3-Hydroxyanthranilic Acid Is an Efficient, Cell-derived Co-antioxidant for α-Tocopherol, Inhibiting Human Low Density Lipoprotein and Plasma Lipid Peroxidation*

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α-Tocopherol (α-TOH) can promote lipid peroxidation in human low density lipoprotein (LDL) unless co-antioxidants are present that eliminate the chain-carrying α-tocopheroxyl radical (α-TO•) (Bowry, V. W., Mohr, D., Cleary, J., and Stocker, R. (1995) J. Biol. Chem. 270, 5756–5763). Interferon-γ inhibits human monocyte/macrophage-facilitated LDL lipid peroxidation via induction of cellular tryptophan degradation and production and release of 3-hydroxyanthranilic acid (3HAA) (Christen, S., Thomas, S. R., Garner, B., and Stocker, R. (1994) J. Clin. Invest. 93, 2149–2158). We now report on the mechanism of antioxidant action of 3HAA. 3HAA directly reduced α-TO• in UV-exposed micellar dispersions of α-TOH or in LDL incubated with soybean 15-lipoxygenase (SLO), as assessed by electron paramagnetic resonance spectroscopy. 3HAA did not inhibit SLO enzyme activity. Anthranilic acid, which lacks the phenoxyl group, was incapable of reducing α-TO•. 3HAA dose-dependently inhibited the peroxidation of surface phospholipids and core cholesteryl esters in LDL exposed to SLO, peroxyl radicals (ROO•), or Cu2⁺; oxidants that convert α-TOH to α-TO•. In all cases, sparing of LDL’s α-TOH, but not ubiquinol-10 (CoQ10H2), was observed until the majority of 3HAA was consumed. Addition of 3HAA or ascorbate prevented further consumption of α-TOH and accumulation of lipid hydroperoxides when added to aqueous or lipophilic ROO•-oxidizing LDL after complete and partial consumption of CoQ10H2 and α-TOH, respectively. In contrast, addition of urate, an efficient ROO• scavenger incapable of scavenging α-TO•, did not efficiently inhibit ongoing lipid peroxidation. Oxidation of 3HAA-supplemented human plasma by aqueous ROO• resulted in the successive consumption of ascorbate, CoQ10H2, 3HAA, bilirubin, α-TOH, and urate. Lipid peroxidation was prevented as long as ascorbate, CoQ10H2, and 3HAA were present, but subsequently proceeded as a free-radical chain reaction concomitant with α-TOH, bilirubin, and urate consumption. Addition of 3HAA to aqueous ROO•-oxidizing plasma, after complete consumption of ascorbate and CoQ10H2, strongly inhibited ongoing lipid peroxidation and consumption of α-TOH, bilirubin, and urate immediately and as efficiently as did ascorbate. These findings demonstrate that 3HAA is a highly efficient co-antioxidant for plasma lipid peroxidation by virtue of its ability to interact with α-TO• in lipoproteins. Since interferon-γ is the principal inducer of tryptophan degradation and release of 3HAA by monocytes/macrophages, this may represent a localized extracellular antioxidant defense against LDL oxidation in inflammation.

Oxidative modification of low density lipoprotein (LDL) is implicated as an important early event in atherogenesis (1, 2). Oxidatively modified LDL may be taken up by macrophages via the scavenger receptor to form lipid-laden or “foam cells,” a hallmark of early atherosclerotic lesions (3). Furthermore, oxidized LDL possesses an array of additional pro-atherogenic properties (1–4), and the formation of lipid hydroperoxides and products derived from them may play a central role in LDL oxidation. In light of the likely pathophysiological relevance of oxidized LDL, a great deal of interest has focused on LDL lipid antioxidation. The “oxidation hypothesis” of atherosclerosis (1) implies that LDL antioxidants are potentially anti-atherogenic. Consistent with this, some (5–8) though not all (9) synthetic lipid-soluble antioxidants slow the progression of atherosclerosis in animal models.

