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Ghrelin as a novel locally produced relaxing peptide of the iris sphincter and dilator muscles

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Abstract

Ghrelin is a recently described acylated peptide, which works as a somatosecretagogue and has described effects on the smooth, skeletal and cardiac muscle. We examined the production and effects of ghrelin on relaxation of the iris muscles. Contractile effects of 1–5 human ghrelin (frGhr, 10^{-9}–6×10^{-5} M) and 1–5 human des-octanoyl-ghrelin (d-frGhr; 10^{-9}–6×10^{-5} M) were tested on iris rabbit sphincter ($n = 11$ frGhr; $n = 7$ d-frGhr), dilator ($n = 6$ frGhr; $n = 6$ d-frGhr) and rat sphincter ($n = 6$ frGhr; $n = 8$ d-frGhr) precontracted muscles. On rabbit sphincter the effect of frGhr was also tested in presence of: i) L-NA (10^{-5} M; $n = 7$); ii) indomethacin (10^{-5} M; $n = 7$); iii) DLys^{3}GHRP6 (10^{-4} M; $n = 6$); and iv) apamin + carybdotoxin (10^{-6} M; $n = 6$). Furthermore, on rabbit dilator the effect of frGhr was tested in presence of DLys^{3}GHRP6 (10^{-4} M; $n = 7$). Finally, ghrelin mRNA production was assessed by "*in situ*" hybridization in Wistar rat eyes ($n = 8$). In all muscles, frGhr promoted a concentration-dependent relaxation, maximal at 6×10^{-5} M, 1.5–3 min after its addition, decreasing tension by $34.1 \pm 12.1\%$, $25.8 \pm 4.8\%$ and $52.1 \pm 10.3\%$ in the rabbit sphincter, dilator and rat sphincter, respectively. In the rabbit sphincter the relaxing effects of frGhr were: (i) enhanced in presence of DLys^{3}GHRP6 ($118.1 \pm 21.1\%$); (ii) blunted by indomethacin; and (iii) not altered by apamin + carybdotoxin ($36.4 \pm 14.4\%$) or L-NA ($52.4 \pm 11.4\%$). Relaxing effects of d-frGhr in rabbit ($43.3 \pm 5.2\%$) and rat ($77.1 \pm 15.3\%$) sphincter muscles were similar to those of frGhr. In rabbit dilator muscle, d-frGhr did not significantly alter active tension and the relaxing effect of frGhr was blunted by GHSR-1a blockage. Ghrelin mRNA was identified in iris posterior epithelium. In conclusion, ghrelin is a novel, locally produced, relaxing agent of iris dilator and sphincter muscles, an effect that is mediated by GHSR-1a in the former, but not in the latter. Furthermore, in the sphincter it seems to be mediated by prostaglandins, but not by NO or K_{Ca} channels.

Keywords: peptide hormones; iris; ciliary epithelium; muscle fibers

1. Introduction

Ghrelin is a recently described acylated peptide, initially isolated from rat stomach, which works as a somatosecretagogue (Kojima et al., 1999). Growth hormone secretagogues (GHS) promote GH release by direct action on the pituitary gland (Smith et al., 1997; Locatelli and Torsello, 1997; Bowers, 1998; Dieguez and Casanueva, 2000), stimulating a specific G Protein coupled receptor (GHS type 1a receptor,

Abbreviations: L-NA, N^{ω}-nitro-L-arginine; GHS, growth hormone somatosecretagogues; GHSR-1a, growth hormone somatosecretagogues receptor type 1a; COX, cycloxygenase; ET-1, endothelin 1; frGhr, 1–5 human ghrelin; d-frGhr, 1–5 human des-octanoyl-ghrelin; NO, nitric oxide; Pg, prostaglandins; EP_2, prostaglandin E receptor 2.

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GHSR-1a), which is different from GHRH receptor (Howard et al., 1996; Muccioli et al., 1998; Smith et al., 1999; Feighner et al., 1998). Binding sites to the somatosecretagogues have been described in several tissues such as (in order of decreasing binding activity) the myocardium, adrenal gland, gonads, arteries, lung, liver, skeletal muscle, kidney, pituitary, thyroid, adipose tissue, veins, uterus, skin and lymphnode (Papotti et al., 2000; Iglesias et al., 2004). Interestingly, in many of these tissues, the specific binding values were described as even higher than in the pituitary gland (Papotti et al., 2000; Bodart et al., 1999; Muccioli et al., 2000).

Several studies investigated the physiological role of ghrelin and GHSR-1a in cardiac, skeletal and smooth muscles. In cardiac muscle, ghrelin is synthesized and secreted by cardiomyocytes (Iglesias et al., 2004), and acts as a negative inotrope. This effect is modulated by cycloxygenase depends on endocardial endothelium and seems to be independent from GHSR-1a (Bedendi et al., 2003). In the skeletal muscle, ghrelin reduces membrane potential, apparently by increasing chloride's permeability. This action is directly dependent of GHSR-1a and is blocked by the specific inhibitor DLys^{3}GHRP6 (Pierno et al., 2003). In human vascular smooth muscle, ghrelin appears as the most potent endothelium-independent vasodilator, reversing effectively endothelin-1 (ET-1) mediated constrictions with potency similar to that of adrenomodullin (Wiley and Davenport, 2002). Ghrelin also has a potent hypotensive effect associated with reduced nitric oxide (NO) availability. This effect, in rats, is reversed by the blockage of the Ca^{2+} activated K^+ channels (Shinde et al., 2005).

