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Original article

Ergothioneine stands out from hercynine in the reaction with singlet oxygen: Resistance to glutathione and TRIS in the generation of specific products indicates high reactivity

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ABSTRACT

The candidate vitamin ergothioneine (ET), an imidazole-2-thione derivative of histidine betaine, is generally considered an antioxidant. However, the precise physiological role of ET is still unresolved. Here, we investigated *in vitro* the hypothesis that ET serves specifically to eradicate noxious singlet oxygen ($^1O_2$). Pure $^1O_2$ was generated by thermolysis at 37 °C of *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide 1,4-endoperoxide ($DHPNO_2$). Assays of $DHPNO_2$ with ET or hercynine (= ET minus sulfur) at pH 7.4 were analyzed by LC-MS in full scan mode to detect products. Based on accurate mass and product ion scan data, several products were identified and then quantitated as a function of time by selected reaction monitoring. All products of hercynine contained, after a [4+2] cycloaddition of $^1O_2$, a carbonyl at position 2 of the imidazole ring. By contrast, because of the doubly bonded sulfur, we infer from the products of ET as the initial intermediates a 4,5-dioxetane (after [2+2] cycloaddition) and hydroperoxides at position 4 and 5 (after Schenck ene reactions). The generation of single products from ET, but not from hercynine, was fully resistant to a large excess of tris (hydroxymethyl)aminomethane (TRIS) or glutathione (GSH). This suggests that $^1O_2$ markedly favors ET over GSH (at least 50-fold) and TRIS (at least 250-fold) for the initial reaction. Loss of ET was almost abolished in 5 mM GSH, but not in 25 mM TRIS. Regeneration of ET seems feasible, since some ET products – by contrast to hercynine products - decomposed easily in the MS collision cell to become aromatic again.

1. Introduction

Ergothioneine (ET) is a natural compound that humans and other vertebrates cannot synthesize; it must be absorbed from food. Most of our contemporary food contains very little ET, but many mushrooms [1,2] and cyanobacteria [3] contain around 1 mg/g dried material. After ingestion, ET is rapidly cleared from the circulation and then retained in the body with minimal metabolism. During the biosynthesis of ET, L-histidine is converted to a betaine and a sulfur atom is attached to position 2 of the imidazole ring [1,4]. ET can be considered a derivative of thiourea. Because of the prevailing thione tautomer [5,6], ET has several properties that are markedly different from ordinary thiols like the ubiquitous glutathione (GSH).

Previously, we discovered an ET transporter (ETT; human gene symbol *SLC22A4*) [7]. ETT is a powerful sodium-driven uptake transporter in the plasma membrane [8,9]. Cells lacking ETT do not accumulate ET since phospholipid bilayers are virtually impermeable to this hydrophilic zwitterion. In the human body ETT is strongly expressed in erythrocyte progenitor cells in bone marrow, the small intestine (ileum), trachea, kidney, cerebellum, lung, and monocytes. The ability to absorb, distribute, and retain ET depends entirely on this highly specific transporter [10–13].

Abbreviations: $^1O_2$, singlet oxygen; DHPN, *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide; $DHPNO_2$, DHPN 1,4-endoperoxide; ET, ergothioneine; ETT, ergothioneine transporter; GSH, glutathione; HPLC, high-performance liquid chromatography; HRMS, accurate ion mass measurement at high resolution; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; SRM, selected reaction monitoring; TMPyP, 5,10,15,20-Tetrakis(*N*-methyl-4-pyridyl)−21,23H-porphyrin tetratosylate; TRIS, tris(hydroxymethyl)aminomethane

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Case-control studies suggest that polymorphisms in the *SLC22A4* gene are associated with susceptibility to chronic inflammatory diseases, such as Crohn's disease, ulcerative colitis, and type I diabetes [12,14], but it is unknown how these mutations promote disease. The mere existence and evolutionary conservation of ETT imply that ET fulfills a beneficial role, perhaps like a vitamin. In general, ET is considered an intracellular antioxidant [5,14]. However, its precise physiological purpose is still unresolved. Why do we accumulate ET in particular tissues or cells despite a 10-fold excess of GSH, the general antioxidant?

Recently, we have reported that in the skin of unstressed ETT knockout zebrafish, the content of 8-oxoguanine (8OG; alias 8-oxo-7,8-dihydroguanine) was increased 4-fold vs. wild-type [15]. This led to the hypothesis that the specific purpose of ET could be to eradicate noxious singlet oxygen ($^1O_2$).

$^1O_2$ is a member of the ROS (reactive oxygen species) quartet; it is less reactive than the hydroxyl radical, but more aggressive than the superoxide anion and hydrogen peroxide [16]. This translates into a relatively long intracellular half-life ($t_{1/2} \approx 3$ μs) [17]. The radius of the sphere of activity of $^1O_2$ from its point of intracellular production was estimated at 155 nm [18]. While the hydroxyl radical reacts with almost anything, $^1O_2$ is selective about its reaction partner. For example, the hydroxyl radical attacks all 4 DNA bases, but $^1O_2$ confines itself to guanine [19]. In guanine, $^1O_2$ reacts via [4+2] cycloaddition with the imidazole ring to create a 4,8-endoperoxide, an 8-hydroperoxide and, after reduction, an 8-carbonyl [20,21]. Interestingly, a doubly bonded oxygen (carbonyl group) at position 2 of the imidazole ring stimulates reactivity very much: 8OG - which bears obvious similarity in the imidazole ring with ET - reacts at least 100-fold faster with $^1O_2$ than guanine [22].

