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Identification of the site of glucocorticoid action on neutral amino acid transport in superficial nephrons of rat kidney

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Summary. Glucocorticoid hormones enhance the reabsorptive capacity of filtered amino acids in rat kidney, as it was shown in previous *in vivo* clearance experiments. In the present study, the site of glucocorticoid action on neutral amino acid transport in superficial nephrons of rat kidney was investigated using *in vivo* micropuncture technique. Adult female Wistar rats were treated with dexamethasone (DEX), and fractional excretion of L-glutamine (L-Gln) and L-leucine (L-Leu) were determined and related to inulin after microinfusion into different nephron segments. DEX reduced fractional excretion of both neutral amino acids as a sign of enhanced reabsorptive capacity. The site of main DEX action on L-Leu reabsorption has been localized in the proximal straight tubule. However, in the case of L-Gln, the inhibition of γ-glutamyltranspeptidase (γ-GT) by administration of acivicin indicated the importance of this brush border enzyme in reduced L-Gln excretion. DEX enhanced γ-GT activity by tubular acidification. It can be presumed a DEX-inducible transport system for neutral amino acids mainly localized in proximal straight tubules of rat kidney.

Keywords: Amino acid transport – Kidney – Glucocorticoid hormone – Glutamine – Leucine – Micropuncture – Gamma-glutamyltranspeptidase – Rat

Introduction

In the last decade several amino acid transporters were cloned and molecularly characterized. By uptake and inhibition experiments the different families of transport proteins could be assigned to the functionally characterized amino acid transport systems (for detailed review see Christensen, 1990; Silbernagl, 1992; Palacin et al., 1998). However, there were only a few studies dealing with the regulation of renal amino acid transport by hormones.

In murine renal cortical slices testosterone and dexamethasone (DEX) stimulated amino acid uptake (Koenig et al., 1982). King et al. (1982) could demonstrate enhanced taurine uptake by the flounder renal tubules after DEX treatment. In isolated perfused kidney of adrenalectomized rats the supplementation of glucocorticoids led to increased glutamine uptake and utilization (Welbourne, 1988). Previous experiments in our laboratory indicated a key role of DEX and triiodothyronine (T3) in maturation of renal amino acid transport in immature rats (Fleck, 1992). In clearance experiments we could demonstrate stimulatory influences of DEX, T3 and epidermal growth factor (EGF) on renal tubular amino acid uptake of neutral amino acids under conditions of overloaded transport capacity of the tubular carrier systems (Fleck et al., 1997; Fleck and Pertsch, 1998). The effects of these substances were more pronounced in 5/6-nephrectomizsed rats used as a model of renal failure, where enhanced amino acid load of single nephron occurs (Fleck et al., 1999). Nevertheless, by means of clearance studies it is not possible to get detailed information on hormone action within the single nephron. Therefore, we performed micropuncture experiments to localize the site of glucocorticoid action on tubular amino acid reabsorption. Because L-glutamine (L-Gln) serves as a major fuel powering tubular transport processes and base generation in the kidneys of acidotic animals, we have chosen this neutral amino acid for our studies (Welbourne, 1989a). Furthermore, we tested L-leucine (L-Leu), another neutral amino acid to know more from the inside of neutral amino acid transport and its regulation. We microinfused ^{14}C-Gln and ^{14}C-Leu in concentrations comparable to glomerular filtrate into early and late proximal or early distal tubules of superficial nephrons, and determined their fractional excretions (FE) relative to co-microinfused ^{3}H-inulin. However, because of the high capacity of the amino acid transporters in the kidney, it is difficult to determine changes in transport function. Therefore, we administered 100-fold higher amino acid concentrations to overload the tubular transport systems. Under these conditions it is known to show hormone-induced changes in transport function (Silbernagl, 1980).

Different studies have shown that the luminal brush border enzyme γ-glutamyltranspeptidase (γ-GT) contributes to L-Gln reabsorption by degradation to L-glutamate (L-Glu) and NH_4^+ and reabsorption of L-Glu via carrier system for acidic amino acids (Silbernagl, 1986; Welbourne, 1989b). Therefore, *in vivo*-inhibition experiments were performed using acivicin, a γ-GT inhibitor, and the *in vitro* γ-GT activity in rat kidney cortex was determined in kidney cortex homogenate of control and DEX-treated animals.

