The role of p38 MAPK in the induction of intestinal inflammation by dietary oxysterols: modulation by wine phenolics

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Abstract

Dietary oxysterols are cholesterol auto-oxidation products widely present in cholesterol-rich foods. They are thought to affect the intestinal barrier function, playing a role in gut inflammation. This study has characterized specific cell signals that are up-regulated in differentiated CaCo-2 colonic epithelial cells by a mixture of oxysterols representative of a hyper-cholesterolemic diet. p38 MAPK activation plays a major role, while other signal branches, i.e. the JNK and ERK pathways, make minor contributions to the intestinal inflammation induced by dietary oxysterols. p38 transduction might be the missing link connecting the known NADPH oxidase activation, and the induction of NF-κB-dependent inflammatory events related to oxysterols' action in the intestine. A NOX1/p38 MAPK/NF-κB signaling axis was demonstrated by the quenched inflammation observed on blocking individual branches of this signal with specific chemical inhibitors. Further, all these signaling sites were prevented when CaCo-2 cell were pre-incubated with phenolic compounds extracted from selected wines made of typical Sardinian grape varieties: red Cannonau and white Vermentino. Notably, Cannonau was more effective than Vermentino. The effect of Sardinian wine extracts on intestinal inflammation induced by dietary oxysterols might mainly be due to their phenolic content, more abundant in Cannonau than in Vermentino. Furthermore, among different phenolic components of both wines, epicatechin and caffeic acid exerted the strongest effects. These findings show a major role of the NOX1/p38 MAPK/NF-κB signaling axis in the activation of oxysterol-dependent intestinal inflammation, and confirm the concept that phenolics act as modulators at different sites of pro-oxidant and pro-inflammatory cell signals.

Keywords: cholesterol oxidation products, diet, cell signaling, polyphenols, NADPH oxidase, interleukins.

1. **Introduction**

The intestinal mucosa provides the first physical and functional defensive barrier against luminal antigens. Among the oxidants present in food, oxysterols, as dietary cholesterol oxidation products, are the most abundant. Cholesterol-rich food, which is exposed to oxygen and high temperature during processing and storage, undergoes auto-oxidation leading to the formation of oxysterols, mainly 7-hydroxycholesterols, 5,6-epoxycholesterols and 7-ketocholesterol.

The role of oxysterols in human pathophysiology has chiefly been linked to cardiovascular and neurodegenerative diseases.[1] Oxysterols have been widely studied in atherosclerotic processes, where they induce inflammation by modulating specific mitogen-activated protein kinases (MAPKs) signaling pathways, and by activating redox-sensitive transcription nuclear factor kappa B (NF-κB) in vascular cells.[2]

Only recently, oxysterols have been demonstrated to affect the intestinal barrier function.[3,4,5] They induce *in vitro* pro-inflammatory and pro-apoptotic effects, mainly by impairing redox balance: they increase reactive oxygen species (ROS) production by up-regulating intestinal NADPH oxidase (NOX) isoform 1.[3,4] However, the transductional and transcriptional signaling pathways that are activated by dietary oxysterols in intestinal cells still await clarification.

An important target for human health is to identify dietary compounds that could counteract oxysterol damage, thus helping to maintain correct gastro-intestinal function.

Most studies in this area, both on experimental models and in humans, have aimed to ascertain the beneficial properties of phenolics naturally present in foods of plant origin; these compounds are involved particularly in protecting against cardiovascular diseases, aging and cancer.[6,7]

Wine phenolic compounds have also been considered protective against diseases characterized by oxidative stress and inflammation.[8, 9] However, little is known about their beneficial effects on intestinal function.[10,11] Phenolics are the most abundant non-alcoholic components of wine; their concentration and composition in wine depend on the grape varieties and winemaking processes employed.[12,13,14] Phenolic compounds include phenolic acids and polyphenols, which are characterized by the presence of different numbers of phenolic rings and hydroxyl groups; they are commonly subdivided into flavonoids and non-flavonoid compounds.[15]

Wine phenolics can be transformed by the gut microflora into biologically-active compounds, which thus reach their highest concentration in the intestinal mucosa, where they exert maximum action.[11] Phenolics are generally considered to be antioxidants, acting directly as free-radical scavengers. However, they can also interfere with specific cellular red-ox signaling pathways, which control cell proliferation and survival.[16,17,18]

Studies on enterocyte-like CaCo-2 cells have demonstrated the antioxidant[19] and anti-inflammatory[20] properties of two phenolic extracts obtained from Sardinian wines, Cannonau red wine and Vermentino white wine. The preventive effects of Sardinian wine extracts against intestinal inflammation were suggested based mainly on the polyphenolic fraction's ability to down-modulate NOX1 activity.[20]

The present study aimed to characterize the MAPKs involved in inflammation-related cell signals, which can be activated by specific oxysterols added to cells in a mixture representative of a hyper-cholesterolemic diet. Differentiated CaCo-2 cells, which display a mature enterocyte phenotype, were used as experimental model. The common activation of NOX1/protein 38 (p38) MAPK/NF-κB appeared to be the principal signaling pathway involved. The potential preventive effect against activation of this signal by the oxysterol mixture was tested by pre-treating the cells with phenolic extracts from Cannonau and Vermentino Sardinian wines. As red wine contains a larger number, and different types, of phenolics than does white wine, the effect of the individual phenolic compounds present in both wine extracts was also studied.

2. Materials and methods

Reagents

Unless otherwise specified, all chemicals were from Sigma–Aldrich (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX (4500 mg/L glucose and 110 mg/L sodium pyruvate) and fetal bovine serum (FBS) were from Invitrogen (S. Giuliano Milanese, Italy). Gallic acid, (+)-catechin were from Fluka (Milan, Italy). The protein assay dye reagent 2-mercaptoethanol and the enhanced chemiluminescence (ECL) substrate for Western Blotting (WB) system were from Bio-Rad (Milan, Italy). Protein A – Sepharose resin and nitrocellulose membrane were from GE Healthcare (Milan, Italy). Mouse

anti-Nox activator 1 (NoxA1) primary polyclonal antibody (ab68523), and mouse anti-fibrillarin primary monoclonal antibody (ab4566) were from Abcam (Cambridge, MA, USA), and rabbit anti-NF-κB p65 primary monoclonal antibody (D14E12) was from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-phospho-extracellular signal regulated kinase (Erk) ½ (p-Erk) primary monoclonal antibody (05-797R) and rabbit anti-MAP Kinase ½ primary polyclonal antibody (06-182; Erk1/2) were from Millipore Corporation (Billerica, MA, USA). Non-fat dry milk (Blotto), goat anti-Nox1 primary polyclonal antibody (sc-5821; Mox1), mouse anti-phospho-tyr (p-tyr) primary monoclonal antibody (sc-7020), rabbit anti-c-Jun N-terminal kinase (JNK) primary polyclonal antibody (sc-572), mouse anti-p38 primary monoclonal antibody (sc-7972), goat anti-mouse (sc-2005) and goat anti-rabbit (sc-2004)-horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz (Tebu-Bio s.r.l., Magenta, Milan, Italy). NuPAGE Novex 10% Bis-Tris Proteins pre-cast polyacrylamide gels were from Life Technologies (Carlsbad, California, U.S.). Human Interleukin (IL)-8 Enzyme Linked Immunosorbent Assay (ELISA) Kit was from BioLegend (San Diego, USA).

