Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis *in vivo* and *in vitro*

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Abstract. Urotensin-II (UII), along its receptor UT-R, is widely expressed in the cardiovascular system, where it exerts regulatory actions under both physiological and pathological conditions. Real-time PCR and immunocytochemistry demonstrated the expression of UII and UT-R as mRNA and protein in rat neuromicrovascular endothelial cells (NECs). UII did not affect the proliferation rate of cultured NECs, but exerted a strong angiogenic action in both an *in vitro* assay on Matrigel and an *in vivo* assay on chorioallantoic membrane. The angiogenic effect of UII was similar to that of FGF-2, and was abolished by the UT-R antagonist Palosuran. Collectively, our findings allow us to include UII in the group of cytokines (e.g. endothelin-1 and adrenomedullin), which are expressed in endothelial cells and exert a pro-angiogenic effect acting in an autocrine-paracrine manner.

Introduction

Urotensin-II (UII) is a cyclic 11- (human) or 15-amino acid peptide (rodent), originally isolated from fish urophysis (1), which exerts a potent systemic vasoconstrictor and hypertensive effect (2,3). UII has been identified as an endogenous ligand of the orphan G protein-coupled receptor (GPR) 14 (2,4,5), that has been renamed urotensin receptor (UT-R) (6).

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UII and UT-R are widely expressed in the heart and large arteries (7-11), and many lines of evidence lead us to conclude that UII plays a role in the physiology and pathophysiology of the cardiovascular system (12-14). UII has also been reported to exert a strong mitogenic action on many cell phenotypes, and the expression of UII and UT-R has been demonstrated in several tumor-derived cell lines (11).

The cardiovascular distribution and the potential tumor growth promoting action of UII prompted us to examine whether UII and UT-R are expressed in endothelial cells (EC) and can affect angiogenesis *in vivo* and *in vitro*.

Materials and methods

Animals and reagents. Male adult Sprague-Dawley rats 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), EC growth medium MV2 from Promo Cell (Heidelberg, Germany), Matrigel® from Becton Dickinson Labware (Bedford, MA) and human recombinant fibroblast growth factor (FGF)-2 from R&D Systems (Abingdon, UK). Goat anti-rat UT-R (E-18, sc-10194) and UII antibodies (C-19, sc-21095) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit anti-goat Cy2- and Cy3-conjugated secondary antibodies from Chemicon (Temecula, CA). Fetal calf serum (FCS), bovine serum albumin (BSA), phosphate-buffered saline (PBS), 5'-bromo-2'-deoxyuridine (BrdU), 4',6'-diamine-2'-phenilindole (DAPI), and all other chemicals and laboratory reagents were provided by Sigma-Aldrich Corp. (St. Louis, MO). The UT-R antagonist (UT-RA) Palosuran (ACT-058362) (15) was a gift from Dr M. Clozel (Actelion Pharmaceutical Ltd., Allschwil, Switzerland).

Neuromicrovascular EC (NEC) culture. Rats were decapitated and their brains were promptly removed. NECs were isolated from the brains, according to Abbot *et al* (16), and cultured and purified by immunoseparation, as

previously described (17,18). In all experiments, cultures from the 2nd to the 4th passage were used.

Real-time RT-PCR. NECs from the 2nd passage were harvested, and total RNA was extracted, purified and reverse transcribed to cDNA (19,21). Real-time PCR was carried out in an I-Cycler iQ detection system (BioRad Laboratories, Milan, Italy), as detailed previously (22-24), using the following primers: i) rat UII: sense-131-5', 5'-AGCTTCCAG TGCTTGAGGAA-3' and antisense-314-3', 5'-GAATCTTGC CCAGTGAGAGC-3' (NM_019160, bp 183); and ii) rat UT-R: sense-80-5', 5'-ACTCCAACGTGTCCCTCAAC-3' and antisense-317-3', 5'-AAGGGAATGCTCAGCAGGTA-3' (NM_020537, bp 237). The PCR program included a denaturation step at 95˚C for 3 min, 35 cycles of two amplification steps at 95˚C for 15 sec and annealing at 60˚C for 30 sec, and melting curve at 60-90˚C with a heating rate of 0.5˚C/10 sec. The specificity of amplification was tested at the end of each run by melting curve analysis, using the I-Cycler software 3.0. The specificity of the PCR was further verified by sequencing analysis, as previously detailed (25).

