**Regular Article**

*Clarification of the Mechanism of Structural Change Induced by Reoxygenation following the Induction of Lipid Peroxidation in Caco-2 Cell Monolayers*

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**Summary:** Recently, we established a system for assessing ischemia/reperfusion injury, specifically the opening of tight junctions (TJ), caused by reoxygenation following the induction of lipid peroxidation by tertiary-butylhydroperoxide (t-BuOOH), using the human intestinal epithelial cell line Caco-2 in order to focus on the barrier function of the epithelium independent of the vascular compartment. In the present study, we attempted to identify factors involved in the structural changes induced by reoxygenation using 0.5 mM t-BuOOH in Caco-2 cell monolayers. Glutathione (GSH) and N-acetylcystein, a precursor of GSH, inhibited the opening of TJ evoked by reoxygenation following the induction of lipid peroxidation by 0.5 mM of t-BuOOH. Tiron, as a cell permeable superoxide anion scavenger and deferoxamine, an iron-chelating agent ameliorated the opening in a dose-dependent manner. Also, Tiron suppressed the apical-to-basal and basal-to-apical permeability of the increased Rhodamine123 by reoxygenation in a concentration-dependent manner. These results collectively suggest that superoxide anion and iron ions play an important role or contribute to structural changes such as the opening of TJ induced by reoxygenation following the induction of lipid peroxidation by 0.5 mM t-BuOOH.

**Key words:** ischemia; reperfusion injury; TJ opening; P-glycoprotein; superoxide anion; iron ions; lipid peroxidation; Caco-2 cell monolayers

**Introduction**

Grafting of the small intestine has attracted a lot of attention in recent years. The transplantation using immunosuppressors such as tacrolimus is an effective strategy for treating patients with intestinal failure.¹ Because the success of the transplantation depends on a delicate balance immunosuppression and rejection, the maintenance of adequate levels of blood of tacrolimus is critical. And it is also known that P-glycoprotein (P-gp) is a drug excretion pump that controls absorption of immunosuppressant such as tacrolimus. However, not only organ rejection but also early ischemia/reperfusion injury must be overcome. One of the causes of ischemia/reperfusion injury is believed to be the opening of tight junctions (TJ).²⁻⁴

The epithelium of the digestive tract separates the vascular system from the lumen of the digestive tract and compromise of the barrier could allow invasion by toxic compounds such as endotoxin. This invasion is physioanatomically referred to as a paracellular invasion, and must be prevented during organ transplantation. However, it is difficult to assess injuries such as the opening of TJ with the *in vivo* models used to study ischemia/reperfusion injury. We have reported that cellular hypoxia caused by ischemia ultimately reduces the barrier function of epithelial cells, and lipid peroxidation plays an important role in this process.⁵ Based on this report, we recently established a system for assessing ischemia/reperfusion injury *in vitro*, in which lipid peroxidation caused by tertiary-butylhydroperoxide (t-BuOOH), a lipid peroxidation inducer, acts as a trigger.⁶ Using the human intestinal epithelial cell line Caco-2, we used this system to focus on the barrier function of the epithelium independent of the vascular compartment.⁶ It was found that reoxygenation following the induction of lipid peroxidation by 0.5 mM t-BuOOH (a low concentration) induced the
opening of TJ.6)

In the present study, we attempted to identify factors involved in structural changes such as the opening of TJ using a low concentration of t-BuOOH in Caco-2 cell monolayers. The permeability of Rhodamine123, as a model P-gp substrate, was investigated during the opening of TJ as mentioned above.

Materials and Methods

Materials:

- t-butylhydroperoxide, glutathione, N-acetyl-L-cystein, 4,5-dihydroxy-1,3-benzene-disulfonic acid, sodium N,N-diethyldithiocarbamate trihydrate, deferoxamine mesylate and Rhodamine123 were purchased from Sigma Co. Ltd. (St. Louis, MO). The other reagents were of analytical grade or better.

