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Amino acid and protein oxidation in cardiovascular disease

Review Article

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Summary. Substantial evidence suggests that oxidative events contribute to the pathogenesis of atherosclerotic heart disease. For example, animal model data and numerous *in vitro* studies point to specific pathways as participants in disease initiation and progression. Moreover, recent clinical studies demonstrate clinical utility in monitoring systemic levels of protein-bound nitrotyrosine as a predictor of risk for coronary artery disease, atherosclerotic burden, and response to statin therapy. However, a definitive cause-and-effect relationship between oxidation and atherosclerosis has yet to be established, and multiple recent large prospective "antioxidant" intervention trials have failed to significantly impact upon disease risk and progression. In this review we highlight why such failures should not be taken as an indictment of the "Oxidation Hypothesis." Emphasis will be placed on discussion of molecular markers whose structures convey information about oxidation pathways leading to their formation, and which appear to be mechanistically linked to the disease process. Only through rational design of targeted interventions aimed at suppressing distinct oxidation pathways, with concomitant monitoring of antioxidant efficacy in human clinical studies, will answers to the role of oxidation in the pathogenesis of human atherosclerosis be defined.

Keywords: Atherosclerosis – Inflammation – Lipid peroxidation – Myeloperoxidase – Nitration – Chlorination – Free Radical

Introduction

Lipoprotein oxidation, atherosclerosis and inflammation

Atherosclerosis is the leading cause of death in industrialized societies. Like other chronic degenerative diseases of aging, oxidative damage of macromolecules is thought to play a role in the disease process. However, the relevant oxidation pathways have only recently begun to be unraveled. A wealth of clinical, pathological, biochemical and genetic data support the notion that atherosclerosis is a chronic inflammatory disorder (Ross, 1993; Libby, 1995). One of the earliest events in the atherosclerotic process is the recruitment of inflammatory cells into subendothelial tissue. A cascade of biochemical events accompanies these changes, leading to the development of a local inflammatory process within the artery wall. Acute phase reactants (e.g. C-reactive protein), sensitive but non-specific markers of inflammation, are enriched in early and late stages of atherosclerotic lesions (Vlaicu, 1985; Reynolds, 1987). Their detection as blood borne markers that predict risk for CAD in epidemiological trials is becoming part of routine preventive risk assessment (Ridker, 1997; Danesh, 1998; Ridker, 2001).

How inflammation contributes to the development of atherosclerosis is unclear, but the oxidation of LDL, the major carrier of cholesterol in blood, is thought to play an important role (Berliner, 1995; Podrez, 2000a). Oxidized LDL and bioactive lipid oxidation products can trigger many of the biological events that occur in early atherosclerosis. Furthermore, LDL recovered from atherosclerotic lesions has numerous biochemical properties consistent with oxidation of the lipoprotein (Berliner, 1995; Hazen, 1999a; Podrez, 1999; Podrez, 2000a). Only recently have some of the chemical pathways involved in LDL, protein and lipid oxidation *in vivo* become established. This information is critical in development of mechanism-based therapies. For example, vitamin E (α-tocopherol) is widely employed as an antioxidant. While it is effective at blocking

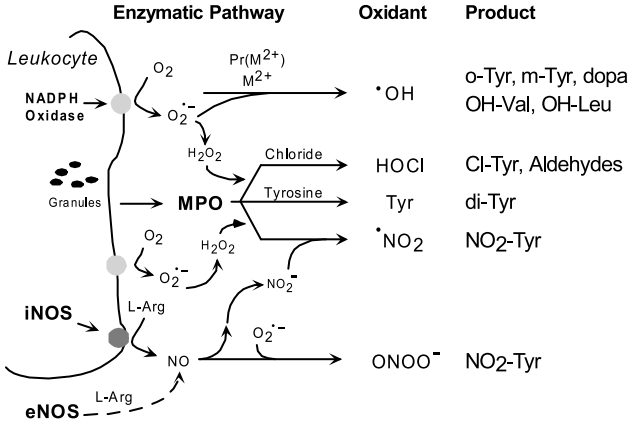

Fig. 1. Pathways of amino acid and protein oxidation that occur in human cardiovascular disease

free metal ion-catalyzed oxidation reactions *in vitro*, it is ineffective at blocking many oxidation pathways that appear to be of more physiological relevance, such as MPO-generated reactive nitrogen or halogenating species, or the nitric oxide-derived oxidant peroxynitrite (Podrez, 1999; Podrez, 2000a; Rubbo, 2000; Heinecke, 2001).

