Inflammatory reaction without endogenous antioxidant response in Caco-2 cells exposed to iron/ascorbate-mediated lipid peroxidation

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FREE RADICALS AND OXIDANTS are potentially deleterious molecules that have been implicated in the pathogenesis of many diseases (27, 28). In humans, normal biological activities and exogenous contributors lead to the formation of a large amount of highly reactive radicals every day (21). These radicals can lead to the oxidation of DNA, proteins, lipids, and other cellular constituents, which play a key role in the pathophysiology of various diseases, including inflammatory diseases and cancer (28). Humans have developed antioxidant defenses and detoxification systems to protect against oxidative stress repercussions and to repair damaged biological components (5, 14, 21). However, in many conditions, overproduction of radicals can overcome antioxidant defense systems and give rise to cellular oxidant burden, rendering tissues susceptible to oxidant-induced damage (8, 41).

Oxidative stress is generally defined as an imbalance between prooxidant and antioxidant species (12, 37). Increased oxidative stress has been implicated in iron overload conditions, such as homozygous hemochromatosis and treatment of β-thalassemia (36, 57) and is thought to be due to iron-catalyzed generation of hydroxyl and alkoxyl radicals through Fenton chemistry: $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^+ + \text{Fe}^{3+} + \text{OH}^-$ and $\text{LOOH} + \text{Fe}^{2+} \rightarrow \text{LO}^+ + \text{Fe}^{3+} + \text{OH}^-$, where $\text{HO}^+$ is an hydroxyl radical, $\text{LOOH}$ is a lipid hydroperoxide, and $\text{LO}^+$ is an alkoxyl radical. Various biomolecules are susceptible to damage by reactive oxygen species generated by the presence of reduced transition metals such as Fe$^{2+}$ (23). When these transition metals specifically bind to a protein, the polypeptide backbone can be cleaved by reactive oxygen species in the presence of $\text{H}_2\text{O}_2$ and/or O$_2$ and a reducing agent such as ascorbate. Vitamin C or ascorbate is a strong antioxidant capable of scavenging a wide variety of reactive oxygen and nitrogen species (22). However, under various in vivo and in vitro conditions, ascorbate can act as a prooxidant by reducing transition metal ions, thereby driving the Fenton reaction $\text{AH}^- + \text{Fe}^{3+} \rightarrow \text{A}^- + \text{Fe}^{2+} + \text{H}^+$, where $\text{AH}^-$ a molecule of ascorbate and $\text{A}^-$ an ascorbyl radical (22). Given this mounting evidence for prooxidant role of ascorbate in the presence of redoxactive transition metal ions, we previously used the Fe$^{2+}$/ascorbate-mediated lipid peroxidation system to investigate the repercussions of lipid peroxidation on lipid metabolism. We found that iron/ascorbate-induced peroxidation provoked profound perturbations in lipid transport, apoprotein biogenesis, lipoprotein assembly, and sterol regulatory enzymes, which play a critical role in cholesterol homeostasis (6, 7, 9).

The lumen of the intestine is lined with a single layer of epithelial cells, mainly represented by absorptive columnar enterocytes. These cells are not only responsible for nutrient transport, but also actively participate in local epithelial immune response, which can...
enhance protection against microbial and dietary antigens (15). With regards to the daily challenge by various diet-derived oxidants, the intestine is endowed with several defense mechanisms that preserve cellular integrity and tissue homeostasis (5, 6, 10, 14, 15, 21, 42). It has become increasingly apparent that patients with Crohn’s disease exhibit elevated free radical levels and an improper antioxidant defense system (20, 25, 29, 39, 51, 54). In this debilitating illness, chronic oxidative stress produces marked elevations of DNA oxidation constituents and cell proteins, resulting in inflammation and tissue destruction (11, 35, 38). Even if the etiology of Crohn’s disease has yet to be fully elucidated, a close relationship has been noted between reactive species and the mucosal inflammatory process. However, the ability of intestinal cells to deal with luminal oxidants by increasing endogenous defense mechanisms has not been thoroughly examined. Furthermore, the mechanisms by which oxidants contribute to inflammation are not entirely clear, but one proposed pathway may be through the activation of the transcription factor NF-κB, which initiates the expression of genes controlling several aspects of the inflammatory, immune, and acute phase responses (4, 40, 48, 50).

