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ORIGINAL ARTICLE

Presence and Distribution of Urocortin and its Receptors in the Epididymis of Alpaca (*Vicugna pacos)*

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With 2 figures

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Summary

Urocortin 1 (UCN) is a 40-amino acid peptide belonging to the corticotrophin-releasing hormone (CRH) family. The biological effects of this peptide are modulated by binding two G-coupled receptors named CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2). CRHR2 has high affinity for UCN. The aim of the present study was to investigate the presence and distribution of UCN, CRHR1 and CRHR2 in the epididymis of the South America camelid Alpaca (*Vicugna pacos*) by Western blotting analysis and immunohistochemistry. Tissue extracts of the organ reacted with the anti-UCN, anti-CRHR1 and anti-CRHR2 antibodies, recognizing in all the cases a single specific protein band. UCN- and CRHR2-immunoreactivities (IRs) were found in the cytoplasm of the principal cells (PCs) of the caput epididymis. A prevalent supranuclear localization of granular-shaped positive material was observed. CRHR1-IR was observed in the fibromuscular stromal cells encircling the tubules and in the smooth musculature of the blood vessels throughout the three epididymal segments. In addition, in the cauda, CRHR1-IR was observed in some apical epithelial cells (ACs) which were morphologically similar to apical mitochondria-rich cells (AMRCs). These results suggest that UCN, CRHR1 and CRHR2 are expressed in the alpaca epididymis and that CRH-related peptides might play multiple roles in maturation and storage of spermatozoa.

Introduction

Urocortin 1 (UCN), a member of corticotrophin-releasing hormone (CRH) family, is a 40-amino acid peptide and was first described in the rat hypothalamic non-preganglionic Edinger–Westphal nucleus (npEW) (Vaughan et al., 1995). Few years later the identification of UCN, other CRH-like peptides were discovered: urocortin2 (UCN 2 or stresscopin-related peptide) and urocortin 3 (UCN 3 or stresscopin) (Hsu and Hsueh, 2001; Reyes et al., 2001; Yamauchi et al., 2005). Both CRH and UCN can stimulate ACTH secretion from anterior pituitary corticotrophs *in vitro* and *in vivo* (Asaba et al., 1998). They contribute to different biological effects by binding two G-protein-coupled transmembrane receptors, named CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2) (Chen et al., 1993; Perrin et al., 1993, 2006; Vita et al., 1993). CRH receptors are 415-amino acid peptides. CRHR1 is highlighted in the central nervous system (CNS) and anterior pituitary, while CRHR2 in extra CNS sites. CRHR1 exhibits high affinity for CRH and UCN, low for UCN 2 and no affinity for UCN 3; CRHR2 exhibits high affinity for UCNs and no affinity for CRH. The expression of UCN and CRH was identified in different organs belonging to digestive, cardiovascular, immune, endocrine and genital tracts (Oki and Sasano, 2004; De Luca et al., 2009; Venkatasubramanian et al., 2010; Yang et al., 2010; Lee et al., 2011; Squillacioti et al., 2011, 2012). In the male genital tract, UCN and CRH receptors have been shown in the rat, mouse and human testis (Tao et al., 2007; Tezval et al., 2009; Lee et al., 2011), in human prostate (Arcuri et al., 2002) and in rat epididymis (De Luca et al., accepted in press). They have been considered to play a role in regulating spermatogenesis and steroidogenesis, sperm motility as well as prostatic and epididymal functions. In particular, in the epididymis, CRH-related

peptides have been suggested to regulate the maturation and the passage of spermatozoa via multiple mechanisms (De Luca et al., accepted in press).

