Free Radical Biology and Medicine 113 (2017) 539–550

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original article

Derangement of intestinal epithelial cell monolayer by dietary cholesterol oxidation products

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ARTICLE INFO

Keywords:
Oxysterols
Diet
JAM-A
Occludin
ZO-1
Metalloproteinases
Epithelial barrier
Intestinal inflammation

ABSTRACT

The emerging role of the diet in the incidence of intestinal inflammatory diseases has stimulated research on the influence of eating habits with pro-inflammatory properties in inducing epithelial barrier disturbance. Cholesterol oxidation products, namely oxysterols, have been shown to promote and sustain oxidative/inflammatory reactions in human digestive tract. This work investigated in an in vitro model the potential ability of a combination of dietary oxysterols representative of a hyper-cholesterol diet to induce the loss of intestinal epithelial layer integrity.

The components of the experimental mixture were the main oxysterols stemming from heat-induced cholesterol auto-oxidation, namely 7-ketocholesterol, 5α,6α-and 5β,6β-epoxycholesterol, 7α- and 7β-hydroxycholesterol. These compounds added to monolayers of differentiated CaCo-2 cells in combination or singularly, caused a time-dependent induction of matrix metalloproteinases (MMP)-2 and -9, also known as gelatinases. The hyperactivation of MMP-2 and -9 was found to be associated with decreased levels of the tight junctions zonula occludens-1 (ZO-1), occludin and Junction Adhesion Molecule-A (JAM-A). Together with such a protein loss, particularly evident for ZO-1, a net perturbation of spatial localization of the three tight junctions was observed.

Cell monolayer pre-treatment with the selective inhibitor of MMPs ARP100 or polyphenol (-)-epicathechin, previously shown to inhibit NADPH oxidase in the same model system, demonstrated that the decrease of the three tight junction proteins was mainly a consequence of MMPs induction, which was in turn dependent on the pro-oxidant property of the oxysterols investigated. Although further investigation on oxysterols intestinal layer damage mechanism is to be carried on, the consequent - but incomplete - prevention of oxysterols-dependent TJs alteration due to MMPs inhibition, avoided the loss of scaffold protein ZO-1, with possible significant recovery of intestinal monolayer integrity.

1. Introduction

Intestinal epithelial barrier damage is a central event in the pathogenesis of important gut diseases, such as inflammatory bowel disease (IBD) and colorectal cancer (CRC), where excessive inflammatory events are triggered by defective exposure of intestinal layer to different luminal antigens [1,2].

Inflammation and immune activation have been well considered to have a major role in the disruption of intestinal epithelial tight junctions (TJs), thus increasing paracellular permeability and favoring tissue injury [3].

TJs, which are located in apical-lateral cell surface, are composed of

Abbreviations: **α-epox**, 5α,6α-epoxycholesterol; **β-epox**, 5β,6β-epoxycholesterol; **7α-OH**, 7α-hydroxycholesterol; **7β-OH**, 7β-Hydroxycholesterol; **7K**, 7-ketocholesterol; **ARP-100**, 2-[((1,1'-Biphenyl)-4-ylsulfonyl)-(1-methylethoxy)amino]-N-hydroxyacetamide; **CRC**, colorectal cancer; **DAPI**, 4',6-diamidino-2-phenylindole; **DMEM**, Dulbecco's modified Eagle's medium; **DPI**, diphenyliodonium; **ECL**, Enhanced chemiluminescence; **ELISA**, Enzyme Linked Immunosorbent Assay; **FBS**, Fetal bovine serum; **HRP**, horseradish peroxidase; **IBD**, inflammatory bowel disease; **IL**, Interleukin; **JAM-A**, Junctional adhesion molecule-A; **LDH**, Lactate dehydrogenase; **MAPK**, Mitogen activated protein kinase; **MMP**, Matrix Metalloproteinase; **NF-κB**, Nuclear Factor-κB; **Oxy-mix**, Oxysterols mixture; **p38**, protein 38; **PBS**, phosphate buffered saline; **SDS**, sodium dodecyl sulphate; **TBS**, tris-buffered saline; **TTBS**, TBS-Tween 20; **TEER**, transepithelial electrical resistance; **TJ**, Tight junctions; **ZO-1**, Zonula occludens-1