Cell Culture:

Caco-2 cells at passage 18 obtained from the American Type Culture Collection (ATCC HTB37; Rockville, MD) were maintained by serial passage in plastic culture dishes (Corning; Becton Dickinson and Co., Lincoln Park, NJ) as described previously.7) The complete medium consisted of Dulbecco’s Modified Eagle’s Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 1% nonessential amino acid (Gibco) without antibiotics. The cells were grown in an atmosphere of 5% CO₂/95% O₂ air at 37°C and subcultured every week using 0.02% EDTA and 0.05% trypsin. Caco-2 cells were seeded on polycarbonate membrane filters (0.4 μm pore size, 1 cm² growth area) inside snapwell cell culture chambers (Corning, Cambridge, MA) at a density of 1 × 10⁴ cells/filter. The culture chambers were fed fresh complete medium every day and were used on the 17th or 18th day for the experiments.

Ischemia/Reperfusion Model:

The Caco-2 cell monolayers on polycarbonate membrane filters were attached to the side-by-side diffusion chamber. The diffusion chamber, gas manifold, and block heater were from Precision Instrument Design, Los Altos, CA. The cells were then brought into contact with 5 ml of Krebs Henselite Bicarbonate Buffer (KHBB) incubation medium in the absence of glucose containing tertiary-butyl hydroperoxide (t-BuOOH), which is a lipid peroxidation inducer,8) without an oxygen supply. After 60 min, the KHBB incubation medium was replaced with the same medium supersaturated with oxygen in the presence of glucose. This was used as an in vitro ischemia/reperfusion model.

Measurement of Transepithelial Electrical Resistance (TEER) and Transport Experiments:

The barrier function of the paracellular route was assessed by measuring transepithelial electrical resistance (TEER) using a Short Circuit Current Amplifier (CEZ-9100, Nihon Koden, Japan). TEER was calculated from the potential difference obtained on loading a small external current (0.01 and 0.1 mA) according to Ohm’s law9) at intervals of 15 min. Decreases in TEER indicate structural changes in the membrane, that is, the opening of TJ.

The compound used for the permeation experiments was Rhodamine123 (Rho123). Rho123 was added at 20 μM to either the apical (A) or basal (B) side at the point of reoxygenation following the induction of lipid peroxidation by t-BuOOH in order to ascertain the permeability (cm/sec) of the cell monolayers to this molecule during the drop in TEER.

To identify the injurious factor, inhibition experiments were performed using glutathione (GSH), N-acetyl-L-cystein (NAC), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), sodium N,N-diethyldithiocarbamate trihydrate (DDC) and deferoxamine mesylate (DFO). 10 mM GSH or 10 mM NAC was incubated with Caco-2 cell monolayers for 15 min prior to the addition of 0.5 mM t-BuOOH. Tiron at 2.5 to 30 mM, DFO at 0.1 mM ~ 1.0 mM or DDC at 1 μM ~ 50 μM was incubated with Caco-2 cell monolayers for 5 min prior to the addition of t-BuOOH at various concentrations.

Statistical Analysis:

A minimum P-value of 0.05 was used as the level of significance for all tests. Turkey tests were performed on permeability data. All plots and columns in the figures are expressed as the means ± standard error (S.E.).

Results

Effects of Glutathione (GSH) and N-acetyl-L-cystein (NAC) as Scavengers of Reactive Oxygen Species on Structural Changes in Caco-2 Cell Monolayers

When cells on both sides were exposed to 0.5 mM t-BuOOH under a hypoxic environment and then supplied with oxygen, TEER decreased markedly (Fig. 1). Because no marked changes were seen in the control, i.e. the presence of oxygen, reoxygenation following the induction of lipid peroxidation appeared to cause TJ to open.

Next, the effects of GSH and NAC on the structural changes induced by reoxygenation following induction of lipid peroxidation by t-BuOOH were examined. GSH and NAC significantly suppressed the reduction in TEER caused by t-BuOOH (Fig. 1).

