Obestatin affords cardioprotection to the ischemic-reperfused isolated rat heart and inhibits apoptosis in cultures of similarly stressed cardiomyocytes

Giuseppe Alloatti,1,4 Elisa Arnoletti,2 Eleonora Bassino,1 Claudia Penna,3 Maria Giulia Perrelli,3 Corrado Ghé,2 and Giampiero Muccio2

1Department of Animal and Human Biology, 2Division of Pharmacology, Department of Anatomy, Pharmacology and Forensic Medicine, and 3Department of Clinical and Biological Sciences, University of Turin, Turin, Italy; and 4Istituto Nazionale per la Ricerca Cardiovascolare, Bologna, Italy

Submitted 24 August 2009; accepted in final form 1 June 2010

Alloatti G, Arnoletti E, Bassino E, Penna C, Perrelli MG, Ghé C, Muccio G. Obestatin affords cardioprotection to the ischemic-reperfused isolated rat heart and inhibits apoptosis in cultures of similarly stressed cardiomyocytes. Am J Physiol Heart Circ Physiol 299: H470–H481, 2010. First published June 4, 2010; doi:10.1152/ajpheart.00800.2009.—Obestatin, a newly discovered peptide encoded by the ghrelin gene, induces the expression of genes regulating pancreatic β-cell differentiation, insulin biosynthesis, and glucose metabolism. It also activates antiapoptotic signaling pathways such as phosphoinositide 3-kinase (PI3K) and ERK1/2 in pancreatic β-cells and human islets. Since these kinases have been shown to protect against myocardial injury, we sought to investigate whether obestatin would exert cardioprotective effects. Both isolated perfused rat heart and cultured cardiomyocyte models of ischemia-reperfusion (I/R) were used to measure infarct size and cell apoptosis as end points of injury. The presence of specific obestatin receptors on cardiac cells as well as the signaling pathways underlying the obestatin effect were also studied. In the isolated heart, the addition of rat obestatin-(1–23) before ischemia reduced infarct size and contractile dysfunction in a concentration-dependent manner, whereas obestatin-(23–1), a synthetic analog with an inverse amino acid sequence, was ineffective. The cardioprotective effect of obestatin-(1–23) was observed at concentrations of 10–50 nmol/l and was abolished by inhibiting PI3K or PKC by the addition of wortmannin (100 nmol/l) or chelerythrine (5 μmol/l), respectively. In rat H9c2 cardiac cells or isolated ventricular myocytes subjected to I/R, 50 nmol/l obestatin-(1–23) reduced cardiomyocyte apoptosis and reduced caspase-3 activation; the antiapoptotic effect was blocked by the inhibition of PKC, PI3K, or ERK1/2 pathways. In keeping with these functional findings, radioreceptor binding results revealed the presence of specific high-affinity obestatin-binding sites, mainly localized on membranes of the ventricular myocardium and cardiomyocytes. Our data suggest that, by acting on specific receptors, obestatin-(1–23) activates PI3K, PKC-ε, PKC-δ, and ERK1/2 signaling and protects cardiac cells against myocardial injury and apoptosis induced by I/R.

ischemia-reperfusion; myocardial infarction; obestatin receptors

obestatin is a recently identified amidated 23-amino acid peptide derived from the carboxy-terminal part of proghrelin, whereas ghrelin derives from the amino-terminal part of the same precursor (52). Originally extracted from the rat stomach, obestatin has been suggested to be a circulating peptide that behaves as a physiological opponent to ghrelin, inhibiting food intake, body weight gain, gastric emptying, jejunal contractility, and growth hormone secretion through interactions with the orphan G protein-coupled receptor 39 (GPR39) (64). However, these findings have been questioned lately, and a number of studies (19, 43, 57) have failed to confirm the anorexigenic effect of obestatin. Furthermore, other groups (13, 27, 34) have been unable to demonstrate that obestatin has agonist properties on GPR39. Therefore, to date, some biological actions of obestatin seem to be controversial and its specific receptor remains unknown.

In addition to effects on feeding, weight gain, and gastrointestinal motility, obestatin has also been shown to influence water intake, memory, anxiety, and sleep via central actions (58) and to stimulate the proliferation of human retinal pigment epithelial and gastric cancer cells by PKC, phosphoinositide 3-kinase (PI3K)/Akt, and ERK1/2 activation (10, 51). Recently, we have demonstrated that obestatin prevents apoptosis in both rodent β-cells and human pancreatic islets by binding to specific obestatin receptors and activation of PI3K/Akt and ERK1/2. This suggests that obestatin may also play a role in cell survival (20). Both PI3K/Akt and ERK1/2 kinases have been demonstrated to protect against apoptosis in several cell types other than pancreatic β-cells, and they, together with PKC, have also been proposed as integral components of antiapoptotic cascades involved in myocardial protection in the setting of ischemia-reperfusion (I/R) as well as myocardial preconditioning (46). In this context, it is important to bear in mind that some ghrelin gene-derived peptides, such as ghrelin and its endogenous derivatives (unacylated ghrelin and des-Gln14 ghrelin), have a wide array of cardiovascular activities (6). Beneficial cardiotropic effects of ghrelin and its analogs have been demonstrated in various experimental models (30, 39). These may result not only from increased growth hormone secretion and appetite, vasodilation, and decreased cytokine production but also from direct receptor-mediated effects of ghrelin and ghrelin mimetic substances on cardiomyocytes (44). Ghrelin decreases cardiac inotropism (5), improves myocardial function after I/R and isoproterenol-induced injury (11, 36), reduces infarct size through a PKC-dependent pathway (16), and inhibits the apoptosis of cardiomyocytes through the activation of PI3K/Akt and ERK1/2 kinases (4). The fact that isolated murine and human cardiomyocytes synthesize and secrete ghrelin suggests that this peptide may also exert protective effects through paracrine/autocrine mechanisms (29). In light of the facts that obestatin originates from the same preprohormone and that circulating levels of the 29- to 94-amino acid proghrelin fragment (C-ghrelin), from which obestatin is cleaved (52), are modulated in cardiovascular diseases (48, 53), it seems logical to hypothesize that obestatin could also have a role in protecting myocardial function under pathological conditions.

