Modulation of Iris Sphincter and Ciliary Muscles by Urocortin 2

Urocortin 2: effects on Iris Sphincter and Ciliary Muscles

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SUMMARY

Urocortin 2 (UCN2) is a peptide related to corticotropin-releasing factor, capable of activating CRF-R2. Among its multisystemic effects, it has actions in all 3 muscle subtypes.

This study's aim was to determine its potential role in two of the intrinsic eye muscle kinetics.

Strips of iris sphincter (rabbit) and ciliary (bovine) muscles were dissected and mounted in isometric force-transducer systems filled with aerated-solutions. Contraction was elicited using carbachol (10^{-6}M for iris sphincter, 10^{-5}M for ciliary muscle), prior to all testing substances.

UCN2 induced relaxation in iris sphincter muscle, being the effect maximal at 10^{-7}M concentrations (-12.2% variation, *versus* control). This effect was abolished with incubation of indomethacin, antisauvagine-30, chelerytrine and SQ22536, but preserved with L-nitro-L-arginine. In carbachol pre-stimulated ciliary muscle, UCN2 (10^{-5}M) enhanced contraction (maximal effect of 18.2% increase, *versus* control).

UCN2 is a new modulator of iris sphincter relaxation, dependent of CRF-R2 activation, synthesis of prostaglandins (COX pathway) and both adenylate cyclase and PKC signaling pathways, but independent of nitric oxide production. Regarding ciliary muscle, UCN2 enhances carbachol-induced contraction, in higher doses.

Keywords: iris sphincter, ciliary muscle, urocortin 2, CRF-R2.

INTRODUCTION

Urocortins are three paralogs of corticotropin-releasing factor (CRF), named as such because the first urocortin identified, urocortin 1 (UCN1), in 1995, had similarities to urotensin and CRF, regarding structure and bioactivity (Vaughan *et al.* 1995, Donaldson *et al.* 1996, Fekete and Zorrilla 2007). There are three isoforms of urocortin: UCN1, urocortin 2 (UCN2, or stresscopin-related peptide) and urocortin 3 (UCN3, or stresscopin) (Hauger *et al.* 2003). UCN1 is widely distributed in the central nervous system (CNS). At the periphery it has been detected in the gastrointestinal tract, placenta, adipose tissue, testis, cardiomyocytes, thymus, spleen and kidney (Florio *et al.* 2004, Boorse and Denver 2006). However, both UCN2 and UCN3 are scarcely expressed in CNS. UCN2 is also expressed in the heart, skeletal muscle, myometrium, adrenal gland, peripheral blood cells and skin. UCN3 expression is considerably lower than that of UCN2 (Boorse and Denver 2006).

CRF related peptides act mainly through two receptors: CRF-R1 and CRF-R2. Both receptors have seven transmembrane domains and belong to the G protein-coupled receptors family. In most cells, these receptors are coupled to a Gs protein, increasing intracellular cyclic adenosine monophosphate (cAMP) levels through the stimulation of adenylate cyclase (Boorse and Denver 2006). The two receptors show significant differences between their pharmacological profile and tissue distribution. Thus, CRF has higher affinity for CRF-R1 receptor, whereas UCN1 binds equally to CRF-R1 and CRF-R2. Both UCN2 and UCN3 are selective agonists of CRF-R2 receptor (Hauger *et al.* 2003). Although UCN3 is more selective for CRF-R2, UCN2 exhibits higher potency (Fekete and Zorrilla 2007). The CRF-R1 receptor is primarily expressed in the CNS (Hauger *et al.* 2003, Boorse and Denver 2006), while CRF-R2 is widely expressed in the peripheral tissues (Florio *et al.* 2004, Boorse and Denver 2006).

UCN2 is a 38-aa peptide (Reyes *et al.* 2001) and its human gene is located at p21.3-4 of chromosome 3 (Hsu and Hsueh 2001). UCN2 seems to play an important role in all three, skeletal, cardiac and smooth muscles. In the former, activation of the CRF-R2 by UCN2 increases contractile force and muscle mass, preventing the loss of skeletal muscle mass (Hinkle *et al.* 2003, Hinkle *et al.* 2004); it also allows skeletal muscle to contract and relax faster (Reutenauer-Patte *et al.* 2012).