The aim of this work was to investigate the presence and distribution of UCN, CRHR1 and CRHR2 in the epididymis of the South America camelid Alpaca (*Vicugna pacos*). The breeding of this 'non-conventional' species plays an important socio-economic role in Andean regions, USA, Australia and Europe. In Italy, the production of alpaca wool is greatly appreciated. Knowledge about the male reproductive system of this species is limited. In literature, only few studies regarding the morpho/functional aspects of the male reproductive system of alpaca are available (Parillo et al., 2009a,b, 2012; Wang et al., 2011; Zerani et al., 2011; Liguori et al., 2012, 2013, 2014). Moreover, the distribution of glycoconjugates residues was described in the epididymis, testis and spermatozoa (Parillo et al., 2009a,b, 2012). Moreover, the presence of orexin A and its receptor 1 was reported in the testis and epididymis (Liguori et al., 2012, 2013, 2014). In the present study, we therefore investigated the presence and distribution of UCN, CRHR1 and CRHR2 in the alpaca epididymis using Western blotting analysis and immunohistochemistry.

Materials and Methods

Animals and tissues processing

Five alpacas (*Vicugna pacos*) of 6–7 years old and sexually mature were used for this research. They were from Domus Alpaca, Pratola Peligna, Italy, and bred in semi-range conditions. These subjects were anaesthetized and surgical castrated according to the procedure described by Fowler (1998). The testes were removed, and epididymis collected and divided in three segments (caput, corpus and cauda): each of them was cut in small pieces. For immunohistochemical technique, specimens were fixed in Bouin's fluid for 24–48 h. For Western blotting analysis, specimens were frozen in liquid nitrogen and stored at −80° C.

Immunoprecipitation and Western blotting analysis

Frozen tissues were homogenized in a homogenization buffer (50 mM Tris-HCl, pH 7.00; 150 mM NaCl; 2% Triton; 5 mM EDTA; 10 mg/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 resulting supernatants were characterized for protein concentration by Bio-Rad protein assay. Equal amounts of proteins were immunoprecipitated overnight at 4°C with anti-UCN, -CRHR1 and -CRH2 (1 μg antibody/200 μg proteins) previously bound to protein A/G-Agarose. A fraction of supernatant was used as an immunoprecipitation input control (as a 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 a 18 and 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gel was transferred to nitrocellulose using a semidry apparatus (Bio-Rad) according to the manufacturer's instructions. 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 RT in the antisera diluted 1:500 in TBST containing 1% BSA. The following antisera were used: polyclonal goat anti-urocortin (sc-1825, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CRHR1 (sc-12383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CRHR2 (sc-1826; Santa Cruz Biotechnology, CA, USA).

The membrane was washed three times with TBST, incubated for 1 h with anti-goat IgG–peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) diluted 1:2000 in TBST-1% BSA and washed three times with TBST. Proteins were visualized by an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Marker proteins (coloured protein molecular weight markers; Prosieve, Lonza) were used to estimate the molecular weight of each band. A fraction of supernatant (50 μg) was separated on SDS–PAGE and immunoblotted with an anti- β-actin primary antibody (Mouse mAb JLA20 cp 01; Calbiochem**,** San Diego, CA; diluted 1:5000) and, successively, with a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; diluted 1: 2000).

Immunohistochemistry

The fixed specimens were dehydrated in a series of ascendant alcohols and embedded in Paraplast. Sections of 6 μm thickness were stained by immunohistochemical avidin–biotin method as it follows. After rehydration, the sections were incubated for 30 min in 3% hydrogen peroxide in water and after three washes in phosphate buffer saline (PBS, pH 7.3), incubated for the same time in 1.5% normal rabbit serum diluted in PBS. Goat polyclonal anti-UCN (sc-1825, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CRHR2 (sc-1826; Santa Cruz Biotechnology, CA, USA) and anti-CRHR1 (sc-12383; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies were diluted 1:200 and applied to the sections

overnight at 4°C. The second day, the sections were washed in PBS and after incubated at room temperature in biotinylated rabbit anti-goat IgG (BA-5000; Vector Laboratories, Burlingame, CA, USA) for 30 min and then in ABC reagent (PK-6105; Vector Laboratories) for the same time. A diaminobenzidine solution was used for the final staining. The specificity of the immunoreaction was detected substituting the primary antibodies with PBS. Control sections resulted negative. The stained sections were finally observed by the light microscope Nikon Eclipse E 600, and pictures were obtained by a Nikon Coolpix 8400 digital camera.

