Vitamin C (ascorbic acid, AA) can act as an antioxidant or a pro-oxidant in vitro, depending on the absence or the presence, respectively, of redox-active metal ions. Some adults with iron-overload and some premature infants have potentially redox-active, bleomycin-detectable iron (BDI) in their plasma. Thus, it has been hypothesized that the combination of AA and BDI causes oxidative damage in vivo. We found that plasma of preterm infants contains high levels of AA and F₂-isoprostanes, stable lipid peroxidation end products. However, F₂-isoprostane levels were not different between those infants with BDI (138 ± 51 pg/ml, n = 19) and those without (126 ± 41 pg/ml, n = 10), and the same was true for protein carbonyls, a marker of protein oxidation (0.77 ± 0.31 and 0.68 ± 0.13 nmol/mg protein, respectively). Incubation of BDI-containing plasma from preterm infants did not result in detectable lipid hydroperoxide formation (>10 nM cholesteryl ester hydroperoxides) as long as AA concentrations remained high. Furthermore, when excess iron was added to adult plasma, BDI became detectable, and endogenous AA was rapidly oxidized. Despite this apparent interaction between excess iron and endogenous AA, there was no detectable lipid peroxidation as long as AA was present at >10% of its initial concentration. Finally, when iron was added to plasma devoid of AA, lipid hydroperoxides were formed immediately, whereas endogenous and exogenous AA delayed the onset of iron-induced lipid peroxidation in a dose-dependent manner. These findings demonstrate that in iron-overloaded plasma, AA acts an antioxidant toward lipids. Furthermore, our data do not support the hypothesis that the combination of high plasma concentrations of AA and BDI, or BDI alone, causes oxidative damage to lipids and proteins in vivo.

Oxidative damage to biomolecules has been implicated as a causal factor in several human diseases (1). For example, retinopathy of prematurity and chronic lung disease in preterm infants have been attributed, in part, to oxidative injury (2). Premature infants may be particularly susceptible to oxidative injury because they are exposed to high levels of oxidants and/or their antioxidant defenses are immature (2–4). The significance of oxidative damage to lipids in preterm infants with respiratory distress is supported by the observations that these infants exhale increased levels of volatile lipid peroxidation products, which is directly correlated with poor respiratory outcome and death (5, 6). Furthermore, high levels of protein carbonyls, a protein oxidation product, in tracheal aspirates of preterm infants are associated with the development of chronic lung disease (7).

Oxidative damage to biomolecules is inhibited by antioxidants (1). Frei et al. (8–10) have shown that vitamin C (ascorbic acid, AA)¹ is a powerful antioxidant preventing lipid peroxidation in plasma exposed to various types of oxidative stress. Interestingly, preterm infants are born with high plasma AA concentrations, which drop precipitously soon after birth (11, 12). In addition, bleomycin-detectable iron (BDI) is present in plasma of some preterm infants (13–15). BDI is a form of iron that is nontransferrin-bound, chelatable by bleomycin, and potentially redox-active (16, 17). Therefore, BDI has been suggested to contribute to oxidative damage in vivo (13–19). BDI has also been detected in patients with iron-overload such as homozygous hemochromatosis (17, 18) and in patients suffering from rheumatoid arthritis (16) or undergoing chemotherapy (19).

It is well known that in the presence of redox-active iron, AA can act as a pro-oxidant in vitro and contribute to the formation of hydroxyl radicals, which in turn may cause lipid, DNA, or protein oxidation (20). Thus, Silvers et al. have suggested that high plasma concentrations of AA in preterm infants are harmful because of the presence of BDI in some of these infants (21). Similarly, there is considerable controversy as to whether vitamin C supplementation in individuals with high iron status and/or BDI in plasma is deleterious because it may cause oxidative damage to biomolecules (15, 22–24). The goal of the present study, therefore, was to determine whether the combination of BDI or excess iron and high concentrations of AA in plasma is associated with increased lipid and protein oxidation in vivo and in vitro.

