Obestatin Plays Beneficial Role in Cardiomyocyte Injury Induced by Ischemia-Reperfusion In Vivo and In Vitro

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Background: Obestatin, primarily recognized as a peptide within the gastrointestinal system, has been shown to benefit the cardiovascular system. We designed this experiment to study the protective role and underlying mechanism of obestatin against ischemia-reperfusion(I/R) injury in myocardial cells.

Material/Methods: In an In vivo experiment, LAD was ligated for 0.5 h and then opened for reperfusion with obestatin for 24 h. Then, the infarction area was shown with TTC staining, and inflammation factors in serum were analyzed by qRT-PCR. In primary cultured cardiomyocytes, we measured the level of LDH, MDA, GSH, and SOD. Finally, we assessed cells apoptosis using flow cytometry and detected the concentrations of caspase-3, Bax, and Bcl-2 using Western blot analysis.

Results: TTC staining showed that in the 3 obestatin groups, the infarct area became smaller with the increase of obestatin concentration. Obestatin also inhibited LDH expression in rat serum and decreased mRNA levels of TNF-α, IL-6, ICAM-1, and iNOS in rat cardiomyocytes after reperfusion. In primary cultured cardiomyocytes, obestatin decreased LDH content and increased GSH level after I/R injury. Obestatin was also found to antagonize the apoptosis of cardiomyocytes in a dose-dependent manner. Western blot analysis showed that obestatin downregulated the expression of caspase-3 and Bax and upregulated the expression of Bcl-2.

Conclusions: Obestatin can protect cardiomyocyte from I/R-induced injury in vitro and in vivo. This beneficial effect is closely related with its properties of anti-inflammation, anti-cytotoxicity, and anti-apoptosis. The protective effect of obestatin might be associated with activation of Bcl-2 and inhibition of caspase-3 and Bax.

MeSH Keywords: Ghrelin ● Myocardial Infarction ● Myocytes, Cardiac ● Reperfusion Injury

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Background

Obestatin is a 23-amino acid peptide encoded by the same gene as ghrelin. It induces the expression of genes mainly regulating pancreatic beta-cell differentiation, insulin biosynthesis, and glucose metabolism. Obestatin is mainly expressed in the gastrointestinal system [1,2], but also exists in other tissues such as brain, pancreatic islets, and epithelial ducts of the mammary gland [2,3].

From its original discovery, obestatin was reported to inhibit gastric emptying, food intake, and body weight gain in adult male rats [4]. Aside from its primary function in the gastrointestinal system, obestatin is also closely related to obesity, diabetes, and the cardiovascular system, especially the latter [5–7]. I/R injury is the leading research topic in the current era of cardiology. It can cause arrhythmia, myocardial stunning, microvascular obstruction, and myocardial necrosis. About 50% of the final infarction area is related to I/R injury. Therefore, I/R injury is strongly related to infarction size and left ventricular remodeling [8].

Although obestatin can ameliorate renal I/R injury and inhibit the development of I/R-induced acute pancreatitis, the study of obestatin and I/R injury in cardiology is limited. Based on previous reports, we hypothesized that obestatin protects myocardial cells under pathological conditions. Therefore, the aim of the present study was to examine the action of obestatin on cardiovascular I/R injury in vivo and in vitro through detection of myocardial infarction, lactate dehydrogenase (LDH), and to study release of inflammation factors and the ratio of apoptotic cardiomyocytes.

Material and Methods

Materials

All medium components needed for cell experiments were purchased from Thermo Fisher Scientific. Obestatin peptide was purchased from Phoenix Pharmaceuticals (USA) and dissolved in DMEM medium. Rabbit polyclonal to alpha smooth muscle actin was purchased from Abcam (ab5694). TRIzol reagent used for mRNA detection was purchased from Takara (Shanghai, China). The PCR primers were purchased from Shanghai Bioengineering (Shanghai, China). We used the Abcam Annexin-V-FITC Apoptotic Detection Kit to detect cell apoptosis. Primary antibodies specific for active caspase-3, Bax, and Bcl-2 were purchased from Chemicon and Cell Signaling Technologies (both in USA). Triphenyltetrazolium chloride (TTC) and assay kits for malondialdehyde (MDA), laccate dehydrogenase (LDH), reduced glutathione (GSH), and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Bioengineering Institute.

