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Nitric oxide attenuates hydrogen peroxide-induced barrier disruption and protein tyrosine phosphorylation in monolayers of intestinal epithelial cell

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Abstract

The intestinal epithelium provides a barrier to the transport of harmful luminal molecules into the systemic circulation. A dysfunctional epithelial barrier is closely associated with the pathogenesis of a variety of intestinal and systemic disorders. We investigated here the effects of nitric oxide (NO) and hydrogen peroxide (H_2O_2) on the barrier function of a human intestinal epithelial cell line, Caco-2. When treated with H_2O_2, Caco-2 cell monolayers grown on permeable supports exhibited several remarkable features of barrier dysfunction as follows: a decrease in transepithelial electrical resistance, an increase in paracellular permeability to dextran, and a disruption of the intercellular junctional localization of the scaffolding protein ZO-1. In addition, an induction of tyrosine phosphorylation of numerous cellular proteins including ZO-1, E-cadherin, and β-catenin, components of tight and adherens junctions, was observed. On the other hand, combined treatment of Caco-2 monolayers with H_2O_2 and an NO donor (NOC5 or NOC12) relieved the damage to the barrier function and suppressed the protein tyrosine phosphorylation induced by H_2O_2 alone. These results suggest that NO protects the barrier function of intestinal epithelia from oxidative stress by modulating some intracellular signaling pathways of protein tyrosine phosphorylation in epithelial cells.

Keywords: Intestinal epithelial cell; Tight junction; Nitric oxide; Hydrogen peroxide; Protein tyrosine phosphorylation; Caco-2 cell

1. Introduction

Epithelia are sheets of cells that provide the interface between masses of cells and a cavity or a lumen. They create a selective barrier that restricts the passage of solutes and maintains the fluid composition of compartments. The tight junctions (TJs), which seal the adjacent cells together to prevent diffusion of macromolecules through the paracellular space, are the most important morphological feature for the epithelial barrier function, while adherens junctions (AJs) are a prerequisite for the formation of TJs [1]. Reactive oxygen species (ROS), which mediate oxidation of cell components, damage cells or perturb intracellular signal transduction cascades [2,3]. Then, ROS can cause epithelial barrier dysfunction and, in turn, the initiation and/or perpetuation of various disorders [4–6].

Nitric oxide (NO) is a free radical with moderate reactivity synthesized from the amino acid L-arginine by the nitric oxide synthetase (NOS) [7]. NO is often produced along with ROS within tissues with inflammation or injury and had been considered a potentially toxic chemical. However, it was also found as an endothelium derived-relaxing factor [8] and has been established to be a diffusible multieffector regulating functions of various tissues such as vascular, neuronal, and immune systems, in recent years [9]. In the vascular system, NO has been revealed to protect the endothelial barrier function from oxidative stress-induced injury [10–14], as well as to regulate vascular tone and interactions between platelets, leukocytes, and the vascular endothelium [15].

The highly selective epithelial barrier within the intestinal mucosa permits the absorption of nutrients but normally restricts the passage of harmful proinflammatory and toxic molecules from the lumen of the intestine into the systemic circulation.

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ROS are generated within the lumen of the intestine from multiple sources including xenobiotics, toxins, catalase-negative bacteria, mycoplasma, bile acids, and desquamated mucosal cells, and affect the intestinal epithelium [6]. The intestinal epithelium is also exposed to ROS from systemic sources, such as activated mucosal neutrophils [5]. Loss of the epithelial barrier function of the intestine appears to contribute to the pathogenesis of a variety of intestinal and systemic disorders [16]. There is robust evidence that constitutive and inducible NO production regulates the integrity of the intestinal mucosa under physiological conditions and counters the increase in mucosal permeability associated with the pathogenesis of various intestinal disorders, although an excess amount of NO may have detrimental effects during chronic inflammation [17,18]. Subepithelial actions of NO within the mucosa, such as maintenance of blood flow, inhibition of platelet and leukocyte adhesion and aggregation within the vasculature, and modulation of mast cell reactivity, had been suggested to be mechanisms for the protective effects of NO against mucosal hyperpermeability [17,18]. However, the direct effect of NO on intestinal epithelia under pathophysiological conditions remains unclear.

To gain an insight into the molecular functions of NO in intestinal epithelia we employed an in vitro culture system, the human intestinal epithelial cell line Caco-2. The effects of exogenous NO donated from NO-generating chemicals such as NOC5 and NOC12 and of hydrogen peroxide (H_2O_2) on the barrier function of Caco-2 monolayers grown on permeable supports were evaluated by measuring the transepithelial electrical resistance (TER) and the paracellular permeability to dextran. Furthermore, the integrity of cell–cell junctions was investigated by immunostaining and immunoblotting of the proteins associated with TJs and AJs. This study demonstrates that exogenous NO attenuates the H_2O_2-induced barrier dysfunction and protein tyrosine phosphorylation. To our knowledge, this is the first report showing that NO acts directly on intestinal epithelial cells to protect the barrier function against oxidative stress.

2. Materials and methods

2.1. Materials

NO donors such as NOC5 and NOC12 were purchased from Dojindo (Kumamoto, Japan). Anti-ZO-1 rabbit polyclonal antibody and anti-β-catenin mouse monoclonal antibody were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Anti-E-cadherin rat monoclonal antibody was purchased from Merck KGaA (Darmstadt, Germany). Anti-phosphotyrosine monoclonal antibody clone 4G10 was from Upstate Biotechnology (Lake Placid, NY, USA). The other reagents and chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless described otherwise.

