Urinary Plasmin Activates Collecting Duct ENaC Current in Preeclampsia

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Abstract—In nephrotic syndrome, plasminogen is aberrantly filtered from plasma to the urinary space and activated along the tubular system. In vitro, plasmin increases ENaC current by proteolytic cleavage of the γ-subunit. It was hypothesized that preeclampsia is associated with plasmin-dependent ability of tubular fluid to activate ENaC. Urine was sampled from 16 preeclamptic (PE) patients and 17 normotensive pregnant women (Ctrl). Urine was analyzed for plasmin(ogen), creatinine, albumin, aldosterone, Na+, K+, proteolytic activity, and for its effect on inward current in cortical collecting duct cells (M1 cells) by whole-cell patch clamp. In PE, urine plasmin(ogen):creatinine ratio was elevated 40-fold (geometric mean, 160 versus 4 µg/g; P<0.0001) and urine aldosterone:creatinine ratio was suppressed to 25% of Ctrl (geometric mean, 27 versus 109 µg/g; P<0.001). A significant negative correlation was found in PE between urinary plasmin(ogen) and aldosterone (P<0.05). In PE, proteolytic activity was detected at 90 to 75 kD by gelatin zymography in 14 of 16 patients and confirmed by serine protease assay. Immunoblotting showed active plasmin in PE urine. Whole-cell inward current increased in M1 cells on exposure to urine from PE (173±21%; n=6; P<0.001). The increase in current was abolished by amiloride (2 µmol/L; P<0.001), α₂-antiplasmin (1 µmol/L; P<0.001), and heat denaturation (P<0.001). Preeclampsia is associated with urinary excretion of plasmin(ogen) and plasmin-dependent activation of ENaC by urine. Proteolytic activation of ENaC by plasmin may contribute to Na+ retention and hypertension in preeclampsia.

Key Words: pregnancy ● hypertension ● kidney ● edema ● renin ● aldosterone

Edema is a hallmark of acute nephrotic syndrome, and recent data favor overfill as the pathophysiological explanation with primary, renal hyperabsorption of NaCl through ENaC.1,2 Because plasma aldosterone is suppressed in nephrotic syndrome,1,2 the alternative pathway of ENaC activation by extracellular proteolysis has been investigated. Soluble proteolytic activity is present in urine samples from nephrotic syndrome patients.4 The major protease was identified as plasmin aberrantly filtered from plasma as plasminogen.5 In vitro, inward amiloride-sensitive current in collecting duct cells is enhanced by exposure to nephrotic urine containing plasmin and by purified plasmin.6 Plasmin was shown to release an inhibitory peptide tract from the exodomain of the γENaC subunit either by direct cleavage at high concentrations or through Glycosylphophatidylinositol-anchored prostasin at low concentrations.5,6,7 Addition of α₂-antiplasmin and aprotinin inhibited the ability of nephrotic urine and plasmin to evoke current.4,6,7 The in vitro cleavage of γENaC is consistent with a distinct shift in migratory pattern of renal tissue γENaC to lower molecular weight isoforms on SDS-PAGE gels in proteinuric conditions.5,9 Sodium retention and ascites formation is mitigated by the ENaC blocker amiloride in experimental nephrotic syndrome in rats and in children with nephrotic syndrome.2 Proteinuria can predict the onset of hypertension and, in a Framingham study cohort, urinary albumin:creatinine ratio predicted the development of hypertension.10 Preeclampsia is a syndrome of pregnancy with abrupt onset of proteinuria and hypertension after 20 weeks of gestation. The cause of preeclampsia is thought to involve release of a soluble fms-like tyrosine kinase (a vascular endothelial growth factor receptor) from placenta leading to decreased bioavailability of circulating vascular endothelial growth factor and endothelial injury, decreased NO formation, vasoconstriction, and glomerular damage with endotheliosis.11,12 Preeclamptic (PE) patients exhibit suppressed renin-angiotensin-aldosterone system activity, in particular aldosterone,13,14 and avid NaCl retention.15 Treatment with furosemide in preeclampsia yields less stimulation of plasma renin concentration.16 These observations are compatible with a primary renal hyperabsorption of NaCl in manifest
preeclampsia. Preeclampsia is associated with fibrin deposition in glomeruli and injury to the glomerular barrier. PE patients display plasmin(ogen) immunoreactivity in urine, to an extent that urine from PE patients has been used as a source to recover human plasminogen and plasmin. These observations led us to hypothesize that manifest preeclampsia is associated with plasmin-dependent protease activity in urine capable of enhancing epithelial ENaC activity. The hypothesis was tested in a cross-sectional design where urine was obtained from patients with preeclampsia and compared with urine obtained from women with uncomplicated pregnancies. The urine samples were tested for their plasmin(ogen) level, proteolytic activity, and ability to activate ENaC.