Effects of Tiron as a Superoxide Anion Scavenger on Structural Changes in Caco-2 Cell Monolayers

Although 30 mM Tiron, a scavenger of superoxide anion, did not suppress the reduction in TEER caused by 5 mM and 10 mM t-BuOOH, it significantly suppressed the reduction caused by 0.5 mM t-BuOOH (Fig. 2). Also, 30 mM Tiron suppressed the reduction...
Fig. 1. Effects of GSH and NAC on structural changes induced by 0.5 mM t-BuOOH in Caco-2 cell monolayers. The concentrations of GSH and NAC used in this study were 10 mM. Without an oxygen supply, cells were brought into contact with 5 ml of incubation medium containing t-BuOOH (i.e.; t-BuOOH). After 60 min, the incubation medium was replaced with a medium supersaturated with oxygen (i.e.; O2 gas addition). Open circle; control (i.e.; no additive), Closed circle; t-BuOOH alone, Closed triangle; NAC+t-BuOOH, Closed square; GSH + t-BuOOH. Results represent means and S.E. (n = 5 ~ 8 for each condition).

Contribution of Reactive Oxygen Metabolites and Iron Ions to TJ Opening caused by 0.5 mM t-BuOOH in a concentration-dependent manner (Fig. 3a).

Under the control conditions, the permeability of Rho123 across the cell monolayers from side B to side A was much greater than that from side A to side B, indicating that the excretion pump was expressed, and it was working in this system (Fig. 3b). Reoxygenation following the induction of lipid peroxidation by 0.5 mM t-BuOOH significantly increased the A-to-B and B-to-A permeability of Rho123 (Fig. 3b). And Tiron suppressed the permeation in both directions in a concentration-dependent manner (Fig. 3b). The effect of Tiron on the permeation corresponded to on the reduction in TEER (Fig. 3a and b).

Effects of DDC on Structural Changes in Caco-2 Cell Monolayers

The reduction in TEER caused by 0.5 mM t-BuOOH was suppressed by DDC in a dose-dependent manner (Fig. 4a), indicating that DDC as well as Tiron

Fig. 2. Effect of Tiron on structural changes induced by t-BuOOH at various concentrations in Caco-2 cell monolayers. (a), (b) and (c) represent the time course of TEER induced by 0.5 mM, 5.0 mM and 10 mM t-BuOOH, respectively. The concentration of Tiron used in this study was 30 mM. Open circle; control (i.e.; no additive), Closed circle; t-BuOOH alone, Closed triangle; Tiron + t-BuOOH. Results represent means and S.E. (n = 8 ~ 18 for each condition).
ameliors the effect of the paracellular barrier such as the opening of TJ induced by t-BuOOH at low concentration. In addition, DDC suppressed both A-to-B and B-to-A permeation in a concentration-dependent manner (Fig. 4b). These findings were similar to those on the suppression of TEER reduction shown in (Fig. 4a).

**Effects of DFO as an Iron-Chelating Agent on Structural Changes and in Caco-2 Cell Monolayers**

Although DFO did not suppress the reduction in TEER caused by 5 mM and 10 mM t-BuOOH, it did significantly suppress that caused by 0.5 mM t-BuOOH (Fig. 5). The reduction caused by 0.5 mM t-BuOOH was suppressed by DFO in a concentration-dependent manner, suggesting that iron ions are involved in the opening of TJ caused by t-BuOOH at low concentrations (Fig. 6a).

Following reoxygenation, DFO suppressed the A-to-B membrane permeation of Rho123 in a concentration-dependent manner (Fig. 6b), similar to the manner in which it suppressed the reduction in TEER (Fig. 6a). On the other hand, DFO did not have any effect on the B-to-A permeation of Rho123 (Fig. 6b).

**Discussion**

Over the past few years, a great deal of information has emerged about oxygen radical-induced cellular injury. However, the role of individual oxygen metabolites is still not clear. *In vitro* studies using vascular endothelial cells, isolated enterocytes and gastric cells showed hydrogen peroxide to be the most likely damaging agent, with superoxide anion and hydroxyl radical less injurious. This information was derived from the experimental findings that superoxide dismutase (SOD) as a superoxide anion scavenger had either no protective effect on the destruction of cells or enhanced the activity of xanthine oxidase (XO). Because SOD converts superoxide anion to hydrogen peroxide, it was speculated that hydrogen peroxide is the oxidant species primarily responsible for the cellular injury.

