Abnormal radiofurosemide binding by Tamm Horsfall glycoprotein of diabetic patients

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Summary. In the present study, 8 Type 1 diabetic patients with normal creatinine clearance and 8 matched controls were examined. Tamm Horsfall glycoprotein was isolated with the NaCl precipitation procedure. Its purity was checked by gel-electrophoresis, immunodiffusion and isoelectric focussing. Tamm Horsfall glycoprotein of diabetic patients had higher glucose ($p < 0.05$) and lower N-acetylneuraminic acid content ($p < 0.01$) than controls. ^{14}C -furosemide binding by Tamm Horsfall glycoprotein was examined using an Amicon ultrafiltration system at 0°C . In nominally sodium-free medium, furosemide binding by Tamm Horsfall glycoprotein was significantly ($p < 0.01$) higher in diabetic patients than in matched controls. The increment of binding capacity with sodium was

similar in controls and diabetic patients so that maximal binding capacity in a NaCl system was 1.7 ± 0.3 in controls and 3.64 ± 0.5 in diabetic patients ($p < 0.025$). Half maximal furosemide binding by Tamm Horsfall glycoprotein occurred at 1.4 ± 0.2 mmol Na/l in controls and 0.52 ± 0.12 in diabetic patients ($p < 0.01$). Abnormal radiofurosemide binding of Tamm Horsfall glycoprotein of diabetic patients may be the consequence of abnormal postribosomal modification of the glycoprotein which is synthesized in an insulin- and glucose-sensitive nephron segment.

Key words: Diabetes mellitus, renal sodium transport, Tamm Horsfall glycoprotein, furosemide.

Tamm Horsfall glycoprotein (THP) is a glycoprotein originally described by Tamm and Horsfall in 1950 [1]. Its participation in some pathological conditions of kidney and urinary tract has recently been discussed [2]. However, its physiological rôle remains unknown.

It is known that THP is present on all plasma membranes of cells in the thick ascending loop of Henle [2]. The topography of its location and phylogenetic and ontogenetic studies suggest a relation to specific functions of the diluting segment [2, 3]. It has recently been demonstrated that THP binds furosemide Na^+ , K^+ , and Cl^- -dependently in a saturable fashion [4]. The sodium concentration needed for half maximal binding was $1.4\text{--}3.3$ mmol/l [4], a figure remarkably similar to the sodium concentration found to half maximally stimulate active transport of sodium in the isolated perfused thick ascending loop of Henle [5]. Furthermore, the concentration of furosemide required for half maximal binding to THP is similar to the concentration that half maximally inhibits Na^+ , K^+ , 2Cl^- cotransport of the thick ascending loop of Henle *in vitro* [6]. Renal sodium handling is abnormal in clinical or experimental states of insulin deficiency or excess [7–9]. Furthermore, we have previously demonstrated abnormal carbohydrate composition of THP in non-proteinuric patients with Type 1 diabetes [10]. These considerations prompted us to investigate whether furosemide binding by THP is abnormal in diabetes mellitus.

Subjects and methods

Subjects

We examined 8 Type 1 diabetic patients, 5 male, 3 female, with a known median duration of diabetes of 17.6 years (range 1–33). Metabolic control varied widely as indicated by HbA_1 values (fast haemoglobin test system, Panchem, Kleinwallstadt, FRG, normal range 5–7.5%). Because albuminuria and bacteriuria may interfere with isolation of THP, patients were selected without albuminuria on first presentation, as tested with a stix (Combur; detection threshold ≥ 30 mg/dl) and without significant bacteriuria (dip slide technique, Uricult). Urinary protein was then measured in 24-h urine collections with Biuret, and endogenous creatinine clearance with standard auto-analyzer technique. None of the patients had secondary complications, e.g. impaired renal function (serum creatinine ≥ 115 $\mu\text{mol/l}$), hypertension (blood pressure $> 140/90$ mmHg) and proliferative retinopathy. Pertinent clinical data are given in Table 1.

Members of the staff of the renal clinic (6 male, 2 female) served as age- and sex-matched controls. None of the patients or probands had medication other than insulin.