Materials
A cross-sectional study was conducted in 16 patients with newly diagnosed preeclampsia. Preeclampsia was defined as pregnancy beyond 20 weeks associated with newly developed hypertension (office systolic blood pressure >140 mm Hg and diastolic blood pressure >90 mm Hg) and urine protein:creatinine ratio >30 mg/mmol. The patients were compared with 17 healthy (normotensive and nonproteinuric) pregnant women. Control urine samples were obtained from 5 nonpregnant healthy women (age 30±2 years). Spot urine samples were frozen immediately after voiding and kept temporarily at −20°C until storage at −80°C. Samples were thawed on ice, and aliquots were made and stored at −80°C until experiments were performed. The study was approved by the Region of Southern Denmark ethical committee system (Project ID: S-VF-20060095). All patients gave written informed consent to participate in the study.

Methods
Blood pressure was measured 3 times after 20 minutes of rest in the seated position using blood pressure monitors from Omron Healthcare Inc (Palatine, IL) and A&D Medical (San Jose, CA). Devices have been approved and validated for clinical use. Before experiments, aliquots of the urine samples were centrifuged at 16,060 G for 30 seconds and the supernatant was used. See the online-only Data Supplement for details concerning measurements of total protein, albumin, aldosterone, creatinine, total plasminogen (plasminogen, plasmin, and plasmin-antiplasmin complexes, abbreviated: plasmin[ogen]), and total soluble urokinase plasminogen activator receptor. Details of urine zymography, immunoprecipitation, Western immunoblotting, and serine protease activity assay can be found in the online-only Data Supplement. Single cell patch-clamp experiments were conducted on the cortical collecting duct cell line (M1), as described.

Statistical Evaluation
Data were tested for normal distribution. If data were not normally distributed, log-transformation was used to achieve normal distribution. Log-normally distributed data are presented in semilogarithmic diagrams with geometric means. Normally distributed data are presented as means±SEM. Controls and PE patients were compared by unpaired Student t test. Correlation was evaluated using Pearson correlation (linear dependence). When comparing means of 3 groups (patch clamp data) 1-way ANOVA was used followed by Bonferroni multiple comparison post hoc test. For all data, P<0.05 were considered significant. For all analyses, GraphPad Prism 5 for Windows version 5.02 was used.

Results
The control group of healthy pregnant women was not different from the group of PE patients with respect to age or gestational age (Figure S1A and S1B in the online-only Data Supplement). PE patients excreted significantly (P<0.0001) more albumin and total protein in the urine and exhibited elevated systolic and diastolic blood pressures compared with normal pregnancies (P<0.0001; Figure S1C–S1F in the online-only Data Supplement). In PE patients, there was a significantly higher urinary excretion of immunoreactive plasmin(ogen), when normalized to urinary concentration of creatinine (P<0.0001; Figure 1A). Urine excretion of soluble urokinase plasminogen activator receptor (U-SuPAR) normalized to urine creatinine concentration in PE patients (n=16) compared with healthy pregnant women (Ctrl, n=17). The thick lines indicate geometric means (160 vs 4 µg/g, respectively) and thin lines the 95% CI. *P<0.0001 by unpaired Student t test. B. Figure shows spot urine concentration of soluble urokinase plasminogen activator receptor (U-SuPAR) normalized to urine creatinine concentration in PE patients (n=16) compared with healthy pregnant women (Ctrl, n=17). The thick lines indicate geometric means (6810 vs 7762 µg/g, respectively) and thin lines the 95% CI. There was no significant difference between the 2 groups.