Cell culture

Human colon adenocarcinoma CaCo-2 cells (20-25 passages; from Cell Bank Interlab Cell Line Collection, Genoa, Italy) were cultured at 1 x 10^6 cells/ml density in DMEM with GlutaMAX supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, 250 ng/ml amphotericin B) and gentamicin (0.04 mg/ml) at 37°C in a 5% CO_2 humidified atmosphere. After reaching 100% confluence (3-4 days after seeding), cells were grown for an additional 18 days with the medium replaced thrice weekly, to allow their full spontaneous differentiation.

Cell treatments

Differentiated CaCo-2 cells were placed in DMEM with 5% FBS and challenged with different dietary oxysterols (Oxy) added as 60 μM mixture, final concentration. The percentage composition of the Oxy used was 42.96% 7-ketocholesterol (7K), 32.3%, 5α,6α-epoxycholesterol (α-epox), 5.76% 5β,6β-epoxycholesterol (β-epox), 4.26% 7α-hydroxycholesterol (7α-OH), 14.71% 7β-hydroxycholesterol (7β-OH) . The concentration of the Oxy was calculated using an average molecular weight of 403 g/mol. Cell were treated with

oxysterols at different times to locate the temporal window for investigating the activation of different cell signaling pathways.

A second set of experiments was performed, pre-treating cells with Cannonau or Vermentino wine extracts (25 µg/ml) or with polyphenols present in the wines, added individually (10 µM caffeic acid, 10 µM gallic acid, 10 µM (+)-catechin, 10 µM (-)-epicatechin, 1 µM quercetin) for 1 hour at 37 °C. The wine extracts were obtained from Sardinian wines produced by Argiolas S.p.A. [Serdiana (Cagliari), Italy]. The wines were less than 6 months of age and neither aged nor fermented in oak casks; their phenolic extracts were obtained from 100 ml of wines as described by Deiana et al.[19] Cells were then incubated with the oxysterols mixture. In certain experiments, cells were also pre-treated with the NADPH oxidase inhibitor diphenyl iodonium (2 µM DPI) for 30 minutes, or with the p38 MAPK inhibitor SB203580 (20 µM) for 1 hour. The inhibitors were added before Oxy treatment and remained in the cell medium throughout the treatment period. The solvent used for diluting DPI was dimethyl sulfoxide (DMSO), while SB203580 was diluted in ethanol.

For the controls, cells were incubated with the same amount of solvent used to dilute the various substances employed.

Cells were then trypsinized, harvested by centrifugation at 173 g for 10 minutes at room temperature, and the medium collected. The cell pellet was then washed in phosphate buffered saline (PBS) 1X, and stored, as was the culture medium, at -20°C for subsequent analyses.

Evaluation of cell death and viability

Cell death was checked in terms of lactate dehydrogenase (LDH) enzyme cell release and the Neutral Red Test. LDH was measured spectrophotometrically in cell culture medium by recording, at 340 nm, NADH consumption during the conversion of pyruvic to lactic acid by this enzyme. Levels of LDH release by cells were expressed as percentages, taking as 100% the amount of enzyme released into the medium when 0.5% Triton X-100 was added to a non-treated culture flask containing the same cell density as treated cells. Percentage of cell death in term of % LDH release (Table 1).

Cell viability was evaluated with the Neutral Red Test by replacing cell culture medium with Neutral Red Solution (0.033% final concentration). After 30 minutes of incubation, the Neutral Red Solution was

carefully removed and the cells quickly rinsed with PBS. The incorporated dye was then dissolved in Neutral Red Solubilization Solution, acetic acid/ethanol/water (1:45:50, v:v:v), and the absorbance measured at 540 nm.[21] Results were expressed as % of cell viability (Table 1).

Evaluation of MAPKs activation

Activation of JNK, p38 and ERK1/2 MAPKs was detected in total cell extracts by Western Blotting (WB). Total cell extracts were obtained as follows: cell pellets were resuspended in lysis buffer [1 M Tris Hcl pH 7.4, 5 M NaCl, 0,5 M ethylenediaminetetraacetic acid (EDTA), 1% Igepal CA-630 (w:v), and 250 µM Na_3VO_4, 0,1% phenylmethylsulfonyl fluoride (PMSF), and 0,1 M dithiothreitol (DTT) as protease inhibitors] for 30 minutes in ice. Cell lysates were subsequently centrifuged at 18,000 x g for 15 minutes at 4°C, and the supernatants collected. Cellular protein content was quantified by the Bio-Rad protein assay dye reagent.[22] JNK and p38 WB analyses were performed upon immunoprecipitation: 50 µg protein total cell extracts were immunoprecipitated with rabbit anti-JNK primary polyclonal antibody (5 µl) or mouse anti-p38 primary monoclonal antibody (5 µl); immunoprecipitation was achieved by 2 hours' incubation at 4°C with Protein A – Sepharose resin. WB analysis of ERK1/2 MAPK was performed without immunoprecipitation. Protein total cell extracts were then incubated with boiling Laemmli buffer [200 mM Tris–HCl, pH 7.4, 36% glycerol (v:v), 7% sodium dodecyl sulphate (SDS; w:v), 1 M 2-mercaptoethanol, 0.1% bromophenol blue (w:v)].

Electrophoresis was run on 10% SDS-polyacrylamide gel (SDS-PAGE) for JNK and p38 analysis, while pre-cast polyacrylamide gels were used for ERK1/2. Gels were blotted to Hybond ECL nitrocellulose membrane. Saturation of nonspecific binding sites was for 2 hours (room temperature) with 5% (w:v) non-fat milk in 0.1M PBS – Tween 0.05% (w:v).

The membranes were incubated overnight at 4°C with different primary antibodies: mouse anti-p-tyr primary monoclonal antibody (1:500 v:v) was used for phosphorylated forms of JNK and p38; rabbit anti-p-ERK1/2 primary monoclonal antibody (1:2000 v:v) was used for ERK ½.

Goat anti-mouse HRP-conjugated secondary antibodies were used for JNK-related p-tyr (1:1000 v:v) and p38-related p-tyr (1:5000 v:v); goat anti-rabbit HRP-conjugated secondary antibody (1:2000 dilution) was used for ERK1/2.

Normalization was done after membrane stripping (incubation with H_20 for 5 minutes, followed by 6 minutes incubation with 0.2N NaOH and 5 minutes incubation with H_2O_2); antibodies used were: mouse anti-p38 primary monoclonal antibody (1:500 v:v), rabbit anti-JNK primary polyclonal antibody (1:500 v:v) and rabbit anti-MAP kinase ½ (Erk1/2) primary polyclonal antibody (1:2000 v:v).

