

Development of Ischemia/Reperfusion Tolerance in the Rat Small Intestine

An Epithelium-independent Event

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Abstract

In stable organ systems, such as the heart and kidneys, an oxidant stress induces an increase in endogenous antioxidant systems resulting in an increased resistance of the tissue to a subsequent oxidant challenge. The development of this oxidant tolerance requires 1.5–6 d. The aim of the present study was to determine whether oxidant tolerance can be induced in the small intestinal mucosa, a labile system whose epithelium turns over every 2–3 d. Ischemia/reperfusion-induced epithelial barrier dysfunction of the small intestinal mucosa was monitored in Sprague-Dawley rats whose intestines had been exposed to an ischemic insult 1, 24, or 72 h previously. At 24 h, but not 1 or 72 h after the initial ischemic insult, the mucosa was more resistant to ischemia/reperfusion-induced barrier dysfunction. The antioxidant status of the mucosa was enhanced at 24 h, but not at 1 or 72 h after the initial ischemic insult. This adaptation appears to be specific for oxidants, since an initial ischemic insult imposed 24 h earlier also protected against H_2O_2 -induced, but not acid- or ethanol-induced, barrier dysfunction. Further studies indicated that the increase in antioxidant status of the mucosa observed 24 h after the initial ischemic insult was a result of adaptational changes in the lamina propria, rather than the epithelium. In vitro studies with isolated epithelial cells also indicated that epithelial cells do not develop oxidant tolerance. We conclude that the development of oxidant tolerance in the small intestinal mucosa does not involve an active participation of the epithelial lining. (*J. Clin. Invest.* 1994. 94:1910–1918.) Key words: glutathione peroxidase • catalase • superoxide dismutase • Caco-2 cells

Introduction

The reactive oxygen metabolites (ROMs)¹, superoxide and/or hydrogen peroxide, are generated as a consequence of normal

cellular aerobic metabolism (1, 2). Since these ROMs can participate in reactions which result in cytotoxicity, detoxification of these ROMs is a prerequisite for successful aerobic life. Mammalian cells contain SOD, which decomposes superoxide, and glutathione peroxidase and catalase, which decompose hydrogen peroxide. These enzymes constitute the major antioxidant systems responsible for preventing intracellular accumulation of cytotoxic levels of superoxide and hydrogen peroxide during normal aerobic metabolism (1). One pathologic event that is believed to disturb this oxidant/antioxidant balance is reperfusion of previously ischemic tissues (2, 3). Generally, it is believed that the burst of ROMs generated upon reperfusion overwhelms the detoxifying capacity of the enzymatic antioxidants and initiates a cascade of events that eventually leads to tissue injury.

Exogenous administration of SOD and/or catalase has been shown to offer protection against ischemia/reperfusion-induced tissue injury in a variety of organ systems (2–4). More recently, efforts have been directed toward enhancing the endogenous antioxidant status of tissues as a means of providing protection from ischemia/reperfusion (I/R) injury. The imposition of a mild oxidant stress in the heart (endotoxin) (5) or the kidney (ischemia/reperfusion) (6) results in the development of I/R tolerance (i.e., the tissue becomes more resistant to a subsequent I/R insult). The protective effect of the initial oxidant stress requires 1.5–6 d to develop, and is associated with enhanced activities of tissue antioxidant enzymes. The brain can also develop ischemic tolerance 1 d after mild ischemic episodes (7).

The organs in which I/R tolerance has been induced represent permanent or stable systems, where little or no cell proliferation occurs after full differentiation. There is no information available on whether a similar phenomenon exists in the more labile organ systems containing tissue with a high cell turnover rate (e.g., the gastrointestinal tract). Thus, the major objective of the present study was to assess whether I/R tolerance can be induced in the small intestine and to define the role of the epithelium in this phenomenon. The results of the present study indicate that I/R tolerance can be induced in the small intestine, and that it is associated with an increase in mucosal antioxidant status. Furthermore, we provide evidence that the intestinal epithelial lining (labile tissue) does not play an active role in the development of I/R tolerance in the intestine.

Methods

In vivo and in vitro approaches were used to systematically characterize the development of I/R tolerance in the small intestine. In vivo studies were designed to determine the time course of the development of I/R tolerance, and whether this phenomenon was associated with an enhanced antioxidant status of the mucosa (i.e., increased activity of superoxide dismutase, catalase, glutathione peroxidase, and its substrate gluta-

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1. Abbreviations used in this paper: CAT, catalase; GSHPX, glutathione peroxidase; I/R, ischemia/reperfusion; LDH, lactic dehydrogenase; ROM, reactive oxygen metabolite; SMA, superior mesenteric artery.

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thione). Furthermore, the *in vivo* model was also used to test the specificity of I/R tolerance for an oxidative stress and to assess whether the improved antioxidant status of the mucosa results from adaptational changes in the epithelial lining or the lamina propria. Finally, *in vitro* studies were undertaken to determine whether oxidant tolerance could be induced in epithelial cells in culture.

In vivo studies

Animal preparation. The experimental preparation used in this study is similar to that previously described (8–10). In brief, male Sprague-Dawley rats (~350 g), previously fasted overnight, were anesthetized with 5-sec-butyl-5-ethyl-2-thiobarbituric acid (Inactin; Research Biochemicals International, Natick, MA; 120 mg/kg, *i.p.*). A tracheotomy was performed, and polyethylene tubing (PE-240) was inserted into the trachea to facilitate breathing. A femoral artery was cannulated for continuous monitoring of systemic arterial pressure. A femoral vein was cannulated for the periodic administration of fluid (5% Ficoll; 70,000 mol wt) to maintain blood volume. A carotid artery was cannulated for obtaining blood samples. After an abdominal incision, a suture (silk 5.0) was passed around the superior mesenteric artery (SMA) and exteriorized through the laparotomy. A proximal segment (10 cm in length) of jejunum (~2 cm distal to the ligament of Treitz) was isolated and cannulated at both ends with soft polyethylene tubing. The proximal catheter was connected to a peristaltic pump (Minipuls; Gilson Medical Electronics, Inc., Middleton, WI) and the intestine perfused with normal saline (pH, ~6.0–6.5) at 1.0 ml/min. The abdomen was irrigated with normal saline and covered with plastic wrap to prevent evaporative fluid loss. The renal blood vessels were ligated to prevent loss of the low molecular weight tracer used to quantitate intestinal mucosal integrity (see below). Body temperature was maintained at 36–37°C with a heating pad and lamp.

