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Probing the substrate specificity of the ergothioneine transporter with methimazole, hercynine, and organic cations

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ABSTRACT

Recently, we have identified the ergothioneine (ET) transporter ETT (gene symbol *SLC22A4*). Much interest in human ETT has been generated by case-control studies that suggest an association of polymorphisms in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases. ETT was originally designated a multispecific novel organic cation transporter (OCTN1). Here we reinvestigated, based on stably transfected 293 cells and with ET as reference substrate, uptake of quinidine, verapamil, and pyrilamine. ETT from human robustly catalyzed transport of ET (68 μl/(min mg protein)), but no transport of organic cations was discernible. With ET as substrate, ETT was relatively resistant to inhibition by selected drugs; the most potent inhibitor was verapamil (K_i = 11 μmol/l). The natural compound hercynine and antithyroid drug methimazole are related in structure to ET. However, efficiency of ETT-mediated transport of methimazole (K_i = 7.5 mmol/l) was 130-fold lower, and transport of hercynine (K_i = 1.4 mmol/l) was 25-fold lower than transport of ET. ETT from mouse, upon expression in 293 cells, catalyzed high affinity, sodium-driven uptake of ET very similar to ETT from human. Additional real-time PCR experiments based on 16 human tissues revealed ETT mRNA levels considerably lower than in bone marrow. Our experiments establish that ETT is highly specific for its physiological substrate ergothioneine. ETT is not a cationic drug transporter, and it does not have high affinity for organic cation inhibitors. Detection of ETT mRNA or protein can therefore be utilized as a specific molecular marker of intracellular ET activity.

1. Introduction

Ergothioneine (ET) is a natural antioxidant which is biosynthesized solely by fungi and mycobacteria [1]. Humans like other mammals absorb it exclusively from food in which it is distributed very unevenly; a distinguished source of ET are mushrooms (0.1–1 mg/g dried material). ET is rapidly cleared from the circulation and then avidly retained with minimal metabolism. The content of ET varies greatly among human tissues [2]. High ET levels

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Abbreviations: ETT, ergothioneine transporter; LC, liquid chromatography; MPP^+, 1-methyl-4-phenylpyridinium; MS, mass spectrometry; TEA, tetraethylammonium

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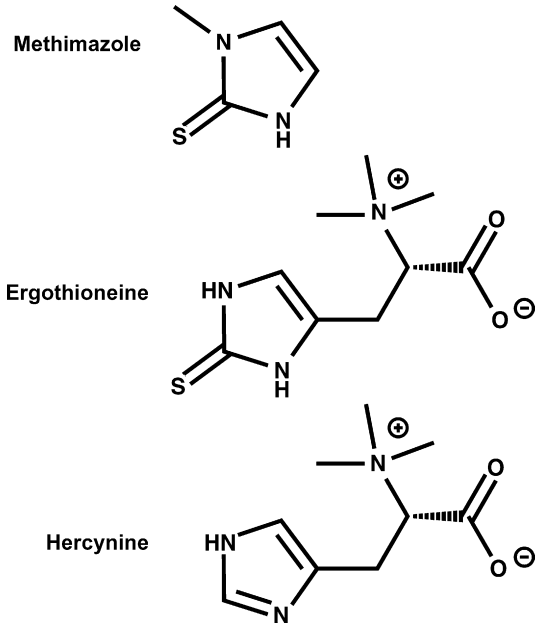

Fig. 1 – Structures of ET, hercynine, and methimazole.

have been found in erythrocytes, bone marrow, seminal fluid and eye.

Chemically, ET is the betaine of histidine with a sulfur atom attached to position 2 of the imidazole ring (Fig. 1). It should not be considered a thiol compound, but rather a thione, a derivative of thiourea. As a consequence of the prevailing thione tautomer, ET is a very stable antioxidant with unique properties; e.g. it does not auto-oxidize at physiological pH and does not promote the generation of hydroxyl radical from H_2O_2 and Fe^{2+} ions (=Fenton reaction) [2]. The precise physiological role of ET is still unclear. Most authors consider it an intracellular antioxidant.

Recently, we have discovered an ET transporter (ETT; gene symbol *SLC22A4*) [3]. ETT from human (ETTh) has high affinity for ET (K_m = 21 μmol/l) and catalyzes cotransport of ET with Na^+. Cells lacking ETT do not accumulate ET, since their plasma membrane is virtually impermeable for this compound. By contrast, cells with expression of ETT accumulate ET to high levels. Based on the expression profile, we judge ETT to be necessary for the supply of ET primarily to erythrocyte progenitor cells and to monocytes.

Much interest in ETT has been generated by case-control studies that suggest an association of polymorphisms in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases. The association with Crohn's disease [4] has been largely confirmed [5–8]. Moreover, associations with ulcerative colitis [9] and Type I diabetes [10] have been reported. There was association with rheumatoid arthritis in a Japanese cohort [11], but this could not be replicated in British [12] and Spanish cohorts [13]. Clearly, in order to elucidate the role of ETT in the genesis of chronic inflammatory diseases, it will be necessary to fully understand substrate specificity and localization of the carrier.

Our previous results suggest that expression of ETT in specific cells indicates intracellular ergothioneine activity. However, the gene product of *SLC22A4* was originally designated a multispecific novel organic cation transporter (OCTN1), because it was reported to transport tetraethylammonium [14], and quinidine, verapamil, and pyrilamine [15]. If ETT also functions as an organic cation transporter, then it cannot serve as a specific molecular marker of ET activity. Thus, it was one of our aims here to clarify, with ET as reference substrate, whether ETT transports organic cations. In addition, we tested organic cations as inhibitiors to see if ETT displays high affinity towards organic cations with ET as substrate.