Human LDL contains a number of endogenous antioxidants, including α-tocopherol (α-TOH) and ubiquinol-10 (CoQ10H2). α-TOH, biologically and chemically the most active form of vitamin E (10), is the most abundant lipid-soluble antioxidant in LDL extracts (11) and as such has received the most interest with respect to research into “antioxidation” of LDL lipids. A number of studies using strongly oxidizing conditions have concluded that α-TOH is an efficient antioxidant for LDL (reviewed in Ref. 11). Indeed, when Cu2⁺ is used at a Cu2⁺/LDL ratio of 10–161, relatively small amounts of lipid hydroperoxides are formed until α-TOH has been consumed (11) after which lipid peroxidation enters a rapid, “uninhibited” phase. Under such oxidizing conditions, enrichment of LDL with vitamin E increases the resistance of the lipoprotein toward oxidation (12, 13). Despite this, the correlation between LDL’s content of α-TOH and its resistance toward copper oxidation is poor (reviewed in Ref. 11).

Recent studies using low fluxes of a variety of oxidants,
including peroxy radicals (ROO·), Cu2+·, Ham’s F-10 medium in the absence and presence of human macrophages, hydroxyl radicals (•OH), 15-lipoxygenase, and horseradish peroxidase/H2O2, have demonstrated that α-TOH, a tocopherol (γ-TOH) in the presence of LDL and ascorbate- and CoQ10H2-free plasma (14–18). That is, lipid peroxidation (i) proceeds via a radical chain reaction in the presence of α-TOH; (ii) is accelerated by enriching the LDL with α-TOH and markedly suppressed in LDL deficient of α-TOH; and (iii) is faster in the presence of α-TOH than immediately after its complete consumption (14–18). These and other unexpected results can be explained readily by tocopherol-mediated peroxidation (TMP) (15, 19).

The TMP model of lipid peroxidation (Scheme I) encompasses the physical constraints and consequences of the reactions taking place in emulsions of peroxidizing LDL (15, 19). It predicts that α-TOH facilitates the entry of a radical from the aqueous phase into LDL (Scheme I, reaction 1). Furthermore, it predicts that the resulting α-TOH is trapped within LDL and thereby physically segregated from α-TOH in other oxidizing LDL particles. Under these conditions, α-TOH can become a radical chain transfer agent, promoting the peroxidation of LDL lipids (Scheme I, reactions 2–4). This pro-oxidant activity of α-TOH is prevented by either entry of a second radical into (XH), compounds capable of efficiently scavenging the α-TOH in oxidizing LDL and exporting the radical into the aqueous phase (Scheme I, reaction 6) (20). Known physiological co-antioxidants include ascorbate (15, 21), CoQ10H2 (16, 22), and bilirubin (23). In contrast, urate (15, 24), reduced glutathione (25), and β-carotene (26) are incapable of efficiently reducing the α-TOH and are not generally potent inhibitors of LDL lipid peroxidation.

Most attention on LDL antioxidation has focused on dietary or synthetic antioxidants that either associate with the lipoprotein or are present in the aqueous phase of plasma. Comparatively, little attention has been given to the idea that cells may produce and release antioxidants and thereby contribute to the prevention of LDL lipid peroxidation in the extracellular space. This type of antioxidant defense may be advantageous as it principally allows for a site-specific and regulated response to, for example, an enhanced requirement for LDL antioxidation in the localized environment of the artery wall. Recently it has been demonstrated that the pro-inflammatory cytokine interferon-γ (IFN-γ) inhibits LDL oxidation facilitated by either murine (27) or human monocyte/macrophages (28). For murine cells, this inhibitory action of the cytokine appears to depend on cellular arginine degradation and production of nitrogen monoxide (NO). In contrast, for human monocytes/macrophages, IFN-γ induced tryptophan degradation along the kynurenine pathway and production and release of micomolar quantities of 3-hydroxyanthranilic acid (3HAA, 2-amino 3-hydroxy benzoic acid, Scheme II) was responsible for the inhibitory action of the cytokine (28).

