Biochemical and Biophysical Research Communications 376 (2008) 154–157

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Oxidative stress induces gastric epithelial permeability through claudin-3

Kayo Hashimoto [a], Tadayuki Oshima [a,*], Toshihiko Tomita [a], Yongmin Kim [a], Takayuki Matsumoto [b], Takashi Joh [c], Hiroto Miwa [a]

[a] Division of Upper Gastroenterology, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan
[b] Division of Lower Gastroenterology, Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan
[c] Department of Internal Medicine and Bioregulation, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho, Nagoya, Aichi 467-8601, Japan

ARTICLE INFO

Article history:
Received 15 August 2008
Available online 5 September 2008

Keywords:
Tight junction
Rebamipide
Permeability
Claudin-3
siRNA

ABSTRACT

Although reactive oxygen species have been implicated as mediators of gastrointestinal injury, their influence on the function of gastric epithelial tight junctions (TJs), which create a paracellular permeability barrier, needs to be fully investigated. H_2O_2 exposure to MKN28 gastric epithelial monolayers caused a significant decrease in trans-epithelial electrical resistance (TEER) and a significant increase in dextran permeability. Oxidant-mediated gastric epithelial permeability was significantly attenuated by a radical scavenger, rebamipide. H_2O_2 decreased the amount of claudin-3 protein but not claudin-4, -7, and JAM-A. Rebamipide significantly attenuated H_2O_2-induced decrease in claudin-3 protein. Small interfering RNA (siRNA) against claudin-3 treatment specifically decreased claudin-3 as seen by immunoblotting and immunofluorescent staining. Gastric TEER was significantly decreased with the treatment of siRNA against claudin-3. This is the first study to demonstrate that claudin-3 is involved in the barrier function of gastric epithelial cells and that rebamipide abolishes the H_2O_2-induced decrease in claudin-3 protein.

Tight junctions (TJs) are the most apical component of the intercellular junctional complexes [1]. TJs separate the apical cell surface domains from the basolateral cell surface domains to establish cell polarity and provide a barrier, regulating paracellular transport of solutes and ions [2]. TJs consist of occludin, the claudin family of proteins, and junctional adhesion molecule (JAM) [3]. Among these TJ molecules, claudins are major integral membrane proteins of TJ strands [3]. Claudin-3 and claudin-4 appear to be phosphorylated by cyclic AMP-dependent kinase (PKA) and protein kinase C, respectively [4]. Phosphorylation of claudin-3 may cause partial redistribution of claudin-3 from the TJs to other membrane or cytoplasmic areas and may provide a mechanism for the decreased TJ strength [4].

Hydrogen peroxide (H_2O_2), a highly toxic oxidizing agent, is constantly generated within all cell types, including gastric epithelial cells, and must be quickly neutralized in order for cell survival [5]. H_2O_2 is also reported to increase epithelial monolayer permeability by disrupting paracellular junctional complexes *in vitro* [6]. *Helicobacter pylori* (*H. pylori*) infection stimulates the accumulation of intracellular reactive oxygen species, and gastric permeability in *H. pylori*-induced chronic gastritis is impaired. This decreased barrier function is paralleled by a decrease in TJ proteins [7].

Rebamipide is a gastroprotective agent used to treat gastritis and gastric ulcers. Rebamipide has been reported to provide gastroprotection through scavenging oxygen-derived free radicals [8] and prevent various experimental models of delayed wound repair *in vitro* [9] as well as *in vivo* [10]. However, involvement of claudins in oxygen radical-mediated gastric epithelial permeability and the effect of rebamipide on the gastric epithelial barrier function have not been fully elucidated.

In the present study, we examined the influence of H_2O_2 on gastric epithelial barrier properties and TJ organization, and clarified which claudins are affected by H_2O_2. We also investigated the effect of rebamipide on H_2O_2-induced increases in gastric epithelial permeability and on TJ organization.

Materials and methods

Cell culture. MKN28, a human gastric adenocarcinoma cell line, was obtained from Immuno-Biological Laboratories Co., Ltd. (Takasaki, Japan). RPMI-1640 (Sigma, St. Louis, MO, USA) was supplemented with 10% FBS. Culture medium was changed every 48–72 h.

