

Hydrogen Peroxide Stimulates Rat Colonic Prostaglandin Production and Alters Electrolyte Transport

S. Selim Karayalcin, Christopher W. Sturbaum, Joseph T. Wachsman, Jih-Ho Cha, and Don W. Powell
Department of Medicine and Core Center in Diarrheal Diseases, University of North Carolina at Chapel Hill,
Chapel Hill, North Carolina 27599

Abstract

The changes in short circuit current (electrogenic Cl^- secretion) of rat colon brought about by xanthine/xanthine oxidase in the Ussing chamber were inhibited by catalase and diethyldithiocarbamate, but not by superoxide dismutase. These results, the reproduction of the response with glucose/glucose oxidase and with exogenous H_2O_2 , and the lack of effect of preincubation with deferoxamine or thiourea implicate H_2O_2 , and not O_2^- or OH^\cdot , as the important reactive oxygen metabolite altering intestinal electrolyte transport. 1 mM H_2O_2 stimulated colonic PGE_2 and PGI_2 production 8- and 15-fold, respectively, inhibited neutral NaCl absorption, and stimulated biphasic electrogenic Cl^- secretion with little effect on enterocyte lactic dehydrogenase release, epithelial conductance, or histology. Cl^- secretion was reduced by cyclooxygenase inhibition. Also, the Cl^- secretion, but not the increase in prostaglandin production, was reduced by enteric nervous system blockade with tetrodotoxin, hexamethonium, or atropine. Thus, H_2O_2 appears to alter electrolyte transport by releasing prostaglandins that activate the enteric nervous system. The change in short circuit current in response to iloprost, but not PGE_2 , was blocked by tetrodotoxin. Therefore, PGI_2 may be the mediator of the H_2O_2 response. H_2O_2 produced in nontoxic concentrations in the inflamed gut could have significant physiologic effects on intestinal water and electrolyte transport. (*J. Clin. Invest.* 1990; 86:60-68.) Key words: intestinal secretion • inflammation • enteric nervous system • prostaglandin • oxygen radical

Introduction

Reactive oxygen metabolites (ROMs)¹ are oxygen-centered free radicals or their reduction products (1-3). The reactivity of oxygen can be increased by excitation or reduction. An

This study was presented in part at the 1988 Annual Meeting of the American Gastroenterological Association, and has appeared in abstract form (1988. *Gastroenterology*. 94:A216; and 1988. *Gastroenterology*. 94:A217).

Address reprint requests to Dr. D. W. Powell, CB #7005, 3029 Old Clinic Bldg., University of North Carolina, Chapel Hill, NC 27599-7005.

Received for publication 17 April 1989 and in revised form 17 January 1990.

1. Abbreviations used in this paper: ATR, atropine; CAT, catalase; DDTc, diethyldithiocarbamate; DES, deferoxamine; ENS, enteric nervous system; G, glucose; GO, glucose oxidase; HEX, hexametho-

excitation product (singlet oxygen) results from an absorption of energy that shifts one of the unpaired electrons of the molecule to an orbit of higher energy with an inversion of its spin. Singlet oxygen may not exist to any extent in biological tissues; however, the reduction products certainly do. The reduction of oxygen by the sequential addition of four electrons creates two products, superoxide (O_2^-) and hydroxyl radical (OH^\cdot), which are free radicals (molecules containing an odd number of electrons, rendering them chemically reactive) as well as hydrogen peroxide (H_2O_2), a potent oxidant. The length of time that H_2O_2 can exist in tissue and the distance to which it can diffuse (much like water) are dependent on the presence of protective enzymes in the tissue (catalase [CAT] and glutathione peroxidase) (1-3). Therefore, H_2O_2 , the least reactive of the metabolites, has the longest half-life and potentially is capable of important biological effects.

The sources of ROMs in the intestine include the mitochondrial and microsomal transport chains, those created by oxidant enzymes such as xanthine oxidase (XO) and the cyclooxygenase and lipoxygenase enzymes of AA metabolism, and the respiratory burst of phagocytic cells (2). Some believe that the most important sources of ROMs in the intestine are resident or newly arrived phagocytes (3). Upon stimulation, a transmembrane electron transport system utilizes NADPH on the cytoplasmic side of the phagocytic cell membrane to reduce O_2 to H_2O_2 . Potentially, millimolar concentrations of H_2O_2 may be secreted in the immediate vicinity of target cells (2, 4). The important stimulants of the formation of ROMs in the intestine, consequently, are those that activate phagocytes (3).

Recent studies in our laboratory have shown that ROMs are capable of altering electrolyte transport in the in vitro rat colon mounted in the Ussing chamber (5). These results led us to postulate that while either ROMs or prostaglandins released by ROMs may have a direct effect on the enterocyte, some cyclooxygenase products like PGI_2 (6, 7) may modulate rat colonic electrolyte transport primarily by activating the enteric nervous system (ENS).

It was unclear from our previous studies, however, which ROM was actually causing this transport response. The xanthine (X)/XO reaction creates O_2^- , which is spontaneously and enzymatically dismutated to H_2O_2 , as well as H_2O_2 itself. In the presence of iron, H_2O_2 can also be metabolized to OH^\cdot (the Haber-Weiss or Fenton reaction) (1, 3). In this investigation we determined that H_2O_2 is the ROM responsible for the alteration in transport and characterized the colonic electrolyte transport response to this oxidant.

nium; H_2O_2 , hydrogen peroxide; INDO, indomethacin; Isc, short circuit current; LDH, lactate dehydrogenase; O_2^- , superoxide; OH^\cdot , hydroxyl radical; PXM, piroxicam; ROM, reactive oxygen metabolite; TH, thiourea; TTX, tetrodotoxin; X, xanthine; XO, xanthine oxidase.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/90/07/0060/09 \$2.00

Volume 86, July 1990, 60-68

Methods

Preparation of intestine. Male Sprague-Dawley rats (250–350 g) with free access to normal rat chow and water were killed by cervical dislocation. The colon was removed and opened along its mesenteric border and rinsed in ice-cold Ringer solution and the serosa and muscularis propria were removed as previously described (5, 8). A 2-cm section of the tissues was fixed with buffered 4% formaldehyde–10% glutaraldehyde and stained with hematoxylin and eosin or with diamine silver for histological demonstration of mucosal and neural structure, respectively (see Fig. 8A). These studies revealed that the plane of division was at the innermost aspect of the circular muscularis propria layer, leaving the muscularis mucosae and submucosal nerve plexus intact. This preparation, consisting of epithelium, lamina propria, muscularis mucosae, and submucosa, was mounted in Ussing-type chambers (0.5 cm² exposed surface area) and bathed in 5–10 ml of glucose-Ringer solution on each side. The glucose-Ringer solution, pH 7.4 when gassed with 95% O₂/5% CO₂, contained 10 mM glucose and ions in the following concentrations (in mM): Na 140; Cl 119.8; K 5.2; HCO₃ 25; Ca 1.2; Mg 1.2; HPO₄ 2.4; and H₂PO₄ 0.4. Solutions were circulated by gas lift and maintained at 37°C in water-jacketed reservoirs.

