Expression and Functional Role of Urotensin-II and Its Receptor in the Adrenal Cortex and Medulla: Novel Insights for the Pathophysiology of Primary Aldosteronism

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Context: The involvement of urotensin II, a vasoactive peptide acting via the G protein-coupled urotensin II receptor, in arterial hypertension remains contentious.

Objective: We investigated the expression of urotensin II and urotensin II receptor in adrenocortical and adrenomedullary tumors and the functional effects of urotensin II receptor activation.

Design: The expression of urotensin II and urotensin II receptor was measured by real time RT-PCR in aldosterone-producing adenoma (n = 22) and pheochromocytoma (n = 10), using histologically normal adrenocortical (n = 6) and normal adrenomedullary (n = 5) tissue as control. Urotensin II peptide and urotensin II receptor protein were investigated with immunohistochemistry and immunoblotting. To identify urotensin II-related and urotensin II receptor-related pathways, a whole transcriptome analysis was used. The adrenocortical effects of urotensin II receptor activation were also assessed by urotensin II infusion with/without the urotensin II receptor antagonist palosuran in rats.

Results: Urotensin II was more expressed in pheochromocytoma than in aldosterone-producing adenoma tissue; the opposite was seen for the urotensin II receptor expression. Urotensin II receptor activation *in vivo* in rats enhanced (by 182 ± 9%; $P < 0.007$) the adrenocortical expression of immunoreactive aldosterone synthase.

Conclusions: Urotensin II is a putative mediator of the effects of the adrenal medulla and pheochromocytoma on the adrenocortical zona glomerulosa. This pathophysiological link might account for the reported causal relationship between pheochromocytoma and primary aldosteronism. **(*J Clin Endocrinol Metab* 94: 684–690, 2009)**

The functional regulation of the adrenal gland involves paracrine interactions of numerous peptides (for review, see Refs. 1 and 2). Urotensin II (UII), a vasoactive peptide acting through specific UII receptors (UT-Rs) (3–6), is widely expressed in many tissues (7–12), including the vasculature and the adrenal gland (13–15). The finding of UII and UT-R gene transcripts in aldosterone-producing adenoma (APA) and pheochromocytoma (Pheo) (14, 16) suggested the participation of UII in the regulation of blood pressure and body fluid homeostasis; however, the quantitative expression of UII and UT-R in APA and Pheo and the biological effects of UII in the adrenal gland were unknown. Therefore, molecular techniques were used to quan-

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Abbreviations: Ang II, Angiotensin II; APA, aldosterone-producing adenoma; CYP11B1, 11β hydroxylase; CYP11B2, aldosterone synthase; GALR3, galanin receptor 3; GPR153, G protein-coupled receptor 153; NAC, normal adrenocortical; NAM, normal adrenomedullary; Pheo, pheochromocytoma; UII, urotensin II; UT-R, UII receptor.

TABLE 1. Anthropometric and biochemical features of the patients with APA and Pheo

	APA (n = 22)	Pheo (n = 10)
Age (yr)	50 ± 11	41 ± 9.6
Gender (♂:♀)	7:15	6:4
Systolic blood pressure (mm Hg)	170 ± 16	149 ± 7
Diastolic blood pressure (mm Hg)	102 ± 11	103 ± 7
Serum K^+ concentration (mmol/liter)	3.0 ± 0.13	3.8 ± 0.2
Plasma renin activity (ng Ang I/ml · h)	0.44 (0.09–1.90)	NA
Plasma aldosterone concentration (ng/dl)	51.2 ± 10.2	NA
Aldosterone renin ratio (ng/dl)/(ng/ml · h)	211 ± 54	NA
Cortisol plasma concentration (ng/ml)	162 (46–275)	NA
Urinary metanephrine (μmol/24 h)	NA	1.9 ± 0.60
Urinary normetanephrine (μmol/24 h)	NA	6.45 ± 4.55

Means ± SEM or (range). Normal values are: for serum K^+ concentration, 3.6–4.5 mmol/liter; for plasma renin activity, 0.65–2.65 ng Ang-I/ml · h; for plasma aldosterone concentration, <11.0 ng/dl; for plasma cortisol concentration, 50–250 ng/ml; for urinary metanephrine, 0.4–1.5 μmol/24 h; for urinary normetanephrine, 0.57–1.9 μmol/24 h. NA, Not available.

tify precisely the UII and UT-R transcripts in APA and Pheo and to pinpoint UII- and UT-R-related pathways in adrenocortical and adrenomedullary tumors. The presence of the UII peptide was investigated by immunoblotting and immunohistochemistry in APA and Pheo. Finally, the role of UT-R activation on the adrenocortical expression of aldosterone synthase (CYP11B2) and 11β hydroxylase (CYP11B1) was investigated by means of an *in vivo* UII infusion taking advantage of palosuran, a specific UT-R antagonist (17–19).