Immunocytochemistry (ICC). NECs from the 2nd passage were seeded on fibronectin-coated cover slides on a 24-well plate, fixed in 4% paraformaldehyde in PBS, treated with 0.1% Triton X-100 in PBS, and incubated with the blocking solution (3% BSA in PBS) for 60 min. The blocking solution was removed, and NECs were incubated overnight with goat anti-UII or anti-UT-R primary antibodies (1:50 dilution) at 4˚C. Cells were washed, and incubated with anti-goat Cy3- (red fluorescence) or Cy2-conjugated secondary antibodies (green fluorescence) for 60 min at room temperature to evidence UII- and UT-R-labeling, respectively. After repeated washing, cells were counterstained with the nuclear dye DAPI. Negative controls were carried out by similarly treating NECs, but omitting the primary antibody (26). Specimens were observed by a Leitz fluorescence microscope.

Cell proliferation assay. NECs from the 3rd passage were seeded ($1.5x10^4$ cells/cm^2) into a 96-well plate, and cultured in MV2 medium, with 1% amphotericin/B-gentamycin, 5% FCS, 0.2 μg/ml hydrocortisone and 1 μg/ml ascorbic acid (basal medium). After 24 h of incubation, the culture medium was replaced with a fresh one containing or not containing FGF-2 (50 ng/ml), and UII (10^{-7} M) and/or Palosuran (10^{-6} M), and incubated at 37˚C. After 6 h of incubation, BrdU (10^{-5} M) was added (17), and NECs were incubated for a further 18 h. The proliferation rate was estimated by the cell proliferation kit of Amersham Pharmacia (Aylesbury, UK). Results were expressed as percent change from baseline and were the mean ± SD of 6 separate experiments.

In vitro angiogenesis assay. Matrigel was thawed on ice overnight, and spread evenly (50 μl) over each well of a 24-well plate. The plates were incubated for 30 min at 37˚C to allow Matrigel to gel, and NECs from the 4th passage were seeded ($2.5x10^4$ cells/cm^2) and cultured in basal medium, containing or not containing i) UII (10^{-7} M) and/or Palosuran (10^{-6} M); and ii) FGF-2 (50 ng/ml) and/or UT-RA (10^{-6} M). After 18 h of incubation at 37˚C, cultures were photographed and phase contrast images were recorded, using a digital camera (DG 200; Leica imaging system, Cambridge, UK) connected to a Laborlux S microscope (Leitz, Wetzlar, Germany) and saved as TIFF files. Image analysis was carried out using the Leica Qwin software, as previously detailed (27). Values were expressed as difference (± Δ) from baseline values, and were the means ± SD of 4 separate experiments.

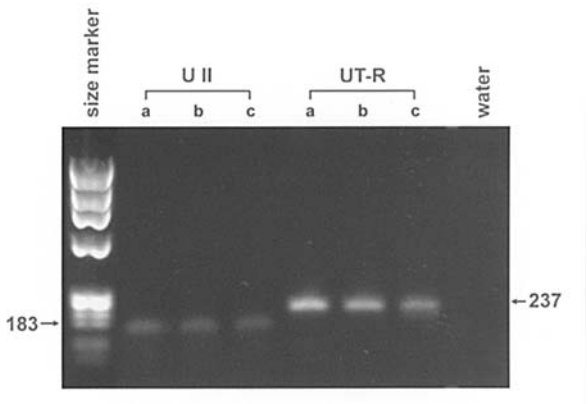

Figure 1. Ethidium bromide-stained 2% agarose gel electrophoresis showing cDNA amplified with rat UII and UT-R specific primers from RNA of three exemplary cultured NECs. The first lane was loaded with Roche marker VIII. No amplification with water instead of RNA is shown as negative control.

