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Dietary Sources and Antioxidant Effects of Ergothioneine

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Ergothioneine is a native membrane-impermeable thiol compound that is specifically accumulated in cells via the organic cation transporter OCTN1. In humans, OCTN1 and ergothioneine have been implicated in the etiopathogenesis of autoimmune disorders. However, available evidence about dietary sources and the functional role of ergothioneine in human physiology is scarce. Here, we analyzed the ergothioneine content in common foods using liquid chromatography tandem-mass spectrometry. Additionally, we assessed the protective potency of ergothioneine against various oxidative stressors in OCTN1-expressing cells in comparison with the main intracellular thiol antioxidant glutathione by evaluating cell viability with the MTT reduction assay. Only some food contained ergothioneine with highest concentrations detected in specialty mushrooms, kidney, liver, black and red beans, and oat bran. Ergothioneine exhibited cell protection only against copper(II)-induced toxicity but was far less potent than glutathione, indicting that ergothioneine is not involved in the intracellular antioxidant thiol defense system.

KEYWORDS: Ergothioneine; glutathione; thiol; OCTN1; antioxidant effects; food

INTRODUCTION

The native, water-soluble thiol compound L-ergothioneine (2-thioimidazole betaine, ESH) has recently attracted attention because of its identification as the biogenic key substrate of the organic cation transporter OCTN1 (gene symbol: SLC22A4) (*1*). OCTN1 has been implicated as a susceptibly factor in the etiopathology of autoimmune disorders such as rheumatoid arthritis (*2*, *3*) and Crohn's disease (*4*, *5*). Remarkably, we could demonstrate that the OCTN1 gene variant 503F, which is strongly associated with Crohn's disease risk among Caucasian individuals, exhibited an increased transport efficiency and intracellular accumulation of ESH compared with those of the wild-type variant 503L (*6*). Moreover, patients with rheumatoid arthritis showed significantly higher levels of ESH in red blood cells than healthy controls or patients with non-autoimmune inflammatory disorders (*7*).

Current evidence suggests that ESH is synthesized in Actinomycetales bacteria (e.g., *Mycobacterium ssp.*) and in various non-yeast-like fungi (including edible genera of the basidiomycota division such as *Boletus* ssp., and human toxic genera of the ascomycota division such as *Claviceps* ssp. and *Aspergillus* ssp.) (*8*). In contrast, no ESH synthesis could be detected in higher plants or any animal species (*9*). Hence, it has been suggested that the incorporation of ESH in plants may result from the absorption of the ESH produced by microorganisms in the soil via the root system and that it is enriched in animals through ESH-containing plant and animal foods (*9*). However, except from a systematic analysis of mushrooms (*10*) and some anecdotal reports of ESH occurrence in grains (*11*, *12*), so far no information has been available about the ESH content in common foods of Western- or Mediterranean-type diets, which is a prerequisite for estimating dietary intake.

Although the existence of a specific transport system indicates a specific role for ESH in human biology, so far no definite molecular mechanisms have been assigned. Because of its thiol function, ESH has been implicated in the intracellular antioxidant thiol defense system along with the prototypic water-soluble thiol antioxidant glutathione (GSH) (*13*). Various antioxidant activities have been proposed for ESH, involving the removal of cell toxic radical species or the chelating of metal ions (*13*−*24*). However, the biological relevance of these findings is unclear because in preceding studies no account has been taken of the specific intracellular accumulation of ESH via OCTN1-mediation.

The study reported here was conducted to determine the major sources of ESH in the human diet and to assess possible protective effects of ESH against oxidative stress applied to OCTN1-expressing cells in comparison with the established thiol antioxidant GSH.

MATERIALS AND METHODS

Foods and Chemicals. Foods were purchased from local retail markets and major supermarket chains between April and June of 2005. If not otherwise stated, only fresh food, food from mature plants, and products from adult animals were used. Only the edible portions (without peels, seeds, bones, etc.) were used and processed immediately without deep-freezing. Representative samples of each food were obtained by pooling 3−5 equally weighted (at least 10 g) portions of different proveniences or brands. Solvents were from VWR (Darmstadt,

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Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Extraction of ESH from Food. ESH was extracted from food samples by adapting our previously developed method (*25*). The pooled samples were minced for 2 min by UltraTurrax (Janke & Kunkel, Staufen, Germany) as appropriate, and afterward, samples (5−10 g) were homogenized in liquid nitrogen. Then, 1.0 g of the micronized powder was spiked with 50 μg of thiamazole as internal standard, extracted in 10 mL of 4 mmol/L perchloric acid by sonication for 10 min with overhead shaking for 15 min at 40 rpm (Heidolph Instruments, Munich, Germany), and centrifugated at 3,000*g* at 4 °C for 10 min. Supernatants were further cleaned up by ultracentrifugation at 40,000*g* at 20 °C for 30 min. The homogeneous beverages and liquids tested were directly spiked with internal standard (50 μg/mL thiamazole).