Quantitative assessment of mucosal epithelial integrity. After surgery, 100 μ Ci of ^{51}Cr -labeled EDTA was administered as a bolus through the femoral venous catheter. Samples of intestinal perfusate were collected from the effluent jejunal cannula at 5-min intervals to monitor ^{51}Cr -EDTA clearance. Once steady-state clearances were obtained (60 min), an additional 40-min perfusion with normal saline (pH, ~6.0–6.5) served as a control. After the control period, the SMA was occluded by providing a standardized tension on the suture around the SMA. After a 20-min ischemic period, the tension on the suture was released, and the intestine was allowed to reperfuse for an additional 2 h (Fig. 1). Blood samples (300 μ l) were taken during the control period, during the ischemic period, and every 40 min during the subsequent reperfusion period. At the end of the experiment, the animal was euthanized, and the segment of jejunum was removed and weighed. ^{51}Cr activity in the perfusate (effluent) and plasma samples were assessed using a gamma counter. The ^{51}Cr -EDTA clearance was calculated using the following formula (8–10):

$$\frac{\text{perfusate (cpm/ml)} \times \text{perfusion rate (ml/min)} \times 100}{\text{plasma (cpm/ml)} \times \text{tissue weight (g)}}$$

^{51}Cr -EDTA clearance (expressed as ml/min per 100 g tissue weight) is a commonly used index of mucosal epithelial integrity (11).

Histological assessment of mucosal injury. The morphologic appearance of the jejunal mucosa was analyzed by light microscopy. Samples of jejunum were immersed in 2.0% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer for ≥ 6 h, dehydrated in graded ethanol up to 95% ethanol, and embedded in glycol methacrylate (JB-4; Polysciences Inc., Warrington, PA). The semithin sections (2.5 μ m) were stained with hematoxylin and eosin and were photographed with an Olympus BH-2 microscope. Mucosal epithelial injury was quantitated by assessing the percent of villi exhibiting a discontinuous epithelium (i.e., “breaks” in the epithelial barrier) (8, 9). Three animals per group were biopsied with 1,500–2,000 villi being examined per group. To prevent observer bias, all mucosal samples were coded on collection and the codes were not made available to the investigator evaluating epithelial integrity.

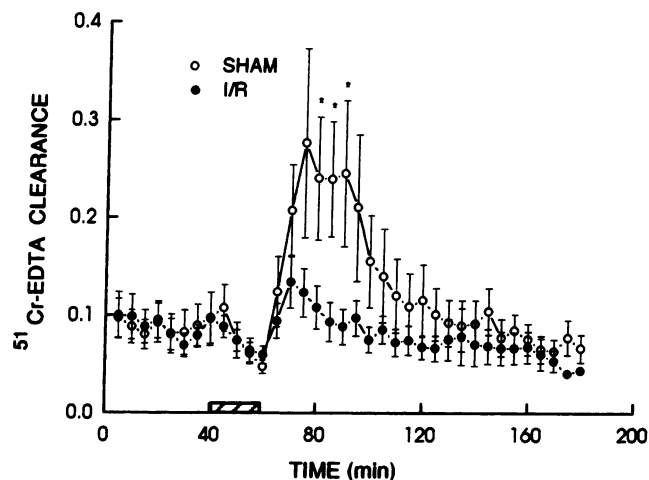


Figure 1. Effects of a 20-min period of intestinal ischemia (hatched bar) on ^{51}Cr -EDTA clearance across the jejunal mucosal epithelium in animals whose SMA was occluded for 15 min (I/R) and animals whose SMA was merely massaged (SHAM) 24 h earlier. All values ($\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$) are mean \pm SEM from nine animals in each group. As the omnibus ANOVA revealed significant interaction between time and treatment (sham vs I/R groups), posthoc comparisons (Duncan's test) for each time point were performed. * $P < 0.05$ compared to corresponding time point in I/R group.

Separation of epithelium from the lamina propria. Epithelial cells were separated from the lamina propria using the method developed by Weiser (12). Jejunal segments were harvested, and the lumen was rinsed with 0.154 mM NaCl containing 1mM DTT and then filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM Na citrate, 8 mM KH_2PO_4 , and 5.6 mM Na_2PO_4 at pH 7.3). After 15 min, solution A was removed and the segment was filled with solution B ($\text{Mg}^{2+}/\text{Ca}^{2+}$ -free PBS, 1.5 mM EDTA, and 0.5 mM DTT). After 8 min, solution B was collected and the segment rinsed and refilled with solution B. After 12 min, the collection procedure was repeated and the segment refilled with solution B. After 37 additional min, the final collection was obtained. Histological examination of the mucosa indicated that the 8-min fraction contained villus tip cells, the 12-min fraction contained mid-villus cells, and the 37-min fraction contained crypt cells.

Mucosal antioxidant status. Jejunal segments were removed to ice-cold saline for removal of contents and the segments opened along their longitudinal axis. Mucosa was scraped from the muscle layers and weighed. In some experiments, the epithelial cells were separated from the lamina propria (as described above), centrifuged at 900 g for 5 min, and the supernatant was removed. The naked lamina propria was then scraped from the muscle layer. The scraped mucosa, epithelial cells, and scraped lamina propria were stored at -70°C until they were analyzed for SOD, catalase (CAT), glutathione peroxidase (GSHPX) activities, or GSH content. For analysis, the tissues or cells were thawed and homogenized as a 10% wt/vol mixture in a 20-mM phosphate buffer (pH 7.4), centrifuged at 8,000 g for 20 min at 4°C (RC-5B; Beckman Instruments, Inc., Fullerton, CA). The resulting supernatant was analyzed for SOD, catalase, and GSHPX activities, and for GSH content.

