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Characterization of urocortin as an anti-apoptotic protein in experimental ischemia-reperfusion model of the rat testis

Kenta Sumii [a, *], Hideaki Miyake [b], Noritoshi Enatsu [a], Koji Chiba [a], Masato Fujisawa [a]

[a] Division of Urology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[b] Department of Urology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

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ABSTRACT

The objective of this study was to investigate the role of urocortin in testicular apoptosis using an experimental ischemia-reperfusion rat model. To evaluate the change in urocortin expression and apoptotic status in the testes following ischemia-reperfusion, the left testes of rats were rotated clockwise by 720° for 1 h, and were then harvested at 0, 1, 3, 6 and 24 h after detorsion (n = 5 in each group). A time-dependent increase in the expression levels of urocortin was noted until 6 h after reperfusion, but the expression of urocortin was markedly decreased 24 h after reperfusion. However, a TUNEL assay showed that the proportion of germ cells undergoing apoptosis significantly increased 24 h after reperfusion compared with that of 6 h after reperfusion. To clarify whether or not urocortin directly regulates the testicular apoptosis induced by ischemia-reperfusion, either astressin, an antagonist of urocortin, or normal saline was injected into the rat testes 15 min before detorsion, followed by the testicular torsion. The testes were then removed 3 h after detorsion (n = 5 in each group). The testicular injection of astressin significantly increased the proportion of TUNEL-positive germ cells, and significantly decreased expression of Bcl-2 and Bcl-xL. In addition, the level of phosphorylated ERK 1/2, but not that of phosphorylated Akt, was significantly reduced by the intratesticular administration of astressin. These findings suggest that urocortin may play a cytoprotective role in the germ cells in response to ischemia-reperfusion injury through the activation of major anti-apoptotic proteins, as well as by the mitogen-activated protein kinase signaling pathway activation.

1. Introduction

Urocortin, also known as urocortin 1, is a 40-amino acid peptide which belongs to the corticotrophin-releasing hormone (CRH) family, and was originally identified in rat and human brain [1]. To date, the expression of urocortin has been observed in several peripheral organs, such as digestive tract and cardiovascular and reproductive systems [2]. In addition to urocortin, the urocortin analogs, urocortin 2 and urocortin 3, were identified in human and rodent heart [3,4]. In the male reproductive system, the expression of urocortin was noted in the germ cells of mouse, dog and human as well as the Leydig cells of rat and human [5–8]. However, Lee et al. reported that the expression levels of urocortin 2 and urocortin 3 in the rat testis were markedly weaker than that of urocortin.

The biological functions of urocortin are exerted through activation of its receptors, CRF receptor type 1 (CRFR1) and type 2 (CRFR2), and are effected in various ways [9]. Among its various reported functions, an anti-apoptotic effect of urocortin on cardiomyocytes after ischemia-reperfusion (I/R) injury has been intensively investigated [10,11]. For example, Brar et al. showed that urocortin played a cardioprotective role after I/R injury by activating both the PI3K/Akt and p44/42 mitogen-activated proteinx kinase (MAPK) pathways through CRFR2 [12,13].

Testicular torsion is a urological emergency occurring especially in newborns, children and adolescents [14]. Surgery is usually required and should be performed as soon as possible after symptoms appear, because a prolonged period of testicular ischemia will result in permanent damage, such as loss of spermatogenesis [15]. However, even if the torsion is successfully corrected surgically, a significant increase in germ cell apoptosis and subsequent damage to spermatogenesis may still occur as a result of testicular I/R injury [16]. As previously described, urocortin and CRFR2 are expressed in male germ cells [5], suggesting the anti-

* Corresponding author.
E-mail address: ksumii@med.kobe-u.ac.jp (K. Sumii).

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apoptotic role of urocortin in germ cells against I/R injury; however, to date, only limited data with respect to the role of urocortin in germ cells is available. Considering these findings, we investigated the functional role of urocortin in the apoptosis of germ cells and its molecular mechanisms using an experimental testicular I/R injury rat model.

2. Materials and methods

2.1. Animals

A total of forty 7-week-old male Sprague-Dawley rats weighting 196–226 g were purchased from Oriental Yeast Co. (Tokyo, Japan) and housed with free access to food and water under a controlled environment at 22 °C on a 12-h light, 12-h dark cycle. Animal experiments were approved by Committee of Animal Experiments at Kobe University School of Medicine and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [17].

2.2. Animal surgical procedure

2.2.1. Experiment 1: to evaluate the time-dependent changes in the expression levels of urocortin and the apoptotic status in the rat testis after I/R injury:

The rats were anesthetized with 50 mg/kg sodium pentobarbital. As described previously [18], left scrotal incision was performed, and then the left spermatic cord was rotated clockwise by 720°, and was maintained by fixing the testis to the scrotum with a 4-zero silk suture placed through the tunica albuginea. After 1 h of ischemia, the testes were counter rotated to the natural position and reinserted in the scrotum. Then, the testes were harvested at 0, 1, 3, 6 and 24 h after reperfusion (n = 5 in each group). In the control rats (n = 5), the left testes were pulled out from the scrotum without rotating, and removed after 1 h.

