In vitro effects of reactive oxygen metabolites, with and without flunixin meglumine, on equine colonic mucosa

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Objective—To determine effects of reactive oxygen metabolites (ROMs), with and without flunixin meglumine, on equine right ventral colon (RVC) in vitro.

Animals—18 healthy horses and ponies.

Procedures—In 3 groups of 6 animals each, short-circuit current and conductance were measured in RVC mucosa in Ussing chambers. The 3 groups received physiologic saline (0.9% NaCl) solution, IV, 10 minutes before euthanasia and tissue incubation in Krebs-Ringer-bicarbonate (KRB) solution; flunixin meglumine (1.1 mg/kg, IV) 10 minutes before euthanasia and tissue incubation in KRB solution; or physiologic saline solution, IV, 10 minutes before euthanasia and incubation in KRB solution with 2.7 X 10⁻⁵ M flunixin meglumine. Incubation conditions included control (no addition) and ROM systems, including addition of 1 mM xanthine and 80 μU of xanthine oxidase (to produce the superoxide radical), 1 mM H₂O₂, and 1 mM H₂O₂ and 0.8 mM ferrous sulfate (to produce the hydroxyl radical).

Results—All ROMs that were added or generated significantly increased the short-circuit current except in tissues coincubated with flunixin meglumine, and they induced mild epithelial vacuolation and apoptosis, but did not disrupt the epithelium nor change conductance, lactate dehydrogenase release, or [³H]mannitol flux.

Conclusions and Clinical Relevance—Responses to ROMs could be attributed to increased chloride secretion and inhibited neutral NaCl absorption in equine RVC, possibly by stimulating prostaglandin production. The ROMs examined under conditions of this study could play a role in prostaglandin-mediated colonic secretion in horses with enterocolitis without causing direct mucosal injury. (Am J Vet Res 2007;68:305–312)

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>ROM</td>
<td>Reactive oxygen metabolite</td>
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<td>PG</td>
<td>Prostaglandin</td>
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<td>RVC</td>
<td>Right ventral colon</td>
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<td>KRB</td>
<td>Krebs-Ringer-bicarbonate</td>
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<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<td>DMPO</td>
<td>5,5-dimethylpyrroline-N-oxide</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
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Common life-threatening diseases of the equine large colon, such as large colon volvulus and infectious enterocolitis, are associated with pathophysiologic changes that could be mediated by ROMs. A potential source of ROMs in these diseases is granulocytes activated in response to reperfusion injury or by an infectious agent. Whereas the role of reperfusion injury in small intestinal mucosa has been reported in many species, whether this process occurs in the colon seems to be controversial. Rat colon can incur a reperfusion injury characterized by generation of free radicals and by antioxidant-responsive increases in vascular permeability, mucosal permeability, tissue xanthine oxidase, thiobarbiturate derivatives, arachidonic acid metabolites, and lactic acid. In another study on rat colon, increased tissue myeloperoxidase, the associated histologic injury, and decreased α-tocopherol concentration in tissues during reperfusion were attributed to neutrophil infiltration and neutrophil-derived ROMs. In addition to granulocytes, other potential sources of ROMs in the colon include aldehyde oxidase, the mitochondrial and microsomal transport chains, and the cyclooxygenase and lipoxygenase enzymes of arachidonic acid metabolism.

In equine colon, reperfusion after 2.5 hours of ischemia was associated with histologic improvement in 1 study and reperfusion injury could not be detected via biochemical measurements of mucosal integrity in another. Compared with the equine jejunum, oxidative processes do not seem to contribute appreciably to damage incurred after 2 hours of ischemia and 2 hours of reperfusion in the colon. Attempts at pharmaco-
logic prevention of reperfusion injury in the colon have been disappointing.\textsuperscript{12} Whereas xanthine oxidase activity plays an important role in initiating oxidative damage in the small intestine, activity of this enzyme is negligible in the colon.\textsuperscript{13} Apparent reperfusion injury in the colon could also be attributed to progressive vascular injury,\textsuperscript{14} hypoperfusion,\textsuperscript{12} or metabolic changes,\textsuperscript{13} rather than to oxidative processes.

