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# Inhibition of cardiac leptin expression after infarction reduces subsequent dysfunction

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## Abstract

Leptin is known to exert cardiodepressive effects and to induce left ventricular (LV) remodelling. Nevertheless, the autocrine and/or paracrine activities of this adipokine in the context of post-infarct dysfunction and remodelling have not yet been elucidated. Therefore, we have investigated the evolution of myocardial leptin expression following myocardial infarction (MI) and evaluated the consequences of specific cardiac leptin inhibition on subsequent LV dysfunction. Anaesthetized rats were subjected to temporary coronary occlusion. An antisense oligodesoxynucleotide (AS ODN) directed against leptin mRNA was injected intramyocardially along the border of the infarct 5 days after surgery. Cardiac morphometry and function were monitored by echocardiography over 11 weeks following MI. Production of myocardial leptin and pro-inflammatory cytokines interleukin (IL)-1β and IL-6 were assessed by ELISA. Our results show that (1) cardiac leptin level peaks 7 days after reperfused MI; (2) intramyocardial injection of leptin-AS ODN reduces early IL-1β and IL-6 overexpression and markedly protects contractile function. In conclusion, our findings demonstrate that cardiac leptin expression after MI could contribute to the evolution towards heart failure through autocrine and/or paracrine actions. The detrimental effect of leptin could be mediated by pro-inflammatory cytokines such as IL-1β and IL-6. Our data could constitute the basis of new therapeutic approaches aimed to improve post-MI outcome.

**Keywords:** reperfused myocardial infarction • leptin • left ventricular dysfunction • cytokines • antisense oligodesoxynucleotides

## Introduction

The adipokine leptin was primarily described as a satiety regulatory metabotropic hormone, mainly produced by adipocytes under physiological conditions. Recently, leptin and its receptors (ObRs) have been shown to be overexpressed in a variety of tissues including the myocardium under conditions of ischemia/reperfusion [1–3]. Several lines of evidence indicate that leptin has powerful cardiac effects [4]. Nevertheless, reports are divided on whether leptin has predominantly adverse or beneficial effects on the heart.

Leptin has been shown to decrease cardiac function through a signal transducer and activator of transcription 3 (STAT-3)-nitric-oxide-p38 mitogen-activated protein kinase (p38 MAPK)-dependent mechanism [5] and to exert a direct hypertrophic effect [6–10]. Moreover, *in vivo* continuous infusion of leptin following myocardial infarction (MI) in mice causes eccentric dilation with increased systolic function [10]. In addition, Purdham *et al.* have recently shown that chronic ObR blockade by systemic injection of specific antibodies limits the development of post-infarct cardiac dysfunction in rats [11]. Conversely, blunting leptin signalling in mice through leptin deficiency or ObR deficiency is associated with progressive cardiac hypertrophy [12] and increased severity of cardiac dysfunction and remodelling after MI [13]. In addition, leptin has been shown to reduce infarct size in isolated perfused rat hearts [14] and to attenuate cardiac apoptosis after ischemia by increasing bcl-2 and survivin gene expression and by reducing

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caspase-3 activity [15]. Therefore, the cardiovascular effects of leptin are very complex, and translating basic research studies to human physiology is very difficult, particularly in this area of research.

Clinical data have reported that elevated circulating leptin levels are associated with greater risk of congestive heart failure and cardiovascular disease at least in elderly [16]. Moreover, it has been demonstrated that human serum leptin level reaches a peak value on the second day of hospitalization after MI [17]. These observations suggest that leptin might be involved in the pathophysiological processes leading to cardiac dysfunction and adverse remodelling after MI in human beings.

The aim of the present study was first to determine the profile of cardiac leptin production in a model of reperfused MI in rats. Therefore, myocardial leptin level was assessed by Chemiarray® at different time-points over 10 days after the surgical induction of temporary cardiac ischemia. This preliminary experiment has allowed us to demonstrate a transient peak of leptin cardiac content, reaching a maximum 7 days after reperfused infarction. The second step of the study has consisted in specifically inhibiting post-infarct cardiac leptin production by use of a specific antisense oligodeoxynucleotide (AS ODN) directed against leptin mRNA and directly injected in the myocardial wall, along the border of infarction. This study was designed to evaluate the autocrine and/or paracrine effects of leptin in the heart on long-term cardiac dysfunction without affecting extracardiac leptin activity.

