Urotensin-II and UII-receptor expression and function in the rat adrenal cortex

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Abstract. Urotensin-II (UII) is a potent hypertensive peptide, which has been recognized as an endogenous ligand of the G protein-coupled receptor (GPR)-14, now named UT-R. Real-time PCR demonstrated the expression of UII and UT-R mRNAs in both dispersed and *in vitro* cultured rat adrenocortical cells. UII concentration-dependently decreased basal, but not ACTH-stimulated, corticosterone secretion from cultured adrenocortical cells, and the effect was abolished by the UT-R antagonist Palosuran. UII did not affect the proliferation rate of cultured cells. Taken together, these findings suggest that UII may be included in the group of peptides (adrenomedullin, atrial natriuretic peptide, neurotensin and beacon), that, acting in an autocrine-paracrine manner, are involved in the inhibitory tuning of adrenocortical secretion.

Introduction

Urotensin-II (UII) is an 11- (human) or 14-amino acid peptide (rat), originally isolated from the fish urophysis (1), that exerts a potent vasoconstrictory activity (2-4). UII has been identified as an endogenous ligand of the G protein-coupled receptor (GPR)-14, now named UT-R (2,5-7). The wide distribution of UII and UT-R in the heart and large arteries, coupled to the strong hypertensive effect of UII, led to the conclusion that this peptide may play an important role in the physiology and pathophysiology of the cardiovascular (CV) system (8-12).

Evidence has been also provided that UII and UT-R are expressed in the adrenal gland and adrenal tumors (13-17), and UII has been reported to enhance proliferative activity of human adrenocortical carcinoma-derived SW-13 cells cultured *in vitro* (14,18). Due to its hypertensive effect and CV and adrenal distribution, UII has been compared to endothelin (ET)-1 (14,19,20).

ET-1 is know to exert not only a strong proliferogenic but also a major secretagogue action on the adrenal cortex (reviewed in ref. 21), and this prompted us to study the expression of UII and UT-R genes in rat adrenocortical cells and to examine whether UII affects their *in vitro* secretion and growth.

Materials and methods

Animals and reagents. Adult male Sprague-Dawley rats (~200 g body weight) were purchased from Charles-River (Como, Italy), and the experiment protocol was approved by the local Ethics Committee for Animal Studies. Rat UII was obtained from Neosystem Laboratoires (Strasbourg, France), and the UT-R antagonist (UT-RA) Palosuran (ACT-058362) (22) was a generous gift of Dr M. Clozel (Actelion Pharmaceuticals Ltd., Allschwil, Switzerland). Collagenase and deoxyribonuclease were provided by Worthington Biochemical Corp. (Lake Wood, NJ), and iTaq DNA polymerase from Bio-Rad Laboratories (Milan, Italy). ACTH, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), bovine serum albumin (BSA), and all other chemicals and reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Dispersed cell. Rats were decapitated, and their adrenals promply removed under sterile conditions. Adrenals were decapsulated to separate zona glomerulosa, and then halved and enucleated to eliminate medullary chromaffin tissue. Dispersed zona fasciculata-reticularis (inner) cells were obtained by sequential enzymatic digestion (collagenase-I, 2 mg/ml and deoxyribonuclease-I, 0.1 mg/ml in DMEM) and mechanical disaggregation.

In vitro culture. Dispersed inner adrenocortical cells were seeded at a density of $2x10^4$ cells/cm^2 in 24-well polystyrene plates, and cultured for 72 h at 37˚C in DMEM (added with 1.125 g/l sodium bicarbonate, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin) in an atmosphere of

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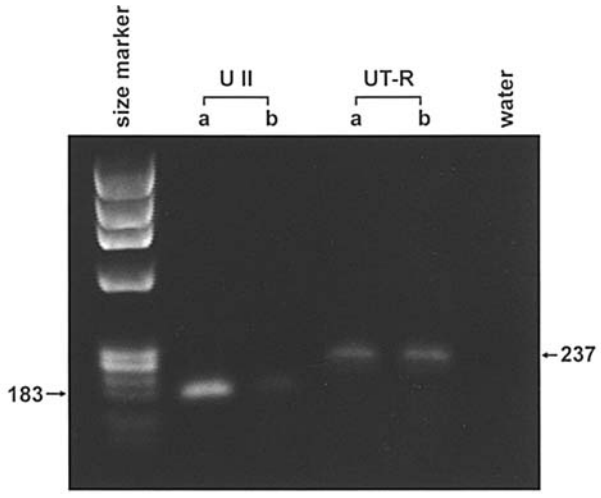

Figure 1. Ethidium bromide-stained 2% agarose gel electrophoresis showing cDNA amplified with rat UII and UT-R specific primers from RNA of exemplary freshly-dispersed (a) and cultured rat adrenocortical-cell samples (b). The first lane was loaded with Roche Marker VIII. No amplification with water instead of RNA is shown as negative control.