2.2.2. Experiment 2: to evaluate the role of urocortin in the apoptosis of the rat testis after I/R injury:

A total of 5 rats (AST group) were treated with intratesticular injection of a CRF receptor antagonist, astressin (Sigma-Aldrich Co., St. Louis, MO, USA) which was dissolved in normal saline and prepared at a dose of 10μg/50μl/testis, 15 min before detorsion, followed by 1 h of testicular torsion. The testes were then harvested 3 h after reperfusion. Intratesticular injection was performed near the rete testis with a 27-gauge needle, with the volume of injection being determined based on the recommendation of a prior study [19]. To exclude the impact of needle puncture on the testicular apoptosis, an additional 5 rats (NS group) were treated with intratesticular injection of normal saline (50μl/testis) under the same surgical procedures as the AST group. The findings of these 2 groups were compared with those of the 5 rats (Co group) whose testes were removed 3 h after reperfusion without any treatment as a control.

Harvested testes were bisected, and one half was immediately snap-frozen and stored at −80 °C until experimental procedures. The other half was fixed in Bouin solution for histopathological examination.

2.3. Quantitative RT-PCR analysis

Total ribonucleic acid (RNA) was extracted from the rat testes using TRIzol reagent (Life Technologies Japan, Tokyo, Japan), and reverse transcription (RT) reactions were performed using a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, California) following the manufacturer's instructions. To quantitatively evaluate the expression levels of urocortin and β-actin mRNAs in each sample, real-time reverse transcription polymerase chain reaction (RT-PCR) analysis using a standard curve method with SYBR Green I (Takara Bio, Tokyo, Japan) was then conducted as previously described [20]. The detailed information of sequence specific primers, synthesized by Eurofins Genomics Inc. (Tokyo, Japan), is presented in previous studies [21,22]. Urocortin mRNA level of each sample was adjusted as the value relative to β-actin mRNA level, and expressed as arbitrary units relative to samples from the control, regarded as 1.

2.4. Western blot analysis

Samples of tissue lysates containing equal amounts of protein (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience Inc., Keene, NH, USA). The membranes were blocked in PBS containing 5% Bovine Serum Albumins (BSA) for 1 h and then incubated with the following primary antibodies overnight at 4 °C: anti-urocortin goat polyclonal antibody, anti-Bcl-2 rabbit polyclonal antibody, anti-Bcl-xL, anti-Bax and anti-Bad mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-phospho-Akt, anti-Akt, anti-phospho-ERK 1/2 and anti-ERK 1/2 rabbit monoclonal antibodies (Cell Signaling Technology, Inc., Tokyo, Japan), and anti-β-actin mouse monoclonal antibody (Sigma-Aldrich Co., St. Louis, MO, USA). The membranes were then incubated for 30 min with horseradish peroxide-conjugated anti-goat, mouse or rabbit secondary antibody (Santa Cruz Biotechnology Inc.), and specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system, Amersham Imager 600 (GE Healthcare, Tokyo, Japan). The relative optical densities of the bands were quantified using Image J Program [23]. Urocortin, Bcl-2, Bcl-xL, Bax and Bad protein levels of each sample were adjusted as the values relative to β-actin and expressed as arbitrary units relative to samples from the control, regarded as 1.

2.5. Immunohistochemistry

The specimens of rat testis were immunohistochemically stained as described previously [24]. Briefly, removed testes were embedded in paraffin and sectioned at a thickness of 5 μm. They were deparaffinised by xylene and rehydrated in decreasing concentrations of ethanol. After blocking endogenous peroxidase with 3% hydrogen peroxidase in methanol, sections were boiled in 0.01 M citrate buffer for 10 min and incubated with 5% normal blocking serum in Tris–buffered saline for 30 min. The sections were then incubated with a polyclonal anti-urocortin antibody (1:100, Santa Cruz Biotechnology Inc.) and were then incubated with biotinylated anti-goat secondary antibody. After being incubated in an avidin–biotin peroxidase complex for 30 min, the samples were exposed to diaminobenzidine tetrahydrochloride solution and counterstained with hematoxylin.

2.6. TUNEL assay

Germ cells undergoing apoptosis were detected by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-labelling (TUNEL) method using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. At least one-hundred seminiferous tubules from each testis were evaluated in circular cross sections using randomly selected microscopic fields (× 200 magnification), and the apoptotic index was defined as the number of TUNEL-positive nuclei per tubule [25].

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