Pathways of protein oxidation in human atherosclerosis and inflammation

Much of the progress in defining the molecular mechanisms accounting for protein and lipoprotein oxidation in human atherosclerotic tissues arises from use of stable molecular markers for distinct oxidation pathways. These ''Molecular Fingerprints'' are informative in determining oxidation pathways operative *in vivo*. Figure 1 represents an overall scheme of pathways for which enrichment in Molecular Fingerprints at all or some of the stages of atherosclerosis have been demonstrated. The pathways leading to their formation are thus implicated in promoting protein and lipoprotein oxidation in human atherosclerotic tissues. We will use this scheme as a road map for discussion of each of the indicated chemical mechanisms likely operative *in vivo*. However, before discussion of each pathway, we will first discuss the source of initial oxidizing equivalents – superoxide ($O_2^{\bullet -}$).

Sources of reduced oxygen species

The respiratory burst of leukocytes serves as a major source of $O_2^{\bullet -}$ at sites of inflammation. Given the many links between monocyte recruitment and monocyte-derived macrophages as major cellular participants in atherosclerotic lesions, the NAD(P)H Oxidase complex of leukocytes has been implicated as a source of $O_2^{\bullet -}$ in human atheroma. While the link between human leukocyte NADPH oxidase complex and development of cardiovascular disease is not easily assessed, it is interesting to note that peripheral white blood cell count, absolute monocyte count, and absolute neutrophil count all serve as independent predictors (small but statistically significant) of atherosclerosis risk in humans (Nieto, 1992; Danesh, 1998; Cannon, 2001). Recent studies with mice with functional deficiencies for NAD(P)H Oxidase complex activity, have proven complex – since in one study no increases in lesion development were noted in the murine model (Kirk, 2000), whereas in another model deficiency of NAD(P)H Oxidase activity was linked to decreases in lesion development (Barry-Lane, 2001). Alternative sources of $O_2^{\bullet -}$ exist however, and deserve mention. A homologous oxidase complex exists in cells of the artery wall, such as vascular smooth muscle and endothelial cells – nox (Sorescu, 2002), and likely also participates in $O_2^{\bullet -}$ formation. Roles for ''uncoupled '' nitric oxide synthase, which produces $O_2^{\bullet -}$ in the absence of L-Arginine and the cofactor tetrahydrobiopterine (Kerr, 1999; Shinozaki, 1999), xanthine oxidase, and perhaps mitochondrial sources, may all potentially lead to $O_2^{\bullet -}$ generation within the complex milieu of an artery wall (Nakazono, 1991; Ohara, 1993; Cardillo, 1997; Hua, 2000).

Redox-active transition metal ions

The most widely studied models for LDL oxidation *in vitro* involve redox active transition metal ions such as iron and copper. Indeed, inhibition in metal-catalyzed LDL oxidation *in vitro* has defined the actions of compounds like α-tocopherol as an antioxidant. Whether free redox active transition metal ions participate in extracellular oxidation events in biological matrices seems doubtful, however, since multiple redundant mechanisms exist for chelating such metal ions *in vivo*, rendering them redox inactive. Albumin, the most abundant protein in plasma, binds free copper and iron avidly, and readily blocks LDL oxidation *in vitro* (Thomas, 1992). Thus, addition of only trace levels ($<$10%) of plasma proteins to reaction mixtures is sufficient to block LDL oxidation *ex vivo* by free copper and iron (Dabbagh, 1995). Whether protein-bound redox active transition metal ions ($Pr(M^{2+})$, Fig. 1) such as ceruloplasmin, the major copper-carrying protein in plasma, contribute to oxidation *in vivo* has not yet been examined. *In vitro* studies with isolated ceruloplasmin in reaction mixtures containing plasma support this as a potential pathway operative *in vivo* (Fox, 2000).

