Sex Differences in Renal Proximal Tubular Cell Homeostasis

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ABSTRACT

Studies in human patients and animals have revealed sex-specific differences in susceptibility to renal diseases. Because actions of female sex hormones on normal renal tissue might protect against damage, we searched for potential influences of the female hormone cycle on basic renal functions by studying excretion of urinary marker proteins in healthy human probands. We collected second morning spot urine samples of unmedicated naturally ovulating women, postmenopausal women, and men daily and determined urinary excretion of the renal tubular enzymes fructose-1,6-bisphosphatase and glutathione-S-transferase–α. Additionally, we quantified urinary excretion of blood plasma proteins α1–microglobulin, albumin, and IgG. Naturally cycling women showed prominent peaks in the temporal pattern of urinary fructose-1,6-bisphosphatase and glutathione-S-transferase–α release exclusively within 7 days after ovulation or onset of menses. In contrast, postmenopausal women and men showed consistently low levels of urinary fructose-1,6-bisphosphatase excretion over comparable periods. We did not detect changes in urinary α1–microglobulin, albumin, or IgG excretion. Results of this study indicate that proximal tubular tissue architecture, representing a nonreproductive organ–derived epithelium, undergoes periodic adaptations phased by the female reproductive hormone cycle. The temporally delimitated higher rate of enzymuria in ovulating women might be a sign of recurring increases of tubular cell turnover that potentially provide enhanced repair capacity and thus, higher resistance to renal damage.


Women are not just small men. Sex differences affect most, if not all, of the organ systems in the body.1 Although the necessity to research this topic has been advocated, there is still a considerable lack of knowledge about sex and gender dimorphism in human physiology and pathophysiology.2,3

With regard to the kidneys, registry data indicate that the incidence of ESRD is significantly lower in women compared with men across all age groups.4,5 The overall lifetime risk for developing ESRD was estimated to be 1:60 for healthy middle-aged women versus 1:40 for men in a Canadian population–based study,6 with similar sex differences found in other populations.7 Sex and gender differences in susceptibility to renal damage and/or differences in the rate of disease progression have been implicated.8–11 In experimental models, females present with less pronounced renal damaging scores compared with males.12 Estrogen was found to be beneficial in prevention of ischemia-reperfusion injury.13–15 Sex differences in nephrotoxin–induced renal injury were also described.16–19 However, although men showed a higher overall incidence of perioperative AKI, the opposite was true for some surgical procedures,
such as cardiothoracic surgery–related AKI and contrast-induced nephropathy.20,21

Progression to ESRD has been reported to be slower in women affected by nondiabetic renal disease on the basis of studies involving human patients or animals with IgA or membranous nephropathy, hypertensive nephropathy, polycystic kidney disease, and others.22–24 In the setting of diabetes, however, potential hormone imbalances in women with diabetes were implicated as a potential reason for the absence of clear sex differences.25 In addition, cohorts from the general population or patients with CKD did not show statistically significant sex differences when eGFRs were correlated with the risk of developing ESRD, questioning the concept of a generally slower progression of renal disease in women.26

A functional role of sex hormones in the kidneys has been suggested by animal and in vitro studies.12,27,28 Sex steroid receptors have been shown to be expressed in renal cells.29–32 Furthermore, transcriptome analysis revealed one of the highest numbers of estrogen-regulated genes in healthy mouse kidneys compared with other organs not involved in reproductive functions.33 Sex hormones were implicated in modulating the renin-angiotensin-aldosterone system,4–7 AA metabolism,38,39 nitric oxide–based regulatory systems,40,41 and renal extracellular matrix proteins.42,43 Vascular endothelial growth factor expression was shown to be increased by estrogen potentially maintaining a healthy intrarenal vasculature.44 In addition, direct and indirect effects on mitochondrial function were implicated in renal protection by estrogens.45,46

In this report, urinary marker protein excretion was measured to study the effects of sex and hormone status on proximal tubular cell function in unmedicated healthy human probands.

### RESULTS

Enzymatic activity of fructose-1,6-bisphosphatase (F-1,6-BPase), a proximal tubular marker protein, was quantified and normalized to creatinine followed by correction for total body creatinine with regular natural menstrual cycles. A pattern with characteristic increments was observed in the F-1,6-BPase enzymatic activity time profile of ovulating women (Figure 1). The prominent F-1,6-BPase peaks appeared to correlate with distinct phases of the menstrual cycle determined by menstruation and ovulation. Men or postmenopausal women excreted consistently lower levels of F-1,6-BPase in the urine over a comparable time period in sharp contrast to the characteristic F-1,6-BPase profile of women of reproductive age with peak excretion at distinct days in close relation to the hormone cycle.

