Oxygen Free Radical Injury of IEC-18 Small Intestinal Epithelial Cell Monolayers

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Oxygen radicals can cause endothelial and epithelial permeability changes and mucosal injury of the small intestine. There is no clear consensus concerning the relative injurious potential of individual oxygen radicals. In this study, the small intestinal cell line IEC-18 was used as an in vitro model to study the relative injurious effects of reactive oxygen metabolites. By introducing different combinations of oxygen metabolite-producing enzymes, xanthine oxidase, superoxide dismutase, and catalase, and an iron chelator, deferoxamine, to the fully confluent monolayers and to proliferating IEC-18 cells, the differential injurious effects of the oxygen metabolites $O_2^-$, $H_2O_2$, and $OH^-$ could be evaluated. The extent of cellular injury was assessed using $[3H]$thymidine uptake, $^{51}Cr$ release, and morphological evaluations. Our results suggest that $OH^-$ produced as a by-product of $O_2^-$ and $H_2O_2$ via the Haber-Weiss reaction was the most injurious oxygen species involved in cellular injury of IEC-18 monolayers induced by xanthine oxidase. $O_2^-$ produced by xanthine oxidase appeared to be only minimally injurious, and $H_2O_2$ produced by xanthine oxidase and as a result of conversion of $O_2^-$ by superoxide dismutase was moderately injurious. Superoxide dismutase and deferoxamine at appropriate concentrations were protective against xanthine/xanthine oxidase-induced monolayer injury. $H_2O_2$ added directly or produced indirectly by glucose oxidase was very injurious to the intestinal monolayers, and this injury was mitigated by catalase.

In the past 20 years, the importance of reactive oxygen species in cellular injury has become more evident. Normally, oxygen radicals play an important role in the defense against foreign microorganisms. Deficient production of oxygen radicals can lead to disorders such as chronic granulomatous disease, in which the inability of polymorphonuclear leukocytes to produce toxic oxygen species results in defective defense against catalase-positive organisms (1). Oxygen metabolites play an important role in many different biological processes, including inflammation (1-5), oxygen-induced lung injury (1-5,8), ischemic/reperfusion injuries (1-5,9,10), aging (1,5,11,12), and carcinogenesis (1,5,13,14).

Recently, there has been increasing interest in the possible role of reactive oxygen radicals in intestinal diseases. Oxygen radicals have been implicated in ischemia-induced permeability changes of the intestine (15-20), in causing intestinal inflammation (21,23), and in playing a role in the etiology of Crohn's disease and ulcerative colitis (21,24,25). The permeability characteristics of the intestine to medium and large molecules (> 4 Å in diameter) may be important in the etiology of inflammatory bowel diseases such as Crohn's disease (26-30). Based on these findings, a possible etiologic association between oxygen radicals, epithelial injury, increased intestinal permeability, and Crohn's disease and ulcerative colitis has been advanced (21,26).

The normal small intestine has a high concentration of xanthine oxidase (XO) and has the potential to produce large amounts of reactive oxygen species (17,31-34). The colon has a much lower concentration of XO and is thought to be less susceptible to oxygen radical-induced injury (17,31). Studies by Granger and Parks and coworkers (15-19,21,22,32,33) have suggested XO to be the important producers of the reactive oxygen species in the intestine (15,18,32-
They showed that allopurinol significantly decreased ischemia/reperfusion-induced intestinal injury. Recent reports by these investigators suggest that XO-derived oxygen species attract granulocytes that further exacerbate intestinal injury (32,35,36). Thus, when the antioxidant mechanisms of the small intestine are overwhelmed, cellular and tissue injury may result. The major reactive oxygen metabolites that have been implicated in cellular injury include superoxide anion \( \text{O}_2^{-} \), hydrogen peroxide \( \text{H}_2\text{O}_2 \), and hydroxyl radical \( \text{OH}^{-} \). Of these, \( \text{H}_2\text{O}_2 \) had been reported to be the oxygen metabolite most injurious to cells (37–39), but some studies have disputed this claim and have suggested \( \text{OH}^{-} \) as being the most damaging (5,15). Superoxide anion is considered to be relatively nontoxic (21,40). Hydrogen peroxide has been shown to cause cellular injury and increases in whole intestinal permeability (41) and permeability of Madin Darby Camino kidney cell line monolayers (38). Hydroxyl radical is the most reactive oxygen radical and can initiate lipid peroxidation leading to damage of cell membrane and cell death (1,5,40,42,43). Recent findings suggest that \( \text{O}_2^{-} \) may have direct toxicity (44,45), but its importance in cellular injury has been mainly attributed to its role as a precursor to more injurious oxygen metabolites (1,21).

