Chapter 11
Hormone-Dependent Changes in Female Urinary Proteome

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Abstract Human urine represents a good source for proteomic research for clinically related studies as it can be collected and processed easily and can give information about kidney-related mechanisms. Little is known about the urinary proteomic changes resulting from physiological (normal), pathological, or environmental variations, and there are few reports on hormone-related modifications of urine proteome. In our study, we highlighted the variations of urinary proteins associated with menstrual cycle or estro-progestin pill in females. We also described an association between some urinary proteins and the renin–angiotensin–aldosterone system, which might help to improve the understanding of physiological and pathological processes when a gender-specific pattern such as the menopause-related hypertension or eclampsia is evident. We therefore support the usefulness of urinary proteomics as a valuable tool for clinically related study as it can provide information on candidate biomarkers which, in turn, need to be confirmed by multiple approaches before the use in a clinical setting.

Keywords Urinary proteomics · Female hormones · Hypertension · Renin–angiotensin–aldosterone system

11.1 Introduction

Urine is one of the most attractive bio-fluids for clinically related proteomic studies due to its intrinsic properties: it retains information from the kidney through the physiological process of protein secretion from tubular cells along all the nephron and the genitourinary tract, and from more distant organs (through its circulation origin), it is easy to collect, even repeatedly and prospectively, it's stable and relatively less complex (in respect to other blood derived body fluids) [9, 19]. The

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urinary proteome, in fact, retains information from either local or distal proteomic changes, and its content could be largely affected by physiological (normal), pathological, or environmental variations [15]. Quite recently, several reports have illustrated some aspects of the normal urinary proteome, either from a methodological point of view [31, 32], or in an attempt to highlight differences in infant–adult male urine [11] and changes between adult and old general population [3]. Despite a few exceptions, proteomic studies on human subjects concern individuals of both sexes even if the gender influence in certain pathologies is a recent matter of debate. Female urinary proteomics, and specifically the influence of hormones on female proteomics, was investigated in healthy subjects, by our group [6], and in pregnant women, by others [4, 5, 7, 20, 40]. Pregnancy status could be considered as a particular hormonal state involving complex physiological mechanisms. Urinary proteomic reports in pregnancy were mainly focused on complications of pregnancy such as preeclampsia [4, 6, 7] and other pregnancy-related hypertensive disorders [20]. These studies were conducted by using a mass spectrometry-based approach and illustrated a set of putative biomarkers useful for prediction and diagnosis of preeclampsia.

In addition, a very recent report focused on an interesting aspect of proteomics, a posttranslational modification (i.e., phosphorylation) [40]; these authors describe a comprehensive urinary proteomic profile of healthy women before, during, and after delivery, giving information on the phosphoproteins that are modulated in pregnancy. This study could be very useful for the understanding of the mechanisms involved in the upkeep of a healthy and uncomplicated pregnancy.

On the other hand, urinary metabolomic research in the field of hormone-related alteration is also gaining importance [27, 36]. It is worth mentioning that, concerning disease related to hormones, many diverse proteomic studies have been conducted on cellular models or tissue [13, 30, 38] among the very recent ones.

Hormones can be of natural origin, such as the physiological modulators of human body functions, or pharmacological products; in the case of females' subjects, sexual cycle-related changes are due mainly to two natural hormones that modulate estrogen and progestogen secretion: follicle-stimulating hormone (FSH) involved in the follicular phase, and luteinizing hormone (LH) involved in the luteal phase. Estrogens and progestogens are also the two pharmacological components of oral contraceptives (OC), widely used by the female population and substantially well tolerated. However, it is important to observe that despite the reduction of undesirable side effects due to the introduction of low-dose combination and progestogen-only pills [14], OC therapy is still associated with hypertensive disease [8, 34], and uncontrolled hypertension remains as a relative contraindication for its use. Moreover, there is a widely recognized association between hormonal therapy (contraceptive or hormonal replacement in menopause) and the burden of hypertension in woman [2]. In detail, female hormones were proved early on to presumably activate the renin–angiotensin–aldosterone system (RAAS) [23], leading to blood pressure (BP) increase. The RAAS regulation is particularly important in some forms of hypertension, such as primary aldosteronism (PA), in which this system is structurally altered.