Materials and methods

Animals and treatment

Adult female Wistar rats (Han: Wist) of our institute's own out-bred stock weighing 141–235 g were fed a standard diet (Altromin 1316) and tap water ad libitum. Animals were housed under standardized conditions in plastic cages, light-dark cycle 12/12 hours, temperature 22 ± 2°C, humidity 50 ± 10%. Dexamethasone (Fortecortin®, Merck,

Darmstadt, Germany) was administered in a dose of 60 μg/100 g b.wt. intraperitoneally for 3 days, once daily at 8 a.m. dissolved in 1 ml 0.9% saline per 100 g b.wt. In this dose range, hormone receptor sites are completely satured by dexamethasone (Rafestin-Oblin et al., 1986). Controls received saline only. Acivicin (ICN Biomedicals, Ohio, U.S.A.) was injected in a dose of 2 mg/100 g b.wt., dissolved in 1 ml Ringer solution, intravenously one hour before the micropuncture experiment. At this dose the γ-GT activity was inhibited completely (Silbernagl, 1986).

Surgical procedures

Rats were anaesthetized by intraperitoneally administration of sodium thiobarbiturate (Inactin®, Byk Gulden, Konstanz, Germany; 12 mg/100 g b.wt.), and were placed on a heated operating table with a thermostat control set at 37°C. The rectal temperature of the animals was controlled during the whole experimental period, which lasted for 6 hours. A tracheotomy was performed, and two polyethylene catheters (outer diameter ~0.5 mm) were inserted into right jugular vein. Ringer solution pH 7.4 (in mmol/l: 156 Na^+; 5.4 K^+; 2.25 Ca^{2+}; 164 Cl^-; 2.4 HCO_3^-) was infused (0.05 ml/min × 100 g b.wt.). After subcostal incision in the left flank the left kidney was exposed and immobilized in a plastic cup intraabdominally. The kidney was continuously superfused with paraffin oil heated to 37°C to avoid desiccation of the kidney surface. Catheters were inserted into left ureter and urinary bladder to collect urine separately.

Micropuncture experiments

Glass micropuncture pipettes with ground, bevelled tips had an outer diameter of 10 to 12 μm. They were filled with Ringer solution pH 7.4, ^{14}C-Gln (Amersham, UK; specific activity 10.8 GBq/mmol) or ^{14}C-Leu (Amersham; specific activity 11.7 GBq/mmol), and ^{3}H-labelled inulin (NEN, Boston, U.S.A.; specific activity 7.55 GBq/mmol) as a volume marker, and were connected to a perfusion pump. The amino acid concentrations in the infusion solution were 0.3 mM and 30 mM L-Gln, respectively, and 0.15 mM or 15 mM L-Leu. These concentrations were reached by supplementation of unlabelled L-Gln or L-Leu (Sigma, Deisenhofen, Germany). The infusion solutions also contained 0.05% lissamine green (Chroma, Köngen, Germany) to uncover the flow in the nephron, and to indicate backflow or extravasate. A high-intensity light, directed to a fiber-optic system, provided light for the inspection of the kidney surface. Early and late proximal, and early distal tubular segments were identified under visual control using a binocular microscope (Zeiss, Jena, Germany) by administration of 30 μl lissamine green (5%; pH 7.4). Micropipettes were inserted using micromanipulator (Leitz, Wetzlar, Germany), and different nephron segments were infused with 15 nl/min for 10 minutes. Urine samples were collected separately for both kidneys for one hour (Andreucci, 1978; Silbernagl et al., 1994).

Determination methods

^{14}C and ^{3}H dpm counts were detected in urine by liquid scintillation counting (Wallac, Turku, Finland). The ^{3}H dpm counts in the urine of the right, unexposed kidney were subtracted from those of the left kidney to correct inulin outflow into systemic circulation. Only visually correct microinfusions with contralateral $^3H < 20\%$ of ipsilateral urine were accepted. Fractional amino acid excretions were determined relative to inulin. For further details see Silbernagl et al. (1994). Sodium and potassium in urine were measured by flame photometry (Zeiss, Jena, Germany). Urine pH was detected with pH-meter (WTW, Weilheim, Germany) and urine osmolality was measured using freezing point method (Knauer, Berlin, Germany). γ-GT activity in kidney homogenate was detected by auto-analyzer (Beckman, Fullerton, U.S.A.) using the method of Szasz (1969). Total protein content of the kidneys was determined using the method of Watters (1978).

