Up-regulation of COX-2 and mPGES-1 by 27-hydroxycholesterol and 4-hydroxynonenal: A crucial role in atherosclerotic plaque instability.

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Abstract

Atherosclerosis is currently understood to be mainly the consequence of a complicated inflammatory process at the different stages of plaque development. Among the several inflammatory molecules involved, up-regulation of the functional cyclooxygenase 2/membrane-bound prostaglandin E synthase 1 (COX-2/mPGES-1) axis plays a key role in plaque development. Excessive production of oxidized lipids, following low-density lipoprotein (LDL) oxidation, is a characteristic feature of atherosclerosis. Among LDLs, the oxysterol 27-hydroxycholesterol (27-OH) and the aldehyde 4-hydroxynonenal (HNE) substantially accumulate in the atherosclerotic plaque, contributing to its progression and instability through a variety of processes. This study shows that 27-OH and HNE promote up-regulation of both the inducible enzymes COX-2 and mPGES-1, leading to increased production of prostaglandin (PG) E2 and inducible nitric oxide synthase, and the subsequent release of nitric oxide in human promonocytic U937 cells. The study also examined the potential involvement of the functionally coupled COX-2/mPGES-1 in enhancing the production of certain pro-inflammatory cytokines and of matrix metalloproteinase 9 by U937 cells. This enhancement is presumably due to the induction of PGE2 synthesis, as a result of the up-regulation of the COX-2/mPGES-1, stimulated by the two oxidized lipids, 27-OH and HNE. Induction of PGE2 synthesis might thus be a mechanism of plaque instability and eventual rupture, contributing to matrix metalloproteinase production by activated macrophages.

Keywords	Atherosclerosis; Inflammation, Plaque instability; 27-Hydroxycholesterol; 4-Hydroxynonenal.
Taxonomy	Molecular Biology, Biochemistry

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Turin, September 26, 2018

Dear Prof. Huiyong Yin
Editor
Free Radical Biology and Medicine

we are re-submitting the second revised version of our manuscript Ref: FRBM_2018_57 *"Up-regulation of COX-2 and mPGES-1 by 27-hydroxycholesterol and 4-hydroxynonenal: a crucial role in atherosclerotic plaque instability"*.

We thank the Reviewer for the thoughtful review and helpful comments, which have guided the revision of the manuscript. With the hope of having answered in a satisfactory way to all points raised by the Reviewer, we are looking forward to hearing from you.

Best regards,
Gabriella Leonarduzzi

Turin, September 26, 2018

We thank the Reviewer for the thoughtful review and helpful comments, which have guided the revision of the manuscript. Hereafter, please find our response to the comments.

Reviewer 3:
1. The title has been changed.
2. Concerning the English language, the manuscript has been reviewed by a native speaker.
3. In the Introduction and in the Discussion sections we have added some comments regarding the missing points suggested by the Reviewer (plus references).

With the hope of having answered in a satisfactory way to all comments raised by the Reviewer, we are looking forward to hearing from you.

Best regards,
Gabriella Leonarduzzi

Up-regulation of COX-2 and mPGES-1 by 27-hydroxycholesterol and 4-hydroxynonenal: a crucial role in atherosclerotic plaque instability

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Abstract

Atherosclerosis is currently understood to be mainly the consequence of a complicated inflammatory process at the different stages of plaque development. Among the several inflammatory molecules involved, up-regulation of the functional cyclooxygenase 2/membrane-bound prostaglandin E synthase 1 (COX-2/mPGES-1) axis plays a key role in plaque development. Excessive production of oxidized lipids, following low-density lipoprotein (LDL) oxidation, is a characteristic feature of atherosclerosis. Among LDLs, the oxysterol 27-hydroxycholesterol (27-OH) and the aldehyde 4-hydroxynonenal (HNE) substantially accumulate in the atherosclerotic plaque, contributing to its progression and instability through a variety of processes. This study shows that 27-OH and HNE promote up-regulation of both the inducible enzymes COX-2 and mPGES-1, leading to increased production of prostaglandin (PG) E_2 and inducible nitric oxide synthase, and the subsequent release of nitric oxide in human promonocytic U937 cells. The study also examined the potential involvement of the functionally coupled COX-2/mPGES-1 in enhancing the production of certain pro-inflammatory cytokines and of matrix metalloproteinase 9 by U937 cells. This enhancement is presumably due to the induction of PGE_2 synthesis, as a result of the up-regulation of the COX-2/mPGES-1, stimulated by the two oxidized lipids, 27-OH and HNE. Induction of PGE_2 synthesis might thus be a mechanism of plaque instability and eventual rupture, contributing to matrix metalloproteinase production by activated macrophages.

***Keywords*:** Atherosclerosis; Inflammation, Plaque instability; 27-Hydroxycholesterol; 4-Hydroxynonenal.

Abbreviations: 27-OH, 27-hydroxycholesterol; COX-2, cyclooxygenase-2; EC, endothelial cell; EGCG, epigallocatechin gallate; ERK, extracellular signal-regulated kinases; HNE, 4-hydroxynonenal; IL, interleukin; iNOS, inducible nitric oxide synthase; LDL, low density

lipoprotein; LXR, liver X receptor; MMP, matrix metalloproteinase; mPGES-1, membrane-bound prostaglandin E synthase-1; NF-κB, nuclear factor-κB; NO, nitric oxide; oxLDL, oxidized LDL; PG, prostaglandin; PK, protein kinase; ROS, reactive oxygen species; SMC, smooth muscle cell; TIMP, tissue inhibitor of MMP; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor.

1. Introduction

Atherosclerosis is a complex chronic inflammatory disease of the vessel wall. Inflammation, as well as innate and adaptive immune responses, and oxidative stress play a central role in all stages of atherosclerosis including the transition of stable atherosclerotic plaques to unstable plaques [1,2].

Development of atherosclerotic lesions is preceded by impaired vascular endothelium function which favors the retention and accumulation of low-density lipoproteins (LDLs) in the sub-endothelial space by specific proteins of extracellular matrix, including collagen and proteoglycan. Here, the lipid moieties of LDL undergo lipid peroxidation resulting in the release of various reactive oxidized lipids, including aldehydes, cholesterol oxidation products, and oxidized phospholipids. Many studies have demonstrated that accumulation of plaque lipids enhances plaque formation, supporting the concept that the plaque is atherogenic. Oxidized LDLs (oxLDLs), which accumulate progressively in atherosclerotic lesions, stimulate smooth muscle cell (SMC) differentiation and proliferation, monocyte/macrophage recruitment and differentiation, platelet aggregation, up-regulation of leukocyte adhesion molecules, and matrix metalloproteinase (MMP) activation. Finally, the intercellular cross-talk that occurs among vascular cells leads to a fibroproliferative response. During this response, the extracellular matrix components, released by differentiated SMCs, play a key role in plaque formation and progression, providing the structural integrity of the plaque itself, as well as contributing to cell migration and proliferation [1].

In particular, the interaction between oxLDLs and monocyte/macrophage plays predominant roles during initiation and progression of atherosclerosis. Activated macrophages not only take up the oxLDLs through the scavenger receptor CD36 to form lipid-loaded foam cells, but also release pro-inflammatory molecules, such as cytokines, chemokines, and adhesion molecules [1]; the release of inflammatory molecules induces an inflammatory state, with subsequent activation of the

other arterial wall cells, resulting in a lesion that is unstable and prone to rupture when exposed to increasing hemodynamic stress. Plaques vulnerable to rupture - the major cause of acute coronary syndrome - are characterized by a lipid-rich necrotic core, accumulation of inflammatory cells, and fibrous cap weakening, due both to ongoing inflammation and to an imbalance between extracellular matrix synthesis and degradation [3-6].

Of the inflammatory molecules involved, cyclooxygenase 2 (COX-2), which is an inducible enzyme and overexpressed by activated macrophages in atherosclerotic plaques, may participate in the pathogenesis of plaque progression and instability through a variety of processes. These comprise activation of chemotaxis, induction of vascular permeability, propagation of inflammatory molecules, stimulation of SMC migration, and the resultant induction of itself. Thus COX-2 is responsible for the production of eicosanoids, mainly prostaglandin (PG) E_2, in inflammatory diseases. PGE_2 is formed from arachidonic acid via the COX-2-catalyzed formation of PGH_2 and further transformation by PGE synthases. The isomerization of the endoperoxide PGH_2 to PGE_2 is catalyzed by three different PGH synthases: a cytosolic PGE synthase (cPGES), and two membrane-bound PGE synthases (mPGES-1 and mPGES-2). mPGES-1 is mainly an induced isomerase, and uses COX-2-derived endoperoxide; it is therefore up-regulated in response to various pro-inflammatory stimuli, with a concomitant increased expression of COX-2 [7-9].
Expression of inducible nitric oxide synthase (iNOS) also occurs in conditions of inflammation, and produces a large amount of nitric oxide (NO), which contributes to the up-regulation of COX-2 and to the subsequent release of PGE_2 upon direct interaction with the COX-2 enzyme. A crucial link between iNOS and COX-2 pathway has thus been suggested [10-12].

Much evidence deriving from *in vitro* and *ex vivo* studies has implicated MMPs, mainly MMP-2 and MMP-9, in plaque instability and rupture, because of their ability to degrade all macromolecular constituents of the extracellular matrix [13-17]. Induction of expression and activity of MMP-2 and MMP-9 by activated macrophages has been shown to occur through a PGE_2/cAMP-dependent pathway, involving the simultaneous up-regulation of COX-2 and mPGES-1 [15,18]. It has been shown that these two enzymes, COX-2 and mPGES-1, are simultaneously up-regulated by pro-inflammatory stimuli, such as the release of growth factors and cytokines [19].

The enhanced production of MMPs in vulnerable regions of unstable atherosclerotic plaques may, thus, be due to the increased release of PGE_2, due in turn to induction of the functionally COX-2/mPGES-1 axis by inflammatory stimuli. In addition to increased biosynthesis of PGE_2-dependent MMPs in the atherosclerotic plaque setting, PGE_2 can also amplify the release of cytokines, and inflammatory cytokines can directly enhance the synthesis of MMPs in activated macrophages [20,21]. Clearly, all these events play critical roles in enhancing and sustaining a

vicious circle that might result in plaque rupture. Of note, among the MMPs, MMP-9 has been consistently implicated in plaque instability and rupture [22]. Various inflammatory molecules, in particular PGE_2 and cytokines, as well as oxidative stress molecules such as reactive oxygen species (ROS), play crucial roles in the cross-talk among vascular cells leading to up-regulation of MMPs [23,24].

It has been extensively demonstrated that lipids carried by oxLDLs activate monocytes and macrophages during atherosclerotic inflammatory processes, and that they contribute to increasing and sustaining pro-inflammatory molecule production [25-27].

Among the oxidized plaque lipids, the oxysterol 27-hydroxycholesterol (27-OH), a cholesterol oxidation product, and the aldehyde 4-hydroxynonenal (HNE), one of the more reactive end products of polyunsaturated fatty acid (PUFA) peroxidation, play key roles in the pathogenesis of atherosclerosis. They act by stimulating various signal transduction pathways that are involved both in the inflammatory response and in the immune response and oxidative stress [28-31].

Based on these considerations, it was decided to investigate whether the oxysterol 27-OH and the aldehyde HNE, which substantially accumulate in the atherosclerotic plaque, may up-regulate COX-2 and mPGES-1, resulting in the induction of PGE_2 synthesis; this might be a mechanism of plaque instability and eventual rupture, contributing to the production of inflammatory cytokines and MMPs by activated macrophages. The study found an enhanced production of certain pro-inflammatory cytokines and of MMP-9, presumably due to the induction of PGE_2 synthesis, as a result of the up-regulation of the functionally coupled inducible COX-2/mPGES-1, in human promonocytic U937 cells stimulated by the oxidized lipids 27-OH and HNE.

2. Methods

2.1. Cell culture and treatments

The human promonocytic cell line U937 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies, Monza, Italy), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (Sigma-Aldrich, Milan, Italy) at 37 °C with 5% CO_2. The cells were dispensed at $1x10^6$/ml and made quiescent through overnight incubation in serum-free medium. They were then placed in RPMI 1640 medium with 2% fetal bovine serum and treated with 6 μM 27-OH (Avanti Polarlipids, Alabaster, AL, USA) or in serum-free RPMI 1640 medium and treated with 5 μM HNE (Alexis, Vinci-Biochem, Vinci, Italy). In certain experiments, cells were pretreated for 1 h with 25 μM of NS-398, a specific inhibitor of COX-2 or with 10 μM

epigallocatechin gallate (EGCG) (Sigma-Aldrich). Final concentrations and incubation times for all experiments are given in the figure legends.

2.2. RNA extraction

Total RNA was extracted from cells using TRIzol reagent (Applied Biosystems, Thermo Fisher Scientific, Monza, Italy), following the manufacturer's instructions, after treatment with 27-OH or HNE. RNA was dissolved in RNase-free water with RNase inhibitors (RNase SUPERase-In; Applied Biosystems, Thermo Fisher Scientific). The amount and purity (A_{260}/A_{280} ratio) of the extracted RNA were assessed spectrophotometrically.