Patients and Methods

Adrenal specimens

Adrenocortical tissues from 22 patients with APA and adrenomedullary tissues from 10 patients with Pheo were investigated. Histologically normal adrenocortical (NAC) and normal adrenomedullary (NAM) tissue obtained from six patients with renal cancer carcinoma undergoing unilateral nephrectomy and ipsilateral adrenalectomy and from five patients adrenalectomized for nonfunctioning incidentally discovered adrenal mass ("incidentaloma") were studied as controls for the APA and Pheo tissues, respectively. The use of tissues followed our institutional guidelines; all patients gave an informed consent. The tissues obtained under sterile conditions at surgery were immediately frozen in liquid nitrogen and stored at −80 C until they were used as described (20). The diagnosis of APA was based on lateralization of aldosterone secretion at adrenal vein sampling (21), surgery, pathology, and follow-up data. At follow-up, demonstration of normokalemia and cure or improvement of hypertension, as previously defined (22, 23), were required. Pheo was diagnosed according to state-of-the-art criteria (24) and confirmed at pathology, histology, and immunostaining for chromogranin A and synaptophysin.

Measurement of UII and UT-R mRNAs

UII and UT-R mRNAs were measured with a real time RT-PCR with Universal ProbeLibrary Probes and Universal ProbeLibrary Assay Design by ProbeFinder Software (Roche, Monza, Italy) (www.lc480.it) in the LightCycler 480 Instrument (Roche). Porphobilinogen deaminase, an accepted housekeeping gene for both adrenal cortex and medulla gene expression studies, was similarly processed to normalize for RNA quantity and reverse transcription efficiency (25). The specificity of the amplicons was verified by sequencing analysis. UII and UT-R expression in APA and Pheo was calculated relative to porphobilinogen deaminase, used as an internal control, and to the control pools of NAC and NAM tissue, used as calibrators, for APA and Pheo, respectively. Quantification of gene expression was carried out by comparative Ct (2-ΔΔCt) method.

Two-color microarray-based gene expression

cRNA was synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification Kit and the Two-Color RNA Spike-In (Agilent Technologies, Palo Alto, CA) (26), as detailed in Supplemental Methods (published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The Labeled/Amplified cRNA was hybridized on an oligomicroarray chip (Whole Human Genome Microarray Kit, 1 × 44K, G4112A; Agilent Technologies) that contains about 44,000 60-mer *in situ* synthesized sequences representing the whole human genome. Details for data analysis are reported in Supplemental Methods.

UII and UT-R protein expression and immunohistochemistry

Immunoblot analysis with a goat polyclonal antibody was used to investigate the expression of UII and UT-R at the protein level, as detailed in Supplemental Methods. The UII antibody is directed against the C-terminal portion of the UII precursor from which mature UII originates. The membranes were incubated overnight at 4 C with a primary antibody against UII (1:500 dilution) or UT-R (1:500), both from Santa Cruz Biotechnology (Santa Cruz, CA) and then with the horseradish peroxidase conjugated secondary antigoat antibody (1:5000; Amersham Biosciences Europe, Freiburg, Germany). Detection was made with the enhanced chemiluminescence system from Pierce (CELBIO, Milan, Italy). Blots were analyzed by the Quantity One Program of VersaDOC 1000 (Bio-Rad, Milan, Italy). Deglycosylation of protein extracts was performed by Enzymatic Protein Deglycosylation Kit (Sigma, Milan, Italy).

