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Quantification and Mechanisms of Oxidative Stress in Chronic Disease †

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† Presented at Natural Products and the Hallmarks of Chronic Diseases—COST Action 16112, Luxemburg, 25–27 March 2019.

Published: 17 April 2019

Abstract: There is now strong evidence that the redox environment inside cells is very different to that outside the cell, and that many extracellular environments are both more oxidizing and also subject to extensive oxidation. This difference in redox environments results in significant changes in oxidation chemistry and biology, altered redox equilibria, and antioxidant defense mechanisms. It is also increasingly apparent that oxidation events both inside and outside cells (extracellular oxidation) play a critical role in driving many diseases. Many extracellular proteins are highly abundant, long-lived and relatively poorly protected against damage. They can therefore accumulate high levels of modification during ageing and chronic disease, resulting in their use as biomarkers of long-term oxidative stress. Furthermore, increasing evidence supports the hypothesis that oxidized extracellular matrix materials play a key role in determining cell function and fate.

Keywords: protein oxidation; post-translation modification; extracellular matrix; cardiovascular disease; atherosclerosis; peroxynitrous acid; nitration; hypochlorous acid; chlorination

Biological materials are exposed to endogenous and exogenous free radicals and two-electron oxidants on a continual basis [1]. The generation and subsequent reactions of these species are limited by defense systems within cells and organisms, including low-molecular-mass materials that act as scavengers (e.g., ascorbic acid, tocopherols, glutathione and other thiols, urate, quinols, carotenoids, polyphenols), enzymes that remove oxidants (e.g., superoxide dismutases), enzymes that remove oxidant precursors (e.g., catalases, glutathione peroxidases and peroxiredoxins), and enzyme systems that repair or remove damaged materials (DNA repair enzymes, phospholipases, methionine sulfoxide reductases, disulfide reductases/isomerases, sulfiredoxins, proteasomes, lysosomes, proteases) [1].

Despite the presence of these mechanisms, elevated levels of oxidative damage have been reported to be present in multiple human, animal, microbial and plant systems [1]. This can be a result of increased oxidant formation, a decrease or failure of defense systems, or both. In many cases both may be important, as defense systems are also subject to damage, or depletion of critical co-factors. Ageing also results in declines in enzyme activity, and a decreased level of metabolites and essential trace elements. This decline can be accelerated by environmental factors and disease.

A large number of oxidants can be generated in vivo, and these vary markedly in their reactivity and specificity [1,2]. Both one-electron (radical, e.g., hydroxyl radicals, HO•) and two-electron oxidants (e.g., hypochlorous acid, HOCl; peroxynitrous acid, ONOOH; singlet oxygen, $^1O_2$; peroxides) can be formed, with these having markedly different reactions kinetics and mechanisms of reaction [2]. Whilst many radicals react with high rate constants (i.e., very rapidly), this is not always true, with some radicals being relatively long-lived, and poorly reactive [2]. A similar pattern is seen with two-electron oxidants, with some having very high rate constants for reaction with

particular targets (e.g., HOCl, $^1O_2$ with sulfur-containing and aromatic amino acids), and others low rate constants [2]. The damage generated by these species is therefore highly variable and complex [2]. HO• is unselective and causes widespread indiscriminate damage, whilst other oxidants (e.g., H_2O_2) may react slowly and with high selectivity (i.e., with very specific targets) [2]. The products generate by these reactions are also numerous and diverse [2–5]. In some cases, the products are generic (e.g., protein carbonyls [6–8]) and are formed by multiple different pathways, whereas in some other cases, the product is specific and diagnostic for a particular type of reactive species. Thus, the species 3-chlorotyrosine formed from the amino acid tyrosine (Tyr) is a specific product generated by the powerful two-electron oxidant, HOCl [9–11]. Highly reactive oxidants only diffuse short distances before reaction, whereas less reactive species have longer half-lives, can diffuse over longer distances, and induce damage at remote locations [2,12–14]. Reactive oxidants therefore usually give rise to site-specific damage at, or near, their sites of formation. Many oxidants can also give rise to secondary oxidants (e.g., radicals formed by the decomposition of lipid peroxides), of different reactivity and lifetimes when compared to the initial species, complicating the analysis of damage [2]. A greater understanding of the nature of potential oxidants, their reactivity, and the patterns and extents of damage that they induce, is therefore of major importance.