SOD activity was analyzed by the method of Crapo (13), in which inhibition of the rate of reduction of cytochrome C by superoxide radicals is used as an indirect measurement of SOD activity. SOD activity measured in this assay represents total tissue SOD activity, and is expressed as 1 U = SOD activity required to inhibit cytochrome C reduction by 50%. Catalase activity was determined by the method of Aebi (14), where the disappearance of hydrogen peroxide was monitored at 240 nm as a measure of catalase activity. 1 U of catalase activity is defined as 1 μ mol of H_2O_2 degraded/minute per mg protein. GSHPX

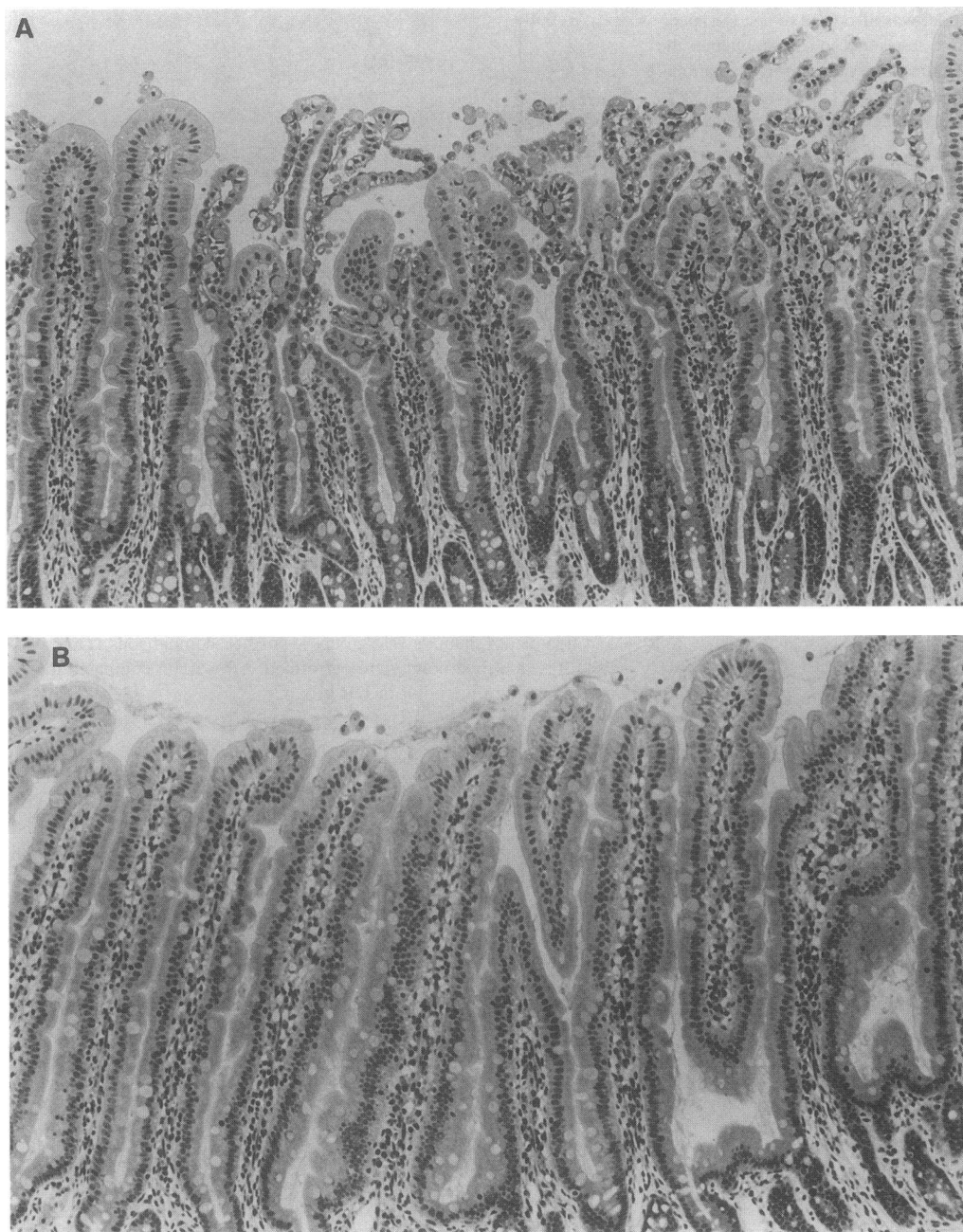


Figure 2. Rat jejunal mucosa after a 20-min period of intestinal ischemia (80-min time point in Fig. 1) from animals whose SMA was occluded for 15 min (**B**) and animals whose SMA was merely massaged (**A**) 24 h earlier. I/R injury was generally characterized by sheets of epithelium detaching from the lamina propria. The I/R group had $13.3 \pm 2.7\%$ of their villi damaged by the second ischemic insult, whereas the sham group had $46.6 \pm 1.1\%$ of their villi damaged.

activity was measured by the method of Tappel (15). This method measures the reduction of glutathione [oxidized] by spectrophotometrically following a coupled reaction involving the oxidation of NADPH where the disappearance of NADPH is monitored at 340 nm. 1 U of GSHPX activity is defined as 1 μ mol of NADPH converted to NADP/min per mg protein. GSH content was measured by the method of Owens and Belcher (16), in which GSH content is determined spectrophotometrically following incubation of tissue lysates with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and measuring absorbance at 412 nm. This method determines total tissue thiol content; however, HPLC analysis in our laboratory indicates that $> 97\%$ of the determined thiol content is GSH. Total tissue GSH content is expressed as micrograms per milligram of protein. All biochemical analyses were standardized per milligram of protein content, as determined by the method of Bradford (17) using gamma globulin as a standard.