Four to six tissues were obtained from distal colon for experiments in which one tissue served as a control (stimulated with the agonist), while the others were used to test the effects of inhibitors. Concentration–response curves were obtained by adding the agonists or inhibitors to the serosal solution of four tissues mounted from a single animal. At various times, 1-ml samples of serosal bathing solutions were placed into polypropylene vials, gassed with argon, and stored at –20°C for future radioimmunoassay of various eicosanoids. In other experiments, aliquots of either the mucosal or serosal solutions were taken for measurement of lactic dehydrogenase (LDH) activity or for concentrations of O₂[–] or H₂O₂. To assess the effect of oxidants on intestinal histology, control and H₂O₂-treated tissues were removed from the Ussing chamber after 2 h of incubation, fixed, and stained as noted above.

Electrical and electrolyte flux measurements. The tissues were mounted in Ussing chambers and the measured transmural PD was continuously short-circuited to zero with the aid of an automatic voltage clamp (DVC-1000 voltage/current clamp; World Precision Instruments, Inc., New Haven, CT) using Ag-AgCl electrodes connected to the bathing solution via agar bridges. After a 30–45-min pre-equilibration period, by which time the PD had stabilized, the tissues were stimulated from the serosal side by addition of various agonists. When inhibitors or stimulants were studied, those agents were added to the serosal bathing solution for at least 20 min before addition of the agonists. Conductances (G) were calculated from the open-circuit PD and the short circuit current (I_{sc}) using Ohm's law. The maximal increase in I_{sc} (ΔI_{sc}) above baseline values after the stimulation was calculated as previously described (5).

Unidirectional electrolyte fluxes of ²²Na and ³⁶Cl were measured as previously described in paired tissues that had electrical conductances differing by no more than 25% (5). The difference between mucosal-to-serosal (J_{ms}) and serosal-to-mucosal (J_{sm}) flux represented net transport (J_{net}) with positive values indicating absorption and negative values representing secretion. The residual flux, J_{net}^R, which probably represents net HCO₃[–] secretion or K absorption, was calculated by the formula $J_{net}^R = I_{sc} - (J_{ms}^{Na} - J_{sm}^{Cl})$.

Prostaglandin assays. The prostaglandin content of incubation solutions was determined by direct immunoassay on 100–300-ml samples according to the method of Granstrom and Kindahl (9). The PG antisera had minimal cross-reactivity (< 1% for most other prostaglandins), except for 6% cross-reactivity of the E₂ antisera with PGA₂ and 2–8% cross-reactivity of the 6-keto-PGF_{1 α} antisera for PGF_{1 α} and PGF_{2 α} . The accuracy and precision of the assays were determined under each experimental condition as previously described (5, 10) and the validity of the assays was checked by high performance liquid chromatography. Prostaglandin production was calculated from the concentrations measured at successive 15-min time periods and ex-

pressed as nanograms per 30 minutes per square centimeter. The values reported are PG production in the first 30 min.

LDH measurements. The release of LDH from the intestinal epithelial cell was measured in aliquots of mucosal bathing solution before, and 1, 5, 15, 30, and 45 min after the serosal addition of 1 mM H₂O₂ or the mucosal addition of 5 mM sodium deoxycholate as a positive control. LDH was measured by a modification of the method of Wroblewski and LaDue (11). H₂O₂ at 1-mM concentrations was demonstrated to have no effect on LDH activity of standards, although 1 M H₂O₂ completely inhibited LDH activity.

H₂O₂ and O₂[–] measurements. The concentration of H₂O₂ in the mucosal or serosal bathing solutions was determined by a modification of the methods of Matalon et al. (12). 125- μ l aliquots of bathing solution were added to 500 μ l of 100-mM KPO₄ (pH 4.0) containing 20 purpurogallin units/ml of horseradish peroxidase, 1.5 mM 4-aminoantipyrine, and 11 mM phenol. The absorption was read at 510 nm and the concentration of H₂O₂ calculated from a standard curve.

The concentration of O₂[–] generated by the X/XO reaction was measured in the Ussing chamber with or without rat colonic tissue present, and with or without the presence of SOD or diethyldithiocarbamate (DDTC), by a modification of the ferricytochrome reduction method as described by Pick and Mizel (13). Ferricytochrome was added directly to the serosal solution of the Ussing chamber to a final concentration of 160 μ M, and 600- μ l aliquots of the serosal solution were taken before and at various time intervals after initiating the X/XO reaction. The aliquots were added directly to the spectrophotometer cuvette and the reduction of ferricytochrome *c* was determined using an ϵ of $21 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Materials. Radioimmunoassay reagents for PGE₂ and 6-keto-PGF_{1 α} analysis were obtained from Advanced Magnetics, Inc. (Cambridge, MA). X, XO, glucose oxidase (GO), SOD, horseradish peroxidase (type II, 200 purpurogallin units/mg), phenol ACS reagent, oxy-purinol, ferricytochrome *c* (type III), DDTC, CAT, thiourea (TH), tetrodotoxin (TTX), hexamethonium bromide (HEX), atropine sulfate (ATR), indomethacin (INDO), piroxicam (PXM), PGE₂, H₂O₂, and sodium deoxycholate were all obtained from Sigma Chemical Co. (St. Louis, MO). Additional lots of XO, SOD, and CAT were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) to confirm results obtained with Sigma enzymes. Deferoxamine mesylate (DES) was obtained from Ciba Pharmaceutical Co. (Summit, NJ). ²²Na and ³⁶Cl were purchased from Amersham Corp. (Arlington Heights, IL) and ICN Radiochemicals (Irvine, CA), respectively. Iloprost was a gift from Berlex Laboratories, Inc. (Cedar Knolls, NJ).

INDO and PXM were dissolved as stock solutions in DMSO such that only 10 μ l was added to 10 ml of Ussing chamber solution, a concentration that has no effect on electrolyte transport. The other reagents were dissolved in Ringer solution. ROMs were created in the Ussing chamber either by adding varying concentrations of XO to the serosal Ringer solution containing 0.7 mM X or by the addition of GO to the glucose-Ringer solution containing 10 mM G.

Statistical evaluation. If one of the four tissues in a set proved unsatisfactory for technical reasons, the entire group of data in that experiment was disregarded. Although ~ 10% of the experiments performed were thus unusable, this design did allow the data to be normalized and presented as "percent of control response," which simplified its presentation and statistical analysis. The statistical significance of paired experiments was determined with the paired *t* test. When multiple comparisons were undertaken, a parametric or nonparametric analysis of variance was used.

