Prooxidative chain transfer activity by thiol groups in biological systems

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ABSTRACT

Cysteine is arguably the best-studied biological amino acid, whose thiol group frequently participates in catalysis or ligand binding by proteins. Still, cysteine’s unusual biological distribution has remained mysterious, being strikingly underrepresented in transmembrane domains and on accessible protein surfaces, particularly in aerobic life forms (“cysteine anomaly”). Noting that lipophilic thiols have been used for decades as radical chain transfer agents in polymer chemistry, we speculated that the rapid formation of thiyl radicals in hydrophobic phases might provide a rationale for the cysteine anomaly. Hence, we have investigated the effects of dodecylthiol and related compounds in isolated biomembranes, cultivated human cells and whole animals (C. elegans). We have found that lipophilic thiols at micromolar concentrations were efficient accelerators, but not inducers of lipid peroxidation, catalyzed fatty acid isomerization to trans-fatty acids, and evoked a massive cellular stress response related to protein and DNA damage. These effects were specific for lipophilic thiols and were absent with thioethers, alcohols or hydrophilic compounds. Catalytic chain transfer activity by thiyl radicals appears to have deeply influenced the structural biology of life as reflected in the cysteine anomaly. Chain transfer agents represent a novel class of biological cytotoxins that selectively accelerate oxidative damage in vivo.

1. Introduction

Cysteine is the most versatile amino acid in terms of reactivity and protein structure control, being widely used for the formation of disulfide bridges [1], redox regulation [2], cofactor ligation [3] and catalysis [4,5]. Nevertheless, a number of properties of cysteine with respect to its usage and conservation are bewildering. For example, functionally relevant and clustered cysteines are strictly conserved as expected [6,7]. However, coincidental cysteines that lack any specific function are much less conserved than all other coincidental amino acids and appear to be rapidly substituted during evolution [6,7]. Hence, we have investigated the effects of dodecylthiol and related compounds in isolated biomembranes, cultivated human cells and whole animals (C. elegans). We have found that lipophilic thiols at micromolar concentrations were efficient accelerators, but not inducers of lipid peroxidation, catalyzed fatty acid isomerization to trans-fatty acids, and evoked a massive cellular stress response related to protein and DNA damage. These effects were specific for lipophilic thiols and were absent with thioethers, alcohols or hydrophilic compounds. Catalytic chain transfer activity by thiyl radicals appears to have deeply influenced the structural biology of life as reflected in the cysteine anomaly. Chain transfer agents represent a novel class of biological cytotoxins that selectively accelerate oxidative damage in vivo.

1 The latter idea is supported by the result that cysteine was the most divergent amino acid in comparisons of aerobic versus anaerobic proteomes. For example, cysteine is significantly depleted in aerobic bacteria, archaea, unicellular eukaryotes and mitochondria [8]. In mitochondria from aerobic animals, but not from anaerobic-parasitic animals, the degree of cysteine depletion predicted animal longevity across phylogenetic boundaries [8]. More detailed analyses have shown that the latter effect was largely attributable to intramembrane cysteine residues [9]. An adverse chemical reactivity of membrane cysteines under oxidizing conditions is also suggested by the extreme avoidance of cysteine in membrane proteins from aerobic archaea living under chemically harsh conditions [10]. Aerobic archaea exhibit average membrane cysteine frequencies of 0.2%, which is fivefold lower than in...
bacteria, and tenfold lower than in eukaryotes. From a comparison of the diverging usage patterns of cysteine and methionine in mitochondria, it has been proposed that thyl radical formation might account for the avoidance of cysteine in aerobic membranes [11]. Here, we have experimentally tested this prediction in vivo using lipophilic thiols like dodecylthiol as model compounds.

Dodecylthiol is a high production volume industrial chemical used to control molecular weight in radical polymerization processes leading to polychloroprene (neoprene), polyacrylic acid or polyvinyl chloride [12]. In these processes, it acts as chain transfer agent, dynamically interrupting radical chains in progress and restarting them elsewhere [13, 14]. Whereas the principles of chain transfer catalysis by thiols have long been established in polymer chemistry [15,16], the effects of classical chain transfer agents in biological systems have not been explored. Since free radical chain reactions play a similarly pivotal role in biology as in polymer chemistry [10,17–19], we wish to do so in the following report.