The aims of this study were first, to determine whether intestinal cells preserve cellular integrity and appropriately respond to iron-catalyzed free radical-mediated lipid peroxidation by enhancing the activity of GSH peroxidase, GSSG reductase, GSH transferase, catalase, and superoxide dismutase, as well as the levels of glutathione. Second, as an attempt to understand the oxidative modulation of inflammatory response, attention was directed toward the status of NF-κB activation and inflammation mediators. The well-characterized Caco-2 cell model was used in the present study to assess the effects of iron-induced lipid peroxidation on antioxidant enzyme systems and innate immune response.

**Materials and Methods**

**Cell culture.** Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C in MEM (GIBCO-BRL, Grand Island, NY) containing 1% penicillin/streptomycin and 1% MEM nonessential amino acids (GIBCO-BRL) supplemented with 10% decomplemented FBS (Flow, McLean, VA). Caco-2 cells (passages 30–40) were maintained with 5% CO2 in T-75 cm2 flasks (Corning, Corning, NY). Cultures were split (1:6) when they reached 75–90% confluence, using 0.05% trypsin-0.5 mM EDTA (GIBCO-BRL). For individual experiments, cells were plated at a density of 1 × 10^4 cells/well on 23.1 mm polyethylene terephthalate Falcon filter inserts with 0.4-μm pores (Becton Dickinson, Franklin Lakes, NJ) in MEM supplemented with 5% FBS. The inserts were placed into six-well culture plates, permitting separate access to the upper and lower compartments of the monolayers. Cultures were maintained for 21 days, a period at which the Caco-2 cells are fully differentiated and suitable for studies on metabolic functions, such as lipid transport (33). Medium was refreshed every second day.

**Estimation of lipid peroxidation.** Caco-2 cells were cultured in the presence or absence of Fe^{3+}/ascorbate (0.2:2 mM; Sigma, St. Louis, MO) for periods varying between 1 and 24 h. The amount of free malondialdehyde (MDA) formed during the reaction was determined by HPLC, as we previously described (6). Proteins were first precipitated with a 10% sodium tungstate (Na2WO4) solution (Aldrich, Milwaukee, WI). The protein-free supernatants were then reacted with an equivalent volume of 0.5% (wt/vol) thiobarbituric acid solution (TBA; Sigma, St. Louis, MO) at 90°C for 60 min. After cooling to room temperature, the pink chromogen [(TBA)_2-MDA] was extracted with 1-butanol and dried over a stream of nitrogen at 37°C. The dry extract was then resuspended in a KH2PO4/methanol mobile phase (70:30, pH 7.0) before MDA detection by HPLC.

**Effect of the butylated hydroxytoluene antioxidant.** To determine whether lipid peroxidation was responsible for the alterations caused by iron/ascorbate, the powerful antioxidant butylated hydroxytoluene (BHT; Sigma) was added to the apical compartment, at a concentration of 150 mM for 1 h before the addition of the iron/ascorbate complex. BHT was dissolved in ethanol and control wells received an equal volume of the alcohol carrier (1% in the medium).

**Brush-border membrane isolation and cell membrane fluidity.** Brush-border membranes were purified from Caco-2 cell homogenates according to the method of Schmitz et al. (47). Briefly, the culture medium was removed, and the cells attached to filters were rinsed twice with PBS (GIBCO-BRL). The cells were then scraped off and homogenized in Tris-1HCl mannitol (2 mM HCl, and 50 mM mannitol, pH 7.0). Following the addition of 10 mM MgCl2, the homogenates were centrifuged (7,700 g, 15 min, 4°C). The supernatants were subsequently centrifuged (20,000 g, 30 min, 4°C) and the resulting pellets were used for the determination of fluidity.