TABLE 2. Relative expression (ΔΔCt) of the UII and UT-R genes and their ratio in the normal adrenal medulla and cortex and in adrenomedullary and adrenocortical tumors

	UII mRNA (arbitrary units)	UT-R mRNA (arbitrary units)	UII/UT-R ratio
Adrenomedullary tissue			
Pheo	1.11 (0.00–3.45)[b]	0.06 (0.00–0.51)[a,c]	4.79 (0.00–69.39)
Normal medulla	1.0 (0.17–1.83)	1.00 (0.15–1.85)	1.00 (0.00–3.78)
Adrenocortical tissue			
APA	0.05 (0.00–0.43)[a,c]	10.5 (0.7–18.9)[c,d]	0.013 (0.00–0.128)
Normal cortex	1.33 (0.00–2.73)	1.0 (0.00–3.5)	1.00 (0.44–1.56)

Data are expressed as median (interquartile range).

[a] $P < 0.01$ *vs.* NAC.

[b] $P < 0.035$ *vs.* APA.

[c] $P < 0.05$ *vs.* NAM.

[d] $P < 0.001$ *vs.* Pheo.

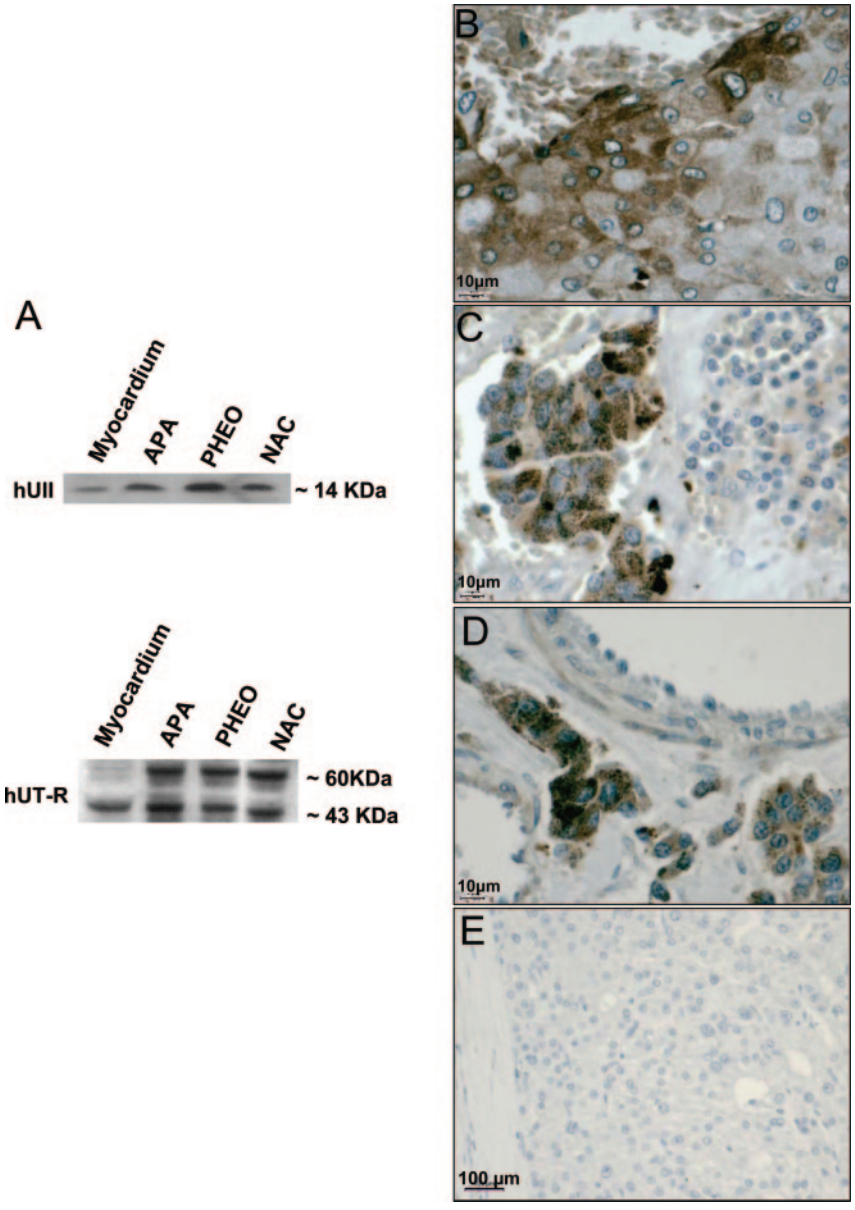

FIG. 1. A, Representative immunoblot analysis of human UII and UT-R protein expression. The *upper panel* shows expression of UII precursor of 124 amino acids with the expected molecular mass of about 14 kDa in the human myocardium (tested as positive control), APA, Pheo, and NAC. The *lower panel* shows expression of UT-R of the expected molecular mass (43 kDa) from homogenates of normal human myocardium. In APA, Pheo, and NAC, an additional 60-kDa band was found. B–E, Immunohistochemistry with a specific antibody against UII showed a staining of Pheo tissue that was more prominent in some islets of cells (B and C) often located perivascularly (D). No staining was found when the tissue section was processed in an identical way but with omission of the primary antibody (E) or after preincubation with UII.

Details for immunohistochemistry are given in Supplemental Methods.

UT-R activation in rats

To investigate whether the UT-R activation up-regulates the adrenocortical expression of the CYP11B2 and CYP11B1, UII (600 pmol/kg · h) (27) was infused by osmotic minipumps (model 2ML2; Alzet, Palo Alto, CA) either alone or on top of the UT-R antagonist palosuran (300 mg/kg) (18) into normotensive male Sprague Dawley rats (of about 250 g body weight; n = 11). Angiotensin II (Ang II) (700 μg/kg · d) was infused in parallel as positive control (28). After 1-wk infusion, the rats were killed, the adrenal gland was snap-frozen in isopentane, precooled on dry ice, and then stored under liquid nitrogen until used for immunohistochemistry and gene expression studies. Specific antibodies against the rat CYP11B2 or CYP11B1 (29) were used for immunohistochemistry. Binding was detected in the tissue sections processed as described in Supplemental Methods. Negative controls were performed by omission of the primary antibody. Sections were mounted and examined at 5× magnification, using a Leica DQ optical microscope. The immunoreactive area was then calculated with a specific routine developed to detect in an operator-independent fashion the brownish staining as percentage of the total adrenal cortex.

