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DOSE-DEPENDENT EFFECT OF RESVERATROL ON HUMAN ENDOTHELIAL CELL FUNCTIONS

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PhD thesis in Biochemistry and Molecular Biology
of PhD Course in Life Sciences and Biotechnologies - University of Sassari

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person except that which appears in the citations and acknowledgements. Nor does it contain material, which to a substantial extent I have submitted for the qualification for any other degree of another university or other institution of higher learning.

Name: Signed:

Nguyen Thi Hieu Dung

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ABBREVIATION

Bax	Bcl-2-associated protein X
BSA	Bovine serum albumin
CAT	Catalase
CDK	Cyclin-dependent kinase
CYP	Cytochrome P450
CVD	Cardiovascular diseases
DMSO	Dimethyl sulfoxide
ETC	Electron transport chain
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
HUVEC	Human umbilical vein endothelial cell
JNK	c-Jun amino-terminal kinase MAPK
MAPK	Mitogen-activated protein kinase
MEK	MAPK ERK cascade
ODC	Ornithine Decarboxylase
PBS	Phosphate buffered saline
PI(3)K	Phosphoinositide-3-kinase
PKC	Protein kinase C
RES	Resveratrol
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide dismutase
TRAIL	TNF-related apoptosis-inducing ligand

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ABSTRACT

Purpose - Resveratrol consumption has been shown to exhibit cardiovascular-preventive and anticancer activities in preclinical studies. The mechanism of Resveratrol effect on human umbilical vein endothelial cell proliferation and apoptosis were not clearly understood. This study was undertaken to study on the dose-dependent effect of Resveratrol on HUVEC function and identify the potential signals involved in this pathway.

Experimental Design and Results - HUVECs were used as described in this study**.** Treatment of HUVECs with the concentrations of 1 and 100 μM RES showed that low concentrations increase cell growth, and decrease intracellular ROS levels while high concentrations cause the opposite effect significantly. The result of DNA fragmentation experiments may indicate cell death by apoptosis in HUVECs related to the expression of Bax and the decrease of the expression of Bcl-2. Using RES 50 μM, is consistent with the significant increase in DNA fragmentation, a typical marker of apoptosis. And at this concentration, RES makes endothelial cell cycle arrest by reducing Cyclin D1 and ODC, c-myc expression. Resveratrol dose-dependently modulates PKC activity on HUVEC proliferation and apoptosis.

Conclusion - Our data indicated that the RES effect on HUVECs is biphasic. PKC appears to be the potential mediator of the observed Resveratrol effects on HUVEC functions (proliferation, apoptosis), hypothesized this linked to the intracellular ROS levels.

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CHAPTER 1

INTRODUCTION

1.1. Reactive oxygen species

1.1.1. Chemistry and sources of ROS

Reactive Oxygen Species (ROS) are a variety of molecules and free radicals derived from molecular oxygen, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO.) and singlet oxygen (O.)…Cellular ROS are generated endogenously as in the process of mitochondrial oxidative phosphorylation or they may arise from interactions with exogenous sources. There are two types of ROS, those of free radicals, which contain unpaired valence shell electrons, and non-radical ROS, which do not have unpaired electron(s) but are chemically reactive and prone to become radical ROS. Examples of radical ROS are superoxide ($O_2^{-\bullet}$), hydroxyl (•OH), peroxyl ($RO_2\bullet$) and alkoxyl (RO•). Non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) [1], all of these oxidants are derived from molecular oxygen [2].

Figure 1.1. Complete and incomplete reduction of molecular oxygen [3].
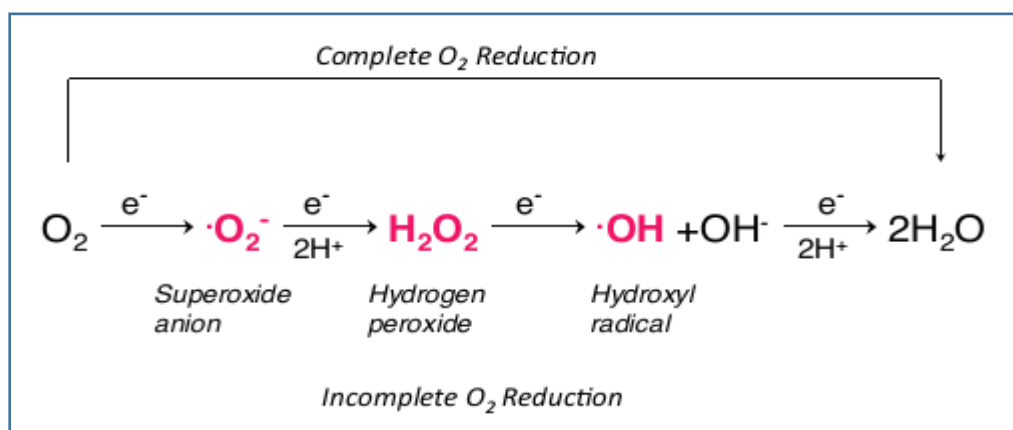
The amount of free radical production is determined by the balance of many factors, and ROS are produced both endogenously and exogenously. In

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mammalian cells the endogenous sources of ROS include mitochondria, plasma membrane, endoplasmic reticulum, cytosol and peroxisomes [4,5,6,7].

Mitochondria (mainly complex I & III, but also mono-amino oxidase, α-keto-glutarate dehydrogenase, glycerol phosphate dehydrogenase, p66[shc] [8]) seem to be quantitatively the most important subcellular site of $O_2^{-\bullet}$ and H_2O_2 production in mammalian organs and the steady state concentration of $O_2^{-\bullet}$ in the mitochondrial matrix is about 5- to 10-fold higher than that in the cytosolic and nuclear spaces [9]. Generation of mitochondrial ROS mainly occurs in the mitochondrial electron transport chain located on the inner mitochondrial membrane during the process of oxidative phosphorylation. Oxidative phosphorylation is involved to energy production in mitochondria. Five big protein complexes are responsible for this process. These mitochondrial electron transport chain complexes are named complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase).
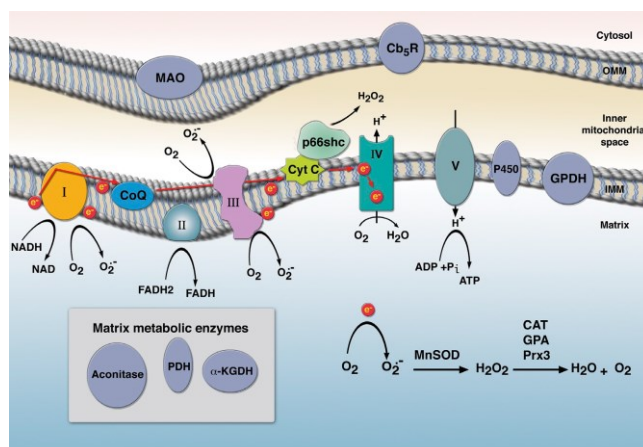

Figure 1.2. Reactive oxygen species generation and disposal in the mitochondria [10]

The endoplasmic reticulum (ER) contains the cytochrome P-450 (CYP) which is also one of the main sources of ROS and b5 enzymes, diamine oxidase, Ero1 [11]. It is a membrane-bound intracellular organelle that is primarily involved in lipid and protein biosynthesis. Smooth ER (lacking

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bound ribosomes) contains enzymes that catalyse a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The most extensively studied of these are the CYP. CYP belongs to a multi-gene family of heme proteins and its name derives from the spectral absorbance peak of their carbon monoxide-bound species at 450 nm [12,13]. They support the oxidative, peroxidative and reductive metabolism of such endogenous and xenobiotic substrates as environmental pollutants, agrochemicals, plant allele-chemicals, steroids, prostaglandins and fatty acids .

CYP has also been proposed as a source of ROS. Through the induction of CYP enzymes, the possibility for the production of ROS, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle [14]. ROS produced by CYP systems can potentially cause lipid peroxidation, cell toxicity, and death [12].

Also peroxisomes are an important source of cellular H_2O_2 production under physiologic conditions [15]. They contain a number of H_2O_2-generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase and fatty acyl-CoA oxidase. In mammalian peroxisomes, the potential sources of H_2O_2 formation are the oxidases that transfer hydrogen from their respective substrates to molecular oxygen. Peroxisomal catalase utilizes H_2O_2 produced by these enzymes to oxidize a variety of other substrates in "peroxidative" reactions [16]. These types of oxidative reactions are particularly important in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules (including ethanol) that enter the circulation. Another major function of the oxidative reactions carried out in peroxisomes is β-oxidation of fatty acids, which in mammalian cells occurs in mitochondria and peroxisomes [17]. Specific signaling roles have not been ascribed to peroxisome-derived oxidants, and only a small fraction of H_2O_2 generated in these intracellular organelles appears to escape peroxisomal catalase.

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In the cytosol, the arachadonic acid cascade, yielding prostaglandins and leukotrienes may generate ROS when the released lipid is metabolized. ROS can be generated as byproducts during metabolism of arachidonic acid, which to some degrees take place in practically every cell. Enzymes participating in the process are cyclooxygenase, lipooxygenase and CYP [18]. Arachidonic acid may be a source of ROS even by a non-enzymatic process.

Additional endogenous sources of cellular ROS are plasma membrane-associated oxidases. One of the best characterized is the NADPH oxidase [6]. It is present in both professional phagocytic cells (macrophages, neutrophils and eosinophils) and non-phagocytic cells (such as endothelial or smooth muscle cells) [19]. NADPH oxidase is considered as a "professional" ROS producer [20], whereas the other enzymes produce ROS only as by-products along with their specific catalytic pathways [21]. This multicomponent enzyme catalyses the one-electron reduction of O_2 to $O_2^{-\bullet}$, with NADPH as the electron donor through the transmembrane protein cytochrome b558 (a heterodimeric complex of gp91[phox] and p22[phox] protein subunits). The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to O_2 on the outside.

In addition to physiological sources of ROS, diverse exogenous agents can contribute to the intracellular production of free radicals. Most of these compounds cause the generation of superoxide ($O_2^{-\bullet}$) and hydrogen peroxide. The mechanism of action of many exogenous agents involves redox cycling whereby an electron is accepted to form a free radical and it is then transferred to oxygen. The exogenous sources of ROS are: xenobiotics, chlorinated compounds, environmental agents, metals (redox and nonredox), ions, and radiation [7]. Some peroxisome-proliferating compounds are among the classes of compounds that induce oxidative stress and damage in vitro and in vivo [4].
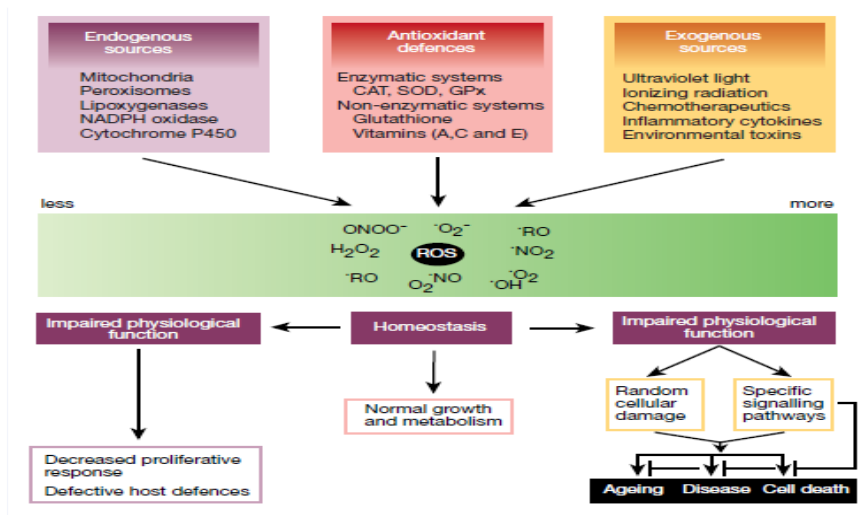
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Figure 1.3. The sources and cellular responses to ROS [10]

1.1.2. Functions of ROS

It has been established that ROS can be both harmful and beneficial in biological systems depending on the environment [22,23]. Beneficial effects of ROS involve, for example, the physiological roles in cellular responses to such as defense against infectious agents, and in the function of a number of cellular signaling systems. In contrast, overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans [24]. It has been reported that deleterious effects of ROS on human cells may end in oxidative injury leading to programmed cell death i.e. apoptosis [25].

- ROS and cell signalling

Although the endogenous generation of ROS are a consequence of metabolic activities, many environmental stimuli including cytokines, ultraviolet radiation, chemotherapeutic agents, hyperthermia and even growth factors generate high levels of ROS that can perturb the normal redox balance and shift cells into a state of oxidative stress. When the stress is severe,

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survival is dependent on the ability of the cell to adapt to or resist the stress, and to repair or replace the damaged molecules. Alternatively, cells may respond to the insult by undergoing apoptosis, a process whereby severely damaged cells are removed from the multicellular host, and which, within limits, preserves the organism [26].
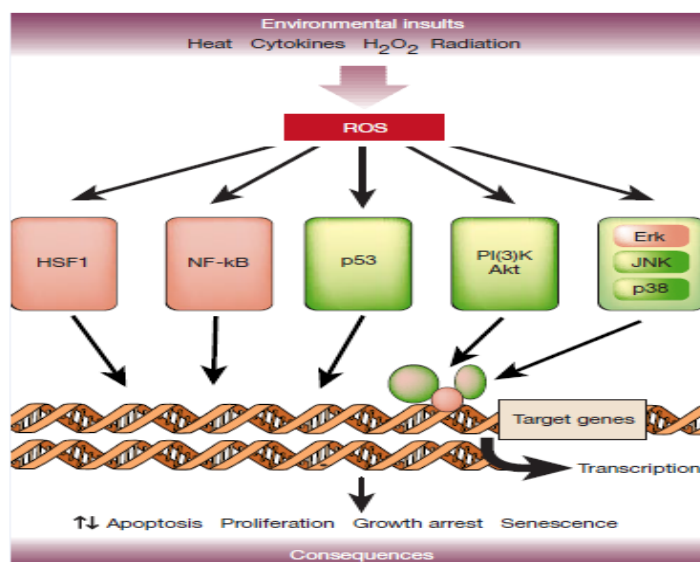

Figure 1.4. Major signalling pathways activated in response to oxidative stress [10]

Activation of these pathways is not unique to oxidative stress, as they are known to have central roles in regulating cellular responses to other stresses as well as regulating normal growth and metabolism. Indeed, in some situations the response to oxidants may involve overstimulation of normal ROS regulated signalling pathways. In general, the heat shock response, ERK, PI(3)K/Akt and NF-kB signalling pathways exert a pro-survival influence during oxidant injury, whereas activation of p53, JNK and p38 are more commonly linked to apoptosis. However, numerous exceptions to these generalities can be found.

The initiating events leading to activation of pathways in response to oxidants are incompletely understood. Although a large number of signalling pathways appear to be regulated by ROS, the signalling molecules targeted by ROS are less clear. There is growing evidence, however, that redox regulation

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might occur at multiple levels in the signalling pathways from receptor to nucleus. Receptor kinases and phosphatases themselves may be targets of oxidative stress. Growth factor receptors are most commonly activated by ligand-induced dimerization or oligomerization that auto-phosphorylates its cytoplasmic kinase domain. Ligand-independent clustering and activation of receptors in response to ultraviolet light have also been well demonstrated, and this effect appears to be mediated by ROS [16].

- ROS and proliferation

ROS can act as a messenger in signalling cascades involved in cell proliferation and differentiation [28]. In mammalian cells, interactions between growth factors and receptors are known to generate ROS, which at low concentrations are required to activate proliferative signalling for cell division [29,30]. Cell division is a series of coordinated events governed by the periodic expression of phase-specific cyclins (G1, S, G2 and M). At each phase, specific cyclins complex with, and activate CDKs to phosphorylate target proteins resulting in their activation or inhibition. Different cyclin-CDK combinations dictate the specificity of protein targets to restrict their activities for the events specific to each phase [31,32]. Activation of cyclin expression is key for progression in the cell-cycle, and transcription factors that activate their expression are modified post-translationally so that cyclin expression can be regulated accordingly.

In mammalian cells, multiple signalling pathways activate cell proliferation. Hormones, growth factors, or cytokines activate signal transduction cascades by the phosphorylation, of mitogen-activated kinases to activate transcription factors to induce expression cyclin D [33]. Entry into the cell-cycle, particularly from G0 to G1, is driven by the expression of cyclin D1, which is the only cyclin able to drive terminally differentiated cells back into the cell-cycle [34]. Transition from G1 to S phase also requires

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cyclin D to complex with Cdk4 and Cdk6, which phosphorylates pRb releasing its inhibition of the transcription factor E2F for gene expression of cyclin E and progression to S phase [31,32].