Peak days were systematically identified in the F-1,6-BPase enzyme activity time course pattern of ovulating women by defining the two maxima per menstrual cycle length not within

![Figure 1. Sex and hormone status–specific differences in the time course of urinary F-1,6-BPase enzyme excretion. (A) Example data series. Second morning urinary samples were collected daily and analyzed for F-1,6-BPase enzyme activity. A time course representative of ovulating women, men, and postmenopausal women is shown. F-1,6-BPase enzyme activity was normalized to urinary creatinine followed by correction for total body creatinine and expressed as units per hour. Ovulation days are labeled by arrows (ovulation) as indicated by the woman on the basis of basal body temperature charting combined with fertile cervical mucus observations. The days marked by arrows as menses were the recorded first days of menstrual flow. (B) Aligned F-1,6-BPase excretion profile. Urinary F-1,6-BPase excretion (units per hour) of ovulating women was measured daily and aligned to the days of the F-1,6-BPase peak maxima associated with ovulation or menses. Peak maxima in the F-1,6-BPase time course were identified as the two maximal values within one cycle length not within 4 days. The analysis window was then shifted over the entire dataset, allowing the identification of F-1,6-BPase peaks within uncompleted menstrual cycles at the beginning or the end of the data series as well. Day 0 was designated as the day of the F-1,6-BPase peak maximum associated with ovulation. The menstrual F-1,6-BPase peaks were aligned to day 13, accounting for the median distance of ovulatory to menstrual F-1,6-BPase peaks (range =10–16). The graph represents the median (solid line) and the 25th and 75th percentiles (dashed lines) of the aligned datasets. The range between the 25th and 75th percentiles of urinary F-1,6-BPase excretion from postmenopausal women and men is shown as the hatched area.

4 days. Days preceding or after the thus-identified maxima with the next highest F-1,6-BPase values of the analysis window were considered as additional peak days, resulting in the identification of peaks with widths of 1–6 days (70% of peaks had a
width from 1 to 3 days). Alternatively, peaks were identified in the profiles of ovulating women by using a peak detection algorithm (greater than the individual’s median +3 SDs of the data of men and postmenopausal women). The peaks detected by the two methods were identical in the time courses of the ovulating women.

To correlate the F-1,6-BPase peaks with distinct phases of the menstrual cycle, all of the data series obtained from ovulating women were analyzed to determine how often peak maxima or days within a peak could be detected on each day of the menstrual cycle. Results shown in Figure 2 indicate F-1,6-BPase peak identification exclusively within days 0–7 or 14–20 after ovulation, with the latter corresponding to days 1–7 of the next cycle marked by menstrual flow start. Most often, peaks were observed about 2–4 days after the respective reference day, namely ovulation or first day of menses. The close correlation of the F-1,6-BPase peak with the female hormonal cycle was also apparent when time distances between peaks were analyzed. Ovulation– to menses–associated peak distances turned out to be quite constant, with a median length of 12 days (range =10–16 days), whereas menstrual–to ovulation–associated peak distances showed a much higher variation, ranging 7–22 days with a median of 15 days (Figure 3A). These lengths remind us of typical follicular and luteal phase lengths of healthy women without reproductive impairment with high fluctuations of the follicular phase lengths and fairly constant luteal phase lengths.

Furthermore, F-1,6-BPase peak distances of ovulating women were compared with their respective follicular or luteal phase length of the corresponding menstrual cycle, showing a statistically significant correlation (Figure 3B). Deviations of the individual data pairs from the regression line were about 1–2 days, which is in the range of the error inherent to the methods used for determination of ovulation (defining follicular and luteal phase lengths) compared with ultrasound-based determination of ovulation as the gold standard. Hence, menses– to ovulation–associated peak distance matches the follicular phase length, and ovulation– to menses–associated peak distance matches the luteal phase, confirming a close correlation between the F-1,6-BPase peaks and the female menstrual cycle.

For a quantitative comparison of F-1,6-BPase measurements, days of maximal excretion were compared between ovulating women, postmenopausal women, and men. As a result, F-1,6-BPase excretion was significantly higher on peak days in ovulating women compared with days of F-1,6-BPase maxima in postmenopausal women or men (Figure 4). Basal F-1,6-BPase excretion, however, determined as excretion rates on days not within a time window of 3 days before to 3 days after the identified maxima was not significantly different. Thus, basal daily urinary F-1,6-BPase excretion was sex independent, whereas significantly increased rates of F-1,6-BPase excretion were observed to correlate with ovulation and menses in women of reproductive age.

The next question was if glutathione-S-transferase–α (GSTα), another proximal tubular cell–specific marker protein, showed similar increases. For this aim, urinary samples from ovulating women were grouped into peak and nonpeak according to the enzymatic F-1,6-BPase measurements and used to quantify GSTα by an enzyme immunoassay. As a result, GSTα was found to be significantly increased on peak versus nonpeak days in ovulating women, with heights of