2.2. Culture of Caco-2 cells and treatment with H_2O_2 and/or NO donors

The human colon carcinoma cell line Caco-2 purchased from RIKEN BioResource Center (Ibaraki, Japan, RCB0988) was derived from the European Collection of Animal Cell Cultures (Wiltshire, U.K., ECA86010202) [19]. Cells were cultured in minimum essential medium (MEM) supplemented with 20% fetal bovine serum, 0.1 mM non-essential amino acids, and 50 μg/ml gentamicin at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity. Cells were seeded into Millicell-HA or −CM culture plate inserts (12-mm diameter, 0.6–cm^2 effective membrane area, Millipore Corp., Bedford, MA, USA) at a density of 1.7×10^5 cells/cm^2 and cultured for 4 days before treatments with H_2O_2 and/or NO donors. The membrane surface of Millicell-CM inserts was coated with Matrigel (BD Biosciences, Bedford, MA, USA) prior to the cell culture. Treatments with H_2O_2 and/or NO donors were started by replacing the culture medium with a fresh one containing appropriate amounts of H_2O_2 and/or NO donors and the cultures were continued under the usual cell culture conditions. Thus, both apical and basolateral sides of Caco-2 monolayers were exposed to H_2O_2 and NO donors.

2.3. Determination of TER

TER was measured using a Millicell-ERS resistance system (Millipore Corp) at 1-h intervals and expressed as a percentage of the corresponding TER immediately after the start of treatments. The resistance of the supporting membrane in Millicell inserts was subtracted from all readings before calculations.

2.4. Determination of paracellular permeability

Paracellular permeability to fluorescein-conjugated dextran (average molecular weight of 3000, Invitrogen Corp.) from the apical- to basolateral-side was measured according to methods described previously with some modifications [20]. Caco-2 cell monolayers on Millicell-HA inserts were treated with H_2O_2 and/or NO donors as described above. After a 3-h treatment, the cultures were rinsed with P-buffer (10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, and 145 mM NaCl) and transferred to a 24-well plate containing 600 μl of P-buffer per well. Then, 200 μl of P-buffer containing 50 μg of fluorescein-dextran (tracer solution) was added to the apical side of Millicell inserts. After a 3-h incubation of the culture, the media were collected separately from both apical and basal compartments and the concentration of fluorescein-dextran was measured on a fluorescence multiplate reader (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer, Wellesley, MA, USA). The permeability of the monolayers was expressed as clearance (Cl), which was calculated using the following formula: Cl (μl/cm^2/h)=FDab/[FD]a/S, where FDab is the apical to basolateral flux of the tracer (μg/h), [FD]a is the concentration of the tracer in the apical compartment (250 μg/ml), and S is the surface area (0.6 cm^2).

2.5. Western blot analyses

Caco-2 cell monolayers on Millicell-HA inserts (30-mm diameter, 4.2-cm^2 effective membrane area) treated with H_2O_2 and/or NOC5 were harvested after a 3-h incubation period, and homogenized in modified RIPA lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.2% SDS, 0.2% sodium deoxycholate, 10 μM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin A, leupeptin, and aprotinin). The homogenate was centrifuged at 15,000×g for 15 min at 4 °C and the supernatant was then further centrifuged at 100,000×g for 30 min at 4 °C. The protein concentration of the resulting cytosolic fraction was quantitated by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and then the fraction was subjected to Western blot analyses as described in our previous report [21]. Densitometric analysis was performed using Image Gauge ver. 3.4 software (Fuji Film, Tokyo, Japan).

2.6. Immunocytochemistry of ZO-1 protein in Caco-2 cells

Caco-2 cell monolayers on Millicell-CM inserts (12-mm diameter) were treated with H_2O_2 and/or NOC5 for 3 h and then fixed, permeabilized, and blocked as described in our previous report [21]. The specimens were treated with the primary antibody diluted in PBS for 1 h at 37 °C. The visualization of antibodies was performed using tetramethylrhodamine B isothiocyanate- or fluorescein isothiocyanate-conjugated secondary antibodies. The nucleus was stained with Hoechst 33258. Specimens were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Immunoprecipitation of TJ and AJ associated proteins

Cytosolic fractions of Caco-2 cells at the same protein concentration (0.5–0.6 mg/ml) were precleared separately by incubating with one-fourth of the settled volume of protein G-Sepharose beads (GE Healthcare Bio-Sciences Corp.) for 1 h at 4 °C and the supernatant was saved as a precleared extract. The precleared extracts (250 μl each) were incubated with 3 μg of anti-E-cadherin or anti-ZO-1 antibody at 4 °C for 15 h. Immune complexes were recovered by incubating with 20 μl of protein-G Sepharose at 4 °C for 3 h with gentle rocking. The beads were then washed three times with cold lysis buffer, treated with SDS-PAGE sample buffer, and processed for Western analysis.

2.8. Statistical analysis

Differences between two groups were evaluated with Student's *t*-test or with Welch's *t*-test after an initial analysis with the *F*-test. The level of statistical significance was taken as $P < 0.05$.