In the current study we investigated the molecular mechanism(s) by which 3HAA efficiently inhibits LDL lipid peroxidation. The results obtained show that this natural aminophenol interacts with α-TOH in peroxidizing LDL and as such represents a cell-derived co-antioxidant, the synthesis and release of which is regulated by IFN-γ.

EXPERIMENTAL PROCEDURES

Materials—2,2′-azobis(2-aminopropanohydrochloride (AAPH) and 2,2′-azobis(2,2-dimethylvaleronitrile) (AMVN) were obtained from Polysciences (Warrington, PA). Anthranilic acid, ascorbic acid, α-TOH, 3-hydroxykynurenine, phosphate-buffered saline (PBS) tablets, soybean 15-lipoxygenase (SLO), and uric acid were from Sigma. 3HAA, cetyltrimethyl ammonium chloride (HTAC) and sodium dodecyl sulfate (SDS) were from Aldrich. Copper(II) sulfate and KBr were from British Drug House (BDH). Nanopure water (MODULAB) was used for all aqueous buffers, which were subsequently treated with Chelex-100 (Bio-Rad) to remove contaminating amounts of redox-active transition metals, as verified by the ascorbate autoxidation assay (29). All organic solvents used (high performance liquid chromatographic (HPLC) quality) were from either Mallinckrodt or Merck.

Preparation of Plasma and LDL—Blood was obtained from healthy male or female donors and drawn into heparinized vacutainers (Becton Dickinson, Mountain View, CA). Plasma was prepared by centrifuging blood at 1000 × g at 4 °C for 15 min. Human LDL (ρ = 1.006 – 1.063 g/ml) was isolated from fresh plasma by rapid density gradient ultracentrifugation (2 h, 15 °C, 100,000 rpm) using a TL-100.4 rotor in a TL-100 benchtop centrifuge (Beckman) [30]. Prior to oxidation, KBr and other low molecular weight contaminants (including aqueous antioxidants) were removed by passage of the lipoprotein solution through two consecutive PD-10 gel filtration columns (Pharmacia Biotech Inc.), equilibrated with chelaxed PBS or 50 mM phosphate buffer (pH 7.4). For electron paramagnetic resonance (EPR) experiments, LDL was concentrated to 3–4 mg of protein/ml using Centricon-30 concentrators with a molecular weight cut-off of 30 kDa (Amicon) before gel filtration.
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Oxidation of Plasma and LDL—Oxidation of plasma and isolated LDL (0.5–0.85 mg of protein/ml) was carried out under air at 37 °C by addition of the thermolabile azo initiators, AAPH or AMVN, which release water- and lipid-soluble ROO', respectively, at the known rates (R): \( R_{\text{AAPH}} = 1.3 \times 10^{-6} \) [AAPH] s\(^{-1}\) (24) and \( R_{\text{AMVN}} = 2.6 \times 10^{-6} \) [AMVN] s\(^{-1}\) (31). AMVN (dissolved in ethanol), was added to LDL solutions or plasma dropwise to avoid precipitation. The final concentration of AMVN did not exceed 2 mM. Where indicated, isolated LDL was oxidized by Cu\(^{2+}\) (at a ratio of 5 mol of Cu\(^{2+}\) per mol of apo B-100) or SLO (8 μg of protein/ml).

**Determination of Aqueous and Lipophilic Antioxidants, Neutral and Oxidized Lipids By HPLC—Aliquot (50–100 μl) of the plasma or LDL reaction mixtures were withdrawn at the indicated times and extracted with 1 ml of acidified methanol (0.1% (v/v) acetic acid) and 5 ml of hexane, mixed, and centrifuged at 4 °C at 1000 × g for 5 min. Extracts were stored at −20 °C for no longer than 48 h prior to analyses. The resulting hexane layer (4 ml) was removed, dried under vacuum, and resuspended immediately in isopropanol (200 μl) for analyses of the lipid-soluble components. The levels of α-TOH and CoQ\(_{10}\), neutral lipids (mainly free cholesterol and cholesteryl esters), and cholesteryl ester hydroperoxides were determined by reversed-phase HPLC with electrochemical, UV\(_{210}\) nm, and post-column chemiluminescence detection (35).