The widely used antithyroid drug methimazole (=1-methyl-imidazole-2-thione) and the side chain of ET have, except for the methyl moiety, identical structures (cf. Fig. 1). In view of the possible involvement of ETT in chronic inflammatory diseases, it is very interesting that methimazole has immunosuppressive [16–18] and powerful anti-inflammatory [19] activity. In order to evaluate whether ETT could provide a specific route of entry of methimazole into those cells that express this carrier, we tested methimazole as a substrate. The substrate specificity of ETT was probed further with the natural precursor of ET, hercynine, which lacks the sulfur atom but is otherwise identical to ET (Fig. 1).

Finally, we expressed and analyzed ETT from mouse in 293 cells to see if key functional properties are conserved over species.

2. Materials and methods

2.1. *Plasmid constructs*

The construction of pEBTetD/ETTh has been described previously [20]. The cDNA of ETTm was inserted into the polylinker of a plasmid related to pEBTet but without the cassette for expression of the Tet repressor; the latter was supplied by a second plasmid [20]. The cDNA sequence of ETTm corresponds to GenBank entry AB016257 except for a single base deviation at position 66 downstream of the stop codon (C > T). The 5′-interface between cDNA and vector is **GTTTAAACTT AAGCTT** CGCGCCGAAT (polylinker in bold, cDNA underlined); the 3′-interface is TCAAAAGCCT **GGATCC ACTA**. The construct was assembled by standard cloning methods; the whole insert was verified by DNA sequencing.

2.2. *Cell culture*

293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were grown at 37 °C in a humidified atmosphere (5% CO_2) in plastic culture flasks (Falcon 3112, Becton Dickinson, Heidelberg, Germany). The growth medium was Dulbecco's Modified Eagle Medium (Life Technologies 31885-023, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Cölbe, Germany). Medium was changed every 2–3 days and the culture was split every 5 days.

Stably transfected cell lines were generated as reported previously for the pEBTetD vector [20]; cell culture medium always contained 3 μg/ml puromycin (PAA Laboratories) to ascertain plasmid maintenance. To turn on protein expression, cells were cultivated for at least 20 h in regular growth

medium supplemented with 1 μg/ml doxycycline (195044, MP Biomedicals, Eschwege, Germany).

2.3. Transport assays

For measurement of solute uptake by LC–ESI-MS/MS, cells were grown in surface culture on 60 mm polystyrol dishes (Nunclon 150288, Nunc, Roskilde, Denmark) precoated with 0.1 g/l poly-L-ornithine in 0.15 M boric acid–NaOH, pH 8.4. Cells were used for uptake experiments at a confluence of at least 70%. Uptake was measured at 37 °C. Uptake buffer contains 125 mmol/l NaCl, 25 mmol/l HEPES–NaOH pH 7.4, 5.6 mmol/l (+)glucose, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $CaCl_2$, and 1.2 mmol/l $MgSO_4$. With inhibitor concentrations >1 mmol/l, the NaCl concentration was isoosmotically reduced. After preincubation for at least 20 min in 4 ml of uptake buffer, the buffer was replaced with 2 ml of substrate in uptake buffer. Incubation was stopped after 1 min by rinsing the cells four times with each 4 ml ice-cold uptake buffer. Subsequently, the cells were solubilized with 4 mmol/l $HClO_4$ and stored at −20 °C. After centrifugation (1 min, 16,000 × *g*, 20 °C) of the thawed lysates, 100 μl of the supernatant was mixed with 10 μl unlabelled MPP^+ iodide (5.0 ng/μl) which served as internal standard. Of this mixture, 20 μl samples were analyzed by LC–MS/MS on a triple quadrupole mass spectrometer (TSQ Quantum, Thermo Electron, Dreieich, Germany). Atmospheric pressure ionization with positive electrospray was used. The LC system consisted of Surveyor LC-pump, autosampler, and Waters Atlantis HILIC silica column (length 100 mm, diameter 3 mm, particle size 5 μm). The solvent for isocratic chromatography (flow rate 250 μl/min) was made of methanol (70%) and 0.1% formic acid (30%). For quantification by SRM (selected reaction monitoring; scan time 0.3 s), at first the optimal collision energy (CE) for argon-induced fragmentation in the second quadrupole was determined for each analyte. From the product ion spectra, the following fragmentations were selected for SRM (*m/z* parent, *m/z* fragment, CE)—ergothioneine: 230, 127, 24 V; hercynine: 198, 95, 20 V; methimazole: 115, 57, 24 V; MPP^+: 170, 128, 25 V; pyrilamine: 286, 121, 17 V; quinidine: 325, 184, 32 V; verapamil: 455, 165, 26 V. For each analyte, the area of the intensity versus time peak was integrated and divided by the area of the MPP^+ peak to yield the analyte response ratio. Linear calibration curves ($R^2 > 0.99$) were constructed from at least six standards which were prepared using control cell lysates as solvent. Sample analyte content was calculated from the analyte response ratio and the slope of the calibration curve, obtained by weighted linear regression.

Protein was measured by the BCA assay with bovine serum albumin as standard. The protein content of MS samples was estimated from the response ratio for proline, which was calibrated against the BCA assay (four to six matched cell dishes) for each MS session.

2.4. Synthesis of hercynine

Since hercynine is not available commercially, it was synthesized from a-*N*,*N*-dimethyl-L-histidine (F3625, Bachem, Bubendorf, Switzerland) and methyl iodide as described [21]. The raw product was purified by preparative thin layer chromatography on a glass backed silica gel plate (cat. no. 113792, Merck, Darmstadt, Germany) with methanol/H_2O/25% (v/v) NH_3 (8:2:1) as developing solvent. Hercynine was eluted with water from the silica gel fraction, dried, and weighed. Our preparation was 91% pure as judged by LC–MS total ion current (positive mode).