3. Results

3.1. Effects of H_2O_2 and NO donors on TER of Caco-2 cell monolayers

The TER of Caco-2 cell monolayers was measured to clarify the effect of H_2O_2 and NO on the integrity of the epithelial barrier. In all experiments shown here, cells were exposed to these reactive oxygen and nitrogen species on both apical and basolateral sides. When cells were exposed to 36 μM or higher doses of H_2O_2, TER declined gradually and reached a minimum plateau level after a 3-h incubation (Fig. 1A, TER is expressed as a percentage of the basal value). This reduction in the TER of Caco-2 monolayers induced by H_2O_2 was dose-dependent, and lasted at least for 6 h. The decomposition of H_2O_2 caused by the addition of catalase to the culture could not fully rescue the reduction in TER even after 10 min of incubation and the decomposition of H_2O_2 by the catalase after 40 min of incubation was no longer effective (Fig. 1B). On the other hand, NOC5 itself did not exhibit a significant reduction in TER at up to 1 mM (Fig. 1C). Interestingly, NOC5 counteracted the reduction in TER when it was administered simultaneously with H_2O_2 (36 μM), although the damage was not prevented totally by 4–30 μM NOC5 (Fig. 1D). Higher doses of NOC5 (60–1000 μM) gave almost the same results with 15 μM NOC5 and any additional protection by excess amounts of NOC5 was not observed (data not shown). Further protection could not be achieved also when 30 μM of NOC5 was administered 20 min prior to H_2O_2 (data not shown). Meanwhile, a protective effect on the H_2O_2-induced reduction in TER was also observed with NOC12, another NO donor with a slower decomposition rate (Fig. 1E). However, when the NO-generating ability of NOC5 was exhausted by incubating for prolonged periods prior to the coadministration of H_2O_2, the protective effects of NOC5 completely disappeared (Fig. 1E).

To confirm the protective effect of NO against the H_2O_2-induced reduction in TER, we repeated the analyses of TER of cell monolayers treated with a combination of 36 μM H_2O_2 and one of several doses of NOC5 or NOC12. Fig. 2 shows the means of relative TER after a 3-h treatment. H_2O_2 alone reduced the relative TER to 28% of the basal level. The simultaneous addition of NOC5 or NOC12 with H_2O_2 restored the TER up to around 50% of the basal level in a dose-dependent manner. Interestingly, the relative TER of mock-treated cell monolayers was reduced to 81% of the basal level, while it was kept at around 100% in the cell monolayers treated with 60 μM NOC12 alone. The relative TER of cell monolayers treated with 15 μM NOC5 alone was slightly higher than that of mock-treated cell monolayers also, although the difference was not statistically significant.

3.2. Effects of H_2O_2 and NO donors on paracellular permeability of Caco-2 cell monolayers

TER measurements not only indicate the integrity of the tight junctional barrier, but also reflect changes in membrane conductance [22]. As an independent measurement of tight junctional barrier integrity, we analyzed the paracellular passage of fluorescein-conjugated dextran with an average molecular weight of 3 kDa, which cannot penetrate the cellular membrane under physiological conditions. Cell monolayers were treated with combinations of 36 μM H_2O_2 and several doses of NO donors for 3 h and then assessed for paracellular permeability to fluorescein-dextran (Fig. 3). Compared to the mock-treatment, the addition of H_2O_2 greatly increased the paracellular permeability. Whereas, cell monolayers treated with H_2O_2 plus NO donors exhibited a paracellular permeability statistically equal to that of the mock-treated control cells. In contrast to the results of TER analyses, 4 μM NOC5 or 15 μM NOC12 was sufficient to totally prevent the H_2O_2-induced increase in paracellular permeability. In addition, administration of 15 μM NOC5 or 60 μM NOC12 alone significantly decreased the paracellular permeability from the control level. These results were consistent with the protective effect of NO on the barrier function of Caco-2 cell monolayers demonstrated by the TER analyses (Fig. 2), although the magnitude of the protective effect of NO on paracellular permeability was remarkably larger than that on TER.

3.3. Effects of H_2O_2 and NOC5 on the morphological integrity of TJs formed in Caco-2 cells

Next, we analyzed the subcellular localization of a tight junctional scaffolding protein ZO-1 to clarify the effects of H_2O_2 and NO on the morphological integrity of TJs in Caco-2 cell monolayers. In mock-treated control monolayers, ZO-1 is distributed continuously at the intercellular junctions (Fig. 4A). A discontinuous distribution of ZO-1 was observed in cell monolayers treated with 36 μM H_2O_2 for 3 h (Fig. 4B), whereas a combination of treatment with NOC5 (15 μM) and H_2O_2 rescued the localization of ZO-1 at intercellular junctions (Fig. 4C). Cell monolayers treated with NOC5 alone did not exhibit any difference in ZO-1 localization from mock-treated control cells (Fig. 4D). We found no morphological change to the nucleus after a 3-h incubation with 36 μM H_2O_2 and/or 15 μM NOC5 (data not shown).

