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# Increase of bikunin and $\alpha_1$-microglobulin concentrations in urine of rats during pregnancy is due to decreased tubular reabsorption

Anna M. Blom [a,*], Cecilia Falkenberg [b], Mats Sjöquist [c], Ulf J. Eriksson [d], Bo Åkerström [b], Erik Fries [a]

[a] *Department of Medical and Physiological Chemistry, S-751 23 Uppsala, Sweden*
[b] *Department of Cell and Molecular Biology, Lund University, S-22100 Lund, Sweden*
[c] *Department of Physiological and Medical Physics, S-751 23 Uppsala, Sweden*
[d] *Department of Cell Biology, Biomedical Center, S-751 23 Uppsala, Sweden*

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**Abstract**

Bikunin and $\alpha_1$-microglobulin are two plasma proteins of about 25 kDa which are made in the liver from a common precursor. The concentration of bikunin in human urine has been shown to increase several fold during various conditions of stress. The mechanism behind this increase is unknown. We have studied pregnant rats and found that the bikunin and $\alpha_1$-microglobulin levels in their urine increased 3-fold towards the end of the pregnancy, whereas those of albumin and orosomucoid did not. There were no significant changes in either the bikunin/$\alpha_1$-microglobulin mRNA level or the concentrations of the two proteins in serum. These findings imply that the synthesis and the clearance rates of bikunin and $\alpha_1$-microglobulin are normal during pregnancy but that the tubular reabsorption of these proteins is decreased. © 1997 Elsevier Science B.V.

*Keywords:* Bikunin; $\alpha_1$-microglobulin; Pregnancy; Urine; Reabsorption

## 1. Introduction

Bikunin is a plasma protein of 25 kDa which consists of two similar domains, each with proteinase inhibitory activity [1,2]. The physiological role of bikunin is unclear but the protein has been shown to affect the metastasis of cancer cells — possibly by inhibiting plasmin [3,4]. Bikunin has also been reported to stimulate cell growth [5,6] and to block the release of intracellular $Ca^{2+}$ induced by lipopolysaccharide [7]. The primary translation product of the mRNA coding for bikunin is a precursor also containing $\alpha_1$-microglobulin [8]. The precursor is cleaved intracellularly and the two proteins are secreted separately. $\alpha_1$-microglobulin has been shown to affect inflammatory and immune reactions [9]. In the blood, most of bikunin and $\alpha_1$-microglobulin occurs in complex with other, larger polypeptides [10–12].

Experiments with radiolabelled bikunin have shown that the protein has a plasma half-life of about 10 min and that the kidneys account for approximately half of the uptake [13]. Bikunin is the major

* Corresponding author. Fax: +46 18 174975. E-mail: anna.blom@medkem.uu.se


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proteinase inhibitor of urine [14] and has often been referred to as the urinary trypsin inhibitor. A protein that might be identical to bikunin has been found to be an inhibitor of kidney stone formation [15]. It has been known for almost a century that the bikunin concentration in human urine (initially measured as antitryptic activity) increases several fold during pregnancy and under various disease conditions such as infection and fever (reviewed by Faarvang [16]); similar observations have been made for $\alpha_1$-microglobulin [9]. It has been suggested that these increases might be due to an increased synthesis of the two proteins (acute phase reaction [17]) or to proteolytic release from high molecular weight complexes [18,19]. Using the rat as a model, we show in this study that the increase of the urinary levels of bikunin and $\alpha_1$-microglobulin during pregnancy is due to decreased tubular reabsorption.

## 2. Materials and methods

### 2.1. Plasma and urine collection

Female virgin Sprague-Dawley rats (200–280 g) were kept in cages with free access to water and standardized diet (R36; Lactamin, Stockholm, Sweden). They were mated overnight and conception was assessed by a vaginal smear on the following morning; this day was taken as day 0 of the pregnancy. For urine collection, the animals were transferred to metabolical cages where they were kept for 16–20 h, unless specified otherwise. Before the rats were returned to standard cages, a blood sample was taken from the tip of the tail.

### 2.2. Protein analysis of plasma and urine samples

The bikunin concentration was measured with a radioimmunoassay as previously described [2]; bikunin linked to other polypeptides was removed by perchloric acid precipitation [2]. The concentrations of $\alpha_1$-microglobulin and orosomucoid [20] were also determined with radioimmunoassays. The creatinine and protein contents of urine were measured with colorimetric assays (Sigma Diagnostics, USA and Protein Assay, BioRad, USA, respectively).