2.5. Calculations and statistics

The clearance is directly proportional to k_{cat}/K_m (k_{cat}: turnover number) and thus a valid measure of efficiency of transport (provided that the substrate concentration is much smaller than the respective K_m) [22]. The clearance equals initial rate of specific uptake (=uptake mediated by expressed carrier) divided by substrate concentration. Specific uptake equals total uptake minus uptake into control cells (=non-specific uptake).

Analysis of saturation curves and calculation of K_i-values have been reported previously [23]. Fitted parameters such as K_m- and K_i-values are given as geometric mean with 95% confidence interval. Arithmetic means are given with S.E.M. Velocity of uptake of ET as a function of Na^+ concentration (Fig. 7) was described with a modified Hill function: $v = V_0 + V_{lim}/(1 + Kc_{Na^+}^{-h})$ with h = Hill coefficient and c_{Na^+} = sodium concentration. In the absence of any sign of inhibition, a K_i-value was estimated as the highest used inhibitor concentration multiplied by 5. The unpaired *t*-test was used to test for significance; two-tailed *P* values are given.

2.6. Drugs

5-Aminosalicylic acid (819019, Merck, Darmstadt, Germany), L-carnitine (C-0283, Sigma–Aldrich, Munich, Germany), L-(+)-ergothioneine (F-3455, Bachem, Bubendorf, Switzerland), methimazole (M-8506, Sigma–Aldrich), 1-methyl-4-phenyl-pyridinium iodide (D-048, Sigma–Aldrich), pyrilamine (P-5514, Sigma–Aldrich), quinidine (Q-0875, Sigma–Aldrich), thioperamide (T-123, RBI, Natick, MA, USA), verapamil-HCl (Knoll AG (Abbott Laboratories), Liestal, Switzerland). Disprocynium24 (1,1′-diisopropyl-2,4-cyanine iodide) was synthesized as described previously [24]. All other chemicals were at least of analytical grade.

3. Results

3.1. Transport of organic cations

Uptake mediated by ETT from human (ETTh) of 1 μmol/l quinidine, verapamil, pyrilamine, and ET was investigated with 293 cells stably transfected with pEBTetD/ETTh. pEBTetD is an Epstein-Barr plasmid vector for doxycycline-inducible protein expression in human cell lines based on the simple tetracycline repressor [20]. Expression is turned on by addition of 1 μg/ml doxycycline to the culture medium for about 20 h. This system provides a high rate of ETTh-mediated transport in the on-state (=100%) and a low rate (4%) in the off-state [20]. The results (Fig. 2) indicate high levels of quinidine, verapamil, and pyrilamine in off-state and on-state cell lysates. However, expression of the transporter did not increase lysate contents ($P = 0.38$, 0.76, and 0.50, respectively; $n = 5$). By contrast, the ET

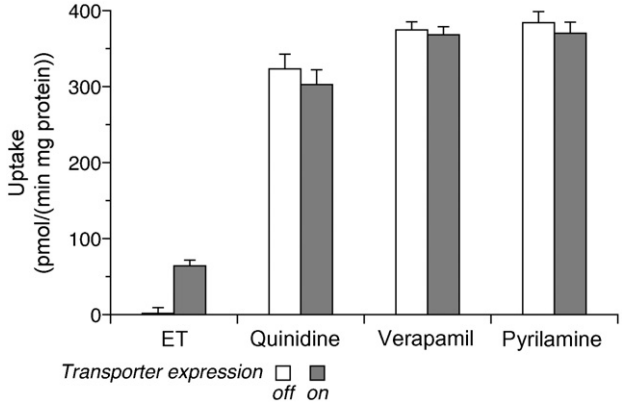

Fig. 2 – Determination by LC–MS/MS of uptake of ET and organic cations into 293 cells with or without expression of ETTh. Cells grown in dishes were incubated for 1 min with 1.0 μmol/l substrate in uptake buffer, washed, and lysed with methanol. The substrate content of cell lysates was determined by LC–MS/MS. Endogenous ET, corresponding to 30 ± 1 pmol/(min mg protein) for off-state cells and 77 ± 4 pmol/(min mg protein) for on-state cells, was subtracted to yield the uptake rates shown. The clearance for ET thus amounts to 62 ± 8 μl/(min mg protein).

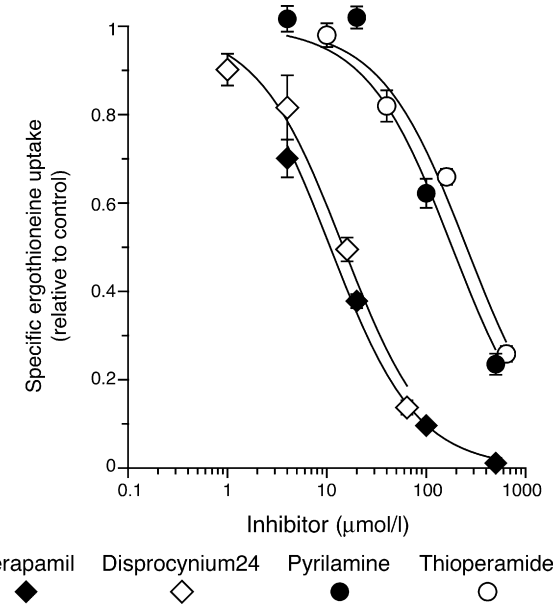

Fig. 3 – Inhibition of ETTh-mediated uptake of ET by selected drugs. An uptake period of 1 min was chosen to approximate initial rates of transport. Shown is mean ± S.E.M. ($n = 3$) of the specific uptake of ET (10 μmol/l) in the presence of inhibitor relative to control. Specific uptake was calculated as total content minus endogenous content of control cells divided by uptake time. A Hill coefficient of 1 was used for non-linear regression.

content was much higher in on-state compared to off-state cell lysates ($P < 0.0001$; $n = 3$), indicating robust expression of functional transporter. Note that for both cell states, ET already present in the cells at the beginning of the uptake period due to uptake from the cell culture medium was subtracted; it was determined with matching dishes incubated in uptake buffer devoid of substrate. For the xenobiotics, no such correction is necessary. Our data suggests that ETT does not transport quinidine, verapamil or pyrilamine.

