JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2011, 62, 4, 411-419
www.jpp.krakow.pl

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PARAMOUNT LEVELS OF ERGOTHIONEINE TRANSPORTER *SLC22A4* MRNA IN BOAR SEMINAL VESICLES AND CROSS-SPECIES ANALYSIS OF ERGOTHIONEINE AND GLUTATHIONE IN SEMINAL PLASMA

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Ergothioneine (ET) is a unique natural antioxidant which mammalia acquire exclusively from their food. Recently, we have discovered an ET transporter (ETT; gene symbol *SLC22A4*). The existence of a specific transporter suggests a beneficial role for ET; however, the precise physiological purpose of ET is still unclear. A conspicuous site of high extracellular ET accumulation is boar seminal plasma. Here, we have investigated whether ETT is responsible for specific accumulation of ET in the boar reproductive tract. The putative ETT from pig (ETTp) was cloned and validated by functional expression in 293 cells. The highest levels of ETTp mRNA were detected by real-time RT-PCR in seminal vesicles, eye, and kidney; much less was present in bulbourethral gland, testis, and prostate. By contrast, there was virtually no ETT mRNA in rat seminal vesicles. ET content in boar reproductive tissues, determined by LC-MS/MS, closely matched the ETT expression profile. Thus, strong and specific expression of ETTp in boar seminal vesicles explains high accumulation of ET in this gland and hence also in seminal plasma. Previous reports suggest that the glutathione (GSH) content of seminal plasma correlates directly with ET content; however, a comprehensive analysis across several species is not available. We have measured ET and GSH in seminal plasma from human, boar, bull, stallion, and rabbit by LC-MS/MS. GSH levels in seminal plasma do not correlate with ET levels. This suggests that the function of ET, at least in this extracellular context, does not depend on redox cycling with GSH.

Key words: *Ergothioneine, antioxidant, glutathione peroxidase, lipid peroxidation, male reproductive tract, reproduction*

Abbreviations: ET, ergothioneine; ETT, ergothioneine transporter; GR, glutathione reductase; GSH, glutathione; LC, liquid chromatography; MS, mass spectrometry; PUFA, polyunsaturated fatty acids

INTRODUCTION

Ergothioneine (ET) is a natural antioxidant which is biosynthesized solely by fungi and mycobacteria (1). Chemically, ET is the betaine of histidine with a sulfur atom attached to the imidazole ring. 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 (2).

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 the plasma membrane is virtually impermeable for this compound. By contrast, cells with expression of ETT accumulate ET to high levels. In humans, ETT is strongly expressed in small intestine, kidney, erythrocyte progenitor cells in bone marrow, and monocytes (3). 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 such as Crohn's disease (4-9), ulcerative colitis (10) and Type I diabetes (11). It is presently unknown how the mutation in the transporter gene promotes disease.

The existence of a specific transporter suggests a beneficial role for ET. Most authors consider ET as an intracellular antioxidant. However, the precise physiological purpose of ET and the consequences of ET deficiency are still unclear. A particularly conspicuous site of ET accumulation is boar semen; in seminal plasma, 0.3-0.8 mmol/l ET has been measured (12, 13). Boar spermatozoa contain, if any at all, much less ET than seminal plasma (14). Thus, in boar semen ET is an extracellular constituent - unlike blood, where nearly all ET is contained within erythrocytes and specific leukocytes. ET apparently enters boar semen at the seminal vesicles. The isolated secretion of this gland contains amazingly high concentrations (average: 3.4 mmol/l; range: 1.3-11 mM; 29-256 mg/100 ml) (12); this level is 10 times higher than in the blood of pig (range: 0.13-1.2 mM; 3-27 mg/100 ml) or other species (1, 14). It is unclear at

present whether ETT is responsible for accumulation of ET in boar seminal vesicles.

Other species with high ET levels in male accessory secretions are horse, donkey (15), mole, and hedgehog (16). In the stallion, ET is predominantly secreted by the ampulla of the vas deferens; ampullar secretion contained on average 1.7 mmol/l (17) or 2.8 mmol/l ET (15). By utter contrast, human, bull, and ram have been reported to contain only traces of ET in semen (14). This remarkable species difference presents a good opportunity to analyze the specific function of ET in seminal plasma. Mammalian spermatozoa contain extraordinarily high levels of polyunsaturated fatty acids (PUFA) in plasma membrane phospholipids (18, 19) and are thus highly susceptible to oxidative damage (lipid peroxidation) caused by reactive oxygen species (ROS) (20). The degradation of membrane PUFA and the concomitant release of toxic fatty acid fragments impair sperm motility and fertility (18, 20). Mammalia apparently have evolved several systems both within spermatozoa and in the surrounding seminal plasma to prevent peroxidation damage; ET, along with anti-oxidant enzymes and other compounds, may be an important factor here.

Interestingly, there is data to suggest that the glutathione (GSH) content of seminal plasma correlates directly with ET content. Spermatozoa of human and bull contain high GSH levels (0.3-0.4 mmol/l (21, 22)), but in seminal plasma the GSH concentration is low (human: 0.5-2.0 µmol/l (21, 23, 24); bull: 0.9 µmol/l, calculated with an estimated seminal plasma protein concentration of 50 mg/ml (22)). By contrast, the GSH concentration in seminal plasma has been reported ≥100 times higher for boar (186 µmol/l (13) and 250 µmol/l (25)) and stallion (2500 µmol/l) (25). Sperm cells of boar contain no GSH (21). A strict correlation of ET and GSH in seminal plasma would suggest a functional link between both compounds; however, a comprehensive analysis across several species is not available. GSH has been suggested previously to reduce and thus recycle ET without enzyme catalysis (26), but no evidence was presented then.

