IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Derangement of intestinal epithelial cell monolayer by dietary cholesterol oxidation products

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1653309 since 2021-12-06T17:01:01Z

Published version:

DOI:10.1016/j.freeradbiomed.2017.10.390

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

05 May 2024

DERANGEMENT OF INTESTINAL EPITHELIAL CELL MONOLAYER BY DIETARY CHOLESTEROL OXIDATION PRODUCTS

Monica Deiana[a], Simone Calfapietra[b], Alessandra Incani[a], Angela Atzeri[a], Daniela Rossin[b], Roberto Loi[a], Barbara Sottero[b], Noemi Iaia[b], Giuseppe Poli[b], Fiorella Biasi[b].

[a] Dept. of Biomedical Sciences, Pathology Section, University of Cagliari, 09124 Cagliari, Italy.

[b]Dept. of Clinical and Biological Sciences, University of Turin, 10043 Orbassano (Turin), Italy.

Authors' e-mail addresses:

Monica Deiana: mdeiana@unica.it; Simone Calfapietra: simo.calfo@hotmail.it; Alessandra Incani: alessandra.incani@gmail.com; Angela Atzeri: aatzeri@unica.it; Daniela Rossin: d.rossin@unito.it; Roberto Loi: rloi@unica.it; Barbara Sottero: barbara.sottero@unito.it; Noemi Iaia: noemi.iaia@edu.unito.it; Giuseppe Poli: giuseppe.poli@unito.it; Fiorella Biasi: fiorella.biasi@unito.it

Corresponding author:

Prof. Fiorella Biasi

Dept. of Clinical and Biological Sciences, University of Turin

San Luigi Hospital, Regione Gonzole 10,

10043 Orbassano (Turin), Italy

Tel.: 0039-011-6705420

Fax: 0039-011-6705424

Email: fiorella.biasi@unito.it

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.

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.

Keywords: Oxysterols, diet, JAM-A, Occludin, ZO-1, Metalloproteinases, epithelial barrier, intestinal inflammation

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 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; 11; 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-oxo-cholesterol 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 (7K) 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 7K, 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 7K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, 8.8μM 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 TEER values >500 Ohm/cm^2 were used.

For treatments, different substances (see section 2.3. Cell treatments) were added on the apical side of each insert. TEER was measured using a Millicell-Electrical Resistance System (Millipore, Bedford, MA, USA). The

resistance of cell-free insert was subtracted from each measurement. TEER was expressed as a negative percentage variation compared with TEER soon after each treatment.

2.5. Cell death evaluation

Extracellular release of lactate dehydrogenase (LDH) was checked as a parameter of cytolysis. LDH was evaluated in the cell medium spectrophotometrically, and enzymatic reaction recorded as ΔAbs/min at a wavelength of 340 nm. LDH was expressed as a percentage, taking 100% as the amount of the total enzyme released into cell medium when 0.5% Triton X-100 was added to culture flask containing the same cell density as the test sample.

2.6. Immunoblotting

At the end of incubation times, cells were scraped and washed with ice-cold phosphate buffer saline (PBS) before the addition of 150 µL lysis buffer for protein extraction (lysis buffer: M-PER™ Mammalian Protein Extraction Reagent and Pierce™ Protease and Phosphatase Inhibitor) to cell pellets. Lysates were incubated for 45 min on ice, centrifuged at 2000g at 4°C for 5 min, and the obtained supernatants were used for the analyses. Total cell protein concentration of extracts was evaluated with a Bio-Rad protein assay dye reagent [18].

ZO-1 levels evaluation was performed upon immunoprecipitation: total cell extracts (80 µg protein) were immunoprecipitated overnight with rabbit anti-ZO-1 polyclonal antibody (2 µL). Immunoprecipitation was achieved by 2 h incubation at 4°C with Protein A - Sepharose resin, and pellets were used for immunoblotting.

All the samples (immunoprecipitated or not) were boiled at 70°C for 10 min in boiling Laemmli buffer [200 mMTris-HCl, pH 7.4, glycerol 36% (v/v), SDS 7% (w/v), 1M 2-mercaptoethanol, bromophenol blue 0.1% (w/v)].

The boiled samples were run on 10% or 4-12% SDS-polyacrylamide gels (20 µg/lane) and proteins were transferred to Hybond ECL nitrocellulose membrane. The membrane was then incubated in a blocking buffer [TBS supplemented with 0.05% (v/v) Tween 20 (TTBS)] containing 5% (w/v) skimmed milk powder for 1 h at room temperature, followed by three 5 min washes in TTBS.

Blots were then incubated with either rabbit anti-ZO-1 (1:1000 dilution), rabbit anti-occludin (1:1000 dilution), rabbit anti-JAM-A (1:500 dilution) polyclonal antibodies, in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer) overnight at room temperature on a three-dimensional rocking table. Blots were washed twice for 10 min in TTBS and then incubated with goat anti-rabbit/mouse IgG conjugated to horseradish peroxidase (1:2000 dilutions) for 1 h in TTBS containing 5% (w/v) skimmed milk powder. Finally, blots were washed twice for 10 min in TTBS and exposed to ECL® reagent for 1–2 min as described in the manufacturer's protocol. Blots were then exposed to Hyperfilm-ECL using a ChemiDoc MP system (Biorad Laboratories, Inc.). The molecular weights of the bands were

calculated by comparison with pre-stained molecular weight markers that were run in parallel with the samples (27,000–180,000 and 6,000–45,000 Da). Protein bands were densitometrically quantified using Image J Software (USA).

2.7. Immunofluorescence of TJ proteins

Immunofluorescent localization of TJ proteins (ZO-1, occludin and JAM-A) was assessed in CaCo-2 cells grown as monolayer on polycarbonate Transwell inserts. After incubation, cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min, and washed twice with PBS. Non-specific binding sites were blocked by 60 min incubation with PBS containing 5% donkey serum and 0.5% bovine serum albumin (BSA). After two further washes with PBS, Caco-2 monolayers were incubated overnight at 4°C with rabbit anti-occludin (1:25), mouse anti-ZO-1 (1:50) or JAM-A (1:100) antibodies. Cells were then washed and incubated with goat IgG-Alexa Fluor 488 anti-rabbit (1:500), for 2 h at room temperature. All antibodies were diluted in PBS containing 0.5% BSA and 0.1% Triton X-100. After two final washes nuclei were counterstained with DAPI (1:1000 dilution v/v in PBS) for 10 min, and mounted with Prolong Gold Antifade Mountant. TJ proteins were examined with a Leica DM 2000 fluorescence microscope (Leica Microsystem, Wetzlar, Germany) at X 400 total magnification. Images were acquired with Leica LAS software.

2.8. Gelatin zymography

MMP-2 and MMP-9 are produced and assembled in the cytoplasm, secreted in zymogenic form as pro-MMPs, and activated extracellularly. Therefore, MMPs activities were assayed in cell medium by direct gelatin (gel) zymography. Proteins (12 μg) were separated by electrophoresis in 8% SDS-PAGE gel containing gelatin (0.8 mg/mL) under non-reducing conditions. Notably, Laemmli buffer used contained 200 mM Tris–HCl, pH 7.4, glycerol 72% (v/v), and SDS 7% (w/v). The gel was washed with Tris buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5, final solution) for 1 h and then incubated overnight at 37°C in a proteolysis buffer (40 mM Tris-HCl, 200 mM NaCl, 10mM $CaCl_2$, 0.02% NaN_3, pH 7.5, final solution). The gel was then stained for 3 h in a Coomassie Blue solution (0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, final solution) and destained with 5% methanol and 7% acetic acid (final solution). MMP-2 and MMP-9 activities were detected as a clear band on a blue background.

The activity of MMPs was quantified by densitometric analysis using Image J Software (USA). Results were expressed as folds compared to control values (taken as 1).

2.9. Evaluation of IL-8 protein levels by ELISA

Collected cell culture medium from each treatment was used for ELISA detection. The total protein concentration was measured in the cell medium using Bio-Rad protein assay dye reagent. Extracellular levels of IL-8 were quantified using commercial ELISA kits (see section 2.1. Materials), and following the manufacturer's instructions. Sample absorbance values were read at 405 nm with a wavelength correction of 650 nm using a microplate reader (Model 680, Bio-Rad), and data analyzed using SlideWrite Plus software (Advanced Graphics Software). Cytokine evaluation was performed in triplicate and expressed as pg/mg of proteins.

2.10. Statistical analyses. Results were expressed as mean ± Standard Deviation (SD). Statistical differences among sets of experimental data were evaluated using the one-way ANOVA test associated with Bonferroni's multiple comparison post hoc test. Data were analyzed with GraphPad InStat software (San Diego, CA, USA).

3. RESULTS

3.1. A representative mixture of dietary oxysterols used in our experiments induces permeability changes - but no cell death - in CaCo-2 cells monolayer

To understand if a diet-compatible mixture of oxysterols can affect paracellular permeability, CaCo-2 cells were grown on Transwell inserts for 3 weeks to reach differentiation, and then treated for different times with increasing concentrations of Oxy-mix. The analysis of intestinal barrier permeability was assessed by measuring CaCo-2 monolayer TEER. In the same experiments the capability of Oxy-mix to induce cell death was monitored in terms of LDH release in the cell medium. The most significant results are shown in Figure 1: 60 µM Oxy-mix was the lowest concentration found to significantly induce TEER decrease (Figure 1A), the latter event being already evident after 48 hours with the highest significance after 72 hours incubation (Figure 1B).

Correspondingly, LDH evaluation was routinely performed at different Oxy-mix concentrations and times incubation up to 72 hours in order to verify possible toxicity of Oxy-mix compounds; 30 and 60 µM concentrations did not exert any necrogenic effect. Conversely, treatments with 90 µM Oxy-mix at the longest time of cell incubation significantly increased LDH release (14,5 ± 3.9 %) (Figure 1 A-B).

Following these observations, in order to exclude necrosis and achieve the maximum biological effects, all subsequent experiments were performed by using 60 µM Oxy-mix.

3.2. A representative mixture of dietary oxysterols stimulates intestinal cell release of active MMP-2 and MMP-9

MMPs are expressed in various pathological conditions associated with inflammation. They have been demonstrated to play a role in regulating the activity of different pro-inflammatory mediators and influence cellular adhesion and motility, thus concurring to epithelial barrier destabilization, which is an important aspect in IBD and CRC.

An unbalanced diet is considered one of the main risk factors in intestinal damage. Therefore, the possible involvement of dietary compounds such as cholesterol oxidation products present in food in the induction of MMPs has been investigated. Of note, oxysterols produced in food by cholesterol auto-oxidation are always present as a mixture [19] and the strong pro-inflammatory effect of such oxysterols combination (at 30-60 μM concentration) has been proven *in vitro* by using differentiated CaCo-2 cells [20; 21]. Indeed, the challenge of differentiated CaCo-2 cells with 60 μM Oxy-mix along time allowed to observe MMP-2 and -9 progressive inductions, particularly MMP-9, as shown by gel-zymography (Figure 2A).

When the different Oxy-mix components were administered singularly, at the relative concentration present in the mixture, they appeared to only slightly modify the activation state of metalloproteinases at 72 h incubation time, but never reaching significance compared with control (Figure 2B).

Such a finding suggests an additive effect of the investigated oxysterols when in combination without the prevalence of any of them in up-regulating the MMP-2 and -9 activities.

3.3. Oxidative and inflammatory reactions are involved in the induction of MMP-2 and MMP-9 by dietary oxysterols mixture

The ability of dietary oxysterols to activate MMP-2 and -9 because of their pro-oxidant and pro-inflammatory properties was tested. Therefore, DPI (2 μM), previously proven to effectively inhibit oxysterols-dependent up-regulation of NADPH oxidase (NOX)1 [22], was added to the cell incubation medium, 30 min prior to Oxy-mix treatment.

Other aliquots of differentiated CaCo-2 cells were pre-treated for 1 h with 10 μM (-)-epicatechin, a flavan-3-ol shown to markedly quench oxidative/inflammatory signaling pathways triggered by oxysterols, in particular inhibiting intestinal NOX [21].

The cytotoxicity of oxysterols in the presence of different inhibitors was tested by evaluating cell LDH release and no significant difference was observed among the experimental groups (Figure 3A).

Cell pre-treatment with DPI and flavan-3-ol compound inhibited the induction of both MMP-2 and MMP-9 observed after 72 h cell incubation in the presence of Oxy-mix (Figure 3A).

In another set of experiments, the possible protection by (-)-epicatechin or MMPs inhibitor ARP 100 against the up-regulation of IL-8 secretion induced in differentiated CaCo-2 cells by oxysterols treatment was investigated (Figure 3B).

Preliminarily, in order to choose the most suitable non toxic concentration of ARP 100, CaCo-2 cells were treated with increasing concentrations of this compound (from 20 nM to 20 μM) 1 h before Oxy-mix incubation (72 h). Following LDH and gel-zymography measurements, 200 nM was considered as the concentration of ARP 100 showing the utmost inhibiting effect on both MMP-2 and MMP-9, without inducing cytotoxicity (data not shown).

As expected, (-)-epicatechin efficiently counteracted IL-8 increased production after Oxy-mix cell incubation. Even more interestingly, the brand new finding was that ARP 100 prevented IL-8 up-regulation induced by Oxy-mix (Figure 3B). A possible link between MMP-2 and -9 induction and pro-inflammatory IL-8 up-regulation mediated by oxysterols in colonic cells was then proposed.

3.4. Dietary oxysterols mixture induce marked loss and delocalization of tight junctions in differentiated CaCo-2 cells

In order to elucidate the molecular processes underlying the potential oxysterols-dependent dysfunction of intestinal epithelial barrier, we examined epithelial TJs in differentiated CaCo-2 cells after Oxy-mix treatment, considering that these proteins regulate paracellular permeability. Protein levels and cellular distribution of tight junctions ZO-1, occludin and JAM-A were evaluated by Western Blotting and immunofluorescence after cell incubation with Oxy-mix for 48 -72 h. Western blot analyses showed that Oxy-mix induced a significant decrease of all TJ levels after 72 h cell treatment, such effect being particularly remarkable on ZO-1 (Figure 4).

More importantly, marked TJs' cytosolic delocalization and spatial derangement in differentiated CaCo-2 cells challenged with 60 μM Oxy-mix were clearly shown by fluorescence microscopy already after 48 h treatment and definitely confirmed after 72 h treatment (Figure 5).

3.5. Inhibition of NADPH oxidase activity prevents Oxy-mix-induced TJs decrease

To investigate the actual involvement of oxysterols-dependent pro-oxidant activity in intestinal layer destabilization, TJs levels were evaluated in cells pre-treated with 2 μM DPI and then treated with Oxy-mix. Such a DPI concentration was shown to not have cytotoxic effect (see Figure 3B). Cell challenge with DPI indeed prevented the Oxy-mix-exerted decrease of TJs, thus supporting a causative association between NOX-dependent pro-oxidant effects of oxysterols and their ability to impair intestinal barrier (Figure 6).

3.6. Hyperactivation of MMPs induced by dietary oxysterols is a primary cause of TJ levels decrease

Aiming at clarifying the possible contribution of MMP-2 and -9 to the Oxy-mix-induced TJs decrease in colonic epithelial cells, a 60 min pre-treatment of differentiated CaCo-2 cells with MMP inhibitor ARP 100 was performed, and ZO-1, occludin and JAM-A quantities were assessed.

Figure 7 shows that 1h pre-treatment with 200 nM ARP 100 prevented TJs loss caused by CaCo-2 cell exposure to the investigated dietary oxysterols combination. As reported in Figure 8 also cell pre-incubation with (-)-epicatechin, which was demonstrated to lower Oxy-mix-dependent MMPs activation, fully prevented the ZO-1, occludin and JAM-A decrease observed in cells treated with oxysterols.

3.7. Preservation of TJ levels by cell pre-treatment with ARP 100 or (-)-epicatechin afforded only partial protection against TJ membrane delocalization.