**Fatty acid analysis.** Aliquots of the brush-border membranes were diluted with 300 mM mannitol, 10 mM Tris-HEPES, and 100 mM KCl (pH 7.5). Fluidity was estimated by the incorporation of the fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene-sulfonate, and measurement of polarization (6). The final probe-to-lipid molar ratio was 1:1,000. Fluorescence was measured in a spectrofluorometer at 25°C with polarization filters parallel and perpendicular to the excitation beam. Excitation was at 360 nm and emission at 420 nm.

**Permeability.** Intestinal permeability was assessed with 51Cr-labeled EDTA as described previously (2).

**Regulatory antioxidant enzyme activity.** The activity of the antioxidant enzymes SOD, catalase, glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-R), and glutathione S-transferase (GST) were measured in cell homogenates after incubation with prooxidants and/or antioxidants. Briefly, a substrate for each enzyme was added to the cell homogenates, and rate of disappearance of the substrate was measured by spectrophotometry. Superoxide dismutase activity was determined essentially as described by Sun et al. (52). The method for the other enzyme quantification was adapted from the protocol reported by Pippenger et al. (43). For catalase activity, cell homogenates were placed in a cuvette that received H2O2 (10 mM) as a substrate, and absorbency was read at 240 nm for 3 min. For GSH-Px activity, cell
homogenates were added to a PBS-based working solution (pH 7.0) containing 1 mM GSH, 0.6 U GSSG-R, and 100 mM NADPH with a small quantity of tert-butyl hydroperoxide (1.5%) to initiate the reaction. For GST activity, cell homogenates were resuspended in a PBS buffer solution (pH 6.5) containing 1 mM 1-chloro-2,4-dinitrobenzene and 15 mM GSH to initiate the reaction. For GSSG-R activity, cell homogenates were mixed with a reagent (1 mM GSSG, 0.1 mM NADPH, and 1% Triton X-100) with an ice for 4 min. For each of the glutathione enzymes, absorbency rate was measured in a spectrometer at 340 nm for 3 min.

Glutathione levels. To measure total glutathione levels, Caco-2 cells were washed twice with PBS, scraped in a buffer (50 mM Tris·HCl, 0.1 mM EDTA-Na2, 10 mM L-serine, and 20 mM Na-borate, pH 7.6), and sonicated on ice. Proteins were precipitated by adding a 10% sulfosalicylic acid solution and centrifuged at 10,000 rpm for 4 min. The supernatants were collected in cuvettes to which were added NADPH as a substrate, DTNB, and a small quantity of exogenous GSSG-R. The rate of disappearance of NADPH was measured in a spectrometer at 412 nm during 8 min, and the concentration of total GSH was calculated using a standard curve.

Immunoblot analysis of COX-2, inhibitor-κB, and ICAM-1. Following incubation with prooxidant and antioxidant, Caco-2 cells were washed twice with PBS and scraped in 0.5 ml lysis buffer (in mM: 50 Tris·HCl, pH 7.5, 150 NaCl, 5 EDTA, 1 PMSF, 1 BHT, and 1 pepstatin, and 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100). Cells were sonicated and aliquots of cell homogenates were mixed with loading buffer (Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE and were transferred onto Hybond nitrocellulose membranes (Amersham, Piscataway, NJ). The latter were then blocked 1 h with 2% skim milk in Tris buffer containing 0.05% Tween (TBST), and the membranes were incubated in 0.5% milk containing NF-κB p65 goat polyclonal IgG (Santa Cruz Biotechnology). After being washed with TBST, membranes were incubated with anti-goat IgG horseradish peroxidase-linked antibody (Biosource). Detection was performed by using an enhanced chemiluminescence system for antigen-antibody complexes.

Nuclear extraction and immunoblot analysis of NF-κB. Nuclear extracts were prepared to verify NF-κB protein expression by Western blotting. Cells were washed twice with 1 mM PBS-EDTA and left on ice for 4 min in a lysis buffer containing (in mM) 10 HEPES, 10 KCl, 1.5 MgCl2, 2 EDTA, 1,4-dithio-β-treithol, and 20% glycerol and antiproteases and left on ice for 1 h with occasional vortexing. They were then centrifuged for 10 min at 10,000 g at 4°C, and the supernatants were collected for protein and Western blot analysis. Nuclear extraction techniques were modified from Egan et al. (17).