Animals and treatment

Forty male Sprague-Dawley (SD) rats (body weight 250–300 g) were provided by the Zhejiang University Animal Center. All rats were housed at 25°C and 55% relative humidity, a fixed 12-h light/dark cycle, and free access to food and water. All animal care and experimental protocols complied with the Animal Management Rules of the People’s Republic of China (document No. 55, 2001) and the Guidelines for the Care and Use of Laboratory Animals. All experiment rats were assigned into 5 groups equally and randomly: (1) control, (2) I/R injury, and (3–5) Obestatin protection: treatment with obestatin 12.5, 25, and 50 nm/kg.

Ischemia-reperfusion-induced injury model

The model of myocardial I/R injury was successfully produced with ligation of the left anterior descending (LAD) coronary artery as described previously [9], with some modifications. The LAD was ligated for 0.5 h, and then opened for treatment with obestatin (local injection in the left myocardium) for 24 h of reperfusion. In the control group, the rats underwent similar surgery with the stitching line passing through the LAD, but without ligation. All surgery was performed under sterile conditions, and buprenorphine hydrochloride (0.05 mg/kg) was administered once after the surgery.

TTC staining

All rats were sacrificed for evaluation of regional myocardial infarct by staining with triphenyltetrazolium chloride (TTC). The left ventricle of each heart was sectioned into several rings (about 5-mm thick) in the short axis. All the heart tissues were stained according to the instructions of the TTC assay kit. After staining, the heart tissues were fixed with 4% paraformaldehyde, after which the stained heart slices were photographed immediately with an HD digital camera, and infarct size was analyzed and calculated with Image-pro Plus Version 6.0.

mRNA measurement of IL-6, IL-1β, TNF-α, ICAM-1, and iNOS

Total RNA of cardiomyocytes was extracted with the TR Izol reagent according to the instructions from the manufacturer. All of the primers were checked using a basic local alignment search tool to determine their selectivity. Real-time PCR cycling was conducted using the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with the following reaction conditions: the PCR mixture consisted of 10.4 μl of SYBR GreenMasterMix, 0.4 μl of both the sense and antisense primers, 2.0 μl of the sample cDNA solution, and distilled water to obtain a final volume of 20 μl. The PCR cycling was carried out using the following program: a denaturation step at 95°C for 30 s, 40 amplification cycles at 95°C for 5 s, 60°C for 30 s, and a melting step at 95°C for 15 s, then detection at 65°C for 1 s. The quantitative analysis was performed with the 2−ΔΔCT method.
propidium iodide and FITC-coupled annexin-V for 30 min. Flow cytometry analysis was carried out using a 488-nm laser coupled with BD Biosciences’ FACS Calibur sorter (USA). Necrotic cells were stained with both annexin-V and propidium iodide, while apoptotic cells were stained only with annexin-V.

**Western blot analysis**

The various groups of cells were rinsed with PBS buffer, then cytoplasmic extracts were prepared with lysis buffer (Bio-Rad, USA) mixed with protease inhibitors (Roche, USA). We determined the protein concentration with Bio-Rad DC Protein Assay Kits (USA) according to the instructions. After boiling at 95°C for 5 min, 40 μg of protein samples mixed with 2× SDS buffer were separated by 10% polyacrylamide gel, and received electrophoresis for 100 min and transferring for 90 min. Then, nitrocellulose membranes were blocked for 1.5 h in TBST solution with 5% non-fat milk power at room temperature. After blocking, the membrane was exposed to the first antibodies (1: 1000 dilution with TBST buffer) overnight at 4°C. The next day, membranes were rinsed with TBST buffer 3 times (5 min each) and then incubated with different secondary antibodies (1: 5000 dilution with TBST buffer) for 1 h. Membranes were rinsed again with TBST buffer 3 times (5 min each), and the bands were visualized with a chemiluminescence detection system (Amersham). The target bands were quantified as the relative ratio between the bands of interest and GAPDH bands.

**Statistical analysis**

For normally distributed continuous variables, all the data are presented as mean ±SEM; otherwise, median and quartile range were used. We used one-way analysis of variance and log rank (Mantel-Cox) tests to analyze all data, using SPSS (version 16.0; Chicago, IL) statistics software. Values of P<0.05 were considered statistically significant.

**Results**

**Obestatin reduced myocardial injury area induced by I/R in rat heart**

In the model group 4 out of 8 rats died and no rats died in the control group. In the 3 obestatin groups, 2 rats died in the obestatin 12.5 nm/kg group, 1 rat died in the obestatin 25 nm/kg group, and no rats died in the 50 nm/kg group. Obestatin (50 nM/kg) improved survival time and decreased the mortality rate (Figure 1A, Table 2).