At present, PA is recognized as the most frequent cause of secondary hypertension, with an incidence varying from 5 to 15 % among hypertensive patients [10]. In PA hypertension, the screening test is represented by aldosterone-to-renin ratio (ARR). Once the result of the screening test is positive, PA diagnosis needs further confirmation to document the autonomous production of aldosterone [12]. The most widely used confirmatory tests are intravenous saline load (ivSLT), oral saline load, and fludrocortisone suppression test [12]. All these tests are time-consuming and expensive, and potentially burdened by the risk of uncontrolled hypertensive peaks [10], so they have to be reserved for carefully selected patients. In different surveys, the percentage of negative confirmatory tests after a positive ARR screening varies from 30 to 50 % [12]. Thus, factors interfering with ARR diagnostic accuracy should be recognized and if possible avoided. We documented an activation of the RAAS in the luteal phase, that is, higher renin and aldosterone values but unchanged ARR in follicular and luteal phases of the menstrual cycle, of healthy normotensive women [26]. Furthermore, we demonstrated that oral contraceptive therapy influences ARR determination in normotensive women [26], with higher ARR values in the luteal phase. This means that endogenous hormones and OC can change the levels of some hormones that are used as tests for pathology screening, leading to false positives and increasing the problem of accuracy.

The demonstration that ARR values are affected by endogenous and exogenous female sex hormones in normotensive individuals suggests the possibility that the initial ARR determination in the workup of hypertensive patients may be sufficient in male patients, but that it should probably be repeated in women. For the same reason, oral contraceptives should be included in the list of drugs to be withdrawn before ARR measurement [26].

Moreover, aldosterone, besides being involved in PA, is the principal human mineralocorticoid hormone, increasingly recognized as playing a significant role in the pathophysiology of renal and cardiovascular disease states.

Although attention has been devoted recently to non-renal action of aldosterone, unfavorable hormone-related effects are traditionally ascribed to salt and water retention at renal level and the relative consequences on extracellular fluid volume and blood pressure. Aldosterone increases the rate of sodium (Na) reabsorption across epithelial cells of the distal nephron by increasing Na transport through the epithelial Na channel (ENaC), the principal physiological target of aldosterone action [29]. In particular, an aldosterone-driven pathway leads to the activation of the ENaC at the distal tubule and collecting duct level, eventually promoting cell Na entry for potassium (K) exit in equimolar proportions. The ENaC is a membrane complex localized not only in the kidney but also in several other tissues and organs involved in Na maintenance (i.e., airways, skin, colon, and salivary glands). The channel is composed of three homologous subunits (α, β, and γ) [1] that are functionally complementary in activating the ENaC by a sequential mechanism of cooperation. In recent years, much attention has been devoted to proteolytic mechanisms able to activate the ENaC by removing inhibitory peptides on the β or γ chains. One of the most interesting proteins investigated in this context is prostasin, a 40-kDa serine protease first detected in the prostate gland and seminal fluid.

Prostasin activates ENaC when expressed in oocytes, and the addition of aldosterone to cultured mouse collecting ducts increases ENaC activity by increasing prostasin secretion and mRNA and protein expressions [24].

Aldosterone induces the activation of prostasin and, in cooperation with other protease(s), such as furin, cleaves (at one or more sites) the γ-subunit of ENaC, contributing to the removal of an inhibitor peptide and increasing the probability of the channel opening. Prostasin is also of interest because it is a glycosylphosphatidylinositol (GPI)-anchored protein, secreted in extracellular fluids such as urine, and the amount released in urine seems to be grossly proportional to the extent of ENaC activation [24]. As regards the influence of hormones on prostasin modulation, it has recently been reported that prostasin is expressed at a relatively high level in human placenta trophoblasts in the early weeks of pregnancy [21], further supporting a possible correlation with sex hormones. As physiological cyclic variations of female hormones alter Na balance and affect salt retention, we hypothesized that urinary prostasin might be accordingly modulated during different phases of the menstrual cycle and/or after OC therapy [25].

