EXPERIMENTAL STUDY

Orexin A suppresses *in vivo* GH secretionL M Seoane, S A Tovar, D Perez², F Mallo², M Lopez, R Señaris, F F Casanueva¹ and C DieguezDepartments of Physiology and ¹Medicine, Endocrine Area Complejo Hospitalario Universitario de Santiago, University of Santiago, Santiago de Compostela, Spain and ²Department of Functional Biology, University of Vigo, Vigo, Spain

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Abstract

Background/aims: Orexins (OXs) are a newly described family of hypothalamic neuropeptides. Based on the distribution of OX neurons and their receptors in the brain, it has been postulated that they could play a role in the regulation of neuroendocrine function. GH secretion is markedly influenced by nutritional status and body weight. To investigate the role OX-A plays in the neuroregulation of GH secretion we have studied its effect on spontaneous GH secretion as well as GH responses to GHRH and ghrelin in freely moving rats. Finally, we also assessed the effect of OX-A on *in vitro* GH secretion.

Methods: We administered OX-A (10 µg, i.c.v.) or vehicle (10 µl, i.c.v.) to freely moving rats. Spontaneous GH secretion was assessed over 6 h with blood samples taken every 15 min.

Results: Administration of OX-A led to a decrease in spontaneous GH secretion in comparison with vehicle-treated rats, as assessed by mean GH levels (means ± S.E.M. 4.2 ± 1.7 ng/ml vs 9.4 ± 2.2 ng/ml; $P < 0.05$), mean GH amplitude (3.6 ± 0.5 ng/ml vs 20.8 ± 5.6 ng/ml; $P < 0.01$) and area under the curve (848 ± 379 ng/ml per 4 h vs 1957 ± 458 ng/ml per 4 h; $P < 0.05$). In contrast, OX-A failed to modify *in vivo* GH responses to GHRH (10 µg/kg, i.v.) although it markedly blunted GH responses to ghrelin (40 µg/kg, i.v.) (mean peak GH levels: 331 ± 71 ng/ml, vehicle, vs 43 ± 11 ng/ml in OX-A-treated rats; $P < 0.01$). Finally, OX-A infusion (10^{-7} , 10^{-8} or 10^{-9} M) failed to modify *in vitro* basal GH secretion or GH responses to GHRH, ghrelin and KCl.

Conclusions: These data indicate that OX-A plays an inhibitory role in GH secretion and may act as a bridge among the regulatory signals that are involved in the control of growth, nutritional status and sleep regulation.

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Introduction

In addition to stimulating body growth, growth hormone (GH) plays an important role in metabolism. In turn, alterations in nutritional status, such as obesity or food deprivation, markedly influence GH secretion (1). As GH secretion is normalized after weight loss in obesity, or after refeeding in states of food deprivation, there is no doubt that altered GH secretion develops as a consequence of altered metabolic status (1, 2).

Two novel hypothalamic neuropeptides, the orexins A and B (OX-A and OX-B), have recently been identified and shown to play a role in the regulation of food intake. Both of them are derived from a common precursor, prepro-OX (also called prehypocretin), which is synthesized in neurons within and around the lateral and posterior hypothalamus in the adult rat brain (3–6). The OX-containing neurons project to multiple sites of the central nervous system (7, 8). These peptides bind and activate two closely related G-protein coupled receptors, called OX receptor 1 (OX1R) and

OX receptor 2 (OX2R), which are expressed in different parts of the central nervous system, as well as in certain peripheral tissues (3, 9–11). This wide distribution of OX fibres and OX receptors indicates that OXs may play an important role in physiological functions other than feeding. Recent data show that OXs stimulate the expression of c-Fos in the paraventricular and arcuate nucleus, and peripheral administration of OX inhibited plasma thyrotrophin levels as well as *in vitro* hypothalamic thyrotrophin-releasing hormone release (12). Furthermore, OX-A and OX-B have been reported to modulate *in vivo* luteinizing hormone secretion (13, 14), the hypothalamic–pituitary–adrenal axis (15) and *in vitro* GH secretion from porcine somatotrophs. Taken together these data suggest that OXs may play an important neuroendocrine role in anterior pituitary hormone secretion.

To investigate the role OX-A plays in the neuroregulation of GH secretion we have studied its effect on spontaneous GH secretion as well as GH responses to GH-releasing hormone (GHRH) and ghrelin in freely

moving rats. Finally, we also assessed the effect of OX-A on *in vitro* GH secretion, since the presence of OX receptors in the human anterior pituitary has been recently reported (10), as well as the activation of voltage-gated Ca^{2+} currents in porcine somatotrophs (16).