# Materials and methods

## Reperfused MI

Adult male Wistar rats (250–350 g body wt; Charles River, L'Arbresle Cedex, France) were maintained on a standard diet and cared for according to the guiding principles in the care and use of animals (European Communities Council Directive L358-86/609/EEC, November 1986). All protocols involving living animals were performed under the license from the French authorities (license number A38018).

Rats were anaesthetized intraperitoneally with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg). Rats were rapidly intubated and mechanically ventilated (tidal volume: 1 ml/100 g body weight; ventilation rate: 65 strokes/min.) with a mixture of isoflurane (0.5%; AErrane®, Lessins, Belgium) and oxygen (20%) in room air (79.5%). Experimental MI was performed as previously described [18]. A left thoracotomy was performed at the fourth intercostal space and the heart was briefly exteriorized by digital pressure on the chest wall. The left coronary artery was ligated 1–2 mm from its origin. The heart was then quickly returned to the chest cavity. After 1 hr of occlusion, the ligation was removed and the left coronary artery reperfused.

## Time course of changes in leptin cardiac content

Following left coronary artery occlusion and reperfusion, rats were killed 3, 5, 7, 8 or 10 days after MI. And heart samples were quickly frozen at liquid nitrogen temperature and stored at –80°C until assay. Frozen samples (200–400 mg) were crushed into liquid nitrogen and homogenized in a Tris (25 mM)-ethylenediaminetetraacetic acid (2 mM) buffer (pH 7.4) adapted from Guo *et al.* [19], and containing a protease inhibitor cocktail 1/200 (P2714; Sigma-Aldrich, L'Isle d'Abeau Chesnes, France), and Triton X-100 (0.5%). After incubation for 2 hrs at 4°C, this suspension was centrifuged for 30 min. at 14,000 × *g* at 4°C to remove cellular debris. Protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Myocardial leptin levels were assessed by Chemiarray™ according to the manufacturer's instructions (Rat Cytokine Antibody Array I; Chemicon-Millipore, Inc., Billerica, MA, USA). Briefly, membranes were incubated for 30 min. in 10 mM Tris buffer (5% bovine serum albumin, 0.15 M NaCl, pH 7.6) and for 1 hr with protein extracts in presence of biotinylated antibodies. Membranes were then washed and incubated for 30 min. with horseradish peroxidase (HRP)-conjugated streptavidin. Membranes were revealed with enhanced chemiluminescence (ECL).

For each group, the same amount of cardiac extract from three different hearts were mixed and incubated with the membrane, as previously described [18]. Membranes were scanned and blot-intensities (arbitrary units) were measured using ImageJ 1.37. Optical density of each spot was expressed as a percentage of a specific positive internal standard (after subtraction of background).

## Cardiac leptin inhibition with antisense oligodeoxynucleotides

Specific anti-leptin AS ODN and inactive random ODN [Control ODN, nearly same length and guanine-cytosine (GC) content] were designed and manufactured by Biognostik (Antisense custom design kit, accession n° : MN-013076; Biognostik, Göttingen, Germany). AS ODN and Control ODN were stored in sterile phosphate buffer at 4°C until use.

Five days after coronary artery occlusion and reperfusion, rats were anaesthetized and ventilated. The fourth intercostal space was reopened and the heart was briefly exteriorized by digital pressure on the chest wall. Control ODN or AS ODN (0.1 mM solution) was rapidly injected in the myocardial wall in the border of the infracted area. Each animal received five intramyocardial micro-injections of ODN solution (total injected volume: 0.15 ml). No mortality was observed during this protocol.

## Echocardiographic follow-up

Two-dimensional targeted M-mode recording was obtained from the short axis view of the heart at the level of the papillary muscle using a 15-7 MHz probe (HP21380A; Agilent Technology, Philips Systèmes Médicaux, France) connected to a Helwett Packard Sonos 5500. TM-mode measurements of the LV end-diastolic (LVEDD) and LV end-systolic (LVESD) dimensions were made according to the recommendations formulated by the American Society of Echocardiography. The percentage of LV fractional shortening (LVFS) was calculated as LVFS (%) = ([LVEDD – LVESD]/LVEDD) × 100.