To determine NF-κB protein expression, nuclear extracts were used instead of whole cell homogenates. Immunoblotting techniques were as described above. Nitrocellulose membranes were incubated in 0.5% milk containing NF-κB p65 goat polyclonal IgG (Santa Cruz Biotechnology). After being washed with TBST, membranes were incubated with anti-goat IgG horseradish peroxidase-linked antibody (Biosource) in 1% milk. Results were obtained by chemiluminescence.

Determination of IL-8. The cellular proinflammatory chemokine IL-8 was measured using an ELISA kit (BD Biosciences, Mississauga, ON, Canada) following the incubation of Caco-2 cells with BHT and/or iron/ascorbate.

Statistical analysis. All values are expressed as means ± SE. Data were analyzed by the one-way ANOVA and the two-tailed Student’s t-test. A P value of ≤ 0.05 was considered significant.

RESULTS

MDA production following iron/ascorbate treatment. Experiments were first performed to examine the effect of iron/ascorbate on MDA production in Caco-2 cells. Differentiated Caco-2 cells were incubated with BHT and/or iron/ascorbate at concentration of 150 μM to neutralize the oxidative stress. Results shown are means ± SE for 4 separate experiments, each carried out in triplicate. CTR, control; Fe, iron/ascorbate. *P < 0.05 vs. control without Fe; **P < 0.05 vs. control, BHT and Fe/BHT.
fects of iron/ascorbate on lipid peroxidation in Caco-2 cells. Iron/ascorbate caused a dose-dependent MDA production (Fig. 1A). On the other hand, incubation of Caco-2 cells with iron/ascorbate over various periods of time resulted in constant cellular lipid peroxidation (Fig. 1B). The addition of the antioxidant BHT at a concentration of 150 μM suppressed the production of MDA, providing direct evidence for the ability of the iron/ascorbate system to provoke profound lipid peroxidation.

Transcellular fluidity. Treatment of Caco-2 cells with iron/ascorbate did not affect the morphology of Caco-2 cells that displayed a high degree of polarization, brush-border microvilli, tight junctions, and interdigitations (results not shown). This was consistent with recovered high transepithelial cell monolayer resistance (Table 1) that represents an indication of cell confluence and monolayer integrity as well as a measure of tight junction formation. Even the transport of $^{51}$Cr-labeled EDTA, a sensitive tool for the estimation of paracellular permeability, was slightly increased in Caco-2 cells supplemented with iron/ascorbate (Table 1), which suggests that only few molecules diffuse to the basolateral side via the paracellular shunt pathway.

Subsequent experiments were carried out to determine the effects of iron/ascorbate on membrane fluidity, which is representative of the relative motional freedom of lipid molecules in the membrane bilayer. Fluidity was measured by the incorporation of the fluorescent probe [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene] (TMA-DPH) in apical membranes isolated from Caco-2 cells. A significant decrease was observed with iron/ascorbate treatment (Table 1). Preincubation with BHT prevented the fluidity decline observed with iron/ascorbate, which suggests the direct involvement of oxidative stress.

Fatty acid composition. In agreement with the membrane fluidity data, differences were observed in cellular fatty acid composition. Overall, polyunsaturated fatty acids (PUFA) were markedly decreased in Caco-2 cells exposed to iron/ascorbate, especially linoleic and arachidonic acids (Table 2). As a result, the ratio of PUFA to saturated fatty acids was significantly lower by 79.4% in iron/ascorbate-treated cells when compared with control cells. In particular, the ratio of eicosatrienoic acid (20:3ω9) arachidonic acid (20:4ω6), a commonly used index for essential fatty acid deficiency, was significantly higher in iron/ascorbate-treated cells. Besides, BHT was capable of normalizing the fatty acid composition abnormalities. Taken together, these data suggest that iron/ascorbate-mediated lipid peroxidation is able to induce a fall in membrane fluidity, probably by attacking polyunsaturated fatty acid bonds.