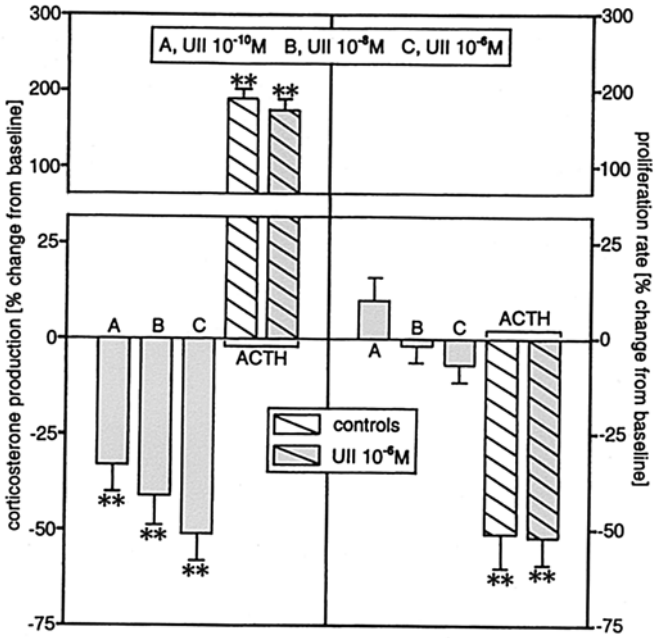

Figure 2. Effects of UII on basal and ACTH-stimulated corticosterone production from (left panel) and basal and ACTH-inhibited proliferative activity of (right panel) rat inner adrenocortical cells cultured *in vitro*. Results are expressed as percent change from baseline and are the mean ± SEM of 6 separate experiments. $^{**}p<0.01$ from the respective baseline value.

95% air-5% CO_2, medium being changed every 24 h (23,24). At day 3 of culture, cells were incubated for 24 h as follows: i) UII (from 10^{-10} to 10^{-6} M); ii) ACTH (10^{-8} M) alone or in the presence of 10^{-6} M UII; and iii) UT-RA (from 10^{-7} to 10^{-5} M) alone or in the presence of 10^{-6} M UII and 10^{-8} M ACTH. Control (baseline) cultures were incubated without any chemical. Medium was collected and stored at -80˚C.

Reverse transcription (RT)-real-time polymerase chain reaction (PCR). Freshly dispersed and cultured control inner adrenocortical cells were harvested and frozen. Total RNA was extracted and reverse transcribed to cDNA (25-28). The RT-reaction solution (1 μl) was added to a mixture (final volume, 25 μl), containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 0.8 mM dNTPs and 25 U/μl iTaq DNA polymerase (29-31). Real-time PCR was performed in a Bio-Rad I-Cycler iQ detection system, using the following protocol: denaturation (95˚C for 3 min), 35 cycles of two steps of amplification (95˚C for 15 sec and annealing for 30 sec), and melting curve (60-90˚C with a heating rate of 0.5˚C/10 sec). Primer sequences, annealing temperature and the predicted size of amplicons were: i) UII (NM_019160): sense-131-5', 5'-AG CTTCCAGTGCTTGAGGAA-3' and antisense-314-3', 5'-GA ATCTTGCCCAGTGAGAGC-3' (60˚C; 183 bp); and ii) UT-R (NM_020537): sense-80-5', 5'-ACTCCAACgTgTCCCTCA AC-3' and antisense-317-3', 5'-AAGGGAATGCTCAGCAG GTA-3' (60˚C; 237 bp). The specifity of amplification was tested at the end of each run by real-time PCR melting analysis, using the I-Cycler iQ software 3.0.

Corticosterone assay. Corticosterone concentration in the incubation media was measured by radioimmune assay, as previously detailed (32). Intra- and inter-assay CVs were 7.0% and 8.3%, respectively.

Cell proliferation. The proliferation rate of cultured cells was assayed by the EZ4U non-radioactive cell proliferation and cytotoxic assay of Biomedica (Vienna, Austria), as described earlier (33,34). Briefly, cultured cells 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 EL-13 (Bio-Tek Instruments, Winooski, VT).

Statistics. Results were expressed as percent change from baseline, and were the mean ± SEM of six separate experiments. The statistical comparison was performed by ANOVA, followed by the multiple range test of Duncan.

Results

RT-PCR revealed UII and UT-R mRNA expression in both freshly dispersed and cultured rat adrenocortical cells (Fig. 1). Real-time PCR melting curve analysis showed well-defined peaks for UII and UT-R genes; thus, ruling out amplification of non-specific products.

UII concentration-dependently lowered basal corticosterone secretion from cultured rat inner adrenocortical cells, without significantly altering the ACTH-stimulated one (Fig. 2). The inhibitory effect of UII was counteracted by UT-RA in a concentration-dependent manner. UT-RA per se neither affected basal corticosterone production nor changed the ACTH-stimulated one (Fig. 3). UII did not induce significant changes in either basal or ACTH-suppressed proliferation rate of cultured adrenocortical cells (Fig. 2).