Statistical analysis

The results are given as means ± S.E.M. with n = 4–6 animals and m = 15–20 single nephron infusions (2–4 per animal) in each group. Significance was tested using hierarchical analysis of variance and multiple t-test with Bonferroni correction, $p \leq 0.05$.

Results

Because amino acid excretion depends on different renal parameters like glomerular filtration rate and urine flow (Silbernagl, 1988), control and DEX treated rats were compared in their physiological renal parameters during micropuncture experiments. No differences were found in urine flow, excretion of osmotically active substances or sodium and potassium excretion (Table 1). The detected parameters correspond to the results of other authors (Kersten and Bräunlich, 1968; Appenroth et al., 1995). Only urine pH was decreased significantly from 6.53 ± 0.26% to 5.88 ± 0.09% after DEX pre-treatment.

In a first series of micropuncture experiments we microinfused L-Gln and L-Leu in concentrations of 0.3 and 0.15 mM, respectively, comparable to glomerular filtrate under physiological conditions into the first accessible loop of proximal tubules of superficial nephrons (Fig. 1A). As shown in former studies (Lingard et al., 1974; Fleck et al., 1997), fractional amino acid excretion was very low: FE (%) L-Gln 3.67 ± 0.38; L-Leu 2.09 ± 0.26, and remained unchanged after DEX treatment. To detect DEX-induced changes of tubular transport capacity, we increased the amino acid load up to 30 mM L-Gln or 15 mM L-Leu. As shown in Fig. 1B, the FE of L-Gln increased only 10-fold, and the FE of L-Leu increased 14-fold, while concentration in infusate was enhanced 100-fold. This was a sign of a high reserve of transport capacity of amino acid reabsorption systems. When rats were treated with DEX, the FE of L-Gln decreased significantly to 24.12 ± 4.17% (about one third). Also the FE of L-Leu was about sixth part, but not significantly reduced. To localize the site of DEX action on neutral amino acid transport within single nephron, both amino acids were microinfused into the last accessible loop of proximal tubules. As shown in Fig. 1C, the FE of both

Table 1. Renal parameters in control and dexamethasone treated rats after standard operative procedure for micropuncture experiments measured in final urine, and γ-glutamyl-transpeptidase activity of kidney cortex. Arithmetic means ± S.E.M; n = 4–6

	Control	Dexamethasone
Urine volume [μl/h × 100 g b.wt.]	427 ± 90	605 ± 194
Osmotically active substances [μosmol/h × 100 g b.wt.]	59.3 ± 9.9	48.7 ± 3.2
pH	6.53 ± 0.26	5.88 ± 0.09*
Na^+-excretion [mval/h × 100 g b.wt.]	0.029 ± 0.010	0.055 ± 0.012
K^+-excretion [maval/h × 100 g b.wt.]	0.017 ± 0.004	0.022 ± 0.001
γ-GT activity [μmol/min × mg protein]	0.99 ± 0.70	1.03 ± 0.03

Asterisk indicates significant differences between both groups ($p \leq 0.05$).