Reagents and antibodies. Rebamipide, 2-(4-chlorobenzoylamino) -3-[2-(1H)-quinolinone-4-yl]propionic acid, was obtained from Otsuka Pharmaceutical Co. (Tokushima, Japan). Rabbit anti-claudin-3, mouse anti-claudin-4, rabbit anti-claudin-7, and rabbit anti-JAM-A antibodies were purchased from Invitrogen Laboratories (South San Francisco, CA, USA). Alexa488-conjugated anti-mouse IgG

* Corresponding author. Fax: +81 798 45 6661.
E-mail address: t-oshima@hyo-med.ac.jp (T. Oshima).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.08.140

antibody was obtained from Invitrogen (Carlsbad, CA, USA). Cy3-conjugated goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (Westgrove, PA, USA).

Trans-epithelial electrical resistance (TEER). MKN28 cells were plated on tissue culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA). The resistance across the monolayer was measured using MILLICELL-ERS (Millipore Corporation, Bedford, MA, USA). At 100% confluence, TEER was measured. The change in electrical resistance was represented by baseline resistance [11]. When resistance was stable (at >280 Ω cm^2), the culture medium from the upper (apical) compartment was replaced with medium containing H_2O_2. In some experiments, monolayers were pretreated with rebamipide for 60 min and H_2O_2 was added in the presence of rebamipide. Cell viability was assessed by measuring the release of lactate dehydrogenase (LDH) (LDH assay; Sigma) into the media. All data represent the average of three identically treated monolayers.

Western analysis of cell lysates. Equal quantities of protein were separated on 8% SDS–PAGE. Gels were transferred to nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL, USA). The membrane was incubated with the appropriate primary and HRP-conjugated secondary antibody. The membrane was developed using the enhanced chemiluminescence (ECL) detection system (Amersham).

Epithelial permeability. Fluorescein isothiocyanate labeled dextran (FITC-dextran) (MW 4000) was used as a permeable tracer that passes across the epithelial monolayer [12]. MKN28 cells were grown on the tissue culture inserts. HBSS containing 5 mg/ml FITC-dextran was put into the luminal chamber. A 100 μl sample was taken from the lower chamber and the absorbance of FITC-dextran was determined at 492 nm using a microplate fluorometer (Fluoroskan Ascent). The data are represented by permeability index [12].

Immunofluorescence staining of junctional proteins. MKN28 cells were seeded onto coverslips. Samples were fixed with ethanol and acetone [13] and stained for claudin-3 and claudin-4. Slides were viewed using a fluorescence microscope.

Small interfering RNA (siRNA). For siRNA silencing of human claudin-3, ON-TARGET *plus SMART*pool and a non-specific control siRNA was purchased from Dharmacon, Inc. (Lafayette, CO, USA). Cells were transfected with 100 nM siRNA using Dharma*FECT*™ 4 (Dharmacon, Inc.). Assays were performed 3 days after MKN28 cells were transfected.

Statistical analysis. All data are expressed as means ± SE. Data were analyzed using one-way ANOVA with Bonferroni's correction for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Effect of rebamipide on H_2O_2-induced gastric epithelial permeability

H_2O_2 treatment of gastric epithelial monolayers caused a significant decrease in TEER at 4, 8, and 24 h (Fig. 1A). There was no evidence of cytotoxic effects with H_2O_2 (1–500 μM) at any time point, as determined by the LDH release assay (data not shown). Pretreatment with rebamipide dose dependently inhibited the H_2O_2-induced decrease in TEER. Treatment of monolayers with rebamipide did not influence the baseline TEER. H_2O_2 significantly increased the FITC-dextran permeability (Fig. 1B). Rebamipide significantly attenuated the H_2O_2-induced increase in permeability. Rebamipide (400 μM, 24 h) itself did not have any effect on the permeability.