2.3. cDNA preparation and real-time RT-PCR

cDNA was synthesized by reverse transcription from 2 μg RNA with a commercial kit and random primers (High-Capacity cDNA reverse transcription kit; Applied Biosystems, Thermo Fisher Scientific), following the manufacturer's instructions. Singleplex real-time RT-PCR was performed on 40 ng of cDNA using TaqMan gene expression assay kits prepared for human mPGES-1, iNOS, interleukin-8 (IL-8), IL-1β, tumor necrosis factor-α (TNF-α), MMP-9, and β-actin and TaqMan Fast Universal PCR master mix, and analyzed by a 7500 Fast real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). The oligonucleotide sequences are not revealed by the manufacturer because of proprietary interests. The cycling parameters were as follows: 20 s at 95 °C for AmpErase UNG activation, 3 s at 95 °C for AmpliTaq Gold DNA polymerase activation, and 40 cycles of 3 s each at 95 °C (melting) and 30 s at 60 °C (annealing/extension). The fractional cycle number at which fluorescence passes the threshold in the amplification plot of fluorescence signal versus cycle number was determined for each gene considered. The results were then normalized to the expression of β-actin, as housekeeping gene. Target gene expression was quantified relatively with a mathematical method proposed by Livak and Schmittgen [32].

2.4. Analysis of mPGES-1, IL-8, IL-1β, and TNF-α by immunofluorescence, and detection by confocal laser microscopy

After treatments, cells were transferred onto glass slides ($8x10^4$ cells/slide) by cytocentrifugation. Specimens were fixed in cold methanol for 10 min and permeabilized for 10

min with 0.1 M PBS-0.4% Triton X-100 solution. Cells were then incubated with a 100 mM sodium cyanoborohydride reducing agent for 10 min at 37 °C. After blocking nonspecific sites of binding with 0.1 M PBS containing 5% goat serum, 3% BSA, and 0.3% Tween 20, slides were incubated in the presence of primary antibodies against mPGES-1 (1:200), IL-8 (1:100), IL-1β (1:50), and TNF-α (1:50) (Santa Cruz Biotechnology, Dallas, TX, USA) and then with specific secondary antibodies (1:200) conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) fluorochromes (Alexa Fluor, Molecular Probes, Thermo Fischer Scientific). Slides mounted with glycerol and distilled water (1:1) were observed through an LSM 510 confocal laser microscope (Carl Zeiss SpA, Arese, Milan, Italy) equipped with an inverted microscope with Plan-NEOFLUAR lenses (40 x/0.75).

2.5. Analysis of COX-2 by Western blotting

Cells were lysed in ice-cold buffer containing 20 mM Hepes, pH 7.9, 0.35M NaCl, 20% glycerol, 1% Igepal CA-630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and protease inhibitors and centrifuged at 14000 rpm for 15 min. For COX-2 protein, 50 µg of total proteins were boiled for 5 min in Laemmli buffer and separated in 10% denaturing SDS/polyacrylamide gels followed by transfer to nitrocellulose membranes. Filters were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) 1X-0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with the appropriate primary antibody against COX-2 (1:250) diluted in 5% nonfat dried milk or in 1% bovine serum albumin in TBS 1X-0.05% Tween 20, followed by specific horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature (Santa Cruz Biotechnology). The membranes were then stripped with the Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and again immunoblotted with anti-actin primary antibody (1:6000) (Sigma-Aldrich) and then incubated with a specific secondary antibody, as described above. Proteins were detected using the Clarity Western ECL subtrate (Bio-Rad Laboratoires, Segrate, Milan, Italy) following the manufacturer's protocol. The immunoreactive bands were scanned and subjected to densitometric analysis using the Image Tool software package. The results are expressed in relative units, determined by normalizing the density of each band of the corresponding reference protein band.

2.6. NO measurement by Griess reaction

NO concentration was determined by quantifying the stable end-product of NO, i.e. nitrite. A colorimetric assay kit (Griess Reagent Kit for Nitrite Determination) based on the Griess reaction was used following the manufacturer's instructions (Molecular Probes, Thermo Fisher Scientific). Briefly, 80 µl of supernatant collected after 48 h cell treatment were sequentially mixed with 80 µl of a 1% solution of sulfanilic acid in 5% phosphoric acid and 80 µl of a 0.1% solution of N-(1-naphthyl) ethylenediamine dihydrochloride. The mixture was kept at room temperature for 30 min. The absorbance was measured at 550 nm in a microplate reader. Nitrite concentrations were calculated from a standard curve obtained by diluting the sodium nitrite stock solution contained in the kit in cell medium.

2.7. PGE_2 determination by enzyme immunoassay (EIA)

After U937 cell treatment with 6 µM 27-OH or 5 µM HNE for 24 h, supernatants were collected, centrifuged, and stored at -80 °C. Supernatants were purified using a C-18 column, as per the manufacturer's protocol. After evaporation of ethyl acetate under nitrogen stream, and resuspension of sample in EIA buffer, levels of PGE_2 were measured using a commercially-available enzyme immunoassay kit (Prostaglandin E_2 ELISA Kit-monoclonal) following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Absorbance was measured at 415 nm in a microplate reader.

2.8. Evaluation of MMP-9 protein levels by enzyme linked immunosorbent assay (ELISA)

After treatments, cells were lysed and cytosolic proteins were stored for ELISA detection. MMP-9 levels were quantified using the DuoSet ELISA kit (R&D System, MN, USA), following the manufacturer's instructions. The plates were read at 450 nm with wavelength correction of 550 nm in a microplate reader (Model 680 Microplate Reader, Bio-Rad). The concentrations of MMP-9 were extrapolated from the standard curve.

2.9. Statistical analysis

All values are expressed as means ± standard deviation (SD). Statistical analysis of the data was by one-way ANOVA with Bonferroni's post-test for multiple comparisons. Differences with $p < 0.05$ were considered statistically significant. Statistical calculations were done with the GraphPad InStat3 software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. The lipid oxidation products 27-OH and HNE up-regulate both COX-2 and mPGES-1 in U937 cells

To investigate whether 27-OH and HNE are involved in the simultaneous up-regulation of COX-2 and mPGES-1, human promonocytic U937 cells were incubated with these two oxidized lipids. The oxysterol 27-OH was used at 6 μM, a concentration mimicking the amount found in atherosclerotic plaques [33], while the aldehyde HNE was used at a concentration of 5 μM, which is below that found in inflamed and diseased tissues [34].

The effects of 27-OH and HNE on the protein levels of COX-2 were analyzed by Western blotting, while the expression and synthesis of m-PGES-1 were quantified by real-time RT-PCR and confocal laser microscopy, respectively.

Both oxidized lipids significantly induced COX-2 protein levels after 24 h cell treatment (Fig. 1 A), compared to untreated cells (control). Further, an approximately time-dependent increase of mPGES-1 mRNA levels was observed from 2 to 6 h of treatment, compared with untreated cells (control), in particular after cell treatment with HNE (5 μM). However, both oxidized lipids significantly induced mPGES-1 expression after 6 h cell treatment (Fig. 1B). An increase of mPGSE-1 protein levels was observed by immunofluorescence analysis, using a confocal laser microscope, after 24 h cell incubation with either 27-OH or HNE (Fig. 1C).

3.2. Induction of PGE_2 production by U937 cells treated with 27-OH or HNE

COX-2 and mPGES-1, induced in response to inflammatory stimuli, are involved in prostaglandin generation in inflammatory diseases, including atherosclerosis [7,9]. Among prostaglandins, PGE_2 plays a key role in the setting of human atherogenesis.

The effects of 27-OH (6 μM) and HNE (5 μM) on PGE_2 production by human promonocytic U937 cells was checked by ELISA after 24 h treatment. PGE_2 production, as a result of induction of the functional COX-2/mPGES-1 axis, was consistently induced by both compounds compared to untreated cells (control) (Fig. 2). As noticed in previous results concerning COX-2/mPGES-1 up-regulation, again in this case the strongest effect on PGE_2 production occurred in cells treated with HNE.

3.3. Both 27-OH and HNE up-regulate iNOS and NO

Expression of iNOS occurs in condition of inflammation, and its activation promotes and maintains the production of NO. Cross-communication between iNOS and COX-2 has been suggested, since NO contributes to the up-regulation of COX-2 and the subsequent release of PGE_2; these play crucial roles in plaque instability.

To investigate whether 27-OH or HNE stimulate iNOS expression, U937 cells were incubated with 6 μM 27-OH or 5 μM HNE for up to 6 h. A time-dependent increase of iNOS mRNA levels was observed from 2 to 6 h of cell treatment with HNE compared with untreated cells (control), while in cells incubated with 27-OH a significant increase of iNOS expression occured only after 6 h treatment (Fig. 3A). As a consequence of the increased expression of iNOS, an increased production of NO (in terms of nitrite) was then observed using the colorimetric assay kit, in cells treated for 48 h with either compound (Fig. 3B).

3.4. Down-regulation of mPGES-1 by the anti-inflammatory effect exerted by EGCG in U937 cells treated with 27-OH or HNE

The pathophysiological role of the functional COX-2/mPGES-1 axis also depends on the induction of mPGES-1 expression by pro-inflammatory stimuli [35,36].

To determine whether natural compounds, such as polyphenols, may act as anti-inflammatory molecules, cells were pretreated with the flavanol EGCG (10 μM). A significant reduction in mPGES-1 expression was observed in the presence of EGCG after 6 h cell incubation with 27-OH (6 μM) or HNE (5 μM), compared with cells treated with 27-OH or HNE alone (Fig. 4). EGCG may act by preventing the inflammatory state that is generated in U937 cells upon incubation with the oxysterol or the aldehyde; this would reduce the expression of COX-2 and mPGES-1, critical enzymes in the progression of atherosclerotic plaque, and in the formation of functionally-coupled COX-2/mPGES-1.

3.5. Up-regulation of IL-8, IL-1β and TNF-α by 27-OH and HNE: implication of functionally-coupled COX-2/mPGES-1 in cytokine release

The enhanced PGE_2 synthesis as a consequence of COX-2/mPGES-1 induction may amplify the release of inflammatory cytokines by activated macrophages. Both PGE_2 and inflammatory

cytokine release contribute to atherosclerotic plaque instability, by increasing MMP production with consequent matrix break-down [37,38].

Among the inflammatory cytokines involved in the pathogenesis of atherosclerosis, the effect of 6 μM 27-OH and of 5 μM HNE on IL-8, IL-1β, and TNF-α expression and synthesis on U937 cells was determined by real-time RT-PCR, or by immunofluorescence analysis using a confocal laser microscope, respectively.

Expression of all the cytokines considered was significantly increased by 27-OH and HNE after 6 h treatment, compared with untreated cells (Fig. 5A). Increased protein levels of these inflammatory cytokines were also clearly detected by confocal microscopy after 24 h cell treatment (Fig. 5B).

To investigate involvement of the COX-2 pathway and of inflammatory stimuli on IL-8, IL-1β, and TNF-α release by U937 cells incubated with 27-OH (6 μM) or HNE (5 μM), the cells were pretreated with 25 μM NS-398, a selective inhibitor of COX-2, or with 10 μM EGCG, an anti-inflammatory molecule. Expression (Fig. 5A) and protein levels (Fig. 5B) of all the cytokines examined were markedly decreased, supporting the involvement of COX-2/mPGES-1 and of the subsequent PGE_2 production, in exacerbating the inflammatory state that contributes to plaque instability.

3.6. Involvement of COX-2 pathway on up-regulation of MMP-9 by 27-OH and HNE in U937 cells

The protease MMP-9 plays a fundamental role in the instability and eventual rupture of atherosclerotic plaques [20-22]. Its expression and proteolytic activity can be up-regulated by different cell signaling pathways, which may be interconnected. Among them, several studies point to MMP-9 release occurring through a COX-2/mPGES-1/PGE_2 pathway [18,39,40].

The effects of 27-OH (6 μM) and HNE (5 μM) on MMP-9 expression and synthesis were thus explored, by real-time RT-PCR after 24 h treatment, and by ELISA after 48 h treatment, respectively. Of note, the ELISA kit employed recognizes both inactive and active forms of MMP-9. In human promonocytic U937 cells, MMP-9 expression (Fig. 6A) and protein levels (Fig. 6B) were consistently induced both by the oxysterol and by the lipid aldehyde, compared with untreated cells (control).

To investigate whether the production of MMP-9 by U937 cells occurs through a COX-2-dependent pathway, cells were pretreated with NS-398 (25 μM) or with EGCG (10 μM). With the specific COX-2 inhibitor or the natural anti-inflammatory flavanol, the up-regulation of MMP-9

expression and synthesis induced by 27-OH or HNE was significantly decreased compared with control cells (Fig. 6A,B).

These data support the potential role of COX-2/mPGES-1 and, presumably, of PGE_2 in MMP-9 up-regulation, in enhancing plaque instability.

4. Discussion

Current understanding of the pathogenesis of atherosclerosis suggests the involvement of complex mechanisms that go beyond the mere storage of oxLDLs: it is now clear that inflammation plays a critical role in the cascade of events that lead to atherosclerotic plaque rupture [1,3,4]. OxLDLs can stimulate inflammatory activation of vascular cells acting as "danger associated molecular patterns" (DAMPs). They also provide oxidation-specific epitopes that are recognized by innate natural immunoglobulin M (IgM) antibodies, as well as by C-reactive protein and complement protein system. Although the natural IgM antibodies can facilitate the clearance of oxLDLs, the overstimulation of the innate immune system can lead to the development of an autoimmune response that includes both immunoglobulin class switching (e.g. to IgG), affinity maturation of antibodies, and the release of T-lymphocyte response to oxLDLs. All these events can contribute to maintain inflammation and the progression of atherosclerotic lesions [41]. In this connection, while the IgM anti-oxLDLs are able to reduce the atherosclerotic events, in turn the IgG antibodies stimulate the secretion of pro-inflammatory cytokines contributing to oxLDL accumulation at sub-endothelial sites and atherosclerotic plaque formation [42].

