

Urotensin II Inhibits Arginine Vasotocin and Stimulates Isotocin Release from Nerve Endings in the Pituitary of Gilthead Sea Bream (*Sparus aurata*)



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ABSTRACT

The aim of this in vitro study was to determine whether arginine vasotocin (AVT) and isotocin (IT) release from nerve endings is affected by urotensin II (UII) in gilthead sea bream pituitary. Primary cultures of pituitary cells were exposed to 10^{-12} , 10^{-10} , and 10^{-8} M UII for 6, 24, and 48 hr. AVT and IT contents were determined in the culture media by high performance liquid chromatography (HPLC). UII at all doses decreased AVT release after 6, 24, and 48 hr of incubation. IT release was increased by UII only after 24 hr of incubation. This study, for the first time, indicates that UII affects AVT and IT release from nerve endings in the pituitary of *Sparus aurata*. It is presumed that UII together with AVT and IT may control response to different salinities in fishes. *J. Exp. Zool.* 9999A: XX–XX, 2014. © 2014 Wiley Periodicals, Inc.

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In fishes, hormonal regulation of water and ion homeostasis requires participation and interaction of many endocrine systems at the various functional levels of the organism (Kulczykowska, 2007). One of them is urotensin II (UII), a cyclic peptide originally isolated from the urophysis of the goby (*Gillichthys mirabilis*) (Pearson et al., '80). In teleosts, UII appears to be involved in the control of osmoregulatory and metabolic functions and also in the cardiovascular and gastrointestinal activities, and immune response (Sheridan et al., '87; Lu et al., 2006; Le Mével et al., 2008; Nobata et al., 2011; Singh and Rai, 2011). In the European flounder (*Platichthys flesus*), urophysial UII content increased over the 24 hr following transfer from sea water to fresh water, whereas plasma UII content and UII receptor expression in key target organs (kidney and gill) decreased, suggesting down-regulation of the UII system (Bond et al., 2002; Lu et al., 2006).

In teleosts, also nonapeptides such as arginine vasotocin (AVT) and isotocin (IT), the homologues of mammalian arginine vasopressin (AVP), and oxytocin (OT), seem to be involved in the maintenance of water and ion homeostasis (McCormick and

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Bradshaw, 2006; Kulczykowska, 2007). There is also evidence for a role of AVT and IT in response to different stress stimuli (Kulczykowska et al., 2001; Mancera et al., 2008). It has been observed that the synthesis of both nonapeptides and their release from the neurohypophysis are sensitive to changes of water salinity. In teleosts, an acute change in water salinity results in altered pro AVT and pro IT mRNA expression in hypothalamic neurons (Hyodo et al., '91; Urano et al., '94; Martos-Sitcha et al., 2013) and in altered content of AVT and IT in the pituitary (Haruta et al., '91; Bond et al., 2002; Martos-Sitcha et al., 2013).

The potential relationship between AVT and other hormonal systems such as UII contributing to the osmoregulation in fishes has been suggested before (Warne and Balment, '95; Winter et al., '99; Bond et al., 2002). AVT and IT are synthesized in the preoptic area (POA) and transported to the neurohypophysis for storage and release into the vascular system via axon terminals (Van den Dungen et al., '82; Holmqvist and Ekström, '95; Saito et al., 2004). UII has been identified in teleost and non-teleost fishes not only in the urophysis but also in the CNS, for example, in caudal spinal cord, medulla oblongata, several hypothalamic nuclei, and most thalamic nuclei (Yulis and Lederis, '86, '88; Waugh and Conlon, '93; Waugh et al., '95). UII and UII receptor mRNA expression has been detected in all brain regions of European flounder, including the telencephalon-preoptic region, hypothalamus, and pituitary (Lu et al., 2006). These results may indicate a site of interaction between the UII and AVT/IT systems within the POA, hypothalamus and pituitary. Moreover, in the European flounder it was observed that both UII and AVT are involved in the hyper- and hypo-osmotic stress (Warne and Balment, '95; Winter et al., '99; Bond et al., 2002). However, to the best of our knowledge, the influence of UII on AVT and IT secretion in teleosts has never been studied before.

The aim of the present study was to determine whether AVT and IT release from nerve endings is affected by UII in the pituitary of gilthead sea bream (*Sparus aurata*).

MATERIALS AND METHODS

Fish

Three-year old gilthead sea bream were held at the National Center for Mariculture, Eilat, Israel. Fish were kept in a 5-m³ circular tank supplied with aeration at ambient seawater temperature (20–26°C), salinity of 40 ± 0.5‰, under natural light conditions 11 hr:13 hr (light/dark). Fish were fed with commercial sea bream diet containing 45% protein. In October, with the reproductive season approaching, 49 fishes of both sexes (560 ± 24 g) were euthanized with an overdose of bicarbonate-buffered MS222 (0.02%; Sigma–Aldrich, St. Louis, MO, USA), and after decapitation, the pituitary glands were immediately collected.

