Up-regulation of PCSK6 by lipid oxidation products: A possible role in atherosclerosis

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Abstract:	Atherosclerosis is a degenerative disease characterized by lesions that develop in the wall of large- and medium-sized arteries due to the accumulation of low-density lipoproteins (LDLs) in the intima. A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and the aldehyde 4-hydroxy-2-nonenal (HNE), the major pro-atherogenic components of oxidized LDLs, significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. The involvement of certain members of the protein convertase subtilisin/kexin proteases (PCSKs) in atherosclerosis has been recently hypothesized. Among them, PCSK6 has been associated with plaque instability, mainly thanks to its ability to stimulate the activity of matrix metalloproteinases (MMPs) involved in extracellular matrix remodeling and to enhance inflammation. In U937 promonocytic cells and in human umbilical vein endothelial cells, an oxysterol mixture and HNE were able to up-regulate the level and activity of PCSK6, resulting in MMP-9 activation as demonstrated by PCSK6 silencing. Inflammation, enhanced by these lipid oxidation products, plays a key role in the up-regulation of PCSK6 activity as demonstrated by cell pretreatment with NS-398, with epigallocatechin gallate or with acetylsalicylic acid, all with anti-inflammatory effects. For the first time, we demonstrated that both oxysterols and HNE, which substantially accumulate in the atherosclerotic plaque, up-regulate the activity of PCSK6. Of note, we also suggest a potential association between PCSK6 activity and MMP-9 activation, pointing out that PCSK6 could contribute to atherosclerotic plaque development.
Opposed Reviewers:	

Prof. Claude Forest
Editor of Biochimie

Turin, December 11, 2020

Dear Prof. Claude Forest,

we are submitting to Biochimie the revised manuscript “Up-regulation of PCSK6 by lipid oxidation products: a possible role in atherosclerosis” (Testa et al.), Ref No BIOCHI-D-20-01027. We have answered to all questions raised by the Reviewers and Editor. With the hope to have made our manuscript acceptable by Biochimie for publication.

Looking forward to hearing from you.

Best regards,
Gabriella Leonarduzzi

We thank the reviewers for their suggestions. We have reviewed the paper based on their requests and comments.

Reviewer 1
Introduction:
- The suggested papers (Ref 24, 25) have been added in the introduction and a new sentence has been written (Line 48-52).

Materials and Methods
- In the paragraph 2.1 we have justified the composition and the concentration of the oxysterol mixture used for the experiments. In the reference 31 a table reports the GC/MS analysis of oxysterols quantified in human atherosclerotic plaques (Line 9-17).
- At the beginning of the paragraph 2.5 the code number of the PCSK6 siRNA and of a non-targeting siRNA have been added. siRNAs sequences are not revealed by the manufacturer because of proprietary interests.

Discussion
- The paper suggested by the Reviewer has been added (Line 11, Ref 44).
- A new comment about the effects of PCSK6 on U937 cells treated with the oxysterol mixture and its possible role in cell death has been added in the Discussion section (end of pag 15 of the revised paper).
This part cannot be more deeply discussed because few data are present in literature about the association of PCSK6 with atherosclerosis, although its involvement in this process is emerging. Because PCSK6 is mainly involved in the cell migration and ECM degradation, we focused our attention on the role of PCSK6 on MMP-9 activity. Preliminary results have been obtained in our laboratory about the mechanism through which oxysterols might stimulate PCSK6 activity by inducing intracellular ROS production and the possible involvement of PCSK6 on cell death. Concerning the last point, PCSK6 does not seem responsible for the apoptotic cell death. However, further investigation is necessary.
- A comment about the potential role of the single oxysterols on PCSK6 expression has been added at the end of the Discussion section and the paper suggested by the Reviewer has been added (Ref 63).

Reviewer 2.
The Figures 3, 4, 6, and 7 have been modified as suggested by the Reviewer 2. The Figure 5 has been modified as well as its legend; the end of the paragraph 3.4 of the Results section has also been modified according to the new figure.

Editor
As suggested by the Editor, we have added the Author contributions and the statement that the authors have approved the final article

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Title of Paper: Up-regulation of PCSK6 by lipid oxidation products: a possible role in atherosclerosis"
Author (s): Gabriella Testa, Erica Staurenghi, Serena Giannelli, Barbara Sottero, Simona Gargiulo, Giuseppe Poli, Paola Gamba and Gabriella Leonarduzzi

We have no conflict of interest to declare.

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ABSTRACT

Atherosclerosis is a degenerative disease characterized by lesions that develop in the wall of large- and medium-sized arteries due to the accumulation of low-density lipoproteins (LDLs) in the intima. A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and the aldehyde 4-hydroxy-2-nonenal (HNE), the major pro-atherogenic components of oxidized LDLs, significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. The involvement of certain members of the protein convertase subtilisin/kexin proteases (PCSKs) in atherosclerosis has been recently hypothesized. Among them, PCSK6 has been associated with plaque instability, mainly thanks to its ability to stimulate the activity of matrix metalloproteinases (MMPs) involved in extracellular matrix remodeling and to enhance inflammation. In U937 promonocytic cells and in human umbilical vein endothelial cells, an oxysterol mixture and HNE were able to up-regulate the level and activity of PCSK6, resulting in MMP-9 activation as demonstrated by PCSK6 silencing. Inflammation, enhanced by these lipid oxidation products, plays a key role in the up-regulation of PCSK6 activity as demonstrated by cell pretreatment with NS-398, with epigallocatechin gallate or with acetylsalicylic acid, all with anti-inflammatory effects.

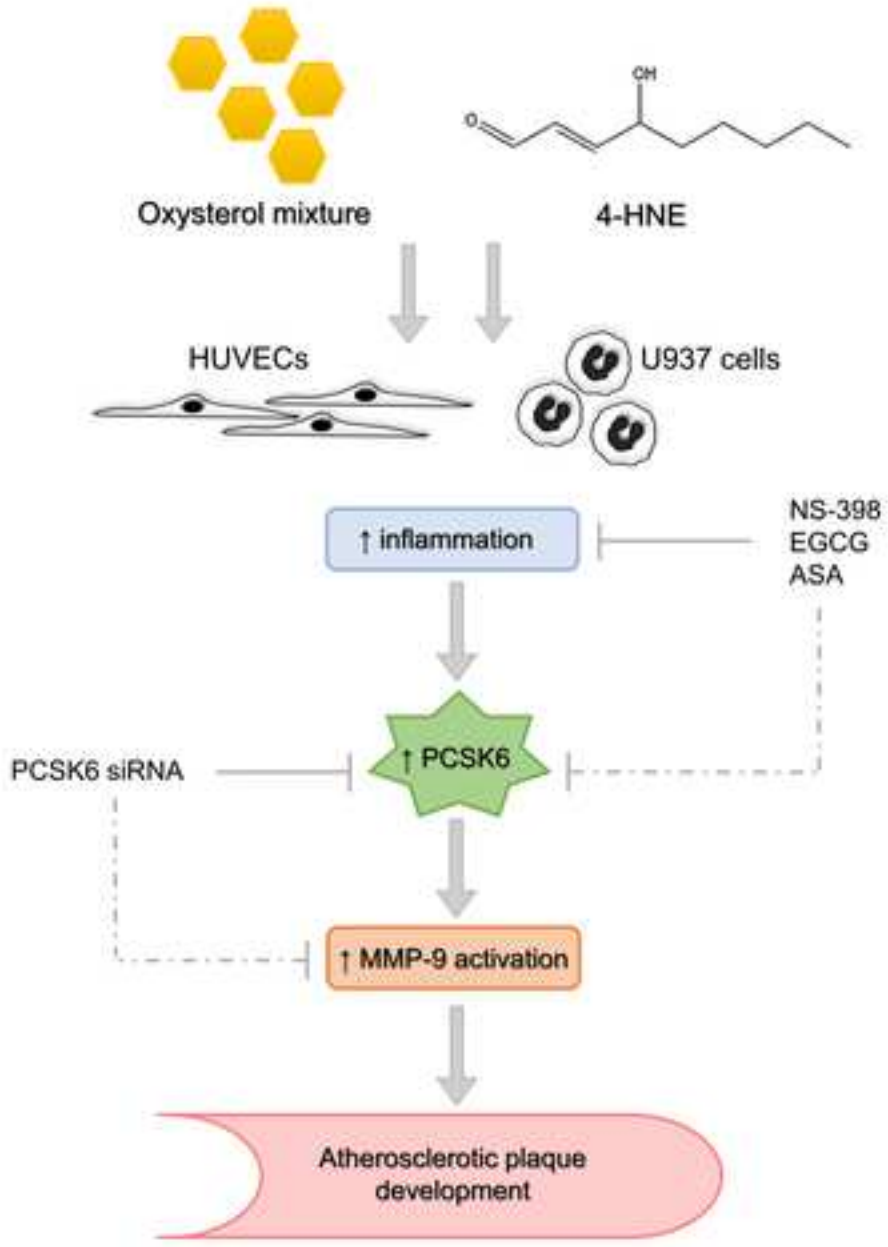
For the first time, we demonstrated that both oxysterols and HNE, which substantially accumulate in the atherosclerotic plaque, up-regulate the activity of PCSK6. Of note, we also suggest a potential association between PCSK6 activity and MMP-9 activation, pointing out that PCSK6 could contribute to atherosclerotic plaque development.

Highlights:

An oxysterol mixture and HNE stimulate PCSK6 expression and activity.

PCSK6 up-regulation induces MMP-9 activation promoting plaque progression.

Inflammation induced by oxysterols and HNE promotes PCSK6 up-regulation.



Up-regulation of PCSK6 by lipid oxidation products: a possible role in atherosclerosis

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ABSTRACT

Atherosclerosis is a degenerative disease characterized by lesions that develop in the wall of large- and medium-sized arteries due to the accumulation of low-density lipoproteins (LDLs) in the intima. A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and the aldehyde 4-hydroxy-2-nonenal (HNE), the major pro-atherogenic components of oxidized LDLs, significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. The involvement of certain members of the protein convertase subtilisin/kexin proteases (PCSKs) in atherosclerosis has been recently hypothesized. Among them, PCSK6 has been associated with plaque instability, mainly thanks to its ability to stimulate the activity of matrix metalloproteinases (MMPs) involved in extracellular matrix remodeling and to enhance inflammation. In U937 promonocytic cells and in human umbilical vein endothelial cells, an oxysterol mixture and HNE were able to up-regulate the level and activity of PCSK6, resulting in MMP-9 activation as demonstrated by PCSK6 silencing. Inflammation, enhanced by these lipid oxidation products, plays a key role in the up-regulation of PCSK6 activity as demonstrated by cell pretreatment with NS-398, with epigallocatechin gallate or with acetylsalicylic acid, all with anti-inflammatory effects.

For the first time, we demonstrated that both oxysterols and HNE, which substantially accumulate in the atherosclerotic plaque, up-regulate the activity of PCSK6. Of note, we also suggest a potential association between PCSK6 activity and MMP-9 activation, pointing out that PCSK6 could contribute to atherosclerotic plaque development.

