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Perspective

trans-Fatty acids and radical stress: What are the real culprits?

Chryssostomos Chatgilialoglu,[a,*] Carla Ferreri,[a] Ioannis N. Lykakis[a] and Peter Wardman[b]

[a]*ISOF, Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129 Bologna, Italy*
[b]*University of Oxford, Gray Cancer Institute, PO Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, UK*

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Abstract—Free radical-catalysed *cis–trans* isomerization of unsaturated lipids in biomimetic models and their significance in eukaryotic cells have been explored in the last few years, an integrating hypothesis being that *trans*-fatty acids have their origins in both dietary sources and from isomerization of natural isomers by an endogenous radical stress. In this perspective, a summary of the achievements and a discussion of the possible biological sources of isomerizing radical species are given, indicating a need for further research on thiyl radical generation in biological systems. In this context, crucial questions remain to be answered by free radical research involving membrane lipids, thus contributing to lipidomics and embracing biology and medicine.
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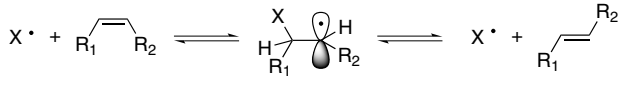
1. Endogenous versus exogenous pathways of *trans* lipids

An increasing number of studies have explored the presence of *trans*-fatty acid residues in living systems. This is a very lively field of interdisciplinary research spanning from chemistry to microbiology, pharmacology, biology, and, of course, medicine. The configuration of isolated double bonds in naturally occurring lipids of eukaryotes is *cis*. However, *cis–trans* isomerization of lipid double bonds occurs in some bacteria enzymatically,[1,2] and *trans* isomers are present in mammalian cells after dietary supplementation of chemically modified fats.[3] The latter is important nutritionally, since *cis*/*trans* isomeric mixtures of fats result from vegetable and fish oils manipulated by partial hydrogenation or deodorization processes employed in the food-processing industry. In modified fats, the structures of *trans*-fatty acid residues consist of geometrical and positional isomers having un-shifted and shifted double bonds, respectively, compared to natural *cis* compounds. Some *trans* geometrical isomers found in living organisms can only arise via an endogenous transformation of the naturally occurring *cis* structures and are correlated with radical stress produced during physiological and pathological processes.[4–8]

2. The free radical path

Several free radicals, including the biologically relevant thiyl radicals ($RS^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$), are known to isomerize double bonds.[9,10] Scheme 1 shows the reaction mechanism, involving reversible addition of a radical $X^{\bullet}$ to the double bond to form a radical-adduct. The reconstitution of the double bond is obtained by β-elimination of $X^{\bullet}$, and the result favours *trans* geometry, the most thermodynamically stable configuration. It should be noted that (i) $X^{\bullet}$ acts as a catalyst for *cis–trans* isomerization and (ii) positional isomers cannot be formed because the mechanism does not allow a double-bond shift.

The *cis–trans* isomerization by $RS^{\bullet}$ is an efficient process and detailed kinetic data for the four reactions in Scheme 1 are available in the case of methyl oleate with $HOCH_2CH_2S^{\bullet}$ radical.[11,12] On the other hand, the available kinetic data for the *cis–trans* isomerization by $NO_2^{\bullet}$ suggest that this radical cannot be very efficient as an isomerizing species, and in a biological environment this reaction should not play a role.[9] In the omega-6 series of poly-unsaturated fatty acids (PUFAs), *cis–trans* isomer-



Scheme 1. Reaction mechanism for the *cis–trans* isomerization catalysed by $X^{\bullet}$ radical.

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* Corresponding author. Tel.: +39 051 639 8309; fax: +39 051 639 8349; e-mail: chrys@isof.cnr.it

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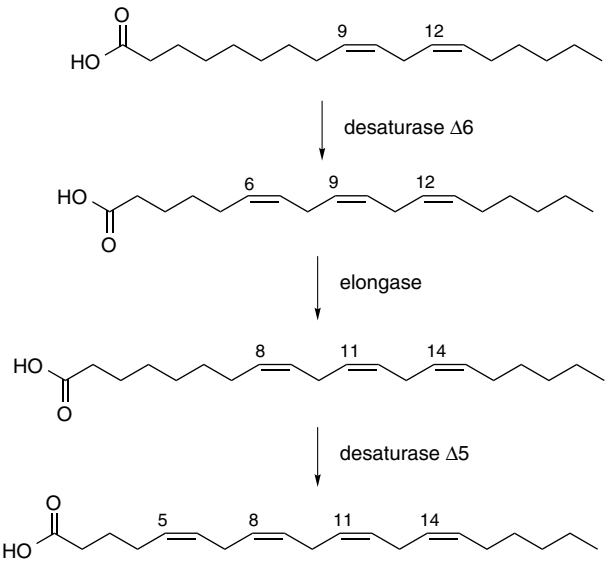
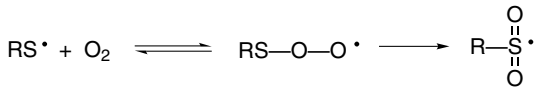
ization of methyl linoleate,[13] γ-linolenate[13] and arachidonate[4] catalysed by RS· has been studied in some detail. Each isolated double bond in the PUFA behaves independently as discussed above. Moreover, isomerization is a stepwise process with the formation of mono-*trans* isomers, followed by di-*trans* isomers and so on, the isomeric composition being regulated by the relative thermodynamic stability.