In a low-flow model of ischemia in the colon, 3 hours of ischemia at 20% of baseline flow was followed by reperfusion injury, characterized by greater mucosal disruption and neutrophil infiltration, compared with a similar ischemic period.\textsuperscript{1,5} Reperfusion for 30 minutes following 2 hours of ischemia significantly increased the osmotic reflection coefficient in the colon, a sensitive measure of microvascular permeability and reperfusion-induced endothelial response.\textsuperscript{7} Two hours of reperfusion after 3 hours of ischemia in the colon increased tissue myeloperoxidase, suggesting neutrophil infiltration during reperfusion.\textsuperscript{18} In similar studies, an increase in lipid peroxidation and a decline in tissue superoxide dismutase after ischemia\textsuperscript{19} and a decrease in tissue myeloperoxidase and mucosal damage in response to a 21-aminosteroid\textsuperscript{20} suggest that some biochemical conditions exist to allow reperfusion injury in the colon. In the latter study,\textsuperscript{20} myeloperoxidase and lipid peroxidation did not increase significantly in the colon during 2 hours of reperfusion, although the high resident population of eosinophils in colonic mucosa can obscure changes in neutrophil-derived myeloperoxidase in that tissue.\textsuperscript{19}

Another approach to examining reperfusion injury or inflammatory responses in a tissue is to study the response to added ROMs, so that susceptibility to different agents can be determined and specific treatments identified.\textsuperscript{21,22} The authors have reported that HOCl at different concentrations could damage equine colonic mucosa in vitro and that this effect was mitigated by ascorbic acid.\textsuperscript{23} When ROMs and various immune system agonists are placed on the serosal side of rat and rabbit colon in Ussing chambers, they alter intestinal epithelial transport through a PG-mediated process without inducing mucosal injury.\textsuperscript{11} This could also be an important route for fluid loss into the intestines during inflammatory diseases, such as enterocolitis.\textsuperscript{24,25} Although histologic changes suggestive of reperfusion injury have been described in ischemia-reperfusion models in the colon of horses,\textsuperscript{3} the potential for effects of ROMs from granulocytes, predominantly eosinophils, appears to be more pronounced in the colon of horses and ponies with naturally acquired and castor oil-induced colitis.\textsuperscript{2} In the castor oil model of acute colitis, increased malondialdehyde in the inflamed tissue was attributed to lipid peroxidation by ROMs from stimulated, recruited, and resident granulocytes, and the associated increase in tissue PGE\textsubscript{2} was considered an important mediator of the secretory response.\textsuperscript{3}

The hypothesis of the study reported here was that the 3 ROMs, superoxide radical, hydrogen peroxide, and hydroxyl radical,\textsuperscript{24,25} could affect ion transport in the equine RVC in vitro. These ROMs could be relevant to intestinal epithelial injury and secretion in vivo and can be generated in the intestinal lumen or released by inflammatory cells in the lamina propria.\textsuperscript{26} To examine the role of PGs on changes in ion transport elicited by ROMs, ROM effects were studied in the presence of flunixin meglumine. The RVC was examined because it is affected by the diseases of interest\textsuperscript{13,16} and it is the segment that has been examined previously on issues relevant to this study, such as effects of another ROM (HOCl)\textsuperscript{23} and flunixin meglumine.\textsuperscript{27} Therefore, the purpose of the study reported here was to determine the effects of ROMs, with and without flunixin meglumine, on equine RVC in vitro.

**Materials and Methods**

**Horses and tissue preparation—**The Institutional Animal Care and Use Committee of the University of Illinois approved the following procedures. Healthy adult horses and ponies, ranging in age from 2 to 8 years and body weight from 128 to 477 kg, were euthanatized by administration of an overdose of pentobarbital sodium. Horses were donated to the University of Illinois to be euthanatized for reasons other than gastrointestinal tract disease, and the ponies had been used in terminal surgical exercises.