In this study, we investigated the possible injurious effects of oxygen metabolites directly on small intestinal epithelial cell (IEC-18) monolayers. We studied the differential injurious effects of reactive oxygen species by selectively introducing different oxygen metabolite–producing enzymes to the monolayers. Because of the potential importance of oxygen metabolites in causing small intestinal injury, the small intestinal cell line IEC-18 was chosen as in vitro model to study the injurious effects of oxygen metabolites. IEC-18 is an established epithelial cell line from normal rat ileum that shows tissue-specific small intestinal surface antigens and numerous surface microvilli (46–48). The cells have well-developed Golgi complexes with a number of membrane-limited granules in the cytoplasm. These cells have been shown to develop tight and gap junctions (46–48). We have successfully grown IEC-18 as confluent monolayers on culture plates and on permeable inserts as an in vitro model of small intestinal epithelium for transport studies (48).

**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle medium, trypsin, and fetal bovine serum were purchased from Gibco Laboratories (Grand Island, NY). Insulin, glutamine, penicillin, streptomycin, and phosphate-buffered saline were purchased from Irvine Scientific (Santa Ana, CA). \(^{3}H\)Thymidine and Na\(_{2}\)CrO\(_{4}\) were purchased from New England Nuclear (Boston, MA). Xanthine (X), Triton X-100, XO, superoxide dismutase (SOD), glucose oxidase (GO), deferoxamine (DEP), and catalase (C) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was obtained from Fisher Scientific (Springfield, NJ). All other chemicals were of reagent grade.

**Cell Cultures**

Cell line IEC-18 was purchased from American Type Culture Collection (Rockville, MD). The stock cultures were grown in an atmosphere of 5% CO\(_{2}\) at 37°C in a culture medium composed of Dulbecco’s modified Eagle medium (with 4.5 mg/mL of glucose) containing 5% fetal bovine serum, 10 μg/mL of insulin, 50 U/mL of penicillin, 50 U/mL of streptomycin, and 4 mmol/L glutamine (46). Culture media were changed every 3 days. Confluent monolayers were subcultured by trypsinization using 0.25% trypsin and 0.9 mmol/L ethylenediaminetetraacetic acid (EDTA) in Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline. For most experiments, IEC-18 was detached from stock cultures by trypsinization, washed once by centrifugation, resuspended, and subcultured in a 0.1 mL medium in 96 well plates at a concentration of 3 × 10\(^4\) cells/mL. Cultures were examined under an inverted light microscope on a regular basis to monitor growth and contamination.

**Assessment of Synthesis of DNA**

Various concentrations of oxygen radical–producing enzymes and 0.25 μCi of \(^{3}H\)thymidine were added to the wells 24 hours or 1 week after IEC-18 had been previously subcultured in 96 well plates (47). At the end of 20 hours of treatment, \(^{3}H\)thymidine-containing media were removed, and the wells were refilled with 0.25% trypsin and 0.9 mmol/L EDTA in Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline. The plates were incubated at 37°C for 20 minutes and frozen at −70°C for at least 1 hour before the trypsintized cultures were thawed and harvested by rapid filtration using a cell harvester (Brandel, Rockville, MD). Radioactivity was counted in a Beckman LS8000 liquid scintillation counter (Beckman Instruments, Palo Alto, CA). For each experimental run, six duplicate wells \((n = 6)\) were used.