2. Results and discussion

Dodecylthiol (12SH), dodecylmethylsulfide (12SMe) and dodecanol (12OH) (Fig. 1A) were applied to primary human fibroblasts (IMR90 cells) and analyzed for cytotoxic effects. Dodecylthiol was toxic at micromolar concentrations, whereas the thioether and the alcohol had no effect (Fig. 1B). Comparison of additional alkyl thiols indicated that a long alkyl chain and thus lipophilicity was prerequisite for toxicity (Fig. 1C). Hydrophilic thiols related to aqueous cysteine were nontoxic, meeting biological expectation (Fig. 1D). Similar results were obtained in more rapidly dividing clonal neuronal HT22 cells (Fig. 1E and F).

Analytical investigation indicated that the reducing chemical dodecylthiol acted as a prooxidative chemical in vivo. In IMR90 cells, it induced a rapid rise in the levels of 8-isoprostane (Fig. 2A), one of the best-validated markers of biological lipid peroxidation [20]. In parallel, the cells suffered from a significant loss of the easily peroxidizable polyunsaturated fatty acids (PUFAs) arachidonic acid (AA) and docosahexaenoic acid (DHA) (Fig. 2B). In the GC fatty acid profile of dodecylthiol-treated cells, a striking extra peak running just ahead of oleic acid was noted (Fig. 2C), which was identified as trans-oleic acid and quantified (Fig. 2D). Notably, cis-trans-isomerization of monounsaturated fatty acid (MUFAs) has been described before as specific footprint of thiyl radical formation in biological systems [21,22]. Consistently, dodecanol at identical concentrations did not induce fatty acid changes in human cells (Fig. 2B,D). Both dodecylthiol and dodecanol had no significant effect on the fractional content of stearic acid or cis-oleic acid (Fig. 2E). To separate any potential cell signaling effects of dodecylthiol from its behavior as plain chemical, the compound was also tested in native, but sonicated and hence biologically “dead” lipid membranes from rat brain. When added together with a biochemically

Fig. 1. Cytotoxicity of lipophilic thiols and related compounds in cultivated cells. (A) Key chemicals investigated in this work. (B) Toxicity of the compounds shown in A after 3 days treatment of IMR90 cells. (C) Toxicity of shorter or longer alkyl thiols in IMR90 cells. (D) Toxicity of hydrophilic thiols in IMR90 cells. (E) Toxicity of alkyl thiols after 3 days treatment of HT22 cells. (F) Toxicity of hydrophilic thiols in HT22 cells. In 4SH, 8SH, 10SH, 12SH, 14SH, the number in indicates the length of the linear alkyl chain. CAM is cysteamine, DES is deamino cysteine (3-mercaptopropionic acid), CYS is cysteine, GSH is glutathione.
common radical initiator (iron/ascorbate), millimolar dodecylthiol rapidly promoted lipid peroxidation measured as malondialdehyde after 15 min (Fig. 2F). Dodecylmethylsulfide was inert, and dodecanol blocked peroxidation presumably by disrupting the native membrane vesicles [23]. In the long term, though, dodecylthiol had only a mild effect on the final level of malondialdehyde after 16 h (Fig. 2F), indicating that lipid peroxidation was accelerated, but not initiated by the compound. To verify this supposition, the rapid effects of different concentrations of dodecylthiol were analyzed with or without radical initiator. In the absence of initiator, dodecylthiol indeed failed to induce malondialdehyde formation (Fig. 2G). In conjunction with initiator, however, lipid peroxidation was strikingly accelerated (Fig. 2H). These data indicate that thiol groups in membranes neither act as direct prooxidants (radical initiators), nor as antioxidants (radical terminators), but rather as accelerators of propagation.

This conclusion was probed using published chemical rate constants $k_r$ from the literature [18,21,24–26] (Fig. 3A). It turns out that of the two steps of radical propagation in biological systems, the attack of a lipid peroxyl radical ($\text{LOO}^\cdot$) towards a native lipid $L_0$ is rate-limiting, by approximately a factor of $10^7$ compared with the subsequent reaction of the generated carbon-centered radical ($L^\cdot$) with molecular oxygen [18]. That rate-limiting step can be bypassed and accelerated by a factor of 10 or more when thiol groups are present (Fig. 3A); the formation of a thiyl radical ($\text{RS}^\cdot$) becomes rate-limiting under these conditions. The thiyl radical then rapidly reacts with the substrate of the chain reaction, native lipid $L^\cdot$. 