Figure 1. A, Figure shows spot urine plasmin(ogen) (U-plg) concentration normalized to urine creatinine concentration in samples obtained from preeclamptic (PE) patients (n=16) and healthy pregnant women (Ctrl, n=17). The thick lines indicate geometric means (160 vs 4 µg/g, respectively) and thin lines the 95% CI. *P<0.0001 by unpaired Student t test. B. Figure shows spot urine concentration of soluble urokinase plasminogen activator receptor (U-SuPAR) normalized to urine creatinine concentration in PE patients (n=16) compared with healthy pregnant women (Ctrl, n=17). The thick lines indicate geometric means (6810 vs 7762 µg/g, respectively) and thin lines the 95% CI. There was no significant difference between the 2 groups.
yielded a product with molecular size that migrated similarly to pure human plasmin (molecular weight \( \approx 80 \) kDa). PE: urine samples from healthy pregnant controls. When cells were treated with amiloride (2 µmol/L) before exposure to PE urine (Figure 3C and 3D). In the presence of amiloride, whole-cell current after the addition of PE urine was significantly lower than current measured with urine from healthy control pregnancies (\( P<0.05 \); Figure 3A and 3D). Treatment with \( \alpha_2 \)-antiplasmin (1 µmol/L) abolished inward current in response to PE urine (\( P<0.001 \); Figure 3D). Heat denaturation of urine samples abolished the ability of PE urine to evoke inward current (\( P<0.001 \); Figure 3D). Inward current was at the level observed after amiloride and \( \alpha_2 \)-antiplasmin treatment and significantly lower than control urine (\( P<0.01 \); Figure 3D). Urine Na/K ratio was not significantly different between Ctrl (2.0±0.3) and PE (1.8±0.3) patients. Urine aldosterone excretion was significantly lower in women with pre-eclampsia compared with healthy controls when normalized for creatinine concentration (Figure 4A) (\( P<0.001 \); geometric means). A significant negative correlation (\( P<0.05 \)) was found between plasmin(ogen) and aldosterone in urine from PE patients (Figure 4B).

**Discussion**

The present set of data shows significant urinary excretion of plasmin(ogen) and active plasmin in patients with preeclampsia compared with healthy pregnant controls, as measured by Western immunoblotting of urine immunoprecipitated with antiplasmin(ogen) antibody and subjected to gel separation and Western blotting. Reaction with an antibody that detects human plasmin(ogen) was observed using urine samples from healthy pregnant controls. Plasmin control demonstrated significant recovery and similar migratory pattern as immunoreactive protein from patient urine.

**Figure 2. A**, Western immunoblot for plasmin(ogen) of spot urine samples from preeclamptic (PE) patients and healthy pregnant controls (control). Pure human plasmin (P) served as a positive control. Urine and pure human plasmin (5 µg in buffer) underwent similar purification by aprotinin-coated beads followed by separation through SDS-PAGE under denaturing conditions and immunoblotting by an antibody directed against human plasmin(ogen). No immunoreactive protein was observed using urine samples from healthy pregnant controls while proteins with molecular size of plasminogen (83, 88 kDa) and plasmin (intact 83, subunits 64, 57, and 23 kDa) appeared in PE samples. Plasmin control demonstrated significant recovery and similar migratory pattern as immunoreactive protein from patient urine.

**B**, Representative gelatin-PAGE zymography of crude urine samples from PE patients and healthy pregnant women (control). The zymogram shows proteolytic activity at 60 to 90 kDa with a major product around 70 kDa, corresponding with the proteolytic activity of pure human plasmin (P) at 1 µg per well. Urine samples from the control group (Control) did not display protease activity.

**C**, Dilution series for pure human plasmin by a fluorescence-substrate serine protease activity assay. The dilution series of pure plasmin generated the line with concentrations indicated at the x axis. Serine proteases were purified from 1-mL urine samples from PE patients and healthy controls by aprotinin-coated sepharose beads. Eluate was used for fluorescence assay (50 µL). Broken line indicates the geometric mean of serine protease activity obtained in 1 mL of PE patient’s urine (n=13). Urine samples from control pregnancies (n=14) yielded activity measurements not significantly different from 0 by this method. RFU indicates relative fluorescence unit.

