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ORIGINAL ARTICLE

Expression of Orexin A and its Receptor 1 in the Epididymis of the South American Camelid Alpaca (*Vicugna pacos*)

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Summary

Orexins A (ox A) and B are two peptides originally discovered in neurons of rat hypothalamus, and later found in different cellular types of the gastrointestinal and genital tracts. They arise from the proteolytic cleavage of a common precursor molecule, prepro-orexin, and bind to two receptors, namely receptor 1 (ox1r) and receptor 2 for orexins, that show different binding affinity. The central role of the two peptides has been extensively studied, whereas their activity in the periphery is still poorly known. Here, we investigated the presence of ox A and ox1r in the epididymis of a South American camelid species, the alpaca, by immunohistochemistry, and we also assessed the expression of prepro-orexin and ox1r in tissue extracts by Western blotting analysis. Ox A- and ox1r-immunoreactivity was found in the cytoplasm of principal cells of the caput epididymis. A prevalent supranuclear localization of granular-shaped positive material was observed. No positivity was present in the other cytotypes of epididymis. The expression of two peptides with molecular weight corresponding to those of prepro-orexin and ox1r, respectively, was detected in the tissue extracts from the organ.

Introduction

Orexins A (ox A) and B are two peptides produced by hypothalamic neurons of many mammalian species (de Lecea et al., 1998; Sakurai et al., 1998) and by nerve and endocrine cells belonging to the gastrointestinal and genital tracts (Kirchgessner and Liu, 1999; Barreiro et al., 2005; Ehrström et al., 2005; Tafuri et al., 2009, 2010). They derive from the proteolytic cleavage of the common precursor molecule, prepro-orexin, and bind to two G-coupled membrane receptors, namely receptor 1 (ox1r) and 2 (ox2r) for orexins. While ox1r is highly selective for ox A, ox2r links the cognate peptides with equal affinity (Sakurai et al., 1998).

Multiple evidence show that the hypothalamic orexins play a major role in relevant physiological functions such as food intake (Sakurai, 1999), sleep/wake cycle (Piper et al., 2000), blood pressure and heart rate (Shirasaka et al., 1999), sexual behaviour and arousal (Gulia et al., 2003), plasma corticosterone levels (Kuru et al., 2000) and adrenal function (Spinazzi et al., 2006). Orexins also play a critical role in the pathogenesis of narcolepsy in mice and human (Chemelli et al., 1999; Peyron et al., 2000).

On the contrary, studies on the localization and activity of orexins in the peripheral organs are limited to the gastrointestinal and genital tracts. In the genital tract, ox A has been shown to be produced by neuroendocrine cells of cattle urethra (Russo et al., 2008), the principal cells of rat epididymis (Tafuri et al., 2009) and Sertoli cells of rat and alpaca testes (Barreiro et al., 2005; Tafuri et al., 2010; Liguori et al., 2012). Furthermore, the presence of ox1r mRNAs has been demonstrated in the seminal vesicles, penis and epididymis of humans (Karteris et al., 2004), in the testis of sheep (Zhang et al., 2005), chicken (Ohkubo et al., 2003) and rats (Barreiro et al., 2004; Assisi et al., 2012), in the cattle prostate (Russo et al., 2008) and in the rat epididymis (Tafuri et al., 2009).

Aim of this work was to investigate the presence of ox A and ox1r in the epididymis of the South American

camelid alpaca (*Vicugna pacos*), a non-traditional species of zootechnical interest in Europe. The breeding of alpaca always held importance in the country of origin, the Andean altitudes of South America from Ecuador to southern Chile, for meat and wool production. In the last years, such breeding extended to USA, Australia and Europe where the characteristics of the wool produced are greatly appreciated. The physiology of alpaca reproduction is still poorly known, while a better knowledge of the reproductive mechanisms of this animal species could provide the basis for an extension of the mating period which is currently seasonal.

The localization of ox A and ox1r in the alpaca epididymis has been investigated using an immunohistochemical technique, and the expression of the precursor molecule, prepro-orexin, and ox1r in tissue extracts from the organ using Western blotting analysis.