Effect of rebamipide on H_2O_2-induced change in TJ proteins

The quantity of claudin-3 but not that of claudin-4 protein was significantly lower after H_2O_2 treatment. (Fig. 2A) The quantities of claudin-3 and -4 were not affected by rebamipide treatment (data not shown). H_2O_2-induced decrease in the quantity of claudin-3

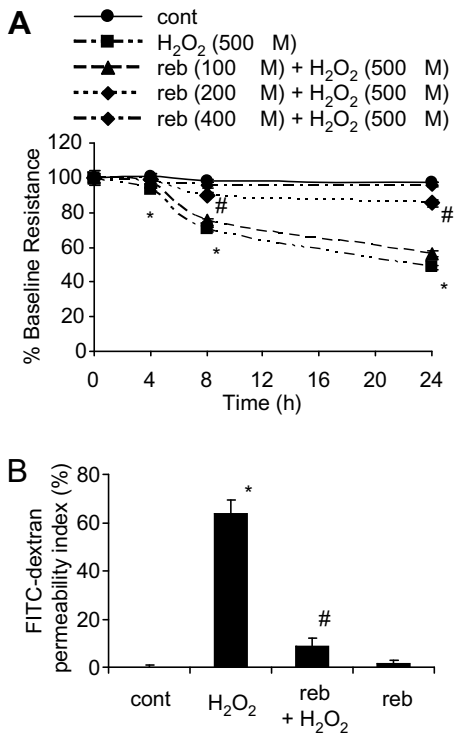

Fig. 1. Effects of rebamipide on H_2O_2-induced gastric epithelial permeability. (A) H_2O_2 treatment of gastric epithelial monolayers caused a significant decrease in TEER at 4, 8, and 24 h. Rebamipide (reb) pretreatment dose dependently and significantly inhibited H_2O_2-induced decrease in TEER ($n = 3$). (B) H_2O_2 (500 μM, 24 h) significantly increased the FITC-dextran permeability. H_2O_2-induced permeability was significantly blocked by rebamipide (400 μM) pretreatment ($n = 3$). $^{*}P < 0.05$ vs. untreated control (cont). $^{\#}P < 0.05$ vs. H_2O_2.

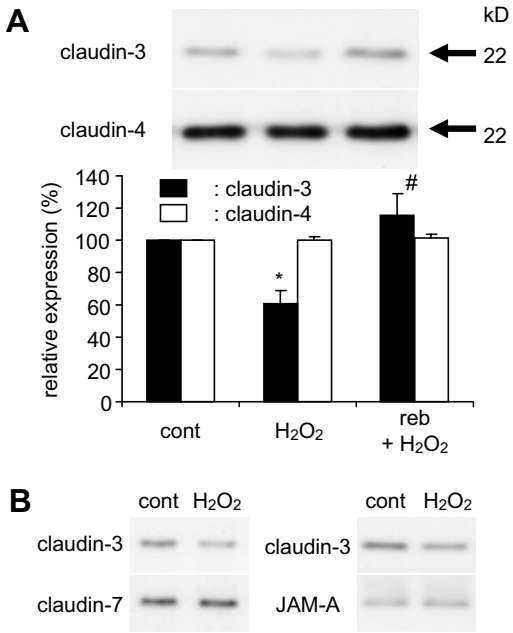

Fig. 2. Effect of rebamipide on H_2O_2-induced changes in TJ proteins. (A) The quantity of claudin-3 protein but not that of claudin-4 was significantly reduced by H_2O_2 (500 μM, 24 h) and this decrease was significantly blocked by rebamipide (400 μM) pretreatment ($n = 3$). $^{*}P < 0.01$ vs. control (cont); $^{\#}P < 0.01$ vs. H_2O_2. (B) Claudin-3 but not claudin-7 or JAM-A protein decreased with H_2O_2 treatment.

was attenuated by rebamipide pretreatment. Neither claudin-7 nor JAM-A was affected by H_2O_2 treatment (Fig. 2B).

Claudin-3 and -4 were stained at junctions under control conditions, as determined by immunofluorescence staining (Fig. 3). Both claudin-3 and -4 were co-localized at the junctions. H_2O_2 treatment decreased claudin-3 staining and this decrease was abolished by rebamipide pretreatment (Fig. 3). Claudin-4 staining was not affected by H_2O_2 treatment.

siRNA against claudin-3

siRNA against claudin-3 treatment specifically decreased claudin-3 protein (Fig. 4A). Claudin-4 protein was not affected by siRNA against claudin-3. Although claudin-3 immunostaining was markedly decreased in monolayers treated with claudin-3 siRNA, this siRNA had no effect on claudin-4 (Fig. 4B). Gastric epithelial TEER was significantly decreased with clauidn-3 siRNA treatment but not by a control siRNA (Fig. 4C).