### 2.3. RNA preparation and northern blot analysis

mRNA was isolated directly from homogenates of liver and kidney tissues obtained from control and pregnant rats (after 16 days of pregnancy) by the use of magnetic particles (PolyATract System 1000; Promega; Madison, USA). The obtained mRNA (1 $\mu$g) was denatured by formaldehyde, separated by electrophoresis and blotted onto a membrane (Hybond-N+; Amersham; Buckinghamshire, UK). A full-length clone of rat cDNA for $\alpha_1$-microglobulin-bikunin was used as a probe; labelling was made with [$\alpha$-$^{32}$P]dCTP (Megaprime DNA labelling kit; Amersham; Buckinghamshire, UK). Hybridization was performed for 2 h at 65°C in Rapid-hyb buffer (Amersham; Buckinghamshire, UK). The membrane was washed for 20 min in 2 × SSC (30 mM $Na_3$ citrate, 300 mM NaCl, pH 7.0), 0.1% SDS at room temperature and then 2 × 15 min in 0.1 × SSC, 0.1% SDS at 65°C. Finally, the membrane was exposed to Fuji RX film overnight. The probe was removed from the membrane by a 10 minute immersion in boiling SDS (0.5%). Subsequently, the relative amounts of mRNA in each lane were determined by hybridization with a 700 bp fragment of cDNA coding for human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [21].

## 3. Results

### 3.1. Protein levels in urine during pregnancy

As an introduction to this study, we measured the urinary level of bikunin of normal rats during a few days after their transfer to metabolic cages. As previously shown for humans [16], we found that the urinary level of bikunin varied greatly between different rats (Fig. 1). The daily variation was also striking; comparison of the patterns from several rats showed that the level during the night was generally higher than during the day. Similarly, the bikunin concentration of human urine has been shown to increase with increasing physical activity [22]. We also noted that the bikunin level was elevated during the first day in the metabolic cage. All subsequent analyses were therefore done with animals which had previously been in this cage for at least one day.

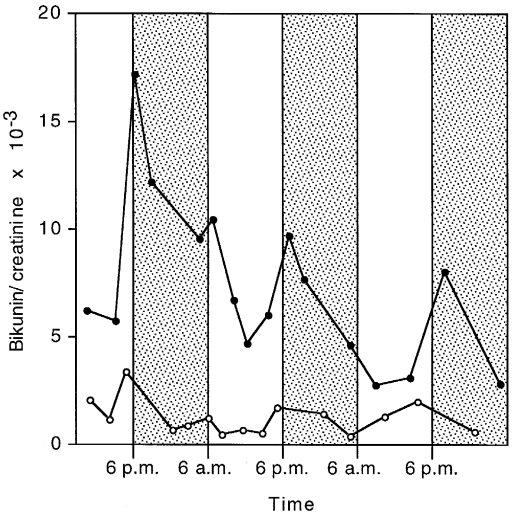


Fig. 1. Bikunin level in urine of normal rats. Two rats were transferred for the first time to metabolic cages and urine was collected. At the times indicated, urine was withdrawn and the concentrations of bikunin and creatinine determined. The effect of variations in the glomerular filtration rate was eliminated by dividing the bikunin value with that of creatinine. The stippled regions indicate time periods without light. The obtained results illustrate the facts that the bikunin level varies during the day and night and that the average level may be very different for different animals.

Fig. 2 (top and middle panels, open circles) shows that the bikunin and $\alpha_1$-microglobulin levels in urine collected from normal animals during 16–20 h intervals were relatively constant during a period of 20 days. During pregnancy, however, the levels increased 2- to 3-fold (closed circles). The individual variation was large: of 12 pregnant rats studied, 3 showed less than 50% of increase and one a 5-fold increase of the bikunin concentration. The individual levels prior to pregnancy varied by $\pm 70\%$ and the results are therefore shown relative to the initial value for each rat.

