PROFESSOR FERRUH ARTUNC (Orcid ID : 0000-0002-3777-9316)

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Rebuttal to Editorial: sodium retention by uPA in nephrotic syndrome?

Bernhard N. Bohnert[1-3], Sandip Kanse[4], Silke Haerteis[5], Christoph Korbmacher[6], Ferruh Artunc[1-3]

[1] Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Disease, Nephrology and Clinical Chemistry, University Hospital Tübingen, Germany

[2] Institute of Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Center Munich at the University Tübingen, Germany

[3] German Center for Diabetes Research (DZD) at the University Tübingen, Germany

[4] Institute of Basic Medical Sciences, University of Oslo, Norway

[5] Institute of Anatomy, University of Regensburg, Germany

[6] Institute of Cellular and Molecular Physiology, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Germany

Address for correspondence:
Ferruh Artunc, MD
University hospital Tuebingen
Department of Internal Medicine
Division of Endocrinology, Diabetology and Nephrology
Otfried-Mueller-Str.10
72076 Tuebingen, Germany
E-mail: ferruh.artunc@med.uni-tuebingen.de
Tel. +49-7071-2982711 / Fax +49-7071-2925215

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Dear editor,

We welcome the opportunity to reply to the elegant editorial of Prof. Ehmke [1] in which he highlights contradicting conclusions reached by Hinrichs et al. [2] and by us [3] in two recently published articles in Acta Physiologica. In our reply, we first comment on some technical aspects discussed by Prof. Ehmke as possible explanations for the discrepant conclusions reached in the two studies. In addition, we highlight some *in vivo* data reported by Hinrichs et al. [2] which in our view do not oppose but rather support our conclusion that urokinase, also known as urokinase-type plasminogen activator (uPA), is not essential for sodium retention in nephrotic syndrome.

As pointed out by Prof. Ehmke, different experimental approaches were used in the two studies to test the hypothesis that uPA mediated plasmin generation from aberrantly filtered plasminogen is a key mechanism responsible for proteolytic ENaC activation and increased renal sodium absorption in nephrotic syndrome. Hinrichs et al. [2] used a monoclonal antibody to inhibit uPA (uPAab) acutely in mice with nephrotic syndrome due to an inducible podocin knockout (Pod KO mice). In contrast, we used a constitutive uPA knockout mouse model in which we induced nephrotic syndrome with doxorubicin. Prof. Ehmke suggests that acute versus chronic inhibition of uPA may be a reason for the apparently discrepant findings of the two studies. For example, in the chronic uPA knockout mouse model other serine proteases may compensate for the loss of uPA, whereas this is less likely to occur with an acute inhibition of uPA by an antibody. We fully agree that, like in any constitutive global knockout model, compensatory mechanisms have to be taken into consideration. However, we can clearly demonstrate that in nephrotic uPA knockout mice the conversion of urinary plasminogen to plasmin is severely impaired compared to control mice. This confirms efficient uPA knockout and the important role of uPA in converting plasminogen to plasmin in nephrotic urine. Moreover, it demonstrates that uPA knockout mice do not compensate the chronic lack of uPA by upregulating other serine proteases with the ability to convert plasminogen into plasmin in nephrotic urine. In this context we would like to emphasize an important strength of the global constitutive uPA knockout model used in our study: it not only abolishes any local tubular uPA activity but also prevents aberrant glomerular filtration of circulating soluble uPA in nephrotic syndrome which is thought to contribute to uPA-mediated plasminogen activation in nephrotic urine [4]. We agree with Prof. Ehmke that in nephrotic mice with genetic uPA deletion the formation of plasmin from plasminogen may not be completely suppressed. We also agree that very low concentrations of plasmin are sufficient to cause proteolytic ENaC activation as we have previously shown *in vitro* [5]. Therefore, we cannot rule out

the possibility that in nephrotic uPA knockout mice very low concentrations of urinary plasmin may contribute to proteolytic ENaC activation. Importantly, this does not challenge our main conclusion that uPA is not essential for sodium retention in nephrotic syndrome.

A second methodological difference between the two studies pointed out by Prof. Ehmke is the use of different strategies to induce nephrotic syndrome. Regarding our model he is concerned that using doxorubicin may cause further renal damage in addition to severe impairment of the glomerular filtration barrier. Doxorubicin-induced nephropathy is primarily a toxic model inducing podocyte ablation without inflammation and resembles human focal segmental glomerulosclerosis [6]. The kinetics of the onset of proteinuria is very similar to that seen in the inducible model used by Hinrichs et al. [2] in which the podocyte foot process protein podocin is specifically deleted. Both models lead to global glomerulosclerosis, interstitial fibrosis, tubular atrophy and finally progressive renal failure leading to death of the nephrotic mice after 40 days [6, 7]. Inflammatory changes occur during the progression period, but are less prominent in the first 15 days during which proteinuria and nephrotic syndrome develop in both models. Subtle differences in the composition or quantity of excreted proteins cannot be ruled out, but in our opinion are unlikely to explain the different conclusions reached in the two studies.