Regarding smooth muscle modulation, in human pregnant and nonpregnant myometrial cells, not only UCN2 and CRF-R2 receptor were found, but also UCN2-treatment induced phosphorylation of myosin light chain, which is associated with smooth muscle contraction (Karteris *et al.* 2004). In the lung, CRF-R2 activation promotes bronchorelaxation (Moffatt *et al.* 2006). In the gastrointestinal tract, CRF-R2 blockade prevents CRF and UCN1 inhibition of both gastric emptying and ileal muscle contractions (Porcher *et al.* 2005).

Concerning the cardiovascular system, UCN2 has positive inotropic and lusitropic effects. It also increases coronary blood flow and myocardial function and exerts potent and consistent relaxation of pulmonary and systemic vessels, reducing the arterial blood pressure (Adao *et al.* 2015).

In the ocular globe, either UCN1, UCN2 and UCN3, CRF-R1 and CRF-R2 are expressed in the retinal pigmented epithelium (Hauger *et al.* 2003, Zmijewski *et al.* 2007). At the anterior segment of the eye CRF-positive inflammatory cells were detected infiltrating the iris and ciliary body epithelia under experimental autoimmune uveoretinitis in rats (Mastorakos *et al.* 1995). Additionally, CRF is capable of inducing relaxation of the iris sphincter muscle in toads, which can be blocked using its antagonist (Carr and Zozzaro 2004).

Regarding its effects in the eye, UCN2 seems to be able to protect retinal degeneration following bilateral common carotid artery occlusion, reducing the damage on retinal layers and increasing the thickness of all layers in 40% and by 55% the number of ganglion cells, in the dose of 40μM (Szabadfi *et al.* 2009). Also, UCN2 treatment protects the retina from monosodium-glutamate-induced retinal degeneration (Szabadfi *et al.* 2014). Finally, both UCN1 and UCN2 have a vasodilator effect in the retinal circulation (Kaneko *et al.* 2007). In this study, UCN2 was used because of its selective effect on CRF-R2.

As the effect of UCN2 in the neurohumoral regulation of the anterior segment of the eye has not been described yet, our purpose is to investigate its role in both rabbit iris sphincter and bovine ciliary muscle kinetics, and the probable subcellular pathways involved.

METHODS

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

FUNCTIONAL STUDIES

Specimens preparation - iris

The study was performed in isolated iris sphincter muscles (n=44) from male New Zealand white rabbits (*Oryctolagus cuniculus*; 2.0-3.0 Kg). Animals were euthanized with a lethal injection of sodium pentobarbital salt (50 mg/Kg) into the marginal ear vein. The eyes were immediately enucleated and placed in a modified Krebs-Ringer (KR) solution at 36ºC, 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; $CaCl_2.2H_20$ 2.5; $NaHCO_3$ 17; $C_3H_3NaO_3$ 15 and

CH_3COONa 5. Once the cornea was removed, the iris sphincter muscles were quickly excised and immersed in the KR solution. After dissecting 5x1 mm strips of iris sphincter muscle, its ends were tied with silk thread for mounting in a 5 ml horizontal organ bath containing the above-described solution, connecting to an electromagnetic length-tension transducer (University of Antwerp, Belgium), which continuously recorded the force variation. All the surgical procedures were performed under microscope (Zeiss, Stemi 2000C, Germany). Solutions were bubbled with a gas mixture containing 95% O_2 and 5% CO_2 and pH was maintained between 7.38-7.42.

Iris sphincter muscles were allowed to extend at a constant preload (0,5 mN) and bathing solutions were continuously replaced until muscle length stabilization. They were then switched to isometric conditions and the protocols initiated when muscle tension remained stable during a minimum of 10 minutes.

Effects of urocortin 2 in pre-contracted iris sphincter muscle

Once stabilized, rabbit iris sphincter muscles were contracted by adding carbachol (10^{-6}M) to the organ bath and stabilization would occur in about 10 minutes. Increasing doses of UCN2 (10^{-10}-10^{-6}M; *n*=6) were added to the organ bath in order to build up a concentration-response curve. The same procedure was recorded with the same UCN2 doses in the presence of: (i) a NO synthase inhibitor, L-nitro-L-arginine (10^{-5}M, *n*=9); (ii) a cyclooxygenase (COX) inhibitor, indomethacin (10^{-5}M, *n*=8); (iii) a CRF-R2 selective antagonist, antisauvagine-30 (10^{-6}M, n=7); (iv) a protein kinase C (PKC) inhibitor, chelerythrine (10^{-5}M, *n*=6) and (v) an adenylate cyclase inhibitor, SQ22536 (10^{-6}M, n=8).

