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THE ROLE OF OXYSTEROLS IN ENDOTHELIAL DYSFUNCTION

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Supervisor
Prof.ssa FRANCESCA LUCHETTI

Ph.D Student
Dott.ssa NASONI MARIA GEMMA

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INDEX

ABSTRACT

The endothelium is a thin monolayer of cells that covers the lumen of blood vessels from the heart to the smallest capillaries, creating a barrier between blood and the surrounding tissue.

Currently, it is considered a real organ with the main function of modulating the vessel tone in response to humoral, nervous and mechanical stimuli. Furthermore, it plays an active role in vascular homeostasis mediating blood fluidity, regulation of inflammation, immune response and neovascularization.

Endothelial dysfunction (ED) is a pathological condition characterized by reduced vasodilation, pro-oxidative state and procoagulant activity. ED has been identified as the main event in the pathogenesis of macrovascular disease including atherosclerosis.

The oxidation of cholesterol in the low-density lipoprotein (LDL) produces a class of compounds called oxysterols. They are able to regulate many biological processes and exert several biochemical effects of potential pathophysiological relevance. Oxysterols are found in human LDL and in atherosclerotic plaques playing a key role in atheroma formation, thanks to their pro-inflammatory, pro-oxidant, pro-apoptotic, and fibrogenic properties.

In the last decade, numerous studies have investigated the mechanisms and *in vivo* relevance of endoplasmic reticulum (ER) stress in the atherosclerosis process. Indeed, a close examination of ER stress and UPR pathways has demonstrated many links to major inflammatory and stress signaling networks.

To this purpose, the present research is aimed at investigating the role of secosterol-B (SEC-B), a new oxysterol found in atherosclerotic plaques, as a possible inducer of endothelial dysfunction and damage affecting ER structure and function in endothelial cells.

In detail, our results highlight that SEC-B is able to induce ER stress, as revealed by significant expansion and change of structure. At low doses cells try to cope with this stress by activating autophagy and the ubiquitin proteasome system in the attempt to restore ER function. However, at higher dose cell apoptosis occurs in a pathway that involves early phosphorylation of $eIF2\alpha$ and NF-kB activation, suggesting that the adaptive program fails and the cell activates the apoptotic program.

Subsequently, to investigate the mechanism involved in SEC-B-induced ER stress, the study was focused on endothelial cell activation, evaluating inflammatory response induced by SEC-B. Our finding demonstrate that SEC-B is able to activate human microvascular endothelial cells by improving oxidative stress and affecting interleukin 1-β (IL1-β) and

intracellular adhesion molecule-1 (ICAM-1) expression. In addition, our data suggest an involvement of SEC-B in TWEAK/Fn14 pathway regulation. The proinflammatory state leads to impairment of HMEC-1 function characterized by decrease of cell viability, down-modulation of intracellular NO bioavailability and enhancement in U937 recruitment on activated HMEC-1 cells.

The results obtained in this study point to an important role of SEC-B in proinflammatory activation and ER damage of human endothelial cells that lead to ED. These findings provide additional insight about the role of oxysterols in ED and its potential implication in atherosclerotic disease.

INTRODUCTION

The vascular endothelium: a functional organ

The vascular endothelium is the thin monolayer of endothelial cells lining the lumen of all blood vessels (Fig.1). However, it would be reductive to define the vascular endothelium simply as a set of cells that act as a barrier between the blood and surrounding tissues. Currently, it is considered a real organ with the main function of modulating the vessel tone and the entity of the blood flow in response to humoral, nervous and mechanical stimuli. Under physiological conditions, the endothelium plays an active role in vascular homeostasis by releasing bioactive factors which allow it to adapt functionally and structurally to changes in the microenvironment (Verma S, et al. 2003).
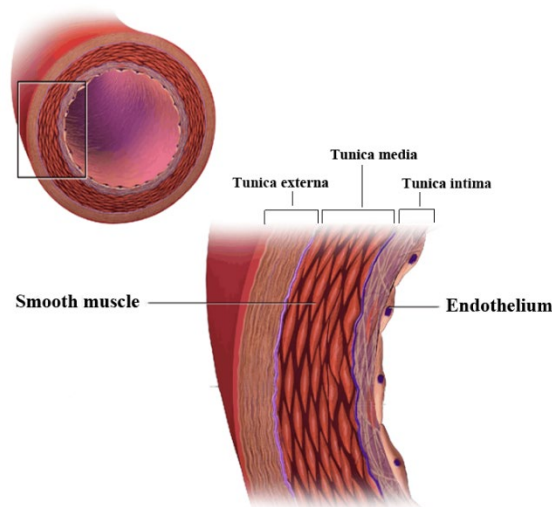

Fig.1. Structure of the vascular wall. The endothelium is interposed in the tunica intima between smooth muscle cells and the lumen of blood vessel. (Adapted from Wikipedia).

The strategic location allows the endothelium to carry out its functions by continuously interacting with the hematopoietic and vascular smooth muscle cells. It's able to regulate vascular tone modulating smooth muscle cells and producing vasodilatation or vasoconstriction. It also contributes to mitogenesis, angiogenesis, vascular permeability, balance of blood fluidity, regulation of inflammation, leukocyte recruitment and platelet

activation (Fig.2). The endothelial function is strictly dependent on continuity anatomy of the cellular monolayer both from its functional integrity (Furchgott RF, et al. 1980).
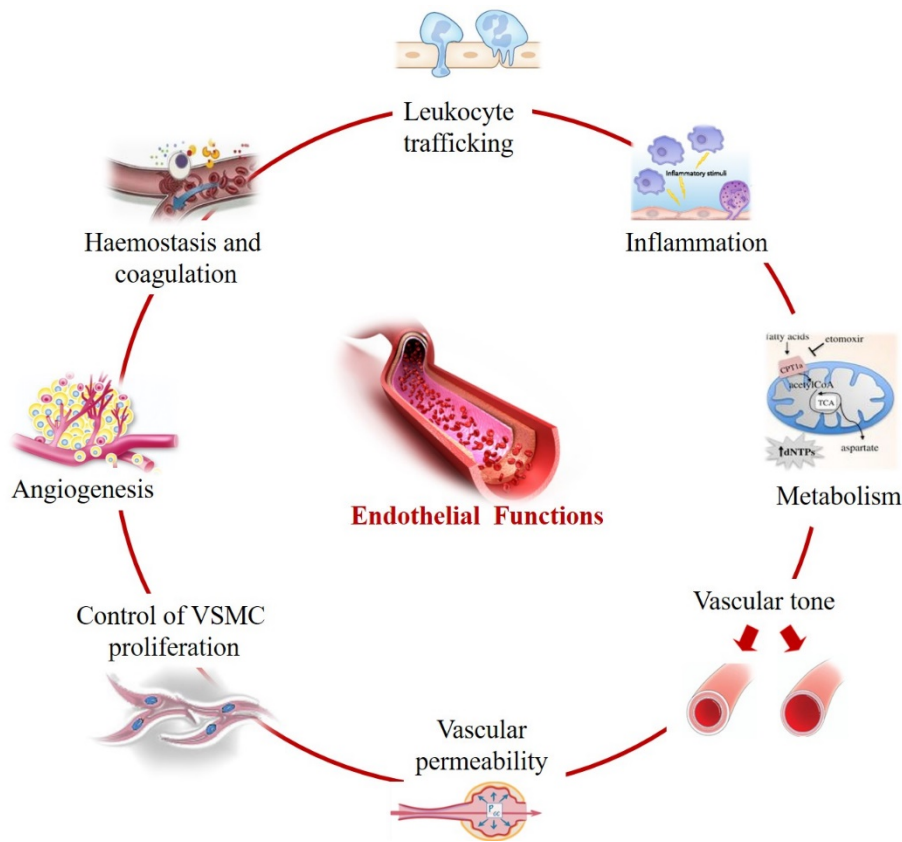

Fig.2. Multiple functions of endothelium.

Nitric oxide (NO) is one of the most important substances produced by the endothelium and it represents the most powerful vasodilator (other synthesized vasodilator substances from endothelial cells are prostacyclin and some endothelial hyperpolarizing factors).

NO is generated by the enzyme nitric oxide synthase (NOS), which converts the amino acid L-arginine to NO. There are three isoforms of NOS: neuronal isoform (nNOS) which acts as a neuronal messenger that regulates synaptic neurotransmitter release, inducible isoform (iNOS) which is only expressed in cells that have been exposed to inflammatory stimuli that activate the macrophages, and endothelial NOS or constitutive NOS (eNOS), which produces NO in the vasculature. In the present discussion will focus on eNOS, given its function of blood vessel regulation.

In unstimulated endothelial cells, eNOS is bound to the caveolin-1 protein and located within caveolae.

eNOS can be activated in calcium-dependent or- independent ways (Fig.3). When intracellular levels of Ca^{2+} increase, it binds to calmodulin (CaM) and leads to the activation of calmodulin-binding domain of eNOS to produce NO (Zhao Y, et al. 2015). It is important to highlight that this mechanism is dependent on the levels of intracellular Ca^{2+} in the endoplasmic reticulum (ER). The short-term increase in NO is dependent on the intracellular Ca^{2+} but once this decreases additional mechanisms are activated to regulate NO production (Sandoo A, et al. 2010). Another mechanism is the phosphorylation of eNOS via protein kinases, such as protein kinase A and B. The responses to hemodynamic shear stress and hormones are mediated mainly through this calcium-independent pathway (Zhao Y, et al. 2015). It is important to emphasize that, the contribution of Ca^{2+} and eNOS phosphorylation to NO production is dependent on the duration of the shear stress.

Once synthesized, NO diffuses across the endothelial cell into the adjacent smooth muscle, decreases smooth muscle tension and also helps to restore Ca^{2+} homeostasis (Fig.4A). The mechanisms described are continuously active to maintain basal vasodilator tone (Sandoo A, et al. 2010).
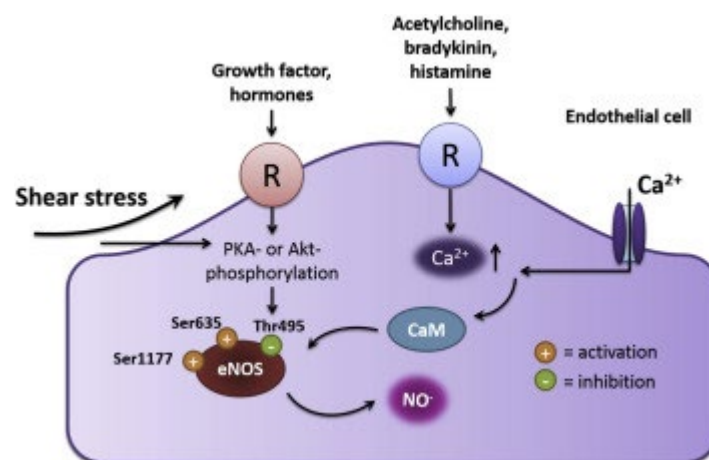
Fig.3. Endothelial nitric oxide synthase (eNOS) can be activated in calcium-dependent or- independent ways *(Zhao Y, et al. 2015).*

In addition, NO functions as an inhibitor of cell growth and inflammation, with antiplatelet effects. Indeed, NO is involved in preventing leukocyte activation and adhesion to the endothelium reducing the inflammatory response (Sandoo A, et al. 2010).

The endothelium also synthesizes vasoconstrictor substances, the most powerful of which is endothelin-1 (ET-1). ET-1 is widely expressed in endothelial cells, vascular smooth muscles,

central nervous system (CNS) and reproductive tissues (Unic A, et al. 2011). It is formed from the precursor molecule called pre-pro-endothelin (pre-pro-ET) which hydrolyzes to "big ET" or pro-endothelin (pro-ET). Finally, endothelin converting enzymes (ECE) converts pro-endothelin to mature endothelin (Unic A, et al. 2011).

This peptide can be synthesized under several stimuli such as local factors (thrombin, interleukin-1, growth factors), hormones and physical factors (hypoxia, hypovolemia). On the other hand, among the inhibitors of ET-1 synthesis there are NO, prostaglandin E2 (PGE2), epidermal growth factor (EGF) and strong shear stress (i.e. increased blood flow) (Kisanuki YY et al. 2010).

ET-1 may be stored in endothelial cells, which are the main biological source of ET-1 (Kisanuki YY et al. 2010). Under appropriate stimuli, ET-1 can be secreted in two ways, via constitutive and regulatory secretion. Constitutive secretion is the most common type of release and involves gene transcription and increased stability of mRNA for pre-pro-ET. In this way the secretion is very slow (Unic A, et al. 2011). On the contrary the regulatory mode leads to a rapid secretion of ET. It acts on ET_A receptors (present only on smooth muscle cells) and ET_B (present both on the endothelium than on smooth muscle cells). On smooth muscle cells it causes vasoconstriction, inflammation and smooth muscle cells proliferation. Activation of ET_B receptors on the endothelium causes vasodilatation by inducing the release of NO which acts as a negative feedback inhibiting the production of ET-1 (Fig.4B) (Sandoo A, et al. 2010).

Accordingly, in response to mechanical stimuli, such as shear stress and hormonal stimuli, endothelial cells can release several factors that regulate vasomotor function, trigger inflammatory processes and affect hemostasis. Among other endothelial production molecules, we find vasoconstrictors factors such as angiotensin II (Ang II), thromboxane A2 and reactive oxygen species (ROS), and inflammation modulators, represented not only by NO, but also by adhesion molecules of the endothelium, such as ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular adhesion molecule-1) and E-selectin and inflammatory mediators such interleukin-1 (IL-1) and chemokines (Endemann DH, et al. 2004).
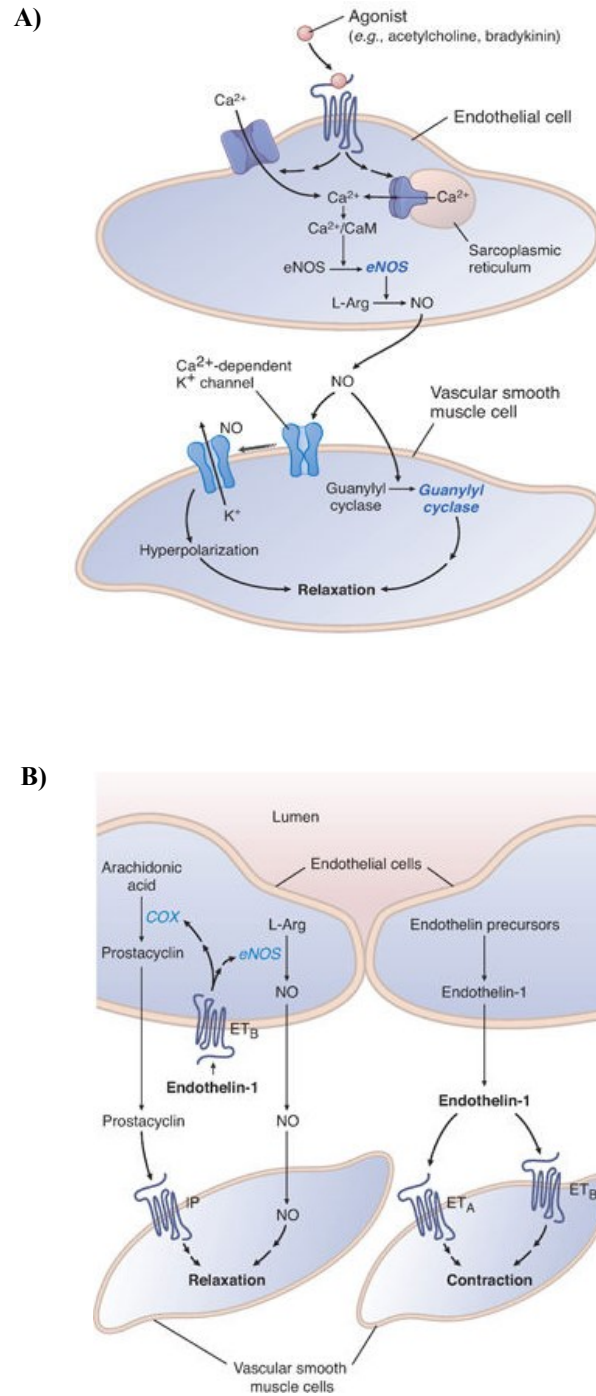
Fig.4. A) Endothelial regulation of nitric oxide-mediated vascular smooth muscle relaxation. **B)** Effect of endothelin-1 on the blood vessel wall. (*Basicmedical Key. Pharmacology of Vascular Tone*)

Patterns of change in endothelial function: cell activation and dysfunction

The endothelium plays a complex role to maintain vascular homeostasis. When endothelial cells lose their ability to maintain this tricky balance, there are important implications for the regulation of hemostasis and thrombosis, local vascular tone and redox balance that can lead acute and chronic inflammatory reactions within the arterial wall.