Address for reprint requests and other correspondence: G. Alloatti, Dipartimento di Biologia Animale e dell’Uomo, Via Accademia Albertina, 13, Torino 10123, Italy (e-mail: giuseppe.alloatti@unito.it).
Therefore, the aim of this study was to examine the effects of obestatin on acute I/R injury in isolated rat hearts and its effects on apoptosis induced in cultured rat cardiomyocytes by an experimental model of I/R. Infarct size, lactate dehydrogenase (LDH) release, and cardiohemodynamic performances were assessed as end points of myocardial damage, whereas morphological nuclear changes detected by Hoechst 33258 staining and caspase-3 activity were evaluated as markers of cardiomyocyte apoptosis. In addition, the involvement of specific obestatin receptors in these cardioprotective effects and the effect of selective inhibitors of the antiapoptotic signaling pathway were investigated.

METHODS

Chemicals. The following peptides were all obtained from NeoMPS (Strasbourg, France) and were produced by conventional solid-phase synthesis and purified (97%) by reverse-phase HPLC (numbers of amino acid residues and their sequences are given in parentheses): rat obestatin-(1–23) (Phe-Asn-Ala-Pro-Phe-Asp-Val-Gly-Ile-Lys-Ala-Glu-Leu-Ser-Ala-Glu-Ash-Ash-Phe-Glu-Val-Gly-Ile-Lys-Leu-Ala-Arg-Ala-Ala-Glu), rabbit obestatin-(1–28) (Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-Gln-Glu-Ash-Glu-Ser-Ser-Pro-Pro-Ala-Lys-Leu-Glu-Pro-Glu), porcine motilin, and rat obestatin-(23–1). The latter is a synthetic obestatin analog containing the same amino acids as obestatin-(1–23) but in an inverse order of sequence. 125I-labeled rat obestatin-(1–23) (125I-obestatin; specific activity: 1,800 –2,000 Ci/mmol) was radioiodinated using a lactoperoxidase method by GE Healthcare (Amersham). Solutions used in isolated heart and cell culture experiments were freshly prepared. Cell culture reagents were purchased from Invitrogen (Milan, Italy). Solutions used in isolated heart and cell culture experiments were freshly prepared. Cell culture reagents were purchased from Invitrogen (Milan, Italy).

Animals. Male rats (250 –300 g body wt) were allowed access to tap water and standard rodent diet ad libitum. Animals received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and in accordance with the Italian law (DL-116, Jan. 27, 1992).

The scientific project was supervised and approved by the Italian Ministry of Health (Rome, Italy) and by the ethical committee of the University of Turin.

Isolated heart preparations. The cardioprotective effect of obestatin was tested on isolated coronary-perfused rat hearts. Rats were anesthetized, heparinized, and killed by cervical dislocation. After death, the heart was rapidly excised, placed in ice-cold buffer solution, and weighed. Isolated hearts were attached to the perfusion apparatus and retrogradely perfused at a constant pressure (80 mmHg) with a 0.9% NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5.5 D-glucose, and 5 HEPES (pH adjusted to 7.38 with NaOH). Hearts were kept in a temperature-controlled chamber (36°C), electrically paced at 280 beats/min through a 302-T Anapulse stimulator connected to a 305-R stimulus isolator (World Precision Instruments, New Haven, CT), and instrumented as previously described (2). Coronary flow (CF) was monitored with an electronic drop counter placed along the perfusion line. Isolated hearts were allowed to stabilize for 30 min, when baseline parameters were recorded. After stabilization, hearts were randomly assigned to one of the treatment groups described below and then subjected to 40 min of global, no-flow ischemia followed by 120 min of reperfusion. Hearts were divided into nine experimental groups (5 hearts/group) and received, before ischemia, an additional 20 min of treatment as follows: control group (perfusion with physiological solution; group 1); treatment with different concentrations (5, 10, 20, or 50 nmol/l) of obestatin-(1–23) (groups 2–5); combined treatment of 50 nmol/l obestatin-(1–23) together with 100 nmol/l wortmannin, a PI3K/Akt inhibitor (62) (group 6); combined treatment of 50 nmol/l obestatin-(1–23) together with 5 μmol/l chereathyrine, a specific PKC inhibitor (25) (group 7); treatment with 50 nmol/l obestatin-(23–1) (group 8); and, finally, treatment with ghrelin (50 nmol/l) to compare the effects of this peptide with those produced by obestatin (group 9).

In preliminary experiments, we showed that in hearts pretreated with wortmannin (100 nmol/l) or chereathyrine (5 μmol/l) alone before ischemia, the recovery of left ventricular (LV) pressure (LVP) during reperfusion and infarct size were not statistically different from those observed in the control group (group 1).

Myocardial injury. To assess myocardial injury, hearts were rapidly removed from the perfusion apparatus at the end of reperfusion, and the ventricular tissue was dissected into 2- to 3-mm circumferential slices, which were then incubated for 20 min at 37°C in a 0.1% solution of nitroblue tetrazolium in phosphate buffer, where the unstained necrotic tissue could be separated from the stained viable tissue. The weights of the necrotic and non-necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total ventricular mass (38). LDH release, an index of cytotoxicity, was measured according to Hollaar and Van der Laarse (26). Samples of coronary effluent (2 ml) were withdrawn from a catheter inserted into the right ventricle via the pulmonary artery immediately before ischemia and at 2, 5, 10, and 20 min of reperfusion. Thereafter, samples were collected every 20 min until the end of reperfusion. Data are expressed as cumulative values for the entire reperfusion period.

Myocardial performance. A polyvinyl chloride balloon was placed into the LV and connected to an electromanometer to record LVP in the same experiments in which measurements of infarct size were performed. LVP was visualized on a Tektronix 2211 digital storage oscilloscope and continuously acquired and recorded by a Power Mac computer using Labview software (National Instruments). LV performance was assessed by measuring LV end-diastolic pressure (LVEDP) and maximum rate of rise of LV developed pressure (LV +dP/dt).