3.2. *Inhibition by organic cations of ET transport*

293 cells expressing ETTh were analyzed for inhibition of ET uptake by selected drugs (Fig. 3, Table 1). None of the drugs displayed high affinity ($K_i < 1$ μmol/l) towards ETTh. Verapamil ($K_i = 11$ μM) was the most potent inhibitor, followed by disprocynium24, pyrilamine, and thioperamide. Lidocaine up to a concentration of 64 μM failed to inhibit ETTh noticeably. ETTh was also fully resistant to inhibition by carnitine and 5-aminosalicylic acid, each up to 640 μM.

3.3. *Methimazole*

Uptake of methimazole and ET was determined in parallel with 293 cells stably transfected with pEBTetD/ETTh as above. Cell lysates were analyzed by LC–MS/MS. Initial rates of specific uptake were calculated as the difference in substrate content of on-state-cells and off-state-cells, divided by uptake time (=1 min). Endogenous ET was determined and subtracted as above. With 100 μmol/l methimazole, a clearance of 0.3 ± 0.2 μl/(min mg protein) was calculated ($n = 3$). With 10 μmol/l ET, measured with paired dishes, the clearance was 39 ± 2 μl/(min mg protein). Thus, transport of methimazole by ETTh is negligible. The affinity of ETTh for methimazole was determined by inhibition of uptake of ET (Fig. 4). A K_i of 7.5 mmol/l was determined from the data (Table 1).

3.4. *Hercynine*

Hercynine was synthesized, purified, and analyzed for purity as described in Section 2. Uptake of hercynine and ET was determined in parallel with 293 cells stably transfected with pEBTetD/ETTh as above. Cell lysates were analyzed by LC–MS/MS. Endogenous substrate was determined and subtracted as above. With 100 μmol/l hercynine, a clearance of 1.1 ± 0.2 μl/(min mg(protein)) was calculated ($n = 4$). With 10 μmol/l ET, measured with paired dishes, the clearance was 28 ± 3 μl/

Table 1 – Inhibition of ETTh-mediated uptake of ergothioneine by selected drugs and compounds

Drug	K_i (μmol/l)	95% CI
Verapamil	10.8	9.1, 12.8
Disprocynium24	14.6	11.2, 19.1
Pyrilamine	182	134, 247
Thioperamide	254	209, 311
Hercynine	1450	700, 2990
Methimazole	7520	5090, 9940

Uptake (1 min) of 10 μmol/l ET was assayed in stably transfected 293 cells ($n = 12$ for each drug). Given is the K_i (assuming a Hill coefficient of 1) with the corresponding 95% confidence interval (CI).

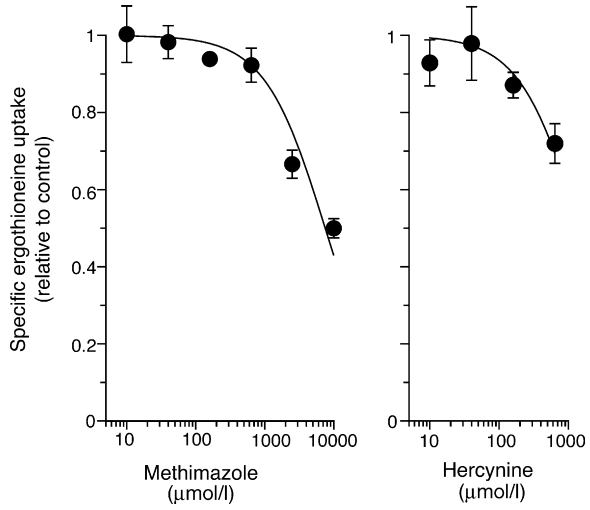

Fig. 4 – Inhibition of ETTh-mediated uptake of ET by methimazole and hercynine. See legend to Fig. 3 for experimental conditions.

(min mg(protein)). It follows that ETT transports hercynine, albeit at low transport efficiency. The affinity of ETTh for hercynine was determined by inhibition of uptake of ET (Fig. 4). A K_i of 1.4 mmol/l was extrapolated from the data (Table 1).

3.5. *Functional characterization of ETT from mouse*

ETT from mouse (ETTm) was expressed in 293 cells and analyzed for uptake of ET. In saturation analysis, a K_m of 50 μmol/l (95% confidence interval, 33–76) was determined

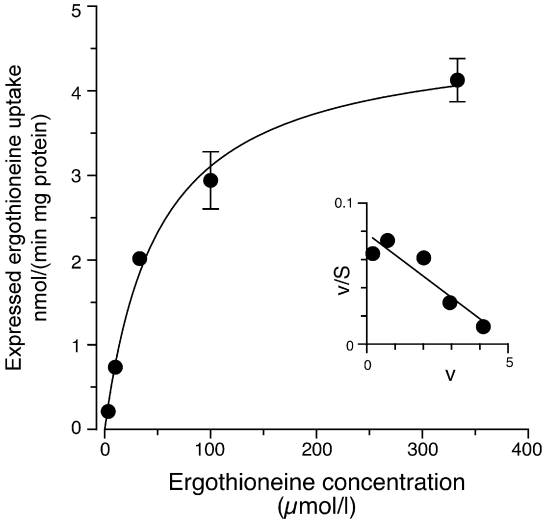

Fig. 5 – Saturation of uptake of ET mediated by ETT from mouse. An uptake period of 1 min was chosen to approximate initial rates of transport. Shown is mean ± S.E.M. (n = 3). Expressed uptake equals total content minus endogenous content divided by uptake time minus non-specific uptake. Non-specific uptake increased linearly with ET concentration, slope = 0.20 μl/(min mg protein). V_{max} = 4.7 ± 0.3 nmol/(min mg protein). Inset: Eadie-Scatchard transformation.