In the iris sphincter and dilator muscles the relaxing effects of several substances have been investigated over the last decade. Geyer et al. (1998) showed that β_3 adrenergic stimulation elicits a significant relaxation of the bovine iris sphincter muscle, an effect that was later shown to be potentiated by muscarinic blockage (Barilan et al., 2003). In the same experimental preparation, it was demonstrated that the endogenous NO-guanylyl cyclase-cGMP cascade mediates the non-adrenergic-non-cholinergic relaxation (Pianka et al., 2000) and that adrenomodullin relaxes it by increasing intracellular cAMP (Yousufzai et al., 1999). Finally, both galanin and somatostantin inhibit the cholinergic response of the rabbit iris sphincter muscle, being galanin more potent than somatostantin in this effect (43% vs 16%) (Yamaji et al., 2003). On the other hand, pituitary adenylate cyclase activating peptide-27 promotes relaxation of rabbit iris dilator muscles submitted to electric field stimulation or pre-contracted by phenylephrine (Yoshitomi et al., 2002).

The effect of some peptides in the iris muscle tone can modify the aqueous humor drainage mechanisms and induce changes in the pupil diameter. With the purpose of studying the effects and physiological relevance of ghrelin in the modulation of iris smooth muscles relaxation we investigated its direct effects on the iris sphincter or dilator muscles of rabbit or rat eyes as well as the presence of its mRNA in the anterior segment of the rat's eye.

2. Methods

All animal procedures were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

2.1. Functional studies

2.1.1. Specimens preparation

The study was performed in isolated iris sphincter ($n = 44$) and iris dilator ($n = 19$) muscles from male New Zealand white rabbits (*Oryctolagus cuniculus*; 2.0–3.0 Kg) and in iris sphincter muscles ($n = 14$) from Wistar rats. Animals were euthanized after an injection of pentobarbital sodium salt (50 mg/kg) into the marginal ear vein (rabbits) or pentobarbital sodium salt (150 mg/kg) intraperitoneal. The eyes were immediately enucleated and placed in modified Krebs-Ringer (KR) solution at 35 °C, with the following composition in mM: NaCl 98; KCl 4.7; $MgSO_4.7H_2O$ 2.4; KH_2PO_4 1.2; glucose 4.5; $CaCl_2.2H_2O$ 1.8 (2.5 for the rat muscles); $NaHCO_3$ 17; $C_3H_3NaO_3$ 15 and CH_3COONa 5. After removal of the cornea, the iris sphincter or dilator muscles were quickly excised and immersed in the KR solution. After dissection, the ends of each piece were tied with silk thread for mounting in a 15 ml plexi glass organ bath containing the above-described solutions. One end of the specimen was connected to an electromagnetic length-tension transducer (University of Antwerp, Belgium), and the other end was secured to a clip at the bottom of the organ bath. In rabbits we tied all the entire strip of the sphincter muscle or a radial strip of the dilator with 2 mm of width; while in rats we tied the entire ring. All the surgical procedures were taken under microscope (Wild M 650, Leica Microsystems, Switzerland). Solutions were bubbled with 95% O_2 and 5% CO_2 and pH was maintained between 7.38–7.42.

Iris muscles were stabilized always at the same preload (1.1 mN for the rabbit sphincter, 0.5 mN for the rabbit dilator and 0.2 mN for the rat sphincter) and bathing solutions replaced every 20 min until muscle length stabilization. They were then switched to isometric conditions and the protocols initiated when muscle tension stabilized (usually after 20 min).

2.1.2. Experimental protocols

2.1.2.1. Rabbit iris sphincter muscles. After stabilization, the rabbit iris sphincter muscles were contracted, by adding Carbachol (10^{-7} M) to the organ bath. When a stable contraction was reached, increasing doses of 1–5 human ghrelin (frGhr; 10^{-9}–6×10^{-5} M) were added to the organ bath to generate concentration-response curves in the absence ($n = 7$) or presence of: i) D-Lys^{3}GHRP6 (10^{-4} M, $n = 6$), a GHS receptor 1a antagonist; ii) N^{ω}-nitro-L-Arginine (LNA: 10^{-5} M, $n = 7$), a NO-synthase inhibitor; iii) indomethacin (10^{-5} M, $n = 7$), a cycloxigenase inhibitor; and iv) apamin and carybdotoxin (10^{-6} M + 10^{-6} M, $n = 6$), inhibitor of the Ca^{2+} activated K^+ channels. In a subset of muscles ($n = 4$) a single dose of

frGhr (6×10^{-5} M) was added and active tension recorded during 20 min. Finally, the effects of increasing concentrations of 1–5 human des-octanoyl-ghrelin (d-frGhr; 10^{-9}–6×10^{-5} M) were tested in another group of muscles ($n = 7$).

In these experiments the muscle from the fellow eye was used as control.

2.1.2.2. Rabbit iris dilator muscles. After stabilization, the muscles were contracted with epinephrine (10^{-4} M). Two contractions, with 30 min duration each, were obtained in the same muscle. In one of the contractions increasing concentrations of 1–5 human ghrelin (frGhr; 10^{-7}–6×10^{-5} M), alone ($n = 6$) or in the presence of DLys^{3}GHRP6 (10^{-4} M, $n = 7$), or 1–5 human des-octanoyl-ghrelin (d-frGhr; 10^{-6}–6×10^{-5} M; $n = 6$) were tested. In the other contraction, which was used as control, a similar volume of the vehicle was added to the bath. The order of test and control contractions was random.