In general, a pattern of various non-adaptive alterations in functional phenotype, it's defined as "endothelial cell dysfunction". The term "endothelial dysfunction" (ED) identifies a pathological condition mainly characterized by a reduction of NO bioavailability and an increase of oxidative stress, correlated by a reduced vasodilation, prothrombic state and by proinflammatory activity. Impairment of endothelial function is attributed to high oxidative stress and inflammation both processes that lead to abnormal NO production, the key mechanism responsible for ED. An excess of ROS results from impaired NO synthase, decreased L-arginine uptake and a reduced superoxide dismutase (SOD). In particular, ROS are known to quench NO with formation of peroxynitrite, which is a cytotoxic oxidant that bring to degradation of the eNOS cofactor tetrahydrobiopterin (BH4) leading to "uncoupling" of eNOS, as a falling in NO production (Endemann DH, et al. 2004). Moreover, an increase activity of Nox (NADPH oxidases), the main source of reactive oxygen and nitrogen species (RONS) in the vascular wall, is associate to eNOS uncoupling leading to ED (Marchio P, et al. 2019).

Oxidative excess is linked to a proinflammatory state of the vessel wall. Increase in intracellular concentration of ROS activates the nuclear factor NF-kB, leading to an increase in the transcription of pro-inflammatory genes and the expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) (Heitzer T, et al. 2001). This condition is defined as "endothelial cell activation", characterized by expression of cell-surface adhesion molecules and typically induced by proinflammatory cytokines, such as TNF-α ,IL-6, IFN-γ, IL-1) or growth factors (e.g. angiotensin II), that facilitate the recruitment and attachment of circulating leukocytes to the vessel wall. Endothelial cell activation by proinflammatory cytokines could clearly lead ED by inhibiting eNOS expression and decreasing NO bioavailability through the induction of ROS (Liao JK. 2013).

Endothelial cells are continually target for a wide stimuli which can induce a cell response in three different ways: 1) stimulation (or "Type I Activation") with a rapid response of endothelial cells, typically do not result in sustained morphological or functional changes

(Pober JS, et al. 1990); 2) activation (or "Type II Activation") with a slower protein synthesis-dependent response initiated by inflammatory cytokine and the activation of pleiotropic transcription factors, such as NF-kB and inducible endothelial-leukocyte adhesion molecule (Pober JS, et al. 1990); 3) injury, a process occurring by strong stimuli that give a "proinflammatory endothelial phenotype" and which can induce either endothelial necrosis or endothelial dysfunction (Djordjević VB, et al. 2011).

However, Liao et al. have been postulated that NO prevented endothelial cell activation through inhibition of NF-κB, limiting therefore leukocyte adhesion to the vessel wall. Accordingly, ED by loss of NO, can leads to increased endothelial cell activation via NF-κB (Liao JK. 2013).

Taken together, both ED and endothelial cell activation lead to increase vasoconstriction, vascular permeability, SMC proliferation, platelet aggregation, and leukocyte adhesion (Fig.5). These two processes are defined as the key events in the development of the main cardiovascular (CV) pathologies, such as atherosclerosis disease. Indeed, it is well-known that CV risk factors such as hypercholesterolemia, hyperglycemia, obesity, hypertension, smoking and aging adversely affect the endothelium, promoting vascular inflammation and ED (Widmer RJ, et al. 2014).
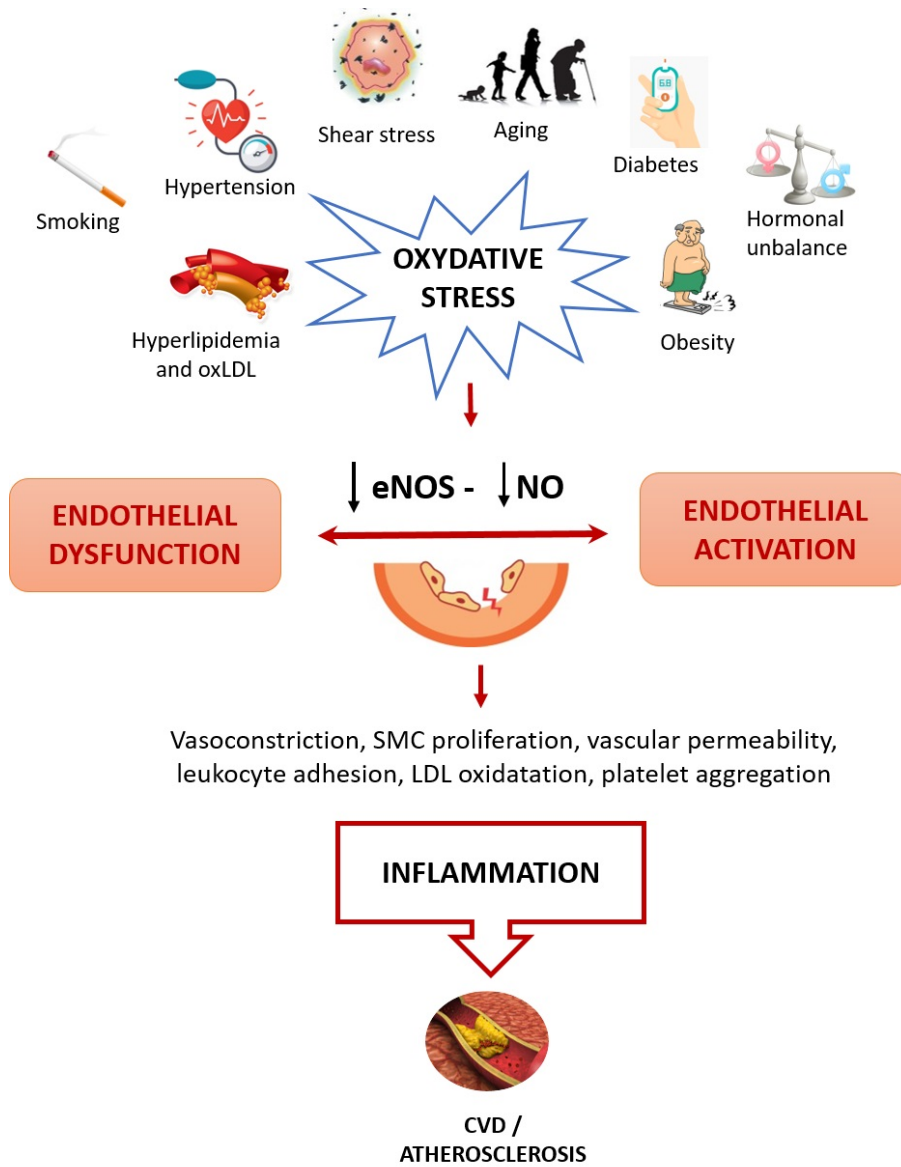
Fig.5. Overview of causes, mechanisms and consequences of endothelial cell activation and dysfunction.

ED in the atherosclerosis disease: implication of oxLDL and ER stress

ED has been identified as the early event in the pathogenesis of atherosclerosis. Atherosclerosis is a progressive inflammatory systemic disease affecting mainly the wall of large and medium arteries, at sites prone to turbulent or oscillatory shear stress (Marchio P, et al.2019). As described above, endothelial cell dysfunction in response to specific pathophysiological stimuli (e.g., hypercholesterolemia and other dyslipidemias, diabetes, hypertension, aging) has important local manifestations within the walls of arteries (Gimbrone MA Jr, et al. 2016). In this condition ROS production has been found to be improved in the vessel walls. Increased ROS species leads to the oxidation of native low-density lipoprotein (LDL) to oxidized LDL (oxLDL), which plays an important role in atherogenesis (Kattoor AJ, et al. 2019). The endothelial atherogenic phenotype has an increased permeability to circulating LDL, and their accumulation in the tunica intima is the first step in plaque formation (Fig.6A) (Marchio P, et al.2019). LDL are exposed to oxidation and act damaging the endothelium and triggering the inflammatory process (Chistiakov DA, et al. 2015; Hansson GK, 2005). Activated endothelial cells express cytokines, chemokines, and adhesion molecules (such as MCP-1, ICAM-1, VCAM-1, E-selectin) promoting the rolling of monocytes to the atherosclerotic lesion, and inducing the maturation of monocytes into proinflammatory macrophages (Chistiakov DA, et al. 2015; Libby P. 2012).

In pathological condition, endothelial cells upregulate the surface expression of oxLDL receptor-1 (LOX-1), that leads to increased lipid uptake by macrophages in the intima media. The accumulation of foam cells and macrophages within the plaque exacerbate the inflammatory signaling by releasing chemokines and cytokines such as IL-1, IL-6, TNF-α, IFN-γ. In addition, ROS production, growth factors and vascular smooth muscle cell proliferation accelerate the development of atherosclerosis (Fig.6B) (Chistiakov DA, et al. 2017). Specifically, the atheroma plaque is characterized by a necrotic lipid core, which is a result of dead foam cells, inflammatory and immune cells (such as T cells and macrophages), damaged endothelial and smooth muscle cells, detritus, connective tissue and fibrous cap surrounding the plaque (Hansson GK, 2005). Accordingly, in this scenario ED results in a cyclical relationship between oxidative stress and inflammation.

Overall, these complex mechanisms and interactions in the arterial wall indicate that alteration of endothelial cell phenotype in to atherogenic phenotype represents the starting point of atherosclerosis which leads to endothelial cell activation and destabilization of atherosclerotic plaque (Closa D, et al. 2004).

A)
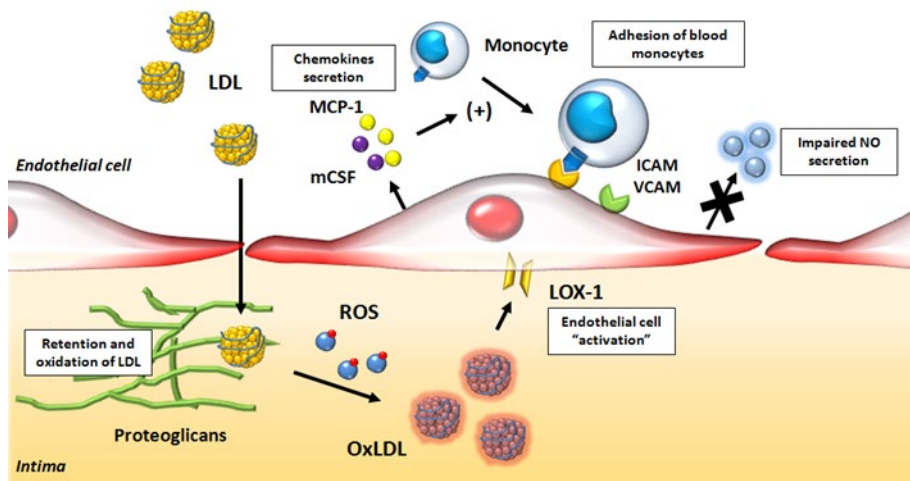
B)
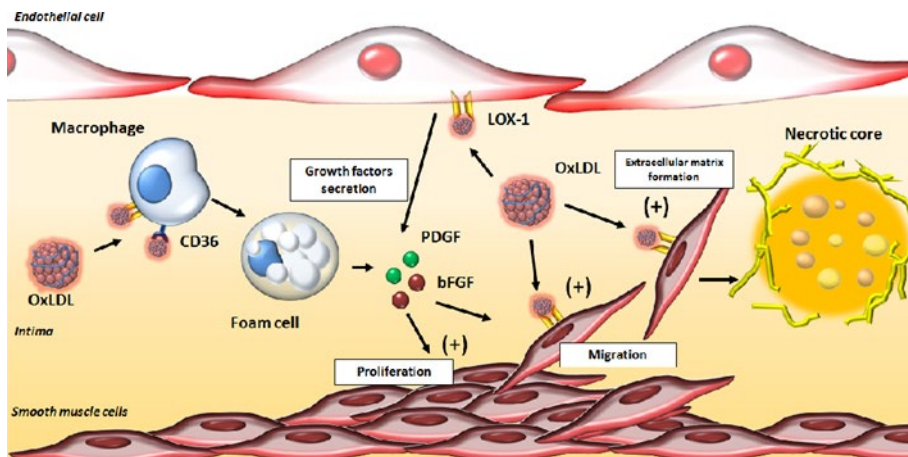
Fig. 6. A) Role of OxLDL in ED. **B)** Role of OxLDL in foam cell formation and in VSMC proliferation (Leiva E, et al. 2015)

Furthermore, it is a relatively recent area of investigation the contribution of ER stress to endothelial disfunction. Accumulating evidence support a role for ER stress in all stages of atherosclerotic lesion, however, it was not clear how ER stress may contribute to disease progression (Huang A, et al. 2018). ER is a eukaryotic organelle with important regulatory functions, such as protein modification, folding, lipid synthesis and transport and regulation of cytosolic Ca^{2+} levels. An impairment in ER function leads to a condition known as ER stress.

Risk factors for CV disease have been linked to increased ER stress in the arterial wall of mouse model systems and unfolded protein response (UPR) activation have been observed in diseased arteries from human patients (Myoishi M, et al. 2007). The UPR is signaled by three ER stress sensors: double-stranded RNA-activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6α (ATF6α) (Fig.7). The activation of UPR aims at reducing protein load on the ER and improving its homeostasis. ER homeostasis occurs through translational and transcriptional changes. Activated PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) leading to a rapid inhibition of protein synthesis (Han J, et al. 2016). Although the UPR was originally identified to maintain the protein homeostasis in the ER, a number of studies suggest that the UPR plays essential roles in maintaining the metabolic and lipid homeostasis (Han J, et al. 2016).

To counterbalance ER stress, the cells can activate two major degradation pathways: the ubiquitin–proteasome system (UPS) and autophagy. In particular, recent investigations reveal a crucial role for the autophagic pathway in inflammation. Interestingly, in a condition of ER stress, ER is able to trigger autophagy through the IRE-1–JNK/p38 and the transcription factor 4 (ATF4) pathways in mammalian cells (Yorimitsu T, et al. 2006).

The ER stress can be associate to autophagy in different ways: the induction of macroautophagy; the formation of autophagosomes at the ER level; and the autophagy of ER itself, a phenomenon also called reticulophagy. This type of autophagy is characterized by the presence of membrane derived from the ER within the autophagosomes. This selective ER sequestration helps cells to counterbalances ER expansion (Bernales S, et al. 2006).

Nevertheless, a chronic activation of UPR is documented as a state of ER stress related with induction of pro-inflammatory and pro-apoptotic pathways (Marciniak SJ, et al. 2006).

Atherosclerotic plaque provides conditions that can trigger ER stress and UPR, including inflammation and presence of oxLDL (Hansson GK., 2005). Indeed, it was demonstrated that oxLDLs induce ER-stress and apoptosis in cultured cells of human arterial wall (Ivanova EA, et al. 2016). Moreover, it was reported that the release of ROS along with inflammatory cytokines could then initiate ER stress at the site of plaque (Frostegard J, 2013). The evidence that ER stress takes part in the apoptotic effect of ox-LDL through the IRE1α/c-Jun N-terminal kinase pathway was demonstrated by the protective effect exerted by specific small interfering RNAs and c-Jun N-terminal kinase inhibitors (Sanson M, et al. 2009; Luchetti F, et al. 2017).