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**Figure 2.** Frequency of F-1,6-BPase peak detection on each day of the menstrual cycle. (A) Relation between days of F-1,6-BPase peak maxima and the day of ovulation. Daily second morning urinary samples of ovulating women were analyzed for F-1,6-BPase enzyme activity normalized to urinary creatinine. F-1,6-BPase peak maxima were defined as the two maxima within a menstrual cycle length, shifting the analysis window over the dataset. The F-1,6-BPase peak days were aligned to the day of ovulation (day 0). The graph represents the count of F-1,6-BPase peak maxima identified on the respective days after ovulation. The dashed line (day 14) represents the median start day of the next cycle marked by the beginning of menstrual flow. (B) Relation between all days within F-1,6-BPase peaks and the day of ovulation. Peak maxima were identified as described in A. The same data series as in A is represented, with the difference that days in addition to the peak maxima were defined as peak days if the next highest F-1,6-BPase values in the analysis window preceded or followed the peak maxima. Thus, not only peak maxima but all of the days within an F-1,6-BPase peak are represented. The dashed line (day 14) represents the median start day of the next cycle marked by the beginning of menstrual flow.
DISCUSSION

Women seem to be less susceptible to renal damage than men.8 Animal and in vitro studies suggest involvement of sex hormones.12 In this report, the novel concept is presented that proximal tubular tissue architecture might be cyclically modulated in accordance with the female reproductive hormone cycle, with men being devoid of a similar rhythm in renal cell function. Evidence is on the basis of a study involving unmedicated, healthy human probands. The proximal tubular marker enzyme F-1,6-BPase is shown to be transiently increased in the urine of naturally ovulating women, closely correlating with ovulation and menses. Opposed to women with natural ovulatory cycles, postmenopausal women and male probands, engaged as controls, excreted low levels of F-1,6-BPase over comparable time periods matching the recordings from women outside the distinct peak days. The F-1,6-BPase peaks in the pattern of ovulating women were accompanied by increases in the urinary excretion of GSTα, another intracellular proximal tubular–specific enzyme. By contrast, urinary α1–microglobulin, albumin, and IgG excretion did not show differences between F-1,6-BPase peak and nonpeak days.

As a referencing system to account for differences in the diuretic status, urinary creatinine was used followed by correcting for differences in total body creatinine content. A comparison of this referencing system with the widely used practice to express urinary analytes as mass or units per gram creatinine revealed that apparent sex–specific differences in the ratio of F-1,6-BPase to creatinine (units per gram creatinine) could be attributed to differences in total body creatinine, whereas intrinsic differences in basic F-1,6-BPase excretion between women and men were not detected (Supplemental Figure 1), with the exception of the F-1,6-BPase peaks in the time courses of the ovulating women. Thus, a similar modification of the referencing system as recommended by Gerber and Mann50 might be useful to express clinically relevant urinary analytes, such as albumin, to avoid sex-specific underdiagnosis or overdiagnosis.

Prerequisite for the detection of the observed transient, menstrual cycle phase–dependent periodic changes in the excretion profile of the urinary proximal tubular cell–specific marker proteins was the use of an extended study protocol: samples were obtained from probands systematically on every day over a time course of more than one menstrual cycle in length. By contrast, typical current experimental designs address female sex hormone effects on human physiology by comparing samples from phases of high- and low-hormone exposure, like the luteal and early follicular phase of the menstrual cycle or treatment with sex hormones.51 If we had used such an approach, we would have missed the here–described relatively narrow peaks in the time course pattern of urinary F-1,6-BPase excretion in healthy, naturally ovulating women. The peaks were exclusively found within a time window of 7 days starting with the day of ovulation or the onset of menses and peak widths were most often found to be between 1 and 3 days.

The two menstrual cycle phases found to be correlated with the F-1,6-BPase peaks do not share a common hormonal status.

Figure 3. F-1,6-BPase peak distances and follicular/luteal phase lengths. (A) Analysis of the distance between consecutive F-1,6-BPase peak maxima in ovulating women. F-1,6-
BPase peak maxima were defined as the two maxima within a menstrual cycle length in the time course profiles of urinary F-1,6-BPase excretion obtained from ovulating women, shifting the analysis window over the dataset. Peak distance was defined as days between consecutive F-1,6-BPase peaks, excluding the peak days themselves. Ovulation to menses–associated F-1,6-BPase peak distance (ov-menses) and menses to ovulation–associated F-1,6-BPase peak distance (menses-ov) are shown. The box plots represent the medians and 25th and 75th percentiles, with outliers showing maximal and minimal values. (B) Correlation of the F-1,6-BPase peak maxima distances and the follicular or luteal phase lengths of ovulating women. The distance between consecutive F-1,6-BPase peak maxima of ovulating women was determined as described in A (x axis). The corresponding follicular or luteal phase length is shown on the y axis. The individual data points and the least square linear regression line (solid line) with the 95% confidence bands of the best fit line (dashed lines) are shown. A Pearson correlation analysis was performed, revealing a significant correlation at P<0.05 shown by the correlation coefficient r marked by an asterisk.

about fourfold over basal compared with roughly twofold increases observed for F-1,6-BPase (Figure 5). To find out if the increases in enzymuria were accompanied by gross changes in tubular or glomerular protein handling, we also quantified α1–microglobulin, albumin, and IgG in the urinary samples by enzyme immunoassays (Figure 5). Again, samples from peak days and nonpeak days were compared, with the result that none of the parameters showed statistically significant differences.