Stable products of amino acid oxidation that are formed by metal-catalyzed generation of hydroxyl radical serve as a potential tool to assess the role of this pathway in human atheroma (Fig. 1). Exposure of the aromatic amino acid phenylalanine to hydroxyl radical generating systems produces multiple isomers of tyrosine (ortho, meta and para) – the former two, nonphysiological isomers of tyrosine, have served as molecular markers for protein oxidative injury by hydroxyl radical-like species (Huggins, 1993; Leeuwenburgh, 1997b). Using stable isotope dilution mass spectrometry approaches, Leeuwenburgh et al. observed that no significant differences in the content of o- and m-tyrosine were present at all stages of lesion evolution, though a trend toward significance was noted with advanced necrotic lesions (Leeuwenburgh, 1997b). Hydroxyl radical oxidation products of aliphatic amino acids like hydroxyvaline (OH-Val), hydroxyleucine (OH-Leu) or tyrosine (dopa) serve as alternative molecular markers for hydroxyl radical like species (Fig. 1). Fu et al. have reported increases in these species in analyses of human atheroma based upon studies employing HPLC with on-line fluorescence detection of derivatized amino acid hydrolysates (Fu, 1998). None of the studies reported thus far (for o-Tyr, m-Tyr, HO-Val, HO-Leu or dopa) employed methods for the simultaneous monitoring of intra-preparative artifactual formation of the analytes, such as are now routinely employed with mass spectrometric analyses of other stable oxidation products (Wu, 1999a, b; Frost, 2000; MacPherson, 2001; Brennan, 2002; Zhang, 2002a). Finally, although not indicated in Fig. 1, it should also be noted that peroxynitrite ($ONOO^-$), a potent oxidant formed by reaction of nitric oxide and $O_2^{\bullet -}$, has hydroxyl radical-like properties (Beckman, 1990; Brennan, 2002) and may thus participate in formation of hydroxylated amino acid oxidation products observed *in vivo*.

Phagocytes and their reactive intermediates implicated in atherosclerosis

Reactive oxidants generated by phagocytes are of central importance in host defenses, tumor surveillance and inflammation (Babior, 1978; Klebanoff, 1980; Weiss, 1989). As noted above, phagocyte activation triggers a membrane-associated NAD(P)H oxidase to reduce oxygen to $O_2^{\bullet -}$), which subsequently dismutates to form hydrogen peroxide (H_2O_2). Both $O_2^{\bullet -}$ and H_2O_2 are relatively weak oxidants and do not directly cause tissue damage. Myeloperoxidase (MPO) plays a central role in catalyzing formation of many leukocyte-derived oxidants using H_2O_2 as substrate. The oxidants generated play a critical role in the destruction of invading pathogens (Klebanoff, 1980). However, reactive intermediates generated by activated phagocytes are also potentially deleterious and have been implicated in the pathogenesis of diseases ranging from atherosclerosis to ischemia-reperfusion injury and cancer (Weiss, 1989; Stadtman, 1992; Ames, 1993).

Reactive chlorinating species

Hypochlorous acid (HOCl) is the best characterized product of MPO (Agner, 1972; Harrison, 1976; Foote, 1983), and MPO is the only human enzyme known to produce reactive chlorinating species under physiological conditions (i.e. $Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O$) (Agner, 1972; Harrison, 1976; Foote, 1983; Weiss, 1986). HOCl is microbicidal and cytotoxic, properties which stem from its chemical reactivity which includes chlorination of amines (Weil, 1949; Thomas, 1982; Weiss, 1982) and unsaturated lipids (Winterbourn, 1992; Hazen, 1996a) and oxidative bleaching of heme groups and iron sulfur centers (Albrich, 1981). HOCl is in equilibrium with secondary chlorinating intermediates, such as *N*-monochloramines (R-NHCl) (Weil, 1949; Thomas, 1982; Weiss, 1982). Gas chromatography mass spectrometry studies demonstrate that chlorine gas (Cl_2), which is in equilibrium with HOCl (i.e. $HOCl + Cl^- + H^+ = Cl_2 + H_2O$), is also generated by the MPO–H_2O_2–Cl^- system (Hazen, 1996b). Activated human neutrophils employ an oxidant with characteristics identical to those of Cl_2 during phagocytosis to promote aromatic chlorination reactions yielding 3-chlorotyrosine (Cl-Tyr), a specific marker for MPO-catalyzed halogenation (Hazen, 1996b, 1997a). In the presence of a molar excess of halogenating species, more advanced oxidation products may also be formed, such as 3,5-dichlorotyrosine (Davies, 1999). The specificity of Cl-Tyr as a MPO-generated oxidation product has now been confirmed using MPO knockout mice and multiple distinct inflammation models (Brennan, 2001, 2002; Zhang, 2002a). Marked increases in the content of 3-chlorotyrosine within both human atheroma and LDL isolated from atherosclerotic intima has been established using stable isotope dilution mass spectrometry approaches, thus confirming that MPO participates in oxidative modification of LDL and protein targets in cardiovascular disease (Hazen, 1997a).