2.1.2.3. Wistar rat iris sphincter muscles. The Wistar rat iris sphincter muscles were initially pre-contracted by Carbachol (10^{-6} M). Two contractions with duration of 30 min each were obtained in the same muscle. In one of the contractions increasing concentrations of 1–5 Human ghrelin (frGhr; 10^{-7}–6×10^{-5} M; $n = 6$) or des-octanoyl-ghrelin (d-frGhr; 10^{-7}–6×10^{-5} M; $n = 8$) were tested, while during the other contraction, which was used as control, a similar volume of the vehicle was added to the bath. The order of test and control contractions was random.

2.1.3. Materials

Human 1–5 ghrelin (frGhr; Gly-Ser-Ser(n-Octanoyl)-Phe-Leu-NH_2) and human 1–5 des-octanoyl-ghrelin (d-frGhr; Gly-Ser-Ser-Phe-Leu-NH_2) were obtained from Peptides International (Lousville, Kentucky). This peptide has the same properties in binding to GHSR-1a as the molecule of Ghrelin (Bednarek et al., 2000). The other chemicals from Sigma Chemical Co (St Louis, Mo). Peptides were prepared in aliquots and stored at −20 °C.

2.2. In situ hybridization (n = 8)

After decapitation, Wistar rats (Charles-River, Spain) eyes were enucleated and fixed immediately. The eye was bisected equatorially, washed briefly in PBS and fixed in freshly prepared phosphate-buffered 4% (w/v) neutral buffered paraformaldeyde during an overnight period. Then the tissues were consecutively dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin. Ten-micrometer sections of the embedded tissue were mounted on SuperFrost® Plus slides (Menzel-Glaser). Some of the sections were stained with hematoxylin and eosin for light microscope examination. All handling of tissues and sections was performed under RNase-free conditions.

Single stranded antisense probe to detect ghrelin was generated from pGEM-T, containing a 247-bp fragment of ghrelin cDNA, kindly offered by Dr. Tena-Sampere, Salamanca University. The riboprobe labeled with digoxigenin was prepared by in vitro transcription using a DIG RNA labelling kit (Roche, Germany), according to manufacturer's instructions. The enzymes used for linearization and transcription were *Nco*I restriction enzyme and SP6 RNA polimerase, respectively.

For *in situ* hybridization, the sections were rehydrated, washed in PBS. Pretreatment included proteinase K digestion (2 μg/ml, pH 7.4, at 37 °C) followed by postfixation in 4% paraformaldeyde for 30 min at room temperature and washing with standard saline citrate (SSC, Promega, USA). Digoxigenin-labeled ghrelin probe was then added to newly prepared hybridization solution (50% deionized formamide, 10% dextran sulphate, 1X Denhardt's reagent, 1 mg/ml of yeast tRNA, 195 mM NaCl, 8.88 mM Tris–HCl, 11 mM Tris, 5 M EDTA, 4.4 mM $Na_2HPO_4.2H_2O$, 1 mM Na_2HPO_4) at a concentration of 1% (v/v). On each slide 100 μl of hybridization solution were applied and hybridization was carried out overnight in a humidified chamber at 70 °C. After hybridization, the slides were washed, and rinsed in maleic acid buffer (10 mM maleic acid, 15 mM NaCl, 1% Tween 20), pH 7.5, at room temperature. The slides were washed at 65 °C with successive changes of 0.5X SSC, 50% formamide, 0.1% Tween 20 (Sigma, U.K.) and rinsed in maleic acid buffer (10 mM maleic acid, 15 mM NaCl, 1% Tween 20), pH 7.5, at room temperature. The block reagent (blocking reagent 20%, goat serum 20% in maleic acid buffer) and anti-DIG antibody (Roche, Germany) at 1:2000 dilution was added to tissue sections and incubated overnight at room temperature. The development reaction was obtained by incubating the slides in NTMT buffer (100 mM NaCl, 100 mM Tris–HCl, 50 mM $MgCl_2$, 1% Tween 20) with BCIP (5-bromo-4-chloride-3-indoil-phosphate; Roche, Germany) and NBT (Nitroblue tetrazolium chloride). This reaction occurred for 15 h at 37 °C.

Negative controls were conducted by the omission of the anti-sense probe and positive controls by hybridizing the anti-sense probe with sections from the gastric fundus. The sections were examined under a light microscope (Axioskop 2 plus, Carl Zeiss, Germany). Photomicrographs were taken with an Axiocam color video camera (Carl Zeiss, Germany).

2.3. Statistical analysis

Data presented as means ± SEM. Active tension was defined as tension developed after muscle contraction minus resting tension at baseline (Fig. 1). Changes in active tension were expressed as percent decrease from the value obtained after stabilization of the contraction elicited by carbachol or epinephrine. Concentration-response curves of Ghrelin in each experimental condition were evaluated with one-way repeated measures ANOVA. Effects of each dose of Ghrelin in different experimental conditions were tested with one-way ANOVA. When significant differences were detected with any of the ANOVA test, the Student-Newman–Keuls

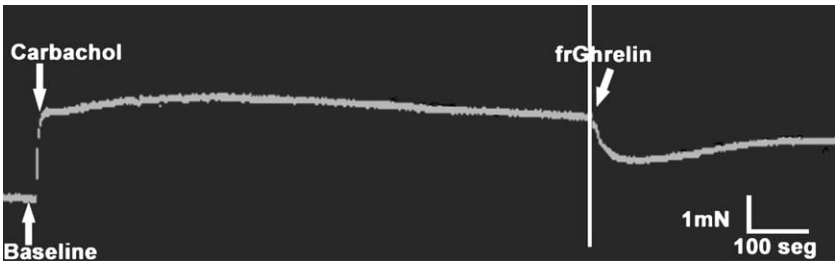

Fig. 1. Representative example of iris sphincter muscle active tension during 30 min. Second arrow marks addition of carbachol (10^{-7} M), which is followed by an increase in active tension. The muscle was allowed to stabilize before addition of frGhr (6×10^{-5} M, third arrow), which abruptly relaxed the iris muscle (maximum effect 1.5–3 min later).

test was selected to perform multiple comparisons. $P < 0.05$ was accepted as significant.