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The two menstrual cycle phases found to be correlated with the F-1,6-BPase peaks do not share a common hormonal status.
The peaks observed in the days after ovulation are localized in the early luteal phase, characterized by relatively high levels of estrogen and progesterone, whereas the peaks observed during the time of menstrual flow are present during a phase hormonally characterized by levels of low estrogen and low progesterone (Figure 6). A common hormonal influence is, however, conceivable if focus is not directed to the absolute hormone levels but to relative changes. Both peaks appear within a few days after estrogen levels have declined significantly: the ovulatory F-1,6-BPase peak followed the first drop of estrogen in the menstrual cycle, which is preceded by peak levels of estrogen immediately before ovulation, and the menstrual F-1,6-BPase peak was detected after estrogen levels declined a second time after luteal phase estrogen heights. Thus, the observed marked and transient increases of F-1,6-BPase and GSTα excretion might depend on relative changes of the hormonal milieu rather than absolute hormone levels.

Urinary tubular enzymes are considered attractive biomarkers for renal damage because of their increased release from the cells into the urine on injury caused by renal disease, ischemia, or nephrotoxin treatment. The nephron is composed of different tubular segments with distinct functions, necessitating a characteristic enzyme configuration. F-1,6-BPase is, for example, a key gluconeogenic enzyme, which is—along the nephron—exclusively expressed in proximal tubular cells. Because F-1,6-BPase from other sources, like liver or muscle, is thought to be excluded from the urinary compartment because of its size, urinary F-1,6-BPase release was suggested as a selective marker for studying proximal tubular cell damage. As another example of nephron-specific expression, GSTα, an enzyme involved in detoxification, was found to be restricted to the proximal tubular part of the nephron. Thus, our finding of an increased urinary excretion of F-1,6-BPase along with GSTα provides evidence for involvement of proximal tubular cells. Intracellular tubular enzymes are released into the urinary space by damaged cells and thus, were established as urinary marker proteins indicative of cellular injury. Urinary enzymes were discussed to enter the urinary space via exfoliating cells, shedding of membrane vesicles containing intracellular material, leakage through damaged cell membranes, or up-regulation within the cell because of, for example, functional overburdening. However, why would this occur in healthy women without evidence of renal damage?

The observed increases of urinary proximal tubular enzyme excretion could be because of a transiently increased rate of cell losses. Homeostasis is, however, maintained whenever increased cell death is accompanied by a higher cell proliferation rate. Thus, the F-1,6-BPase peaks might not be a sign of overall damage but instead, may indicate phases of enhanced proximal tubular cell renewal. If this was the case, then women in their reproductive years would easily get rid of potentially injured, dysfunctional, or simply older proximal tubular cells, replacing them with fresh new cells.

Such an increased proximal tubular cell turnover might be the result of a direct hormonal effect on the cells. Prerequisite for such an action is the presence of hormone receptors on proximal tubular cells. Evidence for proximal tubular segment-specific expression of estrogen receptor-α, nuclear androgen receptor, and membrane progestin receptor—α was recently provided, whereas distal nephron segments showed a different expression pattern with preponderance of G protein-coupled estrogen receptor and nuclear progestin receptor. A proliferative role of estrogens is well established in epithelial cells of reproductive organs, and estrogens are involved in tumor development. With regard to renal tubular cells, hamster and rabbit primary proximal tubular cells were shown to proliferate when treated with estrogen at physiologic concentrations. Recent transcriptome analysis revealed that one of the biologic processes influenced by genes showing a sex-biased expression in human tubuli was found to be regulation of progression through the cell cycle. In addition to their role in proliferation induction, estrogens are also implicated in the prevention of apoptotic cell

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Quantitative comparison of maximal and basal F-1,6-BPase excretion in urines of ovulating women, postmenopausal women, and men. Daily second morning urinary samples were analyzed for F-1,6-BPase enzyme activity normalized to urinary creatinine followed by correction for total body creatinine and expressed as units per hour. F-1,6-BPase maxima were defined as the two maxima within a menstrual cycle length in ovulating women or 29 days in postmenopausal women or men. Urinary F-1,6-BPase enzymatic activity measured on maxima was compared between ovulating women, postmenopausal women, and men (left panel). Basal excretion rates were obtained from datasets excluding maxima ±3 days (right panel). Data are represented as box plots with medians and 25th and 75th percentiles; outliers show maximal and minimal values. Groups were compared by the nonparametric Kruskal–Wallis test followed by the Dunn post-test. Postmenopausal women were defined as the reference group. *Statistically significant differences to this group (P<0.05).
In the kidneys, antiapoptotic actions were attributed to estrogens in renal podocytes and proximal tubular cells.\textsuperscript{70,71} Thus, estrogens might directly provide proliferative and antiapoptotic stimuli to proximal tubular cells. As a consequence, declines of estrogen levels after ovulation or before onset of menses might result in a transient phase of increased cell death.\textsuperscript{69} It is conceivable that an enhanced tubular repair mechanism would confer a higher resistance against kidney damage to women, potentially explaining their reduced susceptibility to develop renal diseases compared with men. Even after cessation of hormone exposure and loss of the cyclical repair phases, it might take time until unrepaired damages sum up to cause irreversible loss of function. Thus, the protective effect in women might emanate into the postmenopausal age as documented by the US Renal Data System\textsuperscript{4} and other international registries.\textsuperscript{5}

