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Identification of orexins and cognate receptors in the lacrimal gland of sheep

Cecilia Dall'Aglio[a,*], Francesca Mercati[a], Margherita Maranesi[b], Cristiano Boiti[b]

[a] Dipartimento di Scienze Biopatologiche ed Igiene delle Produzioni Animali ed Alimentari, Sezione di Anatomia Veterinaria, Via San Costanzo 4, 06126 Perugia, Italy
[b] Dipartimento di Scienze Biopatologiche ed Igiene delle Produzioni Animali ed Alimentari, Laboratorio di Biotecnologie fisiologiche, Sezione di Fisiologia, Facoltà di Medicina Veterinaria, Via San Costanzo 4, 06126 Perugia, Italy

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ABSTRACT

The aim of the present work was to study, by means of immunohistochemical and RT-PCR techniques, the presence and distribution of immunopositivity for orexin A and B (OXA and OXB) and orexin type 1 and 2 receptors (OX_1R and OX_2R) in the lacrimal gland of sheep as well as the gene expressions for prepro-orexin (PPOX) and cognate receptors. In serial sections, positive staining for OXA and OXB were localized in the same nervous fibers within the connective tissue septa. Positive staining for OX_1R was evidenced in the wall of small arteries while that for OX_2R was observed in the secretory portion of the acinar gland cells with a characteristic localization in the apical cytoplasm. RT-PCR analysis showed the presence of transcripts for PPOX, OX_1R and OX_2R in the sheep lacrimal gland; the gene expression of OX_1R was two-fold greater ($p < 0.01$) than that of OX_2R. Taken together the present findings raise intriguing questions on the potential role of the orexinergic system in the regulation of lacrimal gland functions that require further investigations.

1. Introduction

The orexin system is widely distributed in the central nervous system [29] where it controls several functions including feeding and drinking behaviors and sleep-wake cycle [3,28,32]. The orexin system comprises two neuropeptides, orexin A (OXA or hypocretin-1) and orexin B (OXB or hypocretin-2), derived from proteolysis of a common 130-aminoacid precursor, prepro-orexin (PPOX), and two receptors, OX_1R and OX_2R. The two neuropeptides share 40% homology and the sequence of OXA is fully preserved among rats, humans, mice, pigs, and cows [19,32]. Both OX_1R and OX_2R belong to the G-protein-coupled receptor super-family, are 90% homologous and highly preserved across rats and humans [29,32]. While OXA shows equal binding affinity for both receptor types, OXB has 10-fold greater affinity for OX_2R than for OX_1R [32]. In the last decade, many studies in humans, laboratory and domestic animals [5–10,15,23,24,30] have described the presence of orexins in the endocrine cells of the gastro-entero-pancreatic system and in neurones and nervous fibers distributed in the gastrointestinal submucosa and muscular layer. Recently, we have characterized the components of the orexinic system in the nervous structures innervating the mandibular gland of pigs as well as in the secreting structures of the gland [10]. Thus, increasing evidence demonstrates that the orexin system is widely expressed in a variety of tissues and organs in many mammalian species.

The lacrimal gland is a multi-lobular, tubulo-acinar (or tubulo-alveolar) gland; it can be a serous, mucous or mixed type gland, depending on the species [16,25]: serous in pigs, cats, and rabbits, mucous in goats, mixed in dogs, rats, and humans. This gland secretes a complex fluid, composed of water, electrolytes and proteins that contribute to form the aqueous layer of the tear film coating the eye and help to nourish and protect the ocular surface [18]. Tear production is tightly regulated by a neural reflex arc through afferent sensory nerves from the cornea and conjunctiva, the lacrimal nucleus in the brain, and efferent parasympathetic and sympathetic nerves which end within the lacrimal gland in close proximity to the acinar, ductal, and myo-epithelial cells and to local blood vessels as well. The amount and composition of lacrimal gland secretion is qualitatively different depending on species. In sheep, the lacrimal gland is mixed as evidenced by the presence of the three cell types in the acini. Mucous, serous, and seromucous cells may be present in the same acinous in variable proportions or may form completely serous or mucous acini [16]. Secretory granules of various nature were identified not only in the acinar cells, but also in the epithelial cells lining the intra- and inter-lobular ducts [17]. This finding, based on ultra-structural and histochemical studies, indicates that in sheep the duct system of the lacrimal

* Corresponding author at: Sezione di Anatomia Veterinaria, Dipartimento di Scienze Biopatologiche Veterinarie ed Igiene delle Produzioni Animali ed Alimentari, Via San Costanzo 4, 06126, Perugia, Italy. Tel.: +39 075 5857635; fax: +39 075 5857631.
E-mail address: cecilia.dallaglio@unipg.it (C. Dall'Aglio).

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gland may also regulate the protein content of the primary fluid secreted by the acinar cells and not just the balance of water and electrolytes.

Considering the wide distribution of the orexin system among different tissues in several species and the strict anatomical similarity between the major salivary glands and the lacrimal gland regarding their secretory structures and neural control, in this study we aimed to characterize the presence of the whole components of the orexinic system as well as their precise cellular localization in the lacrimal gland of sheep. For this study, we purposely selected the lacrimal gland of sheep because its ultra-structural organization was previously characterized in our laboratory [16,17]. As far as we know, there is only one report that described the immunohistochemical localization of OXB and OX_1R in the lacrimal gland of rats [1].