A full-thickness segment of RVC was removed immediately, rinsed of intestinal contents, and transported to the laboratory in KRB solution at 4°C. The tissue was pinned on a rubber surface with the mucosal surface up and at a temperature of 37°C. While the solution was constantly gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, sharp dissection was used to remove mucosal sheets from the muscle layers,\textsuperscript{29} which were then mounted in Ussing chambers. These chambers had an aperture of 1.13 cm\textsuperscript{2}, and a solution volume of 7 mL bathed each tissue surface. The KRB solution was composed of 112mM NaCl, 25mM NaHCO\textsubscript{3}, 10mM glucose, 5mM KCl, 3mM Na acetate, 3mM Na bicarbonate, 2.5mM CaCl\textsubscript{2}, 1.2mM MgSO\textsubscript{4}, 1.2mM KH\textsubscript{2}PO\textsubscript{4}, and 0.01mM mannitol. This solution was maintained at a pH of 7.4 by constant gassing with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, at a temperature of 37°C by circulation via means of a gas lift through water-jacketed reservoirs.

**Experimental design—**Ponies and horses were equally distributed among 3 groups of 6 horses/group. One group received flunixin meglumine (1.1 mg/kg, IV) 10 minutes before euthanasia, and tissues were collected, dissected, and incubated in plain KRB solution. The second group received physiologic saline (0.9% NaCl) solution, IV, in a volume equivalent on a body weight basis to that received by horses given flunixin meglumine 10 minutes before euthanasia, and tissues were collected, dissected, and incubated in plain KRB solution. The third group received physiologic saline solution, IV, in a volume equivalent on a body weight basis to that received by horses given flunixin meglumine 10 minutes before euthanasia, and tissues were collected, dissected, and incubated in plain KRB solution. The third group received physiologic saline solution, IV, in a volume equivalent on a body weight basis to that received by horses given flunixin meglumine 10 minutes before euthanasia.

**Incubation conditions—**Salt bridges of 0.9% NaCl and 4% agar were used to connect the chambers to an automatic voltage clamp,\textsuperscript{8} and the junction potentials of
Assessment of cytotoxicosis—Samples of mucosal medium were collected at the end of the incubation periods and stored at –20°C to be assayed for LDH.† Absorbance was measured on a spectrophotometer at 490 nm, and results were recorded as absorbance per gram of tissue (dry weight), with dry weight determined for the mucosal surface in the chamber as described. At the end of the experiment, portions of the tissues were placed in Karnovsky fixative and embedded in epoxy resins. Sections were cut to 0.1-µm thickness, stained with 1% toluidine blue, and examined via light microscopy to assess tissue damage.

In all 3 groups, incubation conditions were as follows: control (no additions to the basic incubation solution), 1 mM xanthine† and 80 µU of xanthine oxidase/mL (to produce the superoxide radical), 1 mM H₂O₂,† and 1 mM H₂O₂ and 0.5 mM FeSO₄ † (to produce the hydroxyl radical). Additions were made to both sides of the tissues when steady-state readings for short-circuit current and conductance were obtained (40 to 50 minutes after they were mounted in chambers). Electrical responses were recorded at 5-minute intervals for 60 minutes after oxidants were added to ensure that peak responses were not missed. Tissues were incubated for an additional 105 minutes after additions.

EPR spectroscopy—To generate the hydroxyl radical via the Fenton reaction, 1 mM H₂O₂ and 0.5 mM FeSO₄ were added to KRB solution in water-jacketed beakers at a temperature of 37°C and gassed with 95% O₂ and 5% CO₂. To form a stable adduct with the hydroxyl radical that could be measured by use of EPR spectroscopy (100 mM of the spin trap), DMPO® was added simultaneously. Although concentrations of 10, 25, 50, and 100 mM DMPO were evaluated initially, 100 mM was used in all experiments because the highest concentrations gave the most intense signals. Samples of RVC mucosa of 3 horses were incubated in beakers with 1 mM H₂O₂ and 0.3 mM FeSO₄ and in the spin trap and compared with beakers without tissue to determine the effect of colonic mucosa on hydroxyl generation. Volume ratio of tissue to medium was the same as that for Ussing chambers.

The spin adduct signal was measured by use of an EPR spectrometer (9.2 GHz, X-band) with a modulation frequency of 100 kHz, microwave power of 20 MW, and modulation amplitude adjusted as needed. Each sample was analyzed in a quartz glass flat cell. A scan time of 4 minutes was used, and spectra were collected on software installed on a computer. Signal intensity was measured by use of normalized peak height.
Statistical analysis—Tissue responses were measured in duplicate, and each horse was used as the unit of observation. Results are expressed as mean ± SEM. Effects of treatment and time were examined by use of ANOVA for repeated measures with a general linear models procedure.21,29 The Bonferroni method was used to detect differences between the groups when the treatment by time interactions were significant. Significance was set at \( P < 0.05 \).