For ascorbate and urate analyses, the aqueous methanol phase was removed, filtered (0.2 μm), and analyzed immediately by HPLC with electrochemical detection (33). For bilirubin analysis, 50-μl plasma aliquots were extracted into 200 μl of ice-cold ethanol and analyzed by HPLC with UV detection at 440 nm as described previously (34). For 3HAA analysis, aliquots of plasma or LDL reaction mixtures were deproteinized with 10% trichloroacetic acid (1:1, v/v) and analyzed by HPLC with electrochemical detection (35).

Our previous studies (28) indicated that 3HAA potently inhibited LDL oxidation facilitated by human monocytes/macrophages in Ham’s F-10 medium, a transition metal-dependent oxidizing system. It was first tested whether 3HAA prevents transition metals from participating in redox reactions. 3HAA (10 μM) failed to prevent Ham’s F-10-catalyzed oxidation of ascorbate. Even when added at a 100-fold molar excess over the transition metal, 3HAA failed to inhibit Cu\(^{2+}\) - or Fe\(^{3+}\) -catalyzed oxidation of ascorbate in phosphate buffer, whereas the transition-metal chelator, DETA-PAC, efficiently inhibited such oxidation (not shown). These results indicate that the aminophenol does not prevent transition metals from participating in redox reactions. Furthermore, neither the absorption nor fluorescence spectra of 3HAA in phosphate buffer were altered upon addition of either Cu\(^{2+}\) or Fe\(^{3+}\), indicating that the aminophenol does not chelate transition metals (not shown).

**Reaction of 3HAA with α-TO—**It was next investigated whether 3HAA was able to react with α-TO\(^{-}\), since under mild radical oxidizing conditions α-TO\(^{-}\) mediates the peroxidation of LDL lipids (15, 17). Exposure of micellar dispersions of α-TO\(^{-}\) to UV light (Fig. 1A) or LDL to SLO (Fig. 1C) resulted in the formation of the α-TO\(^{-}\) as characterized by a seven-line EPR spectrum with hyperfine coupling consistent with that previously reported for α-TO\(^{-}\) (37). Addition of 10 μM 3HAA to either UV light-exposed micellar dispersions of α-TO\(^{-}\) (after the UV light was switched off) (Fig. 1B) or SLO-oxidizing LDL (Fig. 1D), resulted in the immediate quenching of the α-TO\(^{-}\) EPR signal to below the limit of detection. No other g ~ 2 radicals were observed on addition of 3HAA to α-TO-containing solutions suggesting that the resulting putative 3-hydroxyanthranil radical was short-lived on the EPR time scale. It is known that radical-mediated oxidation of 3HAA results in production of the phenoxazinone cinnabarinic acid (38). Consistent with this, addition of 3HAA to α-TO-containing micelles resulted in the consumption of the aminophenol and formation of cinnabarinic acid (not shown). Anthranilic acid (2-aminobenzoic acid), which differs from 3HAA only by the absence of the phenoxyl...
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3HAA did not directly inhibit SLO activity, as demonstrated by indistinguishable rates of SLO-catalyzed oxidation of linoleic acid in the absence and presence of the aminophenol (Fig. 2A). However, 3HAA potently and dose-dependently inhibited the accumulation of CE–O(O)H and consumption of α-TOH in LDL exposed to SLO (Fig. 2B), an oxidant that requires α-TOH for efficient LDL lipid peroxidation initiation (17).