11.2 Changes in Female Proteome Due to Cycle-Related Hormones and OC Intake [6]

A group of healthy young women (mean age 28.5 ± 7.9 years) was enrolled for the study, urine samples were taken, and clinical parameters related to RAAS (blood pressure (BP), urinary Na, plasmatic K, aldosterone, renin, FSH, LH, estradiol, and progesterone) were recorded at follicular and luteal phases and after OC intake. Urine samples were processed and pooled for the subsequent proteomic analysis. A classical proteomic approach based on 2D-PAGE was applied to study the urinary proteome of female subjects at different hormonal states, aimed at the identification of proteins undergoing modulation associated with female hormone variations and, in turn, possible connection with sexual hormones to RAAS. Regarding this analysis, samples corresponding to mid-cycle phase (G1), luteal phase (G2), and after two months of contraceptive therapy with a third-generation OC pill (G3) were analyzed; for a complete overview of the mechanisms involved, a fourth comparison was performed, considering G1 and G2 as a whole (G = "untreated samples") *versus* G3 ("treated samples"). Figure 11.1 reports the standard maps (as obtained by PDQuest software) for the four comparisons investigated (G analysis). The software analysis gave an output of 40, 17, 34, and 24 differentially modulated protein spots in the comparisons G1–G2, G2–G3, G1–G3, and G–G3, respectively. After software analysis, the differentially expressed proteins were excised from the gels and analyzed by MS/MS for identification. The differential proteomic analysis performed allowed the detection of many proteins related to the physiological changes or induced by the OC treatment as illustrated in Table 11.1.

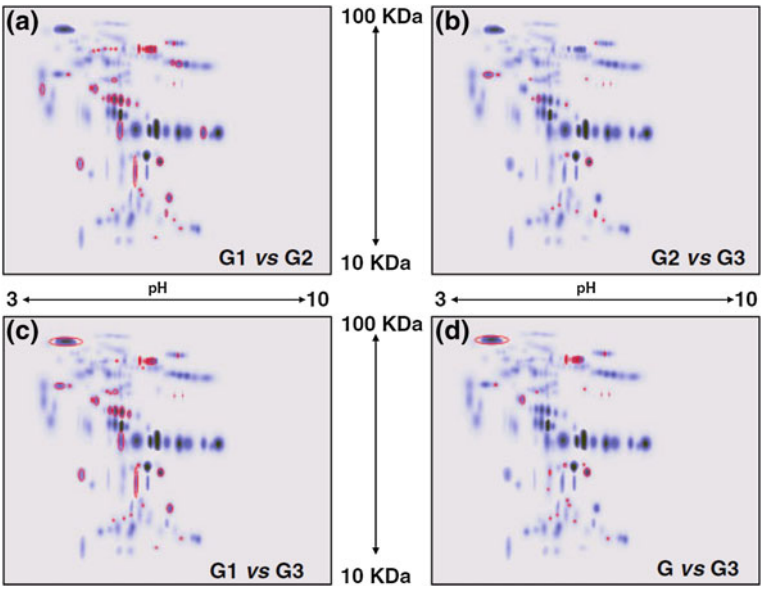

Fig. 11.1 Standard maps obtained with the analysis software PDQuest for the four comparisons investigated. The differentially expressed spots are shown as *red circles* **a** in G1 versus G2; **b** in G2 versus G3; **c** in G1 versus G3; and **d** in G versus G3 comparisons. From Ref. [6] with permission

Regarding the molecular function of the modulated proteins, it is worth noting that the majority could be classified as protein binding, endopeptidase inhibitors, ion binding, or other, while according to their cellular localization, we found that most of the identified proteins were secretion proteins. Others were derived from cytoplasm, lysosomes, or extracellular proteins.

We did not observe severe modulation in protein "expression" as just one protein in one comparison was found so extremely modulated as to be considered newly expressed (cystatin A), but the observed changes are interesting in any case in terms of their association with RAAS activation. The clinical parameters annotated in the three phases under study showed a particular trend: gonadotropin and progesterone were different along the phases of the cycle, and in parallel, renin and aldosterone were increased in the luteal phase. After OC intake, systolic blood pressure (SBP) and diastolic blood pressure (DBP) showed an increase, while FSH, LH, progesterone, and estradiol were lower during the therapy in agreement with the inhibitory effect of OCs on gonadotropin regulation. Aldosterone decreased after OC therapy, whereas urinary sodium excretion and renin increased.