Keywords: oxysterols, 4-hydroxy-2-nonenal, PCSK6, MMP-9, inflammation, atherosclerosis

Abbreviations: AP, activator protein 1; ASA, acetylsalicylic acid; COX-2, cyclooxygenase-2; ECs, endothelial cells; ECM, extracellular matrix; EGCG, epigallocatechin gallate; HDLs, high density lipoproteins; HNE, 4-hydroxy-2-nonenal; HUVECs, human umbilical vein ECs; IL, interleukin; LDLs, low density lipoproteins; MAPKs, mitogen-activated protein kinases; MMPs, matrix metalloproteinases; mPGES-1, membrane-bound prostaglandin E synthase-1; NF-κB, nuclear factor-κB; oxLDLs, oxidized LDLs; PCSKs, protein convertase subtilisin/kexin proteases; PDGF, platelet-derived growth factor subunit B; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; SMCs, smooth muscle cells; TGF-β1, transforming growth factor-β1; TLR-4, Toll-like receptor-4; TNF-α, tumor necrosis factor-α; VSMCs, vascular SMCs.

1. Introduction

Atherosclerosis is a multifactorial and degenerative disease affecting large- and medium-sized arteries, characterized by lesions that develop and progress in the arterial wall due to low density lipoprotein (LDL) accumulation in the arterial intima. Although increased plasma lipids are central to the development of atherosclerosis, this disease is now mainly thought to be the consequence of a complex inflammatory process, involving immune responses as well as oxidative stress, with subsequent activation of all arterial wall cells [1,2]. The intercellular cross-talk that occurs among vascular smooth muscle cells (VSMCs), macrophages, endothelial cells (ECs), and leukocytes leads to a fibroproliferative response. During this response, the extracellular matrix (ECM) plays 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. Alterations in local inflammation may also induce an increased ECM modulation in the shoulder region of the fibrous cap, resulting in plaque destabilization with subsequent risk of rupture [3-5].

Recently, the involvement of certain members of the pro-protein convertase subtilisin/kexin proteases (PCSKs) in atherosclerosis was demonstrated [6,7]. PCSKs are enzymes that cleave and convert their immature target proteins into a biologically active form [8]. Consequently, by controlling the activity of pro-atherosclerotic factors, including metalloproteinases (MMPs) and cytokines, these enzymes may play a central role in the atherosclerotic plaque formation [9]. Members of PCSKs have been identified in human vascular lesions and animal models of atherosclerosis [10], while their inhibition reduces vascular remodeling and lessens progression of atherosclerosis in animal models, by regulating inflammation, including cell proliferation and migration, as well as lipid metabolism [11]. Among them, the involvement of the pro-protein convertase PCSK6 (also known as PACE4) in cancer progression [12-14] and rheumatoid arthritis development [15,16] is well documented but, recently, it has also been associated with atherosclerotic plaque progression and instability, thanks to its ability to stimulate the activity of MMPs responsible for ECM remodeling.

Up-regulation of PCSK6 was demonstrated in patients with symptomatic carotid plaques compared to asymptomatic ones, where it plays a key role in inflammation and ECM remodeling causing plaque instability and rupture [17]. PCSK6 was found localized in VSMCs and in the ECM within the fibrous cap region and the co-localization of PCSK6 and MMP-9 was observed in the fibrous cap and near the necrotic core of carotid plaques. Moreover, it was demonstrated that PCSK6 mRNA positively correlates with markers of macrophages (e.g. CD36 and CD40 receptors) and with inflammatory molecules, in particular growth factors and cytokines. A significant up-regulation of PCSK6 in VSMCs was indeed induced by nuclear factor-κB (NF-κB),

tumor necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1) [17]. Recently, it was also demonstrated that PCSK6 is a key factor in vascular remodeling through regulation of SMC migration and intimal invasion by modulation of MMP-14 activation, particularly upon platelet-derived growth factor subunit B (PDGFB) stimulation [18]. Because of its potential role in atherosclerosis, PCSK6 can be considered as a new marker of plaque development and instability.

One of the major risk factors of atherosclerosis is hypercholesterolemia, which promotes the intramural accumulation of oxidized LDLs (oxLDLs) in susceptible areas of the arterial vasculature, promoting EC dysfunction and, in turn, a chronic inflammatory state [2,19]. It has been shown that a lipid extract of human atherosclerotic plaques induces oxidative stress in mouse macrophages, and decreases the ability of high density lipoproteins (HDLs) to trigger cholesterol efflux from macrophages [20]. Moreover, a lipid extract from human carotid plaques has been shown to increase the expression of pro-inflammatory mediators in human promonocytic THP-1 cells and macrophage-like cells. Of note, the fraction rich in cholesterol oxidation products, named oxysterols, was a major contributor to this effect [21]. Unlike cholesterol, oxysterols, which consistently accumulate in the atherosclerotic plaque, critically contribute to cell dysfunction and death by amplifying oxidative stress and inflammation. The cytotoxicity of oxysterols is due to their capacity to diffuse through membranes where they affect receptor and enzymatic function damaging membranes, and altering pathways and overall cell function. Noteworthy, by this way they can contribute to the development of chronic and age-related diseases such as atherosclerosis [22-25]. Among the several reactive molecules deriving from LDL oxidation, indeed, oxysterols as well as 4-hydroxy-2-nonenal (HNE), one of the more reactive end products of polyunsaturated fatty acid peroxidation, play a pivotal role in all the various steps of atheroma formation, from the initial lesion to the plaque instability [22,23,26,27].

In previous studies performed in human promonocytic U937 cells, we demonstrated that these lipid oxidation products contribute to plaque instability by up-regulating MMP-9 through various interconnected signaling pathways. Moreover, these molecules also enhanced the release of certain cytokines, thereby augmenting the inflammatory response. Of note, the induced inflammatory cytokines were found to act on MMP-9 production, thus sustaining the release of this matrix-degrading enzyme and contributing to plaque vulnerability [28-30].

Based on these previous findings, we investigated whether an oxysterol mixture, of a composition similar to that found in human carotid plaques [31], or the aldehyde HNE may modulate, in U937 cells and in human umbilical vein endothelial cells (HUVECs), the levels and the activity of the pro-protein convertase PCSK6. The compounds increased both levels and

activity of PCSK6, resulting in a significant activation of MMP-9, as demonstrated by transfecting cells with a PCSK6 small interfering RNA (siRNA). Inflammation, enhanced by oxysterols and HNE, also played a key role in the up-regulation of PCSK6 activity; this has been demonstrated by cell pretreatment with NS-398, a selective inhibitor of cyclooxygenase-2 (COX-2), or with epigallocatechin gallate (EGCG), a polyphenol with anti-inflammatory effects, as well as with acetylsalicylic acid (ASA), which has anti-inflammatory, anti-pyretic, and anti-thrombotic effects.

For the first time, the data show that the investigated lipid oxidation derivatives, which substantially accumulate in the atherosclerotic plaque, may up-regulate the activity of PCSK6 suggesting, of note, a potential link between PCSK6 activity and MMP-9 activation pointing out that PCSK6 might contribute to ECM remodeling and plaque development.

2. Materials and Methods

2.1. Cell culture and treatments

The human promonocytic cell line U937 and HUVECs were respectively cultured in RPMI 1640 and DMEM medium, both supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Sigma-Aldrich, Milan, Italy), at 37°C with 5% CO_2 in humidified atmosphere. For U937 and HUVEC cell co-culture, a mixture of RPMI and DMEM (1:1, v/v) was used.

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 or DMEM or in RPMI/DMEM medium with 2% fetal bovine serum and treated with 20 μM oxysterol mixture of a composition similar to that found in human carotid plaques by gas chromatography/mass spectrometry (GC/MS) analysis [31] thus mimicking the challenge of vascular cells by cholesterol oxidized products accumulating within the central core of atheroma. The composition of the oxysterol mixture is: 7α-hydroxycholesterol (4%), 7β-hydroxycholesterol (10%), 5α,6α-epoxycholesterol (8%), 5β,6β-epoxycholesterol (22%), cholestan-3β,5α,6β-triol (6%), 7-ketocholesterol (21%), 25-hydroxycholesterol (1%), and 27-hydroxycholesterol (28%) (Avanti Polar Lipids, Alabaster, AL, USA). The percentage of each individual oxysterol was approximately calculated on the basis of the ratio oxysterol ng/tissue mg. Other cells were placed in serum-free medium (RPMI 1640, DMEM or RPMI/DMEM) and then treated with 5 μM HNE (Alexis, Euroclone).

Concerning the cell co-culture, after the treatments the medium containing the U937 cells was centrifuged to collect them; HUVECs were detached and collected.

In certain experiments, cells were pretreated for 1 h with 25 μM NS-398, a specific inhibitor of COX-2 or with 10 μM EGCG, a polyphenol with antioxidant and anti-inflammatory properties (Sigma-Aldrich); other cells were co-treated with ASA (0.5-5 mM), an anti-inflammatory drug (Sigma-Aldrich); all cells were then incubated with the oxysterol mixture (20 μM) or with HNE (5 μM).

Final concentrations and incubation times for all experiments are given in the Result section or in the figure legends.

2.2. RNA extraction, cDNA preparation, and real-time RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Euroclone), following the manufacturer's instructions. RNA was dissolved in RNase-free water fortified with RNase inhibitors (RNase SUPERase-In; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The amount and purity (A_{260}/A_{280} ratio) of the extracted RNA were assessed spectrophotometrically. The samples were stored at -80°C until the analysis.

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 kit prepared for human PCSK6 and β-actin and TaqMan Fast Universal PCR master mix, and then 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.

Relative quantification of target gene expression was achieved with a mathematical method proposed by Livak and Schmittgen [32].

2.3. Analysis of PCSK6 protein levels by Western blotting

After treatments, cells were lysed in ice-cold buffer (1 ml PBS fortified with 10 μl Triton

X-100, 10 µl SDS 10%, and protease inhibitors) and centrifuged at 14000 rpm for 15 min. Total protein content was spectrophotometrically measured by Bradford's method [33].

Total proteins, 70 µg, were immunoprecipitated with anti-PCSK6 polyclonal primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) in RIPA-buffer (20 mM TRIS, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) added with protease inhibitors and incubated overnight at 4°C. Then, 15 µl of protein A-Sepharose resin were added and the samples were incubated for 2 h at 4°C and centrifuged at 2500 rpm for 5 min.

The samples were boiled for 5 min in Laemmli buffer and separated in 8% 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 a primary antibody against PCSK6 (1:500), diluted in 5% nonfat dried milk followed by a specific anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 2 h at room temperature (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were detected using the Clarity Western ECL substrate (Bio-Rad Laboratoires, Segrate, Milan, Italy) following the manufacturer's protocol.

The bands were quantified by densitometric analysis using the Image Lab software (Bio-Rad Laboratories). The results are expressed in relative units, as percentage versus untreated cells, considered as control.

2.4. Enzymatic activity evaluation of PCSK6 by spectrofluorimetry

To measure PCSK6 enzymatic activity, 40 µg of total proteins were added with 150 µl of substrate buffer (Tris-HCl 50 mM, pH 7.5 and 10 mM $CaCl_2$) and incubated for 30 min at 37°C with 80 µM of the Boc-RVRR-AMC substrate (Enzo Life Sciences, Farmingdale, NY, USA). The reaction was stopped with 5 mM EDTA. PCSK6 activity was evaluated as the release of fluorescent 7-amino-4-methylcoumarin (AMC), using a spectrofluorimeter (λ_{ex} 360 nm, λ_{em} 460 nm) (Kontron SFM 25, USA). Data are expressed as percentage versus untreated cells, considered as control.