Fig. 2. Biochemical effects of lipophilic thiols and related compounds in human IMR90 cells (A–E) and native rat brain membranes (F–H). (A) Induction of 8-isoprostane, a product of radical-mediated arachidonic acid oxidation ($n = 4$). (B) Content of polyunsaturated fatty acids (PUFAs) after 6 h treatment as percentage of all fatty acids. AA is arachidonic acid, DHA is docosahexaenoic acid ($n = 3$). (C) Example of a fatty acid GC of cells treated for 6 h with 100 μM 12SH. The trans-oleic acid peak is marked by a hash sign. (D) Quantification of trans-palmitoleic acid (Trans-POA, C16:1\(\Delta9\)) and trans-oleic acid (Trans-OA, C18:1\(\Delta9\)) after 6 h treatment as fraction of all fatty acids ($n = 3$). (E) Quantification of stearic acid (SA) and oleic acid (OA) after 6 h treatment ($n = 3$). (F) Production of malondialdehyde in rat brain membranes after short-term (0.25 h) or long-term (16 h) treatment with lipophilic compounds and iron/ascorbate as radical initiator ($n = 3$). (G) Malondialdehyde formation in rat brain membranes treated with 12SH without any radical initiator (mean ± range from $n = 2$). (H) Malondialdehyde formation in rat brain membranes treated with 12SH plus radical initiator iron/ascorbate (mean ± range from $n = 2$). CTRL in H represents treatment with the initiator alone. Asterisks denote: $*p = 0.01$, **$p = 0.001$ versus CTRL.
The propagation step of the reaction (red arrow) is the rate-limiting step of biological lipid peroxidation, as the subsequent reaction of the carbon-centered lipid radical (L•) with molecular oxygen to yield a new lipid peroxyl radical (LOO•) is much faster, by approximately a factor of 10^7 (ref. 18). As shown in this report, intramembrane thiol groups accelerate lipid peroxidation by offering a faster bypass to the commonly rate-limiting step via the formation of thyl radicals (RS•). The slowest of the involved bypass rate constants [24,25] is at least one order of magnitude higher than the commonly rate-limiting attack on L• (k_{rel} > 10^6 M^{-1}s^{-1} versus k_1 \approx 10^5 M^{-1}s^{-1}). Beyond their catalytic acceleration of lipid peroxidation, thyl radicals (RS•) are sufficiently reactive [21,26] to induce the oxidation of membrane proteins (Proteins) and the isomerization of monounsaturated fatty acids towards trans-fatty acids via an addition-fragmentation mechanism (RS-MUFAs). Rate constants were adopted from the following sources: k_1 < 10^2 M^{-1}s^{-1} (ref. 18); k_2 > 10^3 M^{-1}s^{-1} (ref. 25); k_3 > 10^6 M^{-1}s^{-1} (ref. 24); k_4 \approx 10^6 M^{-1}s^{-1} (ref. 18); k_5 \approx 10^2-10^3 M^{-1}s^{-1} (ref. 26); k_6 \approx 2 \times 10^2 M^{-1}s^{-1} (ref. 21).

(B) Relative reaction rates of mitochondrial lipid peroxidation determined from specific, applicable rate constants and substrate concentrations in both pathways bear the potential to compare Supplementary Discussion S2 and Supplementary Fig. S3 for details and caveats. Therefore, even minor physiological adjustments of the substrate concentrations in both pathways bear the potential to directly influence the overall rate of propagation, as illustrated in Supplementary Discussion S2 on the examples of fetal rat liver mitochondria (with their lower concentration of bisallylic positions) and C. elegans mitochondria (with their higher concentration of intramembrane cysteine). Thus, none of the two fates of a lipid peroxyl radical (LOO•) appears to be rate-limiting from the outset, which might rationalize why indeed both pathways have been subject to recent evolutionary optimization [8,9,27-29].

