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Review

Ergothioneine; antioxidant potential, physiological function and role in disease ☆

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ABSTRACT

Since its discovery, the unique properties of the naturally occurring amino acid, L-ergothioneine (EGT; 2-mercaptohistidine trimethylbetaine), have intrigued researchers for more than a century. This widely distributed thione is only known to be synthesized by non-yeast fungi, mycobacteria and cyanobacteria but accumulates in higher organisms at up to millimolar levels *via* an organic cation transporter (OCTN1). The physiological role of EGT has yet to be established. Numerous *in vitro* assays have demonstrated the antioxidant and cytoprotective capabilities of EGT against a wide range of cellular stressors, but an antioxidant role has yet to be fully verified *in vivo*. Nevertheless the accumulation, tissue distribution and scavenging properties, all highlight the potential for EGT to function as a physiological antioxidant. This article reviews our current state of knowledge. This article is part of a Special Issue entitled: Antioxidants and Antioxidant Treatment in Disease.

1. Introduction

In 1909, Charles Tanret, whilst investigating the ergot fungus, *Claviceps purpurea*, which devastated rye grain, isolated a unique crystalline sulfur-containing compound, later identified as a trimethylbetaine of 2-thiol-L-histidine [1]. This water soluble thiol, named ergothioneine (EGT) after the ergot fungus, is known to be synthesized only by non-yeast fungi, certain bacteria belonging to the order *Actinomycetales* and recently also by cyanobacteria [2–4]. Yet despite the inability to be synthesized by higher animals, EGT was soon identified in the blood of pigs by two independent groups, Hunter et al. and Benedict et al., as a confounder of uric acid measurements [5,6]. Since then there have been hundreds of reports on EGT regarding its synthesis, abundance in nature, chemical properties, possible physiological functions and potential role as an antioxidant. Many reports have shown that EGT may act as a cytoprotectant *in vitro*, but its role *in vivo* is uncertain. This review aims to shed light on the unique properties of EGT and evaluate the evidence for its potential roles *in vivo*.

1.1. Properties of ergothioneine

In solution, EGT exists as a tautomer between its thiol and thione forms (Fig. 1), however at physiological pH it exists predominantly as the thione. Hence EGT only reacts with some sulfhydryl reagents such as iodoacetamide, but not with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) [7,8]. Another unique attribute of EGT is that the standard redox potential of the thiol–disulfide couple is −0.06 V in contrast to other naturally occurring thiols which typically range between −0.2 and −0.32 V [9]. In combination, these two properties confer greater stability under physiological conditions, hence EGT does not readily undergo auto-oxidation as rapidly as other 'antioxidant' thiols such as glutathione (GSH) which can generate free radicals in the process [10]. For example, the oxidation and generation of H_2O_2 were observed by a wide range of thiols in cell culture media, whereas no significant decrease in EGT was observed following incubation in cell culture media [11] for 4 days at room temperature (unpublished). This stability also accounts for EGT's slow degradation and resistance to disulfide formation [12,13]. Formation of the EGT disulfide has been demonstrated at very low pH in the presence of copper or H_2O_2, but not in neutral or alkaline solutions [14].

1.2. Chemistry and synthesis

EGT is a colorless, odorless compound of relative molecular mass 229.30 and a relatively high solubility in aqueous solutions (aqueous solubility limit of 0.9 M at 25 °C). Naturally occurring EGT possesses the L configuration around the α carbon with optical rotation of $[\alpha]_D$ +116° [15]. The biosynthesis of EGT proceeds from L-histidine through the intermediary precursor hercynine and then to EGT through the incorporation of sulfur derived from cysteine, as determined

Abbreviations: EGT, ergothioneine; GSH, glutathione; GPx, glutathione peroxidase; OCT, organic cation transporter (N1, N2); ROS, reactive oxygen species; TNF, tumor necrosis factor; GSSG, oxidized glutathione; IL, interleukin (1β, 6, 8); RA, rheumatoid arthritis; CD, Crohn's disease; IR, ischemia/reperfusion

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by studies using radiolabelled sulfur [16–18]. The enzymes involved in this conversion are still not known. Early attempts to synthesize EGT chemically resulted in racemization of the product [19]. The synthesis of enantiomerically pure L-(+)-EGT was first described by Xu and Yadan [20].

2. Uptake and distribution

2.1. Abundance in foods and tissues

Despite apparently being exclusively synthesized in fungi, cyanobacteria and mycobacteria (no evidence to date exists for the direct biosynthesis of EGT in animals and higher plants), EGT is ubiquitously present in most cells and tissues of plants and mammals [13]. A wide array of foods contain trace amounts of EGT, however some foods were found to possess particularly high levels including certain species of mushrooms (where it can be synthesized), black and red beans, red meat, liver, kidney, and grains [21].

In humans, EGT has been shown to accumulate in various cells and tissues at high concentrations (100 μM to 2 mM), most abundantly in erythrocytes, bone marrow, liver, kidney, seminal fluid and the lens and cornea of eyes [22–25]. Early on it was recognized that EGT was obtained through dietary means. For example, grain-fed pigs showed very high levels of EGT in blood whereas pigs fed on a purified casein diet had no detectable EGT [26]. Furthermore, synthetic [^{35}S]-EGT fed to rats was observed to enter blood and tissues [27]. Metabolic turnover rate for EGT was also shown to be relatively slow with no appreciable drop in blood and liver levels seen in rats fasted for 1 week [12]. Likewise, this unique pharmacokinetic property of EGT was observed in mice which maintained blood levels of ^{3}H-EGT for more than 14 days following a single oral administration [28]. This high accumulation and slow turnover of EGT have been attributed in part to the renal reabsorption and low urinary excretion of ^{3}H-EGT [28].