Materials and methods

Adult male Sprague–Dawley rats (200–250 g) were housed in a 12 h light:12 h darkness cycle in a temperature- and humidity-controlled room. Chronic i.c.v. and intracardiac cannulae were implanted under ketamine–xylazine anaesthesia, as previously described (17). After surgery, the animals were placed directly in isolation test chambers for 5 days, and were given free access to regular Purina rat chow and tap water. Thereafter, the animals continued to have food available freely. On the day of the experiment, blood samples (0.3 ml) were withdrawn at the appropriate times. The animals ($n = 7–9$ rats/group) received either vehicle or OX-A (10 μg) (Bachem, Basle, Switzerland), through the i.c.v. route (18). The protocols were approved by the ethics committee of the University of Santiago de Compostela, and experiments performed in agreement with the rules of laboratory animal care and international law on animal experimentation.

Hormone assays

Plasma GH concentrations were determined by double-antibody RIA using materials supplied by the National Hormone Pituitary Program, as described previously (17). Values are expressed in terms of the GH reference preparation (GH-RP-2). The intra- and interassay coefficients of variation were 7 and 10 % respectively. Characteristics of the GH secretory pattern (mean GH levels, area under the curve (AUC) and amplitude) were assessed by the ULTRA program (kindly supplied by E Van Cauter (19)); * $P < 0.05$; ** $P < 0.01$ (19).

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Comparison between the different groups was assessed by the Mann–Whitney test.

In vitro experiments

Animals were killed by decapitation. Anterior pituitaries were dissected and washed in sterile Earle's balanced salt solution (EBSS). Enzymatic dispersion was carried out at 37 °C in a solution containing 0.25 % collagenase and 0.005 % DNase, with gentle resuspension every 15 min for a total of 90 min. The dispersed cells were separated by centrifugation (1000 g , 5 min), washed twice with EBSS and resuspended in a culture medium (α -MEN with 2.5 % fetal

calf serum and antibiotics: 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 2.5 $\mu\text{g}/\text{ml}$ Fungizone). Thereafter the cells were mixed with Biogel P-2 (BioRad), previously swollen overnight, and loaded into a 2 ml sterile disposable syringe as previously described (17). The column was placed in a 37 °C water bath and perfused with the culture medium (0.5 ml/min) for 2 h before starting to collect samples every 5 min. At the end of the experiment, the samples were stored and kept frozen until assayed.

Results

Following administration of saline, the normal male rats exhibited the typical ultradian GH rhythm (Fig. 1a) with mean GH levels of 9.4 ± 2.2 ng/ml, a mean GH amplitude of 20.8 ± 5.6 ng/ml and a total GH secretion as assessed by the AUC of 1951 ± 458 ng/ml per 4 h. Administration of OX-A was associated with a marked decrease in mean GH levels (4.2 ± 1.7 ng/ml; $P < 0.05$), mean GH amplitude (3.65 ± 0.5 ng/ml; $P < 0.01$) and AUC (848 ± 379 ng/ml per 4 h; $P < 0.05$) in comparison with rats that received vehicle, without any significant differences in mean nadir levels or frequency (Fig. 1b–d). In contrast, no significant differences were found between control and OX-treated rats in *in vivo* GH responses to GHRH (Fig. 2a). However, while control rats showed a marked response to ghrelin, OX-A administration markedly inhibited this response, the differences being significant at 5 and 10 min ($P < 0.01$) (Fig. 2b).

Finally, we assessed the effects of OX-A on *in vitro* GH secretion. As Figs 3 and 4 display, basal GH secretion (AUC: 162 ± 20 ng/60 min in control cells vs 183 ± 4 ng/60 min after 10^{-7} M OX-A) and after stimulation with GHRH, ghrelin and KCl were similar in perfused anterior pituitary cells during infusion with either vehicle or OX-A (10^{-7} M).

Discussion

In the present work we found that administration of OX-A to normally fed rats led to a clear-cut decrease in spontaneous GH secretion, which indicates that OXs are involved in the neuroregulation of GH. Since OX receptors are present at both the hypothalamic and pituitary (10) level we assessed the effects of OX-A on *in vivo* and *in vitro* GH responses to GHRH. The fact that OX-A failed to modify *in vitro* GH secretion suggests that its inhibitory effect was taking place at the hypothalamic level. This is in contrast to previous data obtained in porcine somatotrophs where it was found that OX increased *in vitro* GH responses to GHRH (16). It is likely that these discrepancies reflect interspecies differences. This inhibitory effect of OX-A on spontaneous GH secretion is unlikely to be mediated by an increase in somatostatinergic tone, since *in vivo*

Figure 1 (a) Representative plasma GH profiles in normally fed male rats ($n = 8$ per group) that received either vehicle ($10 \mu\text{l}$, i.c.v.) or OX-A ($10 \mu\text{g}$, i.c.v.), at 1200 h. (b–d) Characteristics of the GH secretory pattern (mean AUCs, GH levels and amplitudes) in male rats injected with OX-A or vehicle as assessed by the ULTRA program. * $P < 0.05$; ** $P < 0.01$.