**EXPERIMENTAL PROCEDURES**

**Subjects**—The study protocol was approved by the Institutional Review Board of the Brigham and Women’s Hospital, Boston, MA, and the Clinical Research Committee of the Beth Israel Hospital, Boston, MA. Twenty-nine infants with a gestational age of less than 34 weeks born at either hospital were enrolled in the study. Mean gestational age and birth weight were 29 weeks and 1390 grams, respectively (see also Table I). There were no exclusion criteria based on maternal, preg-

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¹ The abbreviations used are: AA, ascorbic acid; BDI, bleomycin-detectable iron; LIBC, latent iron-binding capacity; HPLC, high pressure liquid chromatography.

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nancy, or perinatal history. Umbilical cord blood samples were drawn into heparinized tubes (Vacutainer® PST™ Gel and Lithium Heparin, Becton Dickinson, Rutherford, NJ) immediately after birth. After centrifugation of the blood samples at 1000 x g for 5 min and 4 °C, the plasma was collected and stored at −70 °C until analysis (up to six months). Blood samples from five adult, nonsmoking, healthy volunteers acting as controls were collected by venipuncture and processed and stored in identical fashion.

Measurements—BDI was measured as described (25). Serum total iron and latent iron-binding capacity (LIBC, unbound iron) were measured by optical spectrophotometric analysis using a commercial kit (Sigma procedure 565). This procedure is based upon the determination of iron with ferrozine as described by Persijn et al. (26). Total-iron-binding capacity (total iron + LIBC) and transferrin saturation (total iron/total iron binding capacity) were calculated from these measured values. Plasma levels of nonesterified F2-isoprostanes, stable end products of lipid peroxidation, were measured by gas chromatography negative ion chemical ionization/mass spectrometry with selected ion monitoring (27). Protein carbonyls were determined as described by Lyras et al. (28). For AA determination, plasma (50 μl) was mixed with an equal volume of 5% (w/v) metaphosphoric acid containing 1 mM D,L-dithiothreitol, centrifuged, and the supernatant analyzed by paired-ion reverse-phase HPLC with electrochemical detection at an applied potential of +0.6 V (9). For lipid hydroperoxide analysis, plasma (100 μl) was extracted with 1 ml of methanol and 5 ml of hexane. The hexane phase was collected and evaporated under nitrogen, and cholesteryl ester hydroperoxides were quantitated by reverse-phase HPLC with chemiluminescence detection (29).

Statistical Analysis—The two-sample Student’s t-test was used to compare plasma concentrations of AA, F2-isoprostanes, and protein carbonyls in preterm infants with and without BDI and in adults. Stepwise multivariate linear regression analysis was performed to evaluate correlations between gestational age, BDI, AA, F2-isoprostanes, and protein carbonyl levels. Statistical significance was accepted if the null hypothesis was rejected at the p < 0.05 level.

RESULTS

To investigate the role of BDI in oxidative damage in vivo, we obtained plasma samples from 29 preterm infants and 5 adult controls. BDI was found in plasma of 19 (66%) preterm infants (mean ± SD, 0.75 ± 0.70 μM, range 0.02–5.0 μM) and was not detected in any of the adult control samples (Table I). Plasma concentrations of AA and nonesterified F2-isoprostanes, but not protein carbonyls, were significantly higher in preterm infants than in adults (Table I). There were no significant differences in the levels of AA, F2-isoprostanes, and protein carbonyls between preterm infants with and without BDI (Table I). For all preterm infants (n = 29), multivariate regression analysis revealed that F2-isoprostanes were positively correlated with gestational age (p = 0.002) and AA (p = 0.02), suggesting a pro-oxidant role of AA. However, subgroup analysis of preterm infants with and without BDI showed that the correlation between F2-isoprostanes and AA was not statistically significant in either group. In addition, in those infants with BDI there was no statistically significant correlation between F2-isoprostane and BDI levels. These data suggest that the high plasma concentrations of F2-isoprostanes in preterm infants are not a direct consequence of the presence of BDI nor a pro-oxidant interaction between BDI and AA. Conversely, the data also do not provide support for an antioxidant role of AA in these infants.

