Effects of neuromedin-U on immature rat adrenocortical cells: *In vitro* and *in vivo* studies

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Received November 27, 2007; Accepted December 31, 2007

Abstract. Neuromedin U (NMU) is a brain-gut peptide, that in the peripheral organs and tissues acts via a G protein-coupled receptor, called NMUR1. Reverse transcription-polymerase chain reaction showed the expression of NMUR1 mRNA in either cortex and medulla or dispersed zona glomerulosa and zona fasciculata-reticularis cells of the immature rat adrenals. Accordingly, immunocytochemistry demonstrated the presence of NMUR1-like immunoreactivity in the cortex and medulla of immature adrenals. NMU8 administration to immature rats was found to raise aldosterone, but not corticosterone, plasma concentration, without altering adrenal growth. Conversely, the exposure to NMU8 markedly enhanced the proliferative activity of immature rat inner adrenocortical cells in primary *in vitro* culture, without significantly affecting their corticosterone secretion. Collectively, our findings suggest that adrenals of immature rats may be a target for circulating NMU. However, the physiological significance and relevance of the adrenal effects of NMU remain to be ascertained.

Introduction

Neuromedin-U (NMU) is a brain-gut peptide (23 amino acid residues in the rat) involved in the regulation of energy homeostasis and neuroendocrine functions (1-7; reviewed in ref. 8). NMU acts via two subtypes of G protein-coupled receptors, NMUR1 and NMUR2, the former being the peripheral receptor and the latter the central one (9,10).

As in the case of other peptides modulating energy homeostasis and food intake (e.g. NPY, cholecystokinin, galanin, leptin, orexins, beacon and NPW) (11-17), also NMU appears to regulate the hypothalamic-pituitary-adrenal (HPA) axis. The expression of NMU and $NMUR_2$ was found in the hypothalamus and anterior pituitary (10,18-33), while in the adrenal glands only the expression of NMUR1 was detected (9,27-29,33,34). NMU has been shown to enhance c-fos expression in the hypothalamic paraventricular nucleus (1,2,30,35), to increase CRH and vasopressin output from rat hypothalamic explants, and to stimulate ACTH and corticosterone secretion in the rat (2,4,5,36-38).

The present study aimed to examine the expression of NMUR1 gene in the different components of immature rat adrenal gland, and to investigate the effect of NMU8, that contains the 7-amino acid C-terminal active sequence of the native peptide (39), on the secretion and proliferative activity of cultured adrenocortical cells and adrenal cortex of immature rats.

Materials and methods

Animals and reagents. Immature male Wistar rats (21-day-old), bred in our laboratory facilities, were kept under a 14:10 h light-dark cycle (illumination onset at 6:00 a.m.) at 23˚C, and maintained on a standard diet and tap water *ad libitum*. The experimental protocol was approved by the local Ethics Committee for Animal Studies. NMU8 was purchased from Bachem (Bubbendorf, Switzerland), and vincristine from Gedeon-Richter (Budapest, Hungary). Rabbit anti-rat NMUR1 polyclonal primary antibody was provided by Alpha Diagnostic International (San Antonio, TX), and secondary peroxidase-conjugated anti-rabbit IgG polyclonal antibody by Amersham Biosciences (Piscataway, NJ). Dulbecco's modified minimum essential medium (DMEM)/nutrient mix and fetal calf serum (FCS) were provided by Gibco (Milan, Italy). All other chemicals and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Experimental design. Groups of rats (n=8) were given three subcutaneous injections of 1 or 4 nmoles/100 g body weight of NMU8 dissolved in 0.2 ml 0.9% saline 24, 16 and 8 h before sacrifice. Control rats (n=8) received subcutaneous injections of saline. Three hours before sacrifice all rats were given an intraperitoneal injection of 0.1 mg/100 g body

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Key words: neuromedin U, NMUR1, adrenal gland, steroid secretion, cell prolifeation, immature rat

weight of vincristine. Animals were decapitated at 11:00 a.m., and their trunk blood was collected in the presence of EDTA (1 mg/ml). Plasma was separated and stored at -36˚C for hormone assay. Adrenals were removed, fixed in Bouin's solution and embedded in paraffin for metaphase-index assay and morphometric estimations. Adrenal glands of a number of untreated immature rats were removed, freed of pericapsular fat, fixed in Bouin's solution overnight and embedded in paraffin for immunocytochemistry (ICC). Other adrenals were decapsulated and halved: the cortex and medulla were separated, and dispersed adrenocortical cells were obtained by sequential enzymatic digestion and mechanical disaggregation. Specimens were frozen at -80˚C and used for reverse transcription (RT)-polymerase chain reaction (PCR) assay. Then, adrenal glands of further 12 immature rats were used to obtain primary inner adrenocortical cell cultures (see below).

