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Salinity-dependent *in vitro* effects of homologous natriuretic peptides on the pituitary–interrenal axis in eels

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ABSTRACT

We examined the effects of atrial, B-type, ventricular and C-type natriuretic peptides (ANP, BNP, VNP and CNP1, 3, 4) on cortisol secretion from interrenal tissue *in vitro* in both freshwater (FW) and seawater (SW)-acclimated eels. We first localized the interrenal and chromaffin cells in the eel head kidney using cell specific markers (cholesterol side-chain cleavage enzyme (P450ssc) and tyrosine hydroxylase (TH), respectively) and established the *in vitro* incubation system for eel interrenal tissue. Unexpectedly, none of the NPs given alone to the interrenal tissue of FW and SW eels stimulated cortisol secretion. However, ANP and VNP, but not BNP and three CNPs, enhanced the steroidogenic action of ACTH in SW interrenal preparations, while CNP1 and CNP4, but not ANP, BNP, VNP and CNP3, potentiated the ACTH action in FW preparations. These salinity dependent effects of NPs are consistent with the previous *in vivo* study in the eel where endogenous ACTH can act with the injected NPs. 8-Br–cGMP also enhanced the ACTH action in both FW and SW eel preparations, suggesting that the NP actions were mediated by the guanylyl cyclase-coupled NP receptors (GC-A and B) that were localized in the eel interrenal. Further, ANP and CNP1 stimulated ACTH secretion from isolated pituitary glands of SW and/or FW eels. In summary, the present study revealed complex mechanisms of NP action on corticosteroidogenesis through the pituitary–interrenal axis in eels, thereby providing a deeper insight into the role of the NP family in the acclimation of this euryhaline teleost to diverse salinity environments.

1. Introduction

Cortisol is a pleiotropic hormone important for various aspects of homeostasis in vertebrates, particularly for glucose homeostasis and stress response. In mammals, cortisol acts as a glucocorticoid whereas aldosterone acts as a mineralocorticoid controlling ion and water balance [41,59]. In teleost fishes, however, there is no clear evidence to date for aldosterone production [5,10,47], and 11β-hydroxylase lacks aldosterone synthesizing activity in eels [22]. Consequently, cortisol has been believed to act not only as a glucocorticoid but also as a mineralocorticoid in teleosts and plays a central role in osmoregulation, especially in the acclimation process to both seawater (SW) and freshwater (FW) environments [38]. Thus, the regulation of cortisol secretion is of crucial importance to understand the multiple functions of this hormone.

In relation to the involvement in stress, adrenocorticotropic hormone (ACTH) released from the anterior pituitary has been considered as the main regulator of cortisol secretion through the hypothalamo–pituitary–interrenal (HPI) axis in teleosts [13,59]. However, a number of other hormones secreted from tissues other than the pituitary have also been implicated in cortisol secretion, most likely for fine tuning of its regulation. Some of these hormones are also known to be involved in osmoregulation such as the natriuretic peptides (NPs), angiotensin II and urotensins [2,31].

The natriuretic peptide family consists of atrial, B-type, ventricular and C-type NP (ANP, BNP, VNP and CNP). NPs are known to comprise a family of paralogous peptides that play a pivotal role in both cardiovascular and body fluid homeostasis in vertebrates [14,29,34,52,55]. Interestingly, the NP family consists of seven members (ANP, BNP, VNP and CNP1–4) in teleosts and chondrosteans [19,57]. Among the NP members, ANP, BNP and VNP are cardiac hormones circulating in the blood, while CNPs are basically a paracrine factor in the brain and endothelial cells. Although the relative biological potency of the newly identified NPs has not yet been examined, NPs have been shown to have important functions for the body fluid regulation in fishes and tetrapods [54,55]. Furthermore, the steroidogenic action of NPs has been studied for over 20 years but there are controversies about their endocrine effects among the different vertebrate groups.

In mammals, ANP and BNP inhibit aldosterone and cortisol secretion from the adrenal cortex [4,9,42], whereas CNP (later shown to be an ortholog of CNP4) inhibits aldosterone secretion but stimulates cortisol secretion [60]. As BNP was first isolated

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from the bovine adrenal chromaffin cells and named aldosterone-secretion inhibitory factor [43], it may act as a paracrine factor within the adrenal, although no expression of NPs was detected in the eel interrenal tissue [44]. The difference of the effect between ANP/BNP and CNP in mammals may be accounted for by binding to different receptors: ANP and BNP bind to NP receptor type A (GC-A) with high affinity and CNP to NP receptor type B (GC-B) [18]. The GC-A and GC-B are guanylyl cyclase-coupled receptors and use cyclic guanylyl monophosphate (cGMP) as an intracellular messenger. In non-mammalian tetrapods, mammalian ANP has been shown to inhibit aldosterone and corticosterone secretion in reptiles [8] and amphibians [30].

In contrast to the inhibitory effects of ANP and BNP on corticosteroidogenesis in tetrapods, ANP stimulates cortisol secretion *in vivo* in the flounder [2] and *in vitro* in the carp [31] and rainbow trout [2] using rat and human peptides. As mammalian and fish ANP share only ca. 60% identity, and as teleost NPs share higher identities with each other in the same species, there is a need to compare the effects of all NPs using homologous peptides in one species. Homologous NPs were used for the first time in the eel, which showed that ANP and CNP (now renamed CNP1) enhanced cortisol secretion in conscious SW- and FW-acclimated eels, respectively [35]. These salinity-dependent effects confirmed the earlier *in vitro* study in the trout using human ANP [2]. Recently, we identified six NPs (ANP, BNP, VNP, CNP1, CNP3 and CNP4) in the eel, but the CNP2 gene does not seem to be expressed even in the brain where it is exclusively expressed in other teleost species [44]. In order to understand the role of diversified NPs more thoroughly in teleost osmoregulation, we attempted to examine the effects of all homologous NPs on cortisol secretion *in vitro* using the newly established interrenal preparations from FW and SW eels.