Very interesting results came from the analyses carried out by fluorescence microscopy regarding spatial localization of the three investigated tight junctions in differentiated CaCo-2 cells challenged 72 h with 60 μM Oxy-mix but pre-treated either with the MMPs inhibitor ARP 100 or (-)-epicatechin. Both treatments significantly preserved the localization of ZO-1, i.e. the cytosolic tight junction that can be considered as a scaffold protein between membrane junction proteins and cytoskeleton. On the contrary, with regard to the two trans-membrane TJs, occludin and JAM-A, a correct assembly on cell membranes was maintained only when CaCo2 cells were pre-treated with (-)-epicatechin (Figure 9).

4. DISCUSSION

Derangement of intestinal barrier is certainly a primary event in gut pathology. Increased paracellular permeability can allow permeation of luminal pro-inflammatory and immunogenic molecules, resulting in persistent tissue inflammation and damage. Mucosal layer impairment has been associated with a variety of human diseases affecting gut, including IBD, celiac disease, colorectal cancer, intestinal mucositis due to cancer therapy, irritable bowel syndrome [14; 23; 24; 25].

Data reported here indicate that a high dietary intake of oxysterols, a mix of compounds stemming from cholesterol auto-oxidation, affect the integrity of gut epithelial barrier permeability mainly through the induction of MMP-2 and MMP-9.

Oxysterols percentage distribution has been detected in foods in relation to high or low cholesterol intake [19; 26]. In the adopted *in vitro* experimental model, a pathophysiologically relevant mixture of the main oxysterols detectable in cholesterol-rich food (namely7α-OH, 7β-OH, 7K, α-epox and β-epox) was used at a fixed concentration of

60 μM, an amount reliably corresponding to high cholesterol intake and already characterized for its pro-oxidant and pro-inflammatory effects [20; 21].

Pathophysiological interest on oxysterols comes from intensive research on degenerative diseases associated with hypercholesterolemia [5; 27; 28]. The relationship among the oxysterols consumed in food, their plasma levels and the occurrence of diseases was consistently demonstrated by different authors [29; 30]. A new tumor-supporting function of oxysterols in recruiting pro-tumoral neutrophils that can promote angiogenesis through the release of MMP9 has been demonstrated in the cancer microenvironment [7].

Differentiated CaCo-2 cells cultured in monolayer were employed because recognized to be a reliable model of enterocyte-like cells with absorptive properties similar to those of small intestinal mucosa, thus being extensively adopted to study drug intestinal permeability [31].

The challenge of differentiated CaCo-2 cells with the representative Oxy-mix (60 μM) strikingly induced the enzyme activity of the two gelatinases, MMP-2 and MMP-9, in a time-dependent manner. Notably, none of the single components of the mixture was able to induce a significant up-regulation of the two enzymes, thus suggesting that dietary oxysterols acquire pathological significance especially when in combination, as it actually occurs, because of additive or synergistic interactions among them.

MMP-2 and -9 activation in response to dietary oxysterols appears to be related to their ability to maintain an oxidative environment through intestinal NADPH oxidase up-regulation. This event would directly favor the so called "cysteine switch" necessary to enzymatic activation, and indirectly trigger inflammatory signaling pathways responsible for MMPs activation. The involvement of NADPH oxidase in inducing MMPs through the activation of NF-κB-dependent cell signals has been demonstrated in different cell types [32; 33; 34; 35]. 25-Hydroxycholesterol was shown to enhance release and activation of MMP-2 and -9 by promoting NF-κB nuclear translocation in fibroblasts [36]. Gargiulo and colleagues demonstrated that a mixture of oxysterols, mimicking that accumulating in advanced atherosclerotic lesions, induced MMP-9 expression in macrophages via the induction of NADPH oxidase/MAPKs/NF-κB signaling pathway [37].

We have recently demonstrated that the same dietary combination of oxysterols employed in the present study was able to induce inflammatory reactions by up-regulating IL-8 secretion and its signaling components, such as NADPH oxidase/p38 MAPK/NF-κB, in differentiated CaCo-2 cells [21].

NOX and derived oxygen species that have been recognized to induce inflammatory reactions could mediate the observed MMP-2 and -9 induction by Oxy-mix. This hypothesis was here confirmed through the observed complete abrogation of the oxysterol-dependent enzyme activation by cell pre-treatment with the selective NOX inhibitor DPI, as

well as by (-)-epicatechin. DPI concentration used for our experimental model did not show any cytotoxic effect as evaluated by LDH cell release.

The role of (-)-epicatechin in the inhibition of NOX and related inflammatory signals associated with NF-κB has been widely supported by studies on different cell systems, including intestinal cells [21; 38]. (-)-Epicatechin mechanism of action against oxysterols-dependent induction of NOX-MMPs axis could be due to its capability to limit the accessibility of lipids to the membrane by interacting with lipid rafts, where a number of cell signaling proteins including NADPH oxidase are located. [39; 40]. The possibility that (-)-epicatechin would interfere with oxysterols-related receptors, such as the intestinal cholesterol transporter Niemann-Pick C1-Like 1 [41], cannot be excluded even in absence of supporting evidence [42].

Beside their well-known effects on extracellular matrix degradation, MMPs have long been recognized to mediate cytokine activation, cell migration and differentiation, but also pathophysiological degradation of junctional proteins, all events contributing to modulate cell lining barriers function. In fact, an abnormal increase in MMPs activity has been associated with enhanced permeability of endothelial and epithelial barriers, an event mainly dependent on tight junctions disruption [43; 14**].**

Consistently, MMP-2 protein synthesis and activation were associated with increased intestinal permeability and degradation of TJ protein claudin-5 in CaCo-2 cell monolayers in response to mast cell-specific serine protease chymase treatment [13]. MMP-2 and -3 were found to be responsible for decreased levels and delocalization of occludin and tricellulin in CaCo-2 cells in response to Gram-negative bacteria products such as N-acyl- homoserine lactones [44].

Notably, the deranging effect on intestinal cell monolayer as exerted by dietary oxysterols is reported here for the first time. A significant decrease in TJs primarily involved in the regulation of paracellular permeability, namely ZO-1, occludin and JAM-A, was observed by Western Blotting in differentiated CaCo-2 cell monolayers treated with a representative dietary combination of oxysterols. Moreover, immunostaining analyses allowed to observe TJs' cellular delocalization already after 48 hours treatment, thus stressing that oxysterols may hamper cell-to-cell contact also by affecting protein distribution at cellular junctions.

Cell pre-treatment with a suitable concentration of ARP 100 able to fully inhibit both MMP-2 and MMP-9 without interfering with cell viability, allowed to prevent the Oxy-mix-induced loss of TJs. However, ARP 100 cell pre-treatment was not able to fully prevent TJs delocalization observed in oxysterol-treated cell monolayers, actually only preventing that of ZO-1. The discrepancy actually observed between immunoblotting and immunofluorescence results could be ascribed to the involvement of further mechanisms in addition to MMPs induction. This conclusion is supported by the results obtained in cells pre-treated with (-)-epicatechin that appeared to be more effective than ARP

100, since this flavan-3-ol significantly quenched both decrease and delocalization of ZO-1, JAM-A, occludin as induced by Oxy-mix.

As stated above, current evidence points to the (-)-epicatechin capacity to maintain the integrity of the apical plasma membrane of intestinal cells, thus limiting the accessibility of hydrophobic oxidizing compounds, including oxysterols. Furthermore, (-)-epicatechin can interact with lipid rafts, where enzymes like NADPH oxidase are located. Therefore, (-)-epicatechin-dependent NOX1 activity inhibition can reduce the production of derived oxidant species, which modulate pro-inflammatory signals [39]. A possible direct effect of NOX-1 on the expression of TJs independently of their ability to induce MMPs needs to be taken into consideration. The observation that unlike DPI, (-)-epicatechin can also reduce the TJs delocalization induced by oxysterols, suggests further direct action of these compounds on lipid rafts, consequently on TJ interactions and their compartmentalization, regardless of proteinases activation.

Interestingly, TJs are organized within cholesterol-rich lipid raft membrane domains and cholesterol content can influence TJs focalization and functions, i.e. membrane bioactivity [45]. Cholesterol has been considered for increasing thickness and stiffness of lipid bilayers, where it regulates protein sorting. Even if still not totally clear, cholesterol appears to stabilize the association of tight junctions in TJ strands generation. Claudins, JAM-A and occludin have been demonstrated to be associated with cholesterol rich membranes in CaCo-2 cells. Membrane cholesterol depletion by methyl-beta-cyclodextrin resulted in increased paracellular permeability by altering TJ proteins distribution, in particular by displacing claudins 3, 4 and 7, JAM -A and occludin, thus reducing their interaction [46; 47]. Furthermore, the use of Lovastatin (inhibiting 3-hydroxy-3methylglutaryl-CoA reductase) to lower cellular cholesterol by about 40% was shown to induce a fall in transepithelial resistance associated with occludin displacement from lateral membranes to intracellular compartment [48].

The role of oxysterols in modifying lipid rafts with subsequent changes in their physical and functional properties has been documented. For instance, 7K has been found to modify lipid raft domains in THP-1 monocytes, with increased cytosolic-free Ca^{2+} and apoptosis [49]. The ability of oxysterols to influence domain formation is very sensitive to double bond and hydroxyl group positions present in their chemical structure [for review, see 50; 51]: α-epox, 7β-OH and 7K inhibit lipid rafts formation, whereas the side-chain oxidized compound 25-hydroxycholesterol promotes rafts formation as well as cholesterol does. Notably, α-epox, 7β-OH and 7K are among those produced in foodstuff through cholesterol-autoxidation. Oxysterols direct capacity to insert into membranes by displacing cholesterol can alter biomembranes biophysical properties. In this way, these oxidized compounds can strongly influence dynamic interactions with peripheral membrane proteins, thus irreversibly affecting tight junctions assembly.

In summary, the reported findings using enterocyte-like CaCo-2 cell monolayers strikingly indicate that a combination of oxysterols similar to that found in heat processed cholesterol-rich food can cause intestinal barrier damage by inducing MMP-2 and MMP-9 activation through an enhanced production of oxidant species. Furthermore, the evidence that preventive inhibition of MMP activity does not allow to achieve full preservation of TJ spatial distribution and membrane localization, contrary to that occurring in the case of (-)-epicatechin pre-treatment, suggests that proteolysis is not the only mechanism by which oxysterols affect barrier integrity. The restored TJs synthesis does not apparently imply a recovery of their assembly as well, indicating that oxysterols could also interfere at lipid rafts with TJs association by displacing cholesterol, which is required for the maintenance of tight-junction integrity.

The chemical and/or physical interference with cholesterol function by an excess of oxysterols at the level of lipid rafts of cell intestinal barrier will be the target of further investigation.

Acknowledgements

The authors thank the University of Turin and the University of Cagliari, Italy, for providing financial support for the conduct of this research.

Conflicts of interest: none

REFERENCES

[1] L.R. Lopetuso, F. Scaldaferri, G. Bruno, V. Petito, F. Franceschi, A. Gasbarrini, The therapeutic management of gut barrier leaking: The emerging role for mucosal barrier protectors, Eur. Rev. Med. Pharmacol. Sci. 19 (2015) 1068-1076.

[2] L. Pastorelli, C. De Salvo, J.R. Mercado, M. Vecchi, T.T. Pizarro, Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: Lessons learned from animal models and human genetics, Front. Immunol. 4 (2013) 280. doi:10.3389/fimmu.2013.00280.

[3] A. Michielan, R. D'Incà, Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut, Mediators Inflamm. 2015 (2015) 628157. doi:10.1155/2015/628157.

[4] C.T. Capaldo, A.E. Farkas, A. Nusrat, Epithelial adhesive junctions, F1000 Prime Rep. 6 (2014) 1. doi:10.12703/P6-1.

[5] G. Poli, F. Biasi, G. Leonarduzzi, Oxysterols in the pathogenesis of major chronic diseases, Redox Biol. 1 (2013) 125-130. doi:10.1016/j.redox.2012.12.001.

[6] E. Antoniou, G.A. Margonis, A. Angelou, G.C. Zografos, E. Pikoulis, Cytokine networks in animal models of colitis-associated cancer, Anticancer Res. 35 (2015) 19-24.

[7] L. Raccosta, R. Fontana, D. Maggioni, C. Lanterna, E.J. Villablanca, A. Paniccia, A. Musumeci, E. Chiricozzi, M.L. Trincavelli, S. Daniele, C. Martini, J.A. Gustafsson, C. Doglioni, S.G. Feo, A. Leiva, M.G. Ciampa, L. Mauri, C. Sensi, A. Prinetti, I. Eberini, J.R. Mora, C. Bordignon, K.R. Steffensen, S. Sonnino, S. Sozzani, C. Traversari, V. Russo, The oxysterol-CXCR2 axis plays a key role in the recruitment of tumor-promoting neutrophils, J. Exp. Med. 26 (2013) 1711-1728.

[8] F. Biasi, T. Guina, M. Maina, M. Nano, A. Falcone, E. Aroasio, G.M. Saracco, M. Papotti, G. Leonarduzzi, G. Poli, Progressive increase of matrix metalloprotease-9 and interleukin-8 serum levels during carcinogenic process in human colorectal tract, PLoS ONE. 7 (2012). doi: 10.1371/journal.pone.0041839.

[9] N. Salem, I. Kamal, J. Al-Maghrabi, A. Abuzenadah, A.A. Peer-Zada, Y. Qari, M. Al-Ahwal, M. Al-Qahtani, A. Buhmeid, High expression of matrix metalloproteinases: MMP-2 and MMP-9 predicts poor survival outcome in colorectal carcinoma. Future Oncol. 12 (2016) 323-331. doi: 10.2217/fon.15.325.

[10] M.J. Meijer, M.A. Mieremet-Ooms, A.M. van der Zon , W. van Duijn, R.A. van Hogezand, C.F. Sier, D.W. Hommes, C.B. Lamers, H.W. Verspaget, Increased mucosal matrix metalloproteinase-1, -2, -3 and -9 activity in patients with inflammatory bowel disease and the relation with Crohn's disease phenotype, Dig. Liver Dis. 39 (2007) 733-739. doi:10.1016/j.dld.2007.05.010.

[11] S. O'Sullivan, J.F. Gilmer, C. Medina, Matrix metalloproteinases in inflammatory bowel disease: An update, Mediators Inflamm. 2015 (2015) 964131. doi:10.1155/2015/964131.

[12] P. Nighot, R. Al-Sadi, M. Rawat, S. Guo, D.M. Watterson, T. Ma, Matrix metalloproteinase 9-induced increase in intestinal epithelial tight junction permeability contributes to the severity of experimental DSS colitis, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (2015) G988-G997. doi:10.1152/ajpgi.00256.2015.

[13] K.R. Groschwitz, D. Wu, H. Osterfeld, R. Ahrens, S.P. Hogan, Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism, Am. J. Physiol. Gastrointest. Liver Physiol. 304 (2013) 479-489. doi: 10.1152/ajpgi.00186.2012.

[14] N. Al-Dasooqi, H.R. Wardill, R.J. Gibson, Gastrointestinal mucositis: The role of MMP-tight junction interactions in tissue injury, Pathol. Oncol. Res. 20 (2014) 485-491. doi:10.1007/s12253-013-9733-y.

[15] E. Hadler-Olsen, B. Fadnes, I. Sylte, L. Uhlin-Hansen, J.-O. Winberg, Regulation of matrix metalloproteinase activity in health and disease, FEBS J. 278 (2011) 28-45. doi:10.1111/j.1742-4658.2010.07920.x.

[16] S. Gargiulo, P. Gamba, G. Testa, D. Rossin, F. Biasi, G. Poli, G. Leonarduzzi, Relation between TLR4/NF-κB signaling pathway activation by 27-hydroxycholesterol and 4-hydroxynonenal, and atherosclerotic plaque instability, Aging Cell 14 (2015) 569-581. doi: 10.1111/acel.12322.