**Assessment of Cytotoxicity**

Chromium-release assay was used to determine cytotoxicity of reactive oxygen metabolites to IEC-18 (47). The experiments were performed on 1-week-old confluent monolayer cultures. Growth medium in individual wells was removed and replaced with 0.1 mL of fresh growth medium with 0.5 μCi of Na\(_{2}\)CrO\(_{4}\). After 1 hour of incubation, the radioactively labeled medium was removed, and the cultures were washed twice with fresh culture medium. The wells were then refilled with 0.2 mL of fresh medium (controls), fresh medium with various concentrations of oxygen radical–producing biological enzymes, enzymes, or Triton X-100 (complete release).
At the end of a 20-hour incubation, incubation medium was removed from wells containing control and treated cells. The radioactivity of the samples was counted in a Beckman Gamma 9000 counter. We expressed the data in terms of cytotoxicity, which was calculated as follows:

\[
\text{Cr Release} = \left( \frac{\text{Treated} - \text{Control}}{\text{Triton X-100} - \text{Control}} \right) \times 100\%.
\]

For each experimental run, six duplicate wells \( n = 6 \) were used.

**Light Microscopy**

Phase-contrast light micrographs were taken using a Nikon diaphot inverted microscope equipped with a Nikon GF camera (Nikon, Japan) and Kodak Tri-X-Pan film (Rochester, NY).

**Induction of Cellular Injury With Oxygen Radical-Inducing Biological Enzymes**

Various concentrations of XO (0.25, 2.5, and \( 5.0 \times 10^{-2} \) U/mL) were added to the cells incubated in medium containing \( 2.25 \times 10^{-1} \) mol/L xanthine. Superoxide dismutase (4-336 U/mL) was added before the addition of XO to assess the effect of pretreatment with SOD. When assessing the protective effect of C, it (4.6 U/mL) was added before the addition of any other oxidant-inducing agents. Catalase was always dissolved in cell medium and filtered through 0.22-µm nylon membrane before use. Glucose oxidase (0.0044-4.4 U/mL) was introduced directly into the incubation medium containing 4.5 mg/mL of glucose. Hydrogen peroxide (0.01-1.0 mmol/L) was also added directly to the medium bathing the monolayers. It is important to note that the doses of the oxidant-modifying biological enzymes used in these experiments were less than or equal to the doses used by other investigators previously (18,19,37–39,49).

**Statistics**

All values are reported as mean ± SEM, and comparisons of the experimental data were performed using Student's t test.

**Results**

**Effect of Xanthine Oxidase, Superoxide Dismutase, and Catalase on \[^{3}H\]Thymidine Uptake by IEC-18 Monolayers**

Using methods previously described (43,45, 50,51), we evaluated the relative injurious effect of \( \text{O}_2^- \), \( \text{OH}^- \), and \( \text{H}_2\text{O}_2 \) on IEC 18 monolayers by selectively introducing X/XO, SOD, and C to the medium bathing IEC-18 monolayers (Figure 1). The effect of X/XO-generated oxygen metabolites on cellular metabolism was assessed by quantitating the amount of \[^{3}H\]thymidine uptake by the IEC-18 monolayers. When X (\( 2.25 \times 10^{-1} \) mol/L) and XO (\( 2.5 \times 10^{-2} \) U/mL) were introduced to the IEC-18 monolayers, \([^{3}H]\text{thymidine uptake} \) was greatly reduced compared with the controls (Figure 2). Increasing the concentration of XO resulted in a corresponding decrease in thymidine uptake. When SOD (42 U/mL) was introduced before the addition of XO, there was less alteration of cellular metabolism, as indicated by significantly greater \([^{3}H]\text{thymidine uptake} \) (Figure 3). Decreasing the concentration of SOD resulted in progressively lower thymidine uptake (Figure 4) by the monolayers. The addition of C before XO and SOD led to a marked increase in \([^{3}H]\text{thymidine uptake} \) (Figure 3). There was also a marked increase in thymidine uptake when C (4.6 U/mL) was added before X/XO (Figure 3), indicating protection against XO-induced cellular injury. Similar results were obtained when noncon-
fluent IEC-18 cells were injured using the above oxygen metabolite-catalyzing enzymes.