Materials and Methods

Animals

The subjects utilized for this research were healthy and kept in optimal nutritional and sanitary conditions at the 'Domus Alpaca' farm (Pratola Peligna, Italy) where they were bred following a semi-range conditions. Three males, 6 years old and 60/70 kg in body weight each, underwent surgical castration according to the procedure described by Fowler (1998). This protocol fully adheres to the requirements of the International Guiding Principles for Biomedical Research Involving Animals. Animal care was followed throughout the procedure. All subjects were lacking of preputial adhesions, according to their sexual maturity (Tibary and Vaughan, 2006). The epididymis was subdivided in three segments (cranial, intermediate and caudal ones), and each segment was cut in small pieces. This material was either fixed in Bouin's fluid for 24–48 h or frozen in liquid nitrogen and stored at −80°C.

Immunohistochemistry

The fixed material was dehydrated in a series of ascending alcohols and embedded in Paraplast in vacuum. Six-μm-thick sections were stained by the immunohistochemical avidin–biotin method as it follows. After rehydration, the sections were incubated for 30 min in 3% hydrogen peroxide in water and, after a brief washing, still incubated for the same time in 1.5% normal rabbit serum containing PBS. For the specific step, goat polyclonal anti-ox A (sc-8070, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat polyclonal anti-ox1r (sc-8072, Santa Cruz Biotechnology) antibodies were diluted 1:200 and applied to the sections in a moist chamber overnight at 4°C. The sections were washed and incubated at room temperature (18–22°C) in biotinylated rabbit anti-goat IgG diluted 1:200 (BA-5000, Vector Laboratories, Burlingame, CA, USA) for 30 min and then in freshly prepared ABC reagent (PK-6105, Vector Laboratories) for 30 min. A diaminobenzidine solution was used as final staining. The specificity of the immunoreaction was tested by substituting the primary antibody with PBS or pre-absorbing the antibody with an excess (100 μg/ml) of its relative antigen. Control sections resulted always negative. The preparations were observed by the light microscope Nikon Eclipse E 600, and microphotographs were taken by a Nikon Coolpix 8400 digital camera (Tokyo, Japan).

Western blotting analysis

Frozen tissues were homogenized in buffer (0.05 M Tris HCl, pH 7.0; 0.15 M NaCl; 2% Triton; 0.005 M EDTA; 10 mg/ml leupeptin; 0.1 U/ml aprotinin; 0.001 M PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16 000 × ***g*** for 20 min at 4°C. Aliquots of the supernatant were subjected to electrophoresis on 15% sodium dodecyl sulphate-polyacrylamide gel (SDS PAGE) (Bio-Rad, Hercules, CA, USA) which was then 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) containing Tris-buffered saline with Tween 20 (TBST) (0.15 M NaCl; 0.02 M Tris HCl, pH 7.4; 0.3% Tween-20), washed in the same buffer and again incubated for 2 h at room temperature with rabbit polyclonal anti-prepro-orexin (AB3096; Millipore Corporation, Billerica, MA, USA) or goat polyclonal anti-ox1r antibody diluted 1:500 in 2.5% BSA containing TBST. After washing three times in buffer, the membrane was incubated in 1:2 000 diluted goat anti-rabbit (AP 132P; Millipore Corporation) or rabbit anti-goat (PI-9500; Vector Laboratories) secondary antibody, both peroxidase conjugated, for 1 h at room temperature. Proteins were visualized by an enhanced chemiluminescence kit (RPN 2109; ECL Amersham, Little Chalfont, Buckinghamshire, UK). Marker proteins were used to estimate the molecular weight of each band. To monitor loading of gel lanes, the blots were stripped and re-probed using an anti-tubulin monoclonal antibody (MAB1637; Chemicon International Inc., Temecula, CA, USA).

Results

The cranial segment of the alpaca epididymis showed two distinct histological parts that cannot be distinguished at macroscopical examination. The first portion showed a

monolayered tall lining epithelium encircling a small lumen generally devoid of spermatozoa. The second one was characterized by a lower epithelium and a wider diameter of the tubular cross section. This portion normally contains large masses of clustered spermatozoa, and it is referred in the text as 'caput' or 'head' of the epididymis. The results below described were obtained from three males: no significant differences were found among the three tested animals.