All membranes were developed using ECL WB System (Bio-Rad) to detect chemiluminescence. The intensity of each band was quantitatively determined by densitometry, using Image Tool software. Intensities of samples were expressed as percentages compared to controls (whose band intensity was taken as 100%). All densitometric measurements were normalized against primary antibodies, as stated above.

Analysis of nuclear factor NF-κB

NF-κB activation was detected as p65 subunit increase in nuclear extracts by WB, as follows: cells were incubated for 20 minutes in ice with Buffer A solution [1 M KCl, 1 M 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES), 1 M $MgCl_2$, 0,01 M EDTA, 1% Igepal CA-630 (w:v)], and were centrifuged at 100 g for 10 minutes at 4°C. Pellets were suspended in Buffer B solution [5 M KCl, 1 M HEPES, 0,01 M EDTA] for 5 minutes in ice, and then in Buffer C solution [1 M HEPES, 0,01 M EDTA, 99% glycerol (v:v)] for 15 minutes in ice. Cell lysates were subsequently centrifuged at 18,000 g for 15 minutes at 4°C, and supernatants collected; 75 µg nuclear proteins of each sample were quantified by the Bio-Rad protein assay dye reagent, and used for analysis. Rabbit anti-NF-κB p65 monoclonal primary antibody (1:800 dilution), and goat anti-rabbit HRP-conjugated secondary antibody (1:2000 dilution) were used for WB (the procedure is described in the section “Evaluation of MAPKs activation”). For normalization, mouse anti-fibrillarin primary monoclonal antibody (1:800 dilution) was used.

Evaluation of NOX1 activation

Activation of intestinal NADPH oxidase isoform NOX1 was evaluated by WB.

Cell membrane proteins were extracted using Mem-PER Eukaryotic Protein Extraction Reagent Kit (following the manufacturer's protocol; Thermo Scientific, Rockford, IL, USA).

Considering that Nox1 is the membrane component, and NoxA1 is one of the cytoplasmic subunits of the intestinal NOX1 isoform, membrane extracts (100 µg proteins quantified by the Bio-Rad protein assay dye reagent) were immunoprecipitated with 5 µl goat anti-Nox1 primary polyclonal antibody. Mouse anti-NoxA1 primary polyclonal antibody (1:200 dilution), and goat anti-mouse HRP-conjugated secondary antibody (1:1000 dilution), were used for WB (the procedure is described in the section "Evaluation of MAPKs activation").

Evaluation of IL-8 protein levels

After 24 h cell treatment with Oxy, and 30 minutes' pre-treatment with p38 MAPK inhibitor SB203580 (20µM), the collected culture medium was used for ELISA detection. Total protein concentration of the cell culture medium was measured using Bio-Rad protein assay dye reagent. Levels of IL-8 were quantified using the Human IL-8 ELISA Kit (BioLegend, San Diego, USA) following the manufacturer's instructions. Sample absorbance values were read at 450 nm with wavelength correction of 550 nm in a microplate reader (Model 680 microplate reader Bio-Rad), and data analyzed using SlideWrite Plus software (Advanced Graphics Software). The cytokine profile of each sample was evaluated in duplicate. The values are expressed as pg/mg of proteins.

Statistical analyses

Results are expressed as means ± S.D. (standard deviation). The statistical significance of parametric differences among sets of experimental data was evaluated by the one-way ANOVA test associated with Bonferroni's multiple comparison post test.

3. RESULTS

Analysis of time-course activation of MAPK signaling pathways by the Oxy mixture

To identify MAPKs that are activated by dietary oxysterols in differentiated CaCo-2 cells, time-course experiments were performed in these cells in the presence of 60 μM Oxy, for times from 30 minutes to 4 hours, and p38, JNK and ERK1/2 activation was analyzed.

Among different MAPKs, p38 was highly responsive to Oxy exposure (Fig. 1A), whereas little and no activation, respectively, of JNK and ERK occurred (Fig. 1B, 1C).

The observed effect on p38 phosphorylation began within the first hour, reaching its maximum after 2 hours Oxy mixture treatment, and gradually decreasing thereafter to reach normal values at 4 hours. Statistical significance was maintained throughout the period of p38 activation (Fig. 1A). A significant increase of p-JNK, although lower than that of p38, was observed but only after 2 hours cell incubation with the Oxy mixture.

Different modulation of MAPKs by wine phenolic components

The efficacy of phenolic compounds present in extracts of two Sardinian wines, red Cannonau and white Vermentino, on modulating MAPKs activity was assessed in differentiated CaCo-2 cells. Sardinian wine extracts (25 μg wine extracts / ml cell incubation medium) were added to cell cultures 1 hour before treatment with Oxy. Based on the results of the above time-course analysis, cells were then treated for 2 hours with the Oxy mixture, to monitor the effect of wine phenolics on maximum p38 and JNK activation. Cell pre-treatment with Sardinian wine extracts prevented p38 induction by the Oxy mixture treatment (Fig. 2A), but did not prevent JNK activation (Fig. 2C). In particular, Cannonau extract exerted a protective effect on oxysterols-related p38 activation (52% decrease) more efficiently than Vermentino extract (33% decrease). Notably, the experimental group treated with Vermentino extract alone also showed significant p38 increase versus controls (Fig.2A).

In parallel, the role of each principal phenolic component present in both red and white wines on oxysterols-dependent p38 and JNK activation was studied (Fig. 2B, 2D). Among the phenolic components of wine extracts, catechin and epicatechin (two flavanols), gallic acid (a hydroxybenzoic acid), caffeic acid (a hydroxycinnamic acid) and quercetin (a flavonol) were considered.

As expected from data obtained for total phenolic wine extracts, only epicatechin and caffeic acid down-regulated p38 activation; they did not affect JNK activation, suggesting that wine phenolics exert their action on oxysterols' effects mainly via the p38 MAPK pathway. A slight, but not significant, p38 decrease was also observed in cells pre-treated with gallic acid (Fig. 2B).

Effect of wine extracts on NF-κB induction by the Oxy mixture

Time course experiments treating differentiated CaCo-2 cells with 60 μM Oxy mixture for times up to 4 hours were performed, in order to evaluate activation of the redox sensitive transcription factor NF-κB by this mixture.

NF-κB dimers are normally in the inactive state in the cytoplasm, because of their association with their subunit inhibitors IκBs. Upon cell stimulation, IκB kinase (IKK) phosphorylates IκB proteins, by inducing their ubiquitination and proteosomal degradation, and allowing p50/p65 NF-κB dimers to accumulate in the nucleus and to activate gene transcription. Nucleic accumulation of p65 NF-κB was thus considered as a parameter of NF-κB activation. Cell incubation with the Oxy mixture caused NF-κB activation after 2 hours treatment, and reached maximum increase at 3 hours (Fig. 3A).