Plasma aldosterone concentrations were measured before and after the UII infusion as described (27).

Statistical analysis

Natural logarithm transformation of UII and UT-R genes expression was undertaken to achieve a normal distribution. Comparison of transcript levels between the normal adrenal tissue, the APA, and the Pheo tissue was carried out with ANOVA and Bonferroni *post hoc* test. The statistical analysis was carried out with the SPSS for Windows software (version 15.01; SPSS Italy Inc., Bologna, Italy); the microarray results were analyzed as reported (26).

Results

Patient characteristics

The main features of patients with APA and Pheo are shown in Table 1. The APA patients showed overtly elevated plasma aldosterone levels and aldosterone renin ratio (ARR), and low values of serum K^+ and plasma renin activity (22). The Pheo patients had markedly increased urinary metanephrine and normetanephrine excretion. All patients showed cure of the hyperaldosteronism or the Pheo and a marked fall of blood pressure after adrenalectomy.

Quantitative real time RT-PCR

UII and UT-R gene mRNAs were detected in all human adrenal tissues. The specificity of the amplicons was confirmed by sequencing. The UII and UT-R expression showed intermediate values in both the NAC and NAM tissue; however, UII was significantly more expressed in Pheo than in APA, whereas UT-R showed an opposite expression pattern (Table 2). Thus, the ratio of UII/UT-R mRNA differed markedly between Pheo and APA. No correlation between the UII/UT-R mRNA and plasma aldosterone, the aldosterone renin ratio, and mean blood pressure before adrenalectomy was found in the APA.

Immunoblotting and immunohistochemistry

Immunoblot analysis showed the UII precursor peptide and UT-R protein in the NAC and APA and Pheo tissue (Fig. 1A).

Under denaturing conditions, all adrenal specimens showed, besides the expected UT-R band found in the positive control (myocardium), two additional bands, one of about 60 kDa and one of about 80 kDa. After deglycosylation, the 60-kDa band disappeared.

Immunohistochemistry confirmed the UII (precursor and peptide) expression in Pheo (Fig. 1, B–D); it showed prominent staining of groups of cells arranged in nodules and scattered throughout the tissue and sometimes located perivascularly. Only a weak immunostaining for UII could be occasionally detected in the APA.