## *Ex vivo* determination of LV pressure–volume curves

Seven or 77 days after the surgical induction of infarction, rats were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and passive pressure–volume characteristics of the LV were evaluated post-mortem as previously described [20]. A saturated solution of potassium chloride was injected into the *vena cava* until the heart stopped. The heart was then

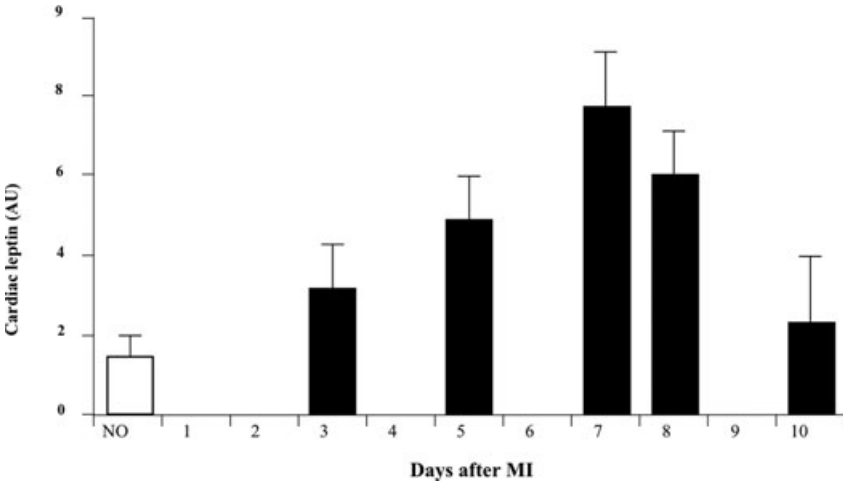


**Fig. 1** Evolution of cardiac leptin level during the first 10 days following reperfused MI. Chemiarray™: Blot intensities are expressed as a percentage of positive specific internal controls. $n = 3$ hearts per point.

excised and a cannula, connected to a pressure transducer (P23XL; Statham Instruments, Hato Ray, Puerto Rico), was inserted 5 mm into the LV through the aorta. The right and left atrioventricular junctions, the pulmonary artery and *vena cava* were ligated and physiological saline was infused in the LV at a constant flow rate of 0.68 ml/min. while intraventricular pressure was continuously monitored from 0 to 40 mmHg. The operating LV end-diastolic volume (OLVEDV) was determined from the LV pressure–volume curve and defined as the volume corresponding to a filling pressure equal to *in vivo* end-diastolic pressure.

### Assessment of infarct size

Hearts were frozen in liquid nitrogen and cut at –20°C with a cryostat (Microm HM505E; Microm International GmbH, Walldorf, Germany). Three 20-μm-thick transverse sections were obtained at 5.36 mm from the basis of the ventricles of each frozen heart. Each frozen section was stained using nitro blue tetrazolium (0.04% in 0.05 mol/l sodium succinate buffer, pH 7.6). Necrotic and non-necrotic tissues were distinguished by the absence or presence of staining respectively, and the endocardial and epicardial lengths, as well as total areas of necrotic and non-infarcted muscles were estimated by planimetry.

### Estimation of myocardial leptin, interleukin (IL)-6 and IL-1β by ELISA

Leptin, IL-6 and IL-1β were assessed using ELISA kits (leptin/IL-6/IL-1β Duoset; R&D Systems, Abingdon, UK), and expressed as pg/mg protein.

### Statistical analysis

Values are expressed as means ± SEM. One way analysis of variance was performed to determine significant differences between groups. The significance of the difference between the means of the groups was tested with Fisher's *a posteriori* protected least significant difference test. $P = 0.05$ was considered as the threshold of statistical significance.

## Results

### Time course of changes in myocardial leptin content after reperfused MI

Myocardial leptin level, measured by cytokine array, progressively increased from day 3 after MI to reach a maximum (+380% *versus* non-operated) at day 7, before returning to non-operated baseline level at day 10 (Fig. 1).