Western blot analysis. To investigate whether obestatin-(1–23) could induce the phosphorylation of different kinases (Akt, PKC-ε, PKC-δ, and ERK1/2) involved in cardioprotection (7, 9, 23, 24, 33, 47, 60), after stabilization, rat hearts were perfused with physiological solution alone for additional 10 min (sham) or treated with 50 nmol/l obestatin-(1–23) for 10 min (in all cases, n = 5). Hearts were then homogenized on ice in RIPA lysis buffer (Santa Cruz Biotechnology) using a polytron tissue grinder. The homogenate was centrifuged at 4°C for 30 min at 13,000 g. The supernatants, corresponding to ~60 μg protein, were subjected to SDS-PAGE on 8% acrylamide gels (for PKC-ε, phospho-PKC-ε, PKC-δ, and phospho-PKC-δ) or 10% acrylamide gels (for Akt, phospho-Akt, ERK1/2, and phospho-ERK1/2) and transferred to polyvinylidene difluoride membranes (GE Healthcare). Membranes were then incubated overnight at 4°C with the following primary antibodies: anti-PKC-ε, anti-Akt, anti-phospho-(Ser473)-Akt, anti-PKC-δ, anti-phospho-(Ser643)-PKC-δ, anti-ERK1/2, and anti-phospho-(Thr202,Tyr204)-ERK1/2 (Cell Signaling), and anti-phospho-(Ser729)-PKC-ε (Upstate). To confirm equal protein loading, membranes were incubated with an anti-β-actin antibody (Sigma). Immunoblotted proteins were visualized using an Immuno-Star HRP Substrate Kit (Bio-Rad) and quantified by Kodak Image Station 440CF. Image analyses were performed by Kodak ID 3.5 software.

Cell cultures and treatment. An embryonic rat heart-derived cell line (H9c2 cells) is a suitable cellular model widely used to investigate the different molecular mechanisms involved in cardiomyocyte survival (4, 14, 42, 54). These cells, obtained (at passage 14) from the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia (Brescia, Italy) were cultured as previously described (54) and exposed to acute simulated I/R to investigate the effect of obestatin on the apoptosis induced by this type of injury. In some I/R experiments, adult rat ventricular myocytes were isolated and purified as previously described (17) and used to test the effect of obestatin on primary cultures of cardiomyocytes. The experimental protocol used to simulate I/R was a modified version of the method proposed by Rakshit et al. (56). Briefly, the serum-containing cell culture medium was replaced with serum-free DMEM 2 h before the start of the experiment.
Cultured cardiomyocytes were then treated with an “ischemic” buffer solution containing (in mmol/l) 118 NaCl, 24 NaHCO3, 1 Na2HPO4, 2.5 CaCl2, 0.5 sodium EDTA, 20 sodium lactate, and 6 KCl (pH 6.2), pregressed with 95% N2-5% CO2 for at least 5 min, and placed for 3 h in a sealed Anaero-Pack pouch system. This system provided near-anoxic conditions with an O2 concentration of <1% and a CO2 concentration of ~5% throughout the 1-h incubation at 37°C and induced a marked increase in hypoxia-inducible factor-1α protein (E. Tamagno, personal communications), the principal transcriptional factor involved in the transcriptional responses to hypoxia, in cardiomyocytes (37). After incubation under such simulated ischemia, cells were incubated in glucose-containing DMEM under normoxic conditions (reperfusion) for 24 h at 37°C and then assayed for apoptosis. To investigate the effect of obestatin-(1–23), cells were treated 20 min before I/R was started with 50 nmol/l obestatin, a concentration that has been found to completely abolish the myocardial damage induced by I/R in the isolated rat heart. To establish the specificity of the effect, obestatin-(23–1), an obestatin analog that has been found inactive in reducing I/R-induced myocardial injury, was also tested in the I/R assay. To examine the effect of obestatin was mediated via the activation of a specific intracellular signaling pathway, cells were pretreated 10 min before 50 nmol/l obestatin-(1–23) with PKC [chelerythrine (5 μmol/l)] and PI3K/Akt [wortmannin (100 nmol/l)] inhibitors, which have previously found to abolish the cardioprotective effect of obestatin in the isolated I/R rat heart, or with PD-98059 (40 μmol/l), a specific inhibitor of the survival kinase ERK1/2 (1). For the I/R control group, cells were treated with the vehicle and subjected to I/R as described above. In the negative control group, cells were kept in the same culture medium as used for I/R and maintained at 37°C under normoxic conditions. All experiments were performed at least three times.

Cardiomyocyte apoptosis. Morphological changes (chromatin condensation and fragmentation) in the nuclei of apoptotic cardiomyocytes were detected by Hoechst 33258 staining, as previously described (20). Stained nuclei were double counted under a fluorescence microscope (4’6-diamidino-2-phenylindole filter). The activity of caspase-3, a central component of the proteolytic cascade during apoptosis (32), was assessed in cell lysates using a caspase-3 colorimetric kit (Assay Designs, Bologna, Italy).

Receptor binding assay. Obestatin binding to membranes of the myocardium (atrium or ventricle), H9c2 cells, and ventricular myocytes was assayed as previously described (20) using 125I-labeled rat obestatin-(1–23) as a ligand. Tissues or cells were homogenized in 0.32 mol/l sucrose containing 50 mmol/l Tris-HCl, 1 mmol/l EDTA, 5 mmol/l MgCl2, 0.002% bacitracin, 0.002% PMSF, and 2,000 KIU/100 ml aprotinin (pH 7.4). The homogenate was first centrifuged at 500 g at 4°C for 10 min, and the supernatant was carefully decanted and subjected to a second centrifugation at 30,000 g for 30 min at 4°C. The resulting membrane pellet was resuspended in ice-cold buffer [50 mmol/l Tris-HCl, 2.5 mmol/l EGTA, and 0.002% bacitracin (pH 7.4)] and immediately used to determine the protein concentration by Bradford’s method (8) for binding studies. For a single-point binding assay, membranes (corresponding to 100 μg membrane protein) were incubated in triplicate at room temperature for 2 h in a final volume of 0.5 ml of assay buffer [50 mmol/l Tris-HCl, 2.5 mmol/l EGTA, 0.002% bacitracin, and 0.1% BSA (pH 7.4)] with 0.5 nmol/l of radiolabeled obestatin without (total binding) or with a 2,000-fold excess of unlabeled rat obestatin-(1–23). At the end of the 2-h incubation period, ice-cold assay buffer was added, and the radioactivity bound to membranes was measured as previously described (45). Specific binding was calculated as the difference between total binding and nonspecific binding and expressed as femtomoles per milligram of protein. Receptor binding saturation experiments were also conducted by incubating tissue or cell membranes (100 μg protein) with increasing concentrations (from 0.035 to 4 nmol/l) of radioligand in the absence and presence of a fixed concentration (2 μmol/l) of unlabeled obestatin. Saturation isotherms were analyzed as previously described (45), and the maximal binding capacity (Bmax) and dissociation constant (Kd) values were calculated by GraphPad Prism 4 analysis (GraphPad Software, San Diego, CA). To establish binding site specificity, increasing concentrations of obestatin-(1–23), obestatin-(23–1), ghrelin, and motilin were tested in competition assays with 1 nmol/l [125I]-obestatin. The concentrations causing half-maximal radioligand displacement (IC50 values) for the various competitors were calculated by an iterative nonlinear curve fitting program.