Reactive chlorinating species as participants in aldehyde formation from free amino acids

Activated phagocytes employ the MPO system to oxidize common plasma α-amino acids into distinct aldehydes in

high yield (Hazen, 1996c; Anderson, 1997; Hazen, 1998a, b). The mechanism involves initial chlorination of the α-amino group by HOCl forming an unstable α-monochloramine intermediate, which rapidly undergoes a deamination and decarboxylation reaction to yield an aldehyde via an imine intermediate under physiological conditions (Hazen, 1998a). Mass spectrometric methods for the analysis of reactive aldehydes in biological matrices as their oxime derivatives following amino acid oxidation have been established (Hsu, 1999). The MPO-generated aldehyde formed during oxidation of tyrosine, p-hydroxyphenylacetaldehdye (pHA) (Hazen, 1996c), forms a Schiff base adduct with protein lysine residues (Hazen, 1997b) and is generated *in vivo* where it is enriched in early stage human atheroma (Hazen, 2000). Amine containing glycerophospholipids (PS and PE) also serve as targets for modification by pHA *in vitro* (Hazen, 1999b) and are enriched in surgically removed diseased vascular tissues (Heller, 2000). Aldehydes formed during oxidation of specific amino acids (glycine and threonine) are of particular interest since they include formaldehyde and acrolein, respectively (Anderson, 1997; Hazen, 1998b), reactive species implicated in cellular injury and cardiovascular disease (reviewed in Podrez, 2000a). Using MPO knockout mice and a myocardial infarction model, a primary role for leukocyte MPO in formation of acrolein *in vivo* has recently been confirmed. Mass spectrometry analyses of ventricular tissues in wild-type and MPO knockout mice demonstrated over an 80% reduction in acrolein generated 72 h following myocardial infarction (Penn, 2003).

Tyrosyl radical generation

The phenoxyl hydrogen atom of protein tyrosine residues is readily abstracted yielding a resonance-stabilized tyrosine radical. This species can initiate lipid oxidation in model systems *in vitro*, and reacts rapidly with additional tyrosyl radicals forming stable cross-links on proteins. A major product formed is o,o′-dityrosine (Heinecke, 1993) (di-Tyr, Fig. 1). Tyrosyl radical formation may occur as a consequence of numerous more potent oxidants, such as hydroxyl radical and $ONOO^-$; however, an enzymatic source that has received considerable interest is the MPO–H_2O_2–Tyr system (Fig. 1) (Heinecke, 1993). Mass spectrometry studies confirm that di-Tyr is enriched in human atheroma and LDL recovered from diseased tissue (Leeuwenburgh, 1997b). Thus, it has been argued that tyrosyl radical may serve as an important pathway for initiation of lipid oxidation in atherosclerosis (Heinecke, 2002). Remarkably, recent studies using MPO knockout mice demonstrated that MPO-generated reactive nitrogen species, not tyrosyl radical, appear more important in initiation of lipid peroxidation in the inflammatory models thus far examined (Zhang, 2002a). It is also interesting to note that oxidative modification of lipoproteins by tyrosyl radical generating systems may lead to beneficial rather than harmful biological activities. Modification of high density lipoprotein (HDL) by exposure to tyrosyl radical generating systems augments the capacity of the lipoprotein to promote reverse cholesterol efflux from cells (Francis, 1993; Wang, 1998). The mechanism appears to involve oxidative cross-linking of apolipoprotein A isoforms, presumably through a dityrosine cross-link (Wang, 1998). The role of tyrosyl radical in atherogenesis is thus complex and not clearly defined.