An earlier study revealed gender differences in blood EGT accumulation, likely associated with testosterone levels, since male and female rats injected with testosterone revealed EGT levels double that of untreated females [29]. However these results have yet to be replicated. A study in humans found that blood EGT levels increase to 1.5–2.0 mg/100 ml from 1 to 10 years of age (y/o) peaking at around 3.7 mg/100 ml by 18 y/o and gradually declining to 2.3–3.0 mg/100 ml between 19 and 50 y/o and plateauing beyond 51+y/o with an average of 2.8 mg/100 ml [30]. Interestingly, similar age-related declines have been observed in the total activity of the antioxidant enzyme, glutathione peroxidase (GPx) [31,32].

However even though EGT has a relatively wide tissue distribution it is not currently considered an essential dietary component and there are no reports of symptoms due to its deficiency. Likewise, rats fed on an EGT-depleted diet did not reveal any obvious physiological changes, nor were there any abnormalities in reproduction, delivery and growth rate of the second generation animals [22,33].

2.2. Analysis of ergothioneine

Since its discovery, the methods employed to quantify EGT in biological samples have constantly evolved to increase specificity and sensitivity. Many earlier methods harnessed chemical assays and colorimetric techniques to isolate and identify EGT from blood [34–38]. However these techniques lacked specificity and hence often gave erroneously high values of EGT in blood [13]. Mayumi et al. [12] first harnessed liquid chromatography to analyze EGT in rat blood and tissue and later Dubost and colleagues [39] applied liquid chromatography-mass spectroscopy for the quantification of EGT in mushrooms. The lack of specificity and sensitivity of certain earlier analytical methods as highlighted in Table 1, may raise doubts as to the reliability of these early estimates of EGT in animal and plant material, therefore such measurements will probably need to be verified using current techniques. Furthermore, a portion of tissue EGT has been found to be associated with proteins, hence previous reports may be inaccurate due to exclusion of protein-bound EGT [33]. This highlights the need for standardized and calibrated, chemically robust, methods to accurately analyze EGT.

2.3. Ergothioneine transport

Even though EGT is present in many cells, plasma membranes seem to be intrinsically impermeable to EGT and hence a mechanism for uptake and accumulation in cells must exist [27]. Furthermore, cellular uptake of EGT exhibits significant temperature and Na^{+}-dependence and is saturable, further supporting involvement of carrier-mediated transport [40]. The gene *SLC22A4* encodes an integral membrane protein, the organic cation transporter (OCTN1) that facilitates pH-dependent transport of specific compounds across the membrane. Unlike OCTN2, which is predominantly involved in carnitine transport, OCTN1 has poor carnitine translocation activity, suggesting there is likely to be another physiological role for the transport [41]. In comparison to OCTN2, little is known about the physiological role of OCTN1. Recently however, Grundemann et al. identified that EGT is a key substrate for OCTN1 using a liquid chromatography mass spectrometry difference shading approach [42]. The specificity of OCTN1 was further demonstrated by the lack of discernible uptake of various organic cations including a 130 and 25-fold lower transport, relative to EGT, of structurally similar, methimazole and hercynine, respectively [43]. While uptake of EGT is both Na^{+} and pH dependent, transport is only lost at unphysiologically low Na^{+} and pH [44,45].

Silencing the gene encoding the OCTN1 in cell cultures inhibits uptake of EGT [46]. Similarly, metabolomic analysis of OCTN1 knockout (*octn1*$^{-/-}$) mice revealed that most tissues of the animal were almost completely deficient of EGT, indicating an absence of alternative mechanisms of uptake [28]. Analysis of various tissues in the body (Fig. 2) revealed a high level of OCTN1 in the bone marrow, which is consistent with high levels of EGT reported in erythrocytes [43]. Likewise intestinal tissue and peripheral blood cell concentrations of EGT are relatively independent of diet but closely correlated to OCTN1 mRNA expression [44,47].

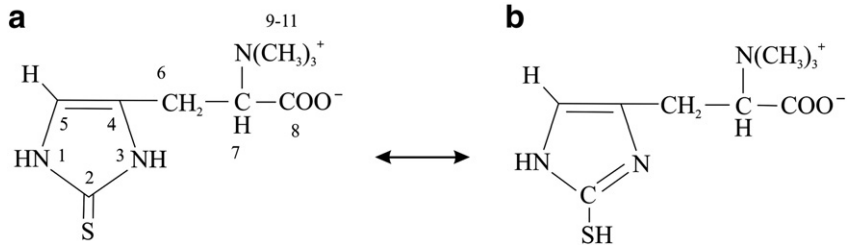

Fig. 1. Structure of thione–thiol tautomers of EGT (2-mercaptohistidine trimethylbetaine). In solution at physiological pH, EGT exists predominantly in the thione (a) rather than the thiol (b) hence conferring greater stability over other simple thiols such as GSH.

Table 1
The following table details the concentration of EGT in human and rat blood and selected rat tissues as determined by different analytical methods in the past. The considerable variation in the observed concentrations highlights the critical need to apply modern analytical techniques to standardize concentrations of EGT in biological samples. Earlier colorimetric methods lacked specificity due to extraneous compounds and many modifications focused on removing impurities. These methods also lacked sensitivity and some may not have considered protein bound EGT. Current analytical techniques such as liquid chromatography–mass spectrometry have yet to be applied to human and animal tissues. ND — not detectable.