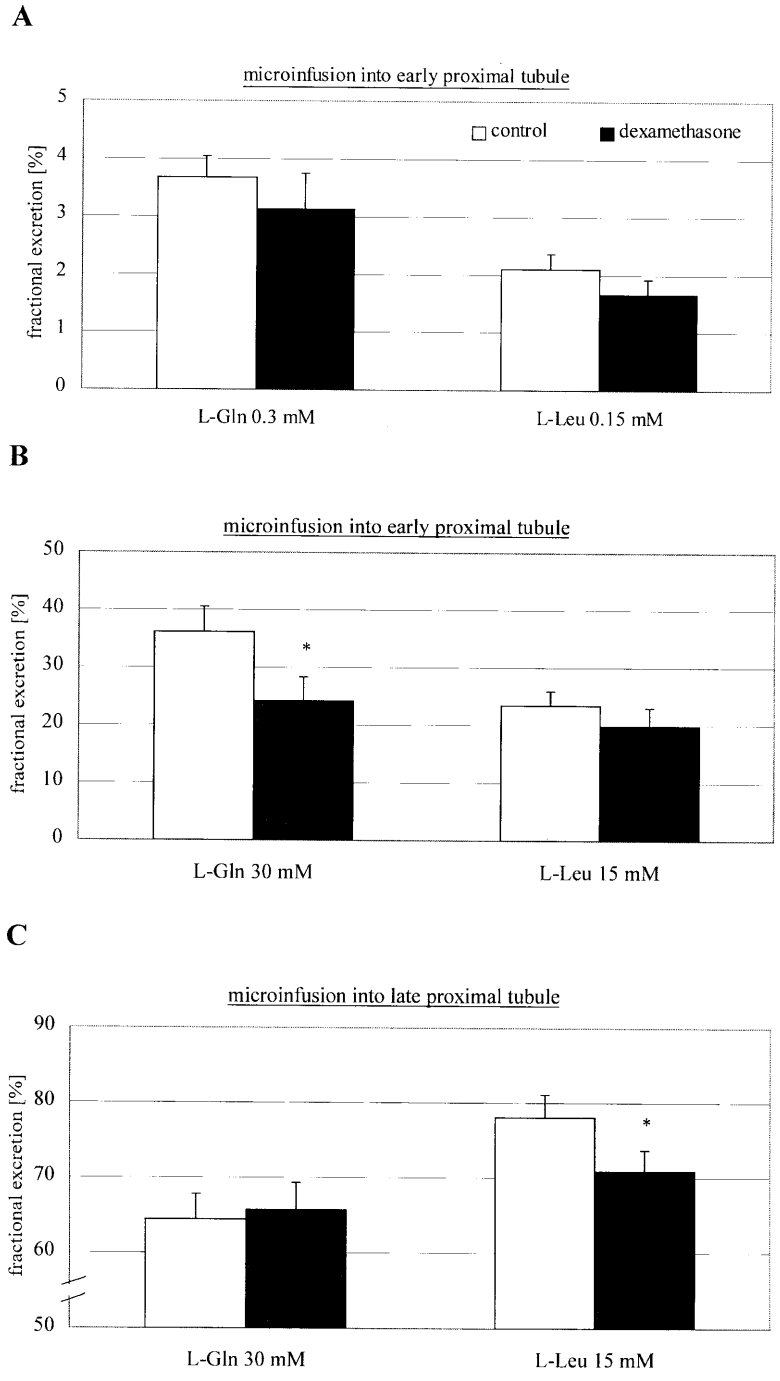

Fig. 1. Fractional excretion of L-glutamine (*L-Gln*) and L-leucine (*L-Leu*) after microinfusion of (**A**) 0.30 mM or 0.15 mM, respectively, into early proximal tubules of superficial nephrons, after microinfusion of 30 mM or 15 mM, respectively, into (**B**) early and (**C**) late proximal tubules of superficial nephrons in control and dexamethasone treated rats. Arithmetic means ± S.E.M., n = 4–6 rats, m = 15–20 single nephron microinfusions (2–5 per rat)* – significantly different from control rats ($p \leq 0.05$)