In vitro culture. Dispersed inner adrenocortical cells were suspended in DMEM/nutrient mix (added with 1.125 g/l sodium bicarbonate, 10% FCS and Sigma penicillin-streptomycin-fungizone mixture) and counted in a CASY-cell counter (Model TT; Schaerfe System, Reutlingen, Germany). They were seeded at a density of 10^4 cells/well into 24- or 96-well cluster dishes, and cultured for 96 h at 37˚C, the medium being changed every 24 h (40). At day 4 of culture, cells were incubated for an additional 24 h with NMU8 (10^{-8} or 10^{-6} M). As positive control, a group of cultures was incubated in the presence of 10^{-8} M ACTH. Culture medium was collected and stored at -36˚C.

RT-PCR. Total RNA was extracted from the frozen specimens, and reverse transcribed to cDNA (41-44). PCR was carried out, as previously detailed (45-47), in a Roche LightCycler 2.0, using the following primers: NMUR1 sense (481-500), 5'-GCC-ATC-TGG-GTC-TTC-GCT-AT-3' and antisense (797-816) 5'-CAC-CTG-TCT-GCG-TTC-CCT-AT-3' (336 bp; accession number, AF-242873). The PCR program was: denaturation step at 94˚C for 10 min, followed by 35 cycles of three amplification steps (94˚C for 30 sec, annealing at 55˚C for 60 sec and extension at 68˚C for 120 sec). To rule out the possibility of amplifying genomic DNA, one PCR was performed without prior RT of the RNA. Detection of the PCR amplification products was carried out by size fractionation on 2% agarose gel electrophoresis.

ICC. Paraffin-embedded adrenals were cut, and the sections (6 μm) were incubated with the primary anti-NMUR1 antibody (1:100 dilution) for 60 min at 4˚C. After washing, sections were incubated overnight at 37˚C with the secondary peroxidase-conjugated antibody, and peroxidase activity was detected using the DAB technique (Dako Liquid DAB substrate-chromogen system; Dako, Glostrup, Denmark). When necessary, nuclei were counterstained with hematoxylin. Negative controls were carried out by similarly treating adjacent sections and omitting the primary antibody (48).

Metaphase index and morphometry. Sections (6 μm) were stained with hematoxylin and eosin, and metaphase index (number of vincristine-arrested metaphase cells per 1,000 cells) was calculated at a magnification of x400, by counting 5,000 cells in the outer cortex of each adrenal (49). The number of nuclei of adrenocortical cells was counted at a magnification of x400 in 50 fields (area, 0.003 mm^2) of the outer cortex of each adrenal, and the number of nuclei per mm^2 was calculated.

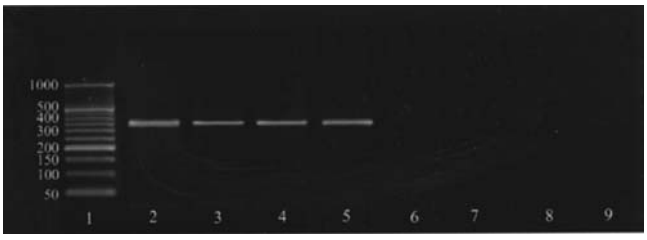

Figure 1. Ethidium bromide-stained 2% agarose gel showing cDNA amplified with rat NMUR1 specific primers from exemplary adrenal cortex (2), adrenal medulla (3), dispersed zona glomerulosa cells (4) and dispersed zona fasciculata-reticularis cells (5) of immature rats. Respective negative controls (no RT of the RNA) are shown in lanes 6-9. Lane 1 was loaded with 200 ng of a DNA size marker (50 bp DNA Ladder; MBI Fermentas, Vilnius, Lithuania).

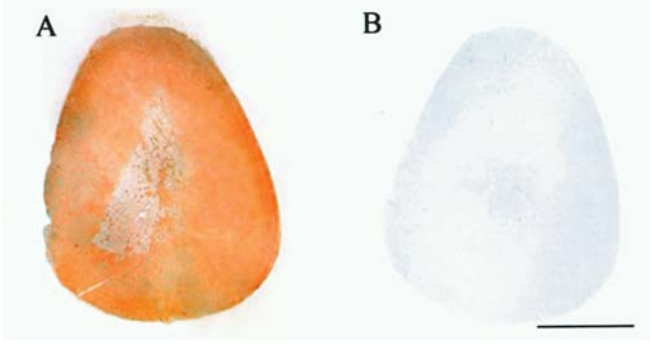

Figure 2. ICC localization of NMUR1-like immunoreactivity in the adrenal gland of immature rats. Staining is more intense in the cortex than in the adrenal medulla (A). Negative control was obtained by omitting the primary antibody (B). Magnification as defined by bar, 1 mm.

