Does vitamin C act as a pro-oxidant under physiological conditions?

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ABSTRACT **Vitamin C readily scavenges reactive oxygen and nitrogen species and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids, and proteins. Vitamin C also reduces redox active transition metal ions in the active sites of specific biosynthetic enzymes. The interaction of vitamin C with 'free', catalytically active metal ions could contribute to oxidative damage through the production of hydroxyl and alkoxyl radicals; whether these mechanisms occur *in vivo*, however, is uncertain. To examine this issue, we reviewed studies that investigated the role of vitamin C, both in the presence and absence of metal ions, in oxidative DNA, lipid, and protein damage. We found compelling evidence for antioxidant protection of lipids by vitamin C in biological fluids, animals, and humans, both with and without iron cosupplementation. Although the data on protein oxidation in humans are sparse and inconclusive, the available data in animals consistently show an antioxidant role of vitamin C. The data on vitamin C and DNA oxidation *in vivo* are inconsistent and conflicting, but some of the discrepancies can be explained by flaws in experimental design and methodology. These and other important issues discussed here need to be addressed in future studies of the role of vitamin C in oxidative damage.—Carr, A., Frei, B. Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J.* 13, 1007–1024 (1999)**

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VITAMIN C (ASCORBATE) acts as a potent water-soluble antioxidant in biological fluids (1, 2) by scavenging physiologically relevant reactive oxygen species and reactive nitrogen species (3). These include free radicals such as hydroxyl radicals, aqueous peroxyl radicals, superoxide anion, and nitrogen dioxide, as well as nonradical species such as hypochlorous acid, ozone, singlet oxygen, nitrosating species (N_2O_3/N_2O_4), nitroxide, and peroxynitrite (**Table 1**). Evidence for the interaction of vitamin C with radicals and oxidants has been found in extracellular fluids such as plasma and respiratory tract lining fluid (13). Ascorbate is depleted in these fluids *in vivo* under conditions of oxidative stress such as smoking (14) and inflammation associated with rheumatoid arthritis and adult respiratory distress syndrome (3).

In addition to scavenging reactive oxygen species and reactive nitrogen species, vitamin C can regenerate other small molecule antioxidants, such as α-tocopherol, glutathione (GSH),[2] urate, and β-carotene, from their respective radical species (3) (Table 1). Interaction of ascorbate with the α-tocopheroxyl radical to regenerate α-tocopherol moves radicals from the lipid phase into the aqueous phase and hence prevents tocopherol-mediated peroxidation (15). Although ascorbate acts as a coantioxidant for α-tocopherol in isolated lipoproteins and cells (16, 17), it is uncertain whether ascorbate recycles, or rather spares, α-tocopherol *in vivo* (18, 19). In contrast, ascorbate has been shown to spare GSH under conditions of increased oxidative stress *in vivo* (20).

Vitamin C is an effective antioxidant for several reasons. First, both ascorbate and the ascorbyl radical, the latter formed by one electron oxidation of ascorbate (**Fig. 1**), have low reduction potentials (21) and can react with most other biologically relevant radicals and oxidants (some of which are listed in Table 1). Second, the ascorbyl radical has a low reactivity due to resonance stabilization of the unpaired electron and readily dismutates ($k_2 = 2 \times 10^5\ M^{-1}\ s^{-1}$) to ascorbate and dehydroascorbic acid (DHA) (Fig. 1) (4). In addition, ascorbate can be regenerated from both the ascorbyl radical and DHA

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[2] Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; BDI, bleomycin-detectable iron; ELISA, enzyme-linked immunoassay; DHA, dehydroascorbic acid; FAPY, formamidopyrimidine; GC-MS, gas chromatography-mass spectroscopy; GSH, glutathione; HNE, 4-hydroxynonenal; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; LDL, low density lipoprotein; $LO^{\bullet}$, lipid alkoxyl radicals; LOOH, lipid hydroperoxides; MDA, malondialdehyde; ODS, osteogenic disorder Shionogi; 8-oxoade, 8-oxoadenine; 8-oxogua, 8-oxoguanine; 8-oxodG, 8-oxo-2′-deoxyguanosine; SIN-1, 3-morpholinosydnonimine; TBARS, thiobarbituric acid reactive substances.

TABLE 1. *Reactive oxygen and nitrogen species which are scavenged by ascorbate*

Chemical species scavenged by ascorbate	Reaction rate (M^{-1} s^{-1})[a]	Ref
Reactive oxygen species		
Hydroxyl radical ($^{\bullet}OH$)	1.1×10^{10}	(4)
Alkoxyl radicals ($RO^{\bullet}$)	1.6×10^{9}	(4)
Peroxyl radicals ($RO_2^{\bullet}$)	$1\text{-}2 \times 10^{6}$	(4)
Superoxide anion/hydroperoxyl radical ($O_2^{\bullet -}/HO_2^{\bullet}$)	1×10^{5}	(4)
Hypochlorous acid (HOCl)		(5)
Ozone (O_3)		(6)
Single oxygen ($^1O_2$)		(7)
Reactive nitrogen species		
Nitrogen dioxide ($NO_2^{\bullet}$)		(8)
Dinitrogen trioxide/dinitrogen tetroxide (N_2O_3/N_2O_4)	1.2×10^{9}	(9)
Nitroxide (NO)		(10)
Peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH)	235	(11)
Antioxidant-derived radicals[b]		
α-Tocopheroxyl radical (α-$TO^{\bullet}$)	2×10^{5}	(4)
Thiyl/sulphenyl radicals ($RS^{\bullet}/RSO^{\bullet}$)	6×10^{8}	(4)
Urate radical ($UH^{\bullet}$)	1×10^{6}	(4)
β-Carotene radical cation (β-$C^{\bullet +}$)		(12)

[a] The approximate rates of reaction (at pH 7.4) are given if known.
[b] A number of other small molecule antioxidants can be regenerated from their respective radical species by ascorbate.

by enzyme-dependent and independent pathways. The ascorbyl radical is reduced by an NADH-dependent semidehydroascorbate reductase (22) and the NADPH-dependent selenoenzyme thioredoxin reductase (23). DHA can be reduced back to ascorbate nonenzymatically by GSH and lipoic acid (22) as well as by thioredoxin reductase (24) and the GSH-dependent enzyme glutaredoxin (25).

Another important biological function of vitamin C is its interaction with redox active transition metal ions, such as iron and copper. Vitamin C acts as a cosubstrate for hydroxylase and oxygenase enzymes involved in the biosynthesis of procollagen, carnitine, and neurotransmitters (26). A deficiency of vitamin C causes scurvy resulting from a decreased activity of these enzymes (26). Ascorbate maintains the active center metal ions of the hydroxylases and oxygenases in a reduced state for optimal enzyme activity. The reduction of iron by vitamin C has also been implicated in the increased dietary absorption of non-heme iron (27).

Paradoxically, the reduction of transition metal ions by ascorbate (reactions 1a and b) could also have deleterious effects via the production of hydroxyl radicals or lipid alkoxyl radicals ($LO^{\bullet}$) by reaction of the reduced metal ions with hydrogen peroxide or lipid hydroperoxides (LOOH) (reactions 2 and 3, respectively) (3, 4). Although this Fenton chemistry occurs readily *in vitro*, its relevance *in vivo* has been a matter of some controversy, the main point of contention being the availability of catalytic metal ions *in vivo* (28). The levels of 'free' metal ions are thought to be very low due to their sequestration by various metal binding proteins such as ferritin, transferrin, and ceruloplasmin (28). However, during tissue injury metal ions may be released from their stores and could subsequently interact with ascorbate (3). The *in vivo* evidence for a prooxidant or an antioxidant role of vitamin C in the presence of redox active metal ions will be discussed.