The color of the myocardial infarction area that received TTC staining changed to white without being dyed, but the normal myocardium area was dyed brick-red. In the 3 obestatin treatment groups, the red area became increasingly larger.

**Primary cardiomyocytes cultures and treatment**

Cardiomyocytes from male SD rats were isolated and cultured as described previously [10], and all the operations were in accordance with the animal care guidelines promulgated by the Chinese government. The removed beating heart was put into cold Hanks buffer as quickly as possible to increase cell survival. The primary cultured cardiomyocytes were passaged for 3–7 generations before being used for the experiments and cultured to 80% confluence before the medium was replaced. Before the formal experiments, immunofluorescence staining was carried out to detect the expression of α-SMA with standard procedures as described previously [11]. Cells were observed using an IX50 fluorescence microscope (Olympus, Tokyo, Japan) after staining.

**Detection of LDH, MDA, GSH and SOD**

The concentrations of LDH, MDA, GSH, and SOD in the supernatants collected from treated cardiomyocytes were detected with ELISA kits according to the instructions provided by the manufacturer. The color absorbance at 450 nm was measured using a Bio-Rad microplate reader. The concentration of LDH in the serum of SD rats was also determined by this method.

**Flow cytometry analysis**

We used flow cytometry analysis to detect propidium iodide staining and Annexin-V binding. Cells were first incubated with 1, 3, or 10 nM obestatin for 2 h, after washing in ice-cold PBS, and different groups of cells were incubated with both propidium iodide and FITC-coupled annexin-V for 30 min. Flow cytometry analysis was carried out using a 488-nm laser coupled with BD Biosciences’ FACS Calibur sorter (USA). Necrotic cells were stained with both annexin-V and propidium iodide, while apoptotic cells were stained only with annexin-V.

**Table 1. The primer sequences.**

| Gene | Forward (F) | Reverse (R) |
|------|-------------|-------------|
| iNOS | (F) GGAGCGAGTTGGATTGTC | (R) GTGAGGCTTGGCTGATTGAG |
| TNF-α | (F) CCAGCGCTCTTCTGTCTACTG | (R) GCTACGGGCTTGGCTGACTC |
| IL-6 | (F) AGGAGCGAGTTGTGGATTGTC | (R) TTGCCATTGACCACTTTC |
| IL-1β | (F) ACTATGGCAACTGTCCTCGAAC | (R) GTGCTGGGTTCTCCATCTG |
| ICAM-1 | (F) CGTGGCGCATGAACTACCTACCT | (R) TTAGGCGCTCTCCTGAGC |
| β-actin | (F) AGGGCCCTCGAAGCTTAAG | (R) CCAGAGGCTATAAGGACAC |

40 s and 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 34 s. The primer sequences are described in Table 1, and β-actin was used as an internal control.
with increased obestatin concentration. When the obestatin concentration reached 50 nm/kg, the red area was the largest (Figure 1B), indicating that obestatin helped myocardial cells recover from I/R injury.

Obestatin decreased the LDH content in rat serum after reperfusion

LDH is a well-known indicator of cytotoxicity and its level significantly increase when cardiomyocytes suffer various kinds of injuries. In this study, the LDH content in rat serum was measured after reperfusion to assess the damage caused by I/R injury. The results showed a significant decrease in LDH content with increasing obestatin concentration. This indicates that obestatin helps protect myocardial cells from I/R injury by reducing cytotoxicity.

**Figure 1.** (A) The difference in survival between model group and obestatin groups. We used log rank analysis to compare the survival difference between the various groups, especially the model group and the 3 obestatin groups. Combined with the data in Table 2, we found survival time was significantly shorter in the model group than in the control group. Obestatin treatment prolonged the survival time and decreased mortality rate, and this improvement was significant in the obestatin 50 nM/kg group. (B) Infarct area of rat heart was reduced by treatment with obestatin. In the model group, infarction size increased significantly compared to the control group, but in the obestatin reperfusion groups, infarction size gradually decreased compared to the model group and this reduction was most obvious when obestatin reached 50 nM/kg. *p<0.05 compared with model, ###p<0.001 compared with control.
Table 2. Log rank analysis results of the difference in rat survival.

| Group               | Model group | Obestatin 12.5 nm/kg | Obestatin 25.0 nm/kg | Obestatin 50.0 nm/kg |
|---------------------|-------------|----------------------|----------------------|----------------------|
|                     | $\chi^2$    | $p$                  | $\chi^2$             | $p$                  | $\chi^2$             | $p$                  |
| Control group       | 5.043       | 0.025                | 2.140                | 0.143                | 1.000                | 0.317                |
| Model group         |             |                      |                      |                      |                      |                      |
| Obestatin 12.5 nm/kg|             |                      | 1.057                | 0.304                | 2.630                | 0.105                | 5.043                | 0.025                |
| Obestatin 25.0 nm/kg|             |                      |                      |                      |                      |                      |                      |                      |
| Obestatin 50.0 nm/kg|             |                      |                      |                      |                      |                      |                      |                      |

Obestatin played beneficial role in cardiomyocyte injury.