Furthermore, as the G1 and G2 phases taken together could mimic a normal situation, we focalized on the comparison taking G1 + G2 as a whole (G) versus G3. The proteins found down-regulated were perlecan, aminoacylase 1, fatty acid-binding protein 5, S100 calcium-binding protein A8, and serum albumin, while the

Table 11.1 List of proteins identified by MS/MS analysis

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Alpha-1-micro-globulin/bikunin	AMBP	403	P02760	gi|4,502,067	2	142	/39,886	/5.95	down 2,7			
Aminoacylase 1	ACY1	6,401	Q03154	gi|4,501,901	9	332	/45,856	/5.77		down 2,13		down 1,78
Annexin A3, iso-form CRA_b	ANXA3	5,302	P12429	gi| 119,626,228	7	236	/39,845	/6.68	up 3,22	down 2,5		
Clusterin	CLU	2,301	P10909	gi|338,305	3	151	/36,997	/5.74	up 3,53		up 4,31	up 2,08
Clusterin	CLU	2,302	P10909	gi|178,855	7	79	48,772	6.27	up 4,16	down 2,78		
Clusterin	CLU	2,402	P10909	gi|178,855	5	197	/49,342	/6.27	up 2,89		up 2,55	
Clusterin	CLU	3,302	P10909	gi|178,855	6	246	/48,772	/6.27	up 3,17	down 1,78	up 1,76	
Clusterin	CLU	3,304	P10909	gi|338,305	3	184	/36,997	/5.74	up 2,43		up 1,69	
Clusterin	CLU	3,401	P10909	gi|178,855	3	101	/49,342	/6.27			up 7,19	
Clusterin	CLU	4,302	P10909	gi|178,855	2	107	/49,342	/6.27	up 2,67		up 1,97	
Clusterin	CLU	4,304	P10909	gi|178,855	2	98	/49,342	/6.27	up 3,64		up 1,71	
Beta-2 microglobulin	B2 M	7,002	P61769	gi|34,616	3	169	/12,905	/5.77	down 3,33	up 1,77		
Complement component C4B	C4B	2,403	P0C0L5	gi|187,771	1	99	/40,795	/5.19		up 3,91		
Cystatin A	CSTA	4,104	P01040	gi|4,885,165	2	96	/11,000	/5.38		new induced	up 9,41	up 15
Fatty acid-binding protein 5	FABP5	5,102	Q01469	gi|4,557,581	4	135	/15,155	/6.60	down 1,89			down 1,69
Fatty acid-binding protein 5	FABP5	5,104	Q01469	gi|4,557,581	4	135	/15,155	/6.60	down 1,61		down 2,78	down 2,13

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Fatty acid-binding protein 5	FABP5	6,104	Q01469	gi|4,557,581	2	84	/15,497	/6.60	down 1,54		down 1.89	
Gelsolin	GSN	1,403	P06396	gi|17,028,367	3	169	/31,052	/4.85	up 3	up 2,5	up 7,56	up 4,07
Gelsolin	GSN	3,403	P06396	gi|17,028,367	3	147	/31,052	/4.85	up 2,84		up 3,36	
Perlecan	HSPG2	5,202	P98160	gi|11,602,963	4	208	/477,024	/6.03		down 2	down 2,78	down 1,89
Perlecan	HSPG2	6,103	P98160	gi|11,602,963	2	141	/477,024	/6.03	down 2,38	up 6,20	up 2,60	up 3,95
Perlecan	HSPG2	6,202	P98160	gi|184,427	3	72	/481,880	/6.05				down 3
Ig heavy chain variable region	IGHV3-23	7,506		gi|112,695,045	2	75	/10,785	/9.06	up 2,38			
Ig heavy chain variable region	IGHV3-23	7,507		gi|112,695,045	2	75	/10,785	/9.06	down 1,67			
Ig kappa light chain 1	IGKV	4,206		gi|4,176,418	6	397	/23,690	/6.92	down 1,89		down 2,22	
Ig superfamily, member 8	IGSF8	5,108	Q969P0	gi|16,445,029	4	206	/65,621	/8.23	down 4		down 2.38	
Mannan-binding lectin-ass. serine protease 2	MASP2	4,103	O00187	gi|3,297,879	4	180	/77,176	/5.43		up 1,83	up 2,01	up 2,1
Prostaglandin H2 D-isomerase	PGDS	8,201	O60760	gi|32,171,249	3	80	/21,299	/7.66	up 3,11			
Calgranulin A	S100A8	7,004	P05109	gi|29,888	2	89	/10,988	/9.19	down 1,70		down 2,86	down 2,1