Sample	[EGT] (mg/[a]100 ml or [b]100 g)	Method	Authors	Ref.
Human erythrocytes[a]	1.25–4.0	Chemical assay (daizo method)	Hunter, 1928	[34]
	7.5	Chemical assay (silver precipitation)	Behre and Benedict, 1929	[148]
	1.9–5.5	Chemical assay (daizo method–strong alkali)	Latner, 1948	[149]
	5.4–5.7	Chemical assay (daizo method–lead acetate)	Hunter, 1949	[36]
	2.0–3.1	Chemical assay (daizo method–iodobismuthous acid precipitation)	Lawson et al., 1950	[150]
	7.3	Chemical assay (bromine water)	Touster, 1951	[151]
	9.6	Chemical assay (daizo method–iodobismuthous acid precipitation)	Fraser, 1951	[152]
	1.8–4.2	Chemical assay (daizo–ion exchange)	Melville, 1953	[37]
	1.0–2.6	Chemical assay (trimethylamine estimation)	Jocelyn et al., 1958	[38]
	1.1–3.2	Chemical assay (2,2′-dipyridyl disulfide)	Carlsson et al., 1974	[153]
	3.2	Cation exchange chromatography	Fahey et al., 1981	[154]
Rat erythrocytes[a]	5.3	Chemical assay (daizo method)	Beatty, 1952	[138]
	4.1	Chemical assay (daizo method)	Mellville, 1954	[22]
	1.6	Chemical (*p*-chloromercuribenzoate (PCMB) method)	Hama et al., 1972	[155]
	0.28	High performance liquid chromatography	Mayumi et al., 1978	[12]
	0.21	Thin-layer chromatography	Kaneko et al., 1980	[116]
	0.67	High performance liquid chromatography	Muda et al., (1988)	[156]
Rat liver[b]	13.3	Chemical assay (daizo method)	Mellville, 1954	[22]
	4.7	Chemical (PCMB method)	Hama et al., 1972	[155]
	4.83	High performance liquid chromatography	Mayumi et al., 1978	[12]
	7.82	Thin-layer chromatography	Kaneko et al., 1980	[116]
Rat kidney[b]	4.3	Chemical assay (daizo method)	Mellville, 1954	[22]
	6.73	Chemical (PCMB method)	Hama et al., 1972	[155]
	1.87	High performance liquid chromatography	Mayumi et al., 1978	[12]
	1.58	Thin-layer chromatography	Kaneko et al., 1980	[116]
Rat testicle[b]	ND	Chemical assay (daizo method)	Mellville, 1954	[22]
	0.24	High performance liquid chromatography	Mayumi et al., 1978	[12]
	0.5	Thin-layer chromatography	Kaneko et al., 1980	[116]
Rat brain[b]	ND	Chemical assay (daizo method)	Mellville, 1954	[22]
	0.21	High performance liquid chromatography	Mayumi et al., 1978	[12]
	0.41	Thin-layer chromatography	Kaneko et al., 1980	[116]

3. Physiological role of ergothioneine

3.1. Antioxidant properties

Numerous rationales have been proposed to explain the ubiquitous presence of EGT and its extensive uptake and accumulation in tissues [48]. These include a role for EGT as a cation chelator [49–52], factor in bioenergetics [53], regulator of gene expression [48] and immune regulator [54,55]. However the most often cited attribute of EGT is its possible function as an antioxidant and cytoprotectant [56–60]. A wide body of evidence suggests that EGT may function as a physiological antioxidant. Closer examination of the distribution of EGT in the body reveals that the compound is preferentially accumulated in organs, cells and secretions predisposed to high levels of oxidative stress and inflammation such as liver, kidneys, erythrocytes, eye lens and seminal fluid [22,23,25]. In addition, depleting HeLa cells of EGT through OCTN1 silencing, leads to increases in oxidative burden on mitochondrial components, visualized through increased protein carbonylation (a general biomarker of oxidative damage to proteins [61]) and mitochondrial DNA damage (measured by quantitative real-time PCR of D-Loop mutations), further emphasizing the possible roles as a physiological antioxidant [46]. Analysis of OCTN1 knockout mice developed by Kato and coworkers, revealed that the animals which were completely deficient in EGT, appeared to be more susceptible to oxidative stress as determined by significantly higher lethality in *octn1*$^{-/-}$ mice following intestinal ischemia and reperfusion injury [28] which is known to involve reactive oxygen species (ROS), relative to wild-type mice [62]. However, no other biomarkers of oxidative damage were reported by the authors.

EGT has been established *in vitro* as a powerful scavenger of hydroxyl radicals (•OH), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) [58,63–65]. EGT was also shown to deactivate singlet oxygen at a higher rate ($k_\Delta = 2.3\times10^7\ M^{-1}s^{-1}$) than other simple thiols including GSH [66]. Indeed, the reactions of EGT with •OH and peroxyl radicals were determined by pulse radiolysis to have almost diffusion-controlled rate constants of $1.2\times10^{10}\ M^{-1}\ s^{-1}$ and $1.2\times10^9\ M^{-1}\ s^{-1}$, respectively [67]. These antioxidant abilities were demonstrated in more complex systems through exposure of α_1-antiproteinase to HOCl and DNA to •OH generated by Cu^{2+} in the presence of H_2O_2 and ascorbate; EGT exerted almost complete protection against damage in each case [58]. Hydroxyl radical scavenging however, is unlikely to be a mechanism of antioxidant action *in vivo*, since almost every biomolecule can react equally fast with •OH and many are present at far greater levels than EGT [62,68].