The urine was also assayed for orosomucoid, an acute phase protein [23] of 45 kDa. The level of this protein did not increase during pregnancy (Fig. 2, bottom left-hand panel) showing that the observed effects were not due to inflammation. We also found that the protein concentration of the urine varied $< \pm 30\%$ during the duration of the experiment (data not shown). Furthermore, analysis by gel electrophoresis showed that albumin was the most abundant urinary protein and that the concentration of this protein did not change significantly. The serum levels of bikunin, $\alpha_1$-microglobulin and orosomucoid were also determined (Fig. 2, right-hand panels) which

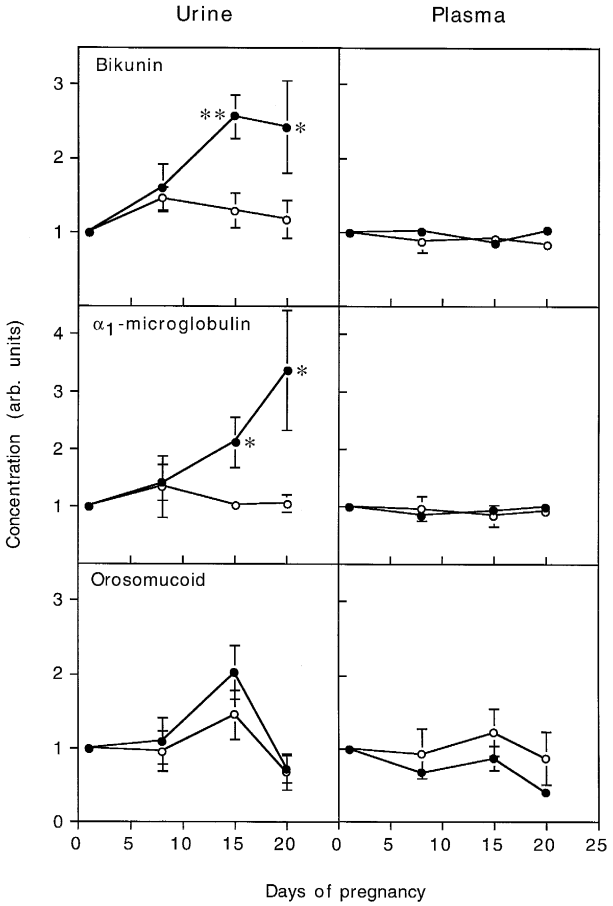


Fig. 2. Effect of pregnancy on the levels of bikunin, $\alpha_1$-microglobulin and orosomucoid in rat urine and plasma. Rats were mated and after the number of days indicated, they were kept for 16–20 h in metabolic cages for urine collection. At the end of this period a blood sample was taken. The concentrations of different proteins were then determined with radioimmunoassays. For the determination of the level of bikunin in free form, complexed bikunin was first removed by precipitation with perchloric acid. The protein concentrations in the urine were divided by the corresponding creatinine concentrations as described in Fig. 1. Shown are the protein levels relative to the initial value for each rat which was set at one. Open and closed circles represent values obtained for control and pregnant rats, respectively; SEMs are indicated by bars. 4–9 animals were used for each time series. A nonparametric, paired *t*-test was used to evaluate the statistical significance of the differences between the values of day 1 and those of days 8, 15 and 20 ($^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$).

showed that these did not significantly increase during pregnancy.

### 3.2. Level of bikunin / $\alpha_1$-microglobulin mRNA during pregnancy

To see if the increase of the bikunin and $\alpha_1$-microglobulin levels in urine upon pregnancy could be caused by an increase in the production of the two proteins, we determined the relative amounts of their mRNA in liver tissue. For this analysis we used rats whose urinary bikunin level were found to increase at least 2-fold towards the end of the pregnancy. Using $\alpha_1$-microglobulin/bikunin cDNA as the probe, we detected one mRNA species of the expected size [24]: 1.3 kb (Fig. 3). The amount of this mRNA relative to that of GAPDH (in arbitrary units) in normal and pregnant rats were $7.1 \pm 0.4$ (mean $\pm$ SEM; 5 animals) and $6.8 \pm 0.5$ (5 animals), respectively. The difference between these values is not statistically significant as judged by an unpaired *t*-test. To see if the increased urinary levels of bikunin/$\alpha_1$-microglobulin was due to synthesis of the two proteins in the kidneys, we also measured the mRNA level in this tissue: there were less than 0.1 U.