The identification of cultured cardiomyocytes and determination of the obestatin working concentration

Having confirmed the beneficial role of obestatin in vivo, we next studied the effect of obestatin in vitro. Immunofluorescence staining with α-SMA proved the effectiveness of primary cultures of isolated rat ventricular myocytes. To determine the effective obestatin concentration, cultured cardiomyocytes were treated with $10^{-11}$–$10^{-8}$ M obestatin. From a concentration of $10^{-7}$ M, obestatin damaged cells, and this damage had significant difference when the concentration reached $10^{-6}$ M (Figure 4). Therefore, we chose 3 concentrations of obestatin (1, 3, and 10 nM) for the following experiments.

Obestatin decreased LDH content and increased GSH level in cardiomyocytes after I/R injury

Oxidative stress resulting from the metabolism of ROS plays an integral role in myocardial injury. Therefore, we searched for cytokines reflecting oxidative stress resulting from I/R injury in this part of the experiment. The levels of LDH and MDA were increased obviously after I/R injury, while GSH and SOD decreased significantly. In the obestatin (10 nM) + I/R group, the level of LDH and MDA both decreased, but only the decrease of LDH was significantly different compared with the I/R group. For the other 2 cytokines, the level of GSH gradually increased in the 3 obestatin pretreatment groups. When the obestatin concentration reached 10 nM, the increase was the most obvious compared with the I/R group (Figure 5). From these results, we conclude that obestatin antagonizes oxidative stress induced by I/R injury.

Obestatin decreased cardiomyocyte apoptosis after I/R injury

In addition to demonstrating inhibition of inflammation reaction and oxidative stress, we performed this experiment...
to investigate whether obestatin had a direct protective effect on cardiomyocyte survival. The number of apoptotic cells in the I/R group increased significantly compared with cells in the control group (41.3±4.36% vs. 5.7±0.69%). When cardiomyocytes were incubated with various concentrations of obestatin (1, 3, and 10 nM) prior to I/R injury, the apoptotic cells decreased to 29.6±3.96%, 29.2±3.77%, and 22.3±4.17%, respectively (Figure 6). This demonstrates that obestatin protects cardiomyocytes against I/R injury directly in a dose-dependent manner.

Obestatin inhibited the caspase-3 and Bax and promoted Bcl-2 expression at the protein level

To elucidate the possible molecular mechanisms by which obestatin protects against I/R injury in cardiomyocytes, we assessed Bcl-2, Bax, and active caspase-3 expression at the protein level using Western blot analysis. We found that obestatin (1, 3, or 10 nM) prevents I/R-induced inhibition of Bcl-2, and it blocked the promotion of Bax and active caspase-3. This protective effect of obestatin was dose-dependent (Figure 7).
Discussion

Obestatin was first described as a bioactive peptide encoded by the same gene as ghrelin [12]. We previously demonstrated that ghrelin protects H9C2 cells from H$_2$O$_2$-induced injury, and this effect is closely related to its antioxidant effect [13]. Other experiments have shown that ghrelin has a beneficial effect in the progression of cardiovascular diseases [14,15], and in this process, some signal molecules (e.g., TNF-α, NF-κB, Akt serine kinases, and extracellular signal-regulated kinase-1/2) were upregulated or activated [16,17]. 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 [18]. For this reason, we decided to study the effect of obestatin in cardiomyocyte viability and metabolism.