The study was carried out on an outpatient basis with patients and probands on their usual diets. Twenty-four hour urine was collected in two 1 polyvinylchloride bottles to which 30 mg Na-azide had been added. Pilot studies had shown that $> 90\%$ of THP added to urine was recovered from the bottles (excluding adsorption to the PVC wall or interference of azide with THP recovery).

Isolation of Tamm Horsfall glycoprotein and tests for purity of preparation

THP was isolated by repeated precipitation with 0.58 M NaCl according to the original method of Tamm and Horsfall [1]. The THP precipi-

Table 1. Clinical data

Diabetic patients							Control subjects					
Sex	Age (years)	Duration of diabetes (years)	Creatinine clearance (ml/min/1.73 m ²)	RR (mmHg)	Urinary protein (mg/24 h)	HbA _{1c} (%)	Sex	Age (years)	Creatinine clearance (ml/min/1.73 m ²)	RR (mmHg)	Urinary protein (mg/24 h)	HbA _{1c} (%)
R.B. f	53	33	85	140/80	500	7.1	R.E. m	48	115	140/80	130	7.1
H.K. m	49	19	94	140/80	70	7.1	D.J. m	28	120	130/70	138	5.5
St.M. m	46	17	140	130/80	67	8.1	R.M. m	31	125	140/80	145	7.2
B.R. m	51	12	130	140/90	125	8.7	R.H. m	54	116	105/75	85	6.3
R.B. f	54	24	98	140/85	47	10.8	E.F. f	22	85	110/80	95	5.8
P.V. m	46	18	134	140/80	700	11.2	N.M. m	31	108	110/80	180	6.1
K.C. m	41	16	140	110/80	800	13.2	E.M. f	42	117	140/85	197	7.3
R.F. f	28	1	125	105/75	67	17.1	N.F. m	39	106	110/70	78	6.5

Fig. 1. SDS PAGE of Tamm Horsfall protein of diabetic and control subjects. Lane 1 reference proteins (fibrinogen 300 kD, transferrin 83 kD, BSA 68 kD, catalase 60 kD, ovalbumin 45 kD, aldolase 40 kD, trypsin 23.5 kD). Lane 2 control THP with contamination of albumin, sample discarded; Lane 3–4 control THP, Lane 5–6 diabetic THP

itate was dialysed (Visking dialysis tubing, type 36/32, Serva Co., Heidelberg, FRG) against distilled water and concentrated by Amicon ultrafiltration (Amicon Co., Ditten/Ruhr, FRG; filter XM 50, transmembrane pressure 5 bar). All steps were carried out at 4°C. The final THP preparation was freeze dried.

To evaluate the purity of the THP preparation used, all samples were subjected to polyacrylamide gelelectrophoresis in sodium-dodecylsulfate (SDS PAGE), Ouchterlony immunodiffusion and isoelectric focusing. Proteins other than THP were not demonstrable in any of the preparations used for the binding studies.

SDS PAGE was carried out according to Laemmli [11]. Ten percent polyacrylamide slab gels (2 mm thick, 12 mm high) were used with 2.5% stacking gel (2.5 cm high). After electrophoresis, the gels were stained for 1 h in a solution of 0.02% Coomassie brilliant blue in a mixture of 25% methanol and 10% acetic acid in water. They were subsequently destained overnight in the same solution without Coomassie-blue. Reference proteins of known molecular weight were added to a parallel gel as reference proteins (Fig. 1). THP migrated as

one single band, staining with Coomassie-blue and PAS, but not with 0.2% Toluidine blue and had an estimated molecular weight of 80 kD.

In parallel, samples were examined by double immunodiffusion according to Ouchterlony. Plates were prepared with 1% immunodiffusion agar in barbiturate buffer (pH 8.6) according to LKB application note 294. Ten mg samples of freeze dried THP were dissolved in 1 ml of Tris-glycine buffer (1.5 M; pH 8.8) and tested against antihuman uromucoid serum (Behring Company, Marburg, FRG, catalogue number 1726 L) and, in addition, against antiserum to human albumin (Behring Company, catalogue number A 10118 B) and to human serum (Behring Company, catalogue number 100109 H). THP yielded one precipitation band with human uromucoid antiserum and did not precipitate with antiserum to human serum or antiserum to human albumin.