Results

Immunoprecipitation and Western blotting

The results of the immunoprecipitation and Western blot analysis are shown in Fig. 1. Tissue extracts of the caput, corpus and cauda of alpaca epididymis reacted with the anti-UCN antibody recognizing one major protein band of approximately 16 kDa. Similarly, anti-CRHRs antibodies each recognized one protein band measuring approximately 55 kDa in tissue extracts of all the epididymal segments.

Immunohistochemical evaluation

UCN-(Fig. 2a) and CRHR2-(Fig. 2b) immunoreactivity (IR) was described in the PCs of the caput epididymis (arrow). The positive material showed a fine granular aspect and was localized in the luminal portion of these cells. This material had a supranuclear localization and was widely diffused in the epithelium of epididymal tubules and rarely intermingled by the presence of few negative elements.

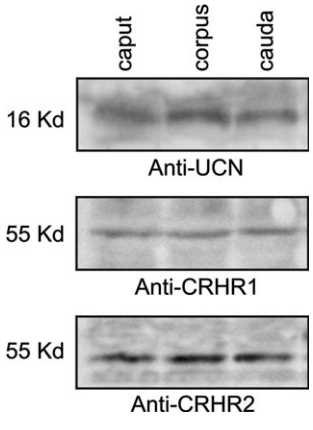

Fig. 1. Expression of UCN, CRH1 and CRHR2 in all segments of the alpaca epididymis by Western blot (WB). Tissue extracts were immunoprecipitated and immunoblotted with anti-UCN, anti-CRHR1 and anti-CRHR2 antibodies. Molecular weight markers are in kDa.

CRHR1-IR was distributed in the fibromuscular cells surrounding the epididymal tubules (double arrow) in all tracts of the organ (Fig. 2c–e) and in the smooth musculature of the blood vessels (Fig. 2c,d, arrowhead). In adding, some ACs (Fig. 2e, arrow) morphologically similar to AMRCs resulted positive. These cells appeared slender without an apparent connection with the basal membrane.

Discussion

In the present study, we have demonstrated the presence of UCN, CRHR1 and CRHR2 proteins in the epididymis of the alpaca. These results confirmed previous data obtained in the rat (De Luca et al., accepted in press). Immunoprecipitation and Western blotting analysis demonstrated that the anti-mammalian UCN antiserum recognized a protein band with a molecular weight of about 16 kDa in all tracts of the alpaca epididymis. This protein is comparable to the mammalian UCN precursor of 122-amino acids (Vaughan et al., 1995; Donaldson et al., 1996). Similarly, the antisera used against CRHR1 and CRHR2 recognized protein bands with molecular weights of approximately 55 kDa. These proteins are comparable to the mammalian CRH receptors (Chen et al., 1993; Perrin et al., 1993). Even though immunoprecipitation and Western blotting analysis recognized UCN and CRHR2 in all epididymal tracts, immunohistochemistry revealed UCN- and CRHR2-IRs only in the caput. These results suggest that UCN and CRHR2 are present in all tracts of the epididymis, but in the corpus and cauda, these peptides are located in smaller quantities and not detectable by immunohistochemistry. The presence of UCN and CRHR2 has been previously described in the rodent epididymis (Perrin et al., 1995; Perrin and Vale, 1999; De Luca et al., accepted in press). UCN and CRHR2 were distributed in the PCs of the caput epididymis. The supranuclear localization of these peptides in the PCs suggested a role in regulation of epithelial functions by an autocrine signalling. It can be hypothesized in alpaca epididymis that urocortin, via CRHR2, participates in regulation of epithelial cell growth and modulation of hormonal secretion, in particular of pituitary propiomelanocortin (POMC)-derived peptides. Corticotrophin-releasing factor (CRF) and urocortin stimulate pituitary POMC gene expression in mammals (Vaughan et al., 1995; Turnbull and Rivier, 1997; Asaba et al., 1998; Hsu and Hsueh, 2001) and POMC-derived secretion in the placenta (Petraglia et al., 1999) and pituitary (Vaughan et al., 1995; Turnbull and Rivier, 1997; Asaba et al., 1998; Hsu and Hsueh, 2001).

