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Novel evidence of ghrelin and growth hormone segretagogue receptor expression by human ocular tissues ☆

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ABSTRACT

Aim of the study: The gastrointestinal peptide hormone ghrelin (Ghr) was discovered in 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHSR-1a). It is a pleiotropic peptide that modulates a wide spectrum of biological activities, such as growth hormone (GH) release, feeding stimulation, adiposity and cardiovascular actions. The presence of Ghr mRNA in the iris and ciliary body (CB) epithelium was recently demonstrated in animal models, where a possible myorelaxing effect on the iris muscles has been suggested. Based on these observations, the aim of our study was to investigate the Ghr and GHSR-1a expression and localization in the normal human eye.

Material: Five different ciliary body/iris samples from normal eyes were subjected to Western blot analysis. Immunohistochemical detection was performed on three enucleated eyes. Twenty aqueous humor (AqH) samples obtained from patients submitted to cataract surgery were analyzed with an ELISA for the presence of Ghr.

Results: Ghr and GHSR-1a were co-expressed by the pigmented epithelium (PE) of the CB, by the retinal pigmented epithelium (RPE) and by the anterior limiting layer (ALL) of the iris. No reaction was detected at the subepithelial level in the ciliary or pupillae smooth muscle cells. The AqH samples were positive for the presence of Ghr.

Conclusion: This study provides the first evidence that Ghr and GHSR-1a are expressed in the human eye by specific cells. The understanding of the functional role of Ghr at the human eye level needs more efforts and investigation, but a hypothetical action on the GH retinal synthesis and/or on the circadian clock system could be suggested.

1. Introduction

Ghrelin (Ghr) is a 28-amino-acid peptide that is mainly secreted by the human gastric oxyntic mucosa. It is derived from the sequential processing of pre-proghrelin (117-amino acids) and proghrelin (94-amino-acids) [1,2]. It is involved in appetite control and induces a potent release of growth hormone (GH) by binding the GH secretagogue receptor type 1a (GHSR-1a). O-n-octanoyl esterification at the serine 3 residue is essential for binding with the GHSR-1a [3]. A non-acetylated form of Ghr that binds GHSR-1a with very low affinity [3,4] was recently identified in the stomach and blood. Ghr is widely expressed in different tissues, such as the small intestine, heart, pancreas, brain, hypothalamic arcuate nucleus, pituitary gland, lung, skeletal and smooth muscle, salivary glands, ovary and testis [5]. In addition to its orexigenic activity, Ghr exerts several non-metabolic effects acting at the endocrine, cardiovascular, musculoskeletal and immune levels [4,6].

Recent studies have demonstrated that Ghr is expressed in the rodent eye in the anterior and posterior segments, with the highest expression levels in the retina and iris [7]. Ghr has been shown to interfere with the kinetics of the iris by reducing the sphincter and dilator muscle tension. The Ghr effect on the iris sphincter muscle appears to be independent from GHSR-1a [8] and species independent [9]. The Ghr–GHSR-1a system is able to modulate retinal angiogenesis in vitro and in vivo and is suggested to play a role in the pathogenesis of the proliferative retinopathies [10]. Ghr has been detected in normal and glaucomatous aqueous humor (AqH) [11,12].

☆ DFA, GB, GCE and ZR designed and performed experiments; LM gave technical support; IP and DAMA analyzed data, GPE conceptual support, ADB wrote the paper.

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Evidence increases for the physiological and pathogenic roles of the Ghr–GHSR-1a system [9] at the ocular level. The aim of our study was to investigate the Ghr and GHSR-1a expression and localization in the normal human eye.

2. Materials and methods

The study was approved by the local institutional Ethical Committee. The research followed the tenets of the Declaration of Helsinki. Informed consent for aqueous humor collection during routine surgery was obtained from the subjects.

2.1. Western blotting

Western blotting analyses were performed on the ciliary body (CB)/iris samples obtained from five patients who underwent routine glaucoma surgery for visual rehabilitation. Samples of the iris/CB were collected during trabeculectomy surgery [Cairns' procedure, alone or associated with phacoemulsification (phaco-trabeculectomy)]. The tissue was homogenized in RIPA buffer (1% Nonidet P40, 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate) in the presence of a protease inhibitor. The protein concentration was evaluated by the Bradford method. The lysate samples (35 μg) were separated by SDS-PAGE and blotted, as previously described [13,14]. The membranes were incubated overnight at 4 °C with a rabbit polyclonal anti GHSR1-a (Abcam, 1:1000) or anti-Ghr (Abcam, 1:500) and probed with a horse-radish-peroxidase-conjugated anti-rabbit secondary antibody (Abcam, 1:2000). After washing, the immunoreactive bands were detected with a long lasting chemiluminescent substrate (LiteAblot®EXTEND, EuroClone) and visualized on a GEL DOC 1000 (Biorad, Milano, Italy). The membranes incubated with the secondary antibody only were used as an internal negative control, and lysates from the gastric oxyntic mucosa and HeLa cells represented the positive controls for Ghr and GHSR1-a expression, respectively.