In the human body, $^1O_2$ can be generated by sunlit photosensitizers in skin and eye. It is clear from the rare human disorder erythropoietic protoporphyria that sunlight does also impinge on erythrocytes in circulating blood, a major site of ET accumulation in all vertebrates. Symptoms include itching, severe pain, swelling, and skin ulcers. Here, sunlight drives $^1O_2$ production from protoporphyrin IX, the iron-free precursor of heme. Binding of Fe^{2+} to the porphyrin ring blocks $^1O_2$ production almost completely [23]. However, if only a small fraction of heme in erythrocytes was degraded to iron-free porphyrin, deleterious $^1O_2$ would be produced. Alternatively, even intact hemoglobin may generate singlet oxygen, because of its peroxidase activity [24–26]. Peroxidases such as myeloperoxidase, eosinophilic peroxidase, lactoperoxidase and thyroid peroxidase are closely related heme proteins. They generate $^1O_2$ as a by-product of enzymatic oxygen conversions [27]. In monocytes and macrophages - another site of prominent ET accumulation - peroxidases produce highly reactive defence molecules (respiratory burst). Here, the collateral production of singlet oxygen [28,29] could also cause cellular damage.

Information on the reaction of ET with $^1O_2$ is scarce. Hartman and coworkers reported conflicting results; at least in an assay with rose bengal, ET was a better quencher than azide [30,31]. Other groups have measured that in simple aqueous solution ET reacts faster than GSH with $^1O_2$ [32,33]. To the best of our knowledge, the reaction products of ET and $^1O_2$, if any, are unknown.

Recently, the reaction of ET in aqueous solution with the oxidants hypochlorite (ClO^-), peroxynitrite ($ONOO^-$), and hydrogen peroxide ± myoglobin was investigated [34]. By LC-MS, 3 products were detected: ET disulfide, ET sulfonic acid, and hercynine (= ET minus sulfur). It was hypothesized that hercynine is generated in a pathway involving hydrolysis of ET disulfide.

The aim of our study was to define by LC-MS the products of ET and $^1O_2$ that are generated in aqueous solution at 37 °C and physiological pH. The reactivity of ET towards $^1O_2$ was compared with hercynine. The responses to the addition of TRIS and GSH were used to devise a model of the reaction pathways.

To generate $^1O_2$ in aqueous solution, photosensitizers like rose bengal, methylene blue, or TMPyP [35] are frequently used. The major drawback of this approach is that superoxide anion, hydrogen peroxide, and hydroxyl radical are generated alongside with singlet oxygen, so results can be ambiguous. A brilliant alternative is based on the reversible binding (Diels-Alder addition) of $^1O_2$ to polycyclic aromatic hydrocarbons like naphthalene and anthracene [36]. The resulting endoperoxides are stable in the cold (≤ 4 °C), but release $^1O_2$ (together with some unreactive triplet oxygen) upon mild warming. A very useful

Table 1
Products from the reaction of ET or hercynine with $^1O_2$.

Compound Code	SRM	Assay	Accurate Mass (Da) Delta (mDa)	Peak Top Elution Time (min)	Full Scan Peak Area
230 early	230:171	hercynine + $^1O_2$	230.1133 0.7	6.1	7.1
230 late	230:153	hercynine + $^1O_2$	230.1134 0.6	7.2	4.2
246:229	246:229	hercynine + $^1O_2$	246.1084 0.5	6.2	3.1
246:143	246:143	ET + $^1O_2$	246.0905 0.7	6.6	−1.9
248	248:188	hercynine + $^1O_2$	248.1241 0.5	7.0	6.2
262	262:245	ET + $^1O_2$	262.0854 0.7	5.4	10
264	264:188	ET + $^1O_2$	264.1012 0.6	6.7	1.3
288	288:212	hercynine + $^1O_2$ + 0.1 mM thiourea	288.1120 1.0	8.5	1.0
333	333:274	hercynine + $^1O_2$ + 5 mM TRIS	333.1767 0.7	6.7	29
365 early 365 late	365:230 365:244	ET + $^1O_2$ + 5 mM TRIS	365.1490 0.4	6.8 7.2	combined 13
519	519:460	hercynine + $^1O_2$ + 0.1 mM GSH	519.1863 1.0	7.9	4.4
535	535:228	hercynine + $^1O_2$ + 1 mM GSH	535.1814 0.8	7.8	6.4
551	551:244	ET + $^1O_2$ + 0.1 mM GSH	551.1583 1.0	7.7	1.7
553	553:246	ET + $^1O_2$ + 1 mM GSH	553.1741 0.9	7.9	0.95

All assays contained 100 μM ET or hercynine plus 10 mM $DHPNO_2$. Full scan peak area (in units of 10^6 counts × min) was calculated as the difference of the area under the curve between 60 min and 0 min samples. SRM states parent and fragment masses. Delta, exact mass of proposed compound minus accurate mass.

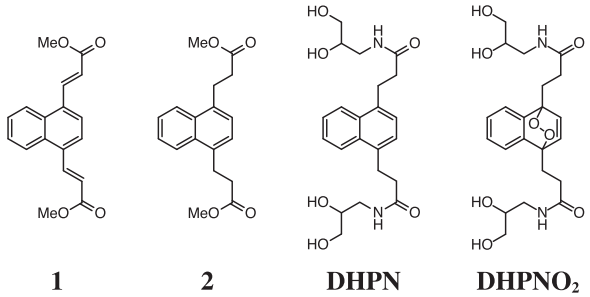

Fig. 1. Synthesis of $DHPNO_2$. Structures of intermediates and products. See Section 2 for details.