To prevent abnormal proliferation, the activity of cyclin-CDK can be inhibited by cyclin-dependent kinase inhibitor (CKI). Upon DNA damage, p53 is phosphorylated by upstream kinases and activates transcription of CKI p21, which sequesters and inhibits the activity of cyclin D1-CDK4/6 and cyclin E-CDK2, hence arresting cells at G1 phase [35]. In response to oxidative stress, sirtuins (SIRT1) deacetylate FOXO transcription factors for activation of oxidative stress response and induce cell-cycle arrest at G1 by the transcription of the CKI p27 [36].
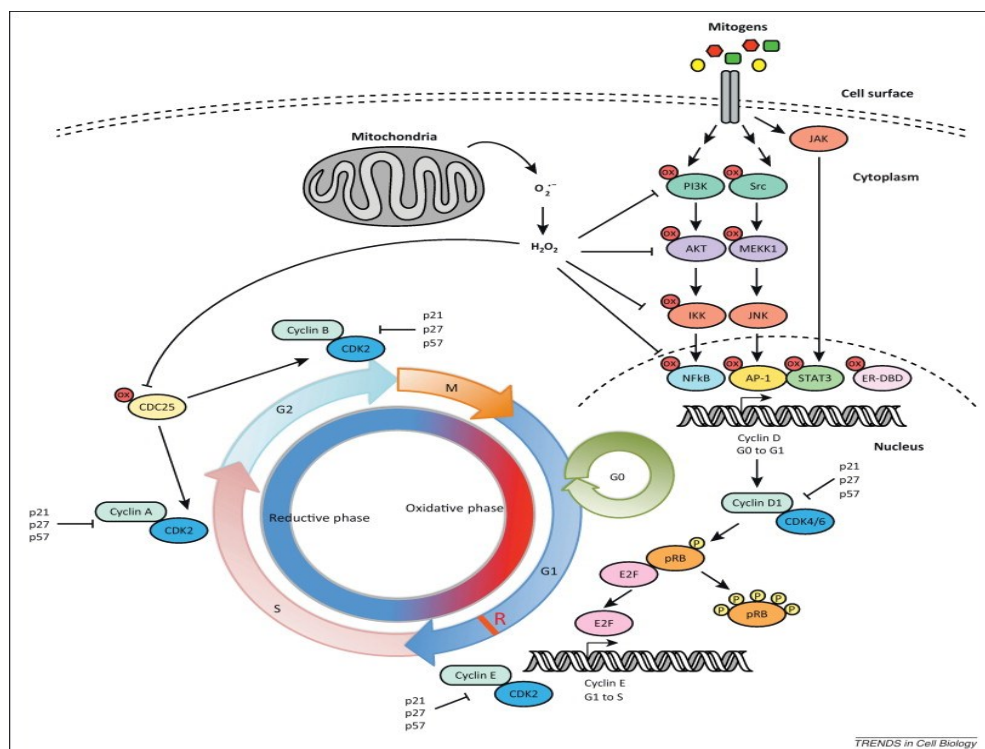
Figure 1.5: Overview of redox control of cell proliferation [37]

- ROS and apoptosis

Although under normal conditions there is a balance between ROS formation and antioxidants, in several pathological scenarios the antioxidant defences become insufficient resulting in oxidative stress leading often to

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apoptosis and cell death. Apoptosis (or programmed cell death) is the mechanism used by mammals, plants and other organisms to eliminate redundant or damaged cells [38]. Apoptosis can be initiated by a variety of stimuli, including hyperthermia, growth-factor or hormone withdrawal, glucocorticoids, oxidants, ionizing radiation and multiple classes of chemotherapeutic agents [39]. In the apoptotic process, initial stress-induced damage does not kill cells directly, rather it causes an apoptotic signaling program that leads to cell death [40].

Apoptotic cell death may be triggered through the extrinsic (receptor-mediated) or the intrinsic (mitochondria-mediated) pathway [41]. The extrinsic pathway of apoptosis is mediated by death receptors in that ligand-receptor binding initiates protein-protein interactions at cell membranes that activate initiator caspases. Major known receptors include Fas, TNF receptor 1, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1, and TRAIL receptor 2 [42]. The death receptors consist of three functional extracellular ligand-binding, transmembrane and intracellular domains. Apoptotic signaling is initiated by the association of death domain-containing adaptor proteins [43].

The intrinsic pathway can be triggered by many stimuli including ROS. Mitochondria are the major site of ROS production and accumulation of ROS may lead to the initiation of apoptosis [41]. Release of cytochrome c is considered as the central event, since it is important for aggregation of the adapter molecule (Apaf). The Bax, a proapoptotic member of the Bcl-2 family can directly induce mitochondria to release cytochrome c. The damage of the mitochondrial pores by ROS may contribute to cytochrome c release due to disruption of the mitochondrial membrane potential. In contrast, it is unclear how the initial ROS is released from mitochondria. If a sequential event is assumed, initial released ROS could directly or indirectly (via ceramide

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generation) increase the gating potential of the pore. Collectively, it seems that mitochondria are both source and target of ROS [44].
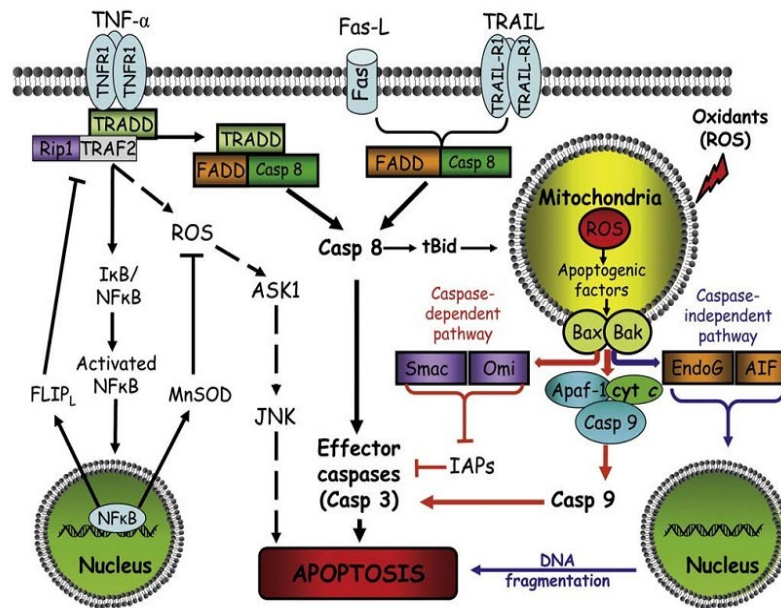

Figure 1.6. Death-receptor-mediated and mitochondrial pathways of cells apoptosis [43].

A complete understanding of the redox control of apoptotic initiation and execution could support the development of therapeutic interventions targeted at oxidative stress-associated disorders [43].

1.2. Endothelial cells

An endothelium lines the entire vascular system and is composed of a monolayer of endothelial cells. In an adult, the endothelium consists of approximately 1×10^{13} cells forming an almost 1 kg "organ". The vascular endothelium is versatile and multifunctional having many synthetic and metabolic properties, including the regulation of thrombosis and thrombolysis, platelet adherence, modulation of vascular tone and blood flow, and regulation of immune and inflammatory responses by controlling leukocyte, monocyte and lymphocyte interactions with the vessel wall. Perturbations of endothelial structure and function may also result in pathological states. Atherosclerosis, hemostatic dysfunction, and altered inflammatory and immune response are examples of situations where the endothelial cell plays a critical role.

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Endothelial cell structure and functional integrity are important in the maintenance of the vessel wall and circulatory function. As a barrier, the endothelium is semi-permeable and controls the transfer of small and large molecules. However, endothelial cells are dynamic and are can conduct variety of metabolic and synthetic functions. These cells exert significant paracrine and endocrine actions through their influence on the underlying smooth muscle cells or on circulating blood elements, such as platelets and white blood cells.

Under basal conditions, endothelial cells are intimately involved in maintaining the non-thrombogenic blood–tissue interface by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow. They produce and release a variety of vasoactive substances, such as prostacyclin and nitric oxide, both of which inhibit platelet aggregation and cause vasodilation. These mediators are released in response to a range of chemical stimuli, such as thrombin, bradykinin, or ADP, as well as changes in hemodynamic forces, such as alterations in blood pressure or flow. Endothelial cells can also be activated by various stimuli, such as thrombin or histamine, marked by a switch in their synthetic profile from basal conditions towards an activated state that is pro-thrombotic, pro-proliferative and vasoconstricting [45].

Figure 1.7. Illustration of blood vessel or artery
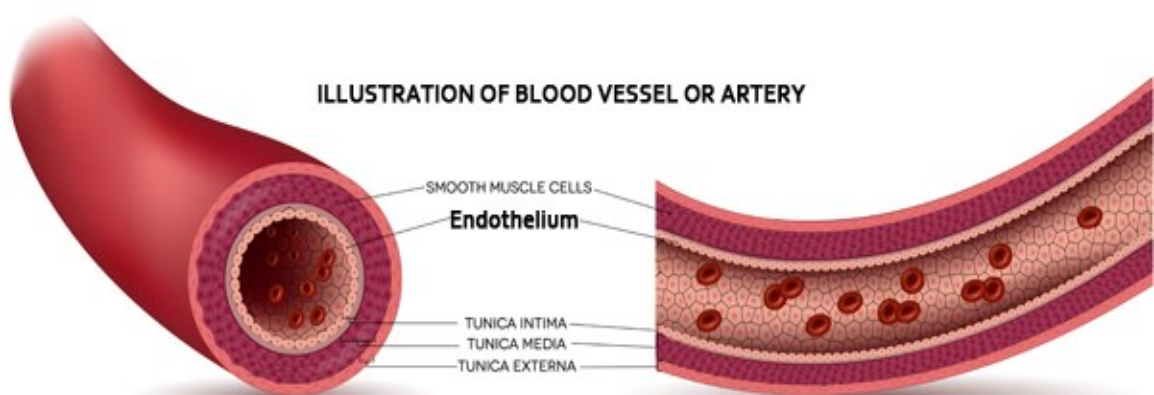
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According to the World Health Organization, from 2014, cardiovascular diseases (CVD) are the number one cause of death worldwide. One of the key players in maintaining proper cardiovascular function is the endothelium, the inner layer of all blood vessels. This monolayer of cells on one hand serves as a barrier between blood and the surrounding tissue and on the other hand regulates many aspects of vessel function. Therefore, it is not surprising that interventions reducing the risk for CVD improve endothelial function. There is a clear correlation between endothelial dysfunction, in which the endothelial homeostasis is disturbed, and the development and progression of many CVD [46].

1.3. Resveratrol

A plant phytoalexin, Resveratrol (RES) (3,5,4-trihydroxystilbene) as illustrated in figure 1.8, is low molecular mass (MW=228.2) natural product produced by secondary metabolites which are generally nonessential for the basic processes of the plant [141]. The first mention of RES was in a Japanese article in 1939 by Michio Takaoka, who isolated it from Veratrum album, variety grandiflorum, and later, in 1963, from the roots of Japanese knotweed: RES (3,5,4'-trihydroxy-trans-stilbene) is a natural compound found in red grape skin, Japanese knotweed (polygonum cuspidatum), peanuts, blueberries and some other berries. It is a powerful antioxidant produced by some plants to protect them against environmental stresses. Antioxidants neutralize free radicals, which are believed to be the cause of aging. Japanese knotweed is the plant source with the highest RES content. RES has been found in at least 72 plant species (distributed in 31 genera and 12 families), a number of which are included in the human diet, such as mulberries, peanuts and grapes. It occurs in two isoforms cis- and trans-RES, but trans-RES is more biologically active than its cis-isoform [142]. The trans-RES structure is similar to that of the synthetic estrogen, diethylstilbestrol. Indeed, it has been

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indicated that the trans-RES has greater anticancer and cardio-protective activities than the cis- isomer [47].
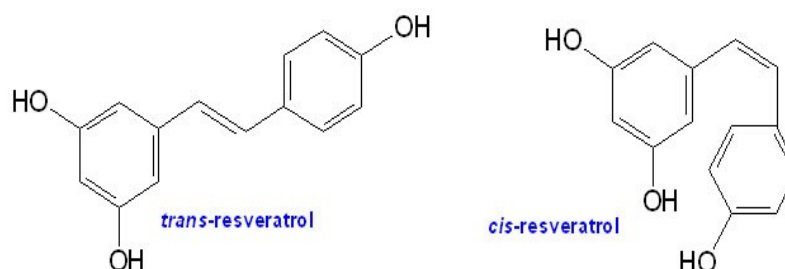

Figure 1.8. Chemical structures of resveratrol

Resveratrol is produced by various plants as a defense against stress, injury, excessive sunlight, ultraviolet radiation, infection, and invading fungi. RES is also considered a nutraceutical present in grapes, peanuts, pine trees, cassia and other plants, and many food products. In wine, the concentration of RES varies: red wines contain between 0.2 and 5.8 mg/l, depending upon the grape variety, whereas white wines contain 0.68 mg/l. This variation derives from the fact that red wine is extracted with the grape skin intact, whereas white wine is fermented after removal of the skin. Red wine contains more trans-resveratrol than white wine, whereas white has a higher concentration of cis-resveratrol. Concentrations of resveratrol in some natural foods are given in Table 1.1 [48].

Source	Resveratrol concentration
100% Natural peanut butter	~0.65 µg/g
Bilberries	~16 ng/g
Blueberries	~32 ng/g
Boiled peanuts	~5.1 µg/g
Cranberry raw juice	~0.2 mg/L
Dry grape skin	~24.06 µg/g
Grapes	0.16–3.54 µg/g
Peanut butter	0.3–1.4 µg/g
Peanuts	0.02–1.92 µg/g
Pistachios	0.09–1.67 µg/g
Ports and sherries	<0.1 mg/L
Ref grape juice	~0.50 mg/L
Red wines	0.1–14.3 mg/L
Roasted peanuts	~0.055 µg/g
White grape juice	~0.05 mg/L
White wines	<0.1–2.1 mg/L

Table 1.1. The amount of resveratrol found in natural foods [49]

RES attracted interest during early nineties in the context of "French

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paradox"; the phenomena wherein certain population of France, in spite of eating a regular high fat diet, was less susceptible to heart diseases [50]. The apparent cardioprotection was attributed to the regular consumption, in their diet, of moderate doses of red wine rich in RES [51].

In last few decades, RES has gained the attention of scientists worldwide; in fact, since then, dozens of reports have been the focus of various in vivo and in vitro studies aimed at investigating its effect on multiple pathophysiological processes and conditions. It has been reported to possess anti-inflammatory [52], vasorelaxing [53] activity and it has been demonstrated to inhibit lipid peroxidation [54,55] and platelet aggregation, which is a major contributor in the process of atherosclerosis [56], ex vivo. Moreover, RES has been shown to possess potential anticancer activity in various cancer cells at the initiation, promotion, and progression stages [57-59]. It is also well known to possess anti-cancer properties in animal model [60].

In the vast majority of cases, RES displays inhibitory/stimulatory effects in the micro-molarity range, which is potentially attainable pharmacologically. It appears that RES, as a pharmacological agent, has a wide spectrum of targets [49].

1.4. Resveratrol effects

There are an abundance of scientific studies and their novelty on multiple effects of RES.

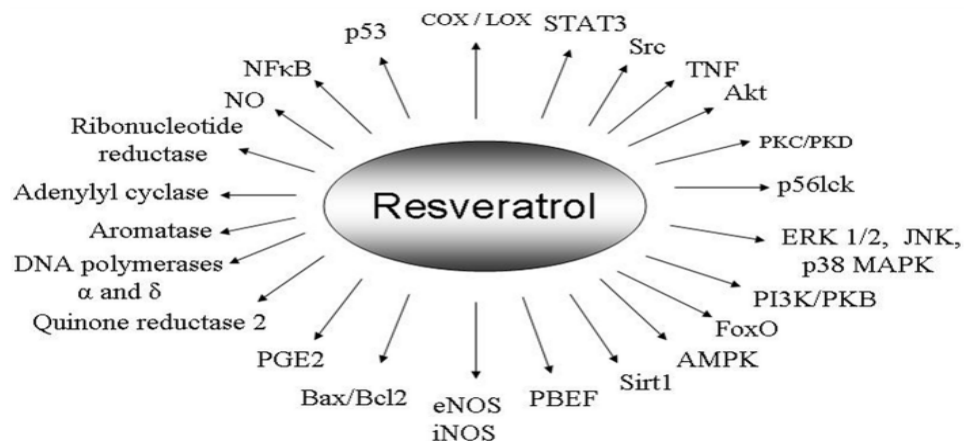Figure 1.9. Molecular targets of RES [131]

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- Cardiovascular protection by RES

- Anti-oxidative properties of RES

RES effectively scavenges free radicals and possesses antioxidant activities. In vitro studies indicate that RES is a poor antioxidant. However, in vivo RES functions as a potent antioxidant [61]. The in vivo anti-oxidative actions of RES, at least in part, is mediated by its ability to stimulate nitric oxide formation. RES can maintain the concentrations of intracellular antioxidants present in biological systems such as glutathione oxidation found in stressed peripheral blood mononuclear cells isolated from healthy humans [62]. Also it has been reported that RES increased glutathione amounts in human lymphocytes that were activated by hydrogen peroxide [63]. In human lymphocytes, RES increased the amounts of several antioxidant enzymes including glutathione peroxidase, glutathione-5 transferase, and glutathione reductase. It has been reported that RES restored glutathione reductase in cells subjected to tissue plasminogen activator (TPA)-mediated oxidative stress [64]. Studies have demonstrated that RES not only has potent antioxidant activity in vivo but also has the ability to inhibit platelet aggregation. These actions may help to prevent free radical formation. The number and position of hydroxyl substituents in RES analogues seems to play an important role in the potency of their inhibition of ROS production. Burkitt et al. provided evidence for hydroxyl-radical scavenging and a novel, glutathione-sparing mechanism of action for RES [65,66].