GH responses to GHRH were similar to control rats. On the other hand, OX-A was able to suppress *in vivo* GH responses to ghrelin. Data gathered in recent years using the endogenous peptide as well as different

synthetic peptides, such as growth hormone releasing peptide-6, have shown that the stimulatory effect of ghrelin is mainly exerted at the hypothalamic level (20–25). Furthermore, it is known that, although

Figure 2 Mean $\pm$ S.E.M. plasma GH levels after administration of: (a) vehicle (control), GHRH ($10 \mu\text{g/kg}$ i.v.) and GHRH + OX-A ($10 \mu\text{g}$, i.c.v.); (b) OX-A ($10 \mu\text{g}$, i.c.v.), ghrelin ($40 \mu\text{g/kg}$, i.v.) and ghrelin + OX-A ($n = 8$ rats per group), ** $P < 0.01$.

Figure 3 *In vitro* GH responses to GHRH or KCl in perfused rat anterior pituitary cells after treatment with (a) vehicle or (b) OX-A 10^{-7} M; $n = 2$ experiments.

GHRH and ghrelin stimulate GH secretion, through different mechanisms, the effect of ghrelin on GH is abolished in the absence of GHRH (20). Since OX-containing neurons project to the arcuate nucleus where most of the GHRH neurons are located, it could be possible that OX-A inhibits endogenous GHRH tone. This would explain the inhibitory effect of OX-A on basal GH secretion and GH responses to ghrelin, as well as its lack of influence on GH responses to exogenously administered GHRH.

Although the physiological relevance is still unclear, several possibilities can be put forward. It is possible that OXs serve as a signal linking metabolic status and GH secretion. It is already known that the levels of prepro-OX are markedly influenced by nutritional status, being up-regulated upon fasting (3, 26). This is particularly interesting, since food-deprivation in the rat is associated with a marked decrease in

spontaneous GH secretion (1). Thus, it is possible that the fasting-induced increase in OX gene expression inhibits GH secretion. On the other hand, ghrelin, the endogenous ligand for the GH secretagogue, has recently been implicated in the control of body weight homeostasis (27–29). Like OXs, ghrelin expression increases with prolonged fasting and its administration rapidly increases food intake and body weight. In contrast, we found that OX-A and ghrelin play opposite roles in the neuroregulation of GH secretion, indicating that the mechanisms mediating their effects on body weight homeostasis and the regulation of anterior pituitary hormone secretion are different.

Finally, the possibility that OXs could act as common regulators of sleep and neuroendocrine function should also be considered. Indeed, recent data suggest that OXs are involved in sleep control. Thus, it was found that knock-out mice lacking the prepro-OX gene exhibited

Figure 4 AUCs of *in vitro* GH responses to (a) ghrelin and (b) GHRH and following infusion of vehicle or OX-A (10^{-7} M); $n = 2-4$ experiments.

sleep abnormalities resembling narcolepsy (30). In keeping with this observation, using the well-characterized canine models of narcolepsy, namely a breed of Doberman and Labrador retriever, two different deletions in the transcripts of the OX2R were found (31). Furthermore, the vast majority of human patients lack OX in their cerebrospinal fluid, and autoimmune-mediated destruction of hypocretin neurons appears to be the cause of human narcolepsy

(32). Interestingly, assessment of GH secretion in narcoleptic patients showed that these patients secreted approximately 50 % of their total production during daytime, whereas controls secreted only 25 % during the day. Also the GH output pattern of narcoleptic patients was less regular (33). Based on this finding it was postulated that the OX system, that is particularly active during the day, may normally inhibit hypothalamic GHRH secretion to promote arousal and reduce GH secretion. Our data showing that OX administration inhibits spontaneous GH secretion provides direct support for this hypothesis (33).

In summary, our data provide direct evidence that OX-A is involved in the control of spontaneous GH secretion in adult male rats. This finding indicates an additional mechanism of adaptation of the hypothalamus to nutritional status and may help to explain the disrupted GH secretion previously found in narcoleptic patients. Further studies are needed in order to integrate these data into the framework of what is known regarding the interrelationship among OXs and the hypothalamic-GH axis in sleep regulation.

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