Peroxynitrite ($ONOO^-$), nitrosoperoxycarbonate ($ONOOCO_2^-$) and carbonate radical are able to cause lipid peroxidation, damage protein and DNA and deplete intracellular antioxidants and have been extensively implicated in many disorders [69–71]. EGT was shown to be a more effective scavenger of peroxynitrite than GSH and Trolox C and dose-dependently prevented nitration of tyrosine and inactivation of α_1-anti-proteinase at concentrations less than 1 mM [65,72]. EGT was also shown to dose-dependently protect PC12 cells from a $ONOO^-$ generating system, 3-morpholinosydnonimine [73]. By comparison, direct reactivity of EGT with superoxide ($\bullet O_2^-$) was found to

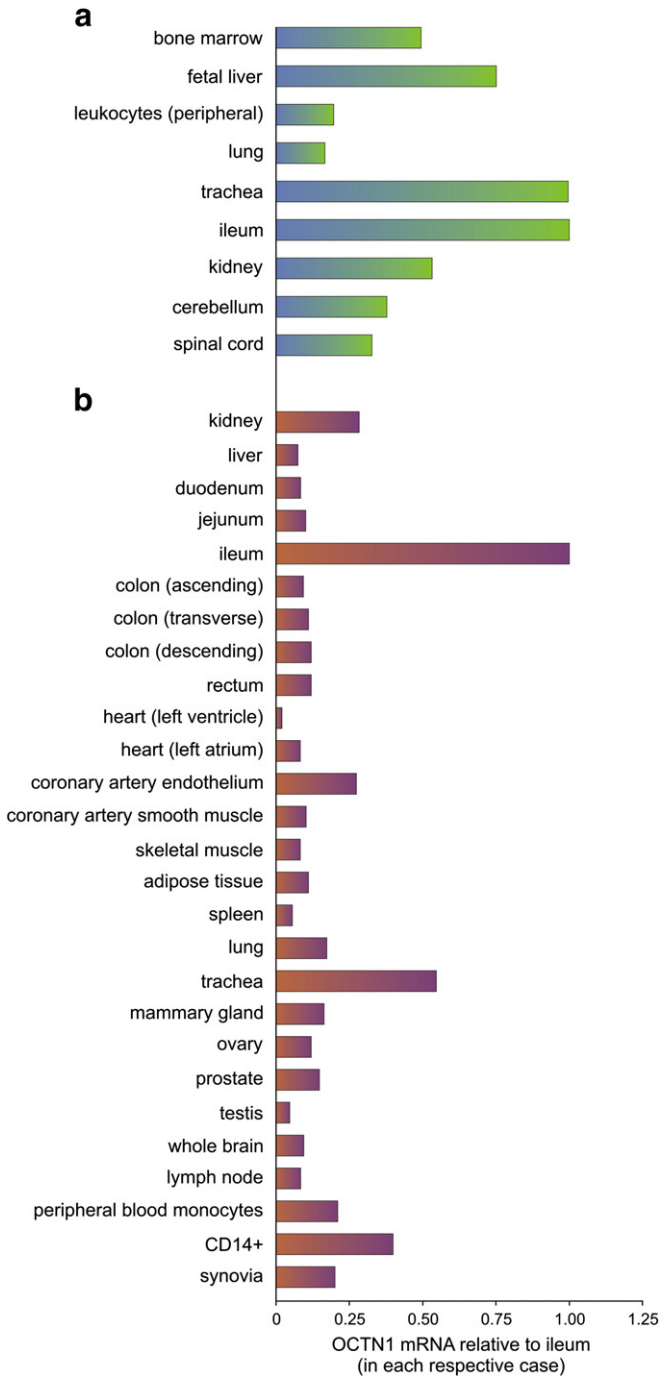

Fig. 2. Tissue expression of the EGT transporter, OCTN1, analyzed by real-time PCR adapted from 2 independent sources (Grundemann et al. 2005; National Academy of Sciences and Taubert et al. 2009; BMJ Journals) [42,47]. Data are relative to mRNA level of the ileum for each set.

be relatively poor with a rate constant estimated to be below $10^3\ M^{-1}\ s^{-1}$ [58]. EGT also had no effect on sodium nitroprusside generated NO•-induced cell death, that is, its reaction with NO•, if any, is also slow [73].

EGT was found to also have relatively poor direct reactivity with H_2O_2 [57,74], although there have been some reports of its cytoprotective effects in cell cultures. In a human neuronal hybridoma cell line EGT was shown to have mild protective effects against H_2O_2. Interestingly however, it synergistically amplified the protective effects of N-acetylcysteine, demonstrating dose-dependent inhibition of H_2O_2-induced cell death [59]. Similarly, EGT protected PC12 cells from H_2O_2-induced cytotoxicity and prevented DNA damage as observed by the comet assay [75]. This protection was shown to involve inhibition of p38 MAPK and Akt activation; hence EGT may play a protective role in H_2O_2-induced apoptosis by activation of intracellular antioxidant pathways involving p38 MAPK rather than by direct scavenging of H_2O_2 [75]. *In vitro* assays have also demonstrated that EGT can protect against DNA damage and peroxidation of arachidonic acid by H_2O_2 and H_2O_2/heme protein mixtures, respectively [58,75]. The reduction of ferrylmyoglobin (a reactive species formed when myoglobin reacts with H_2O_2) by EGT, as demonstrated by Arduini et al., [74] may be the mechanism of protection of arachidonic acid and has been suggested to be the manner by which EGT prevents ischemic myocardial damage [74].

Contradictory to many other studies, Ey et al. [21] found that EGT only protected OCTN1 transfected HEK-293 against copper(II)-induced toxicity, but not against a range of other cellular stressors as opposed to GSH which protected against all stressors tested at equivalent concentrations. Hence, together with the higher observable levels of cellular GSH, they argued that EGT is not involved in the intracellular antioxidant thiol defense system. These contrasting results over previous observations of EGT's effectiveness against ROS were suggested to be attributed to the earlier use of unphysiologically high concentrations, use of cell free systems which differ from *in vivo* and differing experimental conditions. Whilst these authors used a model of perhaps limited physiological relevance, that is an OCTN1 vector transfected HEK-293 cell line with modest EGT uptake (which may differ greatly from high OCTN1 expressing cell types), the results do indicate that GSH may still be the primary thiol defense in cells.