Results

Experimental design to determine the biologically active ROM. Fig. 1 depicts the experimental framework for determining which ROM was responsible for the ΔI_{sc} created by the X/XO reaction. The relative proportions of O₂[–] and H₂O₂ created by the X/XO reaction are dependent on the pH, PO₂,

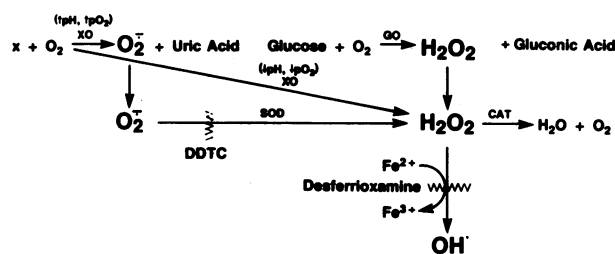


Figure 1. Experimental framework for determining which reactive oxygen metabolite is responsible for X/XO-mediated changes in intestinal electrolyte transport.

and X concentrations present; a higher pH and PO_2 favor the production of O_2^- (14). As shown in Fig. 2, A and B, the peak concentrations of H_2O_2 ($129 \pm 24 \mu\text{M}$) and O_2^- ($26 \pm 1 \mu\text{M}$) represent a ratio of 5:1 in our system. The concentrations of X and XO used were those determined previously by formal concentration-response experiments to give a maximal ΔIsc (5). The Isc response to X/XO was inhibited 90% by 25 μM oxypurinol, provided that the XO was preincubated with oxypurinol at ambient O_2 tension. If O_2^- was responsible for the ΔIsc , then that response might be inhibited by the exogenous addition of SOD, which should hasten its metabolism to H_2O_2 (see Fig. 1). However, the exogenous addition of SOD might or might not have an effect on the ΔIsc , depending on whether endogenous SOD was limiting. Similarly, if O_2^- is the agonist, DDTC should theoretically enhance the response by slowing the conversion of O_2^- to H_2O_2 . Conversely, if H_2O_2 was the biologically active ROM, the ΔIsc might be inhibited by DDTC, which should slow the conversion of O_2^- to H_2O_2 .

As shown in Fig. 2, A and B, while SOD did increase H_2O_2 production by an additional 45 μM , with a corresponding reduction in measurable O_2^- , DDTC actually inhibited the production of both O_2^- and H_2O_2 . This unexpected effect of DDTC might be due to its ability to act as a reducing agent, or perhaps because it is capable of chelating the molybdate ion of XO, thus inactivating the enzyme.

If H_2O_2 was the ROM responsible for the change in Isc , the ΔIsc would be diminished by the exogenous addition of CAT, which rapidly degrades H_2O_2 to O_2 and H_2O (Fig. 1). In addition, the ΔIsc response would be mimicked by the exogenous addition of H_2O_2 or by use of a H_2O_2 generating system (G/GO) (Fig. 2 C).

To rule out the possibility that OH^\cdot is the important ROM resulting in the ΔIsc , the tissues can be preincubated with the iron chelator DES, which will prevent the Haber-Weiss reaction. Furthermore, the effect of scavengers of OH^\cdot , such as thiourea, can be assessed. If Fe chelation or thiourea alters the ΔIsc to either X/XO, G/GO, or H_2O_2 , this would be evidence that OH^\cdot was the responsible species.

ROM responsible for the ΔIsc of rat colon. Fig. 3 depicts the effect of SOD on the ΔIsc response to ROMs created by the X/XO reaction in the serosal bathing solution of rat colon mounted in the Ussing chamber. The response of the tissue to exogenous X/XO is a biphasic, sustained increase in Isc , with peak 1 occurring at 3–6 min and peak 2 being maximal at ~ 30 min. Preincubation with increasing amounts of SOD had no effect on the peak 1 response to X/XO, but diminished peak 2 in a concentration-dependent manner. At 60–120 μM SOD significantly ($P < 0.001$) inhibited the second peak in the

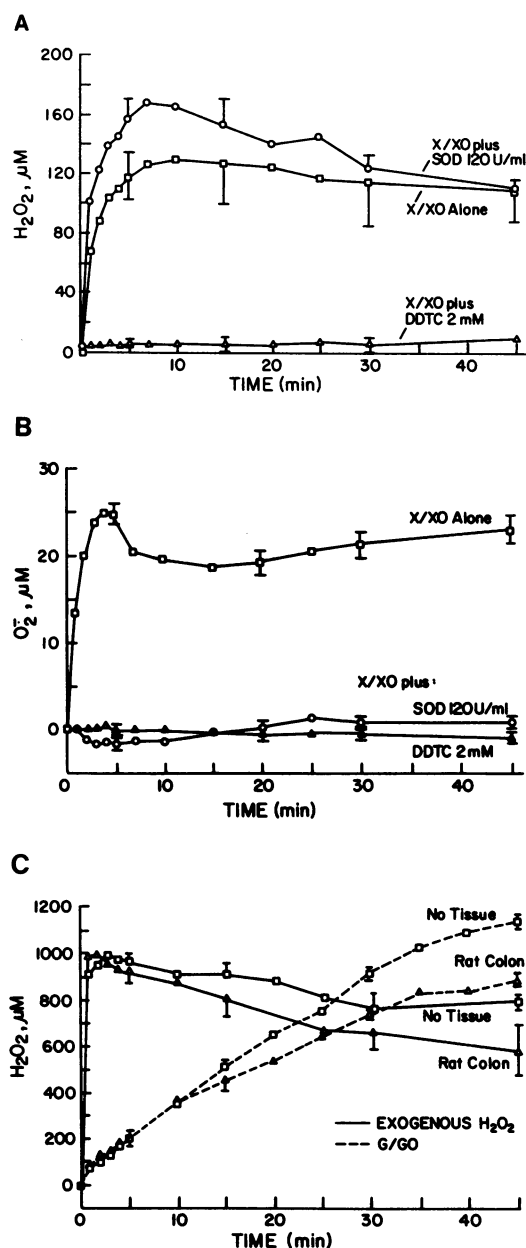


Figure 2. A, The effect of SOD (120 U/ml) and DDTC (2 mM) on H_2O_2 production in the Ussing chamber created by the X (0.7 mM)/XO (20 mU/ml) reaction. B, The effect of SOD and DDTC on O_2^- production created by the X/XO reaction. C, Colonic metabolism of exogenously added H_2O_2 (1 mM) or H_2O_2 generated by the G (10 mM)/GO (200 mU/ml) reaction in the Ussing chamber ($n = 4$ for each experiment). This concentration of GO was shown by formal concentration-response experiments to give a maximal Isc response in the presence of 10 mM G.

group of experiments in Fig. 3, but did not in subsequent experiments with different lots of SOD. This suggests presence of a contaminant in the Sigma enzymes. The stimulatory responses to X/XO was essentially duplicated with enzymes purchased from Boehringer Mannheim Biochemicals, but there was no inhibition with SOD from this source.