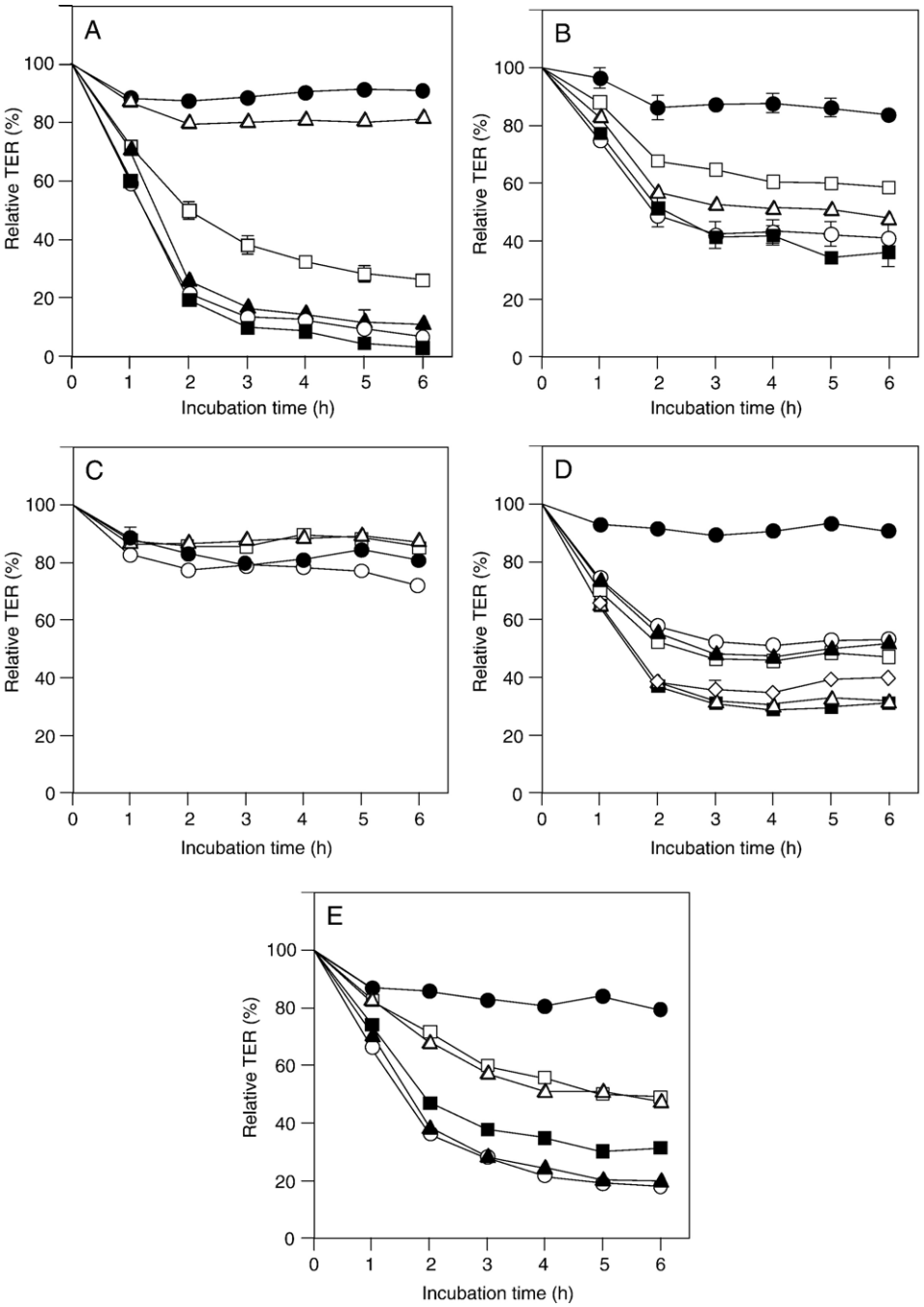

Fig. 1. Effects of H_2O_2 and NO donors on TER of Caco-2 cell monolayers. (A) H_2O_2 was administered to Caco-2 cell monolayers grown on Millicell inserts at a final concentration of either 0 μM (mock-treated control, closed circle), 12 μM (open triangle), 36 μM (open square), 75 μM (open circle), 150 μM (closed square), or 300 μM (closed triangle), and TER was determined at intervals of 1 h. (B) The cell monolayers exposed to H_2O_2 (36 μM) were treated with an excess of catalase in both apical and basolateral compartments to eliminate H_2O_2 after 10 (open square), 20 (open triangle), or 40 (open circle) min. As a reference, a mock treatment without H_2O_2 (control, closed circle) and treatment with 36 μM H_2O_2 without catalase (closed square) were also conducted. (C) NOC5 was added to Caco-2 cell monolayers at final concentrations of 0 μM (mock-treated control, closed circle), 63 μM (open triangle), 125 μM (not shown), 250 μM (open square), 500 μM (not shown), and 1000 μM (open circle), and TER was determined at 1-h intervals. Data for 125 and 500 μM NOC5 were left out of the figure to avoid confusion. (D) Various concentrations of NOC5 were added simultaneously with 36 μM H_2O_2 to Caco-2 cells. Final concentrations of NOC5 administered were 0 μM (closed square), 2 μM (open triangle), 4 μM (open diamond), 8 μM (open square), 15 μM (open circle), and 30 μM (closed triangle). As a reference, a mock treatment without H_2O_2 and NOC5 was also conducted (control, closed circle). (E) Caco-2 cell monolayers were simultaneously treated with NO donors and 36 μM H_2O_2. The NO donors were 15 μM (closed square) or 60 μM (open square) of NOC12, 15 μM of NOC5 (open triangle), and 15 μM of decomposed NOC5 (closed triangle) which could not generate NO any more. As a reference, a mock treatment (control, closed circle) and treatment with 36 μM H_2O_2 (open circle) were also conducted. In all cases, the resistance values were standardized to the initial TER (%) and expressed as the mean ± SE (SE bars smaller than 3% were not shown). $n=2$ for most data points, but $n=4$ for the data points of mock and 36 μM H_2O_2 treatment groups in graph (A).

