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Expression of urocortin and its receptors in the rat epididymis

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ABSTRACT

Urocortin (UCN; 40 aa) is a corticotrophin-releasing hormone (CRH)-related peptide. The biological actions of CRH family peptides are mediated by two types of G-protein-coupled receptors, CRH type 1 receptor (CRHR1) and CRH type 2 receptor (CRHR2). The biological effects of the peptides are mediated and modulated not only by CRH receptors but also by a highly conserved CRH-binding protein (CRHBP). The aim of the present study was to investigate the expression of UCN, CRHR1, CRHR2 and CRHBP by immunohistochemistry, Western blot, RT-PCR and real-time RT-PCR in the rat epididymis. Urocortin, CRHR1 and CRHR2, but not CRHBP, were expressed in all segments of the rat epididymis. Specifically, UCN- and CRHR2-immunoreactivities (IRs) were distributed in epididymal epithelial cells of the caput, corpus and cauda. CRHR1-IR was found in the fibromuscular cells surrounding the epididymal duct and in the smooth musculature of the blood vessels throughout the organ. UCN and CRHR2 mRNA expression levels were higher in the caput and corpus than in the cauda, while CRHR1 mRNA level was higher in the cauda than those in the caput and corpus. In summary, UCN, CRHR1 and CRHR2 are expressed in the rat epididymis. It is suggested that CRH-related peptides might play multiple roles in the maturation and storage of spermatozoa.

1. Introduction

Urocortin (UCN) is a peptide (40 amino acids, aa) belonging to the corticotrophin-releasing hormone (CRH) family and it exhibits 45% homology to CRH [1–3]. Urocortin was identified by Vaughan et al. [4], and similar to CRH, was found to stimulate adrenocorticotrophic hormone (ACTH) *in vitro* and *in vivo* production by corticotrophs [5]. Additional mammalian CRH-like peptides include UCN2 (stresscopin-related peptide, 38 aa) and UCN3 (stresscopin) which share more than 80% of homology [6]. The biological effects of CRH and UCNs

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are mediated by distinct G-protein-coupled receptors, CRH receptor type 1 (CRHR1) and 2 (CRHR2) [7,8]. CRHR1, primarily expressed in the central nervous system (CNS) and the anterior pituitary, is an important mediator of the activation of the hypothalamic–pituitary–axis (HPA) [9]. CRHR2 is expressed in extra-CNS sites and has 70% sequence identity to CRHR1. CRHR2 exhibits high affinity toward UCNs and no toward CRH. Its activation suppresses multiple metabolic functions including feeding in fasted mice [10], feeding-induced edema and gastric emptying [11]. The biological effects of UCN and other CRH-related peptides are also modulated by CRH-binding protein (CRHBP). CRHBP is a 37 kD glycoprotein identified in mammals and non-mammalian vertebrates and is highly conserved during phylogenesis [12]. It was isolated from human plasma [13,14], the rat brain and the anterior pituitary [15,16]. CRHBP binds human CRH (hCRH) and UCN with an equal or greater affinity than CRH receptors [4,17] and modulates the access of CRH to CRHRs [12]. UCN and CRHRs gene expression and/or immunoexpression have been detected in several peripheral organs belonging to the digestive, cardiovascular, immune, endocrine and reproductive systems [18–22].

In the male reproductive system, the presence of UCN was reported in the human prostate, suggesting its role in the autocrine/paracrine regulation of prostatic function [23]. Moreover, expression of UCN and CRH receptors was found in the rat, mouse and human testis [22,24,25] and they are believed to play a role in the regulation of spermatogenesis, sperm motility and testosterone releasing. To highlight other sites of urocortin action and to indicate other possible urocortin functions, we investigated the expression of UCN, CRHR1, CRHR2 and CRHBP in the rat epididymis.