Discussion

Our RT-PCR findings, showing the expression of UII and UT-R in both freshly dispersed and cultured rat adrenocortical

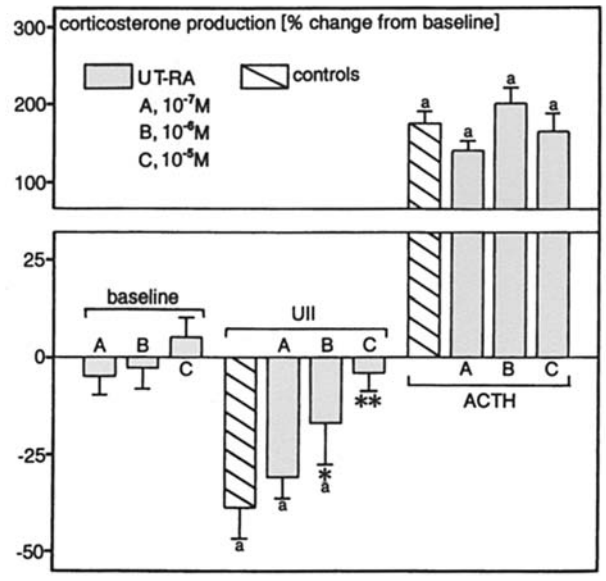

Figure 3. Effect of UT-RA on basal, UII-inhibited and ACTH-stimulated corticosterone production from rat inner adrenocortical cells cultured *in vitro*. Results are expressed as percent change from baseline and are the mean ± SEM of 6 separate experiments. [a]p<0.01 from the respective baseline value; [*]p<0.05 and [**]p<0.01 from the respective control value.

cells, confirm earlier results (13,16) and rule out the possibility that the expression can be ascribed to the extraparenchymal components of the gland (capillary and stromal fibroblasts). Hence, they may suggest a possible autocrine-paracrine mechanism of action of UII in the adrenal cortex, as seems to occur in other peripheral tissues (reviewed in ref. 17). However, our data do not conclusively prove that UII and UT-R genes are expressed at the protein level in adrenals, and further Western blot and immunocytochemical studies are underway to address this issue.

Despite the fact that UII has been claimed to be the new ET-1 (19), we were unable to find a stimulating action of this peptide on the secretory activity of adrenocortical cells. Conversely, our study provides evidence for an inhibitory action of UII on basal, but not ACTH-stimulated, glucocorticoid secretion. The effect appears to be mediated by the UT-R because it is abrogated by the selective UT-RA Palosuran (22). Of interest, the putative UT-RA SB-710411 (35) was ineffective in counteracting the UII antisecretagogue action, suggesting that Palosuran is to be considered the elective drug in the investigations on the adrenal effect of UII.

The comparison of UII to ET-1 was based on the following main lines of evidence: i) UII is the most potent mammalian vasoconstrictor (3), and its plasma concentration was found to be markedly elevated in hypertensive subjects (10,36) and in patients with congestive heart failure (37-40); ii) high UII expression has been detected in coronary atherosclerotic lesions (16,41). Taken together, these observations, coupled to the demonstration that UII induces cardiomyocyte hypertrophy (42,43) and cardiac fibrosis (8,44), led to the conclusion that this peptide, such as ET-1 (45,46), may play a deleterious role in the progression of CV diseases (9-12). However, in the normal heart UII has been shown to exert a strong inotropic effect (47), and a sustained COX- and NO-dependent coronary vasodilatory action (48). Moreover, UII has been recently reported to induce hypotensive responses in both normotensive and spontaneously hypertensive rats (49). These findings are reminiscent of those obtained with the vasodilatory peptide adrenomedullin (AM), which, along with its receptors, is expressed in vessels and heart (50,51) and is thought to exert a major CV protective action (reviewed in ref. 52). This, along with our present findings indicating that UII, such as AM (53), suppresses the secretory activity of the adrenal cortex, could suggest that UII, at least under normal conditions, is to be considered the novel AM, more than the new ET-1.

In keeping with previous findings (32-34, 54), ACTH was found to inhibit *in vitro* growth of rat adrenocortical cells, thereby confirming that the stimulation of specialized secretory functions of adrenocortical cells cultured *in vitro* is coupled with the inhibition of their proliferative activity (reviewed in ref. 55). Although both ET-1 and AM have been shown to exert a clearcut growth-promoting action on adrenocortical cells (reviewed in refs. 21,53), our investigation does not reveal any sizeable effect of UII on the proliferation rate of cultured rat inner cells. This observation disagrees with the reported proliferogenic effect of UII on SW-13 cells (14,18), but it must be taken into account that the physiology of this human adrenocortical carcinoma-derived cell line does not surely reflect that of normal adrenocortical cells (26).

Compelling evidence indicates that several peptides synthesized in adrenal glands are able to control adrenocortical secretion acting in an autocrine-paracrine manner (reviewed in refs. 56,57). Although a large part of these peptides exert a secretagogue effect, others, in addition to AM (53), possess a marked inhibitory action: atrial natriuretic peptide (58-61), neurotensin (62,63), leptin (64-66) and beacon (27,31,33,67). Our study provides the first evidence that UII may be included in this last group of peptides involved in the fine-tuning of adrenocortical secretion.

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