Results

Flunixin meglumine given before euthanasia or included at a concentration of \( 2.7 \times 10^{-5} \) M in all tissue collection and incubation solutions significantly reduced the baseline short-circuit current, compared with saline solution administered IV (Figures 1–3). All chemically generated or added ROMs caused a significant increase in short-circuit current, but the significant increase in short-circuit current was more sustained in tissues from horses that received saline solution with incubation with xanthine plus xanthine oxidase. None of the incubation conditions in plain KRB, in KRB with flunixin meglumine, or after IV administration of flunixin meglumine changed conductance, LDH release, and mannitol fluxes; therefore, data for these measurements were pooled from the 3 groups (Table 1). Flunixin meglumine added to bathing solutions blocked the short-circuit current response to all ROMs, but prior treatment with flunixin meglumine did not.

The adduct formed from DMPO and \( \text{H}_2\text{O}_2 \) and \( \text{FeSO}_4 \) generated a signal characteristic of the hydroxyl radical29,30 (Figure 4). Signal intensity generated during incubation with the colonic mucosa (n = 3) was 17.2 ± 4.9% of the signal intensity without the tissue. On histologic examinations, individual surface epithelial cells appeared to have undergone mild apoptosis with all ROM treatments and had exfoliated without loss of epithelial barrier integrity (Figure 5). Tissues incubated with xanthine plus xanthine oxidase had discrete areas of vacuolation in epithelial cells (Figure 6). Regardless of these histologic changes, the epithelial layer was intact in all tissues.

Discussion

According to results of this study, exposure of equine colonic mucosa to \( \text{H}_2\text{O}_2 \), xanthine plus xanthine oxidase, and a hydroxyl-radical generating system (\( \text{H}_2\text{O}_2 \) and \( \text{FeSO}_4 \)) increased the short-circuit current, possibly through a PG-mediated increase in electrogenic chloride secretion and a concurrent PG-mediated decrease in neutral NaCl absorption.11 The ability of the NSAID, flunixin meglumine, to block this response confirmed the role of PGs in ROM-induced changes in ion transport in equine colon.

Although specific ROMs were generated or added, conditions and enzymatic pathways in the tissues could have produced metabolites different from those intended for study. Superoxide anion generated by xanthine plus xanthine oxidase does not seem to play a role in lipid peroxidation and other cellular responses; however, it can reduce \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \), which could accelerate conversion of \( \text{H}_2\text{O}_2 \) to hydroxyl.

Table 1—Mean ± SEM data (pooled among all groups) for LDH release, conductance, and mannitol permeability in equine RVC mucosa from horses and ponies (n = 6) that received saline solution, IV, 10 minutes before euthanasia (control treatment); flunixin meglumine, IV, 10 minutes before euthanasia; or saline solution, IV, 10 minutes before euthanasia followed by incubation with \( 2.7 \times 10^{-5} \) M flunixin meglumine on both surfaces of the tissue.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>LDH (absorbance/g)</th>
<th>Conductance (millisiemens/cm²)</th>
<th>Mannitol permeability (pmol/cm²/h)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.233 ± 0.04</td>
<td>6.0 ± 0.20</td>
<td>88.7 ± 11.8</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>0.243 ± 0.05</td>
<td>6.9 ± 1.28</td>
<td>81.9 ± 10.3</td>
</tr>
<tr>
<td>( \text{H}_2\text{O}_2 ) + Fe</td>
<td>0.259 ± 0.06</td>
<td>6.8 ± 0.44</td>
<td>87.4 ± 13.9</td>
</tr>
<tr>
<td>X/XO</td>
<td>0.227 ± 0.04</td>
<td>6.9 ± 0.29</td>
<td>81.8 ± 10.1</td>
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*Mucosa was incubated in plain KRB solution (control treatment), and plain KRB with \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2 \) and \( \text{FeSO}_4 \), or xanthine plus xanthine oxidase (X/XO) to generate the superoxide anion. Mannitol permeability data and LDH absorbance data are for 120 minutes of incubation, with 105 minutes of exposure to each treatment. Conductance was recorded at intervals throughout 105 minutes of exposure to each treatment, and the means of values over time are given.