3.2. Other evidence of antioxidant properties

Numerous other *in vitro* assays and data have also demonstrated the potential antioxidant properties of EGT. Alloxan-induced lipid peroxidation of phosphatidylcholine liposomes was inhibited by 67% and 100% with the addition of only 20 μM and 100 μM EGT, respectively, which was double the protective effect of coenzyme Q_{10} [76]. Park et al. [77] investigated the free radical scavenging capacity of rhizome extracts using the radical generators DPPH and ABTS, and found that high radical scavenging values were correlated with the EGT content of the extracts. The *mshA* mutant of *Mycobacterium smegmatis* deficient in mycothiol, a major intracellular detoxification thiol similar to GSH in eukaryotes, was found to overproduce ergothioneine, putatively as a compensatory function [78].

The *in vitro* antioxidant properties of EGT have been extended to the commercial food industry, whereby it has demonstrated the ability to inhibit discoloration of red meats and fish and prevent lipid peroxidation and melanosic activity (melanin blackspot formation due to the action of phenoloxidase) in preservation of post-harvest crustaceans [79,80].

3.3. Chelation of metals

It was also discovered early on that EGT is able to chelate divalent metal ions. Various groups have demonstrated the ability of EGT to form complexes with divalent metal cations including Cu^{2+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} Fe^{2+} and Ni^{2+} [49–51]. These complexes form at a molar ratio of 2:1 of EGT to metal ion, with the most stable of these being with copper, which has the highest complex formation constant [50]. Binding of these metal ions in the body may help prevent their participation in the generation of ROS. EGT was found to protect both DNA and protein against copper-induced oxidative damage (caused by Cu^{2+}, H_2O_2 and ascorbate) through formation of a redox-inactive EGT-Cu complex [52,58]. In animals and humans the high levels of EGT in semen have been shown to prevent Cu^{2+} inhibition of sperm motility [81]. In contrast to the generation of ROS by GSH in the presence of Cu^{2+} (*via* formation of a redox-active Cu(I)–

$[GSH]_2$ complex) [82], the complex of EGT with Cu^{2+} is relatively stable and hence does not decompose to generate radicals [52].

3.4. Intracellular ergothioneine

Kawano and colleagues [33] found that EGT accumulated in the mitochondrial fraction of hepatic cells following injection of radiolabelled EGT into rats. Consistent with this finding was the presence of OCTN1 expression in the mitochondria in mammalian cell lines [41]. This suggests that EGT may function to protect vulnerable mitochondrial DNA and other mitochondrial constituents from damage by ROS generated by the electron transport chain. Supporting this theory are the findings by Paul and Snyder [46], demonstrating increased mitochondrial DNA damage in response to OCTN1 silencing when cells were exposed to H_2O_2. Furthermore the authors found that depleting the cells of EGT also led to an increase in protein carbonyls, lipid peroxidation and susceptibility to H_2O_2-induced cell death [46].

Studies using fractionation of rat hepatic cells in a sucrose gradient revealed very limited levels of EGT in the nucleus relative to the cytoplasm and mitochondria, however later studies by Markova et al. revealed similar levels of EGT in the nucleus as the cytoplasm [83]. Regardless, the presence of EGT within the nuclear fraction suggests OCTN1 mediated translocation across the nuclear membrane which may indicate a possible role in protection of DNA. Although this protective ability has yet to be established, *in vitro* assays have demonstrated that EGT is able to protect DNA from damage against various reactive species [57,58].

4. Does ergothioneine function as an antioxidant *in vivo*?

Of course the literature is replete with examples of compounds that exert excellent antioxidant properties *in vitro* but are ineffective *in vivo*, such as many flavonoids [62,84]. So is there evidence that EGT could act in this way *in vivo*? A study by Deiana et al. [85] demonstrated the potential of EGT to decrease oxidative damage *in vivo*. Rats supplemented with dietary EGT were found to have lower levels of lipid peroxidation and higher levels of glutathione and α-tocopherol in their liver and kidney, following administration of ferric-nitrilotriacetate, a catalyst of Fenton chemistry [85]. However this system imposes severe oxidative stress and the data do not indicate that EGT acts as an antioxidant under normal circumstances.

4.1. Protection against UV and gamma radiation

The presence of OCTN1 has been reported in primary keratinocytes and fibroblast cultures and also by immunohistochemical analysis of neonatal skin sections [76,83]. Although the functional role of EGT in the skin is not known, its presence may suggest a role as a physiological protectant against ultraviolet (UV)-induced ROS generation and damage. Much of the skin damage from UV radiation is mediated through the generation of ROS [86]. Several studies have shown the cytoprotective effects of EGT against UV-induced damage and cell death [76,87–89]. Keratinocytes exposed to UV radiation showed a considerable decrease in cell viability along with a fivefold increase in caspase-9 activity, but addition of EGT was able to significantly increase cell viability and decrease caspase-9 activity [83,87]. Furthermore, EGT was shown to suppress the expression of tumor necrosis factor-α (TNFα) and matrix metalloproteinase-1 resulting from UV irradiation of fibroblasts [90]. EGT is known to absorb light in the UV range and hence this physical property may account for some of its ability to block UV damage [91].

Motohashi et al. [92,93] demonstrated the radioprotective capabilities of EGT. The γ-irradiation of metmyoglobin resulted in marked structural changes, however the addition of 0.5 mM EGT was able to prevent these changes [92]. Similarly, Hartman et al. [94] demonstrated that 1 mM EGT afforded complete protection against T4 bacteriophage inactivation by γ-radiation. However, these results may be through •OH scavenging *in vitro*, and so their physiological relevance is uncertain.