- Anti-inflammatory actions of RES

Numerous lines of evidence suggest that RES is a potent anti-inflammatory agent. The ability of RES to protect cardiovascular disorders mostly comes from the anti-inflammatory property of RES. RES significantly improved post-ischemic ventricular function and reduced myocardial infract size compared to the non-treated control group. Suppressed aberrant

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expression of tissue factor and cytokines in vascular cells achieved by RES is due to the anti-inflammatory role of RES [61,67]. Inflammation is intimately involved in the pathogenesis of atherosclerosis and other cardiovascular adverse events. RES has been shown to influence arachidonic acid metabolism, inflammatory cytokine production, ROS production, and NF-κB activity, all of which contribute to the enhancement of anti-inflammation activity and the exacerbation of cardiovascular complications [68,69]. As already described, RES can suppress the activation of transcription factor NF-κB, which is closely linked with inflammation. It can also suppress the expression of pro-inflammatory cytokines such as TNF, IL-1, IL-6 and IL-8 [70,71]. RES can abrogate the expression of proteins such as iNOS, COX-2 and 5-LOX, that mediate inflammation. Kimura et al. showed that RES inhibits the 5-LOX products 5-hydroxy-6,8,11,14- eicosatetraenoic acid (5-HETE) 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-diHETE) and leukotriene C4 (LTC4) at IC50 of 8.9 μM, 6.7 μM, and 1.37 μM, respectively. RES increases the expression of the tumor suppressor protein p53 prior to NSAID-activated gene -1 (NAG-1) induction, indicating that induction of NAG-1 expression by RES is mediated by p53 expression. These authors also showed that the p53-binding sites within the promoter region of NAG-1 play a pivotal role in controlling induction of NAG-1 expression by RES. RES exerted a strong inhibitory effect on the superoxide radical (O_2^-) and H_2O_2 produced by macrophages stimulated by Lipopolysaccharide (LPS) or Lipopolysaccharide -mimetic activities (PMA). RES also significantly decreased ^{3}H-arachidonic acid release induced by LPS and PMA or by exposure to O_2^- or H_2O_2. RES treatment caused a significant impairment of COX-2 induction stimulated by LPS and PMA or by O_2- or H_2O_2 exposure. These RES effects were correlated with a marked reduction of prostaglandin synthesis. These results indicate that the anti-inflammatory action of RES

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affects arachidonic acid mobilization and COX-2 induction [66,72].

- Anti-proliferative effects of RES

RES has been shown to suppress proliferation of a wide variety of tumor cells, including lymphoid and myeloid cancers, breast, colon, prostate, ovary, liver, lung cancers, melanoma and muscles. Several studies have shown the anti-proliferative effects of RES on B cells. Billard et al. investigated the effects of RES on leukemic cells from patients with chronic B-cell malignancies and found that RES had anti-proliferative effects and induced apoptosis in leukemic B-cells that correlated with activation of caspase-3, a drop in the mitochondrial transmembrane potential, reduction in the expression of the anti-apoptotic protein Bcl-2, and reduction in expression of inducible nitric oxide synthase [73]. In contrast, RES had little effect on the survival of normal peripheral blood mononuclear cells.

Fang demonstrated that RES inhibits NADH: ubiquinone oxidoreductase activity and lowers the level of induced ornithine decarboxylase activity leading to the anti-proliferative and anticancer actions [74]. Fontecave et al showed that RES is a remarkable inhibitor of ribonucleotide reductase and DNA synthesis in mammalian cells. The growth inhibitory and anti-proliferative properties of RES appear to be attributable to its induction of apoptotic cell death as determined by morphological and ultrastructural changes, inter-nucleosomal DNA fragmentation, and increased proportion of the sub-diploid cell population. RES treatment results in a gradual decrease in the expression of anti-apoptotic Bcl-2 [75].

Haider et al showed that RES leads to a reversible arrest in the early S phase of the vascular smooth muscle cell cycle, accompanied by an accumulation of hyper-phosphorylated retinoblastoma protein. In contrast to studies with other cell systems, RES decreases cellular levels of the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1. They also found that RES

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only slightly inhibits phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, Akt and p70S6 kinase upon serum stimulation and that the observed S-phase arrest is not linked to an increase in apoptotic cell death, since there was no detectable increase in apoptotic nuclei and in levels of the pro-apoptotic protein Bax [76,77].

- Resveratrol induces apoptosis

Apoptosis is a mode of cell death that differs from necrosis. While the former is characterized by initiation of cell death from the outside of the cell, the latter is a death mechanism initiated from inside the cell, primarily from the mitochondria [78,79]. Apoptosis is usually mediated through the activation of caspases. Mechanistically, two different types of apoptosis have been described; one that is caspase-8-dependent and receptor-mediated (type I), and the other that is caspase-9-dependent and usually mediated through the mitochondria (type II). RES has been shown to mediate apoptosis through a variety of different pathways as described below:
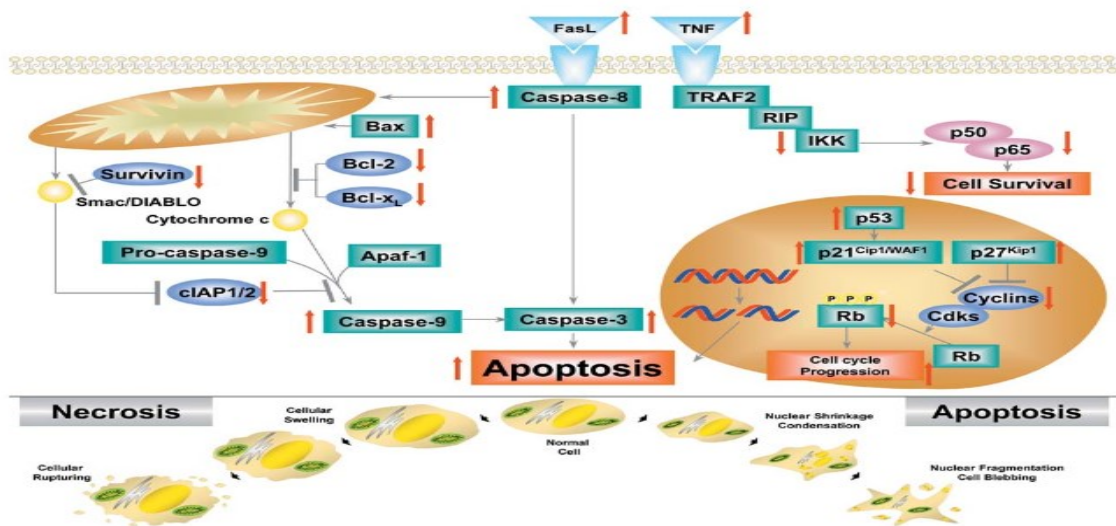
Figure 1.10: Various proposed mechanisms of apoptosis of tumour cells by resveratrol [66]

- Fas pathway: Resveratrol has been shown to induce death receptors, that in turn activate apoptosis, through the type I pathway. Fas is one of the death receptors of the tumor necrosis factor (TNF) superfamily [80]. Clement et al. showed that RES triggered FasL signaling-dependent apoptosis in

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human tumor cells [81]. Altogether, these results indicate that the ability of RES to induce redistribution of the Fas receptor in membrane rafts may contribute to the molecule's ability to trigger apoptosis in colon cancer cells.

- Mitochondrial pathway: RES has also been shown to activate the type II pathway. This pathway for apoptosis is mediated through the activation of the mitochondrial pathway. Dorrie et al. showed that RES induced extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in all cells and that these effects were independent of Fas signaling [78]. Tinhofer et al. showed that RES induced apoptosis via a novel mitochondrial pathway controlled by Bcl-2 [79].

- p53 activation pathway: p53 is a tumor suppressor gene. There are numerous reports about the role of p53 in RES-induced apoptosis [76,82]. Huang et al. found that RES-induced apoptosis occurred only in cells expressing wild-type p53 (p53+/+), but not in p53-deficient (p53-/-) cells, while there was no difference in apoptosis induction between normal lymphoblasts and sphingomyelinase-deficient cell lines [83]. These results demonstrated that RES induces apoptosis through activation of p53 activity, suggesting that RES's antitumor activity may occur through induction of apoptosis. Hsieh et al. showed that RES inhibited proliferation of pulmonary artery endothelial cells, which correlated with suppression of cell progression through the S- and G2-phases of the cell-cycle and was accompanied by increased expression of p53 and elevation of the level of Cdk inhibitor p21Cip1/WAF1 [84]. Lu et al. showed that RES analogues significantly induced expression of p53, GADD45 and Bax genes and concomitantly suppressed expression of the Bcl-2 gene in human fibroblasts transformed with SV40 virus (WI38VA), but not in non-transfected WI38 cells [85]. A large increase in p53 DNA-binding activity and the presence of p53 in the Bax promoter binding complex suggested that p53 was responsible for the

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Bax gene expression induced by RES in transformed cells. She elucidated the potential signaling components underlying RES-induced p53 activation and induction of apoptosis.

- Suppression of MAPK by RES

Three different MAPK have been identified: ERK1/2, JNK and p38 MAPK. While ERK1/2 have been implicated in the proliferation of cells, JNK and p38 MAPK are activated in response to different types of stress stimuli. JNK activation is needed for activation of AP-1; it also mediates apoptosis in some situations. Numerous studies suggest that RES modulates all three of these protein kinases. Miloso et al. showed that RES induced activation of ERK1/2 in human neuroblastoma SH-SY5Y cells. In undifferentiated cells, RES 1 μM induced phosphorylation of ERK1/2, which was already evident at 2 minutes, peaked at 10 minutes and still persisted at 30 minutes. A wide range of RES concentrations (from 1 pM to 10 μM) were able to induce phosphorylation of ERK1/2, while higher concentrations (50-100 μM) inhibited phosphorylation of MAPK [86,87].

*Suppression of protein kinases by RES

Protein kinase C (PKC) has been shown to play a major role in tumorigenesis. The PKC isozyme subfamily consists of cPKC-α, -β, and -γ, nPKC-D and - ε, and αPKC-ζ. Numerous reports indicate that RES can inhibit PKC. Garcia-Garcia et al. showed that RES was incorporated into model membranes and inhibited PKC-α activity [88]. RES activated by phosphatidylcholine/ phosphatidylserine vesicles inhibited PKC-α with an IC50 of 30 μM, whereas that activated by Triton X-100 micelles inhibited PKC-α with an IC50 of 300 μM. These results indicate that the inhibition of PKC-α by RES can be mediated, at least partially, by membrane effects exerted near the lipid-water interface. Stewart et al. showed that RES preferentially inhibited PKC-catalyzed phosphorylation of a cofactor-

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independent, arginine-rich protein substrate by a novel mechanism. While RES has been shown to antagonize both isolated and cellular forms of PKC, the weak inhibitory potency observed against isolated PKC cannot account for the reported efficacy of the polyphenol against PKC in cells. Stewart et al. analyzed the mechanism of PKC inhibition by RES and found that RES has a broad range of inhibitory potencies against purified PKC that depend on the nature of the substrate and the cofactor dependence of the phosphotransferase reaction. RES weakly inhibited the Ca^{2+}/phosphatidylserine-stimulated activity of a purified rat brain PKC isozyme mixture (IC50, 90 μM) by competition with ATP (Ki, 55 μM). Consistent with the kinetic evidence for a catalytic domain-directed mechanism was RES's inhibition of the lipid-dependent activity of PKC isozymes with divergent the regulatory domains, and it was even more effective in inhibiting a cofactor- independent catalytic domain fragment of PKC generated by limited proteolysis. This suggested that regulatory features of PKC might impede RES inhibition of the enzyme. To explore this, the authors examined the effects of RES on PKC-catalyzed phosphorylation of the cofactor-independent substrate protamine sulfate, which is a polybasic protein that activates PKC by a novel mechanism. RES potently inhibited protamine sulfate phosphorylation (IC50, 10 μM) by a mechanism that entailed antagonism of the activation of PKC by protamine sulfate and did not involve competition with either substrate. Protein kinase D (PKD) is a member of the PKC superfamily with distinctive structural, enzymic and regulatory properties. Identification of the cellular function(s) of PKD has been hampered by the absence of a selective inhibitor. Stewart et al. compared the effects of RES against the auto-phosphorylation reactions of PKC isozymes to those against the auto-phosphorylation reactions of the novel phorbolester-responsive kinase PKD [89]. They found that RES inhibited PKD auto-phosphorylation, but had only negligible effects against

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the auto-phosphorylation reactions of representative members of each PKC isozyme subfamily (cPKC-α, -β1 and -γ, nPKC-D and -ε, and αPKC-ζ). Resveratrol was comparably effective against PKD auto-phosphorylation (IC50, 52 μM) and PKD phosphorylation of the exogenous substrate syntide-2 (IC50, 36 μM). The inhibitory potency of RES against PKD is in line with those observed in cellular systems and against other purified enzymes and binding proteins that are implicated in the cancer chemo-preventive activity of the polyphenol. Thus, PKD inhibition may contribute to the cancer chemo-preventive action of RES. Atten et al. demonstrated that RES treatment significantly inhibited PKC activity of KATO-III human gastric adenocarcinoma cells and of human recombinant PKC-α [90]. Woo et al. showed that RES inhibited PMA-mediated PKC-Δ activation, which led to suppression of MMP-9 [91]. The COP9 signalosome (CSN), purified from human erythrocytes, possesses kinase activity that phosphorylates proteins such as c-Jun and p53, with consequences for their ubiquitin-dependent degradation. Uhle et al. showed that RES could block the CSN-associated kinases protein kinase CK2 and PKD and induce degradation of c-Jun in HeLa cells [92].

- Suppression of cell-cycle proteins by RES

Numerous reports indicate that RES inhibits proliferation of cells by inhibiting cell-cycle progress. Various reports indicate that RES inhibits different cells at different stages of the cell-cycle. The arrest of cells in G1-phase [93], S-phase[94], S/G2-phase [84] and G2-phase [95] of the cell-cycle has been reported. Why the effects of RES on different cell types vary so widely is not clear. Which cell-cycle proteins are modulated by RES has been investigated in detail. Wolter et al. showed the down-regulation of the cyclin D1/Cdk4 complex by RES in colon cancer cell lines [96]. Yu et al. showed that, following treatment of H22 tumor-bearing mice with RES at 10 or 15

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mg/kg bodyweight for 10 days, the growth of transplantable liver cancers was inhibited by 36.3% or 49.3%, respectively [97]. The levels of expression of cyclin B1 and Cdc2 protein were decreased in treated tumors, whereas the expression of cyclin D1 protein did not change. Liang et al. showed that RES induced G2 arrest through the inhibition of Cdk7 and Cdc2 kinases in colon carcinoma HT-29 cells [95]. Larrosa et al. showed that RES and the related molecule 4-hydroxystilbene induced S-phase arrest and up-regulation of cyclins A, E and B1 in human SK-Mel-28 melanoma cells. Thus, it is clear that the effects of RES on the cell-cycle are highly variable [98]. Kuwajerwala et al. showed that RES had a dual effect on DNA synthesis [99]. At concentrations of 5-10 μM, it caused a 2- to 3-fold increase in DNA synthesis, and at doses ≥15 μM, it inhibited DNA synthesis. The increase in DNA synthesis was seen only in LNCaP cells, not in the androgen-independent DU145 prostate cancer cells or in NIH/3T3 fibroblast cells. The RES-induced increase in DNA synthesis was associated with enrichment of LNCaP cells in S-phase and concurrent decreases in nuclear p21Cip1/WAF1 and p27Kip1 levels. Furthermore, consistent with the entry of LNCaP cells into the S-phase, there was a dramatic increase in nuclear Cdk2 activity associated with both cyclin A and cyclin E.

- Anti-atherosclerotic effects of RES

Atherosclerosis is defined histologically as the thickening of an arterial wall due to the deposition of lipids. Atherosclerotic plaque formation can be divided into several stages, beginning with the initial lesion, and progressing through the buildup of intimal fatty streaks, appearance of the progressing lesion and finally advancing to an established lesion. Atherosclerosis predominantly affects the intimal layer of the arterial vessel wall. It is characterized by the deposition of extracellular lipids, the proliferation and migration of local smooth muscle cells, and a chronic inflammation. It leads

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to luminal narrowing and/or thrombus formation, resulting in clinical events such as coronary artery disease, peripheral arterial disease or stroke [100]. Due to the involvement of lipids, especially low density lipoproteins (LDLs), in the atherosclerotic process, it could be of interest to improve the lipid profile. Some preclinical studies have shown that RES could modify this profile, notably by decreasing plasma triglyceride and LDL-cholesterol levels, and by increasing HDL-cholesterol. As reported by Cho et al [101], RES could also potentiate the hypocholesterolemiant action of pravastatin, by down-regulating the 3-hydroxy-3-methyl-glutaryl-CoAreductase (HMG-CoA reductase), an enzyme that intervenes in the first steps of cholesterol biosynthesis. Besides, RES could increase the expression of the LDL receptors in hepatocytes in vitro, thereby contributing to further decrease blood LDL-cholesterol levels. In addition, the antioxidant properties of RES resulted in a decrease of LDL oxidation (process directly involved in atherogenesis), an induction of several endogenous antioxidant systems, and anti-inflammatory properties. The inhibition of smooth muscle cell migration also participates to the anti-atherogenic properties of RES. All these properties show that RES acts on the major factors involved in the atherosclerotic process. Accordingly, several potential targets related to the beneficial effects of RES in CVDs have been highlighted. RES especially activates SIRT-1 (a class III histone deacetylase), eNOS, Nrf2 and antioxidant response element, and decreases TNFα production. The global action of RES thus results in a decrease of endothelial apoptosis, endothelial activation and vascular inflammation, and improves the endothelial function. Actions of RES in the first steps of the atherogenic process have been observed. Indeed, RES has been shown to decrease the expression of adhesion molecules (intercellular adhesion molecule-1, ICAM-1, and vascular cell adhesion molecule-1, VCAM-1) via inhibition of NF-κB pathway activation [102].