2.5. Small interfering RNA (siRNA) transfection

PCSK6 siRNA was used for transient gene knockdown study (Silencer Select Pre-designed siRNA s9990, Ambion, Thermo Fisher Scientific). A non-targeting siRNA was used as a negative control (Silencer Select Negative Control #1 siRNA, Ambion). Both siRNAs sequences are not revealed

by the manufacturer because of proprietary interests. Transfection of PCSK6 siRNA was performed following the manufacturer's instructions.

Briefly, 50 nM of siRNA was mixed with 25 µl of transfection reagent solution (and left at room temperature for 10 min in RPMI medium with 1% fetal bovine serum without antibiotics. After 24 h of reverse transfection, the cells (4 x 10^4/500 µl) were incubated with 20 µM oxysterol mixture or 5 µM HNE for 24 h; for gene expression analysis, total RNA was isolated from the cells and used for quantitative RT-PCR as described above. The transfection efficiency, validated by quantitative RT-PCR, was approximately 70-80% (Supplementary Fig. 1).

2.6. Gel zymography

MMP-9 activity was measured by zymography assay on cell protein extracts. After treatment, cells were lysed in a lysis buffer (20 mM Hepes, pH 7.9, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, and 1% Igepal CA-630) and 50 µg of proteins were separated by 8% SDS-PAGE containing gelatin (0.8 mg/ml) under non-reducing conditions. The gels were washed with a washing buffer (50 mM Tris and 2.5% Triton X-100, pH 7.5), for 1 h to remove SDS and then incubated at 37°C overnight in an incubation buffer (40 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3, pH 7.5). Gelatinolytic activity was detected as unstained bands on a blue background after staining with 0.05% Coomassie Blue R250 and destaining with 5% methanol and 7% acetic acid solution. The bands were quantified by densitometric analysis using Image Lab software (Bio-Rad Laboratories).

2.7. Statistical analysis

All values are expressed as means ± standard deviation (SD). Data were analyzed statistically using one-way ANOVA with Bonferroni's post-hoc test for multiple comparisons. Differences at $p<0.05$ were considered statistically significant. Calculations were performed by GraphPad InStat3 software package (GraphPad Software, San Diego, CA, USA).

RESULTS

3.1. The oxysterol mixture and HNE induce PCSK6 expression and synthesis in both U937 cells and HUVECs

To investigate whether lipid oxidation products are involved in the up-regulation of PCSK6, a serine protease, U937 cells and HUVECs were incubated with the oxysterol mixture or HNE, which consistently accumulate in atherosclerotic plaques.

Human promonocytic U937 cells were incubated up to 8 h, either with an oxysterol mixture of composition similar to that found in advanced atherosclerotic plaques or with HNE, at the non-cytotoxic concentrations. The oxysterol mixture was used at 20 μM, a concentration mimicking the amount found in atherosclerotic plaques [31], 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 the oxysterols and HNE on PCSK6 expression and synthesis were analyzed by real-time RT-PCR and Western blotting, respectively. Both lipid oxidation derivatives significantly induced PCSK6 gene expression: in oxysterol-treated cells the induction of the pro-protein convertase was markedly increased, about 30-fold, after 6 h cell treatment (Fig. 1A); in cells treated with HNE, the induction of the pro-protein convertase was less marked compared with the oxysterol-treated cells, about 6-fold with a progressive reduction (Fig. 1C).

Regarding PCSK6 protein levels, the oxysterol mixture significantly induced its synthesis after 18 h cell treatment as shown in Figure 1B, followed by a reduction of PCSK6 protein levels but still above the control. In HNE-treated cells, a progressive increase of PCSK6 protein levels up to 48 h was observed, with a significant increase after 24 and 48 h cell treatment compared to untreated cells considered as control (Fig. 1D).

During atherosclerosis development and progression, the cross-talk among all types of vascular cells plays a crucial role. In particular, ECs play an important role in the initial phase of plaque formation [35]. In order to understand whether the pro-protein convertase PCSK6 might be induced by the oxidation lipid products in HUVECs, both expression and synthesis of the enzyme was investigated also in these cells. HUVECs were incubated with 20 μM oxysterol mixture or 5 μM HNE up to 48 h. In this set of experiments, both trend and increment of PCSK6 mRNA levels, quantified by real-time RT-PCR, were slightly different from that obtained in U937 cells. PCSK6 expression was induced by oxysterols and reached a maximum by 6 h treatment (about 5-fold increase), after which it reduced, although it was still significantly up-regulated (about 2-fold after 8 h cell treatment) (Fig. 2A). In HUVECs treated with HNE, a significant PCSK6 overexpression was observed after 2 and 6 h cell treatment, although the increase was about 2-fold (Fig. 2C).

With regard to PCSK6 protein levels, analyzed by Western blotting, the oxysterol mixture markedly induced the synthesis of PCSK6 from 18 up to 24 h cell treatment, slightly decreasing after 48 h but remaining significantly higher compared to untreated cells (control) (Fig. 2B). HNE

cell treatment was able to induce PCSK6 protein levels in a significant and time-dependent manner from 18 up to 48 h cell incubation (Fig. 2D).

3.2. Induction of PCSK6 activity by the oxysterol mixture and HNE in human promonocytic U937 cells and HUVECs.

Once demonstrated that oxysterols and HNE increase PCSK6 expression and synthesis, we investigated their effect on its enzymatic activity.

PCSK6 activity was quantified through a spectrofluorimetric method in U937 cells and HUVECs incubated with the oxysterol mixture (20 μM) or with HNE (5 μM) up to 48 h. In U937 cells, the activity of PCSK6 significantly increased after 18 h cell treatment with oxysterols, then it was reduced until control levels were reached (48 h) (Fig. 3A). In HNE-treated cells, PCSK6 activity raised in a time-dependent manner with a maximum at 48 h (approximately 2-fold) (Fig. 3B).

In HUVECs, the enzymatic activity of PCSK6 increased in a time-dependent manner after oxysterol mixture cell treatment, and the increase was significant after 24 and 48 h compared to control cells (Fig. 3C). In HNE-treated cells, a significant increase of PCSK6 activity after 18 and 24 h cell incubation was observed, with a slight decrease after 48 h (Fig. 3D).

3.3. Modulation of PCSK6 activity in U937 cell and HUVEC co-culture incubated with the oxysterol mixture or HNE

Since *in vivo* there is a constant cross-talk among all vascular cells, especially between monocytes and ECs, we then investigated whether co-culturing U937 cells and HUVECs might further modulate PCSK6 activity. Co-cultured cells were treated with the oxysterol mixture (20 μM) or HNE (5 μM) up to 48 h, and then analyzed separately. Oxysterol or HNE treatments up-regulated PCSK6 activity in both cells (Fig. 4A-D) with a trend similar to that observed in cells treated individually (Fig. 3A-D) but the enzyme activity induced by the oxysterol mixture was more statistically significant compared to untreated cells considered as control (Fig. 4A and C).

3.4. Induction of MMP-9 activity by the oxysterol mixture and HNE through PSCK6 activation in U937 cells.

The role of PCSK6 in atherosclerosis is still not fully elucidated, while its role in carcinogenesis is well documented. In particular, it has been shown that PCSK6 induces the

activity of MMP-9 to promote the mobility and the invasiveness of cancer cells [36].

Given the importance of MMP-9 in atherosclerosis, particularly for the instability and rupture of plaques due to its involvement in fibrous cap matrix degradation [7,37,38], we investigated whether PCSK6 up-regulation induced by oxysterols and HNE might promote MMP-9 activity in U937 cells, since it is mainly produced by macrophages.

The activity of MMP-9, a gelatinase B, was analyzed by gelatin zymography. The results show a significant increase of MMP-9 activity in cells treated either with the oxysterol mixture (20 µM) or with HNE (5 µM).

Oxysterols promoted a significant and progressive increase of MMP-9 activity in a time-dependant manner up to 48 h, confirming our previous results [28]. To investigate a potential association between PCSK6 up-regulation and MMP-9 over-activation, U937 cells were incubated with oxysterols, after the down-regulation of PCSK6 gene by a specific siRNA. The enzymatic activity of MMP-9 was then analyzed by gelatin zymography. Using PCSK6 siRNA, MMP-9 activity induced by oxysterols was significantly blocked after 24 h cell incubation compared to untreated cells (control) (Fig. 5A).

In HNE-treated cells, MMP-9 activity markedly increased (about 2-fold) after 18 and 24 h cell treatment with a slight reduction at 48 h, although it was still significantly higher compared to untreated cells considered as control. After the down-regulation of PCSK6 gene, the enzymatic activity of MMP-9 was also significantly blocked after 24 h cell incubation with HNE compared to untreated cells (control) (Fig. 5B)

This data suggests a possible link between PCSK6 activity and MMP-9 activation, underlining their involvement in atherosclerotic plaque development.

3.5. Inhibition of inflammation reduces PCSK6 activity in both U937 and HUVECs.

Nowadays, it is clear that inflammation plays a key role in atherosclerotic plaque progression and instability [1,2,4]. It is also well known that the levels of COX-2, a key inflammatory mediator, are increased in atherosclerotic lesions [39]. In this connection, we recently demonstrated that the oxysterol 27-hydroxycholesterol and the aldehyde HNE are able to increase expression and synthesis of COX-2 as well as the levels of some pro-inflammatory cytokines in U937 cells [30]. Further, it has been shown that PCSK6 mRNA levels are increased in SMCs stimulated with pro-inflammatory cytokines [18]. On this basis, we hypothesized that inflammation could be the driving force for PCSK6 activation within the atherosclerotic plaque.

U937 cells and HUVECs were pre-treated (1 h) with 25 µM NS-398, a selective inhibitor

of COX-2, or with 10 μM EGCG, a polyphenol with antioxidant and anti-inflammatory effects, and then incubated with 20 μM oxysterol mixture or 5 μM HNE for 24 h. We also investigated the effect of ASA on PCSK6 activity in the same cells, since ASA acts as an inhibitor of both cyclooxygenases enzymes (COX-1 and COX-2), resulting in the inhibition of prostaglandin biosynthesis. U937 cells were co-treated with different concentration of ASA (0.5 mM, 1 mM, or 5 mM) and with the oxysterol mixture (20 μM) or HNE (5 μM) for 24 h.

PCSK6 activity induced by the lipid oxidation products was inhibited by both NS-398 and EGCG cell pre-treatment in U937 cells (Fig. 6A,B) and in HUVECs (Fig. 6C,D). A marked and significant concentration-dependent inhibition of PCSK6 activity was observed in U937 cells co-treated with ASA and oxysterols (Fig. 7A) or HNE (Fig. 7B), in particular at 5 mM ASA concentration, compared to untreated cells (control).

4. Discussion

It is well known that hypercholesterolemia is an important requisite for the initiation and progression of the atherosclerotic lesion. Indeed, the initial event is the accumulation of oxLDLs in the sub-intima space due to endothelial dysfunction and they play a key role in the pathogenesis of atherosclerosis since their up-take by activated macrophages promotes oxidative stress and chronic inflammation state, leading to the progression and instability of the atherosclerotic plaque [2,5,19,40]. During these processes, the intercellular cross-talk that occurs among the various arterial wall cells leads to a fibroproliferative response [1,2,19].