4.2. Function of EGT in blood

Early studies failed to demonstrate uptake of EGT by mature erythrocytes, however later studies by Mitsuyama and May [60] clearly demonstrated uptake and accumulation beyond basal levels. In addition, OCTN1 has been detected in mature erythrocytes [95].

The highly reactive ferryl-hemoglobin, derived from oxidation of oxyhemoglobin, plays a critical role in lipid peroxidation in erythrocytes [62]. As previously mentioned EGT was able to reduce ferryl-myoglobin/hemoglobin and also prevented the peroxidation of arachidonic acid by a mixture of H_2O_2 and heme protein. Furthermore, Spicer et al. [96] found that the rate of nitrite-induced oxidation of hemoglobin in isolated rabbit blood, to methemoglobin, was inversely proportional to EGT content, and subsequent addition of EGT reduced methemoglobin back to hemoglobin. Thus EGT may act as a protectant against peroxidation in blood [58]. Correspondingly, dietary intake of EGT was shown to retard nitrite-induced methemoglobin formation in rabbits [96]. The ability of EGT to scavenge HOCl [58], may also be relevant in protecting erythrocytes from damage by neutrophils, the principal source of HOCl in the body [62].

Whilst the accumulation of EGT in erythrocytes is often suggested to be due to the predisposition of red blood cells to oxidative burden [62], it has been suggested that the expression of OCTN1 in bone marrow, fetal liver and erythroid cells may play a role in growth and differentiation of erythrocytes [95,97]. In support of this notion, Nakamura et al. [40] reported that depression of OCTN1 expression by siRNA impaired the proliferation and differentiation of human leukemic cell cultures. Further supporting a link between EGT and proliferation, an up-regulation of OCTN1 has been found to occur not only in fetal liver but also in regenerating liver tissue [98].

Additionally EGT has been suggested to play a role in glycolytic metabolism by erythrocytes. Due to the lack of mitochondria in the mature mammalian erythrocyte, energy is derived from the Embden–Meyerhof pathway, resulting in lactate formation. Rats starved for 24 h following EGT administration, maintained lactate levels in their erythrocytes whereas levels in control animals dropped by 60% [53]. Furthermore, when EGT was added directly to intact blood cells *in vitro*, levels of lactate increased whilst levels of glucose-6-phosphate and fructose-6-phosphate decreased relative to controls [53]. Similarly, *in vitro* addition of EGT to human platelets incubated with pyruvate-2-^{14}C significantly increased $^{14}CO_2$ [99]. Whilst it is difficult to ascertain what these observations could mean it may suggest there may be some interaction between EGT and the glycolytic pathway.

4.3. Interaction with other intracellular defenses

Some researchers have suggested that EGT may interact with other cellular stress defenses in times of excessive oxidative burden, for example as a means to reduce oxidized GSH [74]. Kawano et al. [100] whilst studying the inhibition of lipid peroxidation by EGT using isolated mouse liver microsomes *in vitro*, observed a significant dose dependent increase in GPx, glutathione reductase and mitochondrial superoxide dismutase activities on addition of EGT. However this response was seen at unphysiological concentrations, ranging from 12.5 to 50 mM EGT. In mice, EGT was able to restore the cisplatin-induced decrease in the GSH/GSSG ratio in the brain, suggesting that that EGT may directly or indirectly assist in maintaining *in vivo* thiol defenses [101]. *In vitro* studies have shown that oxidized EGT can be reduced by either cysteine or GSH, however it is important to note that this was achieved under conditions which probably would not occur *in vivo* [14]. Arduini et al. [74] also spectrally

observed the metmyoglobin (Mb^{III})-dependent oxidation of EGT by H_2O_2/Mb^{III} *in vitro*, with subsequent regeneration of EGT upon addition of GSH.

Despite the view that EGT may help maintain levels of GSH, this would likely require enzymatic catalysis but there have been no reports of any interaction between EGT and thioredoxin or glutaredoxins. Furthermore, depletion of GSH by oxidative stressors did not affect levels of EGT in erythrocytes, and conversely the auto-oxidation of GSH was not accelerated in the presence of EGT [60]. A more recent study found that EGT had no effect on glutathione transferases [102].

The oxidized form of EGT generated by pulse radiolysis, was demonstrated to be rapidly reduced by ascorbic acid ($k = 6.3 \times 10^8\ M^{-1} s^{-1}$) similar to the regeneration of α-tocopherol in the body [67]. However, this oxidized form of EGT has only been observed *in vitro* under conditions probably not feasible *in vivo*, which raises doubts as to whether these reactions are important *in vivo*.

5. Ergothioneine and disease

5.1. Chronic inflammatory conditions

The role of EGT in inflammation is somewhat controversial. On the one hand mutations of the OCTN1 gene, *SLC22A4* have been implicated in chronic inflammatory conditions including rheumatoid arthritis (RA) [103,104] and Crohn's disease (CD) [105,106]. Patients with mildly active RA were found to have significantly higher erythrocytic and monocytic levels of EGT and these levels were closely correlated to expression of OCTN1 mRNA in $CD14^+$ cells [103]. Mice with collagen-induced arthritis also highly express OCTN1 in hematological and immunological tissues of inflamed joints [104]. Similar to RA, analysis of the inflamed ileal mucosa of CD patients also revealed elevated levels of EGT which corresponded to increases in OCTN1 mRNA expression relative to non-CD subjects [47]. Interestingly, the 503F variant of OCTN1 associated with CD was found to have a 50% greater transport efficiency compared to the normal variant (503L) [45]. Silencing OCTN1 expression in cultured erythroid cells impairing EGT uptake, has been shown to be pro-apoptotic [40]. Hence in these chronic inflammatory conditions, the increased EGT has been postulated to stimulate inflammation by promoting immune cell survival mediated by the anti-apoptotic properties of EGT [45,47].