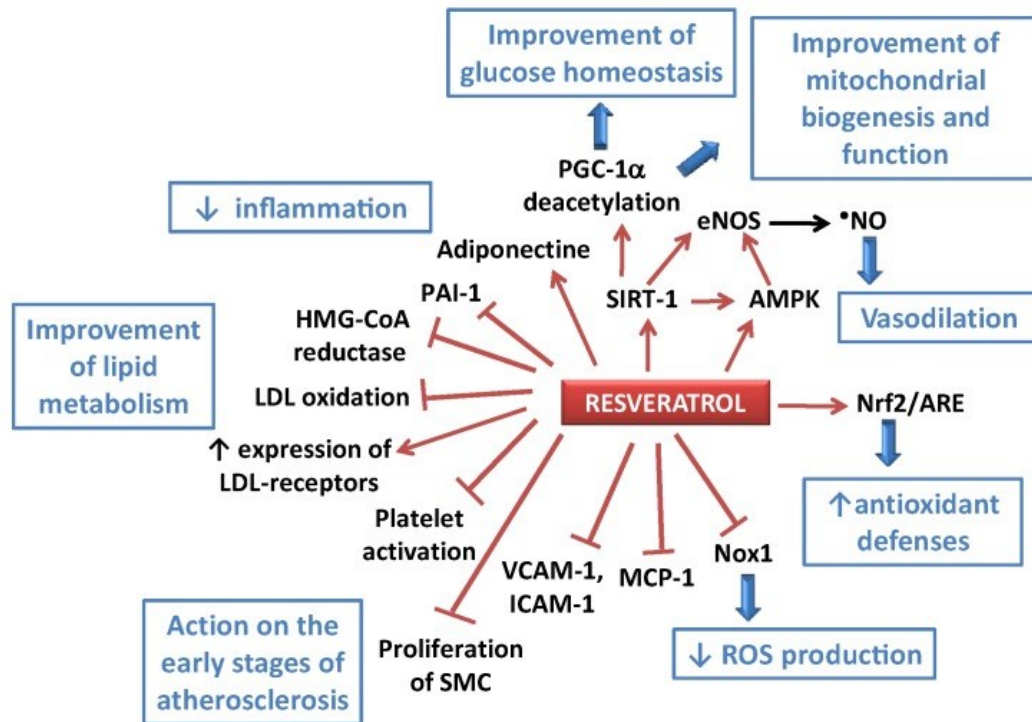Nguyen Thi Hieu Dung – *Dose-dependent effect of Resveratrol on human endothelial cell functions*
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Figure 1.11: Some of the potential effects of RES towards atherogenesis and impaired metabolism.

- Antitumor effects of resveratrol

Numerous reports suggest that RES exerts therapeutic effects against cancer. Carbo et al. found that administration of RES to rats inoculated with a fast-growing tumor (the Yoshida AH-130 ascites hepatoma) caused a very significant decrease (25%) in the tumor cell content [63]. The effects of the diphenol were associated with an increase in the number of cells in the G2/M cell-cycle phase. Interestingly, flow cytometric analysis of the tumor cell population revealed the existence of an aneuploidy peak (representing 28% of total), which suggests that RES decreases tumor cell numbers by inducing apoptosis.

The pharmacokinetic studies have revealed that after absorption of RES the target organs are liver and kidney, where it is concentrated after absorption and is mainly converted to a sulfated form and a glucuronide conjugate. In vivo, RES blocks the multistep process of carcinogenesis at various stages: it blocks

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carcinogen activation by inhibiting aryl hydrocarbon-induced CYP1A1 expression and activity, and suppresses tumor initiation, promotion and progression. Limited data in humans have revealed that RES is pharmacologically quite safe. Currently, structural analogs of RES with improved bioavailability are being pursued as potential therapeutic agents for cancer. It is reported that consumption of red wine is associated with a slight but statistically significant reduction in the development of lung cancer. Red wine consumption, which has been associated with a reduced risk of heart disease, cancer, and the common cold, may also protect against lung cancer [103]. Red wine contains tannins and RES, substances which could explain the drink's anticancer properties. Tannins act as antioxidants, which mop up free radicals-particles that are harmful to cells. RES is known to fight cancer tumor growth [61,104].

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CHAPTER 2
RESEARCH OBJECTIVES

Endothelial cells form a continuous monolayer at the inner wall of all human blood vessels and play a key role in the development of vascular diseases. Under pathological conditions, the tight control over endothelial functions which is critical for vascular homeostasis can be lost, creating a dysfunctional endothelium that contributes to the development of atherosclerosis, cardiovascular diseases and aging. The etiology of these altered endothelial functions is multi-factorial and the mechanisms underlying them are complex and not yet fully elucidated. Today, there is substantial evidence that many endothelial functions are sensitive to the presence of ROS and subsequent oxidative stress.

Free radicals are, indeed, now considered as key regulatory molecules vital for life, but they cause cellular and organ damage when produced in excess or when innate antioxidant defenses are overwhelmed [105]. A variety of pathogenic stimuli can increase ROS production within the endothelial cell triggering biochemical and cellular processes, such as apoptosis and proliferation that eventually result in endothelial dysfunction [106].

In this context, it has been hypothesized that RES consumption, can counteract the effects of ROS by preventing ROS-induced oxidative damage and preserve endothelial function reducing the occurrence of cardiovascular events [107]. In fact their consumption has been associated with a reduced incidence of risk factors for CVD [108]. CVD is of multifactorial etiology associated generally to a variety of risk factors for its development including hyper-cholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity among others. As a result, scientific resources have focused to a large extent on the role that RES could play to delay or prevent

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oxidative stress and consequently the incidence of chronic disorders. Among antioxidants, RES was 95 % efficient at preventing lipid peroxidation, compared with approximately 65 % for Vitamin E and 37 % for Vitamin C [109]. Resveratrol intake has been suggested to play a role in the reduction of risk of coronary heart disease and also have a cancer preventive activity in tumor initiation, promotion and progression.

The widely accepted notion that consumption of RES is useful to counteract oxidative stress and promote good health is at the basis of popular antioxidant rich diets and supplementations. However, the clinical use of RES has generally failed to live up to their early promise; in fact many controlled clinical trials have failed to demonstrate that increased RES consumption has a protective action against CVD [108]. In the endothelium, ROS are not only involved in pathological processes but, by modulating redox-regulated intracellular signals, they also finely tune EC physiology [110]. It has been proposed that ROS might function as dual effectors modulating both prosurvival and antisurvival signals [111]. Indeed, RES effects appear to be concentration dependent [112] and the molecular mechanisms underlying this phenomenon, as well as the associated outcomes in vascular cells, are largely unknown.

Therefore, the purpose of the research was to assess how RES affects differently the (patho)physiology of endothelial cells extracted from human umbilical vein. We initially evaluated the change of intracellular levels of ROS in presence of RES.

To determine whether the observed RES effect on HUVECs may be related to the employed concentrations.

Finally we investigated the molecular mechanism underlying the observed phenomena that modulate RES effects on HUVEC functions.

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CHAPTER 3
MATERIALS AND METHODS

3.1. Reagents

Resveratrol was purchased from Sigma-Aldrich (St Louis, USA; catalog number: R5010), dissolved in DMSO (dimethyl sulfoxide; Sigma-Aldrich), and stored at -20°C. For all experiments, the final concentrations of Resveratrol were prepared by diluting the stock in M199 medium and then, in sterilized filter. The final concentration of DMSO in cultured medium was 0.1% which caused no measurable effect on cell growth.

3.2. Cell culture and treatments

Human endothelial cells were isolated from human umbilical cords, called HUVEC, and cultured as previously described. Briefly, HUVECs were detached from the interior of the umbilical vein of a 30-cm segment of cord by treatment for 10 minutes at 37°C with 0.05% (w/v) collagenase Type II from Clostridium hystolyticum (Gibco BRL, Paisley, UK) in medium M199 (Gibco, BRL, Paisley, UK) containing 100 units/ml of penicillin G sodium salt and 100 µg/ml streptomycin sulphate (Sigma Chemical Co., St. Louis, MO, USA). HUVECs were harvested at 2000 rpm for 10 minutes and finally resuspended in 5 ml of medium M199 supplemented with 10% (v/v) FCS, 10% (v/v) fetal newborn-calf serum (Gibco BRL, Paisley, UK), 2 mM L-glutamine and antibiotics as above. Cells were then plated in 25 cm^2 tissue culture flasks (Falcon, Oxnard, CA, USA) and cultured in an atmosphere of 5% CO_2 /95% air. When confluent, HUVECs were subcultured at a split ratio of 1 :2 by a brief treatment with 0.1% trypsin plus 0.02% EDTA in phosphate buffered saline (PBS) (120 mM NaCl, 2.5 mM KCl, 8.5 mM NaH_2PO_4 , 1.5 mM

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KH$_2$ PO$_4$), pH 7.3. Cultured cells were identified as endothelial cells by their typical cobblestone appearance and production of von Willebrand factor as measured by enzyme-linked immunosorbent assay. Cell viability was checked by the trypan blue-exclusion method and used within three passages. In each experiment, HUVECs were plated at a density of 80 000 cells/ml in 24-well plates (Falcon, Oxnard, CA, USA). After 24 hours, the medium was replaced and cells were synchronised following a 48-hour incubation in serum-free medium M199 containing 0.2% (w/v) bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) [113].

Finally, the cells were stimulated with or without RES (from 1 to 100 μmol/l in DMSO) as indicated in the figures.
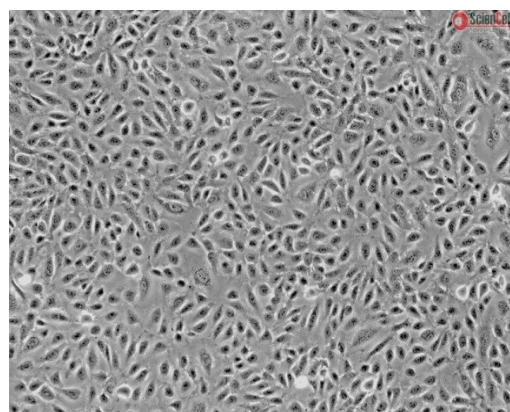

Figure 3.1. Segments of cord　　　　Figure 3.2. Endothelial cells under microscope

3.3. Determination of intracellular ROS levels

Intracellular ROS levels were determined by using the ROS molecular probe 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) (Molecular Probe, Eugene, OR). Within the cell, esterases cleave the acetate groups on H$_2$DCF-DA, thus trapping the reduced form of the probe (H$_2$DCF). Intracellular ROS oxidize H$_2$DCF, yielding the fluorescent product, DCF. After treatments, cells were incubated for 30 minutes with Hanks' Balanced Salt Solution (HBSS) containing 5 μM H$_2$DCF-DA, then washed twice with

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HBSS, and fluorescence was measured by using a GENios plus microplate reader (Tecan, Mannerdorf, CH). Excitation and emission wavelengths used for fluorescence quantification were 485 and 535 nm, respectively. All fluorescence measurements were corrected for background fluorescence and protein concentration. Using untreated cells as a reference, the antioxidant and pro-oxidant outcome was evaluated by comparison of three measurements and expressed as a percentage of untreated controls.

3.4. Determination of cell viability (MTT assay)

Cell metabolic activity was assessed in 96-well plates (BD Falcon) by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI).

Yellow MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. This reduction occurs only when mitochondrial reductase enzymes are active, therefore conversion can be directly related to the number of viable cells.

Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in a large amount of MTT formazan formed and an increase in absorbance.

So after treatments for 24 hours, cells were added with 20 μl MTT solution (0.1 mg/ml) in medium M199 and incubated at 37°C in a cell incubator for 2 hours. At the end of the incubation period, the medium was removed, and the cell monolayer was washed twice with HBSS. The converted dye was solubilized with acidic isopropanol (0,04 N HCl in absolute isopropanol). The absorbance was then read at 570 nm with a multiplate reader (Microplate Reader VERSAmax, Molecular Devices).

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3.5. Determination of DNA synthesis

To determine DNA synthesis, synchronised HUVECs cultured in 12-well plates (Becton Dickinson) were serum-starved following a 24-hour incubation in serum-free medium and then treated as described in figure legends. During the last 24 hours, cells from each experimental group were added with 1 µCi/ml [^{3}H] thymidine (Specific activity 5 Ci/mmol, Amersham) for 1-hour at 3-hour intervals. At the indicated time points, the medium was removed and the cell monolayer in each well was washed twice with PBS (1 ml), exposed to 5% (v/v) trichloroacetic acid (500 µl) for 5 minutes, then fixed in methanol (500 µl). Finally, the cells were digested by the addition of 25 M formic acid (500 µl). Each formic acid digest was transferred with one rinse of PBS (1 ml) to a scintillation vial containing 3.5 ml of INSTA-GEL scintillation fluid (Packard Instruments Co., Meriden, CT, USA), and radioactivity was determined by liquid scintillation counting using a Wallac 1215 RackBeta liquid scintillation counter (LKB Instrument Inc., Gaithersburg, MD, USA. And DNA synthesis was determined as previously described by assessing [^{3}H] thymidine incorporation.
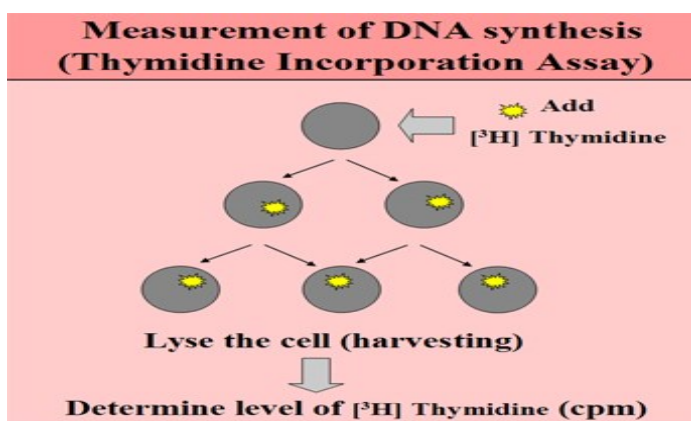
3.6. DNA fragmentation

Cellular DNA was prelabeled with [^{3}H] thymidine (1 µCi/mL) for 24 hours in the presence of different concentrations of RES. At the end of this

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time point, cells were permeabilized on ice for 1 hour with a hypotonic buffer, and centrifuged at 14.000g at 4°C. The supernatants (S1) were collected, and the pellets (P1) were suspended in 2mM EDTA and incubated on ice for 2 hours. Samples were centrifuged as before, the new supernatants (S2) were collected and the final pellets (P2) were hydrolysed with PCA 0,5 M. Finally all the fractions were counted on a liquid scintillation counter, and the results are expressed as the percentage of the total DNA released in the combined supernatants (representing fragmented DNA).

$$\text{Released DNA fragment (\%)} = \frac{\text{cpm S1} + \text{cpm S2}}{\text{cpm S1} + \text{cpm S2} + \text{cpm S3}} \times 100$$

3.7. Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Serum-starved HUVECs cultured in T-25 culture flask (Falcon, Oxnard, CA, U.S.A.) were treated as described in figures legend. At the indicated time points, total RNA was extracted, reverse transcribed and amplified according to the procedure previously described [114].