Despite a well-established link among inflammation, oxLDL and ER stress, the role played by specific products of lipid oxidation into this interaction is still to be defined.
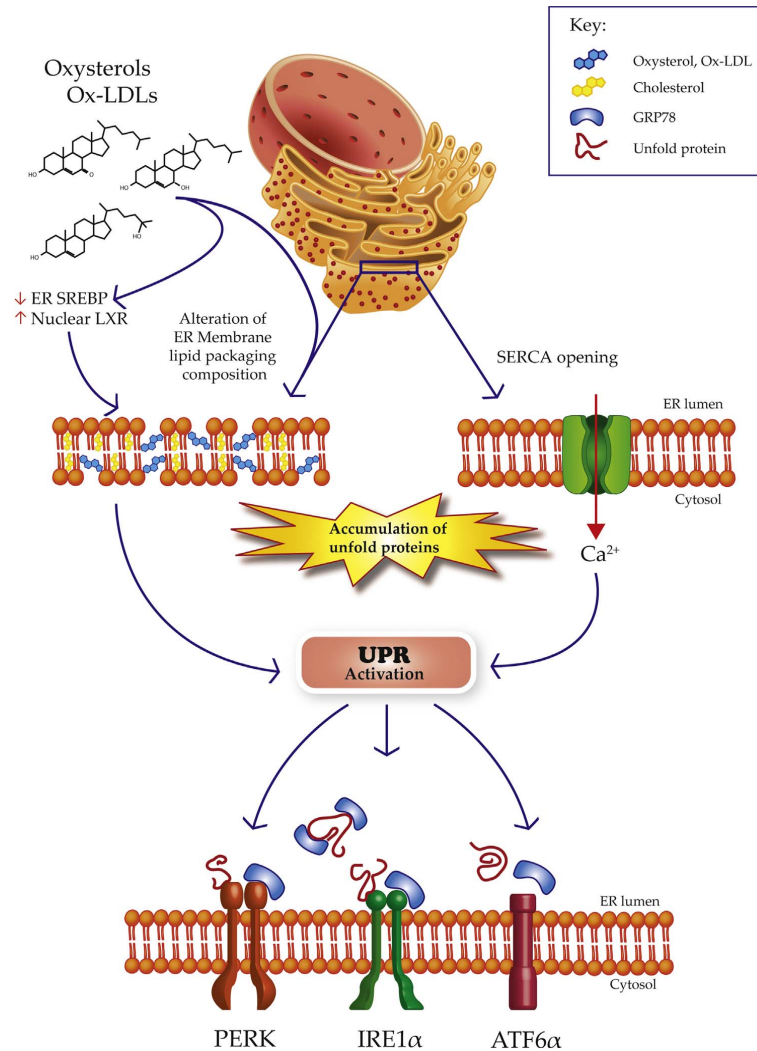
Fig.7. Schematic representation of activation of the UPR signaling pathway in ER stress. (Luchetti F, et al. 2017).

Oxysterols: a current insight about oxidation products of cholesterol

Among the reactive molecules carried in oxLDL, there are oxysterols. Oxysterols are a family of 27-carbon cholesterol oxidation derivatives that have, in the last decades, attracted the attention of many investigators. They are present in tissues and fluids, which are a natural component of the diet or arise enzymatically or non-enzymatically in the human organism.

They represent a numerous group of sterols that are similar in structure to cholesterol but contain an additional hydroxy, ketone or epoxide group in the sterol nucleus and / or a hydroxyl group in the side chain of their molecules. They are generated *in vivo* non-enzymatically following the lipid oxidation process in biological membranes and lipoproteins in a pathway termed "autoxidation", or by enzymatic reaction during cholesterol catabolism via cytochrome P450 (CYP) family and cholesterol-25-hydroxylase (CH25H) (Brown AJ, et al. 2009). In particular, autoxidation of cholesterol can occur through reactions initiated by free radical species, such as those arising from the superoxide/hydrogen peroxide/hydroxyl radical system and by non-radical highly reactive oxygen species such as singlet oxygen, HOCl, and ozone (Iuliano L, 2011).

Oxysterols can be classified in two main categories: those oxygenated in the sterol ring, mainly in position 7 (eg: 7α / β-hydroperoxychesterol, 7-ketocolesterol and 7α / β-hydroxycholesterol) and those oxygenated in the side chain (ex: 24-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol). Generally, oxygenated oxysterols in the ring are prone to form non-enzymatically, while oxygenated sterols in the side chain have an enzymatic origin. However, 25-hydroxycholesterol and 7α-hydroxycholesterol can be produced either enzymatically or non-enzymatically (Fig.8) (Kloudova A, et al. 2017).
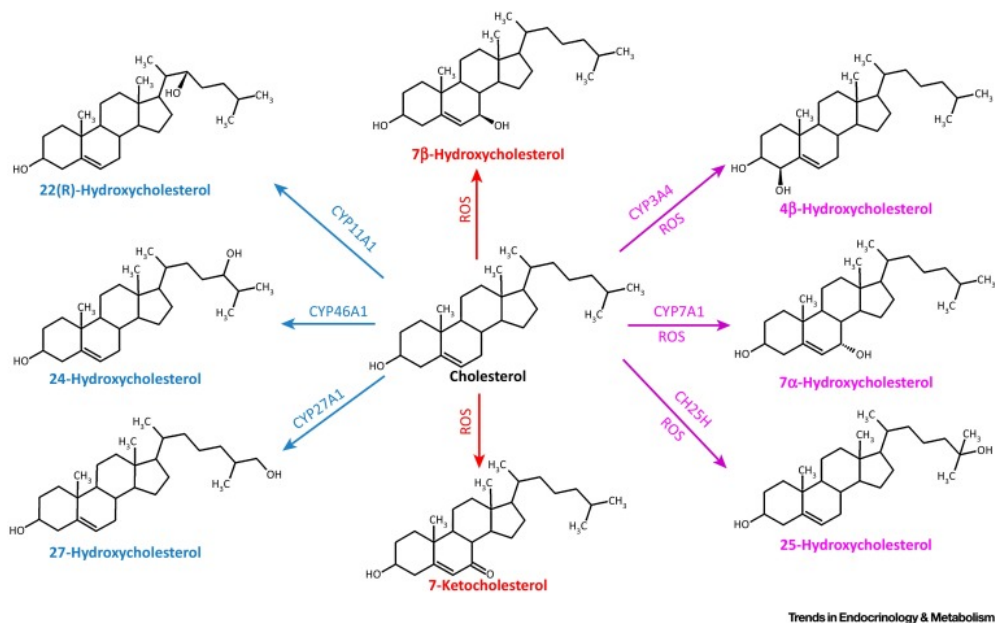
Fig.8. Structures of common oxysterols (Kloudova A, et al. 2017).

In addition, oxysterols can derive from the diet, in particular in foods rich in cholesterol (i.e clarified butter, milk, eggs, dried or preserved fish). Indeed, the products containing cholesterol are usually prone to oxidation, especially products subjected to storage, radiation or high temperatures, due to ROS exposure. The most commonly detected oxysterols in processed foods are 7-oxygenated sterols (i.e. 7-ketocolesterol, 7β-hydroxycholesterol) and 5,6-oxygenated sterols (Leonarduzzi G. et al., 2002). These oxysterols are mainly absorbed as esters in the upper intestinal tract and transported in the plasma by the chylomicrons first and then by the other lipoprotein classes.

Hight levels of oxysterols are present in LDL and to a lesser extent in high density lipoproteins (HDL) and in very low-density lipoproteins (VLDL).

Oxysterols show a biochemical reactivity that is one or even two orders of magnitude higher than cholesterol. They are absorbed more quickly in the intestine, have a faster plasma release and are rapidly taken up by tissues. Furthermore, unlike cholesterol, oxysterols are able to permeate through lipophilic membranes (Wielkoszyński T, et al. 2018; Krut LH, et al., 1997).

In normal condition, they are maintained in low concentrations in the organism and mediate several important physiological functions. They participate in the regulation of cholesterol metabolism, in the biosynthesis of lipids and sterols, as substrates for bile acid formation, in

the transport of sterol between tissues. Oxysterols influence several signaling pathways, membrane fluidity, and the activity of some membrane proteins (Kloudova A, et al. 2017). However, they also show several adverse effects such as cytotoxicity, carcinogenicity, atherotoxicity, angiotoxicity and apoptotic and pro-inflammatory properties (Fig.9) (Vejux A, et al. 2009, Larsson DA, et al. 2006; Ares MPS, et al. 2000; Larsson H, et. 2007).
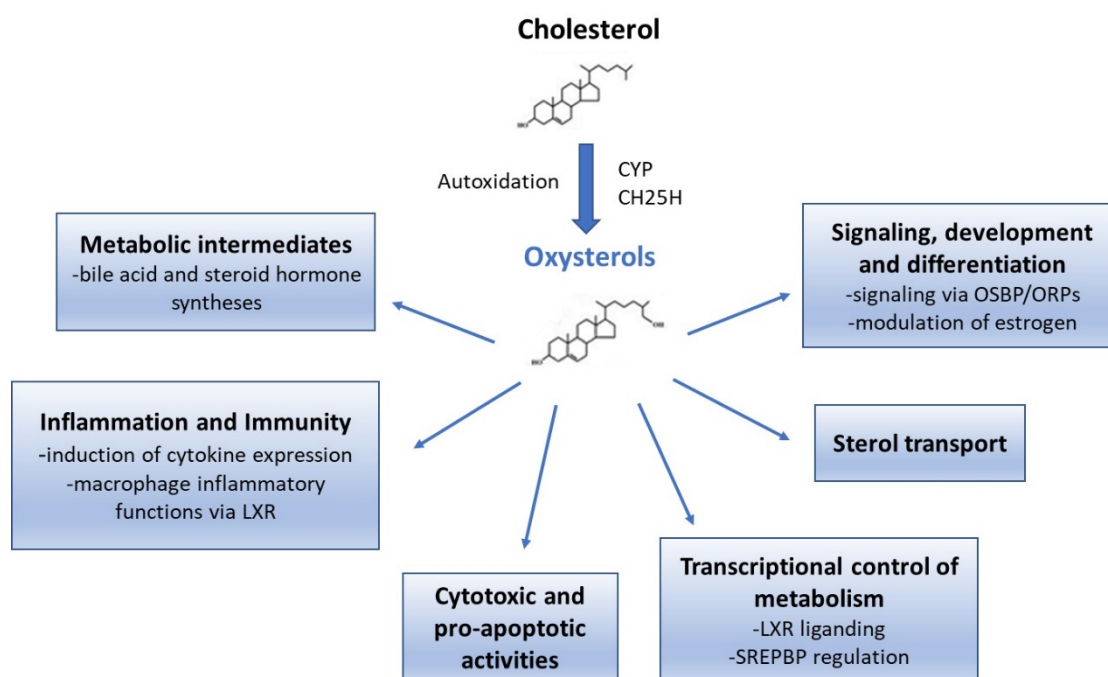

Fig.9. A schematic presentation summarizing the major functions of oxysterols.
(Adapt from Olkkonen VM, et al. 2012)

A pathological level of oxysterols is been associated to several chronic inflammatory human diseases such as atherosclerosis, neurodegenerative processes and diabetes (Zmysłowski A, et al. 2017; Sottero B, et al. 2009). Significantly, they are found in atherosclerotic lesions suggesting a crucial role in atherosclerotic development. Several studies were performed in *in vivo* and *in vitro* models, showing atherosclerotic properties of oxysterols (Meynier A, et al. 2002; Umetani M, et al. 2014; Gargiulo S, et al. 2017).

However, due to the different experimental approaches conducted in *in vivo* studies and the great heterogeneity of this compounds, a final conclusion about pro-atherosclerotic effects and their contribution in vascular diseases it's remained to be defined (Zmysłowski A, et al. 2017).

Secosterol-B, a recent oxysterol found in atherosclerotic plaques

In this study has been investigate the role of a recent oxysterol as a possible inducer of ED and its implication in atherosclerosis development.

The oxysterol taken into consideration is an autoxidation product of cholesterol, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde, also known as Secosterol-B (SEC-B). Elevated levels of SEC -B have been detected in human LDL, in atherosclerotic plaques and brain tissues in neurodegenerative diseases, including Alzheimer's disease and Lewy body dementia, suggesting an involvement of SEC-B in several inflammation-related disorders **(**Miyoshi N, et al. 2013).

SEC-B is one of the major ozonolysis product of cholesterol generated *in vivo* through a myeloperoxidase-dependent pathway by cholesterol reaction with ozone to form secosterol-A that its converted to SEC-B by aldolization (Fig.10). However, Wentworth et al. reported that SEC-B can be generated by reaction of cholesterol with other oxidants, such as singlet oxygen (Wentworth AD, et al 2009; Tomono S, et al. 2011).
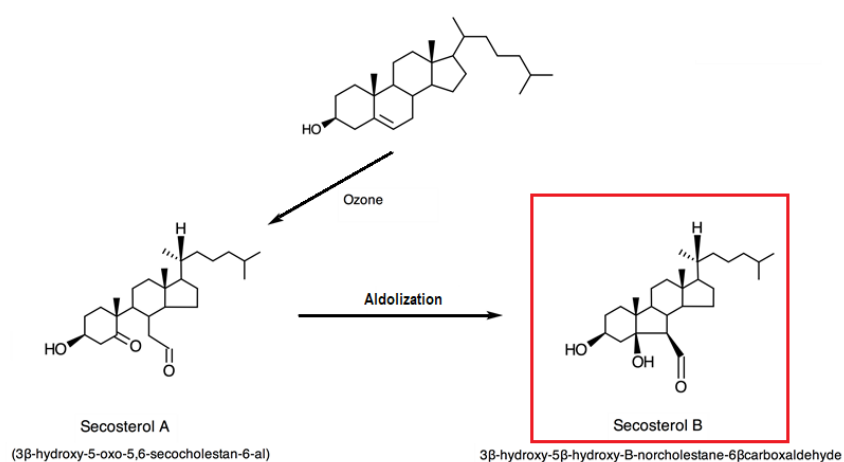
Fig.10. Simplified representation of type II autoxidation of cholesterol mediated by ozone.

Several studies have found that secosterol-A and SEC-B induce cell death in various cultured cells, including human B-lymphocytes (WI-L2), T-lymphocytes (Jurkat), vascular smooth muscle cells (VSMC), abdominal aorta endothelial cells (HAEC), a murine tissue macrophage (J774.1), and an alveolar macrophage cell line (MH-S) (Tomono S, et al.2013). Tomono et al., have demonstrated that the treatment not only with SEC-B but also with its

derivatives (seco-COOH and seco-CH2OH) had strong cytotoxic activities as compared to the other tested cytotoxic oxysterols, including 5β,6β-epoxyCh, 7β-OHCh, 7-KCh, and 25-OHCh (Tomono S, et al. 2013).

Moreover, Wentworth et al. reported that incubation of human LDL with either secosterol-A or SEC-B led to time-dependent changes in the circular dichroism spectra of apoB-100, consistent with an altered secondary structure, and increased atherogenicity, e.g. the secosterol-modified LDL was avidly taken up by macrophage leading to foam cell formation (Wentworth P Jr, et al. 2003; Miyoshi N, et al. 2014).

Accordingly, SEC-B is reported as bioactive molecule but its potential proatherogenic properties and activities raise a number of questions in the context of its role in atherosclerosis. Indeed, the contribution and mechanic involvement of SEC-B in ED still remain unclear.

AIM OF THE STUDY

The research was aimed to investigate the role of SEC-B, a new oxysterol found in atherosclerotic plaques, as a possible inducer of ED.

In detail, the first chapter is the pre-print version (pre-referenced) of the published article (Luchetti F, Crinelli R, Nasoni MG, Cesarini E, Canonico B, Guidi L, Zerbinati C, Magnani M, Papa S, Iuliano L. Secosterol-B affects endoplasmic reticulum structure in endothelial cells. J Steroid Biochem Mol Biol. 2019 Jun; 190:234-241. doi: 10.1016/j.jsbmb.2019.04.014). In this work was described the ability of SEC-B to affect ER structure and function in primary human endothelial cells. ER stress was investigated by analysing the apoptotic and/or autophagic pathways in relationship to the severity of the stress.

The second chapter is the article in preparation with the title: "Secosterol-B induces proinflammatory activation of human microvascular endothelial cells leading to endothelial dysfunction". In this following study, it was investigated the mechanism of cell dysfunction, analysing the proinflammatory activation of endothelial cells exposed to different doses of SEC-B.

Chapter 1

5,6 secosterol aldehyde affects endoplasmic reticulum structure in endothelial cells

Francesca Luchetti [a], Rita Crinelli [a], Maria Gemma Nasoni [a], Erica Cesarini [a], Barbara Canonico [a], Loretta Guidi [a], Chiara Zerbinati [b], Mauro Magnani [a], Stefano Papa [a], Luigi Iuliano [b].

[a] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy.
[b] Department of Medico-Surgical Sciences and Biotechnology, Sapienza University of Rome, Italy.