Cell pre-treatment with wine extracts showed that only Cannonau red wine strongly inhibited the NF-kB activation induced by oxysterols (Fig. 3B). Consistent with previous findings, only epicatechin and caffeic acid, which had been able to decrease p38 MAPK activation, attenuated activation of NF-κB in CaCo-2 cells treated with Oxy mixture (Fig. 3C).

Effect of wine phenolics on inflammation induced by the Oxy mixture might involve NOX1/p38 MAPK/NF-κB pathway

Previous studies have indicated that dietary oxysterols have pro-inflammatory effects, which occur mainly by activating oxidative cell signals through the induction of NADPH oxidase isoform NOX1, and subsequent ROS generation.

Therefore, the relationship between oxysterols-dependent NOX1 activation and p38 MAPK/NF-κB induction was assessed in CaCo-2 cells treated with oxysterols. Further, because wine phenolics have been

considered for their antioxidant and anti-inflammatory effects in different cell types, modulation of this signaling pathway was investigated as a possible target for the anti-inflammatory effect of wine phenolics. In order to explore the NOX1/p38 MAPK/NF-κB signaling axis in oxysterols-induced inflammation, CaCo-2 cells were pre-treated for 1 hour with the selective chemical p38 inhibitor SB203580, or for 30 minutes with the NADPH oxidase inhibitor DPI (Fig. 4). Inhibition of p38 with SB203580 (Fig. 4A1) significantly prevented both the increase of p65 NF-κB in nuclei (Fig. 4A2) and production of IL-8 in cell medium (Fig. 4A3) induced by Oxy mixture treatment. As observed for p38 and NF-κB activation, cell pre-treatment with epicatechin or caffeic acid also exerted protective effects on IL-8 production (Fig. 4A3).

Similarly, inhibition of NOX1 with DPI decreased both p38 (Fig. 4B1) and NF-κB activation (Fig. 4B2) induced by the Oxy mixture. Further, the efficacy on modulating NOX1 activity of cell pre-treatment with epicatechin or with caffeic acid was assessed. NOX1 activity was up-regulated by 30 minutes' cell treatment with 60 μM Oxy mixture (80 % increase vs. control), and both wine phenolics prevented oxysterols-dependent NOX1 hyper-activation: the effect of epicatechin was stronger than that of caffeic acid, i.*e.* 52% *vs*. 37% decrease (Fig. 4B3).

4. DISCUSSION

The intestinal tract is the organ first exposed to the luminal oxidants present in foods; thus a high dietary intake of cholesterol and its oxidation products can likely contribute to the functional impairment of the enteric mucosa. Auto-oxidation of dietary cholesterol generally produces a mixture of oxysterols (mainly 7α-OH, 7β-OH, 7K, α-epoxy and β-epoxy) that has been found in food in concentrations ranging from 10 to 100 μM.[23,24]

Generally, when oxysterols reach the intracellular compartment they behave as potent molecular regulators. Intracellular oxysterols could act as secondary messengers in cell signal transduction pathways, of which ERK1/2, p38 MAPK, and JNK play important roles in coordinating a variety of cellular processes, including cell proliferation, differentiation, and death.

The pathophysiological effects of oxysterols appear to depend specifically on the type of oxysterol and the type of cell.[25] Signaling pathways induced by oxysterols have been investigated in depth in vascular cells. An

oxysterol mixture detected in hypercholesterolemic human plasma has been shown to induce expression of a cluster of differentiation 36 receptors, as well as the activation of protein kinase C (PKC)δ, the MAPK/ERK pathway and peroxisome proliferator-activated receptor γ, in U937 monocytes. In the mixture used in this study, certain oxysterols of dietary origin, namely 7K, 7α-OH, 7β-OH, α- and β-epoxy, were included with cholestan-3β, 5α, 6β-triol and 25-hydroxycholesterol.[26] Marked activation of ERK1/2, p38 MAPK and NF-κB, with increased production of pro-inflammatory cytokines, has been associated to enhanced expression of the NOX4 isoform in THP-1 macrophages treated with 7K or 25-hydroxycholesterol.[27] Similarly, the induction of AKT-PKC, ERK and p38 MAPKs by 7K, which led to NF-κB activation and interleukin production in human retinal ARPE-19, has been reported. However, in these cells NADPH oxidase did not appear to be involved in the inflammatory response induced by 7K.[28] Furthermore, prolonged activation of MAPKs, in particular of p38, by 7K at different concentrations has also been reported in liver cells.[29]

Despite the importance of the intestinal mucosa in oxysterol absorption, current knowledge on inflammation-related molecular mechanisms activated by oxysterols in the enterocytes is still limited.

A previous study showed a dietary oxysterol mixture to significantly enhance IL-6 and IL-8 mRNA expression and synthesis; this mixture was able to trigger inflammation mainly through NOX1 activation.[20] However, signaling events, which link intestinal NOX1 activation and interleukin production induced by oxysterols, have not yet been demonstrated.

The present study first analyzed the MAPK signaling pathways potentially induced in enterocyte-like CaCo-2 cells, by a mixture of oxysterols that closely mimics those present in cholesterol-containing foods;[30] 60 μM of the oxysterol mixture was chosen as the concentration corresponding to a high intake of dietary cholesterol oxidation products, capable of exerting a strong pro-inflammatory effect.[20]

Time-course analyses on MAPKs activation for up to 4 hours, in differentiated CaCo-2 cells treated with the Oxy mixture, indicate increased amounts of p38 and JNK phosphorylated forms, but not of ERK 1/2; maximum activation values were reached at 2 hours' incubation. Notably, activation of p38 was greater than that of JNK, and was already significant after 1 hour's oxysterol incubation. Taking the experimental model of cell treatment with Oxy mixture used as modeling an acute condition, it was hypothesized that p38 was mainly involved during the early phases of inflammatory response induced by oxysterols, because

of its marked and early induction compared to the other MAPKs. The observed decrease of NF-κB activation induced by Oxy mixture in differentiated CaCo-2cells pre-treated with p38 inhibitor SB203580 confirmed this hypothesis.

A previous study found that phenolic extracts from Sardinian wines lowered oxysterol-dependent intestinal NOX1 activation; in particular, the high content of phenolics present in Cannonau red wine exerted a strong anti-inflammatory effect, by lowering interleukin production through NOX1 inhibition. In particular, inhibiting effect of red wine extract was observed on expression and synthesis of IL-6 and IL-8 [20]. The same effect was demonstrated in CaCo-2 cells pre-treated with epigallocatechin gallate, which is the main polyphenol present in green tea.[4]

The potential role of phenolic compounds in preventing MAPKs activation by dietary oxysterols was investigated. Phenolics are normally found in a wide variety of plant-derived foods and beverages, including grains, legumes, fruits, vegetables, green and black tea, and wine; [31] they are present in the form of complex mixtures. Thus the effects on MAPKs of phenolic compounds from two types of Sardinian wines, either as a mixture in extracts, or individually, were tested. Further, the effect on oxysterols-dependent MAPKs activation of Cannonau red wine, which has an abundant polyphenolic fraction, was compared with that of Vermentino white wine, with a smaller polyphenolic fraction.[19] It emerged that, of the two MAPKs, JNK and p38, activated by the Oxy mixture, only p38 was completely prevented by cell pre-treatment with red wine phenolic extract; conversely, the inhibition of p38 by Vermentino extract was moderate. In agreement with available reports[32,11], these data indicate that, apart from their well-known direct antioxidant properties as free radical scavengers, wine phenolics are also able to modulate red-ox signaling pathways implicated in the inflammatory response; in this study they modulated p38 MAPK activity in intestinal cells.