One µg of total RNA from human endothelial cells was reverse-transcribed for 45 minutes at 37°C. The reaction was performed in a solution of 25 µl containing, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.2 mM of each dNTP, 0.1 µg of oligo dT, 200 units of M-MLV reverse transcriptase (Life Technologies, Paisley, U.K.). The reaction mixture was then heated at 95°C for 5 minutes to inactivate the enzyme. PCR amplification was performed in 25 µl of a reaction mixture containing: 5 µl of the reverse transcribed cDNA, 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase (Life Technologies Paisley, U.K), 0.2 mM of each dNTP, and 50 pmoles of each sense and antisense primer that had previously dissolved in TE solutions (Tris 10 mM pH 8.0, EDTA 1 mM pH 8.0). The number of amplification cycles was

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determined experimentally for each primer pairs by using 0.5 μCi of [α - ^{32}P]dCTP (Specific activity 3000Ci/mmol, Amersham Pharmacia Biothech, Buckinghamshire, U.K.) and establishing the point at which exponential accumulation plateaus. Using 30 PCR cycles, the products of glyceradehyde-3-phosphate dehydrogenase (GAPDH) and Bax, Bcl-2 amplification were all within the linear phase of the reaction. Indeed, similar conditions have been previously reported for semiquantitative analysis of gene expression. The position of PCR fragments was evaluated by comparison with a DNA molecular weight marker (Gibco BRL, Paisley, U.K.). GAPDH mRNA was used for each sample as an internal control for mRNA integrity and equal loading. The levels of radioactivity incorporated into ODC product were normalized by comparison with the levels of radioactivity incorporated into the GAPDH product from the same sample. Specific primers directed against human sequences for ODC, c-myc, Cyclin D1 and GAPDH and PCR conditions were previously described [115].

Total RNA was extracted at the indicated time points. The PKC mRNA expression was evaluated by RT-PCR. Total RNA (1 μg) from synchronized subconfluent HUVECs treated as indicated, was reverse-transcribed for 45 minutes at 37^oC. The reaction was performed in a solution of 25 μl, containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.2 mM of each dNTP, 0.1 μg of oligo dT, 200 U of M-MLV reverse transcriptase (Gibco BRL, Paisley, UK). The reaction mixture was then heated at 95^oC for 5 minutes to inactivate the enzyme. PCR amplification was performed in 25 μl of a reaction mixture containing 5 μl of the reverse transcribed cDNA, 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Gibco BRL, Paisley, UK), 0.2 mM of each dNTP and 50 pmoles of each sense and antisense primers that were previously dissolved in TE solutions (Tris 10 mM pH 8.0,

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EDTA, 1 mM pH 8.0). The number of amplification cycles was determined experimentally for each primer pair by establishing the point at which exponential accumulation plateaus. Aliquots of the PCR reactions, including 0.5 μCi of [K-32 P] dCTP (3000 Ci/mmol) were taken after 10, 15, 20, 25, 30, 35 and 40 cycles. Then, [K-32 P] dCTP- labelled PCR products were electrophoresed on 2% agarose gels. Ethidium bromide-stained bands were excized under UV light and the radioactivity incorporated into PCR products was determined by L-scintillation counting. Under these experimental conditions, and using 30 PCR cycles, [K-32 P] dCTP-labelled time course experiments revealed that the products of GAPDH. Indeed, similar conditions are previously reported for a semiquantitative analysis of gene expression.

The position of PCR fragments was evaluated by comparison with a DNA molecular weight marker (Gibco BRL, Paisley, UK). GAPDH mRNA was used for each sample as an internal control for the mRNA integrity and equal loading. The levels of radioactivity incorporated into specific products were normalized by comparison with the levels of radioactivity incorporated into the GAPDH product from the same sample. Specific primers directed against human sequences for some studied genes and GAPDH were previously described. Their sequences, PCR conditions and the size of generated fragments are shown in Table 3.1.

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Gene	Deoxyoligonucleotide sequences	Product base pair	Amplification condition (Denaturing, annealing, extension)
GADPH	Forward, 5'-CCACCCATGGCAAATTCCATGGCA-3' Reverse, 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	598	95ºC for 30 s 58ºC for 30 s 72ºC for 1 min; 30 cycles
c-myc	Forward, 5'- TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT-3' Reverse, 5'- TCTTGACATTCTCCTCGGTGTCCGAGGACCT-3'	479	72ºC for 1 min 95ºC for 40 s 60ºC for 40 s; 35 cycles
ODC	Forward, 5'- GCAGGATCCACCATGAACAACTTTGGTAA-3' Reverse, 5'-GCCGAGATCTCAGAAGAAGAAACTTC-3'	120	72ºC for 1 min 95ºC for 40 s 43ºC for 40 s; 30 cycles
Bax	Forward, 5'-CCTTTTCTACTTTGCCAGCAAAC-3' Reverse, 5'-GAGGCCGTCCCAACCAC-3'	291	95ºC for 45 s 60ºC for 50 s 72ºC for 1 min; 35 cycles
Bcl-2	Forward, 5′-AGGGTCAGATGGACACATGGTG-3′ Reverse, 5′-CGTTGCCTGTGGGTGACTAATC-3	460	95ºC for 45 s 68ºC for 50 s 72ºC for 1 min; 38 cycles
Cyclin D1	Forward, 5'-TACTACCGCCTCACACGCTTC-3' Reverse, 5'-TTCGATCTGCTCCTGGCAG-3'	493	72ºC for 1 min 94ºC for 50 s 60ºC for 45 s; 30 cycles

Table 3.1. Sense and antisense primers of some genes related to our study

3.8. Protein extraction

Serum-starved or growing HUVECs were cultured in T25 culture flask (Falcon, Oxnard, CA, U.S.A.) and treated as described in figures legend. At the end of each experimental point, the medium was removed and cells were detached with 0.1% trypsin plus 0.02% EDTA in PBS, pH 7.3 and pelletted by centrifugation at 1000g for 5 minutes. The pellet was washed with PBS, centrifuged as above and then resuspended in 100 µl of a chilled lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM b-glycerophosphate,0.1mM okadaic acid,1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 50 mg/ml leupeptin, and 10 mM pepstatin). The samples

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were sonicated for 10 seconds (Branson, sonifer B-12,setting3) and incubated at 4°C for 15 minutes, lysates were then centrifuged at 10,000 g for 15 minutes (4°C) and analyzed for the protein content by Lowry method. Following RES treatments, the cells were harvested at 40 minutes, 24 hours post-treatment and then washed with cold PBS. The supernatant (total cell lysate) was collected, aliquoted, and stored at - 80^{0}C.

3.9. Immunoblotting Analysis

Immunoblotting analysis for ERK1/2, MEK1/2 and p-38 was performed as previously described [116]. Each sample was added with Laemmli sample buffer and boiled for 4 minutes. Equal amounts of sample protein (10 to 20 μg/lane) were loaded. Sample and prestained molecular weight markers (Santa Cruz Biotechnology, Inc. Santa Cruz,CA, U.S.A.) were fractionated by SDS-PAGE with a 10% acrylamide separation gel at 200 Volts for 45 minutes. Proteins were transferred to nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol for 2 hours at 350 mA at room temperature. Nitrocellulose membranes were incubated in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.2% Tween 20 (TNT 20) with 5% non-fat dried milk for 1 hour, washed three-times in TNT 20 (3, 3, 5 minutes), and incubated for 1 hour with primary antibody in TNT 20 containing 5% milk at room temperature for nonphospho-antibodies, and overnight at 4°C for phospho-specific antibodies. Proteins of interest were detected using specific antibodies against phospho-ERK1/2, Phospho-MEK1/2 and Phospho-p-38 (New England Biolabs, Inc.Beverly, MA, USA). The following dilutions were used for individual antibodies against different proteins: phospho-ERK1/2 (1:1000), Phospho-MEK1/2 (1:1000), Phospho-p-38 (1:1000). After further washing in TNT 20, membranes were incubated for 1 hour with horseradish peroxidase-linked anti-IgG secondary antibody diluted 1:5000 (Bio-Rad, Hercules, CA, U.S.A.) and immunoreactive proteins were detected by ECL as described by the

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supplier (Amersham Pharmacia Biotech, Buckinghamshire, U.K). The intensities of autoradiographic bands were measured with a laser densitometer (ImageQuant Computing Desitometer 300/325, Molecular Dynamics, Sunnyvalle, CA, USA). Data are representative of three or more independent experiments.

We used specific antibodies against the phosphorylated form of the protein kinase ERK1/2, p42/44MAPK, p-38 (Cell Signaling, Danvers, MA) and β-actin (Sigma-Aldrich, Saint-Louis, MO, USA).

Results were expressed as arbitrary units, and ratios of individual densitometric results were normalized to β-actin immunoreactivity.

3.10. Determination of PKC activity

Serum-starved HUVECs cultured in T25 culture flask (Falcon, Oxnard, CA, U.S.A.), were treated as described in figures legend. At the end of each experimental point, the medium was removed and cells were detached with 0.1% trypsin plus 0.02% EDTA in PBS, pH 7.3 and pelleted by centrifugation at 1000g for 5 minutes. The pellet was washed with PBS, centrifuged as above and then resuspended in 100 µl of a chilled lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 50 mM sodium fluoride, 20 mM b-glycerophosphate, 0.1 mM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 50 mg/ml leupeptin, and 10 mM pepstatin). The samples were sonicated for 10 seconds. (Branson, sonifer B-12, setting 3) and incubated at 4°C for 15 minutes; lysates were then centrifuged at 10.000 g for 15 minutes (4°C) and analyzed for the protein content by Lowry method. Aliquots of the homogenate were taken to measure total PKC activity by a PKC enzyme assay system (PepTag Non-Radioactive Protein Kinase Assay; Promega, Madison, WI,USA), according to the instructions of manufacturer.

Two micrograms of PepTagTMC1 peptide were incubated with the 20

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µg of the samples in a final volume of 25 µl for 30 minutes at 30°C. The reactions were stopped by heating to 95°C for 10 minutes. The samples were loaded on a 0.8% agarose gel and run at 100 Volts for 15 minutes. Phosphorylated peptide migrated towards the anode, while non-phosphorylated peptide migrated towards the cathode. Using a razor blade, the negatively-charged phosphorylated bands were excised from the gel and assayed for PKC activity according to the manufacturer's instructions.

3.11. Statistical analysis

Data are expressed as means ± SDs of three different experiments. One-way ANOVA followed by a post hoc Newman-Keuls Multiple Comparison Test were used to detect differences of means among treatments with significance defined as $p < 0.05$. When appropriate, two-way ANOVA with a Bonferoni post-test was used to assess any differences among the treatments and the times ($p < 0.05$). Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA).

3.12. Ethical approval

Human umbilical cords were collected from normal-term pregnancies after delivering, processing before 24 hours. All the procedures carried out with the umbilical cords were approved by the Ethics Committee from the Faculty of Medicine of the University of Sassari, and the consent of the donor mothers were obtained.

The umbilical cords were severed from the placenta soon after birth and obtained in sterile transported medium (5 % Solution A: NaCl, KCl, $MgSO_4.7H_2O$, $CaCl_2$; 5 % Solution B: Na_2HPO_4, KH_2PO_4, Glucose; Phenol Red Solution 0.5 M 10 X: Phenol Red, $NaHCO_3$; Hepes 0.5 M; Gentamicin 0.5%, Amphotericin 1%). The samples were maintained at 4°C until processing.

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CHAPTER 4
RESULTS

4.1. Dose-dependent effect of Resveratrol on Human umbilical vein endothelial cell ROS levels

The study began with the quantification of ROS levels in HUVECs treated with various concentrations of RES and untreated cells (CTRL) used as control. Intracellular ROS generation was examined in HUVECs in response to RES using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This probe enters the cells and can be oxidized in the presence of ROS, generating the fluorescent compound, DCF. Then to determine the effects of RES on HUVECs, cells were treated as indicated previously and intracellular ROS levels were assessed after treatments. This was evaluated as change in DCF fluorescence. The results from three pooled measurements are shown below and results are expressed as percentage of untreated controls.

Treatment of HUVECs with 0.5µM RES exerted a significant antioxidant effect. However, the exposure of cell cultures to higher concentrations of RES increased intracellular ROS levels in a dose-dependent manner. As a result, the antioxidant effect seen at 0.5µM was lost and a marked pro-oxidant effect was evident at both 5 and 25µM (figure 4.1).
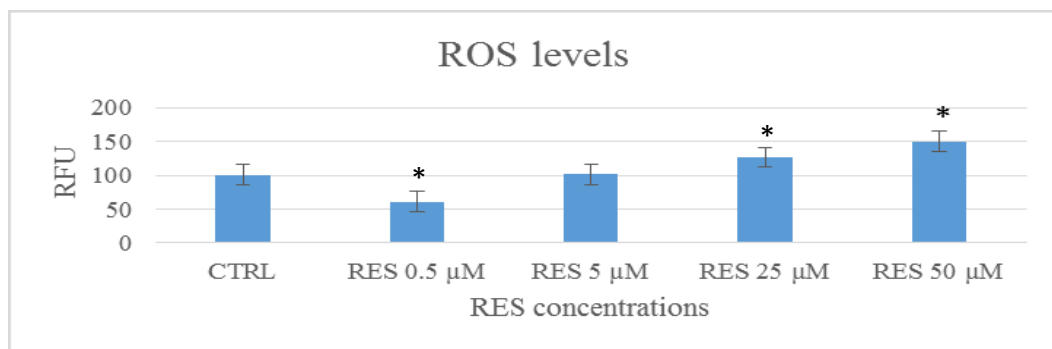

Figure 4.1. Dose-dependent effect of RES on ROS levels. HUVECs were stimulated for 24 hours as indicated in figure and then ROS levels were assessed after treatments. Means ± SD of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).
(RFU: Relative Absorbance Units, % of control)

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4.2. Dose-dependent effect of Resveratrol on Human umbilical vein endothelial cell viability

From the above results of intracellular ROS levels, we studied the effects of RES on the HUVEC viability. This was evaluated by the MTT assay. This test exclusively detects viable cells because tetrazolium salts are reduced only by metabolically active cells to formazan, a colored substance which can be quantified spectrophotometrically. Figure 4.2 shows the results of an experiment in which HUVECs were treated for 24 hours with increasing concentrations of RES (1-100 μM). The effects were compared with two types of control: the first, called FCS, is made by cell growth medium (M199), with 5% FCS, optimal concentration for the best proliferation of these cells (data not shown). The second control is made by M199 with 5% FCS in the presence of 0.1% DMSO, the solvent in which RES has been solubilized. The results of the vitality experiments are overlapped in the two types of control. For this reason, in the subsequent experiments we use only the one without DMSO.

Our results of RES treatment show that low concentrations of RES (1-25 μM) induced an increase in cell viability within 24 hours of treatment, with a peak absorbance at 10 μM (153% compared to the control 100%), while higher concentrations (25-100 μM) determined a decrease in HUVEC vitality, with a minimum at 100 μM RES (66% compared to control, 100%).

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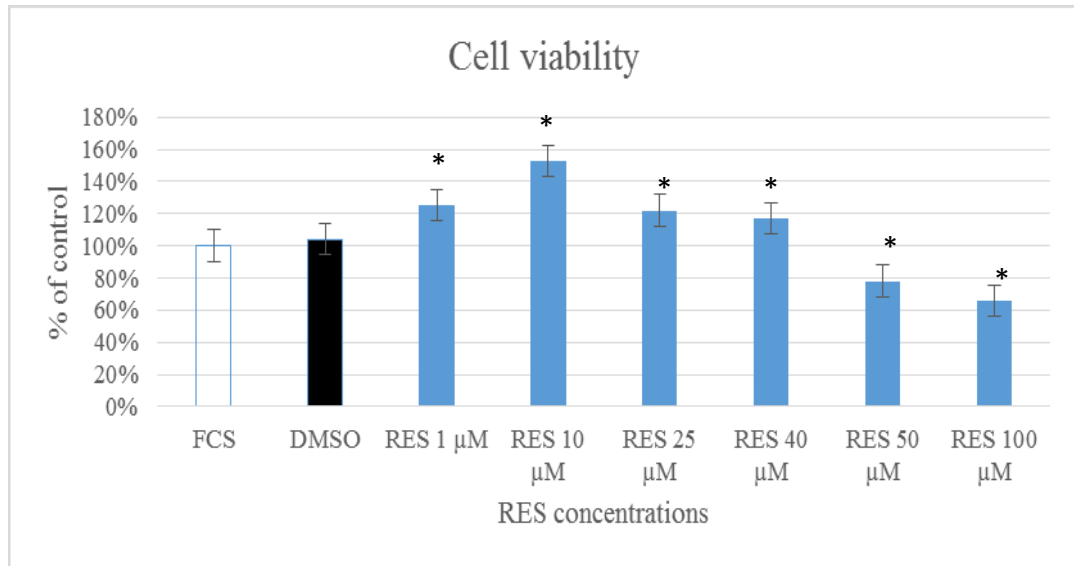of PhD Course in Life Sciences and Biotechnologies - University of Sassari

Figure 4.2: The standard MTT-assay with HUVECs. Cells were grown for 22 hours in the medium M199 with or without (FCS 5%, DMSO=FCS 5%+DMSO 0,1%) the indicated concentrations of RES; subsequently they were incubated with the MTT solution for another 2 hours. Means ± SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).

4.3. Dose-dependent effect of Resveratrol on DNA synthesis

HUVECs were serum-starved following a 24-hour incubation and then treated with increasing concentrations of RES (1-100 μM). During the last 24 hours, HUVECs were added with 1μCi/ml [^{3}H] thymidine. At the end of each experiment, cells were washed twice with ice-cold PBS (1 ml/well) and processed for liquid scintillation counting as above. Figure 4.3 shows low concentrations of RES (1-10 μM) induced an improved DNA synthesis compared to control, but the only 10 μM concentration gave a significant increase. The concentrations greater than 10 μM lead to a significant inhibition of incorporation of [^{3}H] thymidine, with a maximum at 50 μM and 100 μM.