Little information can be gained from the use of SOD and DDTC alone because endogenous stores of SOD might be

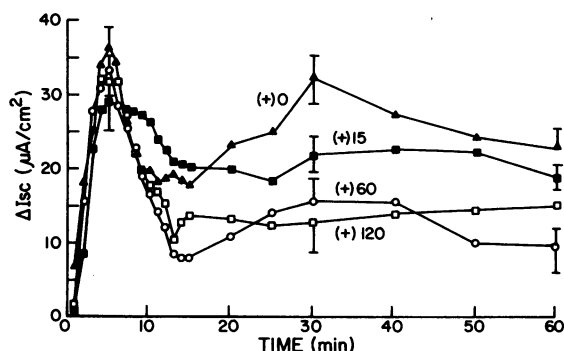


Figure 3. The effect of increasing concentrations of SOD on the ΔI_{sc} to ROMs created by the X (0.7 mM)/XO (20 mU/ml) reaction. SOD concentrations present in the serosal bathing solution were (in U/ml): 0 (▲), 15 (■), 60 (○), and 120 (□) ($n = 5$). The basal I_{sc} ranged from 26 to 31 $\mu A/cm^2$ before initiating the X/XO reaction. The inhibition of the I_{sc} at 30 min (peak 2) with higher concentrations of SOD was not reproduced when a different lot of SOD was used.

sufficient to maximally catalyze the formation of H_2O_2 . Furthermore, the DDTC experiments were not easily interpretable because DDTC abolished the formation of both O_2^- and H_2O_2 (Fig. 2 B). Both peaks 1 and 2 were diminished by preincubation with DDTC in a concentration-dependent manner, and 2 mM was sufficient to essentially abolish the ΔI_{sc} response to X/XO (not shown). This was a specific response relating to DDTC's effect on metabolism of O_2 , rather than some nonspecific effect of DDTC on electrogenic Cl secretion by the colonocyte, because PGE_2 (10^{-6} M) had the same effect on ΔI_{sc} in control tissues ($76 \pm 20 \mu A/cm^2$) as it did in tissues preincubated with 2 mM DDTC ($84 \pm 15 \mu A/cm^2$).

The addition of CAT inhibited the ΔI_{sc} (Fig. 4) and 1,000 U/ml essentially abolished the response. This suggests that H_2O_2 was the species responsible for the ΔI_{sc} to X/XO. Fig. 5 indicates that the creation of ROMs by the G/GO reaction or the exogenous addition of H_2O_2 mimicked the X/XO response, although the time courses and magnitudes of peak 2 are somewhat different for the three ROM-generating systems.

The experiments described above do not rule out the possibility that the change in I_{sc} is due to OH^- . Preincubation of the

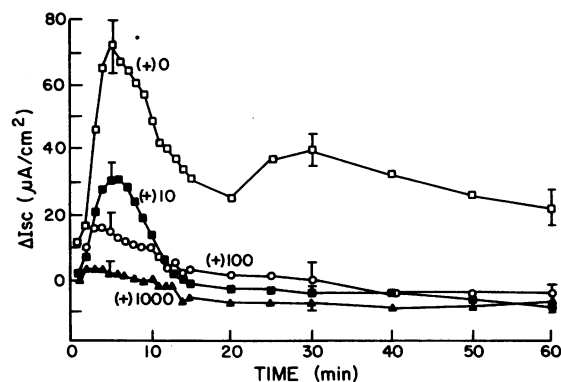


Figure 4. Effect of CAT on ΔI_{sc} response to X/XO. CAT present in the serosal bathing solution before stimulation with X/XO were (in U/ml): 0 (□), 10 (■), 100 (○), and 1,000 (▲) ($n = 4$). The basal I_{sc} ranged from 27 to 35 $\mu A/cm^2$ before initiating the X/XO reaction.

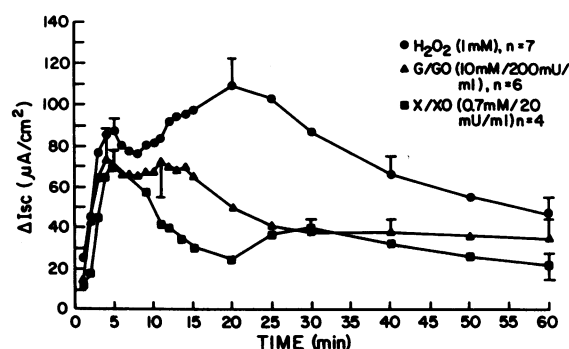


Figure 5. Comparison of the ΔI_{sc} response in rat colon to three ROM-generating systems: H_2O_2 , G/GO, and X/XO. The curves shown for the different agonists represent maximal effective concentrations as determined by formal concentration-response studies. Note that while peak 1 is similar for all three systems, peak 2 varies in time and magnitude. The mean basal I_{sc} concentrations were similar in each experiment before the addition or generation of H_2O_2 .

tissue with DES had no effect on the ΔI_{sc} to G/GO, nor did preincubation with the OH^- scavenger thiourea (Fig. 6). Fig. 6 also summarizes the experiments noted above, indicating the effects of the various agents on either the early (peak 1) or late (peak 2) I_{sc} response. The data are consistent with the idea that H_2O_2 and not OH^- is the responsible ROM.

Mechanism of H_2O_2 effect on colonic electrolyte transport. H_2O_2 added to the mucosal bathing solution of the rat colon in the Ussing chamber stimulated the initial ΔI_{sc} with a time course similar to serosally added H_2O_2 , but the response was less and there was no second ΔI_{sc} peak response. Fig. 7 shows the concentration-response curve to serosal H_2O_2 with a maximal early and late peak response at 1 mM H_2O_2 . There was inhibition of the response at concentrations > 1 mM. This diminished response at higher H_2O_2 concentrations was a toxic effect, as confirmed by histological study (Fig. 8). Only minor histological differences could be discerned between control tissues and those exposed to 1 mM H_2O_2 (Fig. 8 B). Furthermore, 1 mM serosal H_2O_2 addition did not increase the release of LDH into the mucosal bathing solution (at 15 min, control, 227 ± 49 IU/liter, vs. serosal H_2O_2 , 209 ± 33 IU/liter, $n = 4$),

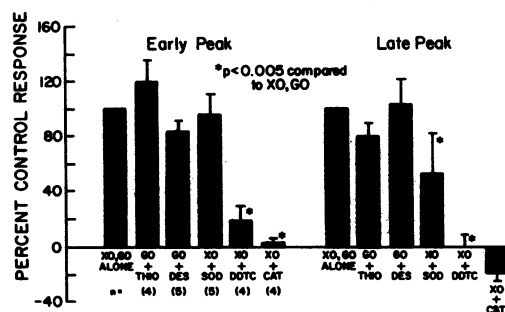


Figure 6. Summary of the effect of various inhibitors or stimulants of oxygen metabolism on the early and late peak ΔI_{sc} response to ROMs created by either the X/XO or the G/GO reaction. The responses in each study are normalized to the percent of control response.

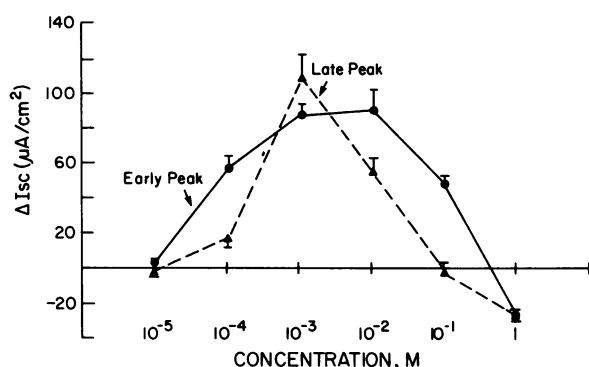


Figure 7. Concentration-response curve for the ΔI_{sc} response of rat colon to H_2O_2 added to the serosal bathing solution. Concentrations > 1 mM diminish the ΔI_{sc} as a result of a toxic effect of the oxidant (see Fig. 8).

whereas 5 mM mucosal deoxycholate significantly ($P < 0.05$, $n = 4$) increased mucosal bathing solution LDH to 403 ± 36 IU/liter. In contrast, exposure of rat colon for 1 h to 1 M serosal H_2O_2 completely destroyed the crypt epithelium (Fig. 8 C).