Endogenous antioxidant defense. To examine whether alterations in oxygen radical metabolizing enzymes occur in Caco-2 cells when incubated with iron/ascorbate, the activities of superoxide dismutase, catalase, glutathione peroxidase, GST, and glutathione reductase were measured. As illustrated in Figs. 2 and 3, there were no significant changes in the activities of all the aforementioned enzymes following the addition of iron/ascorbate or BHT. The incapacity of enzymes to react is also reflected by the unmodified total glutathione levels.

Degradation of inhibitor-κB and activation of NF-κB. To verify whether iron/ascorbate could induce an inflammatory response, the degradation of the inhibitory protein inhibitor-κB α (IkBα) and activation of the transcription factor NF-κB were verified. Following the addition of iron/ascorbate, Caco-2 cell homogenates were analyzed by Western blot, and the expression of the 37-kDa protein IkBα was evaluated. As shown in Fig. 4A, cells treated with iron/ascorbate exhibit decreased quantities of IkBα, which could account for its degradation in the cytosol and the release of NF-κB. In parallel, the translocation of NF-κB to the nucleus was evidenced by the increased levels of the p65 NF-κB subfraction in nuclear extracts from cells treated with iron/ascorbate (Fig. 4A). Both degradation of IkBα and nuclear translocation of NF-κB were inhibited by the addition of the antioxidant BHT, suggesting that the oxidative properties of iron/ascorbate contribute to the activation of this transcription factor. Therefore, the addition of a prooxidant such as iron/ascorbate to intestinal epithelial cells activates NF-κB, which could then lead to the transcription of genes that regulate inflammatory protein synthesis.

Expression of ICAM-1, COX-2, and IL-8. The protein expression of intracellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2) was analyzed in Caco-2 cells. These two proteins are known to have a central role in inflammatory response. The incubation of Caco-2 cells with iron/ascorbate upregulated the protein expression of ICAM-1 and COX-2 (Fig. 4B and 4C). In the presence of BHT, iron/ascorbate was not able to induce the expression of these two proteins. Finally, the exposure of Caco-2 cells to iron/ascorbate

Table 1. Effect of iron/ascorbate on membrane features

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TER (Ω cm$^{-2}$)</th>
<th>Permeability (dpm/mg protein)</th>
<th>Fluidity (1/delay polarization)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2,987 ± 141</td>
<td>1,417 ± 44</td>
<td>3.42 ± 0.21</td>
</tr>
<tr>
<td>BHT</td>
<td>3,124 ± 156</td>
<td>1,566 ± 39</td>
<td>3.48 ± 0.17</td>
</tr>
<tr>
<td>Fe</td>
<td>2,899 ± 163</td>
<td>1,832 ± 48*</td>
<td>2.91 ± 0.13*</td>
</tr>
<tr>
<td>Fe/BHT</td>
<td>3,012 ± 144</td>
<td>1,539 ± 51</td>
<td>3.50 ± 0.14</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 3 separate experiments. Transcellular resistance (TER), permeability, and membrane fluidity were determined in Caco-2 cells following 8-h incubation with 0.2 mM iron/ascorbate (Fe) and/or 0.15 mM butylated hydroxytoluene (BHT). As described in MATERIALS AND METHODS, TER was measured using a millicell epithelial voltmeter apparatus, permeability by quantifying [Cr$^{51}$]-EDTA, and membrane fluidity by the incorporation of a fluorescent probe. *$P < 0.05$ vs. control, BHT, and Fe/BHT.
enhanced the secretion of IL-8 into the basolateral medium, whereas BHT was not capable of eliminating the increased iron/ascorbate-mediated IL-8 output (Fig. 5).

DISCUSSION

Reactive oxygen intermediates represent important mediators in the activation of inflammatory responses. In particular, chronic gut inflammation is characterized by the enhanced production of reactive oxygen metabolites. The main objective of our work was to examine how intestinal epithelial cells endowed with several antioxidant systems behave in front of injurious effects of oxidative stress. Our in vitro studies clearly established the ineptitude of Caco-2 cells to efficiently promote their endogenous antioxidant systems to neutralize, or at least attenuate, iron/ascorbate-mediated lipid peroxidation. Consequently, inflammatory mechanisms were triggered, including the activation of NF-κB and the increased levels of IL-8, ICAM-1, and COX-2.