3. Results

3.1. Effects of frGhr on carbachol induced contraction of rabbit sphincter muscle

Active tension after carbachol-induced contraction of rabbit iris sphincter muscles was similar in all experimental protocols (3.16 ± 0.09 mN; $n = 44$).

Effects of a single dose of frGhr (6×10^{-5} M) on relaxation of carbachol-precontracted muscles are illustrated in a representative example in Fig. 1. After addition of frGhr active tension quickly fell in the first 1.5–3 min from 3.45 ± 0.47 to 1.27 ± 0.76 mN and then recovered, over the following 10 min, almost back to the initial tension.

Fig. 2 (upper panel) shows concentration-response curves obtained 2.5 min after addition of frGhr to the bath. When compared to control, frGhr induced a significant decrease of active tension of $10.7 \pm 5.6\%$, $27.2 \pm 8.9\%$ and $34.1 \pm 12.1\%$ at 10^{-5}, 3×10^{-5} and 6×10^{-5} M respectively.

3.2. Effects of d-frGhr and blockage of the GHSR-1a on carbachol-induced contraction of rabbit sphincter muscle

Fig. 2 (middle panel) shows concentration-response curves obtained 2.5 min after addition of des-octanoyl-ghrelin (d-frGhr) to the bath. D-frGhr significantly decreased active tension by $10.6 \pm 3.8\%$, $20.0 \pm 6.1\%$ and $43.3 \pm 5.2\%$ at 10^{-5}, 3×10^{-5} and 6×10^{-5} M, respectively, when compared to control. So, d-frGhr also significantly relaxes, in the same proportion, the iris sphincter muscle as observed with the addition of frGhr.

With regard to the blockage of the GHSR-1a, D-Lys^{3}GHRP6 did not alter per se the active tension before the addition of frGhr. By pretreating the muscles with D-Lys^{3}GHRP6, we observed a more than threefold bigger relaxing effect of frGhr, which decreased active tension by 49.9 ± 12.5, 94.2 ± 19.4 and $118.6 \pm 21.1\%$, respectively at the concentrations of 10^{-5}, 3×10^{-5} and 6×10^{-5} M (Fig. 2 lower panel). So the relaxing effect of frGhr is not mediated

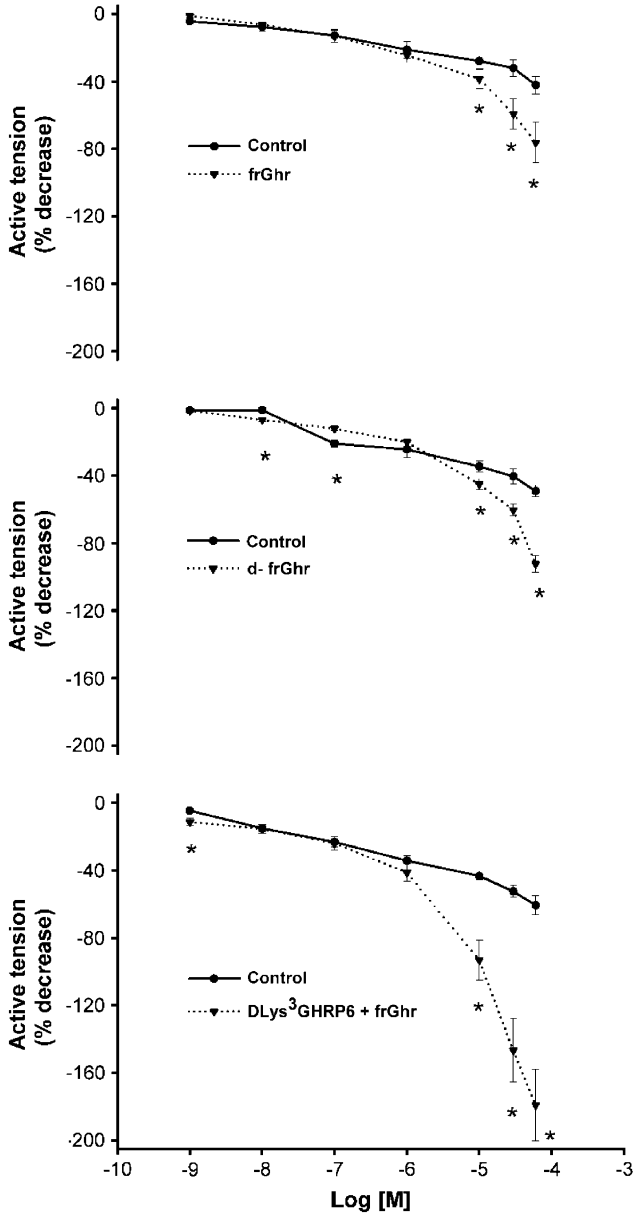

Fig. 2. Concentration-response curves of frGhr (upper panel, dotted line) and d-frGhr (middle panel, dotted line) in carbachol-precontracted iris sphincter muscles. The lower panel shows a concentration- response curve of frGhr in presence of D-Lys^{3}GHRP6 (10^{-4} M), an inhibitor of the GHRS-1a. In all panels, active tension decline with time of the iris sphincter muscles of the fellow eyes (full lines), which were used as control, are also presented. $P < 0.05$: * vs. control.

by GHSR-1a. Also it seams that its blockage can potentiate ghrelin's relaxing effect.