[17] B. Buttari, L. Segoni, E. Profumo, D. D'Arcangelo, S. Rossi, F. Facchiano, R. Businaro, L. Iuliano, R. Riganò, 7-Oxo-cholesterol potentiates pro-inflammatory signaling in human M1 and M2 macrophages, Biochem. Pharmacol. 86 (2013) 130-137. doi:10.1016/j.bcp.2013.04.008.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[19] J. Plat, J.A. Nichols, R.P. Mensink, Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation, J. Lipid. Res. 46 (2005) 2468-2476. doi:10.1194/jlr.M500272-JLR200.

[20] F. Biasi, T. Guina, M. Maina, B. Cabboi, M. Deiana, C.I. Tuberoso, S. Calfapietra, E. Chiarpotto, B. Sottero, P. Gamba, S. Gargiulo, V. Brunetto, G. Testa, M. A. Dessì, G. Poli, G. Leonarduzzi, Phenolic compounds present in Sardinian wine extracts protect against the production of inflammatory cytokines induced by oxysterols in CaCo-2 human enterocyte-like cells, Biochem. Pharmacol. 86 (2013) 138-145. doi:10.1016/j.bcp.2013.03.024.

[21] T. Guina, M. Deiana, S. Calfapietra, B. Cabboi, M. Maina, C.I. Tuberoso, G. Leonarduzzi, P. Gamba, S. Gargiulo, G. Testa, G. Poli, F. Biasi, The role of p38 MAPK in the induction of intestinal inflammation by dietary oxysterols: modulation by wine phenolics. Food Funct. 6 (2015) 1218-1228. doi:10.1039/c4fo01116c.

[22] C. Mascia, M. Maina, E. Chiarpotto, G. Leonarduzzi, G. PolI, F. Biasi, Proinflammatory effect of cholesterol and its oxidation products on CaCo-2 human enterocyte-like cells: effective protection by epigallocatechin-3-gallate, Free Radic. Biol. Med. 49 (2010) 2049-2057. doi: 10.1016/j.freeradbiomed.2010.09.033.

[23] P. Das, P. Goswami, T.K. Das, T. Nag, V. Sreenivas, V. Ahuja, S.K. Panda, S.D. Gupta, G.K. Makharia, Comparative tight junction protein expressions in colonic Crohn’s disease, ulcerative colitis, and tuberculosis: A new perspective, Virchows Arch. 460 (2012) 261-270. doi:10.1007/s00428-012-1195-1.

[24] M. Schumann, B. Siegmund, J.D. Schulzke, M. Fromm, Celiac Disease: Role of the Epithelial Barrier, Cell. Mol. Gastroenterol. Hepatol. 3 (2017) 150-162. doi: 10.1016/j.jcmgh.2016.12.006.

[25] M.J. Waldner, M.F. Neurath, Mechanisms of Immune Signaling in Colitis-Associated Cancer, Cell. Mol. Gastroenterol. Hepatol. 1 (2014) 6-16. doi: 10.1016/j.jcmgh.2014.11.006.

[26] M.I. Khan, J.S. Min, S.O. Lee, D.G. Yim, K.H Seol, M. Lee, C. Jo, Cooking, storage, and reheating effect on the formation of cholesterol oxidation products in processed meat products, Lipids Health Dis. 14 (2015) 89. doi: 10.1186/s12944-015-0091-5.

[27] M. Brzeska, K. Szymczyk, A. Szterk, Current Knowledge about Oxysterols: A Review, J Food Sci. 81 (2016) R2299-R2308. doi: 10.1111/1750-3841.13423.

[28] A. Otaegui-Arrazola, M. Menéndez-Carreño, D. Ansorena, I. Astiasarán, Oxysterols: A world to explore, Food Chem. Toxicol. 48 (2010) 3289-303. doi: 10.1016/j.fct.2010.09.023.

[29] I. Staprans, X.M. Pan, J.H. Rapp, C. Grunfeld, K.R. Feingold, Oxidized cholesterol in
the diet accelerates the development of atherosclerosis in LDL receptor- andapolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 20 (2000) 708-14.

[30] I. Soto-Rodríguez, A. Alexander-Aguilera, A. Zamudio-Pérez, M. Camara-Contreras, G. Hernandez-Diaz, H.S. Garcia, Alteration of some inflammatory biomarkers by dietary oxysterols in rats. Inflammation. 35 (2012) 1302-7. doi: 10.1007/s10753-012-9442-9.

[31] H. Sun, E.C. Chow, S. Liu, Y. Du, K.S. Pang, The Caco-2 cell monolayer: usefulness and limitations, Expert Opin Drug Metab Toxicol. 4 (2008) 395-411. doi:10.1517/17425255.4.4.395.

[32] G. Svineng, C. Ravuri, O. Rikardsen, N.E. Huseby, J.O. Winberg, The role of reactive oxygen species in integrin and matrix metalloproteinase expression and function, Connect. Tissue Res. 49 (2008) 197-202. doi:10.1080/03008200802143166.

[33] N. Tobar, V. Villar, J.F. Santibanez, ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion, Mol. Cell Biochem. 340 (2010) 195-202. doi:10.1007/s11010-010-0418-5.

[34] S. Lian, Y. Xia, P.N. Khoi, T.T. Ung, H.J. Yoon, N.H. Kim, K.K. Kim, Y. Do Jung, Cadmium induces matrix metalloproteinase-9 expression via ROS-dependent EGFR, NF-κB, and AP-1 pathways in human endothelial cells, Toxicology. 338 (2015) 104-116. doi:10.1016/j.tox.2015.10.008.

[35] J. Sarkar, A. Chowdhury, T. Chakraborti, S. Chakraborti, Cross-talk between NADPH oxidase-PKCα-p(38)MAPK and NF-κB-MT1MMP in activating proMMP-2 by ET-1 in pulmonary artery smooth muscle cells, Mol. Cell Biochem. 415 (2016) 13-28. doi: 10.1007/s11010-016-2673-6.

[36] T. Ichikawa, H. Sugiura, A. Koarai, T. Kikuchi, M. Hiramatsu, H. Kawabata, K. Akamatsu, T. Hirano, M. Nakanishi, K. Matsunaga, Y. Minakata, M. Ichinose, 25-Hydroxycholesterol promotes fibroblast-mediated tissue remodeling through NF-κB dependent pathway, Exp. Cell Res. 319 (2013) 1176–1186. doi:10.1016/j.yexcr.2013.02.014.

[37] S. Gargiulo, B. Sottero, P. Gamba, E. Chiarpotto, G. Poli, G. Leonarduzzi, Plaque oxysterols induce unbalanced up-regulation of matrix metalloproteinase-9 in macrophagic cells through redox-sensitive signaling pathways: Implications regarding the vulnerability of atherosclerotic lesions, Free Radic. Biol. Med. 51 (2011) 844-855. doi:10.1016/j.freeradbiomed.2011.05.030.

[38] T.C. Contreras, E. Ricciardi, E. Cremonini, P.I. Oteiza, (-)-Epicatechin in the prevention of tumor necrosis alpha-induced loss of Caco-2 cell barrier integrity, Arch. Biochem. Biophys. 573(2015) 84-91. doi: 10.1016/j.abb.2015.01.024.

[39] C.G. Fraga, P.I. Oteiza, Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. Free Radic. Biol. Med. 51(2011):813-23. doi: 10.1016/j.freeradbiomed.2011.06.002.

[40] S.V. Verstraeten, C.G. Fraga, P.I. Oteiza, Interactions of flavan-3-ols and procyanidins with membranes: mechanisms and the physiological relevance, Food Funct. 6(2015):32-41. doi: 10.1039/c4fo00647j.

[41] F. Karaki, K. Ohgane, K. Dodo, Y. Hashimoto, Structure-activity relationship studies of Niemann-Pick type C1-like 1 (NPC1L1) ligands identified by screening assay monitoring pharmacological chaperone effect, Bioorg. Med. Chem. 21(2013):5297-309. doi: 10.1016/j.bmc.2013.06.022.

[42] M. Nekohashi, M. Ogawa, T. Ogihara, K. Nakazawa, H. Kato, T. Misaka, K. Abe, S. Kobayashi, Luteolin and quercetin affect the cholesterol absorption mediated by epithelial cholesterol transporter niemann-pick c1-like 1 in caco-2 cells and rats, PLoS ONE. 9(2014) e97901. doi: 10.1371/journal.pone.0097901.

[43] P.M. Cummins, Occludin: one protein, many forms, Mol. Cell. Biol. 32 (2012) 242-250. doi: 10.1128/MCB.06029-11.

[44] S.Y. Eum, D. Jaraki, L. Bertrand, I.E. András, M. Toborek, Disruption of epithelial barrier by quorum-sensing N-3-(oxododecanoyl)-homoserine lactone is mediated by matrix metalloproteinases, Am. J. Physiol. Gastrointest. Liver Physiol. 306 (2014) G992-G1001. doi: 10.1152/ajpgi.00016.2014.

[45] B.P. Head, H.H. Patel, P.A. Insel, Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling, Biochim. Biophys. Acta. 1838 (2014) 532-545. doi:10.1016/j.bbamem.2013.07.018.

[46] D. Lambert, C.A. O'Neill, P.J. Padfield, Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins, Biochem J. 387 (2005) 553-560. doi:10.1042/BJ20041377.

[47] D. Lambert, C.A. O'Neill, P.J. Padfield, Methyl-beta-cyclodextrin increases permeability of Caco-2 cell monolayers by displacing specific claudins from cholesterol rich domains associated with tight junctions, Cell. Physiol. Biochem. 20 (2007) 495-506. doi: 10.1159/000107533

[48] A. Nusrat, C.A. Parkos, P. Verkade, C.S. Foley, T.W. Liang, W. Innis-Whitehouse, K.K. Eastburn, J.L. Madara, Tight junctions are membrane microdomains, J. Cell Sci. 113 (2000) 1771-1781.

[49] A. Berthier, S. Lemaire-Ewing, C. Prunet, S. Monier, A. Athias, G. Bessède, J.P. Pais de Barros, A. Laubriet, P. Gambert, G. Lizard, D. Néel, Involvement of a calcium-dependent dephosphorylation of BAD associated with the localization of Trpc-1 within lipid rafts in 7-ketocholesterol-induced THP-1 cell apoptosis, Cell Death Differ. 11 (2004) 897-905. doi:10.1038/sj.cdd.4401434.

[50] J.B. Massey, Membrane and protein interactions of oxysterols, Curr. Opin. Lipidol. 17 (2006) 296-301. doi:10.1097/01.mol.0000226123.17629.ab. doi:10.1097/01.mol.0000226123.17629.ab.

[51] B. Sottero, P. Gamba, S. Gargiulo, G. Leonarduzzi, G. Poli, Cholesterol oxidation products and disease: an emerging topic of interest in medicinal chemistry, Curr. Med. Chem. 16 (2009) 685-705. doi: 10.2174/092986709787458353.

Figure legends

Figure 1. Analysis of permeability alteration and cytolysis in CaCo-2 cell monolayer.

A. TEER was measured in differentiated CaCo-2 cells treated with different concentrations of Oxy-mix (30, 60 or 90 μM) for 72 h (left panel). TEER time-course (24, 48, 72 h) was also performed by incubating differentiated CaCo-2 cells with 60 μM Oxy-mix (right panel). TEER was shown as negative percentage variation compared with TEER soon after each treatment (see baseline).

B. LDH release was evaluated in the culture media of differentiated CaCo-2 cells treated with 30, 60 or 90 μM Oxy-mix (left panel) or incubated with 60 μM Oxy-mix for 24, 48 or 72 h (right panel). LDH was evaluated as percentage referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to cultured cells grown at the same density of other samples. Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *p<0.05, ***p<0.001.

Figure 2. Time-dependent changes in MMP-2 and MMP-9 induction in differentiated CaCo-2 cells treated with dietary oxysterols.

A. The induction of MMP-2 and MMP-9 was evaluated by gel-zymography in CaCo-2 cells treated with 60 μM Oxy-mix for 24, 48 and 72 h.

B. MMPs induction was also evaluated with Oxy-mix individual compounds after 72 h cell incubation. Each oxysterol was added to the cells individually at the same concentration at which they were present in the mixture: 25.8 μM 7K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, 8.8 μM 7β-OH. Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *p < 0.05, **p<0.01 and ***p<0.001; significantly different vs. Oxy-mix: a p<0.05, b p<0.01, c p<0.001.

Figure 3. Involvement of oxidative and inflammatory reactions in Oxy-mix-mediated gelatinases induction.

A. The induction of MMP-2 and MMP-9 was analyzed by gel-zymography in CaCo-2 cells incubated with 60 μM Oxy-mix for 72 h, pretreated or not with DPI (2 μM for 30 min) or (-)-epicatechin (EPC) (10 μM for 1 h).

B. IL-8 levels were measured by ELISA in cell medium of differentiated CaCo-2 cells pre-treated or not with EPC (10 μM for 1h) or ARP 100 (200 nM for 1 h) and incubated with 60 μM Oxy-mix for 72 hrs. At the left, LDH cell release percentage is reported referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to cultured cells grown at the same density of other samples. Data are reported as means ± SD of three independent

experiments. Significantly different vs. controls: **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$, c $p<0.001$.

Figure 4. Oxy-mix induces TJs loss in differentiated CaCo-2 cells.

Decreased levels of ZO-1, occludin and JAM-A were detected by Western Blotting in lysates from CaCo-2 cells incubated with 60 μM Oxy-mix for 48 and 72 h. Data are expressed as percentage of control (100%). Values are means ± SD of three independent experiments. Significantly different vs. controls: **$p<0.01$, ***$p<0.001$.

Figure 5. Cellular distribution of TJs in differentiated CaCo2 cells treated with dietary oxysterols.

Marked delocalization of TJs ZO-1, occludin and JAM-A after cell monolayers incubation with 60 μM Oxy-mix for 48 and 72 h was visualized by immunofluorescence microscopy. For all images TJs were immunostained with green color; CaCo-2 cells nuclei were stained with DAPI and shown in blue color. Original magnification: X 400.

Figure 6. Cell pre-treatment with NADPH oxidase inhibitor restores TJ levels decreased by Oxy-mix cell incubation.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells 1 h pre-treated with NADPH oxidase inhibitor DPI (2 μM), incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *$p<0.05$, **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$.

Figure 7. Effect of ARP 100 on TJ decreased levels induced by Oxy-mix.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells in presence of 200 nM ARP 100 (1 h pre-treatment) incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: **$p<0.01$, *** $p<0.001$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$, c $p<0.001$.

Figure 8. Oxy-mix-dependent decrease of TJ cell levels is prevent by EPC.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells in presence of 10 μM EPC (1 h pre-treatment) incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *$p<0.05$, **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$.

Figure 9. Effect of EPC and ARP 100 on TJs delocalization induced by oxysterols combination.

CaCo-2 cell monolayers were pre-treated or not with 10 μM EPC or 200 nM ARP 100 and incubated with 60 μM Oxy-mix for 72 h. Fluorescence microscopy was used to visualize TJs cellular distribution (X 400 magnification). TJ proteins were visualized as green immunofluorescence. Nuclei were stained with DAPI and shown in blue color.

ZO-1 localization was completely restored in presence of both EPC and ARP 100. Occludin and JAM-A do not appear to recover their membrane distribution with ARP 100 pre-treatment. On the other hand, EPC appears more effective than ARP 100, showing a correct reassembly of all TJs examined.

Revised Manuscript (text with changes Marked)
Click here to view linked References

DERANGEMENT OF INTESTINAL EPITHELIAL CELL MONOLAYER ~~BARRIER~~ BY DIETARY CHOLESTEROL OXIDATION PRODUCTS

Monica Deiana[a], Simone Calfapietra[b], Alessandra Incani[a], Angela Atzeri[a], Daniela Rossin[b], Roberto Loi[a], Barbara Sottero[b], Noemi Iaia[b], Giuseppe Poli[b], Fiorella Biasi[b].