2.2. Morphological and Immunohistochemical analyses

Sections were obtained from formalin-fixed, paraffin-embedded normal and intact human eyeballs. Two eyes were enucleated after a major zygomatic-sphenoid greater wing trauma involving the optic nerve and the Zinn ligament, and a third eye was obtained by an orbit exenteration for maxillary sinus carcinoma without eye invasion. Hematoxylin–eosin staining was performed on the paraffin-embedded sections, as previously described [15]. Immunohistochemistry was performed with the Bond Polymer Refine Red Detection In-Vitro diagnostics kit for the automated Bond system on a Bond Max series automated stainer (Leica, Wetzlar, Germany). High pH antigen retrieval was performed for 20 min. For immunostaining, sections were incubated with rabbit anti-Ghr (Abcam, 1:100, which recognizes the acylated and desacylated forms), anti-GHSR (Abcam, 1:500) or monoclonal anti-Smooth Muscle Actin (SMA) (Dako 1:100) for 30 min, washed and probed with the specific alkaline phosphatase conjugated secondary antibody (Dako, Glostrup, Denmark). The sections that reacted with the secondary antibody only were used for the controls of the procedure.

2.3. AqH analysis

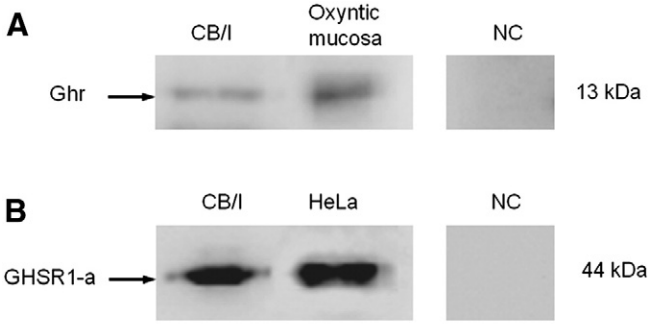
The presence of total Ghr in human AqH was evaluated by an enzyme immunoassay kit to human Ghr (Millipore Corporation, Billerica, MA, USA). The AqH samples were obtained from 20 patients undergoing routine cataract surgery. The AqH samples (40–100 μl) extracted at the beginning of surgery via clear cornea tunnel for phaco in quiet eyes, were not contaminated with blood. The subjects were not affected by other ocular pathologies and did not take medications for chronic or dysmetabolic diseases. Each test was performed analyzing 20 μl of AqH, according to the manufacturer's instructions. All of the samples were processed in duplicate during the same assay session. The sensitivity of the kit was 30 pg/ml.

3. Results

3.1. Western blotting and microscopic analysis

Western blotting performed in human CB/iris samples showed evidence of specific immunoreactive bands for Ghr and GHSR-1a (Fig. 1).

The eye morphological features were identified by hematoxylin–eosin staining (Supplementary figure. The CB is made up of the ciliary epithelium (CE) and the underlying tissues, the stroma and the ciliary muscles. The CE represents the site of AqH secretion and is made up of two polarized neuroepithelial cell layers, the pigmented (PE) and nonpigmented (NPE) epithelium, which oppose each other at their respective apical plasma membranes and extend from the root of the iris (anterior) to the beginning of the retina (posterior). The PE cell layer becomes continuous with the retinal pigmented epithelium (RPE), and the NPE layer is related to the sensory layer of the retina. The immunohistochemical analysis of the serial sections allowed us to localize Ghr and GHSR-1a in the ocular tissues (Fig. 2). At the CB level, Ghr and GHSR-1a were expressed by the outer PE of the double-layered epithelium (Fig. 2H, K, P, S). These cells contain pigment granules of melanin that are synthesized and deposited in membrane-bound organelles, such as melanosomes. These dense granules are abundant, ellipsoidal and concentrated near the apical plasma membrane; they can be easily distinguished by their size and their dark color from Ghr and its receptor, which appeared immune-detected as smaller and red granules in the cytoplasm. The Ghr and GHSR-1a were also detected inside the RPE (Fig. 2J, M, R, S). This positivity faded with distance from the CB along the retinal epithelium, where single dome-shaped positive cells were found scattered inside the RPE. Intense positive cells for Ghr and GHSR-1a were detected in the anterior limiting layer of the iris (ALL), but not in the epithelial cells of the posterior limiting layer (PLL) (Fig. 2I, L, Q, T).



C

Ghr	CB/I	Oxyntic mucosa
	106.552±7.458	123.080±13.538
GHSR1-a	**CB/I**	**HeLa**
	160.971±14.487	185.445± 14.835

Fig. 1. Western blot analysis of Ghr and GHRS1-a expression in CB/iris samples. Human oxyntic mucosa and HeLa cell lysates were used as the internal positive control for Ghr (A) and GHSR1-a (B) expression, respectively. The membranes incubated with the secondary antibody only were used as the internal negative control (NC). (C) Densitometric analysis of immunoreactive bands for Ghr and GHRS1-a respectively. Values are expressed as gray mean value ± SD. The results are representative of five different experiments.