3.3. Influence of NOS, COX or the blockage of K^+ channels on frGhr effects on iris sphincter muscle

Fig. 3 shows concentration-response curves obtained 2.5 min after the addition of frGhr to the bath, either alone or in presence of L-NA (10^{-5} M), indomethacin (10^{-5} M) or Apamin (10^{-6} M) + Carybdotoxin (10^{-6} M). These inhibitors were added to the bath 10 min after the addition of carbachol and 20 min before the addition of frGhr. None of them altered per se the active tension prior to frGhr addition.

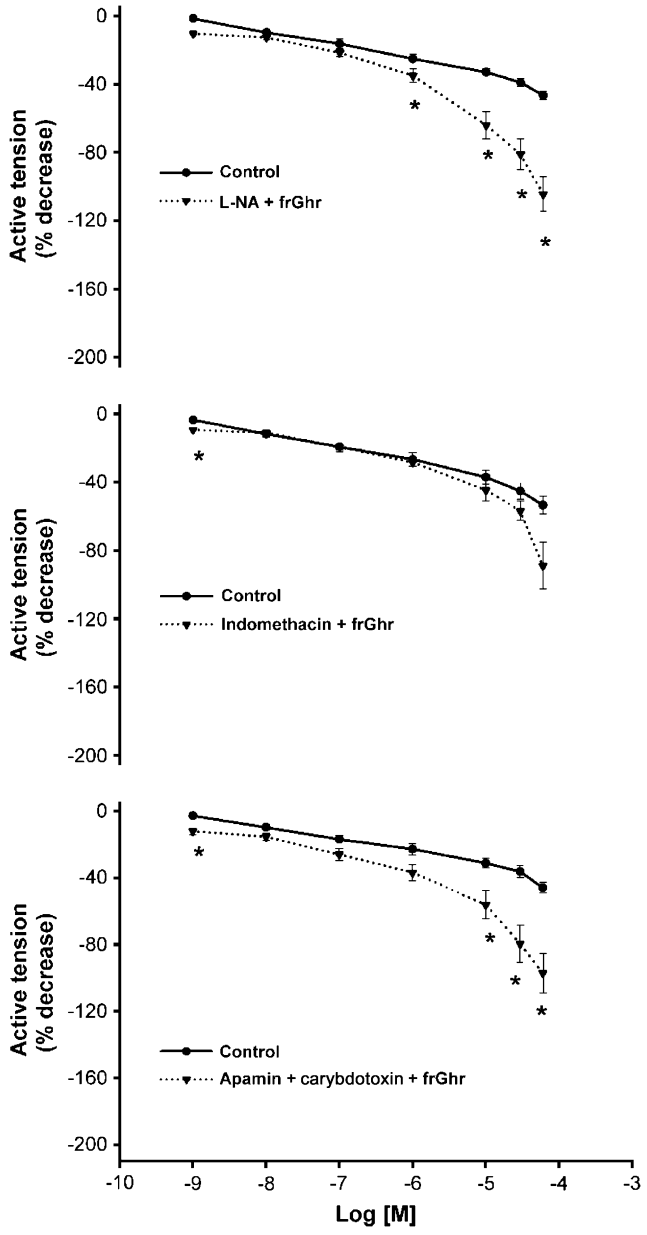

Fig. 3. Concentration-response curves of frGhr in carbachol pre-contracted iris sphincter muscles, in the presence of: i) L-nitro-L-arginine (L-NA; 10^{-5} M; upper panel); ii) indomethacin (10^{-5} M; middle panel); and iii) apamin (10^{-6} M) and carybdotoxin (10^{-6} M; lower panel). In all panels, active tension decline with time of the iris sphincter muscles of the fellow eyes (full lines), which were used as control, are also presented. $P < 0.05$: * vs. control.

The relaxing effect of frGhr was blunted by indomethacin. In these circumstances, active tension only decreased 1.4 ± 6.8, 9.0 ± 6.7 and $20.3 \pm 12.1\%$ in response to 10^{-5}, 3×10^{-5} and 6×10^{-5} M of frGhr, which failed to reach statistical significance when compared to control (Fig. 3, middle panel). On the contrary, in presence of L-NA the relaxing effect of frGhr was preserved (active tension decreased 29.4 ± 6.2, 37.5 ± 9.4 and $52.4 \pm 11.4\%$ at 10^{-5}, 3×10^{-5} and 6×10^{-5} M; Fig. 3 – upper panel). Although these values are as a mean bigger than those obtained with frGhr alone, such difference failed to reach statistical significance.

Apamin and carybdotoxin (Ca^{2+} activated K^+ channels inhibitors) also did not significantly alter the relaxing effect of frGhr. In these conditions, active tension decreased 12.9 ± 10.4, 27.1 ± 13.7 and $36.4 \pm 14.4\%$ at 10^{-5}, 3×10^{-5} and 6×10^{-5} M of frGhr, respectively.

3.4. Effects of frGhr or d-frGhr on epinephrine induced contraction of rabbit dilator muscle

Active tension after epinephrine-induced contraction of rabbit iris dilator muscles was similar in all experimental protocols (1.20 ± 0.05 mN; $n = 19$).