Experimental protocols. The animals were anesthetized with Ethrane/Telazol (14 mg/kg, i.m.) and, after a midline laparotomy, the SMA was isolated. In the experimental (I/R) group, the SMA was occluded for 15 min with a bulldog vascular clamp (Fire Science Tools, Inc., Foster City, CA). After the ischemic insult, the vascular clamp was removed, the laparotomy was closed with staples, and the animals were allowed to recover (water ad lib, no food). In the control (sham) group, the animals were treated in an identical manner to those in the I/R group, except that the SMA was gently massaged, not occluded. 24 h later, animals from each group were anesthetized with inactin and instrumented for monitoring I/R-induced changes in mucosal integrity using of ^{51}Cr -EDTA clearance as described above. The second acute surgery was timed such that the induction of a 20-min period of SMA occlusion occurred 24 h after the initial 15-min occlusion or massaging of the SMA. The animals tolerated the surgery well and remained fairly

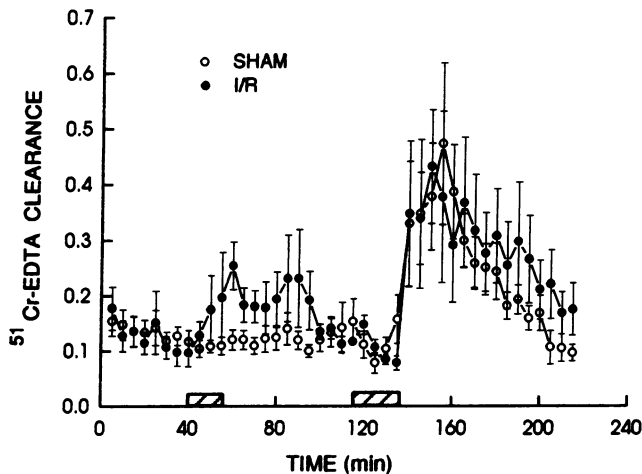


Figure 3. The effects of a 20-min period of intestinal ischemia (hatched bar) on ^{51}Cr -EDTA clearance across the jejunal mucosal epithelium in animals whose SMA was occluded for 15 min (I/R) and animals whose SMA was merely massaged (SHAM) 1 h earlier. All values ($\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$) are expressed as the mean \pm SEM from eight animals in each group.

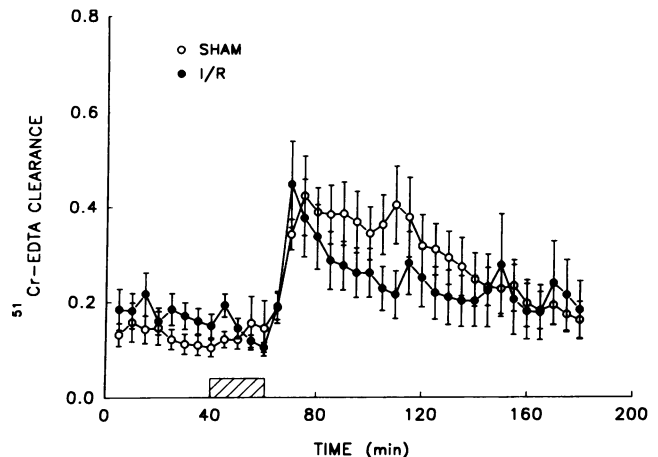


Figure 4. The effects of a 20-min period of intestinal ischemia (hatched bar) on ^{51}Cr -EDTA clearance across the jejunal mucosal epithelium in animals whose SMA was occluded for 15 min (I/R) and animals whose SMA was merely massaged (SHAM) 72 h earlier. All values ($\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$) are expressed as the mean \pm SEM from eight animals in each group.

stable throughout data collection. For example, mean systemic arterial pressure in the sham-operated animals was 108 ± 3 , 115 ± 10 , 102 ± 9 , and 90 ± 9 mmHg at the 40-, 80-, 120-, and 160-min time points in Fig. 1, respectively. In the animals exposed to I/R 24 h earlier, mean systemic arterial pressure was 102 ± 12 , 111 ± 12 , 100 ± 13 , and 91 ± 7 mmHg at the 40-, 80-, 120-, and 160-min time points in Fig. 1, respectively.

The rat intestine is a labile organ system whose epithelium is completely replaced within 2–3 d (18). Thus, we assessed whether I/R tolerance can be demonstrated in the intestine 72 h after the initial I/R insult. The same protocol described above was used to impose the initial I/R insult, except that the animals were allowed to recover for 72 h (food and water ad lib for the first 48 h, water ad lib and no food for the last 24 h). Subsequently, the animals were instrumented for monitoring mucosal epithelial integrity using ^{51}Cr -EDTA clearance during the second I/R insult as described above.

Previous studies in the heart indicate that I/R tolerance can develop within minutes (19). Thus, we assessed whether I/R tolerance can be demonstrated in the intestine 1 h after the initial I/R insult. In these experiments, rats were fasted for 24 h (water ad lib) and anesthetized with Inactin. The animals were instrumented for monitoring I/R-induced changes in mucosal integrity using ^{51}Cr -EDTA clearance as described above. After an initial 40-min control period, the SMA was occluded by providing a standardized tension on the suture around the SMA. After a 15-min I/R period, the suture was released, and the intestine was reperused for 1 h. Subsequently, the SMA was occluded a second time by placing tension on the suture for 20 min. After the second I/R insult, the intestine was allowed to reperuse. As a control (sham), some animals were not exposed to the initial I/R insult.

In another series of experiments, sham and I/R animals were prepared as described above for ^{51}Cr -EDTA studies. Jejunal mucosa was harvested at 1, 24, or 72 h after the initial I/R insult for assessment of mucosal antioxidant status as described above.

The above experiments indicated that a previous I/R insult largely prevented the rise in ^{51}Cr -EDTA clearance induced by a subsequent I/R challenge imposed 24 h later. No protective effect was noted if the hiatus between I/R insults was 1 or 72 h. Similarly, the antioxidant status of the mucosa was enhanced at 24 h, but not at 1 or 72 h, after the initial insult. Thus, subsequent experiments were designed to further characterize the I/R tolerance that was manifest at 24 h after the initial I/R insult.

A group of I/R and sham-operated animals were prepared for histologic verification of the development of I/R tolerance 24 h after the initial I/R insult. Tissue samples were obtained 20 min after reperfusion (80-min time point in Fig. 1), and mucosal integrity was evaluated histologically.

In another series of experiments, we tested the specificity of this I/R tolerance. Animals that had been exposed to intestinal ischemia 24 h earlier were instrumented for measurement of ^{51}Cr -EDTA clearance as described above. However, instead of imposing a second I/R insult, the lumen of the jejunum was perfused with either H_2O_2 (5 mM), HCl (5 mM), or ethanol (15%).