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Calgranulin B	S100A9	5,001	P06702	gi|4,506,773	5	242	/13,291	/5.71		up 4,61		up 4,3
S100 calcium-binding protein A7	S100A7	6,003	P31151	gi|12,053,626	4	68	/10,410	/6.08	down 2	up 1,68		
Serpin peptidase inhibitor, clade B, member 3	Serpin B3	7,304	P29508	gi|239,552	3	185	/44,564	/6.35		up 2,75	up 2,67	up 3,08
Serpin peptidase inhibitor, clade B, member 3	SerpinB3	7,305	P29508	gi|239,552	4	170	/44,564	/6.35	down 1,79	up 4,18	up 2,33	up 2,11
Serum albumin	ALB	2,706	P02768	gi|3,212,456	22	1,082	/68,425	/5.67	down 7,14			
Serum albumin	ALB	2,707	P02768	gi|28,590	12	622	/71,246	/5.92	down 4,17			
Serum albumin	ALB	2,708	P02768	gi|28,592	18	914	/71,316	/6.05	down 20			
Serum albumin	ALB	3,706	P02768	gi|28,592	12	661	/71,316	/6.05	down 8,33		down 3,13	
Serum albumin	ALB	3,707	P02768	gi|28,592	17	866	/71,316	/6.05	down 16,67			
Serum albumin	ALB	5,703	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,82		down 2,44	down 1,82
Serum albumin	ALB	5,704	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,67		down 2,13	down 1,64
Serum albumin	ALB	5,705	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 2		down 3,23	down 2,22
Serum albumin	ALB	6,705	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,79			

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Serum albumin	ALB	6,706	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,75		down 2,1	down 1,59
Serum albumin	ALB	6,707	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,96		down 2,33	down 1,69
Tetranectin	CLEC3B	4,102	P05452	gi|267,108	1	60	/22,552	/5.52				up 1,66
Thioredoxin	TXN	3,103	P10599	gi|1,065,111	2	131	/11,629	/4.82	down 2.63		down 1,78	
Transferrin	TF	6,702	P02787	gi|4,557,871	8	449	/79,280	/6.81	down 3,84	up 2,33		
Transferrin	TF	7,701	P02787	gi|553,788	8	449	/79,280	/6.81	down 1,75			
Transferrin	TF	7,702	P02787	gi|553,788	8	409	/55,207	/6.00			up 3,02	up 2,11
Ubiquitin	UBB	6,004	P62988	gi| 134,105,063	3	161	/8,571	/5.73	down 1,82		down 1,67	
Uromodulin	UMOD	1,801	P07911	gi|340,166	3	165	/72,429	/4.97			up 2,46	up 1,87
Vitelline membrane outer layer 1	VMO1	1,101	Q7Z5L0	gi|32,698,964	2	155	/22,033	/4.90	down 1,89		down 1,89	
Zn-alpha2-glycoprotein	AZGP1	3,306	P25311	gi|38,026	2	73	/34,942	/5.71			up 3,36	up 3,04

Modified from Table 1 in Ref. [7] with permission

Note *: Fold of variation in expression: *increased protein* (Up = in the right-hand group), *decreased protein* (Down = in the right-hand group)

ones up-regulated were clusterin, cystatin A, gelsolin, mannan-binding lectin-associated serine protease 2, S100 calcium-binding protein A9, serpin peptidase inhibitor, clade B, member 3 (serpin B3), tetranectin, uromodulin, and Zn-a2-glycoprotein.

Interestingly, and probably due to different posttranslational regulations, some proteins were present in both groups, as either over- or under-expressed.

We then focused our attention on 3 proteins for further validation by Western immunoblotting (uromodulin, clusterin, and serpin B3) as shown in Fig. 11.2 and ELISA (serpin B3).

Uromodulin represents one of the most abundant proteins present in human urine; it is produced by the thick ascending limb of the loop of Henle of mammalian kidney, and it is a GPI-anchored glycoprotein. This protein is probably involved in many kidney pathologies such as tubulointerstitial nephritis, kidney stones, cast nephropathy, and urolithiasis [33]. In Fig. 11.2, it is possible to notice that uromodulin abundance was increased in the G3 pool, in agreement with the proteomic analysis and with observations that uromodulin could be associated with salt intake in healthy individuals [37], as after OC therapy, sodium excretion was found to be increased (G vs. G3 comparison) in our set of individuals.

Concerning the other two proteins, clusterin and serpin B3, validated by Western immunoblotting, we could confirm the trend observed in the 2D gels regarding their abundance after OC treatment (see Fig. 11.2 panels B and C). The protein clusterin is known to be involved in many biological functions, and it was previously associated with angiotensin II action (which is induced by estro-progestogen treatment) [39].