The effect of 1 mM serosal H_2O_2 on rat colonic electrolyte transport is shown in Table I, section A. H_2O_2 diminished mucosal-to-serosal fluxes of Na and Cl, stimulated serosal-to-mucosal fluxes of Cl, and significantly increased the I_{sc} and PD. As a result of these changes in unidirectional fluxes, there was a significant inhibition of J_{Na}^{net} and a reversal of J_{Cl}^{net} from absorption to secretion. These changes indicate that H_2O_2 was inhibiting neutral NaCl absorption and stimulating electrogenic Cl secretion in rat colon. This concentration of H_2O_2 had no effect on transepithelial electrical conductance (G).

In our previous study with X/XO (5), the ΔI_{sc} response was inhibited by cyclooxygenase inhibitors as well as by inhibitors of ENS activity. Accordingly, we determined the effect of the cyclooxygenase inhibitors INDO and PXM on the early and late peak ΔI_{sc} response to H_2O_2 , and contrasted these with the effects of the ENS inhibitors TTX, HEX, and high-dose ATR (Fig. 9). These inhibitors all reduced the peak 1 ΔI_{sc} by

$\sim 70\%$. INDO (10^{-6} M), but not PXM (10^{-6} M), inhibited the peak 2 response. Because PXM has a chemical structure that should be sensitive to oxidants, we repeated the experiments but preincubated with increasing concentrations of PXM. A concentration-response inhibition of both the early and late peak responses could be demonstrated, and at 10^{-4} M PXM both the early and late peaks were inhibited by $\sim 70\%$ ($P < 0.01$). This suggests that the lack of inhibition of the late peak with lower concentrations of PXM was due to the time-dependent destruction of the drug by the oxidant effect of H_2O_2 . Table I, section B, shows the effect of INDO on the electrolyte transport response to H_2O_2 . The inhibition of neutral NaCl absorption and the stimulation of electrogenic Cl secretion were diminished, but not abolished, by cyclooxygenase blockade.

The experiments shown in Fig. 9 suggest that either the ENS is releasing prostaglandins that alter electrolyte transport or, alternatively, that prostaglandins release neurotransmitters from the ENS and the neurotransmitters are the major agonists involved in the I_{sc} response to H_2O_2 . To determine which of these is more likely, we measured PGE_2 and PGI_2 (assayed as 6-keto- $PGF_{1\alpha}$) production in the experiments depicted in Fig. 9. As shown in Table II, H_2O_2 increased production of $PGE_2 \sim 7$ –8-fold and 6-keto- $PGF_{1\alpha}$ production 15–18-fold. The peak output by PGE_2 was in the first 15-min period after the addition of H_2O_2 , a time course compatible with the stimulation of I_{sc} . This stimulation of prostaglandin production was inhibited by pretreatment with INDO or PXM, but not by preincubation with the neural inhibitors TTX, HEX, and ATR. These results suggest that the prostaglandins produced by the ROMs were initiating Cl secretion in large part by activating the ENS.

To determine if PGE_2 or PGI_2 was a more important stimulant of the ENS, the I_{sc} response to serosal PGE_2 was compared with that of the Iloprost, a stable analogue of PGI_2 , in the presence and absence of TTX. In rat colon preincubated with 10^{-6} M INDO to inhibit endogenous PG production (Table III), the ΔI_{sc} to PGE_2 was not diminished by neural blockade with TTX, whereas the ΔI_{sc} to Iloprost was inhibited 94%. These data suggest that while PGE_2 has its stimulatory

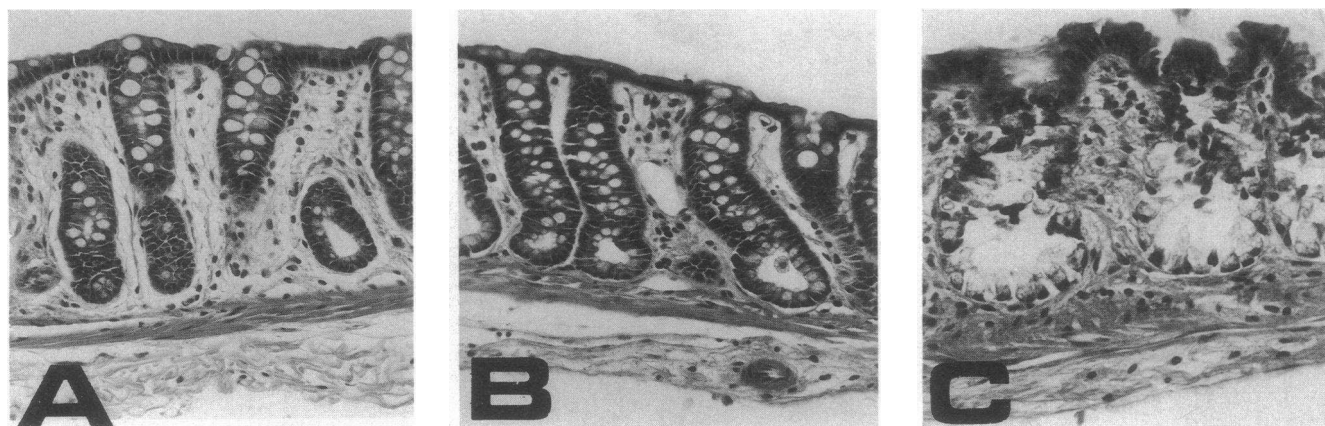


Figure 8. Histology of rat colon after incubation for 1 h in the Ussing chamber. A, control; B, 1 mM H_2O_2 ; C, 1 M H_2O_2 . Note that the submucosa, with its component of the ENS, is present in this preparation of stripped rat colon. There are only minor changes in the histology of tissues as the result of incubation with 1 mM H_2O_2 : slight dilatation of crypts and more cuboidal appearance of the surface colonocytes (B) as compared with control tissues (A). In contrast, after incubation with 1 M H_2O_2 (C), the crypt epithelium is completely destroyed.

