Peptides 60 (2014) 23–31

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Obestatin improves ischemia/reperfusion-induced renal injury in rats via its antioxidant and anti-apoptotic effects: Role of the nitric oxide

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ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 21 July 2014
Accepted 21 July 2014

Keywords:
Obestatin
Renal ischemia-reperfusion injury
Nitric oxide
Oxidative stress

ABSTRACT

Obestatin was shown to have anti-inflammatory effects in several inflammatory models. To elucidate the potential renoprotective effects of obestatin, renal I/R injury was induced in male Sprague Dawley rats by placing a clamp across left renal artery for 60 min following a right nephrectomy. Clamp was released and the rats were injected with either saline or obestatin (10, 30, 100 μg/kg). In some experiments, obestatin (10 μg/kg) was administered with L-NAME (10 mg/kg) or L-Nil (0.36 mg/kg). Following a 24-h reperfusion, the rats were decapitated to measure serum creatinine and nitrite/nitrate levels, renal malondialdehyde (MDA), glutathione (GSH) levels and myeloperoxidase (MPO) activity and to assess cortical necrosis and apoptosis scores. Obestatin treatment reduced I/R-induced increase in creatinine levels, renal MPO activity and renal MDA levels, while renal GSH levels were significantly increased by obestatin. Histological analysis revealed that severe I/R injury and high apoptosis score in the kidney samples of saline-treated rats were significantly reduced and the cortical/medullary injury was ameliorated by obestatin. Expression of eNOS, which was increased by I/R injury, was further increased by obestatin, while serum NO levels were significantly decreased. iNOS inhibitor L-Nil reduced oxidative renal damage and improved the functional and histopathological parameters. I/R-induced elevation in eNOS expression, which was further increased by obestatin, was depressed by L-NAME and L-Nil treatments. The present data demonstrate that obestatin ameliorates renal I/R-injury by its possible antioxidative, anti-inflammatory and anti-apoptotic properties, which appear to involve the suppression of neutrophil accumulation and modulation of NO metabolism.

Introduction

Ischemia/reperfusion (I/R) injury of the kidney may occur in shock, during surgical revascularization of the renal artery or during renal transplantation [4,17]. It is well known that reperfusion following ischemia may cause additional damage and further complicate the injury through accumulation of ions, generation of reactive oxygen species (ROS), endothelial dysfunction, platelet aggregation and immune activation [11]. Specifically, endothelial dysfunction is involved in both initiation and maintenance of tubular epithelial injury [26]. Studies have shown that endothelial injury aggravates the inflammatory response through the loss of normal production of endothelial nitric oxide (NO) synthase (eNOS), while induction of eNOS is effective in improving I/R injury and diminishing the injurious effects of oxidative stress [13]. On the other hand, inducible NOS (iNOS) can be induced by cytokines and lipopolysaccharides in the kidney and becomes abundant during I/R leading to renal cell injury [7,21]. Peroxynitrite generation, formed from NO by reacting with superoxide, is implicated as the major cause of I/R-induced oxidant renal injury [21,31,40]. Accordingly, several in vivo and in vitro studies have demonstrated that the absence of iNOS or the inhibition of iNOS expression/activity can ameliorate or prevent renal I/R injury [22,29].

Obestatin is a recently identified peptide hormone secreted by the cells of the stomach and small intestine of several mammals including humans [42]. Although obestatin and ghrelin are both encoded by the same gene and derived from the

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http://dx.doi.org/10.1016/j.peptides.2014.07.019
0196-9781/

precursor protein proghrelin, obestatin behaves as a physiological opponent to ghrelin in inhibiting food intake, body weight gain, gastric emptying, jejunal contractility, and growth hormone secretion [42]. Obestatin was shown to stimulate the proliferation of human retinal pigment epithelial and gastric cancer cells [6,33], while it was demonstrated to prevent apoptosis in both rodent and human pancreatic islets [14]. Obestatin exerts protection in myocardial I/R injury, in myocardial ischemic preconditioning and in isolated ventricular myocytes [1,27]. We have previously shown that peripheral or central administration of obestatin exerts potent anti-inflammatory and protective effects in subarachnoid hemorrhage-induced brain damage [12], intestinal I/R injury [37] or colitis [32]. Similar to obestatin, ghrelin was previously shown to protect against I/R injury in mice via its antiapoptotic effects [38]. Based on these findings, in the current study, we investigated whether obestatin could alleviate oxidative damage and improve renal I/R injury in rats. Secondly, we elucidated the role of the NO metabolism in the protective action of obestatin.

Material and methods

Animals

Ten-week-old male Sprague–Dawley rats (250–300 g) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature ($22 \pm 2\,^{\circ}C$) and relative humidity (65–70%) were kept constant. Rats were fed with standard laboratory chow with free access to water. All experimental protocols were approved by the Marmara University Animal Care and Use Committee.