The primary aims of the present study were to investigate whether ETT is responsible for specific accumulation of ET in boar seminal vesicles and whether the levels of ET and GSH correlate across species.

MATERIALS AND METHODS

Plasmid constructs

The cDNAs of ETT from pig (ETTp) and chicken (ETTch) were generated by RT-PCR with total RNA from kidney, cloned into pUC19, fully sequenced, and finally inserted into expression vector pEBTetD (27). The cDNAs cloned and used in this work were deposited at EMBL database with accession numbers FN178490 (ETT from chicken) and FN178491 (ETT from pig). The 5'-interface between ETTp cDNA and pEBTetD is **GTTTAAACTTAAGCTT** gccacc ATGCGGGACTACGAC (plasmid polylinker in bold, Kozak motif lowercase, cDNA underlined); the 3'-interface is TAACTTCATTCTGA **GCGGCCGC GGG**. For ETTch cDNA, the 5'-interface is **GTTTAAACTTAAGCTT** gccacc ATGCGGGACTACGAA; the 3'-interface is ACATTGTGA AGAAGGATGCACACTCA GAGGGTTTGG **CTCGAG CGATCGC**.

Cell culture

293 cells (ATCC CRL-1573; also known as HEK-293 cells), 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, Coelbe, 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 (27); 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).

Transport assays

For measurement of solute uptake, 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 $MgSO_4$. After preincubation for at least 20 minutes in 4 ml of uptake buffer, the buffer was replaced with 2 ml of substrate in uptake buffer. The total substrate concentration if not indicated otherwise was 0.1 µmol/l for radiotracer assays (^{14}C-glycine betaine, ^{3}H-carnitine, ^{14}C-tetraethylammonium) and 10 µmol/l for unlabeled compounds (ET, stachydrine). Incubation was stopped after 1 min by rinsing the cells four times each with 4 ml ice-cold uptake buffer. Radioactivity was determined, after cell lysis with 0.1% v/v Triton X-100 in 5 mmol/l TRIS-HCl pH 7.4, by liquid scintillation counting. For LC-electrospray ionization-MS/MS analysis, the cells were solubilized with 4 mmol/l $HClO_4$ or methanol and stored at -20°C. After centrifugation (1 min, 16,000 x g, 20°C) of the thawed lysates, 100 µl of the supernatant was mixed with 10 µl unlabeled 1-methyl-4-phenylpyridinium (MPP^+) iodide (0.5 ng/µl) which served as internal standard. Of this mixture, 20 µl samples were analyzed by LC-MS/MS (flow rate 0.25 ml/min) on a triple quadrupole mass spectrometer (TSQ Quantum, Thermo Electron, Dreieich, Germany or 4000 Q TRAP, Applied Biosystems, Darmstadt, Germany). Atmospheric pressure ionization with positive electrospray was used. Isocratic chromatography (70% methanol and 30% 0.1% formic acid) was employed with a Waters Atlantis HILIC silica column (length 50 mm, diameter 3 mm, particle size 5 µm). For quantification (scan time 0.3 s), the optimal collision energy for nitrogen-induced fragmentation in the second quadrupole was determined for each analyte. From the product ion spectra, the following fragmentations were selected for selected reaction monitoring (m/z parent, m/z fragment, collision energy): ergothioneine: 230, 127, 27 V; MPP^+: 170, 128, 25 V; stachydrine 144, 84, 33 V; tetraethylammonium: 130, 86, 26 V. For each analyte, the area of the intensity *vs*. 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 linear regression.

Protein was measured by the BCA assay (Pierce) with bovine serum albumin as standard. The protein content of MS samples was estimated from 4-6 matched cell dishes.

Analysis of ergothioneine content of tissues

Frozen tissue was disintegrated in 4 mmol/l $HClO_4$ on ice using a Potter S Homogenizer (Sartorius, Goettingen, Germany).

After centrifugation of the homogenate (10 min at 10,000 x g), the ET content of the supernatant was determined by LC-MS/MS as described above.

Analysis of ergothioneine and glutathione content of seminal plasma

In this section, buffer is 10 mM ammonium acetate pH 8.6, 1 mM EDTA. After centrifugation (10 min, 16,000 x g, 20°C) of frozen-thawed ejaculate, 25 µl of the supernatant (=seminal plasma) was mixed with 25 µl buffer or GSH or ET in buffer, 75 µl of either 10 mM DTNB in buffer (GSH assay) or buffer (ET assay), and 125 µl metaphosphoric acid (3%, ice-cold). Mixtures were centrifuged (10 min, 16,000 x g, 4°C), incubated (1 h, 4°C), and frozen. After thawing and centrifugation (2 min, 16000 x g, 20°C), 80 µl of the supernatant was mixed with either 10 µl (GSH assay) or 7 µl (ET assay) ammonia (10% v/v), filled to 200 µl with buffer, and centrifuged (2 min, 16,000 x g, 20°C). Of this mixture, 20 µl samples were analyzed by LC-MS/MS. With every sample, an individual calibration curve was constructed by addition of 3 standards before processing; final concentration increments in the injected samples were 10, 20, and 40 µM for GSH, and 8, 16, and 32 µM for ET.