3. Compartmentalization and isomerizing species

As a model of a biologically occurring process, the *cis–trans* isomerization of unsaturated fatty acid residues catalysed by thiyl radicals was first studied in large unilamellar vesicles prepared by the extrusion technique (LUVET). The choice of an amphiphilic $HOCH_2CH_2S^{\cdot}$ radical, generated from 2-mercaptoethanol, facilitated the exploration of geometric isomerization in model membranes by the action of diffusible thiyl radicals. However, glutathione (GSH) with different lipophilicity was also used and gave similar results. Using vesicles from SAPC (stearoyl arachidonoyl phosphatidylcholine), soybean lecithin or egg yolk lecithin, it was possible to demonstrate that the double bonds closest to the membrane polar region are the most reactive towards attack by diffusing thiyl radicals.[13,14] For example, arachidonic acid residues in vesicles were more reactive than oleic and linoleic acids; two positions, the double bonds at positions 5 and 8 out of the four present in this lipid, were transformed preferentially. From studies to date, arachidonic acid residues in membrane phospholipids emerged as very important pointers to help distinguish endogenous *trans* isomers, formed by radical processes, from the exogenous *trans* isomers derived from dietary contributions.[4,14] The interplay between exogenous and endogenous pathways for isomerization of arachidonic double bonds is shown in Scheme 2, where the biosynthesis from the precursor (linoleic acid) is detailed: the two double bonds of positions 11 and 14



Scheme 2. Enzymatic fatty acid transformations.



Scheme 3. Thiyl radicals in the presence of molecular oxygen.

are provided by dietary linoleic acid (which can be *cis* or *trans*, depending on the food), whereas the other two double bonds (positions 5 and 8) are formed by desaturase enzymes, which produce selectively *cis* unsaturation. Assays on the in vitro desaturation or elongation of mono-*trans* isomers of linoleic acid by rat liver microsomes showed that 9*cis*,12*trans* isomer was better desaturated, whereas 9*trans*,12*cis* isomer was better elongated.[15] In vivo, the double bonds at positions 5 and 8 of arachidonic acid, stored in membrane phospholipids, can only have a *cis* configuration, unless these positions are involved by an isomerization process occurring in membranes by diffusible thiyl radicals.

With a library of *trans* arachidonic isomers available, generated in model systems, research could focus on erythrocyte membrane phospholipids, which are the preferential storage for arachidonic acid after biosynthesis. Monitoring of this omega-6 fatty acid is important also during nutritional studies, which established that *trans*-fatty acids are incorporated in cell membranes, because the *trans* dietary precursor can be processed in vivo.

It is worth pointing out that the presence of 0.2 mM molecular oxygen (a few times higher than the molarity of typical well-oxygenated tissues) did not influence the effectiveness of the geometric isomerization by thiyl radicals. It is well known that thiyl radicals add reversibly to oxygen, the equilibrium constant (K) being ca. 3200 M^{-1}, the adduct rearranging slowly to the corresponding sulfonyl radical (Scheme 3).[16] Under these experimental conditions, these reactions are considered to be unimportant, although we recently found that methanesulfonyl radical ($MeSO_2^{\cdot}$) is also an efficient isomerizing agent of unsaturated phospholipids in LUVET but only in the absence of oxygen. On the contrary, our preliminary results indicate that $NO_2^{\cdot}$ radicals are not efficient isomerizing species in LUVET in the presence or absence of molecular oxygen.[17]

4. Are thiyl radicals the real culprits in the endogenous pathway?

On the basis of the efficiency of the thiyl radical-catalysed *cis–trans* isomerization in vitro and the presence of many sulfur-containing compounds in the cell, two studies were carried out in order to demonstrate that the *trans* geometry of lipid double bonds can be endogenously generated within membrane phospholipids.[6,8] Cultures of human leukaemia cell lines (THP-1) were incubated in the absence and presence of thiol compounds, ensuring that no contribution of *trans* compounds could come from the medium.[6] In parallel experiments, levels of a few millimolar thiol compounds