Reactive nitrogen species

The best-characterized nitric oxide ($^{\bullet}NO$)-derived oxidant that may be formed by activated phagocytes is peroxynitrous acid (ONOOH), which is in equilibrium with its conjugate base, peroxynitrite ($ONOO^-$). This agent is generated at a diffusion-controlled rate by reaction of $O_2^{\bullet -}$ with $^{\bullet}NO$ (Beckman, 1990, 1994a). At neutral pH, 3-nitrotyrosine (NO_2-Tyr, Fig. 1) is a characteristic product of protein oxidation by $ONOO^-$ and other reactive nitrogen species (Beckman, 1994b; Hazen, 1999a; Brennan, 2002). Over the past 5 years additional reactive nitrogen species have been enumerated in leukocyte-dependent oxidative damage by pathways that involve peroxidase and nitrite (NO_2^-), the auto-oxidation product of NO (Eiserich, 1996; van der Vleit, 1997; Jiang, 1997; Eiserich, 1998; Hazen, 1999a; Wu, 1999a; MacPherson, 2001; Brennan, 2002). Leukocyte peroxidases (MPO and eosinophil peroxidase) can directly utilize NO_2^- as a co-substrate with H_2O_2 to mediate nitration of phenolic compounds (van der Vleit, 1997; Wu, 1999a). The nitrating species formed is the one electron oxidation product of NO_2^-, nitrogen dioxide ($^{\bullet}NO_2$, Fig. 1) (Brennan, 2002). A related nitrogen-centered oxidant derived from HOCl and NO_2^-, nitryl chloride (NO_2Cl), has also been reported (Eiserich, 1996); however, its role in nitration of biological targets has been questioned based upon both kinetic considerations (Jiang, 1997) and experimental data using isolated human leukocytes (Hazen, 1999a).

Both immunohistochemical and mass spectrometry studies demonstrate that protein-bound NO_2-Tyr is markedly enriched in human atheroma and LDL recovered from atherosclerotic aorta (Beckman, 1994; Leeuwenburgh, 1997a). Studies using MPO and eosinophil peroxidase

knock out mice demonstrate that multiple pathways exist for generation of NO_2Tyr *in vivo*, including peroxidase-mediate nitration of extracellular proteins (Brennan, 2002). LDL modified by reactive nitrogen species is converted into a high uptake form leading to cholesterol accumulation and foam cell formation. Both peroxynitrite (Grahman, 1993) and the MPO–H_2O_2–NO_2^- system of monocytes (Podrez, 1999) have been implicated in rendering LDL atherogenic in this manner. Further, the latter pathway has been shown to promote lipid peroxidation in whole plasma using leukocytes isolated from subjects with MPO deficiency and mass spectrometric detection of multiple distinct lipid peroxidation products (Zhang, 2002b). LDL modified by the MPO–H_2O_2–NO_2^- system of monocytes is converted into a ligand for the scavenger receptor CD36 (Podrez, 2000b), a receptor implicated in atherosclerosis (Febbraio, 2000). Unambiguous evidence that MPO plays a major role as an enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation was recently shown using murine models of inflammation and MPO knockout mice (Zhang, 2002a).

Nitrotyrosine and cardiovascular disease – recent clinical studies

In a recent clinical study, systemic levels of protein-bound nitrotyrosine were assessed as predictors of cardiovascular disease risk and burden. Stable isotope dilution electrospray ionization tandem mass spectrometry-based analyses in a case control study with over 200 patients revealed protein-bound nitrotyrosine as a robust predictor of cardiovascular risk, independent of established risk factors and other inflammatory markers like C-reactive protein (Shishehbor, 2003). In addition, the strength of the association between nitrotyrosine levels and atherosclerosis increased with increasing clinical evidence of atherosclerotic burden (Shishehbor, 2003). In this same report, a separate interventional study revealed that a therapy known to reduce cardiovascular risk, treatment with hydroxymethylglutaryl coenzyme A reductase inhibitors (e.g. atorvastatin; trade-name Lipitor), resulted in marked reductions in plasma protein-content of nitrotyrosine. The reduction in nitrotyrosine levels was comparable in magnitude to, but independent of, reductions in total cholesterol and LDL particle number elicited by the drug, suggesting that nitrotyrosine may prove a viable target for statin therapies (Shishehbor, 2003). The mechanism for the systemic antioxidant effect of statin therapy likely involves inhibition of isoprenylation of key proteins involved in superoxide and nitric oxide formation (Shishehbor, 2003).