[a] Dept. of Biomedical Sciences, Pathology Section, University of Cagliari, 09124 Cagliari, Italy.

[b]Dept. of Clinical and Biological Sciences, University of Turin, 10043 Orbassano (Turin), Italy.

Authors' e-mail addresses:

Monica Deiana: mdeiana@unica.it; Simone Calfapietra: simo.calfo@hotmail.it; Alessandra Incani: alessandra.incani@gmail.com; Angela Atzeri: aatzeri@unica.it; Daniela Rossin: d.rossin@unito.it; Roberto Loi: rloi@unica.it; Barbara Sottero: barbara.sottero@unito.it; Noemi Iaia: noemi.iaia@edu.unito.it; Giuseppe Poli: giuseppe.poli@unito.it; Fiorella Biasi: fiorella.biasi@unito.it

Corresponding author:

Prof. Fiorella Biasi

Dept. of Clinical and Biological Sciences, University of Turin

San Luigi Hospital, Regione Gonzole 10,

10043 Orbassano (Turin), Italy

Tel.: 0039-011-6705420

Fax: 0039-011-6705424

Email: fiorella.biasi@unito.it

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.

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.

Keywords: Oxysterols, diet, JAM-A, Occludin, ZO-1, Metalloproteinases, epithelial barrier, intestinal inflammation

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 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; 11; 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-oxo-cholesterol 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 (7K) 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 7K, 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 7K, 19.4 µM α-epox, 3.4 µM β-epox, 2.6 µM 7α-OH, 8.8µM 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. ~~Cell death was routinely checked for each treatment up to 72 h by lactate dehydrogenase (LDH) enzyme release into cell medium [18]. Percentages of LDH cell release reached at the latest incubation time were 2.2 ± 0.4 in controls, 4.7 ± 0.5 in cells treated with oxysterols, and 3.4 ± 1.2 in cells treated with oxysterols plus different inhibitors, but no significant differences were observed among experimental groups.~~

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 TEER values >500 Ohm/cm^2 were used.

For treatments, different substances (see section 2.3. Cell treatments) were added on the apical side of each insert. TEER was measured using a Millicell-Electrical Resistance System (Millipore, Bedford, MA, USA). The resistance of cell-free insert was subtracted from each measurement. TEER was expressed as a negative percentage variation compared with TEER soon after each treatment.

2.5. Cell death evaluation

Extracellular release of lactate dehydrogenase (LDH) was checked as a parameter of cytolysis. LDH was evaluated in the cell medium spectrophotometrically, and enzymatic reaction recorded as ΔAbs/min at a wavelength of 340 nm. LDH was expressed as a percentage, taking 100% as the amount of the total enzyme released into cell medium when 0.5% Triton X-100 was added to culture flask containing the same cell density as the test sample.

2.6. Immunoblotting

At the end of incubation times, cells were scraped and washed with ice-cold phosphate buffer saline (PBS) before the addition of 150 µL lysis buffer for protein extraction (lysis buffer: M-PER™ Mammalian Protein Extraction Reagent and Pierce™ Protease and Phosphatase Inhibitor) to cell pellets. Lysates were incubated for 45 min on ice, centrifuged at 2000g at 4°C for 5 min, and the obtained supernatants were used for the analyses. Total cell protein concentration of extracts was evaluated with a Bio-Rad protein assay dye reagent [18].

ZO-1 levels evaluation was performed upon immunoprecipitation: total cell extracts (80 µg protein) were immunoprecipitated overnight with rabbit anti-ZO-1 polyclonal antibody (2 µL). Immunoprecipitation was achieved by 2 h incubation at 4°C with Protein A - Sepharose resin, and pellets were used for immunoblotting.

All the samples (immunoprecipitated or not) were boiled at 70°C for 10 min in boiling Laemmli buffer [200 mMTris-HCl, pH 7.4, glycerol 36% (v/v), SDS 7% (w/v), 1M 2-mercaptoethanol, bromophenol blue 0.1% (w/v)].

The boiled samples were run on 10% or 4-12% SDS-polyacrylamide gels (20 µg/lane) and proteins were transferred to Hybond ECL nitrocellulose membrane. The membrane was then incubated in a blocking buffer [TBS supplemented with 0.05% (v/v) Tween 20 (TTBS)] containing 5% (w/v) skimmed milk powder for 1 h at room temperature, followed by three 5 min washes in TTBS.

Blots were then incubated with either rabbit anti-ZO-1 (1:1000 dilution), rabbit anti-occludin (1:1000 dilution), rabbit anti-JAM-A (1:500 dilution) polyclonal antibodies, in TTBS containing 1% (w/v) skimmed milk powder

(antibody buffer) overnight at room temperature on a three-dimensional rocking table. Blots were washed twice for 10 min in TTBS and then incubated with goat anti-rabbit/mouse IgG conjugated to horseradish peroxidase (1:2000 dilutions) for 1 h in TTBS containing 5% (w/v) skimmed milk powder. Finally, blots were washed twice for 10 min in TTBS and exposed to ECL® reagent for 1–2 min as described in the manufacturer's protocol. Blots were then exposed to Hyperfilm-ECL using a ChemiDoc MP system (Biorad Laboratories, Inc.). The molecular weights of the bands were calculated by comparison with pre-stained molecular weight markers that were run in parallel with the samples (27,000–180,000 and 6,000–45,000 Da). Protein bands were densitometrically quantified using Image J Software (USA).

2.7. Immunofluorescence of TJ proteins

Immunofluorescent localization of TJ proteins (ZO-1, occludin and JAM-A) was assessed in CaCo-2 cells grown as monolayer on polycarbonate Transwell inserts. After incubation, cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min, and washed twice with PBS. Non-specific binding sites were blocked by 60 min incubation with PBS containing 5% donkey serum and 0.5% bovine serum albumin (BSA). After two further washes with PBS, Caco-2 monolayers were incubated overnight at 4°C with rabbit anti-occludin (1:25), mouse anti-ZO-1 (1:50) or JAM-A (1:100) antibodies. Cells were then washed and incubated with goat IgG-Alexa Fluor 488 anti-rabbit (1:500), for 2 h at room temperature. All antibodies were diluted in PBS containing 0.5% BSA and 0.1% Triton X-100. After two final washes nuclei were counterstained with DAPI (1:1000 dilution v/v in PBS) for 10 min, and mounted with Prolong Gold Antifade Mountant. TJ proteins were examined with a Leica DM 2000 fluorescence microscope (Leica Microsystem, Wetzlar, Germany) at X 400 total magnification. Images were acquired with Leica LAS software.

2.8. Gelatin zymography

MMP-2 and MMP-9 are produced and assembled in the cytoplasm, secreted in zymogenic form as pro-MMPs, and activated extracellularly. Therefore, MMPs activities were assayed in cell medium by direct gelatin (gel) zymography. Proteins (12 μg) were separated by electrophoresis in 8% SDS-PAGE gel containing gelatin (0.8 mg/mL) under non-reducing conditions. Notably, Laemmli buffer used contained 200 mM Tris–HCl, pH 7.4, glycerol 72% (v/v), and SDS 7% (w/v). The gel was washed with Tris buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5, final solution) for 1 h and then incubated overnight at 37°C in a proteolysis buffer (40 mM Tris-HCl, 200 mM NaCl, 10mM $CaCl_2$, 0.02% NaN_3, pH 7.5, final solution). The gel was then stained for 3 h in a Coomassie Blue solution (0.05%

Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, final solution) and destained with 5% methanol and 7% acetic acid (final solution). MMP-2 and MMP-9 activities were detected as a clear band on a blue background.

The activity of MMPs was quantified by densitometric analysis using Image J Software (USA). Results were expressed as folds compared to control values (taken as 1).

2.9. Evaluation of IL-8 protein levels by ELISA

Collected cell culture medium from each treatment was used for ELISA detection. The total protein concentration was measured in the cell medium using Bio-Rad protein assay dye reagent. Extracellular levels of IL-8 were quantified using commercial ELISA kits (see section 2.1. Materials), and following the manufacturer's instructions. Sample absorbance values were read at 405 nm with a wavelength correction of 650 nm using a microplate reader (Model 680, Bio-Rad), and data analyzed using SlideWrite Plus software (Advanced Graphics Software). Cytokine evaluation was performed in triplicate and expressed as pg/mg of proteins.

2.10. Statistical analyses. Results were expressed as mean ± Standard Deviation (SD). Statistical differences among sets of experimental data were evaluated using the one-way ANOVA test associated with Bonferroni's multiple comparison post hoc test. Data were analyzed with GraphPad InStat software (San Diego, CA, USA).

3. RESULTS

3.1. A representative mixture of dietary oxysterols used in our experiments induces permeability changes - but no cell death - in CaCo-2 cells monolayer

To understand if a diet-compatible mixture of oxysterols can affect paracellular permeability, CaCo-2 cells were grown on Transwell inserts for 3 weeks to reach differentiation, and then treated for different times with increasing concentrations of Oxy-mix. The analysis of intestinal barrier permeability was assessed by measuring CaCo-2 monolayer TEER. In the same experiments the capability of Oxy-mix to induce cell death was monitored in terms of LDH release in the cell medium. The most significant results are shown in Figure 1: 60 µM Oxy-mix was the lowest concentration found to significantly induce TEER decrease (Figure 1A), the latter event being already evident after 48 hours with the highest significance after 72 hours incubation (Figure 1B).

Correspondingly, LDH evaluation was routinely performed at different Oxy-mix concentrations and times incubation up to 72 hours in order to verify possible toxicity of Oxy-mix compounds; 30 and 60 µM concentrations did not exert any necrogenic effect. Conversely, treatments with 90 µM Oxy-mix at the longest time of cell incubation significantly increased LDH release (14,5 ± 3.9 %) (Figure 1 A-B).

Following these observations, in order to exclude necrosis and achieve the maximum biological effects, all subsequent experiments were performed by using 60 µM Oxy-mix.

3.2. A representative mixture of dietary oxysterols stimulates intestinal cell release of active MMP-2 and MMP-9

MMPs are expressed in various pathological conditions associated with inflammation. They have been demonstrated to play a role in regulating the activity of different pro-inflammatory mediators and influence cellular adhesion and motility, thus concurring to epithelial barrier destabilization, which is an important aspect in IBD and CRC.

An unbalanced diet is considered one of the main risk factors in intestinal damage. Therefore, the possible involvement of dietary compounds such as cholesterol oxidation products present in food in the induction of MMPs has been investigated. Of note, oxysterols produced in food by cholesterol auto-oxidation are always present as a mixture [19] and the strong pro-inflammatory effect of such oxysterols combination (at 30-60 µM concentration) has been proven *in vitro* by using differentiated CaCo-2 cells [20; 21]. Indeed, the challenge of differentiated CaCo-2 cells with 60 µM Oxy-mix along time allowed to observe MMP-2 and -9 progressive inductions, particularly MMP-9, as shown by gel-zymography (Figure 2A).

When the different Oxy-mix components were administered singularly, at the relative concentration present in the mixture, they appeared to only slightly modify the activation state of metalloproteinases at 72 h incubation time, but never reaching significance compared with control (Figure 2B).

Such a finding suggests an additive effect of the investigated oxysterols when in combination without the prevalence of any of them in up-regulating the MMP-2 and -9 activities.

3.3. Oxidative and inflammatory reactions are involved in the induction of MMP-2 and MMP-9 by dietary oxysterols mixture

The ability of dietary oxysterols to activate MMP-2 and -9 because of their pro-oxidant and pro-inflammatory properties was tested. Therefore, DPI (2 µM), previously proven to effectively inhibit oxysterols-dependent up-regulation of NADPH oxidase (NOX)1 [22], was added to the cell incubation medium, 30 min prior to Oxy-mix treatment.

Other aliquots of differentiated CaCo-2 cells were pre-treated for 1 h with 10 µM (-)-epicatechin, a flavan-3-ol shown to markedly quench oxidative/inflammatory signaling pathways triggered by oxysterols, in particular inhibiting intestinal NOX [21].

The cytotoxicity of oxysterols in the presence of different inhibitors was tested by evaluating cell LDH release and no significant difference was observed among the experimental groups (Figure 3A).

Cell pre-treatment with DPI and flavan-3-ol compound inhibited the induction of both MMP-2 and MMP-9 observed after 72 h cell incubation in the presence of Oxy-mix (Figure 3A).

In another set of experiments, the possible protection by (-)-epicatechin or MMPs inhibitor ARP 100 against the up-regulation of IL-8 secretion induced in differentiated CaCo-2 cells by oxysterols treatment was investigated (Figure 3B).

Preliminarily, in order to choose the most suitable non toxic concentration of ARP 100, CaCo-2 cells were treated with increasing concentrations of this compound (from 20 nM to 20 μM) 1 h before Oxy-mix incubation (72 h). Following LDH and gel-zymography measurements, 200 nM was considered as the concentration of ARP 100 showing the utmost inhibiting effect on both MMP-2 and MMP-9, without inducing cytotoxicity (data not shown).

As expected, (-)-epicatechin efficiently counteracted IL-8 increased production after Oxy-mix cell incubation. Even more interestingly, the brand new finding was that ARP 100 prevented IL-8 up-regulation induced by Oxy-mix (Figure 3B). A possible link between MMP-2 and -9 induction and pro-inflammatory IL-8 up-regulation mediated by oxysterols in colonic cells was then proposed.

3.4. Dietary oxysterols mixture induce marked loss and delocalization of tight junctions in differentiated CaCo-2 cells

In order to elucidate the molecular processes underlying the potential oxysterols-dependent dysfunction of intestinal epithelial barrier, we examined epithelial TJs in differentiated CaCo-2 cells after Oxy-mix treatment, considering that these proteins regulate paracellular permeability. Protein levels and cellular distribution of tight junctions ZO-1, occludin and JAM-A were evaluated by Western Blotting and immunofluorescence after cell incubation with Oxy-mix for 48 -72 h. Western blot analyses showed that Oxy-mix induced a significant decrease of all TJ levels after 72 h cell treatment, such effect being particularly remarkable on ZO-1 (Figure 4).

More importantly, marked TJs' cytosolic delocalization and spatial derangement in differentiated CaCo-2 cells challenged with 60 μM Oxy-mix were clearly shown by fluorescence microscopy already after 48 h treatment and definitely confirmed after 72 h treatment (Figure 5).

3.5. Inhibition of NADPH oxidase activity prevents Oxy-mix-induced TJs decrease

To investigate the actual involvement of oxysterols-dependent pro-oxidant activity in intestinal layer destabilization, TJs levels were evaluated in cells pre-treated with 2 μM DPI and then treated with Oxy-mix. Such a

DPI concentration was shown to not have cytotoxic effect (see Figure 3B). Cell challenge with DPI indeed prevented the Oxy-mix-exerted decrease of TJs, thus supporting a causative association between NOX-dependent pro-oxidant effects of oxysterols and their ability to impair intestinal barrier (Figure 6).

3.6. Hyperactivation of ~~gelatinases~~ MMPs induced by dietary oxysterols is a primary cause of TJ levels decrease

Aiming at clarifying the possible contribution of MMP-2 and -9 to the Oxy-mix-induced TJs decrease in colonic epithelial cells, a 60 min pre-treatment of differentiated CaCo-2 cells with MMP inhibitor ARP 100 was performed, and ZO-1, occludin and JAM-A quantities were assessed.

Figure 7 shows that 1h pre-treatment with 200 nM ARP 100 prevented TJs loss caused by CaCo-2 cell exposure to the investigated dietary oxysterols combination. As reported in Figure 8 also cell pre-incubation with (-)-epicatechin, which was demonstrated to lower Oxy-mix-dependent MMPs activation, fully prevented the ZO-1, occludin and JAM-A decrease observed in cells treated with oxysterols.