**D**, Correlation between concentrations of albumin (U-alb mg/L) and plasminogen (U-plg ug/L). Data were log-transformed to obtain normal distribution. (Pearson correlation; \( P<0.05 \); \( R^2=0.2931 \)).
Plasmin activity confers to urine samples the ability to activate inward current in single collecting duct cells through ENaC as judged by sensitivity to α₂-antiplasmin, amiloride, and heat denaturation. There was a significant inverse correlation between urine aldosterone and plasmin(ogen) and a direct correlation between urine plasmin(ogen) and albumin, which suggests aberrant filtration from plasma. The new information gained on ENaC activation by plasmin in urine from PE patients suggests that ENaC may contribute to an inappropriate renal conservation of Na⁺ and water and suppression of plasma aldosterone and contribute to hypertension in preeclampsia. Liddle syndrome of hypertension with suppressed aldosterone in which ENaC remains membrane-associated illustrates the significance of dysregulated ENaC for blood pressure. A pathological mechanism that includes ENaC-mediated, excessive renal conservation of NaCl in preeclampsia is consistent with a number of previous observations: (1) plasma renin and aldosterone is suppressed with temporal correlation between onset of proteinuria and suppression of renin and aldosterone; (2) extracellular volume expansion with elevated atrial natriuretic peptide; (3) more avid retention of NaCl after NaCl infusion; and (4) diminished responsiveness of renin secretion to furosemide but normal reaction of renin.

Figure 3. A to C, Original current traces obtained in single cortical collecting duct cells (M1 line) by patch-clamp recordings. In A to C, gray traces display recordings from cells before superfusion with urine, whereas black traces show recordings from the same cells after superfusion with urine. A, A healthy pregnant control; B, a patient with preeclampsia; and C, urine from the same preeclamptic (PE) patient as in B but after the addition of amiloride. D, Bar graph showing the average changes in current obtained in patch-clamp experiments with single M1 cells in response to superfusion with urine from healthy pregnant controls (Ctrl) and urine from PE patients. The same PE patient urine samples were tested after addition of amiloride (PE+A, 2 µmol/L), α₂-antiplasmin (PE+AP, 1 µmol/L), and heat denaturation of the samples (PE+HD). n=6 in all experiments. *P<0.0001; **P<0.001; ***P<0.05, by ANOVA followed by unpaired Student t test with Bonferroni correction.

Figure 4. A, Aldosterone concentration (U-aldo) normalized to creatinine concentration (U-crea) in spot urine samples from patients suffering from preeclampsia (PE, n=16) and healthy pregnant women (Ctrl, n=17). The thick lines indicate geometric means and thin lines the 95% CI (27 vs 109, µg/g, respectively). *P<0.001, unpaired Student t test. B, Correlation between plasmin(ogen)–(U-plg)/creatinine ratio and aldosterone (U-aldo)/creatinine ratio in spot urine samples from PE patients (n=16). Correlation was estimated by Pearson test; P<0.05; R²=0.2581.
release to postural changes. The K+-sparring antihypertensive drug amiloride normalizes sodium balance and resolves edema in a rat model of nephrotic syndrome and in children with nephrotic syndrome. Previous trials with thiazides and loop diuretics summarized in a Cochrane review have failed to show attenuation of preeclampsia. This could be because of exaggerated Na+ reabsorption along the collecting duct that might abrogate the effect of diuretics that target Na+ transport proteins in more proximal nephron segments. Proof-of-concept for ENaC involvement in clinical manifestations of preeclampsia requires a randomized intervention study. So far, only casuistic reports are available: a pregnant patient with Conn syndrome and 2 pregnant women with Bartter syndrome received amiloride therapy throughout pregnancy or beyond week 17 and in doses ≤ 15 to 20 mg/day in one of the cases. These pregnancies resulted in healthy offspring.