**Cytotoxic Effect of Xanthine Oxidase on IEC-18 Monolayers**

To determine the effect of X/XO-generated oxygen metabolites on cellular membrane integrity, various combinations of X/XO, SOD, and C were added to the IEC-18 monolayers, and \(^{51}\text{Cr}\) release was measured from monolayers preloaded with \(^{51}\text{Cr}\). Addition of X \((2.25 \times 10^{-4} \text{ mol/L})\) and XO \((2.5 \times 10^{-2} \text{ U/mL})\) resulted in significant cytotoxicity with increase in release of \(^{51}\text{Cr}\) compared with the controls (Figure 5). The addition of SOD \((42 \text{ U/mL})\) before X/XO resulted in protection against X/XO-induced cytotoxicity. Pretreatment of cells with C \((4.6 \text{ U/mL})\) before the addition of X/XO and XO and SOD resulted in a marked decrease in \(^{51}\text{Cr}\) release. The pattern of X/XO-induced cytotoxicity was consistent with X/XO-

**Effect of Deferoxamine on Xanthine/Xanthine Oxidase–Induced Cytotoxicity**

The effect of the iron chelator DEF on X/XO-induced monolayer injury was assessed by measuring \(^{51}\text{Cr}\) release from preloaded cells. IEC-18 monolayers were pretreated with varying concentrations \((0.05, 0.1, 0.5, 1.0, \text{ and } 5.0 \text{ mmol/L})\) of DEF before X \((2.25 \times 10^{-4} \text{ mol/L})/\text{XO} (5.0 \times 10^{-2} \text{ U/mL})\) addition. Pretreatment of monolayers with higher concentrations of DEF \((0.5, 1.0, \text{ and } 5.0 \text{ mmol/L})\) resulted in a marked protective effect against X/XO-induced \(^{51}\text{Cr}\) release, whereas lower doses \((0.05 \text{ and } 0.1 \text{ mmol/L})\) had no significant protective effect on \(^{51}\text{Cr}\) release by the preloaded IEC-18 monolayers (Figure 6). Morphological assessment using phase-contrast light micro-

![Figure 3. Effect of SOD (42 U/mL), C (4.6 U/mL), and SOD (42 U/mL) and C (4.6 U/mL) on X \((2.25 \times 10^{-4} \text{ mol/L})/\text{XO} (2.5 \times 10^{-2} \text{ U/mL})\)-induced alterations of \(^{3}\text{H}\)thymidine uptake by the IEC-18 monolayers. Bars represent \(^{3}\text{H}\)thymidine uptake (± SEM) by the 1-week-old IEC-18 monolayers at the end of the 20-hour treatment period. *Statistically significant \((P < 0.01)\) increase in thymidine uptake compared with XO-treated group.](image)

![Figure 4. Effect of increasing concentrations of SOD on X \((2.25 \times 10^{-4} \text{ mol/L})/\text{XO} (2.5 \times 10^{-2} \text{ U/mL})\)-induced alterations of \(^{3}\text{H}\)thymidine uptake (± SEM) by the IEC-18 monolayers. Increasing doses of SOD to 42 U/mL resulted in a dose-related increase in thymidine uptake by the IEC-18 monolayers.](image)

![Figure 5. Effect of SOD (42 U/mL), C (4.6 U/mL), and SOD (42 U/mL) and C (4.6 U/mL) on XO \((2.5 \times 10^{-2} \text{ U/mL})\)-induced cytotoxicity of IEC-18 monolayers as measured by \(^{51}\text{Cr}\) release. Bars represent mean percent of \(^{51}\text{Cr}\) release (± SEM) from 1-week-old monolayers at the end of the 20-hour treatment period. *Statistically significant \((P < 0.01)\) decrease in monolayer cytotoxicity from the XO-treated group. **Statistically significant \((P < 0.05)\) decrease in monolayer cytotoxicity from the XO-treated group.](image)

![Figure 6. Effect of increasing concentration of DEF (0.05 5.0 mmol/L) on X \((2.25 \times 10^{-4} \text{ mol/L})/\text{XO} (2.5 \times 10^{-2} \text{ U/mL})\)-induced cytotoxicity of IEC-18 monolayers as measured by \(^{51}\text{Cr}\) release. Bars represent mean percent of \(^{51}\text{Cr}\) release (± SEM) from 1-week-old monolayers at the end of the 20-hour treatment period. *Statistically significant \((P < 0.01)\) decrease in monolayer cytotoxicity from the XO-treated group.](image)
copy showed the dose-related protective effect of DEF against X/X0-induced monolayer injury (Figure 7).