Statistical analysis. Values are expressed as means ± SE. Statistical analysis was carried out with one-way ANOVA followed by Student’s t-test or Newman-Keuls multiple-range test depending on the experiments. Significance was accepted when P < 0.05.

RESULTS

Obestatin reduces myocardial injury induced by I/R in the isolated rat heart. In control I/R hearts, myocardial injury, as assessed by measuring infarct size at the end of reperfusion, was 56.3 ± 3.6% of the area at risk (Fig. 1). When administered 20 min before ischemia, rat obestatin caused a significant dose-dependent reduction of the infarct size within a range of concentrations from 10 to 50 nmol/l, with a maximal anti-ischemic effect (a 70% reduction compared with control values) at the highest concentration tested (Fig. 1). The anti-ischemic effect of 50 nmol/l obestatin was superimposable to that displayed by the same concentration of rat ghrelin-(1–28) (Fig. 1), an acylated peptide cleaved from the amino-terminal of the same obestatin precursor (proghrelin), which has already been shown to exert cardioprotective actions (for a review, see Ref. 35). In contrast, 50 nmol/l obestatin-(23–1), a synthetic obestatin analog with an inverse amino acid sequence, was devoid of any protective effect against cardiac damage induced by I/R (Fig. 1). The protective effect of obestatin was also confirmed by measuring the release of LDH, a well-known indicator of cytotoxicity, in the coronary venous effluent. During reperfusion, LDH release into the effluent (1,295 ± 84 U/g wet wt in the control group) was significantly reduced by pretreatment with 10 or 50 nmol/l obestatin (917 ± 106 and

![Infarct Size %](https://example.com/infarct_size_graph.png)

Fig. 1. Infarct size detected at the end of reperfusion (120 min) in Langendorff-perfused rat hearts subjected to 40 min of ischemia and treated with vehicle alone (control), different concentrations (5, 10, 20, or 50 nmol/l) of rat obestatin-(1–23) [OB-(1–23)], 50 nmol/l of rat obestatin-(23–1) [OB-(23–1)], or 50 nmol/l of rat ghrelin. Values are expressed as percentages of ventricular mass and are means ± SE. n = 5 hearts/group. *P < 0.05, **P < 0.01, and ***P < 0.001 for OB-(1–23)- or ghrelin-treated hearts vs. control hearts.
contracture developed during ischemia, reaching a peak within 20 min. In control hearts, LV end-diastolic pressure (LVEDP) was comparable in all the experimental groups (Fig. 2A). Ischemia caused an increase of LVEDP (from 10 to 50 nmol/l) of obestatin-(1–23). Ischemia significantly reduced compared with control hearts, with the values of LVEDP recorded at the end of reperfusion being only between 10 and 20 mmHg over preischemic values (Fig. 2A). Obestatin also improved, in a concentration-dependent manner, the recovery of LV +dP/dt during reperfusion (Fig. 2B). These data indicate that obestatin significantly reduces the contractile dysfunction induced by I/R in the isolated rat heart, with the extent of the protective effect being concentration dependent.

The protective effect of obestatin-(1–23) was likely independent from alterations in the CF rate, since this peptide did not significantly modify CF under baseline conditions (in hearts from group 5, CF = 7.2 ± 2.8 and 7.9 ± 2.6 ml·min⁻¹·g wet wt⁻¹ before and after treatment) or during reperfusion. As shown in Table 1, CF, after a transient increase at the beginning of reperfusion, showed a progressive decline later on during the reperfusion period in the hearts of the control group (reaching CF = 79.8 ± 5.3% of the preischemic value at 120 min). The time course of CF values measured in the hearts from group 5 (at 120 min, CF = 86.2 ± 2.6% of the preischemic value) was comparable with that recorded in the control group. The fact that the protective effect of obestatin did not involve a better recovery of CF during reperfusion suggests a direct action of this mediator at the cellular level.

Obestatin prevents cardiomyocyte apoptosis induced by simulated I/R. We performed additional in vitro experiments to investigate whether the obestatin-induced protection observed in I/R isolated rat hearts was due to a direct effect on cardiomyocyte survival. Rat H9c2 cells, which maintain a cardiac muscle phenotype, were exposed to a simulated I/R condition to induce cell apoptosis (54) and were treated with vehicle (control group) or with obestatin-(1–23). To test the specificity of the effect, the action of obestatin-(23–1) was also assayed. In H9c2 cells, apoptosis, as assessed by Hoechst 33258 staining of apoptotic nuclei (Fig. 3, A–E) and by the activation of caspase-3, a major mediator of apoptosis (Fig. 3F), markedly increased under I/R. Obestatin-(1–23), at the concentration (50

Table 1. Coronary flow values measured in the hearts of the control group and obestatin treatment group 5 during reperfusion