The following LC conditions were used: GSH, Atlantis T3 column (particle size 5 µm, diameter x length = 3.0 x 100 mm; Waters, Eschborn, Germany); A: 0.1% formic acid, B: 0.1% formic acid in acetonitrile; gradient: 0.4 ml/min; 60% B at 0 min, 60% B at 9.5 min, 0% B at 10 min, stop at 11 min; ET, Atlantis HILIC silica column; A: 0.1% formic acid, B: 0.1% formic acid in acetonitrile; gradient: 0.4 ml/min; 90% B at 0 min, 90% B at 0.25 min, 10% B at 2 min, 10% B at 4 min, 90% B at 5 min, stop at 6 min. The following fragmentations were selected for selected reaction monitoring (positive electrospray; m/z parent, m/z fragment, collision energy (V)): GSH-DTNB: 505, 273, 31; ET: 230, 127, 27. Sample analyte content was calculated from the analyte peak area and the slope of the calibration curve, obtained by weighted ($1/y^2$) linear regression.

Expression profiling by real-time RT-PCR

Total RNA was isolated by the method of Chomczynski and Sacchi (28) from frozen (-80°C) tissues. Reverse transcription was performed as detailed previously (29) with the following modifications: i) RQ1-DNase (Promega, Mannheim, Germany) was used at 1 U/µg total RNA; ii) Random nonamers were used for priming; iii) cDNA synthesis was performed at 42°C.

A LightCycler 1.0 apparatus with system 2.0 software (Roche, Mannheim, Germany) was used for real-time PCR. Product accumulation was detected with locked nucleic acid hydrolysis probes (TaqMan principle (30)) from the human Universal ProbeLibrary (Roche). A single reaction (total volume 10 µl) contained 1 µl master mix (5 x concentration; LightCycler TaqMan Master; Roche 04735536001), 1 µmol/l each of forward and reverse primer, 50 nmol/l probe, and 1 µl of cDNA. Contamination controls contained water instead of DNA. After enzyme activation (10 min, 95°C), thermocycling consisted of 45 cycles of 10 s at 95°C, 30 s at 55°C, and 1 s at 72°C; velocity of temperature change was 1.1°C/s.

Primers and probes are shown in Table 1.

Calculations and statistics

The clearance equals initial rate of specific uptake (=uptake mediated by expressed carrier) divided by substrate concentration; it 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) (32, 33). Specific uptake equals total uptake minus uptake into control cells (=non-specific uptake).

Analysis of the time course of substrate accumulation was based on a one-compartment model (34). Analysis of saturation curves has been reported previously (35). K_m-values are given as geometric mean with 95% confidence interval. In the figures, symbols and bars represent arithmetic mean ±S.E.M. (n=3) if not indicated otherwise in the legend. The unpaired t-test was used to test for significance; two-tailed *P* values are given.

Animals and semen samples

Chicken organs were kindly provided by a local chicken farm, and pig organs by a slaughterhouse in Jena, Germany, and by the Institute of Experimental Medicine, University of Cologne; rat organs were from a local stock of adult male Wistar rats (*Rattus norvegicus*).

Semen samples were obtained from the following German institutions: stallion, Landesgestut Celle, Spoerckenstrasse 10, 29221 Celle; boar, Schweinezuchtverband Baden-Wuerttemberg e.V., Besamungsstation Herbertingen, Oelkofer Strasse 33,

Table 1. Fluorescence curves were analyzed by non-linear simultaneous fitting as described previously (31) to yield the relative mRNA level. To normalize the amount of cDNA per assay, β-actin was measured in parallel assays.

Target accession number	**Forward primer**	**Reverse primer**	**Probe**
ETT pig FN178491	GGTGTGTGAGGATGATTGGA	CCAACGAGCACACCTATGAA	CCACCTCC (#10)
beta-actin pig XM_003124280	TCAAGGAGAAGCTGTGCTACG	TCCAGGGAGGAGGAGGAC	GAGCAGGA (#15)
ETT chicken FN178490	GCCCCTGTTTGCTTACTTCA	GATCCACCAGAGCGGAAC	GGATGCTG (#89)
beta-actin chicken NM_205518	CCACAGCTGCCTCTAGCTCT	TGACCTGACCATCAGGGAGT	CCTGGAGA (#80)
ETT rat NM_022270	TTTCGTGTACCTGGGTGCTT	GTCAGACTGCCCATGAGGAT	CTCCTGCC (#51)
beta-actin rat NM_031144	CATCACTATCGGCAATGAGC	TTCCTGGGTATGGAATCCTG	CTTCCAGC (#11)

88518 Herbertingen; rabbit, Dr. Zimmermann GbR, Billingshalden 1, 73453 Abtsgmuend-Untergroeningen; bull, Rinderunion Baden-Wuerttemberg e.V., Oelkoferstr. 41, 88518 Herbertingen; and MASTERRIND GmbH Verden, Osterkrug 20, 27283 Verden; human, Cryostore Deutschland GmbH, Akazienalle 8-12, 45127 Essen.