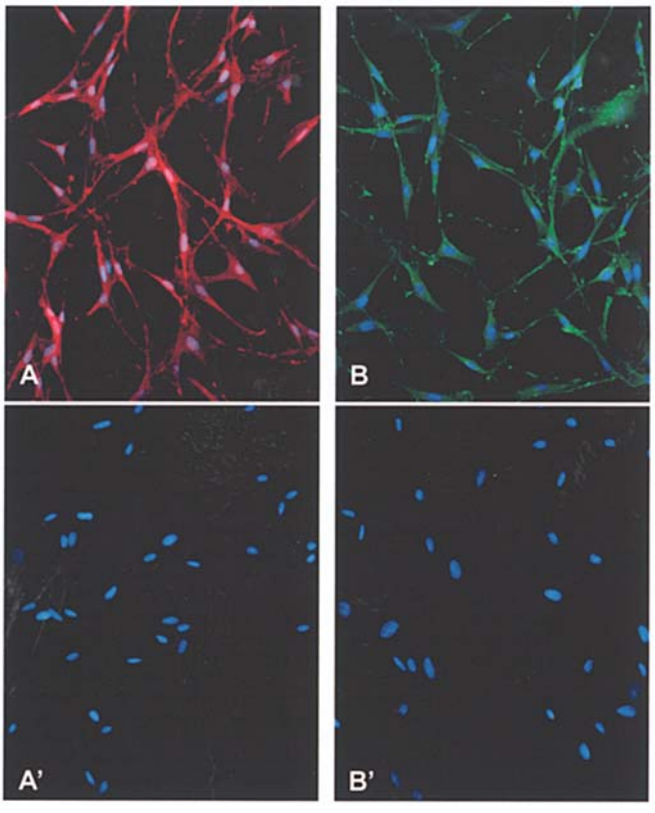

Figure 2. ICC demonstration of UII (red immunofluorescence) (A) and UT-R (green immunofluorescence) (B) in NECs. The respective negative controls are shown in the lower panels (A' and B'). DAPI-stained nuclei appear as blue structures. Magnification x180.

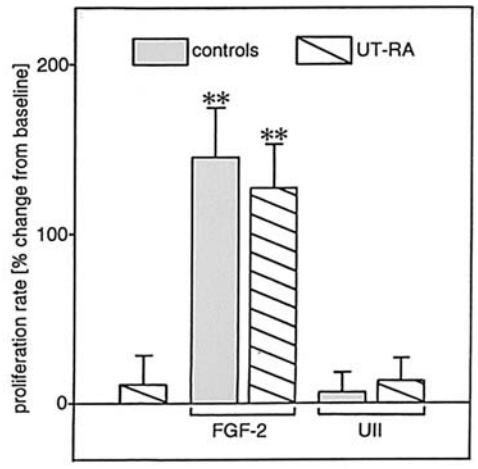

Figure 3. Effects of FGF-2 (50 ng/ml), UII (10^{-7} M) and the UT-RA Palosuran (10^{-6} M) on the proliferation rate of cultured NECs. Bars are means ± SD of six separate experiments. **P<0.01 from baseline.