On the other hand, serpin B3, a protease inhibitor of the serpin family, was further investigated by means of a commercial ELISA kit adapted for urine analysis. Our data on individual samples provided a further confirmation of serpin B3 increase after OC treatment. Other reports in mouse described the association of serpins with angiotensin II [28] or the regulation of serpin genes by gonadotropins [22]. In addition to these data, we also observed a further increase in serpin B3

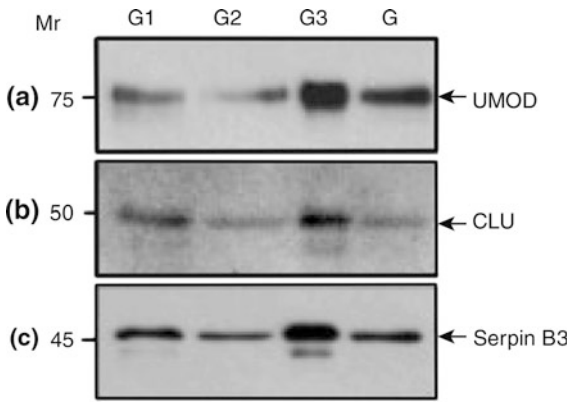

Fig. 11.2 Western immunoblotting obtained after SDS-PAGE and incubation with antibodies anti-uromodulin (**a**), clusterin (**b**), and serpin B3 (**c**). From Ref. [7] with permission

expression in a group of 5 pregnant women, with a gestational range of 20–-25 weeks, indicating a hormonal control on serpin B3 expression, or secretion, in urine [6].

Our work on urinary proteomics of healthy women enabled the identification of some proteins likely to be associated with RAAS changes induced by hormones: uromodulin, clusterin, perlecan, and serpin B3. Further investigations in the field of contraceptive- or menopause-associated hypertension could benefit from our preliminary discoveries.

11.3 Changes in A Specific Protein (Prostasin) Related to ENaC Activation in Urine of Healthy Subjects According to Different Phases of the Menstrual Cycle and/or After OC Therapy [25]

Urinary prostasin was assessed in a subset of healthy women in the same phases (G1, G2, and G3) of the comparative proteomic illustrated in paragraph 3. In fertile women, the comparison between the follicular and luteal phases confirmed the activation of the aldosterone–renin axis during the menstrual cycle, with raised luteal aldosterone and renin concentrations but without concordant modifications of their ratio (aldosterone range 167 ± 90 to 341 ± 175 pg ml^{-1}, $P = 0.01$; renin range 12 ± 8.3 to 20 ± 12.4 pg/ml, $P = 0.05$; ARR range 17.4 ± 11.3 to 20.7 ± 11, P = NS). Similar to the ARR, the urinary prostasin level did not substantially vary in the luteal compared with the follicular phase (prostasin range 2.97 ± 3.58 to 2.35 ± 1.23 nM; data not shown). After OC therapy, the aldosterone and renin values were significantly reduced (aldosterone range 341.0 ± 175.5 to 201.0 ± 99.7 pg ml^{-1}; renin range 19.7 ± 12.5 to 6.9 ± 3.3 pg ml^{-1}), whereas the ARR increased (from 20.7 ± 11.0 to 33.7 ± 18.3). Urinary prostasin also tended to increase (2.37 ± 1.27 to 4.85 ± 5.28 nM) consistent with the ARR; however, this increase was not statistically significant ($P = 0.07$; Table 11.2).

In healthy women, the prostasin level, unlike the aldosterone level, did not substantially change following the menstrual cycle phases. Therefore, although sexual hormone fluctuations are indeed able to affect aldosterone and renin concentrations, the release of prostasin in urine does not appear to be sufficiently sensitive to these variations, or speculatively, ENaC activity does not actually change in a significant way. Estro-progestinic therapy had a greater capability to alter such a balance. In fact, despite slight changes in the aldosterone and renin levels (both decreased), the ARR and the urine concentration of prostasin increased, but the increases did not reach statistical significance. Moreover, although the BP values did not reach pathological levels, they increased substantially after OC therapy and accompanied a significantly increased U-Na.