<table>
<thead>
<tr>
<th>Time</th>
<th>Control Group</th>
<th>Group 5 [Obestatin (50 nmol)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 min</td>
<td>8.1 ± 0.4</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>20 min</td>
<td>7.4 ± 0.4</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>40 min</td>
<td>6.2 ± 0.3</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>60 min</td>
<td>6.0 ± 0.3</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>80 min</td>
<td>6.0 ± 0.4</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>100 min</td>
<td>5.9 ± 0.4</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>120 min</td>
<td>5.9 ± 0.4</td>
<td>6.8 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE (in ml·min⁻¹·g⁻¹). Preischemic coronary flow values were 7.4 ± 2.2 and 7.9 ± 2.6 ml·min⁻¹·g⁻¹ in control and group 5 hearts, respectively.
nmol/l) that caused the highest cardioprotective effect, significantly reduced apoptosis (Fig. 3, C and E) and counteracted caspase-3 activation (Fig. 3F). This effect was dose dependent, with 10 nmol/l being the lowest concentration exerting a significant effect (data not shown), in agreement with previous results in isolated heart preparations. In contrast, obestatin-(23–1), when added to the culture medium at a concentration of 50 nmol/l, was ineffective in reducing both apoptosis (Fig. 3, D and E) and caspase-3 activation (Fig. 3F) induced by I/R. Additional evidence of the protective effect of obestatin was obtained in primary cultures of isolated rat ventricular myocytes exposed to simulated I/R conditions in the absence (control group) or presence of 50 nmol/l obestatin-(1–23). Also in these cells, obestatin significantly promoted cell survival (Fig. 4A) and prevented caspase-3 activation (Fig. 4B). Taken together, these results therefore suggest that obestatin prevents apoptosis and promotes the survival of cardiomyocytes undergoing I/R.

The cardioprotective and antiapoptotic effect of obestatin is abolished by specific PKC, PI3K/Akt, or ERK1/2 inhibitors. When given concomitantly with 50 nmol/l obestatin-(1–23), chelerythrine (5 μmol/l) completely abolished the protective effect exerted by obestatin against I/R-induced myocardial injury, as judged both by infarct size (Fig. 5) and contractile dysfunction, i.e., a rise of LVEDP (Fig. 6A) and a decrease of LV +dP/dt (Fig. 6B). The obestatin-mediated myocardial pro-
Detection was also abolished by the PI3K/Akt inhibitor wortmannin (100 nmol/l; Figs. 5 and 6). None of these inhibitors had any effect on mechanical performance indexes (LVEDP or LV/dP/dt) or on infarct size when given alone (data not shown). These findings suggest that both these pathways may be involved in the cytoprotective, antiapoptotic effect of obestatin.

Evidence for obestatin receptors in the rat myocardium and cultured cardiomyocytes. To identify the cardiac receptor mediating the cardioprotective and antiapoptotic effects of obestatin, the binding of 125I-labeled rat obestatin-(1–23) to membranes of rat heart tissue was studied using the H9c2 cardiac muscle cell line and isolated ventricular myocytes. In single-point binding assay experiments, considerable specific obestatin-binding values were found in all specimens of cardiac tissue (Table 2). The highest specific binding activity was observed in the ventricular myocardium, where it represented ~55–66% of total radioactivity bound. In contrast, moderate specific binding values were recorded in all specimens of the atrial myocardium, with values that were ~73% lower (P < 0.05) than those found in the ventricular myocardium. A clear specific binding of radiolabeled obestatin was also present in H9c2 cardiac muscle cells and in primary ventricular myocytes, with values that were comparable with those found in the ventricular myocardium.

In keeping with the effect of inhibitors in the isolated heart, the PKC inhibitor chelerythrine and the PI3K inhibitor wortmannin both abolished the antiapoptotic effect of obestatin in H9c2 cells. Similar results were also obtained with the specific ERK1/2 kinase inhibitor PD-98059 (40 μmol/l; Fig. 8, A and B). None of these inhibitors had any effect on cardiomyocyte apoptosis when given alone (data not shown). These findings suggest that both these pathways may be involved in the cytoprotective, antiapoptotic effect of obestatin.

Fig. 5. Infarct size detected at the end of reperfusion (120 min) in Langendorff-perfused rat hearts subjected to 40 min of ischemia and treated with vehicle (control), 50 nmol/l OB-(1–23) alone, or with 50 nmol/l OB-(1–23) in the presence of 5 μmol/l chelerythrine (+CHE) or 100 nmol/l wortmannin (+W). Values are expressed as percentages of ventricular mass and are means ± SE; n = 5 hearts/group. *P < 0.001, OB-(1–23)-treated hearts vs. control hearts or vs. OB-(1–23) + chelerythrine-treated or OB-(1–23) + wortmannin-treated hearts.

Evidence for obestatin receptors in the rat myocardium and cultured cardiomyocytes. To identify the cardiac receptor mediating the cardioprotective and antiapoptotic effects of obestatin, the binding of 125I-labeled rat obestatin-(1–23) to membranes of rat heart tissue was studied using the H9c2 cardiac muscle cell line and isolated ventricular myocytes. In single-point binding assay experiments, considerable specific obestatin-binding values were found in all specimens of cardiac tissue (Table 2). The highest specific binding activity was observed in the ventricular myocardium, where it represented ~55–66% of total radioactivity bound. In contrast, moderate specific binding values were recorded in all specimens of the atrial myocardium, with values that were ~73% lower (P < 0.05) than those found in the ventricular myocardium. A clear specific binding of radiolabeled obestatin was also present in H9c2 cardiac muscle cells and in primary ventricular myocytes, with values that were comparable with those found in the ventricular myocardium.

Experiments using increasing concentrations of 125I-obestatin, ranging from 0.035 to 4 nmol/l, provided evidence for saturable specific binding in all tissue or cell types that had proved positive for obestatin binding in single-point assay experiments (Fig. 9A). Scatchard analysis of these saturation binding data (Fig. 9B) demonstrated the existence of a single class of high-affinity sites in all cardiac tissues or cells examined, with Bmax values greater in H9c2 cells, in the ventricular myocardium, or in myocytes (P < 0.05) than those found in the atrial myocardium. The calculated Bmax values (n = 3) were 14.3 ± 1.0 fmol/mg protein for H9c2 cells, 12.3 ± 0.6 fmol/mg protein for the ventricular myocardium,
obestatin receptors and suggest that obestatin may act directly on the cardiac tissue to promote its cardioprotective effects.

DISCUSSION

The present study shows that obestatin exerts a concentration-dependent protective effect against I/R injury in the isolated rat heart, cultured H9c2 cardiac cells, or isolated ventricular myocytes, which depends on the activation of specific membrane receptors. Moreover, this study indicates that the signaling pathways downstream of the stimulation of obestatin receptors involve the activation of PI3K, PKC-ε, PKC-δ, and ERK1/2.