In each muscle, two carbachol-induced contractions were studied. One of them was randomly selected to test the effects of UCN2, while the other was used as control (a similar volume of vehicle solution was added to the bath).

Specimens preparation – ciliary muscle

Bovine globes were collected from the local slaughterhouse and transported to the laboratory within one hour in a container filled with ice. Briefly, one globe was circumferentially cut 5 mm posterior to the iridocorneal angle. The lens was carefully taken and the anterior compartment was cut in half. Under the microscope, the ciliary body was dissected under cold PSS solution (composition in mM: NaCl 118; KCl 4.7; KH_2PO_4 1.2; glucose 11; $CaCl_2.2H_20$ 2.5; $MgCl_2.6H_2O$ 1.2; $NaHCO_3$ 25), continuously aerated with the gas mixture mentioned above. Finally, four contiguous 2 mm strips of ciliary muscle were isolated and two tight 6/0 silk thread knots were tied 2.5 mm apart – one at the most centrifugal end and the other in the scleral spur residues.

The four muscle strips were horizontally mounted in a four-chamber isometric-force transducer system (DMT, Tissue Bath System 720MO, Denmark). Each chamber was filled with 5 ml of continuously-aerated PSS solution (T=36ºC, pH=7,40-7,45). The strips were slowly stretched, stabilizing at 1 mN passive force, while several PSS washes were done. After 20 minutes, the baths were filled with a potassium-rich PSS solution (KPSS) to elicit a 3-minute contraction in order to test viability and enhance contractility, followed by PSS wash and another KPSS-PSS cycle. The muscles were then stabilized for 1 hour, with PSS substitution every 15 minutes.

Effects of urocortin 2 in ciliary muscle

Carbachol (10^{-5}M) was added and only the muscle-strips which duplicated their force were studied. Each chamber was randomly assigned to test UCN2 or water (control),

n=10 pairs. After contractility stabilization, increasing doses of UCN2 (10^{-10}M-10^{-5}M) or same volume water were subsequently added every 5 minutes. One hour was waited after the last dose. Following, sodium nitroprusside (10^{-4}M) was added to the chambers which tested UCN2, in order to verify viability and relaxation capability.

To test if UCN2 had a precontractility effect, we subjected the muscle strips to a second carbachol-induced contraction: all the chambers were washed with PSS solution several times and the muscles were again subjected to 1 mN passive force. Randomly, two chambers (one of each previous group) were assigned to test 10^{-6}M UCN2 (*versus* water), 20 minutes prior to the second contraction (n=7 pairs). The active force of both peak and 10 minutes after were compared to the prior contraction. Also, the effects of UCN2 (10^{-6}M) on resting muscle were analyzed (n=7 pairs).

MATERIALS

Most chemicals were obtained from Sigma Chemical Co (St Louis, Mo). UCN2 was purchased from Bachem. The peptides were prepared in aliquots and stored at -20°C for 3 months.

STATISTICAL ANALYSIS

Data are presented as mean±SEM. Concentration-response curves of UCN2 in carbachol pre-contracted muscles in each experimental condition were evaluated with two-way repeated measures ANOVA. $p<0.05$ was accepted as significant. The software used was GraphPad Prism 6®.

RESULTS

Relaxing effects of UCN2 in iris sphincter muscle

Active force developed after carbachol-induced contractions of rabbit's iris sphincter muscle-samples was similar between active and control contractions.

Figure 1 shows concentration-response curves obtained after adding UCN2 to pre-contracted rabbit iris sphincter muscles. When compared to control, UCN2 induced a significant decrease of active force at 10^{-7}M and 10^{-6}M concentrations, being the effect maximal at 10^{-7}M (-12.2±2.6% *versus* control).

Subcellular pathways studies

In order to test the probable subcellular pathways involved in UCN2-mediated relaxation of iris sphincter muscle, several inhibitors of specific mediators were added to the bath 10 min after carbachol-contraction and 5 min before UCN2. Neither of these altered *per se* the active force prior to UCN2 addition.