3.7. Preservation of TJ levels by cell pre-treatment with ARP 100 or (-)-epicatechin afforded only partial protection against TJ membrane delocalization.

Very interesting results came from the analyses carried out by fluorescence microscopy regarding spatial localization of the three investigated tight junctions in differentiated CaCo-2 cells challenged 72 h with 60 μM Oxy-mix but pre-treated either with the MMPs inhibitor ARP 100 or (-)-epicatechin. Both treatments significantly preserved the localization of ZO-1, i.e. the cytosolic tight junction that can be considered as a scaffold protein between membrane junction proteins and cytoskeleton. On the contrary, with regard to the two trans-membrane TJs, occludin and JAM-A, a correct assembly on cell membranes was maintained only when CaCo2 cells were pre-treated with (-)-epicatechin (Figure 9).

4. DISCUSSION

Derangement of intestinal barrier is certainly a primary event in gut pathology. Increased paracellular permeability can allow permeation of luminal pro-inflammatory and immunogenic molecules, resulting in persistent tissue inflammation and damage. Mucosal layer impairment has been associated with a variety of human diseases affecting gut, including IBD, celiac disease, colorectal cancer, intestinal mucositis due to cancer therapy, irritable bowel syndrome [14; 23; 24; 25].

Data reported here indicate that a high dietary intake of oxysterols, a mix of compounds stemming from cholesterol auto-oxidation, affect the integrity of gut epithelial barrier permeability mainly through the induction of MMP-2 and MMP-9.

Oxysterols percentage distribution has been detected in foods in relation to high or low cholesterol intake [19; 26]. In the adopted *in vitro* experimental model, a pathophysiologically relevant mixture of the main oxysterols detectable in cholesterol-rich food (namely7α-OH, 7β-OH, 7K, α-epox and β-epox) was used at a fixed concentration of 60 μM, an amount reliably corresponding to high cholesterol intake and already characterized for its pro-oxidant and pro-inflammatory effects [20; 21].

Pathophysiological interest on oxysterols comes from intensive research on degenerative diseases associated with hypercholesterolemia [5; 27; 28]. The relationship among the oxysterols consumed in food, their plasma levels and the occurrence of diseases was consistently demonstrated by different authors [29; 30]. A new tumor-supporting function of oxysterols in recruiting pro-tumoral neutrophils that can promote angiogenesis through the release of MMP9 has been demonstrated in the cancer microenvironment [7].

Differentiated CaCo-2 cells cultured in monolayer were employed because recognized to be a reliable model of enterocyte-like cells with absorptive properties similar to those of small intestinal mucosa, thus being extensively adopted to study drug intestinal permeability [31].

The challenge of differentiated CaCo-2 cells with the representative Oxy-mix (60 μM) strikingly induced the enzyme activity of the two gelatinases, MMP-2 and MMP-9, in a time-dependent manner. Notably, none of the single components of the mixture was able to induce a significant up-regulation of the two enzymes, thus suggesting that dietary oxysterols acquire pathological significance especially when in combination, as it actually occurs, because of additive or synergistic interactions among them.

MMP-2 and -9 activation in response to dietary oxysterols appears to be related to their ability to maintain an oxidative environment through intestinal NADPH oxidase up-regulation. This event would directly favor the so called "cysteine switch" necessary to enzymatic activation, and indirectly trigger inflammatory signaling pathways responsible for MMPs activation. The involvement of NADPH oxidase in inducing MMPs through the activation of NF-κB-dependent cell signals has been demonstrated in different cell types [32; 33; 34; 35]. 25-Hydroxycholesterol was shown to enhance release and activation of MMP-2 and -9 by promoting NF-κB nuclear translocation in fibroblasts [36]. Gargiulo and colleagues demonstrated that a mixture of oxysterols, mimicking that accumulating in advanced atherosclerotic lesions, induced MMP-9 expression in macrophages via the induction of NADPH oxidase/MAPKs/NF-κB signaling pathway [37].

We have recently demonstrated that the same dietary combination of oxysterols employed in the present study was able to induce inflammatory reactions by up-regulating IL-8 secretion and its signaling components, such as NADPH oxidase/p38 MAPK/NF-κB, in differentiated CaCo-2 cells [21].

NOX and derived oxygen species that have been recognized to induce inflammatory reactions could mediate the observed MMP-2 and -9 induction by Oxy-mix. This hypothesis was here confirmed through the observed complete abrogation of the oxysterol-dependent enzyme activation by cell pre-treatment with the selective NOX inhibitor DPI, as well as by (-)-epicatechin. ~~, a pleiotropic polyphenol provided with antioxidant and anti-inflammatory properties, including a marked inhibitory effect on intestinal NADPH oxidase activation~~. DPI concentration used for our experimental model did not show any cytotoxic effect as evaluated by LDH cell release.

The role of (-)-epicatechin in the inhibition of NOX and related inflammatory signals associated with NF-κB has been widely supported by studies on different cell systems, including intestinal cells [21; 38]. (-)-Epicatechin mechanism of action against oxysterols-dependent induction of NOX-MMPs axis could be due to its capability to limit the accessibility of lipids to the membrane by interacting with lipid rafts, where a number of cell signaling proteins including NADPH oxidase are located. [39; 40]. The possibility that (-)-epicatechin would interfere with oxysterols-related receptors, such as the intestinal cholesterol transporter Niemann-Pick C1-Like 1 [41], cannot be excluded even in absence of supporting evidence [42].

Beside their well-known effects on extracellular matrix degradation, MMPs have long been recognized to mediate cytokine activation, cell migration and differentiation, but also pathophysiological degradation of junctional proteins, all events contributing to modulate cell lining barriers function. In fact, an abnormal increase in MMPs activity has been associated with enhanced permeability of endothelial and epithelial barriers, an event mainly dependent on tight junctions disruption [43; 14**].**

Consistently, MMP-2 protein synthesis and activation were associated with increased intestinal permeability and degradation of TJ protein claudin-5 in CaCo-2 cell monolayers in response to mast cell-specific serine protease chymase treatment [13]. MMP-2 and -3 were found to be responsible for decreased levels and delocalization of occludin and tricellulin in CaCo-2 cells in response to Gram-negative bacteria products such as N-acyl- homoserine lactones [44].

Notably, the deranging effect on intestinal cell monolayer as exerted by dietary oxysterols is reported here for the first time. ~~The same applies to the involvement of MMP-2 and MMP-9 in the pathogenesis of such a barrier impairment. Convincing demonstration that MMPs hyperactivation induced by Oxy-mix can have a role in the observed decreased levels of TJs comes from experiments performed by pre-treating cells with a suitable concentration of ARP 100 able to fully inhibit both MMP-2 and MMP-9 without interfering with cell viability.~~ A significant decrease in TJs

primarily involved in the regulation of paracellular permeability, namely ZO-1, occludin and JAM-A, was observed by Western Blotting in differentiated CaCo-2 cell monolayers treated with a representative dietary combination of oxysterols. Moreover, immunostaining analyses allowed to observe TJs' cellular delocalization already after 48 hours treatment, thus stressing that oxysterols may hamper cell-to-cell contact also by affecting protein distribution at cellular junctions.

Cell pre-treatment with a suitable concentration of ARP 100 able to fully inhibit both MMP-2 and MMP-9 without interfering with cell viability, allowed to prevent the Oxy-mix-induced loss of TJs. However, ARP 100 cell pre-treatment was not able to fully prevent TJs delocalization observed in oxysterol-treated cell monolayers, actually only preventing that of ZO-1. The discrepancy actually observed between immunoblotting and immunofluorescence results could be ascribed to the involvement of further mechanisms in addition to MMPs induction. This conclusion is supported by the results obtained in cells pre-treated with (-)-epicatechin that appeared to be more effective than ARP 100, since this flavan-3-ol significantly quenched both decrease and delocalization of ZO-1, JAM-A, occludin as induced by Oxy-mix.

As stated above, current evidence points to the (-)-epicatechin capacity to maintain the integrity of the apical plasma membrane of intestinal cells, thus limiting the accessibility of hydrophobic oxidizing compounds, including oxysterols. Furthermore, (-)-epicatechin can interact with lipid rafts, where enzymes like NADPH oxidase are located. Therefore, (-)-epicatechin-dependent NOX1 activity inhibition can reduce the production of derived oxidant species, which modulate pro-inflammatory signals [39]. A possible direct effect of NOX-1 on the expression of TJs independently of their ability to induce MMPs needs to be taken into consideration. The observation that unlike DPI, (-)-epicatechin can also reduce the TJs delocalization induced by oxysterols, suggests further direct action of these compounds on lipid rafts, consequently on TJ interactions and their compartmentalization, regardless of proteinases activation.

Interestingly, TJs are organized within cholesterol-rich lipid raft membrane domains and cholesterol content can influence TJs focalization and functions, i.e. membrane bioactivity [45]. Cholesterol has been considered for increasing thickness and stiffness of lipid bilayers, where it regulates protein sorting. Even if still not totally clear, cholesterol appears to stabilize the association of tight junctions in TJ strands generation. Claudins, JAM-A and occludin have been demonstrated to be associated with cholesterol rich membranes in CaCo-2 cells. Membrane cholesterol depletion by methyl-beta-cyclodextrin resulted in increased paracellular permeability by altering TJ proteins distribution, in particular by displacing claudins 3, 4 and 7, JAM -A and occludin, thus reducing their interaction [46; 47]. Furthermore, the use of Lovastatin (inhibiting 3-hydroxy-3methylglutaryl-CoA reductase) to lower cellular cholesterol by about 40%

was shown to induce a fall in transepithelial resistance associated with occludin displacement from lateral membranes to intracellular compartment [48].

The role of oxysterols in modifying lipid rafts with subsequent changes in their physical and functional properties has been documented. For instance, 7K has been found to modify lipid raft domains in THP-1 monocytes, with increased cytosolic-free Ca^{2+} and apoptosis [49]. The ability of oxysterols to influence domain formation is very sensitive to double bond and hydroxyl group positions present in their chemical structure [for review, see 50; 51]: α-epox, 7β-OH and 7K inhibit lipid rafts formation, whereas the side-chain oxidized compound 25-hydroxycholesterol promotes rafts formation as well as cholesterol does. Notably, α-epox, 7β-OH and 7K are among those produced in foodstuff through cholesterol-autoxidation. Oxysterols direct capacity to insert into membranes by displacing cholesterol can alter biomembranes biophysical properties. In this way, these oxidized compounds can strongly influence dynamic interactions with peripheral membrane proteins, thus irreversibly affecting tight junctions assembly.

In summary, the reported findings using enterocyte-like CaCo-2 cell monolayers strikingly indicate that a combination of oxysterols similar to that found in heat processed cholesterol-rich food can cause intestinal barrier damage by inducing MMP-2 and MMP-9 activation through an enhanced production of oxidant species. Furthermore, the evidence that preventive inhibition of MMP activity does not allow to achieve full preservation of TJ spatial distribution and membrane localization, contrary to that occurring in the case of (-)-epicatechin pre-treatment, suggests that proteolysis is not the only mechanism by which oxysterols affect barrier integrity. The restored TJs synthesis does not apparently imply a recovery of their assembly as well, indicating that oxysterols could also interfere at lipid rafts with TJs association by displacing cholesterol, which is required for the maintenance of tight-junction integrity.

The chemical and/or physical interference with cholesterol function by an excess of oxysterols at the level of lipid rafts of cell intestinal barrier will be the target of further investigation.

Acknowledgements

The authors thank the University of Turin and the University of Cagliari, Italy, for providing financial support for the conduct of this research.

Conflicts of interest: none

REFERENCES

[1] L.R. Lopetuso, F. Scaldaferri, G. Bruno, V. Petito, F. Franceschi, A. Gasbarrini, The therapeutic management of gut barrier leaking: The emerging role for mucosal barrier protectors, Eur. Rev. Med. Pharmacol. Sci. 19 (2015) 1068-1076.

[2] L. Pastorelli, C. De Salvo, J.R. Mercado, M. Vecchi, T.T. Pizarro, Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: Lessons learned from animal models and human genetics, Front. Immunol. 4 (2013) 280. doi:10.3389/fimmu.2013.00280.

[3] A. Michielan, R. D'Incà, Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut, Mediators Inflamm. 2015 (2015) 628157. doi:10.1155/2015/628157.

[4] C.T. Capaldo, A.E. Farkas, A. Nusrat, Epithelial adhesive junctions, F1000 Prime Rep. 6 (2014) 1. doi:10.12703/P6-1.

[5] G. Poli, F. Biasi, G. Leonarduzzi, Oxysterols in the pathogenesis of major chronic diseases, Redox Biol. 1 (2013) 125-130. doi:10.1016/j.redox.2012.12.001.

[6] E. Antoniou, G.A. Margonis, A. Angelou, G.C. Zografos, E. Pikoulis, Cytokine networks in animal models of colitis-associated cancer, Anticancer Res. 35 (2015) 19-24.

[7] L. Raccosta, R. Fontana, D. Maggioni, C. Lanterna, E.J. Villablanca, A. Paniccia, A. Musumeci, E. Chiricozzi, M.L. Trincavelli, S. Daniele, C. Martini, J.A. Gustafsson, C. Doglioni, S.G. Feo, A. Leiva, M.G. Ciampa, L. Mauri, C. Sensi, A. Prinetti, I. Eberini, J.R. Mora, C. Bordignon, K.R. Steffensen, S. Sonnino, S. Sozzani, C. Traversari, V. Russo, The oxysterol-CXCR2 axis plays a key role in the recruitment of tumor-promoting neutrophils, J. Exp. Med. 26 (2013) 1711-1728.

[8] F. Biasi, T. Guina, M. Maina, M. Nano, A. Falcone, E. Aroasio, G.M. Saracco, M. Papotti, G. Leonarduzzi, G. Poli, Progressive increase of matrix metalloprotease-9 and interleukin-8 serum levels during carcinogenic process in human colorectal tract, PLoS ONE. 7 (2012). doi: 10.1371/journal.pone.0041839.

[9] N. Salem, I. Kamal, J. Al-Maghrabi, A. Abuzenadah, A.A. Peer-Zada, Y. Qari, M. Al-Ahwal, M. Al-Qahtani, A. Buhmeid, High expression of matrix metalloproteinases: MMP-2 and MMP-9 predicts poor survival outcome in colorectal carcinoma. Future Oncol. 12 (2016) 323-331. doi: 10.2217/fon.15.325.

[10] M.J. Meijer, M.A. Mieremet-Ooms, A.M. van der Zon , W. van Duijn, R.A. van Hogezand, C.F. Sier, D.W. Hommes, C.B. Lamers, H.W. Verspaget, Increased mucosal matrix metalloproteinase-1, -2, -3 and -9 activity in patients with inflammatory bowel disease and the relation with Crohn's disease phenotype, Dig. Liver Dis. 39 (2007) 733-739. doi:10.1016/j.dld.2007.05.010.

[11] S. O'Sullivan, J.F. Gilmer, C. Medina, Matrix metalloproteinases in inflammatory bowel disease: An update, Mediators Inflamm. 2015 (2015) 964131. doi:10.1155/2015/964131.

[12] P. Nighot, R. Al-Sadi, M. Rawat, S. Guo, D.M. Watterson, T. Ma, Matrix metalloproteinase 9-induced increase in intestinal epithelial tight junction permeability contributes to the severity of experimental DSS colitis, Am. J. Physiol. Gastrointest. Liver Physiol. 309 (2015) G988-G997. doi:10.1152/ajpgi.00256.2015.

[13] K.R. Groschwitz, D. Wu, H. Osterfeld, R. Ahrens, S.P. Hogan, Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism, Am. J. Physiol. Gastrointest. Liver Physiol. 304 (2013) 479-489. doi: 10.1152/ajpgi.00186.2012.

[14] N. Al-Dasooqi, H.R. Wardill, R.J. Gibson, Gastrointestinal mucositis: The role of MMP-tight junction interactions in tissue injury, Pathol. Oncol. Res. 20 (2014) 485-491. doi:10.1007/s12253-013-9733-y.