To assess whether the epithelial cells lining the villi played an active role in the development of I/R tolerance, the following two series of experiments were performed. Sham and I/R animals were prepared, and 24 h after the initial insult, the epithelial cells were separated from the lamina propria as described above. Both the isolated epithelial cells and the lamina propria were biochemically evaluated for changes in their antioxidant status. In another group of sham and I/R animals, epithelial cells were harvested from the villus tips 24 h after the initial I/R insult, and their susceptibility to oxidant stress was evaluated. The isolated epithelial cells were exposed to H_2O_2 (5 mM) for 10–20 min, and their viability was assessed by endogenous lactic dehydrogenase (LDH) release. LDH release was expressed as the percent of maximal LDH released from cells lysed with 0.05% Triton X-100, as described by Bergmeyer et al. (20).

In vitro studies

Since the viability of rat enterocytes decreases within minutes after isolation from the villi (21), long-term (hours) experiments to study the development of oxidant tolerance were not feasible. Thus, we used the human carcinoma cell line, Caco-2 (American Type Culture Collection, Rockville, MD), to study this phenomenon. Caco-2 cells are malignant, not normal, cells, and they are colonic, not small intestinal, cells. Nonetheless, we chose to use this cell line because it has been well characterized, and it exhibits morphological (establishment of polarity, microvilli, etc.) and functional (brush-border enzymes, ionic transport, etc.) characteristics of mature small intestinal enterocytes (22).

Cell culture. The Caco-2 cells were grown in high glucose DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 20% FBS, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. The cell cultures were

Table I. Jejunal Mucosal Antioxidant Status 1, 24, and 72 h after an I/R Insult

	1 h		24 h		72 h	
	SHAM	I/R	SHAM	I/R	SHAM	I/R
SOD	5.0±0.1	4.9±0.4	4.9±0.4	5.2±0.7	5.2±0.1	5.1±0.3
CAT	3.1±0.2	3.1±0.2	3.1±0.2	4.1±0.2*	3.1±0.5	3.4±0.4
GSHPX	38±2	42±2	44±2	54±4*	38±2	39±4
GSH	2.9±0.3	2.9±0.2	3.3±0.2	5.0±0.9*	3.8±0.2	6.7±0.7*

I/R, animals whose superior mesenteric artery was occluded for 15 min at 1, 24, or 72 h before biopsy; SHAM, animals whose superior mesenteric artery was only massaged at 1, 24, or 72 h before biopsy. Enzyme activities are expressed in milliunits per milligram, while GSH content is expressed in micrograms per milligram of protein. Values represent mean±SEM of assays on mucosal samples obtained from six to eight rats.

* $P < 0.05$ compared to SHAM. (Student's t test).

maintained in a humidified atmosphere with 5% CO₂ at 37°C, and were expanded by brief trypsinization (0.25% trypsin in Ca²⁺/Mg²⁺-free BSS containing 0.02% EDTA). The epithelial cells were seeded into collagen-coated (collagen IV; Sigma Chemical Co., St. Louis, MO) 48-well plates, and were used in experiments at 7 d after confluence (8).

Experimental protocol. To assess whether oxidant tolerance could be induced in Caco-2 cells, confluent monolayers were exposed to 0.4–1.6 mM H₂O₂ for 1 h. Subsequently, the monolayers were washed with HBSS, DME containing ⁵¹Cr was added, and the cells were incubated at standard conditions for 24 h. The monolayers were washed with HBSS to remove media containing ⁵¹Cr, and incubated with H₂O₂ (1.2–7.2 mM) for 4 h. Subsequently, an aliquot (450 µl) of the supernatant (500 µl) was obtained, and the remainder was aspirated. Then, 500 µl of PBS was added to the wells, the monolayers were washed gently, and an aliquot (450 µl) of the wash was obtained. Finally, 500 µl of 2N NaOH was added to the wells to lyse the cells, and 2 h later a sample of the lysate was obtained. The ⁵¹Cr activity of supernatant, wash, and lysate was determined with a gamma counter. Two indices of epithelial cell injury were calculated according to the following equations.

percent of ⁵¹Cr release

$$= \frac{\text{supernatant (cpm)} \times 100}{\text{supernatant (cpm)} + \text{wash (cpm)} + \text{lysate (cpm)}}$$

Percent of ⁵¹Cr release into the supernatant was taken as an index of epithelial cell lysis (23–25).

percent of cell detachment

$$= \frac{\text{wash (cpm)} \times 100}{\text{supernatant (cpm)} + \text{wash (cpm)} + \text{lysate (cpm)}}$$

Cell detachment represents the cells that were viable but were unable to maintain adhesion to the underlying matrix when exposed to the sheer stress of washing. Cell detachment is frequently used as an index of cell dysfunction (23–25).

Statistics. All values are presented as means±SEM. The EDTA clearance data over time and treatment (sham and I/R groups) were analyzed with repeated measures ANOVA followed by appropriate post-hoc comparisons.

Results

The effects of I/R on jejunal clearance of ⁵¹Cr-EDTA are shown in Fig. 1. In control (sham-operated) animals, reperfusion of the jejunal segment resulted in an increase in EDTA clearance that returned to control levels within 1 h. In animals that had been exposed to a previous (24 h earlier) I/R insult, there was

very little effect on jejunal EDTA clearance upon reperfusion. Morphological analyses (Fig. 2) confirmed that the mucosal epithelium was injured upon reperfusion in the sham-operated rats (46.6±1.3% of the villi had a discontinuous epithelial lining). In general, the injury was characterized by sheets of epithelium detaching from the lamina propria. Reperfusion-induced epithelial injury was dramatically reduced by a previous exposure of the intestine to an I/R insult (13.3±2.7% of the villi had a discontinuous epithelium). Taken together, these findings indicate that I/R-induced injury to the small intestine can be ameliorated by a previous I/R insult (i.e., I/R tolerance can be induced in the small intestine).

When the interval between I/R insults was reduced to 1 h, I/R tolerance did not develop. As shown in Fig. 3, it required ~ 1 h for the membrane defect (i.e., increase in EDTA clearance) to resolve itself after the initial I/R insult. The subsequent I/R-induced increase in EDTA clearance was no different from that observed in the animals not exposed to the initial I/R insult. When the interval between I/R insults was extended to 72 h, I/R tolerance could no longer be demonstrated. As shown in Fig. 4, the I/R-induced increases in EDTA clearance in the sham-operated animals and in animals exposed to a previous (72 h earlier) I/R insult were similar.