Beyond the experimentally confirmed acceleration of lipid peroxidation (Fig. 2A,F,H) and the generation of trans-fatty acids (Fig. 2C and D), published rate constants [26,30] also predict the attack of thyl radicals on proteins, involving complex secondary rearrangements [30,31] (Fig. 3A). Hence, protein carbonyl formation and the cellular stress response to protein damage were investigated in docosahexaenol-treated human IMR90 fibroblasts. Protein carbonyls are a widely consulted, nonspecific marker of protein oxidation reflecting the oxidation of various amino acids [32,33]. Protein carbonyls were significantly induced by single docosahexaenol administration, peaking after 24 h of treatment (Fig. 4A). Somewhat earlier, but on about the same time scale as for lipid peroxidation, a significant induction of protein poly-ubiquitination was observed (Fig. 4B), which is the prime cellular protein degradation signal and often used as general marker of protein physicochemical damage. Consistent with damage from lipophilic thyl radicals, membrane proteins were much more affected by poly-ubiquitination than cytosolic proteins (Supplementary Fig. 5A), resembling the pattern of protein oxidation in human post mortem samples [33]. Again, lipophilicity and the presence of a thiol group were prerequisite for increased protein poly-ubiquitination (Supplementary Fig. 5B). Docosahexaenol treatment also led to an induction and activation of the LC3B protein involved in autophagic vesicle formation to degrade proteins, whereas the investigated autophagic cargo adaptors remained constant (Supplementary Figs. 5A and D). In addition to protein degradative pathways, the protective protein-refolding heat-shock response (HSP70) was strongly induced (Fig. 4C). Pro-apoptotic cell death cascades were also activated to some extent, as reflected by caspase 3 cleavage (Fig. 4D) and a loss of the caspase 3 substrate PARP1 (Fig. 4E). A dual strategy like this is not uncommon in acutely damaged cells that need to “decide” whether to defend or to self-destruct for the good of the whole organism. The latter decision is especially relevant when a cell is permanently damaged on the DNA level and might become a tumor cell. Significantly higher levels of the specific DNA damage response (HSP70) were investigated in dodecylthiol-treated rat liver mitochondria (Fig. 4F). This indicates that the case DNA damage is a common consequence of lipid peroxidation and thought to accrue via electrophilic peroxidation products such as malondialdehyde [34,35].

To better appreciate the observation that MUFAs, PUFA and membrane proteins were all measurably affected by the presence of lipophilic thiols, a survey of the main thyl radical reaction pathways in the lipid bilayer and in the aqueous space was assembled (Supplementary Fig. S3). Comparing reaction rates v_{rel} calculated from rate constants and substrate concentrations in rat liver mitochondria as in Fig. 3B, the attack on PUFA emerged as fastest pathway of thyl radical dissipation in the IMM (v_{red} = 9.8 \times 10^5 s^{-1}), followed by the attack on proteins (v_{red} = 2.0 \times 10^3 s^{-1}) and MUFAs (v_{red} = 1.8 \times 10^3 s^{-1}). Phenolic antioxidants such as ubiquinone appear to be inefficient in the detoxification of thyl
radicals ($v_{red} = 3.3 \times 10^3 \text{s}^{-1}$), in agreement with experimental observations [36]. In contrast, thiyl radicals with access to aqueous ascorbate or glutathione were predicted to be efficiently reduced [26,30,31] by these agents (ascorbate: $v_{red} = 3.4 \times 10^5 \text{s}^{-1}$; glutathione: $v_{red} = 3.9 \times 10^5 \text{s}^{-1}$). This might explain why experimental thiol toxicity in cell culture required pervasive lipophilicity (Fig. 1). Radicals with only moderate lipophilicity, polar moieties close to the thiol group, or highly volatile compounds may still be reduced by ascorbate in the outer contact layer of the membrane, i.e. the region of perturbed water. Precedents for such an interphase one-electron reduction by ascorbate are the recycling of tocopherol radicals [37] and membrane protein tyrosine and tryptophan radicals [38]. The well-known and very rapid reaction of thiyl radicals with molecular oxygen [30,31,39] to form thylperoxyl radicals appears to be of only minor importance ($v_{red} = 4.5 \times 10^3 \text{s}^{-1}$) in the respiring mitochondrion due to its low oxygen concentration of 2 μM or less [40]. The calculations in Supplementary Fig. S3 provide a quantitative rationale for the much higher toxicity of thiyl radicals inside lipid bilayers despite a generally marginal influence of solvent properties on thiyl radical formation and reactivity [24,39,41]: the heightened toxicity of thiyl radicals in membranes rests upon the absence of an
effective thyl radical scavenger in the lipid bilayer.