Chemicals

Unlabeled compounds were as follows: L-carnitine (C-0283, Sigma-Aldrich, Munich, Germany), L-(+)-ergothioneine (F-3455, Bachem, Bubendorf, Switzerland), 1-methyl-4-phenylpyridinium iodide (D-048, Sigma-Aldrich), stachydrine hydrochloride (0604, Extrasynthese, Genay, France). All other chemicals were at least of analytical grade. Radiotracers (all from ARC, St. Louis, MO, USA) were as follows: L-carnitine hydrochloride (H-3, 3.1 kBq/pmol, ART-293), glycine betaine (C-14, 1.85 Bq/pmol, ARC-1432), tetraethylammonium (C-14, 2.04 Bq/pmol, ARC-577).

RESULTS

Cloning of ergothioneine transporter from pig and chicken

The cDNA of the putative ergothioneine transporter from pig (ETTp) was cloned by RT-PCR with total RNA isolated from *Sus scrofa* kidney. Since there was no full-length cDNA record in the public databases, we used several EST (expressed sequence tag) and genomic DNA records with similarity to ETT from human (ETTh; gene symbol *SLC22A4*) to design the PCR primers. At least 12 independent clones were sequenced for each position to generate a consensus cDNA. The amino acid sequence of ETTp is clearly homologous to ETT from human (88% identity, 91% similarity).

In order to compare ET transport in a non-mammalian vertebrate, the cDNA of the putative ETT from chicken (ETTch) was cloned with total RNA isolated from *Gallus gallus* kidney by RT-PCR based on genomic DNA records. Alignment of amino acid sequences of ETTch and ETTh scored 77% identity and 81% similarity.

Validation of transporter function

In order to validate the above assignments of ergothioneine transporters, it was necessary to demonstrate efficient and specific transport of ET. Both cDNAs were separately inserted into the pEBTetD vector and then stably transfected into 293 cells. pEBTetD is an episomal Epstein-Barr plasmid vector for doxycycline-inducible protein expression in human cell lines based on the simple tetracycline repressor (27). Expression is turned on by addition of 1 µg/ml doxycycline to the culture medium for about 20 h. With *e.g.* ETTh, this system provides a high rate of carrier-mediated transport in the on-state (=100%) and a low rate (4%) in the off-state (27).

With the stably transfected cell lines, uptake of ET was measured. The ET content of cell lysates was determined by LC-MS/MS. The time course of uptake (*Fig. 1*) revealed that ET accumulates to much higher levels in induced cells compared to uninduced control cells. At an extracellular ET concentration of 10 µmol/l, cells with expression of ETTp turned on reached an intracellular concentration of 1.0 mmol/l after 60 min (calculated with an intracellular water space of 6.7 µl/mg protein (36) and a conjectured rate of carrier-expressing cells of 100%). Thus, ETT from pig catalyzed intracellular accumulation of ET by a factor of about 100 (or higher, since the actual expression rate will be lower than 100%) over the external medium. By contrast, off-state-cells reached an intracellular concentration of 0.11 mmol/l only; this accumulation was probably caused by leak expression of the cloned transporter (see above), because in 293 cells there is no endogenous expression of ETTh (3). Similar results were obtained with ETT from chicken (not shown). To approximate

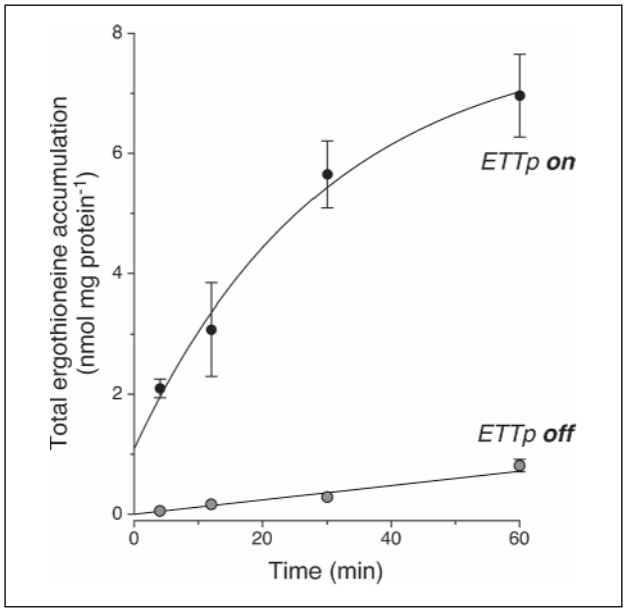

Fig. 1. Time course of ET uptake into cells with and without heterologous expression of ETTp. Cells grown in dishes were incubated at 37°C with 10 µmol/l ET for the indicated time, washed, and lysed with methanol. The substrate content of cell lysates was determined by LC-MS/MS. Exponential functions plus offset (to allow for ET accumulation from the culture medium) were fitted to experimental data (mean ±S.E.M.; n=3).