Biochemical analyses of the antioxidant status of the jejunal mucosa at 1, 24, and 72 h after the initial I/R insult are shown in Table I. At 24 h after the initial insult, mucosal GSHPX and catalase activities were increased, while SOD activity was unchanged when compared to sham-operated controls. Mucosal levels of GSH were also increased at 24 h after the initial I/R insult. HPLC analysis confirmed that 24 h after the insult, 97% of the total mucosal thiol content was GSH in both sham and I/R-treated animals. At 1 and 72 h after the initial insult, there were no significant changes in any of the measured antioxidants, as compared to the sham-operated controls. These findings indicate that the development of I/R tolerance in the small intestine is associated with an increase in the mucosal antioxidant status.

Based on the above findings, we tested the hypothesis that I/R tolerance was specific for oxidant-mediated injury. As shown in Fig. 5 A, perfusion of the jejunal lumen with H₂O₂ produced an increase in EDTA clearance in the sham-operated animals, but not in the animals exposed to a previous (24 h earlier) I/R insult. By contrast, perfusion of the lumen with hydrochloric acid produced similar increases in EDTA clearances in the sham-operated animals and in animals exposed to

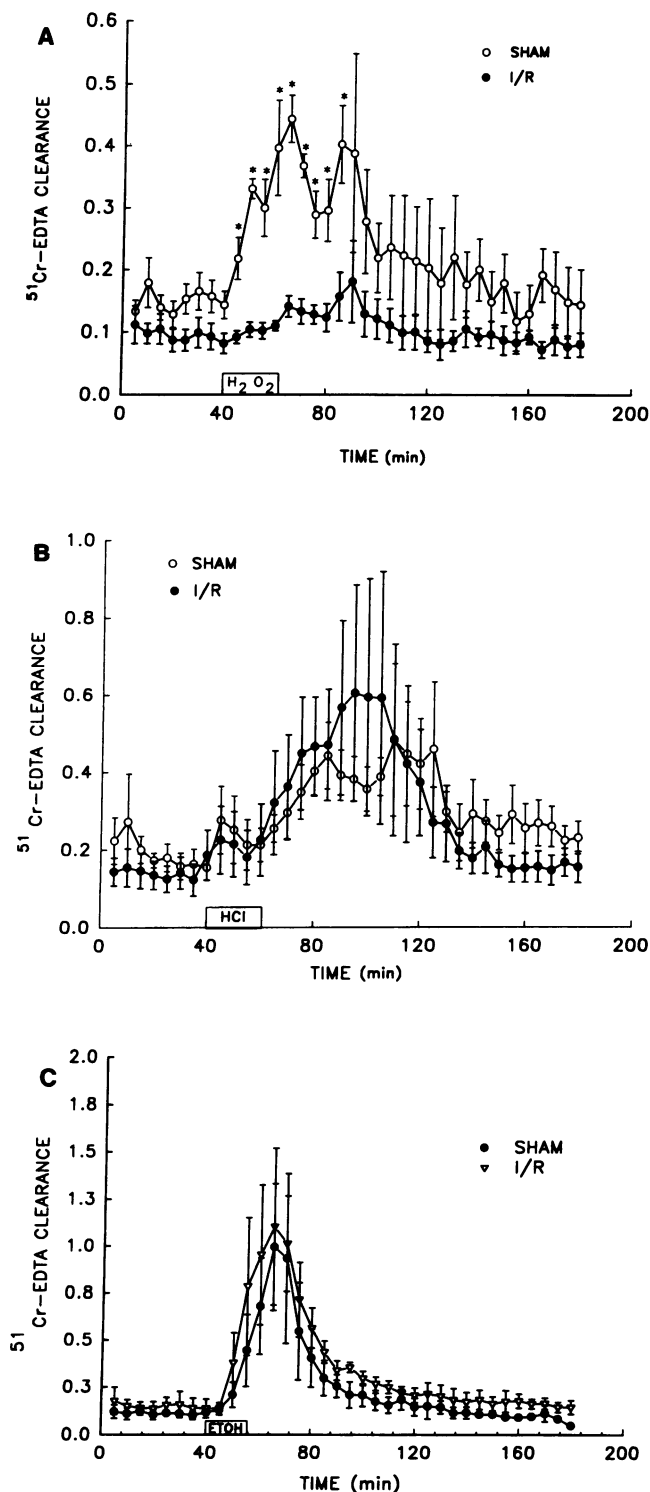


Figure 5. The effects of luminal perfusion with 5 mM H_2O_2 (A), HCl (B), or ethanol (C) on ^{51}Cr -EDTA clearance across the jejunal mucosal epithelium in animals whose SMA was occluded for 15 min (I/R) and animals whose SMA was merely massaged (SHAM) 24 h earlier. All values (ml \cdot min $^{-1}$ \cdot 100 g $^{-1}$) are expressed as the mean \pm SEM from three to five animals in each group. As the omnibus ANOVA revealed significant interaction between time and treatment (sham vs I/R groups) in the experiments with H_2O_2 posthoc comparisons (Duncan's test) for each time point were performed. * $P < 0.05$ compared to corresponding time point in I/R group.

a previous I/R episode (Fig. 5 B). Similarly, perfusion of the lumen with ethanol produced similar increases in EDTA clearances in sham-operated and I/R-treated animals (Fig. 5 C). Taken together, these findings indicate that a previous I/R insult to the small intestine renders the intestine less susceptible to oxidant-induced injury, but does not affect the susceptibility of the intestine to acid- or ethanol-induced injury.

To assess whether the epithelial cells lining the villi actively participate in the development of oxidant tolerance, they were separated from the lamina propria 24 h after the initial I/R insult, and their antioxidant status was assessed. As shown in Table II, there were no significant changes in any of the measured antioxidants, compared with the sham-operated controls. By contrast, 24 h after the initial I/R insult, the activities of SOD, catalase, and GSHPX in the lamina propria were significantly increased compared with sham-operated controls. These observations suggest that epithelial cells do not participate in the development of oxidant tolerance in the small intestine.