There is substantial experimental evidence for the concept that proteolytic ENaC activation by proteasuria contributes to renal sodium retention in nephrotic syndrome [8]. In particular, two key observations support this hypothesis: firstly, treatment with the ENaC inhibitor amiloride greatly attenuates renal sodium retention in rat [9] and mouse [3, 10] models of nephrotic syndrome. Secondly, inhibiting urinary protease activity by treating mice with aprotinin reduces renal sodium retention to a similar extent as amiloride treatment [10]. As pointed out by Prof. Ehmke, two authors of our present study contributed to the publication by Svenningsen et al. [11] proposing the concept that in nephrotic syndrome uPA converts aberrantly filtered plasminogen to plasmin which can be readily detected in nephrotic urine. Moreover, this latter study and several subsequent reports clearly established that plasmin can proteolytically activate ENaC *in vitro* by cleaving the γ-subunit of the channel at specific sites [12, 13]. In our present study, we confirmed that in the oocyte expression system a combination of uPA and plasminogen is needed for the proteolytic activation of ENaC. In contrast, uPA or plasminogen alone had no stimulatory effect. These *in vitro* data support the hypothesis that uPA is needed to generate plasmin which in turn can proteolytically activate ENaC. However, several other serine proteases have been shown to activate ENaC *in vitro* by cleaving γENaC at the same cleavage sites as plasmin or at several alternative cleavage sites [14, 15].

Therefore, it has to be kept in mind that in addition to plasmin nephrotic urine may contain several other serine proteases with the ability to mediate proteolytic ENaC activation. In this context the question raised by Prof. Ehmke, whether the antibody against uPA may inhibit other serine proteases, is obviously relevant for the interpretation of the *in vivo* data reported by Hinrichs et al. [2].

Hinrichs et al. [2] demonstrate that the anti-uPA antibody essentially abolished the conversion of plasminogen to active plasmin in nephrotic urine which supports the conclusion that it effectively inhibits uPA. As stated by the authors "This was not associated with altered delta weight and did not mitigate ascites formation (Figure 7C,D), however, it was associated with a non-significant increase in day-by-day urinary sodium excretion (Figure 7E) and a significant attenuation of accumulated sodium balance (Figure 7F)." A close inspection of figure 7E reveals that up to day 14 cumulative sodium balance in uPAab-treated Pod KO mice was very similar to that in vehicle treated Pod KO mice. A significant difference of cumulative sodium balance was only observed at day 18/19 after induction of nephrotic syndrome. In our view, these data do not provide compelling evidence that the anti-uPA antibody substantially reduces sodium retention, at least not in the most relevant initial phase of nephrotic syndrome. Sodium balance studies in mice are technically challenging and have to be interpreted with caution because they are error-prone. This is highlighted by the experiments of Hinrichs et al. in which they observed a significant weight increase (Figure 2A) and also significant ascites formation (Figure 2C) in Pod KO versus wild-type mice, but failed to observe a significant difference in sodium balance (Figure 2B). They also did not detect a significantly higher daily sodium excretion in the uPAab-treated Pod KO mice compared to vehicle treated Pod KO mice. Importantly, in the uPAab-treated Pod KO mice the weight gain (Figure 7C) and ascites formation (Figure 7D) were very similar to those observed in the vehicle treated Pod KO mice. Maximal body weight gain occurred on day 14 in both groups of animals. These measurements are likely to be more robust and reliable than the sodium balance studies and provide strong evidence that sodium and fluid retention are largely preserved in the anti-uPA antibody treated animals. Thus, in our view the *in vivo* findings of Hinrichs et al. [2] do not support their conclusion that inhibiting uPA reduces sodium retention in nephrotic syndrome. On the contrary, we feel that their *in vivo* data are nicely consistent with our finding that after induction of nephrotic syndrome the body weight curve in uPA knock-out mice is essentially identical to that observed in wild-type littermates (Figure 5G) [2]. Taken together the findings of the two studies support the conclusion that uPA is important for mediating the conversion of

plasminogen to plasmin but does not play a critical role in the pathogenesis of sodium and fluid retention in nephrotic syndrome.

In conclusion, we would like to thank Prof. Ehmke for his careful analysis of the two studies, his insightful comments and his general appreciation of the pathophysiological relevance of this topic. We hope that with our reply we can contribute to the discussion and offer a reconciling perspective by focusing on the *in vivo* findings of the two studies which are largely consistent and do not support a major role of urokinase in the pathophysiology of sodium retention in nephrotic syndrome. In our view, both studies question the current concept of uPA-mediated plasmin generation as a key mechanism of proteolytic ENaC activation and sodium retention in nephrotic syndrome. Therefore, they also challenge the idea that specific inhibition of uPA may be a useful therapeutic strategy in patients with nephrotic syndrome. This does not rule out that inhibiting other serine proteases may have beneficial effects in nephrotic syndrome as suggested by our finding that aprotinin treatment substantially reduces weight gain and ascites formation in mice with doxorubicin induced proteinuria [10]. However, more research is needed to identify the relevant proteases and to investigate their potential as therapeutic targets.

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