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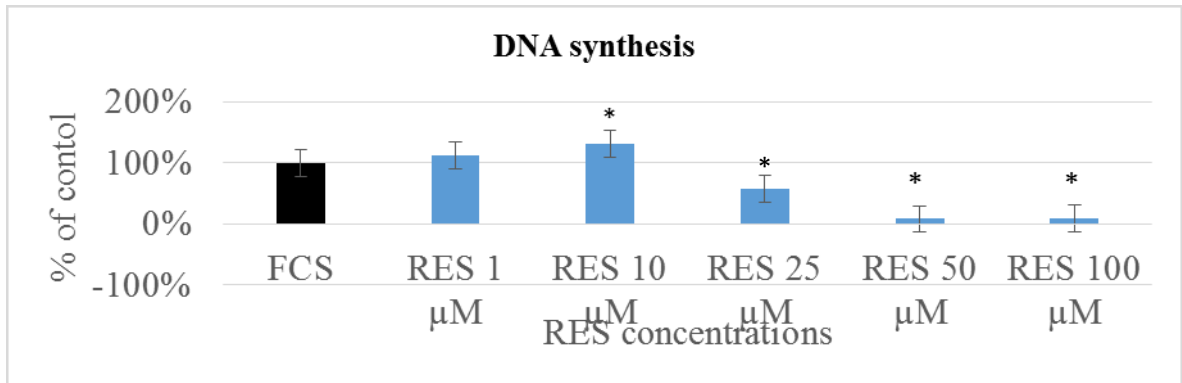of PhD Course in Life Sciences and Biotechnologies - University of Sassari

Figure 4.3: RES modulates HUVEC proliferation. Serum-starved HUVECs were treated for the 48 h with the medium M199, in the absence (FCS 5%) or presence of growing concentrations of RES. At the end, cells were processed for liquid scintillation counting as described. Data are expressed as % of the control. *, significantly different from the control (p< 0.01). Means ± SD of a representative experiment, performed in triplicate, are shown.

4.4. Dose-dependent effect of Resveratrol on DNA fragmentation

To determine whether the inhibition of HUVEC proliferation by RES was due to the induction of apoptosis, we evaluated the rate of programed cell death by measuring the degree of DNA fragmentation, using a radioisotopic technique with [³H] thymidine.

Cellular DNA was prelabeled with 1 μCi/ml [³H] thymidine for 24 hours in the presence of different concentrations of RES ranging from 1 – 100 μM.
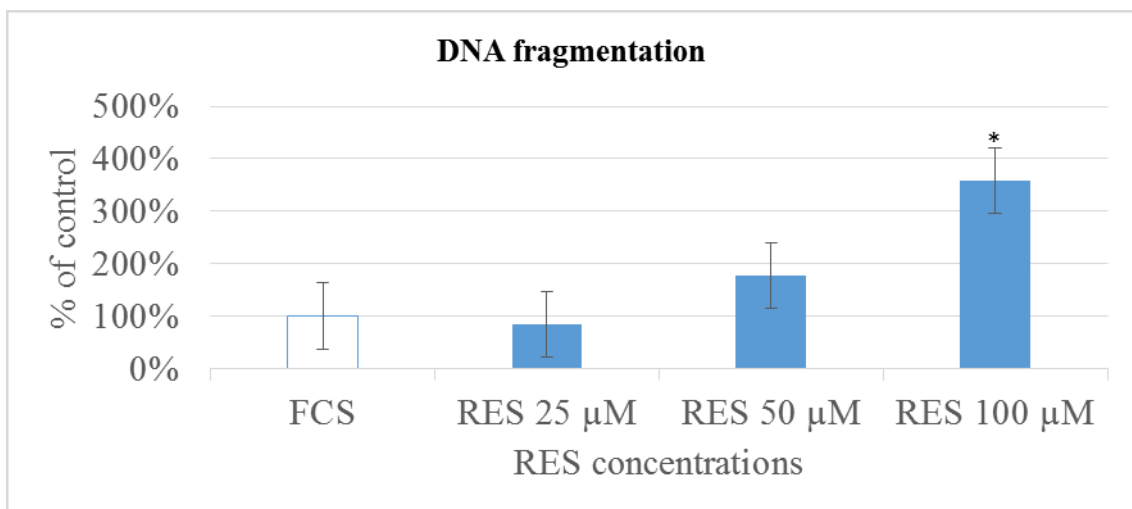

Figure 4.4: Effect of RES on HUVEC DNA fragmentation. Growing cells were treated for 24 hours with medium M199, containing 5% FCS in the absence or presence of growing concentrations of RES. At the end, cells were processed for liquid scintillation counting as described. Data are expressed as percentage of control (FCS) 100% and represent the mean ± SDs of three different experiments. *, significantly different from untreated.

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As shown in figure 4.4, whereas the concentration 25 μM decreases slowly the DNA fragmentation compared to the control (which has been given the arbitrary value of 100%), the higher concentrations induce an higher DNA fragmentation, especially after 100 μM treatment (358% compared to the control). Such results have encouraged us to hypothesize that the observed anti-proliferative phenomena using high concentrations of RES, could be correlated with an activation of an apoptotic program in HUVECs from RES. For this reason, we evaluated the expression of some genes involved in the programmed cell death.

4.5. Evaluation of gene expressions involved in cell death and cell survival by Reverse-transcription Polymerase Chain Reaction

Total RNA was extracted at the indicated time points, according to the procedure described in ''Materials and Methods''. The position of PCR fragments was evaluated by comparison with a DNA molecular weight marker. GAPDH mRNA was used for each sample as an internal control for the mRNA integrity and equal loading. The levels of radioactivity incorporated into specific products were normalized by comparison with the levels of radioactivity incorporated into the GAPDH product from the same sample. Specific primers directed against human sequences for Bcl-2, Bax, c-myc, ODC, Cyclin D1 and GAPDH were previously described. Their sequences, PCR conditions and the size of generated fragments are shown in Table 3.1.

Apoptosis is a programmed form of cell death, morphologically characterized by cell shrinkage and condensation of both nuclear chromatin and cytoplasm. On a molecular basis, two distinct signaling pathways are known to be involved, the mitochondrial pathway and the death receptor (Fas-Fas ligand) pathway. Bcl-2 is a major anti-apoptotic protein residing in the mitochondrial membrane, which is now widely recognized to protect cells

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against various forms of apoptotic stimuli. Bax, on the other hand, is a pro-apoptotic factor, which exerts its function by homodimerizing and accumulating in the mitochondrial membrane. Bcl-2 family proteins regulate mitochondrial membrane stability. Disruption of the mitochondrial membrane leads to cytochrome C release, activation of caspase-9, and eventually apoptotic cell death.

Using a semi-quantitative RT-PCR, we studied Bcl-2, Bax gene related to cell apoptosis. HUVECs were treated for 24 hours with those concentrations of RES (25, 40 and 50 µM) at which cells have showed a reduced viability.

In this experiment, the results were compared with two types of control: in the first, time 0, RNA was extracted at the end of the cells synchronization, and in the second, after 24 hours without RES. As internal control, we used the constitutive gene GAPDH. The levels of Bcl-2 mRNA decreased with the augmentation of RES concentration up to 50 µM and this result agrees with previous experiments. So in another group of experiment (Figure 4.6) we assess if RES 40 µM treatment for times less than 24 hours, would be already able to reduce the expression of Bcl-2 compared to control. The gene expression does not indicate any significantly difference between the control and the treated cells after 16 hours. This means RES effect is also time-depending.

Then we evaluated the expression of the pro-apoptotic gene Bax. On the basis of the results obtained with the Bcl-2 gene, we studied a dose-response assessment (Figure 4.7); so the HUVECs were treated for 24 hours with increasing concentrations of RES (25, 40 and 50 µM). Bax gene expression had a strong augmentation after 50 µM treatment, according to Bcl-2 results, an anti-apoptotic gene, that at the same concentration had shown the lower gene expression.

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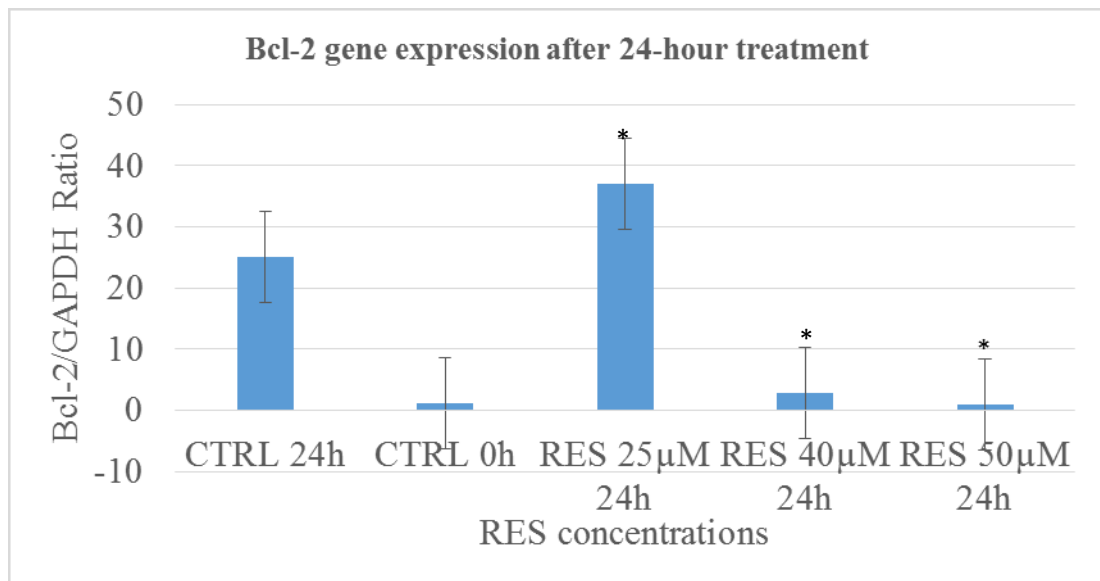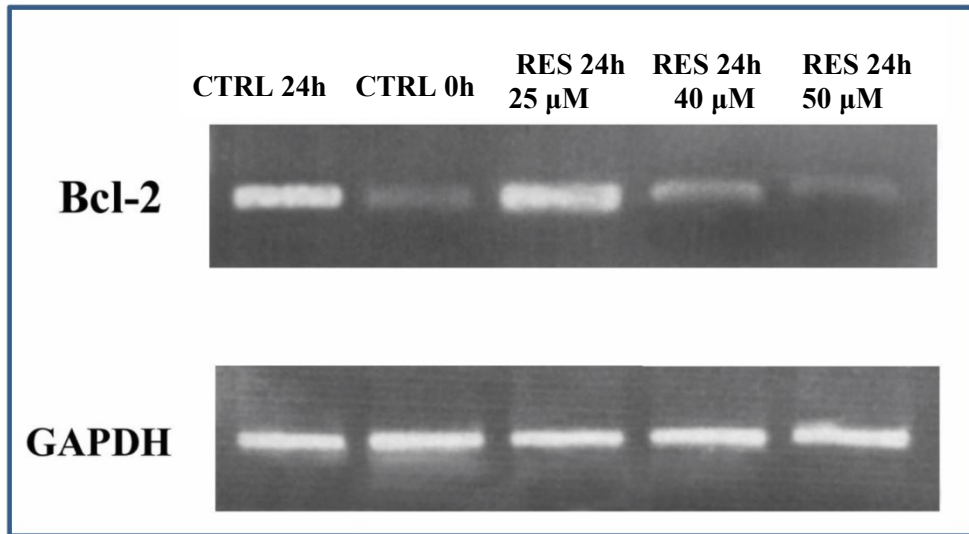of PhD Course in Life Sciences and Biotechnologies - University of Sassari

Figure 4.5: RES induces Bcl-2 gene expression. Serum-starved HUVECs were stimulated for the indicated times with M199 containing different concentrations of RES as indicated. Means ± SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).

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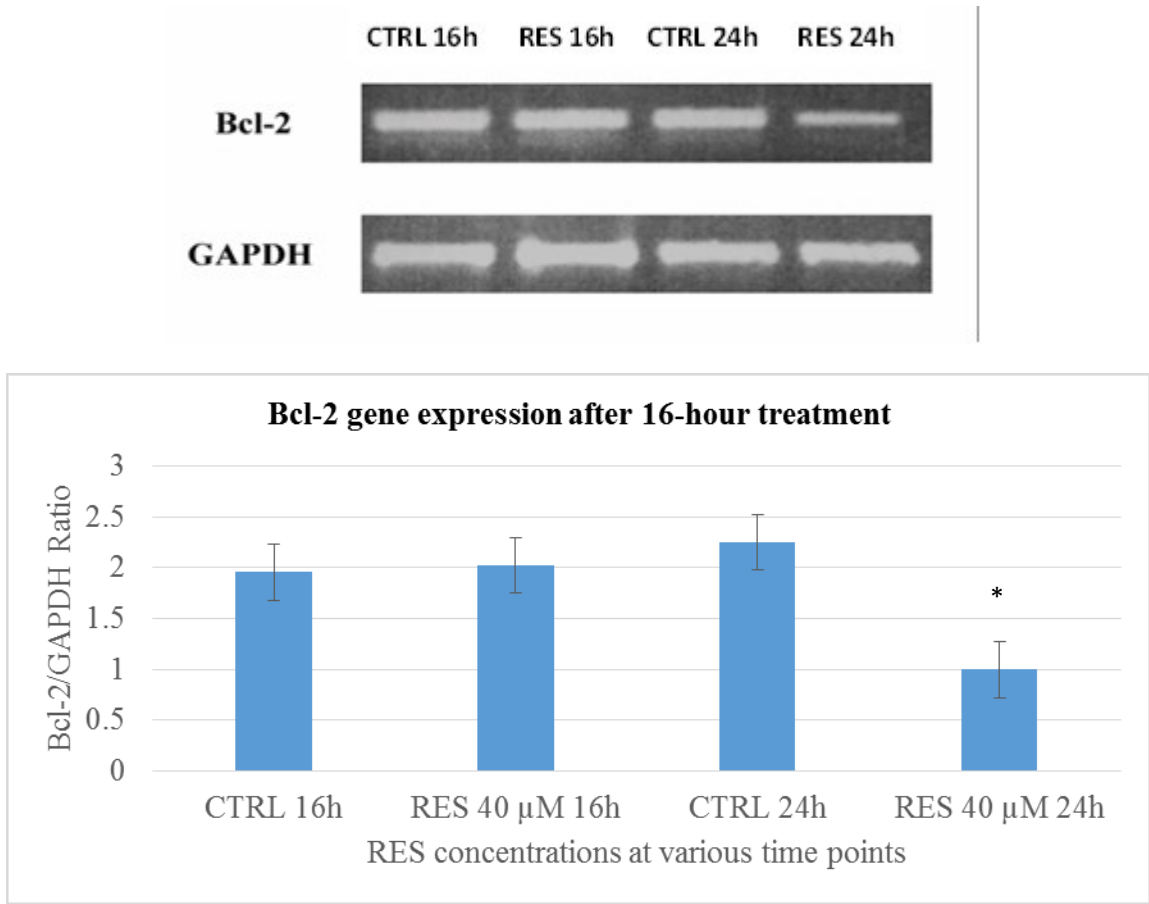of PhD Course in Life Sciences and Biotechnologies - University of Sassari

Figure 4.6: RES induces Bcl-2 gene expression. Serum-starved HUVECs were stimulated for the indicated times with the medium M199 containing RES 40 µM as indicated. Means + SD of a representative experiment, performed in triplicate, are shown. *, significantly different from the control (p< 0.01).

Then we evaluated the expression of the pro-apoptotic gene Bax.