Cell proliferation assay. Proliferation rate of cultured adrenocortical cells was assayed by the EZ4U non-radioactive cell proliferation and cytotoxic assay of Biomedica (Vienna, Austria), as detailed earlier (40,50). Briefly, cultures were incubated for the last 5 h with EZ4U and formazan production, which is linearly related to the cell number, was assayed by measuring absorbance at 490 nm wavelength in a microplate autoreader EL13 (Bio-Tek Instruments, Winooski, VT).

Hormone assay. Aldosterone and corticosterone were extracted from plasma, and corticosterone from culture incubation media, and measured by RIA, as detailed earlier (51,52). Aldosterone RIA: sensitivity, 5 pg/ml, intra- and interassay CVs, 5 and 7%, respectively. Corticosterone RIA: sensitivity, 50 pg/ml, intra- and interassay CVs, 7 and 9%, respectively.

Statistics. Data were expressed as means ± SEM, and the statistical significance of the differences among experimental groups was estimated using ANOVA, followed by the Duncan's multiple range test.

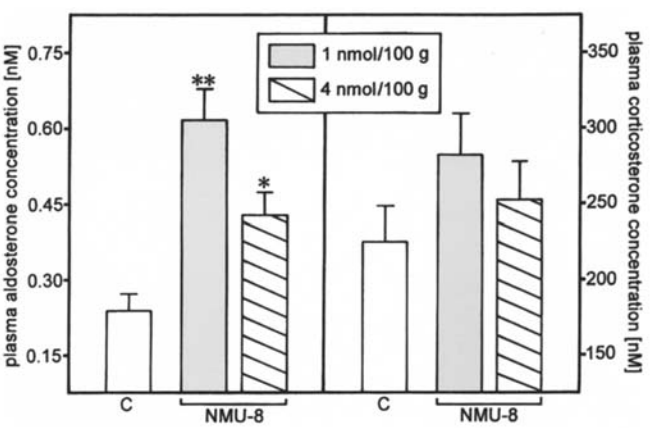

Figure 3. Effects of NMU8 administration on aldosterone and corticosterone plasma concentrations in immature rats. Bars are means ± SEM (n=8). *P<0.05 and **P<0.01 from control (C) rats.

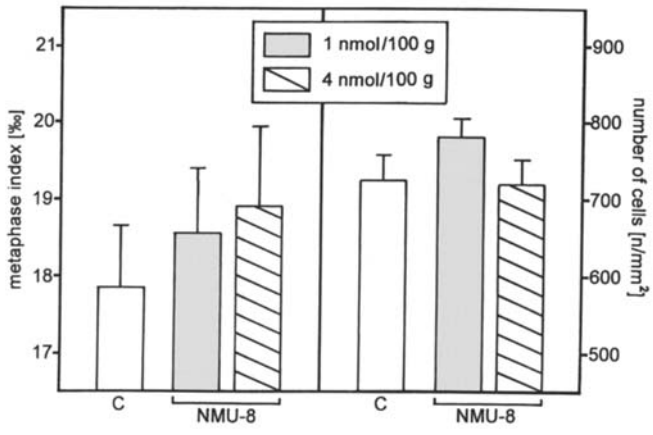

Figure 4. Lack of effect of NMU8 administration on the growth of the adrenal in immature rats. Bars are means ± SEM (n=8).

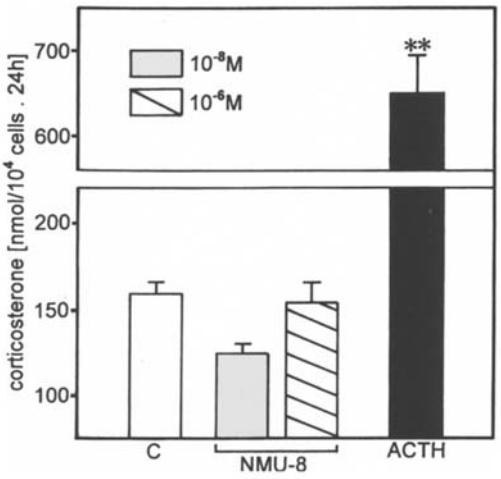

Figure 5. Effects of NMU8 and ACTH on corticosterone secretion from immature rat inner adrenocortical cells in primary *in vitro* culture. Bars are means ± SEM (n=4), **P<0.01 from control (C) cultures.