Histological evaluation of ox A and ox1r

Ox A (Fig. 1a–d)- and ox1r-immunoreactivity (Figs 1e–f) was found in the principal cells of the epididymis head. The positive material showed a fine granular aspect and was mostly localized in the supranuclear cytoplasm (Fig. 1a,b,e,f), which sometimes appeared to be almost entirely filled. Rarely, clusters of ox A reactive granules were also found in the infranuclear portion of the cell (Fig. 1c). Throughout the head, the positive cells had a focal distribution being clustered in small zones. In the areas of wide diffusion, such cells lined almost entirely the circular profile of the epididymal tubule, and their sequence was only rarely discontinued by the presence of single or few negative elements.

Cytotypes different from the principal one were always found to be negative. Fig. 1d shows the profile of basal cells and 'apical mitochondria-rich' (AMR) cells which are clearly unstained. This latter cytotype has been described some decades ago in the rat (Brown and Montesano, 1980) and human (Regadera et al., 1993) epididymis and only recently was also found in the alpaca organ (Parillo et al., 2009).

Western blotting analysis of tissue extracts

The presence of prepro-orexin and ox1r in tissue extracts of the alpaca epididymis was demonstrated using, respectively, a rabbit polyclonal antibody raised against a peptide mapping near the C-terminus of mouse prepro-orexin and a goat polyclonal antibody raised against a peptide mapping near the C-terminus of rat ox1r. The molecular weight of the detected prepro-orexin was 16 kDa, whereas that of ox1r was 50 kDa (Fig. 2). These molecular weight values correspond to those of prepro-orexin and ox1r found in the brain of the rat, respectively (Jöhren et al., 2001). Both the peptides were detected in rat brain homogenates which were used as positive control. The specificity of the response was confirmed by pre-incubation of the prepro-orexin and ox1r antibodies with respective blocking peptides. There was no expression of prepro-orexin and ox1r in these preparations. The stripping of the blots and their re-probing with a monoclonal anti-tubulin antibody demonstrated equal loading of proteins in all lanes (Fig. 2).

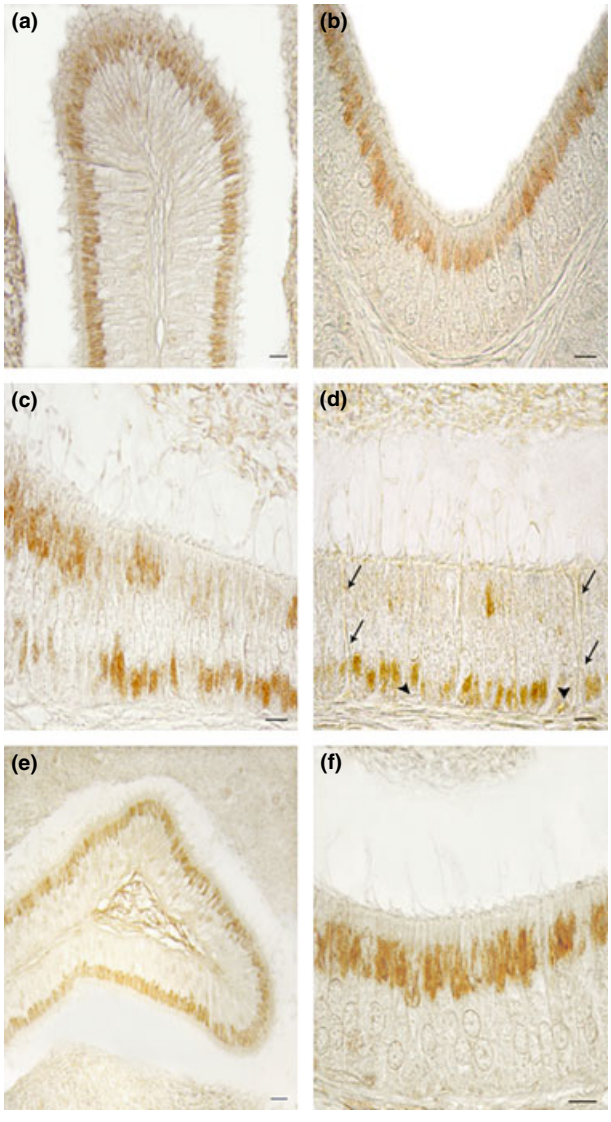

Fig. 1. Ox A (a–d)- and ox1r-immunoreactivity (e,f) in the principal cell cytoplasm of alpaca caput epididymis. (a): a finger-like, intraluminal protrusion of the tubular wall lined by a sequence of positive cells. (b,c): supranuclear (b) and infranuclear (c) localization of ox A-immunopositive material. (d): negative AMR (arrows) and basal (arrowheads) cells intermingled among slightly positive principal cells. (e): irregular intraluminal protrusion of the tubular wall lined by ox1r-immunoreactive cells. (f): supranuclear localization of ox1r-immunoreactivity in the principal cell cytoplasm. Avidin–biotin immunohistochemical method. Bars: 10 μm.