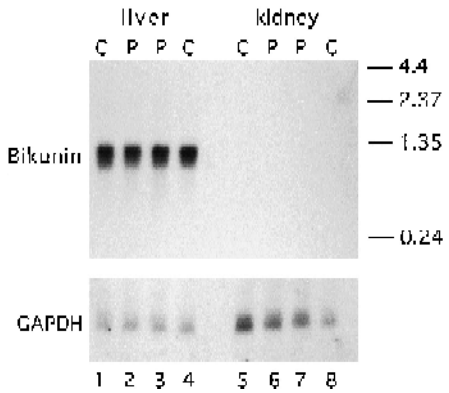


Fig. 3. Determination of mRNA levels for bikunin/$\alpha$1-microglobulin in rat liver and kidney. mRNA was isolated from the homogenates of liver and kidney, fractionated by gel electrophoresis and transferred to a membrane. mRNA for bikunin/$\alpha$1-microglobulin was detected with a specific probe labelled with $^{32}$P (upper panel); the positions of reference mRNA molecules run in parallel are shown to the right with their sizes in kilobases. The amount of GAPDH mRNA — an indicator for the amount of mRNA loaded on each lane — was determined by the same technique (lower panel).

## 4. Discussion

In this study we have found that the concentrations of bikunin and $\alpha_1$-microglobulin in the urine of rats increase during pregnancy. We also show that pregnancy does not affect the level of the bikunin/$\alpha_1$-microglobulin mRNA indicating that the production of the two proteins is constant. Furthermore, we found that the serum concentrations of the two proteins were unaffected implying that their clearance rates are not affected by pregnancy. We have previously shown that approximately half of the uptake of bikunin occurs in the kidneys and that bikunin is filtered through the glomeruli [13]. Proteins that pass through the glomeruli are efficiently reabsorbed in the proximal tubuli with the result that only a small fraction of the amount in the filtrate appears in the urine [25]. This fact together with our experimental observations suggests that the cause for the increase of urinary bikunin and $\alpha_1$-microglobulin levels during pregnancy is that the efficiency of their reabsorption decreases. An alternative explanation would be that there is an increased rate of translation of the bikunin/$\alpha_1$-microglobulin mRNA during pregnancy which would be balanced by an increased clearance rate. However, we have previously shown that the rate at which bikunin is filtered through the kidneys is remarkably high considering the fact that the hydrodynamic size of bikunin is close to that of bikunin [26]; if the kidneys only would account for the clearance, the half-life of bikunin would be about 20 min [13]. A several fold increase of the clearance rate of bikunin therefore seems very unlikely. Furthermore, we found that the plasma half-life of i.v. injected bikunin labelled with $^{125}$I was the same in pregnant and control animals (unpublished observation). A second alternative explanation for the increase of $\alpha_1$-microglobulin and bikunin in urine would be that pregnancy induces synthesis of the two proteins in the kidneys. However, this possibility can be ruled out since we could not detect a significant amount of the corresponding mRNA in this tissue.

During the intracellular transport of newly synthesized bikunin approximately two thirds becomes covalently linked to one or two different polypeptides of 80–90 kDa, named the heavy chains [2,27]. In rat plasma, the major bikunin-containing protein is pre-$\alpha$-inhibitor (unpublished observation), which consists

of bikunin and one heavy chain [28]. Similarly, half of the plasma $\alpha_1$-microglobulin occurs in a complex with fibronectin and $\alpha_1$-inhibitor 3 [10,11]. It is possible that part of the bikunin and $\alpha_1$-microglobulin in the urine is formed by proteolytic degradation of these complex molecules [29]. However, if this process would be the major cause of the increased urinary levels during pregnancy, the plasma concentrations of the two proteins in free form would increase proportionally, which was not seen in this study. However, for patients with inflammatory diseases this appears to be the case [18].

Based on the analysis of various proteins in human urine, Bernard et al. [30] concluded that pregnancy leads to an increase of the concentration of low molecular weight proteins. Our results are consistent with this finding; thus the levels of albumin and orosomucoid were unchanged whereas those of bikunin and $\alpha_1$-microglobulin increased. It seems that this size specific effect is also evoked by other situations of stress. Thus, the urinary secretion of retinol binding protein, like that of bikunin, has been shown to increase upon fever and inflammation [31,32]. The mechanism behind this change is unknown but it seems to be mediated in part by the cortisol production [33,34].