Corresponding author: Francesca Luchetti, Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino Italy email: francesca.luchetti@uniurb.it

Abstract

Oxysterols, oxidized derivatives of cholesterol found in LDL and atherosclerotic plaques, trigger several biological responses involved in the initiation and progression of atherosclerosis. Endothelial dysfunction, which occurs when vascular homeostasis is altered, plays a key role in the pathogenesis of several metabolic diseases. The contribution of endoplasmic reticulum (ER) stress to endothelial disfunction is a relatively recent area of investigation. There is a well-established link between LDL oxidation and ER stress but the role played by specific products of lipid oxidation into this interaction is still to be defined. The present study shows that 5,6 secosterol aldehyde (SEC), recently identified in the atherosclerotic plaque, is able to induce ER stress in HUVEC cells, as revealed by significant expansion and change of structure.

At low doses, i.e. 1 and 5 μM, cells try to cope with this stress by activating autophagy and the ubiquitin proteasome system in the attempt to restore ER function. However, at higher doses, i.e. 20 μM, cell apoptosis occurs in a pathway that involves early phosphorylation of eIF2α and NF-kB activation, suggesting that the adaptive program fails and the cell activates the apoptotic program. These findings provide additional insight about the role of oxysterols in endothelial dysfunction and its potential involvement in atherosclerotic pathophysiology.

Graphical abstract
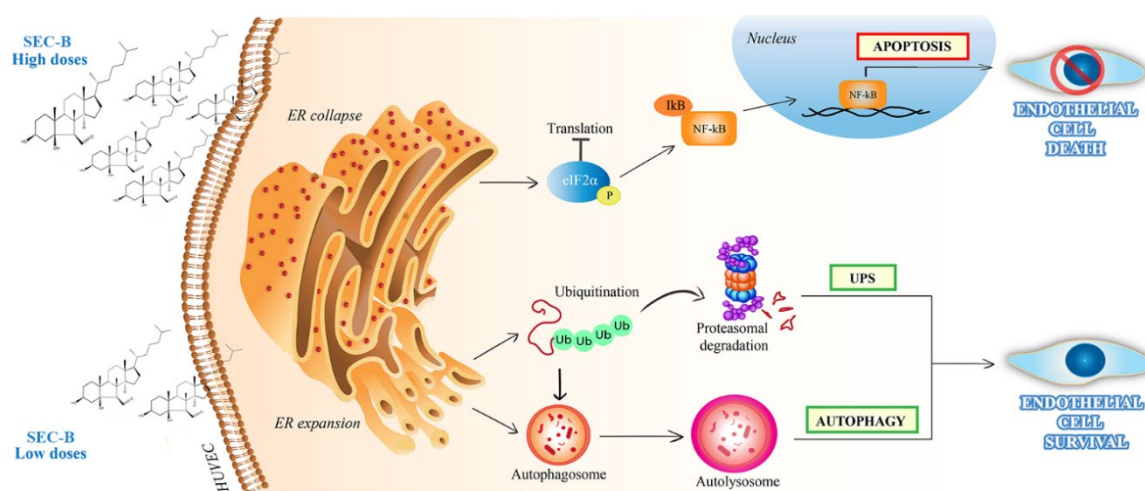
Highlights

- 5,6-secosterol aldehyde (SEC) induces ER stress in HUVEC

- SEC triggers pro-survival signaling through autophagy and UPS

- eIF2α phosphorylation and NF-kB activation involved in the SEC-induced apoptosis

Abbreviations: ANNEX-V, AnnexinV; EC, endothelial cells; eIF2α, translation eukaryotic initiation factor 2; ER, endoplasmic reticulum; FC, flow cytometry; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; ISR, integrated stress response; MDC, monodansylcadaverine; OxLDL, oxidized low-density lipoprotein; PI, propidium iodide; SEC, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde, 5,6 secosterol aldehyde; SDS, Sodium Dodecyl Sulfate; UPR, the unfolded protein response; UPS, ubiquitin/proteasome system

1. Introduction

Endothelial cells (EC) are important vessel components that control the transfer of plasma components and cells across the vessel wall [1], and their alteration contribute to the different phases of atherosclerosis. The formation and progression of atherosclerotic plaque are largely influenced by genetic and environmental factors categorized as local factors, i.e. turbulent blood flow, and modifiable- and non-modifiable systemic factors [2]. It is well established that LDL oxidation plays a crucial role in initiation and progression of atherosclerosis [3]. Products formed during LDL oxidation (oxLDL) - including oxysterols, lysophospholipids and isoprostanes, are able to influence cell signaling and gene expression [4]. Oxysterols are a family of 27-carbon molecules originated from cholesterol oxidation by both enzymatic and non-enzymatic mechanisms [5]. A mounting body of evidence indicates that oxysterols, which are found in atherosclerotic plaques [6], are implicated in atherosclerosis by promoting endothelial cell dysfunction and death [7]. To this regard, 7-ketocholesterol have been shown to induce endoplasmic reticulum (ER) stress that is involved in oxLDL-induced apoptosis in endothelial cells [8-10]. ER has diverse key functions, including protein folding and modification, lipid synthesis and transport, and regulation of cytosolic calcium levels [11]. Perturbation of ER function, which leads to the accumulation of unfolded and misfolded proteins in the ER lumen, induces the activation of a cellular response called ER stress. In this context, the cell triggers the unfolded protein response (UPR) in order to restore ER homeostasis. To mitigate ER stress and promote functional recovery, the UPR leads to translational attenuation and upregulates the expression of ER chaperon genes. Enhanced membrane synthesis and the consequent increase in ER size are key factors in the ability of cells to cope with ER stress [12]. Proteins that do not properly fold are targeted for ER-associated degradation, which efficiently retro-translocate them from the ER into the cytosol for degradation via the ubiquitin/proteasome system (UPS) [13]. The condition of ER stress is also linked to autophagy in different ways: i) severe ER stress triggers macroautophagy in both yeast and mammalian cells; ii) the autophagosomes form at the ER level; and iii) the ER itself can become subject to autophagy, a phenomenon also called reticulophagy.

In the case the insult is severe or prolonged over time, ER stress becomes irreversible and intracellular signaling switches from adaptive to cell death programs. The mechanisms that initiate ER stress-dependent apoptosis have been only partially elucidated. Recently, an

important role has been attributed to the transcription factor NF-kB, which regulates the transcription of genes involved in immunity, inflammation, cell growth and apoptosis [14]. There is considerable interest in oxysterols mediated effects on ER [15], a place where oxysterols regulate the trafficking of proteins that control cholesterol synthesis [16]. We investigated 5,6 secosterol aldehyde (SEC), an oxysterol generated by cholesterol autoxidation that is found in atherosclerotic plaques [17], as a potential candidate of endothelial dysfunction and damage. Our results highlight that SEC is able to induce ER stress, as revealed by significant changes in ER size and shape. At low doses (i.e. 1 and 5 µM) cells activate the autophagic pathway surviving the stress, while at the higher (i.e. 20 µM) cells undergo apoptosis, which is preceded by phosphorylation of eIF2α and nuclear translocation of the transcription factor NF-kB.

2. Materials and Methods

2.1. Chemicals

Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (LGC Standards, Milan, Italy). EndoGRO-LS complete culture media kit was purchased from Millipore (USA), L-glutamine and Penicillin/Streptomycin were purchased from Lonza (Basel, Switzerland). 5,6 secosterol aldehyde (3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde), was synthesized by cholesterol ozonation [18]. AnnexinV (Annex-V)/propidium iodide (PI) kit was from Immunostep (Salamanca, Spain). ER-Tracker Green and Premo Autophagy Sensor LC3B-GFP were from Molecular Probes (Eugene, OR). Monodansylcadaverine (MDC) and anti actin (A 2066) were from Sigma-Aldrich (Milan, Italy). The antibodies against p65 (C-20) and IkBα (C-21) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti phospho-eIF2α (Ser51, D9G8) was from Cell Signaling Technologies (Leiden, The Netherlands). Anti Ubiquitin was kindly provided by Prof. A.L. Haas (Louisiana State University).

2.2. Cell cultures and treatments

HUVEC cells were cultured in 25 cm^2 flasks in EndoGRO-LS, supplemented with low serum medium for human endothelial cells, L-Glutamine (100mM) and 1% antibiotics

(penicillin, streptomycin). The cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C. At 50% confluence cells were detached with trypsin–EDTA, washed and sub-cultivated in new flasks for 1–2 days before the experiments. SEC was dissolved in ethanol. Cells were incubated at 37 °C with different concentrations of SEC (1, 5, 20 μM f.c.) or ethanol (as vehicle, 0.1% f.c.). At the end of the incubation time, cells were washed with PBS and stained with fluorophores or submitted to cell lysis as detailed below.

2.3. Detection of cell death

Apoptotic cells were detected by fluorescein isothiocyanate-conjugated Annexin V (FITC Annex-V). Annex-V fluorescence is a standard method for measuring the amount of phosphatidylserine exposed on the outer face of the plasma membrane following caspase activation. Briefly, cells were resuspended in binding buffer (1x) and stained with Annex-V FITC. Cells were washed with phosphate-buffered saline (PBS), stained with PI and analyzed by flow cytometry (FC). Early apoptotic cells were identified as Annex-V+ population, whereas Annex-V and PI staining include the early and late apoptotic cells [19]. Cytometric experiments were carried out with a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA) equipped with blue (488 nm, air-cooled, 20 mW solid state), red (633 nm, 17 mW HeNe), and violet (405 nm, 30 mW solid state) lasers. Analyses were performed with FACSDivaTM software (BD Biosciences, San Diego, CA). Flow cytometry data were collected by accumulating at least 10,000 events for each tube.

2.4. Autophagy detection

Autophagic vacuoles were labeled with monodansylcadaverine (MDC) by incubating cells grown on six-well plates with 0.05 mM MDC in PBS at 37°C for 10 min. After incubation, cells were trypsinized washed two times with PBS and immediately analyzed by flow cytometry [20]. Autophagy was also detected by measuring the aggregation of LC3B protein coupled to green fluorescence protein (GFP) using the Premo Autophagy Sensor Kit. Briefly, HUVEC cells were transduced with BacMam LC3B-GFP with a multiplicity of infection (MOI) equal to 30, using 1×10^5 cells in glass bottom chambers (MatTek Corporation). Mutated LC3B (G120A)-GFP was used as a negative control and chloroquine treatment (30 μM f.c.) was used as a positive control. Twenty-four hours after transduction, cells were treated as previously described. The appearance of LC3B-GFP aggregates was observed and

photographed using confocal microscope [21]. Fluorescence microscopy was performed with a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany) with 488, 543 and 633 nm illumination and oil-immersed objectives. Image analyses were performed using ImageJ software (NIH, Bethesda, MD). Single horizontal optical sections are illustrated in the captions.

2.5. Endoplasmic reticulum staining

Cells were seeded at a concentration of 2×10^5 cells/well, and 24 hours after plating they were treated with SEC and incubated for 4 or 24 hours. Cells were harvested, washed, and re-suspended in Hank's Balanced Salt Solution (HBSS) containing the ER-tracker Green probe (f.c. 100 nM). After 30 minutes of incubation at 37°C, cells were washed, resuspended in HBSS and analyzed by flow cytometry. For confocal imaging analysis, cells were seeded in glass bottom chambers (MatTek Corporation) at a density of 1×10^5 cells/mL, and 24 hours after plating they were treated with SEC and incubated for different times. Then, ER-tracker was added to the cells and incubated for 30 minutes before the end of the incubation under the same growth conditions.

2.6. Transmission Electron Microscopy

For transmission electron microscopy, cells were seeded in multiwell plates. 24 hours after plating, they were treated with SEC and incubated for different times. At the end of the incubation, the medium was removed and the cells were fixed for 24 hours by immersion in glutaraldehyde solution (2% in 0.1 M phosphate buffer, pH 7.4). Cells were afterwards washed in phosphate buffer and post-fixed in osmium tetroxide (1% in 0.1 M phosphate buffer, pH 7.4) for two hours. At the end of incubation, cells were repeatedly washed in phosphate buffer, dehydrated in a graded ethanol series and embedded in Araldite. Semi-thin (2 μm thick) and ultrathin sections (70 nm thick) were cut with an LKB Ultrotome 2088V. The semithin sections were stained with toluidine blue while the ultrathin sections were contrasted with a saturated solution of uranyl acetate in ethanol 50%, followed by treatment with lead citrate solution [22]. The ultrathin sections were observed under a Philips CM10 transmission electron microscope [23].

2.7. Cell lysis and fractionation

Cytoplasmic and nuclear extracts were obtained by low salt/detergent cell lysis followed by high salt extraction of nuclei, as previously described with some modifications [24]. Briefly, cells were lysed with Buffer A containing: 10 mM Hepes/KOH pH 7.9, 10 mM KCl, 0.1% (v/v) Nonidet-P40, supplemented with a cocktail of protease (cOmplete™ Roche, Basilea, SWZ) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). After 10 min incubation on ice and centrifugation at 14,000 x g at 4°C, the supernatant, corresponding to the cytosolic fraction, was transferred to a new tube, while the resultant pellet was suspended in Buffer B (20 mM Hepes/KOH pH 7.9, 25% glycerol, 0.42 M NaCl, supplemented with protease and phosphatase inhibitors as above) and kept on ice for 20 min. Nuclear proteins were recovered by centrifugation at 14,000 x g in the supernatant. Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard.

For whole-cell extract preparations, cells were directly harvested in Sodium Dodecyl Sulfate (SDS) buffer (50 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 2% w/v SDS, 10 mM N-ethylmaleimide, and supplemented with protease and phosphatase inhibitors as above). Lysates were boiled for 5 min, then sonicated at 100 Watts for 20 sec to shear DNA. Cell debris was removed by centrifugation at 14,000 x g. Protein content was determined by the Lowry assay.

2.8. Western immunoblotting analysis

Proteins were resolved by SDS-PAGE and gels were electroblotted onto a nitrocellulose membrane (0.2 μm pore size) (BioRad laboratories Inc., Milano, Italy). The blots were probed with the following primary antibodies: anti p65 (RelA), anti IkBα, anti [P]eIF2α, anti-Ubiquitin (Ub). Anti actin was used to check equal protein loading. Bands were detected by horseradish peroxidase (HRP)-conjugated secondary antibody (BioRad) and the enhanced chemiluminescence detection kit WesternBright ECL (Advasta). Densitometric quantitation of immunoreactive bands was performed in a Chemidoc apparatus (BioRad) equipped with the Quantity One software.

2.9. Statistical analysis

In protein quantification by significance was evaluated on densitometric data after normalization to actin. Analysis of variance (ANOVA) was used to compare values among different experimental groups for data that met the normality assumption. Differences

between groups were analysed by Bonferroni and Tukey *post hoc* analysis. P values less than 0.05 were considered statistically significant. Data are shown as mean ± standard deviation (SD) of at least three independent experiments. All statistical analysis was performed using Prism 5.0 (GraphPad software).

3. Results

3.1. The ER modification in HUVEC cells treated with SEC

To determine whether SEC treatment alters ER structure we loaded HUVEC cells with ER-tracker green that selectively stains the ER region, which consist of the perinuclear ER and the peripheral ER that extends throughout the cytoplasm. As shown in Fig.1A an increase in fluorescence intensity was evident with 1 and 5 μM until 4 hours of incubation (P<0.05). When the analyses were performed at 24 hours the significant increase of fluorescence was observed only at 1 μM (P<0.01). The overall fluorescence intensity in response to 20 μM SEC did not change after 4 h incubation but was significantly reduced at 24h if compared to control condition (P<0.01). Confocal microscopy (Fig. 1B) images show a dose and time-dependent expansion of the ER region detectable after 4 hours of incubation following the addition of 1 and 5 μM SEC to the incubation medium. In contrast, a sharp decrease in fluorescence coupled with a disorganization of ER membranes was observed in cells treated with 20 μM for 4 h. This behaviour becomes even more evident for the doses of 5 and 20 μM when the treatment is prolonged up to 24 hours.