Discussion

In this work, we describe the immunohistochemical detection of the peptide ox A and its receptor 1 in the

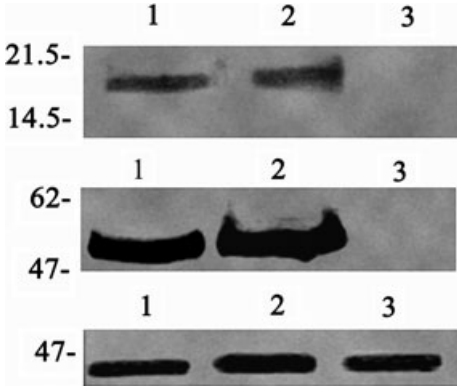

Fig. 2. Prepro-orexin (upper blot) and ox1r (middle blot) expression by Western blotting analysis. Lane 1: rat brain tissue (positive control). Lane 2: alpaca epididymis tissue. Lane 3: alpaca epididymis tissue treated with anti-prepro-orexin and anti-ox1r antibodies pre-absorbed with their respective blocking peptides (negative control). The upper and middle blots were stripped and reprobed with an anti-tubulin antibody to ensure equal loading of protein in all lanes (lower blot). Molecular weight markers are expressed in kDa.

epididymis of the South American camelid alpaca. This finding is supported by the results of Western blotting analysis of tissue extracts which show the presence of the ox A precursor molecule, prepro-orexin, and ox1r, respectively. Similar results have been described in rat epididymis, where the presence of mRNAs coding for both prepro-orexin and ox1r was also shown (Tafuri et al., 2009). Previously, the presence of 519 bp DNA fragments specific for prepro-orexin was found in human epididymis (Karteris et al., 2004). Overall, these findings suggest that mammalian epididymis may be able to synthesise ox A and its specific receptor 1, and the principal cells of the lining epithelium represent the site of their production.

The peptide ox A and its receptor 1 appear to share some similarities with three other endocrine peptides and their respective receptors: calcitonin gene-related peptide (CGRP) (Leung et al., 1992; Brian and Cox, 2006), secretin (Chow et al., 2004) and pituitary adenilate cyclase-activating polypeptide (PACAP) (Zhou et al., 1997; Leung et al., 1998). All these peptides have been found in the principal cells of rat epididymis. Furthermore, the receptors of the four substances are polypeptides composed by seven trans-membrane domains which bind to 'G' proteins in the cytoplasm as first step of their metabolic pathway.

The supranuclear localization of these peptides and their receptors in the principal cells suggests that they may act by either autocrine or paracrine signalling. This latter allows the peptide to be rapidly spread to targets also distant from its source.

Ox A-mRNAs (Tafuri et al., 2009) and secretin-mRNAs (Chow et al., 2004) have been found in the rat epididymis. The functional role of secretin in the epididymis includes the opening of CFTR-Cl^- channels located in the apical membrane of the principal cells. Through these channels, chloride and bicarbonate anions are transferred from the cytoplasm into the intratubular milieu which rapidly hydrates for the cell water loss caused by the changing of local osmolarity (Chow et al., 2004). The principal cells use this mechanism to influence intratubular environment fluidity which is one of the main factors regulating spermatozoa motility and fertility (Cornwall, 2009). The peptides CGRP (Leung et al., 1992; Brian and Cox, 2006) and PACAP (Zhou et al., 1997; Leung et al., 1998) play the same role of secretin with the only difference that the anion transfer is restricted to chloride and bicarbonate, respectively. Therefore, studies are in progress in our laboratory to establish whether and how ox A might contribute to the formation of environmental conditions essential for sperm capacitation.

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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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