Notably, as was observed in the cells pre-treated with NOX 1 and p38 chemical inhibitors, wine phenolics also exerted a blocking effect on the induction of oxysterol-related NOX1 and p38, thus preventing the NF-κB pro-inflammatory signal and interleukin production triggered by dietary oxysterols. In addition, preliminary results from our laboratory have indicated similar behavior in the expression and synthesis of other cytokines than IL-8, such as IL-6 and Monocytes Chemoattractant Protein-1 (data not shown). Even if

these results strongly suggest NOX1-dependent p38 activation, other NOX targets are protein tyrosine phosphatases, whose function can be p38 degradation. Therefore, NOX1 could inactivate theses redox-sensitive phosphatases, consequently indirectly maintaining p38 up-regulated ([33]Chen et al., 2009). Therefore, prevention of p38 activation by phenolic compounds through this pathway needs to be further elucidated.

Furthermore, the stronger inhibitory effect exerted by Cannonau extract might be due to the larger quantities of certain phenolics present in this red wine.

It was thus decided to concentrate on the effect of cell pre-treatment with the individual phenolic components present in both red and white wine extracts: the effects of catechin, epicatechin, gallic acid, caffeic acid and quercetin on Oxy-dependent JNK and p38 activation were evaluated. The effects of anthocyanins and stilbenes were not examined because they are only present in red wine. Among the different phenolics analyzed, only the flavanol epicatechin, and the hydroxycinnamic acid caffeic acid, significantly decreased Oxy-dependent p38 activation. Notably, none of the phenolics analyzed were involved in JNK modulation, at least in this experimental model.

Epicatechin is abundantly present in red wine, as well as in cocoa.[19,34] Its biological action in different experimental models has been extensively reviewed.[35] Transcriptomic analyses on endothelial cells have shown that epicatechin, and its metabolites found in human plasma after epicatechin consumption, regulate a wide range of genes responsible for inflammation, particularly those involved in cell adhesion, chemotaxis, and cytoskeleton organization. Expression of these genes appeared to be mainly regulated by this flavanol through the p38 and p65-NF-κB pathways.[36] In adipocytes, (-)-epicatechin has been found to completely inhibit tumor necrosis factor α (TNFα)-induced activation of adipocyte cell signals, including ERK1/2 and p38 phosphorylation, and NF-κB -DNA binding, both of which are chiefly involved in obesity, insulin resistance, and inflammation processes.[37] Modulation of the red-ox balance with down-regulation of NOX activity by epicatechin and its metabolites, preventing inflammatory processes, have been observed in both *in-vivo* and in-cell models, including intestinal ones.[38,39,20] Recently, the biological effects of flavanols have attracted great interest in gastrointestinal release studies for nutraceutical applications: niosome

carriers have recently been used to enhance the uptake and transport of (+)-epicatechin and (-)-epigallocatechin gallate in CaCo-2 cells.[40]

The majority of studies on caffeic acid in wine have concerned its quantification in different wine types, and have tested its antioxidant capacity. However, little is known on its effect on cell signaling pathways related to intestinal inflammation. A component of propolis, caffeic acid phenethyl ester, has been found to exert an anticolitic effect by attenuating NF-κB activation, as well as TNFα and IL-1β production, in rat-macrophage and human colonic epithelial cell lines stimulated with a peptidoglycan-polysaccharide. Caffeic ester was found to produce a significant reduction in gross colonic injury in female Lewis rats, whose colitis had been induced by bacterial peptidoglycan-polysaccharide.[41] The anti-inflammatory effect of caffeic acid, accompanied by a reduction in the severity of colitis, has recently been confirmed in other experimental models of colitis induced by dextran sodium sulphate in C57BL/6[42] and C3H/HeOuJ mice.[43] The attenuating effect of caffeic acid, as well as that of clorogenic acid, was evidenced at the transcriptional level on IL-8 production in CaCo-2 cells treated with TNFα and H_2O_2.[42] In C3H/ HeOuJ colitic mice, caffeic acid was found to attenuate IL-17 gene expression, with a related decrease in inducible nitric oxide synthase. The study authors suggested that caffeic acid action might occur through modulation of NF-κB and activation of detoxifying cytochrome CYP4B1, a gene that is possibly associated with inflammatory response.[43]

5. CONCLUSIONS

The present study demonstrated that a dietary mixture of oxysterols, in a dose typical of a high intake of dietary cholesterol oxidation products, exerts pro-inflammatory effects in intestinal cells, chiefly by activating specific signaling pathways, which temporally involve NOX1, p38 MAPK and NF-kB. The transduction molecule p38 might be the link between oxidative reactions induced by NOX1 up-regulation, and inflammatory events activated by NF-κB. However, the involvement of phosphatases, which can be lowered by NOX may represent another mechanism that ensure p38 activation, and should not be excluded.

Wine phenolics lower NF-κB activity in intestinal inflammation, thanks to their ability to down-modulate the signaling axis involving NOX1 and p38 MAPK activation, triggered by oxysterols in intestinal cells.

Experiments performed on red and white wine extracts, containing different amounts of phenolics, confirm that the activity of these compounds depends on the quantity and type present in wine.

Abbreviations

α-epox: 5α,6α-epoxycholesterol

β-epox: 5β,6β-epoxycholesterol

7α-OH: 7α-hydroxycholesterol

7β-OH: 7β-hydroxycholesterol

7K: 7-ketocholesterol

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DPI: diphenyl iodonium

DTT: Dithiothreitol

ECL: enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme Linked Immunosorbent Assay

Erk: extracellular signal regulated kinase

FBS: fetal bovine serum

HEPES: (4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid)

HRP: horseradish peroxidase

IκB: inhibitor of κB

IKK: IκB kinase

IL**:** interleukin

JNK: c-Jun N-terminal kinase

LDH: lactate dehydrogenase

MAPK: mitogen activated protein kinase

NOX: NADPH oxidase

NF-κB: nuclear factor-κB

NoxA1: Nox activator 1

Oxy: oxysterols

p38: protein 38

PBS: phosphate buffered saline

PKC: protein kinase C

PMSF: phenylmethylsulfonyl fluoride

ROS: reactive oxygen species

SB203580: 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole

SDS: sodium dodecyl sulphate

TNFα: tumor necrosis factor α

WB: Western blotting

Acknowledgements

The authors thank the CRT Foundation, Turin, and the University of Turin, Italy, for supporting this work.

Conflicts of interest

The authors state that there is no conflict of interest affecting any of the authors or any product named in this research.