LC-MS/MS Quantification of ESH, GSH, and GSSG. Analysis was performed on a triple-quadrupole tandem mass spectrometer (TSQ Quantum, Thermo Electron, Dreieich, Germany) equipped with a thermostated (10 °C) Surveyor autosampler and a thermostated (30 °C) Surveyor HPLC system (Thermo Electron) operating in positive electrospray ionization (ESI^+) mode. Spray voltage was set at 4000 V, and capillary temperature was kept at 350 °C. Nitrogen sheath gas and auxiliary gas pressure were 40 and 4 psi, respectively. Argon collision gas pressure was 1.0 mTorr. Twenty microliter aliquots of samples (liquid nitrogen extracts) were injected onto a 5 μm Aquasil C_{18} column (100 × 3 mm; Thermo Electron) and eluted isocratically at a flow rate of 0.25 mL/min (run time 5.0 min). The mobile phase consisted of 20% (v/v) acetonitrile/0.1% formic acid and 80% (v/v) deionized water/0.1% formic acid. Precursor ion $[M + H]^+ \rightarrow$ product ion transition (single reaction monitoring, SRM) used for the quantification of ESH was *m*/*z* 230 → 127 (collision energy 24 eV). Detection of the internal standard (IS) thiamazole was performed by monitoring the *m*/*z* 115 → 57 transition (collision energy 24 eV). In incubation experiments and for stability assessments, reduced and oxidized glutathione were quantified against thiamazole by monitoring the *m*/*z* 308 → 162 and *m*/*z* 613 → 231 transitions (collision energy 25 eV) in ESI^+-SRM mode as described (*26*).

Validation of the ESH Quantification Method. The intraday precision of the ESH quantification (maximum coefficient of variation of six repeated measurements of six different samples) was 5.9%, the interday precision (maximum coefficient of variation of repeated measurements of six different samples at six days) was 8.8%. All determinations were performed in the linearity range of the calibration function (checked by triplicate determinations of 10 different standard dilutions of pure ESH reference in pure methanol solvent from 50 nmol/L to 500 μmol/L; Mandel test, $r^2 > 0.992$). The detection limit (three times the standard deviation of the measured value of the sample blank divided by the slope of the calibration curve) was 52 nmol/L (12 ng/mL) ESH. Rates of recovery for spiking an ESH-free sample (boiled mashed potatoes) with 100 *n*g/g, 1,000 *n*g/g, and 10,000 *n*g/g ESH were 95.6 ± 4.8, 97.0 ± 3.1, and 101.7 ± 2.7%, respectively ($n = 6$).

Cloning and Cell Culture. The cDNA encoding the organic cation transporter OCTN1 (GenBank accession no. NM_003059) was amplified by RT-PCR from human kidney total RNA and subcloned into the eukaryotic expression vector pcDNA3. Human embryonic kidney cells (HEK-293) lacking endogenous OCTN1 mRNA expression were stably transfected with the OCTN1 vector and the empty vector, respectively, and selected with Geneticin as described (*26*). HEK-293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen).

Transport Experiments. The uptake of ESH and GSH was assessed as described (*26*). Briefly, cells were seeded onto polystyrene dishes of 60 mm diameter (1,000,000 cells per dish) and grown to at least 80% confluence. Prior to the addition of ESH or GSH (at concentrations of 10 μmol/L), the culture medium was replaced by 3 mL of HEPES-modified Krebs buffer (140.0 mmol/L NaCl, 5.0 mmol/L KCl, 2.0 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 10.0 mmol/L HEPES, and 5.0 mmol/L D-glucose, adjusted to pH 7.40), and cells were left to equilibrate for 30 min at 37 °C. Uptake was terminated after indicated times (1 min or 60 min) by washing cells three times with 3 mL of ice-cold buffer. Cells were lysed with 1 mL of 4 mmol/L perchloric acid and lysates subjected to ESI-LC-MS/MS quantification. Cellular protein content was determined after solubilization of perchloric acid precipitates with 1 mL of 0.1 mmol/L NaOH using the bicinchoninic assay (BCA Protein Assay Kit, Pierce, Rockford, IL). Uptake in OCTN1-transfected cells and control cells was reported in nmol/mg protein minus the baseline content of ESH or GSH.

MTT Reduction Assay. Cell viability was assessed in HEK-293 cells by MTT test as described (*26*). Briefly, cells were seeded onto 96-well plates (10,000 cells per well in 100 μL medium), and, after 48−72 h of culture (80−90% confluence), incubated for 60 min with ESH or GSH. After washing three times with PBS and exchanging the medium to remove all extracellular ESH or GSH, cells were subjected to various inducers of oxidative stress at their approximate 50% toxic concentrations (TC_{50}) for 48 h. Specifically, cell damage was induced by hydrogen peroxide (H_2O_2, 100 μmol/L); by free radicals generated from thermal decomposition of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, 5 mmol/L), which induce lipid peroxidation and endogenous production of reactive oxygen species (ROS) (*27*, *28*); by covalent modification of biomolecules with 4-hydroxy-2-nonenal (HNE, 100 μmol/L), a toxic intermediate produced during lipid peroxidation (*29*); by doxorubicin (100 μmol/L)-induced formation of superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($HO^{\bullet}$), and semiquinone radicals (*30*); by the formation of hydroxyl radicals in a Fenton type reaction between Fe^{2+} (50 μmol/L) and ascorbic acid (500 μmol/L) or with Cu^{2+} (50 μmol/L); and by sodium nitrite (5 mmol/L)-induced oxidative and nitrosative stress involving the generation of reactive nirosyl species (RNOS), such as nitric oxide (NO) and nitrosyl cations (NO^+) (*31*).

To indicate metabolic activity, cells were treated with MTT reagent (15 μL of 1 mg/mL stock solution per well) for 1 h, and after drying culture plates for 1 h at 37 °C, the resulting formazan dye was extracted with isopropanol/HCl (100 μL 0.04 mol/L per well), and absorbance was determined spectrophotometrically at 568 nm. The results were in agreement with the data obtained by counting viable cells in the Neubauer chamber according to the tryptan blue (0.04% (w/v)) exclusion method (data not shown).