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http://dx.doi.org/10.1016/j.freeradbiomed.2017.10.390
Received 20 July 2017; Received in revised form 23 October 2017; Accepted 31 October 2017
Available online 02 November 2017

transmembrane proteins, the most important being junctional adhesion molecules (JAMs), tissue-specific occludin and claudins, and cytoplasmic proteins including zonula occludens-1 (ZO-1). TJs form junctional complexes together with adherens junctions, cadherins and catenins, and desmosomes; they maintain a strong inter-cellular linkage acting as a physical barrier, but also regulate intracellular signals of immune and inflammatory responses against pathogens [4].

Intestinal mucosal integrity strongly depends on dietary habits, which, if incorrect, represent important risk factors for IBD and CRC development. Indeed, dietary animal fats widely used in Western countries have been suggested to trigger inflammatory and oxidative reactions in intestinal mucosa. Processed cholesterol rich foods tend to auto-oxidize by yielding high amount of oxysterols. Oxysterols have been demonstrated to exert a wide variety of biochemical effects, and contribute to the development and progression of chronic diseases associated with inflammation, including IBD [5].

Besides dietary factors, inflammation of intestinal mucosa is considered strongly promoting intestinal cancer development [6]. In this relation, various oxysterols with pro-inflammatory properties were observed being released by tumor microenvironment. They have been shown to recruit neutrophils that in turn are tumor promoters by releasing different immunosuppressive and pro-angiogenic factors, as well as matrix metalloproteinases (MMPs) [7]. MMPs represent the most prominent family of enzymes participating to extracellular matrix degradation, and are associated with the aggressiveness of several cancers. In fact, a significant increase of MMP-2 and -9 has been found in serum of patients affected by CRC during the latest tumor malignancy stages [8,9].

Importantly, among different types of MMPs involved in the pathogenesis of IBD by mediating intestinal barrier destabilization during intestinal inflammation, MMP-2 and -9 are definitely implicated [10–12]. Indeed, these gelatinases have been previously linked to TJ proteins degradation in different pathologies, mainly related to blood–brain barrier disruption, but also to the gastrointestinal tract [13,14].

With regard to MMPs activation, oxidant species may directly interact with the conserved cysteine residue in the enzyme prodomain [15]. Oxidative reactions can also act indirectly by inducing specific cell signaling pathways. For instance, lipid oxidation products such as 4-hydroxynonenal and 27-hydroxycholesterol, which accumulate into atherosclerotic lesions, can contribute to the induction of plaque destabilization and rupture by sustaining inflammatory cells recruitment and up-regulation of the gelatinase MMP-9 [16]. Furthermore, 7-oxocholesterol was found to induce M1/M2 macrophage polarization with MMPs strong cell release [17].

However, mechanistic information regarding the role of oxysterols in intestinal layer destabilization is entirely missing. Bearing this in mind, differentiated enterocyte-like CaCo-2 cells were challenged with a pathophysiologically relevant combination of dietary oxysterols. The results obtained point out these oxidized lipids as able to induce a marked colonic epithelial permeabilization by damaging main tight junctions components through human gelatinases hyperactivation, namely MMP-2 and MMP-9.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all reagents and chemicals, including 5α,6α-epoxycholesterol (α-epox), were from Sigma–Aldrich (Milan, Italy). Oxysterols 7-ketocholesterol (7 K) and 5β,6β-epoxycholesterol (β-epox) were from Steraloids Inc. (Newport, RI, USA); 7α-hydroxycholesterol (7α-OH) and 7β-hydroxycholesterol (7β-OH) were from Avanti Polar Lipids (Alabaster, AL, USA). Hydrogen chloride and $CaCl_2$ were from Merck (Millipore Corporation, Darmstadt, Germany); methanol from VWR International (Milan, Italy). The protein assay dye reagent, 2-mercaptoethanol and ECL® Western Blotting System were from Bio-Rad (Milan, Italy). Hybond ECL nitrocellulose membrane was from GE Healthcare (Milan, Italy).