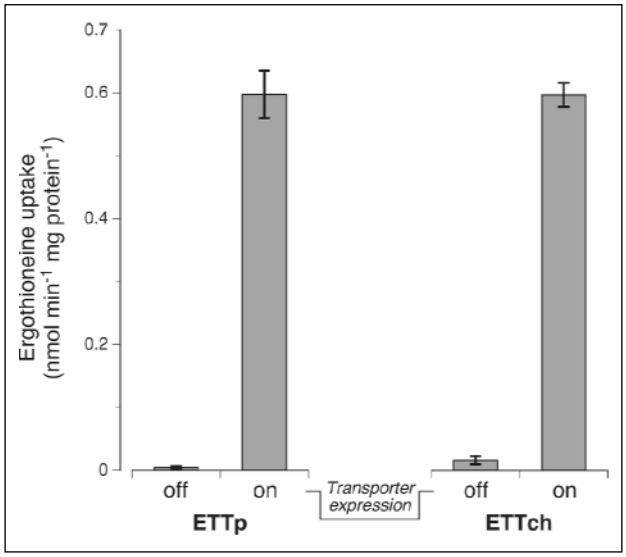

Fig. 2. Uptake of ET into 293 cells with or without expression of ETT from pig and chicken. Cells grown in dishes with ("on") and without ("off") expression of ETTp or ETTch were incubated for 1 min with 10 µmol/l ET in uptake buffer (paired assays, 37°C). Endogenous ET, corresponding *e.g.* for ETTp cells to 21±2 pmol min^{-1} mg $protein^{-1}$ for off-state cells and 68±2 pmol min^{-1} mg $protein^{-1}$ for on-state cells, was determined by incubation with uptake buffer alone and subtracted to yield the uptake rates shown (mean±S.E.M.; n=3).

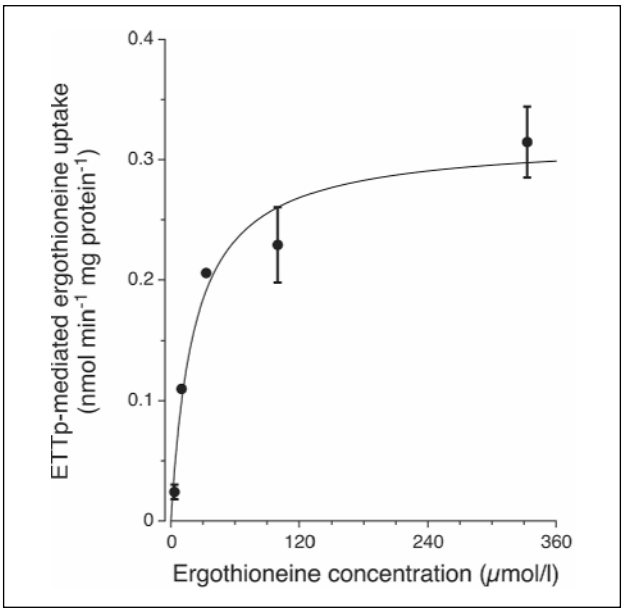

Fig. 3. Saturation of specific uptake of ET mediated by ETTp. An uptake period of 1 min was used to approximate initial rates of transport. Endogenous ET content was determined with uptake buffer incubation and subtracted. Specific uptake was calculated as the difference of total uptake between cells with and without heterologous carrier expression. Uptake into control cells (=ETTp expression off) could be described as a linear function of ET concentration; the slope was 0.15 µl min^{-1} mg protein^{-1}.

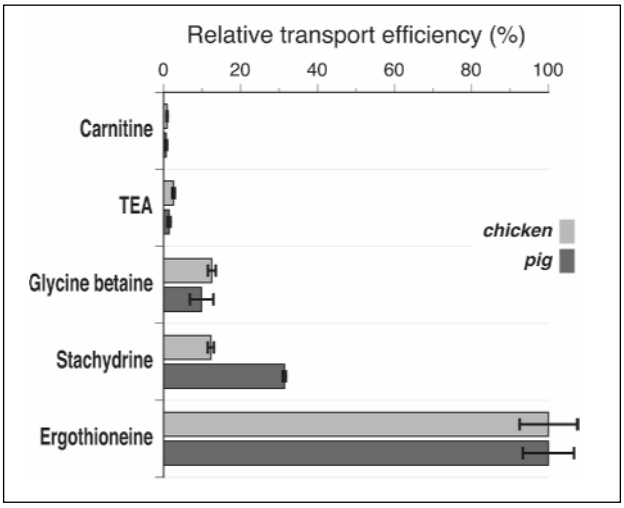

Fig. 4. Substrate specificity of ETT from pig and chicken. Cells grown in dishes were incubated for 1 min at 37°C with the indicated compounds in uptake buffer, washed, and lysed. With ET or stachydrine (10 µmol/l), the content of cell lysates was determined by LC-MS/MS, and endogenous compound was corrected as described in the legend of *Fig. 2*. Uptake of carnitine (0.1 µM), TEA (0.2 µM), and glycine betaine (0.4 µM) was measured by radiotracer assay. Actual transporter-mediated uptake was calculated as the difference of uptake between cells with and without heterologous carrier expression (paired assays). For each compound, the clearance was divided by the corresponding ET clearance.

initial rates of uptake, an uptake time of 1 min was used in subsequent experiments. Efficiency of transport of ET, calculated from the difference of uptake rates shown in *Fig. 2* divided by the substrate concentration, was high both for ETTp (59±4 µl min^{-1} mg protein^{-1}) and ETTch (58±2 µl min^{-1} mg protein^{-1}). Note the excellent signal-to-noise ratio of the expression system for both carriers apparent from *Fig. 2* (P<0.0001; n=3).