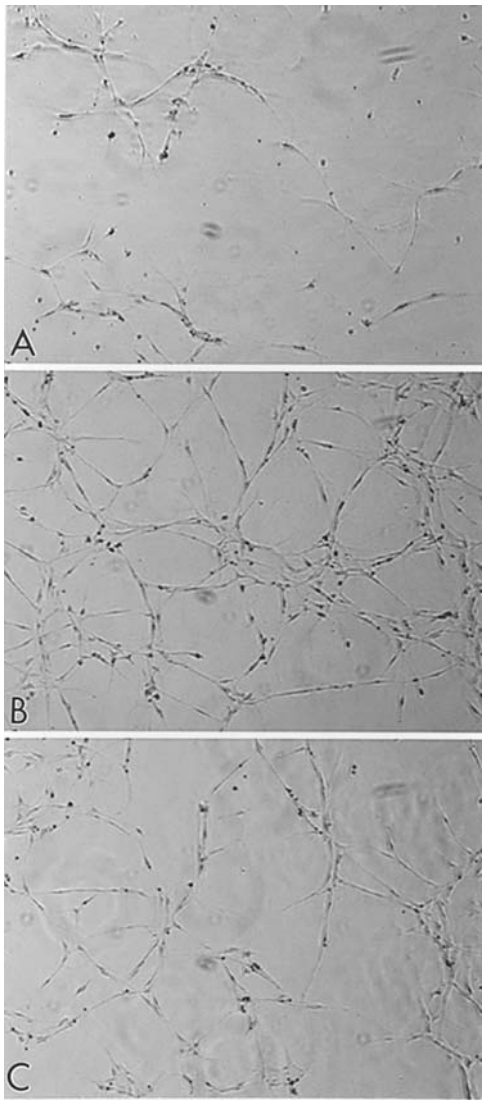

Figure 4. Phase contrast micrographs illustrating the arrangement of NECs into a meshwork of capillary-like tubular structures when cultured on Matrigel for 24 h (A). UII (10^{-7} M) increases the density of the meshwork (B), and the UT-RA Palosuran (10^{-6} M) counteracts this effect (C). Magnification x60.