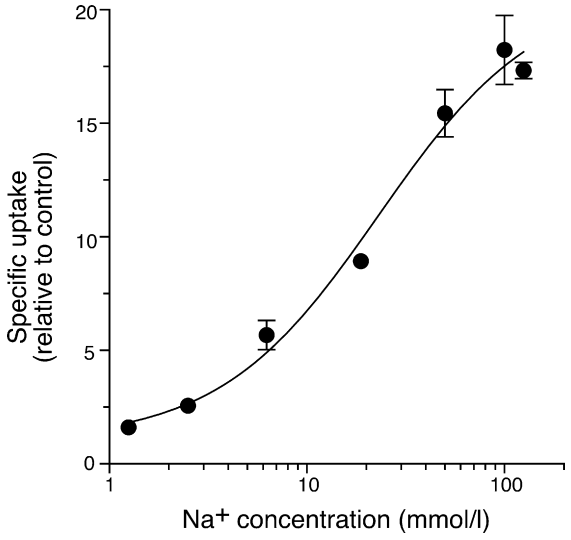

Fig. 6 – Sodium dependence of uptake of ET mediated by ETTm. Cells were rapidly washed with modified uptake buffers in which N-methyl-D-glucosamine isoosmotically substituted Na^+ as indicated and then assayed for uptake in the same buffer. Control uptake buffer was completely free of Na^+. Shown is mean ± S.E.M. (n = 3) of specific initial rates of uptake relative to control (uptake period = 1 min). A Hill coefficient of 1.1 ± 0.4 resulted from curve fitting.

(Fig. 5). Replacement of Na^+ in the uptake buffer by N-methyl-D-glucosamine demonstrated that uptake of ET by ETTm is strongly stimulated by extracellular sodium ions (Fig. 6).

3.6. *Analysis of ETTh mRNA levels*

The expression of ETT was investigated by real-time PCR as described previously [3] in several additional human tissues (Fig. 7). mRNA levels in all tested tissues were much lower than in bone marrow, with the highest levels still in mammary gland, prostate and ovary (>0.2 relative to bone marrow). Note that there is virtually no ETTh mRNA in thyroid and salivary gland.

4. Discussion

There is an enduring notion that ETT (OCTN1) functions as a proton/organic cation antiporter or organic cation/organic cation antiporter [25,26]. For the most part, this notion is probably based on inhibition experiments [15,27], but it must be stressed that it is impossible to tell whether an inhibitor is actually a substrate [28]. In fact, there is only a single report that actually states transport of organic cations other than TEA, i.e. quinidine, verapamil, and pyrilamine [15]. All other functional assays were based on transport of radiolabeled TEA or carnitine. Recently, we have demonstrated that ET is transported >100 times more efficiently than TEA and carnitine [3]. With the discovery of ET as a high performance substrate, it has become possible to test the relevance of

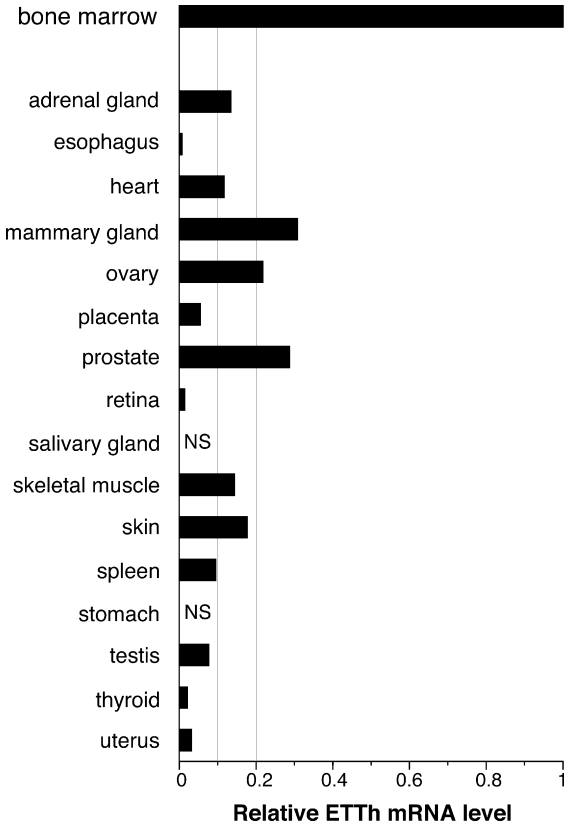

Fig. 7 – Tissue distribution of ETTh analyzed by real-time PCR. Results are given relative to the mRNA level of bone marrow. NS: no signal.

transport of organic cations. 293 cells rapidly accumulate the highly lipophilic organic cations, but expression of ETT does not increase accumulation (Fig. 2). Functional expression of ETTh was verified by robust uptake of ET. Thus, ETT does not discernibly transport quinidine, verapamil, and pyrilamine. By contrast, Yabuuchi et al. recorded significantly higher radioactivity (by a factor of 2.3, 1.6, and 2.2, respectively) with transporter-expressing versus water-injected oocytes from *Xenopus laevis* (see Fig. 3 in the cited work). One possible explanation would be, rather than transport, mere binding of radiolabel to the carrier [24]; this binding may not be apparent in a mammalian cell line since transporter overexpression in the plasma membrane of the oocyte appears to be exceptionally high [29].