Using transmission electron microscopy ER appears located in proximity of the nucleus, and its cisternae appear as flattened tubes with ribosomes. The closest to the external nuclear membrane are in continuity with the nuclear envelope (Fig. 1C-a). Upon incubation of HUVEC with 1μM SEC for 4 hours, a considerable increase in the number of cisternae of ER in the perinuclear area and extension to the peripheral areas has been observed (Fig. 1C-b). By increasing the incubation time up to 24 hours a reduction in the number of cisternae is observed, with swollen and fragmented appearance consistent with a drastic morphological change (Fig. 1 C-c). Higher SEC concentrations, i.e. 5 and 20 μM, after 24 hours of incubation, cause a further deterioration of ER: the swelling of the cisternae proceeds and leads to the detachment of the ribosomes from the ER membranes. This detachment is morphologically evidence by electron transparent space between the

ribosomes and ER membrane (Fig. 1C-d). Moreover, in several area of the cytoplasm the complete detachment of ribosomes occurred (Fig. 1C-e).
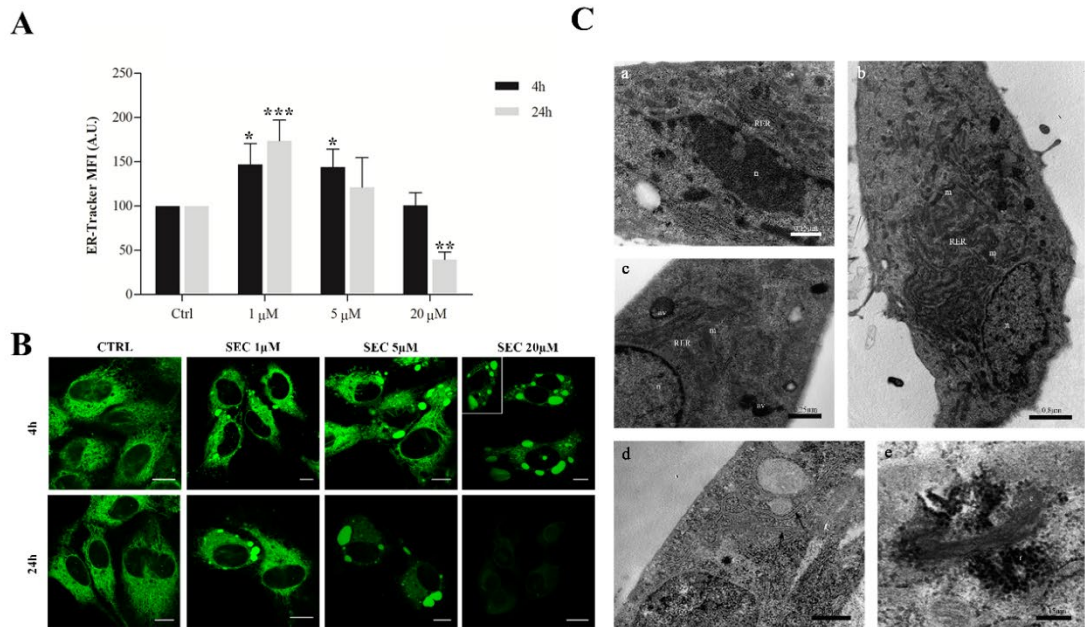

Fig. 1. Effect of SEC treatment on ER structure. (A) Statistical histogram is related to ER-Tracker Green MFI in HUVEC cells on control condition after 4 and 24h of treatment with SEC 1, 5 and 20μM. Each value is expressed as a mean ± SD (n = 3); *P < 0.05, **P < 0.01, ***P < 0.001 vs control. (B) Confocal images of endoplasmic reticulum morphology in HUVEC cells treated with 1, 5 and 20μM of SEC for 4 and 24h and labelled with ER-Tracker Green. Bars: 10μm. (C) (a) Control cells. The ER is formed by cisternae shows the typical flattened tubules with ribosomes. (b) Cell treated with SEC (1μM, 4h). Numerous elongated and convoluted cisternae of ER are distributed in the perinuclear area. Many elongated mitochondria are visible between them. (c) Cell treated with SEC (1μM, 24h). The ER cisternae appear less numerous, swollen and fragmented. Several autophagic vacuoles are visible in the cytoplasm. (d, e) Cell treated with SEC (5 and 20μM, 24h). The cisternae show a greatly damaged morphology and an electron transparent space (arrows) is also evident between the ribosomes and the membranes. The mitochondria are always more and more swollen and damaged (d). The complete detachment of ribosomes from the cisternae is visible (e).

3.2. Evaluation of early and late apoptotic cells

Under a condition of stress, ER increases in size in the attempt, according to several authors [25], to counteract the stress by providing a larger surface and luminal area. If cell is not able to cope the stress condition, in particular when it is extensive or sustained, a shift towards cell death occurs. Our results show that SEC at concentration of 20 µM significantly increased (7.1 folds, p<0.001) early apoptotic cells after 24 h of treatment (Fig. 2A). In addition, our data provide evidence that also 5µM, after 24 hours of incubation, induced a significant increase in the number of early and late apoptotic cells (Fig. 2B). On the other hand, 20 µM SEC induced a significant increase of early and late apoptosis both at 4 and 24 hours of incubation (5.1 and 6.5 folds, p < 0.001, respectively).
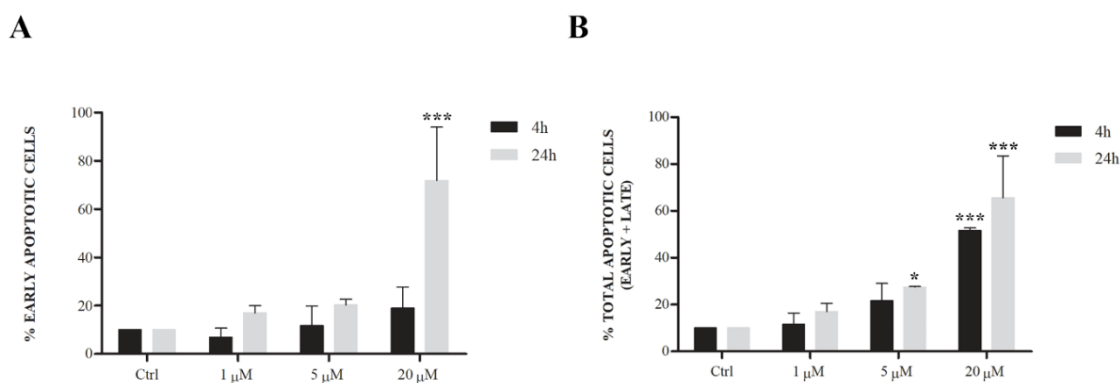

Fig. 2. Assessment of apoptosis by Annex-V and PI staining. Cells were cultured for 4h and 24 h with increasing concentrations of SEC, stained with specific probes and analysed by flow cytometry. (A) Percentage of Annex-V positive cells; (B) percentage of Annex-V plus PI positive cells. Data are reported as mean ± SD (n =3); *P < 0.05, ***P <0.001.

3.3. Induction of autophagy and accumulation of free and protein-conjugated ubiquitin.

A growing body of evidence suggest that ER stress, starvation and viral infection are able to induce the autophagic pathway [26]. To study autophagy we used the MDC dye, as specific marker for autophagic vacuoles that accumulates in mature autophagic vacuoles, such as autophago-lysosomes, but not in the early endosome compartment. In HUVEC cells challenged with SEC we observed the activation of the autophagic pathway occurring after

24 h of incubation of cells with 1 and 5 µM (P<0.01, P<0.001, respectively) (Fig. 3A). To further investigate autophagy in response to SEC cells were transfected with GFP-LC3B to visualize autophagosome formation in cells.

HUVEC cells treated with SEC exhibited a dose-dependent increase in a bright LC3B-positive puncta (Fig. 3B) indicating the presence of autophagosomes. Electron microscopy imaging highlights numerous autophagic vacuoles in the cytoplasm, in particular near the fragmented cisternae, already in the cells treated with 5 µM of SEC for 4h (Fig. 3C-b). At high magnifications, membrane residues likely derived from ER are observed inside the autophagic vacuoles (Fig. 3C-c, d). Our results suggest that selective ER engulfment by autophagosomes may help cells to counterbalance the ER expansion. The ubiquitin–proteasome system and autophagy interact in a coordinated manner to alleviate ER stress by preventing accumulation of misfolded proteins [27]. Consistent with this notion, western immunoblotting analysis of whole cell extracts showed that an accumulation of ubiquitin-conjugated proteins and free ubiquitin occurred in HUVEC cells treated with 1 and 5 µM SEC (Fig. 3D). By contrast, 20 µM induced a decrease in protein ubiquitination, while the levels of the free ubiquitin monomer were essentially unchanged compared to untreated cells (Fig. 3D).
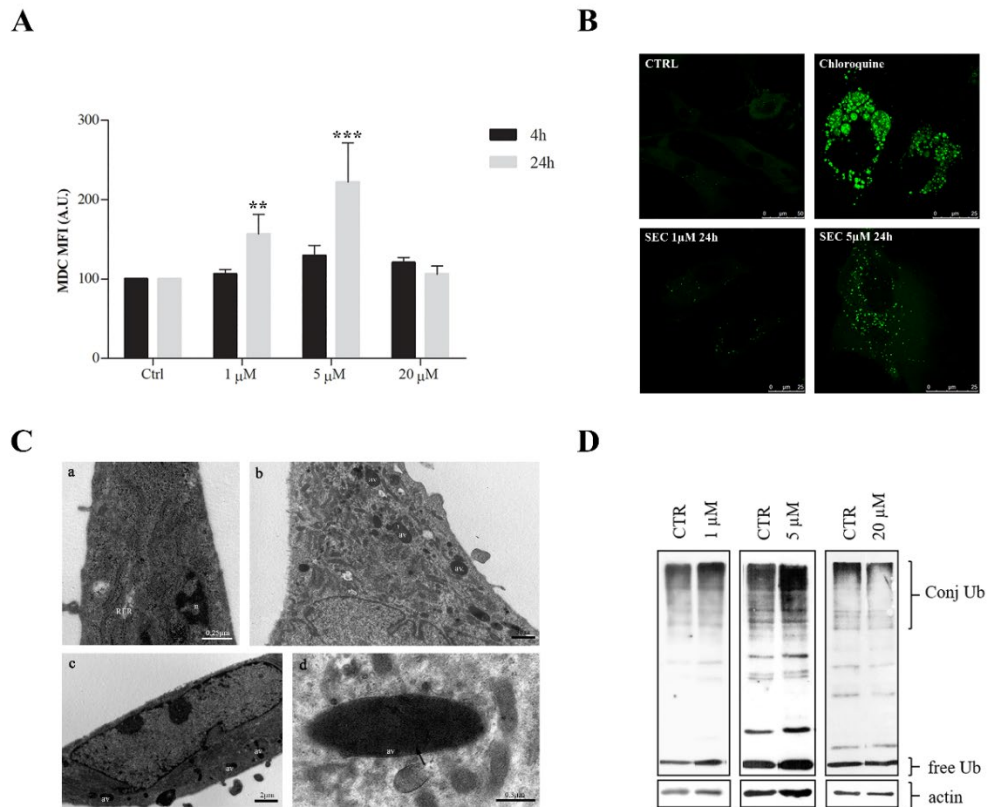
Fig. 3. Autophagic vacuoles were analyzed by calculating MDC-stained HUVEC using flow cytometry. (A) The statistical histogram is related to MDC MFI values in HUVEC cells after 4 and 24h of treatment with different concentration of SEC (1, 5 and 20 μM). Data are reported as mean ±SD; **P<0.01, ***P<0.001. (B) Single confocal optical sections (~0.8 μm thickness) showing LC3B-GFP fluorescence from control, chloroquine, SEC 1μM 24h and SEC 5μM 24h treated cells. Increase in the number of LC3B-GFP positive puncta are evident in SEC treated cells in a dose-dependent manner. Chloroquine treatment represent a positive control. Bars: 50 μm for control, 25 μm for chloroquine and SEC treated HUVEC cells. (C) The cells were analyzed by a transmission microscope to visualize autophagic vacuoles. The typical ER cisternae formed by flattened tubules with ribosomes are present in control cells (a). The autophagic vacuoles are present near the fragmented cisternae in cells treated with SEC (b: 5μM, 4h; c: 5μM, 24h) (b, c). Inside the autophagic vacuoles residues of membranes in cross and longitudinal section (arrows) are clearly visible (d). (D) Western immunoblotting analysis of free ubiquitin (Ub) and protein-conjugated ubiquitin (Conj-Ub). Whole extracts (5 μg) obtained from cells treated with 1, 5 and 20 μM SEC for

24h were loaded onto a 13% (w/v) polyacrylamide gel, electroblotted and stained with an anti-ubiquitin antibody. Actin was stained as loading control.

3.4. Phosphorylation of the translation factor eIF2α and nuclear accumulation of the transcription factor –kB (NF-kB)

To investigate the molecular mechanisms activated during the early phases of SEC treatment we focused on eIF2α and NF-kB. HUVEC cells were harvested at 1, 2 and 4 hours of incubation and submitted to cytoplasmic/nuclear extraction. Western immunoblotting analysis of the cytosolic fraction with an antibody against the phosphorylated form of the translation initiation factor 2 ([P]eIF2α) demonstrated that the factor was already phosphorylated under basal conditions (Fig. 4, lane 1,5,9). Phosphorylation significantly increased at 2 and 4 h of treatment with 20 µM SEC (Fig. 4, lane 11,12), while did not significantly changed at the lower concentrations tested (Fig. 4, lane 2-4, 6-8, 10). In the latter case, the trend was towards a slow reduction, occurring already at 1h for the 5 µM dose, which was completely compensated at 4 hours. eIF2α can be phosphorylated by a number of stress-activated kinases leading to downregulation of the overall rate of protein synthesis. Increased levels of [P]eIF2α serves as an important 'checkpoint', for the cells to either recuperate from stress or be eliminated if the damage is beyond repair [28].
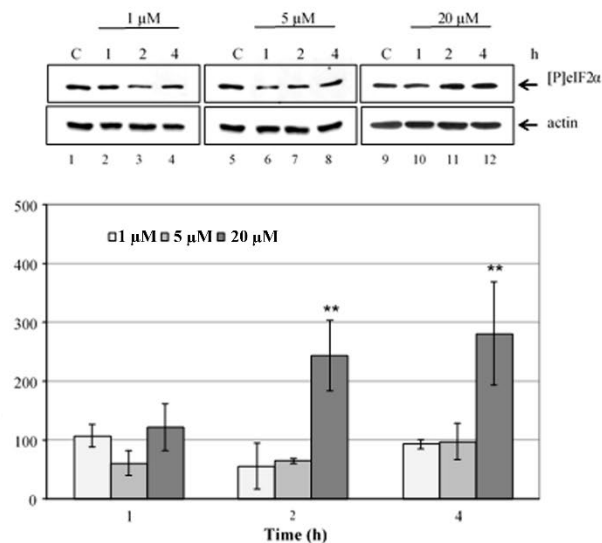

Fig. 4. Western immunoblotting analysis of eIF2α phosphorylation levels in cells untreated (denoted as C) or treated with 1, 5 and 20 µM SEC for different times. Cytosolic extracts

(10 µg) were resolved by SDS-PAGE onto 8% (w/v) polyacrylamide gels and then submitted to western immunoblotting with an antibody which specifically recognizes the phosphorylated form of eIF2α ([P]eIF2α). Actin was stained as loading control. [P]eIF2α levels, normalized to actin content, were determined by quantifying band intensity in a Chemidoc system and reported in the graph as percent of basal levels. Values are the mean ± S.D. of three independent experiments. *p<0.05, **p<0.01.