$AH^- + Fe^{3+} \rightarrow A^{\bullet -} + Fe^{2+} + H^+$ [1a] ($k_2 \approx 10^2\ M^{-1}s^{-1}$) (4)

$AH^- + Cu^{2+} \rightarrow A^{\bullet -} + Cu^+ + H^+$ [1b]

$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + Fe^{3+} + {}^-OH$ [2a] ($k_2 = 76\ M^{-1}s^{-1}$) (29)

$H_2O_2 + Cu^+ \rightarrow HO^{\bullet} + Cu^{2+} + {}^-OH$ [2b] ($k_2 = 4.7 \times 10^3\ M^{-1}s^{-1}$) (29)

$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+} + {}^-OH$ [3a] ($k_2 = 1.5 \times 10^3\ M^{-1}s^{-1}$) (29)

$LOOH + Cu^+ \rightarrow LO^{\bullet} + Cu^{2+} + {}^-OH$ [3b]

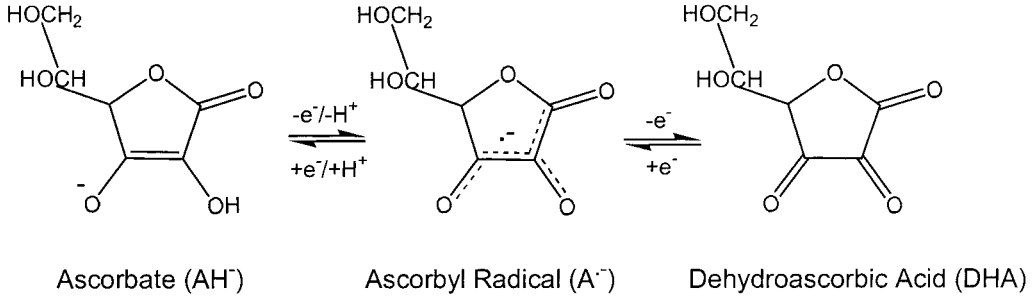

Figure 1. Oxidation of ascorbate (AH^-) by two successive one electron oxidation steps to give the ascorbyl radical ($A^{\bullet -}$) and dehydroascorbic acid (DHA), respectively.

VITAMIN C AND BIOMARKERS OF DNA OXIDATION

Oxidative DNA damage in humans has been estimated to occur at a rate of $\sim 10^4$ to 10^5 hits per cell per day (30, 31). Repair of these oxidative lesions by specific DNA glycosylases and other repair mechanisms is not 100% efficient, therefore lesions accumulate with age and may result in mutations and cancer (30, 31). Of the more than 20 different oxidative DNA lesions, 8-oxoguanine (8-oxogua) and its respective nucleoside 8-oxo-2′-deoxyguanosine (8-oxodG) appear to be the most abundant and most mutagenic (31). 8-Oxogua is formed by attack of guanine by hydroxyl radicals, peroxynitrite, or singlet oxygen and causes a transversion mutation after replication (30, 31). Although there is little direct evidence linking DNA oxidation with cancer (31), numerous epidemiological studies have shown an inverse association between vitamin C intake, or plasma status, and the risk from different types of cancers (32, 33).

The three most commonly used methods for detecting oxidative DNA lesions are gas chromatography-mass spectroscopy (GC-MS), high-performance liquid chromatography with electrochemical detection (HPLC-ECD), and single-cell gel electrophoresis (the comet assay). GC-MS can be used to detect numerous lesions, but it is particularly prone to artifactual oxidation if strenuous preventative measures are not taken (34). HPLC-ECD typically gives levels of lesions 10- to 100-fold lower than GC-MS, most likely due to the milder analytical conditions involved (35, 36). Similarly, the comet assay, although only an indirect method, commonly gives baseline levels of oxidative DNA damage 1000-fold lower than those obtained by GC-MS (35). Lesion-specific glycosylase repair enzymes such as formamidopyrimidine (FAPY) glycosylase and endonuclease III, which are sensitive to oxidized purines and pyrimidines, respectively, can be used in conjunction with the comet assay to improve its specificity (37).

Studies using purified DNA and cells

Addition of vitamin C to purified DNA or isolated nuclei in the presence of redox active metal ions results in single-strand breaks and base modifications such as 8-oxodG (38–40). This is thought to be due to binding of the metal ions to the DNA and resultant site-specific hydroxyl radical production and oxidative damage (38). In the absence of added metal ions, however, vitamin C inhibits the formation of 8-oxodG in purified DNA exposed to peroxynitrite or UV light (39, 41, 42). Vitamin C also acts as an antioxidant in cells (**Table 2**), inhibiting oxidative DNA damage in isolated and cultured cells exposed to hydrogen peroxide and UV-visible light (43–45). In contrast, several studies have shown increased formation of oxidative DNA damage in cultured cells and isolated human lymphocytes in the presence of added vitamin C (46–48). In view of the above findings (38–40), however, this pro-oxidant effect of vitamin C is most likely due to the presence of 'contaminating' metal ions in the media.

Animal supplementation studies

Two recent vitamin C supplementation studies have been conducted in animals to determine the effects on oxidative DNA damage (49, 50) (Table 2). Vitamin C manipulation in guinea pigs, equivalent to marginal deficiency, optimum intake, and megadose intake, had no effect on the hepatic steady-state levels of 8-oxodG, as determined by HPLC-ECD, despite up to a 60-fold variation in vitamin C levels in the liver (49). In contrast, a UV challenge to the eyes of guinea pigs and rats showed a decrease in single-strand breaks in vitamin C-supplemented animals and a corresponding increase in vitamin C-deficient animals (50).

Human supplementation studies

1998 saw the publication of a highly controversial and well-publicized paper in *Nature* entitled "Vitamin C exhibits pro-oxidant properties" (51) (Table 2). In this study, Podmore and colleagues supplemented 30 healthy volunteers with 500 mg of vitamin C daily for 6 wk following 3 wk each of baseline and placebo periods. The plasma concentration of vitamin C was elevated by 60% after vitamin C supplementation. The levels of oxidized DNA bases [8-oxogua and 8-oxoadenine (8-oxoade)], were measured in peripheral blood lymphocytes using GC-MS. The baseline levels of 8-oxogua and 8-oxoade were reported to be 30 and 8 lesions per 10^5 unoxidized bases, respectively (51). After vitamin C supplementation, 8-oxogua levels were significantly reduced relative to baseline and placebo, whereas the levels of 8-oxoade were significantly elevated. The reduced 8-oxogua and the elevated 8-oxoade levels returned to baseline levels after a vitamin C washout period of 7 wk.

Serious issues have been raised about this study (59, 60). First, as mentioned above, GC-MS is prone to artifactual *ex vivo* oxidation, particularly during DNA isolation, extraction, and derivatization for analysis. The levels of 8-oxogua reported in this study are ~10- to 100-fold higher than those reported by others for human lymphocytes (35). Second, 8-oxoade is thought to be at least 10-fold less mutagenic than 8-oxogua (61). Third, lymphocyte vitamin C levels were not determined, even though this was the tissue in which the oxidative DNA damage was

TABLE 2. *Effects of vitamin C supplementation on biomarkers of DNA oxidation*

Study system	Challenge	Effects of vitamin C	Ref.
In vitro			
Human lymphocytes	Hydrogen peroxide	↓ Oxidative DNA damage[a]	(43)
V79 Chinese hamster cells	UV-visible light	↓ 8-oxodG[b]	(44)
L1210 mouse leukemia cells	Visible light	↓ Single-strand breaks, endonuclease-sensitive modifications[a]	(45)
Human lymphocytes, neonatal fibroblasts, Molt-4 T-cells	None	↑ Single-strand breaks[a]	(46)
Human lymphocytes	None	↑ Oxidative DNA damage[a]	(47)
Human lymphocytes	None	↑ Single-strand breaks[a]	(48)
In vivo (animals)			
Guinea pig liver	None	X 8-oxodG[b]	(49)
Guinea pig eye, rat eye	UV light	↓ Single strand breaks[a]	(50)
In vivo (humans)			
Lymphocytes	None	↓ 8-oxoguanine[c] ↑ 8-oxoadenine[c]	(51)
Lymphocytes	None	↓ 8-oxoguanine, 8-oxodG[b]	(52)
Serum	None	↑ 8-oxodG[d]	(52)
Urine	None	↑ 8-oxodG[d] (ns)	(52)
Leukocytes	Iron (12 wk)	↓ 8-oxoguanine, 8-oxoadenine, 5-hydroxyuracil, 5-chlorouracil ↑ Thymine glycol, 5-hydroxycytosine[c]	(53)
Sperm	None	↓ 8-oxodG[b]	(54)
White blood cells	(Smokers), none	↓ 8-oxodG[b] (ns)	(55)
Urine	(Smokers), none	X 8-oxodG[b]	(56)
Lymphocytes	None, *ex vivo* H_2O_2	X Median tail moments[a]	(57)
Lymphocytes	*Ex vivo* H_2O_2	↓ Single-strand breaks[a]	(58)
Whole blood, mononuclear cells	*Ex vivo* γ-irradiation	↓ Mean comet length[a]	(48)

[a] Measured by single cell gel electrophoresis (the comet assay).
[b] Measured by HPLC with electrochemical detection (HPLC-ECD).
[c] Measured by gas chromatography-mass spectroscopy (GC-MS).
[d] Measured by competitive ELISA. ↑ = Increased damage, ↓ = decreased damage, X = no change, 8-oxodG = 8-oxo-2′-deoxyguanosine, ns = not significant.

assessed. The baseline level of vitamin C in plasma, which was 51 μmol/l (62), is already saturating with respect to intracellular lymphocyte vitamin C levels (60); as such, supplementation with 500 mg/day of vitamin C could not have affected these levels. Last, the experimental design is questionable, since it was without a proper placebo group throughout the entire duration of the study.