**Effect of Hydrogen Peroxide on[^H]Thymidine Uptake by IEC-18 Monolayers**

Hydrogen peroxide was added to the medium bathing IEC-18 monolayers to examine the injurious effect of H$_2$O$_2$. Increasing H$_2$O$_2$ concentration resulted in decreased[^H]thymidine uptake compared with controls. Pretreatment of the monolayers with C resulted in significantly less injury to the cells (Table 1).

**Direct Cytotoxicity of Hydrogen Peroxide**

Addition of H$_2$O$_2$ to IEC-18 monolayers resulted in very high $^{99}$Cr release (Table 2). Decreasing concentrations of H$_2$O$_2$ resulted in less $^{99}$Cr release, and pretreatment of the monolayers with C led to significant protection from cell death.

**Effect of Glucose Oxidase on[^H]Thymidine Uptake by IEC-18 Monolayers**

Glucose oxidase, in the presence of glucose, enzymatically generates H$_2$O$_2$ (Figure 1). To study the injurious effect of GO, it was added in varying concentrations to the IEC-18 monolayers bathed in glucose (4.5 mg/mL) containing media. Glucose oxidase even in very low concentrations (Table 3) resulted in extensive interference of cellular metabolism. Increasing concentrations of GO resulted in further decreases in thymidine uptake by the cells. The addition of C provided protective effect (Table 3).

**Cytotoxicity of Glucose Oxidase–Induced Hydrogen Peroxide**

Glucose oxidase–induced production of H$_2$O$_2$ in the presence of 4.5 mg/mL of glucose was very toxic to IEC-18 cells, and low concentrations of GO resulted in very high $^{99}$Cr release. Pretreatment with catalase resulted in a significant decrease in $^{99}$Cr release (Table 2).

**Morphological Assessment of Oxygen Metabolite–Induced Cellular Injury**

The induction of oxygen metabolites using biological enzymes led to extensive damage to the monolayers. The addition of GO and H$_2$O$_2$ resulted in diffusely homogeneous injury with severe shrinkage of cells, detachment of the cells from adjacent cells and the culture plates, and distortion of shape of the cells (Figure 8). The addition of $2.5 \times 10^{-2}$ U/mL of XO resulted in more heterogeneous injury with sloughing of layer of cells from the isolated areas interspersed with well-preserved areas and minimal morphological changes in the central portions of the plate. The injury induced by GO and H$_2$O$_2$ appeared to involve the cells diffusely throughout. The addition of C in both cases led to decrease in morphological injury (Figure 8). The protective effect of DEF against a higher concentration of XO ($5.0 \times 10^{-2}$ U/mL)–induced injury is shown in Figure 7.

**Discussion**

Over the past few years there has been a great deal of new information about oxygen radical–induced cellular injury. However, there has been no clear consensus as to the comparative role of individual oxygen metabolites in cellular injury. Ager and Gordon (37), Baker and Campbell (52), and Hiraishi et al. (49) in their in vitro studies of oxygen radical–induced injury of vascular endothelial cells, isolated enterocytes, and gastric cells have concluded that H$_2$O$_2$ was the most damaging agent and that O$_2^-$ and OH$^-$ were less injurious. They derived their conclusion from their experimental observations that SOD had either no protective effect or resulted in increase in X/X0-mediated destruction of cells. Because SOD converts O$_2^-$ into H$_2$O$_2$, they reasoned that H$_2$O$_2$ must be the oxidant species primarily responsible for the cellular injury. Other investigators, using in vivo models, have found SOD to be protective against X/X0- or oxidant-induced injury (15,19) and have suggested O$_2^-$ or possibly its derivative, OH$^-$, to be the most injurious oxygen radical.