[15] E. Hadler-Olsen, B. Fadnes, I. Sylte, L. Uhlin-Hansen, J.-O. Winberg, Regulation of matrix metalloproteinase activity in health and disease, FEBS J. 278 (2011) 28-45. doi:10.1111/j.1742-4658.2010.07920.x.

[16] S. Gargiulo, P. Gamba, G. Testa, D. Rossin, F. Biasi, G. Poli, G. Leonarduzzi, Relation between TLR4/NF-κB signaling pathway activation by 27-hydroxycholesterol and 4-hydroxynonenal, and atherosclerotic plaque instability, Aging Cell 14 (2015) 569-581. doi: 10.1111/acel.12322.

[17] B. Buttari, L. Segoni, E. Profumo, D. D'Arcangelo, S. Rossi, F. Facchiano, R. Businaro, L. Iuliano, R. Riganò, 7-Oxo-cholesterol potentiates pro-inflammatory signaling in human M1 and M2 macrophages, Biochem. Pharmacol. 86 (2013) 130-137. doi:10.1016/j.bcp.2013.04.008.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

[19] J. Plat, J.A. Nichols, R.P. Mensink, Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation, J. Lipid. Res. 46 (2005) 2468-2476. doi:10.1194/jlr.M500272-JLR200.

[20] F. Biasi, T. Guina, M. Maina, B. Cabboi, M. Deiana, C.I. Tuberoso, S. Calfapietra, E. Chiarpotto, B. Sottero, P. Gamba, S. Gargiulo, V. Brunetto, G. Testa, M. A. Dessì, G. Poli, G. Leonarduzzi, Phenolic compounds present in Sardinian wine extracts protect against the production of inflammatory cytokines induced by oxysterols in CaCo-2 human enterocyte-like cells, Biochem. Pharmacol. 86 (2013) 138-145. doi:10.1016/j.bcp.2013.03.024.

[21] T. Guina, M. Deiana, S. Calfapietra, B. Cabboi, M. Maina, C.I. Tuberoso, G. Leonarduzzi, P. Gamba, S. Gargiulo, G. Testa, G. Poli, F. Biasi, The role of p38 MAPK in the induction of intestinal inflammation by dietary oxysterols: modulation by wine phenolics. Food Funct. 6 (2015) 1218-1228. doi:10.1039/c4fo01116c.

[22] C. Mascia, M. Maina, E. Chiarpotto, G. Leonarduzzi, G. PolI, F. Biasi, Proinflammatory effect of cholesterol and its oxidation products on CaCo-2 human enterocyte-like cells: effective protection by epigallocatechin-3-gallate, Free Radic. Biol. Med. 49 (2010) 2049-2057. doi: 10.1016/j.freeradbiomed.2010.09.033.

[23] P. Das, P. Goswami, T.K. Das, T. Nag, V. Sreenivas, V. Ahuja, S.K. Panda, S.D. Gupta, G.K. Makharia, Comparative tight junction protein expressions in colonic Crohn’s disease, ulcerative colitis, and tuberculosis: A new perspective, Virchows Arch. 460 (2012) 261-270. doi:10.1007/s00428-012-1195-1.

[24] M. Schumann, B. Siegmund, J.D. Schulzke, M. Fromm, Celiac Disease: Role of the Epithelial Barrier, Cell. Mol. Gastroenterol. Hepatol. 3 (2017) 150-162. doi: 10.1016/j.jcmgh.2016.12.006.

[25] M.J. Waldner, M.F. Neurath, Mechanisms of Immune Signaling in Colitis-Associated Cancer, Cell. Mol. Gastroenterol. Hepatol. 1 (2014) 6-16. doi: 10.1016/j.jcmgh.2014.11.006.

[26] M.I. Khan, J.S. Min, S.O. Lee, D.G. Yim, K.H Seol, M. Lee, C. Jo, Cooking, storage, and reheating effect on the formation of cholesterol oxidation products in processed meat products, Lipids Health Dis. 14 (2015) 89. doi: 10.1186/s12944-015-0091-5.

[27] M. Brzeska, K. Szymczyk, A. Szterk, Current Knowledge about Oxysterols: A Review, J Food Sci. 81 (2016) R2299-R2308. doi: 10.1111/1750-3841.13423.

[28] A. Otaegui-Arrazola, M. Menéndez-Carreño, D. Ansorena, I. Astiasarán, Oxysterols: A world to explore, Food Chem. Toxicol. 48 (2010) 3289-303. doi: 10.1016/j.fct.2010.09.023.

[29] I. Staprans, X.M. Pan, J.H. Rapp, C. Grunfeld, K.R. Feingold, Oxidized cholesterol in the diet accelerates the development of atherosclerosis in LDL receptor- andapolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 20 (2000) 708-14.

[30] I. Soto-Rodríguez, A. Alexander-Aguilera, A. Zamudio-Pérez, M. Camara-Contreras, G. Hernandez-Diaz, H.S. Garcia, Alteration of some inflammatory biomarkers by dietary oxysterols in rats. Inflammation. 35 (2012) 1302-7. doi: 10.1007/s10753-012-9442-9.

[31] H. Sun, E.C. Chow, S. Liu, Y. Du, K.S. Pang, The Caco-2 cell monolayer: usefulness and limitations, Expert Opin Drug Metab Toxicol. 4 (2008) 395-411. doi:10.1517/17425255.4.4.395.

[32] G. Svineng, C. Ravuri, O. Rikardsen, N.E. Huseby, J.O. Winberg, The role of reactive oxygen species in integrin and matrix metalloproteinase expression and function, Connect. Tissue Res. 49 (2008) 197-202. doi:10.1080/03008200802143166.

[33] N. Tobar, V. Villar, J.F. Santibanez, ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion, Mol. Cell Biochem. 340 (2010) 195-202. doi:10.1007/s11010-010-0418-5.

[34] S. Lian, Y. Xia, P.N. Khoi, T.T. Ung, H.J. Yoon, N.H. Kim, K.K. Kim, Y. Do Jung, Cadmium induces matrix metalloproteinase-9 expression via ROS-dependent EGFR, NF-κB, and AP-1 pathways in human endothelial cells, Toxicology. 338 (2015) 104-116. doi:10.1016/j.tox.2015.10.008.

[35] J. Sarkar, A. Chowdhury, T. Chakraborti, S. Chakraborti, Cross-talk between NADPH oxidase-PKCα-p(38)MAPK and NF-κB-MT1MMP in activating proMMP-2 by ET-1 in pulmonary artery smooth muscle cells, Mol. Cell Biochem. 415 (2016) 13-28. doi: 10.1007/s11010-016-2673-6.

[36] T. Ichikawa, H. Sugiura, A. Koarai, T. Kikuchi, M. Hiramatsu, H. Kawabata, K. Akamatsu, T. Hirano, M. Nakanishi, K. Matsunaga, Y. Minakata, M. Ichinose, 25-Hydroxycholesterol promotes fibroblast-mediated tissue remodeling through NF-κB dependent pathway, Exp. Cell Res. 319 (2013) 1176–1186. doi:10.1016/j.yexcr.2013.02.014.

[37] S. Gargiulo, B. Sottero, P. Gamba, E. Chiarpotto, G. Poli, G. Leonarduzzi, Plaque oxysterols induce unbalanced up-regulation of matrix metalloproteinase-9 in macrophagic cells through redox-sensitive signaling pathways: Implications regarding the vulnerability of atherosclerotic lesions, Free Radic. Biol. Med. 51 (2011) 844-855. doi:10.1016/j.freeradbiomed.2011.05.030.

[38] T.C. Contreras, E. Ricciardi, E. Cremonini, P.I. Oteiza, (-)-Epicatechin in the prevention of tumor necrosis alpha-induced loss of Caco-2 cell barrier integrity, Arch. Biochem. Biophys. 573(2015) 84-91. doi: 10.1016/j.abb.2015.01.024.

[39] C.G. Fraga, P.I. Oteiza, Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. Free Radic. Biol. Med. 51(2011):813-23. doi: 10.1016/j.freeradbiomed.2011.06.002.

[40] S.V. Verstraeten, C.G. Fraga, P.I. Oteiza, Interactions of flavan-3-ols and procyanidins with membranes: mechanisms and the physiological relevance, Food Funct. 6(2015):32-41. doi: 10.1039/c4fo00647j.

[41] F. Karaki, K. Ohgane, K. Dodo, Y. Hashimoto, Structure-activity relationship studies of Niemann-Pick type C1-like 1 (NPC1L1) ligands identified by screening assay monitoring pharmacological chaperone effect, Bioorg. Med. Chem. 21(2013):5297-309. doi: 10.1016/j.bmc.2013.06.022.

[42] M. Nekohashi, M. Ogawa, T. Ogihara, K. Nakazawa, H. Kato, T. Misaka, K. Abe, S. Kobayashi, Luteolin and quercetin affect the cholesterol absorption mediated by epithelial cholesterol transporter niemann-pick c1-like 1 in caco-2 cells and rats, PLoS ONE. 9(2014) e97901. doi: 10.1371/journal.pone.0097901.

[43] P.M. Cummins, Occludin: one protein, many forms, Mol. Cell. Biol. 32 (2012) 242-250. doi: 10.1128/MCB.06029-11.

[44] S.Y. Eum, D. Jaraki, L. Bertrand, I.E. András, M. Toborek, Disruption of epithelial barrier by quorum-sensing N-3-(oxododecanoyl)-homoserine lactone is mediated by matrix metalloproteinases, Am. J. Physiol. Gastrointest. Liver Physiol. 306 (2014) G992-G1001. doi: 10.1152/ajpgi.00016.2014.

[45] B.P. Head, H.H. Patel, P.A. Insel, Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling, Biochim. Biophys. Acta. 1838 (2014) 532-545. doi:10.1016/j.bbamem.2013.07.018.

[46] D. Lambert, C.A. O'Neill, P.J. Padfield, Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins, Biochem J. 387 (2005) 553-560. doi:10.1042/BJ20041377.

[47] D. Lambert, C.A. O'Neill, P.J. Padfield, Methyl-beta-cyclodextrin increases permeability of Caco-2 cell monolayers by displacing specific claudins from cholesterol rich domains associated with tight junctions, Cell. Physiol. Biochem. 20 (2007) 495-506. doi: 10.1159/000107533

[48] A. Nusrat, C.A. Parkos, P. Verkade, C.S. Foley, T.W. Liang, W. Innis-Whitehouse, K.K. Eastburn, J.L. Madara, Tight junctions are membrane microdomains, J. Cell Sci. 113 (2000) 1771-1781.

[49] A. Berthier, S. Lemaire-Ewing, C. Prunet, S. Monier, A. Athias, G. Bessède, J.P. Pais de Barros, A. Laubriet, P. Gambert, G. Lizard, D. Néel, Involvement of a calcium-dependent dephosphorylation of BAD associated with the localization of Trpc-1 within lipid rafts in 7-ketocholesterol-induced THP-1 cell apoptosis, Cell Death Differ. 11 (2004) 897-905. doi:10.1038/sj.cdd.4401434.

[50] J.B. Massey, Membrane and protein interactions of oxysterols, Curr. Opin. Lipidol. 17 (2006) 296-301. doi:10.1097/01.mol.0000226123.17629.ab. doi:10.1097/01.mol.0000226123.17629.ab.

[51] B. Sottero, P. Gamba, S. Gargiulo, G. Leonarduzzi, G. Poli, Cholesterol oxidation products and disease: an emerging topic of interest in medicinal chemistry, Curr. Med. Chem. 16 (2009) 685-705. doi: 10.2174/092986709787458353.

Figure legends

Figure 1. Analysis of permeability alteration and cytolysis in CaCo-2 cell monolayer.

A. TEER was measured in differentiated CaCo-2 cells treated with different concentrations of Oxy-mix (30, 60 or 90 μM) for 72 h (left panel). TEER time-course (24, 48, 72 h) was also performed by incubating differentiated CaCo-2 cells with 60 μM Oxy-mix (right panel). TEER was shown as negative percentage variation compared with TEER soon after each treatment (see baseline).

B. LDH release was evaluated in the culture media of differentiated CaCo-2 cells treated with 30, 60 or 90 μM Oxy-mix (left panel) or incubated with 60 μM Oxy-mix for 24, 48 or 72 h (right panel). LDH was evaluated as percentage referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to cultured cells grown at the same density of other samples. Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *p<0.05, ***p<0.001.

Figure 2. Time-dependent changes in MMP-2 and MMP-9 induction in differentiated CaCo-2 cells treated with dietary oxysterols.

A. The induction of MMP-2 and MMP-9 was evaluated by gel-zymography in CaCo-2 cells treated with 60 μM Oxy-mix for 24, 48 and 72 h.

B. MMPs induction was also evaluated with Oxy-mix individual compounds after 72 h cell incubation. Each oxysterol was added to the cells individually at the same concentration at which they were present in the mixture: 25.8 μM 7K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, 8.8 μM 7β-OH. Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *p < 0.05, **p<0.01 and ***p<0.001; significantly different vs. Oxy-mix: a p<0.05, b p<0.01, c p<0.001.

Figure 3. Involvement of oxidative and inflammatory reactions in Oxy-mix-mediated gelatinases induction.

A. The induction of MMP-2 and MMP-9 was analyzed by gel-zymography in CaCo-2 cells incubated with 60 μM Oxy-mix for 72 h, pretreated or not with DPI (2 μM for 30 min) or (-)-epicatechin (EPC) (10 μM for 1 h).

B. IL-8 levels were measured by ELISA in cell medium of differentiated CaCo-2 cells pre-treated or not with EPC (10 μM for 1h) or ARP 100 (200 nM for 1 h) and incubated with 60 μM Oxy-mix for 72 hrs. At the left, LDH cell release percentage is reported referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to cultured cells grown at the same density of other samples. Data are reported as means ± SD of three independent

experiments. Significantly different vs. controls: **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$, c $p<0.001$.

Figure 4. Oxy-mix induces TJs loss in differentiated CaCo-2 cells.

Decreased levels of ZO-1, occludin and JAM-A were detected by Western Blotting in lysates from CaCo-2 cells incubated with 60 μM Oxy-mix for 48 and 72 h. Data are expressed as percentage of control (100%). Values are means ± SD of three independent experiments. Significantly different vs. controls: **$p<0.01$, ***$p<0.001$.

Figure 5. Cellular distribution of TJs in differentiated CaCo2 cells treated with dietary oxysterols.

Marked delocalization of TJs ZO-1, occludin and JAM-A after cell monolayers incubation with 60 μM Oxy-mix for 48 and 72 h was visualized by immunofluorescence microscopy. For all images TJs were immunostained with green color; CaCo-2 cells nuclei were stained with DAPI and shown in blue color. Original magnification: X 400.

Figure 6. Cell pre-treatment with NADPH oxidase inhibitor restores TJ levels decreased by Oxy-mix cell incubation.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells 1 h pre-treated with NADPH oxidase inhibitor DPI (2 μM), incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *$p<0.05$, **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$.

Figure 7. Effect of ARP 100 on TJ decreased levels induced by Oxy-mix.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells in presence of 200 nM ARP 100 (1 h pre-treatment) incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: **$p<0.01$, *** $p<0.001$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$, c $p<0.001$.

Figure 8. Oxy-mix-dependent decrease of TJ cell levels is prevent by EPC.

ZO-1, occludin and JAM-A levels were evaluated in lysates from differentiated CaCo-2 cells in presence of 10 μM EPC (1 h pre-treatment) incubated or not with 60 μM Oxy-mix for 72 h. Protein levels were expressed as percentage of control (100%).