In conclusion, our study provides the first indication for the novel concept that proximal tubular tissue architecture—representing a nonreproductive organ—derived epithelium—might undergo periodical adaptations phased by the female reproductive hormone cycle. The observed effects of the menstrual cycle–driven hormone changes on normal renal tissue might be linked to an enhanced repair capacity and a higher resistance against damage. Our findings imply that any attempt to explain the protection of women from renal diseases should not only focus on the overall preponderance of exposure to estrogens and/or progesterone in women compared with androgens in men. It should also take into account the changing hormonal death.\textsuperscript{69} In the kidneys, antiapoptotic actions were attributed to estrogens in renal podocytes and proximal tubular cells.\textsuperscript{70,71} Thus, estrogens might directly provide proliferative and antiapoptotic stimuli to proximal tubular cells. As a consequence, declines of estrogen levels after ovulation or before onset of menses might result in a transient phase of increased cell death.\textsuperscript{69} It is conceivable that an enhanced tubular repair mechanism would confer a higher resistance against kidney damage to women, potentially explaining their reduced susceptibility to develop renal diseases compared with men. Even after cessation of hormone exposure and loss of the cyclical repair phases, it might take time until unrepaired damages sum up to cause irreversible loss of function. Thus, the protective effect in women might emanate into the postmenopausal age as documented by the US Renal Data System\textsuperscript{4} and other international registries.\textsuperscript{5}

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**Figure 5.** Urinary excretion of GST\textsubscript{α}, α1-microglobulin, albumin, and IgG on F-1,6-BPase peak and nonpeak days in ovulating women. Urinary protein concentrates from ovulating women obtained by ultrafiltration were grouped into peak and nonpeak according to their time profile of urinary F-1,6-BPase enzyme activity. F-1,6-BPase peak maxima (peak) were defined as the two maxima within a menstrual cycle length. Days outside the F-1,6-BPase peaks (±3 days) were designated nonpeak. The thus-identified peak and nonpeak samples were used to measure GST\textsubscript{α}, α1-microglobulin, albumin, and IgG. Proteins are expressed as micrograms per hour or milligrams per hour obtained by referencing urinary creatinine followed by correction with the proband–specific creatinine excretion rate. The data are represented as box plots with medians and 25th and 75th percentiles, with the outliers showing maximal and minimal values. *Statistically significant differences (P<0.05) between measurements on peak and nonpeak days were determined by the nonparametric Mann–Whitney test.
environment that female organs are exposed to time and time again over several decades of women’s reproductive lives.

CONCISE METHODS

Study Design

Eleven apparently healthy, unmedicated, naturally ovulating women with usual menstrual cycle lengths between 23 and 35 days, aged 24–44 years, provided daily second morning urinary spot samples over a time course of about 2–3 months. Six postmenopausal women, 57–64 years old, and eight men, 24–73 years old, served as controls. All of the probands were of Central European origin. Exclusion criteria were known acute or chronic diseases, pregnancy, lactation, regular intake of pharmaceuticals including medications interfering with reproductive functions (e.g., hormonal contraception, fertility treatment, or menopausal hormone therapy), weight reduction diet, habitual heavy alcohol consumption, and heavy sports activities. Of the ovulating women, seven were nulliparous, and four were para 1–3. The last pregnancy/lactation episode or the last intake of hormones was at least 6 months before inclusion.

Oral and written instructions were provided on how to collect midstream urine without contamination. Each proband was asked to fill out daily data protocols to obtain information on date and time of micturition, time of the preceding micturition, alcohol intake, changes of habitual dietary intake, illnesses, medication intake, and strenuous sports activities. Ovulating women were instructed to label the first day of bright red flow as the first day of menses and give daily information on relative menstrual flow intensities. They were asked to use tampons during menstruation and recorded this on the data protocol. Informed written consent was provided by each individual. At least once during a collection period, a urinary strip test (Combur-10-Test, Cobas; Roche Diagnostics, Indianapolis, IN) was performed for semiquantitative detection of specific gravity, pH, leukocytes, nitrite, protein, glucose, ketone bodies, urobilinogen, bilirubin, and blood in fresh urine. The study was carried out following the ethical standards established by the Declaration of Helsinki. The study design was judged and positively approved by the Ethics Committee of the Medical University of Innsbruck.