In a subsequent report by these authors (52), samples collected during the vitamin C supplementation study discussed above (51) were reanalyzed using HPLC-ECD. Again, a significant decrease in lymphocyte 8-oxoguanine and 8-oxodG levels was observed after vitamin C supplementation. However, it is impossible to compare directly the data from these two reports since only the relative, but not absolute, amounts of oxidative DNA damage were given in the second paper (52). In the same paper, serum and urine 8-oxodG levels were also measured using a competitive enzyme-linked immunoassay (ELISA) method, and an increase in oxidative DNA damage was observed in these fluids after supplementation with vitamin C. This finding was interpreted as being due to stimulation of DNA repair enzymes by vitamin C. However, the ELISA method gave higher basal levels of 8-oxodG than HPLC-ECD, possibly due to recognition of other oxidative DNA products by the antibody (52).

Another study was published recently on "the effects of iron and vitamin C cosupplementation on oxidative damage to DNA in healthy volunteers" (53) (Table 2). In this study, healthy nonsmoking individuals were supplemented with iron sulfate (14 mg/day) and either 60 or 260 mg/day of vitamin C. The levels of 13 different types of oxidized DNA bases in white blood cells were measured using GC-MS. One group of volunteers had a mean baseline plasma vitamin C concentration of 72 ± 14 μmol/l, which did not change significantly after supplementation. Although there was a transient rise in the levels of some DNA oxidation products after 6 wk of supplementation (e.g., 5-hydroxyhydantoin and FAPY guanine), levels of these products returned to baseline after 12 wk of supplementation. The levels of other DNA oxidation products either significantly decreased (e.g., 8-oxogua and 8-oxoade) or increased (e.g., thymine glycol and 5-hydroxycytosine) after 12 wk of supplementation. Another group of volunteers had a lower mean plasma vitamin C concentration at baseline (50 ± 14 μmol/l), which increased signifi-

cantly after supplementation. Baseline levels of oxidized DNA damage were higher in this group and these levels decreased after supplementation with iron and vitamin C (53).

As in the study by Podmore and colleagues (51), the baseline levels of 8-oxogua and 8-oxoade were very high (53), presumably due to artifactual oxidation as a result of GC-MS analysis. DNA was isolated from whole blood rather than purified lymphocytes; therefore, activation of phagocytes during sample preparation could also lead to artifactual DNA oxidation. Furthermore, there were no control groups given either iron or vitamin C alone, nor was there a placebo group. Reanalysis of the published results (53) reveals an inverse correlation (R^2 = 0.49) between mean plasma vitamin C concentrations and total oxidative DNA damage (as determined by the sum of all 13 oxidative DNA lesions measured), despite an increase in transferrin saturation (**Fig. 2***a, b*). Similar inverse correlations were observed for individual oxidized bases: 8-oxogua (R^2 = 0.51), 8-oxoade (R^2 = 0.39), and FAPY guanine (R^2 = 0.44). Therefore, this study (53) does not provide compelling evidence for a pro-oxidant effect of vitamin C and iron cosupplementation on DNA damage, but supports an antioxidant effect.

HPLC-ECD has been used as an alternative to GC-MS to determine the effects of vitamin C supplementation on oxidative DNA damage in humans (54–56) (Table 2). An early study by Fraga and co-workers (54), using HPLC-ECD, showed significantly increased levels of 8-oxodG in sperm DNA from vitamin C depleted (5 mg/day) or marginally deficient (10–20 mg/day) men. After repletion with 60–250 mg/day of vitamin C, the levels of sperm DNA damage decreased significantly. The same group also found significantly increased levels of sperm 8-oxodG and decreased plasma vitamin C levels in smokers compared with nonsmokers (63). Consistent with these observations, it was recently reported that supplementation of smokers with 500 mg/day of vitamin C for 4 wk resulted in a nonsignificant decrease in 8-oxodG levels in white blood cells (55). Furthermore, a recent randomized placebo controlled trial (56) was carried out with smoking men receiving either 500 mg/day of vitamin C as a normal or slow-release supplement. The 24 h urinary excretion rate of 8-oxodG was measured by HPLC-ECD. No significant change was observed in any treatment group even though the plasma vitamin C concentration increased by 30 to 53%. However, this study is difficult to interpret, since 8-oxodG is a nucleotide excision rather than glycosylase repair product, and can also arise in urine from normal cell and mitochondrial turnover (64) as well as dietary sources.

More recently, the comet assay has been gaining

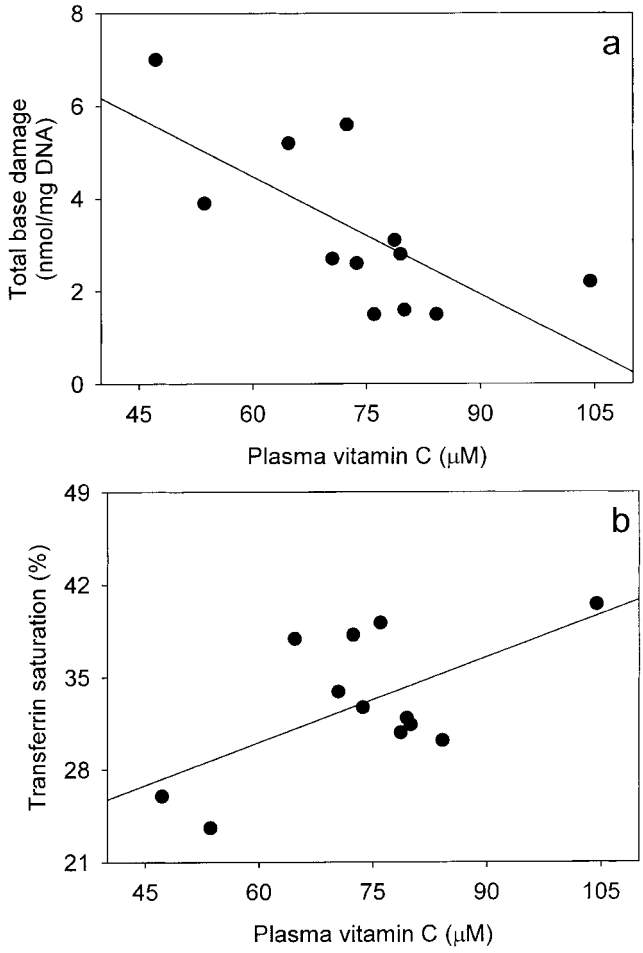

Figure 2. Effects of cosupplementation with vitamin C (60 or 260 mg/day) and iron (14 mg/day) in 38 healthy human volunteers on DNA base damage *(a)* and transferrin saturation *(b)*. Data for mean plasma vitamin C levels, total leukocyte DNA base damage, and transferrin saturation were replotted from ref 53. Linear regression analysis of vitamin C vs. total base damage gave R^2 = 0.49; and vitamin C vs. transferrin saturation gave R^2 = 0.37.

acceptance due to its capacity to detect low levels of basal DNA damage without artifactual oxidation (48, 57, 58) (Table 2). A recent study examined DNA damage in lymphocytes with this assay (57). Nonsmoking subjects were given placebo, 60 and 6000 mg/day of vitamin C for 2 wk each, with 6 wk in between treatment periods. Vitamin C supplementation significantly elevated plasma vitamin C concentration (by 24 and 80% for 60 and 6000 mg/day, respectively), but had no effect on oxidative DNA damage either with or without an *ex vivo* hydrogen peroxide challenge. In contrast, two other studies have shown reduced strand breakage, as determined by the comet assay, in lymphocytes and mixed white blood cells from vitamin C supplemented individuals after an *ex vivo* challenge with either hydrogen peroxide or ionizing radiation (48, 58). In one of these studies (48) vitamin C acted as a pro-oxidant when added to isolated lymphocytes *in vitro*.