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Table 1 – Cell death and viability in differentiated CaCo-2 cells treated with Oxy mixture in presence or absence of wine phenolics.

Experimental Groups	**LDH (% cell death)**	**Neutral Red (% cell viability)**
Control	0,9 ±0,4	100,00
Oxy	3,1 ± 2,1	96,17 ± 1,87
Cannonau	3,3 ± 1,1	100,17 ± 5,86
Cannonau + Oxy	2,6 ± 1,6	100,55 ± 14,36
Vermentino	3,4 ± 1,2	104,32 ± 3,43
Vermentino + Oxy	4,2 ± 2,1	95,28 ± 3,69
Catechin	2,8 ± 1,7	102,00 ± 7,64
Catechin + Oxy	4,3 ± 2,1	104,83 ± 10,69
Epicatechin	3,6 ± 0,3	100,22 ± 12,21
Epicatechin + Oxy	3,6 ± 1,1	95,34 ± 15,00
Gallic acid	5,5 ± 2,3	102,11 ± 10,53
Gallic acid + Oxy	5,4 ± 2,1	96,39 ± 19,81
Caffeic acid	4,6 ± 1,7	98,45 ± 7,12
Caffeic acid + Oxy	3,4 ± 1,4	98,16 ± 9,81
Quercetin	3,0 ± 1,6	99,37 ± 5,80
Quercetin + Oxy	4,9 ± 1,8	101,10 ± 10,17

Differentiated CaCo-2 cells were treated with 60 µM oxysterol mixture (Oxy) or solvent alone (control), or pre-treated for 1 hour with Cannonau or Vermentino wine extracts (25 µg/ml) or with individual phenolic compounds (10 µM Catechin, 10 µM Epicatechin, 10 µM Gallic acid, 10 µM Caffeic acid or 1 µM Quercetin). Cell death and viability were detected after 24 hours cell treatment. Cell death has been expressed as the percentage of LDH released in the medium compared to that present in the medium of cells cultured at the same density and treated with 0.5% Triton X-100 (taken as 100%). Cell viability has been expressed as

percentage of Neutral Red staining compared to control (taken as 100%). Data are means ± SD of 5 different experiments. Statistical difference within experimental groups was calculated using ANOVA associated with the Bonferroni post test.

Figure Legends

Figure 1. Time course of p38, JNK and ERK1/2 MAPKs activation by Oxy mixture in CaCo-2 cells.

Differentiated CaCo-2 cells were treated with 60 µM oxysterol mixture or solvent alone (control) for 30 minutes, 1, 2, 3 or 4 hours. The phosphorylated forms of p38 **(panel A)**, JNK **(panel B)** and ERK1/2 **(panel C)** were analyzed by WB (see materials and methods for details), and compared to the corresponding total unphosphorylated forms. A representative time course WB of each MAPK is shown. Protein levels were evaluated by densitometry and are reported as % increase compared to controls (100%). Data are reported as means ± SD of 3 experiments. Statistical differences within experimental groups were calculated using ANOVA associated with the Bonferroni post test: significantly different versus control (^{a} $p<0.001$).

Figure 2. Sardinian wine extracts and their principal phenolic components modulate p38 MAPK and JNK activation induced by Oxy mixture in CaCo-2 cells.

Action of wine phenolics on p38 MAPK and JNK activation was evaluated in differentiated CaCo-2 cells pre-treated for 1 hour with Cannonau or Vermentino wine extracts (25 µg/ml) or with individual phenolic compounds (10 µM Catechin, 10 µM Epicatechin, 10 µM Gallic acid, 10 µM Caffeic acid or 1 µM Quercetin); cells were then treated for 2 hours with 60 µM Oxy mixture or solvent alone (control).

Panel A - p38 activation in cells pre-treated with wine extracts.

Panel B - p38 activation in cells pre-treated with wine phenolics added individually.

Panel C - JNK activation in cells pre-treated with wine extracts.

Panel D - JNK activation in cells pre-treated with wine phenolics added individually.

The activation of different MAPKs was calculated as stated in Fig. 1, and is reported as % increase compared to control (100%). Data are reported as means ± SD of 5 experiments for each experimental condition. Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: significantly different versus control ($^{a}p<0.001$; ^{b} $p<0.05$); significantly different versus Oxy mixture ($^{\#}p<0.001$; $^{\&}p<0.05$; $^{@}p<0.01$). Representative WB of each treatment is shown. EPC = epicatechin.

Figure 3. Oxy mixture activates NF-κB in CaCo-2 cells; modulation by phenolic wine extracts.

NF-κB activation was analyzed by WB as p65 subunit increase in the nuclear fraction (see materials and methods for details). p65 protein levels were evaluated by densitometry and calculated as % increase compared to control (100%). Fibrillarin was used to normalize nuclear protein levels. A representative WB of each treatment is shown.

Panel A -Time course of NF-κB p65 activation in differentiated Caco-2 cells treated with 60 μM Oxy mixture or solvent alone (control) for 30 minutes, 1, 2, 3, or 4 hours. Data are reported as means ± SD of 4 experiments.

Panel B - Modulation by Sardinian wine extracts of Oxy-dependent NF-κB activation was evaluated by pre-treating differentiated CaCo-2 cells for 1 hour with Cannonau or Vermentino wine extracts (25 μg/ml); cells were then treated for the next 3 hours with 60 μM Oxy mixture. Data are reported as means ± SD of 4 experiments.

Panel C - Activity of epicatechin or caffeic acid on oxysterol-dependent NF-κB increase was analyzed in differentiated Caco-2 cells pre-treated with 10 μM phenolics (1 hour) and treated with 60 μM Oxy mixture (3 hours) or solvent alone (control). Data are reported as means ± SD of 5 experiments.

Statistical differences within the experimental groups were calculated using ANOVA associated with the Bonferroni post test: significantly different versus control ($^{a}p<0.001$; $^{b}p<0.05$; $^{c}p<0.01$); significantly different versus Oxy mixture ($^{\#}p<0.001$; $^{\&}p<0.05$; $^{@}p<0.01$). EPC = epicatechin.

Figure 4. p38 MAPK and NOX1 chemical inhibitors modulate inflammatory signals induced by the Oxy mixture in CaCo-2 cells: comparison with wine phenolic compounds.

The role of p38 MAPK in inflammation induced by the Oxy mixture was verified by evaluating NF-κB activation and IL-8 production after cell pre-treatment with the p38 inhibitor SB203580. Cell pre-incubation with the NADPH oxidase inhibitor DPI further confirmed NOX1 involvement in initiation of the cascade of inflammatory responses to oxysterols.