Experimental design and surgery

Surgical procedures were performed under anesthesia (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine; intraperitoneally (i.p.)). The rats were divided into ischemia-reperfusion (I/R) and sham-operated groups. In the sham control group, rats were subjected to right nephrectomy and a left flank incision without occluding renal vessels ($n = 8$). The rats in the I/R groups were subjected to right nephrectomy through a right flank incision, while an additional left flank incision was made to occlude the left renal artery and vein with a non-traumatic vascular clamp for 60 min. At the end of the ischemic period, the clamp was released for blood reperfusion for 24 h. Immediately after the release of vascular clamps, the I/R groups were treated i.p. with either saline ($n = 8$) or obestatin (10, 30 or 100 μg/kg; Phoenix Pharmaceuticals, cat no: 031-90, dissolved in saline; $n = 8$ for each dose). Likewise, some I/R groups were treated i.p. with a NOS inhibitor (L-NAME; *N-nitro-L-arginine methyl ester*, 10 mg/kg; Sigma Chemical Co., St. Louis, MO, $n = 8$) or a selective iNOS inhibitor (L-Nil; *L-N^6-(1-iminoethyl) lysine*; 0.36 mg/kg; Sigma Chemical Co., St. Louis, MO, $n = 8$) after the release of their vascular clamps. In another set of the experiments, obestatin (10 μg/kg) in combination with either L-NAME (10 mg/kg; $n = 8$) or L-Nil (0.36 mg/kg; $n = 8$) was administered immediately after the release of vascular clamps. The rationale for the doses of obestatin [12,32,37] and NOS inhibitors [24,30] are based on previous studies.

Biochemical analysis of serum

At the end of the 24-h reperfusion period, trunk blood samples were collected for the analysis of blood urea nitrogen, creatinine and LDH levels in the serum. BUN, creatinine and LDH levels in the serum were determined spectrophotometrically using an automated analyzer (Cobas 8000, Roche Diagnostics, Basel, Switzerland).

Renal malondialdehyde (MDA) and glutathione (GSH) assays

Renal tissue samples obtained from each animal were stored at −70 °C for the measurement of malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) activity. Additional kidney samples were placed in 10% formaldehyde for histopathological evaluation of renal injury. Renal tissue samples were homogenized with ice cold trichloroacetic acid (1 g tissue plus 10 ml 10% TCA) an Ultra Turrax tissue homogenizer. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously [39]. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and the results are expressed as nmol MDA/g tissue. Glutathione measurements were performed using a modification of the Ellman procedure [39]. Briefly, after centrifugation at $2000 \times g$ for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4{\cdot}2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels were calculated using an extinction coefficient of $1.36 \times 10^5\ M^{-1}\ cm^{-1}$. The results are expressed in μmol GSH/g tissue.

Measurement of serum nitrite/nitrate concentrations

A nitric oxide analyzer (NOA280i; Sievers Instruments, Boulder, CO, USA) was used for the determination of NO from nitrite/nitrate metabolites (NOx). Collected de-proteinized serum samples were injected into a nitrogen-purge chamber containing vanadium chloride in hydrochloric acid, which converts NOx to NO. The NO-ozone chemiluminescence was detected with the nitric oxide analyzer [19].

Measurement of myeloperoxidase (MPO) activity

Activity of tissue MPO, an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN), is used as an indication of accumulation of neutrophils, because tissue MPO activity correlates with the number of PMN determined histochemically in inflamed tissues. Tissue MPO activity was assessed by measuring the H_2O_2 dependent oxidation of *o*-dianizidine 2HCl. Briefly, tissue samples (0.2–0.3 g) were homogenized in 10 volume of ice-cold potassium phosphate buffer (50 mmol/l K_2HPO_4, pH 6.0) containing hexadecyl-trimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at $41{,}400 \times g$ for 10 min at 4 °C, and the supernatant was discarded. The pellet was then re-homogenized with an equivalent volume of 50 mmol/l K_2HPO_4 containing 0.5% (w/v) hexadecyl-trimethylammonium bromide and 10 mmol/l ethylenediaminetetraacetic acid (EDTA, Sigma). MPO activity was assessed by measuring the H_2O_2 dependent oxidation of *o*-dianizidine 2HCl. One unit of enzyme activity was defined as the amount of the MPO present per gram of tissue weight that caused a change in absorbance of $1.0\ min^{-1}$ at 460 nm and 37 °C [39].

Histopathological examination

For light microscopic investigations, renal tissue specimens were fixed in 10% neutral-buffered formalin, dehydrated in alcohol series, cleared in xylene and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined under a photomicroscope (Olympus BH 2, Tokyo, Japan). Examinations and scorings of sections from each kidney were carried out in a blinded fashion by two independent researchers (S. Cetinel, N. Ozkan) blinded to the drug administration groups.