In the present study, our major objective was to examine and clarify the relative injurious potential of oxygen free radicals: H$_2$O$_2$, OH$^-$, and O$_2^-$, by selectively introducing different oxidant-producing biological enzymes as previously described (45,50,51), we optimized conditions for production of different oxygen free radicals. Figure 1 shows the catalytic reactions of the enzymes used in these experiments. Xanthine oxidase enzymatically generates O$_2^-$ and H$_2$O$_2$ from X (Figure 1). The relative proportion of O$_2^-$ and H$_2$O$_2$ generated by X/XO is dependent on both pH and oxygen tension (53), and the relative ratio of H$_2$O$_2$ and O$_2^-$ generated under our reaction conditions may be as high as 5:1. The superoxide anion generates dismutates spontaneously to form H$_2$O$_2$ and O$_2^-$, Superoxide dismutase enhances the rate of dismutation of O$_2^-$ by a factor of approximately $10^6$ (15,21). H$_2$O$_2$ is enzymatically converted to H$_2$O and O$_2$ by C. Hydroxyl radical is rapidly generated by a stepwise interaction of O$_2^-$ and H$_2$O$_2$ in the presence of metals such as iron or copper via the Haber–Weiss reaction or the Fenton reaction (43,45,50,51). The reduction
and oxidation requirement of iron metal require that both $O_2^-$ and $H_2O_2$ be present in sufficient quantities for optimal production of $OH^-$. Recent studies have suggested that the actual reactive oxygen radical generated by the Haber–Weiss reaction may not be hydroxyl radical (54) but rather an iron-$H_2O_2$ complex referred to as "ferryl complex." Glucose oxidase enzymatically generates $H_2O_2$ in the presence of glucose.

To determine the relative injurious potency of $O_2^-$, $OH^-$, and $H_2O_2$, X/XO, SOD, and C were introduced to IEC-18 monolayers in various combinations and sequences. IEC-18 monolayer injury was assessed by combination of $[^3H]$thymidine uptake, $^{51}Cr$ release, and morphological assessment. Radioactive $^{51}Cr$ release is an excellent quantitative method of measuring cytotoxicity. It measures the intactness of cell membranes, and increases in $^{51}Cr$ release indicate

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**Figure 7.** Phase-contrast light micrographs (original magnification $\times 250$) of confluent 1-week-old IEC-18 monolayers (A), IEC-18 monolayers injured with $2.25 \times 10^{-5}$ mol/L of X and $5.0 \times 10^{-2}$ U/mL of XO (B), and monolayers pretreated with 0.05 (C), 0.1 (D), 0.5 (E), and 1.0 (F) mmol/L of DEF before addition of X/XO.
leakiness or disintegration of cell membrane. The \(^{35}\)Cr-release method is superior to trypan blue exclusion in that trypan blue exclusion tends to overestimate cell viability and relies on subjective observation of cells (55). Measurement of \([	ext{H}]\)thymidine uptake reflects metabolic alteration in the ability of cells to take up thymidine and is an alternative method of assessing cellular injury (47).

The addition of X and XO resulted in severe injury to IEC-18 monolayers with marked increase in \(^{35}\)Cr-release and marked reduction in \([	ext{H}]\)thymidine uptake (Figures 2, 3, and 5). Xanthine/xanthine oxidase generates both \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\), and under our reaction conditions (pH 7.4, 95% \(\text{O}_2\)) as discussed above. When SOD was introduced before the addition of XO, SOD was mildly protective against X/XO-induced injury (Figures 3–5). Because the addition of XO alone was more injurious than XO and SOD together, it implied that \(\text{O}_2^-\) or their derivatives were more injurious to the cells than \(\text{H}_2\text{O}_2\), \(\text{H}_2\text{O}_2\) being the major product formed by the catalytic action of SOD.

The addition of C before XO and SOD should lead to rapid conversion of \(\text{H}_2\text{O}_2\) to \(\text{O}_2\) and \(\text{H}_2\text{O}\) (Figure 1) with a decrease in cellular injury, and this was the case when C was added (Figures 3 and 5) before X/O and SOD addition. There was also marked protection against cellular injury (Figures 3 and 5) when C was added before XO. Because C enzymatically promotes the conversion of \(\text{H}_2\text{O}_2\) to \(\text{H}_2\text{O}\) and \(\text{O}_2\) (Figure 1), the

### Table 1. Effect of Hydrogen Peroxide on \([	ext{H}]\)Thymidine Uptake by IEC-18 Monolayers

<table>
<thead>
<tr>
<th>(\text{H}_2\text{O}_2) (mmol/L)</th>
<th>Catalase (U/mL)</th>
<th>([	ext{H}])Thymidine uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>100.0 ± 4.9</td>
</tr>
<tr>
<td>0.01</td>
<td>81.7 ± 6.8</td>
<td>62.3 ± 4.5</td>
</tr>
<tr>
<td>1.0</td>
<td>7.3 ± 1.5</td>
<td>68.9 ± 6.4</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\)Statistically significant (\(P < 0.01\)) changes in monolayer injury from the controls as reflected by \([	ext{H}]\)thymidine uptake.