Plasminogen is produced predominantly by the liver and circulates at slightly elevated plasma concentrations in normal human pregnancies. The present study confirms significant excretion of plasminogen and shows active plasmin in urine from PE patients. An integral membrane protein plasminogen receptor, Plg-Rγ, has been identified. Urokinase-type plasminogen activator (uPAR) and colocalizes with urokinase-type plasminogen activator receptor (uPAR). Urokinase-type plasminogen activator is abundant in urine and requires activation by uPAR. The present data show urinary excretion of soluble uPAR at similar levels between PE patients and healthy pregnant women. This is compatible with an epithelial origin of soluble uPAR immunoreactivity likely caused by shedding rather than aberrant filtration from plasma where it is slightly elevated in preeclampsia. Our previous data have shown the existence of a cell-attached cascade whereby low concentrations of plasmin (≤ 1 µg/mL) activates cell-anchored prostasin. At the measured concentrations of active plasmin in urine, involvement of amplification, that is, prostasin, in the activation of ENaC, is likely. Adjacent cleavage sites for plasmin and prostasin have been identified in the N terminal of γENaC. According to the stepwise proteolytic activation concept proposed by Kleyman et al, plasmin and prostasin elicit extracellular second hit proteolysis subsequent to intracellular furin to release an inhibitory peptide from γENaC. This causes full activation of the channel.

**References**

Novelty and Significance

What Is New?

• The study provides a new disease mechanism for an old disease, toxemia of pregnancy, that can be linked to the renin-aldosterone system and inappropriate renal NaCl retention.

What Is Relevant?

• Preeclampsia affects globally 3% to 5% of all pregnancies and is the leading cause of maternal and fetal mortality. No causal treatment exists for preeclampsia. The study provides a range of potential new drug targets to alleviate symptoms.

Summary

The study shows a new coupling between aberrant urine enzyme activity and inappropriate renal NaCl retention in preeclampsia. This mechanism may explain the development of hypertension and edema and provide new drug targets to alleviate symptoms.

ONLINE SUPPLEMENT

URINARY PLASMIN ACTIVATES COLLECTING DUCT ENaC CURRENT IN PREECLAMPSIA

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Supplementary Methods

Urinalysis

Before experiments, aliquots of the urine samples were centrifuged at 13,000 rpm for 30 seconds and experiments were done using the supernatant. Concentrations of urinary total protein and urinary albumin were determined on the Cobas Mira Plus device using ABX Pentra reagents (urinary total protein CP, ref: A11A01642 and Micro-albumin CP, ref: A11A01623; Triolab A/S, Brøndby, Denmark) following the manufacturer’s protocol. Urine aldosterone concentration was measured using Coat-A-Count® Aldosterone kit (Siemens Healthcare Diagnostics Inc. Los Angeles, CA, USA) following the manufacturer’s instructions. Urinary creatinine concentrations were determined spectrophotometrically using Microlab 300 (Vital Scientific BV, AC Dieren, NL).

Measurement of total plasminogen (plasminogen, plasmin and plasmin-anti-plasmin complexes, abbreviated: plasmin(ogen)) content in urine samples was performed using a commercial ELISA kit (Human Plasminogen Total Antigen Assay (Cat# IHPLGKT-TOT, Innovative Research, Novi, Michigan, USA). Measurement of total soluble urokinase plasminogen activator receptor (SuPAR) content in urine samples was performed using a commercial ELISA assay (SuPARnostic® Standard Kit, Virogates A/S, Birkeroed, DK). Urinary serine protease activity was tested using 10% Zymogram (gelatin) gel. For quantitative protease assay and additional western immunoblotting, urine samples were pretreated using aprotinin (USB, Cleveland, OH, USA) coupled to CNBr-activated Sepharose 4B (Amersham Bioscience, Hillerod, DK) as previously described1 using 1 mL of urine.

Immunoprecipitation Plasmin(ogen) was immunoprecipitated from 85 µl urine using polyclonal goat anti-plasminogen (ab 6189-100, Abcam, Cambridge, UK) and the Protein G immunoprecipitation kit (IP50, Sigma, St. Louis, Missouri, USA) following the manufacturer’s protocol.

Western Immunoblotting The immunoprecipitate or the aprotinin plasmin(ogen) purification eluate (see quantitative protease assay below) was mixed with NuPAGE® Sample Reducing Agent (10X) (Invitrogen, Carlsbad, CA, USA), NuPAGE® LDS Sample Buffer (4X) (Invitrogen), and heat denatured. Samples were run on a Ready Gel 7.5% Tris-HCl (BioRad Laboratories Inc. Hercules, CA, USA), and subsequently blotted onto an Immobilon™ polyvinylidene difluoride membrane (Millipore, Immobilon-P transfer membrane, pore size 0.45µm, Millipore Corporation, Bedford, MA, USA). The polyclonal goat anti-human plasminogen ab 6189-100 (Abcam) was used as primary antibody and HRP-conjugated anti-goat (DakoCytomation, Glostrup, Denmark) was used as secondary antibody. Blots were developed using AmershamTM ECL™ system.