Detection of Disulfides and Metal Ion Complexes of ESH. The possible formation of ESH disulfides and metal ion complexes of ESH was assessed in methanolic HEK-293 cell extracts by ESI^+-MS/MS monitoring specific collision-induced dissociation (CID) spectra of the respective precursor ions, $[M + H]^+$, at 1.5 mTorr collision gas pressure and 20 eV collision energy. CID scans were run at the precursor ion mass *m*/*z* 457 corresponding to the ESH disulfide ESSE, at *m*/*z* 535 corresponding to the mixed disulfide of ESH with glutathione ESSG, at *m*/*z* 292 corresponding to the 1:1 complex of ESH with copper(I or II) ES-Cu, at *m*/*z* 520 corresponding to the 2:1 complex of ESH with copper(I or II) $(ES)_2$-Cu, and at *m*/*z* 285 and 513 corresponding to the 1:1 and 2:1 complexes with iron(II or III).

ESSE was generated by reacting equimolar concentrations (50 μmol/L) of ESH and Cu(II)SO_4 at 25 °C for 30 min in 0.1 mol/L formic acid and extracting with methanol.

Statistical Analysis. The statistical significance of differences between two treatments was evaluated by the unpaired two-tailed *t*-test or Mann−Whitney U-test, differences between more than two treatments by one-way ANOVA or the Kruskal−Wallis test, as applicable, using SigmaStat 3.0 software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

ESH Content in Common Foods. A broad selection of different food groups was tested for its content of ESH by using a validated LC-MS/MS quantification technique (**Table 1**). The highest ESH concentrations were found in the specialty mushrooms *Boletus edulis* and *Pleurotus ostreatus*. Further food sources rich in ESH were certain meat products (liver and kidney) and specialty plant products (black and red beans and oat bran). In contrast, the majority of commonly consumed foods contained no ESH or only traces of ESH. Concentrations of ESH showed a high variability between different food groups but also within a certain food group. Moreover, no species

Table 1. Ergothioneine Concentrations in Selected Foods and Beverages[a]

food groups with selected food items	ergothioneine (mg/kg wet weight or mg/L)
mushrooms	
- king bolete (*Boletus* edulis)	528.14 (457.92/588.63)
- oyster mushroom (*Pleurotus ostreatus)*	118.91 (97.35/123.42)
- portabella mushroom (*Agaricus bisporus*, brown strain)	0.93 (0.86/1.05)
- button mushroom (*Agaricus bisporus*, white strain)	0.46 (0.38/0.55)
- chanterelle (*Cantharellus cibarius*)	0.06 (0.04/0.09)
- shiitake mushroom (*Lentinula edodes*), baker's yeast (*Saccharomyces cerevisiae*)	b.l.d.
meats, sausages	
- chicken, liver	10.78 (9.73/11.42)
- pork, liver	8.71 (8.06/9.84)
- pork, kidney	7.66 (6.86/8.33)
- pork, loin fillet	1.68 (1.54/1.82)
- beef, loin steak	1.33 (1.09/1.43)
- lamb, loin fillet	1.20 (0.97/1.38)
- chicken, breast	1.15 (1.02/1.24)
- ham	1.12 (0.88/1.31)
- blood sausage (from pork)	1.08 (0.93/1.18)
- liver sausage (from pork)	1.03 (0.79/1.26)
- salami (from pork)	0.51 (0.31/0.64)
- aspic (from pork)	0.46 (0.29/0.53)
fish and seafood	
- trout	0.07 (0.05/0.07)
- salmon, tuna fish, cutlefish, halibut, gamba, shrimp, oyster mussel	b.l.d.
dairy products, eggs	
- egg yolk	0.68 (0.62/0.74)
- egg white	0.38 (0.33/0.41)
- milk, butter, cheese (camembert, edam, mozarella, feta, cottage), yogurt	b.l.d.
grains and grain products, seeds, nuts	
- oat bran	4.41 (4.01/5.87)
- pumpkin seed	1.49 (1.34/1.62)
- wheat bran	0.84 (0.74/0.98)
- wheat germ	0.63 (0.46/0.85)
- spelt	0.61 (0.51/0.77)
- whole grain wheat bread	0.53 (0.44/0.58)
- whole grain rye bread	0.47 (0.27/0.67)
- brown rice	0.04 (0.04/0.04)
- wheat flour (refined), rye flour(refined), barley flour(refined), cornmeal (refined), oatmeal (refined), rye bread, white bread, biscuit, durum wheat, sorghum, white rice, walnut, hazlenut, peanut, chestnut, coconut	b.l.d.
vegetables, fruits	
- black turtle bean	13.49 (12.51/15.68)
- red kidney bean	4.52 (4.17/5.21)
- garlic	3.11 (2.57/3.85)
- broccoli	0.24 (0.18/0.28)
- onion	0.23 (0.13/0.34)
- spinach	0.11 (0.06/0.14)
- celery	0.08 (0.05/0.10)
- green beans, white beans, green peas, lentils, tomato, potato, carrot, beetroot, red and green bell pepper, cabbage turnip, horseradish, white and green asparagus, cauliflower, Brussel sprouts, eggplant, lettuce, soja beans, avocado, capers (canned), green and black olive (canned), parsley, apple, pear, apricot, plum, orange, lemon, lime, grapefruit, red and white grape, banana, pineapple, mango, papaya, cherry, strawberry, raspberry, blackberry, blueberry, fig	b.l.d.
beverages	
- beer	0.02 (b.l.d./0.04)
- coffea, black tea, green tea, cocoa, cola, red wine, white wine, purple grape juice, orange juice, apple juice, cherry juice, tomato juice	b.l.d.