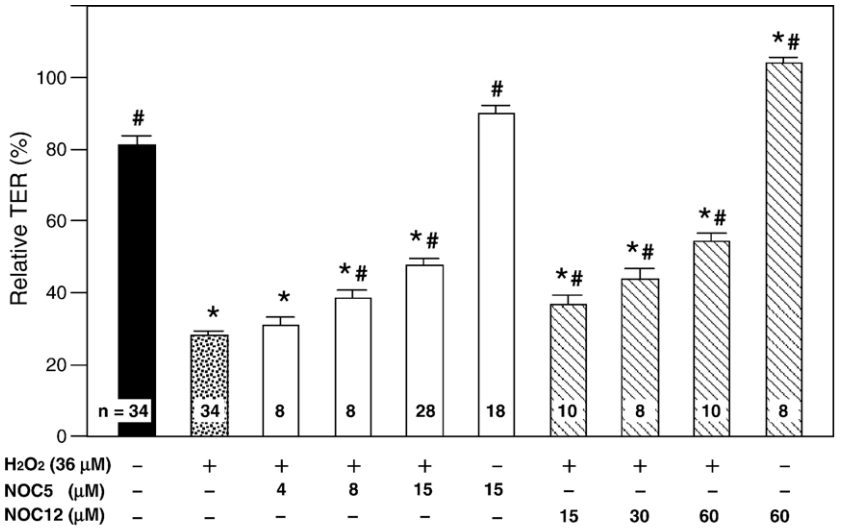

Fig. 2. Dose-dependent attenuation of H_2O_2-induced reduction in TER by NOC5 and NOC12. Caco-2 cell monolayers were treated with a combination of 36 μM H_2O_2 and one of several doses of NOC5 or NOC12. Every combination of H_2O_2 and NO donor was analyzed in at least three independent experiments with duplicate or triplicate samples. The mean of relative TER values after a 3-h treatment is shown with the SE. Total numbers of Millicell insets analyzed are shown at the bottom of each column. Concentrations of H_2O_2, NOC5, and NOC12 added are shown below the columns. ∗: significant difference from the mock treatment (filled column), $P < 0.05$. #: significant difference from H_2O_2 (36 μM) treatment (dotted column), $P < 0.05$.

3.4. Effects of H_2O_2 and NOC5 on protein tyrosine phosphorylation

Oxidative stress had been revealed to associate with increased tyrosine phosphorylation of cellular proteins in several tissues and cells [23–25]. Thus we asked if H_2O_2 induces protein tyrosine phosphorylation in Caco-2 cell monolayers. Fig. 5 shows a Western blot analysis of the phosphorylation of tyrosine residues in cellular proteins after treatment with H_2O_2 and/or NOC5. While the tyrosine phosphorylation level in mock-treated cells was low (lane 1), intense protein tyrosine phosphorylation of numerous cellular proteins was induced in cells treated with 36 μM H_2O_2 for 3 h (lane 2). On the other hand, the protein tyrosine phosphorylation

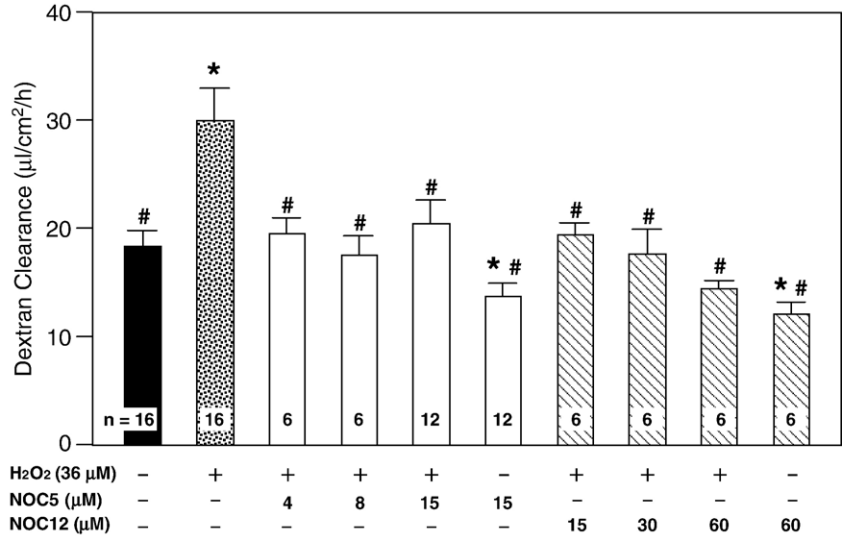

Fig. 3. Paracellular permeability of Caco-2 cell monolayers treated with H_2O_2 and/or NO donors. Cell monolayers on Millicell-HA were treated with a combination of 36 μM H_2O_2 and one of several dose of NOC5 or NOC12 for 3 h and then the apical-to-basolateral flux of fluorescein-dextran (3 kDa) was measured. Every combination of H_2O_2 and NO donor was analyzed in at least two independent experiments with duplicate or triplicate samples and the mean values of dextran clearance are shown. Total numbers of Millicell insets analyzed are shown at the bottom of each column. Concentrations of H_2O_2, NOC5, and NOC12 added are shown below the columns. The values were expressed as the mean±SE. ∗: significant difference from the mock treatment (filled column), $P < 0.05$. #: significant difference from H_2O_2 (36 μM) treatment (dotted column), $P < 0.05$.