We have measured inhibition of ET transport by selected drugs to see if ETT displays, in conjunction with its physiological substrate, high affinity towards organic cations. In a previous study with TEA as substrate [4], the most potent inhibitor of the wild-type carrier (=OCTN1-Leu503) was reported as the antiarrhythmic and local anaesthetic lidocaine (K_i = 0.83 μmol/l), followed by calcium channel antagonist verapamil (8.4 μmol/l) and carnitine (24 μmol/l). By contrast, we can extrapolate from our experiments – also with the wild-type carrier, but with ET as substrate – K_i-values of ≥320 μmol/l for lidocaine and ≥3200 μmol/l for carnitine. To explain the discrepancies one could assume that ET and TEA bind to different transport sites and thus interact differently with the tested inihibitors. TEA, for example, could bind to the sodium-binding site. However, our K_i for verapamil (11 μmol/l) agrees with the previous report. Verapamil represents the most potent inhibitor in our assays, but we rate its affinity only as moderate. Disprocynium24 displayed similar affinity (K_i = 15 μmol/l); note that it is by three orders more potent on the organic cation transporter type 2 (OCT2, gene symbol *SLC22A2*) and on the extra-neuronal monoamine transporter (EMT, gene symbol *SLC22A3*) [30,31]. Low affinity was observed for pyrilamine (K_i = 180 μmol/l), a histamine H_1 receptor antagonist also known as mepyramine, and thioperamide (K_i = 250 μmol/l), a histamine H_3 receptor antagonist that contains, similar to ET, both thiourea and imidazole structures. The anti-inflammatory drug 5-aminosalicylic acid, also known as mesalazine or mesalamine, is considered the active moiety of sulfasalazine. Since it shows predominant actions in the gut, it is used to treat Crohn's disease and mild to moderate ulcerative colitis. Its mechanism of action is not entirely clear. With an extrapolated K_i of ≥3200 μmol/l, our data suggest that ETT is not a target of 5-aminosalicylic acid. Altogether, it appears from our data that ETT is largely resistant to inhibition by organic cations, at least when assayed with its physiological substrate. In other words, currently no potent inhibitor is available for ETT.

The substrate specificity of ETT was probed further with hercynine. Hercynine is a precursor in the biosynthesis of ET [1]. It lacks the sulfur atom, but is otherwise identical in structure to ET. Our results indicate that hercynine is transported, albeit at low efficiency; ET is transported 25-fold better than hercynine. Concordantly, hercynine inhibited uptake of ET with low affinity (K_i = 1.4 mmol/l).

The glycine betaine moiety of ET may be regarded a hydrophilic handle that minimizes membrane permeability. The imidazole-2-thione moiety likely is responsible for the physiological activity of ET. Hence, the widely used antithyroid drug methimazole (Fig. 1) with its highly similiar structure might supply important clues to the function of ET. In the human body, methimazole is concentrated by cells in the thyroid, salivary glands, and polymorphonuclear leucocytes [32]. It is well documented that methimazole has immunosuppressive activity. This was traced back to monocytes and macrophages, the tissue-resident derivatives of monocytes [32]. In monocytes, methimazole inhibits the respiratory burst, i.e. the production of oxygen radicals and H_2O_2 [16,17]. This was interpreted as inhibition of peroxidase or scavenging of free oxygen radicals. Interestingly, monocytes and macrophages, like neutrophils and thyroid cells, accumulate methimazole. These cells apparently express a specific transport protein, since lymphocytes and two cell lines did not accumulate methimazole [32]. It is clear from previous work that ETT is strongly expressed in monocytes [3,11]. We therefore have tested whether ETT could provide a specific route of entry of methimazole into monocytes. Our results indicate that methimazole is virtually no substrate of ETT. Consistently, with a K_i of 7.5 mmol/l (Fig. 4, Table 1), ETTh has very low affinity for methimazole. It follows that methimazole must use a carrier other than ETT to enter cells. We expect that this putative methimazole transporter will not transport ET, but this cannot be tested at present since no such carrier has been molecularly identified. Interestingly, our real-time PCR

data suggest that methimazole is accumulated in cells that negligibly express ETT, if at all, i.e. salivary glands and thyroid. This makes good sense, since lack of ETT protects the thyroid from import of ET, a potential inhibitor of thyroid peroxidase (see below). It is likely therefore that ET and methimazole in general distribute to different cells. Still, in monocytes, because of concurrent expression of transporters, methimazole could indeed mimic a beneficial effect of ET. Altogether, previous reports on the *in vivo* effects of methimazole cannot be used directly to understand the function of ET. However, *in vitro*, without any membrane barrier, methimazole can be considered a valid surrogate of ET.

The existence of a specific transporter suggests that ergothioneine is advantageous for our long-term health. The key question to understand the purpose of ET is—what is the unique intracellular benefit from ET in the presence of 10-fold higher concentrations of the ubiquitous hydrophilic antioxidants glutathione (GSH) and ascorbate? There are many reports that imidazole-2-thiones like ET and methimazole are potent antioxidants *in vitro* [33,34]; however, there is no comprehensive evidence that imidazole-2-thiones are much more potent than GSH, ascorbate, or e.g. trolox, the water-soluble analog of vitamin E [35]. Thus, ET clearly has properties of a general antioxidant or radical scavenger, but other intracellular antioxidants could probably replace this function.