Panel A - Differentiated CaCo-2 cells were pre-treated with 20 μM SB 203580 for 1 hour and then treated with 60 μM Oxy mixture or solvent alone (control) for a further 2, 3 or 24 hours, in order to evaluate p38

(**A1**), p65 NF-κB (**A2**) and IL-8 increase (**A3**), respectively. IL-8 production was also evaluated by 1 hour cell pre-treatment with 10 µM epicatechin or caffeic acid and 24 hours with the Oxy mixture (**A3**). The phosphorylated forms of p38 and nuclear p65 were analyzed by WB, and compared to the corresponding total unphosphorylated form of p38 and nuclear protein fibrillarin. IL-8 release into the cell medium was detected by ELISA (see materials and methods for details). Data of A1 and A2 are reported as means ± SD of 4 experiments; data of A3 are reported as means ± SD of 5 experiments.

Panel B: Differentiated CaCo-2 cells were pre-treated with 2 µM diphenyl iodonium (DPI) for 1 hour and then treated with 60 µM Oxy mixture or solvent alone (control) for a further 2 or 3hours, in order to evaluate p38 (**B1**) or p65 NF-κB (**B2**), respectively. NOX1 activation was evaluated in differentiated CaCo-2 cells pre-treated for 1 hour with 10 µM epicatechin or caffeic acid and then treated for 30 minutes with the Oxy mixture (**B3**) as described in the materials and methods section. Data are reported as means ± SD of 4 experiments for each experimental condition.

Statistical differences within experimental groups were calculated using ANOVA associated with the Bonferroni post test: significantly different versus control ($^{a}p<0.001$; $^{b}p<0.05$; $^{c}p<0.01$); significantly different versus the Oxy mixture ($^{\#}p<0.001$; $^{\&}p<0.05$; $^{@}p<0.01$). EPC = epicatechin.

Graphical Abstract

Dietary oxysterols mainly act through the p38 MAPK/NF-κB signaling axis to promote inflammation in intestinal cells. This pathway activation appears to be closely related to NOX1 induction by oxysterols. Phenolic components present in both white and red wine, such as epicatechin and caffeic acid, can exert their anti-inflammatory effects, by targeting each branch of this signaling axis.

IL-8**:** interleukin 8; NF-κB: nuclear factor-κB; NOX: NADPH oxidase; p38MAPK: protein 38 mitogen activated protein kinase; ROS: reactive oxygen species; SB203580: 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole.

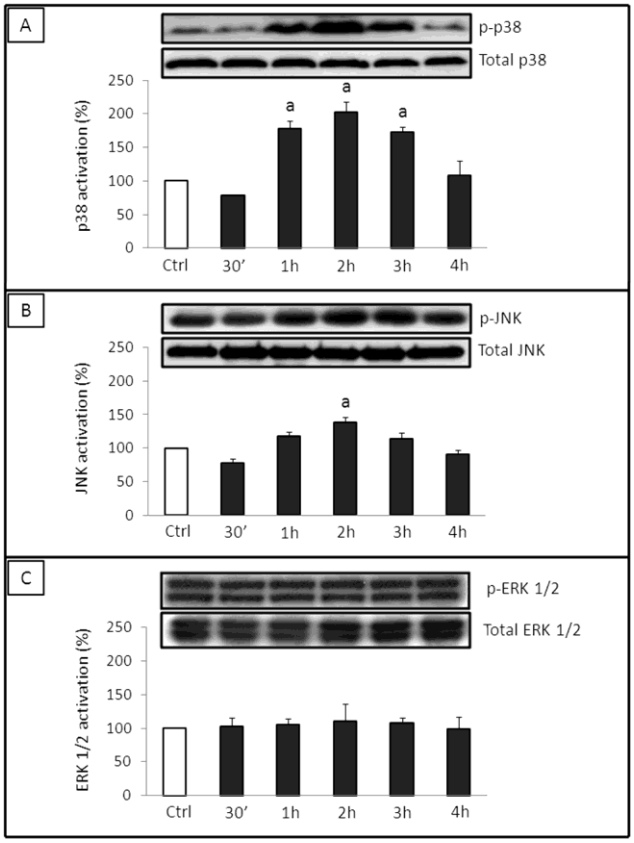
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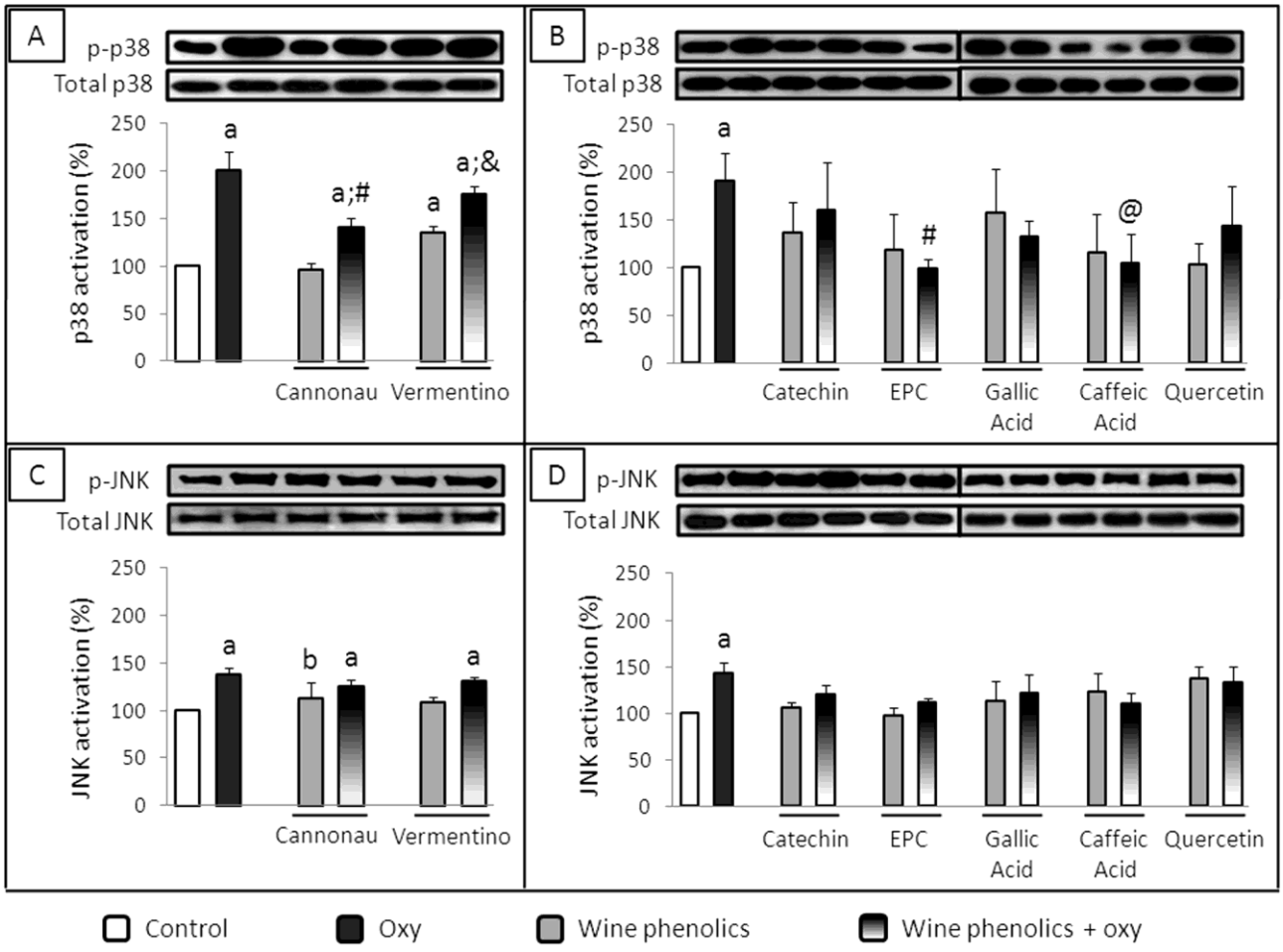
Figure 1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p38 activation (%)
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Quercetin
Control
Oxy
Wine phenolics
Wine phenolics + oxy