Uptake of ET by ETTp was saturable (*Fig. 3*) with a K_m of 22 (95% confidence interval, 12-39) µmol/l which for a transporter signifies high affinity. Similarly, the K_m of ETTch was 11 (8 17) µmol/l (not shown). Maximal uptake rates (V_{max}) were 320±20 (ETTp) and 700±60 pmol min^{-1} mg protein^{-1}, respectively.

As shown in *Fig. 4*, ET was transported much more efficiently (=100%) by ETTp than stachydrine (alias proline betaine; 31%) and glycine betaine (10%). Transport of tetraethylammonium (TEA; 1%) and carnitine (0.7%) was negligible. Hence, the substrate selectivity of ETTp is similar to the profile of ETT from human (3). Concordant results were obtained with ETT from chicken (*Fig. 4*). Both carriers are thus distinguished from the related carnitine transporter (*SLC22A5*) (37, 38). Overall, our functional characterization data clearly validate the assignment of ETTp and ETTch cDNAs.

Expression profile of ergothioneine transporter across species

Based on the pig and chicken cDNAs identified above and on ETT from rat (3), the expression profiles were investigated by real-time RT-PCR as described previously (31) with selected tissues (*Fig. 5*). With pig, the highest levels of ETT mRNA were found in seminal vesicles, eye, and kidney. By contrast, much less was detected in bulbourethral gland, testis, bone marrow, trachea, liver, and prostate. The rat and chicken profiles congruently suggest strong expression of ETT in kidney, but also establish fundamental species differences: by contrast to pig, there was virtually no ETT mRNA in rat seminal vesicles; moreover, there was little ETT mRNA in rat eye, but much in rat liver. In both species, levels in bone marrow were higher than in pig.

Ergothioneine content in tissues of the reproductive tract of the boar

The ET content of boar testis and accessory glands was determined by LC-MS/MS (*Fig. 6*). In excellent agreement with the ETT expression profile, ET content was very high for seminal vesicles and low for testis, prostate and bulbourethral gland.

Ergothioneine and glutathione content of seminal plasma across species

Seminal plasma from human, boar, bull, stallion, and rabbit was isolated as the supernatant after centrifugation (16,000 x g) of frozen-thawed ejaculates; note that this preparation may contain residual spermatozoa or components thereof. The ET and GSH content of seminal plasma was determined by LC-MS/MS (*Table 2*). By contrast to ET, which is very stable, GSH must first be modified covalently at the thiol group to avoid oxidation and thus loss of signal. With our seminal plasma samples, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (39) generated better peaks in LC-MS/MS and better calibration curves than N-ethylmaleimide or iodoacetic acid (not shown). Note that seminal plasma composition varies vastly across species (12); to avoid matrix effects (*alias* ion suppression) in MS analysis, an individual calibration curve was constructed with every sample by addition of standards before processing. The data of *Table 2* indicate high ET levels (around 500 µmol/l) for boar and stallion, >20 times more than for human and bull, and >500 times more than for rabbit (comparison of medians). GSH levels were

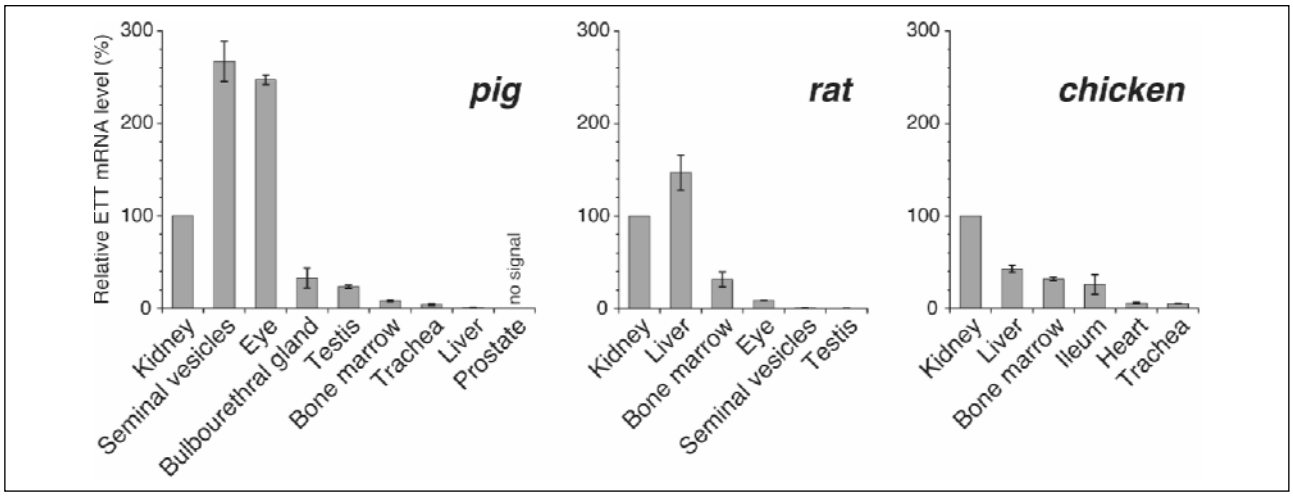

Fig. 5. Tissue distribution of ETT mRNA from pig, rat, and chicken analyzed by real-time RT-PCR. Results (mean±S.E.M.; n=3) are given relative to the mRNA level of kidney; the latter was set to 100% for each series and thus has no error bar. The mRNA of β-actin was measured in parallel assays to normalize the amount of cDNA per assay. Porcine tissues were obtained from several female and male pigs; tissues of other species were from single animals.