Some studies have indicated that the elevated levels of EGT may be secondarily due to the cytokine-mediated up-regulation of OCTN1. Incubation of Caco-2 cells with TNFα was shown to increase OCTN1 expression through TNFα receptor-1 activation, which would hence increase EGT and might in turn promote further inflammation through the aforementioned prolonging of immune cell survival [47]. Similarly OCTN1 expression was found to be up-regulated by RA-associated inflammatory cytokines such as interleukin-1β (IL), and by the transcription factor RUNX1 [107]. However whether this response is involved in pathogenesis or is a counteractive response as a cellular defense, and the molecular actions of EGT, are still undetermined.

Conversely a study undertaken by Kato et al. [28] in a cohort of Japanese CD patients revealed that blood concentrations of EGT were significantly reduced relative to healthy volunteers. Kato et al. likened their observations to OCTN1 knockout mice whereby animals deficient of EGT were predisposed to intestinal inflammation, hence suggesting that EGT may play a role in counteracting intestinal inflammation. In support of this anti-inflammatory effect, EGT has been shown to inhibit the TNFα-mediated increase of IL-8 in epithelial cell cultures [55]. Likewise EGT was shown to protect against free fatty acid induced cell death through activation of p38 and JNK signaling pathways and inhibition of IL-6 expression [54].

This presence of specific *SLC22A4* mutations allegedly associated with CD or RA in specific populations, suggests the existence of ethnic differences in OCTN1 polymorphisms and the possibility of differential disease susceptibility in specific populations. Indeed numerous studies have identified that specific polymorphisms linked to certain conditions may exist only in a particular ethnic population [108–113]. Certain polymorphisms of *SLC22A4* also have a significant impact on EGT uptake similar to the aforementioned 503F *SLC22A4* [45]. Interestingly however, a study undertaken by Petermann et al. [114] in subjects possessing the 503F variant with increased susceptibility to CD, identified an association between increased CD symptoms and the intake of EGT-rich mushrooms. This may highlight a negative impact of excessive EGT uptake, however, the exact role of EGT in chronic inflammatory conditions and other disease has yet to be ascertained.

5.2. Potential effects against neurodegeneration

The expression of OCTN1 was detected in the brain of mice and rats, *albeit* at lower levels than liver, kidney and intestinal tissue, using RT-PCR [95,115]. Consistent with this are numerous reports of EGT levels in mouse, rat, guinea pig, rabbit, cat, sheep and ox brain of between 0.3 and 1 mg per 100 g brain tissue, indicating the capability of EGT to permeate the blood brain barrier [116–118]. Several reports have demonstrated the cytoprotective effects of EGT in neuronal cells against various oxidative challenges. The neurotoxic effects of β-amyloid and cisplatin are well recognized and exposure of neuronal cultures to either compound results in decreased cell viability and apoptosis [119–122]. PC12 (rat pheochromocytoma) cells are commonly used as a model for neurobiological studies, possessing neuronal-like features such as neurite formation and terminal differentiation in response to nerve growth factor. However, these cells are not a true substitute for neurons [123]. Addition of EGT was shown to dose-dependently rescue PC12 cells (which are known to express OCTN1 [44]), from β-amyloid-induced apoptotic death as demonstrated through decreases in TUNEL staining and pro-apoptotic Bax and caspase 3 expression and a concomitant increase in PARP (normally cleaved by caspases) [73]. The effects of Aβ are believed to be mediated through oxidative and nitrosative damage by •OH and $ONOO^-$, since pre-incubation of Aβ with EGT prior to addition to cells had no significant effect on the cytotoxicity of the peptide [73]. Similarly the neurotoxic effects of cisplatin are believed to be mediated *via* ROS generation, and treatment of PC12 cells with 0.1–10 μM EGT prevented the antiproliferative effects of cisplatin and restored neuritic outgrowth in rat cortical neurons [101]. *In vivo* treatment of mice with cisplatin significantly decreased brain EGT levels and body weight, increased brain lipid peroxidation (determined *via* measurement of malondialdehyde using the thiobarbituric acid assay, which is a rather questionable method [62,124]) and has been suggested to impair cognitive functions in the animals. Supplementation with EGT *via* oral gavage, restored levels of EGT in the brain, body weight and cognitive function and inhibited cisplatin-induced 'lipid peroxidation' [101].

Similar to cisplatin and Aβ, the over-stimulation of *N*-methyl-D-aspartate (NMDA) glutamate receptors leads to neuronal cell death *via* increased production of free radicals [125]. Intravitreal injection of NMDA in rats leads to significant loss of retinal neurons, however intraperitoneal injection of EGT significantly protected neurons from NMDA excitotoxicity [126].

5.3. EGT in cardiovascular disease

The immunomodulatory effects of EGT might be beneficial in inhibiting the atherogenic induction of pro-inflammatory cytokines and adhesion molecules. Studies in human aortic endothelial cells revealed that EGT was able to decrease expression of the adhesion molecules VCAM-1, ICAM-1 and E-selectin and inhibit binding of monocytes to the endothelium [127]. ROS have been implicated as