Obestatin is a newly discovered amidated peptide that is derived from the ghrelin peptide precursor preproghrelin (59, 64). Although it has been suggested that both obestatin and ghrelin participate in a complex regulatory system, the intracellular pathways activated by obestatin and the role that it plays in physiological and pathophysiological conditions are largely unknown (58). Several studies (11, 16) have already indicated that ghrelin and other growth hormone secretagogues (GHSs) exert protective effects on cardiac muscle undergoing I/R. Moreover, it has been shown that ghrelin exerts protective effects against oxidative stress and increased production of ROS (15, 31, 65), which are biological events that have been implicated in various pathologies, including hypertension, cardiac ischemia, and myocardial fibrosis (3, 50). In primary cardiomyocytes as well as in the H9c2 cardiomyoblastic cell line and endothelial cells, ghrelin inhibits doxorubicin-induced apoptosis via ERK1/2- and PI3K/Akt-dependent mechanisms (4).

It has been initially suggested that obestatin may antagonize the physiological effects of ghrelin (64). However, more recent findings have shown that obestatin may, by interacting with specific receptors, exert PI3K/Akt- and ERK1/2-mediated antiapoptotic, prosurvival effects in both rodent β-cells and human pancreatic islets (20). Therefore, we hypothesized that, in common with ghrelin, obestatin may also play a protective role against cardiac dysfunctions induced by I/R. Both PI3K/Akt and ERK1/2 kinases have been demonstrated to protect against apoptosis in several cell types and, together with PKC, have also been proposed as integral components of an antiapoptotic cascade involved in myocardial preconditioning against I/R (46).

In the heart, I/R injury includes cell apoptosis and necrosis accompanied by contractile dysfunction. To verify whether obestatin was able to counteract these alterations, we performed experiments on different cardiac preparations using the isolated coronary-perfused heart as a cardiac multicellular model as well as isolated H9c2 cells and adult ventricular myocytes as single cell myocardial models. Nanomolar concentrations of obestatin were able to reduce infarct size and LDH release induced by I/R in the isolated rat heart in a concentration-dependent manner. Furthermore, the same range of concentrations of obestatin improved contractile performance during reperfusion. This effect was evident, in particular, for diastolic contracture and reduction of LV +dP/dt, which have been suggested as very good indicators of I/R injury in isolated rat hearts subjected to global ischemia (18). The range of concentrations (10–50 nmol/l) at which this peptide exhibited a protective effect was comparable with that

Fig. 6. LVEDP (A) and LV +dP/dt (B) during reperfusion in rat hearts subjected to I/R and treated with vehicle (control), 50 nmol/l OB-(1–23) alone, or in the presence of 5 μmol/l chelerythrine or 100 nmol/l wortmannin. Values are means ± SE; n = 5 hearts/group. *P < 0.05, #P < 0.01, and +P < 0.001 for OB-(1–23)-treated hearts vs. OB-(1–23) + chelerythrine-treated or OB-(1–23) + wortmannin-treated hearts.
already shown for ghrelin (11, 16). These concentrations of obestatin were chosen by taking into account its short plasma half-life (2 min) as well as its rapid rate of cellular uptake and degradation (48). In light of the fact that the residual quantity of obestatin detected after 1-h incubation at 37°C was 1% of the total added peptide (G. Muccioli, unpublished observations), we can argue that the concentrations used here are consistent with the physiological circulating levels (0.25–0.50 nmol/l) of obestatin in the rat (12, 22, 64). Obestatin is also likely to play a significant role in cardiac function in humans, both under physiological and pathological conditions. In preliminary studies, saliva obestatin levels were found to be significantly decreased in the serum of subjects with type 2 diabetes mellitus (55) and obesity (28), two pathological conditions that are

Fig. 7. Western blot analysis. OB-(1–23) enhanced the phosphorylation of kinases involved in cardioprotection (Akt, PKC-ε, PKC-δ, and ERK1/2). After stabilization, rat hearts were perfused with physiological solution alone for additional 10 min [sham (S)] or treated with 50 nmol/l OB-(1–23) (OB) for 10 min (in all the cases, n = 5). A: densitometric results for Akt [ratio of phosphorylated (p-)Akt to Akt] with representative blots and the loading control (β-actin). B: densitometry results for PKC-ε (ratio of p-PKC-ε to PKC-ε) with representative blots and the loading control (β-actin). C: densitometric results for PKC-δ (ratio of p-PKC-δ to PKC-δ) with representative blots and the loading control (β-actin). D: densitometric results for ERK1/2 (ratio of p-ERK1/2 to ERK1/2) with representative blots and the loading control (β-actin). Values are means ± SE. *P < 0.05, OB-(1–23)-treated hearts vs. sham hearts.

AJP-Heart Circ Physiol • VOL 299 • AUGUST 2010 • www.ajpheart.org
H478 CARDIOPROTECTIVE EFFECTS OF OBESTATIN

The earliest receptor-initiated mechanisms involved in the action of obestatin are still unknown. Obestatin was initially claimed to activate GPR39 (64), an orphan seven-transmembrane receptor showing high structural similarity to the type 1a ghrelin/GHS receptor (41). However, subsequent studies (27, 34, 61) from a number of independent groups were unable to demonstrate the binding of obestatin to GPR39 or a stimulatory function of the obestatin peptide on GPR39-transfected cells. More recently, the original proposal that obestatin acts as a ligand of GPR39 has been retracted by Zhang et al. (13). Our own data obtained on cardiac tissue show that, despite the low or negligible level of mRNA expression of GPR39 found in the rat heart (27), both rat atrial and ventricular myocardial tissues display high-affinity saturable 125I-obestatin specific binding, which is displaced by rat obestatin but not by ghrelin or motilin. Most significantly, the specific radioligand binding was mainly detected in the ventricular myocardium and ventricular cardiomyocytes, with a receptor density and binding affinity quite close to those previously found in rodent and human pancreatic β-cells, where obestatin promotes cell survival and induces the expression of genes involved in the regulation of β-cell mass and function (20). The nature of this receptor is still unknown. However, our binding experiments, together with the observation that obestatin-(23–1), a synthetic obestatin analog that contains the same amino acids of the natural peptide but in an inverse order of sequence, was devoid of any protective effect, demonstrate for the first time that obestatin recognizes high-affinity binding sites on cardiac cell membranes. This can be taken as strong evidence that obestatin, in its cardioprotective effect against I/R injury, may act directly on the myocardium.