Urine Sample Processing

Test persons were provided with 10-ml urine collection devices (Monovettes; Sarstedt, Numbrecht, Germany) supplemented with 24 μl 0.5 M EDTA (Sigma-Aldrich, St. Louis, MO) and a cooling box. They were instructed to fill two monovettes for each urinary sample and immediately cool the urine samples in their home refrigerator. On the basis of published data on stability of F-1,6-BPase in EDTA-supplemented urine, samples were collected and processed by laboratory personnel within 4 days using cooling gel packs for transportation. pH of the samples was determined. Then, the samples were subjected to a 24°C water bath for

![Figure 6](https://www.jasn.org)

Figure 6. Hypothetical model. (A and B) Daily urinary estrone-3-glucuronide and pregnanediol-3-glucuronide profile in ovulating women. Daily second morning urinary sample sets of two ovulating women encompassing six menstrual cycles were analyzed for estrone-3-glucuronide and pregnanediol-3-glucuronide concentrations normalized to urinary creatinine followed by correction for total body creatinine. The medians (solid lines) and 25th and 75th percentiles (dashed lines) of estrone-3-glucuronide and pregnanediol-3-glucuronide measurements (nanomoles per hour) are shown in A and B, respectively. The results match published urinary estrone-3-glucuronide and pregnanediol-3-glucuronide profiles of ovulating women without reproductive impairment, which were shown to correlate with the respective plasma concentrations of the active hormones.\(^8^1\) Ovulation day is set as day 0. Day 14 represents the median first day of menstrual flow, marking the beginning of the next menstrual cycle. The hatched areas represent the two time windows, in which the F-1,6-BPase peaks were exclusively detected. Both phases are characterized by a preceding drop of the estrogen level. (C) The graph represents a hypothetical model developed to provide a potential explanation for the observed increase of urinary F-1,6-BPase in correlation with ovulation and menses. The schematic represents the proximal tubular epithelium with cells linked through junctional complexes. F-1,6-BPase within the cells represents intracellular cytoplasmatic expression of F-1,6-BPase, which is released into the urine on plasma membrane damage. Because estrogen was proposed to act as a proliferative and antiapoptotic factor on tubular epithelium,\(^1^6,6^7,7^0\) a decline of the estrogen level even if partial—might lead to an increased cell death rate, restoring original cell numbers again.
medium until confluence. After a day in serum-free medium, the cells were treated with 100 ng/ml EGF, which induced an additional proliferative boost within the monolayer. Cells in S phase were increased (12.6% ± 1.6%) after 24 hours of EGF treatment versus time-matched controls without EGF treatment (4.8% ± 1.3%) shown by us in a previous publication. Release of intracellular enzymes by dead cells was quantified by measuring the enzymatic activity of LDH in the cell culture supernatant medium. Medium was collected before starting EGF treatment (before), during EGF treatment (during), and 1 or 2 days after EGF treatment (1 day after and 2 days after, respectively). Controls were exposed to the same medium without EGF for the same time. Data are expressed as fold over time-matched controls. Means ± SDs of data measured in duplicates from five cell culture wells are shown. *Statistically significant difference to time-matched control at P < 0.05 determined by an unpaired t test.

5 minutes and inspected for precipitations afterward. The samples showing precipitations were diluted 1:1 or 1:2 with sterile Aqua bidest. If this was not sufficient for complete solubilization, pH was neutralized by adding 1 N NaOH or 1 N HCl (Sigma-Aldrich). Solubilized samples were then centrifuged for 10 minutes at 5000 × g and room temperature. The supernatant was carefully collected, and 2 ml were frozen in aliquots at −80°C for determination of creatinine and hormones. For enzymatic determination of F-1,6-BPase, potential low molecular weight enzyme inhibitors were removed by Sephadex G-25 Columns according to the instructions of the manufacturer (PD-10 Columns; GE Healthcare, Vienna, Austria) using 120 mM Tris-HCl (pH 7.5) and 12 mM MgCl₂ as elution buffer (chemicals were from Sigma-Aldrich). To concentrate urinary proteins to allow quantification of additional marker proteins, Vivaspin 20 polyethersulfone ultrafiltration units (molecular weight cutoff of 10,000; Sartorius, Gottingen, Germany) were used. Low molecular weight components were eliminated by at least 1:100 dilution with 120 mM Tris-HCl (pH 7.5) and 12 mM MgCl₂ followed by recentration. Ultrafiltration was driven by continuously applying 4-bar pressurized air using an in house–built pressure application apparatus. The concentration factor was determined individually for each sample by weighing the applied urinary supernatant and the retrieved concentrate. Samples were stored in aliquots at −80°C.

**Correction of Differences in Diuretic Status**

To correct for differences in the diuretic status, all of the urinary analytes measured were normalized to 1 g urinary creatinine. In addition, the obtained values were multiplied with the daily creatinine excretion rate calculated for each proband by using the formula by Gerber and Mann, taking into account sex, age, weight, and race. The thus–resulting timed excretion was expressed as excretion per hour.