In another series of experiments, epithelial cells were removed from the villus tips 24 h after the initial insult, and their sensitivity to H_2O_2 was assessed by LDH release. As shown in Table III, the epithelial cells obtained from previously insulted intestines were just as insensitive to H_2O_2 as those obtained from sham-operated controls.

In vitro studies were performed to determine whether oxidant tolerance could be induced in an epithelial cell line, Caco-2. As shown in Fig. 6, exposure of the monolayers to H_2O_2 for 4 h did not produce any detectable injury to the monolayers until concentrations of ~ 5 mM were used. The injury was nonlytic since there was no increase in chromate release; only cell detachment was observed. Pretreatment of the monolayers with 400–1,600 μM H_2O_2 24 h earlier did not alter the degree of cell detachment induced by H_2O_2 .

Discussion

There is a growing body of evidence indicating that a brief I/R episode can render tissue more resistant to injury from a subsequent I/R challenge (6, 7, 19, 26–32). Collectively, the available evidence indicates that the development of I/R tolerance can be subdivided into two distinctly different phenomena, based on the time course and mechanisms involved in the protection. In the heart, the protective effect of a previous I/R episode is apparent within minutes after the initial insult and persists for ≤ 1 –2 hours, after which the susceptibility of the tissue to I/R injury returns to normal (9, 26–32). This phenomenon, referred to as “preconditioning,” is independent of protein synthesis (32), and it is not associated with alterations in the activity of endogenous antioxidant enzymes (31). By contrast, studies in the brain and kidney indicate that the protective effect of a previous I/R insult requires days to develop (6, 7). For example, in the kidney, it requires up to 6 d after the initial I/R insult before the tissue becomes less susceptible to a second I/R challenge (6). The development of this I/R tolerance is associated with an enhanced antioxidant status of the tissue. The results of the present study indicate that the development of I/R tolerance in the small intestine is analogous to the latter phenomenon. In the small intestine, I/R tolerance is present at 24 h, but not at 1 h, after the initial I/R insult. We also show that the development of I/R tolerance in the intestine is associated with an improved antioxidant status of the mucosa.

Table II. Localization of the Enhanced Antioxidant Status within the Jejunal Mucosa 24 h after an I/R Insult

	Villus tip epithelium		Mid-villus epithelium		Crypt epithelium		Lamina propria	
	SHAM	ISCHEMIC	SHAM	IR	SHAM	IR	SHAM	IR
SOD	4.3±0.5	4.5±0.3	4.1±0.3	4.4±0.6	3.9±0.2	4.0±0.5	4.3±0.1	6.1±0.2*
CAT	5.5±0.9	6.9±0.1	5.0±0.9	6.2±1.0	4.1±0.6	4.8±0.7	3.6±0.1	5.1±0.1*
GSHPX	29±6	27±4	32±6	27±6	32±8	30±6	54±2	60±2*
GSH	6.7±0.3	7.5±0.5	6.5±1.2	7.2±0.7	4.7±0.7	5.1±0.9	5.9±0.6	6.5±0.6

I/R, animals whose superior mesenteric artery was occluded for 15 min at 24 h before biopsy; SHAM, animals whose superior mesenteric artery was only massaged at 24 h before biopsy. Enzyme activities are expressed in milliunits per milligram, while GSH content is expressed in micrograms per milligram of protein. Values represent mean±SEM of assays on samples obtained from six to eight rats. * $P < 0.05$ compared to SHAM. (Student's *t* test).

It is generally believed that reperfusion of ischemic tissue results in increased production of ROM. The ROM generated overwhelm the endogenous antioxidants (both enzymatic and nonenzymatic) and produce tissue injury (1, 2, 4). Our observations would indicate that these ROM may also increase tissue levels of antioxidant enzymes via substrate induction and, thereby, confer protection against a subsequent oxidant challenge (i.e., I/R insult). In support of this possibility are the observations that a previous I/R insult offered protection against H₂O₂-induced, but not HCl- or ethanol-induced, mucosal injury. Previous studies (6) have also shown that a mild I/R insult to the kidney increases the activities of endogenous antioxidant enzymes and renders the kidney less susceptible to a subsequent I/R insult or H₂O₂ challenge. Taken together, these findings indicate that a mild I/R insult can lead to development of oxidant tolerance by enhancing the antioxidant status of the tissue.

The present study represents the first attempt to demonstrate that oxidant tolerance can be induced in a labile organ system (i.e., the small intestine). In the small intestine, the entire epithelium is replaced every 2–3 d by migration of cells up the villi and subsequent loss into the lumen (18). Interestingly, I/R tolerance disappears along with a return to a normal mucosal antioxidant status within 3 d after the initial I/R insult. These findings suggest that an adaptive response by the epithelial cells present at the time of the initial insult is necessary for oxidant tolerance to develop. Once these cells have been replaced by a new population of cells not exposed to I/R insults, the mucosa is no longer resistant to I/R challenges. However, further experimentation to test this hypothesis indicated that epithelial cells

do not play an active role in the development of oxidant tolerance in the intestinal mucosa.

When the epithelial cells were separated from the lamina propria 24 h after the initial I/R insult and their antioxidant status was assessed, it was found to be unchanged. However, the antioxidant status of the lamina propria was enhanced 24 h after the initial I/R insult. Catalase and GSHPX activities, as well as GSH, were increased in both whole mucosal scrapings and isolated lamina propria. By contrast, SOD activity was increased in the lamina propria, but not whole mucosa, 24 h after the initial insult. The reasons for these differences are not readily apparent, but may reflect animal and seasonal variations in basal antioxidant status and/or responsiveness to an I/R insult. Despite the latter discrepancies, the data collectively indicate that the increase in the antioxidant status of the mucosa observed 24 h after the initial I/R insult is a result of adaptational changes in the lamina propria, rather than in the epithelium.

Further evidence negating an active role for the epithelium in the development of oxidant tolerance was provided by experiments performed with isolated epithelial cells. Intestinal epithelial cells removed from the villus tips 24 h after the initial I/R insult were just as insensitive to H₂O₂ as epithelial cells obtained from sham-operated controls. This observation indicates that, after the initial I/R insult, the epithelial cells lining the villus did not mount an adaptive response that would render them more resistant to the cytotoxic effects of H₂O₂. Furthermore, pretreatment of Caco-2 cell monolayers with H₂O₂ did not render them less sensitive to a H₂O₂ challenge 24 h later. Taken together, these findings indicate that the epithelium plays only a passive role in the development of oxidant tolerance in the intestinal mucosa.