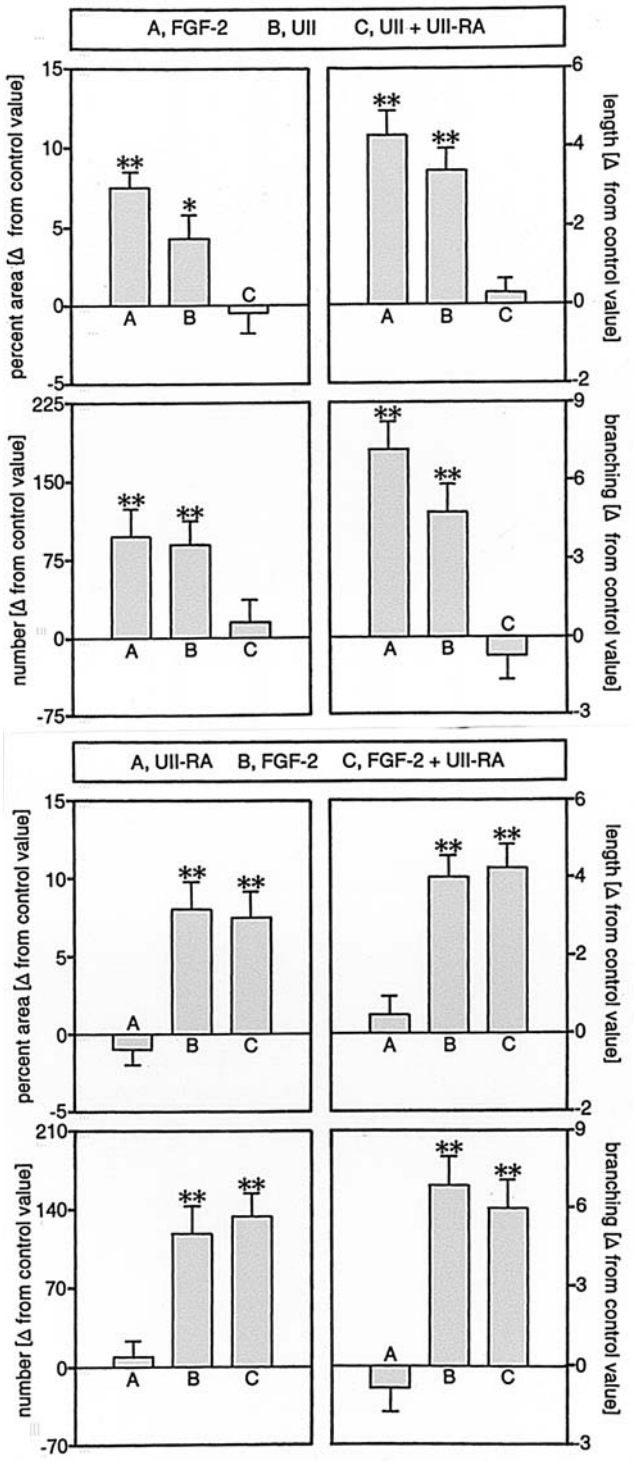

Figure 5. Quantitative analysis of the effects of FGF-2 (50 ng/ml), UII (10^{-7} M) and the UT-RA Palosuran (10^{-6} M) on the dimensional [percent area covered by NECs and total length per field (length)] and topological parameters [number of meshes per field (number) and number of branching points per field (branching)] of the NEC meshwork. Bars are means ± SD of four separate experiments. *P<0.05 and **P<0.01 from control value.

In vivo angiogenesis assay. Chorioallantoic membrane (CAM) assay was carried out as previously described (28). Briefly, fertilized white-leghorn chicken eggs (10 per group) were incubated at 37˚C under constant humidity. On incubation day 3, a square window was opened in the shell to detach the developing CAM after removal of 2-3 ml of albumen. The

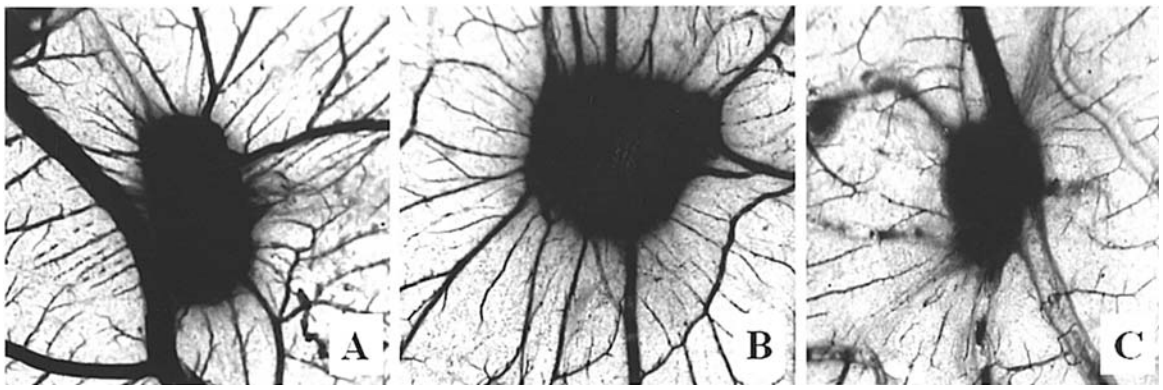

Figure 6. Macroscopic pictures of gelatin sponges soaked with UII (A), FGF-2 (B) and UII plus the UT-RA Palosuran (C), implanted onto the chick embryo chorioallantoic membrane on day 12 of incubation. Note that in A and B numerous allantoic vessels are radially arranged toward the implants, while in C few vessels are recognizable. Magnifications x36.

window was sealed with a glass and eggs were returned to the incubator. On incubation day 8, a 1 mm^3 sterilized gelatin sponge (Gelfoam Upjohn, Kalamazoo, MI) was placed on the top of the growing CAM. Sponges were loaded with 1 μl vehicle (PBS), containing or not containing UII (10^{-7} M), UII plus UT-RA (10^{-6} M), and 500 μg FGF-2. CAM were examined daily until day 12 and photographed *in ovo* with a stereomicroscope equipped with a MC63 camera system (Zeiss, Oberkochen, Germany). On day 12, blood vessels entering the sponge within the focal plane of the CAM were counted by two observers in a double-blind fashion at a magnification of x50, and mean values ± SD were determined.

Statistical analysis. The statistical comparison of results was performed by ANOVA, followed by Student's t-test for unpaired data.