**Creatinine Assay**

Creatinine was measured using a modified Jaffe assay. Centrifuged urinary samples were diluted with Aqua bidest and mixed with an alkaline picrate solution containing 5.67 mM picric acid and 0.12 M NaOH (Sigma-Aldrich). The picrate-creatinine complex was quantified after 40 minutes of reaction time at room temperature against a creatinine (Sigma-Aldrich) standard curve by absorbance measurement at 490 nm in a Tecan Infinite 200Pro Fluorescence and Absorbance Microplate Reader (Tecan Deutschland GmbH, Crailsheim, Germany). Potential differences in unspecified internal absorbance of the urinary samples were controlled for by adding acetic acid (end concentration of 1.7%; Sigma-Aldrich) at the end of the assay, which leads to dissolution of the picate-creatinine complex as suggested in the guidelines on standard operating procedures published by the World Health Organization for creatinine measurement (http://apps.searo.who.int/PDS_DOCS/B0218.pdf). Centrifuged urine samples were compared with the protein–depleted Vivaspin flow through of the same samples. Because potential influences of urinary proteins on the assay results were not detectable in our healthy proband population, as a routine, centrifuged urine was used as sample material.

**Enzymatic F-1,6-BPase Assay**

F-1,6-BPase was assayed by an enzymatic assay as described in the work by Pfaller et al., which was adapted to a 96-well format using polystyrene 96-well plates with bottoms of μClear Film (Greiner Bio-One, Frickenhausen, Germany). Urinary samples desalted by Sephadex G-25 Columns or protein concentrates obtained by Vivaspin ultrafiltration were used as samples. In brief, 400 μM fructose-1,6-bisphosphate (Sigma-Aldrich) and 800 μM NADP⁺ (Sigma-Aldrich) in 100 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂ were used as enzyme substrates, and Phosphoglucoisomerase (1 U/ml; Roche Diagnostics) and Glucose-6-phosphate dehydrogenase (about 0.5 U/ml; Sigma-Aldrich) were added in excess to provide 6-Phosphogluconate and NADPH as end products. Urinary F-1,6-BPase enzyme activity was quantified by monitoring the conversion of NADPH to NADP⁺ over time via spectrophotometry. The rate of NADPH generation was determined by a Tecan Infinite 200Pro Fluorescence and Absorbance Microplate Reader (Tecan Deutschland GmbH). Absorbance at 340 nm was recorded every 1 minute over a time course of 60 minutes after equilibration of plate and spectrophotometer to 37°C. Enzymatic activity was determined by using the slope of the absorbance increase (ΔOD per minute) and the extinction coefficient of NADPH.
(6.3×10^3 L mol⁻¹ cm⁻¹) to calculate units per liter as micromoles NADPH per minute per liter using the Lambert–Beer equation.

**Identification of Peak Days in the F-1,6-BPase Enzyme Activity Time Course Pattern**

Peak days were systematically identified in the F-1,6-BPase enzyme activity time course pattern of ovulating women by defining the two maxima per menstrual cycle length not within 4 days. Days preceding or after the thus-identified maxima with the next highest F-1,6-BPase values of the analysis window were considered as additional peak days. The analysis window was then shifted over the entire dataset, allowing the identification of F-1,6-BPase peaks within uncompleted menstrual cycles at the beginning or the end of the data series as well. Basal urinary F-1,6-BPase excretion on days outside the F-1,6-BPase peaks ±3 days was designated nonpeak.

Alternatively, peaks were identified in the profiles of ovulating women by using a peak detection algorithm used in analytical chemistry to discriminate peaks from baseline: values higher than the median of the individual data series plus 3 SDs of all of the data obtained from men or postmenopausal women (used as controls for baseline excretion) were defined as peaks. The peaks detected by the two methods were identical in the time courses of the ovulating women. Preanalytical factors (diuretic status as determined by the urinary creatinine concentration, urinary pH, and time from micturition to sample processing) were not statistically significantly different between the thus–identified peak and nonpeak samples (Supplemental Figure 2).

For analogous analyses in postmenopausal women and men, an analysis window of 29 days was chosen representing the median cycle length recorded by the ovulating women. Basal excretion was determined as F-1,6-BPase measured on days excluding maxima and the 3 preceding and following days. Application of the above–described peak detection algorithm revealed that 75% and 90% of the maxima were identified with the next highest F-1,6-BPase values of the analysis window of 29 days was chosen representing the median cycle length not within 4 days. Days preceding or after the thus-identified maxima within the next highest F-1,6-BPase values of the analysis window were considered as additional peak days. The analysis window was then shifted over the entire dataset, allowing the identification of F-1,6-BPase peaks within uncompleted menstrual cycles at the beginning or the end of the data series as well. Basal urinary F-1,6-BPase excretion on days outside the F-1,6-BPase peaks ±3 days was designated nonpeak.

Human Albumin and IgG Enzyme Immunoassay

Urinary albumin and IgG were quantified by ELISA using commercially available matched ELISA antibody pairs (Human Albumin or IgG ELISA Quantitation Sets; Bethyl Laboratories) following the manufacturer’s protocol with a modified sample dilution buffer (2% skim milk, 50 mM Tris [pH 8.0], and 140 mM NaCl). Centrifuged urine or Vivaspin concentrates, both stored at −80°C, were used as sample material. All of the samples were adjusted to the same urinary creatinine amount by dilution with 120 mM Tris-HCl (pH 7.5) and 12 mM MgCl₂ before additional dilution in the provided sample buffer. For the standards, the sample buffer was similarly diluted with 120 mM Tris-HCl (pH 7.5) and 12 mM MgCl₂. The results are corrected for differences in diuretic status as described above and expressed as micrograms per hour (GSTα) or milligrams per hour (α1-microglobulin).