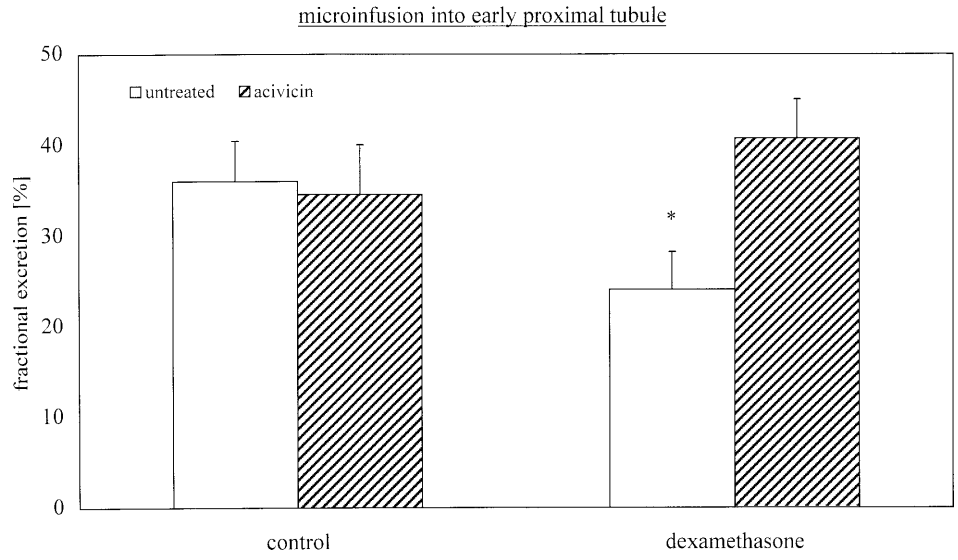

Fig. 2. Effect of acivicin (2 mg/100 g b.wt.) on fractional excretion of L-glutamine after microinfusion of 30 mM into early proximal tubules of superficial nephrons in control and dexamethasone treated rats. Arithmetic means ± S.E.M., n = 4 rats, m = 15–20 single nephron microinfusions (3–5 per rat). * – significantly different from control rats ($p \leq 0.05$)

neutral amino acids were much higher, because the main site of reabsorption is the proximal convoluted tubule. Distal to the proximal convoluted tubule L-Gln- excretion remained unchanged after DEX. The FE were about 65% in controls and DEX-treated rats, respectively. In contrast, FE of L-Leu was reduced significantly from 78.07 ± 3.03% to 70.87 ± 2.85% as a sign of glucocorticoid-induced enhancement of tubular reabsorptive capacity distal to the proximal convoluted tubule. No measurable L-Leu flux was found in distal nephron segments after microinfusion into first accessible loop of distal tubules (FE control 98.64 ± 3.76%; DEX 97.37 ± 2.22%; not shown).

All data obtained from literature exhibited that L-Gln and L-Leu are reabsorbed via the same carrier systems in rat kidney (Silbernagl, 1988). To clarify this discrepancy to our data we investigated the role of γ-GT. This membrane bound enzyme has high activity in the renal brush border membrane of proximal tubules. By conversion of L-Gln to L-Glu, γ-GT could contribute to reduced L-Gln excretion. The results after complete inhibition of γ-GT by administration of acivicin are depicted in Fig. 2. After microinfusion of 30 mM L-Gln into the first accessible loop of proximal convoluted tubule, the DEX-induced reduction in FE of L-Gln was fully abolished.

From these results the question arose, whether or not DEX could influence γ-GT. Detection of γ-GT activity in kidney cortex homogenate exhibited no differences in enzyme activity of control and DEX treated rats (Table 1) indicating no DEX-induced *de novo*-synthesis of γ-GT. However, the decreased urine pH after DEX-treatment pointed out DEX-induced changes in γ-GT activity by tubular acidification.

Discussion

In previous clearance experiments it was shown that renal amino acid transport is under regulation of hormones and growth factors like DEX, T_3 and EGF (Fleck et al., 1997; Fleck and Pertsch, 1998). However, because the reabsorption of amino acids within the tubules is nearly complete, changes in transport capacity were only detectable under conditions of overloaded tubular transport systems. Only in rats with renal failure (5/6-nephrectomy), where tubular amino acid load is enhanced because of diminished number of nephrons, changes in transport capacity were detectable under pathophysiologically occurring amino acid load (Fleck et al., 1999). In these studies, DEX effects were most pronounced in comparison to the other hormones. However, the site of DEX action could not been localized by means of clearance experiments. Using micropuncture technique it was possible to detect the site of hormone action within the single nephron. Microinfusion of physiological concentrations of L-Gln (0.3 mM) and L-Leu (0.15 mM), comparable to ultrafiltrate, into the first accessible loop of superficial nephrons resulted, as expected, in low fractional excretion of the neutral amino acids. Comparing these results with clearance experiments *in vivo*, the fractional excretions were enhanced. The discrepancy between our data and literature could be caused by internephron heterogeneity. Dantzler and Silbernagl (1988) could show that the amino acid transport in deeper, i.e. juxtamedullary nephrons is more effective because of enhanced distal reabsorption. Another explanation for differences in FE could be the rat strain. In comparison to Munich Wistar rats, HAN: Wistar rats do not have superficial glomeruli, therefore, the first accessible loop of proximal tubule is not really close to the glomerulum.