2. Materials and methods

2.1. Animals and tissues

For this study, the lacrimal glands were obtained from 5 adult sheep at the slaughterhouse in Ponte San Giovanni, Perugia (Italy). Immediately after slaughtering, the lacrimal glands were promptly removed from each animal, thorough washed with saline and subdivided transversally into two pieces: one was rinsed with RNAse free phosphate buffered saline and frozen at −80 °C for later evaluation of gene expression, the other was fixed by immersion in Bouin's fluid at room temperature for 24 h and then processed for the immunohistochemical (IHC) studies. Samples of kidney tissue were removed from two sheep and processed for IHC as described above and used as control for assessing the expression of OX_1R in renal arteries.

2.2. Reagents

The mouse monoclonal antibodies anti-OXA and anti-OXB (MAB763 and MAB734 respectively) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA), whereas anti-OX_1R rabbit polyclonal antibody (O 4514) was from Sigma–Aldrich (St. Louis, MO, USA) and anti-OX_2R rabbit polyclonal antibodies (AB 3094) was obtained from Millipore (Billerica, MA, USA). The secondary biotin-conjugated goat anti-mouse IgG antibody for OXA and OXB was purchased from Vector Laboratories (Burlingame, CA, USA), the goat anti-rabbit IgG antibody for OX_1R and OX_2R was supplied by Millipore (AP 132B). The avidin–biotin complex (ABC, PK 6100, Vectastain ABC kit) and the chromogen 3,3′-diaminobenzidine tetrachloride (DAB, Sk 4100) were from Vector Laboratories. Canada Balsam Natural was purchased from VWR International Ltd (Lutterworth, Leicesterschire, UK).

Random hexamer primers, deoxyribonuclease I (DNAase I Amp. Grade), RNAse H-reverse transcriptase (SuperScript III Reverse Transcriptase), *E. coli* RNase H and DNA ladders were obtained from Invitrogen (S. Giuliano Milanese, Milan, Italy) as well as reagent for isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), 10× PCR Buffer, 50 mM $MgCl_2$, RNAse-free tubes and RNAse-free water, deoxy-NTPs, primers for 18S rRNA and corresponding competimers (QuantumRNA 18S Internal Standards) as well as primers for mRNAs of PPOX, OX_1R, and OX_2R. The Nucleospin Extract II kit was purchased from Macherey Nagel Inc. (Bethlehem, PA, USA).

2.3. Immunohistochemistry

After fixation the tissue samples were dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin. The immunohistochemical reaction was visualized on 5 μm serial sections, mounted on poly-L-lysine coated glass slides, utilizing the avidin–biotin-complex and the 3,3′ diaminobenzidine-4-HCl as the chromogen. Briefly, dewaxed sections were microwaved for 15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. All subsequent steps were carried out in a moist chamber at room temperature. To prevent non-specific binding of primary antibodies, after a proper cooling the sections were pre-incubated for 30 min with the normal serum. Subsequently, serial sections were incubated overnight, each with one of the following primary antibodies: anti-OXA and anti-OXB mouse monoclonal antibody (1:100), anti-OX_1R and anti-OX_2R rabbit polyclonal antibody (1:100).

The next day, after washing in phosphate-buffered saline (PBS), the sections were incubated for 30 min at room temperature with the secondary biotin-conjugated goat anti-mouse IgG antibody for OXA and OXB (1:200) with the secondary biotin-conjugated goat anti-rabbit IgG antibody for OX_1R and OX_2R (1:200) and then processed for 30 min using the Vectastain ABC kit. Subsequently, the tissue samples were repeatedly rinsed with PBS and developed with the chromogen solution. After several rinses in PBS, the sections were dehydrated and mounted in Canada Balsam Natural.

Sections in which the primary antibodies were omitted or substituted with pre-immune gamma globulin were used as control of unspecific staining. All tissue analyses and cell counts were carried out on coded slides using a light microscope (Nikon Eclipse E800, Nikon Corporation, Tokyo, Japan) connected to a digital camera (Dxm 1200 Nikon digital camera). Images were processed using an image analysis system (Lucia, Laboratory Imaging Ltd, Praha).

2.4. RNA extraction and reverse transcription

Total RNA was extracted from 100 mg of each lacrimal gland tissue sample. The tissue sample was homogenized in 1 ml of trizol and genomic DNA contamination was prevented by treatment with Dnase I according to the instructions of the manufacturer. Five micrograms of total RNA were reverse-transcribed in 20 μl of Superscript III Reverse transcriptase cDNA synthesis mix using random hexamers. Genomic DNA contamination was checked by performing the PCR procedure without reverse transcriptase.

2.5. Multiplex RT-PCR amplification and data analysis

Multiplex PCR amplification was carried out as previously described [21]. An aliquot of 1 μl of the obtained lacrimal gland cDNA was used as template for subsequent PCR amplification containing two primer sets, one for each target gene (PPOX, OX1R, and OX2R) and the other for the 18S housekeeping gene. The primers sequences are listed in Table 1. Cycling conditions consisted of an initial denaturing cycle at 94 °C for 75 s, followed by 35 cycles for each target gene at 94 °C for 15 s, 62 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min. Within each experiment and for each gene analyzed, the complete set of cDNA samples ($n = 5$) was processed in parallel in a single PCR, using aliquots of the same PCR master mix. Each set of determination was performed in quadruplicate. For every PCR, two negative controls were included, without template and without reverse transcriptase, respectively. The amplified PCR-generated products (20 μL of 25 μL total reaction volume) were analyzed by electrophoresis on 2% agarose gel using ethidium bromide staining. The amplified products, collected from agarose gel after electrophoresis, were purified with the Nucleospin Extract II kit and their identity was confirmed by DNA sequencing with Sanger's method. To evaluate the relative levels of mRNAs, the band intensities for the target genes of interest obtained from each aliquot of PCR products were normalized against those of the housekeeping 18S mRNA co-amplified