Table 11.2 Clinical and biochemical characteristics of the group of healthy women before and after estro-progestinic therapy

	Before OC therapy	After OC therapy	*P*
SBP (mm Hg)	108 ± 7,7	114 ± 11,5	0,05
DBP (mm Hg)	70 ± 5,6	73 ± 7,7	NS
Aldosterone (pg/ml)	341,0 ± 175,5	201,0 ± 99,7	0,01
Renin (pg/ml)	19,7 ± 12,5	6,9 ± 3,3	0,002
ARR	20,7 ± 11,0	33,7 ± 18,3	0,001
P–K (mmol/L	3,79 ± 0,3	3,85 ± 0,2	NS
U–Na (mmol/L)	108,8 ± 44	146,0 ± 34,8	0,014
U–prostasin (nM)	2,41 ± 1,27	4,85 ± 5,28	0,07
U–prostasin/U–Na (nM/mmol*L^{-1})	0,023 ± 0,014	0,033 ± 0,03	NS

From Ref. [25] with permission
Data are means ± SD
SBP systolic blood pressure; *DBP* diastolic blood pressure; *ARR* aldosterone-to-renin ratio (pg.mL^{-1} /pg.mL^{-1}); U-prostasin/U-Na = ratio between urinary prostasin concentration in nM and urinary Na in mmol/L

In conclusion, in healthy subjects, urinary prostasin is similarly present in both genders, and it is not affected by the different phases of the menstrual cycle. Prostasin is modulated by urinary Na, and prostasin levels appear to be correlated with the ARR rather than with individual aldosterone or renin levels. Although a urinary excess of prostasin seems to be associated with ARR, which is the hallmark of conditions such as low-renin hypertension and primary aldosteronism, the precise relationship linking the renin–angiotensin−aldosterone axis, Na, and prostasin in hypertensive patients needs to be further investigated.

11.4 Methods and Protocols

11.4.1 Healthy Fertile Women on Free and OC-Induced Menstrual Cycles

Young healthy normotensive women, with no history of disease and referred for contraceptive counseling to a single physician at the Obstetric and Gynecology Department of the University of Verona, were enrolled in the study. Urine and blood samples were collected in the follicular phase of their menstrual cycles (between 14th and 16th day), in the luteal phase (between 20th and 22nd day) and after 2 months of OC therapy. All patients were prescribed the same third-generation pill, which consists of 0.075 mg gestodene and 0.02 mg estradiol. At each of the three time points, BP was measured and the average values were recorded. The protocol was approved by our institutional review committee, and written informed consent

was obtained from each subject before initiation of the study. Systolic BP and diastolic BP were carefully measured in all enrolled study subjects. BP was evaluated twice in both arms, with the subject remaining in the sitting position for 5 min and the subject avoiding coffee intake and smoking during the preceding 30 min. Further measurements (when necessary) were taken if the differences in BP values were X5 mm Hg.

11.4.2 Urine Sample Collection and Processing

Fresh urine samples were collected in the morning, immediately chilled on ice, and processed according to the previously established protocol [24]. An aliquot of urine was examined for white and red blood cells by a urinalysis test, and the samples of individuals in whom significant microematuria and/or leukocyturia (>10–20 cells) had been detected were excluded from any further analysis. When not immediately concentrated, the processed urine was stored at −80°C until required. Protein concentration was evaluated with Bradford assay (Bio-Rad).

11.4.3 Prostasin and Serpin ELISA Assays

Urine processed as described above was processed in duplicates for both assays.

11.4.3.1 Prostasin

A competitive enzyme-linked immunosorbent assay (ELISA) test was set up by exploiting the rabbit response toward a specific, highly immunogenic, prostasin peptide (amino sequence: AHQLDSYSEDAKVSTLKDI). Polyclonal antibodies were obtained from rabbits inoculated according to the method previously described by Koda et al., and the assay was conducted accordingly [17].

11.4.3.2 Serpin B3

The quantitative measurement of serpin B3 inhibitor was taken with a commercial ELISA kit (SCCA-LISA) purchased from Xeptagen according to manufacturer's recommendations. As the kit was originally designed for serpin B3 assay in serum, several modifications were introduced to adapt the assay and to obtain reproducible and reliable results also in urine [6].

11.4.4 Biochemical Analysis

The patient blood samples were collected after an overnight fast. All of the biochemical tests were performed in the Laboratory of Clinical Chemistry of the University of Verona. Aldosterone and direct active renin levels were determined using commercially available methods (DiaSorin Diagnostics, Turin, Italy) and expressed in pg/ml.

11.4.5 Statistical Analysis

Statistical analyses were performed with SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). The quantitative values were expressed as means ± SD. When necessary, logarithmic transformation was applied to obtain a normal distribution, and the variability of the data was expressed as 95 % confidence intervals. Student's t test for paired data was performed by comparing data from the same subjects. Correlations were tested by simple bivariate correlation analysis.