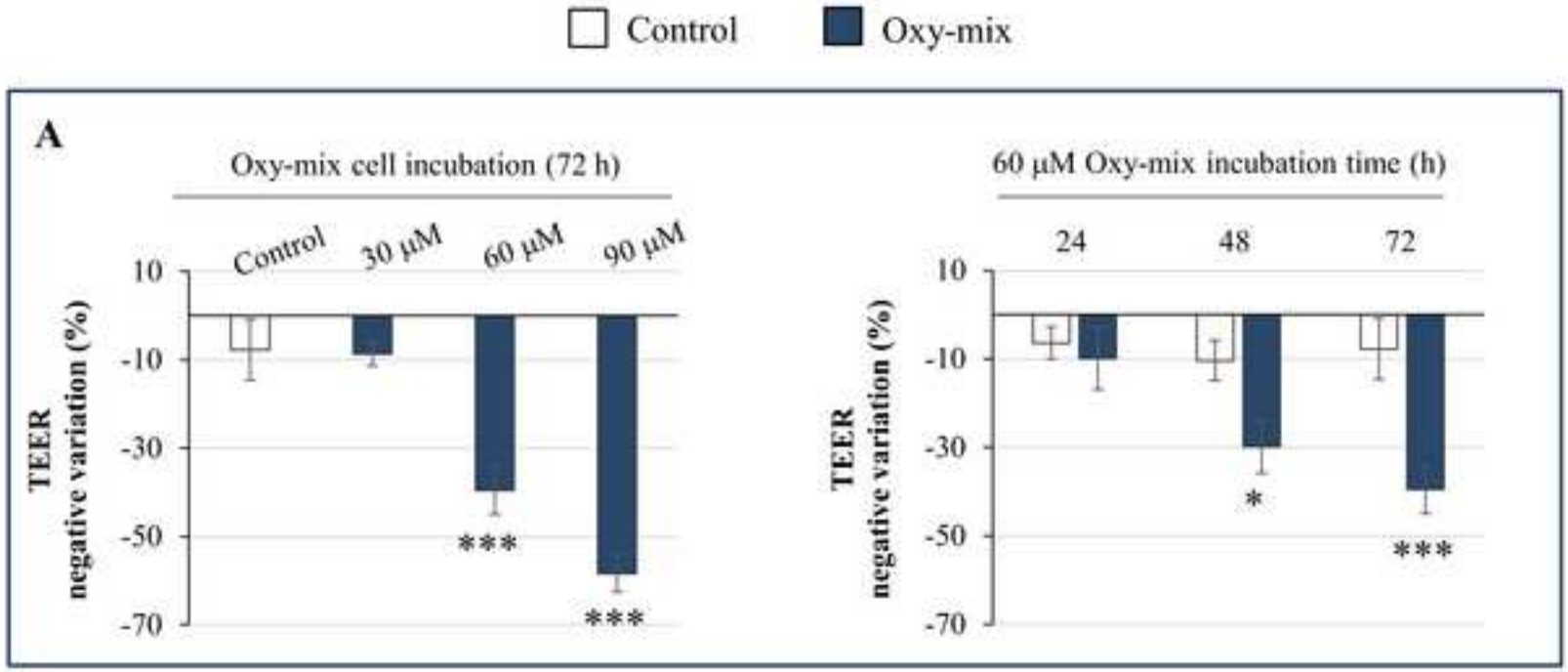
Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *$p<0.05$, **$p<0.01$; significantly different vs. Oxy-mix: a $p<0.05$, b $p<0.01$.

Figure 9. Effect of EPC and ARP 100 on TJs delocalization induced by oxysterols combination.

CaCo-2 cell monolayers were pre-treated or not with 10 μM EPC or 200 nM ARP 100 and incubated with 60 μM Oxy-mix for 72 h. Fluorescence microscopy was used to visualize TJs cellular distribution (X 400 magnification). TJ proteins were visualized as green immunofluorescence. Nuclei were stained with DAPI and shown in blue color.

ZO-1 localization was completely restored in presence of both EPC and ARP 100. Occludin and JAM-A do not appear to recover their membrane distribution with ARP 100 pre-treatment. On the other hand, EPC appears more effective than ARP 100, showing a correct reassembly of all TJs examined.

Figure 1
Click here to download high resolution image



B

Oxy-mix	LDH (%)
Control	2,12 ± 0,45
30 µM	2,65 ± 1,42
60 µM	4,67 ± 0,51
90 µM	14,51 ± 3,89*

Oxy-mix	LDH (%)
Control	1,95 ± 0,29
24 h	3,70 ± 0,71
48 h	2,57 ± 0,59
72 h	4,67 ± 0,51

Figure 2
Click here to download high resolution image

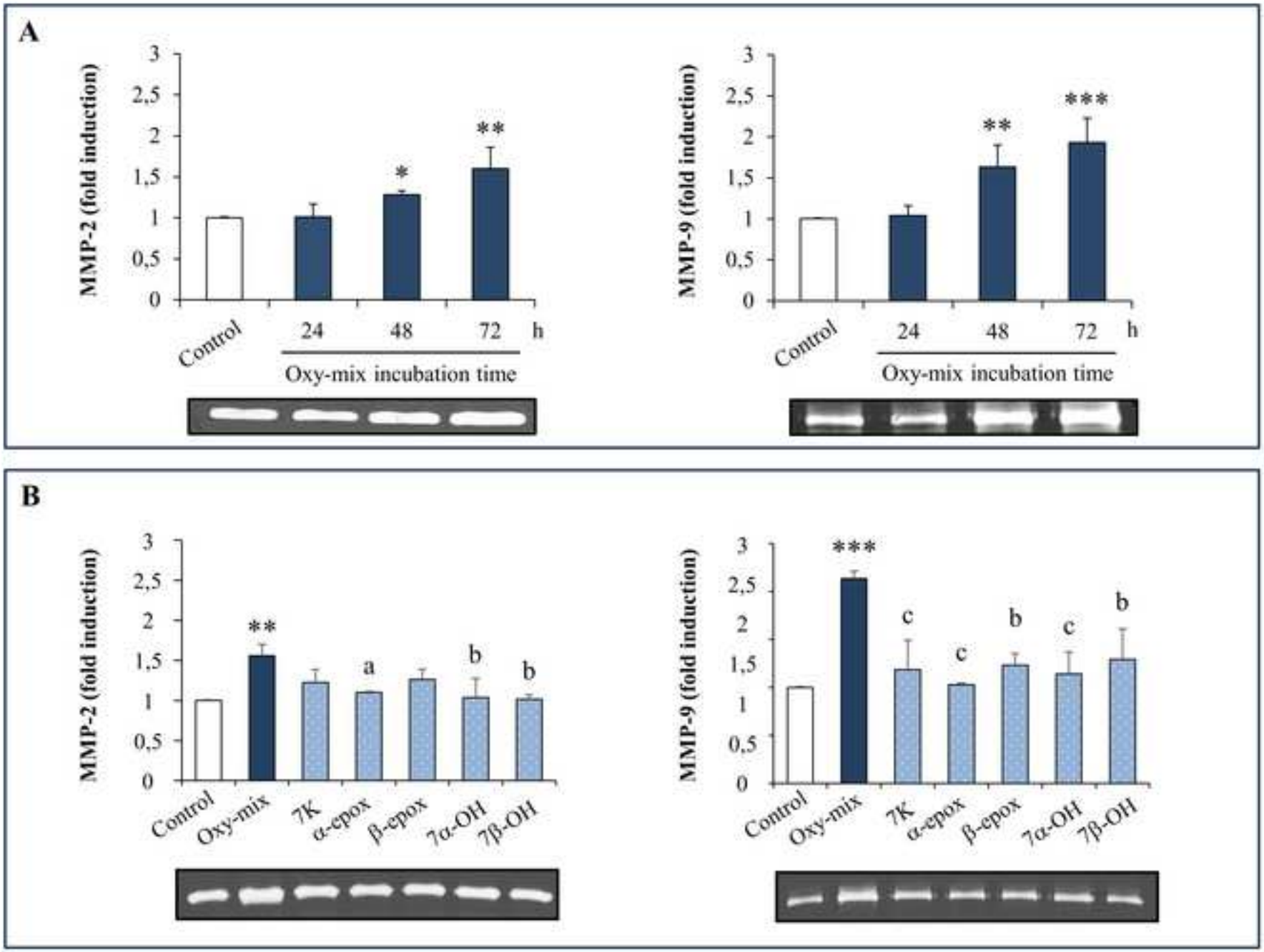
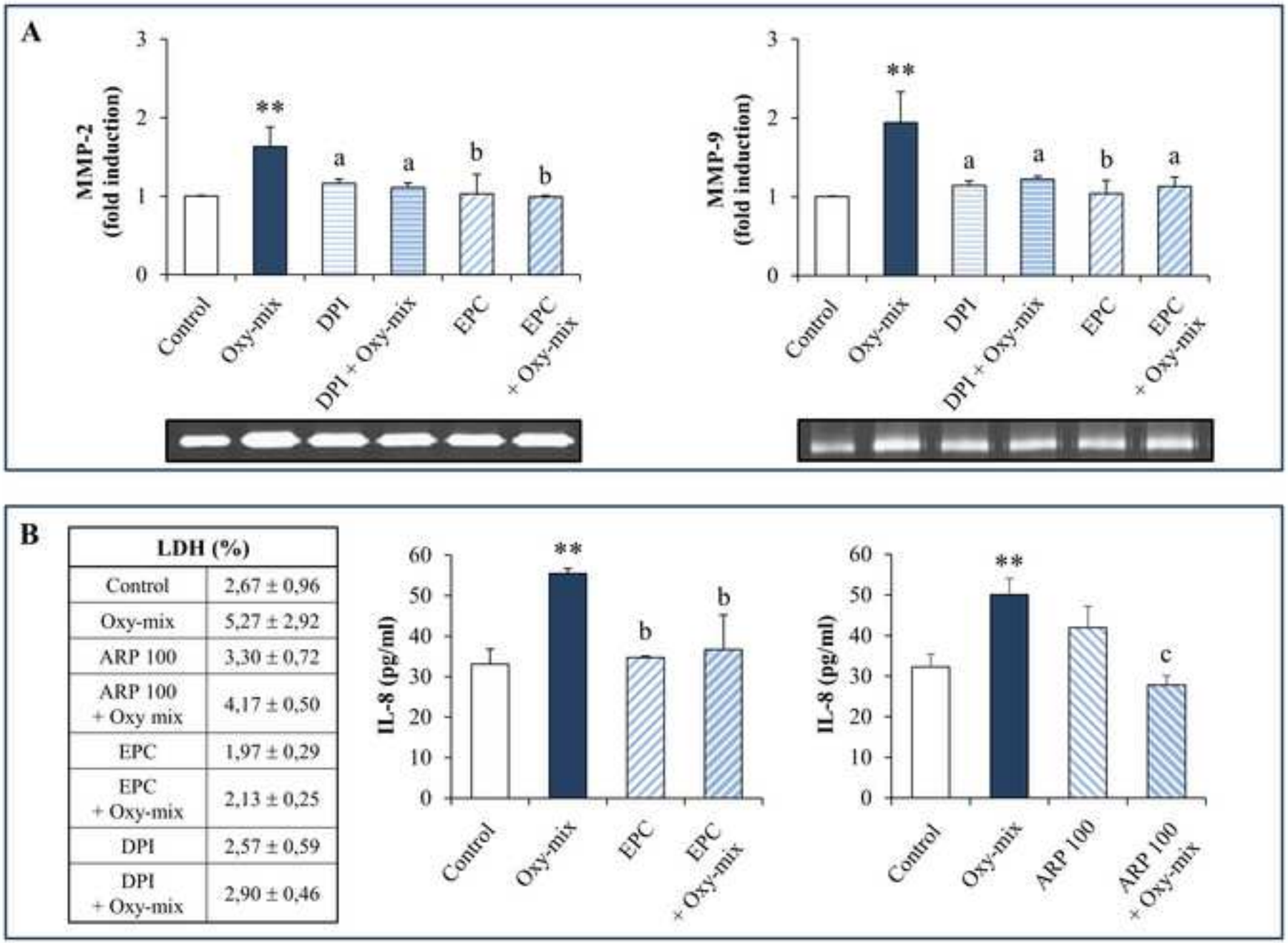


Figure 3
Click here to download high resolution image



LDH (%)	
Control	2,67 ± 0,96
Oxy-mix	5,27 ± 2,92
ARP 100	3,30 ± 0,72
ARP 100 + Oxy mix	4,17 ± 0,50
EPC	1,97 ± 0,29
EPC + Oxy-mix	2,13 ± 0,25
DPI	2,57 ± 0,59
DPI + Oxy-mix	2,90 ± 0,46