The specificity of UII detection was confirmed by lack of staining upon tissue exposure to the secondary antibody and the detection reagents after omission of the primary antibody (Fig. 1E) and after preabsorption with UII.

Gene expression profiling by microarrays

With a whole transcriptome analysis, we found marked differences of expressed sequences between APA and Pheo (relative to their respective controls): 30 sequences were overexpressed and 435 underexpressed in APA; 3213 were overexpressed and 1064 underexpressed in Pheo. APA showed a lower UII gene expression than NAC. By contrast, Pheo exhibited over- and underexpression of UII and the UT-R, respectively, compared with NAM tissue.

We used the same whole transcriptome approach to pinpoint genes whose expression clustered with the UII and UT-R genes in adrenocortical tissues. An agglomerative clustering algorithm was applied to the APA and Pheo transcriptome profiles to identify putative mediators of UT-R signaling. This allowed pinpointing 100 sequences that clustered with the UT-R in either APA or Pheo. Of these, 18 sequences exhibited a similar expression trend in both APA and Pheo (Table 3). Few sequences corresponded to as yet functionally unknown genes; among them we could identify two G protein-coupled receptors [G protein-coupled receptor 153 and galanin receptor 3 (GALR3)], nine transcription mediators, two regulators of cell differentiation/apoptosis (BMP8A and CARD9), and three genes encoding for enzymes implicated in protein metabolism processes (ADAMTS7, DUSP15, and MSRA).

Effect of chronic activation of the UT-R on the rat adrenal cortex

UII infusion markedly increased the CYP11B2 expression in the adrenocortical zona glomerulosa of normotensive rats. This increase (Fig. 2, A and C) was associated with a significant increase of plasma aldosterone concentration (Fig. 3); it was more striking than that induced by a high dose of Ang II (Fig. 3B) and was blunted by palosuran (Fig. 3D). Quantitative analysis of CYP11B2 immunostaining confirmed that the effect of UII occurred via UT-R (Fig. 2E). Both UII and Ang II increased also CYP11B1 expression, but for UII this effect was less marked than that on CYP11B1 (data not shown) and nonspecific because it was unaffected by palosuran. Therefore, the specific stimulation of CYP11B2 by UII was more evident after correction for the effect on CYP11B1 (Fig. 2E).

Discussion

The ubiquitous expression of UII and UT-R in vascular tissue (30) and the intimate relationship of the vasculature with the adrenal cortex and medulla suggested that blood vessel wall-derived UII could modulate the release of adrenal hormones and thereby regulate blood pressure.

Takahashi *et al.* (14) reported the transcripts of UII and its receptor in four APA and seven Pheo and an increased UII-like immunoreactivity in one of six Pheo. Morimoto *et al.* (31) confirmed immunostaining for both UII and UT-R in Pheo, and for UII in the adrenal medulla and to a lesser extent in the cortex and in adrenocortical tumors. At variance, Zeng *et al.* (16) described a lower UII and UT-R expression in Pheo tissue, compared with

TABLE 3. *Homo Sapiens* genes that cluster with the UT-R in APA and Pheo by oligomicroarray analysis

Gene name	Accession no.	Description	Function
ADAMTS7	NM_014272	ADAM metallopeptidase with thrombospondin type 1 motif, 7	Proteolysis
BHLHB4	NM_080606	Basic helix-loop-helix domain containing, class B, 4	Transcription
BMP8A	NM_181809	Bone morphogenetic protein 8a	Cell differentiation
CARD9	BC070091	Caspase recruitment domain family, member 9	Regulation of apoptosis
CEBPA	NM_004364	CCAAT/enhancer binding protein	Transcription
DUSP15	NM_080611	Dual specificity phosphatase 15	Protein amino acid dephosphorylation
EN2	NM_001427	Engrailed homolog 2	Transcription
FOXQ1	NM_033260	Forkhead box Q1	Transcription
GALR3	NM_003614	Galanin receptor 3	Neuropeptide signaling pathway
GPR153	NM_207370	G protein-coupled receptor 153	G protein-coupled receptor protein signaling
GRIN1	NM_021569	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	Ion transport
MLL3	NM_170606	Myeloid/lymphoid or mixed-lineage leukemia 3	Transcription
MSRA	NM_012331	Methionine sulfoxide reductase A	Protein metabolic process
PHOX2A	NM_005169	Paired-like (aristaless) homeobox 2a	Transcription
SOX1	NM_005986	SRY (sex-determining region Y)-box 1	Transcription
SOX17	NM_022454	SRY (sex-determining region Y)-box 17	Transcription
TSPAN10	NM_031945	Tetraspanin 10	Unknown
ZNF579	NM_152600	Zinc finger protein 579	Transcription