The role of the epithelium in oxidant-induced injury to the mucosal barrier may also be passive. In vivo studies indicated that within 10–20 min of luminal perfusion with 5 mM H₂O₂, mucosal permeability to EDTA increased three- to fourfold. However, exposure of epithelial cells harvested from the villus tips to 5 mM H₂O₂ for the same period of time did not result in cell lysis as evidenced by LDH release (Table III). Caco-2 cells were also resistant to H₂O₂, with monolayer disruption occurring at high concentrations (6–7 mM) and prolonged exposure (4 h). Even then, the injury was primarily nonlytic, i.e., the cells were simply more readily detached by imposing a shear stress (wash) across the monolayer (Fig. 6). Taken to-

Table III. Effect of 5 mM H₂O₂ on LDH Release from Epithelial Cells Isolated from Rat Jejunal Villi 24 h after an I/R Insult

Duration of exposure	SHAM		I/R	
	PBS	H ₂ O ₂	PBS	H ₂ O ₂
10 min	24.5±3.5	25.1±3.2	20.3±2.4	26.3±2.3
20 min	43.3±11.2	39.0±12.7	31.6±6.9	40.9±14.1

I/R, animals whose superior mesenteric artery was occluded for 15 min and released; SHAM, animals whose superior mesenteric artery was only massaged. Values represent mean±SEM of percent of total releasable LDH from Triton-treated epithelial cells isolated from four to six rats.

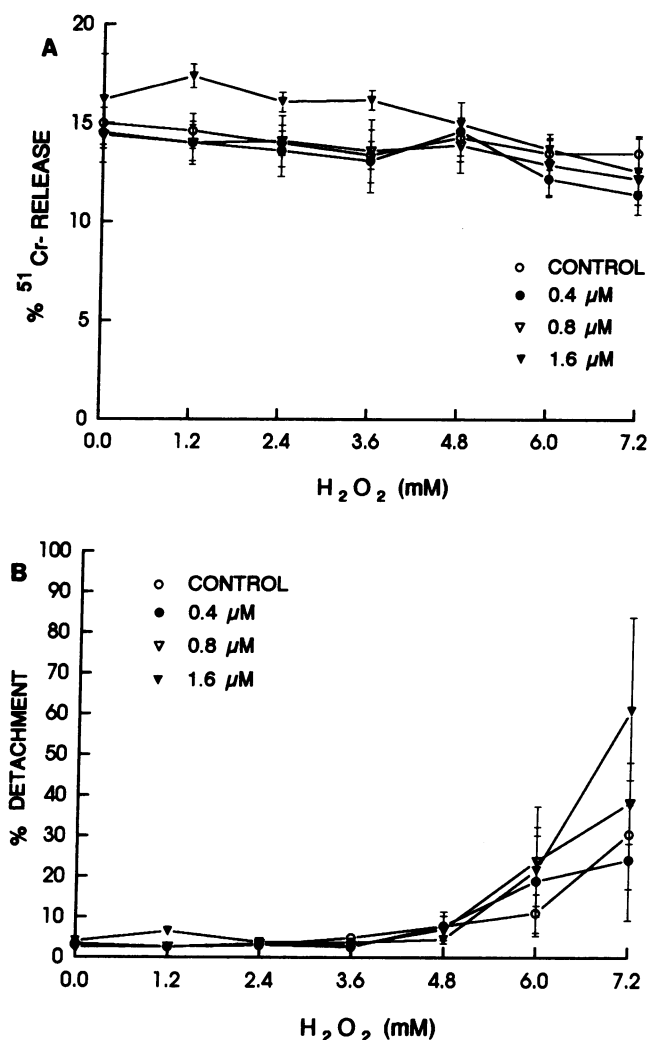


Figure 6. H₂O₂-induced disruption of Caco-2 cell monolayers in untreated and H₂O₂-pretreated monolayers. Caco-2 monolayers were pretreated with 400, 800, or 1,600 μM H₂O₂ 24 h before assessment of H₂O₂-induced ⁵¹Cr-release (A) and epithelial cell detachment (B).

gether, these findings suggest that, in vivo, H₂O₂ may be altering epithelial membrane permeability indirectly by an action on resident cells or structural elements (e.g., basement membrane) within the lamina propria, which ultimately leads to cell detachment. However, it is also possible that H₂O₂-induced cell detachment may be caused by induction of epithelial cell apoptosis.

The identity of the cell type(s) in the lamina propria that respond to an I/R insult by enhancing their antioxidant status is not clear. One likely candidate is the endothelium. Endothelial cells in culture can develop oxidant tolerance in response to an oxidant stress (33). Exposure of pulmonary artery endothelial cells to nontoxic levels of H₂O₂ for 18–24 h increased (a) their endogenous activities of SOD, catalase, and GSHPX; and (b) their resistance to H₂O₂-induced cytotoxicity. In vivo studies in the kidney indicate that an I/R insult results in an increased tolerance to a second I/R insult or H₂O₂ challenge 6 d later (6). The development of this oxidant tolerance is associated with increased activity of SOD, catalase, and GSHPX in renal glomeruli, a segment of the nephron consisting primarily of

endothelium. Although endothelial cells can mount an adaptive response to an I/R insult or oxidant challenge, we cannot exclude other resident cell populations in the lamina propria from contributing to the development of oxidant tolerance in the intestine. Further studies are warranted to definitively identify the cell types in the lamina propria that increase their antioxidant status in response to an I/R insult, and how this adaptive response renders the epithelium more resistant to an oxidant challenge.

In summary, the results of the present study indicate that oxidant tolerance can be induced in a labile organ system, the small intestinal mucosa. 24 h after an initial ischemia insult, the epithelial barrier is less susceptible to injury induced by a subsequent I/R episode or luminal perfusion with H₂O₂. The development of this oxidant tolerance is associated with an enhanced antioxidant status of the mucosa, indicating that induction of antioxidant enzymes is an important mechanism in this adaptational response. However, we cannot exclude the possibility that other factors, such as induction of heat shock proteins, may also contribute to the development of oxidant tolerance (33).

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