Isoelectric focusing (IEF) was carried out in LKB 2117 Multiphor apparatus at 10°C and 6 W with a maximum voltage of 1200 V for 3 h. The preparations failed to migrate in the absence of 6 M urea. Samples were prepared with Servalyte pH 2–11 (Serva Company, Heidelberg, FRG). The suspension was degassed, poured into a 23 × 11 cm tray and dried according to Radola [12]. IEF showed presence of one poorly resolved band with a pI of approximately 4 and no contaminating bands between pH 2.5 to 10.0.

Chemical analysis of Tamm Horsfall glycoprotein

THP protein concentration was determined according to Lowry [13]. Neutral sugars were determined as their alditol acetate [14] by gas-liquid chromatography. Analyses of amino-sugars were carried out using an aminoacid analyser (LKB 440). N-acetyl-neuraminic acid (NANA) was determined colorimetrically using the thiobarbiturate method of Warren [15].

¹⁴C-furosemide binding

The method described by Greven et al. [4] was used. In brief, 8×10^{-5} M/l ¹⁴C-furosemide (specific activity 25 Ci/mmol, kindly supplied by Hoechst Company, Frankfurt/Main, FRG) was incubated for 30 min at 0°C in a medium consisting of 2.5 mg/ml THP, 4 mmol/l Tris-buffer (pH 8) and ions. Bound and free ¹⁴C-furosemide were separated by 5 min centrifugation through an Amicon filter (YM 5Ø 14 mm). All measurements were carried out at least in duplicate. ¹⁴C activity in the filtrate was determined using scintillation counting techniques. Reproducibility of measurements in replicate determinations was typically better $\pm 7\%$.

Statistics

Data are given $\bar{x} \pm \text{SEM}$. Differences between groups were analyzed using both non-parametric Wilcoxon test and analysis of variance. Correlations were examined using linear regression analysis.

Fig. 2. Sodium-dependent ¹⁴C-furosemide binding of THP of diabetic patients and control subjects. Data as $\bar{x} \pm \text{SEM}$. 8 individuals per group. Values for each individual measured at least in duplicate. Significant ($p < 0.01$) difference between diabetic patients and controls (analysis of variance). The insert shows the activating sodium concentration required for half maximal binding; $y = \text{furosemide bound (nmol/mg THP) at given } [\text{Na}^+]$ minus furosemide bound in nominally sodium-free medium; $x = \text{bound furosemide (nmol/mg THP)}; \text{Na}^+$ (mmol/l), $\bigcirc$ ----- $\bigcirc$ diabetic patients; $\bullet$ ----- $\bullet$ control subjects

Fig. 3. Displacement of bound ¹⁴C-furosemide from Tamm Horsfall glycoprotein by Cl⁻. Data as $\bar{x} \pm \text{SEM}$. $n = 8$ per group. Values for each individual measured at least in duplicate. Na⁺ concentration was kept constant at 10 mmol/l and Cl⁻ concentration was adjusted by addition of choline chloride. $\bigcirc$ ----- $\bigcirc$ Diabetic patients; $\bullet$ ----- $\bullet$ control subjects

Table 2. Chemical composition of Tamm Horsfall glycoprotein of diabetic patients

(mg/100 mg THP)	Control Subjects ($n = 38$)	Diabetic Patients ($n = 8$)	Difference
Protein	77.3 ± 1.5	77.7 ± 1.9	NS
Galactose	4.51 ± 0.38	4.33 ± 0.21	NS
Glucose	0.65 ± 0.21	1.71 ± 0.21	0.01
N-acetyl-neuraminic acid	5.10 ± 0.19	3.28 ± 0.24	0.001

Results

Chemical analysis of Tamm Horsfall glycoprotein

As shown in Table 2, protein content of THP was similar in diabetic patients and control subjects. In contrast, glucose content was slightly increased while N-acetyl-neuraminic acid content was markedly decreased.