Several compounds deriving from oxidation of the LDL lipid fraction are responsible for LDL pro-atherogenic effects and, among them, there is no longer any doubt that oxysterols, present at high concentrations in atherosclerotic plaques, play a pivotal role in all the various steps of atheroma development [23,26,41-44]. Another important molecule, deriving from peroxidation of polyunsaturated fatty acids, is HNE, one of the more reactive aldehydes that contributes to atherosclerotic plaque progression [23,27,45]. HNE is also present at high concentrations in plasma of patients affected by acute myocardial infarction [46].

ECM deposition plays a key role in plaque formation, providing the structural integrity of the plaque itself, as well as contributing to cell migration and proliferation. Therefore, the gradual loss of SMCs leads to a lesser synthesis of collagen and the imbalance in the MMPs/TIMPs (tissue inhibitor of metalloproteinases) activity ratio to an uncontrolled ECM degradation and thus to the thinning of the fibrous cap [3,5,37,38]. MMPs have been found at high concentrations within atherosclerotic unstable plaques and in the plasma of patients affected by acute coronary

syndromes [47,48]. It has also been demonstrated that MMP-9 is the most abundant gelatinase released by activated macrophages, and that the production of pro-inflammatory cytokines, such as interleukin-8 (IL-8) and TNF-α, can further stimulate its synthesis [29,49]. In this connection, in previous studies we have demonstrated that various oxysterols and the aldehyde HNE contribute to plaque instability by up-regulating MMP-9. In human promonocytic U937 cells, an oxysterol mixture, of composition similar to that found in advanced atherosclerotic plaques, was shown to up-regulate MMP-9 without affecting its inhibitors TIMP-1 and TIMP-2 [28]. In addition, it has been shown that, among the oxysterols of the mixture, 27-hydroxycholesterol markedly induces MMP-9 expression in U937 cells, through the Toll-like receptor 4 (TLR4)/NF-κB pathway. It also enhanced the release of IL-8, IL-1β and TNF-α, thereby augmenting the inflammatory response. Of note, these inflammatory cytokines have also been found to act on MMP-9 production, thus sustaining the release of this matrix-degrading enzyme and contributing to plaque vulnerability [29]. Further, in the same cell type, we also demonstrated that the most abundant circulating oxysterol 27-hydroxycholesterol and the aldehyde HNE promote the up-regulation of both the inducible enzymes COX-2 and membrane-bound prostaglandin E synthase-1 (mPGES-1), leading to increased production of prostaglandin E_2 (PGE_2). As a result of COX-2/mPGES-1 up-regulation, the production of certain pro-inflammatory cytokines and of MMP-9 by activated macrophages was also observed; the enhancement of cytokines and MMP-9 is presumably due to the induction of PGE_2 synthesis which might thus be a mechanism of plaque instability [30]. HNE has also been demonstrated to up-regulate MMP-9 levels in murine macrophages [50].

Because patients with coronary atherosclerosis show relatively unpredictable events that could strike without warning, there is a great effort in discovering new biomarkers in order to identify patients at high risk of developing atherosclerosis.

Besides the plasma levels of inflammatory molecules, such as cytokines and MMPs, certain members of PCSK family could be suggested as new promising biomarkers of atherosclerosis [7]. For example, PCSK3 (furin) and PCSK5 have been suggested to regulate inflammation in atherosclerosis. Specifically, in VSMCs and macrophages, PCSK3 and PCSK5 have been shown to activate integrins and/or MMPs, suggesting a role for these enzymes in cell proliferation and migration during atherogenesis [51,52]; PCSK expression has also been shown to increase during macrophage differentiation [10]. In addition, PCSK3, PCSK5, and PCSK6 cleave and inactivate both endothelial and lipoprotein lipases, which play a critical role in the metabolism of HDLs, very low density lipoproteins, and chylomicrons [53]. Thanks to microarray and quantitative real-time PCR analyses, it has been observed that PCSK3 is the most dysregulated PCSK in advanced human atherosclerotic plaques, and that its up-regulation is localized in the plaque lymphocytes

and macrophages [9]. Although very little is known on the role of PCSK6 in atherosclerosis development, it was reported that higher serum PCSK6 levels may be a marker for higher cardiovascular risk in patients without a chronic kidney disease [54]. Moreover, polymorphisms in the PCSK6 gene have also been associated with hypertension and cardiovascular risk [55], whereas single nucleotide polymorphisms in PCSK5 have been found to correlate with HDL levels [56]. In human carotid lesions, up-regulation of PCSK6, but also of PCSK7, was also demonstrated, whereas no significant change in expression levels was observed for PCSK2 and PCSK9; PCSK9 is, indeed, involved in the modulation of cholesterol homeostasis rather than plaque vulnerability [17]. PCSK6 has been previously linked to increased cancer cell mobility and invasiveness by enhancing the activity of various ECM-associated pro-proteins, such as MMP-9 [36] but, recently, PCSK6 has also been associated with plaque instability thanks to its ability to stimulate the activity of MMPs involved in ECM remodeling. Perisic and colleagues investigated the expression of various members of the PCSK family: RNA samples extracted from 127 human symptomatic carotid plaques were analyzed using microarray technology. Among various genes involved in the pathogenesis of atherosclerosis, a significant down-regulation of PCSK3 and PCSK5 was found, whereas PCSK6 and PCSK7 were up-regulated in carotid plaques in comparison with control iliac arteries: an increase of about 19-fold of PCSK6 mRNA was observed in plaques in comparison with controls, and an increase of about 8-fold in symptomatic carotid plaques compared to asymptomatic ones. In the same study, by high-throughput immunohistochemistry, it has been shown that PCSK6 is highly expressed in SMCs and ECM within the fibrous cap region. Moreover, a positive correlation between PCSK6 expression and genes associated with inflammation, apoptosis, and matrix degradation was demonstrated. Of note, it has also been shown that PCSK6 and MMP-9 co-localize in the fibrous cap, adjacent to the necrotic core [17]. Very recently, it was also demonstrated that PCSK6 is a key factor in vascular remodeling. PCSK6 was located to fibrous caps and expressed by proliferating SMCs with positive correlation to PDGFB and MMP-2/MMP-14. In particular, it was demonstrated that PCSK6, through the regulation of SMC migration in the intima by MMP-14 activation, plays a key role in vascular remodeling [18]. All data highly sustain the association between high PSCK6 levels and unstable atherosclerotic plaques.

On this basis, we hypothesized that this pro-protein convertase might be involved in plaque progression, given that PCSK6 can modulate MMP-9 expression and its release by inflammatory cells. On this basis, we carried out *in vitro* experiments to assess whether oxysterols and HNE might up-regulate PCSK6 expression and enzymatic activity leading to MMP-9 activation. Considering that during atherosclerosis development the cross-talk between vascular and

inflammatory cells is fundamental, we performed our experiments using two types of cells involved in the atherosclerotic plaque formation: U937 cells and HUVECs.

In U937 cells, a significant increase of PCSK6 expression and synthesis was found after cell treatment with the oxysterol mixture (20 μM) or with HNE (5 μM) (Fig. 1). A similar trend of PCSK6 expression and protein levels was observed in HUVECs incubated with oxysterols or HNE (Fig. 2). Following the increase in PCSK6 levels, the enzymatic activity of PCSK6 was also investigated in the same cells treated either with the oxysterol mixture or with HNE. The activity of the pro-protein convertase was significantly increased in all set of experiments (Fig. 3). Since plaque development is influenced by vascular cell interactions, we then investigated whether a co-culture of U937 cells and HUVECs might further modulate PCSK6 activity. Both oxysterol and HNE treatments induced a similar trend of PCSK6 activity in the cells (Fig. 4), but the enzyme activity induced by the oxysterol mixture was more statistically significant. However, further investigations are needed to better understand how these cells can act on each other and if molecules released in the medium can eventually be responsible for the effect.

Given the crucial role of MMP-9 in atherosclerosis and in particular in plaque progression and instability [7,37,38], we also analyzed the effect of the oxysterol mixture and HNE on its activity in U937 cells, since MMP-9 is mainly produced by macrophages. Our data show a significant increase in MMP-9 activity, following U937 cell incubation with these oxidized lipids (Fig. 5).

Several studies reported an association between PCSK6 and MMPs' activity in various cancer cell models [12], and a co-localization between PCSK6 and MMP-9 in the fibrous cap of carotid plaques [17]; thus, we investigated whether the up-regulation of PCSK6 expression, induced by oxysterols and HNE, might promote MMP-9 activity. PCSK6 silencing in U937 cells significantly inhibited the increased MMP-9 activity induced by oxysterols or HNE (Fig. 5). Although the involved pathways need further investigation, data clearly suggest a potential link between PCSK6 activity and MMP-9 activation that might contribute to plaque progression and instability in according with the finding that PCSK6 and MMP-9 co-localize in the fibrous cap of human symptomatic carotid plaques [17] and that PCSK6 is highly expressed not only by macrophages but also by proliferating SMCs [17,18]. Although vascular and inflammatory cell death might contribute to plaque instability and rupture, preliminary data (not shown) suggest that PCSK6 is not involved in this event. In this connection, PCSK6 was demonstrated to exert an anti-apoptotic effect in prostate cancer cells; in particular, PCSK6 siRNA-induced apoptosis of prostate cancer cells, with consequent antitumoral effect, is mediated through the ER stress and mitochondrial signaling pathways [57].

It is also now clear that inflammation is the driving force of atherosclerosis [1,2]. In this connection, it has been shown that PCSK6 exacerbates inflammation by regulating the expression or secretion of various cytokines [15,16] and, in turn, PCSK6 gene is increased in SMCs stimulated with pro-inflammatory cytokines [17].

With the aim of investigating in our *in vitro* experimental models whether the up-regulation of PCSK6 might depend on inflammation, cells were incubated with some anti-inflammatory compounds: NS-398 or ASA, both inhibitors of cyclooxygenases [58], or EGCG, a polyphenol with well-known antioxidant and anti-inflammatory properties [59,60]. Of note, ASA has anti-inflammatory and anti-pyretic properties; moreover, it has anti-thrombotic property thanks to its ability to inhibit platelet aggregation preventing thrombus formation [61,62]. Using these anti-inflammatory molecules, the enzymatic activity of PCSK6 induced by the used lipid oxidation products was clearly prevented, in particular after cell incubation with ASA, pointing out that inflammation plays a key role in stimulating the activity of the pro-protein convertase PCSK6 (Fig 6,7).

In this study, we investigated the role of an oxysterol mixture on the activity of PCSK6 instead of analyzing the contribution of each of the cholesterol oxidized compounds, since various oxysterols accumulate in the atheroma. However, we cannot exclude that some of them could contribute more to stimulate the activity of PCSK6 and consequently to the release of MMP-9 by vascular cells. In a previous work we have indeed shown that, among the oxysterols of the mixture, 27-hydroxycholesterol and the 7α-hydroxycholesterol as well as 25-hydroxycholesterol were the more effective oxysterols on MMP-9 expression [28]. In contrast to the well documented cytotoxic effect of the oxysterols 7-ketocholesterol and 7β-hydroxycholesterol [63], their action on MMP-9 up-regulation was not significantly increased [28].