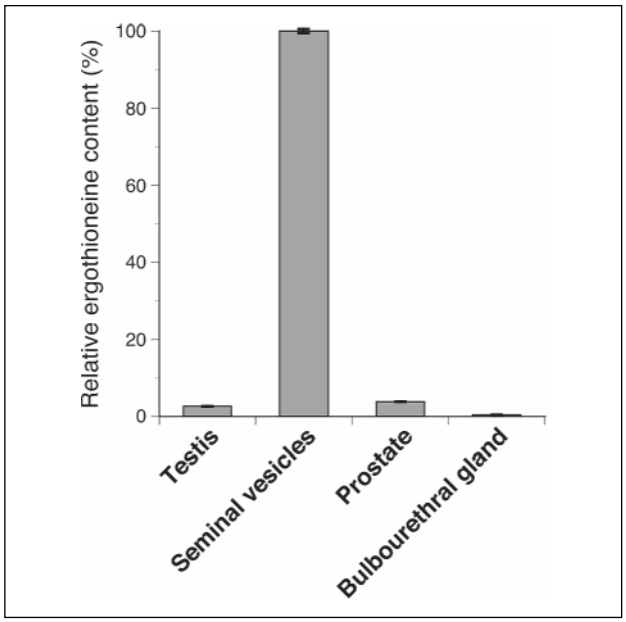

Fig. 6. Relative ET content of boar reproductive tract tissue. The ET content of tissue lysates was determined by LC-MS/MS. Results (mean±S.E.M.; n=3) are given relative to the ET content of seminal vesicles. All tissues were from a single boar. The data shown were calculated from the response ratio for ET divided by the response ratio for proline which served as cytosol volume reference (3); for both ratios, the 1-methyl-4-phenyl pyridinium peak area was used as internal standard.

uniformly low across species (3-5 µmol/l). By contrast, the BIOXYTECH® GSH-400™ colorimetric assay (OXIS Research, Portland, OR, U.S.A.), yielded GSH levels of 500 µmol/l for human and 2200 µmol/l for stallion seminal plasma.

DISCUSSION

In the present study, we have cloned and functionally validated the ergothioneine transporter from pig. We have shown that ETTp mRNA is highly abundant in boar seminal vesicles, but is almost absent from the other investigated parts of the boar reproductive tract and also from rat seminal vesicles. The distribution of ET, measured by LC-MS/MS, matches the ETT expression pattern; this correlation further strengthens the notion that ETT mRNA serves as a marker for ET activity (40). Our data agree with earlier results that were based on a less specific ET detection method (14). We assume that ETTp is located in the basolateral membrane to catalyze sodium-driven uptake of ET from blood into the glandular cells. Apical exit into the seminal fluid probably occurs by non-specific apocrine secretion (41).

Our real-time RT-PCR profile for the rat carrier agrees with and extends previous Northern blot data which indicated high levels in small intestine, liver, and kidney (42). ETT from chicken was also cloned and validated in the present report. Together, our ETT expression profiles of pig, rat, and chicken support the notion that ETT is essential for absorption (intestine) and retention (kidney) of ET in every species. However, the profiles also reveal fundamental species differences; this resembles vast differences in ET content of some organs of different species (1). It appears that different species benefit from ET in different contexts. In other words, high accumulation of ET (*e.g.* rat liver, pig eye and seminal fluid) is not restricted to a single organ or tissue across species.

All vertebrates examined so far depend completely on food for their ET supply; for example, pigs normally have ET blood levels of 200 µmol/l (14), but pigs fed on purified casein diet had no detectable ET in their blood (43). Thus, our lack of control of diet may explain some variability within a species (*Table 2*); anyhow, our LC-MS/MS data on ET in seminal plasma of boar (median 0.6 mmol/l) and stallion (0.4 mmol/l) agree well - despite the use of other methods - with previous reports, *i.e.* 0.76 mmol/l (12) or 0.29 mmol/l (13) for boar; and 0.70 mmol/l (44) or 0.51 mmol/l (25) for stallion. Our data disagree with the notion that seminal plasma of man and bull contain at most traces of ET (12-15); it is safe to attest, however, that boar and stallion contain much more ET in seminal plasma than other species.

Our LC-MS/MS data on GSH (*Table 2*) establish that seminal plasma of boar and stallion contains much less GSH than reported previously (see Introduction). In those reports, a colorimetric assay was used; the same assay also suggested high GSH content in our hands. However, since the LC-MS/MS assay is much more specific than the colorimetric assay (39), we

Table 2. Cross-species analysis of ergothioneine and glutathione in seminal plasma. Samples from 8 indiviuals were analyzed as described in Methods.