It was initially suggested that obestatin antagonizes the physiological effects of ghrelin [4]. However, more recent findings about ischemic-reperfusion have shown that obestatin, by interacting with specific receptors, exerts prosurvival effects against I/R. One study showed that administration of either obestatin or ghrelin exerted similar protective effects against I/R-induced ileal and pulmonary injury [19]. Other research demonstrated that obestatin ameliorates renal I/R injury by anti-oxidative, anti-inflammatory and anti-apoptotic properties, which appear to involve the suppression of neutrophil accumulation and modulation of NO metabolism [20]. Bukowczan et al. reported that treatment with exogenous obestatin reduces the severity of ischemia/reperfusion-induced acute pancreatitis and accelerates recovery in this disease [21]. Alloatti et al. suggested that, by acting on specific receptors, obestatin activated PI3K, PKC-ε, PKC-δ, and ERK1/2 signaling and protected cardiac cells against myocardial injury and apoptosis induced by ischemia-reperfusion [22]. In the clinical setting, there appears to be no correlation between ischemic heart disease and plasma obestatin [23]. However, there is a report of the disturbance of circulating ghrelin and obestatin in chronic heart failure patients, especially those with cachexia, which may have a role in the pathogenesis of cardiac cachexia in chronic heart failure [24].

We hypothesized that in common with ghrelin, obestatin may also play a protective role against cardiac dysfunctions induced
We investigated the protective effects of obestatin on heart I/R injury in rat at 2 levels: in vivo LAD ligation and in vitro myocardial cell culture. The LAD was ligated for 0.5 h, and then opened for treatment with obestatin for 4 h of reperfusion. Rats were sacrificed for evaluation of regional myocardial infarct by staining with TTC. We found that obestatin was able to reduce infarct size induced by I/R in rat hearts. The protective effect of obestatin was also confirmed by simulated I/R experiments on myocardial cells, in which reduced apoptosis was observed after treatment with obestatin through flow cytometry analysis.

Inflammation reaction is the most common response accompanying injury of cardiomyocytes, and it is also a vital pathological mechanism underlying the propagation of I/R-induced myocardial injury. In order to examine possible anti-inflammatory properties of obestatin, the cardiomyocytes mRNA of IL-1β, IL-6, TNF-α, iNOS, and ICAM-1 were measured in an in vitro research model.
Figure 7. Proteins levels of active caspase-3, Bcl-2, and Bax in treated cardiomyocytes were quantified via normalization to GAPDH. Obestatin decreased the level of active caspase-3 and Bax, and increased the level of Bcl-2. According to the results of densitometric analysis, this change showed a positive correlation with obestatin concentration. When obestatin concentration reached 10 nM, the inhibition or promotion had statistical significance. * p<0.05 compared with model, # p<0.05 compared with control, ## p<0.01 compared with control.

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Apoptosis is an active process performed mainly by the caspase family, which can be activated by other signaling molecules. There are studies showing that mitochondria can play an outstanding role in the amplification progress of apoptosis re-action [25]. I/R stimulation obviously increases the permeability of mitochondria, specifically the opening of mitochondrial permeability transition pores (mPTP), which increases release of cytochrome c and destroys the mitochondrial trans-membrane potential. Protein molecules of the Bcl-2 family can regulate the release of cytochrome c in cardiomyocytes during the course of oxidative stress. The high expression of Bcl-2 increases cell survival, while Bax decreases it. According to the signal transduction pathway in the apoptotic cascade, the caspase family lies in the downstream position behind the Bcl-2 family. The activated caspase-9 cleaves pro-caspase-3 to form the active caspase-3, which is the true executioner caspase, along with caspases-6 and caspase-7. Then the caspase complex activates DNase, which can break the oligonucleosome DNA into pieces, an event called DNA fragmentation. Our experiment showed that obestatin exerted a completely opposite effect (inhibiting Bcl-2 and stimulating Bax and caspase-3) in the process of I/R. These phenomena show that the anti-apoptotic effect of obestatin is tightly associated with the mitochondrial dysfunction evoked by I/R.

As the powerhouses of the cell, mitochondria play pivotal roles in I/R injury. mPTP opening damages the trans-membrane potential and other mitochondrial activities. Due to research constraints, we did not further study the relationship between mPTP and obestatin, but we have reason to believe that in the process of protecting cardiomyocytes, obestatin might interact...
with mPTP or its molecular core Fo ATP synthase C subunit, which was detected in ST-segment elevation myocardial infarction patients and is significantly related to several surrogate markers of myocardial reperfusion [26]. These will be a focus of our future research.

Conclusions

Our experiments show that obestatin can rescue cardiomyocytes from I/R-induced injury in vitro and in vivo, and obestatin exerts this protective effect on cardiomyocytes through its properties of anti-inflammation, anti-cytotoxicity, and anti-apoptosis. This protection provided by obestatin also has a close relationship with the upregulation of Bcl-2 and down-regulations of Bax and active caspase-3. These findings suggest that obestatin plays a positive role by inhibiting activation of the mitochondrial pathway.

Conflict of interest

The authors declare no financial conflicts of interest.

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