Intestinal epithelial cells represent a physiological barrier for luminal antigens, microbes, and toxins. They are also active participants in the gut immune response with regard to the numerous cytokine receptors on their membrane surface and their capacity to secrete cytokines, chemokines, and leukocyte adhesion molecules (16, 26, 56). Therefore, the disruption of this interface could have serious consequences on barrier and immune functions. To evaluate the effects of oxidative stress on antioxidant defenses and immune reactions, we used the human colon adenocarcinoma Caco-2 cell line. The observations of our studies confirm that, at confluence, these cells differentiate into a highly polarized monolayer and manifest many of the features of small intestinal cells. Furthermore, we previously reported that Caco-2 cells display important functional characteristics such as lipid transport, lipoprotein synthesis, and apolipoprotein biogenesis, which were all affected by iron/ascorbate-mediated lipid peroxidation (9, 32, 33). Therefore, we utilized this intestinal epithelial cell model in the current investigation together with the well-established iron/ascorbate-generating oxidative stress (7, 55). As was shown by our data, the addition of this prooxidant resulted in increased lipid peroxidation in a dose-dependent fashion. Accordingly, treatment with BHT, a lipid-peroxide scavenger, inhibited iron/ascorbate-mediated lipid peroxidation, thus pointing out the direct implication of iron/ascorbate in MDA production. The occurrence of lipid peroxidation was accompanied with

Table 2. Fatty acids and index of EFA deficiency in Caco-2 cells

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Control</th>
<th>Fe</th>
<th>BHT</th>
<th>Fe/BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>39.74 ± 0.42</td>
<td>42.63 ± 0.27</td>
<td>40.22 ± 0.39</td>
<td>37.32 ± 0.47</td>
</tr>
<tr>
<td>PUFA</td>
<td>13.48 ± 0.36</td>
<td>2.82 ± 0.17a</td>
<td>14.47 ± 0.44†</td>
<td>12.95 ± 0.25†</td>
</tr>
<tr>
<td>PUFA/SFA</td>
<td>0.345 ± 0.112</td>
<td>0.059 ± 0.081*</td>
<td>0.371 ± 0.023†</td>
<td>0.339 ± 0.177††</td>
</tr>
<tr>
<td>20:3 ω 9/20:4 ω 6</td>
<td>0.093 ± 0.04</td>
<td>1.519 ± 0.125*</td>
<td>0.105 ± 0.04†</td>
<td>0.104 ± 0.06†</td>
</tr>
</tbody>
</table>

Data represents means ± SE of 3 separate experiments. Caco-2 cells were cultured for 24 hours with 0.2 mM Fe and/or 0.15 mM BHT. At the end of the incubation period, cells were washed, scraped in PBS, and centrifuged, and the resulting pellets were stored at −40°C until analysis. Cellular fatty acid content was analyzed by gas liquid chromatography after direct transesterification. Results are expressed as percentage of total fatty acid content. On the basis of previous reports, criteria for essential fatty acid deficiency included a cellular ratio of 20:3 ω 9/20:4 ω 6 superior to 0.2, in addition to decrease in total cell content of essential fatty acid. SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; EFA, essential fatty acids. *P < 0.005 vs. control, BHT, and Fe/BHT. †P < 0.01 vs. Fe.
essential fatty acid deficiency, as well as changes in membrane fluidity and permeability. Thus the integrity of the intestinal epithelial barrier seems compromised under the iron/ascorbate prooxidant effect. noteworthy are the observations related to levels of lipid peroxidation in the blood circulation and mucosal biopsies of CD patients (1, 34). Available data in the scientific literature have also reported increased intestinal permeability in patients with Crohn’s disease (13). It is therefore reasonable to propose that reactive oxygen species can contribute to mucosal barrier disruption, thereby allowing various luminal antigens, bacteria, and toxins to reach the lamina propria, activate immune cells, and elicit an excessive inflammatory response.