As part of the inflammatory response within atheroma, both the inducible enzymes, COX-2 and mPGES-1, are simultaneously up-regulated in activated macrophages, by inflammatory stimuli including cytokines; this leads to the subsequent production of eicosanoids, mainly PGE_2 which participate in the pathogenesis of atherosclerotic plaque formation and instability through a variety of processes [7,19,43-45].

Both pharmacologic and direct genetic evidence implicate COX-2 over-expression in atherosclerotic lesion formation [21,46,47]. COX-2 has been detected in the fatty streaks of both humans and mice, and its pro-atherogenic role has been demonstrated using rofecoxib, a highly selective COX-2 inhibitor, which reduced the formation of fatty streaks in the vascular wall [46,48]. COX-2 is also extensively expressed, alongside mPGES-1, by activated macrophages within advanced human atherosclerotic plaques, and to a lesser extent by SMCs and ECs, whereas low or even undetectable levels of COX-2 are found in normal human arteries. Of note, macrophages of the shoulder region contain most of the COX-2 protein level within the atherosclerotic lesion

[15,44]. However, it has been observed that macrophage foam cells of atherosclerotic plaques do not express COX-2, thus suggesting that the enzyme is down-regulated in mature foam cells [15,46]. Interestingly, it has also been shown that mice lacking COX-2, mice expressing modified COX-2, and mice treated with a COX-2 inhibitor, all produce significantly less prostacyclin than normal animals whereas thromboxane formation remains unaltered, and the animals develop thrombosis and hypertension [49]. Moreover, in mPGES-1 knockout mice, a 30% reduction in macrophage foam cells was observed in the atherosclerotic plaques, which also displayed less necrosis [50].

The enzyme iNOS is also induced by inflammatory stimuli, such as cytokines, and it potentially regulates the development of atherosclerosis, thanks to its ability to produce peroxynitrite ($ONOO^-$), which contributes to endothelial dysfunction [51], and to produce excess NO [11]. The involvement of iNOS up-regulation in the pathogenesis of atherosclerosis is supported by the observation, in apolipoprotein E-deficient mice, that genetic iNOS deficiency reduced diet-induced atherosclerotic lesion formation [52]. Nanomolar amounts of NO are continuously generated by iNOS, which is regulated by many cell types, including macrophages, neutrophils, ECs and SMCs. Excess NO can impair cholesterol efflux in macrophage foam cells, suggesting that iNOS may reduce plaque healing [53], but it can also contribute to COX-2 up-regulation, supporting cross-talk among the iNOS/NO/COX-2 pathways, leading to an increased release of PGE_2 as a consequence of direct interaction with the COX-2 enzyme [11,12]. The relationship between iNOS and COX-2 is also supported by the co-localization of iNOS and COX-2, predominantly in macrophages/foam cells, in both native and transplanted human coronary arteries [54]. The enhancing effects of pro-inflammatory cytokines on iNOS and NO expression has been demonstrated by inhibiting iNOS and COX-2 induction, using anti-inflammatory steroids that, in parallel, reduced both NO and PGE_2 generation [55,56], as well as by employing the selective iNOS inhibitor aminoguanidine [57].

It has also been shown that oxLDLs induce COX-2 expression by extracellular signal-regulated kinase (ERK) activation, and that COX-2 is expressed in atherosclerotic lesions, where it promotes inflammation [15,58]. Increased COX-2 expression was found in LDL receptor$^{-/-}$ mice, in which it contributed to atherogenesis [46]. Oxysterols and aldehydes play a crucial role among lipids carried by oxLDLs that activate macrophages, contributing and sustaining inflammation during atherosclerotic plaque progression. Of note, it has been demonstrated that accumulation in the vessel walls of foam cell-derived HNE might promote vascular EC senescence [59]. Moreover, HNE accumulate in an age-related manner in the arterial walls contributing to elastin degradation thus favoring the development of the atherosclerotic lesions [60]. However, in a recent study, it has

been reported that low levels of the aldehydes HNE and 4-hydroxy-2E-,6Z-dodecadienal (4-HDDE) induce an hormetic response which counteract stressful stimuli allowing vascular endothelial cells to restore their normal functions [61].

The present study demonstrated that 27-OH, the most abundant circulating oxysterol, and HNE, the most reactive aldehyde, both of which accumulate markedly in atherosclerotic plaques, are able to increase COX-2 (Fig. 1A) and mPGES-1 (Fig. 1B and C) levels, resulting in the induction of PGE_2 release (Fig. 3) in human promonocytic U937 cells. In U937 cells, increased iNOS expression (Fig. 2A) and NO levels (Fig. 2B) were also observed after cell treatment with 27-OH or with HNE. The data indicate that oxidized lipids may stimulate these inducible enzymes, thus triggering release of their products which contribute to inflammation and plaque instability.

During inflammation, abundant pro-inflammatory cytokines are produced by macrophages, following activation of several mechanisms, including up-regulation of COX-2/mPGES-1. In turn, cytokines are themselves responsible for both COX-2 and mPGES-1 up-regulation. In particular, it has been shown that interferon-γ (IFN-γ) in combination with TNF-α induces a synergistic increase of COX-2 mRNA in human macrophages [19]. Moreover, a mixture of IL-1β, TNF-α, and IFN-γ enhances the release of PGE_2 and COX-2 activity in human pulmonary epithelial cells [62]. IL-1β is also a potent inducer of COX-2 expression in various cell types, including osteoblasts [63], while stimulation of human microglia with IL-8 effectively increases expression of pro-inflammatory cytokines and COX-2 [64]. The expression of mPGES-1 is also markedly increased in various cells and tissues by the same pro-inflammatory stimuli (i.e. IL-1β, TNF-α) involved in COX-2 induction [65].

In this connection, it was observed that cell pretreatment with the flavanol EGCG, a polyphenol with anti-inflammatory and antioxidant properties, significantly decreased the expression of mPGES-1 induced by 27-OH or HNE (Fig. 4). The release of certain cytokines, mainly involved in atherosclerotic plaque instability, which is induced by these oxidized lipids, markedly decreased in cells preincubated with NS-398, a specific inhibitor of COX-2, or with EGCG (Fig. 5).

Of note, the simultaneous up-regulation of functionally-coupled COX-2/mPGES-1, during the inflammatory response in macrophages, may also be due to PGE_2 itself, via a positive-feedback regulation of both the PGE_2-synthesizing enzymes. PGE_2 is able to induce transcriptional activation of the two enzymes, by over-expressing its receptor EP2, with consequent activation of the cAMP/protein kinase A/cAMP-response element-binding protein (cAMP/PKA/CREB) signaling pathway [66].

With regard to atherosclerotic plaque instability, increased expression and activity of MMPs, in particular of MMP-2 and MMP-9, has been observed in unstable human carotid plaques, in association with macrophages [13,67]. The increase of MMPs in advanced atherosclerotic plaques has the potential to cause acute plaque rupture and the subsequent thrombotic events. Expression and proteolytic activities of MMPs are induced by several stimuli or mediators, through activation of various interconnected signaling pathways [16,17].

Several cytokines, chemokines, and growth factors regulate MMP expression at the transcriptional level, and also regulate their cell release [23,24]. For example, it has been reported that IL-1β and IL-8 can release a local imbalance between MMPs and tissue inhibitor of MMPs (TIMPs) by inhibiting TIMP-1 expression in macrophages [68,69]. In this connection, in human promonocytic U937 cells, it has been demonstrated that the oxysterol 27-OH and the reactive aldehyde HNE may promote plaque instability through activation of Toll like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway, which seems to up-regulate MMP-9 by increasing local inflammatory cytokine release, or even directly [17]. 27-OH also contributes to inflammation by enhancing the production of IL-8 in macrophages through activation of NF-κB [70] as well as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1), and MMP-9 in THP-1 cells [71]. To confirm that an inflammatory state can contribute to MMP-9 expression, specific antibodies were used to block the action of IL-8, IL-1β, and TNF-α, which are released following cell treatment with 27-OH or HNE and TLR4/NF-κB pathway activation; both MMP-9 mRNA and protein levels were consistently decreased, confirming the importance of these cytokines in regulating the protease levels induced by both the oxidized lipids [17].

Although 27-OH has been studied for its involvement in pro-inflammatory processes, it is now well known that it plays also a role as ligand for liver X receptors (LXRs), transcription factors that regulate an array of genes, including genes involved in anti-inflammatory response [72]. The dual effect of 27-OH on inflammatory process depends on the fact that it is a weak LXR agonist and that 27-OH can bind signaling molecules different from LXRs, thus inducing expression of inflammatory markers. Several studies on LXR ligands, deriving from the use of synthetic agonists of LXR which fully activate it, demonstrated anti-inflammatory effects of LXR [73]. In this connection, treatment with LXR agonists (e.g. T0901317) reduces atherosclerosis in vivo through inhibition of various inflammatory gene expression, including TNF-α, IL-1β, IL-6, COX-2, iNOS, and NF-κB [74]. Of note, LXR activation induces the expression of genes different from those stimulated by endogenous oxysterols. Therefore, although LXR-activating oxysterols might reduce inflammation, they can also act by activating opposing pathways and inducing expression of inflammation markers independently of LXRs [73]. Another beneficial effect of 27-OH is due to its

ability to protect human macrophages from cholesterol overload [75] since oxysterols, by binding to LXR, might act as "cholesterol sensors", increasing the expression of target genes associated with reverse cholesterol transport [74].

Oxidative-stress-associated inflammation provokes vascular events in atherogenesis, and it has been reported that vascular cells in atheroma produce an excess of ROS, which may lead to MMP over-expression and activation, in particular macrophage-derived ROS [76-79]. It has also been shown that an oxysterol mixture of composition similar to that found in advanced human carotid plaques is able to induce MMP-9 expression in U937 cells through ROS over-production; this leads to activation of ERK and c-Jun N-terminal kinase (JNK) signaling via PKC and, consequently, enhancement of DNA binding of NF-κB and activator protein (AP)-1. These findings emerged using specific inhibitors, siRNAs, or specific antibodies. The involvement of ROS in MMP-9 up-regulation has also been proven by pre-treating U937 cells with quercetin or with EGCG, which are both flavonoids acting as antioxidants [16].

The increased production of PGE_2 also appears to be involved in MMP production in the progression and rupture of atherosclerotic plaques. Co-localization of COX-2 and PGES was demonstrated in symptomatic lesions and associated with acute ischemic syndromes, presumably due to MMP-induced plaque rupture [15,44]. This observation was confirmed by the finding of concomitant higher expressions of COX-2, PGES, MMP-2 and MMP-9, in specimens from carotid lesions of patients with transient ischemic attack or stroke, compared with specimens from asymptomatic patients [15]. These molecules co-localize in activated macrophages, which are their major source. Moreover, co-distribution of COX-2 and MMPs was found in advanced atherosclerotic plaques, in a study on hypercholesterolemic rabbits whose pathological features are relevant to human atherosclerosis [80]. The final evidence that induction of COX-2/mPGES expression is associated with MMP-induced plaque rupture comes from human patients with symptomatic carotid artery stenosis: administration of simvastatin decreases inflammation and inhibits both COX-2/mPGES and MMP expression, and in turn contributes to plaque stabilization [81]. It has been also reported that PGE_2 is capable of inducing expression and activity of MMP-2 and MMP-9 in plaque macrophages, through a PGE_2/cAMP-dependent pathway [18]. Of note, incubation of macrophages with the selective COX-2 inhibitor NS-398 blocks MMP-9 expression. Moreover, PGE_2 and MMP-9 expression by COX-$2^{-/-}$ macrophages was markedly lower than by either COX-$2^{+/-}$ or COX-$2^{+/+}$ macrophages [82]. The association between COX-2/PGE_2 and MMPs was also confirmed in animal models, by inhibiting or deleting the enzymes involved in the biosynthesis pathway of PGE_2 [83,84].

Expression of COX-2 and MMP-9 is also mediated by ROS through PKC activity, one of its potential down-stream targets, in response to different stimuli [85,86]. Indeed, conventional PKCα and PKCβ1 appear to be involved in the signal transduction up-stream of COX-2 and MMP-9, as demonstrated upon their inhibition in human monocytes incubated with hydroxytyrosol, an olive oil polyphenol with anti-inflammatory properties [87].

In addition to increased biosynthesis of PGE_2-dependent MMPs in the setting of atherosclerotic plaques, PGE_2 also stimulates cytokine release by vascular cells; inflammatory cytokines, in turn, can directly enhance the synthesis of MMPs in activated macrophages [68]. Clearly, all these events play critical roles in enhancing and sustaining a vicious circle, which might result in plaque rupture.

The *in vitro* data found here are in agreement with the latter considerations. Expression and protein levels of MMP-9 were significantly increased in cells incubated with 27-OH or HNE. It can be hypothesized that the effect of both oxidized lipids on MMP-9 up-regulation might be due to up-regulation of COX-2/mPGES-1, leading to PGE_2 production and to pro-inflammatory cytokine release. This hypothesis is supported by the evidence that, after preincubation of U937 cells with NS-398, a specific inhibitor of the inducible COX-2 enzyme, or with EGCG, an anti-inflammatory polyphenol, both expression and protein levels of MMP-9 were markedly decreased (Fig. 6).