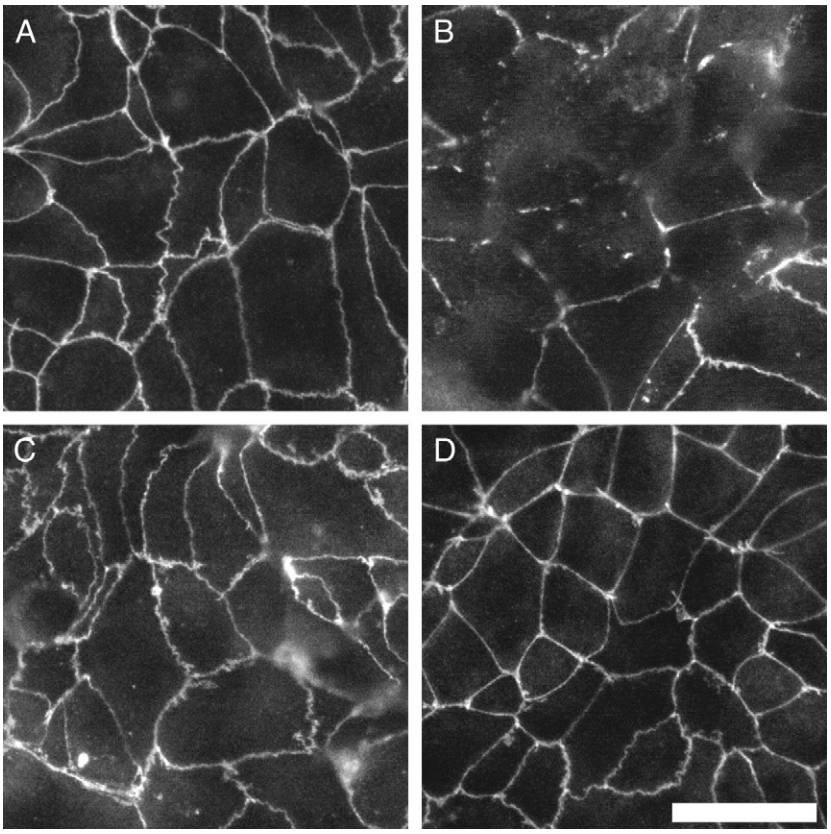

Fig. 4. Subcellular localization of a tight junctional scaffolding protein ZO-1 in H_2O_2 and/or NOC5-treated Caco-2 cells. Monolayers of Caco-2 cells on Millicell inserts were treated with H_2O_2 (36 μM) and/or NOC5 (15 μM) for 3 h and then processed for immunostaining of the ZO-1 protein. Cells treated with saline (A, mock treated control), H_2O_2 (B), H_2O_2 plus NOC5 (C), and NOC5 (D) are shown. Bar, 25 μm.

was remarkably suppressed in the cells administered H_2O_2 plus NOC5 (lane 3). NOC5 alone showed no effect on the tyrosine phosphorylation of cellular proteins (lane 4).

To further determine the effects of H_2O_2 and NO on cell–cell junctional proteins, cellular protein extracts of Caco-2 cell monolayers treated with H_2O_2 and/or NOC5 were subjected to immunoprecipitation using anti-E-cadherin or anti-ZO-1 antibodies and subsequently processed for Western blot analyses of tyrosine phosphorylation (Fig. 6). In anti-E-cadherin immunoprecipitates of either mock- or NOC5-treated cells, no proteins with tyrosine phosphorylation were detected (lanes 1 and 4 of Fig. 6A). In contrast, tyrosine phosphorylation of two proteins with the molecular masses expected for E-cadherin (120 kDa) and associated β-catenin (100 kDa) was induced in anti-E-cadherin immunoprecipitates of H_2O_2-treated cells (lane 2 of Fig. 6A). The H_2O_2-induced tyrosine phosphorylation of these two proteins was apparently diminished by simultaneous administration of NOC5 (lane 3 of Fig. 6A). Coimmunoprecipitation of the adherens junctional proteins E-cadherin and β-catenin was confirmed by reprobing the same filter with respective antibodies (Fig. 6B and C). Western blot analysis of anti-ZO-1 immunoprecipitates revealed that tyrosine phosphorylation of this tight junctional scaffolding protein was stimulated in response to H_2O_2 treatment (lane 2 of Fig. 6D), but not to mock or NOC5 treatments (lanes 1 and 4 of Fig. 6D). Furthermore, this H_2O_2-induced tyrosine phosphorylation of ZO-1 was mostly prevented by the simultaneous administration of NOC5, again (lane 3 of Fig. 6D).

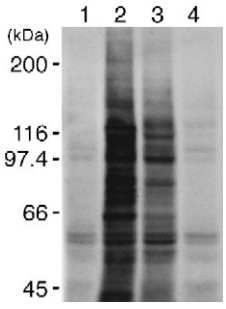

Fig. 5. Effect of H_2O_2 and NO on tyrosine phosphorylation of cellular proteins. Caco-2 cells on Millicell inserts were treated with H_2O_2 (36 μM) and/or NOC5 (15 μM) for 3 h and then lysed for cellular protein extract. Equal amounts of cellular proteins of mock- (lane 1), H_2O_2- (lane 2), H_2O_2 plus NOC5- (lane 3), and NOC5-treated (lane 4) cells were loaded and blotted with anti-phosphotyrosine antibody.

4. Discussion

4.1. Effects of H_2O_2 on intestinal barrier function

In this study, we examined the effects of H_2O_2 and NO on epithelial barrier function in Caco-2 cell monolayers cultured on

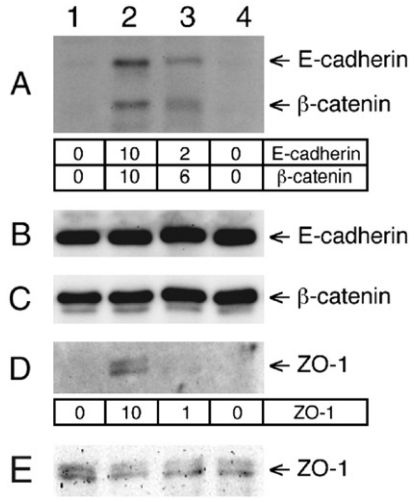

Fig. 6. Tyrosine phosphorylation of cell–cell junctional proteins in H_2O_2 and/or NOC5-treated cells. Caco-2 cells on Millicell inserts were treated with H_2O_2 (36 μM) and/or NOC5 (15 μM) for 3 h and then processed for immunoprecipitation of E-cadherin or ZO-1. The E-cadherin immunoprecipitate was analyzed by Western blotting using anti-phosphotyrosine antibody (A). The same filter was reprobed with anti-E-cadherin (B) and anti-β-catenin (C) antibodies. The ZO-1 immunoprecipitate was analyzed with anti-phosphotyrosine antibody (D) and reprobed with anti-ZO-1 antibody (E). The relative band intensities of tyrosine phosphorylated forms of E-cadherin, β-catenin, and ZO-1 were standardized to those in H_2O_2 treated cells as a value of 10 and are shown below each lane of (A) and (D). Lane 1, mock-treated cells; lane 2, H_2O_2-treated cells; lane 3, H_2O_2 plus NOC5-treated cells; lane 4, NOC5-treated cells.