[a] b.l.d., below limit of detection. The botanical names of mushrooms are given in italics. The ESH values of each food are the means from three to five equally pooled samples of different proveniences or brands. The concentration range with minimum and maximum values of individual samples is given in brackets.

consistency was observed. For example, black and red bean varieties of *Phaseolus vulgaris* had a high ESH content, whereas green and white strains contained no ESH. The occurrence of ESH in whole grain products but not in refined grains indicates that ESH is incorporated only in the outer endosperm layer and not in the starch granules.

The high levels of ESH that we found in *Pleurotus ostreatus* are in keeping with a recent analysis of ESH in cultivated

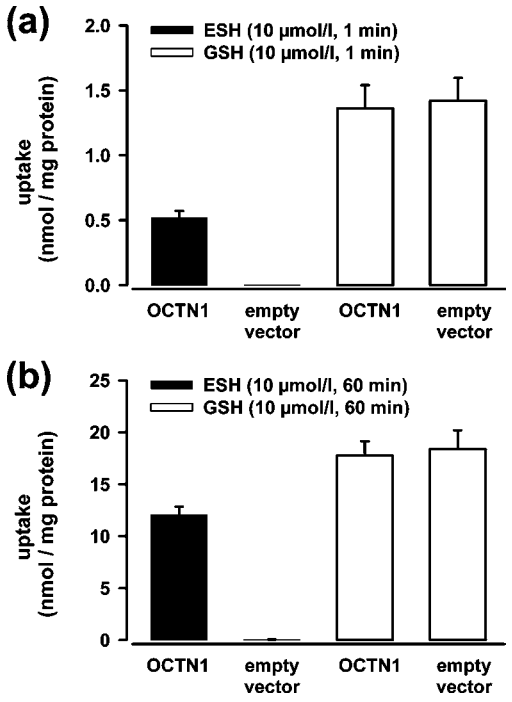

Figure 1. Uptake of ergothioneine (ESH) and glutathione (GSH) into HEK-293 cells transfected with OCTN1 or empty vector. (**a**) Intracellular accumulation after 1 min of incubation; (**b**) after 60 min incubation with 10 μmol/L ESH or GSH. Baseline concentrations of ESH and ESH disulfide (ESSE) in OCTN1 and control cells were below the detection limit. Baseline concentrations of GSH in OCTN1 and control cells were 5.7 ± 0.4 and 5.6 ± 0.3 nmol/mg protein, respectively; baseline concentrations of GSH disulfide (GSSG) in OCTN1 and control cells were 1.0 ± 0.2 and 0.9 ± 0.2 nmol/mg protein, respectively. Uptake of ESH was significantly different between OCTN1 and control cells after 1 and 60 min of incubation ($P < 0.001$); GSH uptake was not different between OCTN1 and control cells ($P > 0.5$). Results represent the mean and SEM of $n = 3$ independent experiments each.

mushrooms of Dubost et al. (*10*) using a HPLC-UV detection method. However, the lack of ESH in pooled samples ($n = 4$) of *Lentinus edodes* and the at least 10-fold lower ESH levels we detected in white and brown strains of *Agaricus bisporus* ($n = 5$) contrasts with the results of Dubost et al. (*10*), who determined ESH concentrations of 400−2000 mg/kg dry weight in these mushroom species. The discordance may be due to the influence of different cultural factors (e.g., different supply with histidine precursor for ESH biosynthesis) (*32*) or the harvesting of mushrooms in different maturation stages of the fungal life cycle. Of note, it has been reported that only the fungal spores and not the mycelium has the capability to synthesize ESH, indicating that the ESH content of the spore-bearing or spore-failing fruitbodies may differ considerably (*33*−*35*).

Specific Transport of ESH via OCTN1. No evidence has been found for the synthesis of ESH in any higher plants or animals (*35*, *36*). However, OCTN1-independent ESH uptake across the cell membrane through passive diffusion was negligible (**Figure 1**). Hence, it is likely that any significant incorporation of ESH observed in non-fungal food samples required transporter-mediated intracellular uptake. Considering the high specificity of OCTN1 for translocating ESH (*1*) and the evolutionary conservation of OCTN1 (*37*), it appears that a prerequisite for ESH accumulation in a certain animal or plant organ is the expression of an orthologue to the human OCTN1 transporter.

Previously conducted food surveys of other biogenic thiols, in particular of the predominant intracellular antioxidant GSH, had established that GSH occurred in most common foods (*38*). But there was no indication of a consistent (negative or positive) correlation between levels of GSH and ESH. For example, very high GSH levels but no ESH were found in asparagus, avocado, or papaya, whereas king bolete showed a very high GSH and ESH content (*39*), and broccoli contained very low GSH and ESH levels (*38*). This is in accord with the finding that cellular uptake of GSH was completely independent of the expression of OCTN1 (**Figure 1**) and is also in agreement with the observation that ESH levels in red blood cells were independent of respective GSH levels (*7*).

In humans, ESH was detected in OCTN1-carrying tissues or cell lineages derived from OCTN1-expressing progenitors (*1*, *40*), specifically in cells of the myeloic lineage such as monocytes or erythrocytes at mean concentrations of 10−15 μmol/L (*6*), whereas no ESH was found in the extracellular fluid (*7*). Upon preloading for 60 min, HEK-293 cells with expression of OCTN1 exhibited similar levels of ESH and GSH accumulation, whereas only GSH was significantly incorporated in HEK-293 cells lacking OCTN1 (**Figure 1**).