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## References

[1] M.W. Swaim, S.V. Pizzo, Biochem. J. 254 (1988) 171–178.
[2] A. Slota, M. Sjöquist, M. Wolgast, J. Alston-Smith, E. Fries, Biol. Chem. Hoppe-Seyler 375 (1994) 127–133.
[3] H. Kobayashi, H. Shinohara, K. Takeuchi, M. Itoh, M. Fujie, M. Saitoh, T. Tearo, Cancer Res. 54 (1994) 844–849.
[4] H. Kobayashi, M. Fuije, H. Shinohara, H. Ohi, M. Sugimura, T. Terao, Int. J. Cancer 57 (1994) 378–384.
[5] W.L. McKeehan, Y. Sakagami, H. Hoshi, K.A. McKeehan, J. Biol. Chem. 261 (1986) 5378–5383.
[6] J.K. Perry, G.K. Scott, C.A. Tse, Biochim. Biophys. Acta 1221 (1994) 145–152.
[7] N. Kanayama, A. Halim, K. Maehara, Y. Kajiwara, M. Fujie, T. Terao, Biochem. Biophys. Res. Comm. 207 (1995) 324–330.
[8] J.F. Kaumeyer, J.O. Polazzi, M.P. Kotick, Nucleic Acids Res. 14 (1986) 7839–7850.
[9] B. Åkerström, L. Lögdberg, TIBS 15 (1990) 240–243.
[10] C. Falkenberg, A. Grubb, B. Åkerström, J. Biol. Chem. 265 (1990) 16150–16157.
[11] C. Falkenberg, J.J. Enghild, I.B. Thogersen, G. Salvesen, B. Åkerström, Biochem. J. 301 (1994) 745–751.
[12] J.-P. Salier, P. Rouet, G. Raguenez, M. Daveau, Biochem. J. 315 (1996) 1–9.
[13] E. Sjöberg, A. Blom, M. Sjöquist, M. Wolgast, J. Alston-Smith, E. Fries, Biochem. J. 308 (1995) 881–887.
[14] B.J. Bromke, F. Kueppers, Biochem. Med. 27 (1982) 56–67.
[15] F. Atmani, S.R. Khan, Urol. Res. 23 (1995) 95–101.
[16] H.J. Faarvang, Scand. J. Clin. Lab. Invest. 17 (1965) 1–78.
[17] P. Pierzchalski, H. Rokita, A. Koj, E. Fries, B. Akerstrom, FEBS Lett. 298 (1992) 165–168.
[18] C. Franck, J.Z. Pedersen, Scand. J. Clin. Lab. Invest. 43 (1983) 151–155.
[19] K. Hochstrasser, G. Bretzel, H. Feuth, W. Hilla, K. Lempart, Hoppe-Seyler's Z. Physiol. Chem. 357 (1976) 153–162.
[20] B. Åkerström, J. Biol. Chem. 260 (1985) 4839–4844.
[21] J.Y. Tso, X. Sun, T. Kao, K.S. Reece, R. Wu, Nucl. Acids Res. 13 (1985) 2485–2502.
[22] L.K. Christensen, Scand. J. Clin. Lab. Invest. 10 (1958) 177–181.
[23] G. Schreiber, G. Howlett, M. Nagashima, A. Millership, H. Martin, J. Urban, L. Kotler, J. Biol. Chem. 257 (1982) 10271–10277.
[24] H. Vetr, W. Gebhard, Biol. Chem. Hoppe-Seyler 371 (1990) 1185–1196.
[25] R.G. Galaske, J.B. v. Liew, L.G. Feld, Kidney Int. 16 (1979) 394–403.
[26] K.E. Lindström, A. Blom, E. Johnsson, B. Haraldsson, E. Fries, Kidney Int. 51 (1997) 00–00.
[27] E.M. Sjöberg, E. Fries, Arch. Biochem. Biophys. 295 (1992) 217–222.
[28] J.J. Enghild, G. Salvesen, S.A. Hefta, I.B. Thogersen, S. Rutherfurd, S.V. Pizzo, J. Biol. Chem. 266 (1991) 747–751.
[29] K. Hochstrasser, J. Niebel, H. Feuth, K. Lempart, Klin. Wschr. 55 (1977) 337–342.
[30] A. Bernard, N. Thielemans, R. Lauwerys, M. v. Lierde, Scand. J. CLin. Lab. Invest. 52 (1992) 871–878.
[31] T. Sandberg, E.H. Cooper, G. Lidin-Janson, H. Yu, Nephron 41 (1985) 39–44.
[32] C.B. Stephensen, J.O. Alvarez, J. Kohatsu, R. Hardmeier, J.I. Kennedy, R.B. Gammon, Am. J. Clin. Nutr. 60 (1994) 388–392.
[33] H.J. Faarvang, Acta Endocrinol. 43 (1963) 484–492.
[34] T. Shikimi, S. Suzuki, M. Takahashi, H. Kaneto, Scand. J. Clin. Lab. Invest. 50 (1990) 1–8.