Influence of NO synthase and COX pathways were tested using L-nitro-L-arginine and indomethacin, respectively. With the former, the UCN2-relaxing effect was preserved, reaching statistical significance at 10^{-9}M-10^{-6}M (figure 2, box a)). However, UCN2-effect was blunted by indomethacin – there were no statistically significant differences when compared to vehicle solution (figure 2, box b)).

Influence of the CRF-R2 receptor was ascertained with antisauvagine-30. This treatment canceled UCN2-relaxing effect (figure 2, box c)).

Influence of protein kinase C and adenylate cyclase signaling pathways were determined using their specific inhibitors: chelerythrine and SQ22536, respectively. Both

incubations completely inhibited UCN2-relaxation (figure 2, boxes d) and e), respectively).

Effects of UCN2 in ciliary muscle

Active force developed after carbachol-induced contractions of bovine ciliary muscles was similar between active and control groups.

Figure 3 shows concentration-response curves obtained after addition of UCN2 (10^{-10}M-10^{-5}M) to pre-contracted bovine ciliary muscle. Figure 4 explores the time-dependent effects of UCN2 upon the maximum concentration used (10^{-5}M) during 1 hour. Only the last dose led to a statistically significant lower fall of the active force, *versus* control. Thus, UCN2 (10^{-5}M) raised the active force by 18.6% (after 5 minutes), 16.1% (after 10 minutes), 18.0% (after 30 minutes) and 15.5% (after 1 hour).

Additionally, UCN2 had no effects on ciliary muscle's resting force, compared to control.

Regarding the pre-contraction studies, there were no statistically significant differences on the second carbachol-elicited contraction between the group treated with UCN2 10^{-6}M and its control, when compared to the first carbachol-elicited contraction (Figure 5).

DISCUSSION

This study describes UCN2 as a new neurohumoral factor that modulates relaxation of iris sphincter muscle. This effect is dependent on CRF-R2 activation, as well as the synthesis of prostaglandins (COX pathway), adenylate cyclase and PKC signaling pathways. It is independent of nitric oxide production. Therefore, this relaxation is mainly a postsynaptic effect.

The addition of UCN2 to carbachol pre-contracted iris sphincter muscles promoted a significant decrease in force for the highest concentrations of UCN2 (10^{-7}-10^{-6}M), being maximal using UCN2 at 10^{-7}M, which is clearly physiological. Because the iris sphincter muscle is a circular muscle and small variations of its radius determine relevant changes in strength, this force variation can lead to a significant change in pupil diameter and tonicity.

Searching new agents capable of modulating iris muscles' tone (in this case, iris sphincter) is very important. The find of new options for intraoperative mydriasis, without significant systemic side effects, (such as those characteristic of phenylephrine or acetylcholine antagonists) is very important. One of the most common ocular surgeries, the cataract surgery, requires maintenance of mydriasis, which is critical to the safety and surgical ease of the procedure (Grob *et al.* 2014).

Many substances have shown to be effective in inducing relaxation of this muscle. Following the same protocol, the selective stimulation of ET_{B2} receptors produced similar results, either in magnitude or in dosing (Rocha-Sousa *et al.* 2009). Additionally, ghrelin promoted a more exuberant relaxation of rabbit's iris sphincter muscle (34.1±12.1% at 10^{-5}M) (Rocha-Sousa *et al.* 2006). Other molecules involved in iris sphincter relaxation are beta-adrenergic receptors agonists, with an important role played by β_2- and β_3-receptors (Topalkara *et al.* 2006). These effects are potentiated by pre-treatment with muscarinic blockers, such as atropine and ipratropium bromide (Barilan *et al.* 2003). Moreover, serotonin – possibly through 5-HT_{1A} receptors (Barnett and Osborne 1993) -, phosphodiesterase inhibitors (PDEI) (type 1 to 5, which emphasizes the role of cAMP and cGMP pathways) (Yogo *et al.* 2009), adrenomodulin (Uchikawa *et al.* 2005), sodium nitroprusside and c-type natriuretic peptide (Ding and Abdel-Latif 1997) and okadaic acid (Wang *et al.* 1994) treatment can too lead to iris sphincter relaxation.