Discussion

Reactive oxygen species (ROS), e.g., H_2O_2 and the superoxide radical (O_2^-), play a causative role in gastric injury and cancer. *H. pylori* infection stimulates the accumulation of the intracellular ROS in different human gastric epithelial cell lines [14,15]. The loss of TJ barrier function by deleterious inflammatory mechanisms is an important concept in gastrointestinal pathophysiology. Epithelial cell TJs from different origins are affected by H_2O_2 [16,17]. Thus, this study aims to define the mechanism behind oxidative stress-induced barrier dysfunction and to determine whether the radical scavenger, rebamipide, can prevent epithelial barrier dysfunction induced by oxidative stress. Among ROS, H_2O_2 can remain inside the cell for a long time and often performs the role of a second messenger for various physiological stimuli such as inflammatory cytokines and growth factors [18].

There are no reports of H_2O_2 changing the amount of claudins. Interestingly, in this study the amount of claudin-3, but not of claudin-4, -7 or JAM-A was decreased by H_2O_2 in gastric epithelial cells. Claudin-4, -7 or JAM-A may be still important for the barrier function of gastric epithelial cells. Epithelial barrier disruption is reportedly induced by claudin-4 removal from TJs [19]. We previously reported that H_2O_2-induced colonic epithelial permeability was developed by the dislocation of claudin-4 protein from TJ through the activation of p38 MAPK [16]. Also, JAM-A has been recently reported to regulate intestinal permeability *in vivo* and *in vitro* [20]. Loss of claudin-4 or -7, but not of claudin-1 or -3, by siRNA significantly decreased TEER in Madin–Darby canine kidney (MDCK) cells [21]. Concerning gastric epithelial cell TJ proteins, claudin-7 is reportedly to be increased in gastric cancer [22]. However, the physiological function of claudin-7 in gastric epithelial cells is still unclear. Moreover, there has been no report investigating the function of JAM-A in gastric epithelial cells.

There are no reports that claudin-3 is directly involved in gastric mucosal permeability. However, EGF-induced increases in claudin-1, -3, and -4 proteins provides a threefold increase in TEER [23] and indirectly indicates that claudin-3 is involved