Oxidant formation can occur both *within* cells and *external* to them [1]. Electron leakage from mitochondria (and to a lesser extent other electron transport chains) is a major source of oxidants within cells, with this resulting in significant fluxes of superoxide radical anions (O_2^{-}•), and subsequently H_2O_2 as a result of the rapid spontaneous, and enzyme-catalyzed dismutation (by superoxide dismutases) of O_2^{-}• [1]. Acute and chronic inflammation is a major source of extracellular oxidant formation, as stimulation of neutrophils, monocytes and macrophages can result in the enzymatic formation of multiple oxidants including O_2^{-}•, H_2O_2, HOCl and ONOOH. HOCl is generated from H_2O_2 and chloride ions by the heme enzyme myeloperoxidase, which is released from intra-cellular storage granules of neutrophils, monocytes and some tissue macrophages [9–11]. The precursor of ONOOH, NO•, is formed via the inducible nitric oxide synthase of macrophages [12]. Lower levels of NO• are also generated by the constitutive nitric oxide synthase isoforms present in many cells. Concomitant with the formation of NO•, O_2^{-}•is generated at relatively high levels by plasma membrane NADPH oxidases (at the expense of intracellular NADPH), with subsequent rapid (diffusion-controlled) reaction of NO• with O_2^{-}• giving peroxynitrous acid, ONOOH [12,15].

Proteins have been shown to be major targets for many oxidants due to their high abundance and the high rate constants for reaction with many oxidants, both within cells and in extracellular compartments (e.g., the extracellular matrix, and in plasma and other fluids) [2,16]. With reactive species, such as HOCl and ONOOH, damage can occur at both side-chain (mainly at Cys, Met, Trp, Tyr and His) and backbone sites. In contrast, less reactive species, such as O_2^{-}• and H_2O_2, give rise to damage that is both limited and highly selective [2,12–14,16]. Thus, H_2O_2 primarily modifies Cys, and to a lesser extent Met, residues on proteins.

Protein damage *within* cells is often rapidly repaired (but *only* in the case of damage to Cys and Met), or removed via catabolism [2]. The situation outside (external) to cells is different, as extracellular materials are usually poorly protected against damage [17], and there is limited capacity for repair. The high abundance of extracellular proteins, their proximity to sites of oxidant formation, their (in general) long half-lives, and poor repair mechanisms, are likely to result in an accumulation of damage on these materials, particularly with increasing age, with this enhanced by disease.

Elastin is the most abundant extracellular matrix protein in elastic tissues (e.g., skin, lungs, arteries), and comprises 30–57% of the aorta by dry mass. Most elastin synthesis occurs during the early years of life, with limited synthesis in adults, therefore most elastin is as old as the host. It is therefore believed to accumulate high levels of modifications over time. Mature elastin is synthesized from monomeric tropoelastin (TE), with the latter undergoing complex processing to form mature elastic fibers. As considerable evidence supports ONOOH formation in the inflamed artery wall (e.g., [18–22]), we hypothesized that TE would be highly susceptible to modification and structural alteration by ONOOH, with consequences for the function of TE. This damage to TE may play a role

in the development of cardiovascular disease, as modified matrix species have been implicated in atherosclerotic lesion development and rupture [18–28].

We have shown that TE is highly sensitive to ONOOH, with the formation of dimers and higher oligomers and fragments (detected by SDS-PAGE with silver staining or Western blotting) and nitration of tyrosine (Tyr) residues to give 3-nitroTyr, detected by both amino acid analysis and mass spectrometry (MS) peptide mass mapping [22]. This damage can be detected with equimolar (i.e., on average, single oxidant hits) or greater levels of oxidant and increases in a dose-dependent manner [22]. Quantification of Tyr loss and 3-nitroTyr formation (by UPLC) indicates that extensive Tyr modification can occur with up to two modified Tyr formed per protein molecule, and up to 8% conversion of the added ONOOH converted to 3-nitroTyr [22]. These effects were modulated by bicarbonate, a competitive target for ONOOH. 3-nitroTyr formation was detected at 12 of 15 Tyr sites in TE treated with equimolar or higher levels of ONOOH. Label-free MS quantification has revealed extensive nitration (>50% modification) at some sites. Four Tyr residues have also been shown to be involved in the formation of inter-and intra-molecular di-tyrosine cross-links, with these characterized using an ^{18}O labelling MS approach [22,29,30].

TE treatment with ONOOH lowered the concentration at which TE assembles to give larger structures (as determined by turbidity and sedimentation experiments; Lorentzen et al., unpublished data), increased the rate of this process, and markedly impaired the reversibility of this process (as determined by dynamic light scattering; Lorentzen et al., unpublished data)). Scanning electron microscopy indicated the presence of abnormal TE structures (Lorentzen et al., unpublished data). Studies on human atherosclerotic lesions using immunohistochemistry showed colocalization of 3-nitroTyr with elastin epitopes, which is consistent with the generation of these modifications in vivo. In addition, a significant association of 3-nitroTyr-containing proteins and elastin was detected with lipid deposits in the artery wall [22]. Together these data suggest that exposure of TE to ONOOH gives rise to marked chemical, structural and functional changes to TE and alters extracellular matrix assembly. This damage appears to accumulate in human arteries with age, and during the development of atherosclerosis.

Funding: This article is based upon work from COST Action NutRedOx-CA16112 supported by COST (European Cooperation in Science and Technology) and the Novo Nordisk Foundation (grant: NNF13OC0004294 to MJD).

Acknowledgments: The author is grateful to the many colleagues and collaborators who have contributed to the studies reviewed in this article.

Conflicts of Interest: The author declares no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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