5. Conclusion

The results obtained underline that both oxysterols and HNE, which significantly accumulate in the atheroma, are able to induce PCSK6 levels and its enzymatic activity, and that up-regulation of PCSK6 might depend on inflammation. In addition, PCSK6 might contribute to the fibrous cap degradation through activation of MMP-9, a key enzyme in matrix remodeling and plaque destabilization. Although further investigations are needed, the involvement of PCSK6 in atherosclerosis could represent the discovery of a novel protein differentially regulated in atherosclerotic plaques and a candidate target for future drug development to prevent atherosclerotic plaque progression and instability.

Author contribution

GT, ES, SG, BS, and SG contributed to the conception of the work, collection and analysis of data; GP and PG contributed to supervise the work; GL supervised all works and wrote the manuscript.

All authors have approved the final version of the article.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] R. Ross, Atherosclerosis- an inflammatory disease, N. Engl. J. Med. 340 (1999) 115–126.

[2] P. Libby, Y. Okamoto, V.Z. Rocha, E. Folco, Inflammation in atherosclerosis: transition from theory to practice, Circ. J. 74 (2010) 213–220.

[3] R. Virmani, A.P. Burke, A. Farb, F.D. Kolodgie, Pathology of the vulnerable plaque, J. Am. Coll. Cardiol. 47 (2006) C13–C18.

[4] P. Libby, The molecular mechanisms of the thrombotic complications of atherosclerosis, J. Intern. Med. 263 (2008) 517–527.

[5] P. Libby, M. Aikawa, Stabilization of atherosclerotic plaques: New mechanisms and clinical targets, Nat. Med. 8 (2002) 1257–1262.

[6] P. Stawowy, H. Kallisch, N. Borges Pereira Stawowy, D. Stibenz, J.P. Veinot, M. Gräfe, N.G. Seidah, M. Chrétien, E. Fleck, K. Graf, Immunohistochemical localization of subtilisin/kexin-like proprotein convertases in human atherosclerosis, Virchows Arch. 446 (2005) 351–359.

[7] P. Stawowy, Proprotein convertases in atherogenesis, Curr. Opin. Lipidol. 26 (2015) 338-344.

[8] N.A. Taylor, W.J. Van De Ven, J.W. Creemers, Curbing activation: proprotein convertases in homeostasis and pathology, FASEB J. 17 (2003) 1215–1227.

[9] H. Turpeinen, E. Raitoharju, A. Oksanen, N. Oksala, M. Levula, L.-P. Lyytikäinen, O. Järvinen, J.W. Creemers, M. Kähönen, R. Laaksonen, M. Pelto-Huikko, T. Lehtimäki, M. Pesu, Proprotein convertases in human atherosclerotic plaques: The overexpression of FURIN and its substrate cytokines BAFF and APRIL, Atherosclerosis 219 (2011) 799–806.

[10] P. Stawowy, E. Fleck, Proprotein convertases furin and PC5: targeting atherosclerosis and restenosis at multiple levels, J. Mol. Md. (Berl) 83 (2005) 865– 875.

[11] X. Lei, D. Basu, Z. Li, M. Zhang, R.D. Rudic, X.C. Jiang, W. Jin, Hepatic overexpression of the prodomain of furin lessens progression of atherosclerosis and reduces vascular remodeling in response to injury, Atherosclerosis 236 (2014) 121–130.

[12] F. Wang, L. Wang, J. Pan, PACE4 regulates proliferation, migration and invasion in human breast cancer MDA-MB-231 cells, Mol. Med. Rep. 11 (2015) 698–704.

[13] C. Levesque, F. Couture, A. Kwiatkowska, R. Desjardins, B. Guérin, W.A. Neugebauer, R. Day, PACE4 inhibitors and their peptidomimetic analogs block prostate cancer tumor progression through quiescence induction, increased apoptosis and impaired neovascularization, Oncotarget 6 (2015) 3680–3693.

[14] D.E. Bassi, J. Fu, R.L. De Cicco, A.J. Klein-Szanto, Proprotein convertases: "Master switches" in the regulation of tumor growth and progression, Mol. Carcinog. 44 (2005) 151–161.

[15] F. Wang, L. Wang, H. Jiang, X. Chang, J. Pan, Inhibition of PCSK6 may play a protective role in the development of rheumatoid arthritis, J. Rheumatol. 42 (2015) 161–169.

[16] H. Jiang, L. Wang, F. Wang, J. Pan., Proprotein convertase subtilisin/kexin type 6 promotes in vitro proliferation, migration and inflammatory cytokine secretion of synovial fibroblast-like cells from rheumatoid arthritis via nuclear-κB, signal transducer and activator of transcription 3 and extracellular signal regulated 1/2 pathways, Mol. Med. Rep. 16 (2017) 8477–8484.

[17] L. Perisic, E. Hedin, A. Razuvaev, M. Lengquist, C. Osterholm, L. Folkersen, P. Gillgren, G. Paulsson-Berne, F. Ponten, J. Odeberg, U. Hedin, Profiling of atherosclerotic lesions by gene and tissue microarrays reveals PCSK6 as a novel protease in unstable carotid atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 33 (2013) 2432–2443.

[18] U. Rykaczewska, B.E. Suur, S. Röhl, A. Razuvaev, M. Lengquist, M. Sabater-Lleal, S.W. Van Der Laan, C.L. Miller, R.C. Wirka, M. Kronqvist, M. Gonzalez Diez, M. Vesterlund, P. Gillgren, J. Odeberg, J.H. Lindeman, F. Veglia, S.E. Humphries, U. de Faire, D. Baldassarre, E. Tremoli, IMPROVE study group, J. Lehtiö, G.K. Hansson, G. Paulsson-Berne, G. Pasterkamp, T. Quertermous, A. Hamsten, P. Eriksson, U. Hedin, L. Matic, PCSK6 Is a Key Protease in the Control of Smooth Muscle Cell Function in Vascular Remodeling, Circ. Res. 126 (2020) 571–585.

[19] D. Steinberg, J.L. Witztum, Oxidized low-density lipoprotein and atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 2311–2316.

[20] H. Tavori, M. Aviram, S. Khatib, R. Musa, S. Nitecki, A. Hoffman, J. Vaya, Human carotid atherosclerotic plaque increases oxidative state of macrophages and low-density lipoproteins, whereas paraoxonase 1 (PON1) decreases such atherogenic effects, Free Radic. Biol. Med. 46

(2009) 607–615.

[21] H. Yehuda, A. Szuchman-Sapir, S. Khatib, R. Musa, S. Tamir, Human atherosclerotic plaque lipid extract promotes expression of proinflammatory factors in human monocytes and macrophage-like cells, Atherosclerosis 218 (2011) 339–343.

[22] S. Gargiulo, P. Gamba, G. Testa, G. Leonarduzzi, G. Poli, The role of oxysterols in vascular ageing, J. Physiol. (London) 594 (2016) 2095–2113.

[23] S. Gargiulo, G. Testa, P. Gamba, E. Staurenghi, G. Poli, G. Leonarduzzi, Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization, Free Radic. Biol. Med. 111 (2017) 140-150.

[24] A. Anderson, A. Campo, E. Fulton, A. Corwin, W.G. Jerome 3rd, M. S O'Connor, 7-Ketocholesterol in disease and aging, Redox Biol. 29 (2020) 101380.

[25] A. Zarrouk, A. Vejux, J. Mackrill, Y. O'Callaghan, M. Hammami, N. O'Brien, G. Lizard, Involvement of oxysterols in age-related diseases and ageing processes, Ageing Res. Rev. 18 (2014) 148-162.

[26] A. Zmyslowski, A. Szterk, Current Knowledge on the mechanism of atherosclerosis and pro-atherosclerotic properties of oxysterols, Lipids Health Dis. 16 (2017) 188.

[27] A. Nègre-Salvayre, S. Garoby-Salom, A. Swiader, M. Rouahi, M. Pucelle, R. Salvayre, Proatherogenic effects of 4-hydroxynonenal, Free Radic. Biol. Med. 111 (2017) 127–139.

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

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

[30] S. Gargiulo, D. Rossin, G. Testa, P. Gamba, E. Staurenghi, F. Biasi, G. Poli, G. Leonarduzzi, Up-regulation of COX-2 and mPGES-1 by 27-hydroxycholesterol and 4-hydroxynonenal: A crucial role in atherosclerotic plaque instability, Free Radic. Biol. Med. 129 (2018) 354–363.

[31] G. Leonarduzzi, G. Poli, B. Sottero, F. Biasi, Activation of the mitochondrial pathway of apoptosis by oxysterols, Front. Biosci. 12 (2007) 791–799.

[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.

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

[34] H. Esterbauer, R.J. Schaur, H. Zollner, Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes, Free Radic. Biol. Med. 11 (1991) 81–128

[35] M.M. Pirvulescu, A.M. Gan, D. Stan, V. Simion, M. Calin, E. Butoi, I. Manduteanu, Subendothelial resistin enhances monocyte transmigration in a co-culture of human endothelial and smooth muscle cells by mechanisms involving fractalkine, MCP-1 and activation of TLR4 and Gi/o proteins signaling, Int. J. Biochem. Cell. Biol. 50 (2014) 29–37.

[36] A. Tsuji, K. Sakurai, E. Kiyokage, T. Yamazaki, S. Koide, K. Toida, K. Ishimura, Y. Matsuda, Secretory proprotein convertases PACE4 and PC6A are heparin-binding proteins which are localized in the extracellular matrix. Potential role of PACE4 in the activation of proproteins in the extracellular matrix, Biochim. Biophys. Acta. 1645 (1) (2003) 95–104.

[37] A.C. Newby, Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture, Physiol. Rev. 85 (2005) 1–31.

[38] A.C. Newby, Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 2108–2114.

[39] F. Cipollone, M.L. Fazia, COX-2 and atherosclerosis, J. Cardiovasc. Pharmacol. 47 (Suppl 1) (2006) S26–36.

[40] S. Mitra, A. Deshmukh, R. Sachdeva, J. Lu, J.L. Mehta, Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy, Am. J. Med. Sci. 342 (2) (2011) 135–142.

[41] A.J. Brown, W. Jessup, Oxysterols and atherosclerosis, Atherosclerosis 142 (1) (1999) 1-28.

[42] G. Leonarduzzi, B. Sottero, G. Poli, Oxidized products of cholesterol: dietary and metabolic origin, and proatherosclerotic effects (review), J. Nutr. Biochem. 13 (12) (2002) 700–710.

[43] C. Helmschrodt, S. Becker, J. Schröter, M. Hecht, G. Aust, J. Thiery, U. Ceglarek, Fast LC-MS/MS analysis of free oxysterols derived from reactive oxygen species in human plasma and carotid plaque, Clin. Chim. Acta. 425 (2013) 3–8.

[44] A. Vejux, G. Lizard, Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis, Mol. Aspects Med. 30 (2009) 153-170.

[45] S.J. Chapple, X. Cheng, G.E. Mann, Effects of 4-hydroxynonenal on vascular endothelial and smooth muscle cell redox signaling and function in health and disease, Redox Biol. 1 (2013) 319–331.

[46] A. Dominguez-Rodriguez, P. Abreu-Gonzalez, A. de la Rosa, M. Vargas, J. Ferrer, M. Garcia, Role of endogenous interleukin-10 production and lipid peroxidation in patients with acute myocardial infarction treated with primary percutaneous transluminal coronary angioplasty,

interleukin-10 and primary angioplasty, Int. J. Cardiol. 99 (1) (2005) 77–81.