The sequences reported showed an expression profile similar to UT-R, *e.g.* high in APA and low in Pheo.

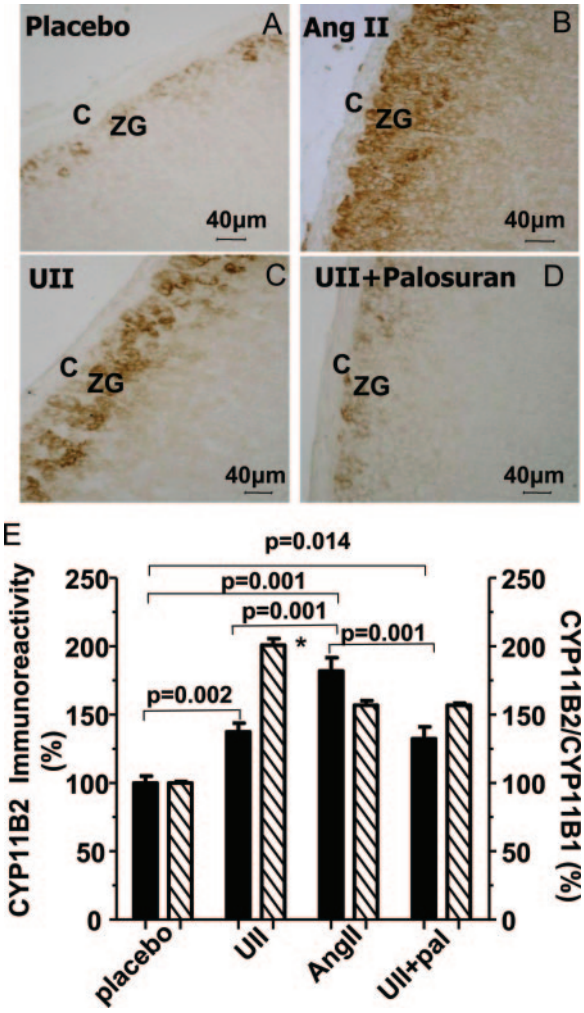

FIG. 2. Immunohistochemistry shows staining of the rat adrenocortical zona glomerulosa (ZG) with a specific antibody for the CYP11B2 (A–D), after 1-wk infusion of vehicle (placebo, A), Ang II (B), UII (C), or UII on top of the UT-R antagonist palosuran (D). C, Capsula; ZG, zona glomerulosa. E, The histogram shows results of quantification of the percentage immunostaining performed in an operator-independent way. Results for CYP11B2 are expressed by attributing 100% to the staining observed with vehicle. The ratio of CYP11B2/CYP11B1 immunostaining (*dashed bars, right y-axis*) showed a significant (*, $P < 0.05$) increase only for the group infused with UII compared with all the other groups.

the normal adrenal tissue, but they provided neither peptide measurement nor comparison with NAM tissue as referent. Therefore, it was not only contentious whether UII was differentially expressed between the normal adrenal cortex and medulla and between Pheo and APA, but also, more importantly, whether UII played any functional role in the adrenal gland.

The present results can fill this gap of knowledge; notwithstanding a relatively large spread of the data, the Pheo exhibited higher UII mRNA content than the APA (Table 2), which showed the lowest level among all adrenal tissues examined.

It is noteworthy that an opposite expression profile of UT-R transcripts across adrenal tumors was detected: the APA showed higher mRNA levels than the Pheo and the normal adrenal medulla, suggesting down-regulation of UT-R when UII expression is enhanced and vice versa. These differences suggest a pathophysiological role of UII in the adrenal gland.

