high dose, consistently elevated plasma IGF-1 concentrations. The increase in plasma IGF-1 in our studies is likely a reflection of the hepatic response to the GH, however, the presence of cardiac IGF-1 receptors (Toyozaki et al., 1993) suggests that circulating IGF-1 may influence the heart in a hormonal fashion. The IGF-1 and IGF-1 receptor expression in the heart is also influenced by GH. The local effects of IGF-1 acting in a paracrine/autocrine fashion may influence the myocyte survival in response to ischemia/reperfusion (Isgaard et al., 1999). Indeed, it has been shown that pharmacologic doses of IGF-1 protect the heart from ischemia/reperfusion injury (Buerke et al., 1995) as the overexpression of IGF-1 in the heart did (Li et al., 1997). The mechanism by which IGF-1 confers cardioprotection has been ascribed to its anti-apoptotic effects (Fujio et al., 2000;

Wang et al., 1998a,b). Thus, CP-424,391-mediated increases in plasma IGF-1 concentrations may have promoted cell survival pathways in the heart and affected a component of the cardioprotection by an anti-apoptotic mechanism. This mechanistic possibility remains to be confirmed by ongoing studies of this compound in cardiac ischemia/reperfusion paradigms.

Our current studies demonstrate once again that experimental ischemic and reperfused myocardium can be pharmacologically protected against cell death. There have been many studies over the last 15 years that have demonstrated this pharmacology, and mechanisms involving neutrophils, oxygen-derived radical species, complement, ion exchange mechanisms (i.e. sodium–hydrogen exchange) and opiate or adenosine receptors have been implicated to play a significant role in mediating cardiac injury (cell death) (Park and Lucchesi, 1999). Although the experimental data have been convincing in almost all cases through confirmation by independent laboratories, clinical trials have not been as successful. However, the recent findings of the AMISTAD trial indicate that human myocardium can be pharmacologically protected from ischemia/reperfusion injury (i.e. infarct size reduced) through the administration of adenosine (Mahaffey et al., 1999). In our studies of the GHS CP-424,391, we employed a 7-day dosing paradigm to effect the cardioprotection in the rabbit. While this dosing paradigm is not apropos for the treatment of an acute myocardial infarction, there may be a therapeutic opportunity for GHS in treating patients with scheduled cardiac surgery where the risk of ischemia is high (i.e. peri-operative myocardial ischemia). In this regard, it has been shown clinically that although cariporide (a sodium hydrogen exchanger-1 inhibitor) did not produce significant cardioprotection in all the patients studied, the compound was effective in a subgroup of patients undergoing CABG (Theroux et al., 2000). Thus, in relation to the currently employed 7-day dosing paradigm, GHS may be effective in some but not in all patients undergoing ischemia/reperfusion injury.

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