\(^{\text{b}}\)Statistically significant (\(P < 0.01\)) increase in \([	ext{H}]\)thymidine uptake compared with the non-C-treated monolayers that received same dose of \(\text{H}_2\text{O}_2\) (1 mmol/L).

### Table 2. Cytotoxicity of Hydrogen Peroxide and Glucose Oxidase–Induced Hydrogen Peroxide As Measured by \(^{51}\)Cr Release

<table>
<thead>
<tr>
<th>(\text{H}_2\text{O}_2) (mmol/L)</th>
<th>C (U/mL)</th>
<th>GO (U/mL)</th>
<th>(^{51})Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 4.2</td>
</tr>
<tr>
<td>0.1</td>
<td>10.8 ± 1.3</td>
<td>80.3 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>4.6</td>
<td>3.13</td>
<td>11.3 ± 1.4</td>
</tr>
<tr>
<td>0.044</td>
<td>0.041</td>
<td>0.044</td>
<td>11.3 ± 0.04</td>
</tr>
<tr>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>92.5 ± 7.1</td>
</tr>
<tr>
<td>4.5</td>
<td>0.0044</td>
<td>0.0044</td>
<td>29.8 ± 2.5</td>
</tr>
<tr>
<td>4.5</td>
<td>0.044</td>
<td>0.044</td>
<td>35.7 ± 2.2</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\)Statistically significant (\(P < 0.01\)) changes in monolayer cytotoxicity from the controls as reflected by \(^{51}\)Cr release.

\(^{\text{b}}\)Statistically significant (\(P < 0.01\)) decrease in monolayer cytotoxicity from the non-C-treated monolayers that received same dose of GO or \(\text{H}_2\text{O}_2\).

Figure 7 (cont'd.).
protection of C against X0-induced injury probably resulted from rapid removal of H2O2 from the incubation media, suggesting that H2O2 also has a significant role in cellular injury. Because C removes H2O2 from the incubation medium by its catalytic action, O2- is the major oxygen metabolite left in the reaction medium. The marked protective effect of C against X/X0-induced injury suggests that O2- by itself is only minimally injurious. Of all the combinations of oxidant-modulating enzymes used, the addition of X/X0 was most injurious to the monolayers. Our experimental findings suggest that the presence of adequate concentrations of both O2- and H2O2 was important in cell injury and that the combination of the two oxygen species was more damaging than either H2O2 or O2- alone, presumably by promoting formation of OH- through the Haber-Weiss reaction.

It has been previously shown that in the presence of Fe3+, X/X0 generates large amounts of OH- through the Haber-Weiss reaction (43,45,50) and catalytic removal of H2O2 from the reaction solution by catalase inhibits formation of OH- (45,50).

To further assess the possible role of OH- in X/X0 induced IEC-18 monolayer injury, the possible protective effect of DEF was examined. The addition of sufficient concentration of the iron chelator DEF should eliminate the participation of iron in the Haber-Weiss reaction and prevent OH- generation. In the present work, pretreatment of IEC-18 monolayers with DEF markedly protected these cells against X/X0 induced injury (Figures 6 and 7), suggesting that the presence of iron in the reaction medium was very important in promoting cellular injury, presumably by generating OH-. The protection of DEF against X/X0-induced injury further substantiates the involvement of OH- in oxidant injury.

Protective effect of SOD against oxidant injury has been questioned by some investigators (37,39,49,52) because they either did not find SOD to be protective or found SOD to increase injury in X/X0-induced cellular injury. In our experiments, we found SOD to protect against cellular injury caused by X0 (Figures 3–5). The discrepancies that exist in the literature concerning the protective effect of SOD on X0-induced oxidant injury may in part have resulted from different investigators using “impure” preparations of SOD. Baker and Campbell (52), Ager and Gordon (37), Weiss et al. (39), and Hiraishi et al. (49) used Sigma SOD. Because Sigma SOD are only partially purified, they are contaminated with other biological enzymes. A recent report by Yagoda et al. (56) also indicates some of the commercial preparations of SOD including Sigma SOD to be contaminated with endotoxin as well. We have examined the direct cytotoxic effect of higher concentrations of Sigma SOD and have found them to be toxic to IEC-18 monolayers when assessed by 31Cr release and [3H]thymidine uptake (unpublished observations). When “more pure” SOD from another supplier was used, the SOD cytotoxicity was no longer observed.