The findings confirm the link between the inducible functionally coupled COX-2/mPGSE-1 and plaque instability, based on the increased release of MMP-9 by macrophages incubated with 27-OH or with HNE, the most representative oxidized lipids in the atherosclerotic plaque. The increased production of MMP-9 seems to be due to the increase release of PGE_2 and pro-inflammatory cytokines, such as IL-8, IL-1β, and TNF-α. In connection with the latter point, the importance of these cytokines in regulating the protease levels induced by the two oxidized lipids has also been demonstrated elsewhere [17].

The data reported here, which are in agreement with other studies, indicate a key role for COX-2/mPGES-1-generated PGE_2 in atherosclerotic plaque development and instability. These inducible enzymes may thus be potential targets for the development of new anti-inflammatory therapeutic agents aimed at increasing the stability of vulnerable plaques. Besides the classical drugs, new natural anti-inflammatory drugs and/or selective COX-2 inhibitors may offer new therapeutic approaches, in the hope that these agents may provide a good efficacy while not producing side effects.

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Conflict of interest

None

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Legends of figures

Fig. 1. 27-OH and HNE induce mPGES-1 and COX-2 levels. U937 cells were treated with 27-OH (6 μM) or HNE (5 μM) and (A) COX-2 protein levels were analyzed by Western blotting after 24 h of incubation. One blot representative of three experiments is shown. The histograms represent the mean values ± SD of three experiments; COX-2 was normalized against the corresponding β-actin levels and expressed as a percentage of the control (untreated cells). **$p<0.01$ and *$p<0.05$ vs. control. (B) Gene expression of mPGES-1 was quantified by real-time RT-PCR in cells treated for up to 6 h with 27-OH or HNE. The histograms represent the mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. ***$p<0.001$, and *$p<0.05$ vs. control. (C) After 24 h cell treatment, mPGES-1 protein levels were detected by confocal laser microscopy using a FITC-conjugated secondary antibody (488-nm exciting laser band and emission passing through a long-pass 505-550 filter, lens 40x/0.75). The images are representative of three experiments.

Fig. 2. Release of PGE_2 following activation of mPGES-1/COX-2 pathway by 27-OH and HNE. U937 cells were incubated for 24 h with 27-OH (6 μM) or HNE (5 μM) and PGE_2 levels were quantified in the medium by enzyme immunoassay (EIA). The histogram represents the values ± SD of three experiments. PGE_2 concentrations (pg/mg total protein) were extrapolated from the standard curve. **$p<0.01$, and *$p<0.05$ vs. control.

Fig. 3. Effect of 27-OH and HNE on nitric oxide production. U937 cells were treated with 27-OH (6 μM) or HNE (5 μM) and (A) iNOS expression levels were quantified by real-time RT-PCR after 6 h cell incubation. The histograms represent the mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. **$p<0.01$, and *$p<0.05$ vs. control. (B) NO production in cell medium was evaluated by Griess reaction after 48 h cell treatment. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control. NO levels expressed as μM were extrapolated from nitrite standard curve. **$p<0.05$ vs. control.

Fig. 4. EGCG reduces mPGES-1 levels induced by the oxidized lipids. U937 cells were pretreated for 1 h with 10 μM epigallocatechin gallate (EGCG) and then for 6 h with 27-OH (6 μM) or HNE (5 μM). mPGES-1 expression was quantified by real-time RT-PCR. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. ***$p<0.001$, and **$p<0.01$ vs. control; §§§$p<0.001$ vs. 27-OH; ###$p<0.001$ vs. HNE.

Fig. 5. Inhibition of COX-2 and cell pretreatment with the anti-inflammatory EGCG decrease the inflammatory cytokine release induced by 27-OH or HNE. Cells were pretreated for 1 h with 25 μM NS-398 or with 10 μM EGCG and (A) expression of IL-8, IL-1β, and TNF-α was quantified by

real-time RT-PCR after 6 h cell treatment with 27-OH (6 μM) or HNE (5 μM). The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control (untreated cells) and normalized to β-actin. **p<0.01, and *p<0.05 vs. control; §§§p<0.001, §§p<0.01, and §p<0.05 vs. 27-OH; ##p<0.01, and #p<0.05 vs. HNE. (B) After 24 h cell incubation with 27-OH or HNE, immunopositive cells were detected by confocal laser microscopy: IL-8 and IL-1β using a TRITC-conjugated secondary antibody (532-nm exciting laser band, 572-nm long-pass emission filter, and 40x/0.75 lens); TNF-α using a FITC-conjugated secondary antibody (488-nm exciting laser band and emission passing through a long-pass 505-550 filter, lens 40x/0.75). The images are representative of three experiments.

Fig. 6. COX-2 inhibition and EGCG cell preincubation decrease MMP-9 levels. Cells were pretreated for 1 h with 25 μM NS-398 or with 10 μM EGCG and then with 27-OH (6 μM) or with HNE (5 μM) for 24 h. (A) MMP-9 gene expression was evaluated by real-time RT-PCR. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control and normalized to β-actin. ***p<0.001, and *p<0.05 vs. control; §§§p<0.001 vs. 27-OH; #p<0.05 vs. HNE. (B) MMP-9 protein levels were analyzed using the ELISA method after 48 h of cell incubation with 27-OH or HNE. MMP-9 concentrations (pg/ml) were extrapolated from the standard curve. The histograms represent mean values ± SD of three experiments. **p<0.01, and *p<0.05 vs. control; §§p<0.01 vs. 27-OH; ###p<0.001 vs. HNE.

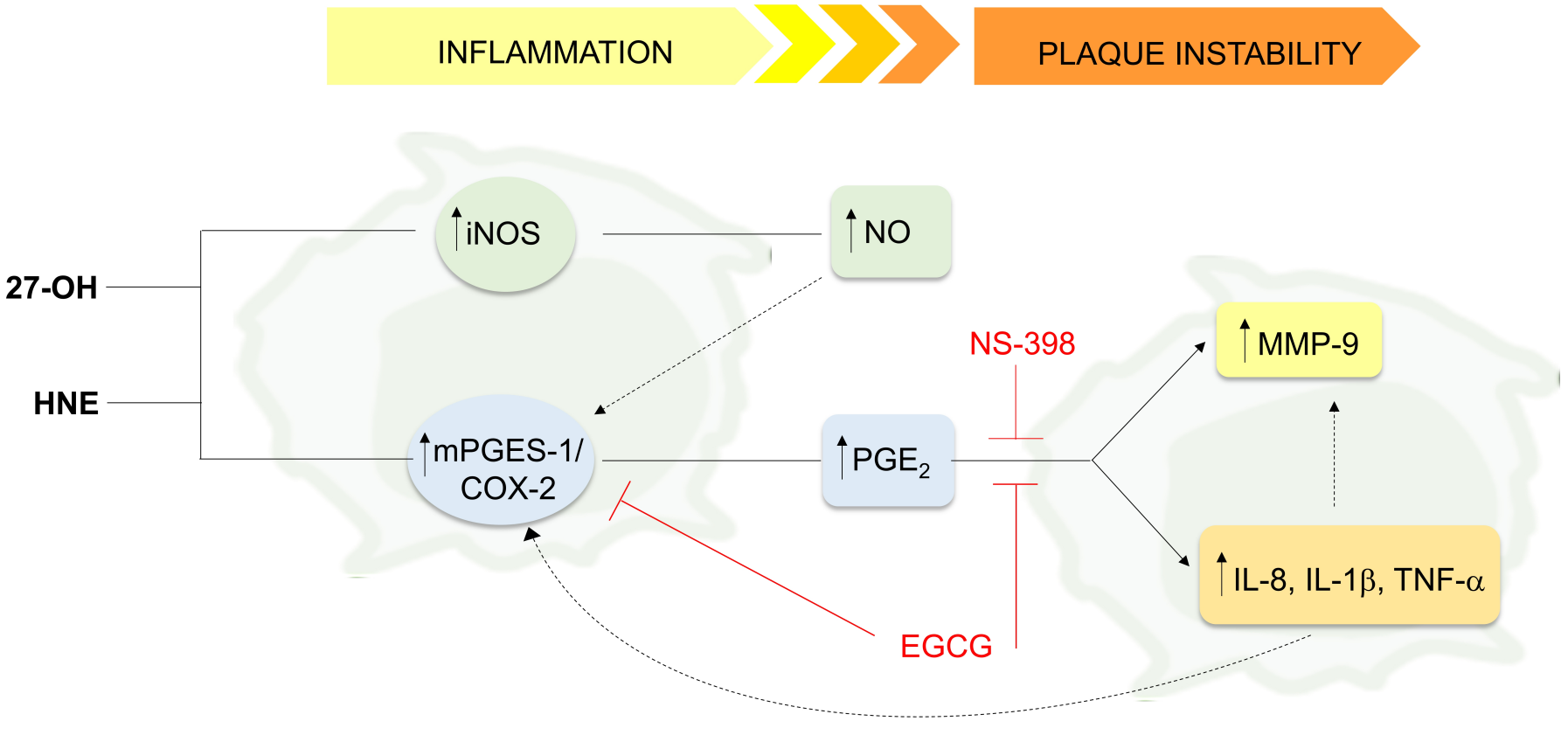
Highlights

27-OH and HNE stimulate inflammation by COX-2/mPGES-1 axis and PGE_2 release.

27-OH and HNE induce NO production through iNOS up-regulation.

Inflammation promoted by 27-OH and HNE may be involved in plaque instability.

Inhibition of inflammation induced by 27-OH and HNE reduces MMP-9 up-regulation.


INFLAMMATION
PLAQUE INSTABILITY
27-OH
HNE
iNOS
NO
mPGES-1/
COX-2
PGE2
NS-398
MMP-9
IL-8, IL-1β, TNF-α
EGCG

Up-regulation of COX-2 and mPGES-1 by 27-hydroxycholesterol and 4-hydroxynonenal: a crucial role in atherosclerotic plaque instability

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Abstract

Atherosclerosis is currently understood to be mainly the consequence of a complicated inflammatory process at the different stages of plaque development. Among the several inflammatory molecules involved, up-regulation of the functional cyclooxygenase 2/membrane-bound prostaglandin E synthase 1 (COX-2/mPGES-1) axis plays a key role in plaque development. Excessive production of oxidized lipids, following low-density lipoprotein (LDL) oxidation, is a characteristic feature of atherosclerosis. Among LDLs, the oxysterol 27-hydroxycholesterol (27-OH) and the aldehyde 4-hydroxynonenal (HNE) substantially accumulate in the atherosclerotic plaque, contributing to its progression and instability through a variety of processes. This study shows that 27-OH and HNE promote up-regulation of both the inducible enzymes COX-2 and mPGES-1, leading to increased production of prostaglandin (PG) E_2 and inducible nitric oxide synthase, and the subsequent release of nitric oxide in human promonocytic U937 cells. The study also examined the potential involvement of the functionally coupled COX-2/mPGES-1 in enhancing the production of certain pro-inflammatory cytokines and of matrix metalloproteinase 9 by U937 cells. This enhancement is presumably due to the induction of PGE_2 synthesis, as a result of the up-regulation of the COX-2/mPGES-1, stimulated by the two oxidized lipids, 27-OH and HNE. Induction of PGE_2 synthesis might thus be a mechanism of plaque instability and eventual rupture, contributing to matrix metalloproteinase production by activated macrophages.

***Keywords*:** Atherosclerosis; Inflammation, Plaque instability; 27-Hydroxycholesterol; 4-Hydroxynonenal.

Abbreviations: 27-OH, 27-hydroxycholesterol; COX-2, cyclooxygenase-2; EC, endothelial cell; EGCG, epigallocatechin gallate; ERK, extracellular signal-regulated kinases; HNE, 4-hydroxynonenal; IL, interleukin; iNOS, inducible nitric oxide synthase; LDL, low density

lipoprotein; LXR, liver X receptor; MMP, matrix metalloproteinase; mPGES-1, membrane-bound prostaglandin E synthase-1; NF-κB, nuclear factor-κB; NO, nitric oxide; oxLDL, oxidized LDL; PG, prostaglandin; PK, protein kinase; ROS, reactive oxygen species; SMC, smooth muscle cell; TIMP, tissue inhibitor of MMP; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor.

1. Introduction

Atherosclerosis is a complex chronic inflammatory disease of the vessel wall. Inflammation, as well as innate and adaptive immune responses, and oxidative stress play a central role in all stages of atherosclerosis including the transition of stable atherosclerotic plaques to unstable plaques [1,2].

Development of atherosclerotic lesions is preceded by impaired vascular endothelium function which favors the retention and accumulation of low-density lipoproteins (LDLs) in the sub-endothelial space by specific proteins of extracellular matrix, including collagen and proteoglycan. Here, the lipid moieties of LDL undergo lipid peroxidation resulting in the release of various reactive oxidized lipids, including aldehydes, cholesterol oxidation products, and oxidized phospholipids. Many studies have demonstrated that accumulation of plaque lipids enhances plaque formation, supporting the concept that the plaque is atherogenic. Oxidized LDLs (oxLDLs), which accumulate progressively in atherosclerotic lesions, stimulate smooth muscle cell (SMC) differentiation and proliferation, monocyte/macrophage recruitment and differentiation, platelet aggregation, up-regulation of leukocyte adhesion molecules, and matrix metalloproteinase (MMP) activation. Finally, the intercellular cross-talk that occurs among vascular cells leads to a fibroproliferative response. During this response, the extracellular matrix components, released by differentiated SMCs, play a key role in plaque formation and progression, providing the structural integrity of the plaque itself, as well as contributing to cell migration and proliferation [1].