Protection of ESH and GSH Against Oxidative Cell Stress. The present study assessed the protective potency of physiological concentrations of intracellular ESH in relation to GSH against oxidative stress. For adequate comparison of antioxidant efficacy before exposure to oxidative stressors, HEK-293 cells were preloaded to similar intracellular levels of ESH and GSH by incubation for 60 min (**Figures 2** and **3**).

ESH exhibited protective effects only against the Cu^{2+}-induced cytotoxicity in OCTN1-expressing HEK-293 cells but not in HEK-293 cells lacking OCTN1 and thus intracellular ESH accumulation (**Figure 3**). In contrast, GSH pretreatment could prevent the toxic effects of all stressors investigated, and in accordance with results from the uptake experiments, its protective potency was independent of OCTN1 expression (**Figures 2** and **3**). Concomitant preloading of OCTN1-expressing cells with ESH and GSH did not change the protective efficacy of GSH (**Figure 2**); the effects of ESH and GSH against Cu^{2+}-mediated cell death were additive (**Figure 3**). This argues against a redox coupling between ESH and GSH under physiological conditions in the way that constant levels of reduced ESH are maintained by the steady oxidation of GSH to GSSG, as has been suggested previously (*13*, *41*). Further support of the failing interaction between ESH and GSH came from the finding that ESH was stable at physiological pH, that auto-oxidation of GSH was not accelerated by the presence of ESH, and that no ESH disulfides or mixed ESH/GSH disulfides were detected (**Figure 4**). In addition, enzymatic metabolism of GSH was found to be unaffected by ESH (*42*).

The apparent ineffectiveness of ESH against free radical species is in contrast to several previous observations (*13*−*23*), in particular to a report indicating higher radical-scavenging capacities of ESH compared with that of GSH (*24*). These differences from our findings may be attributed to different experimental models. The results derived from controlled reactions in cell-free systems (*13*−*16*, *24*) may not apply to complex interactions in cellular systems. In cell toxicity assays (*17*, *19*, *21*, *23*), ESH concentrations were higher or incubation times were longer than those in our tests, and the ESH doses applied in animal experiments (*18*, *20*) by far exceeded the possible dietary intake. Accordingly, at lower concentrations, such as those used in our study, ESH failed to protect rabbit hearts from oxidative injury or prevent GSH oxidation (*43*).