In summary, most of the studies reviewed (Table 2) showed a vitamin C-dependent reduction in oxidative DNA damage, whereas some studies found either no change or an increase in the levels of selected DNA lesions. Experiments using purified DNA or isolated nuclei (38–40) confirm that in the presence of added metal ions, vitamin C acts as a pro-oxidant *in vitro* (see reactions 1–3). In the absence of added metal ions, however, vitamin C inhibits oxidative DNA damage in purified DNA and cells (39, 41–45), although there are a few exceptions (46–48). The latter results are likely explained by 'contaminating' metal ions in the cell culture media. Of the two animal supplementation studies discussed (Table 2), one showed protection by vitamin C against UV-induced DNA damage in the eye (50) and the other reported no change in the steady-state levels of oxidative DNA damage in the liver (49). Of nine human vitamin C supplementation studies (Table 2), four showed a reduction in *ex vivo* or *in vivo* DNA oxidation (48, 54, 55, 58), whereas two showed no change (56, 57); another three showed a decrease in some markers and an increase in others (51–53). Two of the latter studies (51, 53), however, suffer from serious shortcomings, primarily artifactual DNA oxidation during GC-MS analysis and high baseline levels of vitamin C, and thus are difficult, if not impossible, to interpret.

VITAMIN C AND BIOMARKERS OF LIPID OXIDATION

Considerable evidence has accumulated implicating lipid peroxidation and oxidative modification of low density lipoprotein (LDL) in atherosclerotic lesion development (65, 66). F_2-isoprostanes, which are specific lipid peroxidation biomarkers formed from nonenzymatic, radical-mediated oxidation of arachidonyl-containing lipids (67), have been detected in human atherosclerotic lesions (68, 69), as have other oxidized lipids (70). Indirect evidence for the oxidative modification hypothesis of atherosclerosis has come from numerous animal and human studies that have shown an inverse association between antioxidant vitamin intake and atherosclerosis or the risk of cardiovascular diseases (32, 71, 72).

The most commonly measured markers of lipid peroxidation are the aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (HNE), the former usually as thiobarbituric acid reactive substances (TBARS) (67). Accumulation of conjugated dienes and lipid hydroperoxides are often measured to assess the 'oxidizability' of LDL (73). The oxidizability of LDL, which is dependent on its antioxidant content and lipid composition, is determined by measuring the lag time and propagation rate of lipid peroxidation after exposure to copper ions or other oxidants (74). Recently F_2-isoprostanes such as 8-epi$PGF_{2\alpha}$ have gained acceptance as specific biomarkers of lipid peroxidation (see above) (67). Finally, release of volatile hydrocarbons, such as pentane and ethane, have also been used as indicators of *in vivo* lipid peroxidation (67).

Studies using isolated lipoproteins and plasma

Vitamin C protects isolated LDL against oxidation by many different types of oxidative stress, including metal ion-independent and -dependent processes, and we have recently reviewed the evidence (66). Several studies have also shown that endogenous vitamin C in plasma protects against lipid hydroperoxide and F_2-isoprostane formation induced by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-derived aqueous peroxyl radicals (1, 75, 76), peroxynitrite or SIN-1 (77), cigarette smoke (78, 79), or activated neutrophils (75) (**Table 3**). What is perhaps surprising is the effect of vitamin C on plasma lipid oxidation in the presence of redox active transition metal ions. Endogenous and exogenous vitamin C was found to inhibit the formation of lipid hydroperoxides in iron-overloaded human plasma (80) rather than enhance oxidation, as would be expected from Fenton chemistry (reactions 1–3). Similarly, when vitamin C was added to human serum supplemented with copper, antioxidant rather than prooxidant effects were observed (81).

Animal supplementation studies

The earliest studies on the effects of vitamin C supplementation on lipid peroxidation *in vivo* were performed by Tappel and co-workers (82, 83) (Table 3). Expiratory pentane and ethane levels were found to be reduced in guinea pigs supplemented with vitamin C prior to a carbon tetrachloride challenge (82), whereas rats challenged with alloxan showed increased breath pentane and ethane levels, as well as plasma and tissue TBARS, after supplementation with vitamin C (83). A recent animal study found reduced expiratory ethane levels when vitamin C was administered to rats prior to a challenge by paraquat and increased levels when vitamin C was administered after the challenge (84). Other studies have shown reduced endogenous levels of MDA and TBARS in guinea pigs or genetically scorbutic (osteogenic disorder Shionogi, or ODS) rats after supplementation with vitamin C (85, 88, 89) (Table 3). Similar protective effects of vitamin C were reported for animals exposed to enhanced oxidative stress, such as

TABLE 3. *Effects of vitamin C supplementation on biomarkers of lipid oxidation*

Study system	Challenge	Effects of vitamin C	Ref
In vitro			
Human plasma	AAPH	↓ LOOH, F_2-isoprostanes	(48)
Human plasma	AAPH, neutrophils	↓ LOOH	(1, 75)
Human plasma	Peroxynitrite, SIN-1	↓ LOOH	(77)
Human plasma	Cigarette smoke	↓ LOOH	(78, 79)
Human plasma	Iron	↓ LOOH	(80)
Human serum	Copper	↓ TBARS	(81)
In vivo (animals)			
Guinea pig breath	Carbon tetrachloride	↓ Pentane, ethane	(82)
Rat breath, plasma, tissues	Alloxan	↑ Pentane, ethane, TBARS	(83)
Rat breath	Paraquat	↓ or ↑ ethane[a]	(84)
Guinea pig liver	None	↓ MDA	(85)
	Ex vivo iron	↓ TBARS	
Guinea pig liver	Endotoxin + *ex vivo* iron	↓ TBARS	(86)
ODS rat plasma, liver	None	X TBARS	(87)
ODS rat plasma, liver	None	↓ TBARS	(88)
ODS rat plasma, liver	None	↓ TBARS	(89)
Rat liver	Cigarette smoke	↓ MDA, CD, LOOH	(90)
Guinea pig liver	Iron + *ex vivo* autoxidation	↓ MDA	(91)
Guinea pig liver, plasma	Iron	↓ F_2-isoprostanes	unpub.[b]
In vivo (humans)			
Urine	(Smokers), none	↓ 8-epi-$PGF_{2\alpha}$	(92)
Plasma	(CAD patients), none	X 8-epi-$PGF_{2\alpha}$	(93)
LDL	(Smokers), *ex vivo* copper	X CD	(94)
Plasma, LDL	(Smokers), none	↑ MDA	(95)
	Ex vivo copper, hemin/H_2O_2	X CD	
LDL	(Smokers), *ex vivo* copper	↓ TBARS, CD	(96)
Serum	(Smokers), none	X TBARS	(97)
Plasma, LDL	Smoking	↓ TBARS	(98)
Plasma	None	↓ MDA	(99)
Plasma	None	↓ MDA/HNE (ns)	(57)
Urine	None	X TBARS	(100)
Plasma, LDL	None	↓ MDA	(101)
	Ex vivo copper	X TBARS, CD	
LDL	*Ex vivo* copper	↓ CD	(102)
LDL	*Ex vivo* copper	↓ TBARS	(103)
Plasma	Exercise	↓ TBARS	(104)
Plasma, LDL	Exercise	X TBARS	(105)
	Ex vivo copper	↓ CD	

[a] Vitamin C protected against damage when given before paraquat challenge and aggravated damage when given after the challenge.

[b] K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations. ↑ = Increased damage, ↓ = decreased damage, X = no change, AAPH = 2,2′-azobis(2-amidinopropane) hydrochloride, CAD = coronary artery disease, CD = conjugated dienes, HNE = 4-hydroxynonenal, LDL = low density lipoprotein, LOOH = lipid hydroperoxides, MDA = malondialdehyde, ns = not significant, ODS = osteogenic disorder Shionogi, SIN-1 = 3-morpholinosydnonimine, TBARS = thiobarbituric acid reactive substances.

endotoxin or cigarette smoke (86, 90). A single study reported no effect of vitamin C supplementation on lipid peroxidation in ODS rats (87). Two of the studies reported an interaction between vitamin C and vitamin E (86, 89). Finally, two recent studies in which guinea pigs were cosupplemented with vitamin C and iron showed reduced *ex vivo* MDA accumulation (91) and reduced tissue levels of F_2-isoprostanes (K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations), supporting an antioxidant, rather than a pro-oxidant, role of vitamin C *in vivo* in the presence of iron.