Our experiments with specific inhibitors and Western blot analysis indicate that the protective effects induced by obestatin against cell death and contractile dysfunction induced by I/R involve the activation of the PI3K, PKC-ε, PKC-δ, and ERK1/2 pathways, which are known to exert a protective role against I/R injury (9, 23, 33). Thus, the myocardial protection exerted by obestatin is comparable with that observed with various endogenous substances, such as adenosine, bradykinin, insulin, erythropoietin, opioids (21), and glucagon-like peptide-1 (7), which, at least under experimental conditions, all produce a cardioprotective effect via the activation of PI3K and downstream enzymes, such as PKB/Akt, endothelial nitric oxide synthase, and PKC (7, 24, 47, 60). Although the role of PKC in the cardioprotective effect exerted by GHSs has been already shown (16), to our knowledge, this is the first time that the protective action of obestatin on cardiac cells has been shown to be related to the activation of the PI3K and ERK1/2 pathway.

Table 2. Binding of 125I-labeled obestatin to membranes of the rat myocardium, ventricular myocytes, and the H9c2 muscle cardiac cell line

<table>
<thead>
<tr>
<th>Tissue or Cells</th>
<th>No. of Tissue Samples or Cell Preparations</th>
<th>Specific Binding, fmol/0.1 mg Membrane Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial myocardium</td>
<td>6</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Ventricular myocardium</td>
<td>6</td>
<td>0.52 ± 0.01*</td>
</tr>
<tr>
<td>Ventricular myocytes</td>
<td>3</td>
<td>0.44 ± 0.08*</td>
</tr>
<tr>
<td>H9c2 cardiac muscle cell line</td>
<td>3</td>
<td>0.55 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with the atrial myocardium.

associated with a number of clinical manifestations including cardiovascular workload, hemodynamic stress, cardiovascular lability, and myocardial ischemia (40, 63).

The protective effect of obestatin was confirmed by simulated I/R experiments on isolated H9c2 cells and adult ventricular myocytes, in which reduced apoptosis and caspase-3 activation were observed after treatment with obestatin at a concentration (50 nmol/l) that caused the highest cardioprotective action.

We have also provided evidence in the present work that specific obestatin receptors are present in cardiac cells and the myocardium, suggesting that, as already shown for ghrelin, the cardioprotective effects exerted by obestatin against myocardial I/R injury are also initiated by binding of the hormone to specific cardiac receptors.

Table 2. Binding of 125I-labeled obestatin to membranes of the rat myocardium, ventricular myocytes, and the H9c2 muscle cardiac cell line

The earliest receptor-initiated mechanisms involved in the action of obestatin are still unknown. Obestatin was initially claimed to activate GPR39 (64), an orphan seven-transmembrane receptor showing high structural similarity to the type 1a ghrelin/GHS receptor (41). However, subsequent studies (27, 34, 61) from a number of independent groups were unable to demonstrate the binding of obestatin to GPR39 or a stimulatory function of the obestatin peptide on GPR39-transfected cells. More recently, the original proposal that obestatin acts as a ligand of GPR39 has been retracted by Zhang et al. (13). Our own data obtained on cardiac tissue show that, despite the low or negligible level of mRNA expression of GPR39 found in the rat heart (27), both rat atrial and ventricular myocardial tissues display high-affinity saturable 125I-obestatin specific binding, which is displaced by rat obestatin but not by ghrelin or motilin. Most significantly, the specific radioligand binding was mainly detected in the ventricular myocardium and ventricular cardiomyocytes, with a receptor density and binding affinity quite close to those previously found in rodent and human pancreatic β-cells, where obestatin promotes cell survival and induces the expression of genes involved in the regulation of β-cell mass and function (20). The nature of this receptor is still unknown. However, our binding experiments, together with the observation that obestatin-(23–1), a synthetic obestatin analog that contains the same amino acids of the natural peptide but in an inverse order of sequence, was devoid of any protective effect, demonstrate for the first time that obestatin recognizes high-affinity binding sites on cardiac cell membranes. This can be taken as strong evidence that obestatin, in its cardioprotective effect against I/R injury, may act directly on the myocardium.

Our experiments with specific inhibitors and Western blot analysis indicate that the protective effects induced by obestatin against cell death and contractile dysfunction induced by I/R involve the activation of the PI3K, PKC-ε, PKC-δ, and ERK1/2 pathways, which are known to exert a protective role against I/R injury (9, 23, 33). Thus, the myocardial protection exerted by obestatin is comparable with that observed with various endogenous substances, such as adenosine, bradykinin, insulin, erythropoietin, opioids (21), and glucagon-like peptide-1 (7), which, at least under experimental conditions, all produce a cardioprotective effect via the activation of PI3K and downstream enzymes, such as PKB/Akt, endothelial nitric oxide synthase, and PKC (7, 24, 47, 60). Although the role of PKC in the cardioprotective effect exerted by GHSs has been already shown (16), to our knowledge, this is the first time that the protective action of obestatin on cardiac cells has been shown to be related to the activation of the PI3K and ERK1/2 pathway.

Table 2. Binding of 125I-labeled obestatin to membranes of the rat myocardium, ventricular myocytes, and the H9c2 muscle cardiac cell line

The earliest receptor-initiated mechanisms involved in the action of obestatin are still unknown. Obestatin was initially claimed to activate GPR39 (64), an orphan seven-transmembrane receptor showing high structural similarity to the type 1a ghrelin/GHS receptor (41). However, subsequent studies (27, 34, 61) from a number of independent groups were unable to demonstrate the binding of obestatin to GPR39 or a stimulatory function of the obestatin peptide on GPR39-transfected cells. More recently, the original proposal that obestatin acts as a ligand of GPR39 has been retracted by Zhang et al. (13). Our own data obtained on cardiac tissue show that, despite the low or negligible level of mRNA expression of GPR39 found in the rat heart (27), both rat atrial and ventricular myocardial tissues display high-affinity saturable 125I-obestatin specific binding, which is displaced by rat obestatin but not by ghrelin or motilin. Most significantly, the specific radioligand binding was mainly detected in the ventricular myocardium and ventricular cardiomyocytes, with a receptor density and binding affinity quite close to those previously found in rodent and human pancreatic β-cells, where obestatin promotes cell survival and induces the expression of genes involved in the regulation of β-cell mass and function (20). The nature of this receptor is still unknown. However, our binding experiments, together with the observation that obestatin-(23–1), a synthetic obestatin analog that contains the same amino acids of the natural peptide but in an inverse order of sequence, was devoid of any protective effect, demonstrate for the first time that obestatin recognizes high-affinity binding sites on cardiac cell membranes. This can be taken as strong evidence that obestatin, in its cardioprotective effect against I/R injury, may act directly on the myocardium.