In particular, the interaction between oxLDLs and monocyte/macrophage plays predominant roles during initiation and progression of atherosclerosis. Activated macrophages not only take up the oxLDLs through the scavenger receptor CD36 to form lipid-loaded foam cells, but also release pro-inflammatory molecules, such as cytokines, chemokines, and adhesion molecules [1]; the release of inflammatory molecules induces an inflammatory state, with subsequent activation of the

other arterial wall cells, resulting in a lesion that is unstable and prone to rupture when exposed to increasing hemodynamic stress. Plaques vulnerable to rupture - the major cause of acute coronary syndrome - are characterized by a lipid-rich necrotic core, accumulation of inflammatory cells, and fibrous cap weakening, due both to ongoing inflammation and to an imbalance between extracellular matrix synthesis and degradation [3-6].

Of the inflammatory molecules involved, cyclooxygenase 2 (COX-2), which is an inducible enzyme and overexpressed by activated macrophages in atherosclerotic plaques, may participate in the pathogenesis of plaque progression and instability through a variety of processes. These comprise activation of chemotaxis, induction of vascular permeability, propagation of inflammatory molecules, stimulation of SMC migration, and the resultant induction of itself. Thus COX-2 is responsible for the production of eicosanoids, mainly prostaglandin (PG) E_2, in inflammatory diseases. PGE_2 is formed from arachidonic acid via the COX-2-catalyzed formation of PGH_2 and further transformation by PGE synthases. The isomerization of the endoperoxide PGH_2 to PGE_2 is catalyzed by three different PGH synthases: a cytosolic PGE synthase (cPGES), and two membrane-bound PGE synthases (mPGES-1 and mPGES-2). mPGES-1 is mainly an induced isomerase, and uses COX-2-derived endoperoxide; it is therefore up-regulated in response to various pro-inflammatory stimuli, with a concomitant increased expression of COX-2 [7-9].
Expression of inducible nitric oxide synthase (iNOS) also occurs in conditions of inflammation, and produces a large amount of nitric oxide (NO), which contributes to the up-regulation of COX-2 and to the subsequent release of PGE_2 upon direct interaction with the COX-2 enzyme. A crucial link between iNOS and COX-2 pathway has thus been suggested [10-12].

Much evidence deriving from *in vitro* and *ex vivo* studies has implicated MMPs, mainly MMP-2 and MMP-9, in plaque instability and rupture, because of their ability to degrade all macromolecular constituents of the extracellular matrix [13-17]. Induction of expression and activity of MMP-2 and MMP-9 by activated macrophages has been shown to occur through a PGE_2/cAMP-dependent pathway, involving the simultaneous up-regulation of COX-2 and mPGES-1 [15,18]. It has been shown that these two enzymes, COX-2 and mPGES-1, are simultaneously up-regulated by pro-inflammatory stimuli, such as the release of growth factors and cytokines [19].

The enhanced production of MMPs in vulnerable regions of unstable atherosclerotic plaques may, thus, be due to the increased release of PGE_2, due in turn to induction of the functionally COX-2/mPGES-1 axis by inflammatory stimuli. In addition to increased biosynthesis of PGE_2-dependent MMPs in the atherosclerotic plaque setting, PGE_2 can also amplify the release of cytokines, and inflammatory cytokines can directly enhance the synthesis of MMPs in activated macrophages [20,21]. Clearly, all these events play critical roles in enhancing and sustaining a

vicious circle that might result in plaque rupture. Of note, among the MMPs, MMP-9 has been consistently implicated in plaque instability and rupture [22]. Various inflammatory molecules, in particular PGE_2 and cytokines, as well as oxidative stress molecules such as reactive oxygen species (ROS), play crucial roles in the cross-talk among vascular cells leading to up-regulation of MMPs [23,24].

It has been extensively demonstrated that lipids carried by oxLDLs activate monocytes and macrophages during atherosclerotic inflammatory processes, and that they contribute to increasing and sustaining pro-inflammatory molecule production [25-27].

Among the oxidized plaque lipids, the oxysterol 27-hydroxycholesterol (27-OH), a cholesterol oxidation product, and the aldehyde 4-hydroxynonenal (HNE), one of the more reactive end products of polyunsaturated fatty acid (PUFA) peroxidation, play key roles in the pathogenesis of atherosclerosis. They act by stimulating various signal transduction pathways that are involved both in the inflammatory response and in the immune response and oxidative stress [28-31].

Based on these considerations, it was decided to investigate whether the oxysterol 27-OH and the aldehyde HNE, which substantially accumulate in the atherosclerotic plaque, may up-regulate COX-2 and mPGES-1, resulting in the induction of PGE_2 synthesis; this might be a mechanism of plaque instability and eventual rupture, contributing to the production of inflammatory cytokines and MMPs by activated macrophages. The study found an enhanced production of certain pro-inflammatory cytokines and of MMP-9, presumably due to the induction of PGE_2 synthesis, as a result of the up-regulation of the functionally coupled inducible COX-2/mPGES-1, in human promonocytic U937 cells stimulated by the oxidized lipids 27-OH and HNE.

2. Methods

2.1. Cell culture and treatments

The human promonocytic cell line U937 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies, Monza, Italy), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (Sigma-Aldrich, Milan, Italy) at 37 °C with 5% CO_2. The cells were dispensed at $1x10^6$/ml and made quiescent through overnight incubation in serum-free medium. They were then placed in RPMI 1640 medium with 2% fetal bovine serum and treated with 6 μM 27-OH (Avanti Polarlipids, Alabaster, AL, USA) or in serum-free RPMI 1640 medium and treated with 5 μM HNE (Alexis, Vinci-Biochem, Vinci, Italy). In certain experiments, cells were pretreated for 1 h with 25 μM of NS-398, a specific inhibitor of COX-2 or with 10 μM

epigallocatechin gallate (EGCG) (Sigma-Aldrich). Final concentrations and incubation times for all experiments are given in the figure legends.

2.2. RNA extraction

Total RNA was extracted from cells using TRIzol reagent (Applied Biosystems, Thermo Fisher Scientific, Monza, Italy), following the manufacturer's instructions, after treatment with 27-OH or HNE. RNA was dissolved in RNase-free water with RNase inhibitors (RNase SUPERase-In; Applied Biosystems, Thermo Fisher Scientific). The amount and purity (A_{260}/A_{280} ratio) of the extracted RNA were assessed spectrophotometrically.

2.3. cDNA preparation and real-time RT-PCR

cDNA was synthesized by reverse transcription from 2 μg RNA with a commercial kit and random primers (High-Capacity cDNA reverse transcription kit; Applied Biosystems, Thermo Fisher Scientific), following the manufacturer's instructions. Singleplex real-time RT-PCR was performed on 40 ng of cDNA using TaqMan gene expression assay kits prepared for human mPGES-1, iNOS, interleukin-8 (IL-8), IL-1β, tumor necrosis factor-α (TNF-α), MMP-9, and β-actin and TaqMan Fast Universal PCR master mix, and analyzed by a 7500 Fast real-time PCR system (Applied Biosystems, Thermo Fisher Scientific). The oligonucleotide sequences are not revealed by the manufacturer because of proprietary interests. The cycling parameters were as follows: 20 s at 95 °C for AmpErase UNG activation, 3 s at 95 °C for AmpliTaq Gold DNA polymerase activation, and 40 cycles of 3 s each at 95 °C (melting) and 30 s at 60 °C (annealing/extension). The fractional cycle number at which fluorescence passes the threshold in the amplification plot of fluorescence signal versus cycle number was determined for each gene considered. The results were then normalized to the expression of β-actin, as housekeeping gene. Target gene expression was quantified relatively with a mathematical method proposed by Livak and Schmittgen [32].

2.4. Analysis of mPGES-1, IL-8, IL-1β, and TNF-α by immunofluorescence, and detection by confocal laser microscopy

After treatments, cells were transferred onto glass slides ($8x10^4$ cells/slide) by cytocentrifugation. Specimens were fixed in cold methanol for 10 min and permeabilized for 10

min with 0.1 M PBS-0.4% Triton X-100 solution. Cells were then incubated with a 100 mM sodium cyanoborohydride reducing agent for 10 min at 37 °C. After blocking nonspecific sites of binding with 0.1 M PBS containing 5% goat serum, 3% BSA, and 0.3% Tween 20, slides were incubated in the presence of primary antibodies against mPGES-1 (1:200), IL-8 (1:100), IL-1β (1:50), and TNF-α (1:50) (Santa Cruz Biotechnology, Dallas, TX, USA) and then with specific secondary antibodies (1:200) conjugated with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) fluorochromes (Alexa Fluor, Molecular Probes, Thermo Fischer Scientific). Slides mounted with glycerol and distilled water (1:1) were observed through an LSM 510 confocal laser microscope (Carl Zeiss SpA, Arese, Milan, Italy) equipped with an inverted microscope with Plan-NEOFLUAR lenses (40 x/0.75).

2.5. Analysis of COX-2 by Western blotting

Cells were lysed in ice-cold buffer containing 20 mM Hepes, pH 7.9, 0.35M NaCl, 20% glycerol, 1% Igepal CA-630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, and protease inhibitors and centrifuged at 14000 rpm for 15 min. For COX-2 protein, 50 µg of total proteins were boiled for 5 min in Laemmli buffer and separated in 10% denaturing SDS/polyacrylamide gels followed by transfer to nitrocellulose membranes. Filters were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) 1X-0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with the appropriate primary antibody against COX-2 (1:250) diluted in 5% nonfat dried milk or in 1% bovine serum albumin in TBS 1X-0.05% Tween 20, followed by specific horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature (Santa Cruz Biotechnology). The membranes were then stripped with the Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and again immunoblotted with anti-actin primary antibody (1:6000) (Sigma-Aldrich) and then incubated with a specific secondary antibody, as described above. Proteins were detected using the Clarity Western ECL subtrate (Bio-Rad Laboratoires, Segrate, Milan, Italy) following the manufacturer's protocol. The immunoreactive bands were scanned and subjected to densitometric analysis using the Image Tool software package. The results are expressed in relative units, determined by normalizing the density of each band of the corresponding reference protein band.

2.6. NO measurement by Griess reaction

NO concentration was determined by quantifying the stable end-product of NO, i.e. nitrite. A colorimetric assay kit (Griess Reagent Kit for Nitrite Determination) based on the Griess reaction was used following the manufacturer's instructions (Molecular Probes, Thermo Fisher Scientific). Briefly, 80 µl of supernatant collected after 48 h cell treatment were sequentially mixed with 80 µl of a 1% solution of sulfanilic acid in 5% phosphoric acid and 80 µl of a 0.1% solution of N-(1-naphthyl) ethylenediamine dihydrochloride. The mixture was kept at room temperature for 30 min. The absorbance was measured at 550 nm in a microplate reader. Nitrite concentrations were calculated from a standard curve obtained by diluting the sodium nitrite stock solution contained in the kit in cell medium.

2.7. PGE_2 determination by enzyme immunoassay (EIA)

After U937 cell treatment with 6 µM 27-OH or 5 µM HNE for 24 h, supernatants were collected, centrifuged, and stored at -80 °C. Supernatants were purified using a C-18 column, as per the manufacturer's protocol. After evaporation of ethyl acetate under nitrogen stream, and resuspension of sample in EIA buffer, levels of PGE_2 were measured using a commercially-available enzyme immunoassay kit (Prostaglandin E_2 ELISA Kit-monoclonal) following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Absorbance was measured at 415 nm in a microplate reader.

2.8. Evaluation of MMP-9 protein levels by enzyme linked immunosorbent assay (ELISA)

After treatments, cells were lysed and cytosolic proteins were stored for ELISA detection. MMP-9 levels were quantified using the DuoSet ELISA kit (R&D System, MN, USA), following the manufacturer's instructions. The plates were read at 450 nm with wavelength correction of 550 nm in a microplate reader (Model 680 Microplate Reader, Bio-Rad). The concentrations of MMP-9 were extrapolated from the standard curve.

2.9. Statistical analysis

All values are expressed as means ± standard deviation (SD). Statistical analysis of the data was by one-way ANOVA with Bonferroni's post-test for multiple comparisons. Differences with $p < 0.05$ were considered statistically significant. Statistical calculations were done with the GraphPad InStat3 software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. The lipid oxidation products 27-OH and HNE up-regulate both COX-2 and mPGES-1 in U937 cells

To investigate whether 27-OH and HNE are involved in the simultaneous up-regulation of COX-2 and mPGES-1, human promonocytic U937 cells were incubated with these two oxidized lipids. The oxysterol 27-OH was used at 6 μM, a concentration mimicking the amount found in atherosclerotic plaques [33], while the aldehyde HNE was used at a concentration of 5 μM, which is below that found in inflamed and diseased tissues [34].

The effects of 27-OH and HNE on the protein levels of COX-2 were analyzed by Western blotting, while the expression and synthesis of m-PGES-1 were quantified by real-time RT-PCR and confocal laser microscopy, respectively.

Both oxidized lipids significantly induced COX-2 protein levels after 24 h cell treatment (Fig. 1 A), compared to untreated cells (control). Further, an approximately time-dependent increase of mPGES-1 mRNA levels was observed from 2 to 6 h of treatment, compared with untreated cells (control), in particular after cell treatment with HNE (5 μM). However, both oxidized lipids significantly induced mPGES-1 expression after 6 h cell treatment (Fig. 1B). An increase of mPGSE-1 protein levels was observed by immunofluorescence analysis, using a confocal laser microscope, after 24 h cell incubation with either 27-OH or HNE (Fig. 1C).

3.2. Induction of PGE_2 production by U937 cells treated with 27-OH or HNE

COX-2 and mPGES-1, induced in response to inflammatory stimuli, are involved in prostaglandin generation in inflammatory diseases, including atherosclerosis [7,9]. Among prostaglandins, PGE_2 plays a key role in the setting of human atherogenesis.