Figure 4
Click here to download high resolution image

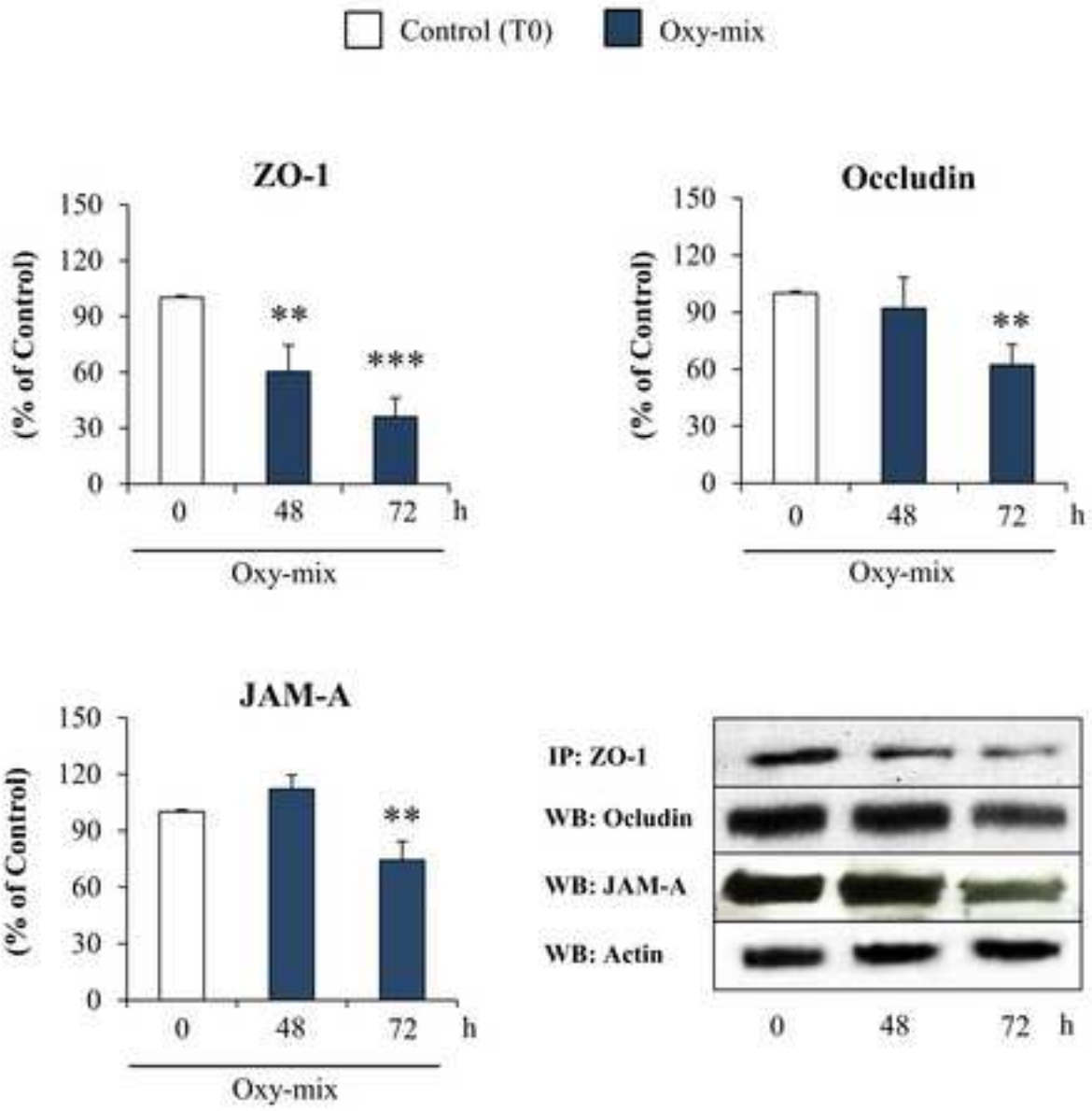


Figure 5
Click here to download high resolution image

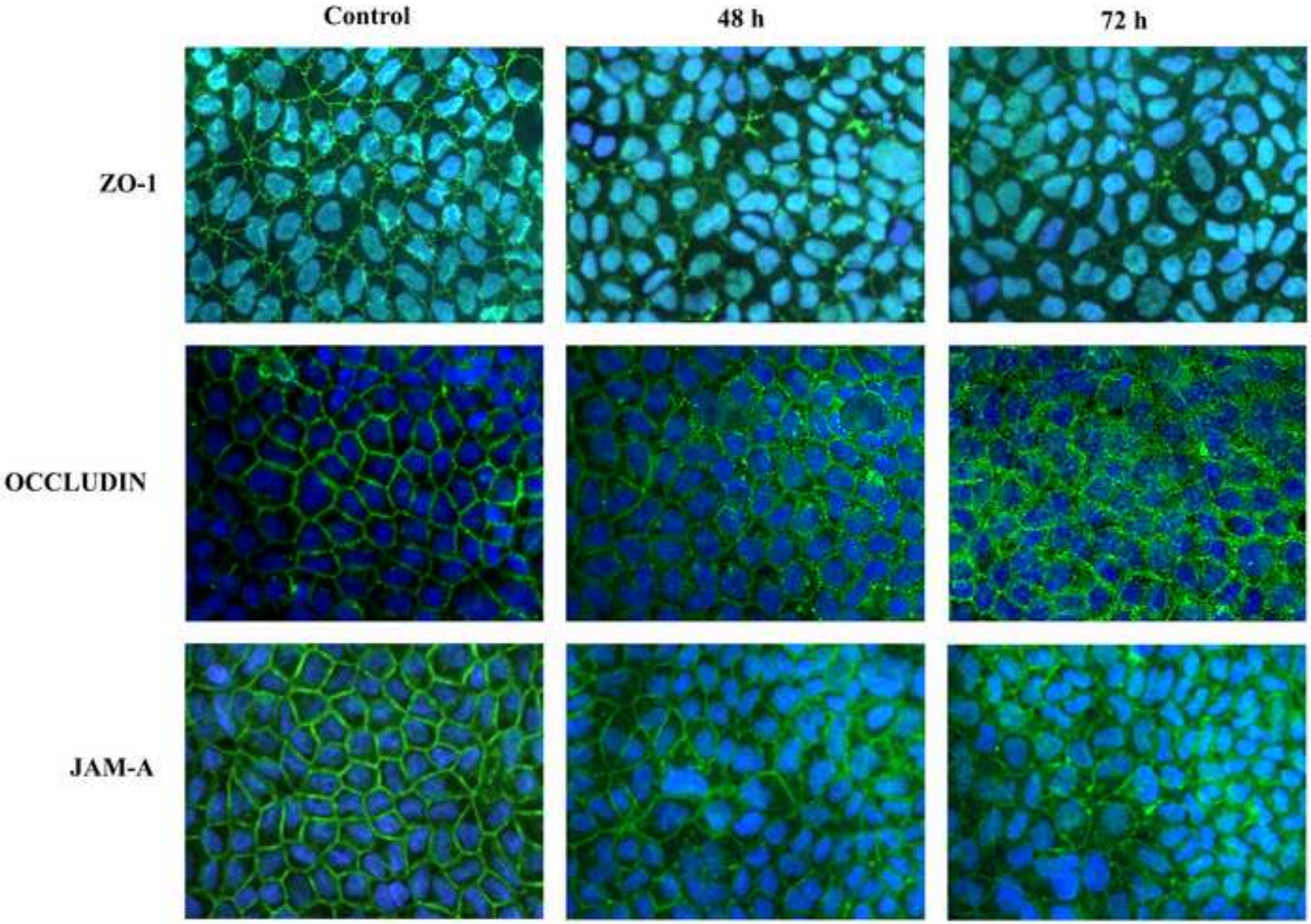


Figure 6
Click here to download high resolution image

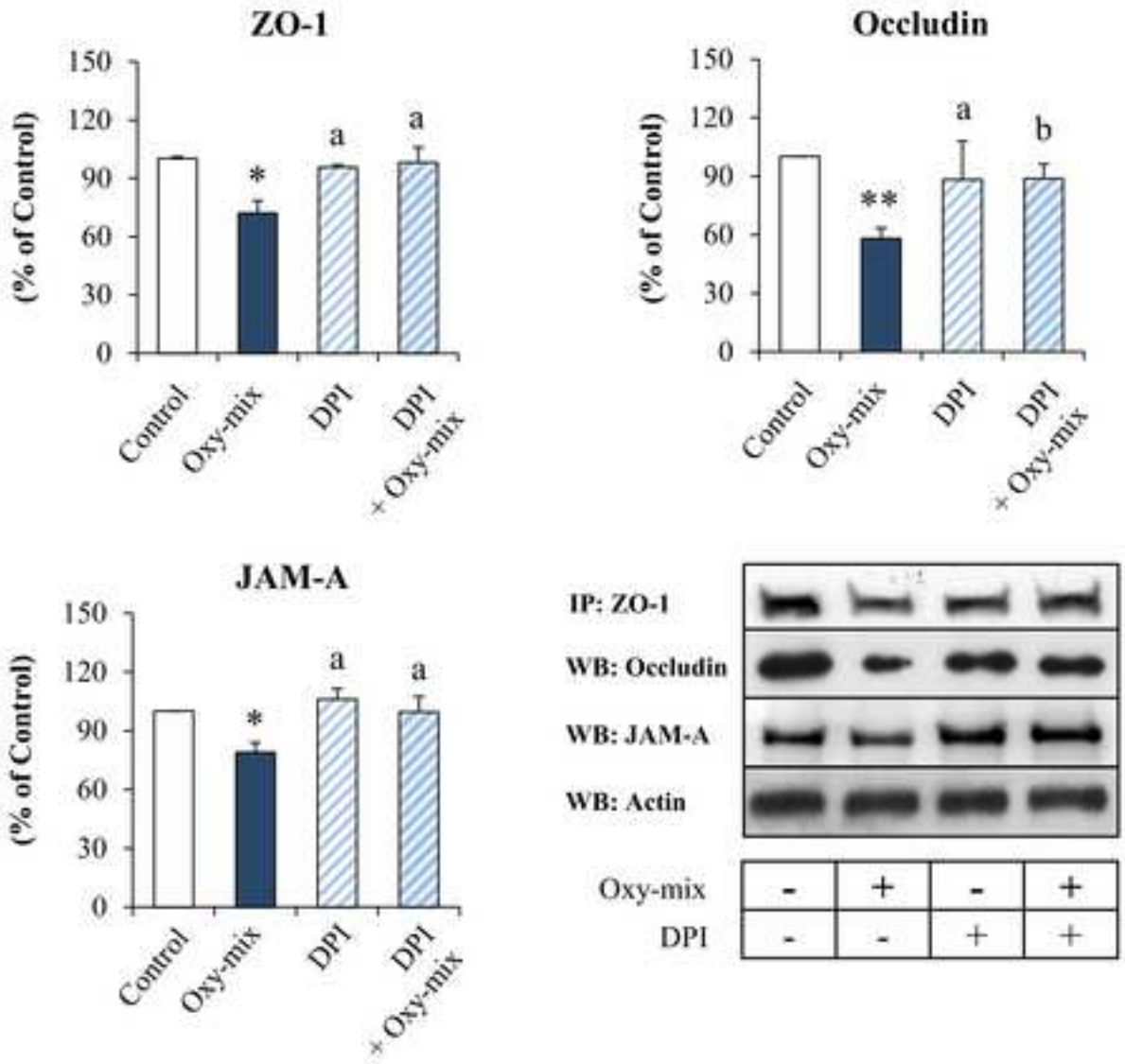


Figure 7
Click here to download high resolution image

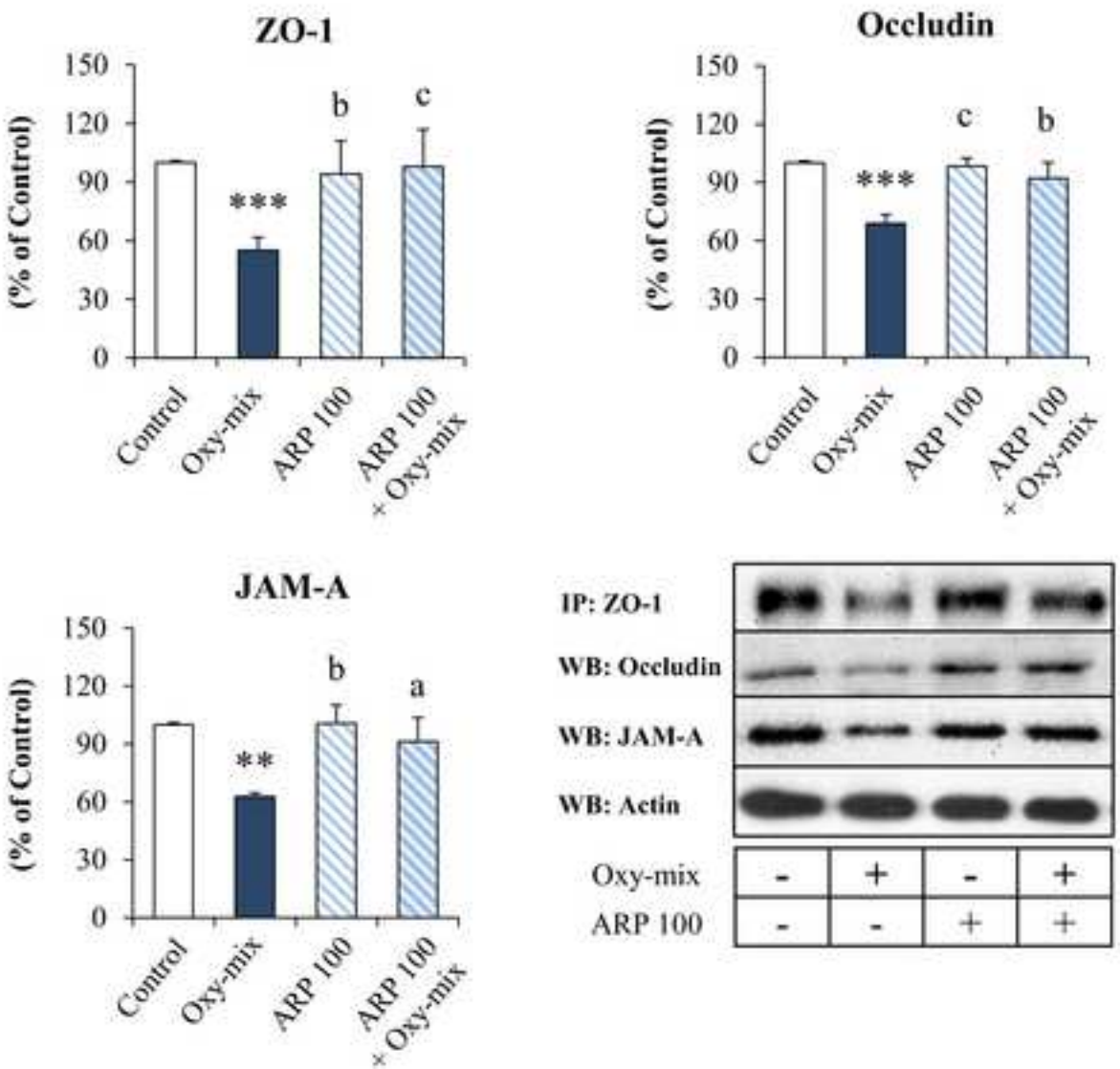


Figure 8
Click here to download high resolution image

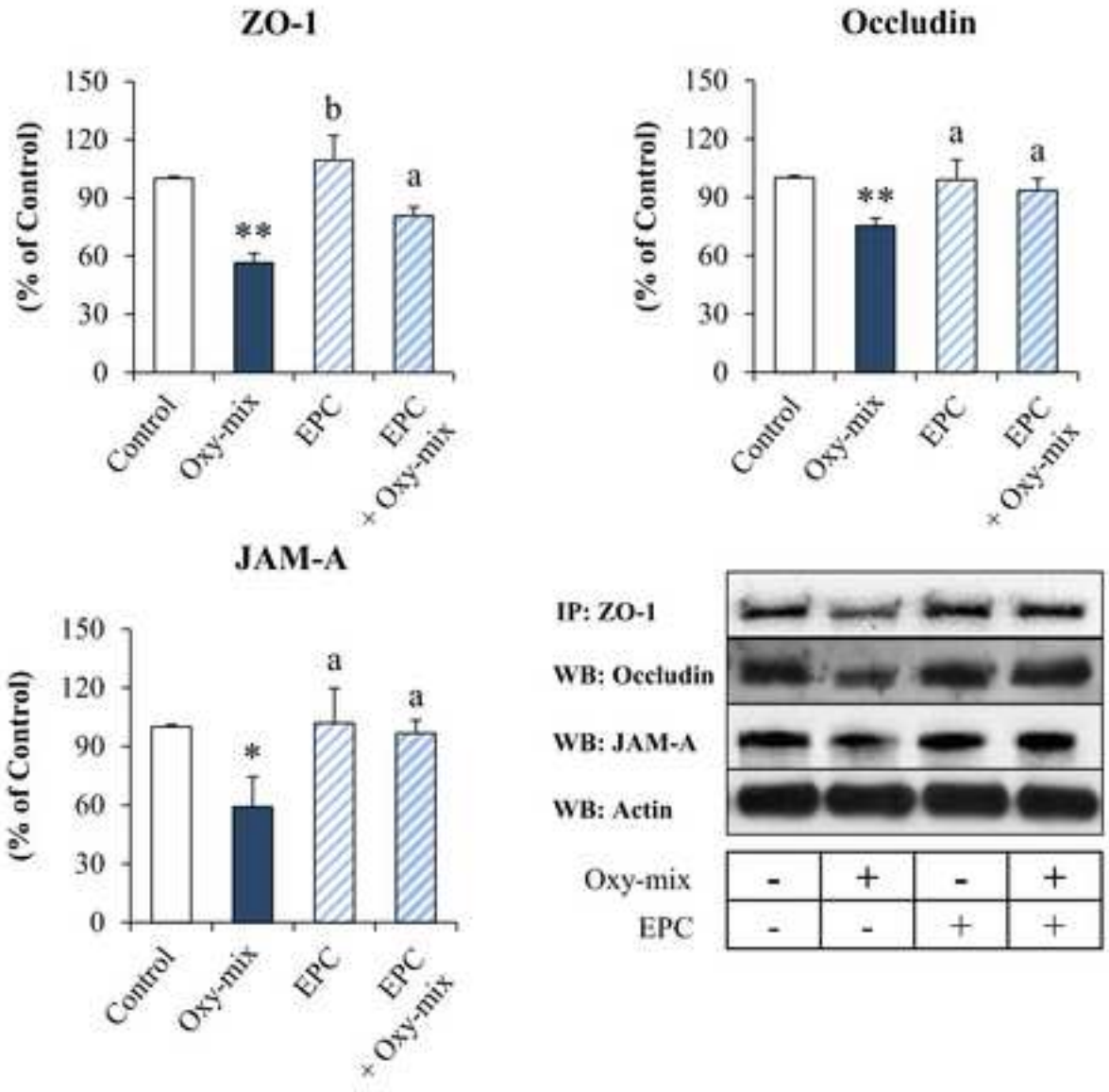


Figure 9
Click here to download high resolution image