Human supplementation studies

Over a dozen studies have been carried out with humans to determine the effects of vitamin C supplementation (500–1000 mg/day) on *in vivo* and *ex vivo* lipid peroxidation (Table 3). Smokers are known to be under enhanced oxidative stress as evidenced by not only reduced plasma levels of ascorbate (14), but also increased levels of circulating lipid oxidation products such as F_2-isoprostanes (92, 106). Six studies have investigated the effect of supplemental vitamin C on lipid peroxidation in smokers (92, 94–98), and one of these studies mea-

sured the specific biomarker 8-epiPGF$_{2\alpha}$ (92). Urinary levels of 8-epiPGF$_{2\alpha}$ in five heavy smokers were decreased by one-third after supplementation with 2000 mg/day of vitamin C for only 5 days. However, another study in which coronary artery disease patients were supplemented with 500 mg/day of vitamin C showed no change in plasma 8-epiPGF$_{2\alpha}$ levels (93). Plasma levels of TBARS have been used as a marker of lipid oxidation in three studies of smokers (95, 97, 98). Of these, one reported a reduction (98), one no change (97), and the third an increase (95) in plasma TBARS levels after vitamin C supplementation. This latter study also showed no effect of vitamin C supplementation on *ex vivo* copper-stimulated LDL oxidation, in agreement with another study (94). However, a third *ex vivo* study (96) found a reduction in LDL oxidation after supplementation of smokers with vitamin C.

Of the vitamin C intervention trials carried out with healthy individuals or nonsmokers (57, 99–105) (Table 3), two reported a significant reduction in plasma MDA levels after supplementation with vitamin C (99, 101). These investigators also reported reduced plasma levels of allantoin, an oxidation product of urate (99), and increased levels of red cell-associated vitamin E and GSH (101). In addition, one of these studies (101) reported reduced *ex vivo* LDL oxidation after vitamin C supplementation, and this finding has been confirmed by another two studies (102, 103). A trial in which nonsmokers were supplemented with 6000 mg/day of vitamin C (57) showed a nonsignificant trend toward reduced plasma levels of MDA and HNE. Another study (100), however, found no change in urinary TBARS levels after supplementation with vitamin C. Last, two recent intervention studies have been conducted to determine the effect of vitamin C consumption on exercise-induced oxidative stress (104, 105). One of these trials (104) showed reduced plasma TBARS levels after exercise in healthy individuals supplemented with vitamin C. The other study (105) showed no change in plasma or LDL TBARS levels in runners supplemented with a single dose of vitamin C, but did show reduced *ex vivo* LDL oxidation.

An important point to note about the *ex vivo* LDL oxidation studies (94–96, 101–103, 105) is that vitamin C, being a water-soluble molecule, is removed from LDL during isolation from plasma. Therefore, no change in *ex vivo* LDL oxidation would be expected, as was observed in three of the studies discussed (94, 95, 101). The decrease observed in LDL oxidation after vitamin C supplementation in some other studies (96, 102, 103, 105) may be explained by 'contamination' of the LDL preparation with vitamin C, which has been observed previously (107) or by sparing, or regeneration, of LDL-associated vitamin E by vitamin C, as was proposed by Harats and co-workers (102). However, these investigators did not observe a change in vitamin E levels after supplementation with vitamin C.

Overall, *in vitro* experiments consistently show that vitamin C protects isolated LDL (66) and plasma from lipid peroxidation induced by various radical or oxidant generating systems, including AAPH (1, 48, 75), SIN-1 (77), cigarette smoke (78, 79), and activated neutrophils (75). Even in the presence of redox active transition metal ions, which normally catalyze Fenton chemistry, vitamin C was found to protect isolated LDL (66) and plasma (80, 81) from lipid peroxidation. With two exceptions (83, 87), all of the animal studies discussed (Table 3) showed a reduction in lipid peroxidation after vitamin C supplementation, either without (85, 88, 89) or with an oxidative challenge (82, 86, 90, 108) or iron loading (91; K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations). Over half of the human supplementation studies discussed (Table 3) showed reduced *in vivo* accumulation of lipid peroxidation products (57, 92, 98, 99, 101, 104), whereas several showed no change (93, 97, 100, 105) and one showed an increase in plasma TBARS levels (95). With regard to the *ex vivo* LDL oxidation studies, an equal number showed no change (94, 95, 101) or a reduction (96, 102, 105) after vitamin C supplementation. However, these latter studies are difficult to interpret due to the experimental design.

VITAMIN C AND BIOMARKERS OF PROTEIN OXIDATION

Oxidative modifications to proteins have been implicated in numerous conditions including aging, cataract, atherosclerosis, diabetes, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (109). Oxidation can result in protein cross-linking and aggregation, as is the case with lens proteins, as well as loss of enzyme activity (110). Epidemiological studies have shown an inverse relationship between antioxidant vitamin intake and the risk from cataract (110).

The most commonly measured markers of protein oxidation are carbonyl groups (111). Protein carbonyls can be formed by several mechanisms: direct oxidative cleavage of the peptide chain, oxidation of specific amino acid residues such as lysine, arginine, proline, and threonine, or modification of lysine, histidine, and cysteine residues by aldehydes, such as MDA and HNE (111). Advanced glycation end-products are another common marker of protein modification (112). These products are generated by reaction of reducing sugars with lysine residues and are commonly found in diabetics (112). Cysteine and methionine residues are very sensitive to oxida-

TABLE 4. *Effects of vitamin C supplementation on biomarkers of protein oxidation*

Study system	Challenge	Effects of vitamin C	Ref
In vitro			
Human plasma	Cigarette smoke	X Plasma carbonyls, thiol oxidation	(78)
Human plasma	Cigarette smoke, aldehydes	X Protein carbonyls	(122)
Human plasma	AAPH	X Thiol oxidation	(1, 75)
Human plasma	Hypochlorous acid	X Thiol oxidation	(123)
In vivo (animals)			
Guinea pig liver	Endotoxin	↓ Protein carbonyls	(86)
Guinea pig liver	None	↓ Protein carbonyls	(85)
ODS rat liver	Endotoxin	↓ Bilirubin oxidation	(124)
Guinea pig lens	*Ex vivo* UV light	↓ Protein aggregation	(125)
Guinea pig lens	*Ex vivo* heat	↓ Protein aggregation	(126)
In vivo (humans)			
Gastric biopsies	(Gastritis patients), none	↓ Nitrotyrosine	(127)
Urine	(CAD patients), none	X o-Tyrosine and o,o′-dityrosine	(93)
Peripheral blood globin	(Smokers), none	X Protein carbonyls	(55)

↓ = Decreased damage, X = no change, AAPH = 2,2′-azobis(2-amidinopropane) hydrochloride, CAD = coronary artery disease.

tive modification; however, the respective disulfide and methionine sulfoxide products can be reduced via enzymatic means (109). Due to the use of improved analytical techniques such as mass spectrometry, specific amino acid biomarkers of protein oxidation are now being measured; these include *o*- and *m*-tyrosine, *o,o′*-dityrosine, and 3-chloro- and 3-nitrotyrosine (109). Modified amino acid residues have been detected in atherosclerotic plaques (113–115) and brains from Alzheimer's disease patients (116).

Studies using isolated proteins and plasma

Like glucose, vitamin C can slowly glycate proteins such as lens crystallins, forming advanced glycation endproducts (112, 117–119), which have been implicated in cataract formation. However, oxidation of ascorbate to DHA (e.g., by metal ions) was required for the glycation reactions to occur. In contrast, two studies have shown that vitamin C can protect eye proteins against oxidative modification by UV light as measured by histidine and thiol oxidation (120, 121). Plasma exposed to cigarette smoke showed increased formation of protein carbonyls; however, added vitamin C had no effect on carbonyl formation (78, 122) (**Table 4**) although it did inhibit lipid peroxidation (78) (see Table 3). In addition, endogenous vitamin C in plasma was not able to protect plasma thiols from oxidation by AAPH-derived aqueous peroxyl radicals (1, 75), cigarette smoke (78), or hypochlorous acid (123). Vitamin C was effective, however, against carbonyl formation in bovine serum albumin exposed to hypochlorous acid (128). Vitamin C was also shown to inhibit peroxynitrite-induced oxidation of specific amino acid residues in isolated LDL, although it was less efficient than urate (77). Last, addition of vitamin C to isolated LDL, which had been oxidized with HOCl, reversed a majority of the lysine modifications (129).