The effects of 27-OH (6 μM) and HNE (5 μM) on PGE_2 production by human promonocytic U937 cells was checked by ELISA after 24 h treatment. PGE_2 production, as a result of induction of the functional COX-2/mPGES-1 axis, was consistently induced by both compounds compared to untreated cells (control) (Fig. 2). As noticed in previous results concerning COX-2/mPGES-1 up-regulation, again in this case the strongest effect on PGE_2 production occurred in cells treated with HNE.

3.3. Both 27-OH and HNE up-regulate iNOS and NO

Expression of iNOS occurs in condition of inflammation, and its activation promotes and maintains the production of NO. Cross-communication between iNOS and COX-2 has been suggested, since NO contributes to the up-regulation of COX-2 and the subsequent release of PGE_2; these play crucial roles in plaque instability.

To investigate whether 27-OH or HNE stimulate iNOS expression, U937 cells were incubated with 6 μM 27-OH or 5 μM HNE for up to 6 h. A time-dependent increase of iNOS mRNA levels was observed from 2 to 6 h of cell treatment with HNE compared with untreated cells (control), while in cells incubated with 27-OH a significant increase of iNOS expression occured only after 6 h treatment (Fig. 3A). As a consequence of the increased expression of iNOS, an increased production of NO (in terms of nitrite) was then observed using the colorimetric assay kit, in cells treated for 48 h with either compound (Fig. 3B).

3.4. Down-regulation of mPGES-1 by the anti-inflammatory effect exerted by EGCG in U937 cells treated with 27-OH or HNE

The pathophysiological role of the functional COX-2/mPGES-1 axis also depends on the induction of mPGES-1 expression by pro-inflammatory stimuli [35,36].

To determine whether natural compounds, such as polyphenols, may act as anti-inflammatory molecules, cells were pretreated with the flavanol EGCG (10 μM). A significant reduction in mPGES-1 expression was observed in the presence of EGCG after 6 h cell incubation with 27-OH (6 μM) or HNE (5 μM), compared with cells treated with 27-OH or HNE alone (Fig. 4). EGCG may act by preventing the inflammatory state that is generated in U937 cells upon incubation with the oxysterol or the aldehyde; this would reduce the expression of COX-2 and mPGES-1, critical enzymes in the progression of atherosclerotic plaque, and in the formation of functionally-coupled COX-2/mPGES-1.

3.5. Up-regulation of IL-8, IL-1β and TNF-α by 27-OH and HNE: implication of functionally-coupled COX-2/mPGES-1 in cytokine release

The enhanced PGE_2 synthesis as a consequence of COX-2/mPGES-1 induction may amplify the release of inflammatory cytokines by activated macrophages. Both PGE_2 and inflammatory

cytokine release contribute to atherosclerotic plaque instability, by increasing MMP production with consequent matrix break-down [37,38].

Among the inflammatory cytokines involved in the pathogenesis of atherosclerosis, the effect of 6 μM 27-OH and of 5 μM HNE on IL-8, IL-1β, and TNF-α expression and synthesis on U937 cells was determined by real-time RT-PCR, or by immunofluorescence analysis using a confocal laser microscope, respectively.

Expression of all the cytokines considered was significantly increased by 27-OH and HNE after 6 h treatment, compared with untreated cells (Fig. 5A). Increased protein levels of these inflammatory cytokines were also clearly detected by confocal microscopy after 24 h cell treatment (Fig. 5B).

To investigate involvement of the COX-2 pathway and of inflammatory stimuli on IL-8, IL-1β, and TNF-α release by U937 cells incubated with 27-OH (6 μM) or HNE (5 μM), the cells were pretreated with 25 μM NS-398, a selective inhibitor of COX-2, or with 10 μM EGCG, an anti-inflammatory molecule. Expression (Fig. 5A) and protein levels (Fig. 5B) of all the cytokines examined were markedly decreased, supporting the involvement of COX-2/mPGES-1 and of the subsequent PGE_2 production, in exacerbating the inflammatory state that contributes to plaque instability.

3.6. Involvement of COX-2 pathway on up-regulation of MMP-9 by 27-OH and HNE in U937 cells

The protease MMP-9 plays a fundamental role in the instability and eventual rupture of atherosclerotic plaques [20-22]. Its expression and proteolytic activity can be up-regulated by different cell signaling pathways, which may be interconnected. Among them, several studies point to MMP-9 release occurring through a COX-2/mPGES-1/PGE_2 pathway [18,39,40].

The effects of 27-OH (6 μM) and HNE (5 μM) on MMP-9 expression and synthesis were thus explored, by real-time RT-PCR after 24 h treatment, and by ELISA after 48 h treatment, respectively. Of note, the ELISA kit employed recognizes both inactive and active forms of MMP-9. In human promonocytic U937 cells, MMP-9 expression (Fig. 6A) and protein levels (Fig. 6B) were consistently induced both by the oxysterol and by the lipid aldehyde, compared with untreated cells (control).

To investigate whether the production of MMP-9 by U937 cells occurs through a COX-2-dependent pathway, cells were pretreated with NS-398 (25 μM) or with EGCG (10 μM). With the specific COX-2 inhibitor or the natural anti-inflammatory flavanol, the up-regulation of MMP-9

expression and synthesis induced by 27-OH or HNE was significantly decreased compared with control cells (Fig. 6A,B).

These data support the potential role of COX-2/mPGES-1 and, presumably, of PGE_2 in MMP-9 up-regulation, in enhancing plaque instability.

4. Discussion

Current understanding of the pathogenesis of atherosclerosis suggests the involvement of complex mechanisms that go beyond the mere storage of oxLDLs: it is now clear that inflammation plays a critical role in the cascade of events that lead to atherosclerotic plaque rupture [1,3,4]. OxLDLs can stimulate inflammatory activation of vascular cells acting as "danger associated molecular patterns" (DAMPs). They also provide oxidation-specific epitopes that are recognized by innate natural immunoglobulin M (IgM) antibodies, as well as by C-reactive protein and complement protein system. Although the natural IgM antibodies can facilitate the clearance of oxLDLs, the overstimulation of the innate immune system can lead to the development of an autoimmune response that includes both immunoglobulin class switching (e.g. to IgG), affinity maturation of antibodies, and the release of T-lymphocyte response to oxLDLs. All these events can contribute to maintain inflammation and the progression of atherosclerotic lesions [41]. In this connection, while the IgM anti-oxLDLs are able to reduce the atherosclerotic events, in turn the IgG antibodies stimulate the secretion of pro-inflammatory cytokines contributing to oxLDL accumulation at sub-endothelial sites and atherosclerotic plaque formation [42].

As part of the inflammatory response within atheroma, both the inducible enzymes, COX-2 and mPGES-1, are simultaneously up-regulated in activated macrophages, by inflammatory stimuli including cytokines; this leads to the subsequent production of eicosanoids, mainly PGE_2 which participate in the pathogenesis of atherosclerotic plaque formation and instability through a variety of processes [7,19,43-45].

Both pharmacologic and direct genetic evidence implicate COX-2 over-expression in atherosclerotic lesion formation [21,46,47]. COX-2 has been detected in the fatty streaks of both humans and mice, and its pro-atherogenic role has been demonstrated using rofecoxib, a highly selective COX-2 inhibitor, which reduced the formation of fatty streaks in the vascular wall [46,48]. COX-2 is also extensively expressed, alongside mPGES-1, by activated macrophages within advanced human atherosclerotic plaques, and to a lesser extent by SMCs and ECs, whereas low or even undetectable levels of COX-2 are found in normal human arteries. Of note, macrophages of the shoulder region contain most of the COX-2 protein level within the atherosclerotic lesion

[15,44]. However, it has been observed that macrophage foam cells of atherosclerotic plaques do not express COX-2, thus suggesting that the enzyme is down-regulated in mature foam cells [15,46]. Interestingly, it has also been shown that mice lacking COX-2, mice expressing modified COX-2, and mice treated with a COX-2 inhibitor, all produce significantly less prostacyclin than normal animals whereas thromboxane formation remains unaltered, and the animals develop thrombosis and hypertension [49]. Moreover, in mPGES-1 knockout mice, a 30% reduction in macrophage foam cells was observed in the atherosclerotic plaques, which also displayed less necrosis [50].

The enzyme iNOS is also induced by inflammatory stimuli, such as cytokines, and it potentially regulates the development of atherosclerosis, thanks to its ability to produce peroxynitrite ($ONOO^-$), which contributes to endothelial dysfunction [51], and to produce excess NO [11]. The involvement of iNOS up-regulation in the pathogenesis of atherosclerosis is supported by the observation, in apolipoprotein E-deficient mice, that genetic iNOS deficiency reduced diet-induced atherosclerotic lesion formation [52]. Nanomolar amounts of NO are continuously generated by iNOS, which is regulated by many cell types, including macrophages, neutrophils, ECs and SMCs. Excess NO can impair cholesterol efflux in macrophage foam cells, suggesting that iNOS may reduce plaque healing [53], but it can also contribute to COX-2 up-regulation, supporting cross-talk among the iNOS/NO/COX-2 pathways, leading to an increased release of PGE_2 as a consequence of direct interaction with the COX-2 enzyme [11,12]. The relationship between iNOS and COX-2 is also supported by the co-localization of iNOS and COX-2, predominantly in macrophages/foam cells, in both native and transplanted human coronary arteries [54]. The enhancing effects of pro-inflammatory cytokines on iNOS and NO expression has been demonstrated by inhibiting iNOS and COX-2 induction, using anti-inflammatory steroids that, in parallel, reduced both NO and PGE_2 generation [55,56], as well as by employing the selective iNOS inhibitor aminoguanidine [57].

It has also been shown that oxLDLs induce COX-2 expression by extracellular signal-regulated kinase (ERK) activation, and that COX-2 is expressed in atherosclerotic lesions, where it promotes inflammation [15,58]. Increased COX-2 expression was found in LDL receptor$^{-/-}$ mice, in which it contributed to atherogenesis [46]. Oxysterols and aldehydes play a crucial role among lipids carried by oxLDLs that activate macrophages, contributing and sustaining inflammation during atherosclerotic plaque progression. Of note, it has been demonstrated that accumulation in the vessel walls of foam cell-derived HNE might promote vascular EC senescence [59]. Moreover, HNE accumulate in an age-related manner in the arterial walls contributing to elastin degradation thus favoring the development of the atherosclerotic lesions [60]. However, in a recent study, it has

been reported that low levels of the aldehydes HNE and 4-hydroxy-2E-,6Z-dodecadienal (4-HDDE) induce an hormetic response which counteract stressful stimuli allowing vascular endothelial cells to restore their normal functions [61].

The present study demonstrated that 27-OH, the most abundant circulating oxysterol, and HNE, the most reactive aldehyde, both of which accumulate markedly in atherosclerotic plaques, are able to increase COX-2 (Fig. 1A) and mPGES-1 (Fig. 1B and C) levels, resulting in the induction of PGE_2 release (Fig. 3) in human promonocytic U937 cells. In U937 cells, increased iNOS expression (Fig. 2A) and NO levels (Fig. 2B) were also observed after cell treatment with 27-OH or with HNE. The data indicate that oxidized lipids may stimulate these inducible enzymes, thus triggering release of their products which contribute to inflammation and plaque instability.

During inflammation, abundant pro-inflammatory cytokines are produced by macrophages, following activation of several mechanisms, including up-regulation of COX-2/mPGES-1. In turn, cytokines are themselves responsible for both COX-2 and mPGES-1 up-regulation. In particular, it has been shown that interferon-γ (IFN-γ) in combination with TNF-α induces a synergistic increase of COX-2 mRNA in human macrophages [19]. Moreover, a mixture of IL-1β, TNF-α, and IFN-γ enhances the release of PGE_2 and COX-2 activity in human pulmonary epithelial cells [62]. IL-1β is also a potent inducer of COX-2 expression in various cell types, including osteoblasts [63], while stimulation of human microglia with IL-8 effectively increases expression of pro-inflammatory cytokines and COX-2 [64]. The expression of mPGES-1 is also markedly increased in various cells and tissues by the same pro-inflammatory stimuli (i.e. IL-1β, TNF-α) involved in COX-2 induction [65].

In this connection, it was observed that cell pretreatment with the flavanol EGCG, a polyphenol with anti-inflammatory and antioxidant properties, significantly decreased the expression of mPGES-1 induced by 27-OH or HNE (Fig. 4). The release of certain cytokines, mainly involved in atherosclerotic plaque instability, which is induced by these oxidized lipids, markedly decreased in cells preincubated with NS-398, a specific inhibitor of COX-2, or with EGCG (Fig. 5).

Of note, the simultaneous up-regulation of functionally-coupled COX-2/mPGES-1, during the inflammatory response in macrophages, may also be due to PGE_2 itself, via a positive-feedback regulation of both the PGE_2-synthesizing enzymes. PGE_2 is able to induce transcriptional activation of the two enzymes, by over-expressing its receptor EP2, with consequent activation of the cAMP/protein kinase A/cAMP-response element-binding protein (cAMP/PKA/CREB) signaling pathway [66].

With regard to atherosclerotic plaque instability, increased expression and activity of MMPs, in particular of MMP-2 and MMP-9, has been observed in unstable human carotid plaques, in association with macrophages [13,67]. The increase of MMPs in advanced atherosclerotic plaques has the potential to cause acute plaque rupture and the subsequent thrombotic events. Expression and proteolytic activities of MMPs are induced by several stimuli or mediators, through activation of various interconnected signaling pathways [16,17].