Our present results with ETT from mouse confirm that ETT catalyzes high affinity, sodium-driven uptake of ET (Figs. 5 and 6). Our real-time PCR data emphasize strong expression of ETT in bone marrow [36], since all other tissues in Fig. 7 had considerably lower mRNA levels. More precisely, ETT is strongly expressed in $CD71^+$ (= transferrin receptor) cells [3]. These findings suggest that ETT charges developing erythrocytes with available ET. We entertain the hypothesis that what really distinguishes ET from other antioxidants is its interaction with protein-bound heme [3]. As detailed previously, we expect ET not to affect native hemoglobin ($HbFe^{II}$), but only to bind to or react with ferryl hemoglobin ($HbFe^{IV}{=}O$). The $HbFe^{IV}{=}O$ species is a highly reactive intermediate in the autocatalytic oxidation, caused by many xenobiotics, of $HbFe^{II}O_2$ to methemoglobin ($HbFe^{III}$) and is also considered a starting point for detrimental radical reactions including heme degradation [37]. Thus, the primary function of ET can be considered as protecting erythrocytes against damage related to $HbFe^{IV}{=}O$ [38]. Monocytes do not express hemoglobin, so there must be another target for ET. A critical clue comes from the fact that hemoglobin has peroxidase activity, analogous to that of horseradish peroxidase [39]. Peroxidases like myeloperoxidase, eosinophil peroxidase, lactoperoxidase, and thyroid peroxidase (TPO) are closely related in structure and function. The second important clue comes from a detailed cell-free analysis of the reaction of methimazole, the *in vitro* substitute of ET, with its pharmacological target TPO. Methimazole does not react with reduced heme. However, it is highly interesting that methimazole binds to and covalently inactivates the heme group of TPO only if the heme is in an oxidized state (=compound II) [40]. Compound II corresponds directly to ferryl hemoglobin [39]. Most notably, GSH was inactive in this assay. Thus, affinity towards oxidized peroxidase may be the key feature of imidazole-2-thione compounds. Indeed, it has been shown that potent inhibition of the prostaglandin H synthase complex by methimazole is not caused by inhibition of the cyclooxygenase but rather by inhibition of the hydroperoxidase component [19]. From these pieces of evidence we infer that ET, by analogy with methimazole, may provide protection for monocytes by specific interaction with peroxidase(s). A lack of ET may thus represent a precipitating factor in the genesis of chronic inflammatory diseases.

In conclusion, our experiments with hercynine and methimazole establish that ETT is highly specific for its physiological substrate ergothioneine. ETT is not a ''multispecific'' cationic drug transporter, it does not show broad substrate specificity, and it does not even have high affinity for organic cation inhibitors. Detection of ETT mRNA or protein in cells can therefore be utilized as an accurate and specific molecular marker of ET activity. Methimazole, in view of its strong antithyroid activity, cannot be employed as an anti-inflammatory agent. However, since methimazole and ET rely on disparate uptake mechanisms for cellular uptake, and since ETT is not expressed in the thyroid (Fig. 7), supplementation of ET to correct a dietary deficit could provide a new therapeutic strategy for chronic inflammatory diseases. Finally, our results provide a basis for creating a knock-out mouse model to further investigate the role of ETT in chronic inflammation.

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REFERENCES

[1] Melville DB. Ergothioneine. Vitam Horm 1958;17:155–204.
[2] Hartman PE. Ergothioneine as antioxidant. Methods in enzymology, vol. 186. Academic Press; 1990. p. 310–18.
[3] Gründemann D, Harlfinger S, Golz S, Geerts A, Lazar A, Berkels R, et al. Discovery of the ergothioneine transporter. Proc Natl Acad Sci USA 2005;102:5256–61.
[4] Peltekova VD, Wintle RF, Rubin LA, Amos CI, Huang Q, Gu X, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nat Genet 2004;36:471–5.
[5] Martinez A, Del Carmen Martin M, Mendoza JL, Taxonera C, Diaz-Rubio M, de la Concha EG, et al. Association of the organic cation transporter OCTN genes with Crohn's disease in the Spanish population. Eur J Hum Genet 2006;14:222–6.
[6] Leung E, Hong J, Fraser AG, Merriman TR, Vishnu P, Krissansen GW. Polymorphisms in the organic cation transporter genes SLC22A4 and SLC22A5 and Crohn's disease in a New Zealand Caucasian cohort. Immunol Cell Biol 2006;84:233–6.
[7] Babusukumar U, Wang T, McGuire E, Broeckel U, Kugathasan S. Contribution of OCTN variants within the IBD5 locus to pediatric onset Crohn's disease. Am J Gastroenterol 2006;101:1354–61.
[8] Fisher SA, Hampe J, Onnie CM, Daly MJ, Curley C, Purcell S, et al. Direct or indirect association in a complex disease: the