key mediators in the pathogenesis of atherosclerosis and other cardiovascular disorders, however their exact involvement is still uncertain [128]. Evidence for the protective role of EGT against cardiovascular disease comes from studies of ischemia/reperfusion (IR), a process involving oxidative stress [62,129]. EGT has been shown to protect cardiac and liver tissue from damage *in vivo* during IR [74,130,131]. The mechanisms by which this protective action is achieved may be through the previously mentioned (Section 3.1) reduction of ferrylmyoglobin protecting tissue from oxidative injury, evident through decreased tissue malondialdehyde levels and also through modulation of heat shock protein 70 and proinflammatory cytokines (TNFα and IL-1β) [74,131]. In addition, chelators of iron and copper have been shown to be protective by decreasing free radical production during IR, suggesting another mechanism by which EGT may protect [132–134]. Conflicting results were obtained by Cargnoni and colleagues [135] who failed to see reduction in IR damage in rabbit hearts following administration of EGT *ex vivo*. Different methodologies employed by the authors could explain these differences. The negative result employed an *ex vivo* model of rabbit heart IR with the addition of 10 or 100 μM EGT into the perfusion solution only 60 min prior to IR, limiting opportunity for uptake by tissues [135]. The independent studies showing IR protection by EGT, both utilized rats supplemented with EGT *via* oral gavage or through diet, daily for 2 to 3 weeks prior to *in vivo* liver or intestinal IR injury, allowing a far greater period of time for uptake and accumulation [130,131]. The conflicting results could also be due to dissimilar expression of OCTN1 by different animals and tissues (much greater expression in ileum and liver compared to heart) and hence varying degrees of uptake and accumulation.

Earlier investigations revealed elevated levels of EGT in diabetic patients which were suggested to contribute to the onset of diabetes mellitus through chelation of zinc, which is important for storage of insulin and glucagon [24,136,137]. However this is unlikely as no significant elevation of EGT was observed in alloxan-induced diabetic rats and studies of metal chelation by EGT have revealed no inhibition of zinc metalloenzymes [49,138]. On the contrary, in mouse myoblast cultures, EGT was shown to inhibit palmitic acid-induction of IL-6, which has been correlated to insulin resistance and type 2 diabetes [54,139,140]. Furthermore, supplementation of diabetic pregnant rats with EGT reduced the rate of embryo malformations to that of non-diabetic controls, which was attributed to modulation of hyperglycemia-dependent oxidative stress [141].

6. Conclusions

Many advances in our understanding of EGT have been achieved in recent years including the identification of a highly specific transporter in higher organisms and humans and also better understanding of its distribution owing to advances in analytical techniques. Whilst the true physiological role of EGT has yet to be fully elucidated, EGT has been shown to possess numerous antioxidant and cytoprotective effects *in vitro* and a few *in vivo* (Fig. 3), including free radical scavenger activity [57,58,67,72], radioprotective properties [93,94], anti-inflammatory actions [54] and protection against UV radiation [87–89,142] or neuronal injury [73,101]. The molecular mechanisms underlying these cytoprotective actions still remain largely undetermined.

However, despite the wide body of evidence implicating its protective role, the high levels of EGT identified in populations suffering from the chronic inflammatory conditions such as RA and CD suggest it may play a role in the progression of these conditions. Interestingly, conflicting levels of EGT in different subgroups of CD may in part be due to differing diets or it may highlight other genetic factors which have yet to be identified.

Conflicting results on antioxidant properties of EGT by various groups, likely owing to differing experimental conditions and widely differing concentrations used, often not physiological, have served to further confuse the role of EGT. Many factors need to be considered when assessing the result from these assays. Firstly the use of different cell types in different studies could also give rise to variations based on the differences in expression of OCTN1. Studies have shown that EGT uptake is related to the expression of OCTN1 which may also limit uptake by some cell types and hence demonstrate a lower protective effect [46]. Secondly, in many cell culture assays, the reaction of cellular stressors with EGT may occur outside the cell giving a false impression of intracellular cytoprotective effects. Finally, whilst numerous assays demonstrate the unique reactivity of EGT *in vitro*, such as scavenging of •OH or ability of "oxidized" EGT to be regenerated by GSH, it is important to note that these are often far from the conditions *in vivo* and hence may not be relevant. Very few *in vivo* studies with EGT have been undertaken and this will need to be addressed further with use of validated biomarkers of oxidative stress optimized for humans [124,143–147]. Our understanding of EGT in the body is still relatively limited and much remains to be learned about this unique compound.

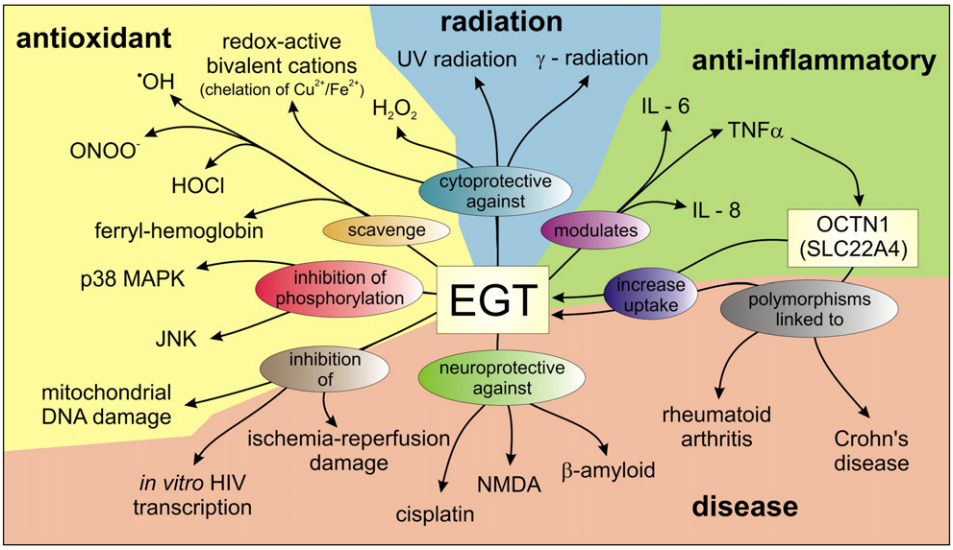

Fig. 3. Possible roles and interactions of EGT *in vivo*. Functional roles as an antioxidant are highlighted in yellow, anti-inflammatory agent in green, protectant against radiation in blue and roles in disease in orange.

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