Several cytokines, chemokines, and growth factors regulate MMP expression at the transcriptional level, and also regulate their cell release [23,24]. For example, it has been reported that IL-1β and IL-8 can release a local imbalance between MMPs and tissue inhibitor of MMPs (TIMPs) by inhibiting TIMP-1 expression in macrophages [68,69]. In this connection, in human promonocytic U937 cells, it has been demonstrated that the oxysterol 27-OH and the reactive aldehyde HNE may promote plaque instability through activation of Toll like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway, which seems to up-regulate MMP-9 by increasing local inflammatory cytokine release, or even directly [17]. 27-OH also contributes to inflammation by enhancing the production of IL-8 in macrophages through activation of NF-κB [70] as well as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1), and MMP-9 in THP-1 cells [71]. To confirm that an inflammatory state can contribute to MMP-9 expression, specific antibodies were used to block the action of IL-8, IL-1β, and TNF-α, which are released following cell treatment with 27-OH or HNE and TLR4/NF-κB pathway activation; both MMP-9 mRNA and protein levels were consistently decreased, confirming the importance of these cytokines in regulating the protease levels induced by both the oxidized lipids [17].

Although 27-OH has been studied for its involvement in pro-inflammatory processes, it is now well known that it plays also a role as ligand for liver X receptors (LXRs), transcription factors that regulate an array of genes, including genes involved in anti-inflammatory response [72]. The dual effect of 27-OH on inflammatory process depends on the fact that it is a weak LXR agonist and that 27-OH can bind signaling molecules different from LXRs, thus inducing expression of inflammatory markers. Several studies on LXR ligands, deriving from the use of synthetic agonists of LXR which fully activate it, demonstrated anti-inflammatory effects of LXR [73]. In this connection, treatment with LXR agonists (e.g. T0901317) reduces atherosclerosis in vivo through inhibition of various inflammatory gene expression, including TNF-α, IL-1β, IL-6, COX-2, iNOS, and NF-κB [74]. Of note, LXR activation induces the expression of genes different from those stimulated by endogenous oxysterols. Therefore, although LXR-activating oxysterols might reduce inflammation, they can also act by activating opposing pathways and inducing expression of inflammation markers independently of LXRs [73]. Another beneficial effect of 27-OH is due to its

ability to protect human macrophages from cholesterol overload [75] since oxysterols, by binding to LXR, might act as "cholesterol sensors", increasing the expression of target genes associated with reverse cholesterol transport [74].

Oxidative-stress-associated inflammation provokes vascular events in atherogenesis, and it has been reported that vascular cells in atheroma produce an excess of ROS, which may lead to MMP over-expression and activation, in particular macrophage-derived ROS [76-79]. It has also been shown that an oxysterol mixture of composition similar to that found in advanced human carotid plaques is able to induce MMP-9 expression in U937 cells through ROS over-production; this leads to activation of ERK and c-Jun N-terminal kinase (JNK) signaling via PKC and, consequently, enhancement of DNA binding of NF-κB and activator protein (AP)-1. These findings emerged using specific inhibitors, siRNAs, or specific antibodies. The involvement of ROS in MMP-9 up-regulation has also been proven by pre-treating U937 cells with quercetin or with EGCG, which are both flavonoids acting as antioxidants [16].

The increased production of PGE_2 also appears to be involved in MMP production in the progression and rupture of atherosclerotic plaques. Co-localization of COX-2 and PGES was demonstrated in symptomatic lesions and associated with acute ischemic syndromes, presumably due to MMP-induced plaque rupture [15,44]. This observation was confirmed by the finding of concomitant higher expressions of COX-2, PGES, MMP-2 and MMP-9, in specimens from carotid lesions of patients with transient ischemic attack or stroke, compared with specimens from asymptomatic patients [15]. These molecules co-localize in activated macrophages, which are their major source. Moreover, co-distribution of COX-2 and MMPs was found in advanced atherosclerotic plaques, in a study on hypercholesterolemic rabbits whose pathological features are relevant to human atherosclerosis [80]. The final evidence that induction of COX-2/mPGES expression is associated with MMP-induced plaque rupture comes from human patients with symptomatic carotid artery stenosis: administration of simvastatin decreases inflammation and inhibits both COX-2/mPGES and MMP expression, and in turn contributes to plaque stabilization [81]. It has been also reported that PGE_2 is capable of inducing expression and activity of MMP-2 and MMP-9 in plaque macrophages, through a PGE_2/cAMP-dependent pathway [18]. Of note, incubation of macrophages with the selective COX-2 inhibitor NS-398 blocks MMP-9 expression. Moreover, PGE_2 and MMP-9 expression by COX-$2^{-/-}$ macrophages was markedly lower than by either COX-$2^{+/-}$ or COX-$2^{+/+}$ macrophages [82]. The association between COX-2/PGE_2 and MMPs was also confirmed in animal models, by inhibiting or deleting the enzymes involved in the biosynthesis pathway of PGE_2 [83,84].

Expression of COX-2 and MMP-9 is also mediated by ROS through PKC activity, one of its potential down-stream targets, in response to different stimuli [85,86]. Indeed, conventional PKCα and PKCβ1 appear to be involved in the signal transduction up-stream of COX-2 and MMP-9, as demonstrated upon their inhibition in human monocytes incubated with hydroxytyrosol, an olive oil polyphenol with anti-inflammatory properties [87].

In addition to increased biosynthesis of PGE_2-dependent MMPs in the setting of atherosclerotic plaques, PGE_2 also stimulates cytokine release by vascular cells; inflammatory cytokines, in turn, can directly enhance the synthesis of MMPs in activated macrophages [68]. Clearly, all these events play critical roles in enhancing and sustaining a vicious circle, which might result in plaque rupture.

The *in vitro* data found here are in agreement with the latter considerations. Expression and protein levels of MMP-9 were significantly increased in cells incubated with 27-OH or HNE. It can be hypothesized that the effect of both oxidized lipids on MMP-9 up-regulation might be due to up-regulation of COX-2/mPGES-1, leading to PGE_2 production and to pro-inflammatory cytokine release. This hypothesis is supported by the evidence that, after preincubation of U937 cells with NS-398, a specific inhibitor of the inducible COX-2 enzyme, or with EGCG, an anti-inflammatory polyphenol, both expression and protein levels of MMP-9 were markedly decreased (Fig. 6).

The findings confirm the link between the inducible functionally coupled COX-2/mPGSE-1 and plaque instability, based on the increased release of MMP-9 by macrophages incubated with 27-OH or with HNE, the most representative oxidized lipids in the atherosclerotic plaque. The increased production of MMP-9 seems to be due to the increase release of PGE_2 and pro-inflammatory cytokines, such as IL-8, IL-1β, and TNF-α. In connection with the latter point, the importance of these cytokines in regulating the protease levels induced by the two oxidized lipids has also been demonstrated elsewhere [17].

The data reported here, which are in agreement with other studies, indicate a key role for COX-2/mPGES-1-generated PGE_2 in atherosclerotic plaque development and instability. These inducible enzymes may thus be potential targets for the development of new anti-inflammatory therapeutic agents aimed at increasing the stability of vulnerable plaques. Besides the classical drugs, new natural anti-inflammatory drugs and/or selective COX-2 inhibitors may offer new therapeutic approaches, in the hope that these agents may provide a good efficacy while not producing side effects.

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Conflict of interest

None

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Legends of figures

Fig. 1. 27-OH and HNE induce mPGES-1 and COX-2 levels. U937 cells were treated with 27-OH (6 μM) or HNE (5 μM) and (A) COX-2 protein levels were analyzed by Western blotting after 24 h of incubation. One blot representative of three experiments is shown. The histograms represent the mean values ± SD of three experiments; COX-2 was normalized against the corresponding β-actin levels and expressed as a percentage of the control (untreated cells). **$p<0.01$ and *$p<0.05$ vs. control. (B) Gene expression of mPGES-1 was quantified by real-time RT-PCR in cells treated for up to 6 h with 27-OH or HNE. The histograms represent the mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. ***$p<0.001$, and *$p<0.05$ vs. control. (C) After 24 h cell treatment, mPGES-1 protein levels were detected by confocal laser microscopy using a FITC-conjugated secondary antibody (488-nm exciting laser band and emission passing through a long-pass 505-550 filter, lens 40x/0.75). The images are representative of three experiments.

Fig. 2. Release of PGE_2 following activation of mPGES-1/COX-2 pathway by 27-OH and HNE. U937 cells were incubated for 24 h with 27-OH (6 μM) or HNE (5 μM) and PGE_2 levels were quantified in the medium by enzyme immunoassay (EIA). The histogram represents the values ± SD of three experiments. PGE_2 concentrations (pg/mg total protein) were extrapolated from the standard curve. **$p<0.01$, and *$p<0.05$ vs. control.

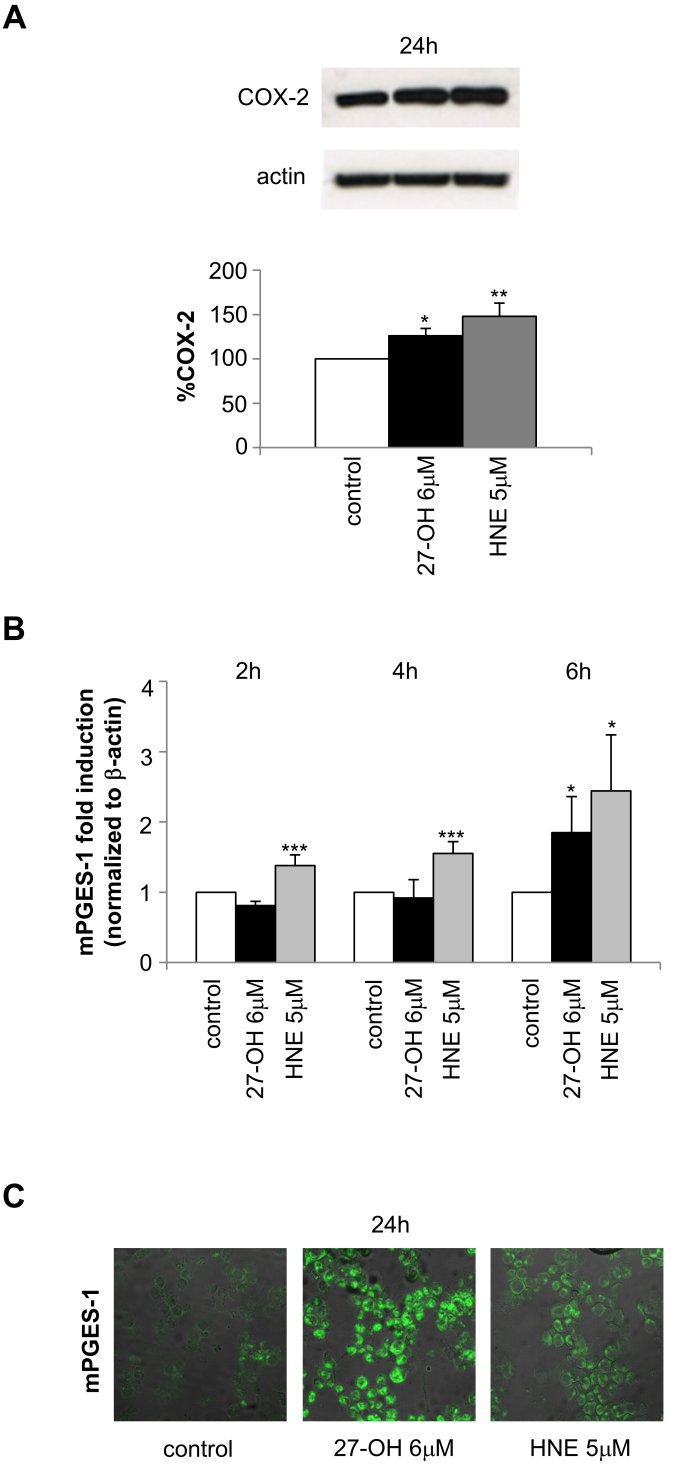
Fig. 3. Effect of 27-OH and HNE on nitric oxide production. U937 cells were treated with 27-OH (6 μM) or HNE (5 μM) and (A) iNOS expression levels were quantified by real-time RT-PCR after 6 h cell incubation. The histograms represent the mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. **$p<0.01$, and *$p<0.05$ vs. control. (B) NO production in cell medium was evaluated by Griess reaction after 48 h cell treatment. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control. NO levels expressed as μM were extrapolated from nitrite standard curve. **$p<0.05$ vs. control.

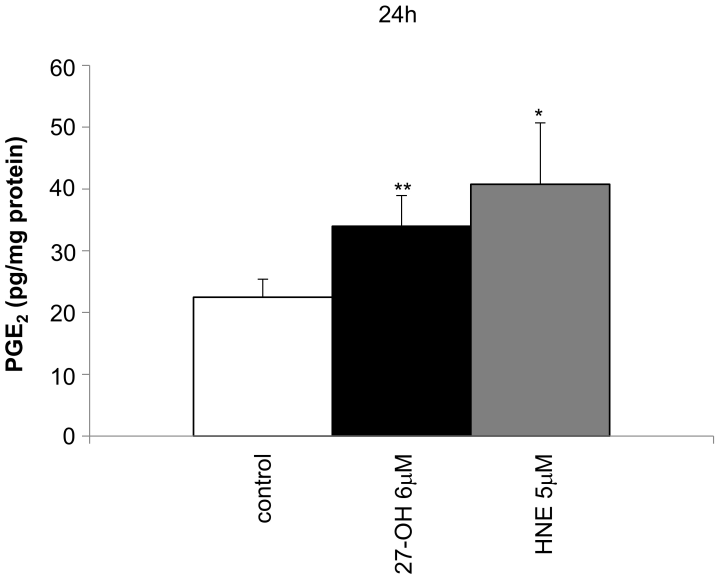
Fig. 4. EGCG reduces mPGES-1 levels induced by the oxidized lipids. U937 cells were pretreated for 1 h with 10 μM epigallocatechin gallate (EGCG) and then for 6 h with 27-OH (6 μM) or HNE (5 μM). mPGES-1 expression was quantified by real-time RT-PCR. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control. Data were normalized to corresponding β-actin levels. ***$p<0.001$, and **$p<0.01$ vs. control; §§§$p<0.001$ vs. 27-OH; ###$p<0.001$ vs. HNE.