role of SLC22A4 and SLC22A5 functional variants in Crohn disease. Hum Mutat 2006;27:778–85.
[9] Waller S, Tremelling M, Bredin F, Godfrey L, Howson J, Parkes M. Evidence for association of OCTN genes and IBD5 with ulcerative colitis. Gut 2006;55:809–14.
[10] Santiago JL, Martinez A, de la Calle H, Fernandez-Arquero M, Figueredo MA, de la Concha EG, et al. Evidence for the association of the SLC22A4 and SLC22A5 genes with type 1 diabetes: a case control study. BMC Med Genet 2006;7:54 (Electronic resource).
[11] Tokuhiro S, Yamada R, Chang X, Suzuki A, Kochi Y, Sawada T, et al. An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. Nat Genet 2003;35:341–8.
[12] Barton A, Eyre S, Bowes J, Ho P, John S, Worthington J. Investigation of the SLC22A4 gene (associated with rheumatoid arthritis in a Japanese population) in a United Kingdom population of rheumatoid arthritis patients. Arthritis Rheum 2005;52:752–8.
[13] Martinez A, Valdivia A, Pascual-Salcedo D, Balsa A, Fernandez-Gutierrez B, De la Concha E, et al. Role of SLC22A4, SLC22A5, and RUNX1 genes in rheumatoid arthritis. J Rheumatol 2006;33:842–6.
[14] Tamai I, Yabuuchi H, Nezu J-I, Sai Y, Oku A, Shimane M, et al. Cloning and characterization of a novel human pH-dependent organic cation transporter OCTN1. FEBS Lett 1997;419:107–11.
[15] Yabuuchi H, Tamai I, Nezu J-I, Sakamoto K, Oku A, Shimane M, et al. Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations. J Pharmacol Exp Ther 1999;289:768–73.
[16] Balazs C, Kiss E, Leovey A, Farid NR. The immunosuppressive effect of methimazole on cell-mediated immunity is mediated by its capacity to inhibit peroxidase and to scavenge free oxygen radicals. Clin Endocrinol 1986;25:7–16.
[17] Weetman AP, Holt ME, Campbell AK, Hall R, McGregor AM. Methimazole and generation of oxygen radicals by monocytes: potential role in immunosuppression. Br Med J (Clin Res Ed) 1984;288:518–20.
[18] Lechpammer M, Lukac J, Lechpammer S, Kusic Z. Antithyroid drug-induced immunomodulation in Graves' disease patients. Acta Med Croatica 2002;56:21–6.
[19] Lagorce JF, Moulard T, Rousseau A, Comby F, Buxeraud J, Raby C. Anti-inflammatory action of methimazole. Pharmacology 1997;55:173–8.
[20] Bach M, Grigat S, Pawlik B, Fork C, Utermöhlen O, Pal S, et al. Fast set-up of doxycycline-inducible protein expression in human cell lines with a single plasmid based on Epstein-Barr virus replication and the simple tetracycline repressor. FEBS J 2007;274:783–90.
[21] Reinhold VN, Ishikawa Y, Melville DB. Synthesis of alpha-N-methylated histidines. J Med Chem 1968;11:258–60.
[22] Gründemann D, Liebich G, Kiefer N, Köster S, Schömig E. Selective substrates for non-neuronal monoamine transporters. Mol Pharmacol 1999;56:1–10.
[23] Schömig E, Babin-Ebell J, Russ H. 11′-Diethyl-2,2′-cyanine (decynium22) potently inhibits the renal transport of organic cations. Naunyn-Schmiedebergs Arch Pharmacol 1993;347:379–83.
[24] Harlfinger S, Fork C, Lazar A, Schömig E, Gründemann D. Are organic cation transporters capable of transporting prostaglandins? Naunyn-Schmiedebergs Arch Pharmacol 2005;372:125–30.
[25] Watanabe C, Kato Y, Sugiura T, Kubo Y, Wakayama T, Iseki S, et al. PDZ Adaptor protein PDZK2 stimulates transport activity of organic cation/carnitine transporter OCTN2 by modulating cell surface expression. Drug Metab Dispos 2006;34:1927–34.
[26] Inazu M, Takeda H, Maehara K, Miyashita K, Tomoda A, Matsumiya T. Functional expression of the organic cation/carnitine transporter 2 in rat astrocytes. J Neurochem 2006;97:424–34.
[27] Wu X, George RL, Huang W, Wang H, Conway SJ, Leibach FH, et al. Structural and functional characteristics and tissue distribution pattern of rat OCTN1, an organic cation transporter, cloned from placenta. Biochim Biophys Acta 2000;1466:315–27.
[28] Schömig E, Lazar A, Gründemann D. Extraneuronal monoamine transporter and organic cation transporters 1 and 2—a review of transport efficiency. In: Sitte HH, Freissmuth M, editors. Handbook of experimental pharmacology-neurotransmitter transporters. Heidelberg: Springer; 2006. p. 151–80.
[29] Eskandari S, Wright EM, Kreman M, Starace DM, Zampighi GA. Structural analysis of cloned plasma membrane proteins by freeze- fracture electron microscopy. Proc Natl Acad Sci USA 1998;95:11235–40.
[30] Gründemann D, Schechinger B, Rappold GA, Schömig E. Molecular identification of the corticosterone-sensitive extraneuronal monoamine transporter. Nature Neurosci 1998;1:349–51.
[31] Gründemann D, Babin-Ebell J, Martel F, Örding N, Schmidt A, Schömig E. Primary structure and functional expression of the apical organic cation transporter from kidney epithelial LLC-PK1 cells. J Biol Chem 1997;272:10408–13.
[32] Weetman AP, Gunn C, Hall R, McGregor AM. The accumulation of [35S]methimazole by monocytes and macrophages. Acta Endocrinol 1984;107:366–70.
[33] Smith RC, Reeves JC, Dage RC, Schnettler RA. Antioxidant properties of 2-imidazolones and 2-imidazolthiones. Biochem Pharmacol 1987;36:1457–60.
[34] Akanmu D, Cecchini R, Aruoma OI, Halliwell B. The antioxidant action of ergothioneine. Arch Biochem Biophys 1991;288:10–6.
[35] Franzoni F, Colognato R, Galetta F, Laurenza I, Barsotti M, Di Stefano R, et al. An in vitro study on the free radical scavenging capacity of ergothioneine: comparison with reduced glutathione, uric acid and trolox. Biomed Pharmacother 2006;60:453–7.
[36] Kobayashi D, Aizawa S, Maeda T, Tsuboi I, Yabuuchi H, Nezu J, et al. Expression of organic cation transporter OCTN1 in hematopoietic cells during erythroid differentiation. Exp Hematol 2004;32:1156–62.
[37] Alayash AI. Oxygen therapeutics: can we tame haemoglobin? Nat Rev 2004;3:152–9.
[38] Arduini A, Mancinelli G, Radatti GL, Hochstein P, Cadenas E. Possible mechanism of inhibition of nitrite-induced oxidation of oxyhemoglobin by ergothioneine and uric acid. Arch Biochem Biophys 1992;294:398–402.
[39] Nagababu E, Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. Biochemistry 2000;39:12503–11.
[40] Engler H, Taurog A, Nakashima T. Mechanism of inactivation of thyroid peroxidase by thioureylene drugs. Biochem Pharmacol 1982;31:3801–6.