3HAA Effectively Reduces α-TOH Consumption and LDL Lipid Peroxidation Initiated By Lipophilic ROO∗—LDL lipid peroxidation initiated by the lipophilic azo-compound AMVN has been demonstrated to occur by TMP (14, 15, 17). Hence, for an aqueous compound to efficiently inhibit such lipid peroxidation, it must reduce α-TO∗ (see “urate paradox” in Ref. 15). Fig. 3 shows the effect of exogenous 3HAA on the oxidation and antioxidation of freshly isolated LDL incubated with AMVN. Independent of the presence of 3HAA, CoQ10H2 was the first antioxidant consumed, and the accumulation of significant amounts of lipid hydroperoxides was not observed as long as CoQ10H2 was present. In the absence of 3HAA, the initial rapid consumption of CoQ10H2 was followed by concomitant consumption of α-TOH and oxidation of LDL's cholesteryl esters and phospholipids. Under these conditions, LDL’s lipids oxidized as a radical-chain reaction with ~25–30 and 7–8 molecules of CE–O(O)H and PC–OOH, respectively, formed per molecule of α-TOH consumed. These findings are in agreement with previous reports (14, 15, 21, 39) and shows that AMVN-initiated LDL lipid peroxidation proceeds via TMP only after CoQ10H2 consumption. In the presence of 3HAA (5 μM), LDL's α-TOH was spared from consumption (Fig. 3A) and the accumulation of CE–O(O)H and PC–OOH was inhibited strongly until the majority of the aminophenol was consumed (Fig. 3B). In contrast, CoQ10H2 was almost completely consumed before any significant loss of 3HAA was detected, indicating a lack of interaction between these two antioxidants (Fig. 3A). Another natural aminophenolic antioxidant, 3-hydroxykynurenine (40), exhibited a similar inhibitory efficacy as 3HAA, while urate (10 μM) was not capable of inhibiting AMVN-initiated lipid peroxidation (not shown).

3HAA Dose-dependently Inhibits AMVN- and Cu2+-initiated LDL Lipid Peroxidation—As with SLO, 3HAA dose-dependently inhibited AMVN- (Fig. 4, A and B) and Cu2+-initiated (Fig. 4, C and D) LDL lipid peroxidation, as indicated by inhibition of both the consumption of α-TOH and the accumulation of CE–O(O)H. In each case, inhibition was noted until the majority of the aminophenol was consumed (not shown). The observation that 3HAA also spared α-TOH from consumption during Cu2+-initiated LDL oxidation provided further support for an interaction between the two antioxidants; Cu2+ rapidly converts LDL's α-TOH to α-TO∗ (41).

Effect of Addition of 3HAA to LDL during AAPH- or AMVN-initiated Oxidation—If 3HAA inhibits LDL lipid peroxidation by reductive elimination of the α-TO∗, it would be expected that addition of the aqueous aminophenol to oxidizing LDL, after partial consumption of α-TOH, would result in an immediate cessation of both α-TOH consumption and lipid peroxidation, regardless of whether the oxidation is initiated from within (i.e. by AMVN-derived lipophilic ROO∗) or outside (i.e. by AAPH-derived aqueous ROO∗) the lipoprotein particle. Indeed, addition of 3HAA to either AAPH- or AMVN-oxidizing LDL, pre-
ventilated further consumption of α-TOH and completely inhibited the accumulation of CE–O(O)H (Fig. 5). 3HAA inhibited both processes as efficiently as the known co-antioxidant, ascorbate. Also, a small though reproducible increase in the amount of α-TOH was noted upon addition of 3HAA or ascorbate (Fig. 5, A and C). In contrast to 3HAA and ascorbate, urate was incapable of inhibiting both CE–O(O)H formation and α-TOH consumption in AMVN-oxidizing LDL (Fig. 5, A and B). When added to AAPH-oxidizing LDL, urate almost completely inhibited consumption of α-TOH (Fig. 5C), yet it failed to efficiently inhibit CE–O(O)H formation (Fig. 5D). This “urate-paradox” (15) is consistent with urate’s inability to scavenge preformed α-TO’ in oxidizing LDL even though it is an efficient scavenger of aqueous RO’.