To study the interactions between BDI, AA, and lipids in plasma, selected samples from preterm infants with high (>0.5 μM), low (0.08–0.5 μM) or nondetectable BDI levels were pooled. Plasma samples from adults with relatively high AA levels were also pooled. BDI levels in the pooled samples were re-determined (Table II). Although F2-isoprostanes could be detected (see Table I), none of the samples contained detectable amounts (≤10 nM) of cholesteryl ester hydroperoxides. It should be noted that F2-isoprostanes are stable end products of lipid peroxidation (27). We have shown previously that authentic lipid hydroperoxides cannot be detected (≤10 nm) in plasma of healthy adults (29), in contrast to nonesterified F2-isoprostanes, which are usually present at levels of about 0.1 nm or 35 pg/ml (see Table I and Ref. 27).

The pooled plasma samples were incubated at 37 °C for 8 days, and concentrations of AA and lipid hydroperoxides (cholesteryl ester hydroperoxides) were followed. As shown in Fig. 1 (A–D), AA was depleted in all samples over the first 4 days of incubation. The maximal absolute rate of AA oxidation was lowest in the adult control sample, and it was higher and apparently related to BDI levels in the samples from premature infants (Table II). However, the fractional AA oxidation rates were not different between the three samples from premature infants (Table II), indicating that the varying absolute rates are a reflection of the initial AA concentrations and not the different BDI levels. In all incubations, there was initially no detectable lipid peroxidation (Fig. 1). Interestingly, in the samples from preterm infants, lipid hydroperoxides became detectable before the complete consumption of AA, but the time point at which detectable lipid peroxidation began was not related to BDI levels (Fig. 1, A–C). In adult plasma, the completion of AA oxidation coincided with the onset of detectable lipid peroxidation (Fig. 1D), in agreement with our previous data (8–10). Once started, lipid peroxidation in all samples occurred at a rate not related to BDI levels (Table II).

The above findings suggest that neither BDI itself nor an interaction between BDI and AA causes lipid oxidative damage in plasma. To further investigate these notions, we added iron (in the form of ferrous ammonium sulfate) to heparinized plasma from a healthy adult male at concentrations below, near, or above the latent iron-binding capacity (LIBC). When iron was added at levels exceeding the LIBC of the plasma sample (40.9 μM), transferrin was 100% saturated, and BDI became detectable (Table III). Interestingly, the sample to which 33 μM iron had been added also contained BDI, although transferrin was only 80% saturated (Table III). This finding is in agreement with previous observations that in plasma of some hemochromatosis patients and preterm infants, BDI is present even when transferrin is not fully iron-saturated (13, 30). In the control plasma sample without added iron and the sample to which 16 μM iron had been added, no BDI was detected before the completion of AA oxidation.
oxidation was observed (earlier than in the control incubation (Fig. 2). When iron was rapidly (containing added iron below the LIBC, AA was oxidized more 2), in agreement with the above data (Fig. 1 see legend to Fig. 1.