Table I. Effect of 1.0 mM Hydrogen Peroxide on Rat Colonic Electrolyte Transport (A) and Response to 10^{-6} M Indomethacin (B) ($n = 8$)

	J_{ms}^{Na}	J_{sm}^{Na}	J_{net}^{Na}	J_{ms}^{Cl}	J_{sm}^{Cl}	J_{net}^{Cl}	Isc	J_{net}^R	PD	G
	$\mu eq/h\ per\ cm^2$								mV	mS/cm^2
A.										
(1) Basal	15.3±1.4	10.5±0.8	4.8±0.9	19.8±1.2	15.9±1.0	3.9±1.0	1.3±0.1	0.4±0.7	−1.8±0.3	20.1±1.7
(2) H ₂ O ₂	13.5±1.0	11.2±1.3	2.2±0.5	16.8±1.0	17.3±1.4	−0.6±1.0	3.1±0.3	0.3±0.8	−4.1±0.7	21.7±2.0
(2 vs. 1)	$P < 0.01$	NS	0.01	0.001	0.05	0.001	0.001	NS	0.001	NS
B.										
(3) INDO	17.3±1.1	10.6±0.7	6.7±0.8	21.9±1.2	15.3±0.9	6.6±1.1	0.5±0.1	0.5±1.3	−0.8±0.2	19.4±1.9
(4) INDO + H ₂ O ₂	15.4±1.3	10.3±0.8	5.1±0.9	19.4±0.9	15.6±0.9	3.8±1.0	1.6±0.3	0.3±0.8	−2.3±0.3	20.0±2.4
(4 vs. 3)	$P < 0.01$	NS	0.01	0.05	NS	0.01	0.001	NS	0.001	NS
(3 vs. 1)	$P < NS$	NS	NS	NS	NS	0.05	0.001	NS	0.001	NS
(4 vs. 2)	$P < NS$	NS	0.01	0.05	NS	0.01	0.01	NS	0.01	NS

Four tissues from each animal were studied simultaneously. Two pairs had INDO in the Ringer solution. After a basal measurement (mean of two 15-min flux periods) the agonist (H₂O₂) was added to the serosal solution, and after a 15-min equilibration period fluxes were measured for two additional periods and meaned. 2 vs. 1 = response to control colon to H₂O₂; 4 vs. 3 = response of the INDO-treated colon to the H₂O₂; 3 vs. 1 = response of the control colon to INDO; 4 vs. 2 = effect of INDO on the H₂O₂ response.

effect on electrogenic Cl secretion through colonocyte receptors, PGI₂ stimulates Cl secretion via receptors on the ENS.

Discussion

The focus of oxygen radicals and oxidants in biology has been on ROMs as injurious agents (1–3). In the gastrointestinal tract a role for ROMs has been postulated in various forms of enteritis (1, 2, 15) and gastric ulceration (16, 17). Ischemic injury has been the best studied model where ROMs created by an endogenous X/XO reaction and from the respiratory burst of neutrophils seem to be responsible for reperfusion injury, especially through the creation of OH[•] (18–21). Recent studies indicating that sulfasalazine and 5-aminosalicylic acid, two important drugs in the treatment of inflammatory bowel

disease, are also reactive oxygen scavengers (22–24) have furthered the idea that ROMs play a fundamental role in gastrointestinal inflammation. The present investigation, however, suggests that ROMs might have a physiologic, protective effect in the intestine, if one considers intestinal secretion and the resulting diarrhea a protective event that might wash offending microorganisms or antigens from the intestinal tract. Prostaglandin release might also be protective to the intestinal mucosa.

Our previous studies using X/XO demonstrated the ability of ROMs to stimulate electrogenic Cl secretion and inhibit neutral NaCl absorption (5). The experiments reported here indicate that H₂O₂ is the important ROM altering intestinal electrolyte transport. The inhibition of the Δ Isc response to X/XO by CAT, coupled with reproduction of the X/XO response with G/GO and exogenous H₂O₂, provide strong evidence that H₂O₂ is the active species altering transport. The possibility that OH[•], formed from H₂O₂ by the Haber-Weiss

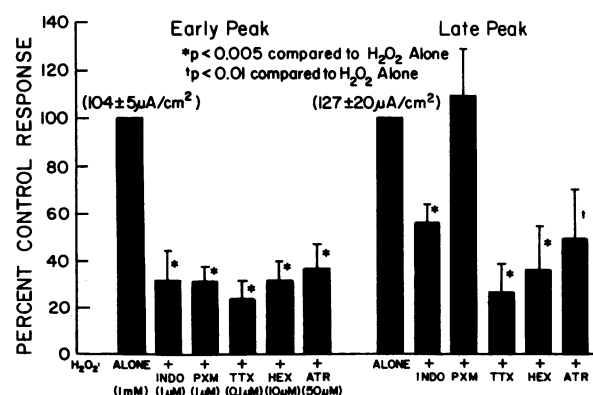


Figure 9. Summary of the effect of preincubation with cyclooxygenase inhibitors (INDO and PXM) and various neural inhibitors or antagonists (TTX, HEX, and ATR) on both the early and late peak Isc response to 1 mM H₂O₂. The data in each study are normalized to the percent of control response. When studied in higher concentrations, PXM was capable of inhibiting the late peak response as well.

Table II. Prostaglandin E₂ and Prostacyclin (Measured as 6-keto-PGF_{1α}) Production by Rat Colon in Response to Hydrogen Peroxide (10^{-3} M) and Effect of Cyclooxygenase and Neural Inhibitors ($n = 5$)

Agonist + inhibitors	PGE ₂	6-keto-PGF _{1α}
	ng/30 min per cm ²	
Control	1.2±0.3	4.1±0.9
H ₂ O ₂ alone	8.0±1.5*	73.6±6.4*
+TTX (10^{-7} M)	8.3±1.3*	62.0±4.5*
+HEX (10^{-5} M)	9.0±0.7*	75.7±12.1*
+ATR (5×10^{-5} M)	9.6±2.5*	75.1±15.5*
+INDO (10^{-6} M)	1.9±0.4‡	17.5±2.9‡
+PXM (10^{-6} M)	1.7±0.8‡	33.8±7.2‡

* $P < 0.005$ control vs. agonist or agonist + inhibitor; ‡ $P < 0.005$ agonist vs. agonist + inhibitor.

Table III. Effect of 10^{-7} M TTX on the ΔI_{sc} to Maximally Effective Concentrations of PGE₂ or the PGI₂ Analogue, Iloprost ($n = 6$)

Stimulus/inhibitor (concentration)	At 5 min	At 30 min
	$\mu A/cm^2$	
PGE ₂ (2.8×10^{-8} M)		
Alone	59±14.6	38±10.0
+TTX (10^{-7} M)	52±11.7	39±9.7
Iloprost (2.8×10^{-7} M)		
Alone	72±17.2	66±17.7
+TTX (10^{-7} M)	4±1.0 [‡]	7±2.6 [‡]

The concentrations of PGE₂ and Iloprost uses were those giving maximal responses in formal concentration-response studies. In these experiments tissues were preincubated for 20 min with 10^{-6} M indomethacin to inhibit endogenous prostaglandin production before stimulation with PGE₂ or Iloprost.

* $P < 0.002$.

reaction, was responsible for the changes in transport was effectively ruled out by the studies with DES and TH, agents that would prevent the formation of or decrease the concentration of OH[•].