were added to the cell cultures during incubation, and the comparison of isomeric trends was carried out. Under standard growth conditions in the absence of thiols, a baseline content of *trans* lipids, not less than 1.2% up to 3.9% of the total fatty acid residues in membrane phospholipids, was measured. After the addition of the amphiphilic 2-mercaptoethanol, it increased up to 5.6% of the main fatty acid residues. Moreover, when a radical stress by γ-irradiation is artificially produced in the cell cultures with thiol present, a larger isomerization effect could be seen with *trans* lipid formation, up to 15.5% in membrane phospholipids. The fatty acid residues most involved in this transformation were arachidonate moieties, as expected. In the second study, the spontaneous occurrence of endogenous *trans*-fatty acids in tissue and erythrocyte phospholipids of young adult rats fed a diet completely free of *trans* isomers showed patterns similar to the one induced by radical stress (γ-irradiation) where the *trans*-fatty acids can reach very high amounts (about 8% of total fatty acids).[8]

The role of $NO_2^{\cdot}$ in radical-mediated isomerization of all-*cis* arachidonic acid was first reported by Jiang et al.[18] They described the formation of *trans*-arachidonic acids by exposure of either arachidonic acid or human platelets to $NO_2^{\cdot}$. Although recognized only by MS spectra and unsatisfactorily separated by HPLC and GC analyses, the authors claimed that mono-*trans* isomers were produced in the $NO_2^{\cdot}$-mediated reaction, and used them to detect *trans*-arachidonic acid in vivo. Studies involved plasma and urine of human that had received deuterium-labelled *trans*-arachidonic acid supplements.

Kermorvant-Duchemin et al. recently described *trans*-arachidonic acid isomers (TAAs) as products of 'nitrative' stress in mice exposed to hyperoxia,[7] ascribing TAAs to the reaction of $NO_2^{\cdot}$ with arachidonic acid, on the basis of a series of reactions stimulated by hyperoxia including the enhanced oxidation of nitric oxide ($NO^{\cdot}$) to $NO_2^{\cdot}$ and increased formation of peroxynitrite formed via reaction of $NO^{\cdot}$ with superoxide. Less TAAs were detected on inhibiting $NO^{\cdot}$ synthase (NOS). However, whether $NO_2^{\cdot}$ is the isomerizing radical seems doubtful.

The ability of the $NO_2^{\cdot}$ radical to initiate *cis–trans* isomerization has indeed been recognized for over 40 years.[9] However, consideration of kinetic data and product studies on the reaction of $NO_2^{\cdot}$ with olefins led to the conclusion that in well-oxygenated cells, catalysis of *cis–trans* isomerization by $NO_2^{\cdot}$ should be an unimportant process, because oxygen reacts rapidly with a carbon-centred radical intermediate, thus leading mostly to lipid peroxidation.[9] Furthermore, $NO_2^{\cdot}$ has a high reactivity towards both thiols and urate, the former antioxidant generating thiyl radicals on reaction with $NO_2^{\cdot}$ and the latter being an important 'sink' for $NO_2^{\cdot}$ in the vascular compartment where the thiol concentration is very low except within the erythrocyte.[19] It could thus be confidently predicted that within the cell (e.g., the cytosolic compartment), production of $NO_2^{\cdot}$ is followed within a few tens of microseconds by reaction with (mainly) glutathione and the formation of thiyl radicals. Therefore, it is more likely in the recent study that the latter is the isomerizing species, that transforms arachidonic acids to the *trans* isomers, and not $NO_2^{\cdot}$,[7] for which it was also estimated that the diffusion distance at typical intracellular thiol levels is as low as ~0.2 μm.[19]

In a commentary that accompanies the study of Kermorvant-Duchemin et al,[7] Smith and Connor also interpret the role of $NO_2^{\cdot}$ as being critical to the isomerization of arachidonic acid in the vascular compartment.[20] However, the overall reactivity of $NO_2^{\cdot}$ was not taken into account; literature data suggested that the high levels of urate in circulating plasma, together with the high reactivity of $NO_2^{\cdot}$ towards urate, result in a diffusion distance of $NO_2^{\cdot}$ in vascular space of <1 μm,[19] much less than the diameter of a single endothelial cell. Further, $NO_2^{\cdot}$ is also very reactive towards other antioxidants, notably ascorbate,[21] which must also be considered, along with glutathione and urate, when discussing pathological effects of nitrative stress in conditions such as retinopathy. The outcome of free radical generation is frequently a balance among several competitive processes, and is certainly the case with species as reactive as $NO_2^{\cdot}$.