Iron status of adult plasma supplemented in vitro with different concentrations of iron

Ferrous ammonium sulfate hexahydrate \((\text{NH}_4)_2\text{Fe(SO}_4)_2 \cdot 6\text{H}_2\text{O}\) was added to plasma at the concentrations indicated in the first column, and BDI, total iron, latent iron-binding capacity (LIBC), total iron-binding capacity (TIBC), and transferrin saturation (%) were determined as described under “Experimental Procedures.” In order to minimize dilution of the plasma, the added volume of the \((\text{NH}_4)_2\text{Fe(SO}_4)_2 \cdot 6\text{H}_2\text{O}\) stock solution was \(\approx 2\% (v/v)\) of the final volume. For further details, see legend to Fig. 2. ND, not detected.

| Sample  | n | Bleomycin-detectable iron | Maximal rate of AA oxidation | Rate of lipid peroxidation |
|---------|---|----------------------------|-----------------------------|---------------------------|
|         |   | \(\mu\mu\text{M}\) | \(\mu\mu\text{M/h}\) % AA/h | % CE/h |
| Preterm I | 4 | 0.7                        | 4.41                        | 2.27 | 0.022 |
| Preterm II | 4 | 0.3                        | 4.11                        | 2.30 | 0.016 |
| Preterm III | 4 | not detected               | 3.24                        | 2.23 | 0.032 |
| Adults | 3 | not detected               | 1.86                        | 1.60 | 0.043 |

FIG. 1. Ascorbic acid oxidation and cholesteryl ester hydroperoxide formation in plasma of preterm infants (panels A C) and adult controls (panel D). Pooled samples were prepared as described in the text and incubated at \(37^\circ\text{C}\) for 8 days. Aliquots were withdrawn at time intervals and analyzed for ascorbic acid (C) and cholesteryl ester hydroperoxides (●) as described under “Experimental Procedures.” Levels of BDI and maximal rates of ascorbic acid oxidation and rates of lipid peroxidation are given in Table II. Levels of CE in (mM) were as follows: 3.58 (A), 4.17 (B), 3.27 (C), and 5.48 (D).

detected, and transferrin saturation was \(< 50\%\) (Table III).

The above plasma samples were incubated at \(37^\circ\text{C}\), and AA consumption and lipid hydroperoxide formation were followed. In the control sample, AA was oxidized slowly \((t_{1/2} = 34.7 \text{ h})\), and lipid peroxidation began after AA had been depleted (Fig. 2), in agreement with the above data (Fig. 1D). In the samples containing added iron below the LIBC, AA was oxidized more rapidly \((t_{1/2} = 4.3 \text{ h})\), and lipid hydroperoxides were detected earlier than in the control incubation (Fig. 2). When iron was added above the LIBC, a dramatic increase in the rate of AA oxidation was observed \((t_{1/2} = 5.7 \text{ min})\), and detectable lipid peroxidation started within the first 2 h of incubation. Despite the presence of \(\geq 1.4 \mu\mu\text{M}\) BDI in these samples (Table II), however, there was initially no detectable lipid peroxidation, and lipid hydroperoxides were formed only after \(> 90\%\) of endogenous AA had been depleted (Fig. 2). The amount of lipid hydroperoxides formed in each sample at the end of the incubation was directly related to the amount of added iron (Fig. 2B) but not BDI nor the initial AA concentration, which was the same in all samples. These data indicate that the added iron interacted with AA, causing its rapid oxidation; however, despite this apparent interaction AA did not exert a pro-oxidant effect toward lipids but rather prevented detectable lipid peroxidation until its depletion.

Finally, to conclusively show that AA acts as an antioxidant, not a pro-oxidant, in plasma containing excess iron, normal adult plasma was either treated with ascorbate oxidase (to oxidize endogenous AA), left untreated, or supplemented with 200 \(\mu\mu\text{M}\) AA, resulting in AA concentrations of \(< 1, 58, \text{ and } 260 \mu\mu\text{M}\) AA, respectively. These samples were incubated at \(37^\circ\text{C}\) with 33 or 75 \(\mu\mu\text{M}\) added iron. In confirmation of the above data (Fig. 2A), the rate of AA oxidation was higher in the presence of 75 than 33 \(\mu\mu\text{M}\) iron \((t_{1/2} = 8.3 \text{ min} \text{ and } 2.6 \text{ h})\), respectively, for the sample containing 58 \(\mu\mu\text{M}\) AA and 22.4 min and 2.2 h, respectively, for the sample containing 260 \(\mu\mu\text{M}\) AA. Importantly, those plasma samples containing the highest levels of AA were most effectively protected against lipid peroxidation, whereas in those samples devoid of detectable AA, lipid peroxidation began immediately after the addition of iron (Fig. 3, A and B). These data demonstrate that AA inhibits rather than promotes iron-induced lipid peroxidation in plasma.