Other investigators, using the above *in vitro* models, found SOD to be protective and suggested superoxide anion or possibly its derivative, the hydroxyl radical, to be the most likely damaging oxygen radical. In fact, it is reported that SOD suppressed ischemia/reperfusion injury in rabbit intestine and mouse intestine. These findings are therefore consistent with the conclusion of Park et al., that in the feline intestine, hydroxyl radical was the most important damaging agent causing membrane leakage via lipid peroxidation.

In our study, the addition of 10 mM GSH, considered a defensive factor (endogenous scavenger of reactive oxygen species: ROS), to our system suppressed the
Contribution of Reactive Oxygen Metabolites and Iron Ions to TJ Opening

reduction in TEER induced by reoxygenation following the induction of lipid peroxidation by 0.5 mM \( t\)-BuOOH (Fig. 1). And NAC as a precursor to GSH having the ability to scavenge hydrogen peroxide, hydroxyl radical and superoxide anion\(^{20}\) also effectively suppressed the reduction in TEER (Fig. 1). These results suggest that ROS contribute to the paracellular barrier dysfunction such as the opening of TJ induced by reoxygenation.

We also conducted another experiment using Tiron, which is a cell permeable superoxide anion scavenger,\(^{21,22}\) in order to clarify the contribution of superoxide anion to the opening of TJ in more detail. The reduction in TEER caused by 0.5 mM \( t\)-BuOOH was significantly ameliorated by Tiron (Fig. 2), in a concentration-dependent manner (Fig. 3a), suggesting that superoxide anion is involved in the opening of TJ evoked by \( t\)-BuOOH at low concentration. Since the inhibitory effects of Tiron are reportedly greater than those of SOD, a cell impermeable superoxide anion,\(^{23–25}\) the suppression of TEER reduction may result from the elimination of superoxide anion by Tiron. Accordingly, we emphasize that intracellular rather than extracellular superoxide anion plays an important role in the structural changes to TJ. On the other hand, no marked effect of Tiron on TEER reduction was seen at 5 mM and 10 mM \( t\)-BuOOH when compared with 0.5 mM \( t\)-BuOOH (Fig. 2). These findings suggest that factors other than the intracellular superoxide anion are involved in the opening of TJ caused by a high concentration of \( t\)-BuOOH.

In contrast, Tiron decreased not only the permeability of Rho123 from side A to B but also the reverse flux (Fig. 3b) to a degree corresponding to the suppression of the reduction in TEER (Fig. 3a). Because 0.5 mM \( t\)-BuOOH had no effect on the activity of P-gp,\(^{6}\) the decrease in both the A-to-B and B-to-A flux of Rho123 caused by Tiron reflected a recovery of TEER.

DDC irreversibly inhibits the activity of SOD by chelating with bivalent ions such as Cu\(^{2+}\) and Zn\(^{2+}\) which are included in SOD.\(^{26,27}\) Therefore, when cells are exposed to DDC and thereafter supplied with \( t\)-BuOOH, TEER should decrease further than after treatment with \( t\)-BuOOH alone, that is, DDC should accelerate the opening of TJ induced by \( t\)-BuOOH. In fact, it has been reported that the increase in the concentration of superoxide anion caused by the suppression of SOD activity by DDC further potentiated ischemia/reperfusion injury.\(^{28–30}\)

The opening of TJ and the increase in both the A-to-B and B-to-A permeability of Rho123 caused by 0.5 mM \( t\)-BuOOH were inhibited by DDC in a dose-dependent manner in the present study (Fig. 4a and b). This finding appears to contradict the results obtained in our investi-
Fig. 5. Effect of DFO on structural changes induced by various concentrations of t-BuOOH in Caco-2 cell monolayers. (a), (b) and (c) represent the time course of TEER induced by 0.5 mM, 5.0 mM and 10 mM t-BuOOH, respectively. The concentration of DFO used in this study was 1.0 mM. Results represent means and S.E. (n = 8-18 for each condition). Open circle; control (i.e.; no additive), Closed circle; t-BuOOH alone, Closed triangle; DFO + t-BuOOH.