Regarding subcellular pathways, cAMP and PKA pathways were too involved in relaxation of rat's coronary arteries and ginea pig smooth muscle of the gastric antrum, positive inotropic and lusitropic effects in ventricular myocytes, and possibly bronchorelaxation in mice, through activation of CRF-R2 (Petkova-Kirova *et al.* 2000, Moffatt *et al.* 2006, Smani *et al.* 2007, Adao *et al.* 2015). On the other hand, PKC pathway was responsible for UCN2 effects observed in human pregnant myometrium and rat ventricular myocytes (Karteris *et al.* 2004, Smani *et al.* 2010).

The effects of UCN2 in rabbit's iris sphincter muscle were not replicated in bovine ciliary muscle. A similar scenario happens with ghrelin: although it is capable of inducing relaxation of pre-contracted samples of rabbit's iris sphincter and dilator muscles, and rat's iris sphincter muscle (Rocha-Sousa *et al.* 2006), it is not able to do so in bovine ciliary muscle (unpublished data by our group). The dose-response curve was similar to control from 10^{-10}M to 10^{-6}M. Interestingly, administration of UCN2 (10^{-5}M) was capable of rising carbachol-contracted ciliary muscle force relative to control, being the effect maximal 5 minutes later (18.6% higher), but statistically significant throughout the whole period studied (1 hour). Two possible explanations arise for this pattern of response: 1) only a higher dose was sufficient to produce a measurable effect, through CRF-R2, which may be repressed, downregulated or competitively antagonized by an endogenous substance, or 2) perhaps UCN2 in higher doses loses specificity for CRF-R2 and interferes with other local receptors or systems. However, it should be noted that, in other study, the effects of retinal degeneration protection following bilateral common carotid artery occlusion were achieved using a $4x10^{-5}$M concentration of UCN2 (Szabadfi *et al.* 2009), which was higher than the maximum dose used in this study.

Finally, UCN2 (10^{-6}M) incubated prior to a second carbachol-induced contraction was incapable of changing the magnitude of the second contraction and it did not alter the force generated by resting ciliary muscle.

In fact, not all studies report a relaxation effect provided by UCN2. A good example is its cardiovascular effects, where it exerts positive inotropic effects in isolated ventricular myocytes (maximal at 10^{-7}M) (Yang *et al.* 2006). Moreover, in human myometrial cells, UCN2 is able to induce phosphorylation of myosin light chain (which is involved in the initiation of smooth muscle contraction), being the effect maximal with UCN2 at 10^{-7}M after 5 min of treatment and returning to basal levels after 45 min (Karteris *et al.* 2004). Finally, in the skeletal muscle, activation of CRF-R2 by UCN2 increases contractile force and muscle mass (Hinkle *et al.* 2003, Hinkle *et al.* 2004).

Studying possible molecules that modulate ciliary muscle tension is of crucial importance, given its modulation of aqueous humor outflow. Ciliary muscle contraction increases the outflow of aqueous humor through the conventional route, via its attachment to the scleral spur, widening the functional spaces of the trabecular meshwork (Wiederholt *et al.* 2000), but it also lowers the outflow facility through the unconventional one; the converse occurs with its relaxation (Rasmussen and Kaufman 2014, Johnson *et al.* 2016). It seems that ciliary muscle and myofibroblast-like cells of the trabecular outflow system act as functional antagonists while modulating outflow resistance, being the resistance of this route lowered through contraction of the former and relaxation of the latter (Tamm *et al.* 2015). In fact, eliciting ciliary muscle contraction is the basis of the pharmacologically-induced reduction of IOP through cholinergic drugs (Akaishi *et al.* 2009, Overby *et al.* 2014, Braunger *et al.* 2015), which is one of the aqueous humour drainage mechanisms that supports the glaucoma pharmacotherapy.