Myeloperoxidase and cardiovascular disease – recent clinical studies

The presence of MPO in human atheroma (Daugherty, 1994; Sugiyama, 2001) and the detection of multiple distinct oxidation products generated by this heme protein within both diseased human atheroma and LDL recovered from atherosclerotic aorta (reviewed in Podrez, 2000a), establishes the presence of catalytically active enzyme during cardiovascular disease in humans. However, such associations do not establish a cause and effect relationship between MPO-catalyzed oxidation and development of cardiovascular disease. In an effort to address this issue, MPO knockout mice were generated, and examined in atherosclerosis models. Contrary to expectations, a modest increase in lesion development was observed in the MPO knockout mice (Brennan, 2001). However, further biochemical examination of the murine models demonstrated that, unlike the human disease, neither MPO nor its oxidation products are detected in mouse atheroma (Brennan, 2001). Thus, the significance of MPO in murine models of atherosclerosis is unclear, and it has been suggested that to study the role of MPO in atherosclerosis, one needs either to humanize existing mouse models, or study the disease in humans (Brennan, 2001; Nauseef, 2001).

Recent clinical studies in humans strongly support a role for MPO in atherogenesis. In a study examining nearly 100 individuals with MPO deficiency, MPO deficient subjects were shown to have a significantly reduced rate of cardiovascular disease (Kutter, 2000). Further, subjects with a functional polymorphism in the promoter region of the MPO gene that leads to decrease expression in reporter constructs conferred significant cardioprotective effects in subjects harboring the mutation (Nikpoor, 2001). In a recent clinical trial looking at sequential patients undergoing coronary angiography, blood and leukocyte levels of MPO served as strong independent predictors of significant coronary artery disease risk (Zhang, 2001). Thus, increasing evidence points to a link between MPO and development of atherosclerosis.

Evidence also suggests a role for MPO in development of unstable plaque and acute coronary syndromes. MPO-generated oxidants have been linked to activation of protease cascades, both through oxidative inactivation of protease inhibitors (e.g. α-1 antitrypsin, TIMPs, and plasminogen activator inhibitor-1), and activation of latent forms of proteases, such as pro-elastase and matrix

metalloproteases (Peppin, 1986; Springman, 1990; Shabani, 1998; Fu, 2001). A central role for MPO in the oxidative inactivation of plasminogen activator inhibitor-1 (PAI-1) following acute myocardial infarction, leading to activation of the plasminogen – plasmin protease cascade and left ventricular dilation, a major cause of congestive heart failure and sudden cardiac death, has recently been reported (Askari, 2003). This may also lead to activation of matrix metalloprotease cascades. MPO-catalyzed site-specific oxidative modification of a critical cysteine residue of matrix metalloproteases has also been linked to functional activation of latent protease activity (Fu, 2001). MPO may thus contribute to acute coronary syndromes through plaque destabilization and rupture, and altered ventricular remodeling following myocardial infarction. Consistent with this hypothesis, recent immunohistochemical studies reveal MPO is enriched within human atheroma that undergo fissuring or plaque rupture in subjects with fatal myocardial infarction (Sugiyama, 2001).

The antioxidant trials that weren't

The CHAOS trial was the first prospective interventional trial using α-tocopherol supplementation as an antioxidant for prevention of cardiovascular events. A remarkable 80% reduction in non-fatal myocardial infarction was noted (Stephens, 1996). However, since that initial study, a host of larger prospective interventional trials have come and gone – all have failed to demonstrate a clinical benefit to α-tocopherol supplementation (reviewed in Diaz, 1997; Witztum, 2001; Heinecke, 2001). This has raised questions about the validity of the hypothesis that oxidation is a critical participant in the atherosclerotic disease process.