The physiological relevance and scope of the described toxic activity of lipophilic thiols was further explored. Prooxidative mechanisms are often exaggerated in cell culture due to the encountered high oxygen partial pressure of 20% (2%–14% in vivo). However, dodecylthiol was similarly toxic at 1% oxygen in the incubator as it was at 20% oxygen (Fig. 5A), in accordance with the basic expectation from kinetic rate constants: the rate-limiting step of propagation (Fig. 3A) would still be expected to be RS• formation (k₂ > 10⁷ M⁻¹ s⁻¹) rather than oxygen addition to L• (k₄ ≈ 10⁸ M⁻¹ s⁻¹). In respiring mitochondria, though, the very low concentration of oxygen as compared to cysteine (approximately 1/1000) substantially scales down this difference (Fig. 3B).

Therefore, to probe the potential relevance of chain transfer catalysis under physiological conditions in vivo, C. elegans nematodes were administered with different concentrations of dodecylthiol in the feed (Fig. 5B–D). In these animals, dodecylthiol also induced lethality, but only after a significant retardation period of about 6 days (Fig. 5B). Nevertheless, a rapid prooxidative effect in vivo was observed whose apparent steady-state maximum was already reached after 6 h (Fig. 5C). Survival curves recorded in chronically dodecylthiol-fed C. elegans revealed late-life toxicity starting at 10 μM (Fig. 5D), similarly as in cell culture (Fig. 1). With escalating doses, however, the toxic effect reached a plateau (at 250 μM and 500 μM), as the drug always required at least 5 days of administration before the first worm died through the drug. In other words, the minimum lifespan was constant and distinctly higher than zero, whereas mean and maximum lifespan exhibited conventional hyperbolic dose-responses (Fig. 5D, inlay). These observations match with a drug that does not initiate radical chain reactions (as peroxides would have done), but rather accelerates ongoing radical chain propagation. Accelerated propagation is prooxidative because it makes any interception by antioxidants, physical separation or repair mechanisms less effective. Hence, the prooxidative toxicity of lipophilic thiols is capped and appears to depend entirely on the level of endogenous radical initiation (Fig. 2G and H). Potentially, this behavior offers applicability as cytostatic drugs in tumors that are characterized by significantly elevated initiator radical production [42–44].

The lifespan-shortening effect of dodecylthiol in C. elegans provides an unsophisticated explanation why long-lived animals proportionally avoid cysteine in the cellular membrane with the highest initiation rate, i.e. the inner mitochondrial membrane [8,9]. Especially here, radical

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Fig. 5. Toxicity of lipophilic thiols under hypoxic conditions and in vivo. (A) HT22 cells were treated with the indicated compounds for 3 days at 1% O₂ or 20% O₂. (B) Toxicity of 100 μM 12SH in C. elegans nematodes under different feeding conditions (variable amounts of E. coli bacteria; 53 ± 12 worms per group; mean ± range from n = 2). (C) 8-Isoprostane formation in C. elegans animals treated as designated (~10,000 worms per group; mean ± range from n = 21). (D) Survival analysis of C. elegans worms administered with varying concentrations of 12SH in the feed starting at day 1 (marked by an arrow; 75 ± 6 worms per group). The arrowheads identify the times of 8-isoprostane analysis as provided in C. The inlay shows the mean lifespan, the minimum lifespan and the maximum lifespan of the animals determined from the survival plot.
propagation rather than radical initiation may be rate-limiting for the overall rate of lipid peroxidation which predicts the biological rate of aging [29,45]. We propose that protein polysulfidation (in the lipid bilayer) and protein glutathionylation (in the aqueous space) are functional complements of mitochondrial cysteine avoidance since both effectively reduce the number of free thiol groups in proteins and appear to be selectively engaged by mitochondria [46,47]. Adapting these mechanisms, an even higher reduction in protein free thiol groups could be achieved than the permanent ~80% reduction realized in mitochondrially encoded proteins through codon changes [8,9].

Declaration of competing interest

The University Medical Center of the Johannes Gutenberg University has filed a patent regarding the use of chain transfer agents as medicinal drugs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101628.

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