11.4.6 SDS-PAGE and Western Immunoblotting

Aliquots with the same amount of total protein content (25 μg) were loaded onto 12 %T SDS-polyacrylamide gels, and 1D SDS-PAGE was performed according to Laemmli [18] as previously described [24]. After electrophoresis, resolved proteins were blotted to a polyvinylidene difluoride (PVDF) membranes (Bio-Rad) as previously described [24]. Membranes were stained with amido black (Sigma-Aldrich) for a few minutes, and unbound stains were removed with destaining buffer (25 % isopropanol, 10 % acetic acid) for 30 min at room temperature. Proteins stained in PVDF membrane were captured using Bio-Rad Chemidoc XRS Imaging System for normalization purposes. In order to prevent non-specific binding of primary antibody, PVDF membranes were blocked overnight at 4 °C with 5 % non-fat dried milk (Sigma-Aldrich) in 0.01 % Tween-20 Tris-buffered saline. Then, membranes were probed with primary antibody against clusterin, uromodulin, and serpin B3 at the appropriate dilutions (1:500; 1:500; 1:2,000) in 1 % non-fat dried milk in 0.05 % Tween-20 Tris-buffered saline for 2 h at room temperature. Unbound primary antibody was removed with 3 washes of 10 min each with 0.05 % Tween-20 Tris-buffered saline. Blots were then incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 dilutions, GE Healthcare). The immunocomplex was detected by enhanced chemiluminescence (ECL, GE Healthcare) on X-Omat AR film (Kodak). The intensity of chemiluminescence response was measured by scanning films and processing the image using Quantity One software version 4.4 (Bio-Rad).

11.4.7 2D-PAGE

Pooled samples were used for the 2D-PAGE separations. The desired volume of each sample was precipitated overnight at 4 °C according to the protocol illustrated in Ref. [16].

The precipitated pellets were resuspended in 150 μl of 2D solubilizing solution, 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 2 % w/v CHAPS (Sigma-Aldrich), and 40 mM Tris (Sigma-Aldrich) and incubated with 5 mM tributylphosphine and 10 mM acrylamide (Bio-Rad) for 60 min at room temperature to reduce protein disulfide bonds and alkylate the cysteine thiolic groups. Later, 10 mM DTT (Sigma-Aldrich) was added in order to stop the reaction. Then, pH 3–10 immobilized pH gradient strips (IPG; Bio-Rad) were rehydrated for 4 h with 150 μl of 2D solubilizing solution (containing 2.5 mg/ml of total urinary proteins), and then, proteins were focused till the total product time x voltage applied was 25,000 Vh for each strip. The IPG strips were equilibrated with 6 M urea (Sigma-Aldrich), 2 % w/v SDS (Sigma-Aldrich), 20 % v/v glycerol (Sigma-Aldrich), and 375 mM Tris–HCl pH 8.8 for 26 min in rocker. For the second dimension, 12 %T SDS-PAGE was used, with the following protocol: 5 mA for each gel for 1 h, then 10 mA/gel for 1 h, and 20 mA/gel until the tracking dye, bromophenol blue, reached the anodic end of the gels. The protein zones were finally revealed with Sypro Ruby protein gel stain (Bio-Rad).

11.4.8 Protein Pattern Differential Analysis and Further Processing of Protein Spots (In-Gel Digestion and Peptide Sequencing by Nano-RP-HPLC-ESI-MS/MS)

Gels were scanned using a Bio-Rad VersaDoc 1,000 Imaging System. 2D gel analysis was performed by PDQuest software (Bio-Rad), version 7.3, as illustrated in Ref. [6].

Spots showing a statistically significant differential expression were carefully cut out from 2D Sypro Ruby-stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al. with minor modifications [35]. Peptide sequencing by nano-RP-HPLC-ESI-MS/MS was performed as previously described as well as protein identification using either a nanoflow HPLC system (Agilent 1,200 series) coupled with an ion trap (model Esquire 6,000 Bruker Daltonik) or a nanoflow HPLC system (Ultimate; Switchos; Famos; LC Packings) coupled with a high-capacity ion trap (model HCTplus Bruker Daltonik) [6]. Protein identification was performed by searching in the National Center for Biotechnology Information non-redundant database (NCBInr) with the Mascot program (Matrix Sciences).

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