DISCUSSION

The aim of this study was to determine whether AA in conjunction with BDI or excess iron acts as a pro-oxidant in human plasma \(\text{in vivo}\) and \(\text{in vitro}\). To this end, we compared BDI and AA concentrations in plasma of 29 preterm infants and 5 adult controls with the levels of nonesterified F2-isoprostanes and protein carbonyls, \(\text{in vivo}\) markers of lipid and protein oxidative damage, respectively. In addition, we measured AA oxidation and lipid hydroperoxide formation in incubations of pooled plasma samples from preterm infants, as well as adult plasma to which iron had been added. All those studies we consistently observed that in plasma containing BDI or excess iron, vitamin C does not act as a pro-oxidant toward lipids or proteins; to the contrary, it strongly inhibits lipid peroxidation \(\text{in vitro}\).

BDI is a form of potentially redox-active iron present in plasma of some human neonates and certain adult patients,
mented in vitro panel B hydroperoxide formation (ammonium sulfate was added to plasma at 0 (11, 12, 21). The observed mother-infant gradient ising higher plasma AA concentrations in neonates than their preterm infants than in adults, consistent with studies show-
those reported by other investigators for preterm infants (14, 15) but somewhat lower than those reported by Evans et al. (13) but nonetheless suggest oxidative damage to biomol-
eules in vivo (13–19). We found BDI in plasma of 19 out of 29 preterm infants, and the concentrations were comparable with those reported by Minetti and colleagues (24), who found that addition of iron to plasma in excess of the LIBC leads to rapid AA oxidation but not forma-
due to the presence of BDI and high concentrations of AA (13–15, 21–23). We observed a statistically significant positive correlation between F2-isoprostanes and AA in all preterm infants. However, if AA were to act as a pro-oxidant, this correlation should be observed only in those infants with BDI, which was not the case. In addition, F2-isoprostane and protein carbonyl levels were not different between preterm infants with and without BDI. These observations do not support the notions that AA acts as a pro-oxidant in preterm infants and that BDI causes oxidative damage in vivo (14, 15, 21, 22).

Conclusive evidence that AA does not act as a pro-oxidant toward lipids in plasma containing BDI or excess iron, and in fact acts as an antioxidant, is provided in the present paper by three different types of experiments: (i) Incubation of plasma from preterm infants containing BDI does not result in detect-
able lipid hydroperoxide formation as long as AA concentra-
tions are high (Fig. 1, A and B). (ii) When iron is added to adult plasma in amounts exceeding the LIBC, BDI is detected, and the rate of AA oxidation is increased dramatically. However, despite this apparent interaction between AA and added iron, detectable lipid peroxidation is prevented as long as AA is present at >10% of its initial concentration (Fig. 2). (iii) Iron-
induced lipid peroxidation in plasma is inhibited, not pro-
Rered, in a concentration-dependent manner by AA (Fig. 3).

These findings are in agreement with our previous observa-
tions that AA strongly inhibits iron- and copper-induced oxida-
tion of low density lipoprotein, presumably by destroying met-
al-binding sites on apolipoprotein B-100 (35–37). Thus, the mechanism by which AA prevents iron-induced lipid peroxidation in plasma may involve the inhibition of iron-binding to plasma lipoproteins. In addition, it has been reported that the simultaneous presence of Fe2+ and Fe3+ is required for the initiation of lipid peroxidation and that there is no lipid per-
oxidation as long as AA is present at concentrations high enough to keep iron in the reduced state (38). Such a mecha-
nism would explain, at least in part, the antioxidant activity of AA in the presence of excess iron observed in the present study. However, the notion that both Fe2+ and Fe3+ are required for initiation of lipid peroxidation has been disputed (39).