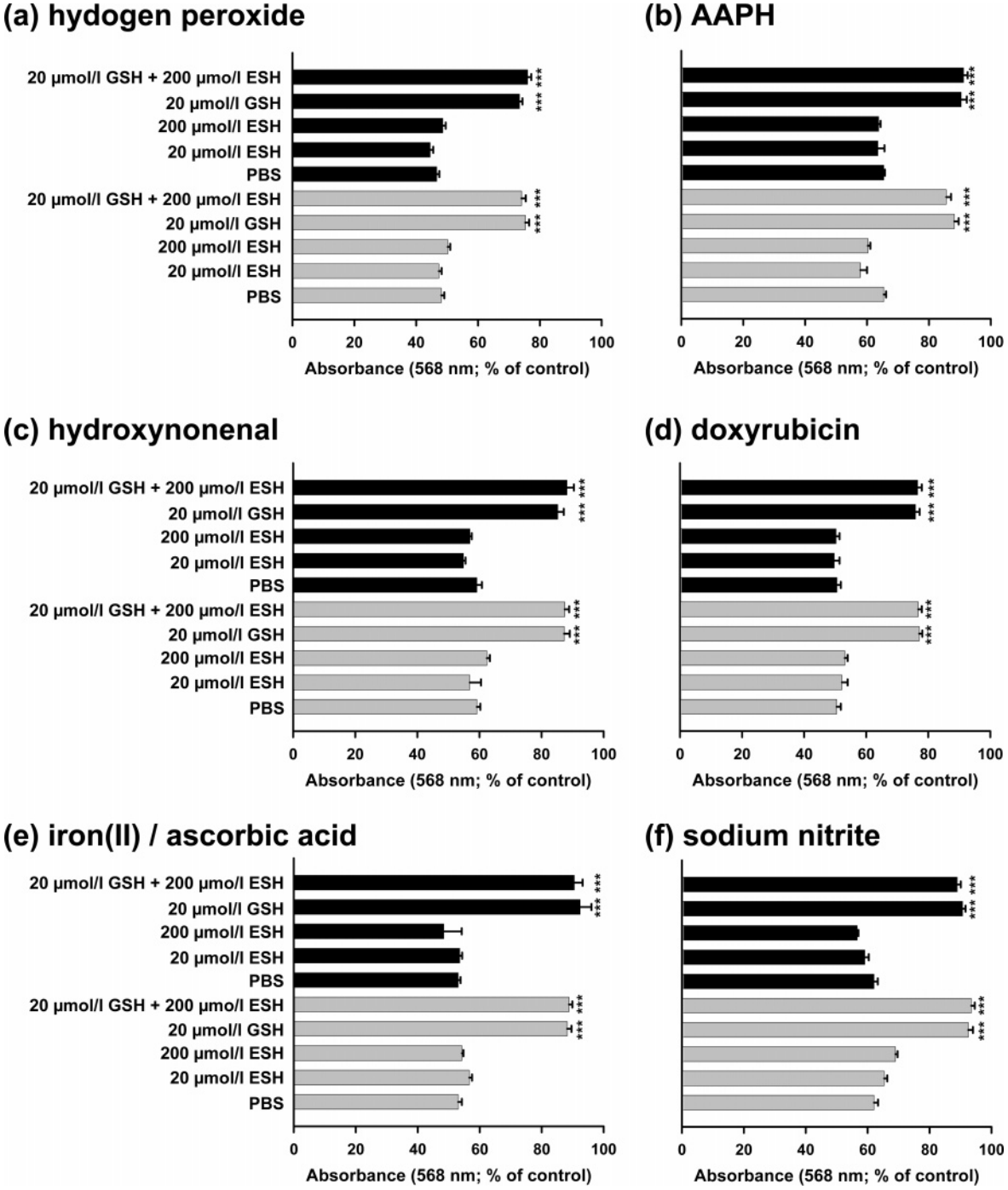

Figure 2. Protective effects of ergothioneine (ESH) and glutathione (GSH) against cell stressors. MTT cell viability assay of HEK-293 cells treated for 48 h with various stressors at the approximate 50% toxic concentration (TC_{50}) after 60 min of preincubation with ESH or GSH or coincubation with ESH and GSH at indicated concentrations. The resulting formazan formation was determined by absorbance at 568 nm. Gray bars, HEK-293 cells transfected with OCTN1; black bars, HEK-293 cells transfected with empty expression vector: (**a**) hydrogen peroxide (100 μmol/L); (**b**) 2,2′-azobis(2-amidinopropane)-dihydrochloride (5 mmol/L); (**c**) 4-hydroxy-2-nonenal (100 μmol/L); (**d**) doxorubicin (100 μmol/L); (**e**) iron(II) sulfate (50 μmol/L) and ascorbic acid (500 μmol/L); and (**f**) sodium nitrite (5 mmol/L). Data represent the mean and SEM of 8–16 independent experiments each. ***$P < 0.001$ indicates a significant difference compared to that of the phosphate-buffered saline (PBS) control as determined by one-way ANOVA with Holm–Sidak correction ($\alpha = 0.05$).

However, the principal difference from our controlled investigation of intracellular ESH activity is that in most incubation studies cells or tissues were exposed to oxidative stressors in the presence of extracellular ESH, and none of these studies controlled intracellular ESH uptake, although the lack of ESH in extracellular space (*7*) and the rapid influx clearance via OCTN1 (*6*) imply that ESH acts only intracellularly in vivo.

As opposed to ESH, the high antioxidant efficacy of GSH is established and also explains the protective activities of GSH in our assay. GSH rapidly detoxifies free radical species by one-electron transfer and oxidation to GSSG ($E_0' = -250$ mV for the ESH/ESSE redox couple) (*36*). Because of the nucleophilic properties of the aliphatic thiol group, GSH reacts with HNE and related α,β-unsaturated carbonyl functions by Michael addition (*29*) and forms stable complexes with transition metal ions such as Cu(II), Zn(II), Co(II), Ni(II), Cd(II), and Fe(II,III). GSH is capable of detoxifying intracellular Cu(II) by two different mechanisms: one-electron reduction of Cu(II) to Cu(I)

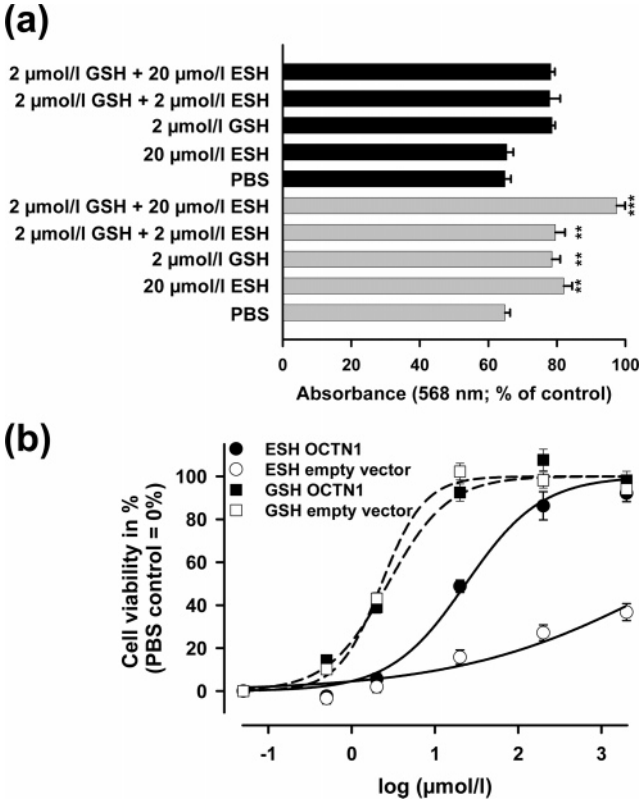

Figure 3. Influence of ergothioneine (ESH) and glutathione (GSH) on copper(II)-induced cell toxicity. (**a**) MTT viability assay of HEK-293 cells treated for 48 h with copper(II) sulfate at the approximate TC_{50} (50 μmol/L) after 60 min of preincubation with ESH or GSH or coincubation with ESH and GSH at indicated concentrations. Data represent the mean and SEM of 16 independent experiments each. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to those of phosphate-buffered saline (PBS) controls as determined by one-way ANOVA with Holm–Sidak correction ($\alpha = 0.05$). (**b**) Concentration–response curves (sigmoidal 3-parametric regression) of ESH and GSH protection against copper(II) toxicity were assessed by MTT assay with the PBS control set to 0% viability. The EC_{50} (95% CI) obtained for ESH was 23.5 (14.6 to 38.1) μmol/L in OCTN1-transfected cells and >2000 μmol/L in OCTN1-free cells. The EC_{50} (95% CI) obtained for GSH was 2.7 (1.8 to 4.1) μmol/L in OCTN1-transfected cells and 2.3 (1.7 to 3.1) μmol/L in OCTN1-free cells.