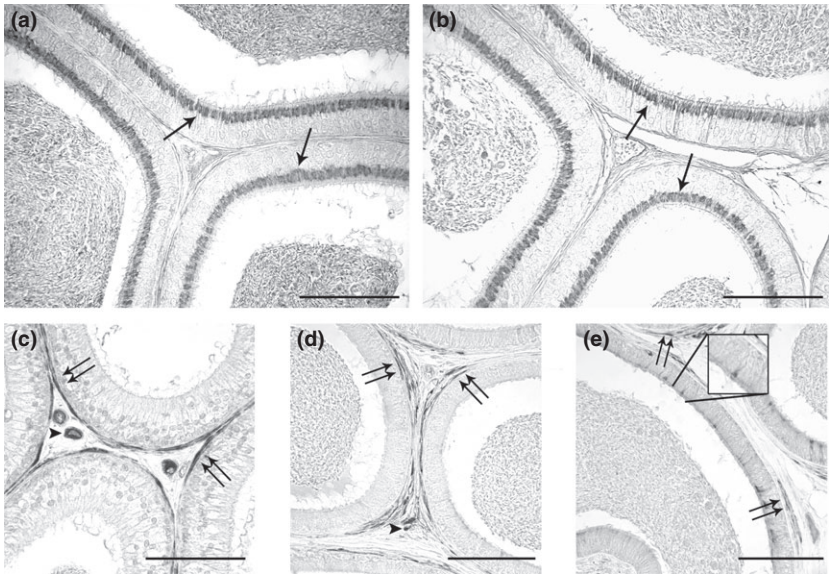

Fig. 2. UCN-IR (a) and CRHR2-IR (b) in the PC cytoplasm of alpaca caput epididymis (arrow). The positive material located in the apical portion of this cell type. CRHR1-IR (c–e) in the fibromuscular stromal cells surrounding the epididymal tubules from the caput to the cauda (double arrow), smooth musculature of the blood vessels (c,d, arrowhead) and epithelial cells similar to AMRCs in the cauda (e, arrow). Avidin–biotin immunohistochemical technique. Bars: 100 μm.

CRHR1-IR was observed in the fibromuscular stromal cells encircling the epididymal tubules of the three tracts of the alpaca epididymis. These results suggest a role of CRH-related peptides in the regulation of the contractility of the epididymis. Among reproductive organs, CRH-related peptides have been demonstrated to modulate uterine contractility during pregnancy (Linton et al., 2001; Grammatopoulos, 2007). Moreover, evidences support that, although the epididymis has a rich innervation, other local, non-neuronal factors participate in the nerve-independent epididymal contractility (Corona et al., 2012). In particular, the cauda epididymis, in which the fibromuscular pattern is higher than in the caput and corpus, is the major site for storage of spermatozoa in the male reproductive tract. It might be hypothesized that CRH-related peptides can facilitate the storage of spermatozoa in the cauda by inhibiting the contractility of epididymal fibromuscular stromal cells via CRHR1. The localization of CRHR1 in the smooth musculature of blood vessels suggests a role in modulating the local blood flow. This hypothesis is corroborated by the finding that UCN is a dilator of arteries (Lubomirov et al., 2001, 2006; Abdelrahman et al., 2005). In the cauda, CRHR1-IR has been detected in some epithelial cells which were morphologically similar to AMRCs. These cells are less frequent than PCs and basal cells (BCs). AMRCs were described like slender cells extending from the basement membrane to the lumen or as apical cells without apparent contact with basement membrane (Regadera et al., 1993). Although the role of AMRCs was unknown, the following functions have been suggested: holocrine secretion (Martan and Risley, 1963), cooperation with the PCs in reabsorption of testicular fluid (Sun and Flickinger, 1980) and acidification of epididymal fluid (Kierszenbaum et al., 1981). UCN therefore like other substances (S100, angiotensin-converting enzyme and galactosyltransferase) expressed in the AMRCs (Alkafafy et al., 2011) might influence the absorptive and secretory activities in the epididymis of camelids.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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