Figure 2—Short-circuit current (\( \mu \text{A/cm}^2 \)) response (mean ± SEM) in mucosa isolated from the RVC of horses and ponies (n = 6) that received saline solution, IV, 10 minutes before euthanasia. Mucosa was isolated, dissected, and incubated in KRB solution with \( 2.7 \times 10^{-5} \) M flunixin meglumine on both surfaces of the tissue. Incubation conditions were as in Figure 1, except for inclusion of flunixin meglumine in all collection, dissection, and incubation media.
radical (Haber Weiss or Fenton reaction), or it could generate peroxynitrite, another toxic end product.\textsuperscript{31} The superoxide anion can spontaneously dismutate to H$_2$O$_2$, or this conversion can be catalyzed by superoxide dismutase,\textsuperscript{31} a process that can peak at approximately 4 to 20 minutes.\textsuperscript{26,31} The combination of xanthine plus xanthine oxidase can theoretically produce a 4:1 excess of H$_2$O$_2$, compared with superoxide anion.\textsuperscript{26,31} Although most or all of the cytotoxicosis caused by superoxide anion and H$_2$O$_2$ is usually attributed to conversion to the more harmful hydroxyl radical,\textsuperscript{33,34} H$_2$O$_2$ might play an important role in ROM-induced tissue injury and biochemical responses.\textsuperscript{11,26,31}

Hydrogen peroxide differs from most oxidants because its slow reaction with other organic substrates allows it to diffuse large distances and its small size, lack of charge, and lipophilic nature facilitate its movement across plasma membranes.\textsuperscript{35,36} It is the major stable metabolite produced by various inflammatory cells, and in micromolar concentrations, it can alter various intracellular functions.\textsuperscript{37} Stimulated neutrophils can produce H$_2$O$_2$ to mediate cytotoxicosis without conversion to H$\text{OCl}$, and the proportion assigned to this use is apparently determined by the triggering agent.\textsuperscript{35} Low-flow hypoxia causes production of H$_2$O$_2$ in hepatocytes in vivo.\textsuperscript{38} Hydrogen peroxide can cause a dose-dependent increase in arachidonic acid metabolites and submicroscopic cell membrane damage in rat colon in vitro through a phospholipase A$_2$–cyclooxygenase and catalase-susceptible pathway, without involvement of the hydroxyl radical.\textsuperscript{37} The concentration of H$_2$O$_2$ used in the present study was within ranges of theoretical concentrations produced within the vicinity of activated neutrophils\textsuperscript{37} and resident phagocytes in mammalian colon.\textsuperscript{11,35} When measurements of H$_2$O$_2$ from activated human neutrophils\textsuperscript{35} are extrapolated to neutrophil numbers per cubic centimeter of equine colon after low-flow ischemia and reperfusion,\textsuperscript{1} by use of the same approach as described for H$\text{OCl}$,\textsuperscript{23} the concentration of H$_2$O$_2$ would be at least one third of what could be generated in the tissue water around such a volume of neutrophils.

Figure 4—Electron paramagnetic resonance spectra (Ångström units [AU]) produced by 100mM DMPO, 1mM H$_2$O$_2$, and 0.5mM FeSO$_4$ incubated in KRB solution for 20 minutes. Spectrometer settings were center field of 3,340 Gauss, sweep rate of 400 Gauss/min, modulation amplitude of 2 Gauss, microwave frequency of 9.4 GHz, and gain of 4.