Another important signal for an alarm phase is the activation of transcription factor NF-Kb, which has been reported to induce apoptosis in the face of a prolonged/detrimental ER stress, transforming an initially compensatory mechanism into a maladaptive one [29]. The nuclear levels of NF-kB were determined by western immunoblotting using an antibody against the p65 subunit (RelA). The transcription factor was already present in the nucleus of control cells (Fig. 5A, lanes 1,5,9). This basal level tended to fluctuate during the incubation with 1 and 5 µM SEC, to decrease at 4 hours, although the decrement was not statistically significant (Fig. 5A, lane 4,8). By contrast, a strong NF-kB nuclear accumulation was observed after 2 and 4 hours of treatment with 20 µM SEC (Fig. 5A, lane 11,12). Notably, this accumulation was not accompanied by degradation of the cytosolic inhibitor IkBα (Fig. 5B). Indeed, the levels of IkBα did not change within the 4 hours of incubation with SEC, at any of the doses tested.
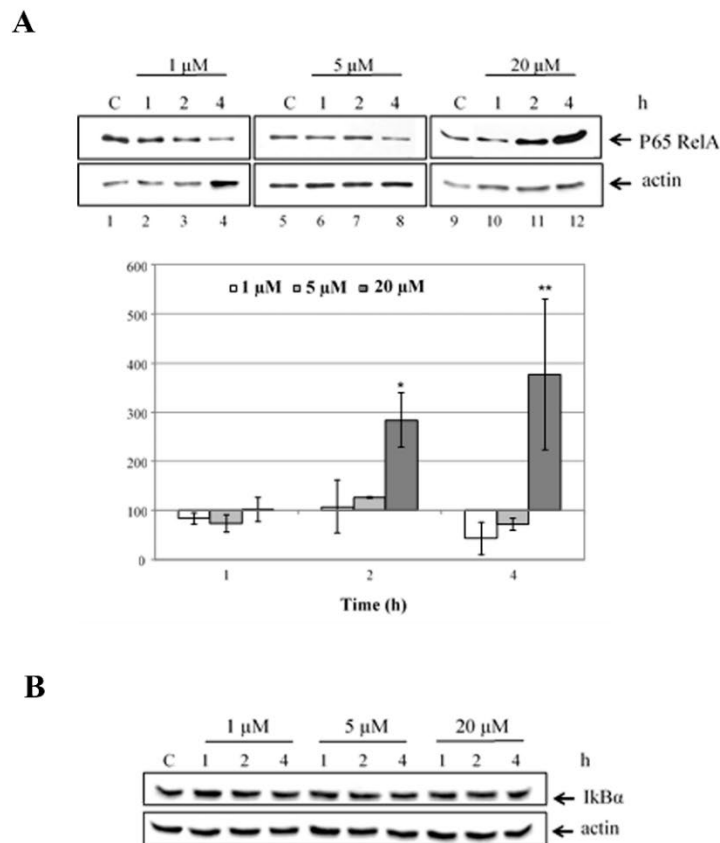
A

B

Fig. 5. Western immunoblotting analysis of p65 (RelA) nuclear levels and IkBα cytosolic levels in cells untreated (denoted as C) or treated with 1, 5 and 20 μM SEC for different times. Nuclear (5 μg) and cytosolic (10 μg) extracts were resolved by SDS-PAGE onto 8% (w/v) and 10% (w/v) polyacrylamide gels, respectively, and then submitted to western immunoblotting with an antibody against p65 (A) and IkBα (B). Actin was stained as loading control. p65 levels in cell nuclei, normalized to actin content, were determined by quantifying band intensity in a Chemidoc system and reported in the graph as percent of control levels. Values are the mean ± S.D. of three independent experiments. *p<0.05, **p<0.01.

4. Discussion

We have found that SEC, an oxysterol found in atherosclerotic plaque, affects ER structure and physiology in HUVEC cells. In particular, 1 μM and 5 μM SEC induced a significant

expansion of ER membranes while higher concentrations, i.e. 20μM, led to the collapse of the entire structure. ER expansion has been observed under several conditions, including cell differentiation, viral infection and certain drugs [30, 31]. Sanson and coworkers [10] reported that 7-ketocholesterol is able to induce ER stress markers in endothelial cells. Recently, ER expansion has been linked to ER stress but the underlying molecular mechanisms are not fully elucidated. Whether activation of the UPR is necessary to promote membrane expansion is still a matter of debate [32].

Notably, at 1 and 5 μM SEC, accumulation of Ub-conjugated proteins and induction of autophagy was observed, indicating that cells are able to activate compensatory mechanisms to relieve the stress. In addition, we identified that autophagosomes are packed with membrane stacks derived from the ER, which according to Bernales [22] represents selective ER sequestration that help cells to counterbalances ER stress. This mechanism was not activated with SEC 20 μM, a concentration that led most of the cells to apoptosis along with massive cell death observed after 24 hours of treatment. Our results are consistent with a recent report showing a key role of autophagy in survival response induced by 27-hydroxycholesterol in U937 cells [33].

Significantly, we found that apoptosis was accompanied by activation of NF-kB and phosphorylation of eIF2α. Phosphorylation of eIF2α is a central mechanism regulating translation initiation in response to different environmental stress, leading to a decrease in global protein synthesis. Although integrated stress response (ISR) is primarily a pro-survival expedient to severe stress, it can drive signaling toward cell death [34]. Jiang and coworkers [28] reported that the phosphorylation of eIF2α is central to the activation of NF-kB by promoting translational inhibition and rapid reduction in IκBα levels. Under our experimental conditions the levels of IkB remain unchanged. This is consistent with previous observations indicating that eIF2α phosphorylation is required for NF-kB activation, but the mechanism of NF-kB induction entails the release and not the degradation of IkBα [28].

Thus, eIF2alpha phosphorylation and NF-kB activation in response to SEC may be correlated events in the same cellular program leading to cell death. NF-kB is a transcription factor that plays a key role in several cell functions being anti or proapoptotic depending on the type of stress and cell line [35]. Recently, inhibition of the NF-κB pathway decreased autophagy and apoptosis, and attenuated cervical tumor cell death induced by brefeldin, indicating a role in cell death during acute ER stress [36].

5. Conclusion

In conclusion, data reported in this paper point to an important role of the ER damage as one of the mechanisms through which SEC may induce endothelial cell dysfunction. Depending on the dose and thus the severity of the stress, cells activate protective mechanisms or death signaling pathways leading to cell survival or death. Future experiments will be necessary to better understand the molecular mechanisms mediated and /or modulated by SEC to affect ER structure and function.

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Chapter 2

Secosterol-B induces proinflammatory activation of human microvascular endothelial cells leading to endothelial dysfunction

Maria Gemma Nasoni [a], Serena Benedetti [a], Francesco Palma [a], Barbara Canonico [a], Erica Cesarini [a], Stefano Papa [a], Luigi Iuliano [b] and Francesca Luchetti [a].

[a] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy.
[b] Department of Medico-Surgical Sciences and Biotechnology, Sapienza University of Rome, Italy.

Abstract

Oxysterols are a family of 27-carbon cholesterol oxidation derivatives found in LDL and atherosclerotic plaques where they trigger several biological responses involved in the initiation and progression of atherosclerosis. Endothelial dysfunction (ED) is an early step in the development of atherosclerosis and is characterized by a change in the balance of vasomotor factors released by the endothelium, expression of inflammatory cytokines and adhesion molecules. Several evidences suggest that oxysterols contribute to ED due to their ability to alter membrane fluidity and cell permeability leading to inflammation, oxidative stress and apoptosis. The aim of the present study was to investigate the role of Secosterol-B (SEC-B) -a recently autoxidation product of cholesterol found in atherosclerotic plaques- on proinflammatory activation and dysfunction of human microvascular endothelial cells (HMEC-1). Our results highlight that SEC-B is able to activate HMEC-1 by improving oxidative stress and expression of proinflammatory molecules, such as IL1-β, TWEAK and ICAM-1. However, this proinflammatory state leads to impairment of endothelial function characterized by decrease of cell viability, down-regulation in NO production and enhancement in monocyte recruitment on activated HMEC-1 cells. Our finding improves a new knowledge about the role of SEC-B in ED and its implication in progression of atherosclerotic disease.

Graphical abstract
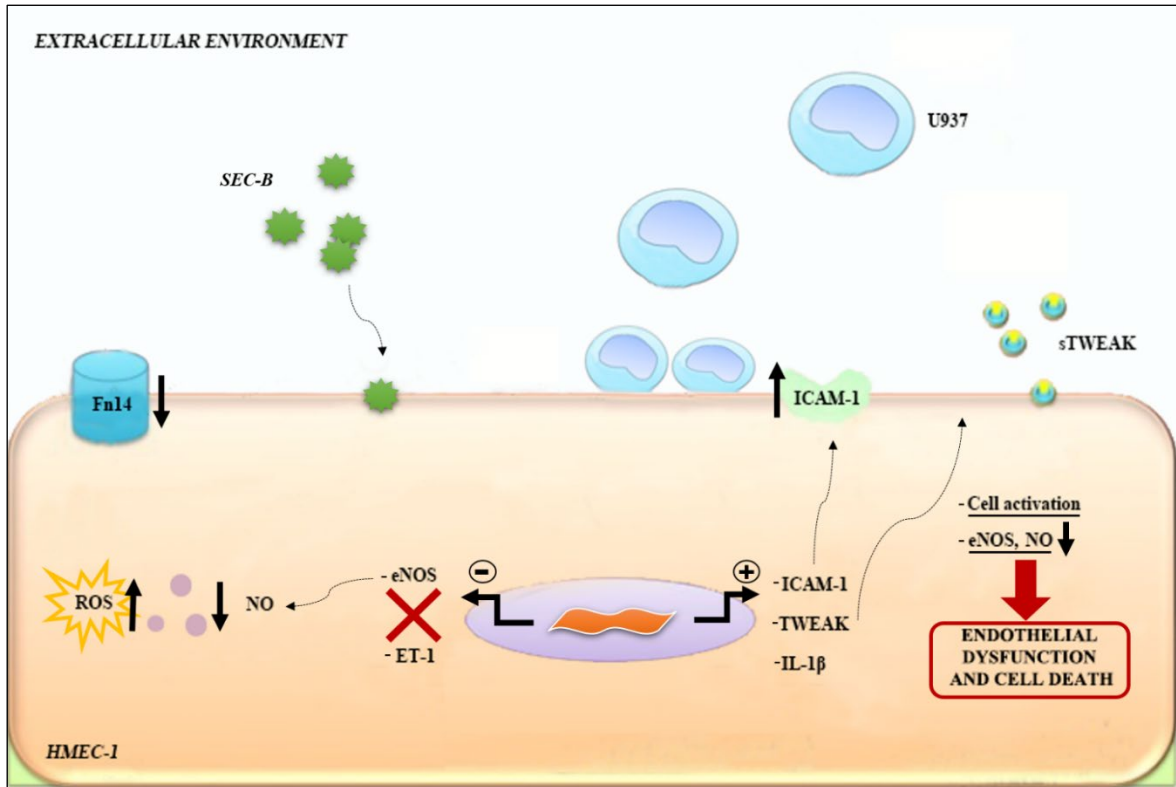
Introduction

The endothelium is the thin monolayer of endothelial cells lining the lumen of all blood vessels. The strategic location allows it to regulate vascular homeostasis modulating the vessel tone in response to humoral, nervous and mechanical stimuli. The endothelium acts as gatekeeper of blood vessel integrity, so the trafficking between endothelium and extracellular environment can mediate several vascular pathologies. Nitric oxide (NO) and endothelin-1 (ET-1) are two most important vasoactive mediators produced by endothelial cells that act as the most powerful endogenous vasodilator and vasoconstrictor, respectively. A reduction of NO bioavailability is a crucial feature of endothelial dysfunction (ED) and it is important hallmark in the atherosclerosis development (Marchio P, et al.2019; Herman AG, et al. 2005; Sandoo A, et al. 2010).

It is well documented that oxidative stress and inflammation have been recognized as partners in crime in bringing about ED and progression of the atherosclerotic plaque. Indeed, an excess of reactive oxygen species (ROS) inactivates NO production triggering to an impairment of endothelium-dependent vasodilation (Endemann DH, et al. 2004; Liao JK, et al. 2013; Incalza MA, et al. 2018).

Oxysterols are cholesterol oxidation products present in oxidized-low density lipoproteins (ox-LDLs) that regulate many biological processes and exert several biochemical effects of potential pathophysiological relevance. A growing body of evidence highlights that oxysterols are involved in proliferation and metabolism and also exert proinflammatory and proapoptotic effects (Vurusaner B, et al. 2014; Gargiulo S, et al. 2016). Accordingly, there is considerable interest in oxysterols mediated effects on ED and their implication in atherosclerosis development.

In our previous study, we demonstrated that Secosterol-B (SEC-B), a recent cholesterol oxidation product found in atherosclerotic plaques, is able to induce endothelial damage and death in HUVEC cells, affecting endoplasmic reticulum (ER) morphology and function (Luchetti F, et al. 2019). However, the mechanism involved in SEC-B-induced ER stress still needs to be clarified. In this scenario, our study was aimed to evaluate the ability of SEC-B to induce prooxidant and proinflammatory effects in human microvascular endothelial cells (HMEC-1). HMEC-1 cells were treated with different doses of SEC-B (5, 10, 20 μM) at the concentration range detectable within the atherosclerotic plaques. The proinflammatory action of SEC-B was evaluated as ability to modulate cellular redox status and NO content. Moreover, the endothelial proinflammatory activation is characterized by

the expression and release of several cytokines and proinflammatory factors, such as cell-surface adhesion molecules or growth factors. To this regard, we investigate the expression of interleukin 1-β (IL-1β) and intracellular adhesion molecule-1 (ICAM-1) expression. Despite IL-1β is produced predominantly by immune-derived cells, it has been demonstrated that it is express in endothelial cells in response to different cytokine stimulations (Wilson HL, et al. 2007). ICAM-1 overexpression is able to mediate adhesion of leukocytes to endothelial cells promoting the transmigration of blood cell in subendothelial tunica (Zhao W, et al. 2017).

In the proinflammatory context, emerging data suggests that TWEAK (tumour necrosis factor-like weak inducer of apoptosis), a cytokine belonging to the tumor necrosis factor (TNF) superfamily, is involved in the inflammation and remodeling of the endothelium (Dohi T, et al. 2012). Although different articles have reported the proinflammatory actions of TWEAK in both VSMCs and renal cells in vitro (Muñoz-García B, et al. 2009), no study has been reported in the context oxysterols and endothelium.

In the present study was investigated the role of SEC-B on proinflammatory endothelial activation that leads to HMEC-1 dysfunction. Our results highlight that SEC-B is able to activate HMEC-1 cells by improving oxidative stress and affecting (IL1-β) and intracellular adhesion molecule-1 (ICAM-1) expression. In addition, our data suggest an involvement of SEC-B in TWEAK/Fn14 pathway regulation. This proinflammatory state leads to impairment of HMEC-1 function characterized by decrease cell viability and down-modulation of NO production.

Materials and methods

Cell culture and treatments

Cell culture of human microvascular endothelial cells HMEC-1 (ATCC, No CRL-3243) was obtained from American Type Culture Collection (Teddington, UK). Cells were grown in MCDB-131 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 10mM Hepes, 10 ng/mL Epidermal Growth Factor (EGF) and 1 µg/mL hydrocortisone at 37 °C in a humidified 5 % CO_2 incubator. 3-hydroxy-5-hydroxy-B-norcholestane-6-carboxaldehyde (secosterol-B) was synthesized by cholesterol ozonation (Wang K, et al. 1993) and was dissolved in ethanol. In order to evaluate the effect of SEC-

B on HMEC-1, cells were incubated with increasing doses of SEC-B (5, 10, 20 µM f.c.) or ethanol (as vehicle, 0.1% f.c.) for indicated time.

WST-8 cell viability assay

The effect of SEC-B on HMEC-1 cell viability was analysed by WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Sigma-Aldrich, Milan, Italy). The assay was based on the cleavage of the tetrazolium salt WST-8 by cellular dehydrogenases in viable cells. Briefly, cells (5000/well) were incubated in clear 96-well plates with SEC-B for 24, 48 and 72 h. After incubation, WST-8 (1:10 final dilution) was added to each well, and cells were further incubated at 37 °C up to 4 h. Colour development was monitored at 450 nm in a multiwell plate reader (Thermo Fisher Scientific, Milan, Italy).

Intracellular ROS evaluation

Intracellular ROS were analysed in HMEC-1 by 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy), which is a cell-permeable non-fluorescent probe turning to highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation. Briefly, cells (5000/well) in black 96-well plates were incubated with DCFH-DA (5 µM) for 30 min at 37 °C. After excess probe removal, cells were treated with SEC-B and the fluorescence emission upon probe oxidation was monitored for 4h at ex/em 485/520 nm in the multiwell plate reader FluoStar Optima (BMG Labtech, Germany).

Measurement of NO production

NO production was quantified intracellularly in HMEC-1 after stimulation with SEC-B by using the cell permeable probe 4,5-diaminofluorescein diacetate (DAF2-DA, Thermo Fisher). DAF2-DA is hydrolyzed to cell impermeable DAF2 by intracellular esterases and reacts with NO and O_2 to the fluorescent DAF2-T. Briefly, cells (5000/well) were incubated in black 96-well plates and were loaded with 5 µmol/l DAF2- DA for 30 min at 37 °C. DAF2-T fluorescence was measured within cells at 485 nm excitation and 535 nm emission in the multiwell plate reader FluoStar Optima (BMG Labtech, Germany).