No changes in fractional excretion were detectable after pre-treatment with DEX. However, when the tubular carrier systems were loaded by microinfusion of high L-Gln or L-Leu concentrations (30 mM and 15 mM, respectively) into early proximal tubule, the FE of both amino acids was enhanced. But the FE was enhanced only 10- to 14-fold, while concentration in infusate was enhanced 100-fold. These results confirmed a high reserve capacity of amino acid transport systems in rat nephrons. In this series of experiments DEX was able to reduce fractional excretion of L-Gln, whereas that of L-Leu was not significantly reduced. A fractional L-Gln excretion of about 65% after microinfusion into last accessible loop of the proximal convoluted tubules indicated L-Gln flux in proximal straight tubule or later nephron parts as described by Dantzler and Silbernagl (1988), but a DEX effect was not detectable in this nephron segment. Thus the site of DEX action on L-Gln reabsorption should be localized in the proximal convoluted tubule. Different results were found in the case of L-Leu reabsorption. DEX reduced FE of L-Leu after microinfusion into the late proximal tubule indicating that the main site of action is localized in the proximal straight tubule or in Henle's loop, since no measurable reabsorption took place in the distal parts of the nephron. The results of *in vivo* clearance experiments were confirmed by these investigations. Glucocorticoid hormones seem to be able

to stimulate tubular amino acid reabsorption, and the main site of action could be localized within the proximal tubule in superficial nephrons. But it was indefinite, why there was a discrepancy between handling of L-Gln and L-Leu, while it was thought that both amino acids are transported via the same carrier systems (Silbernagl, 1988). One reason for the different handling of L-Gln and L-Leu might be an involvement of luminal γ-GT. By conversion of L-Gln to L-Glu this enzyme contributes to reduced fractional excretion after DEX treatment when γ-GT-activity is enhanced (Silbernagl, 1986). To clarify this question we investigated the role of γ-GT, and inhibited the enzyme by administration of its inhibitor acivicin. The inhibition of γ-GT fully abolished the DEX effect on L-Gln FE. Therefore the DEX-induced reduction of fractional L-Gln excretion was induced, at least in part, by conversion of L-Gln to L-Glu and its reabsorption via the carrier systems for acidic amino acids. Direct competition of acivicin with L-Gln at the site of reabsorption could be excluded, because the substance was administered one hour before the clearance experiments.

The determination of γ-GT activity in kidney homogenate showed no differences in untreated and DEX treated rats. Therefore, induction of γ-GT synthesis by DEX could be excluded. For this reason we suppose an activation of γ-GT by tubular acidification. Other authors have demonstrated enhanced glucocorticoid-induced tubular acidification by activation of Na^+-H^+-countertransport (Baum and Quigley, 1993). Curthoys and Hughey (1979) could show that γ-GT in renal brush border membrane has a pH optimum of 8.6 for transpeptidase activity, whereas at a pH of <6.0 the enzyme exclusively has glutaminase activity. This hypothesis was supported by decreased pH value measured in the urine of DEX treated rats.

Another mechanism could be responsible for DEX-induced L-Leu uptake. We assume a stimulated transport system mainly localized in the proximal straight tubule with different affinities to L-Leu and L-Gln. A general effect on different sodium-dependent transport systems by well-known activation of basolateral Na^+-K^+-ATPase could be excluded, because in clearance experiments the DEX effect was restricted to neutral amino acids (Sacktor and Kinsella, 1985; Fleck and Langner, 1998). Other amino acids and the sulfonic acid taurine, which shares a separate transport system with β-amino acids, were unaffected in their fractional excretion (Fleck and Langner, 1998). The question, which transport system is involved in DEX-induced reabsorption could not be fully clarified from our experimental data. Because the tubular L-Gln uptake is absolutely dependent on an existing transmembrane Na^+ gradient, shown in isolated perfused rat kidney and brush border membrane vesicles (Weiss et al., 1978; Welbourne, 1989b), a possible candidate is the Na^+-independent transport system $b^{0,+}$. This transport system belongs to the new family of heteromultimeric amino acid transporters consisting of a heavy chain rBAT and a light chain BAT1 linked by a disulfide bond (Bertran et al., 1992; Mastroberardino et al., 1998; Chairoungdua et al., 1999). It was shown by Mosckovitz et al. (1993) with immunocytochemical methods that the heavy chain rBAT of this amino acid transporter was strongly expressed in brush border membrane of nephron S3

segment. However, further investigations are necessary to clarify the mechanism of glucocorticoid action on amino acid transport systems on cellular level.

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