Fig. 4 shows concentration-response curves obtained 2.5 min after addition of frGhr (upper panel), d-frGhr (middle panel) and frGhr plus the GHSR-1a inhibitor D-Lys^{3}GHRP6 (lower panel), to the iris dilator muscle precontracted by epinephrine. The addition of D-Lys^{3}GHRP6 to the muscle preparation did not alter per se active tension.

When compared to control, only the maximal concentration of frGhr (6×10^{-5} M) significantly decreased active tension by $25.8 \pm 4.8\%$, while in presence D-Lys^{3}GHRP6 fGhr did not significantly alter active tension. Also d-frGhr did not significantly decrease active tension of the precontracted dilator muscle, at any of the studied concentrations.

3.5. Effects of frGhr or d-frGhr on carbachol-induced contraction of rat sphincter muscle

Active tension after carbachol-induced contraction of rat iris sphincter muscles was similar in all experimental protocols (1.76 ± 0.22 mN; $n = 14$).

In these protocols frGhr (Fig. 5; upper panel) or d-frGhr (Fig. 5; lower panel) were added to the bathing solution of rat iris constrictor muscle precontracted by carbachol. When compared to control, frGhr decreased active tension by $15.0 \pm 7.2\%$, $36.1 \pm 10.4\%$ and $52.1 \pm 10.3\%$ at 10^{-5}, 3×10^{-5} and 6×10^{-5} M, respectively, while d-frGhr decreased it by $29.4 \pm 13.1\%$ and $77.1 \pm 15.3\%$ at 3×10^{-5} and 6×10^{-5} M, respectively.

3.6. ''In situ'' hybridization for ghrelin mRNA in the anterior segment

Production of ghrelin could be identified in the anterior segment of rat eyes by ''in situ'' hybridization for its mRNA.

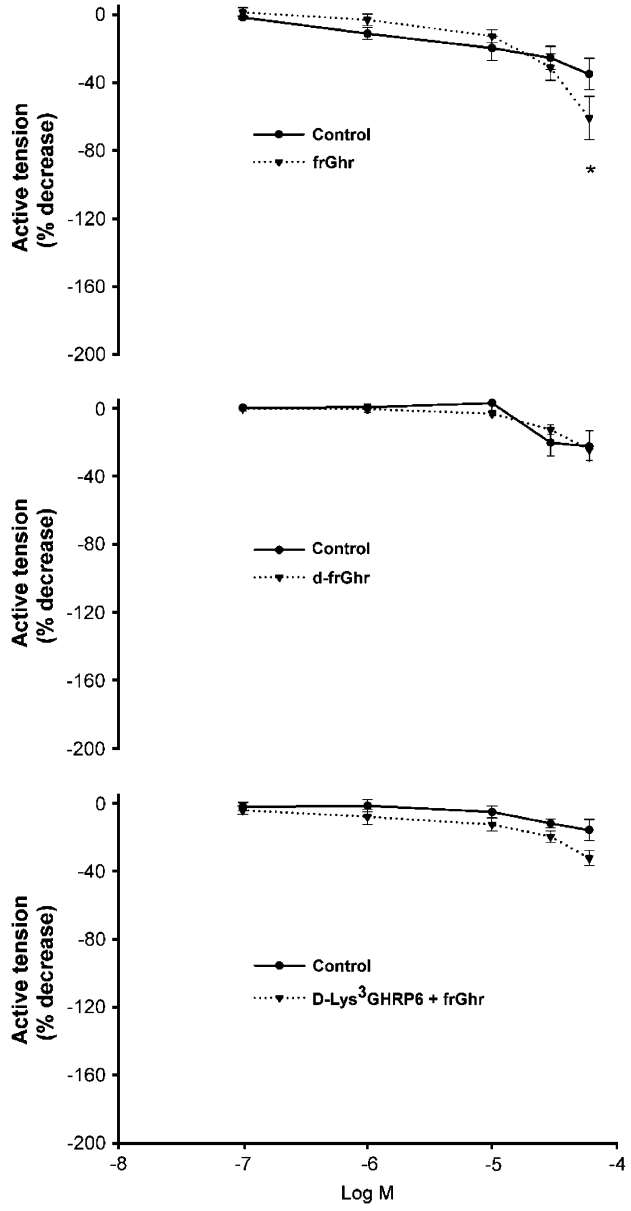

Fig. 4. Concentration-response curves of frGhr (dotted line, upper panel) or d-frGhr (dotted line, middle panel) in epinephrine (10^{-4} M) pre-contracted rabbit iris dilator muscles. The lower panel shows, in similar muscles, a concentration-response curve of frGhr in presence of D-Lys^{3}GHRP6 (10^{-4} M; dotted line). Control curves are presented as full lines. $^*P < 0.05$: vs. control.