### Effects of cardiac leptin inhibition on after MI

There was no significant difference in infarct size between Control ODN-treated (35.16 ± 1.86) and AS ODN-treated (35.74 ± 2.26) groups 1 week and 11 weeks after reperfused infarction (1 week after MI: Cont ODN = 35.16 ± 1.86 *versus* AS ODN = 35.74 ± 2.26, ns; 11 weeks after MI: Cont ODN = 36.60 ± 4.16 *versus* AS ODN = 3.88 ± 6.99, ns). Body weight and food intake were equivalent in both groups (data not shown) over the 11 week follow up.

In order to verify that ODNs were still present in the myocardium 2 days after injection, control rats were injected with Fluoresceine isothiocyanate (FITC)-labelled ODNs, 5 days after MI. Two days after FITC-ODN injection, rats were killed and their hearts were placed under a fluorescence camera (EMCCD 512 × 512 Hamamatsu Orca – Macro Fluo 512 × 512; Hamamatsu Photonics, Massy, France) and illuminated at 488 nm (Cube FITC Leica, Leica Mycrosystemes SAS, Nanterre, France). Compared to untreated hearts (Fig. 2A), FITC-ODN injected hearts exhibited diffuse fluorescence as well as focal hot spots corresponding to the points of injection (Fig. 2B and C).

Myocardial leptin, measured by ELISA, was significantly reduced 7 days after the initial surgical protocol in AS ODN-Treated rats compared to Control ODN-treated rats (Fig. 3A). Myocardial IL-1β (Fig. 3B) and IL-6 (Fig. 3C), measured by ELISA,

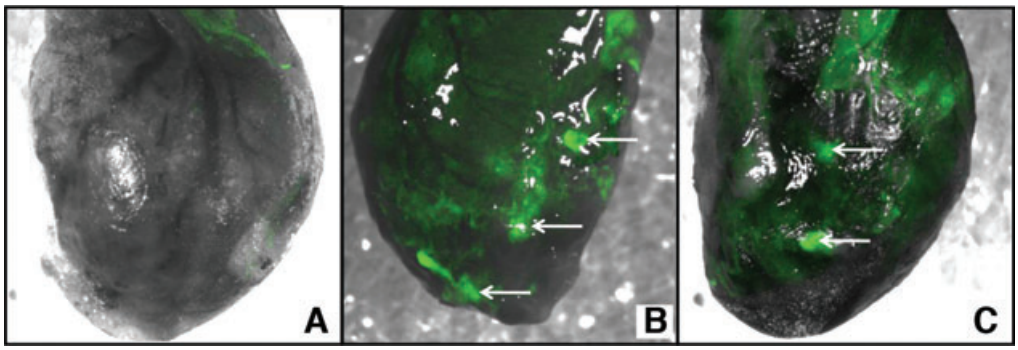


**Fig. 2** Localization of ODNs two days after intramyocardial injection. Rats were subjected to reperfused MI. Control unlabelled ODNs or FITC-labelled ODNs were injected in the border of the infarct, five days after surgery. Two days after ODN injection, rats were killed and their hearts were placed under a fluorescence camera (EMCCD 512 × 512 Hamamatsu Orca – Macro Fluo 512 × 512) and illuminated at 488 nm (Cube FITC Leica). (**A**) Rat heart injected with Control unlabelled ODNs; Rat heart injected with FITC-labelled ODNs: left view (**B**), right view (**C**).

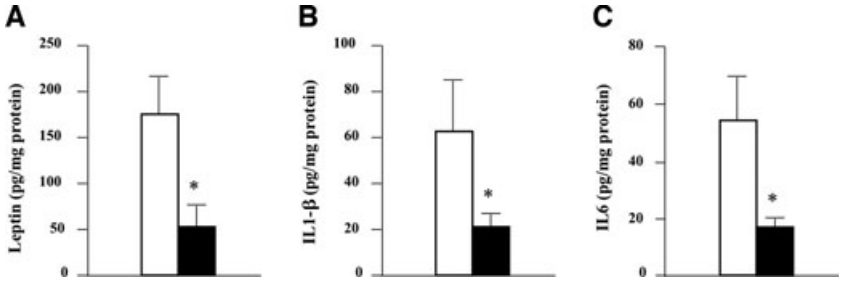


**Fig. 3** Effect of antisense leptin inhibition on leptin, IL-1β and IL-6 myocardial levels, 7 days after reperfused MI. Leptin (**A**), IL-1β (**B**) and IL-6 (**C**) ELISA assays on six to seven hearts per group. Open bars: Control ODN-treated group; Full bars: AS ODN-treated group. Means ± S.E.M. $^{*}P < 0.05$ *versus* Control ODN-treated group.

were markedly reduced 7 days after reperfused infarction in the AS ODN-treated group compared to the Control ODN-treated group.