Measurement of TWEAK level by ELISA

Supernatants of confluent monolayers of HMEC-1 cells treated for different times with increasing concentrations of SEC-B were centrifuged at 1000 g for 10 min, collected and stored at -80° C until assayed. Soluble TWEAK level from supernatants of cells was assessed by a specific immunoassay from Bender MedSystems GmbH (Vienna, Austria) (detection limit 9.7 pg/ml, CV intra-assay 7.9%, CV inter-assay 9.2%).

Western blot analysis

After washing, cells were resuspended in 800 µl of 1X SDS-PAGE loading buffer. Forty microliters of cell lysate were loaded in a 15% polyacrylamide mini gel. Western blotting analysis was performed as described in **(**Catalani S, et al. 2013) using the following antibodies: anti-FN14 (anti-FN14, #4403 Cell Signaling) diluted 1:500 and β-actin monoclonal antibody (AC-15, Sigma) diluted 1:400.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from treated or untreated HMEC-1 cells using RNeasy Mini Kit (Qiagen) and 2 µg was reverse transcribed into cDNA using the SuperScript® First-Strand Synthesis System kit (Invitrogen) as described in the manufacturer's protocol. The cDNA was diluted 1:5 with water and 1 µl used in qPCR analysis using the PCRBIO HS TAQ MIX RED and the following primers pairs: β-actin: GCGAGAAGATGACCCAGATC and GGATAGCACAGCCTGGATAG; FN14: CCAAGCTCCTCCAACCACAA and TGGGGCCTAGTGTCAAGTCT; TWEAK: GCCCATTATGAAGTTCATCCACGACC and GCAGAGGGCTGGAGCTGTTGATTCT; IL1b: CTCGCCAGTGAAATGATGGCT and GTCGGAGATTCGTAGCTGGAT; eNOS: AGGAACCTGTGTGACCCTCA and CGAGGTGGTCCGGGTATCC; ICAM1: GCCGGCCAGCTTATACACAA and CAATCCCTCTCGTCCAGTCG; ETH1: GCTCGTCCCTGATGGATAAA and TTCCTGCTTGGCAAAAATTC. Real-time was run in an Applied Biosystems™ 7500 Real-Time PCR System assays. The expression levels of target genes were normalized to that of β-actin and the relative quantification analysis was based on the 2-ΔΔCt method. Thermal cycling parameters for Real-Time qPCR were: 1 cycle at 95°C for 5 min and 40 cycles at 95°C for 15 sec, 60°C for 31 sec and 72°C for 15 sec.

Measurement of ICAM-1 expression by flow cytometry

To evaluate the surface expression of ICAM-1, HMEC-1 were treated for 24h with SEC-B and then incubated for 1 h with PE-conjugated mouse antihuman ICAM-1 antibody (eBioscience, clone HA58). After washing, the cells were analyzed with FACSCanto II flow cytometer (BD Biosciences, San Diego, CA) using FACSDivaTM software (BD Biosciences, San Diego, CA). Flow cytometry data were collected by accumulating at least 10,000 events for each tube and mean fluorescence intensity (MFI) was measured as an indicator of ICAM-1 surface protein expression.

Flow-Cytometric analysis of supravital Propidium Iodide (PI) staining

HMEC-1 (50.000 cells/well) were plated in 12-well plates for 72h. At the end of the treatment, the cells were centrifuged and suspended in MCB131 containing Propidium Iodide 50 µg/ml (PI; Sigma-Aldrich) (Canonico B, et al. 2016). After incubation for 30 min at room temperature in the dark, the samples were analyzed by flow cytometry (FACSCanto II BD Biosciences). For each sample, 10,000 events were collected. Data acquisition and analysis were performed using FACSDivaTM software (BD Biosciences, San Diego, CA).

Cell adhesion assay

HMEC-1 were cultured at a density 10×10^5 cells/well on six well plates in MCB131 with 10% FBS. U937 cells were grown in RPMI-1640 medium containing 10% FBS and were labelled with 5 µM calcein-AM (Molecular Probes) for 30 min at 37°C. After labelling, cells were washed and 50×10^5 cells/well were seeded onto SEC-B-treated HMEC-1 monolayer for 24h and incubated for 1 h at 37°C and 5% CO_2. Co-cultured cells were washed and the images were obtained at 485 nm excitation and 538 nm emission using a digital camera-attached fluorescence microscope with data acquisition software (Nikon software).

Statistical analysis

Statistical analyses were performed using Prism version 5.00 (GraphPad Software, USA). Assays were carried out in triplicate and the results were expressed as the mean values ± SD.

Differences between samples were assessed by analysis of variance (ANOVA) with Bonferroni *post hoc* test. The results were considered statistically significant when p < 0.05.

Results

Effect of SEC-B on HMEC-1 viability

The effect of SEC-B on cell viability was evaluated by WST-8 and propidium iodide assays. As shown in Fig. 1A, SEC-B-treated cells exhibited dose and time dependent decrease in cell viability within 72h of treatment. In particular, SEC-B at 24h induces a slight reduction of cell viability at doses of 5 µM and 10 µM, that becomes statistically significant when the treatment was prolonged up to 72h (***p<0.001 *vs* control). On the contrary, the highest dose of 20 µM is able to reduce significantly cell viability within 24h (*p<0.05 *vs* control). This behavior is even more evident at 72h, showing a sharp decrease of 46% if compared to the control condition. In addition, as shown in representative contour plots SEC-B induces a dose dependent increase of PI positive cells (Fig. 1B) after 72h of treatment. In particular, apoptotic (region P2) and necrotic (region P3) cells are well detected only in HMEC-1 treated with highest concentrations (10 and 20 µM).
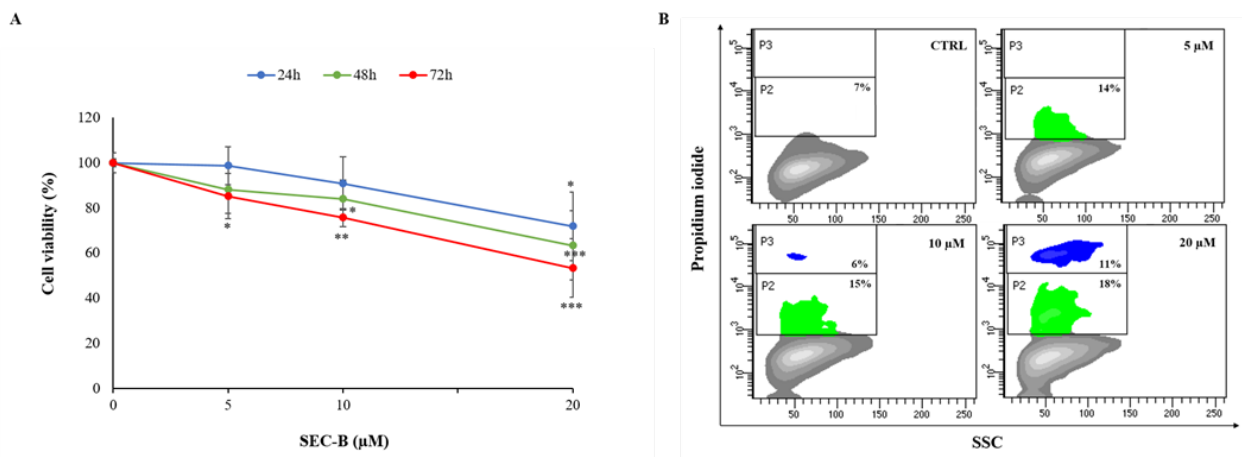

FIG. 1: (A) Cell viability evaluation by WST-8 colorimetric assay upon SEC-B administration (5, 10, 20 µM) for 24, 48, and 72 h. Data are expressed as mean ± SD (n = 3). *p<0.05; **p<0.01; ***p<0.001 vs. untreated

cells. **(B)** Representative supravital PI contour plots of treated HMEC-1 at 72h showing apoptotic (green) and necrotic (blue) populations.

SEC-B induces an increase of intracellular ROS level

Oxidative stress has been largely identified as the one of the main alterations involved in the pathogenesis of macrovascular diseases, playing a key role in the inflammatory response (Incalza MA, et al. 2018). The effect of different concentrations of SEC-B on ROS production was assessed in DCFDA-loaded HMEC-1 cells. SEC-B stimulates ROS generation within 4h of treatment as evidenced by DCFDA oxidation (Fig. 2A). At each time point indicated in Fig. 2A, a significant increase in intracellular ROS content was observed at doses of 10 and 20 µM. In particular, a maximum rise of 33% of ROS compared to the control condition was detected at 4h in HMEC-1 treated with 20 µM (Fig. 2A). Representative confocal images of treated-HMEC-1 cells (Fig. 2B) confirm the data described above. In detail, a diffuse fluorescence was observed in the cytoplasm of treated cells mainly evident at the highest dose.
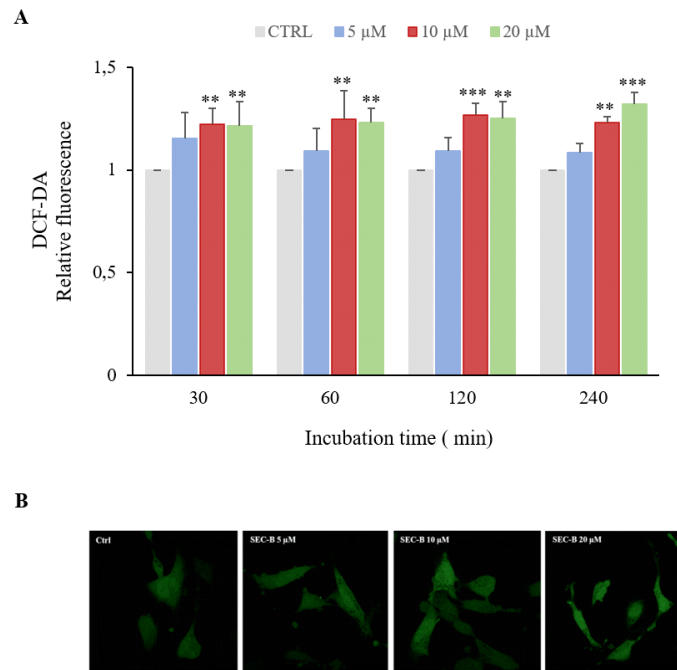

FIG. 2: (A) Intracellular ROS increase during 4 h of SEC-B administration (5, 10, 20 µM) to HMEC-1 cells. Data are expressed as mean ± SD (n = 3). **p<0.01; ***p<0.001 vs. untreated cells. **(B)** Representative confocal microscopy images of treated-HMEC-1 cells labelled with DCF-DA probe. Bars = 25 µm.

Modulation of NO content and ET-1 expression in HMEC-1 exposed to SEC-B

In endothelial cells, NO is the major responsible for the maintenance of vascular homeostasis. Reduction in NO bioavailability and excess of ROS mark the onset of ED (Incalza MA, et al. 2018). Accordingly, we monitored the modulation of intracellular NO production in response to SEC-B administration in HMEC-1 cells. The amount of NO production was quantified after stimulation with SEC-B by cell permeable DAF2-DA probe. At early time, we found that SEC-B induces a dose-dependent increase of NO content, which is maximum (+34%) at 2h with 20 µM compared to untreated cells (*p<0.05; **p<0.01; *vs* control, Fig. 3A). On the contrary, prolonged exposure shows a dose-dependent decrease in intracellular NO levels, with a significant reduction in NO bioavailability after 24h of treatment with the highest doses of SEC-B (*p<0.05; **p<0.01 for 10 and 20 µM *vs* control). In particular, our results demonstrate a decrease in the intracellular NO content of 23% at 20 µM (Fig. 3B). Downstream of NO production is eNOS regulated. Notably, eNOS expression was significantly reduced in the treatment with 20 µM after 24h of incubation (Fig. 3C), whereas at early time the expression is unchanged. Moreover, to study the effect of SEC-B on ED we investigated the modulation of mRNA ET-1 expression by RT-PCR. ET-1 is a vasoconstrictor secreted by endothelial cells, which acts as the natural counterpart of the vasodilator NO. Our results show that SEC-B leads to a significant decrease of ET-1 expression early at 4h of 20 µM of treatment (*p<0.05 *vs* control) (Fig. 3D), probably associated with the early increase in intracellular NO content. However, at 24h of SEC-B exposure ET-1 expression becomes drastically down-regulated, revealing an impairment of HMEC-1 functions to counterbalance the effect of SEC-B.
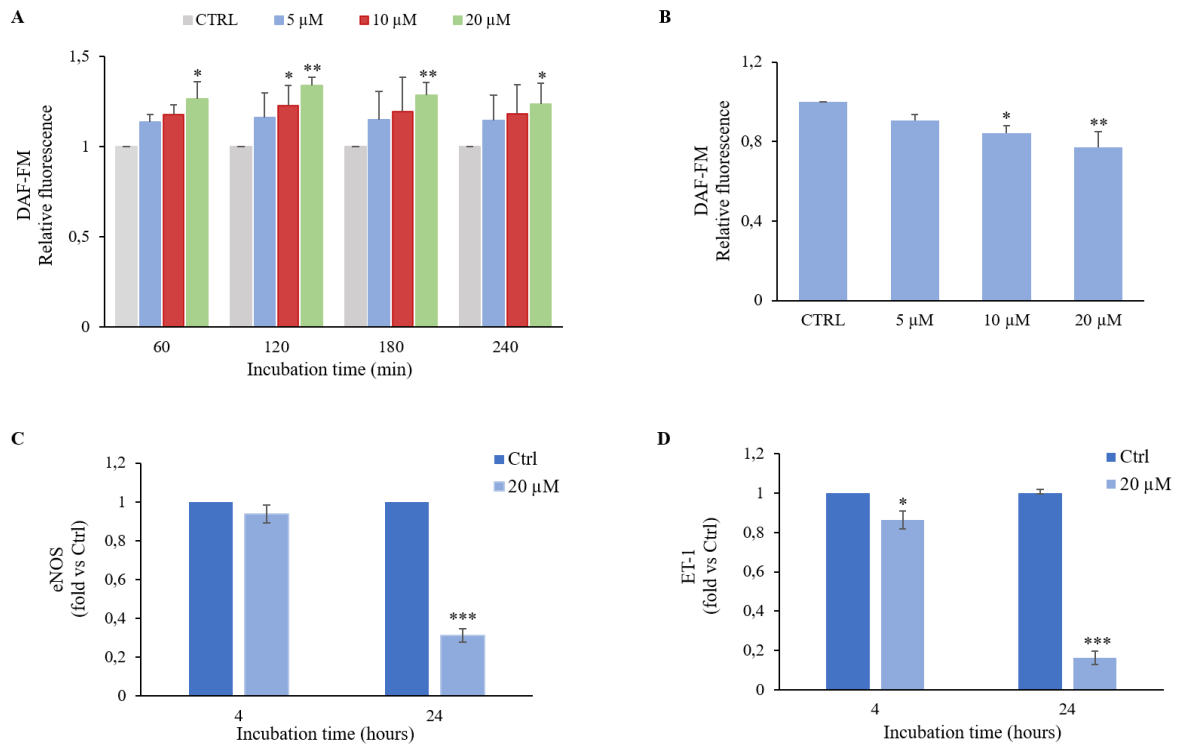
Fig. 3: Effect of SEC-B on NO and ET-1 modulation in HMEC-1 cells. Intracellular NO production at 4 h **(A)** and 24h **(B)** in HMEC-1 exposed to SEC-B (5, 10, 20 µM). **(C,D)** Quantitative real-time PCR of eNOS and ET-1 mRNA expression at 4 and 24h post-treatment in cells treated with 20 µM. Data are expressed as mean ± SD (n = 3). *p<0.05; **p<0.01; ***p<0.001 vs. untreated cells.