Our data are in close agreement with those of Minetti and colleagues (24), who found that addition of iron to plasma in excess of the LIBC leads to rapid AA oxidation but not forma-

![Figure 2](Image 80x364 to 275x729)

**FIG. 2.** Ascorbic acid oxidation (panel A) and cholesteryl ester hydroperoxide formation (panel B) in adult plasma supplemented in vitro with different concentrations of iron. Ferrous ammonium sulfate was added to plasma at 0 (○), 16 (●), 50 (○), 75 (△), and 100 μM (▲). The iron status of these samples (bleomycin-detectable iron, total iron, latent iron-binding capacity, total iron-bind-
ing capacity, and transferrin saturation) are given in Table III. The samples were incubated at 37 °C for 100 h, and aliquots were with-
drawn at time intervals for ascorbic acid and cholesteryl ester hydroper-
oxide (CEOOH) analysis as described under “Experimental Proce-
dures.” Note the different scales for the ordinates in panels A (0–1 μM) and B (0–8 μM).

![Figure 3](Image 313x602 to 559x729)

**FIG. 3.** The effects of ascorbic acid depletion or supplementation on cholesteryl ester hydroperoxide formation in adult plasma incubated with 33 (panel A) or 75 μM iron (panel B). Plasma was depleted of endogenous AA by treatment with 0.5 unit/ml of ascorbate oxidase for 15 min at 25 °C, left untreated, or supple-
mented with 200 μM AA from a freshly prepared 5 mM stock solution of AA in 10 mM phosphate-buffered saline, resulting in measured plasma AA concentrations of < 1 (●), 58 (○), and 260 μM (▲), respectively. Ferrous ammonium sulfate was added at 33 (panel A) or 75 μM (panel B), and the samples were incubated at 37 °C for 42 h. Aliquots were withdrawn at time intervals and analyzed for cholesteryl ester hy-
droperoxides (CEOOH) as described under “Experimental Procedures.” Note the different scales for the ordinates in panels A (0–1 μM) and B (0–8 μM).
tion of hydroxyl radicals, as monitored by spin trapping epr spectroscopy. Even when these researchers attempted to promote hydroxyl radical formation in iron-overloaded plasma by adding hydrogen peroxide and azide, the latter an inhibitor of plasma ferroxidase (Fe^{2+} \rightarrow Fe^{3+}) and catalase activities, no spin trap adducts with hydroxyl radicals were formed (24). The authors speculated, therefore, that iron in excess of the amount bound to transferrin may not support the formation of hydroxyl radicals or that hydroxyl radicals were formed but effectively scavenged at their site of formation by plasma constituents, particularly proteins, before they could react with the spin trap molecules (24). A similar interpretation was suggested by Gutteridge et al. (17), who were unable to detect hydroxyl radical formation in serum of a hemochromatosis patient containing 19.4 μM BDI, a very high concentration of BDI. It is interesting to note that numerous studies have reported hydroxyl radical scavenging at their site of formation by plasma constituents, particularly proteins, before they could react with the spin trap adducts with hydroxyl radicals were formed (24). The conditions significantly alter the protein concentration and antioxidant capacity of these fluids and also lead to the redistribution of iron (18). The results obtained by these studies, therefore, are unlikely to reflect reactions occurring in these fluids in vivo. In contrast, in the present study using nondiluted plasma we did not find evidence for a pro-oxidant role of BDI itself or in combination with AA, and in vitro AA clearly acted as an antioxidant, not a pro-oxidant, toward lipids in iron-overloaded plasma. It remains possible, of course, that pro-oxidant effects are exerted upon other biomolecules, such as DNA.

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