### Echocardiographic measurements

In rats treated with Control ODNs, MI induced a rapid decrease in LV contractility (Fig. 4) which slowed down after the second week after surgery, giving a characteristic exponential curve. The treatment with anti-leptin ODNs completely stopped the deterioration of LV contractility. As a consequence, 77 days after the initial reperfused infarction, LVFS (%) in AS ODN-treated rats was 80% higher than LVFS in Control ODN rats (Fig. 4A).

### Pressure–volume curves

LV pressure–volume curves measured *ex vivo* in potassium arrested hearts are shown in Figure 5A. Pressure–volume curves were equivalent in Control ODN and AS ODN-treated groups at day 7 after reperfused MI. After 77 days of reperfusion, a significant rightward shift of the curves for both groups was found compared with the curves obtained at day 7, indicating an increase in LV compliance. At day 77, the increase in LV compliance was less severe in AS ODN-treated rats than in Control ODN-treated rats. In Control ODN-treated rats, OLVEDV was also significantly increased 77 days after reperfused MI compared with 7 days after surgery (Fig. 5B) indicating an increase in LV volume. AS ODN-treatment totally prevented the increase in OLVEDV between the 7th and the 77th day after MI (Fig. 5B).

## Discussion

Our experimental model of temporary left coronary artery ligation in rats induces a transient increase in myocardial leptin level, 7 days after reperfused MI. The inhibition of this peak of leptin production by specific AS ODNs reduces the expression of cardiac pro-inflammatory cytokines IL-1β and IL-6 and markedly reduces LV dysfunction.

### Leptin neutralization after myocardial infarction

Leptin has been shown to exert a negative inotropic effect on ventricular myocytes *via* increased nitric oxide production [5, 21], as well as to induce cardiac hypertrophy [6, 12]. On this basis some authors have suggested that ObR blockade could reduce cardiac hypertrophy and improve post-infarct cardiac function [11]. Therefore, they have tested the effects of chronic systemic administration