Plasma Experiments—The relative efficacy of 3HAA to inhibit AAPH-initiated lipid peroxidation in human blood plasma was next examined. In the absence of 3HAA, ascorbate (Fig. 6A) and CoQ10H2 (Fig. 6B) were initially consumed, during which time detectable lipid peroxidation was prevented (Fig. 6C). This is consistent with a previous observation (42), although CoQ10H2 consumption was not reported. After complete consumption of ascorbate and CoQ10H2, lipid peroxidation proceeded as a radical chain in the presence of α-TOH, bilirubin, and urate as reported previously (17, 42). Approximately, 22 CE–O(O)H molecules were formed per molecule of α-TOH consumed. 3HAA neither affected the time to onset nor rate of disappearance of ascorbate or CoQ10H2, and its consumption commenced immediately after complete depletion of these antioxidants (Fig. 6, A and B). More importantly, 3HAA efficiently inhibited the accumulation of CE–O(O)H and consumption of α-TOH, bilirubin, and urate for as long as the aminophenol was present (Fig. 6). Thus, in 3HAA-supplemented plasma, the sequential AAPH-initiated consumption of antioxidants was ascorbate > CoQ10H2 > 3HAA > bilirubin > α-TOH ~ urate.

To more clearly delineate 3HAA’s antioxidant activity from that of ascorbate and CoQ10H2, the aminophenol was added to AAPH-oxidizing plasma following complete consumption of ascorbate and CoQ10H2, i.e. at a time where lipid peroxidation and consumption of bilirubin, α-TOH, and urate were ongoing. Addition of 3HAA resulted in an immediate and complete inhibition of accumulation of CE–O(O)H (Fig. 7C) and PC–OOH (Fig. 7B). Furthermore, addition of 3HAA also efficiently inhibited the consumption of α-TOH (Fig. 7B), bilirubin, and urate (Fig. 7A). After complete consumption of the added 3HAA, peroxidation of plasma lipids and consumption of antioxidants proceeded at rates similar to those prior to addition of the aminophenol. Interestingly, the time required for complete consumption of 100 μM 3HAA was greater than that for 100 μM ascorbate (not shown), resulting in a comparatively longer period of inhibition of lipid peroxidation and antioxidant consumption. This is consistent with 3HAA scavenging 2.7 radicals per 3HAA molecule (38) versus 2.0 radicals per ascorbate molecule. Qualitatively similar results were obtained when 3HAA-supplemented plasma was oxidized by AMVN, except that CoQ10H2 consumption preceded that of ascorbate (not shown) (see also Ref. 23). Together these results show that 3HAA, but neither α-TOH, bilirubin, or urate, provided a “second line” of preventative antioxidant defense, behind ascorbate and CoQ10H2 for ROO’-oxidizing human plasma.

**DISCUSSION**

The current study demonstrates that 3HAA is a potent co-antioxidant for lipoprotein-associated α-TOH. Thus, 3HAA (i) efficiently quenched the α-TO’ generated in LDL or micellar dispersions of α-TOH; (ii) efficiently inhibited lipid peroxidation and consumption of α-TOH in isolated LDL oxidized by ROO’, SLO, and Cu2+ (oxidants that require α-TOH for efficient initiation of LDL lipid peroxidation); (iii) completely pre-
vented ongoing lipid peroxidation and α-TOH consumption when added to oxidizing LDL; and (iv) potently inhibited ROO⁻-initiated plasma lipid peroxidation. Together, these results demonstrate that 3HAA is the only physiological compound to date shown to be capable of acting as a preventative, second line of antioxidant defense behind ascorbate and CoQ₁₀H₂ for plasma lipids.