permeable supports. Caco-2 is a human colonic epithelial cell line, which retains features of differentiated intestinal epithelial cells such as defined brush borders and formation of TJs, and has been used for a number of studies on the formation, regulation, and disorganization of the intestinal epithelial barrier [26,27]. Because this in vitro cell culture system is devoid of subepithelial cells, we are able to focus on the intrinsic functions of epithelial cells regarding the formation and dissociation of the intestinal barrier.

We showed here that H_2O_2 (36 μM) caused a reduction in TER, an increase in paracellular permeability, and a disruption of the morphological integrity of TJs in Caco-2 cell monolayers. These are remarkable features of barrier dysfunction. The damage to barrier function became irreversible within the initial 40 min after the administration of H_2O_2 (Fig. 1B). Our preliminary data indicated that H_2O_2, which was administered into a MEM-based medium at a final concentration of 36 μM, was completely eliminated within a half hour of incubation probably because of the H_2O_2 scavenging activity of an amino acid cysteine (data not shown) [28]. Thus, the initial 30 min after the administration of H_2O_2 is crucial to induce the barrier dysfunction of Caco-2 monolayers. On the other hand, nuclear staining with Hoechst 33258 revealed no typical signs of cell death induction among Caco-2 cells treated with 36 μM H_2O_2 (data not shown). Rao et al. reported that a 2-h treatment with 10 mM H_2O_2 caused a reduction in TER but did not cause cell lysis of Caco-2 cell monolayers [23]. Thus, a gross disruption of cell monolayers originating from cytotoxic oxidative damage to cellular components, such as proteins, lipids, sugars and nucleic acids, was not a probable cause for the barrier disruption induced by micromolar levels of H_2O_2.

We also showed that the H_2O_2-induced barrier dysfunction of Caco-2 monolayers was associated with an induction of protein tyrosine phosphorylation of numerous cellular proteins including components of TJs (ZO-1) and AJs (E-cadherin and β-catenin). Consistent with our observations, Rao et al. showed that the H_2O_2-induced barrier dysfunction is associated with tyrosine phosphorylation of a wide spectrum of proteins, including occludin (a component of TJs), ZO-1, E-cadherin, and β-catenin [23,29]. Moreover, they also showed that the H_2O_2-induced barrier dysfunction was inhibited by protein tyrosine kinase (PTK) inhibitors, and potentiated by a phosphotyrosine phosphatase (PTP) inhibitor [23]. It had been revealed previously that H_2O_2 can stimulate protein tyrosine phosphorylation by activating PTKs as well as by inhibiting PTPs [30] and that protein tyrosine phosphorylation is a major mechanism regulating the formation of TJs and AJs [31,32]. Taking these observations together, it is plausible that H_2O_2 modulates the intracellular signaling pathways of PTKs and/or PTPs, and stimulates tyrosine phosphorylation of numerous cellular proteins including occludin, ZO-1, E-cadherin, and β-catenin. The protein tyrosine phosphorylation might lead to the disassembly of TJs and AJs thereby causing the epithelial barrier dysfunction.

4.2. Effects of NO on intestinal barrier function

We demonstrated here that two types of NO donors attenuated the H_2O_2-induced barrier dysfunction in Caco-2 epithelial cell monolayers (Figs. 1D, E, 2, 3 and 4). The preventive effect of NO donors on the reduction in TER was dose dependent. A two to four times higher dose was necessary for NOC12 than NOC5 to achieve similar levels of prevention in the reduction of TER (Fig. 2). Although two molecules of NO are released from one molecule of either NOC5 or NOC12, the NO-releasing rate of NOC5 is nearly 5 times faster than that of NOC12 (half-lives are about 25 min and 120 min, respectively). Then, the gross amounts of NO released during the initial 20–30 min were nearly equivalent, when NOC12 was administered at a 4 times higher dose than NOC5. As described above, the initial 30 min after the administration of H_2O_2 is crucial to induce the barrier dysfunction. Furthermore, the administration of NOC5 prior to H_2O_2 did not have any additional effect concerning the protection of TER (data not shown). Thus, the protective effect against the H_2O_2-mediated reduction in TER was most likely dependent on the amount of NO released from the NO donors during the initial 20–30 min after the administration of H_2O_2. On the other hand, treatment with NOC5 or NOC12 alone seemed to increase TER as compared to the mock-treated control cell monolayers (Fig. 2). In fact, the relative TER of cells treated with either of the NO donors alone was never lower than that of the mock-treated control cells in any of the experiments. It is most likely attributable to protection from a stress involved in the mock treatment itself rather than to an enhancement of the basal barrier function for the following two reasons. First, TER was substantially reduced

from the basal level by the mock treatment itself in most experiments. Second, TER was kept at the basal level by NO donors in some experiments but never increased beyond the basal level even when cells were treated with relatively high doses of NOC5 (63 μM–1.0 mM, Fig. 1C).