[47] Z.S. Galis, G.K. Sukhova, M.W. Lark, P. Libby, Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques, J. Clin. Invest. 94 (1994) 2493–2503.

[48] B. Zeng, A. Prasan, K.C. Fung, V. Solanki, D. Bruce, S.B. Freedman, D. Brieger, Elevated circulating levels of matrix metalloproteinase-9 and -2 in patients with symptomatic coronary artery disease, Intern. Med. J. 35 (6) (2005) 331–335.

[49] M. Nold, A. Goede, W. Eberhardt, J. Pfeilschifter, H. Mühl, IL-18 initiates release of matrix metalloproteinase-9 from peripheral blood mononuclear cells without affecting tissue inhibitor of matrix metalloproteinases-1: suppression by TNF alpha blockage and modulation by IL-10, Naunyn Schmiedebergs Arch. Pharmacol. 367 (1) (2003) 68–75.

[50] S.J. Lee, C.E. Kim, M.R. Yun, K.W. Seo, H.M. Park, J.W. Yun, H.K. Shin, S.S. Bae, C.D. Kim, 4-Hydroxynonenal enhances MMP-9 production in murine macrophages via 5-lipoxygenase-mediated activation of ERK and p38 MAPK, Toxicol. Appl. Pharmacol. 242 (2) (2010) 191–198.

[51] P. Stawowy, H. Kallisch, J.P. Veinot, A. Kilimnik, W. Prichett, S. Goetze, N.G. Seidah, M. Chrétien, E. Fleck, K. Graf, Endoproteolytic activation of alpha(v) integrin by proprotein convertase PC5 is required for vascular smooth muscle cell adhesion to vitronectin and integrin-dependent signaling, Circulation 109 (2004) 770–776.

[52] P. Stawowy, H. Meyborg, D. Stibenz, N. Borges Pereira Stawowy, M. Roser, U. Thanabalasingam, J.P. Veinot, M. Chrétien, N.G. Seidah, E. Fleck, K. Graf, Furin-like proprotein convertases are central regulators of the membrane type matrix metalloproteinase-pro-matrix metalloproteinase-2 proteolytic cascade in atherosclerosis, Circulation 111 (2005) 2820–2827.

[53] W. Jin, I.V. Fuki, N.G. Seidah, S. Benjannet, J.M. Glick, D.J. Rader, Proprotein convertases [corrected] are responsible for proteolysis and inactivation of endothelial lipase, J. Biol. Chem. 280 (44) (2005) 36551–36559.

[54] S.-F. Yang, C.R.-H. Chou, S.-J. Lin, S.-Y. Li, P.-H. Huang, Serum PCSK6 and corin levels are not associated with cardiovascular outcomes in patients undergoing coronary angiography, Plos One 2019, 14, e0226129.

[55] J.P. Li, X.B. Wang, C.Z. Chen, X. Xu, X.M. Hong, X.P. Xu, W. Gao, Y. Huo, The association between paired basic amino acid cleaving enzyme 4 gene haplotype and diastolic blood pressure, Chin. Med. J. (Engl). 117 (3) (2004) 382–388.

[56] I. Iatan, Z. Dastani, R. Do, D. Weissglas-Volkov, I. Ruel, J.C. Lee, A. Huertas-Vazquez, M.R. Taskinen, A. Prat, N.G. Seidah, P. Pajukanta, J.C. Engert, J. Genest, Genetic variation at the

proprotein convertase subtilisin/kexin type 5 gene modulates high-density lipoprotein cholesterol levels, Circ Cardiovasc Genet. 2 (5) (2009) 467–475.

[57] Y. Zhiyong, S. Bin Sun, H. Quan, Y. Jingmin, M. Dawei, L. Jianye, S. Haibo, G. Heqing, PACE4 regulates apoptosis in human prostate cancer cells via endoplasmic reticulum stress and mitochondrial signaling pathways, Drug Des. Devel. Ther. 9 (2015) 5911-5923.

[58] Y.C. Chi, C.L. Shi, M. Zhou, Y. Liu, G. Zhang, S.A. Hou, Selective cyclooxygenase-2 inhibitor NS-398 attenuates myocardial fibrosis in mice after myocardial infarction via Snail signaling pathway, Eur. Rev. Med. Pharmacol. Sci. 21(2017) 5805–5812.

[59] T. Ohishi, S. Goto, P. Monira, M. Isemura, Y. Nakamura, Anti-inflammatory Action of Green Tea, Antiinflamm. Antiallergy Agents Med. Chem. 15 (2) (2016) 74–90.

[60] C. Chu, J. Deng, Y. Man, Y. Qu, Green Tea Extracts Epigallocatechin-3-gallate for Different Treatments, Biomed. Res. Int. 2017 (2017) 5615647.

[61] A. Aboonabi, I. Singh, The effectiveness of antioxidant therapy in aspirin resistance, diabetes population for prevention of thrombosis, Biomed. Pharmacother. 83 (2016) 277–282.

[62] J. Paris, P. Fournau, M. Granero, C. Viens, Anti-thrombotic effect of very low doses of acetyl salicylic acid in rats, Thromb. Res. 29 (1983) 313–321.

[63] A. Vejux, D. Abed-Vieillard, K. Hajji, A. Zarrouk, J.J. Mackrill, S. Ghosh, T. Nury, A. Yammine, M. Zaibi, W. Mihoubi, H. Bouchab, B. Nasser, Y. Grosjean, G. Lizard, 7-Ketocholesterol and 7β-hydroxycholesterol: In vitro and animal models used to characterize their activities and to identify molecules preventing their toxicity, Biochem. Pharmacol. 173 (2020) 113648.

Figure legends

Fig. 1. Up-regulation of PCSK6 levels by the oxysterol mixture and HNE in U937 cells. U937 cells were incubated with 20 µM oxysterol mixture or with 5 µM HNE up to 8 or 48 h. (A, C) PCSK6 expression levels were quantified by real-time RT-PCR. Data, normalized to corresponding β-actin levels, are means ± SD of three experiments, expressed as fold induction vs control. ***$p<0.001$, **$p<0.01$, and *$p<0.05$ vs control. (B, D) PCSK6 protein levels were analyzed by Western blotting after protein immunoprecipitation. One blot representative of three experiments is shown. The histograms represent the mean values ± SD of three experiments; PCSK6 levels are expressed as a percentage of the control value. ***$p<0.001$ and **$p<0.01$ vs control.

Fig. 2. Up-regulation of PCSK6 levels by oxysterol mixture and HNE in HUVECs. HUVECs were

incubated with 20 µM oxysterol mixture or with 5 µM HNE up to 8 or 48 h. (A, C) PCSK6 expression levels were quantified by real-time RT-PCR. Data, normalized to corresponding β-actin levels, are means ± SD of three experiments, expressed as fold induction vs control. ***p<0.001, **p<0.01, and *p<0.05 vs control. (B, D) PCSK6 protein levels were analyzed by Western blotting after protein immunoprecipitation. One blot representative of three experiments is shown. The histograms represent the mean values ± SD of three experiments; PCSK6 levels are expressed as a percentage of the control value. ***p<0.001 and **p<0.01 vs control.

Fig. 3. PCSK6 activity induced by oxysterol mixture and HNE in U937 cells and HUVECs. The enzymatic activity of PCSK6 was analyzed in U937 cells (A, B) and HUVECs (C, D) after incubation with 20 µM oxysterol mixture or with 5 µM HNE up to 48 h, following cell incubation with the fluorogenic substrate Boc-RVRR-AMC and measurement of AMC release by spectrofluorimetry (see Methods). The histograms represent mean values ± SD of three experiments. PCSK6 activity is expressed as a percentage of the control value. ***p<0.001, **p<0.01, and *p<0.05 vs control.

Fig. 4. PCSK6 activity in co-culture of U937 cells and HUVECs. U937 cells and HUVECs were co-cultured and incubated up to 48 h with 20 µM oxysterol mixture or with 5 µM HNE. After separation of the two cell types, PCSK6 enzymatic activity was measured by spectrofluorimetry in U937 cells (A, B) and HUVECs (C, D). The histograms represent mean values ± SD of three experiments. PCSK6 activity is expressed as a percentage of the control value. ***p<0.001 and **p<0.01 vs control.

Fig. 5. Down-regulation of PCSK6 gene prevents MMP-9 activity induced by oxysterols and HNE. MMP-9 gelatinolytic activity was measured by gelatin zymography after incubation of U937 cells with 20 µM oxysterol mixture (A) or with 5 µM HNE (B). Untreated cells are taken as control. (A) Cells were incubated up to 48 h; one zymogram of three experiments is shown. The histograms represent mean values ± SD of three experiments. ***p<0.001, **p<0.01, and *p<0.05 vs control. After 24 h of reverse transfection with PCSK6 siRNA, U937 cells were incubated with 20 µM oxysterol mixture. The histograms represent mean values ± SD of three experiments. ***p<0.001 vs control; ###p<0.001 vs cells treated with oxysterol mixture. (B) Cells were incubated up to 48 h; one zymogram of three experiments is shown. The histograms represent mean values ± SD of three experiments. ***p<0.001 and **p<0.01 vs control. After 24 h of reverse transfection with PCSK6 siRNA, U937 cells were incubated with 5 µM HNE. The histograms represent mean values

± SD of three experiments. *p<0.05 vs control; ##p<0.01 vs cells treated with HNE.

Fig. 6. Modulation of PCSK6 enzymatic activity induced by oxysterols and HNE in U937 cells and HUVECs. Enzymatic activity of PCSK6 was evaluated by spectrofluorimetry in U937 cells (A,B) and HUVECs (C,D) incubated for 24 h with 20 μM oxysterol mixture or with 5 μM HNE; some cells were pre-incubated with 25 μM NS-398, a selective inhibitor of COX-2, or with 10 μM EGCG. The histograms represent mean values ± SD of three experiments. **p<0.01, *p<0.05 vs control (untreated cells); ##p<0.01, #p<0.05 vs cells treated with oxysterol mixture or HNE.

Fig. 7. Modulation of PCSK6 enzymatic activity by acetylsalicylic acid. Enzymatic activity of PCSK6 was evaluated by spectrofluorimetry in U937 cells (A) and HUVECs (B) co-treated for 24 h with acetylsalicylic acid (ASA), at a final concentration of 0.5 mM, 1 mM, or 5 mM, and with oxysterol mixture (20 μM) or with HNE (5 μM). The histograms represent mean values ± SD of three experiments. **p<0.01 vs control (untreated cells); ##p<0.01 vs cells treated with oxysterol mixture or HNE.

Supplementary Fig. 1. Validation of PCSK6 silencing efficiency. U937 cells (**A)** and HUVECs cells (**B**) were transfected for 24 h with PCSK6 or scrambled siRNA. Transient PCSK6 gene knockdown was evaluated by real-time RT-PCR. Data, normalized to corresponding β-actin levels, are means ± SD of three different experiments, expressed as fold induction vs control. ******p<0.01 vs control.

Graphical Abstract: A hypothetical scheme of the effects of an oxysterol mixture or HNE on PCSK6 up-regulation, triggering plaque instability.