A common feature of the oxidants used in the current study (i.e. ROO⁻, Cu²⁺, and SLO) is that they all require α-TOH for efficient initiation of peroxidation of lipids in peroxide-free LDL (17) and they all generate α-TO (26, 37, 41), which propagates LDL lipid peroxidation (Scheme I, reactions 4–6) (15, 17). As 3HAA potently inhibited LDL lipid peroxidation and α-TOH consumption by all these oxidants, it seems that it is the ability of 3HAA to efficiently eliminate the α-TO from an oxidizing LDL that makes it such an efficient antioxidant. Indeed, the efficient ROO⁻ scavenger urate, which cannot react with α-TO, was not able to efficiently inhibit LDL lipid peroxidation initiated by these oxidants, demonstrating that simple ROO⁻ scav-

![Fig. 6.](image1.png) **FIG. 6.** 3HAA inhibits AAPH-initiated oxidation of whole blood plasma. Plasma was incubated with 15 mM AAPH at 37 °C in the absence (closed symbols) and presence (open symbols) of 3HAA. Aliquots were removed at the indicated time points and analyzed for (A) the aqueous antioxidants ascorbate (circles), 3HAA (hatched squares), bilirubin (inverted triangles), and urate (diamonds); (B) the lipophilic antioxidants CoQ₁₀H₂ (triangles) and α-TOH (circles); (C) CE-O(O)H (squares). The 100% values for the antioxidants were 43 μM ascorbate, 1.1 μM CoQ₁₀H₂, 30 μM 3HAA, 18 μM α-TOH, 8 μM bilirubin, and 384 μM urate. The results shown are representative of three independent experiments.

![Fig. 7.](image2.png) **FIG. 7.** Inhibition by 3HAA of ongoing lipid peroxidation and antioxidant consumption in plasma exposed to AAPH. Plasma was incubated with 50 mM AAPH at 37 °C. After 150-min oxidation 100 μM 3HAA was added. An appropriate volume of buffer was added to control plasma. At the indicated time points aliquots were removed and analyzed in control plasma (closed symbols) or 3HAA-supplemented plasma (open symbols) for bilirubin (A, triangles), urate (A, diamonds), α-TOH (B, inverted triangles), PC-O(O)H (B, triangles), ascorbate (C, circles), CoQ₁₀H₂ (C, triangles), 3HAA (C, hatched squares), and CE-O(O)H (C, squares). The 100% values for the antioxidants were 48 μM ascorbate, 0.9 μM CoQ₁₀H₂, 100 μM 3HAA, 15 μM α-TOH, 7 μM bilirubin, and 461 μM urate. The results shown are representative of three independent experiments.

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lated by the efficient inhibition of lipid peroxidation in the presence of 3HAA. Instead, the putative 3-hydroxyanthranilic radical appears to give rise to cinnaric acid, as addition of 3HAA to α-TOH in micelles resulted in formation of the phenoxazone. The oxidative dimerization of 3HAA to cinnaric acid requires each of the two molecules of 3HAA to undergo three successive one-electron oxidation reactions (38), and this may explain why 3HAA exhibited greater inhibitory activity than equimolar amounts of ascorbate. 3HAA has a theoretical \(n\) value of 3.0, which is consistent with the experimentally observed value of 2.7 (38), while that for ascorbate is \(\sim 2.0\). In any case, 3HAA, like ascorbate, appears to remove radicals from within oxidizing LDL particles via export into the aqueous phase (Scheme I, reaction 6; XII = 3HAA or ascorbate).

3HAA exhibits a number of structural features that make it an efficient co-antioxidant (Scheme II). 3HAA is a phenol, a group of compounds known to be potent antioxidants. Indeed, the requirement for the phenoxyl group is implied by the fact that anthranilic acid does not exhibit (co-)antioxidant activity (this work and Refs. 36 and 40). Both 2- and 4-aminophenol, but not 3-aminophenol, are also potent co-antioxidants (36), suggesting that a phenoxyl group in ortho- or para-position to the amino group is essential for efficient co-antioxidation. In addition, using cyclic voltammetry, we previously reported reduction potentials for 3HAA and 3-hydroxykynurenine of 0.33 and 0.36 V, respectively (40). These values are slightly higher than those of ascorbate (0.3 V) and \(\text{Co}_{2}\text{H}_2\text{O}_2\) (0.2 V), but significantly lower than that of α-TOH (0.5 V) and urate (0.6 V). It is thus thermodynamically feasible that both 3HAA and 3-hydroxykynurenine directly reduce α-TOH.