Serine protease activity Urinary protease activity was tested using 10% Zymogram (gelatin) gel, 1.0 mm x 10 well (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol loading 10 µL urine and 10 µL Novonex ® Tris-Glycine SDS Sample buffer (x2) (Invitrogen, Carlsbad, CA, USA) per well. For quantitative protease assay, urine samples were pretreated using Aprotinin (USB, Cleveland, OH, USA) coupled to CNBr-activated Sepharose 4B (Amersham Bioscience, Hillerod, DK) as previously described (Svenningsen et al 2009) using 1 mL of urine. The beads were pelleted and washed thoroughly. Bound proteins were eluted using 100µL PBS, pH 2 added 10 µL 1 mol/L Trizma per sample. Quantitative measurements of serine protease activity in the urine samples were conducted using EnzChek® Peptidase/Protease Assay Kit (E33758), (Molecular Probes™, Eugene, OR, USA) following the manufacturer’s protocol. Readings were done using Fusion™, Packard using software Fusion Instrument Control application v4.0, Perkin Elmer®.
Single cell (CCD M1) Patch-clamp experiments The experiments were conducted on the cortical collecting duct cell line (M1) from ATCC (Boras, Sweden) as previously described. Briefly, M-1 cells were grown to confluence in 25 cm² flasks (Nunc, Roskilde, Denmark) followed by trypsination and seeding onto coverslips in DMEM:F12 (Life Technologies, Taastrup, Denmark) added 5 µmol/L dexamethasone (Sigma, Brøndby, Denmark). M-1 cells incubated at 37°C/5% CO₂ and single cell patch clamp experiments were performed 24 to 48 hours after the cells were seeded. Experiments were performed at room temperature in the tight-seal whole-cell configuration of the patch-clamp technique with heat-polished patch pipettes with resistances of 5-7 MΩ. Seal resistances were in the range of 2-15 GΩ. High resolution membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA) controlled by PULSE v8.11 software on a Power Macintosh G3 computer. The current was monitored by the response to a voltage step of -160 mV for 200 ms from a holding potential of -60 mV (this pulse was repeated every 3rd second throughout the entire experiment). After 30-60 seconds, the cell was gently flushed with urine and the current monitored.

List of References

Figure S1

A

Gestational Age Days

B

Age years

C

Systolic BP mmHg

D

Diastolic BP mmHg

E

ACR mg/g

F

U-protein total/U-crea mg/g
**Figure legends S1**

Description of the two groups showing values from preeclamptic patients and healthy pregnant controls with respect to gestational age, age, arterial blood pressure, urine albumin and protein. Preeclamptic patients (PE, n=17) compared to healthy pregnant controls (Ctrl, n=16).

**A-B,** Figures show gestational age (GA) in days (A) and age of each individual in years (B) for preeclamptic patients (PE) and healthy pregnant controls (Ctrl). Mean values GA: PE 242.4 ± 6.5 days vs. Ctrl 241.9 ± 4.8 days, Age: PE 32.0 ± 1.6 years vs. Ctrl 31.6 ± 1.4 years.

**C-D,** Figures show distribution of systolic (C) and diastolic (D) blood pressures in preeclamptic patients (PE) and healthy pregnant controls (Ctrl). Systolic blood pressure means; PE: 158.5 ± 3.1 mmHg vs. Ctrl: 116.9 ± 2.1 mmHg, diastolic blood pressure means; PE: 100.5 ± 2.2 mmHg vs. Ctrl: 74.9 ± 1.8 mmHg, *P < 0.0001, unpaired Student’s t-test.*

**E,** The figure shows distribution of the urine albumin-creatinine-ratio (ACR) in the two groups PE and Ctrl. (Geometric mean 367.3 mg/g vs. 3.6 mg/g), *P < 0.0001, unpaired Student’s t-test.*

**F,** The figure shows distribution of total urine protein concentration normalized to urine creatinine concentration in the two groups PE patients and control. (Geometric mean: 1002.3 mg/g vs. 89.9 mg/g), *P < 0.0001, unpaired Student’s t-test.*