So far, many components have been studied regarding their ability to induce contraction of ciliary muscle samples. These include muscarinic agonists, mainly M_3 subtype (acting through phospholipase C activity and intracellular calcium accumulation, in a $G_{q/11}$-coupled pathway) (Yasui *et al.* 2008); CB_1-agonist, anandamide, which induces contraction (dependent on phospholipase C and βγ subunit of G_i/G_o proteins), alone or synergistically with carbachol (Lograno and Romano 2004, Romano and Lograno 2013); endothelin-1, through ET_A receptor activation (though, in low-doses, it elicits relaxation, via ET_B receptors) (Kamikawatoko *et al.* 1995); serotonin, through 5-HT_2 and 5-HT_3 receptors (Lograno and Romano 2003) and histamine, via H_1 receptors, which activates phospholipase C and increases intracellular calcium (Markwardt *et al.* 1997). Moreover, ciliary muscle contraction can be provoked through membrane-bound PKC stimulation (using phorbol-12-myristate13-acetate) (Thieme *et al.* 1999) or inhibition of PKC, using staurosporin or H7, causing potentiation of carbachol contraction (Lograno *et al.* 1991, Daniele *et al.* 1997); PDEI (N-ethylmaleimide and iodoacetic acid) (Yoshino and Suzuki 1992); epidermal growth factor-urogastrone (EGF) (Wiederholt *et al.* 1998); substance P (Lograno and Daniele 1988) and 4-aminopyridine, a potassium channel blocking drug, which appears to potentiate the evoked ciliary muscle contractions without changing resting tension (Zacharias and Guerrero 1985). Finally, platelet-activating factor, iloprost, PGI_2, PGE_2, PGE_1, latanoprost, travoprost, $PGF_{2\alpha}$, 17-phenyl-$PGF_{2\alpha}$ and bitamoprost, a prostamide derivate - using both CB_1 and FP receptors -, have all been implied in raising ciliary muscle tension (Lograno *et al.* 1992, Krauss *et al.* 1997, Yoshitomi *et al.* 2002, Romano and Lograno 2007).

As far as the neurohumoral regulation of the eye's anterior segment is concerned, a new potential modulator of intra-ocular muscles kinetics has been described - UCN2

stimulation represents a new mechanism of relaxation of the iris sphincter muscle and seems to raise the tension of carbachol-contracted ciliary muscle.

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FIGURES

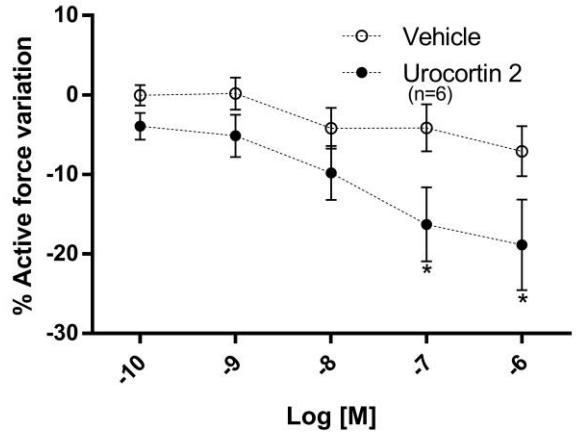

Figure 1. Concentration-response curves of urocortin 2 (10^{-10}-10^{-6}M) in carbachol pre-contracted rabbit iris sphincter muscles, compared with vehicle alone. $p<0.05$: * vs. control.