Figure 2

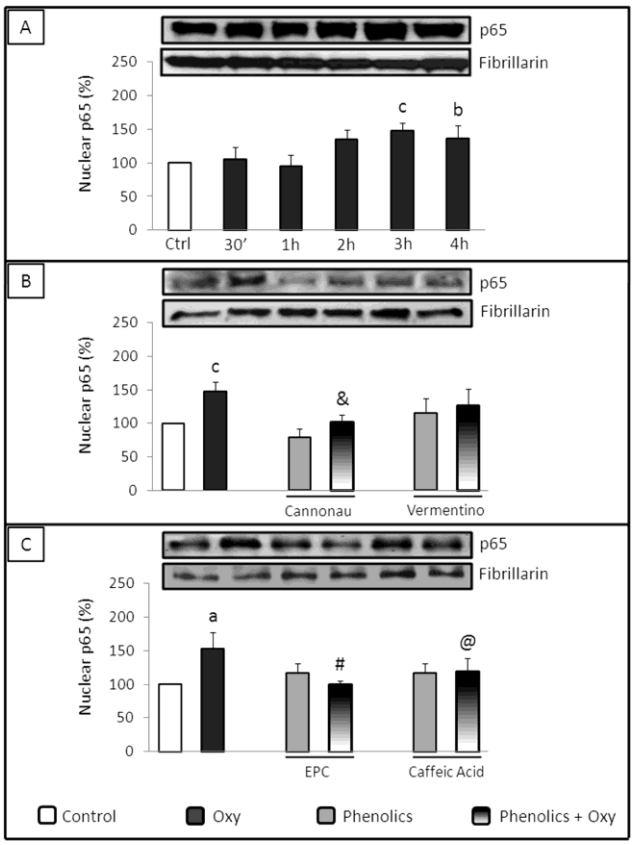


Figure 3

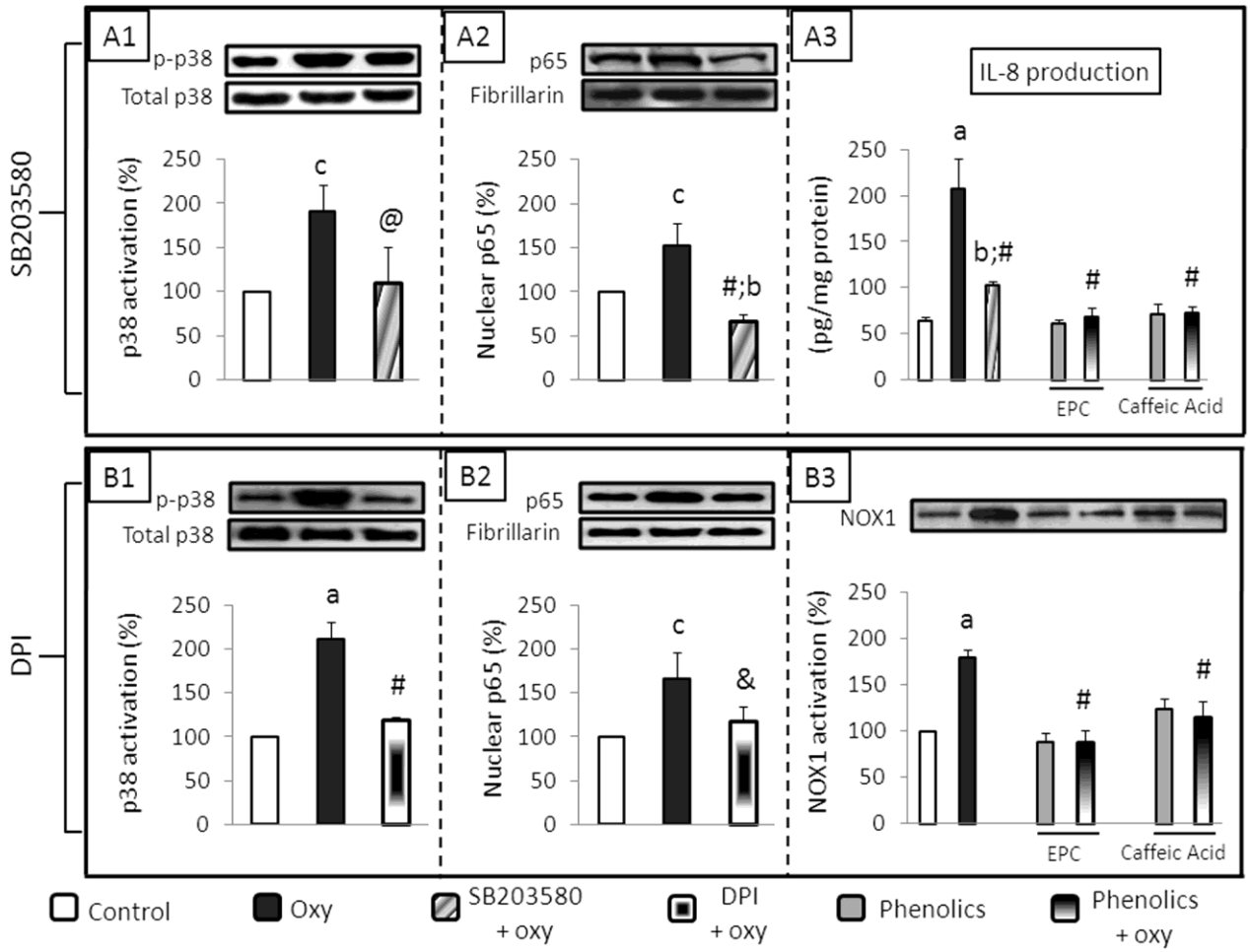
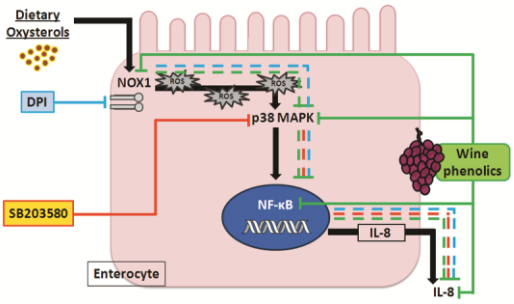


Figure 4



Graphical Abstract