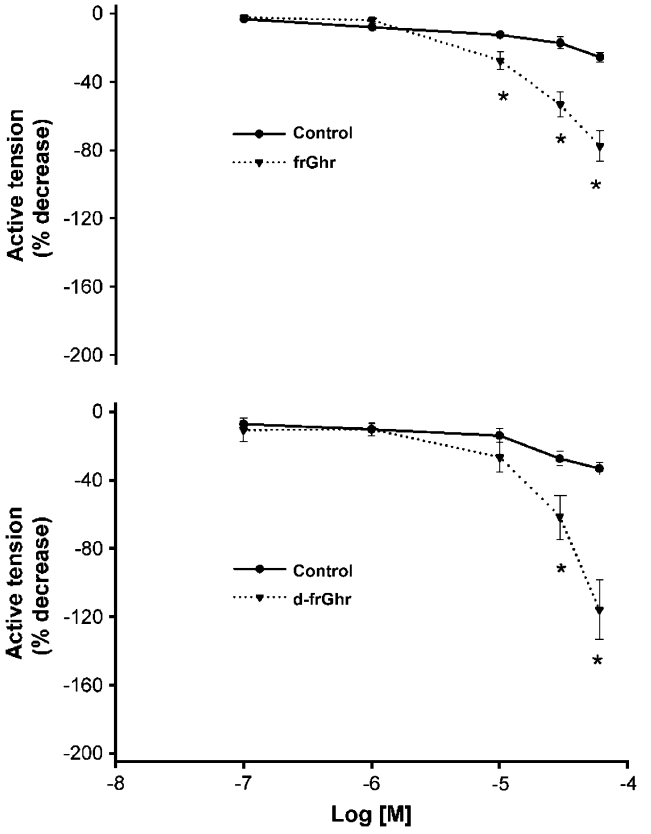

Fig. 5. Concentration-response curves of frGhr (dotted line, upper panel) or d-frGhr (dotted line, lower panel) in carbachol (10^{-6} M) precontracted rat iris sphincter muscles. Control curves are presented as full lines. $P < 0.05$: * vs. control.

Ghrelin transcripts were present in the iris posterior epithelium (Fig. 6A), as shown by the examination at a higher magnification (Fig. 6C), and in some clusters of the iris stroma. Also in the ciliary epithelium we observed transcripts of Ghrelin's mRNA, mainly in the outer face of the ciliary epithelium, which could be morphologically the non-pigmented ciliary epithelium (Figs. 6B,D).

4. Discussion

The present study described a novel relaxing effect of ghrelin in the iris sphincter and dilator muscles, which is mediated by prostaglandins. In addition, we provided evidence for the involvement of different receptor subtypes in the mediation of this relaxing effect, which seems to be distinct in the sphincter and dilator, as well as, for the local production of ghrelin in the anterior segment of the eye.

Addition of frGhr to precontracted iris sphincter muscles abruptly decreased active muscle tension, which reached its minimal value 1.5 to 3 min later. This is a profound relaxing effect with a magnitude similar to the relaxing effect of ghrelin on ET-1 precontracted smooth muscle from Human internal mammary artery, considered one of the most potent relaxing agents in the vasculature (Wiley and Davenport, 2002). However, in iris sphincter muscle, relaxation elicited by ghrelin was abrupt with a maximal effect in the first 3 min that almost disappeared 20 min later, whereas in the arterial smooth muscle the relaxing effect of ghrelin was slow and progressive, reaching a plateau 10 to 20 min after its addition (Wiley and Davenport, 2002). This might reflect either a different behavior of vascular and iris smooth muscles, or a distinct response due to the use of carbachol instead of endothelin-1.

The potent relaxing effect of ghrelin reported in the present study was observed both in terms of inhibition of the cholinergic response in the iris sphincter and of the adrenergic response in the dilator muscles. Ghrelin therefore adds to a list of substances whose relaxing effects of the iris sphincter and dilator muscles have been shown over the last decade

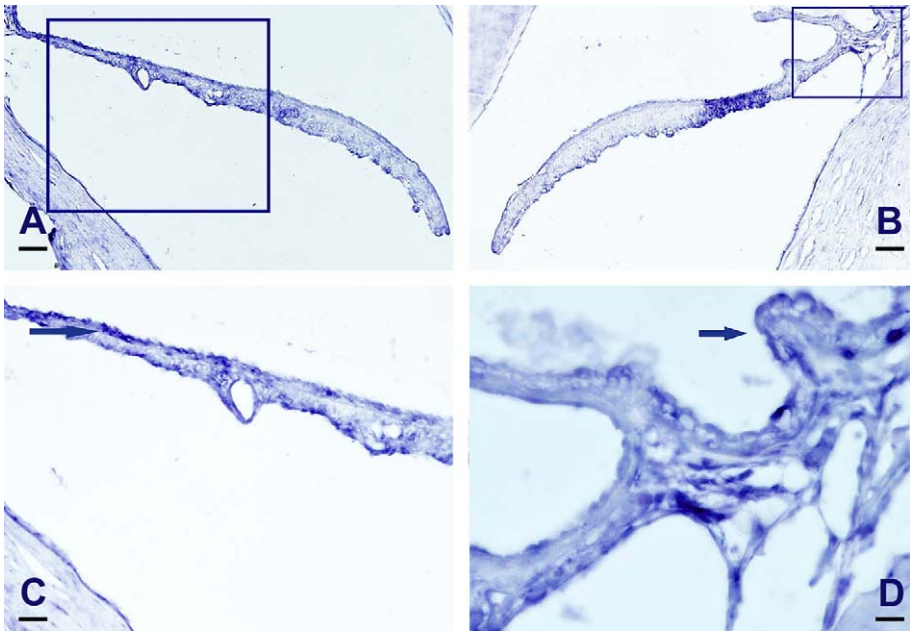

Fig. 6. Ghrelin expression pattern in the iris and ciliary body. In situ hybridization revealed a positive signal widely expressed through the iris and ciliary body (A and B). High levels of ghrelin transcripts were detected in the posterior layer of the iris and some in the iris stroma (panel C arrow), and in the ciliary epithelium mainly in the non-pigmented cells (panel D; arrow). Scale bar corresponds to 100 μm in panel A and B; 50 μm in panel C and 25 μm in panel D.

(Geyer et al., 1998; Yousufzai et al., 1999; Pianka et al., 2000; Barilan et al., 2003; Hourani et al., 1997; Yamaji et al., 2003).