Animal supplementation studies

A recent animal supplementation study (86) showed that consumption of vitamin C reduced protein carbonyl formation in guinea pigs exposed to an endotoxin challenge (Table 4). Similar findings were reported earlier by the same group (85) whereby reduced protein carbonyl formation and reduced lipid peroxidation were observed in guinea pigs receiving supplemental vitamin C. In another study using ODS rats, vitamin C supplementation was shown to reduce bilirubin oxidation after an endotoxin challenge (124). Two other studies have shown that supplementation of guinea pigs with vitamin C reduced aggregation of lens proteins after *ex vivo* exposure to UV light or heat (125, 126).

Human supplementation studies

Very few studies have been conducted with humans to investigate the effects of supplemental vitamin C on *in vivo* protein oxidation (Table 4). Vitamin C supplementation (2000 mg/day for 4–12 months) of patients with *Helicobacter pylori* gastritis led to a significant reduction in nitrotyrosine levels, as determined immunohistochemically in biopsy tissue (127). Another recent study, however, found no change in urinary *o*-tyrosine or *o,o′*-dityrosine levels in coronary artery disease patients supplemented with 500 mg/day of vitamin C for one month (93). Similarly, supplementation of smokers with 500 mg/day of vitamin C for 4 wk showed no decrease in protein carbonyl levels (55). More studies are required that investigate the effects of vitamin C sup-

TABLE 5. *Role of vitamin C in iron-mediated oxidative damage*

Study system	Challenge	Effects of vitamin C	Ref
In vitro			
Human plasma	Iron	↓ LOOH	(80)
Human plasma, lymph, synovial fluid	None	X Hydroxyl radical	(135)
	Iron-EDTA/H_2O_2	↑ Hydroxyl radical	
Human plasma	Iron/H_2O_2	X Hydroxyl radical	(136)
	Iron-EDTA	↑ Hydroxyl radical	
3T3 fibroblasts	Iron	X MDA	(137)
In vivo (animals)			
Guinea pig liver	Iron + *ex vivo* autoxidation	↓ MDA	(91)
Guinea pig plasma, liver	Iron	↓ F_2-isoprostanes	unpub.[a]
In vivo (humans)			
Leukocytes	Iron (12 wk)	↓ 8-oxoguanine, 8-oxoadenine, 5-hydroxyuracil, 5-chlorouracil	(53)
		↑ Thymine glycol, 5-hydroxycytosine	
Preterm infant plasma	(BDI), none	X F_2-isoprostanes, protein carbonyls	(80)

[a] K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations. ↑ = Increased damage, ↓ = decreased damage, X = no change, BDI = bleomycin-detectable iron, EDTA = ethylenediaminetetraacetic acid, LOOH = lipid hydroperoxides, MDA = malondialdehyde.

plementation on different markers of protein oxidation in humans.

In summary, numerous *in vitro* studies have shown that vitamin C can slowly glycate proteins under oxidizing conditions (112, 117–119). However, other *in vitro* studies have shown that vitamin C protects against UV-induced protein oxidation (120, 121). In plasma, vitamin C was not able to protect proteins from oxidation by a number of radical and oxidant generating systems (1, 75, 78, 122, 123), although it was able to protect isolated (lipo)proteins from oxidation (77, 128, 129). In contrast, all of the animal studies discussed (Table 4) showed reduced *in vivo* protein oxidation after vitamin C supplementation (85, 86, 124). Aggregation of lens proteins *ex vivo* was also reduced by *in vivo* vitamin C supplementation of animals (125, 126), questioning the relevance of the above *in vitro* findings (112, 117–119). Of three human studies (93, 127) (Table 4), one showed a reduction in protein oxidation after vitamin C supplementation (127) whereas the other two showed no effect (55, 93).

DOES VITAMIN C ACT AS A PRO-OXIDANT *IN VIVO* IN THE PRESENCE OF IRON?

Vitamin C is known to increase the gastrointestinal absorption of nonheme iron by reducing it to a form that is more easily absorbed (27). It appears, however, that even at high intakes of vitamin C, iron uptake is tightly regulated in healthy people (27). Nevertheless, low dietary levels of vitamin C may be advantageous in cases of iron overload, such as homozygous hemochromatosis and treatment of β-thalassemia, due to potential iron-induced tissue damage (130, 131). Individuals with iron overload generally have low plasma levels of vitamin C, possibly due to interaction with the elevated levels of 'catalytic' iron in these individuals, and therefore vitamin C administration has been proposed to be harmful in these people (3, 132). Iron overload has also been implicated in the sequelae of atherosclerosis, although the data are conflicting and inconsistent, and individuals with iron overload do not suffer from premature atherosclerosis (133, 134). In addition, several vitamin C and iron cosupplementation studies, both in animals and humans, indicate that vitamin C inhibits rather than promotes iron-dependent oxidative damage (summarized in **Table 5**).

Studies using plasma and cultured cells

In vitro experiments have shown that human serum and interstitial fluid strongly inhibit metal ion-dependent lipoprotein oxidation (138). These findings were attributed to the presence of metal binding proteins in these fluids rather than vitamin C, since enzymatic removal of endogenous vitamin C did not affect the results. However, as mentioned (see Table 3), when sufficient exogenous iron (as ferrous ammonium sulfate) is added to plasma to saturate transferrin and result in nonprotein-bound, bleomycin-detectable iron (BDI), endogenous and exogenous vitamin C inhibits rather than promotes lipid peroxidation (80) (Table 5). This is supported by an earlier study in which vitamin C acted as an antioxidant in serum to which excess copper had been added (81). Two other studies carried out with plasma, lymph, and synovial fluid showed that vitamin C can catalyze the formation of hydroxyl radicals, but only when a catalytically active form of iron, iron-EDTA, was added (135, 136), not ferrous ammonium sulfate (136). Finally, oxidative damage as

assessed by elevated MDA levels was observed in fibroblasts exposed to iron; however, cosupplementation with vitamin C did not exacerbate the pro-oxidant effect of the added iron (137).

Animal supplementation studies

As mentioned (see Table 3), two animal studies have reported an antioxidant role for vitamin C in guinea pigs cosupplemented with vitamin C and iron (**Table 5**): *ex vivo* autoxidation of liver microsomes obtained from iron-supplemented guinea pigs resulted in increased accumulation of MDA compared with control animals or animals cosupplemented with iron and vitamin C (91); and plasma and liver F_2-isoprostane levels were increased in vitamin C deficient guinea pigs supplemented with iron and were reduced by vitamin C cosupplementation (K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations). In the latter study, hepatic vitamin C levels, in contrast to iron levels, were inversely associated with hepatic F_2-isoprostane levels ($R^2 = 0.32$, $P=0.003$ for vitamin C vs. $R^2 = 0.003$, $P=0.73$ for iron) (**Fig. 3***a, b*). Another recent study using rats challenged with paraquat showed an antioxidant role for vitamin C when given before paraquat treatment, but a pro-oxidant role when given after the challenge, as determined by expiratory ethane (84) (see Table 3). The pro-oxidant effect was attributed to the release of metal ions from damaged cells. This study (84), therefore, suggests that vitamin C may have different effects depending on when it is added to the system under study, as has been observed previously with copper-dependent lipid peroxidation in LDL (139, 140).

Human supplementation studies

As discussed (see Table 2), a study carried out in humans to assess the effects of simultaneous iron and vitamin C supplementation has yielded mixed results with respect to various types of oxidized DNA bases in leukocytes (Table 5). Reanalysis of the data from this study (53) shows an inverse association between the plasma concentration of vitamin C and total DNA base damage ($R^2 = 0.49$) (see Fig. 2*a*). In addition, there was a positive correlation between the concentration of plasma vitamin C and the percent transferrin saturation ($R^2 = 0.37$) (see Fig. 2*b*), possibly due to a vitamin C-dependent increase in iron bioavailability (27), but no correlation was observed between percent transferrin saturation and total base damage ($R^2 = 0.043$) (not shown). These correlations are analogous to those observed in the above study using guinea pigs (see Fig. 3) and suggest that vitamin C acts as an antioxidant, rather than a pro-oxidant, *in vivo* in the presence of iron.