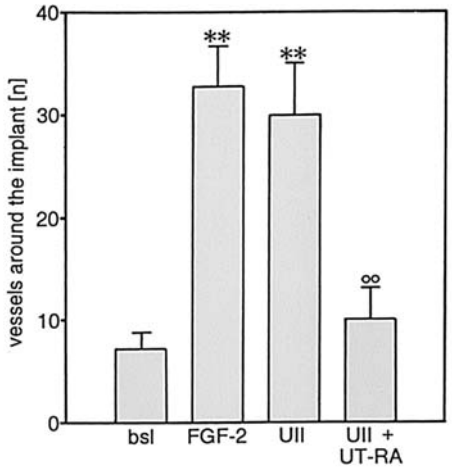

Figure 7. Quantitative analysis of the effects of FGF-2 (50 ng/ml), UII (10^{-7} M) and the UT-RA Palosuran (10^{-6} M) on the angiogenesis in the CAM. Bars are means ± SD of eight separate experiments. $^{**}P<0.01$ from baseline value; $^{\circ\circ}P<0.01$ from FGF-2 and UII values.

Results

RT-PCR revealed a low expression of UII mRNA and an elevated expression of UT-R mRNA in all NEC specimens examined (Fig. 1). The melting curve analysis showed clean defined peaks at temperatures of 91 and 90˚C for UII and UT-R genes, respectively, thus ruling out the amplification of non-specific products. ICC showed intense immunostaining for both UII and UT-R in NECs (Fig. 2).

FGF-2 significantly raised the proliferation rate of cultured NECs, while UII or Palosuran were ineffective (Fig. 3). After seeding on Matrigel, NECs spread and alligned with each other to form branching anastomosing tubes with multicentric junctions that gave rise within 24 h to a meshwork of capillary-like structures (Fig. 4A). UII (10^{-7} M) increased the density of the meshwork, and Palosuran counteracted this effect (Fig. 4B and C). Image analysis confirmed these observations, showing that UII (10^{-7} M), like FGF-2, significantly increased both dimensional (percent area covered by NECs and total length of the network per field) and topological parameters (number of meshes and branching points per field) of the capillary-like meshwork, and Palosuran annulled this effect (Fig. 5). The UT-RA Palosuran was *per se* ineffective, and did not alter the pro-angiogenic action of FGF-2 (Fig. 5). UII (10^{-7} M), added to the CAM, induced a strong angiogenic response, comparable to that of FGF-2. When Palosuran was added together with UII, a significant inhibition of the angiogenic response was observed in 70% of the treated embryos (Figs. 6 and 7).

Discussion

Our angiogenic assays provide unequivocal evidence that UII exerts a potent angiogenic action *in vitro* and *in vivo*, comparable to that of one of the main classic angiogenic cytokines, FGF-2 (29). This effect of UII appears to be selectively mediated by UT-R, because it is annulled by the specific UT-RA Palosuran (15). Palosuran neither *per se* affects basal angiogenesis nor alters the pro-angiogenic action of FGF-2, thereby ruling out the possibility of its non-specific toxic effect. Remarkably, the pro-angiogenic action of UII, in contrast with that of FGF-2, is not associated with a proliferogenic effect on NECs. This would suggest that different mechanisms underlie the *in vitro* actions of these two cytokines.

The present coupled RT-PCR and ICC findings indicating that not only UT-R, but also UII is expressed in NECs, are consistent with a possible autocrine-paracrine mechanism of action. In this regard, it is to be noted that there is evidence of an autocrine-paracrine action of UII in the adrenal glands, where this peptide negatively modulates *in vitro* glucocorticoid secretion (30). Hence, our study suggests that UII may be included in the group of non-classic pro-angiogenic cytokines expressed in ECs, which, in addition to regulating cardiovascular function, also exert a tumor growth-promoting action.

The main members of this cytokine family are endothelin-1 (31) and adrenomedullin (32), which are both known to be potent angiogenic peptides (33,34) and are thought to be the potential targets of antineoplastic therapies (35,36). Of interest, the putative UT-RA SB-710411 (37) was not found to block the *in vitro* and *in vivo* angiogenic action of UII (data not shown), suggesting that Palosuran may be an elective drug of potential relevance in anti-angiogenic therapeutic strategies.

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