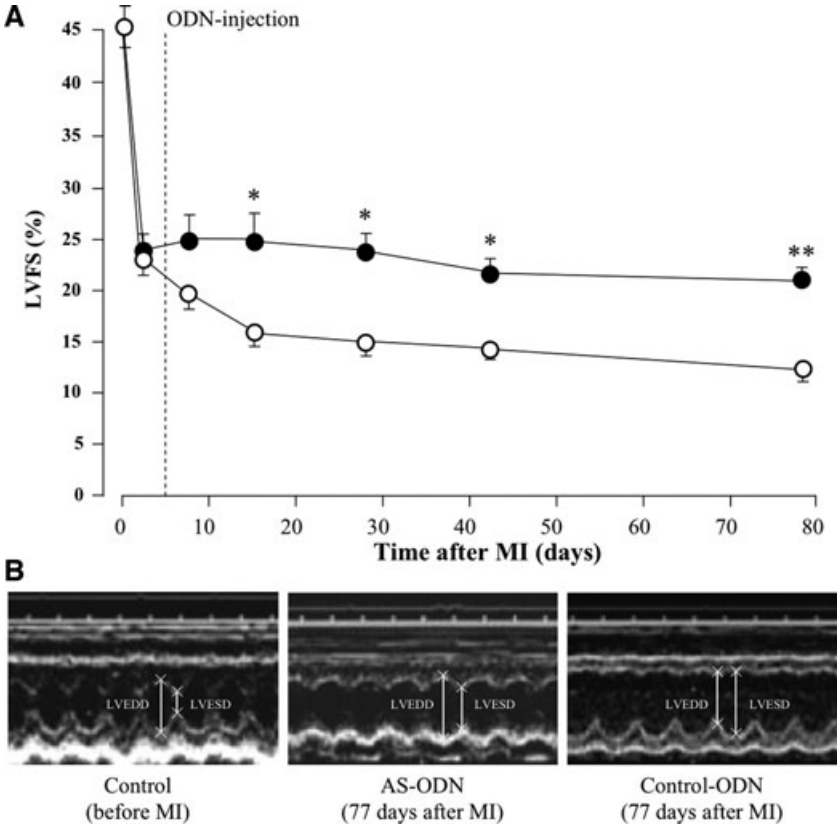


**Fig. 4** Effects of antisense leptin inhibition on cardiac contractility during the 11 week follow-up. (**A**) Echocardiographic measurement of LVFS; open circles: Control ODN-treated group; full circles: AS ODN-treated group; means ± S.E.M.; $n = 6$–10 rats per group; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* Control ODN-treated group. (**B**) Representative M-mode echocardiographic images. LVEDD = left ventricular end diastolic diameter; LVESD = left ventricular end systolic diameter.

(daily i.v. injection) of anti-ObR antibodies, in a rat model of permanent coronary occlusion, over a time period of 4 weeks after MI [11]. Although this study has reported beneficial effects of the treatment, its translation to the human pathology of infarction is very difficult for several reasons. First, if leptin contributes to post-infarct cardiac dysfunction and heart failure, it is probably *via* an autocrine or paracrine mechanism [4]. Therefore, the anti-leptin treatment should have been targeted to the heart to avoid any interference with the physiological endocrine activity of the hormone. Second, the treatment should have been limited in duration to the time period during which endogenously produced leptin exerts its autocrine or paracrine detrimental action to the heart. Contrarily to this previous systemic approach, our study fulfils these two criteria since the AS ODN-treatment is restricted to the myocardium and limited to the period of time during which leptin cardiac content was found to increase after reperfused MI. Indeed, phosphorothioate oligodeoxynucleotides have been shown to be metabolized in living tissues and slowly eliminated within 2–7 days, depending on the organ [22]. Therefore, the AS ODNs used in our study might not affect the endocrine activity of leptin since our treatment does not modify food intake. Nevertheless, since we have not monitored leptin expression throughout the protocol, we cannot exclude the possibility that our short-term ODN treatment could have induced profound phenotypic modifications influencing long-term cardiac expression of leptin.

## Leptin and proinflammatory cytokines

ObRs have been shown to be induced locally in the heart after a sequence of ischemia and reperfusion [1, 3]. Consistently with these previous reports, the present study shows that after temporary left coronary artery ligation in the rat, cardiac leptin level is increased up to 4-fold compared with basal values. Moreover, our study of the time course of cardiac leptin production after reperfusion indicates that this phenomenon peaks 7 days after reperfused MI and decreases thereafter. Interestingly, several experimental studies have reported a transient myocardial increase in the expression of diverse proinflammatory cytokines 1 week after MI in the same rat model [18, 19, 23]. In addition, Berthonneche *et al.* [20] have demonstrated that some of these cytokines contribute significantly to the development of subsequent LV dysfunction and heart failure since their inhibition durably protects cardiac function after MI. A direct link between leptin and pro-inflammatory cytokine production has already been demonstrated in several experimental studies. Notably, leptin induces IL-1β and IL-6 in different cell types [24–27]. In the rat model of reperfused MI, IL-6 and IL-1β are transiently over produced 8 days after surgery [18]. In our study, the injection of leptin AS ODNs 5 days after surgery totally prevented the increase in cardiac IL-6 and IL-1β measured 7 days after reperfused MI. Our results therefore suggest that leptin accumulation in the myocardium after reperfused MI could