with concomitant formation of GSSG and chelation of Cu(II) or Cu(I) with GSH or GSSG (*44*, *45*). Moreover, GSH serves as a substrate for diverse antioxidant and detoxifying enzymes such as GSH peroxidase or GSH transferases, and the reduced thiol form is steadily regenerated by NAD(P)H-dependent GSH reductase. On the basis of these chemical and biochemical properties and the high intracellular GSH concentrations found in human tissues (up to 10 mmol/L), GSH is the predominant intracellular antioxidant defense mechanism (*22*).

Conversely, the low radical scavenging capacity of ESH can be explained by its extremely low reducing potency ($E_0' = -60$ mV for the ESH/ESSE redox couple) (*36*) and the weak nucleophilic properties of the aromatic thiol group, resulting from the thiol/thione tautomerism of ESH that exists almost completely in the thione form under physiological conditions (*36*). Thus, we observed the generation of ESH disulfide only under artificial experimental conditions at low pH (**Figure 5**), but no stable ESSE species were formed in physiological buffer solution (pH 7.4), and no ESSE could be detected in any of the extracts obtained from ESH-pretreated OCTN1-expressing HEK-293 cells or in native erythrocytes (*7*). The efficacy of

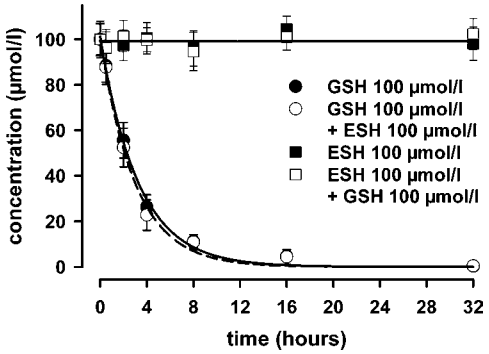

Figure 4. Auto-oxidation stability of ergothioneine (ESH) and glutathione (GSH) during 32 h of incubation in phosphate-buffered saline (PBS) at 37 °C. ESH concentrations remained unchanged. GSH concentrations decayed exponentially with a half-life $t_{1/2}$ of 2.1 h (95% CI; 1.8 to 2.6). Degradation of GSH was not affected by the presence of ESH ($t_{1/2} = 1.9$ h (95% CI; 1.6 to 2.5)). The formation of the ESH disulfide (ESSE) or the mixed disulfide of ESH and GSH (ESSG) was not observed at any of the time-points of incubation by monitoring the CID scans of the parent ion masses $[M + H]^+$ at *m*/*z* 457 and *m*/*z* 535. Data are the means and SEM of $n = 3$ independent experiments.

ESH in diminishing copper(II)-induced toxicity probably lies in the formation of a 2:1 chelate complex that could be isolated from OCTN1-transfected HEK-293 cells after treatment with ESH and copper(II) (**Figure 5**). Also, the formation of a stable $(ES)_2$−Cu(II) complex by applying a chemical titration technique has been proposed from earlier studies (*46*). Conversely, the lack of ESH protection against iron(II)-mediated cell death (**Figure 2**) is in accord with the failure to detect 1:1 or 2:1 complexes between ESH and iron in our study and is also in line with previous reports that observed no chelating potential of ESH for iron ions (*13*). Though ESH was shown to be principally capable of inhibiting toxic Cu(II) effects in OCTN1-expressing cells or their congeners, it appears questionable whether this action significantly accounts for cell protection in vivo. Compared with GSH, ESH exhibited a 10-fold lower efficacy against copper(II) toxicity (**Figure 3**), whereas intracellular ESH concentrations are around 100-fold lower than GSH (*7*). Even under conditions with enhanced oxidative stress with depletion of GSH concentrations such as rheumatoid arthritis, chelating potency of remaining GSH levels (*7*) as well as the chelating capability of GSSG (*44*) by far exceed the copper-detoxifying potential of ESH. Still, instead of protecting against toxic levels of copper(II), ESH may have a physiological role in intracellular copper transfer to metallothioneins, the cysteine-rich metal-binding proteins serving as cellular copper donators to other proteins such as superoxide dismutase or ceruloplasmin (*47*). Although the majority of the cytoplasmatic copper is initially complexed by GSH before incorporation in metallothioneins (*45*), in OCTN1-expressing cells ESH may serve as the preferential chelator and temporary storage form for the copper fraction at the membrane because ESH has been reported to accumulate by nonbonded association on the inner cell membrane surface (*48*). However, this assumption requires further testing.