Human IL-8 ELISA Kits were from PeProtech (DBA Italia, s.r.l., Segrate, Milan, Italy).

Rabbit anti-ZO-1 (SC-10804) and rabbit anti-JAM-A (SC-25629) polyclonal primary antibodies, goat anti-rabbit HRP-conjugated secondary antibody (SC-2004), goat anti-mouse HRP-conjugated secondary antibody (SC-2005), ARP100 (SC-203522) were from Santa Cruz (Tebu-Bio s.r.l., Magenta, Milan, Italy); rabbit anti-occludin (GTX85016) polyclonal antibody was from Gene Tex Inc. (Prodotti Gianni S.p.A., Milan, Italy). Goat anti-rabbit IgG-Alexa Fluor 488, Prolong Gold Antifade Mountant (P36930), Pierce Biotechnology M-PER™ (Mammalian Protein Extraction Reagent) lysis buffer (78501), Pierce™ Protease and Phosphatase Inhibitor (88668), Invitrogen Dulbecco's modified Eagle's medium (DMEM), Invitrogen fetal bovine serum (FBS) and 4',6'-diamidin-2-fenilindol (DAPI) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Twenty four-well polycarbonate Transwell inserts, 0.4 μm mean pore size, were from Millipore (Bedford, MA, USA); 25 cm^2 plastic flasks and 96 multi-well plates were from Falcon, Becton Dickinson Labware Europe (Meylan Cedex, France).

2.2. CaCo-2 cell culture and differentiation

Human colon adenocarcinoma cells CaCo-2 (passages 15–20; from European Collection of Cell Cultures - ECACC – Salisbury, UK) were plated at 1×10^5/mL density and cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution (100 U/mL penicillin, 0.1 mg/mL streptomycin, 250 ng/mL amphotericin B and 0.04 mg/mL gentamicin) at 37 °C in a 5% CO_2 humidified atmosphere. After reaching 100% confluence (day 3–4 after seeding), they were grown for additional 18 days, replacing the medium thrice weekly, to allow their spontaneous differentiation into enterocyte-like phenotype. Twenty-five cm^2 plastic flasks were used for the majority of experiments, except for fluorescence immunocytochemical analyses, in which 24-well polycarbonate Transwell inserts were used.

2.3. Cell treatments

Before each treatment, differentiated CaCo-2 cells were brought to quiescence through overnight incubation in serum-free medium. Cells were then placed in DMEM with 5% FBS and challenged with oxysterol mixture (Oxy-mix) (60 μM final concentration) for 24, 48 or 72 h, at 37 °C depending on different analyses. The percentage composition of the Oxy-mix used was 42.96% for 7 K, 32.3% for α-epox, 5.76% for β-epox, 4.26% for 7α-OH, and 14.71% for 7β-OH. The concentration of the Oxy-mix was calculated using an average molecular weight of 403 g/mol, the molarity of each component resulting: 25.8 μM 7 K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, 8.8 μM 7β-OH. In some experiments cells were pre-treated for 1 h at 37 °C with the MMPs inhibitor N-hydroxy-2-[(4-phenylphenyl)sulfonyl-propan-2-yloxyamino] acetamide (ARP 100).

In another set of experiments, cells were pre-treated with 10 μM (-)-epicatechin at 37 °C for 1 h, or with the 2 μM NADPH oxidase inhibitor diphenylene iodonium (DPI) for 30 min before the addition of Oxy-mix; chemicals remained in the cell medium throughout the treatment period. For the controls, cells were incubated with the same amount of solvent used to dilute the various substances employed.

2.4. Transepithelial electrical resistance

CaCo-2 cells were plated at 10^5 cells/mL density and cultured on Transwell inserts with 12 mm diameter and 0.4 μm pore size. The integrity of the Caco-2 cell monolayer was determined by measuring Transepithelial electrical resistance (TEER) value; only inserts with