Figure 5—Photomicrographs of sections of equine colonic mucosa after 160 minutes of incubation under control conditions (A) and after 105 minutes of incubation with 1mM H$_2$O$_2$ on both surfaces of the tissue (B). Tissues were not exposed to flunixin meglumine in vivo or in vitro. Notice 3 apoptotic bodies on the surface of B (arrow and arrowheads); one contains a nucleus with marginalized chromat (arrow). Toluidine blue stain; bar = 50 µm.
The absence of microscopic evidence of epithelial disruption and the absence of changes in LDH release, tissue conductance, and permeability to mannitol suggest that the equine colon is not susceptible to direct injury from the ROMs examined, although some minor damage at the subcellular level cannot be excluded. These findings cannot be extrapolated directly to reperfusion injury because this system eliminated a contribution from neutrophil influx and activated resident neutrophils, used viable epithelial cells rather than cells rendered susceptible to ROM by anoxia, and used a single application of ROM to the medium versus the sustained delivery that might be expected in vivo. Also, damaged endothelial cells are central to ROM generation and the pathophysiology of reperfusion injury, and the microvascular changes caused by ischemia and reperfusion could lead to epithelial damage in equine colon. In addition, rectal infusion of ROM-generating systems in rats in vivo can induce mucosal inflammation. Nonetheless, in vitro methods have been used to detect ROM-induced tissue damage in monolayers from various cell lines, including equine colon.

In previous Ussing chamber studies on ROMs in rat colon and ileum, the concentration of H$_2$O$_2$ used on the equine colon (1mM) did not induce any cytotoxic effect when H$_2$O$_2$ was added to the serosal surface of tissues. Because mucosal epithelial cells would be exposed to ROMs from subepithelial and intraepithelial granulocytes during reperfusion injury and colitis, ROMs were applied to the luminal and serosal surfaces in the study described here. Although this is a severe model, it is realistic, especially because direct luminal application would be particularly critical with the short-lived hydroxyl radical. Mucosa exposed to ROMs had some rare apoptosis in surface epithelial cells in the expected location for exfoliation of effete cells at the end of their expected life span, and these changes were not observed in control tissues. Apoptosis was also reported in rat small intestine after ischemia and was exacerbated by reperfusion, and 0.5mM H$_2$O$_2$ induced apoptosis selectively in liver fragments in vitro. However, apoptosis seemed insufficient in the study reported here to disrupt epithelial integrity on the basis of all measurements used for tissue damage.

One explanation for lack of cell damage by ROMs in the equine colon is that colonocytes contain endogenous antioxidants, superoxide dismutase, glutathione peroxidase, and catalase to a greater extent than the submucosa and other layers. Scavenging by equine colonocytes was supported by the finding of an 82.8% reduction in hydroxyl signal intensity from the spin-trap reaction in the presence of the colon mucosa, compared with a tissue-free medium. Concentrations of individual oxidants were not measured and which species (the hydroxyl radical or H$_2$O$_2$) was scavenged could not be detected. Hydrogen peroxide can be detoxified by catalase via conversion to water and molecular oxygen in peroxisomes and by glutathione peroxidase in mitochondria and cytosol. Oxygen gas and water produced from this process could cause the discrete clusters of vacuolation in colonocytes exposed to xanthine plus xanthine oxidase.

Consistent with previous reports, all oxidant systems used in the present study caused a sharp increase in short-circuit current that was blocked by coincubation with flunixin meglumine in the bathing medium. On the basis of inhibition studies, H$_2$O$_2$ and not superoxide or the hydroxyl radical was implicated in the same short-circuit response to ROMs in rat colon. The secretory response to H$_2$O$_2$ appears to be concentration dependent in rat colon at concentrations up to 1mM, and > 1mM, these responses decline, presumably because of tissue damage. The response observed with xanthine plus xanthine oxidase in horses that received saline solution IV seemed more pronounced than with the other ROMs and followed a more sustained or biphasic pattern, similar to that recorded in rat colon. Possibly, the concentration of reagent H$_2$O$_2$ added to the chambers was greater than that generated by xanthine plus xanthine oxidase and might have induced a toxic effect that blunted the biphasic response. Alternatively, the concentration of H$_2$O$_2$ added was less than that generated by xanthine plus xanthine oxidase and was therefore insufficient to achieve a biphasic response. Dose-response studies could be used to address this issue, but were not done.