Figure 1

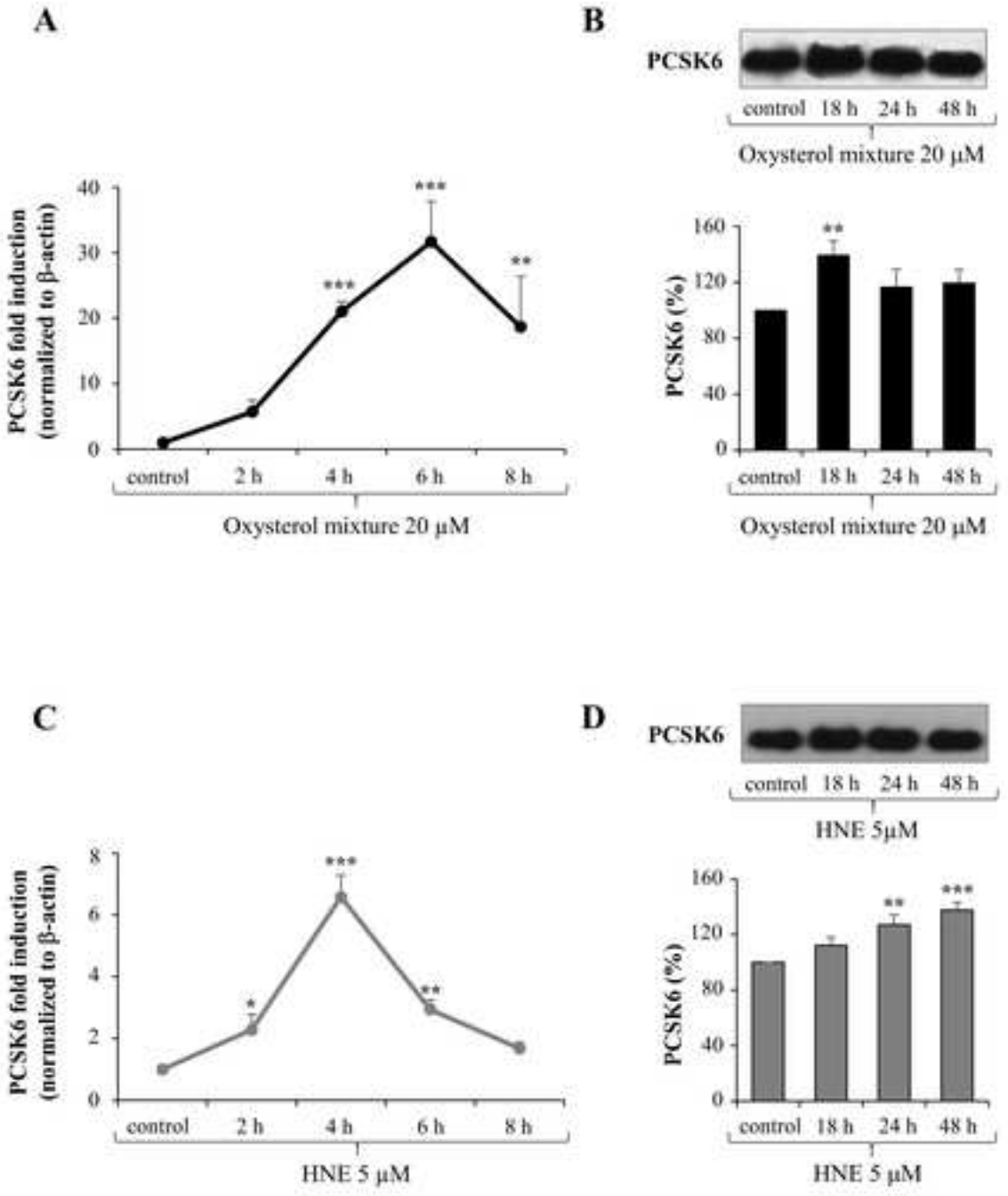

Figure 2

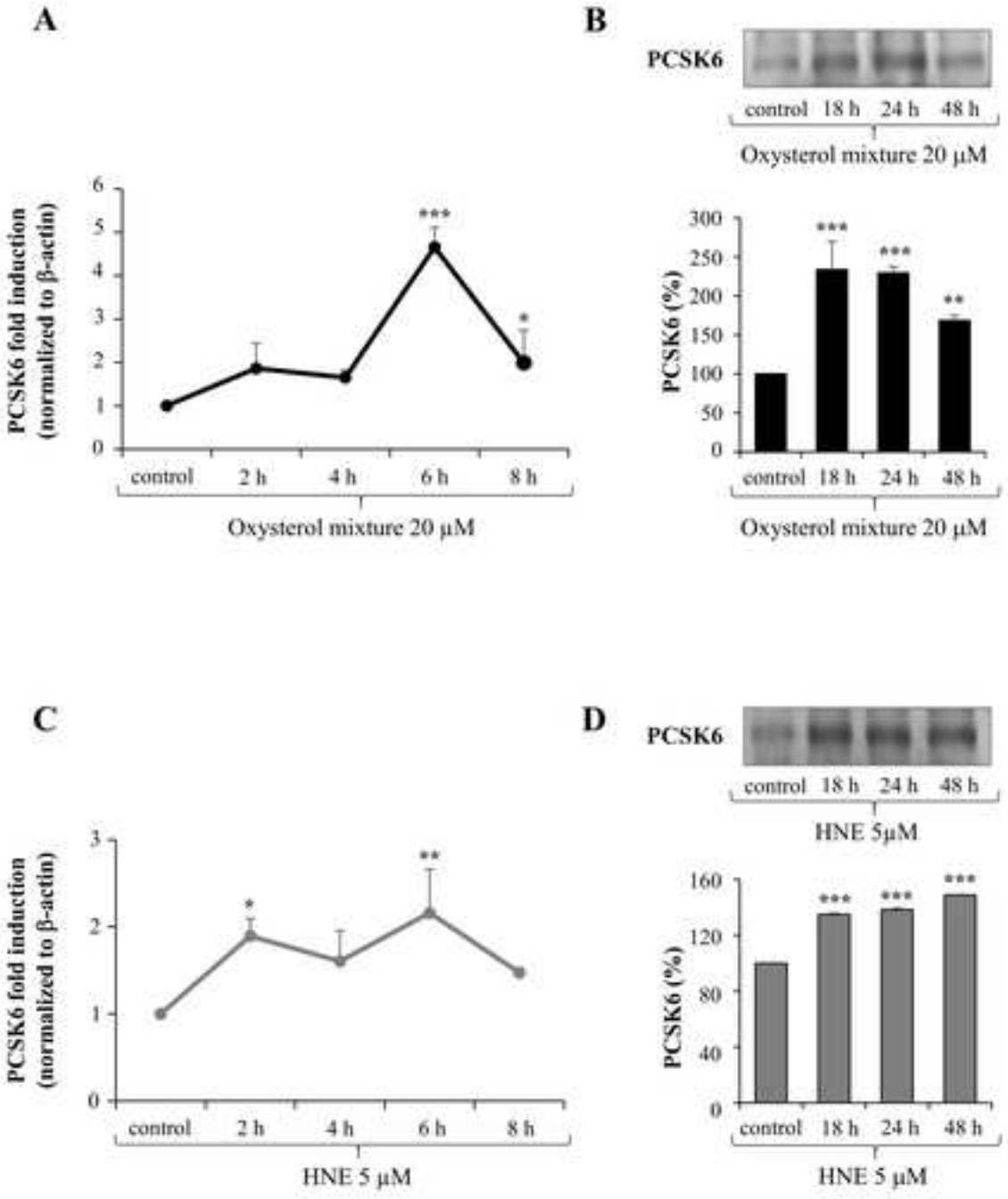

Figure 3

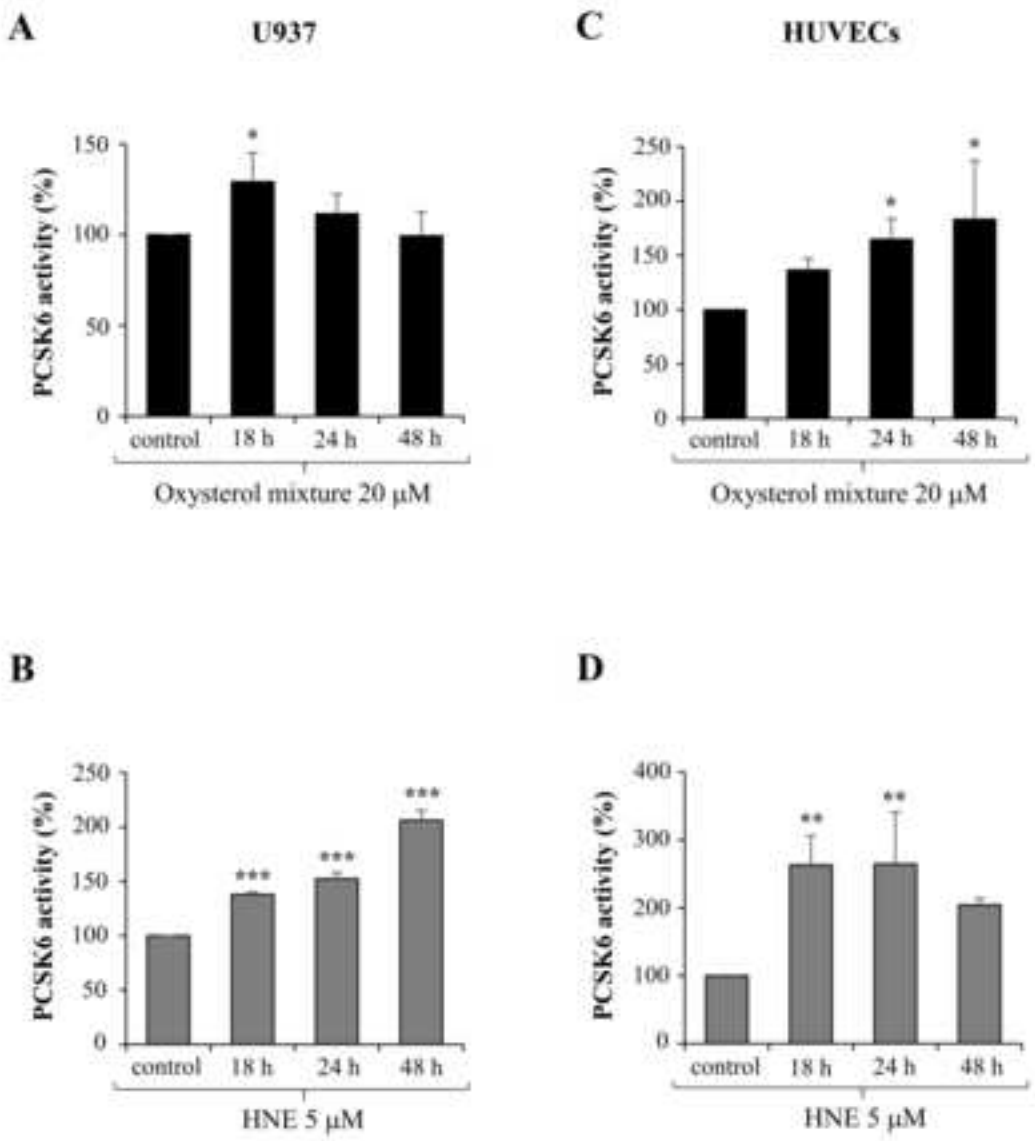

Figure 4