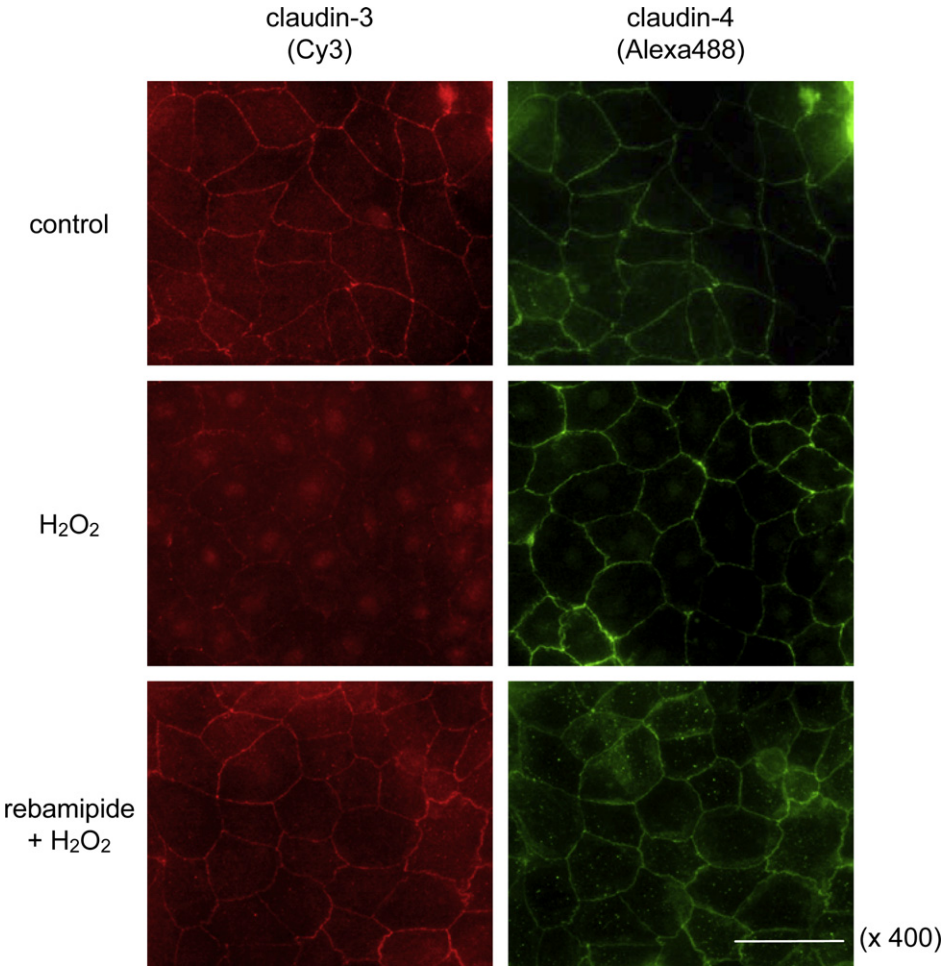

Fig. 3. Immunofluorescent staining of claudin-3 and claudin-4. Both claudin-3 and claudin-4 were co-localized at the junctions. H_2O_2 treatment decreased the staining of claudin-3 but not that of claudin-4. Rebamipide pretreatment blocked the H_2O_2-induced decrease in claudin-3 staining. Bar = 50 μm.

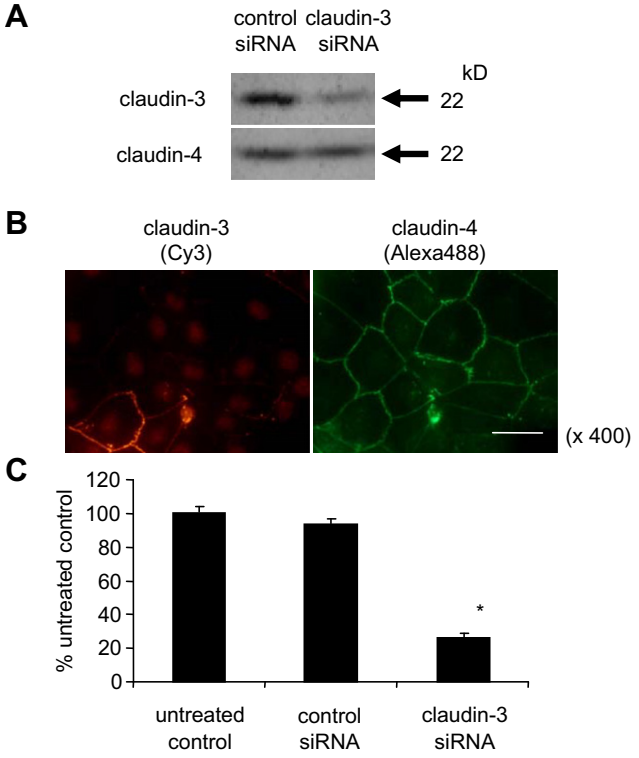

Fig. 4. siRNA against claudin-3. Claudin-3 siRNA specifically reduced the amount of claudin-3 protein shown by immunoblotting (A) and reduced the staining of claudin-3 (B). Claudin-4 protein was not affected by claudin-3 siRNA. (C) Claudin-3 siRNA significantly reduced the gastric epithelial TEER ($n = 3$). Bar = 50 μm. $^{*}P < 0.01$ vs. untreated control or control siRNA.

in barrier function. In the present study, we demonstrated claudin-3 knockdown by siRNA actually decreased the amount of claudin-3 and gastric epithelial TEER. These data indicate that claudin-3 is indeed involved in the TJ formation of gastric epithelial cells. It has been suggested that H_2O_2 can activate PKA and claudin-3 but not claudin-4 can be phosphorylated by PKA [4,24]. Phosphorylation of claudin-3 may provide a mechanism for the decreased TJ strength. The interaction of phosphorylation status and the degradation of claudins was not investigated in this study. The mechanisms of H_2O_2-induced decreases in claudin-3, but not other claudins, are still unclear and need to be elucidated.