The experiment was conducted following the guidelines of the Ministry of Agriculture (Committee for Animal Welfare) after the

completion of training by courses given by the Committee for Animal Welfare (both the Ministry of Agriculture and Ben-Gurion University of the Negev, Israel).

Culture of Dispersed Pituitary Cells

Primary cultures of dispersed pituitary cells were performed according to the procedure described previously by Kalamarz-Kubiak et al. (2014). Dispersed pituitary cells were plated on 24 multi-well plates (Greiner Bio-One, Frickenhausen, Germany) at density of 1.105×10^6 cells/well in 1.5 mL growth medium (Medium 199, 10% FCS, 10 mM HEPES, and 1% Pen–Strep–Nystatin solution; Biological Industries, Beit Haemek, Israel). After 3 days of culture at 28°C in 5% CO₂ atmosphere, growth medium was replaced with harvest medium (Medium 199 supplemented with 0.1% BSA, 10 μM HEPES, and 1% Pen–Strep–Nystatin solution) and UII (10^{-12} , 10^{-10} , and 10^{-8} M) (goby sequence; Bachem, Bubendorf, Switzerland) was added. Each plate consisted of a cell culture supplemented with UII and a cell culture without treatment as control. The plates were incubated at 28°C in 5% CO₂ atmosphere for 6, 24, and 48 hr, respectively. Six replicas were done for each UII concentration and control. After 6, 24, and 48 hr, the media were collected and stored at –70°C until high performance liquid chromatography (HPLC) analysis.

AVT and IT Analysis

Nonapeptides from culture media were extracted by solid-phase extraction (SPE) as described previously by Kalamarz-Kubiak et al. (2011). The separation and detection of AVT and IT were performed with HPLC with fluorescence and UV detection according to a modified procedure by Kulczykowska ('95). Dried samples were reconstituted with 50 μL of 0.1% trifluoroacetic acid (TFA) in 15% acetonitrile and then injected to HPLC system [1200 series Quaternary HPLC system (Agilent Technology, Santa Clara, CA, USA)] with fluorescence detector and Diode Array Detector. The chromatographic separations of peptides were carried out on ZORBAX Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm) (Agilent Technology) using linear gradient system: 20–40% mobile phase B (0.1% TFA in acetonitrile: H₂O (3:1) in mobile phase A (0.1% TFA in H₂O) for 15 min at the flow rate 0.7 mL/min. UV detection was performed at 215 nm, fluorescence detection at 312 nm with excitation at 280 nm. Recovery of AVT and IT was in the 89–93% range. The detection limit was defined as 100 fmol/mL. Intra-day repeatability expressed as relative standard deviation (RSD) was in the 2–4.5% and 5.3–8.2% range for AVT and IT, respectively; inter-day repeatability was in the 2.5–5.5% and 5.5–8.5% range for AVT and IT, respectively.

Statistical Analysis

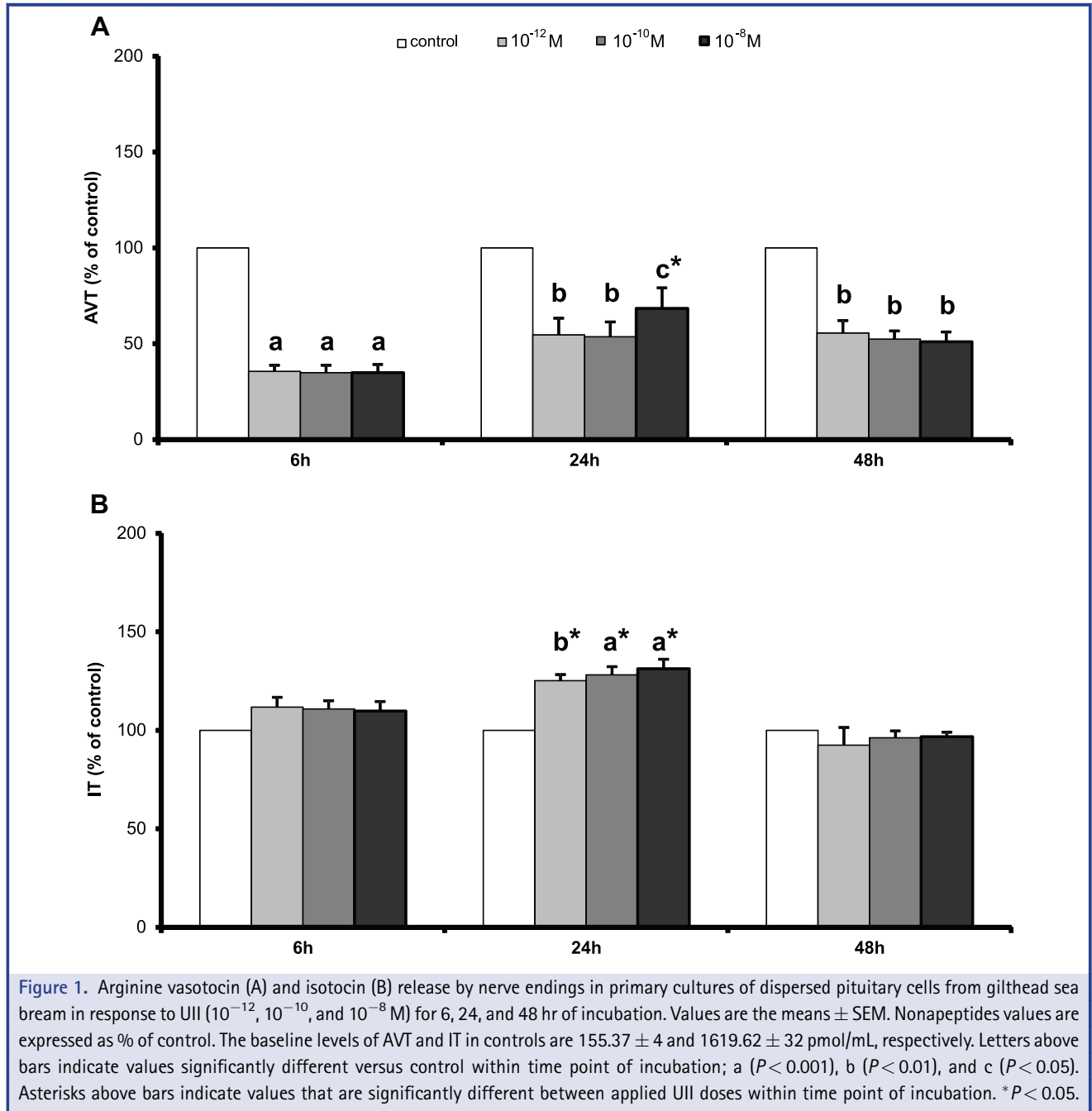
Contents of AVT and IT are presented as % of control. For multiple comparisons, two-way ANOVA was used. Post hoc comparisons were made with the Newman–Keuls test. Significance was taken at $P < 0.05$. Statistical analysis was performed using STATISTICA 7.1.

