Food and Chemical Toxicology 108 (2017) 203–213

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Oxyresveratrol improves tight junction integrity through the PKC and MAPK signaling pathways in Caco-2 cells

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ARTICLE INFO

Article history:
Received 20 May 2017
Received in revised form
14 July 2017
Accepted 1 August 2017
Available online 2 August 2017

Keywords:
Oxyresveratrol
Tight junctions
Protein kinase C
Mitogen-activated protein kinase
Intestinal permeability

ABSTRACT

Strengthening intestinal tight junctions (TJ) provides an effective barrier from the external environment and is important for recovery from inflammatory bowel disease. Oxyresveratrol (OXY), an isomer of hydroxylated resveratrol, is isolated from many plants. The aim of this study was to investigate the effect of OXY on intestinal TJ and to elucidate the mechanism underlying the OXY-mediated increase in TJ integrity in human intestinal Caco-2 cells. OXY-treated Caco-2 cell monolayers showed decreased monolayer permeability as evaluated by paracellular transport assay. The results showed that OXY significantly increased the levels of TJ-related genes and proteins (Claudin-1, Occludin and ZO-1) compared with those of the negative control. OXY activated protein kinase C (PKC) and increased expression levels of mitogen-activated protein kinase (MAPK) genes. OXY also increased gene and protein levels of the transcription factor Cdx-2. Expression levels of TJ, PKC and Cdx-2 proteins and trans-epithelial electrical resistance (TEER) value decreased in OXY-treated Caco-2 cells following treatment with a pan-PKC inhibitor compared with those of the untreated control. In conclusion, OXY strengthens the integrity of the intestinal TJ barrier via activation of the PKC and MAPK pathways.

1. Introduction

The intestinal barrier is involved in the movement of nutrients in food and water or ions, and it plays a defensive role against inflammation (Groschwitz and Hogan, 2009). Solute movement through the intestinal barrier occurs via two major mechanisms, transepithelial/transcellular and paracellular pathways (Tsukita et al., 2001). The transepithelial/transcellular pathway regulates the transport of amino acids, which is controlled by selective transporters, through epithelial cells (Bröer, 2008). The paracellular pathway plays an important role in solute movement between the apical/mucosal and basolateral/serosal fluid compartments through regulation of tight junctions (TJ) (Van Itallie and Anderson, 2006). Although it is not clear whether large molecules are freely transported through the intestinal barrier under inflammatory conditions, the flow of large molecules is restricted by the intestinal barrier in a healthy state (Menard et al., 2010). In addition, it maintains immune response homeostasis by regulating the transport of food-derived antigens, microorganisms and toxins (Izcue et al., 2006; Strobel and Mowat, 1998). Intestinal barrier deficiencies could lead to an increase in permeability, causing uptake of intestinal microbial antigens and pathogenic factors (Cani et al., 2008). These functional defects induce gastrointestinal diseases, such as inflammatory bowel disease (IBD), which consists of two major types, Crohn's disease (CD) and ulcerative colitis (UC), and another functional bowel disorders such as irritable bowel syndrome (IBS) (Camilleri et al., 2012). However, the mechanisms underlying epithelial breakdown-induced diseases remain unclear.

Intestinal epithelial cells (IECs) form a barrier between the intestinal lumen and host connective tissue. Furthermore, IECs secrete various antimicrobial peptides, cathelicidins and calprotectins, which play a role in maintaining intestinal immune homeostasis (Artis, 2008). IECs also secrete pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-

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http://dx.doi.org/10.1016/j.fct.2017.08.002
0278-6915/

1 (IL-1) and chemokines, including IL-8, growth-regulated oncogenes (GROs) and epithelial neutrophil-activating peptide 78 (ENA-78), in response to pathogenic stimuli. These responses increase host resistance against invading pathogens and host mucosal defense (Kagnoff and Eckmann, 1997). IEC monolayers in the intestine are sealed by different protein complexes, including adherens junctions (AJs), desmosomes and TJ, which have different functions (Suzuki, 2013). AJs and desmosomes in the barrier provide mechanical connections for intestinal epithelial cells and determine each cell's apical-basal axis. In contrast, TJ form a continuous barrier between adjacent cells, which regulates the permeability of molecules through the paracellular space (Gumbiner, 1993). In particular, TJ have an important role in sealing the paracellular space and are dynamic structures controlled by cytoskeleton activity (Ivanov, 2012; Schneeberger and Lynch, 1992). Development of TJ is induced by a family of TJ-associated protein complexes, such as Claudin (Furuse et al., 1998), Occludin (Furuse et al., 1993) and Zona Occluden (ZO-1) (Itoh et al., 1999). These complexes are degraded by bacterial secreted proteases (Berkes et al., 2003), resulting in an increase in monolayer permeability. Additionally, luminal pro-inflammatory molecules are expressed, resulting in mucosal tissue damage and leading to an increase in IBD (Su et al., 2009; Turner, 2009). In recent studies, IBD patients have shown decreased TJ-associated protein expression (Bruewer et al., 2006) and increased intestinal permeability due to reduced epithelial barrier function (Lee, 2015; Robinson et al., 2015). In addition, IBD patients have been reported to exhibit enhanced pro-inflammatory cytokine production and immune dysregulation (Buhner et al., 2006; Katz et al., 1989).

Oxyresveratrol (OXY) is a stilbene compound isolated from various plants and has multiple effects, such as anti-inflammatory activity (Chen et al., 2013; Wei et al., 2017), potent neuroprotective activity (Andrabi et al., 2004), protection against hepatic damage (Chao et al., 2008; Zhang et al., 2008) and antibacterial activity (Joung et al., 2016). In this study, the effect of OXY on TJ was investigated, and its mechanism was elucidated. For analysis of the effect of OXY on intestinal integrity, the human intestinal Caco-2 cell line was selected because these cells have been used as *in vitro* models for monolayer integrity and transport studies.