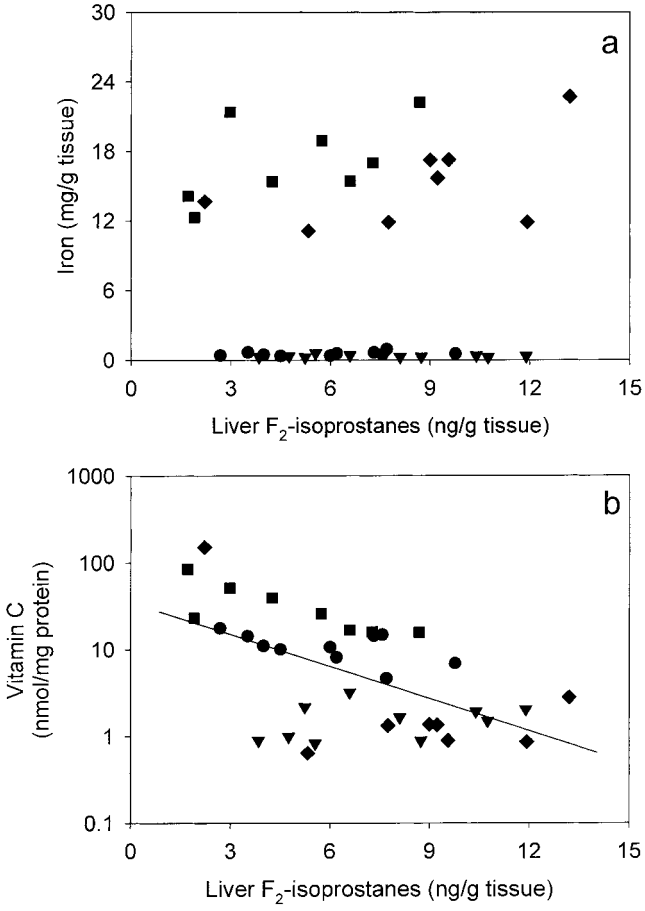

Figure 3. Scatter plot of hepatic iron *(a)* and vitamin C levels *(b)* vs. F_2-isoprostane levels in 38 guinea pigs. Guinea pigs were loaded with iron dextran (1.5 g of iron/kg body weight) or dextran alone (control), then fed a diet with a high (50 mg/day) or low dose (0.5 mg/day) of vitamin C: high vitamin C/control (●), low vitamin C/control (▼), high vitamin C/iron (■), and low vitamin C/iron (◆). Linear regression analysis of iron vs. F_2-isoprostanes gave $R^2 = 0.003$, $P=0.73$; and vitamin C vs. F_2-isoprostanes gave $R^2 = 0.32$, $P=0.003$.

Decreased levels of serum vitamin C and increased levels of oxidative lipid and protein products have been detected in hemochromatosis and β-thalassemia patients (130, 131), which was attributed to the iron overload condition. However, these conclusions are not supported by another recent study with preterm infants with BDI (80). In this study, plasma levels of F_2-isoprostanes and protein carbonyls were not correlated with BDI, even in the presence of high plasma concentrations of vitamin C (Table 5). Although multivariate regression analysis of the data (**Fig. 4**) showed that F_2-isoprostanes were positively correlated with ascorbate ($P=0.02$), this correlation was not statistically significant in either subgroup, i.e., preterm infants with or without BDI. In addition, in those infants with BDI there was no significant correlation between F_2-isoprostane and BDI levels (80).

Overall, *in vitro* studies have shown that vitamin C either has no effect (138) or inhibits (80, 81) metal

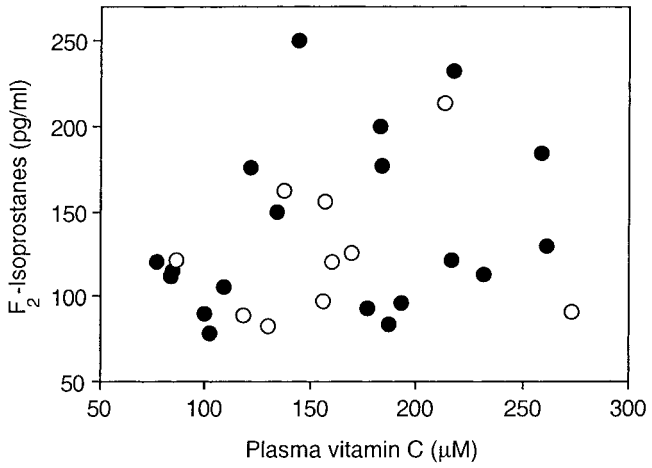

Figure 4. Scatter plot of F_2-isoprostane vs. vitamin C levels in plasma of 29 preterm infants. Ten plasma samples contained no bleomycin-detectable iron (BDI) (○), and 19 contained BDI in the range of 0.02–5.0 μM (●). Multivariate regression analysis showed that F_2-isoprostanes were positively correlated with vitamin C (P=0.02), but this correlation was not statistically significant in either subgroup, i.e., preterm infants with or without BDI. For further details, see (80).

ion-dependent lipid oxidation in plasma and other biological fluids. In contrast, vitamin C may be able to promote metal ion-dependent hydroxyl radical production in biological fluids, but only under certain unphysiological conditions (116, 135). In fibroblast cell cultures vitamin C did not promote the pro-oxidant effect of added iron (137). In addition, two recent animal studies showed an antioxidant role for vitamin C when cosupplemented with iron (91; K. Chen, J. Suh, A. Carr, J. Morrow, J. Zeind, B. Frei, unpublished observations). A third study, in which animals were challenged with paraquat, showed mixed results depending on when vitamin C was administered (84). A recent human study, in which vitamin C and iron were given as cosupplements to healthy adults, showed mixed results with respect to various oxidative DNA lesions, but no overall increase in total base damage (53). Finally, another human study found no correlation between lipid and protein oxidation and the amount of BDI and vitamin C in plasma of preterm infants (80).

DISCUSSION

To answer the question—'does vitamin C act as a pro-oxidant under physiological conditions?'—we have analyzed the data from *in vitro* and *in vivo* studies in which specific biomarkers of oxidative DNA, lipid, and protein damage were measured. *In vitro* studies of oxidative DNA damage indicate that vitamin C acts as an antioxidant unless added or endogenous metal ions are present (Table 2). This result is expected based on the known pro-oxidant role of vitamin C in the presence of metal ions *in vitro* (reactions 1–3). In contrast, *in vitro* studies of lipid peroxidation in plasma and LDL overwhelmingly demonstrate an antioxidant role for vitamin C, even in the presence of added metal ions (Table 3). One specific mechanism for this effect has been postulated by Frei and co-workers: site-specific oxidation of histidine residues and other metal binding sites on lipoproteins and consequent release of the metal ions (141). The *in vitro* data on protein oxidation suggest that ascorbate cannot inhibit thiol oxidation or protein carbonyl formation in biological fluids (Table 4).

A vast majority of the animal studies reviewed show that vitamin C acts as an antioxidant *in vivo* and *ex vivo* toward both lipids (Table 3) and proteins (Table 4), with and without oxidative challenge. There are, however, insufficient animal studies of DNA oxidation to draw conclusions (Table 2). An important point to note about studies in animals that can synthesize vitamin C, such as rats, is that the results may not reflect the situation in humans. Supplementation of these animals with vitamin C may even reduce endogenous levels of ascorbate (142). More appropriate animal models are guinea pigs or genetically scorbutic (ODS) rats (87–89, 124), which, like humans, lack a functional enzyme, L-gulono-γ-lactone oxidase.