Such considerations prompt us to comment on the common misconception that the role of antioxidants is simply to 'scavenge' free radicals. Particularly in the case of scavenging by thiols, the product (thiyl) radical—an inevitable feature of spin conservation—may well be reactive, indeed damaging.[22] It is conceivable and suggested that in the experiments of Kermorvant-Duchemin et al., the trapping of $NO_2^{\cdot}$ radicals by endogenous thiols, such as glutathione, occurred with formation of thiyl radicals, the 'real' isomerizing species. Indeed, this hypothesis is supported by the previously mentioned measurements of TAA formation in THP-1 cells in vitro, which increases under non-nitrosative (thiyl radical) stress.[6] It is important not to overlook literature data when a new biological pathway is discussed such as in the case of *trans* lipid formation. The endogenous pathway by radical stress has been only recently suggested and the need of further and more detailed investigations on the isomerizing species has been emphasized. Therefore, the emphasis in both the article by Kermorvant-Duchemin et al. and the commentary on the modification of lipids achieved only by reactive oxygen and nitrogen species appears to be incorrect. The role of antioxidants must not be neglected. As we have described here, antioxidants have potential to be either protective (ascorbate, urate) or can actually amplify damage (thiols), as well as defining or limiting the site of action of radicals or their spatial (diffusional) characteristics.

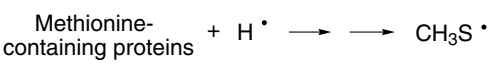
5. Small diffusible sulfur-centred radicals as possible candidates

A crucial question is what are the candidates for the biological production of diffusible thiyl radicals? Experiments designed to answer this question contribute to

the lipidomics of radical stress, and also provide a chemical biology approach for improving the library of *trans* isomers, including some regiospecific isomers formed in vivo. Understanding the molecular mechanisms provides a route to discovering inhibitory pathways, which can perhaps act in vivo as protective systems for preserving lipid geometry.

Possible scenarios for the production of diffusible thiyl radicals were expanded by the discovery of free-radical damage to sulfur-containing proteins resulting in consequent isomerization of membrane lipids. Such tandem protein–lipid damage was studied in methionine-containing proteins, using a biomimetic model of a *cis*-monounsaturated lipid vesicle (DOPC or POPC) and a sulfur-containing peptide or protein, such as amyloid-β or RNase A.[23,24] The protein was added at micromolar levels to a millimolar vesicle suspension. γ-Irradiation induced protein damage, with the release of a low-molecular-weight thiol (CH_3SH) from methionine residues which produces thiyl radicals ($CH_3S^{\bullet}$) that diffuse rapidly in the lipid bilayer to catalyse isomerization of double bonds (Scheme 4). Thus, the formation of *trans* residues in vesicles can be sensitive to even nanomolar levels of protein damage via a catalytic mechanism initiated by thiyl radicals. Therefore, detection of lipid isomerization is also a convenient tool for signalling protein radical damage, with sensitivity not easily achieved with other techniques. From these biomimetic chemical studies a conclusive picture emerged that thiyl radicals are efficient catalysts for *cis–trans* isomerization of lipids in bilayers, and are a potentially important route to radical damage to biological components as well as providing sensitive tool to detect early radical stress in cells.

Considering biologically relevant sulfur species, the simplest thiol, hydrogen sulfide (H_2S), is emerging as a naturally occurring gas with roles in nervous and cardiovascular systems, and pathological conditions such as inflammation. At least three enzymes are responsible for hydrogen sulfide formation in vivo.[25–27] Chemically, the reactions of the simplest S-centred radical ($HS^{\bullet}$) are not as well known as those of other thiyl radicals, although a few papers have characterized some key reactions in the aqueous phase.[28,29] Different radicals (e.g., $HS^{\bullet}$ and $HSS^{\bullet}$) and radical anions (e.g., $S^{\bullet -}$, $HSS^{\bullet 2-}$ and $HSSH^{\bullet -}$) can be produced, but their roles as isomerizing agents are not known. From the biological perspective, involvement of H_2S both in inflammation linked to radical stress, and in signalling between neuronal cells, is suggestive of complex reactivity. The role of hydrogen sulfide as precursor of diffusible S-centered radicals that act as isomerizing agents merits further research in life sciences, free radicals and lipidomics to establish the molecular basis of its biological effects.



Scheme 4. Proposed mechanism for the formation of thiyl radicals from methionine-containing protein.

6. Conclusions

The work to date has pointed out the relevance of *cis–trans* isomerization by a free radical pathway. The outcome of generating any radical in biological milieu is likely to be complex, with several potential targets and antioxidants competing at both primary and secondary levels, as well as antioxidant distribution varying enormously in the different compartments, viewed from both a microscopic and macroscopic level. Therefore, selective generation and reactivity of different radicals are topics needing further work, with particular emphasis on competitive processes due to relative reactivity and concentration of substrates in the microenvironment. Production of protein-bound thiyl radicals, which is regarded as a physiologically occurring process, should also be evaluated.

The involvement of free radicals in biological and pathological processes is an exciting and dynamic area of research. With the growing sophistication of models for characterizing these processes, it is important not to neglect the established body of quantitative data concerning free-radical reactivity.

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