2. Materials and methods

2.1. Chemicals

Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin, nonessential amino acids and sodium pyruvate for the cultivation of cells were obtained from HyClone (Logan, UT, USA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). OXY, Hanks' balanced salt solution (HBSS) and fluorescein isothiocyanate (FITC)-dextran (FD-4) (4000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GF109203X was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Cell culture

Caco-2, a human colonic epithelial cell line, was purchased from the Korean Cell Line Bank (Seoul, Korea). Caco-2 cells were maintained in MEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.1% nonessential amino acids and 0.1% sodium pyruvate. The cells were incubated at 37 °C under 5% CO_2–95% air for 14 days. The culture medium was changed every two days.

2.3. Cytotoxicity of oxyresveratrol

The cells were seeded on 96-well plates at a density of 2.5×10^4 cells/mL and incubated overnight. Subsequently, the cells were treated with various concentrations of OXY (10, 20 and 30 μg/mL) for 24 h at 37 °C in an atmosphere of 5% CO_2–95% air. After treatment, the culture medium was removed and 100 μL of 1:40 diluted MTT in medium was added to each well. The cells were then incubated for 1 h at 37 °C with 5% CO_2. Unreacted dye was removed, and the formazan crystals were solubilized in 100 μL DMSO for 1 h at room temperature. The absorbance was measured at 540 nm using a microplate reader (SpectraMax 340PC384, Molecular Devices Sunnyvale, CA, USA). Cell viability was expressed as the percentage relative to untreated negative control cells.

2.4. Paracellular permeability (FD-4 flux assay)

For measurement of FD-4 transport, Caco-2 cells were seeded at a density of 7×10^4 cells/mL into a 0.3-cm^2 high pore density 0.4 μm insert in a 24-well plate (BD Biosciences) for 10–14 days. The cells were washed three times per week with medium. When the cells reached confluence (>650 Ω of TEER), the cells were treaded with various concentrations of OXY (10, 20 and 30 μg/mL) for 12 h. Then, FD-4 (1 mg/mL) was added to the apical well. After 6 h, the basolateral medium was collected. The fluorescence was measured using a SpectraMax Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm excitation and 520 nm emission.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

The cells were seeded on 6-well plates at a seeding density of 2×10^5 cells/mL and incubated overnight. OXY (10, 20 and 30 μg/mL) was added to the 6-well plate and incubated for 24 h. For inhibition assays, GF109203X (8 μM) was added for 2 h before treatment with 30 μg/mL OXY. Cells were harvested using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's protocol. PCR primers were purchased from Bioneer (Seoul, Korea) as follows: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (sense: 5′- AAG GGT CAT CTC TGC CC -3′, antisense: 5′- GTG ATG GCA TGG ACT GTG GT -3′); *Claudin-1* (sense: 5′- CGA TGA GGT GCA GAA GAT GA -3′, antisense: 5′- CCA GTG AAG AGA GCC TGA CC -3′); *Occludin* (sense: 5′- TTT GTG GGA CAA GGA ACA CA -3′, antisense: 5′- TCA TTC ACT TTG CCA TTG GA T -3′); *ZO-1* (sense: 5′- TGA GGC AGC TCA CAT AAT GC -3′, antisense: 5′- GGT CTC TGC TGG CTT GTT TC -3′); *PKC-α* (sense: 5′- AGC CCA AAG TGT GTG GCA AA -3′, antisense: 5′- AGG TGT TTG TTC TCG CTG GT -3′); *PKC-β* (sense: 5′- AGC CAA AAG CTA GAG ACA AGA GA -3′, antisense: 5′- GGC TCA ACG ATG GAG TTT GC -3′); *PKC-γ* (sense: 5′- CAC TAG GTG TCC CCA ACG TC -3′, antisense: 5′- CGG CTG TAG AGG CTG TAT GG -3′); *PKC-ι* (sense: 5′- AAT GTG GGC GGC ATT CTT TG -3′, antisense: 5′- TTT GCC ACT TTC CCT GGT GT -3′); *PKC-ε* (sense: 5′- GAA CCC GGC GAG GAA ATA CA -3′, antisense: 5′- AGG GCA GGA ATG AAG AAC CG -3′); *PKC-θ* (sense: 5′- ATG TCG AAT CAG AAC GGG CA -3′, antisense: 5′- TAG CAT TCG GCC TTG AGG TT -3′); *PKC-δ* (sense: 5′- CCC TTC TGT GCC GTG AAG AT -3′, antisense: 5′- GCC CGC ATT AGC ACA ATC TG -3′); *ERK-1* (sense: 5′- TCA GAC TCC AAA GCC CTT GAC -3′, antisense: 5′- CGT GCT GTC TCC TGG AAG ATG -3′); *ERK-2* (sense: 5′- TCC AAC AGG CCC ATC TTT CC -3′, antisense: 5′- CCA GAG CTT TGG AGT CAG CA -3′); *JNK-α1* (sense: 5′- GCT TGG AAC ACC ATG TCC TGA -3′, antisense: 5′- GTA CGG GTG TTG GAG AGC TT -3′); *P38-α* (sense: 5′- ATG CAT AAT GGC CGA GCT GT -3′, antisense: 5′- GGT CAA CTT ACC CAG GGG ATT -3′); *Cdx-2* (sense: 5′- GCA GCC AAG TGA AAA CCA GG -3′, antisense: 5′- TTC CTC TCC TTT GCT CTG CG -3′) for Caco-2 cells. The reaction was