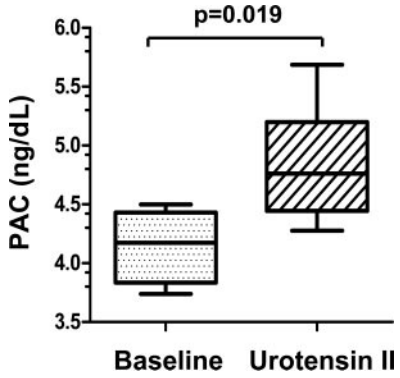

FIG. 3. Plasma aldosterone concentration (PAC) in the male Sprague Dawley rats before and after the infusion of UII (600 pmol/kg · h) for 1 wk via osmotic mini-pumps.

Our studies also showed the UII peptide and the UT-R protein in the normal adrenal cortex and in the APA and Pheo tissue. At immunoblotting, besides the expected 43-kDa band (detected in the human myocardium), we found two additional bands of 60 and 80 kDa in all adrenal specimens (Fig. 1A, *bottom*). The disappearance of the 60-kDa band after protein deglycosylation indicates the occurrence of a glycosylated form of UT-R in the adrenal. The persistence of the 80 kDa after this treatment in APA suggests the presence of UT-R dimerization, a finding that deserves further specific research. Overall, the UII overexpression in Pheo suggests a major role of UII in the pathophysiology of these tumors and particularly in a subset of them that have been causally associated with primary aldosteronism (32–36).

Detection and localization of UII in Pheo tissue and related pathway

Immunohistochemistry confirmed that UII peptide is synthesized in Pheo (31) and furnished novel information on its localization. It showed a specific staining of clusters of Pheo cells, sometimes arranged in nests and located in perivascular regions (Fig. 1, B–D).

The differences of UII and UT-R transcripts between APA and Pheo detected with a whole transcriptome analysis and using the NAC and NAM as reference could be confirmed by RT-PCR, thus extending previous findings (14, 16). Moreover, we identified 18 sequences (Table 3) with an expression profile similar to that of UT-R, *e.g.* high in APA and low in Pheo. Of these, two pertain to G protein-coupled receptors: the galanin receptor 3 (GALR3) and G protein-coupled receptor 153 (GPR153). GALR3 binds galanin, a neuropeptide that is synthesized in the adrenal medulla, and Pheo and is involved in the regulation of adrenocortical function (37). GPR153 encodes an orphan receptor not characterized as yet (38). Of the other sequences, SOX1 and SOX17 entail transcription factors belonging to a class of genes known to be expressed during adrenal fetal development (39). Therefore, these findings emphasize the importance of the interactions between the medulla and cortex and implicate these novel mediators in the UII-related molecular mechanisms.

Effects of UT-R activation in the rat adrenal cortex

We explored the functional effect of a chronic UT-R overactivation on CYP11B2 or CYP11B1 expression with a 1-wk in-

fusion of UII in the presence or absence of an UT-R antagonist in normotensive rats. This experiment was made possible by two recent accomplishments: 1) the development of specific antibodies for these enzymes (29); and 2) the discovery of the UT-R antagonist palosuran (18). UII markedly increased the plasma concentration of aldosterone (Fig. 3) and the CYP11B2 expression in the zona glomerulosa (Fig. 2); this latter effect was blunted by palosuran, thus indicating that it occurred via UT-R activation. Moreover, the UII-induced stimulation of CYP11B2 is likely to be physiologically relevant because the result was more potent than that elicited by a high dose of Ang II, the most widely known secretagogue for aldosterone. Hence, our data support the hypothesis that UII synthesized in the adrenal medulla, and to a larger extent in Pheo, stimulates aldosterone secretion by specifically turning on the expression of the CYP11B2 gene in the zona glomerulosa.

Conclusions

The present results showed the expression of UII in the normal human adrenal gland, in APA and, at a higher level, in Pheo tissue. The opposite trend of expression of UII receptor between APA and Pheo, along with the differences of genes implicated in UII signaling, supports a role of UII in the paracrine interactions between the adrenal medulla and cortex (1, 2). Being relevant for the regulation of adrenal gland function and for the pathophysiology of Pheo and APA, these interactions might provide a mechanistic explanation for the Pheo that presented clinically as aldosteronism (32–36).

Acknowledgments

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Disclosure Statement: The authors of this manuscript have nothing to declare.

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