Analyte (µmol/l)	Species	#1	#2	#3	#4	#5	#6	#7	#8	Mean	SEM	Median
ET	Boar	252	252	454	540	600	701	774	1081	582	98	**570**
	Stallion	93	168	369	397	416	586	608	783	428	81	**407**
	Human	7.0	11	15	18	19	21	23	26	18	2	**19**
	Bull	8.4	10	11	12	13	17	25	29	16	3	**13**
	Rabbit	0.25	0.25	0.40	0.58	0.89	1.8	7.1	9.5	2.6	1.3	**0.74**
GSH	Boar	2.8	3.2	3.5	3.5	4.5	6.4	6.7	7.3	4.7	0.6	**4.0**
	Stallion	2.8	3.5	3.8	4.3	4.4	4.5	4.9	5.1	4.2	0.3	**4.3**
	Human	2.0	2.8	2.9	3.0	3.2	3.2	3.7	4.8	3.2	0.3	**3.1**
	Bull	3.2	3.7	4.0	4.8	5.3	5.6	6.4	7.9	5.1	0.5	**5.0**
	Rabbit	2.9	3.5	3.6	3.7	3.8	4.0	4.6	5.8	4.0	0.3	**3.8**

conclude that the colorimetric assay results overestimate the GSH content. It is a key finding of the present study that in seminal plasma GSH levels do not correlate with ET levels across species. This suggests that the function of ET, at least in this extracellular context, does not depend on the presence of stoichiometric amounts of GSH; multiple oxidation reduction cycles of ET thus seem unlikely.

What is the benefit of having high ET in seminal plasma? Is there anything unique about boar and stallion semen in terms of anti-oxidant enzymes?

Catalase activity in seminal plasma was reported high both for bull (22) and stallion (45). Thus, ET content (high for stallion and low for bull, see above) does not correlate with catalase activity across species. Catalase in sperm cells also does not correlate with ET in seminal plasma, since no activity was seen with cells of boar (46) and bull (22).

Superoxide dismutase (SOD) activity in seminal plasma was high for stallion, but 50-fold lower for boar; bull, ram, and man were intermediate (47). With isolated spermatozoa, SOD activity was similar for boar, stallion, ram and man. Thus, ET content of seminal plasma (see above) does not correlate with SOD activity in seminal plasma or sperm cells across species.

Glutathione (GSH) peroxidases (GPX) are considered key enzymes to destroy not only H_2O_2 (48, 49) but also lipid peroxides (26, 50). There are several reports about GPX activity in seminal plasma and sperm cells, and some of these estimates are plainly conflicting. Measurement of enzyme activity may be inaccurate because of interference by unnoticed compounds (51) or insufficient washing of sperm cells (52). Yet, with a comparison of enzyme activity across several species based on the same preparation and assay, at least the rank order should be correct. It is thus highly interesting that seminal plasma of boar and stallion contained zero GPX acitivity, while obvious activity (in units of nmol of NADPH oxidized min^{-1} mg of $protein^{-1}$) was measured in parallel with seminal plasma of human (16), ram (21), and especially bull (410) (53). As independent confirmation, GPX activity in seminal plasma of boar (0.008) (54) and stallion (1.3) (52) was very low.

With isolated spermatozoa, an analogous trend across several species was observed: by contrast to human, ram, and dog, spermatozoa from boar contained no GPX and no GSH reductase (GR) activity (21). In independent studies, low GPX activity (0.99 (25) and 2.2 (52)) was measured with stallion spermatozoa, and low GPX (and GR) activity (0.21) was measured with boar spermatozoa (46). Recent reports confirm very low GPX activity in boar sperm cells (0.002) (51), seminal plasma (0.006) (51), and fluids of the cauda epididymidis, vesicular and prostate glands (55).

Altogether, boar and stallion may use a special strategy for anti-oxidant protection of PUFA that is based on high levels of ET only outside of sperm cells; here, GPX (and GSH regeneration system) are virtually absent. By contrast, human and bull (and probably others like ram, dog, rat, and mouse) use high levels of GSH plus GPX and GSH regeneration system (GR, NADPH, NADPH-generating enzymes) inside the spermatozoa but little or no ET in seminal plasma. These fundamentally different strategies may be linked to ejaculate volume, which is very large for boar (on average 250 ml) and stallion (70 ml), but small for human (3.5 ml) and bull (4 ml) (12). It may be more effective or robust to provide large volume protection by ET without enzyme involvement. Interestingly, boars and stallions, by contrast to *e.g.* bull and human, produce sperm that is very vulnerable to cold shock or freezing (19, 56). It has to be tested whether keeping boar and stallion sperm cells after centrifugation always in high ET (*e.g.* at 1 mmol/l) is advantageous.

Finally, it can be speculated that the general function of ET in different organs in different species may be related to the function of GPX, *i.e.* prevention of lipid peroxidation. Notably, because of frequent exposure to UV light there is a high risk for lipid peroxidation in the eye of diurnal species; high ET concentrations as reported previously (57, 58) may provide protection.

Conclusions

We have cloned and functionally verified the ergothioneine transporter from pig. Strong and specific expression of this carrier in seminal vesicles fully explains high accumulation of ergothioneine in this gland and hence also in boar seminal plasma. GSH levels in seminal plasma do not correlate with ET levels across species. This suggests that the function of ET, at least in this extracellular context, does not depend on redox cycling with GSH.

Acknowledgements: We thank Simone Kalis, Beatrix Steinrucken, and Regina Baucks for skillful technical assistance, Melanie Simon for procuring boar organs, and Gunilla Martinsson, Gabriele Bullan, Judith Weirich, Anne-Rose Fischer, Mr. Osmers, and Mr. Kolodziej for providing semen samples.

This work was supported by Deutsche Forschungsgemeinschaft (GR 1681/4-1); and Koeln Fortune Program, Faculty of Medicine, University of Cologne (99/2008).

Conflict of interests: None declared.

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Received: May 16, 2011
Accepted: August 17, 2011

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