As shown previously (42, 43), urate and albumin-bound bilirubin were not capable of efficiently preventing plasma lipid peroxidation initiated by aqueous ROO⁻, as lipid peroxidation proceeded as a free radical-chain reaction in their presence. Both compounds are considerably less reactive than ascorbate and α-TOH toward ROO⁻ (44), thus neither compound would be expected to prevent ROO⁻-mediated generation of α-TOO⁻. Second, urate is unable to react with α-TOH (15, 24), while bilirubin, when bound to albumin, is a relatively poor co-antioxidant, at least when compared to 3HAA, ascorbate, and \(\text{Co}_{2}\text{H}_2\text{O}_2\) (36). Interestingly, though presently not understood, albumin-bound bilirubin more efficiently inhibits lipid peroxidation initiated by lipophilic ROO⁻ than aqueous ROO⁻ (20, 23). Free bilirubin, however, is an efficient inhibitor of both aqueous and lipophilic ROO⁻ (20).

Although 3HAA and ascorbate appear to exhibit similar antioxidant action they do differ with respect to the regulation of their availability. In humans, ascorbate is not synthesized and hence is derived exclusively from dietary sources; plasma levels are therefore dependent on the dietary intake of ascorbate. In contrast, 3HAA is not present at significant levels under normal conditions. However, 3HAA and 3-hydroxykynurenine and other tryptophan metabolites along the kynurenine pathway are detected in experimental animals and humans under inflammatory conditions (40, 45–47). 3HAA is produced and released by human monocytes/macrophages, and IFNγ is the principal inducer of tryptophan degradation along the kynurenine pathway via induction of indoleamine 2,3-dioxygenase (IDO). Therefore, formation and release of 3HAA by monocytes/macrophages may represent a regulated and localized antioxidant defense at sites of inflammation (40), perhaps to bolster the antioxidant defense at sites where extracellular levels of ascorbate are transiently depleted. Indeed, it has been demonstrated that stimulated or oxidant exposed neutrophils show enhanced uptake and intracellular accumulation of ascorbate (48). Furthermore, we have noted previously that mature human macrophages at cell numbers resembling that of monocytes in human blood can produce and release up to 30 μM 3HAA (28), depending on the availability of tryptophan. Thus it appears possible that in vivo localized levels of 3HAA could approach plasma levels of ascorbate, however this needs to be addressed in more detail in future studies.

In addition to 3HAA, NO' represents another cell-derived resistant capable of inhibiting LDL oxidation. NO’ is released by nitric oxide (27), though not human macrophages (49) treated with IFNγ. The mechanism by which 3HAA and NO’ inhibit LDL oxidation are different. NO’ appears to be able to scavenge lipid peroxidation radicals and as such inhibits LDL oxidation initiated by either high concentrations of Cu²⁺ or SLO (50). However, NO’ is not an efficient co-antioxidant and hence anti-TMP agent. In addition, NO’ can have deleterious effects via its rapid reaction with O₂⁻ to form peroxynitrite, which potently oxidizes LDL (27), while 3HAA, like ascorbate, can exhibit pro-oxidant activity in the presence of transition metals in vitro (51).

3HAA could conceivably be available as an antioxidant for lipoproteins in a developing atherosclerotic lesion, where monocyte/macrophages and IFNγ are present (52, 53). Also, preliminary findings using a monoclonal antibody for IDO suggest the presence of the enzyme in human atherosclerotic plaque. Future studies may show whether active IDO is present in human lesions and whether this does result in local formation of 3HAA.

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