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Figure 4.7: RES induces Bax gene expression. Serum-starved HUVEC were stimulated for the indicated times with M199 containing different concentrations of RES as indicated. Means ± SD of a representative experiment, performed in triplicate, are shown. *, significantly different from the control (p< 0.01).
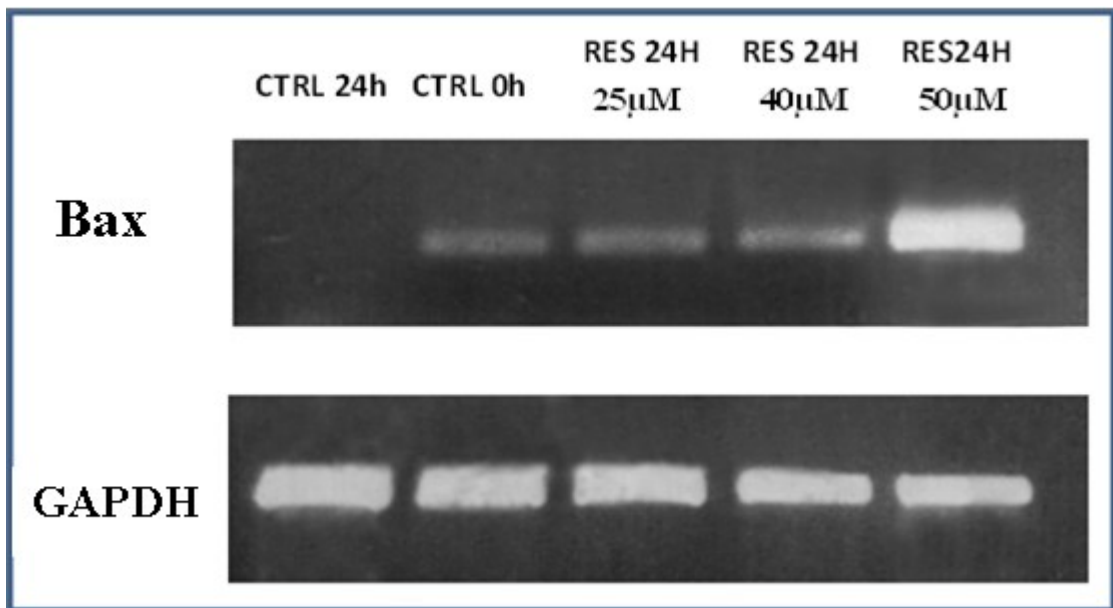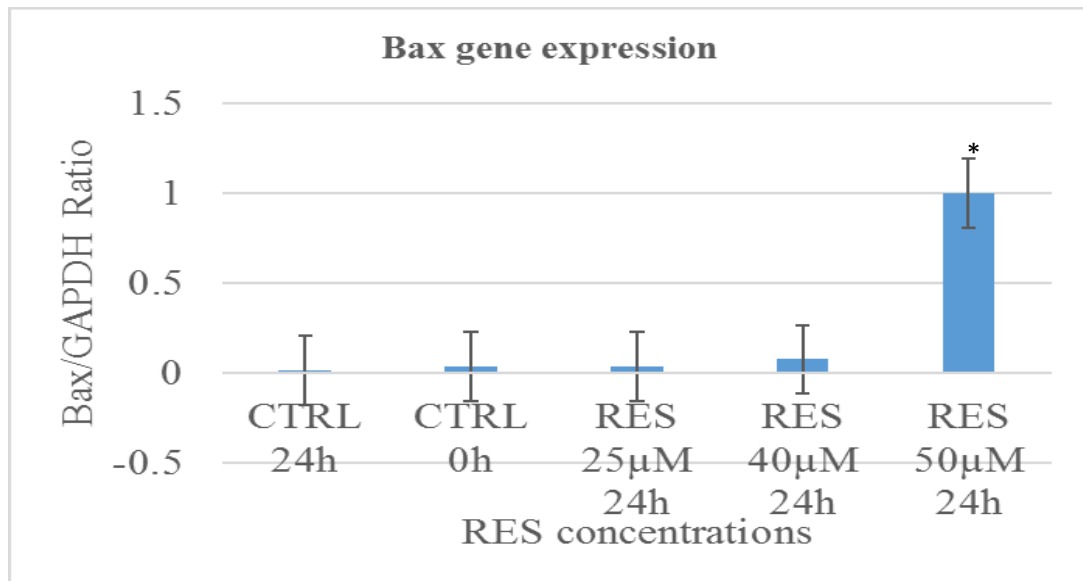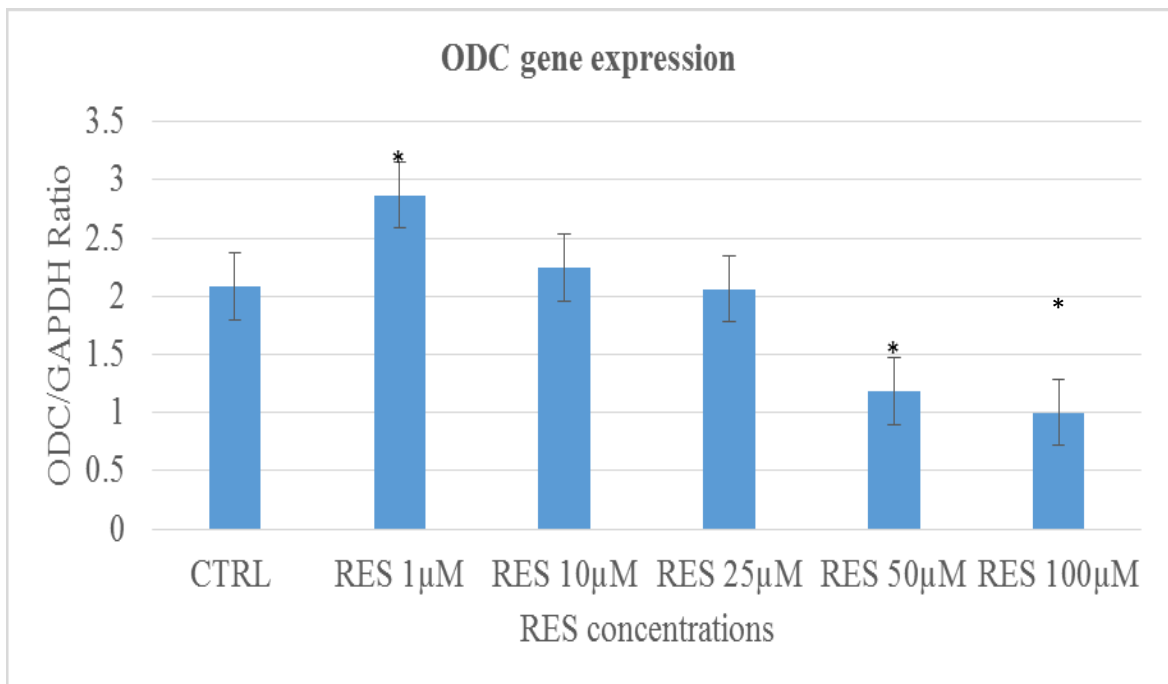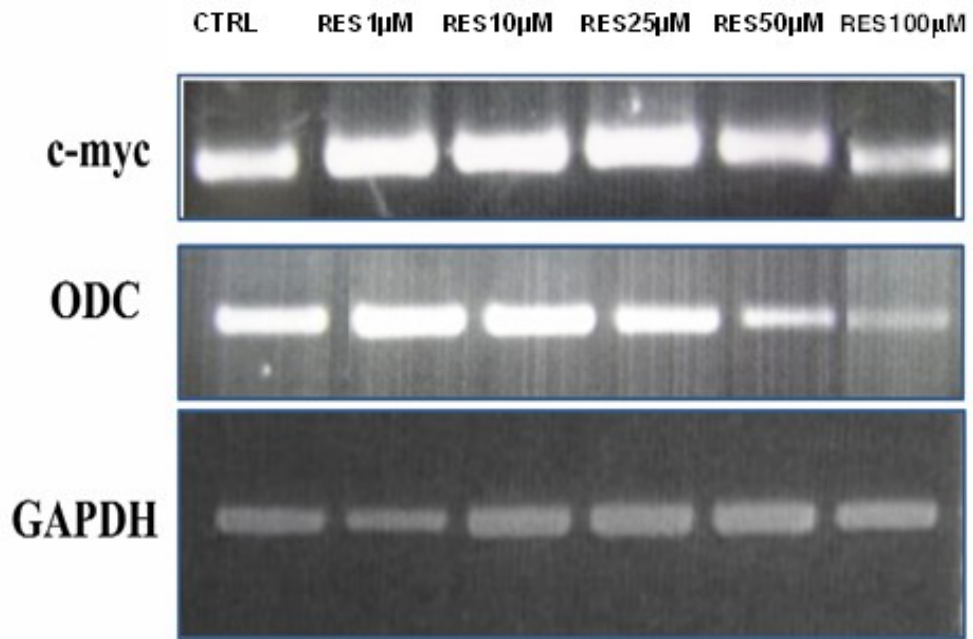
Bax gene expression had a strong augmentation after 50 μM treatment, according to Bcl-2 results, an anti-apoptotic gene, that at the same concentration had shown the lower gene expression.

DNA fragmentation assay showed that the progression of apoptosis was also inhibited by the enhanced expression of Ornithine Decarboxylase (ODC). Therefore, we postulate that the protective function of c-myc is the result of ODC induction. c-myc plays a central role in the regulation of cell cycle progression, differentiation, and apoptosis. ODC is an important mediator of c-myc-induced apoptosis and suggest that ODC mediates other c-myc functions.

Reverse-transcription PCR was carried out for c-myc and ODC in RES-treated HUVECs. Cells were treated for 40 minutes with increasing concentrations of RES (1; 10; 25; 50 and 100 μM). Sense and antisense primers directed against human sequences for c-myc and ODC and PCR conditions were previously described in "Materials and Methods".

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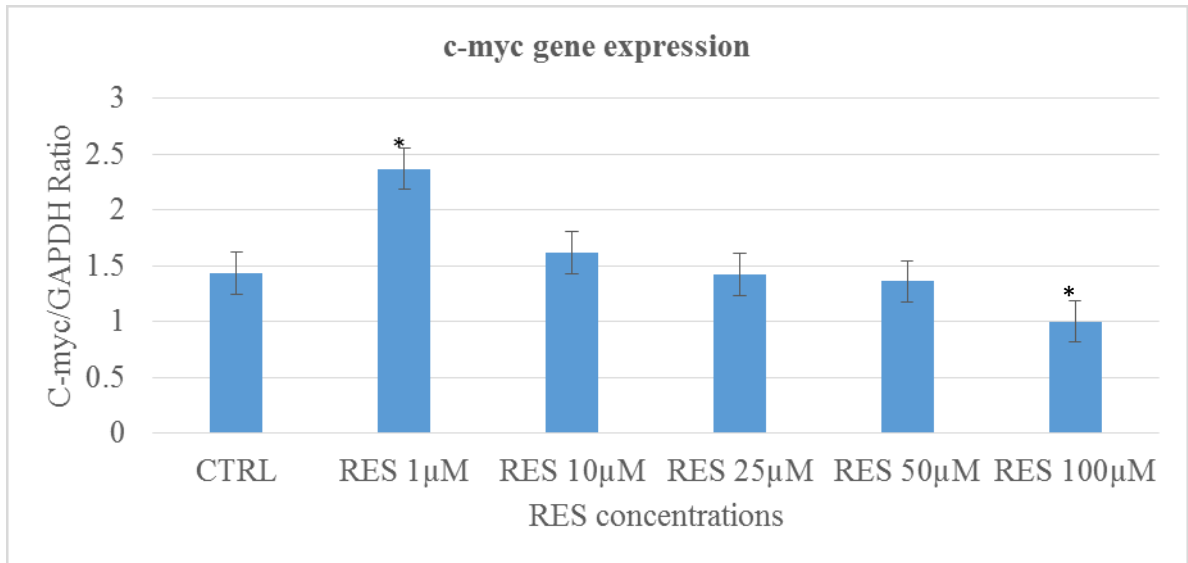of PhD Course in Life Sciences and Biotechnologies - University of Sassari

Figure 4.8: RES modulates c-myc and ODC gene expression. Serum-starved HUVECs were stimulated for 40 minutes with M199 containing different concentrations of RES. Means ± SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control (p< 0.01).

Those figures 4.6, 4.7 and 4.8 showed that high concentrations of RES (50 and 100 μM) lead to reveal significantly down-regulated c-myc, ODC gene expression. So c-myc and ODC expression was suppressed in RES-treated cells, accompanied with apoptotic cell death and whether arrest at S phase or not. We assessed the influence of RES on cell cycle regulator, Cyclin D1, in treated endothelial cells.

Cyclin D1 is known transcription targets of c-myc. Therefore, to investigate whether c-myc induction controls the expression of cell-cycle positive regulators, RT-PCR is used for treated HUVECs [140]. Higher concentrations of RES (50 and 100 μM) determined a decrease in Cyclin D1 expression, with a minimum at 100 μM, that at the same concentration had shown lower c-myc, ODC gene expressions.

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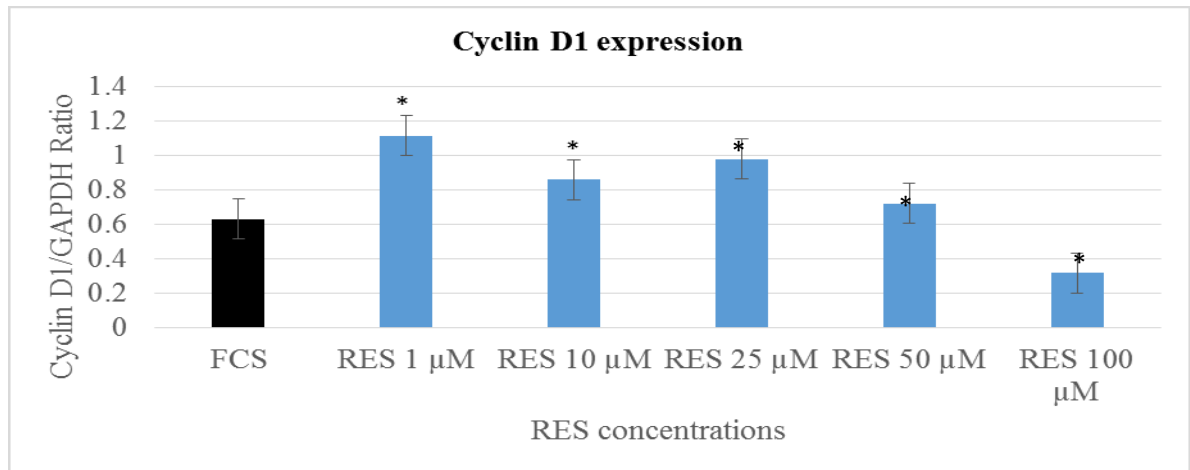of PhD Course in Life Sciences and Biotechnologies - University of Sassari
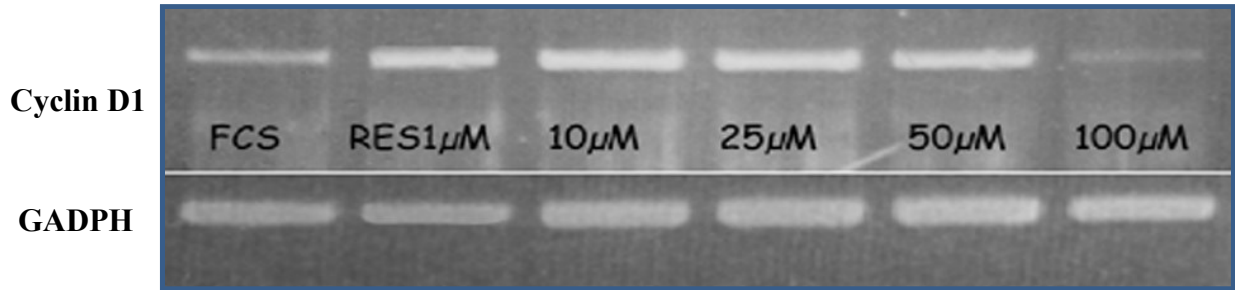
Cyclin D1

GADPH

Figure 4.9: RES modulates Cyclin D1 expression. Serum-starved HUVECs were stimulated for 40 minutes with M199 containing different concentrations of RES as indicated. Means ± SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).

4.6. Immunoblotting Analysis

To identify the signalling pathways related to RES-treated HUVEC proliferation and apoptosis, immunoblotting analysis for ERK1/2, MEK1/2 and p-38 was performed as previously described. Serum-starved HUVECs were cultured in T25 culture flask and treated with RES concentrations ranging from 1; 10; 50 and 100 μM for different time points (40 minutes, 16 hours and 24 hours).

At the time points of 40 minutes and 16 hours, we obtained no bands. After 24 hours treated with RES, we found that there were not any significant differences between the control and treated cells, so RES does not influence on these studied protein kinases. That means another protein kinase participated in the pathway related to expression of Cyclin D1, Bcl-2, Bax, c-myc and ODC gene.

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Figure 4.10: RES doesn't influence MEK/ERK (p-42-44)/p-38 phosphorylation. Serum-starved HUVECs were stimulated for the indicated times with medium M199 in the absence (FCS 5%) or presence of different concentrations of RES. Means ± SDs of a representative experiment, performed in triplicate, are shown.

4.7. Determination of PKC activity

The protein kinase C family consists of more than ten structurally related serine/threonine protein kinases. PKC isoforms are critical regulators of cell proliferation and survival and their expression or activity is altered in some human diseases. Numerous studies recently identified PKC isotype-selective functions in signaling pathways such as: apoptosis, proliferation, migration,

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differentiation. In order to investigate the mechanism of the effects of RES on PKC activities, in vitro assays were utilized in which the levels of RES were systematically varied.

Serum-starved HUVECs were treated for 40 minutes with indicated concentrations of RES. PKC activity are expressed as % of control. It was found that PKC activity was inhibited at relatively high RES concentrations, especially highest dose, 100 μM.