Determination of the Day of Ovulation

The day of ovulation was determined as described in the work by Ecochard et al. on the basis of the initial rise of urinary luteinizing hormone (LH), cervical mucus, or basal body temperature recordings. LH was quantified using the supernatants of the first centrifugation step of the urinary samples. An enzyme immune assay on the basis of magnetic bead separation was applied according to the instructions of the manufacturer (Immunometrics, London, United Kingdom) with the exception that optical density was determined in a standard polystyrene 96-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany) instead of cuvettes. Results were normalized for the diuretic status and expressed as units per hour as stated above. The day of ovulation was defined as the day after an at least threefold increase of urinary LH over the mean of the preceding six measurements. Some women were experienced in fertility awareness methods, providing an estimation of the day of ovulation using data from fertile cervical mucus recordings, determination of the last day of hypothermia on the basis of recordings of the daily awaking basal body temperature, and/or their private home use ovulation prediction kits. The luteal phase lengths given by the number of days between the thus–defined ovulation day and the first day of menses were 12–13 days for all of the recorded menstrual cycles, matching published ranges of luteal phase lengths in women without reproductive impairment, which are described to be fairly constant.

**Estrone-3-Glucuronide and Pregnanediol-3-Glucuronide Enzyme Immunoassay**

Estrone-3-glucuronide and pregnanediol-3-glucuronide were determined by a competitive enzyme immunoassay on the basis of magnetic bead separation according to the instructions of the manufacturer (Immunometrics), with the exception that OD was determined in a standard polystyrene 96-well plate (Greiner Bio-One GmbH) instead of cuvettes. The assay was performed using centrifuged second morning urines as sample material. Results were corrected for differences in diuretic status as stated above.

**Release of Intracellular Enzymes into the Cell Culture Medium In Vitro**

LLC-PK1 porcine proximal tubular cells were maintained in culture as described in the work by Lechner et al. For experiments, the cells were cultivated in FBS containing DMEM (BioWhittaker/Lonza, Verviers, Belgium) containing 5.5 mM glucose, 7% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from...
Life Technologies, Carlsbad, CA) on tissue culture plastic dishes (Greiner Bio-One) in a humidified 5% CO₂ atmosphere at 37°C. Experiments were started a few days after the cells had reached confluence, and the formation of domes confirmed the presence of a functional transporting epithelial monolayer. Cell culture medium was replaced every day. After preincubation in serum-free medium for 1 day, the cells were treated with 100 ng/ml human recombinant EGF (BioLegend, San Diego, CA), which induced an additional proliferative boost within the monolayer. Cells in S phase were increased (12.6%±1.6%) after 24 hours of EGF treatment versus time-matched controls without EGF treatment (4.8%±1.3%) shown previously. Release of intracellular enzymes by dead cells was quantified by measuring the enzymatic activity of LDH in the cell culture supernatant medium as in the work by Lechner et al. For this purpose, cell culture medium was centrifuged before starting EGF treatment, after 1 day of EGF treatment, and 1 or 2 days after EGF treatment for 2 days. Time-matched controls were exposed to the same medium without EGF. The collected cell culture medium was centrifuged for 5 minutes at 200xg and room temperature. LDH was quantified in the supernatant by using an enzymatic assay. The centrifuged culture medium was incubated in 2 mM pyruvate and 0.5 mM NADH in a 50 mM phosphate buffer (pH 7.5; all from Sigma-Aldrich). LDH enzyme activity was quantified by monitoring the conversion of NADH to NAD⁺ over time via spectrophotometry. The rate of NAD⁺ generation was determined by a Tecan Infinite 200Pro Fluorescence and Absorbance Microplate Reader (Tecan Deutschland GmbH). Absorbance at 340 nm was recorded every 1 minute over a time course of 60 minutes after equilibration of plate and spectrophotometer to 37°C. Enzymatic activity was determined by using the slope of the absorbance decrease (ΔOD per minute) and the extinction coefficient of NADH (6.3x10⁻³ L·mol⁻¹·cm⁻¹) to calculate units per liter as micromoles NADH conversion per minute per liter using the Lambert–Beer equation. Data were expressed as fold over the mean of time-matched controls.

Statistical Analyses

For statistical evaluation and construction of graphs, GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used. Datasets were compared by using the nonparametric Kruskal–Wallis test followed by the Dunn multiple comparison test, the nonparametric two-sided Mann–Whitney test, or the parametric two-sided t test. For correlation analysis, a Pearson test was performed. Differences were considered significant at P<0.05.

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DISCLOSURES

None.

REFERENCES


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