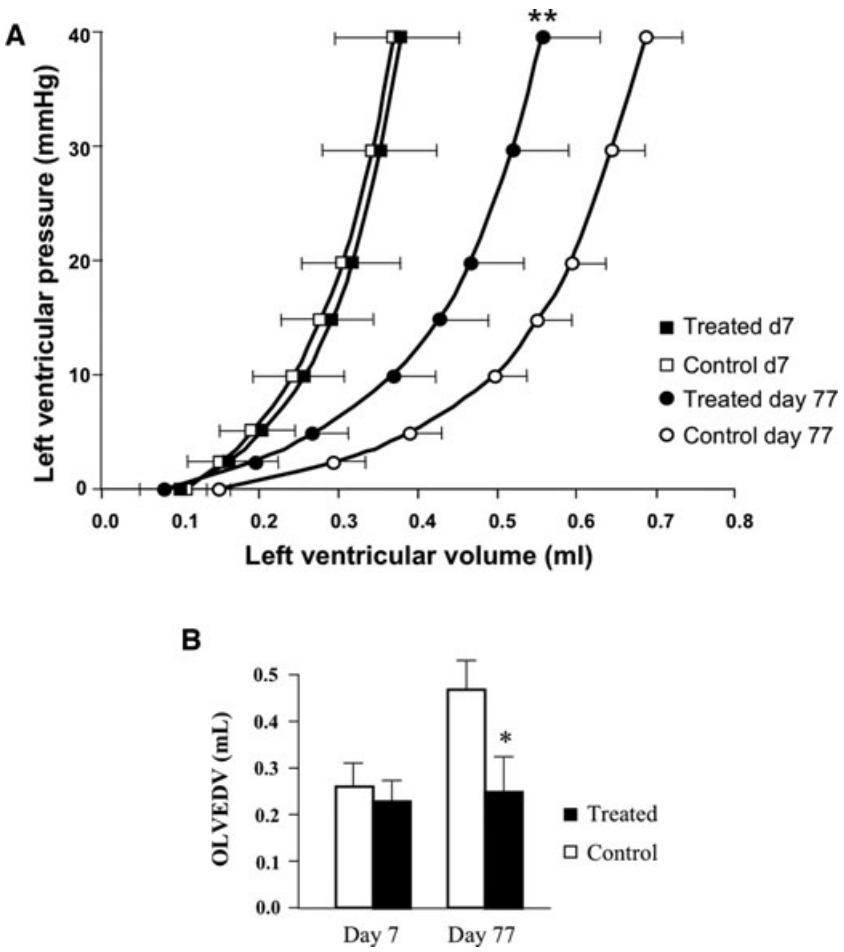


**Fig. 5** LV pressure–volume curves (**A**) and OLVEDVs (**B**) obtained *ex vivo* after potassium arrest during saline infusion over a range of pressure of 0–40 mmHg 7 or 77 days after reperfused MI. OLVEDV: operating LV end-diastolic volume; means ± S.E.M.; $n = 5$–9 rats per group; $^{**}P < 0.01$ *versus* corresponding Control ODN-treated group.

contribute to LV dysfunction and subsequent heart failure, at least in part through the induction of pro-inflammatory cytokine expression.

## Cardiac sources of leptin

The present study demonstrates that reperfused MI induces a transient increase in cardiac leptin content within the first week after surgery. Nevertheless, the cell types responsible for leptin production as well as the mechanisms by which leptin expression is induced still remain to be determined. Leptin is traditionally viewed as an adipocyte-released hormone with endocrine effects. Nevertheless, several peripheral tissues, including the heart, are known to produce leptin which can then exert autocrine or paracine activities [1, 7]. Several *in vitro* studies have demonstrated that cardiomyocytes are able to produce leptin under diverse conditions of stress. Nevertheless, under pathophysiological situations, other cell types such as pericardial (epicardial plus paracardial adipose tissue [28]) and perivascular adipocytes, can significantly contribute to cardiac leptin production. In small rodents, pericardial fat is only present in obese animals and is limited to a localized area around the pericardium, without any direct contact with the cardiomyocytes [4]. Therefore, the pericardial adipose tissue is probably not significantly involved in the transient cardiac leptin expression that we have observed in our study. In pathological states, perivascular fat can also secrete a deleterious profile of adipokines, and leptin levels in this tissue are thought to increase [29]. Nevertheless, the fact that direct intramyocardial injections of anti-leptin ODNs totally abolished the overproduction of leptin in our study strongly suggests that the origin of leptin is within the myocardium rather than perivascular.

In conclusion, our study shows that transient production of leptin in the myocardium after reperfused MI, contributes to the development of LV dysfunction and subsequent heart failure, through an autocrine or paracrine mechanism. Moreover, our results suggest that the mechanism of action of leptin in this context might involve the overexpression of proinflammatory cytokines.

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## Conflict of interest

The authors confirm that there are no conflicts of interest.

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