Results which support the suppression by DDC of cellular injury such as the damage caused by ischemia/reperfusion have been reported in Caco-2, mongolian gerbil and rat gut.\textsuperscript{31,32} Gebhardt et al. also reported that DDC suppressed the extent of membrane damage including the release of lactate dehydrogenase (LDH) in Caco-2 cell monolayers.\textsuperscript{33} The inhibitory effect of DDC, an inhibitor of SOD activity, on the post-hypoxic LDH results from a deceleration of production of hydrogen peroxide, a precursor of the hydroxyl radical that is a more reactive oxygen radical than superoxide anion.\textsuperscript{33} Accordingly, Gebhardt et al. have emphasized that superoxide anion is not responsible for membrane peroxidation, as it is an electron donor and thus unable to react as an oxidant.\textsuperscript{33} Therefore, DCC can not accelerate the opening of TJ if DDC inhibits the activity of SOD and the production of superoxide anion then increases. However, the efficacy of SOD\textsuperscript{21,22} and aggravation by DDC\textsuperscript{28–30} during ischemia/reperfusion injury do not support such a conclusion. Nor do the effects of Tiron shown in (Fig. 2) and (Fig. 3). In conclusion, we hypothesized that DDC, as a chelating agent, suppressed the opening of TJ through chelation with bivalent ions other than Cu\textsuperscript{2+} and Zn\textsuperscript{2+} in SOD during hypoxia-reoxygenation injury.

It is considered that ischemia/reperfusion injury is induced by a drop in the intracellular level of ATP through a decrease in energy production by inactivation of Fe–S oxidation, the central role of aconitase in mitochondria, caused by superoxide anion.\textsuperscript{34–36} And it is known that superoxide anion is a precursor in the generation of more reactive oxygen radicals, such as the hydroxyl radical released from the iron-catalyzed Fenton’s reaction.\textsuperscript{37} Therefore, we investigated the effect of DFO as an iron chelator\textsuperscript{38,39} on structural changes induced by 0.5 mM t-BuOOH in Caco-2 cell monolayers. The reduction in TEER caused by 0.5 mM t-BuOOH was significantly suppressed by DFO in a concentration-dependent manner, suggesting that iron ions are involved in the mechanism or process to open TJ (Fig. 5 and 6a), but other factors are involved in the opening caused by t-BuOOH at higher concentrations (Fig. 5). These results support previous reports that iron ions participated in lipid peroxidation and a chelating agent of iron ions suppressed reperfusion injury \textit{in vivo}.\textsuperscript{40,41} In contrast, it is reported that the availability of free iron is strictly limited in part by the iron-binding protein, transferrin.\textsuperscript{42} The decrease in TEER induced by reoxygenation following the induction of lipid peroxidation could be induced by a release of free iron ions through conformational changes to the iron-transferrin complex. It is therefore considered that DFO as an iron ion chelating agent has an inhibitory effect on the drop in TEER.
The increased A-to-B permeability of Rho123 was suppressed by DFO in a concentration-dependent manner (Fig. 6b), to a similar extent as the suppression of the reduction in TEER (Fig. 6a), but the B-to-A permeability was not (Fig. 6b). The reason for this is not clear and remains to be investigated.

We discussed the role of superoxide anion as an inducing factor after hypoxia and reoxygenation. Since the anion donates one electron during the red/ox reaction, it should be distinguished from other oxidative radicals such as the hydroxyl radical or hydrogen peroxide. It is not yet clear whether the superoxide anion itself or metabolites cause injuries such as the opening of TJ in Caco-2 cell monolayers, but our results, indicate that iron ions participate in the opening of TJ in Caco-2 cell monolayers.

In conclusion, we succeeded in establishing a system for assessing ischemia/reperfusion injury in vitro, in which lipid peroxidation acts as a trigger. Using this model, we showed that reoxygenation induces structural changes such as the opening of TJ in the cell membrane. Also, we were able to show that superoxide anion and iron ions participate in the process by which t-BuOOH at a low concentration causes TJ to open.

We plan to investigate further the mechanism of ischemia/reperfusion injury and overcome the damage during organ transplantation using medical treatments.

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