Na⁺-dependent binding of ¹⁴C-furosemide to Tamm Horsfall glycoprotein

Fig. 2 shows binding of ¹⁴C-furosemide ($8 \times 10^{-5} \text{M/l}$) to THP with increasing concentrations of NaCl in the incubation medium. In nominally NaCl-free medium, binding of ¹⁴C-furosemide to THP was $0.4 \pm 0.2 \text{ nmol/mg THP}$ in controls and 2.0 ± 0.3 in diabetic patients ($p < 0.01$). Binding increased with increasing NaCl concentrations and reached saturation at NaCl concentrations above 5 mmol/l. The amount of ¹⁴C furosemide bound was significantly higher in diabetic patients at any given ambient Na⁺ concentration. However, the increment of binding capacity above basal binding in nominally Na⁺ free medium was similar for controls and diabetic patients ($\Delta 1.3 \pm 0.2 \text{ nmol/mg THP}$, and 1.64 ± 0.32 in controls and diabetic patients, respectively).

Linear transformation of the binding isotherm (insert to Fig. 2) revealed that half maximal binding was reached at a Na⁺ concentration of 1.4 mmol/l for THP in control subjects, and 0.52 mmol/l for THP in diabetic patients.

No significant correlation between ¹⁴C furosemide binding and serum HbA_{1c}, urinary glucose or duration of diabetes was found.

Displacement of bound ¹⁴C-furosemide from Tamm Horsfall glycoprotein by Cl⁻

As shown in Fig. 3, the amount of ¹⁴C-furosemide bound was a function of ambient Cl⁻ concentration for both THP in controls and THP in diabetic patients. In the experiment depicted, Cl⁻ concentration was varied through addition of choline chloride at constant Na⁺ concentration (10 mmol/l). THP in control subjects showed significantly ($p < 0.01$) lower binding at 10 mmol/l Cl⁻ than THP in diabetic patients. The binding maximum occurred at lower ambient Cl⁻ concentration (30 vs 50 mmol/l Cl⁻). At higher Cl⁻ concentrations, binding was lower for THP in control subjects at any given Cl⁻ concentration than for THP in diabetic patients.

Discussion

The present study clearly demonstrates that THP of patients with Type 1 diabetes exhibits altered radiofurose-

mid binding characteristics when compared with THP of age-matched controls.

Kinetic analyses revealed higher basal sodium independent binding capacity for THP of diabetic patients in nominally Na^+ -free medium, but a similar increment of binding capacity with sodium resulting in increased maximal binding capacity at saturating concentrations of Na^+ .

Such abnormal ^{14}C -furosemide binding cannot be explained as an artefact of THP protein content, since diabetic and control THP contained similar amounts of protein as evaluated by the Lowry technique. The cause of abnormal binding may be related to altered composition of the carbohydrate moiety of THP. Chemical analyses showed that THP of diabetic patients had increased glucose, unchanged galactose and diminished N-acetyl-neuraminic acid content. Such compositional abnormalities of THP are of interest in view of the recent demonstration of Lloyd et al. [16] of increased non-dialysable conjugates of glucose with unchanged galactose content in urines of diabetic patients. This may be a reflection of the above change of composition of THP, perhaps in parallel with other urinary glycoproteins, in diabetes mellitus.

The functional consequences of altered THP carbohydrate composition are unknown. In contrast to the almost invariant amino acid composition of THP from different species, the THP carbohydrate component is remarkably variable [2, 17, 18]. It is unknown whether changes of carbohydrate composition have major functional consequences. The Tamm Horsfall glycoprotein is an exclusive product of tubular cells in the thick ascending loop of Henle [2, 3]. These cells are known to phenotypically express insulin receptors [19] and to depend on uptake and aerobic metabolism of glucose [20]. In the genesis of abnormal carbohydrate composition of THP of diabetic patients, a role must be discussed for either insulin deficiency or abnormal pericellular glucose concentration (hyperglycemia and/or increased tubular delivery of glucose) or both.

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