Human tissues are equipped with powerful defense mechanisms capable of withstanding oxidative attacks and limiting the damage to organs. The intestine possesses numerous endogenous antioxidant enzymes: SOD removes superoxide anions, catalase and glutathione peroxidase eliminate peroxides, glutathione reductase converts oxidized glutathione back to its reduced form, and GST transfers oxidized glutathione to the extracellular medium. Surprisingly, the activities of these enzymes remained unchanged despite the marked lipid peroxidation produced by iron/ascorbate, which may explain the membrane alterations reported herein. A progressive fall in enzyme activities and depletion of glutathione content was expected to occur before the onset of any damage to cell constituents and functions. However, no upregulation in these antioxidants was noted despite the persistent oxidative stimuli. This interesting aspect of our work deserves further investigation in humans, and if it is confirmed, therapeutic maneuvers should be designed to enhance mucosal antioxidant defense that may be useful to tackle the negative effects of oxidative stress. It should be noted that most antioxidant enzymes are located in the epithelial cells, whereas the underlying lamina propria is practically devoid of antioxidant enzymes (19).

A potential connection between oxidative stress and proinflammatory gene expression has been established (45, 48, 50). It was therefore reasonable to determine
whether iron/ascorbate-mediated lipid peroxidation could elicit an inflammatory response in Caco-2 cells. NF-κB represents a family of transcription factors that serve as important regulators of the host immune and inflammatory responses. Under resting conditions, NF-κB is sequestered in the cytoplasm by an inhibitory protein known as IκB (18, 53). Following activation by a wide array of external stimuli, a cascade of events occur, resulting in the degradation of IκB and the release of NF-κB, which subsequently translocates to the nucleus and transcriptionally modulates cellular genes implicated in early immune, acute phase, and inflammatory responses (3). Our findings emphasize that the treatment of Caco-2 cells with iron/ascorbate induced the degradation of cytosolic IκB with the ensuing translocation of NF-κB to the nucleus. Furthermore, the presence of BHT inhibited the activation of NF-κB, pointing out that this powerful transcription factor is subject to redox regulation in Caco-2 cells. Increased activation of NF-κB has been reported in both macrophages and intestinal epithelial cells in patients with Crohn’s disease (44, 46). Collectively, these data indicate that in addition to luminal antigens and toxins that are deleterious to intestinal epithelial cells, prooxidants can overcome cell antioxidant defense, destabilize cell integrity, and activate proinflammatory transcription factors.

Activation of the transcription factor results in the induction of inflammatory genes leading to the protein expression of proinflammatory cytokines. We therefore decided to examine whether the exposure of Caco-2 cells to iron/ascorbate triggers IL-8, ICAM-1, and COX-2. IL-8 functions as a potent neutrophil chemoattractant and activator, whereas ICAM-1 is a strong cell surface adhesion molecule (24, 30). The incubation of Caco-2 cells with iron/ascorbate led to augmented cellular production of IL-8 and expression of ICAM-1. Surprisingly, BHT produced the same effect, which did not allow us to highlight whether iron/ascorbate per se or iron/ascorbate-mediated peroxidation provoked the elevation of these two proinflammatory agents. Additional work with other exogenous antioxidants is needed to elucidate this interesting aspect. As to the COX-2 isoenzyme, mounting evidence suggests its direct implication in the production of proinflammatory prostaglandins (49). In the present investigation, COX-2 was found to be induced by iron/ascorbate, whereas BHT was effective in returning COX-2 protein levels back to control values. These findings reflect the need to restore prooxidant/antioxidant balance in an attempt to reduce the impact of powerful proinflammatory agents such as COX-2.

In summary, our data support an oxidative role of iron ascorbate in cell integrity and inflammatory response. It may therefore initiate intestinal cell damage and aggravate ongoing injury, particularly because the gut is not equipped with adequate antioxidant defense. From the elucidation of the involved mechanisms will emerge ways to test their in vivo relevance and to identify potential targets for intervention.
LIPID PEROXIDATION AND INFLAMMATION IN CACO-2 CELLS

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DISCLOSURES

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