Collectively, our data suggest that ESH does not significantly contribute to the antioxidant thiol defense system in vivo. In search of different biological activities of ESH, we have demonstrated that ESH stimulated proliferation in human intestinal mucosa cells with native OCTN1 expression, whereas GSH inhibited proliferation in an antioxidant-type fashion (*6*). Hence, rather than antioxidant activities, proliferative or anti-apoptotic effects may constitute the functional role of ESH, but

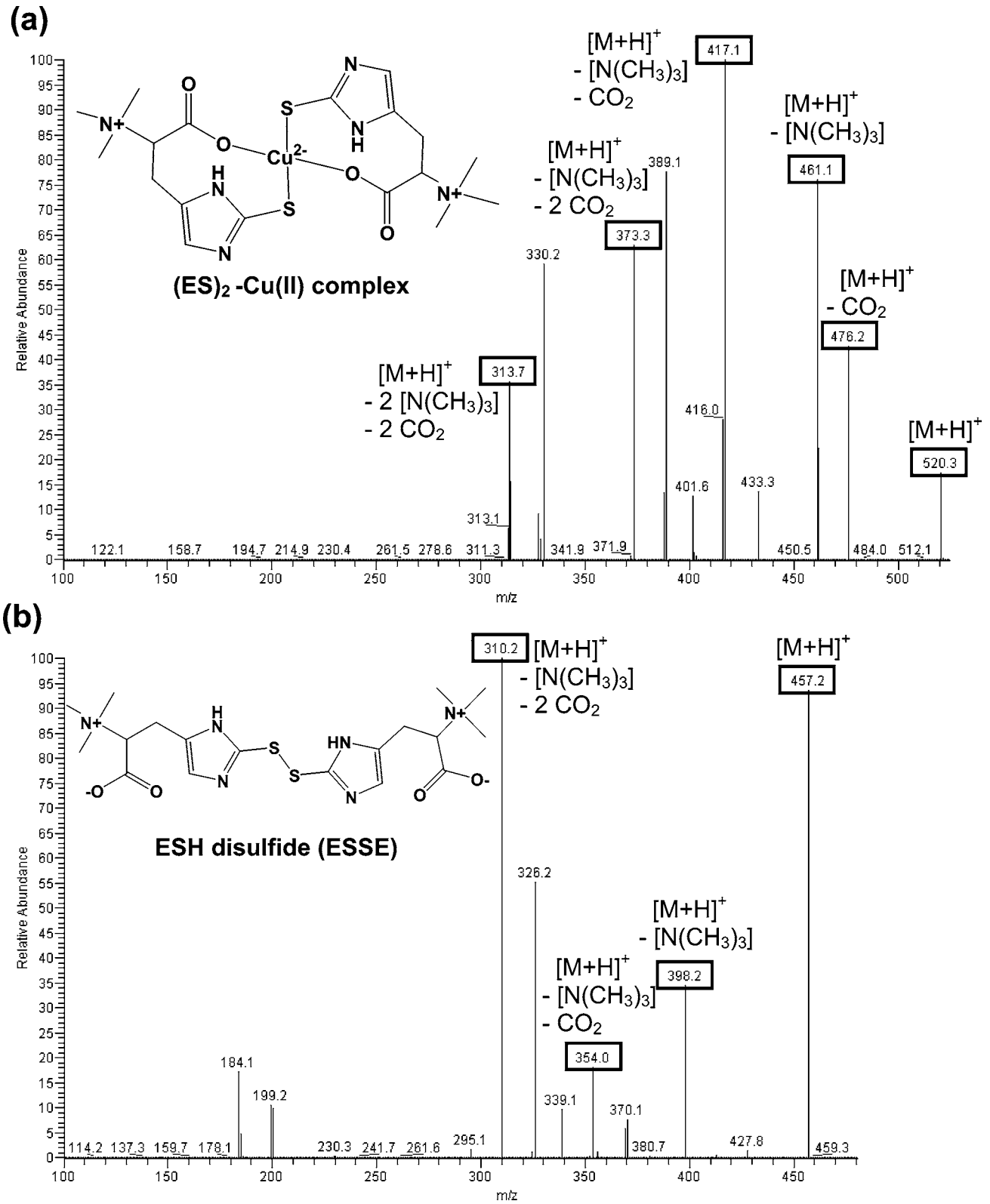

Figure 5. Collision induced dissociation (CID) spectra obtained by positive electrospray tandem mass spectrometry. (**a**) 2:1 complex of ESH with copper-(II); methanolic extract of OCTN1-transfected cells after 60 min of pretreatment with 10 μmol/L ESH and subsequent 48 h of incubation with 50 μmol/L copper(II) sulfate. (**b**) ESH disulfide; methanolic extract of the reaction between 50 μmol/L ESH and 50 μmol/L copper(II) sulfate in 0.1 mmol/L formic acid for 30 min at 25 °C. Characteristic fragment losses and proposed chemical structures are depicted.

the exact molecular mechanism of ESH and its contribution to human health and disease have to be explored in subsequent trials.

Moreover, because no ESH deficiency disorders have been reported so far, there is no substantial basis to recommend an increased intake of ESH rich food or a specific ESH supplementation. Conversely, enhanced ESH accumulation in certain immunological disorders indicates that dietary ESH restriction could be rational advise (e.g., by mitigating disease progression) under pathophysiological conditions such as rheumatoid arthritis.

Conclusions. Certain mushrooms (bolete and oyster), some meat products (liver and kidney), and some plant products (black

and red beans and oat bran) are major dietary sources of ESH, whereas more common foods of the typical Western or Mediterranean diets contain no ESH or only traces of ESH. ESH effectively accumulates in cells carrying the OCTN1 transporter and protects from copper-induced toxicity by complex formation, but its antioxidant potency is much lower compared with that of the predominant intracellular thiol GSH, questioning whether ESH at the concentrations present in human tissues significantly contributes to the antioxidant thiol defense system.

ABBREVIATIONS USED

ESH, ergothioneine; ESSE, ergothioneine disulfide; GSH, glutathione; GSSG, glutathione disulfide; EC_{50}, 50% effective concentration; TC_{50}, 50% toxic concentration.

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