The antiulcer effect of rebamipide is believed to be related to the inhibition of the production of ROS. Rebamipide was one of the most potent hydroxyl radical scavengers studied by electron spin resonance [8]. In this study rebamipide significantly reduced gastric epithelial permeability induced by H_2O_2 and rebamipide itself does not have any effect on the TJ proteins. Although we did not directly show that rebamipide scavenges hydroxyl radicals, it is possible that hydroxyl radicals generated from H_2O_2 in gastric mucosa might be related to these changes and that hydroxyl radical scavenging by rebamipide might contribute to its protective effect. Recently it has been suggested that aspirin-induced gastric epithelial permeability and disruption of ZO-1 are prevented by rebamipide [25]. ZO-1 is a scaffold protein of TJ and may influences the TJ proteins on the cell surface including claudins. The report, however, did not mention about claudins.

To our knowledge, this is the first report demonstrating that H_2O_2 increases gastric epithelial permeability through claudin-3 degradation and that H_2O_2-induced increases in gastric permeability and claudin-3 degradation are abolished by rebamipide.

References

[1] M.G. Farquhar, G.E. Palade, Junctional complexes in various epithelia, J. Cell Biol. 17 (1963) 375–412.
[2] S. Tsukita, M. Furuse, Pores in the wall: claudins constitute tight junction strands containing aqueous pores, J. Cell Biol. 149 (2000) 13–16.
[3] S. Tsukita, M. Furuse, M. Itoh, Multifunctional strands in tight junctions, Nat. Rev. Mol. Cell Biol. 2 (2001) 285–293.
[4] T. D'Souza, R. Agarwal, P.J. Morin, Phosphorylation of claudin-3 at threonine 192 by cAMP-dependent protein kinase regulates tight junction barrier function in ovarian cancer cells, J. Biol. Chem. 280 (2005) 26233–26240.
[5] D.A. Parks, Oxygen radicals: mediators of gastrointestinal pathophysiology, Gut 30 (1989) 293–298.
[6] R.K. Rao, R.D. Baker, S.S. Baker, A. Gupta, M. Holycross, Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation, Am. J. Physiol. 273 (1997) G812–G823.
[7] J.P. Fedwick, T.K. Lapointe, J.B. Meddings, P.M. Sherman, A.G. Buret, *Helicobacter pylori* activates myosin light-chain kinase to disrupt claudin-4 and claudin-5 and increase epithelial permeability, Infect. Immun. 73 (2005) 7844–7852.
[8] T. Yoshikawa, Y. Naito, S. Nakamura, S. Nishimura, T. Kaneko, S. Iinuma, S. Takahashi, M. Kondo, K. Yamasaki, Effect of rebamipide on lipid peroxidation and gastric mucosal injury induced by indometacin in rats, Arzneimittelforschung 43 (1993) 1327–1330.
[9] S. Watanabe, X.E. Wang, M. Hirose, T. Osada, H. Tanaka, N. Sato, Rebamipide prevented delay of wound repair induced by hydrogen peroxide and suppressed apoptosis of gastric epithelial cells in vitro, Dig. Dis. Sci. 43 (1998) 107S–112S.
[10] M. Suzuki, S. Miura, M. Mori, A. Kai, H. Suzuki, D. Fukumura, M. Suematsu, M. Tsuchiya, Rebamipide, a novel antiulcer agent, attenuates *Helicobacter pylori* induced gastric mucosal cell injury associated with neutrophil derived oxidants, Gut 35 (1994) 1375–1378.