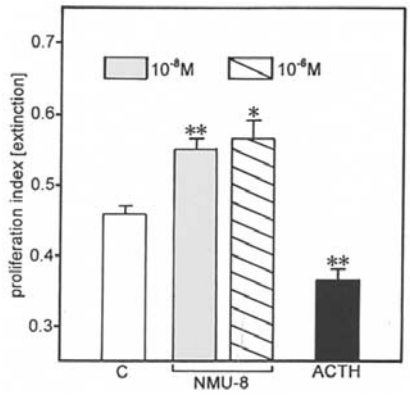

Figure 6. Effects of NMU8 and ACTH on the proliferative activity of immature rat inner adrenocortical cells in primary *in vitro* culture. Bars are means ± SEM (n=4). *P<0.05 and **P<0.01 from control (C) cultures.

Results

RT-PCR detected the expression of NMUR1 mRNA in either the cortex and medulla or in dispersed zona glomerulosa and zona fasciculata-reticularis cells of the immature rat adrenal gland (Fig. 1). ICC revealed the presence of NMUR1-like immunoreactivity in the immature rat adrenals, which was intense in the cortex and weak in the medulla (Fig. 2).

NMU8 administration to immature rats raised plasma aldosterone, but not corticosterone, concentrations (Fig. 3). Neither metaphase index nor the number of adrenocortical cells per unit area were affected (Fig. 4).

As expected, ACTH markedly increased corticosterone secretion from and decreased proliferation rate of cultured adrenocortical cells of immature rats. NMU8 did not alter corticosterone output from cultured cells (Fig. 5), but significantly increased their proliferation rate (Fig. 6).

Discussion

Our present RT-PCR and ICC studies clearly demonstrate the expression of NMUR1 as mRNA and protein in the cortex of immature rat adrenals, thereby confirming earlier findings obtained in adult animals (9,27-29,33,34). Moreover, the detection of NMUR1 mRNA in dispersed zona glomerulosa and zona fasciculata-reticularis cells rules out the possibility that the expression was due to the presence in the specimens assayed of the non-parenchymal components of the gland (connective tissue and capillaries). In contrast, they strongly suggest the involvement of NMU in the physiological regulation of the biological activity of immature rat adrenocortical cells. It has been suggested that NMU exerts its biological effects mainly via autocrine-paracrine mechanisms (53). However, the expression of NMU gene was not detected in the rat adrenals (33). Phoenix Pharmaceuticals has recently made available kits for NMU assay, that showed that this peptide may attain in human plasma concentrations of ~10^{-8} M (30 pg/ml) (33). Hence, it is reasonable to conceive that NMU may activate adrenocortical NMUR1 acting as a true circulating hormone.

Our *in vivo* and *in vitro* experiments gave rather contrasting results. At 10^{-8} and 10^{-6} M concentrations, NMU8 did not affect corticosterone secretion from ACTH-responsive inner

adrenocortical cells in primary culture, but did stimulate their proliferative activity. In contrast, the repeated subcutaneous administration of 1 and 4 nmoles of NMU8, which can give rise to a blood concentration ranging from 10^{-8} to 10^{-7} M (54), was found to increase blood aldosterone, but not the corticosterone level, without significantly altering the proliferative activity of adrenocortical cells. The obvious differences in the biology between *in vivo* and *in vitro* cultured adrenocortical cells may explain these discrepancies.

In vivo findings do not agree with the earlier contention that NMU8 stimulates HPA axis in the rat (see Introduction): neither the ACTH-dependent corticosterone secretion nor adrenal growth were enhanced. Probably the regulation of adrenal function of immature rats differs from that of adult animals. The growth of adrenals connected with the body growth seems to be mainly mediated by the GH-IGF/IGFB system (55), that could not be influenced by exogenous NMU. The NMU8-induced rise in aldosterone plasma level could ensue from either a direct NMUR1-mediated stimulating action on zona glomerulosa (but not zona fasciculata-reticularis) cells or the reported enhanced release of vasopressin (3), which is well known to be a potent aldosterone secretagogue (56). The refractoriness of zona fasciculata-reticularis cell secretion to NMU8 may explain the lack of effect of this peptide on corticosterone secretion from inner adrenocortical-cell primary cultures. However, these cells, which are provided with NMUR1, display a clear-cut proliferative response to NMU8. This observation is not surprising because the growth behavior of adrenocortical cells cultured *in vitro* profoundly differs from that of their *in vivo* counterpart, as evidenced by the fact that ACTH stimulates adrenocortical cell growth *in vivo*, but inhibits proliferation of adrenocortical cells cultured *in vitro* (57).

In conclusion, our present study demonstrates the presence of NMUR1 in immature rat adrenocortical cells, and suggests that NMU may affect their biological activity via an endocrine route. However, the physiological significance and relevance of the adrenal effects of NMU remain to be ascertained.

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