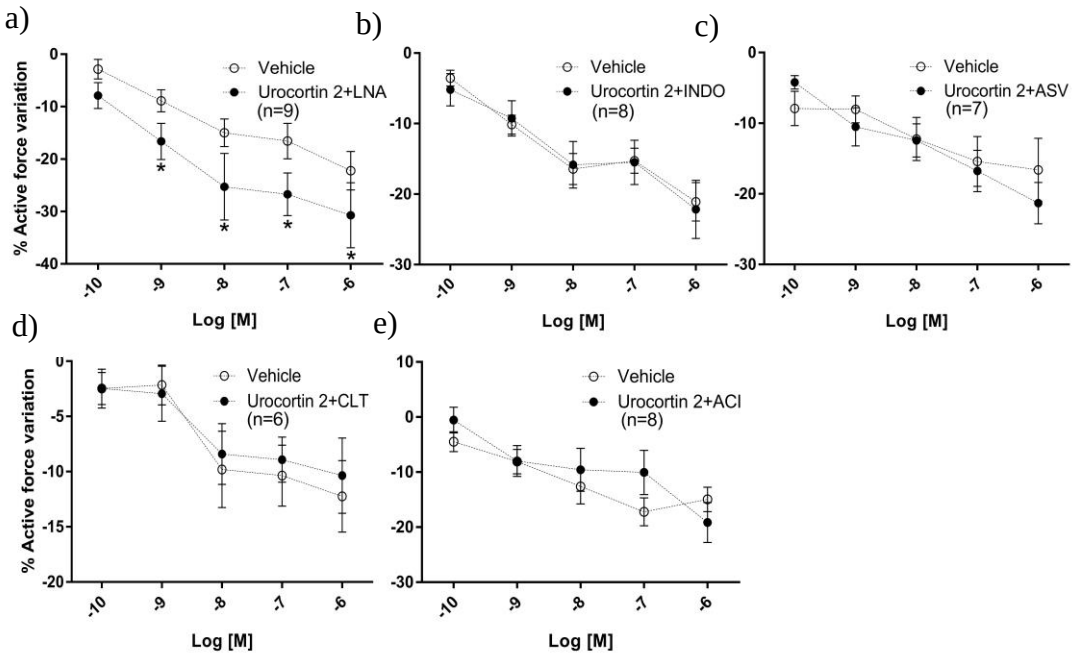

Figure 2. Concentration-response curves of urocortin 2 (10^{-10}-10^{-6}M) in carbachol pre-contracted rabbit iris sphincter muscles, in the presence of either: a) L-nitro-L-arginine (LNA, 10^{-5}M); b) indomethacin (INDO, 10^{-5}M); c) antisauvagine-30 (ASV, 10^{-6} M); d) chelerythrine (CLT, 10^{-5} M); e) SQ22536 (ACI, 10^{-6} M). Comparison with treatment with vehicle alone. $p<0.05$: * vs. control.

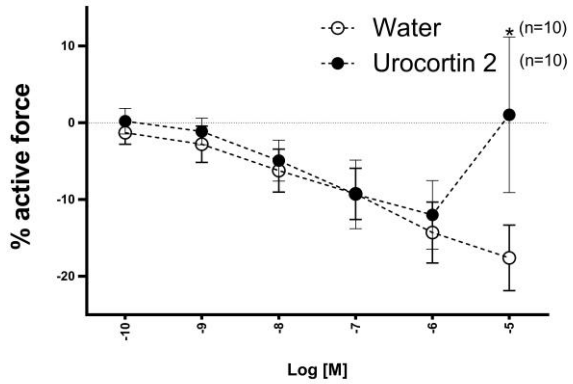

Figure 3. Concentration-response curves of urocortin 2 (10^{-10}-10^{-5}M) in carbachol pre-contracted bovine ciliary muscles, compared with water alone. p<0.05: * vs. control.

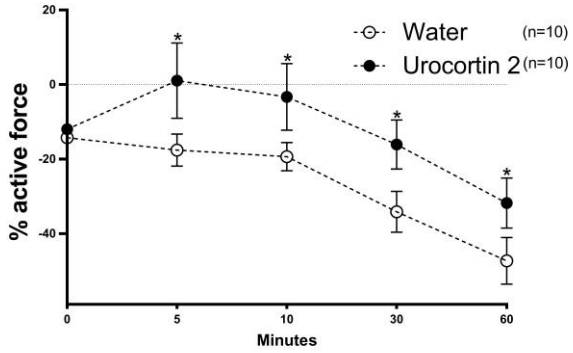

Figure 4. Response curve of urocortin 2 upon adding the final dose (10^{-5}M), measured for 1 hour. p<0.05: * vs. control.

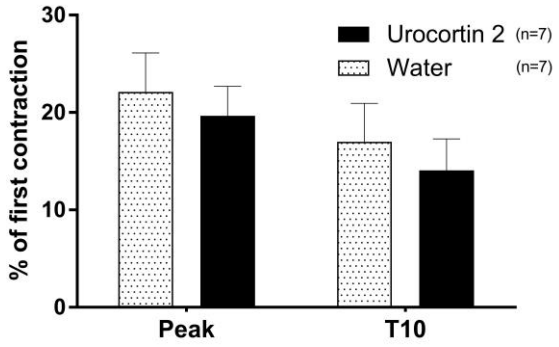

Figure 5. Comparison of the magnitude of the second carbachol-induced contraction (related to the first contraction) between the 20 minutes' pre-treatment with urocortin 2 or water. Both peak contraction and 10 minutes after were analyzed. No differences reached significance.