[11] T. Oshima, F.S. Laroux, L.L. Coe, Z. Morise, S. Kawachi, P. Bauer, M.B. Grisham, R.D. Specian, P. Carter, S. Jennings, D.N. Granger, T. Joh, J.S. Alexander, Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function, Microvasc. Res. 61 (2001) 130–143.
[12] T. Oshima, S.C. Flores, G. Vaitaitis, L.L. Coe, T. Joh, J.H. Park, Y. Zhu, B. Alexander, J.S. Alexander, HIV-1 Tat increases endothelial solute permeability through tyrosine kinase and mitogen-activated protein kinase-dependent pathways, Aids 14 (2000) 475–482.
[13] T. Oshima, O. Blaschuk, B. Gour, M. Symonds, J.W. Elrod, M. Sasaki, T.H. Jackson, J.S. Alexander, Tight junction peptide antagonists enhance neutrophil trans-endothelial chemotaxis, Life Sci. 73 (2003) 1729–1740.
[14] D. Bagchi, T.R. McGinn, X. Ye, M. Bagchi, R.L. Krohn, A. Chatterjee, S.J. Stohs, *Helicobacter pylori*-induced oxidative stress and DNA damage in a primary culture of human gastric mucosal cells, Dig. Dis. Sci. 47 (2002) 1405–1412.
[15] S.Z. Ding, Y. Minohara, X.J. Fan, J. Wang, V.E. Reyes, J. Patel, B. Dirden-Kramer, I. Boldogh, P.B. Ernst, S.E. Crowe, *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells, Infect. Immun. 75 (2007) 4030–4039.
[16] T. Oshima, M. Sasaki, H. Kataoka, H. Miwa, T. Takeuchi, T. Joh, Wip1 protects hydrogen peroxide-induced colonic epithelial barrier dysfunction, Cell. Mol. Life Sci. 64 (2007) 3139–3147.
[17] M.A. Jepson, Disruption of epithelial barrier function by H2O2: distinct responses of Caco-2 and Madin–Darby canine kidney (MDCK) strains, Cell. Mol. Biol. (Noisy-le-grand) 49 (2003) 101–112.
[18] Y.S. Bae, S.W. Kang, M.S. Seo, I.C. Baines, E. Tekle, P.B. Chock, S.G. Rhee, Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation, J. Biol. Chem. 272 (1997) 217–221.
[19] N. Sonoda, M. Furuse, H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, S. Tsukita, Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier, J. Cell Biol. 147 (1999) 195–204.
[20] M.G. Laukoetter, P. Nava, W.Y. Lee, E.A. Severson, C.T. Capaldo, B.A. Babbin, I.R. Williams, M. Koval, E. Peatman, J.A. Campbell, T.S. Dermody, A. Nusrat, C.A. Parkos, JAM-A regulates permeability and inflammation in the intestine in vivo, J. Exp. Med. 204 (2007) 3067–3076.
[21] J. Hou, A.S. Gomes, D.L. Paul, D.A. Goodenough, Study of claudin function by RNA interference, J. Biol. Chem. 281 (2006) 36117–36123.
[22] J.Y. Park, K.H. Park, T.Y. Oh, S.P. Hong, T.J. Jeon, C.H. Kim, S.W. Park, J.B. Chung, S.Y. Song, S. Bang, Up-regulated claudin 7 expression in intestinal-type gastric carcinoma, Oncol. Rep. 18 (2007) 377–382.
[23] A.B. Singh, R.C. Harris, Epidermal growth factor receptor activation differentially regulates claudin expression and enhances transepithelial resistance in Madin–Darby canine kidney cells, J. Biol. Chem. 279 (2004) 3543–3552.
[24] X.C. Bai, D. Lu, A.L. Liu, Z.M. Zhang, X.M. Li, Z.P. Zou, W.S. Zeng, B.L. Cheng, S.Q. Luo, Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast, J. Biol. Chem. 280 (2005) 17497–17506.
[25] T. Suzuki, N. Yoshida, N. Nakabe, Y. Isozaki, H. Kajikawa, T. Takagi, O. Handa, S. Kokura, H. Ichikawa, Y. Naito, H. Matsui, T. Yoshikawa, Prophylactic effect of rebamipide on aspirin-induced gastric lesions and disruption of tight junctional protein zonula occludens-1 distribution, J. Pharmacol. Sci. 106 (2008) 469–477.