In the vascular smooth muscle, the hypotensive effect of ghrelin was recently shown to be mediated by the activation of the Ca^{2+} activated K^{+} channels and to be potentiated by the inhibition of NO release (Shinde et al., 2005).

In addition to the smooth muscle, previous studies showed that ghrelin also decrease contractility of cardiac (Bedendi et al., 2003; Soares et al., 2006) and skeletal muscles (Pierno et al., 2003). In the myocardium, PGI_2 release from endocardial endothelial cells was proposed to mediate the inotropic and lusitropic effects of ghrelin (Bedendi et al., 2003; Soares et al., 2006).

In the present study, the relaxing effect of ghrelin in the iris sphincter muscle also seems to be dependent of prostaglandins, as inhibition of its production blocked it. Prostaglandin receptors are widely distributed in various regions of ocular tissues including iris sphincter muscle, being the EP2 the most abundant prostaglandin receptor in the ciliary body and iris (Biswas et al., 2004; Sharif et al., 2004). Interestingly, EP2 receptors stimulate intracellular cAMP accumulation and muscle relaxation (Abdel-Latif, 2001), suggesting that this might be one of the mechanisms underlying ghrelin's effects.

Blockage of Ca^{2+} activated K^{+} channels or NO synthesis, however, did not significantly modify ghrelin's effect in our study.

Almost all actions of GHS are believed to be mediated through a specific seven transmembrane G-protein-coupled receptor, called GHS receptor type 1a (GHSR-1a), which was identified in the pituitary and the hypothalamus (Howard et al., 1996; Muccioli et al., 1998; Pong et al., 1996; McKee et al., 1997). This receptor is also expressed in thyroid gland, pancreas, spleen, adrenal gland, heart, rat testis, ovary and prostate cancer cells (Papotti et al., 2000; Iglesias et al., 2004; Gnanapavan et al., 2002; Tena-Sempere et al., 2002; Jeffery et al., 2002; Gaytan et al., 2003). In the heart, the negative inotropic effect of ghrelin has been shown, however, to be independent of GHSR-1a (Bedendi et al., 2003; Sharif et al., 2004). Such conclusion was based on the fact that des-octanoyl-ghrelin, a molecule that does not bind to GHSR-1a (Hosoda et al., 2000; Bednarek et al., 2000; Kojima et al., 2001; Torsello et al., 2002; Broglio et al., 2003), has a negative inotropic effect similar to that of ghrelin (Bedendi et al., 2003). Also in the fetal development des-octanoyl-ghrelin has some capacity to bind to a different GHS receptor, which has an important role in the embryologic process (Santos et al., 2006). In these conditions the authors presumed that fetal tissue could express a GHSR different subtype that binds to des-octanoyl-ghrelin (Nakahara et al., 2006). The results of the present study also suggest that the relaxing effects of ghrelin on the iris sphincter muscle are not mediated by GHSR-1a, as they were also observed in response to des-octanoyl-ghrelin. Furthermore, in presence of $D\text{-}Lys^3$ GHRP6, an inhibitor of that receptor (Veeraragavan et al., 1992), the relaxing effect of ghrelin was not inhibited, but rather enhanced more than threefold. It should be, however, stressed that even if GHSR-1a does not seem to mediate the relaxing effect of ghrelin, it presumably modulates it, as inhibition of this receptor enhanced the degree of active tension decline. On the contrary, in the iris dilator muscle GHSR-1a seems to mediate the relaxing effect of ghrelin, as no relaxation was observed in response to des-octanoyl-ghrelin and the effect of ghrelin was inhibited after the blockage of the GHSR-1a. So, in the iris muscles the effect of ghrelin is mediated by more than one receptor,

the GHSR-1a for the dilator and a different one for the sphincter muscle.

The relaxing effects of ghrelin, at least in the sphincter iris muscle, do not seem to be species dependent as similar effects were observed in the present study in rabbits and rats.

Recent works supports the view that the ciliary body is a ''neuroendocrine gland'', which contributes to the aqueous secretion, regulation of intra-ocular pressure, metabolism of steroid hormones and production of many biological peptides (Escribano and Coca-Prados, 2002). Some of these peptides, synthesized and released by the ciliary epithelium, are neuropeptides and hormones usually restricted to endocrine tissues and cells (Ortego et al., 1996a,b; Ortego and Coca-Prados, 1997, 1999). Production and receptors for neurotensin (Ortego and Coca-Prados, 1997), galanin (Ortego and Coca-Prados, 1998), natriuretics peptides (Ortega and Coca-Prados, 1999; Fernandez-Durango et al., 1995), angiotensin II (Cullinane et al., 2002) and endothelins (Fernandez-Durango et al., 2003) have been identified in the pigmented and non-pigmented ciliary epithelium and in cells of the trabecular meshwork (Escribano and Coca-Prados, 2002). Having shown that ghrelin's mRNA is present in the posterior ephitelium of the iris and in the non-pigmented epithelium of the ciliary body the present study indicates that ghrelin possibly is also a product of that ''neuroendocrine gland''. Ghrelin production in the posterior epithelium of the iris and its action in the adjacent muscular tissue, suggests that it presumably plays an important role in the paracrine regulation of muscular kinetics of the iris. Local concentration of ghrelin is therefore presumably much higher than its circulating levels, indicating that the relatively higher concentrations at which the maximal relaxing effects of ghrelin were observed might be physiologically relevant. This effect represents a potential novel mechanism through which, besides adrenergic, cholinergic and trigeminal systems, ocular tissues acutely modulate iris tension, pupillary diameter and therefore trabecular aqueous drainage.

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