RESULTS

Urostensin II at all doses significantly decreased AVT release after 6, 24, and 48 hr of culture in comparison to control (Fig. 1A). UII caused approximately 50% of inhibition in AVT release. In contrast to AVT, IT secretion was significantly higher only after 24 hr of culture in exposure to all tested doses of UII (Fig. 1B). There U II caused approximately 30% of stimulation in IT release.

DISCUSSION

This study provides evidence for the first time that AVT and IT release from nerve endings is influenced by UII in gilthead sea bream pituitary. Although it had been observed that AVT and UII are both linked to osmoregulation (Warne and Balment, '95; Winter et al., '99; Bond et al., 2002), their interactions had not been studied before. The doses of UII used in this in vitro study were



determined on the basis of the literature, considering its concentration in different fish tissues (Kobayashi et al., '86; Kelsall and Balment, '98; Winter et al., '99). The results presented here indicate that UII inhibits AVT release from nerve endings. It has been shown that AVT is an antidiuretic hormone reducing urine production in eels (Henderson and Wales, '74; Babiker and Rankin, '78). Thus, by inhibiting AVT secretion, UII may have a diuretic effect. Furthermore, it is known, that UII administered in vivo increases renal blood flow, glomerular filtration rate, and consequently enhances diuresis and natriuresis in rat (Zhang et al., 2003; Abdel-Razik et al., 2008). The mammalian paradigm could be helpful in interpretation of fish data. Our results indicate that UII strong inhibitory action on AVT release is independent of the time of exposure and tested doses. After 24 hr of incubation, the inhibition of AVT was lower and persisted to the end of experiment. Disinhibition in AVT secretion after a long time of culture may suggest the desensitization of UII receptors as it was observed in human cell lines (Proulx et al., 2008; Batuwangala et al., 2009). In contrast to AVT, UII significantly increased IT release from nerve endings after 24 hr of culture. This stimulatory effect of UII appeared to be independent of tested doses. In a variety of mammalian systems, UII is a naturally occurring somatostatin analog sharing some functional similarities with somatostatin (Pearson et al., '80; Conlon et al., '97). These results are consistent with data in mammals that show that the intracerebroventricular (ICV) somatostatin infusion significantly increases plasma OT secretion in virgin and pregnant rats (Meddle et al., 2010). The opposite response of AVT and IT to UII exposure in pituitary cell culture showed an independent regulation of nonapeptides secretion which has been documented previously in rainbow trout (*Oncorhynchus mykiss*) (Saito and Urano, 2001). An apparent difference in response of AVT and IT to rapid and long-term osmotic changes which have been shown in rainbow trout also supports this idea (Kulczykowska, '97; Kulczykowska et al., 2001).

This study indicates that UII affects AVT and IT release from nerve endings in the pituitary of gilthead sea bream. It seems that UII together with AVT and IT may control response to different salinities in fishes.

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