SEC-B enhances IL-1β and ICAM-1 expression leading to adhesion of U937 cells

It has been established that the dysfunction of NO production and secretion of proinflammatory cytokines are connected to changes in the expression of adhesion molecules which further affect the ability of leukocytes to adhere to the endothelium (Lai TS, et al. 2017; Kubes P, et al. 1991). To this purpose, we investigated the endothelial cell activation through the analysis of IL-1β and ICAM-1 expression in HMEC-1 cells at 24h after SEC-B treatment. Our data demonstrated that SEC-B is able to upregulate mRNA expression of IL-1β after 24h of treatment in a dose dependent manner (Fig. 4A). In addition, we observed a slight dose-dependent increase of ICAM-1 surface expression (Fig. 4B). The enhanced content of protein is coupled with a significant up-regulation of ICAM-1 expression at 20 µM (Fig. 4C). Given the well-known role of ICAM-1 as an endothelial cell adhesion molecule mediating leukocyte recruitment into endothelial tissue, we evaluated whether SEC-B is able to stimulate monocyte adhesion on HMEC-1. We determined the

effect of SEC-B in the adhesion of U937 cells to endothelial cells pre-treated with 5, 10 and 20 µM for 24h. Cell adhesion was evaluated by quantification of the adherent calcein-labelled U937 cells to HMEC-1 (Fig. 5A). The control condition showed a slight binding of U937 to HMEC-1, but cell adhesion dose-dependently increases when HMEC-1 were treated with SEC-B (Fig. 5A,B). As shown in Fig. 5A, the highest dose of 20 µM significantly promotes U937 adhesion to HMEC-1 cells.
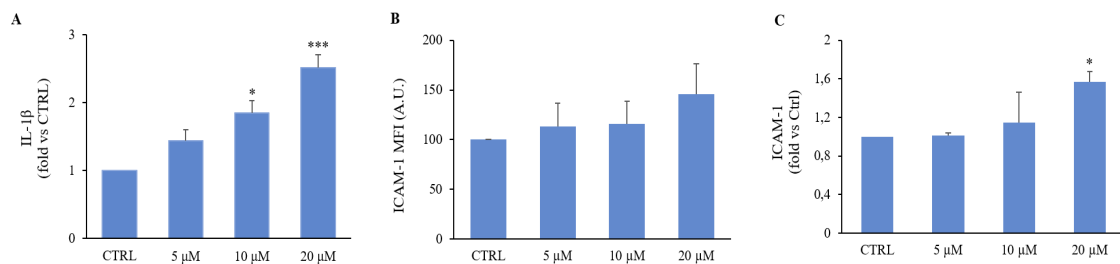

Fig. 4: (A) mRNA expression of IL-1β at 24h in cells treated with increasing concentrations of SEC-B. **(B)** Evaluation of ICAM-1 surface expression at 24h of SEC-B treatment. **(C)** Quantitative real-time PCR of mRNA of ICAM-1 expression after 24h of treatment. Data are expressed as mean ± SD (n = 3). *p<0.05; ***p<0.001 vs. untreated cells.
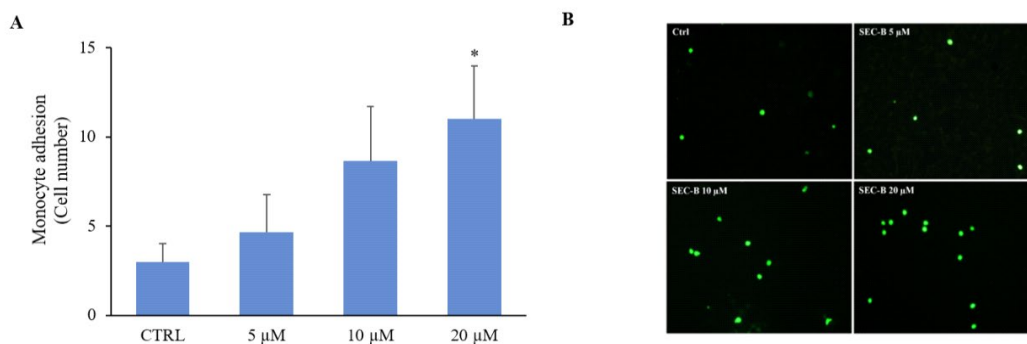

Fig. 5: (A) The number of bound U937 cells with 10X magnification in six randomly selected images was counted. **(B)** Representative fluorescence images showing the effects of SEC-B on adhesion of calcein-AM-labelled U937 cells to HMEC-1 pre-treated for 24h. Data are expressed as mean ± SD (n = 3). *p<0.05; vs. untreated cells

SEC-B up-regulates TWEAK expression followed by down modulation of Fn14 receptor

To further investigate endothelial activation and inflammatory response induced by SEC-B, we focused on TWEAK expression, because this cytokine is involved in the endothelium remodeling under chronic and acute inflammation (Liu H, et al. 2017). We found that SEC-B induces a release of soluble TWEAK in the medium after 4h of treatment. This finding was observed only in treated cells with 20 µM (Fig. 6A) showing a rise of 67% of sTWEAK compared to the untreated condition. To determine whether this observed changes in TWEAK secretion may reflect changes in its gene transcription, we measured mRNA level using real-time RT-PCR. Our analyses performed after 4h of stimulation with SEC-B, show an up-regulation of TWEAK mRNA in cells treated with 20 µM, suggesting that the sTWEAK release is coupled with a marked increase in its expression (Fig. 6B). On the other hand, prolonged exposure of SEC-B leads a significantly decrease (***$p<0.001$ vs. untreated cells) in TWEAK mRNA expression.

To elucidated the involvement of SEC-B in the TWEAK pathway, we analysed the expression of its receptor Fn14 (fibroblast growth factor-inducible 14). Our results demonstrate that HMEC-1 cells exhibit protein expression of Fn14 (Fig. 6C) that is down-modulated following SEC-B treatment. In addition, our data display a sharp decrease in Fn14 total mRNA expression at 24h in cells treated with 20 µM (Fig. 6D). This effect was probably coupled with the down-regulation of TWEAK expression detected at 24h, suggesting a potential involvement of TWEAK on regulation of Fn14 expression.
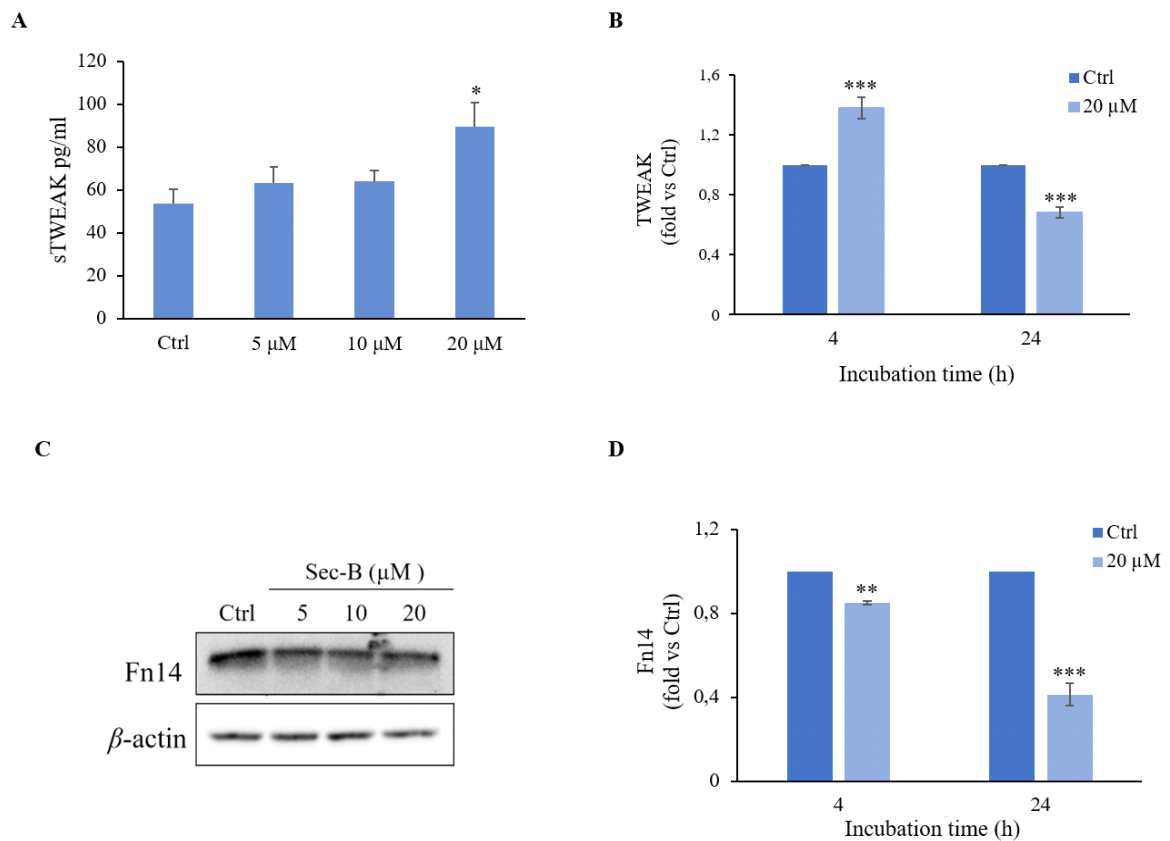
Fig. 6: (A) Dosage of soluble TWEAK in the medium after 4h of SEC-B treatment by ELISA. **(B)** Real-time PCR quantification of TWEAK at 4h and 24h in cells treated with 20 μM SEC-B. **(C)** Western blot result of Fn14 at 24h of SEC-B treatment in HMEC-1 cells, normalized with β-actin. **(D)** mRNA transcript of Fn14 in HMEC-1 treated with 20 μM SEC-B. Data are expressed as mean ± SD (n = 3). *p<0.05; **p<0.01; ***p<0.001 vs. untreated cells.

Discussion

ED is a pathological condition characterized by reduced vasodilation, pro-oxidative state and procoagulant activity. ED has been identified as the main event in the pathogenesis of macrovascular diseases including atherosclerosis (Widmer RJ, et al. 2014; Davignon J, et al. 2004).

Among the several proatherogenic factors, oxysterols were recently identified as potential candidates in atheroma formation, thanks to their proinflammatory and proapoptotic properties (Gargiulo S, et al. 2016). However, little is known about the mechanisms involved in development of atherosclerosis mediated by oxysterols.

In the present study we demonstrated that SEC-B, a recent oxysterol found in atherosclerotic plaque, is able to induce endothelial activation and dysfunction in HMEC-1 cells.

We firstly found that SEC-B increases intracellular ROS accumulation coupled with an enhancement of NO production. These results indicate that cells are able to activate compensatory mechanisms to counterbalance the oxidative stress. However, in our experimental model, we observed that prolonged exposure at the highest doses of SEC-B leads to reduce intracellular NO bioavailability associated with a drastic down-modulation of eNOS. Our findings are in agreement with previously data showing that a high amount of ROS is involved in decrease NO synthesis and production. In particular, ROS are known to quench NO with formation of peroxynitrite, which is a cytotoxic oxidant that bring to degradation of the eNOS cofactor tetrahydrobiopterin (BH4) leading to "uncoupling" of eNOS falling in NO production (Montezano AC, et. 2012; Endemann DH, et al. 2004).

In our study we also found a drastic downregulation of ET-1 expression. This last modification seems to suggest an impairment of HMEC-1 functions to counterbalance the effect of SEC-B. In literature, there are few data about the effects of oxysterols on ET-1 modulation in *in vitro* models. However, it was found that different degrees of LDL oxidation can display different effects on ET-1 regulation. In fact, He Y. and coworkers, demonstrated that extensively oxidized LDL inhibited ET-1 secretion from cultured endothelial cells. The author speculated that the inhibition of ET-1 release could exert a protective effect reducing vessel wall tone near atherosclerotic lesions (He Y, et al. 1996). Moreover, it also recently reported that the liver X receptors (LXR), a nuclear receptor bound by oxysterols, downregulates ET-1 gene expression by interfering with the AP-1/NF-kB signaling pathways (Gao M, et al. 2019).

Several studies have been reported that ED elicited in a persistent proinflammatory state can finally lead to cell death (Taniyama Y, et al. 2003; Wang X, et al. 2019). Consistent with this finding, SEC-B treatment in our study was associated with a decrease in cell viability and an increase in apoptotic and necrotic population.

Significantly, we found that cell dysfunction and death were associated by activation of endothelial cells. Indeed, in this condition, originally defined "endothelial activation" (Pober JS. 1988) endothelial cells responding to various stimuli, acquire the ability to perform new functions inducing expression of numerous cytokines. Notably, our results demonstrate that SEC-B is able to induce the expression of two important factors implicated in several aspects of vascular inflammation, such as IL-1β and ICAM-1 (Alfaidi M, et al. 2015; Zhao W, et al. 2017). Despite IL-1β is mainly produced by immune-derived cells, it has also been

demonstrated that it is expressed in atherosclerotic coronary arteries (Galea J, et al. 1996) and in cultured endothelial cells in response to different cytokine stimulations (Wilson HL, et al. 2007). Furthermore, ICAM-1 overexpression and subsequent adhesion of leukocytes to endothelial cells play a critical role in the early stage of atherosclerosis (Zhao W, et al. 2017). In our in *vitro* model, we demonstrated the ability of SEC-B to enhance U937 recruitment on HMEC-1 cells, thus contributing to endothelial activation and dysfunction. TWEAK is a cytokine of TNF superfamily and has been described in both membrane and soluble forms. Increasing amounts of data support the role of TWEAK in regulation of multiple cellular responses, including proinflammatory activity, angiogenesis and cell proliferation (Liu H, et al. 2017). To date, it is well established that endothelial cells are targets of sTWEAK (Lynch C, et al. 1999; Saas P, et al. 2000). When sTWEAK binds its receptor Fn14, it exerts adverse biological functions in atherosclerosis, inducing dysfunction of endothelial cells and inflammatory responses of monocytes/macrophages (Liu H, et al. 2017). Therefore, the activity of the TWEAK-Fn14 axis is greatly dependent on the induction of Fn14 expression (Raue U, et al. 2015).

However, there is little knowledge about the expression and secretion of this cytokine in the endothelial cells (Stephan D, et al 2013). In the present study, we firstly suggest that the highest dose of SEC-B (20 μM) is able to activate TWEAK expression coupled with a following secretion of its soluble form. On the contrary, prolonged exposure promotes a down modulation of TWEAK expression and a drastic down-regulation of its receptor. These observations are consistent with Chicheportiche et al., which have shown an inverse correlation between TWEAK mRNA level and inflammation after LPS treatment (Chicheportiche Y, et al. 2000). Accordingly, it is also possible that down-regulation both in protein and gene expression of Fn14 occurred at 24h of SEC-B treatment is correlated with the reduction of TWEAK expression. This finding suggests that TWEAK could be an important factor, along with TNF, implicated in inflammation and dysfunction mediated by SEC-B. However, further investigations are necessary to elucidate the involvement of SEC-B in TWEAK/Fn14 axis.

Conclusion

In summary, in this work it has been investigated the proinflammatory activation by SEC-B, which may be an important initiating mechanism to turn on the endothelial inflammatory

response leading to ED. Our data indicate that SEC-B is an oxidative and inflammatory factor that promotes HMEC-1 dysfunction by improving oxidative stress, cytokines expression and leucocyte-endothelial interaction. Thus, our findings provide novel insights on SEC-B-induced ED via pro inflammatory activation that may thereby contribute to atherogenesis.

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CONCLUSIONS

Atherosclerosis represents the most important vascular disorder which account for most CV morbidity and mortality. It is a chronic inflammatory disease that occurs within the arterial wall, affecting the vascular endothelium. Accordingly, the role of the vascular endothelium in preventing and counteracted the adverse factors becomes of crucial importance.
Nowadays, among the common proatherogenic factors, oxysterols are increasingly drawing attention of scientists to their biochemical properties relevant for the atherosclerosis disease (Arca M, et al. 2007). Despite growing *in vivo* studies, the final conclusion about the pro-atherosclerotic properties of oxysterols must still be clarified.

This study investigated the role of SEC-B, a recent oxysterol found in atherosclerotic lesions, as a possible inducer of ED. The finding reported in this study demonstrate that SEC-B is able to affect ER function and induce prooxidant and proinflammatory response in human endothelial cells. It exerts its effect inducing autophagy or apoptotic signaling leading to cell survival or death, dependently on the severity of the stress. Hight doses of SEC-B, that reflect a strong accumulation in arterial wall, trigger a severe activation of endothelial cells and a downmodulation in NO regulatory pathway bring to ED.

Finally, based on the results obtained, it is possible conclude that SEC-B is a proinflammatory oxysterol which adversely affects endothelial function. For this reason, SEC-B plays an important role in atherosclerosis development and may represent a potential biomarker in CV disease.

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