The biphasic response to xanthine plus xanthine oxidase was evident only in colon from horses that received saline solution IV and not in tissues from horses.
that received flunixin meglumine IV. Serosal addition of an ROM is required to induce the sustained biphasic response in rat colon, whereas mucosal application will induce only a single peak.\(^1\)\(^2\) If this is true in equine colon, then possibly flunixin meglumine administered IV continued to block a source of cyclooxygenase that was accessible to oxidants from the serosal surface in vitro but did not block the enzyme accessible from the mucosal surface. Flunixin meglumine was used IV to block PG synthesis in response to tissue preparation and, thereby, lower the baseline short-circuit current.\(^2\)\(^7\) Presumably, there was insufficient flunixin meglumine in the tissue in vitro after IV administration to inhibit PG synthesis in response to ROMs, compared with tissues incubated in flunixin meglumine. Although the in vitro concentration used was based on pharmacologic data,\(^2\) this was not corrected for protein binding and therefore could be much higher than the true concentration in plasma.\(^4\) Alternatively, any residual flunixin meglumine in mucosa after IV administration could have been considerably diluted in the bathing medium.

In rat colon in vitro, \(\text{H}_2\text{O}_2\) and not superoxide or the hydroxyl radical, is the main ROM that can alter electrolyte transport through a large increase in colonic production of PGE\(_\alpha\) and PGI\(_\alpha\), which then activates the enteric nervous system.\(^3\) In rat colonic fragments, \(\text{H}_2\text{O}_2\) causes a time- and dose-dependent release of 6-keto-PGF\(_\alpha\) and thromboxane B\(_2\) through a catalase-susceptible pathway and not through nonspecific cytotoxicosis.\(^3\) Although net prostanooid release is 8 to 10 times greater from the seromuscular layer than from the mucosa,\(^3\) the colonic mucosa alone is capable of intense and rapid (<15 minutes) synthesis of PGE\(_\alpha\). Oxidants can also evoke the release of other secretagogues, such as histamine, 5-hydroxytryptamine, bradykinin, substance P, and acetylcholine, through injury to mast cells, nerves, and fibroblasts.\(^2\) In addition to the PG-mediated, cyclic adenosine monophosphate–stimulated CIC secretion, oxidants can also prolong and amplify the secretory response in monolayers of human colon adenocarcinoma by lowering the calcium-activation threshold of the basolateral Na\(+\)-K\(+\)-2Cl cotransporter.\(^3\) Therefore, ROMs can be potent secretagogues through PGs and prostanoid-independent mechanisms in inflamed intestinal mucosa.\(^3\)

Results of the present study and others suggest that reactive metabolites of oxygen and nitrogen can be key players in a variety of diarrheal diseases through complex direct and indirect interactions with cholinergic neurons, enterocytes, prostanoids, and other possible secretagogues.\(^1\)\(^2\)\(^4\)\(^5\)\(^6\) The secretory components associated with salmonellosis, amebiasis, and cryptosporidiosis have been attributed to PGs and WBCs, which would suggest a benefit with NSAIDs in the treatment of secretory diarrheal diseases.\(^2\)\(^4\)\(^6\) Findings in the present study were consistent with the role of granulocytes and associated inflammatory responses proposed for naturally acquired and induced colitis in equids.\(^2\)\(^3\)

Combined with other data, results of the study reported here also provide some information on the relative importance of different ROMs in tissue injury and, hence, some guidelines on pharmacologic protection. Cytotoxicosis caused by hydroxyl radical generation was not detected, which was consistent with evidence that potential scavengers of this radical fail to mitigate reperfusion injury in equine colon in vivo.\(^5\) Alternatively, the concentrations generated were insufficient to cause tissue damage. However, spin-trapping experiments reveal that neutrophils, monocytes, and macrophages lack the endogenous capacity to generate the hydroxyl radical,\(^6\) and hence, the importance of this radical could be underestimated. Other products from activated neutrophils might be more important in mucosal damage caused by reperfusion injury, such as HOCl, NH\(_2\)Cl, and proteases.\(^5\) On the basis of results of an in vitro study, HOCl is damaging to equine colon at concentrations of 1 to 7 mM, as indicated by increased conductance, increased permeability to mannitol, and damage to surface epithelial cells.\(^6\) Therefore, it would seem that future studies on reperfusion in equine colon should concentrate on HOCl as the possible mediator of reperfusion injury. In studies\(^2\)\(^3\)\(^6\) to date, ascorbic acid and N-acetylcysteine both seem capable of protecting equine colon in vitro against damage from this ROM.

**References**