Concerning the effects of NO on the barrier function, the results of the paracellular permeability analyses were mostly consistent with those of the TER analyses, except that NO was much more efficacious in preventing the increase in paracellular permeability than the reduction in TER. Hence, dose-dependency of the protective effect of NO donors on paracellular permeability could not be observed clearly over the dose ranges examined (Fig. 3). Although, both TER and paracellular permeability are considered as measures of epithelial barrier function, the former reflects the permeability to ions at a given time point whereas the latter reflects the permeability to relatively large molecules over a period of time. These two parameters are regulated in a different manner under some circumstances [20,33,34]. It is possible that H_2O_2 induces paracellular pores, which are wide enough to allow ions as well as large molecules to diffuse through, and that NO makes the pores narrower and prevents the diffusion of large molecules but partly allows the diffusion of ions.

NO is a pleiotropic free radical messenger molecule synthesized by the three isoforms of NOS [7,35,36]. The two constitutive isoforms, nNOS and eNOS, were originally identified in neuronal and endothelial tissues, respectively, and are regulated at the post-translational level. The third isoform iNOS is regulated at the transcriptional level and induced in response to certain cytokines, microbes, and other activating stimuli. Both constitutive and inducible NOSs are expressed in many cells within the intestinal mucosa including epithelial cells [37]. NO participates in numerous essential functions of the intestinal mucosa [17,18]. However, in the presence of mucosal inflammation, NO can be either detrimental or beneficial probably depending on the amount, duration, and anatomical site of synthesis. Recent studies on animals, including genetically modified mice deficient in various NOS isoforms, have shown that constitutive and inducible NO production seems to be beneficial during acute inflammation probably because of its subepithelial activities that maintain mucosal blood flow, inhibit platelet and leukocyte adhesion and aggregation within the vasculature, and modulate mast cell reactivity [17]. The results presented here strongly suggest that in addition to such actions on subepithelial cells, a direct action of NO on epithelial cells may also be involved in the protective effects of NO against epithelial barrier dysfunction during acute inflammation.

4.3. Possible mechanisms for protective effects of NO on epithelial barrier function

As discussed above, modulation of the intracellular signaling pathways of PTKs and/or PTPs is most likely a major mechanism for the H_2O_2-induced barrier dysfunction of Caco-2 monolayers. We revealed here that NO could attenuate the H_2O_2-induced protein tyrosine phosphorylation as well as the H_2O_2-induced barrier dysfunction of Caco-2 monolayers. Since NO is diffusible and can react directly with signaling proteins [7], it might mediate intracellular signaling that inhibits or interrupts the H_2O_2-mediated PTK and/or PTP signaling pathways leading to barrier dysfunction, although the target signaling molecules of NO remain obscure. Activation of soluble guanylate cyclase (sGC) by binding to its heme prosthetic group is the most widely recognized mechanism for NO-mediated signal transduction [38]. Activation of sGC results in an increase in production of guanosine 3′,5′-cyclic monophosphate (cGMP) leading to the activation of cGMP-dependent protein kinases. Therefore we examined whether cGMP is involved in the NO-mediated protection of the epithelial barrier of Caco-2 monolayers using a permeable analogue of cGMP and obtained negative results (our unpublished data). S-nitrosylation of cysteine and nitration of tyrosine are other possible modes for NO-mediated modulation of signaling proteins [7,39]. PTPs have been reported to be at least partially regulated by S-nitrosylation and may be the direct targets of NO-mediated signal transduction opposing barrier dysfunction [40].

Alternatively, NO may protect the epithelial barrier by inhibiting the formation of toxic oxidants originating from H_2O_2 or by scavenging them. It was shown that NO could scavenge lipid radicals, hence terminating the lipid peroxidation chain reaction [41–43]. As mentioned above, a gross disruption of cell monolayers by cell lysis and/or cell death arising from oxidative damage to lipids was not a probable cause for H_2O_2-induced barrier disruption. However, it is possible that lipid peroxidation might associate with the intracellular signaling pathways of the PTKs and/or PTPs and modulate the state of protein tyrosine phosphorylation, which ultimately leads to barrier dysfunction. NO was also shown to be able to inhibit the iron-catalyzed oxidation reaction (Fenton reaction) that produces powerful ROS such as hydroxyl radicals ($^{\cdot}OH$) by acting as an iron chelator [42,44,45]. Although the toxicity of H_2O_2 and superoxide anion ($^{\cdot}O_2^-$) had been often attributed to their conversion to $^{\cdot}OH$, it was shown that H_2O_2 itself is responsible for the barrier dysfunction of Caco-2 monolayers and that neither $^{\cdot}O_2^-$ nor $^{\cdot}OH$ affects the barrier [46]. Thus, the inhibition of the iron-catalyzed oxidation reaction by NO is not likely the cause of the protective effect of NO on H_2O_2-induced barrier dysfunction.

The involvement of NO in biology was assumed for many years to be restricted to damaging reactions. Because NO is often produced along with ROS within tissues with inflammation or injury, a synergistic relationship between the cytotoxic effects of NO and ROS is assumed. However, NO has been established as a pleiotropic messenger in recent years. It also appears that NO can function as a protective agent against ROS-induced cellular damage in some circumstances. The present study demonstrates that NO protects the barrier of Caco-2 intestinal epithelial cells against H_2O_2-induced damage. Since NO also reduces induction of tyrosine phosphorylation of numerous cellular proteins including components of TJs and AJs, NO-mediated intracellular signal transduction must be involved in this protective function. Because our in vitro

experimental system is very useful for analyzing cellular and molecular effects of NO on intestinal epithelia, further studies on this system will provide new insights into the molecular mechanisms of the beneficial function of NO in inflammatory bowel diseases.

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