Our preliminary results showed that all NPs given alone failed to stimulate cortisol secretion in both FW and SW eel interrenal preparations, but some NP types enhanced the ACTH effect on cortisol secretion. Therefore, we are interested in the NP effects on the HPI axis. Although there is no report on the NP action on ACTH secretion in teleost fishes, inhibitory effects have been reported in mammals [21,28]. As ACTH is a major regulator for cortisol secretion throughout vertebrate species, the regulatory action of NPs on ACTH secretion may affect their steroidogenic action *in vivo*. Thus, we further examined the effects of NPs on ACTH secretion from the isolated pituitary *in vitro* to evaluate the multiple actions of NPs on the HPI axis.

2. Materials and methods

2.1. Animals

Cultured immature Japanese eels (*Anguilla japonica*) of approximately 200 g body weight were purchased from a local dealer. Fish were maintained under natural photoperiod in either FW or SW tanks for more than 2 weeks before experimental use. Water in the tank was continuously circulated, aerated and regulated at 18 °C. Aged tap water (almost 0‰ salinity) and natural SW from North Pacific Ocean (35‰) were used as FW and SW, respectively. Animal care and use for the experiments were approved by the Animal Experiment Committee of the University of Tokyo.

2.2. Localization of interrenal cells and chromaffin cells in eel head kidney

In order to establish the *in vitro* system to investigate the effects of NPs on interrenal cells, the precise location of the interrenal and chromaffin cells was analyzed throughout the eel head kidney along with the NP receptors (GC-A and -B). Cholesterol side-chain cleavage enzyme (P450scc) and tyrosine hydroxylase (TH) were used as markers for interrenal cells and chromaffin cells, respectively. The distribution of their gene expression in the head kidney was examined by reverse transcription polymerase chain reaction (RT-PCR). The expression of NP receptor genes was also analyzed by RT-PCR. FW acclimated eels ($n = 3$) and SW acclimated eels ($n = 3$) were anesthetized in 0.1% (w/v) 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO) for 10 min. A pair of head kidneys (approximately 10 cm) were dissected out and cut from the anterior end (opposite to the kidney) in segments of 1 cm (Fig. 1a). The segments were quickly frozen in liquid nitrogen and kept at −80 °C until use. RNA was extracted using Isogen as described by the manufacturer (Nippon Gene, Tokyo, Japan). Single strand cDNAs for each 1 cm segment was prepared from 1.0 μg of total RNA using SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR amplifications were performed using Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan) under the following conditions: an initial denaturation at 94 °C for 3 min was followed by 35 cycles consisting in denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 90 s. β-Actin was used as an internal control. Primers used were P450sccS1 "TGATGGTGGAGTGATGGTTC" and P450sccA1 "TATGTGGTCATGCAGCATGC" for eel P450scc (Accession No. AY654741); GCAs1 "AATTGGAAAGAACATCACCG" and GCAa1 "TACTCAGGATTCTGCGGCTC" for eel GC-A (Accession No. AB012869); GCBs2 "GATGCTTCCAGACAATCACC" and GCBa2 "TAGTAGGGTCTATCATCCAC" for eel GC-B (Accession No. D25417.1); THs1 "AGTAAAGGCTCAGGATAACC" and THa1 "GATGAACACAGAAGCGCATA" for eel TH (Accession No. AB618554); ACTs1 "CTCCCTGGAGAAGAGCTACG" and ACTa1 "GA CGGAGTATTTGCGCTCAGG" for eel β-actin (Accession No. AB0748 46). These primers gave rise to an amplicon of 799 bp for P450 scc, 430 bp for GC-A, 492 bp for GC-B, 615 bp for TH and 370 bp for eel β-actin. The amplified products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

2.3. Immunohistochemistry of eel P450scc and TH

To examine the topographical distribution of interrenal and chromaffin cells in the anterior region of head kidney, the tissues were dissected out and fixed in Bouin's solution overnight at 4 °C. Tissues were then dehydrated and embedded in paraffin. Serial sections of 8 μm thickness were placed on gelatin coated slides and left to dry at 37 °C overnight. Sections were dewaxed by xylene and rehydrated through a graded series of ethanol. The samples were washed three times with PBS (pH 7.4) and then incubated overnight at 4 °C in a humid chamber with a primary monoclonal antibody raised in mouse against rat whole TH sequence (Chemicon MAB5280, Billerica, MA) diluted at 1:4000 in PBS containing 2% normal goat serum (NGS) and 1% sodium azide. The TH antibody has been successfully used in zebrafish brain [61]. After washing three times for 5 min in PBS (pH 7.4), the slide was further processed using a Vectastain ABC Kit (Vector Labs, Burligame, CA) and an alkaline phosphatase substrate (Vector labs) with a development reaction of 15 min at room temperature (blue color). The same slide was washed three times with PBS (pH 7.4) and incubated overnight at 4 °C in a humid chamber with a polyclonal antibody specific to eel P450scc diluted at 1:8000, further processed using Vectastain ABC Kit (Vector Labs) and developed using 3,3′-diaminobenzidine (DAB) for 15 min at room temperature (brown color). The antibody was raised in rabbit and successfully used in the eel gonad [20]. The staining of TH and P450scc was performed separately and showed the same staining pattern as in the double staining experiment with a low background, showing the specificity of both antibodies (data not shown). The eel P450scc antibody was kindly provided by Drs. Shigeho Ijiri and Shinji