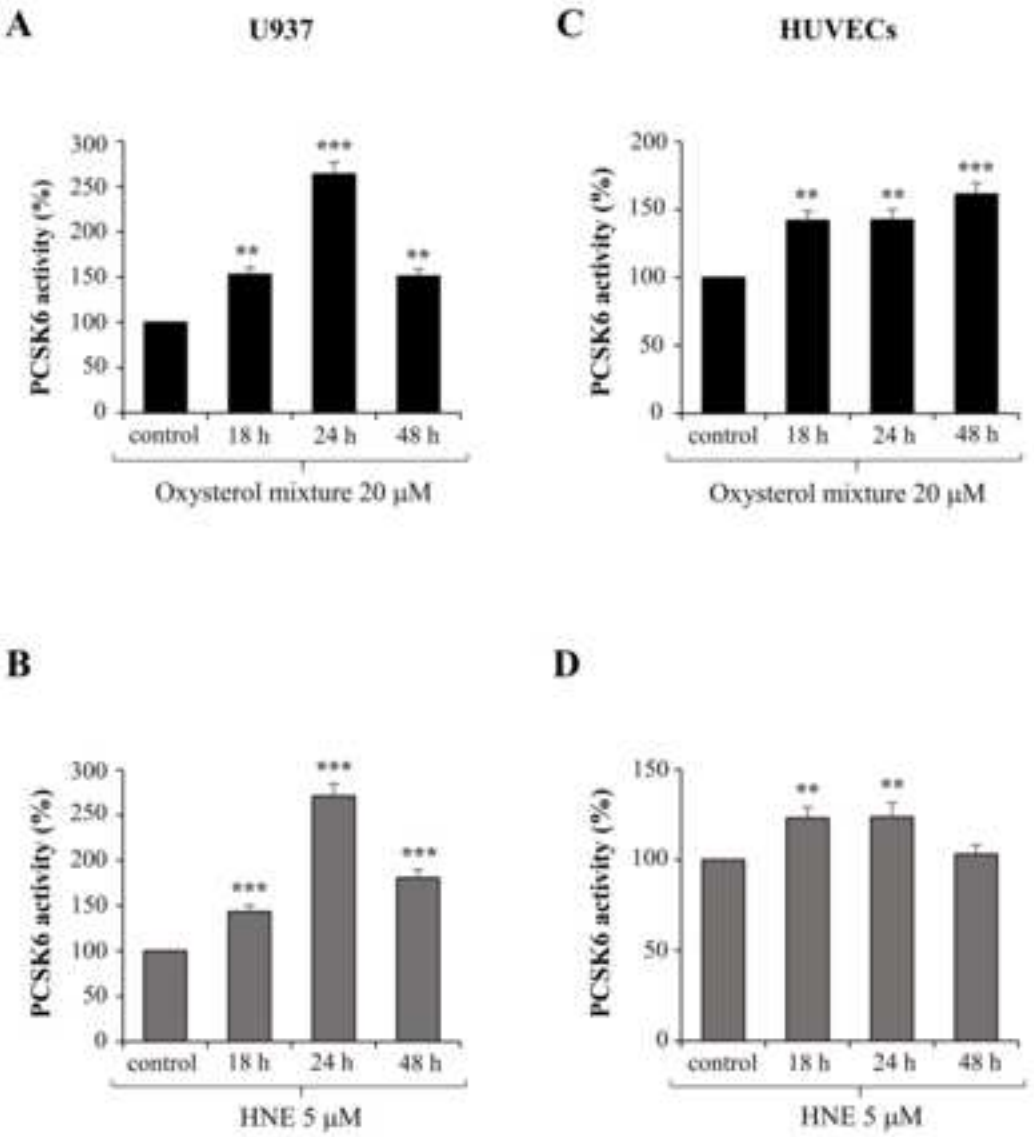

Figure 5

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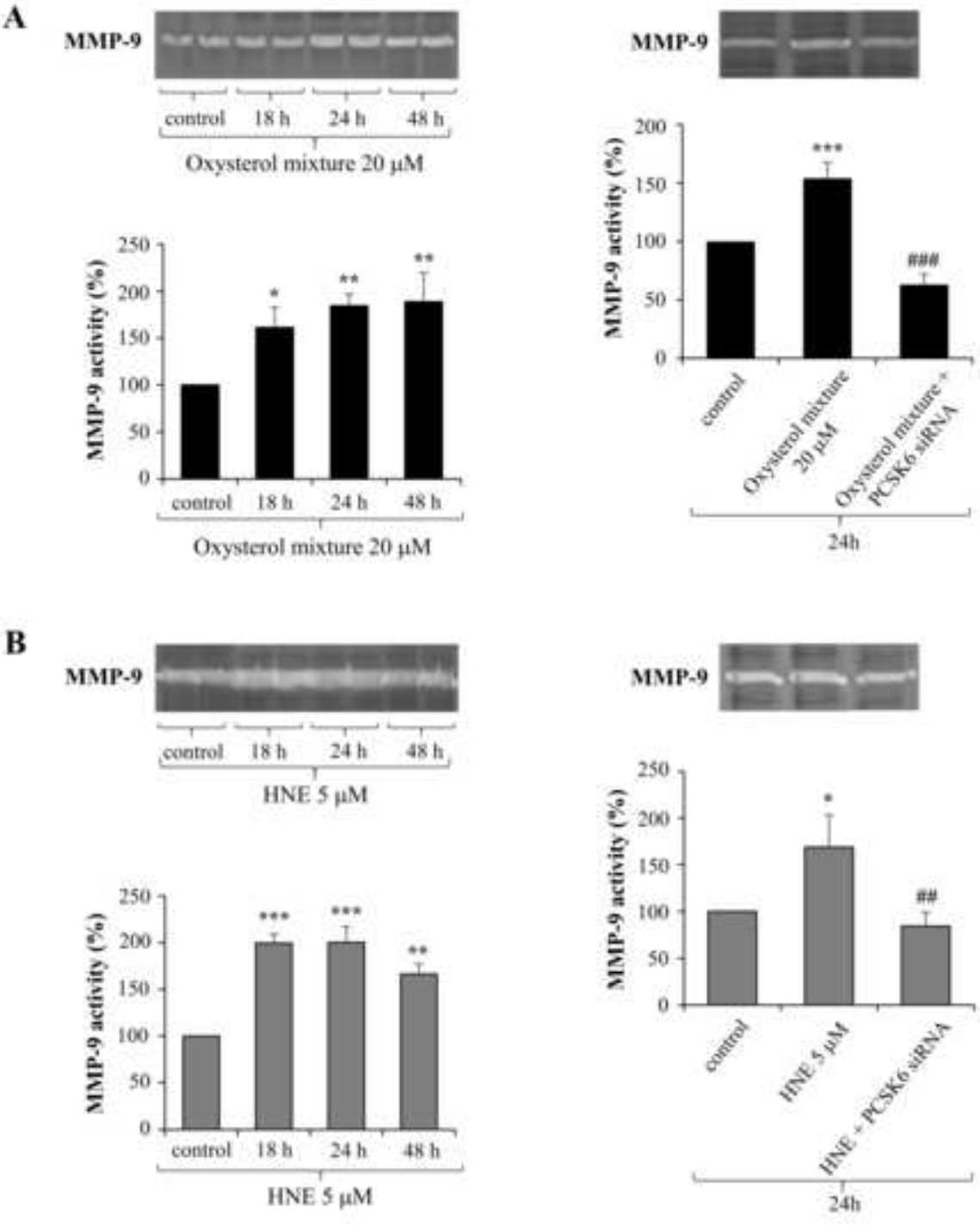

Figure 6

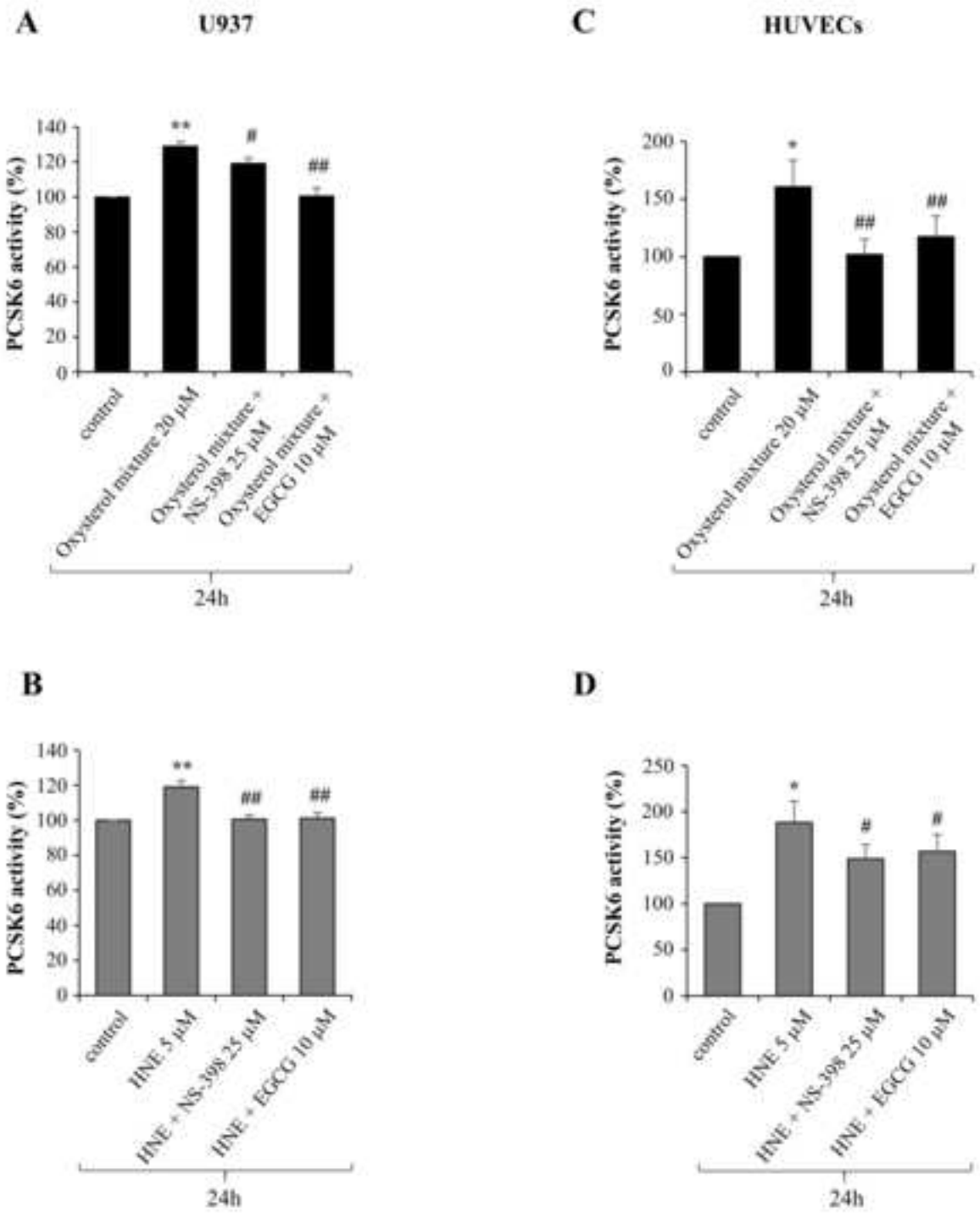

Figure 7