For an antioxidant to be effective as a supplement, it has to interrupt pathways operative in promoting oxidation in tissues involved in the disease process. It is thus remarkable the oxidation pathways known to occur in human cardiovascular disease (e.g. Fig. 1: peroxynitrite mediated oxidation, nitration, MPO-catalyzed nitration, chlorination, tyrosyl radical-initiated oxidative cross-linking) are not efficiently blocked by α-tocopherol (Podrez, 1999, 2000a; Rubbo, 2000; Heinecke, 2001). Further, it is all the more remarkable that recent studies with α-tocopherol supplementation (at massive doses (2000IU) for $1/3$ y) failed to demonstrate reduction in several systemic markers of oxidant stress (Meagher, 2001). In view of these findings, it is hard to interpret recent clinical trials using α-tocopherol supplementation as meaningful antioxidant interventions. Indeed, numerous studies have reported pro-oxidant effects of α-tocopherol in model systems through a process termed ''tocopherol-mediated peroxidation'' (reviewed in Thomas, 2000). Moreover, analyses of tocopherol content in vascular tissues at different stages of lesion evolution reveals that the ''antioxidant'' is not consumed (Thomas, 2000; Upston, 2002a, b).

It is thus clear that α-tocopherol supplementation does not promote substantial clinical benefit in terms of cardiovascular risk reduction in the general population. While it may be true that α-tocopherol supplementation could still decrease markers of oxidant stress in a subset of subjects with enhanced levels of oxidant stress, or following specialized instructions for ingestion to optimize absorbance of the vitamin, it seems unlikely that widespread use of this vitamin for cardiac protection will prove useful in clinical practice.

Future studies need to rely upon alternative antioxidant interventions, in combination with simultaneous monitoring of markers of oxidant stress *in vivo*. One might think that an alternative antioxidant to employ as a supplement in intervention trials is ascorbic acid (vitamin C). However, the pro- vs. anti-oxidant actions of ascorbate are likewise complex, and a role for the vitamin in both further oxidation of pre-existing lipid hydroperoxides (Lee, 2001) and promoting a pro-oxidant state following supplementation in humans (Podmore, 1998) have been reported. Regardless of the antioxidant intervention tried, simultaneous monitoring of systemic markers of protein and lipid peroxidation will be required to ensure net antioxidant action *in vivo*.

Obstacles to overcome in utilizing oxidation products as diagnostics

Perhaps the greatest obstacle for antioxidant trials will not be the choice of antioxidant – but the practical issues surrounding the methods for both sample storage and the analyses chosen for monitoring anti-oxidant efficacy. Amino acid oxidation products are superior to their lipid oxidation product brethren in terms of stability during sample storage. Species like F_2Isoprostanes are readily generated during sample storage, processing, and analysis. It has been suggested that F_2Isoprostanes are not formed in urine because of its dilute nature and low levels of arachidonate. This argument is fatuous. There is nothing unique about urine that makes it an anaerobic environment, and the content of arachidonate in urine is orders of magnitude in excess of F_2Isoprostanes. Indeed, addition of uniformly deuterated arachidonate to urine stored under air at $-80\,^{\circ}$C for several months has significant

levels of deuterated F_2Isoprostanes upon analyses. Further, unlike mass spectrometry methods for quantifying amino acid oxidation products, assays for lipid oxidation products have not yet been reported that routinely monitor for artifactual formation during sample storage and analysis, such as through incorporation of isotopically labeled parent lipids. The costs of such an assay would be prohibitive for widespread use.

Thus, a significant advantage of amino acid oxidation products as markers of disease risk is that they are more readily usable in banked specimens. Archival specimens from pre-existing clinical studies, which will undoubtedly play a critical role in validating the clinical utility of any oxidation marker, cannot be employed for examining lipid oxidation products, unless extensive precautionary measures were taken to prevent artificial oxidation. Stable species like chlorotyrosine and nitrotyrosine, which are not formed during prolonged storage in a freezer, are thus ideally suited from a stability standpoint for serving as potential markers of oxidant stress *in vivo*. They suffer, however, from the rather complex analytical methods currently required for their accurate quantification using mass spectrometry. While commercial ELISA kits are currently available for nitrotyrosine quantification, it has been our experience that such assays are far from accurate, with values typically over an order of magnitude off from those determined from more rigorous quantification methods like stable isotope dilution tandem mass spectrometry. Further advances in the development of high throughput screening methods for amino acid oxidation markers that are validated side-by-side against ''gold standard'' mass spectrometry-based assays are clearly needed.

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