2. Materials and methods

2.1. Animals and tissue collection

A total of 20 adult male Sprague-Dawley rats (13 weeks of age; body weight: 380 ± 20 g; purchased from Harlan Italy) were used. The rats were housed in temperature- and light-controlled rooms and were given *ad libitum* access to food and water. The animals received humane care, and the study protocol was in compliance with our institution's ethical guidelines. All procedures followed Italian laws regarding animal use in research (art. 7 D. Lgs. 116/92). All surgical procedures were carried out aseptically under anesthesia induced with urethane (1.2 g/kg). The animals were terminated under urethane anesthesia, and the removed epididymides were divided into the following three segments: caput (including initial segment), corpus and cauda. In addition, the brain was collected as a positive control. For Western blot and real-time RT-PCR, the samples were immediately frozen on dry ice and stored at −80 °C. For immunohistochemical studies, the samples were immediately fixed in Bouin's fluid.

2.2. Immunoprecipitation and Western blot

Frozen tissues were homogenized in buffer (50 mM Tris–HCl, pH 7.00; 150 mM NaCl; 2% Triton; 5 mM EDTA; 10 μg/mL leupeptin; 0.1 U/mL aprotinin; 1 mM PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16 000 × *g* for 20 min at 4 °C. The protein concentration in the resulting supernatants was determined by a Bio-Rad assay (Hercules, CA, USA). Equal amounts of proteins were immunoprecipitated overnight at 4 °C with anti-UCN, -CRHR1, -CRHR2 and -CRHBP antisera (1 μg antibody/200 μg protein) previously bound to protein A/G agarose beads. A fraction of the supernatant was used as an immunoprecipitation input control (loading control). Beads were sedimented by brief centrifugation and washed extensively with ice-cold homogenization buffer. Proteins solubilized in boiling sodium dodecyl sulphate (SDS) sample buffer (2% SDS; 5% L-mercaptoethanol; 66 mM Tris, pH 7.5; 10 mM EDTA) were separated on 18% and 12% SDS-polyacrylamide gels (Bio-Rad), After electrophoresis, the proteins were transferred to nitrocellulose using a semi-dry apparatus (Bio-Rad). The membrane was incubated for 1 h at 42 °C in 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in TBST (150 mM NaCl; 20 mM Tris–HCl, pH 7.4; 0.3% Tween-20), washed with TBST and incubated for 2 h at room temperature (RT) in antisera diluted 1:500 in TBST containing 1% BSA. The following primary antisera were used: polyclonal goat anti-UCN (sc-1825, Santa Cruz Biotechnology, CA, USA; diluted 1:1000), anti-CRHR1 (sc-12383, Santa Cruz Biotechnology; diluted 1:1000), anti-CRHR2 (sc-1826, Santa Cruz Biotechnology; diluted 1:1000) and polyclonal rabbit anti-CRHBP (SAB1300862, Sigma; diluted 1:1000). The membrane was washed three times with TBST, incubated for 1 h with anti-goat or anti-rabbit IgG conjugated to peroxidase (Vector Laboratories, Burlingame, CA, USA) diluted 1:2000 in 1% BSA containing TBST and washed three times with TBST. Proteins were visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (Prosieve, Lonza) were used to estimate the molecular weight of each band. β-Actin acted as the loading control for the immunoprecipitated proteins. A fraction of the supernatant (50 μg) was separated by SDS-PAGE and immunoblotted with an anti-β-actin primary antibody (Mouse mAb JLA20 cp 01; Calbiochem, San Diego, CA; diluted 1:5000) followed by a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Vector Laboratories; diluted 1: 2000).

2.3. Immunohistochemistry

Fresh tissue fragments were fixed by immersion in Bouin's fixative (6–24 h), processed for paraffin embedding in a vacuum and cut at a thickness of 3–6 μm. The avidin–biotin–peroxidase complex (ABC) method was performed by using the Vectastain ABC kit (Vector Laboratories) [20]. Primary antisera were the same as those used in the previous section and were directed against UCN, CRHR1, CRHR2 and CRHBP. The first three were diluted 1:200 and the fourth 1:500. The secondary antibodies were biotinylated anti-goat or anti-rabbit IgG (Vector Laboratories; diluted 1:200). The specificity of the primary immunoreactions was tested by replacing each antibody with a buffer, preabsorbing the antibody with an excess of the appropriate antigen (100 μg antigen/ml antiserum as the final dilution) or using a dot-blot assay [20]. No immunoreaction was detected in control tests. The slides were