Our experiments with specific inhibitors and Western blot analysis indicate that the protective effects induced by obestatin against cell death and contractile dysfunction induced by I/R involve the activation of the PI3K, PKC-ε, PKC-δ, and ERK1/2 pathways, which are known to exert a protective role against I/R injury (9, 23, 33). Thus, the myocardial protection exerted by obestatin is comparable with that observed with various endogenous substances, such as adenosine, bradykinin, insulin, erythropoietin, opioids (21), and glucagon-like peptide-1 (7), which, at least under experimental conditions, all produce a cardioprotective effect via the activation of PI3K and downstream enzymes, such as PKB/Akt, endothelial nitric oxide synthase, and PKC (7, 24, 47, 60). Although the role of PKC in the cardioprotective effect exerted by GHSs has been already shown (16), to our knowledge, this is the first time that the protective action of obestatin on cardiac cells has been shown to be related to the activation of the PI3K and ERK1/2 pathway.

Table 2. Binding of 125I-labeled obestatin to membranes of the rat myocardium, ventricular myocytes, and the H9c2 muscle cardiac cell line

<table>
<thead>
<tr>
<th>Tissue or Cells</th>
<th>No. of Tissue Samples or Cell Preparations</th>
<th>Specific Binding, fmol/0.1 mg Membrane Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrial myocardium</td>
<td>6</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Ventricular myocardium</td>
<td>6</td>
<td>0.52 ± 0.01*</td>
</tr>
<tr>
<td>Ventricular myocytes</td>
<td>3</td>
<td>0.44 ± 0.08*</td>
</tr>
<tr>
<td>H9c2 cardiac muscle cell line</td>
<td>3</td>
<td>0.55 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 compared with the atrial myocardium.
PKC-ε and PKC-δ have distinct temporal and opposing roles in regulating the myocardial damage induced by I/R. The activation of PKC-ε before ischemia protects mitochondrial function and diminishes apoptosis, mimicking preconditioning, whereas the inhibition of PKC-δ during reperfusion protects the heart from reperfusion-induced damage (9). However, conflicting studies have suggested that PKC-δ activation plays a cardioprotective role in I/R damage and that PKC-δ knockout mice exhibit increased myocardial ischemic damage (9). Moreover, a recent report by Kwak et al. (33) directly demonstrated that the activation of PKC-δ before ischemia exerts a cardioprotective effect against I/R alterations.

Fig. 9. Representative saturation isotherms (A) and Scatchard plots (B) of rat 
$^{125}$I-labeled OB binding to membranes from the rat myocardium, isolated
ventricular myocytes, and the H9c2 cardiac muscle cell line. Experiments were
performed by incubating a fixed amount of membrane protein (100 μg/tube)
with increasing concentrations of radiolabeled OB alone (total binding) or plus
1 μmol/l unlabeled OB to define nonspecific binding. Specific binding values
were obtained by subtracting nonspecific from total binding. The saturation
curves of specific binding were analyzed by Scatchard analysis to calculate
maximal binding capacity and dissociation constant values.

Fig. 10. Competition for binding to membranes obtained from the rat ventricu-
lar myocardium (A) or from H9c2 cells (B) between rat $^{125}$I-labeled OB and
either unlabeled rat OB-(1–23), OB-(23–1), ghrelin, or motilin. The ordinate
represents binding as a percentage of control (specific binding in the absence
of unlabeled competitor). Values are means ± SE of three independent
experiments.
There are limitations of the present study that should be noted. One potential limitation is the applicability of simulated I/R in isolated cultured cells versus I/R in the perfused heart. Although the experimental conditions are different, we think that the experiments performed in isolated cells allowed us to evaluate the direct effect of obestatin on cardiac myocytes at the cellular level, without possible interferences from the other cell types present in multicellular preparations. Our results, obtained in cultured H9c2 cardiac cells or isolated ventricular myocytes, are in line with the demonstration that in other cell types, such as rodent β-cells and human pancreatic islets, obestatin activates PI3K/Akt and ERK1/2, thus preventing apoptosis and promoting cell survival (20).

In summary, we have now shown that nanomolar concentrations of obestatin are able to trigger cardioprotective effects, as shown by a concentration-dependent reduction of the cell death and cardiac dysfunction induced by I/R in the rat heart and isolated cardiac cells. Furthermore, we provided evidence that such obestatin effects are likely to be initiated by specific obestatin binding to receptors present on cardiomyocytes and involve the activation of PI3K, PKC-ε, PKC-β, and ERK1/2 pathways. Our results also suggest that circulating (or local intracardiac) levels of obestatin might play a crucial role in protecting the myocardium from prolonged and excessive stress by triggering the same intracellular pathways involved in ischemic preconditioning. This may have potentially important implications in clinical conditions of cardiodegenerative disease and/or ischemic injury, where cell protection and the improvement of ventricular function are highly desirable.

ACKNOWLEDGMENTS
The authors express sincere thanks to Prof. Francesco De Matteis (Birkbeck College, University of London, London, UK) for expert comments and editorial help and Dr. Elena Tamagno (Department of Experimental medicine and Oncology, University of Turin, Turin, Italy) for hypoxia-inducible factor-1α detection in cardiomyocytes.

GRANTS
G. Alloatti was supported by a grant from the Italian Ministry of University and Research (FIN-60% 2007 and 2008). G. Muccioli was supported by grants from the Regione Piemonte (FIN-60% 2007 and 2008; Grants A58-2004, 2007 and 2008). E. Arnoletti was supported by a Regione Piemonte fellowship (Grant A58-2004).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
18. Gelpi RJ, Morales C, Cohen VM, Downey JM. Xanthine oxidase contributes to preconditioning’s preservation of left ventricular developed pressure in isolated rat heart: developed pressure may not be an appropriate end-point for studies of preconditioning. Basic Res Cardiol 97: 40–46, 2002.