The inhibition of the H₂O₂ effect on electrolyte transport by indomethacin and piroxicam indicates the important role that cyclooxygenase products play in the electrolyte transport effect of oxidants. H₂O₂ was a potent stimulus of PGE₂ and 6-keto-PGF_{1 α} production. In fact, 6-keto-PGF_{1 α} production by H₂O₂ was the highest of any agonist we have studied, including platelet activating factor, chemotactic peptide (FMLP), and anti-IgE (5). These results do not rule out the possibility that ROMs might also directly stimulate the enterocyte as suggested by Olson et al. (25) in cultured monolayers of T84 colon carcinoma cells.

There are several potential mechanisms whereby H₂O₂ might stimulate production of prostaglandins in the intestine. First, peroxides are important cofactors ("peroxide tone") for prostaglandin biosynthesis, as demonstrated by the exogenous addition of either H₂O₂ or lipid peroxides to tissues (26–28). However, there may be an important dose-response effect in that high levels of lipoperoxides, perhaps through OH[•] formation, will inactivate prostaglandin and prostacyclin synthases (28). The ability of low concentrations of hydroperoxides to activate PGH synthase is not just a laboratory phenomenon; it can be shown that activation of phagocytic leukocytes will cause hydroperoxide release that is sufficient to stimulate prostaglandin production from cocultured lymphoma cells (29). These experiments suggest a second possible mechanism for peroxide stimulation of prostaglandin production: the activation of inflammatory or mesenchymal cells. H₂O₂, myeloperoxidase, and eosinophilic peroxidase will stimulate PGE₂ and/or PGI₂ production in mast cells (30–33), fibroblasts (34, 35), renal glomerular mesangial cells (36), and cultured endothelial cells (37, 38). The mechanism of this stimulation is unclear, but in the endothelial cells it seems to involve gating of Ca²⁺ across the cell membrane either as the result of a receptor-mediated event or secondary to lipid peroxidation of the cell

membrane, making it more permeable to Ca²⁺ with subsequent liberation of AA from membrane stores (38).

Increased prostaglandins in the lamina propria of the intestine might initiate changes in enterocyte electrolyte transport by directly stimulating colonocyte adenylate cyclase or, as has recently been suggested by Goerg et al. (6) and Diener et al. (7), could activate receptors on the ENS with resulting release of secretory neurotransmitters. We have confirmed their studies that indicate PGI₂ is an important potential neuromodulator in the rat colon. The effect of Iloprost, but not PGE₂, on electrolyte transport was essentially abolished by pretreatment with TTX. Furthermore, the high concentrations of PGI₂ produced by H₂O₂ and the inhibition of the H₂O₂ effect with TTX, HEX, and high-dose ATR all implicate PGI₂ as an important intermediate in H₂O₂-stimulated Cl secretion in rat colon.

An issue for consideration is whether the prostaglandin synthesis and altered electrolyte transport we report here, artificially created in an in vitro system, might occur in vivo in either normal or inflamed intestine. Studies of isolated neutrophils clearly demonstrate that 10^5 – 10^6 neutrophils can generate concentrations of H₂O₂ in the range of 10–100 μ M/ml (4, 39). This is certainly within the dose-response range for the changes in electrolyte transport demonstrated here. In the normal mammalian colon, phagocytes (eosinophils, neutrophils, and macrophages) are present in numbers approaching 10^6 cells/g of tissue (40). In inflammatory diseases of intestine, the concentration of these cells would increase even more. In our studies H₂O₂ concentrations < 1 mM caused very little damage to the intestinal mucosa; it would appear that the intestinal oxidase defense mechanisms are sufficient to handle this level of oxidant stress. At 1 mM H₂O₂ we could not detect release of LDH or changes in paracellular permeability, a finding similar to that reported by Welsh et al. in cultured Madin-Darby Canine Kidney cells (41), and there was no effect on epithelial histology. At concentrations > 1 mM, we and others (41, 42) find irreversible permeability changes in the epithelium and, at still higher concentrations, severe damage to the epithelium (see Fig. 8).

In summary, this investigation demonstrates H₂O₂ to be a potent agonist for electrogenic Cl secretion and for inhibition of neutral NaCl absorption in the rat colon. This process is mediated by release of prostaglandins from the lamina propria. PGI₂ is a prominent prostaglandin elaborated in response to H₂O₂ and it appears to have its major effect on electrolyte transport by activating the ENS. The specific neurotransmitters acting at the level of the colonocyte remain to be determined. Intestinal secretion that may result in diarrhea can be viewed as a protective mechanism in the intestine, as may the release of prostaglandin. Furthermore, recently it has been suggested that ROMs might be important stimulants of cell division in the damaged intestine, and thus might play an important initiating role in epithelial repair (43). Thus ROMs, though clearly damaging agents at high concentration, also appear to play protective and reparative roles in the intestine.

Acknowledgments

We wish to thank Mrs. Patricia Brown and Ms. Marie A. Jost for excellent secretarial assistance, Dr. Frederic Askin for photomicro-

graphs of colonic tissue, and Dr. Myron Cohen and Dr. Irwin Fridovich for helpful discussion of oxygen radical and oxidant biochemistry.

The work was supported by grants DK-15350 and DK-34987 from the National Institutes of Health.