Consistent with the *in vitro* and animal data on lipid oxidation, human studies on lipid oxidation also indicate an antioxidant role of vitamin C (Table 3), whereas the data on protein oxidation are sparse and inconclusive (Table 4). The human data on the role of vitamin C in DNA oxidation are controversial and appear inconsistent (Table 2), but this inconsistency is likely attributed to technical problems associated with GC-MS analysis in some studies (51, 53). Another crucial point with regard to human supplementation studies, which could explain a lack of an effect of vitamin C supplementation, is the presupplementation, or baseline, levels of vitamin C in plasma or tissues. Levine and co-workers (143) investigated the pharmacokinetics of vitamin C and found that in healthy men, tissue saturation (measured in peripheral blood leukocytes) occurred at vitamin C intakes of ~100 mg/day, which corresponds to a plasma concentration of ~50 μmol/l. If tissues are already saturated due to an adequate intake of vitamin C at baseline, subsequent supplementation cannot have an effect on tissue vitamin C levels and thus oxidative biomarkers.

A majority of the studies that specifically addressed the interaction of vitamin C with iron in physiological fluids and *in vivo* (Table 5) found either no effect of vitamin C or decreased oxidative damage. This result is contrary to what one would expect based on the known pro-oxidant role of vitamin C in Fenton

chemistry *in vitro* (reactions 1–3). Vitamin C played a pro-oxidant role *in vitro* only in biological fluids to which iron-EDTA was added (135, 136). An important point of distinction between vitamin C acting as a pro-oxidant or an antioxidant is the time when the vitamin is added to the system (84, 140). For example, vitamin C acts as an antioxidant if added before initiation of LDL oxidation by copper, but acts as a pro-oxidant if added to LDL that is already (mildly) oxidized (140). However, in a physiological situation, vitamin C would be expected to be present at all times. Another factor that may affect the pro-oxidant vs. antioxidant properties of vitamin C is its concentration, since *in vitro* data suggest that at low levels vitamin C can act as a pro-oxidant, but as an antioxidant at high levels (4). However, *in vivo* evidence for this contention is lacking.

More studies are warranted in which the effects of vitamin C supplementation on more than one biomarker of oxidative damage are determined. This is particularly important because several studies in which more than one oxidative biomarker was measured showed an antioxidant role of vitamin C with respect to lipid oxidation, but not DNA oxidation (40) or protein oxidation (1, 75, 78). These discrepancies may be due to the differential ability of the various macromolecules, i.e., DNA, lipids, and proteins, to bind metal ions and the redox activity of the bound metal ions (28). Both DNA and some membrane lipids can bind metal ions, as can proteins, either as part of their active site (e.g., hemoglobin and superoxide dismutase), biological function (e.g., ferritin, transferrin and ceruloplasmin), or as a general, nonspecific property (e.g., binding of copper by albumin and LDL) (28, 141, 144). Nevertheless, before more studies are carried out, some important points should be considered: *1)* use of appropriate biomarkers, *2)* use of appropriate methodologies, *3)* use of appropriate study systems, and *4)* proper experimental design.

Use of appropriate biomarkers

Numerous different oxidative biomarkers have been used in the studies reviewed, some of which are more specific and hence preferred. Analysis of single-strand breaks by the comet assay as a marker of oxidative DNA damage is less specific than measurement of individual base modifications, such as 8-oxogua and 8-oxodG. Nevertheless, the specificity of single-strand breaks as a marker of oxidative damage can be improved by using lesion-specific glycosylase repair enzymes (37). Similarly, analysis of TBARS is a commonly used assay for measuring lipid peroxidation, but it is nonspecific, particularly in complex biological fluids or tissues (67). In contrast, F_2-isoprostanes such as 8-epi$PGF_{2\alpha}$ are specific biomarkers of nonenzymatic lipid peroxidation (67). A similar argument can be made for markers of protein oxidation: protein carbonyls are fairly nonspecific, being formed by different mechanisms (111), whereas direct measurement of specific amino acid modifications, such as *o*- or *m*-tyrosine, *o,o′*-dityrosine, and 3-chloro- or 3-nitrotyrosine, provides specific information on the oxidative mechanisms involved (109). Although 3-nitrotyrosine was once thought to be a specific marker for peroxynitrite-mediated damage, it is now known that it can also be formed by myeloperoxidase-dependent processes (145).

Use of appropriate methodologies

This issue is particularly important in studies analyzing DNA oxidation, as has already been discussed in detail. What is perhaps surprising is that although improvements have been made to GC-MS methodology to reduce artifactual oxidation (34, 146), these appear to have been ignored in a recent controversial study claiming to observe pro-oxidant properties of vitamin C in humans (51). In contrast, the comet assay and HPLC-ECD appear to be gaining favor as a result of their minimal *ex vivo* oxidation artifacts and low basal levels of oxidative DNA damage (35, 36). Measurement of lipid peroxidation using the relatively nonspecific TBARS assay has been moderately improved by detecting the specific MDA-TBA adduct using HPLC (67). Aldehydes derived from lipid peroxidation have also been measured using GC-MS, which is significantly more sensitive and specific than the TBARS assay (67). Similarly, detection of F_2-isoprostanes by enzyme immunoassays is less specific than analysis of these products by GC-MS, whereby individual F_2-isoprostane isomers can be identified and quantified (67). Last, analysis of vitamin C itself using HPLC-ECD (1) is significantly superior to colorimetric assays (147), which can be subject to interference, analogous to the TBARS assay.

Use of appropriate study systems

With regard to analysis of oxidative DNA damage, tissue levels of DNA oxidation, which reflect a steady state between oxidation and cellular repair, are preferred over urinary levels, which reflect the net rate of damage and repair. This is because urinary levels of oxidized DNA, in contrast to tissue levels, can also result from nonspecific cell turnover (64) and dietary sources, and therefore are difficult, if not impossible, to interpret. In the case of lipid oxidation, the use of *ex vivo* LDL oxidizability to determine the effect of vitamin C supplementation is *not* appropriate because vitamin C, unlike vitamin E, is not LDL associated. *Ex vivo* oxidation of LDL is also

dependent on the rate of radical production, since tocopherol-mediated peroxidation is increased with a low radical flux and decreased with a high radical flux (15). Tocopherol-mediated peroxidation is also affected by the availability of coantioxidants, such as ubiquinol-10 (148). The choice of animal models is important since some models used for vitamin C supplementation studies (rats, for example) produce their own vitamin C endogenously. A study by Tsao and colleagues (142) found that certain intakes of vitamin C lowered tissue ascorbate levels in mice, possibly through feedback inhibition of endogenous ascorbate biosynthesis. Therefore, guinea pigs and ODS rats should be used. Whether or not the animals are to be exposed to an oxidative challenge also needs to be taken into consideration with respect to the specific actions of the challenge.

Proper experimental design

Probably the most important point with respect to study design is the baseline vitamin C levels in human subjects prior to supplementation. As has already been discussed, if the subjects have a dietary intake that is already saturating, i.e., as little as 100 mg of vitamin C per day (143), then (further) supplementation will have no effect. Therefore, future studies need to be conducted with individuals with low initial vitamin C status, and vitamin C levels before and after intervention need to be measured in the tissue or fluid in which oxidative damage is assessed. Whether or not subjects smoke is another important consideration, since smoking is a known oxidative stress that lowers plasma and tissue ascorbate levels (14). Other confounding factors with respect to oxidative stress include alcohol consumption, fruit and vegetable intake, vitamin supplements, pregnancy, oral contraceptive use, and use of antihistamines, antibiotics, or antiinflammatory drugs. In addition, proper placebo controls and other control groups, which often are not included (51, 53), must be used.

CONCLUSION

Does vitamin C act as a pro-oxidant under physiological conditions? The answer appears to be 'no'. Of the 44 *in vivo* studies discussed (Tables 2–4), 38 showed a reduction in markers of oxidative DNA, lipid, and protein damage, 14 showed no change and only 6 showed an increase in oxidative damage after supplementation with vitamin C. Several of the studies showed a combination of effects depending on the study systems or experimental design. Even in the presence of iron (summarized in Table 5), vitamin C predominantly reduced *in vivo* oxidative damage, despite its well known pro-oxidant properties *in vitro* in buffer systems containing iron. In more complex and physiologically relevant *in vitro* systems, such as isolated or cultured cells (Table 2) and biological fluids (Tables 3 and 4), an antioxidant role, or no effect of vitamin C, predominated over a pro-oxidant role. Studies that report a pro-oxidant role for vitamin C need to be evaluated carefully as to their choice of biomarkers, methodology, study system, and experimental design to rule out any oxidation artifacts. It is hoped that these four important considerations will be taken into account in all future studies of the role of vitamin C in oxidative damage. FJ

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