Fig. 5. Inhibition of COX-2 and cell pretreatment with the anti-inflammatory EGCG decrease the inflammatory cytokine release induced by 27-OH or HNE. Cells were pretreated for 1 h with 25 μM NS-398 or with 10 μM EGCG and (A) expression of IL-8, IL-1β, and TNF-α was quantified by

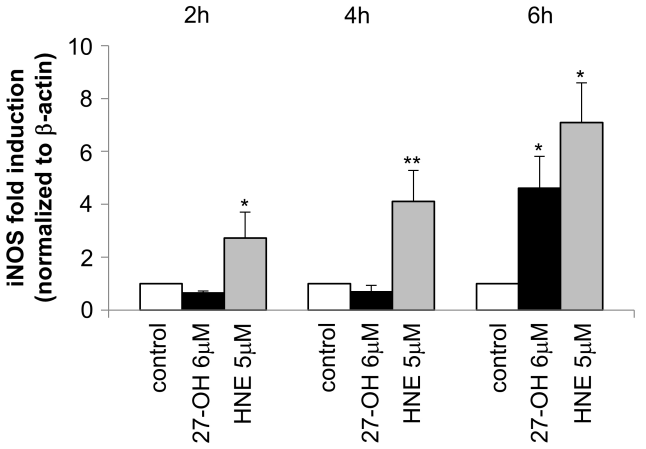
real-time RT-PCR after 6 h cell treatment with 27-OH (6 μM) or HNE (5 μM). The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control (untreated cells) and normalized to β-actin. **p<0.01, and *p<0.05 vs. control; §§§p<0.001, §§p<0.01, and §p<0.05 vs. 27-OH; ##p<0.01, and #p<0.05 vs. HNE. (B) After 24 h cell incubation with 27-OH or HNE, immunopositive cells were detected by confocal laser microscopy: IL-8 and IL-1β using a TRITC-conjugated secondary antibody (532-nm exciting laser band, 572-nm long-pass emission filter, and 40x/0.75 lens); TNF-α using a FITC-conjugated secondary antibody (488-nm exciting laser band and emission passing through a long-pass 505-550 filter, lens 40x/0.75). The images are representative of three experiments.

Fig. 6. COX-2 inhibition and EGCG cell preincubation decrease MMP-9 levels. Cells were pretreated for 1 h with 25 μM NS-398 or with 10 μM EGCG and then with 27-OH (6 μM) or with HNE (5 μM) for 24 h. (A) MMP-9 gene expression was evaluated by real-time RT-PCR. The histograms represent mean values ± SD of three experiments, expressed as fold induction versus control and normalized to β-actin. ***p<0.001, and *p<0.05 vs. control; §§§p<0.001 vs. 27-OH; #p<0.05 vs. HNE. (B) MMP-9 protein levels were analyzed using the ELISA method after 48 h of cell incubation with 27-OH or HNE. MMP-9 concentrations (pg/ml) were extrapolated from the standard curve. The histograms represent mean values ± SD of three experiments. **p<0.01, and *p<0.05 vs. control; §§p<0.01 vs. 27-OH; ###p<0.001 vs. HNE.

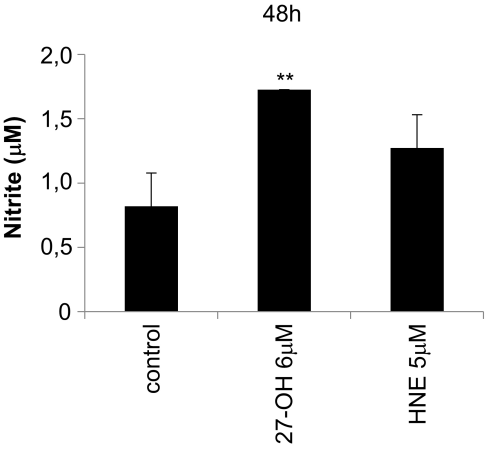

A
24h
COX-2
actin
%COX-2
200
150
100
50
0
*
**
control
27-OH 6μM
HNE 5μM
B
2h
4h
6h
mPGES-1 fold induction
(normalized to β-actin)
4
3
2
1
0
***
***
*
*
control
27-OH 6μM
HNE 5μM
control
27-OH 6μM
HNE 5μM
control
27-OH 6μM
HNE 5μM
C
24h
mPGES-1
control
27-OH 6μM
HNE 5μM


24h
PGE2 (pg/mg protein)
60
50
40
30
20
10
0
**
*
control
27-OH 6μM
HNE 5μM

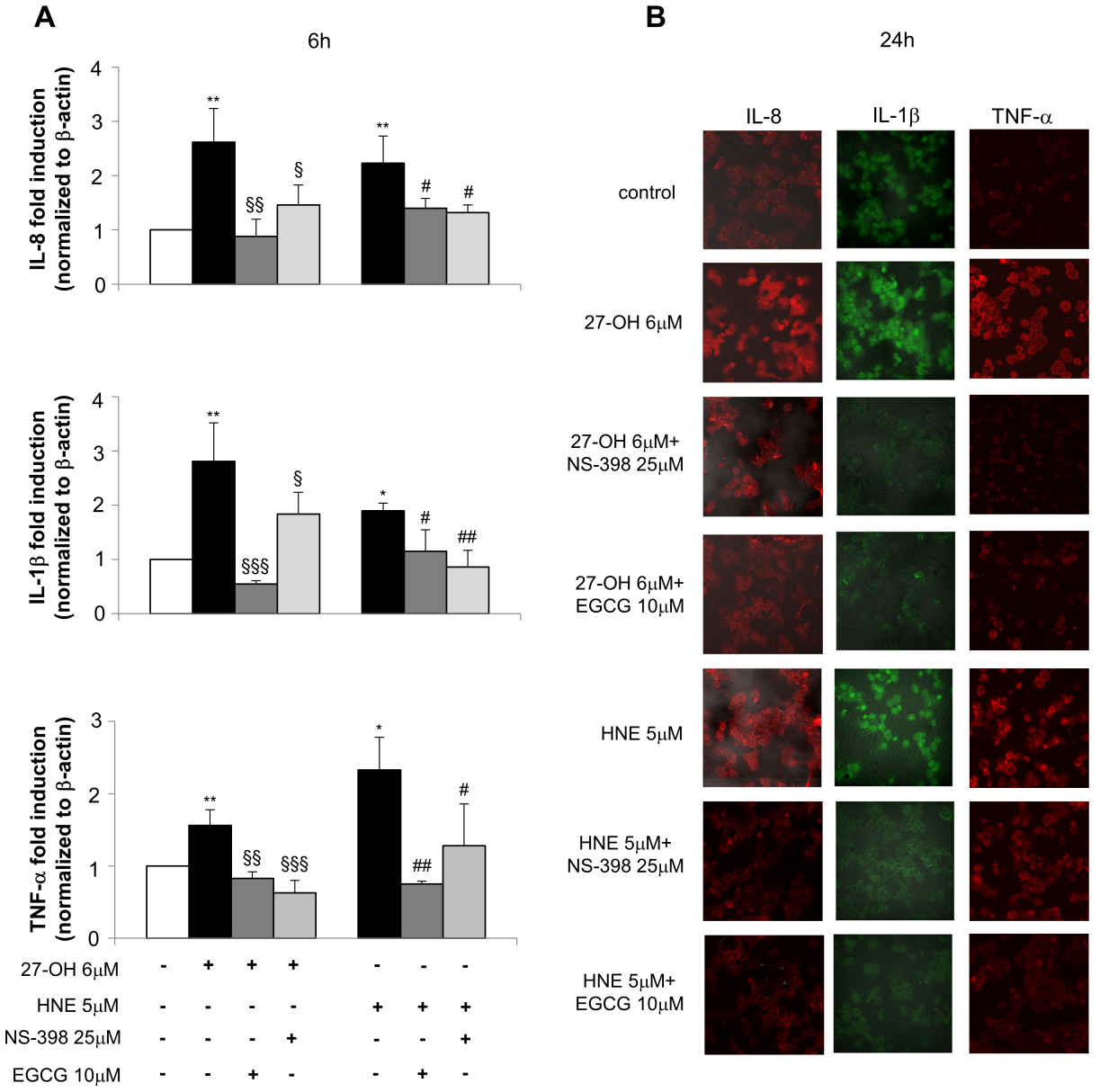
A

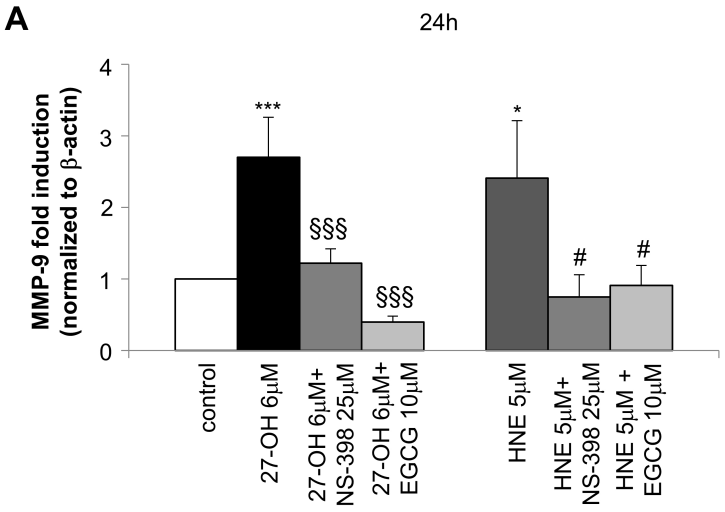

iNOS fold induction
(normalized to β-actin)
10
8
6
4
2
0
2h
4h
6h
*
**
*
*
control
27-OH 6μM
HNE 5μM
control
27-OH 6μM
HNE 5μM
control
27-OH 6μM
HNE 5μM

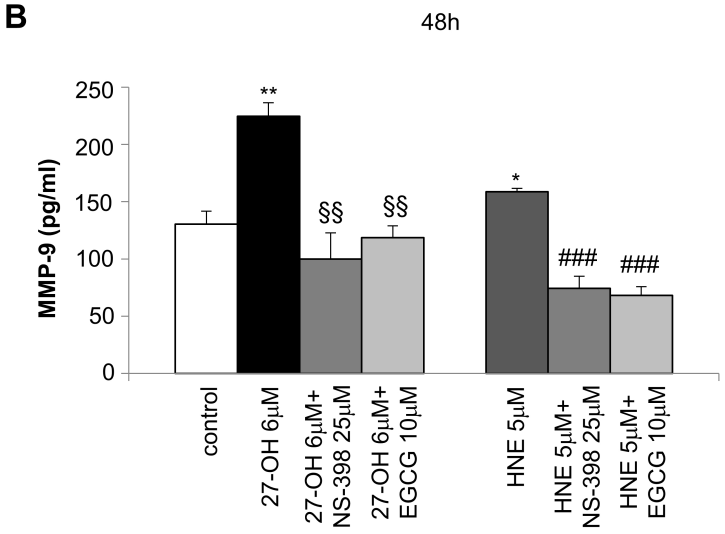
B


48h
Nitrite (μM)
2,0
1,5
1,0
0,5
0
**
control
27-OH 6μM
HNE 5μM

6h
mPGES-1 fold induction
(normalized to β-actin)
5
4
3
2
1
0
**
§§§
***
###
control
27-OH 6μM
27-OH 6μM
+EGCG 10μM
HNE 5μM
HNE 5μM
+EGCG 10μM


A
6h
IL-8 fold induction (normalized to β-actin)
IL-1β fold induction (normalized to β-actin)
TNF-α fold induction (normalized to β-actin)
27-OH 6μM
HNE 5μM
NS-398 25μM
EGCG 10μM
B
24h
IL-8
IL-1β
TNF-α
control
27-OH 6μM
27-OH 6μM+ NS-398 25μM
27-OH 6μM+ EGCG 10μM
HNE 5μM
HNE 5μM+ NS-398 25μM
HNE 5μM+ EGCG 10μM


A
24h
MMP-9 fold induction
(normalized to β-actin)
4
3
2
1
0
***
§§§
§§§
*
#
#
control
27-OH 6μM
27-OH 6μM+
NS-398 25μM
27-OH 6μM+
EGCG 10μM
HNE 5μM
HNE 5μM+
NS-398 25μM
HNE 5μM +
EGCG 10μM


B
48h
MMP-9 (pg/ml)
250
200
150
100
50
0
**
§§
§§
*
###
###
control
27-OH 6μM
27-OH 6μM+
NS-398 25μM
27-OH 6μM+
EGCG 10μM
HNE 5μM
HNE 5μM+
NS-398 25μM
HNE 5μM+
EGCG 10μM