References

1. Granger, D. N., L. A. Hernandez, and M. B. Grisham. 1986. Reactive oxygen metabolites: mediators of cell injury in the digestive system. *Viewpoints on Digestive Diseases*. 18:13-16.
2. Cross, C. E., B. Halliwell, E. T. Borish, W. A. Pryor, B. N. Ames, R. L. Saul, J. M. McCord, and D. Harman. 1987. Oxygen radicals and human disease. *Ann. Intern. Med.* 107:526-545.
3. Klebanoff, S. J. 1988. Phagocytic cells: products of oxygen metabolism. In *Inflammation: Basic Principles and Clinical Correlates*. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, Ltd., New York. 391-444.
4. Test, S. T., and S. J. Weiss. 1984. Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. *J. Biol. Chem.* 259:399-405.
5. Bern, M. J., C. W. Sturbaum, S. S. Karayalcin, H. M. Berschneider, J. T. Wachsmann, and D. W. Powell. 1989. Immune system control of rat and rabbit colonic electrolyte transport: role of prostaglandins and enteric nervous system. *J. Clin. Invest.* 83:1810-1820.
6. Goerg, K. J., M. Roux, R. Wanitschke, and K. H. Meyer zum Buschenfelde. 1986. Die Bedeutung des Meissnerschen Plexus für die sekretorische Funktion der Colonschleimhaut. *Z. Gastroenterol.* 24:70-78.
7. Diener, M., R. J. Bridges, S. F. Knobloch, and W. Rummel. 1988. Neuronally mediated and direct effects of prostaglandins on ion transport in rat colon descendens. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 337:74-78.
8. Binder, H. J., and C. L. Rawlins. 1973. Electrolyte transport across isolated large intestinal mucosa. *Am. J. Physiol.* 225:1232-1239.
9. Granstrom, E., and H. Kindahl. 1978. Radioimmunoassay of prostaglandins and thromboxanes. *Adv. Prostaglandin Thromboxane Res.* 5:119-210.
10. Lawson, L. D., and D. W. Powell. 1987. Bradykinin-stimulated eicosanoid synthesis and secretion by rabbit ileal components. *Am. J. Physiol.* 252 (Gastrointest. Liver Physiol. 15):G783-G790.
11. Wroblewski, F., and J. S. LaDue. 1955. Lactate dehydrogenase activity in blood. *Proc. Soc. Exp. Biol. Med.* 90:210-213.
12. Matalon, S., J. S. Beckman, M. E. Duffey, and B. A. Freeman. 1989. Oxidant inhibition of epithelial active sodium transport. *Free Radical Biol. & Med.* 6:557-564.
13. Pick, E., and D. Mizel. 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay. *J. Immunol. Methods*. 46:211-226.
14. Fridovich, I. 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.* 16:4053-4057.
15. Granger, D. N., M. B. Grisham, B. J. Zimmerman, and C. Von Ritter. 1988. Reactive oxygen metabolites as mediators of cell injury in the intestine. In *Inflammatory Bowel Disease: Current Status and Future Approach*. R. P. MacDermott, editor. Elsevier Science Publishers B. V., Amsterdam. 255-260.
16. Smith, S. M., L. Holm-Rutili, M. A. Perry, M. B. Grisham, K. E. Arfors, D. N. Granger, and P. R. Kvietys. 1987. Role of neutrophils in hemorrhagic shock-induced gastric mucosal injury in the rat. *Gastroenterology*. 93:466-471.
17. Olson, C. E. 1988. Glutathione modulates toxic oxygen metabolite injury of canine chief cell monolayers in primary culture. *Am. J. Physiol.* 254 (Gastrointest. Liver Physiol. 17):G49-G56.
18. Parks, D. A., G. B. Bulkley, D. N. Granger, S. R. Hamilton, and J. M. McCord. 1982. Ischemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology*. 82:9-15.
19. Grisham, M. B., L. A. Hernandez, and D. N. Granger. 1986. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. *Am. J. Physiol.* 251 (Gastrointest. Liver Physiol. 14):G567-G574.
20. Hernandez, L. A., M. B. Grisham, B. Twohig, K. E. Arfors, J. M. Harlan, and D. N. Granger. 1987. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am. J. Physiol.* 253 (Heart Circ. Physiol. 22):H699-H703.
21. Hernandez, L. A., M. B. Grisham, and D. N. Granger. 1987. A role for iron in oxidant-mediated ischemic injury to intestinal microvasculature. *Am. J. Physiol.* 253 (Gastrointest. Liver Physiol. 16):G49-G53.
22. Miyachi, Y., A. Yoshioka, S. Imamura, and Y. Niwa. 1987. Effect of sulphasalazine and its metabolites on the generation of reactive oxygen species. *Gut*. 28:190-195.
23. Craven, P. A., J. Pfanstiel, R. Saito, and F. R. DeRubertis. 1987. Actions of sulphasalazine and 5-aminosalicylic acid as reactive oxygen scavengers in the suppression of bile acid-induced increases in colonic epithelial cell loss and proliferative activity. *Gastroenterology*. 92:1998-2008.
24. Kvietys, P. R., S. M. Smith, M. B. Grisham, and E. A. Mancini. 1988. 5-Aminosalicylic acid protects against ischemia/reperfusion-induced gastric bleeding in the rat. *Gastroenterology*. 94:733-738.
25. Olson, C. E., P. Geshelin, and A. H. Soll. 1987. Hydrogen peroxide increases short circuit current in a cultured colon crypt cell line. *Fed. Proc.* 46:547. (Abstr.)
26. Hemler, M. E., H. W. Cook, and W. E. M. Lands. 1979. Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch. Biochem. Biophys.* 193:340-345.
27. Hemler, M. E., and W. E. M. Lands. 1980. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* 255:6253-6261.
28. Weiss, S. J., J. Turk, and P. Needleman. 1979. A mechanism for the hydroperoxide-mediated inactivation of prostacyclin synthetase. *Blood*. 53:1191-1196.
29. Marshall, P. J., and W. E. M. Lands. 1986. *In vitro* formation of activators for prostaglandin synthesis by neutrophils and macrophages from humans and guinea pigs. *J. Lab. Clin. Med.* 108:525-534.
30. C. Stendahl, L. Molin, and M. Lindroth. 1983. Granulocyte-mediated release of histamine from mast cells: effect of myeloperoxidase and its inhibition by antiinflammatory sulfone compounds. *Int. Arch. Allergy Appl. Immunol.* 70:277-284.
31. Coble, B. I., M. Lindroth, L. Molin, and O. Stendahl. 1984. Histamine release from mast cells during phagocytosis and interaction with activated neutrophils. *Int. Arch. Allergy Appl. Immunol.* 75:32-37.
32. Henderson, W. R., E. Y. Chi, and S. J. Klebanoff. 1980. Eosinophil peroxidase-induced mast cell secretion. *J. Exp. Med.* 152:265-279.
33. Chi, E. Y., and W. R. Henderson. 1984. Ultrastructure of mast cell degranulation induced by eosinophil peroxidase: use of diamino-benzidine cytochemistry by scanning electron microscopy. *J. Histochem. Cytochem.* 32:337-341.
34. Taylor, L., M. J. Menconi, and P. Polgar. 1983. The participation of hydroperoxides and oxygen radicals in the control of prostaglandin synthesis. *J. Biol. Chem.* 258:6855-6857.
35. Berschneider, H. M., and D. W. Powell. 1989. Fibroblasts mediate intestinal secretory response to bradykinin, H_2O_2 and serotonin. *Gastroenterology*. 96:A41. (Abstr.)
36. Baud, L., J. Hagege, J. Sraer, E. Rondeau, J. Perez, and R. Ardaillou. 1983. Reactive oxygen production by cultured rat glomerular mesangial cells during phagocytosis is associated with stimulation of lipoxygenase activity. *J. Exp. Med.* 158:1836-1852.

37. Harlan, J. M., and K. S. Callahan. 1984. Role of hydrogen peroxide in the neutrophil-mediated release of prostacyclin from cultured endothelial cells. *J. Clin. Invest.* 74:442-448.
38. Lewis, M. S., R. E. Whatley, P. Cain, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1988. Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. *J. Clin. Invest.* 82:2045-2055.
39. Nathan, C. F. 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J. Clin. Invest.* 80:1550-1560.
40. Beeken, W., I. Northwood, C. Beliveau, and D. Gump. 1987. Phagocytes in cell suspensions of human colon mucosa. *Gut.* 28:976-980.
41. Welsh, M. J., D. M. Shasby, and R. M. Husted. 1985. Oxidants increase paracellular permeability in a cultured epithelial cell line. *J. Clin. Invest.* 76:1155-1168.
42. Shasby, D. M., M. Winter, and S. S. Shasby. 1988. Oxidants and conductance of cultured epithelial cell monolayers: inositol phospholipid hydrolysis. *Am. J. Physiol.* 255 (Cell Physiol. 24): C781-C788.
43. Craven, P. A., J. Pfanstiel, and F. R. DeRubertis. 1986. Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. *J. Clin. Invest.* 77:850-859.