Parks and Granger (15) and Groggaard et al. (19) using in vivo models studied oxygen radical-induced intestinal injury by inducing ischemia or enzymatic induction using X0. They found that SOD and allopurinol were protective against ischemia or X0-induced injury (15,17–19). These in vivo studies used the IV route for administration of SOD. Parks et al. suggest that the OH- radical was the major oxygen radical causing permeability changes in the in vivo model (15,17). There appears to be a “line” of discrepancy between in vivo and in vitro studies concerning the protective role of SOD against oxygen radical injury. In vivo studies have administered SOD IV and have shown SOD to have protective effect against X0-induced intestinal injury (15,19). Our experimental findings are consistent with the in vivo studies (15,17–19) in showing that SOD is protective against X0-induced oxidant injury. It is interesting to note that in the in vivo studies, the addition of SOD alone is as protective as the addition of SOD and C against ischemic/reperfusion injury. Additionally, in the in vivo studies, ischemic/reperfusion intestinal injury is markedly attenuated by inhibition of neutrophil accumulation and activation. These findings highlight some of the differences between in vivo and in vitro models of oxidant injury. In the in vivo models, the net injurious effect of ischemic/reperfusion injury reflect combined contributions of hormonal, neuronal, vascular, and immunologic factors present in the animal. For instance, the observed in vivo protective effect of SOD may also in part reflect the intrinsic antioxidant mechanisms present in the animal, such as the actions of circulating erythrocyte C. Also, the observed in vivo ischemic/reperfusion injury not only reflect

Table 3. Effect of Glucose Oxidase on [3H]Thymidine Uptake by IEC-18 Monolayers

| GO (U/mL) | C (U/mL) | [3H]Thymidine uptake (%)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>0.0</td>
<td>100.0 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>0.0044</td>
<td>79.3 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>0.014</td>
<td>10.7 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>0.044</td>
<td>7.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td>5.6 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>6.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>0.0044</td>
<td>4.6</td>
<td>93.8 ± 1.1</td>
</tr>
<tr>
<td>0.044</td>
<td>4.6</td>
<td>40.8 ± 1.3</td>
</tr>
<tr>
<td>0.44</td>
<td>4.6</td>
<td>26.3 ± 0.9</td>
</tr>
</tbody>
</table>

*Statistically significant (P < 0.01) changes in monolayer injury from the controls as reflected by [3H]thymidine uptake.
*Statistically significant (P < 0.01) increase in [3H]thymidine uptake compared with the non-C-treated monolayers that received same doses of GO.
injury secondary to activation of XO but also injury induced by activation of neutrophils and other systems. In the in vitro models these contributory factors are absent, and the observed cellular injury more accurately reflect the injurious effect of oxygen species directly on the cells.

Hydrogen peroxide added directly or derived indirectly via GO-generated reactions were very injurious and cytotoxic to IEC-18 cells. This injury was concentration dependent and was mitigated by C (Tables 1-3). The H$_2$O$_2$-induced cellular injury was easily produced in a predictable fashion in these cells.

Our findings suggest that OH$^-$ or perhaps "ferryl complex" as a by-product of H$_2$O$_2$ and O$_2^-$ is the most injurious oxygen species involved in cellular injury induced by XO. H$_2$O$_2$ was moderately injurious.
whereas $O_2^-$ appeared to be minimally injurious. Catalase was markedly protective against X/X0 injury, presumably by both preventing $H_2O_2$ from causing direct cellular injury and by eliminating its participation in the Haber-Weiss reaction. Superoxide dismutase appears to have its protective effect against X/X0 injury by the enzymatic conversion of $O_2^-$ into $H_2O_2$, eliminating its participating in the Haber-Weiss reaction. Deferoxamine protected against X/X0 injury also presumably by preventing iron from participation in the Haber-Weiss reaction.

References

34. Granger DN, McCord JM, Parks DA. Hollwarth Ml. Xanthine oxidase inhibitors attenuate ischemia induced vascular permeability changes in the cat intestine. Gastroenterology 1986;90:80-84.

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