Through figure 4.11, at the time point of 40 minutes, higher concentrations of RES (50 and 100 μM) lead to significant decrease of PKC activity, with a minimum at 100 μM, which at the same concentration had shown lower c-myc, ODC gene expression and Cyclin D1 expression in nucleus. That means RES modulates PKC activity in HUVEC proliferation.
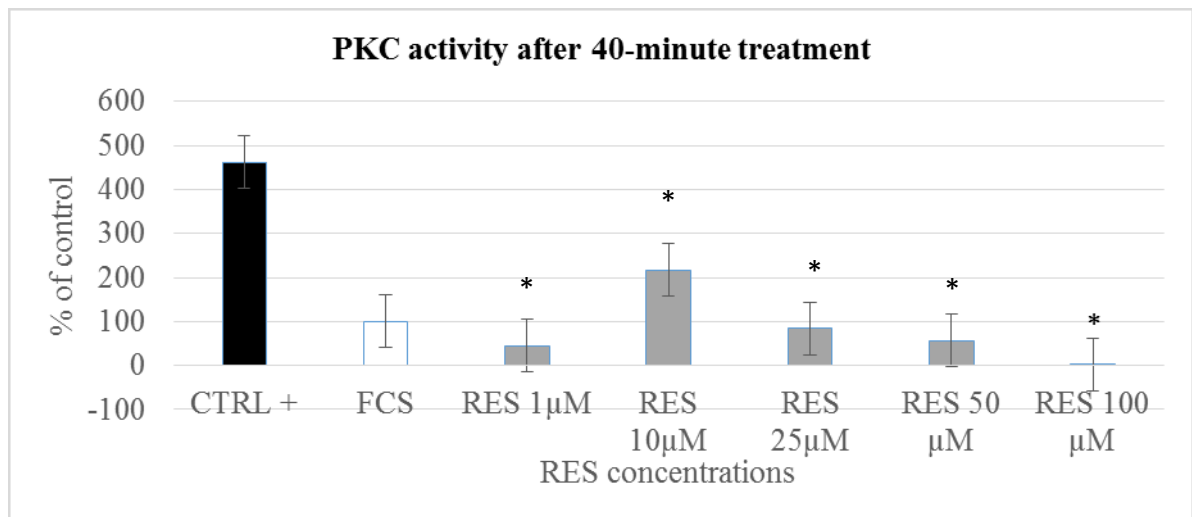
Figure 4.11: RES modulates PKC activity. Serum-starved HUVECs were treated for 40 minutes with medium M199 in the absence (FCS 5%) or presence of the indicated concentrations of RES. PKC activities are expressed as % of control. Means ± SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).

Figure 4.12 showed PKC activity on HUVECs at the other point of time (24 hours). PKC activity are expressed highest at the concentration of 25 μM, lowest at 100 μM, which at the same concentration had shown highest Bcl-2 gene expression and lowest Bax gene expression. That means RES modulates PKC activity in HUVEC apoptosis.

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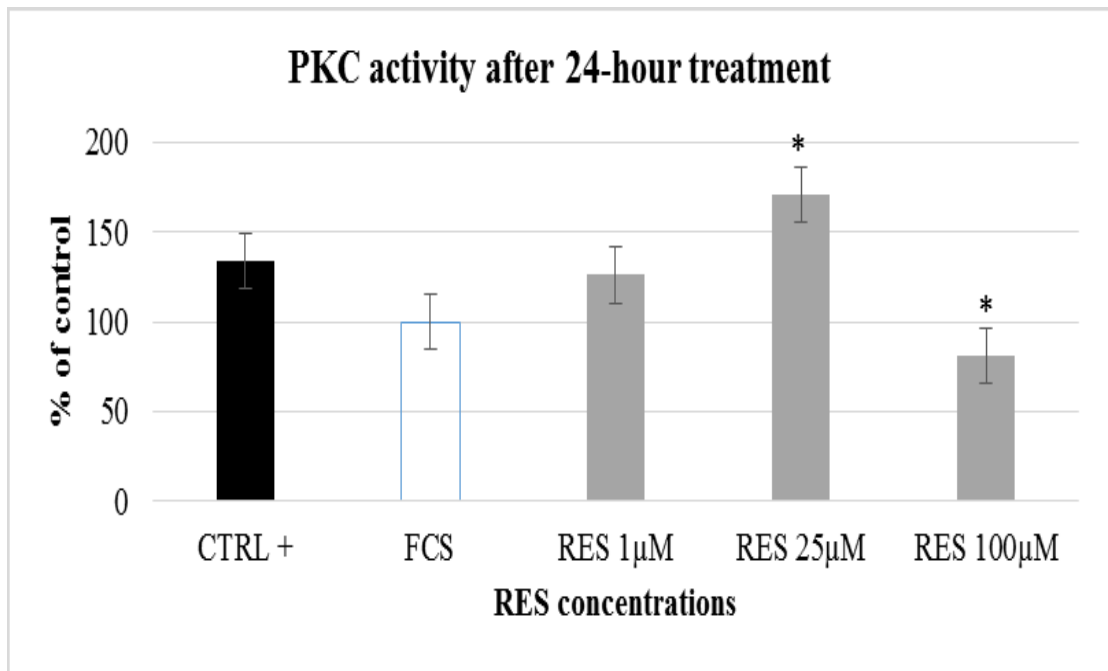
Figure 4.12: RES modulates PKC activity. Serum-starved HUVECs were treated for 24 hours with medium M199 in the absence (FCS 5%) or presence of the indicated concentrations of RES. PKC activities are expressed as % of control. Mean±SDs of a representative experiment, performed in triplicate, are shown. *, significantly different from the control ($p < 0.01$).

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CHAPTER 5
DISCUSSION

The endothelial cell plays a crucial role in the integration and modulation of signals within the vascular wall. Perturbation of such homeostasis by oxidative damage is the trigger for the development of CVD [106]. For this reason, the endothelium has become a widely and useful model to study the effects of natural antioxidants in general and RES in particular on the vascular (patho)physiology. HUVECs were used for our experimentation. Indeed, this vascular cell model has been used for years in the field of cardiovascular research since it has been proven to be extremely reliable and affordable, also easy to grow and to isolate, as compared to other vascular cells [117]. Besides, the anticancer property of RES is the most studied. Since the growth of tumors requires the creation of new blood vessels, this work was intended to evaluate the influence of RES on the proliferation of human endothelial cells, which are essential for the new angiogenesis.

Our results show that for RES, the concentration will determine the overall effects on endothelium. While low doses of RES showed an antioxidant effect, surprisingly, an increase in concentration induced a completely opposite result, resulting in cell damage. However, since RES effects can vary depending on cell types and molecules used, the mechanisms involved in antioxidant-mediated cellular responses are still largely unknown.

Firstly, to validate our ROS assay, we tested its capability to detect variations of intracellular ROS levels in response to increasing doses of RES ranging from 0.5, 5, 25 and 50 µM in cultured HUVECs. Data reported in figure 4.1 show that the probe has the significant dynamic range and responds linearly to increasing doses of RES in this cellular model. To determine the effects of RES on HUVECs, cells were treated as indicated and intracellular

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ROS levels were assessed after treatments. The results from 3 measurements are shown and results are expressed as a percentage of untreated controls. Treatment of HUVECs with 0.5 µM RES exerted a significant antioxidant effect confirming the protective role of RES previously described and validating our experimental system. However, the exposure of cell culture to higher RES concentrations increased intracellular ROS levels in a dose-dependent manner. As a result, the antioxidant effect seen at 0.5 µM was lost and a marked pro-oxidant effect was evident at both 25 and 50 µM. To gain further information on the antioxidant and pro-oxidant effect of RES, we assessed variations of cell viability content in response to RES treatment ranging from 1, 10, 25, 40, 50 and 100 µM in order to identify which the suitable concentration for antioxidant and pro-oxidant is.

The cell viability results suggest that low concentrations (1-25 µM) of RES seem to increase vitality of the cells compared with control (evaluation with MTT), increase the DNA synthesis (thymidine incorporation) and decrease the percentage of DNA fragmented. These results contribute to assume that at these low concentrations, RES has positive effects on the HUVECs proliferation. Results completely opposed are obtained with higher concentrations of this polyphenol (40-100 µM). The most significant effect is the almost complete inhibition of cell proliferation at 50 and 100 µM, and the corresponding increase of DNA fragmentation, respectively 178% and 358%, compared to the control. The result of DNA fragmentation experiments may indicate cell death by apoptosis or necrosis. We studied apoptosis because it is known that RES is able to induce this phenomena, although in other cell lines.

The study of natural compounds with pharmacological activity, such as polyphenols, has become an emerging trend in nutritional and pharmacologic research. Resveratrol (trans-3,5,4′-trihydroxystilbene) is a natural polyphenol synthesized by plants as a phytoalexin that protects against ultraviolet

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radiation and fungal infection. It is found in high concentrations in grapes, berries, nuts and red wine with potentially beneficial anti-carcinogenic, anti-inflammatory, antioxidant and cardio-protective properties. These properties of resveratrol have been linked to the inhibition of proliferation in association with cell cycle arrest and apoptotic cell death typically observed in vitro at concentrations in the range of 25–400 μM [118,119].

The present study demonstrates RES inhibits the cell proliferation and promotes the apoptosis of HUVECs by: (i) limiting DNA synthesis and cell viability, (ii) increasing DNA fragmentation and (iii) inducing gene expressions related to apoptotic and proliferative functions. To our knowledge, this study is the first report evaluating the effects of RES on these signaling pathways.

Over the past decade, RES has emerged as a very promising natural compound with immense therapeutic potential. In several experimental models of carcinogenesis, RES inhibits cancer initiation, promotion and progression.

In addition, RES also exerts its effects by interacting with multiple cellular targets and modulating various signal transduction pathways. However, to date, the studies evaluating the effects of RES on HUVECs are limited, with data indicating that RES exerts various effects.

Furthermore, RES has also gained prominence as a potent chemo-preventive agent that interferes with signaling pathways regulating cell death and survival.

In the next part of this work we studied the effects of RES on the endothelial cell viability. In our study, through MTT assay we found that low concentrations of RES increase cell proliferation and high concentrations lead to opposite effects on exposed cells. This suggests that the RES effect on HUVECs is biphasic, that is low concentrations increase cell growth, high

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concentrations cause the opposite effect. Based on this first result we studied the influence of RES on HUVEC DNA synthesis. For this purpose, the analysis of incorporation with [3H] thymidine in the cellular DNA was done with increasing concentrations of RES (1; 10; 25; 50 and 100 μM) for 48 hours of treatment, the time of an entire cell cycle of HUVECs. The figure on DNA synthesis showed us there was an increase in DNA synthesis at low concentrations of RES. Meanwhile, higher doses lead to a significant inhibition of incorporation of [3H] thymidine, with a maximum at 50 μM and 100 μM. These results suggest that RES, acting on HUVECs in a dose-related fashion, blocking the synthesis of DNA, could be considered as an inducer of the cell cycle blockage at the higher concentrations used. This confirms a dose-dependently effect for RES.

To better understand the mechanisms observed, we decided to measure the degree of DNA fragmentation, using a radioisotopic technique with [3H] thymidine. This group of experiments were conducted in HUVECs treated for 24 hours with different concentrations of RES between 25; 50 and 100 μM. As shown in figure 4.4, the higher concentrations induce an higher DNA fragmentation, especially after 100 μM treatment. This has encouraged us to hypothesize that the observed anti-proliferative phenomena using high concentrations of RES, could be correlated with an activation of an apoptotic program in HUVECs from RES.

The other authors' studies on endothelial cells showed the same results as ours. For examples, results of Bela Szendel show dose-dependent effect on normal endothelial cells in culture. The cell number of HUVECs in culture is decreased drastically at 10 and 100 μg/ml concentration of trans-RES for 24 to 48 hours. One μg/ml of RES exerted a slight anti-proliferative effect. However, a significant, well-measurable proliferation promoting effect on endothelial cells in culture for 24 to 48 h was detected if the amount

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of RES applied was very small (0.1 µg/ml). The apoptotic index decreased with the decrease of the concentration of RES and this change was near to zero at the 0.1 µg/ml dose [120].

One study of Ferry-Dumazet from France shows that at higher dose (60 µM), RES inhibits the growth and induces apoptosis in case of both normal (60 µM) and leukemic (5-43 µM) hematopoietic cells. In this study, the authors have shown that RES induced a dose-dependent decrease in cell number and cell proliferation by inducing apoptosis via activation of caspase cascade. Thus, RES can be considered as an inhibitor for the human myeloid of lymphoid leukemic cell growth at high dose (43 µM). RES can reduce cell growth and induce apoptosis in case of normal cells when administered in high dose (60 µM). Thus, RES has biphasic effects over low to high spectrum of concentrations. At low concentration 5 µM, RES appears to increase cell proliferation, whereas apoptosis is induced in various cancer cells at 15 µM or higher concentration [121].

In a recent study of Kyungmin from Korea, the authors have shown that RES induced apoptosis in endothelial cells with increasing concentration (2.5-100 µM) [122] and clearly indicates that RES at a high concentration has a strong pro-apoptotic effect on endothelial cells.

It is important to notice that diverse activities of RES are dose-dependent. For instance, we demonstrated that RES induced apoptosis in the same concentration range that inhibited cell proliferation. The induction of apoptosis is a possible explanation for the anti-proliferative effect of RES.

For this reason, we evaluated the expressions of some genes involved in the programmed cell death and cell cycle. Using a semi-quantitative RT-PCR, we studied Bcl-2, Bax, c-myc, ODC gene and Cyclin D1 expression.

Firstly, to understand if our results could be correlated with the activation of apoptosis, we evaluated the gene expressions of the pro-apoptotic Bax gene and anti-apoptotic Bcl-2 gene. HUVECs were treated for 24 hours with those

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concentrations of RES (25; 40 and 50 μM) at which cells have showed a reduced viability. The results show that increasing doses of RES lead to a strong inhibition of Bcl-2 and a strong increase of Bax gene expression. These results confirm the data obtained in the last three experiments cell viability, DNA synthesis and DNA fragmentation. In a study of Paula M. Brito on RES disruption in apoptotic pathway, RES, at concentrations so low as 10 μM, inhibited biochemical and cellular mechanisms underlying apoptosis and that RES preferentially disrupted the mitochondrial apoptotic pathway than the death receptor apoptotic pathway, through modulation of Bcl-2 intracellular levels, considerably decreasing the Bax/Bcl-2 ratio [123]. Low showed same results on tumor cells: to investigate the effect of Bcl-2 expression on RES-induced cell death, human leukemia cells were exposed to increasing concentrations of RES and cell viability was determined by the MTT assay. To provide evidence that the growth inhibitory effect of RES was a function of the execution of the apoptotic machinery, the activities of three major caspases (8,9 and 3) were determined. A dose-dependent induction of caspase 8,9 and 3 was detected in cells subjected to RES treatment, while Bcl-2 overexpression significantly blunted the induction of caspase [124]. In the study on mechanisms involved in RES-induced apoptosis and cell cycle arrest in prostate cancer-derived cell lines of Benitez et al, in LNCaP cells, pro-apoptotic regulator Bax increased with RES treatments, whereas anti-apoptotic Bcl-2 protein markedly decreased even at low concentrations of RES. In contrast, in PC-3, cells only Bcl-2 significantly decreased by RES treatment with no important changes in Bax level. So the biochemical mechanisms underlying apoptosis and cyto-protection afforded by RES are not yet fully understood, it is necessary to deeply cell death insights [125].

Myc family proteins are important regulators of cell growth and are frequently activated in tumor-genesis. C-myc is a transcription factor, promotes cell proliferation, and inhibits differentiation where this is normally

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associated with a withdrawal from the cell cycle. The ODC gene is a transcriptional target of c-myc. C-myc and ODC have similar biologic effects. Both are required for entry into S phase of the cell cycle [126]. In this study, serum-starved HUVECs were stimulated for 40 minutes with medium M199 containing different concentrations of RES (1; 10; 25; 50 and 100 µM). We found that c-myc and ODC gene expressions were inhibited significantly at the concentrations of 50 and 100 µM, that means treated HUVECs have a high concentration of ODC protein in endothelial cell cytoplasm. These changes confirm that increase of ODC protein was higher in cells treated with high dose of RES. RT-PCR showed that the ODC mRNA is higher compared to its expression in low doses of RES that play an important role in the mechanisms of apoptotic program in endothelial cells. Therefore, at the concentration of RES 50 µM, Bcl-2 and Bax gene expressions are relevant to the ones of c-myc and ODC mentioned above. Zhang P. 's study showed that the total c-myc protein in Western Blotting and RT-PCR especially its nuclear fraction were decreased distinctively in the two cancer cell lines (UW228-2/3) upon RES treatment of 100 µM [127]. So c-myc regulation of ODC contributes to the induction of apoptosis by c-myc. ODC is generally associated with cell proliferation, as it is required for cell cycle progression.

The present study was designed to test whether RES may effect on endothelial cell cycle. Based on last results on c-myc and ODC gene expression, this prompted us to investigate in further detail the mechanism of action of RES on the cell cycle machinery. RT-PCR analysis of positive cell cycle regulator – Cyclin D1 showed a dose-dependent decrease in Cyclin D1 levels at concentrations up to 100 µM, suggesting the presence of an S to G2 block. In Wolter 's study, Wolter found that as a positive regulator of cdk4 and cdk6, cyclin D1 at high concentration of RES has been implicated in controlling the G1 phase of the cell cycle and is frequently overexpressed in

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human colon adenocarcinomas. Overexpression of antisense cyclin D1 cDNA construct in a human colon carcinoma cell line leads to impaired cell growth and tumorigenicity, implying that cyclin D1 presents an oncogene [96]. This result is the same as our result on RES-treated HUVECs. Higher concentrations of RES (50 and 100 μM) determined a decrease in Cyclin D1 expression with a minimum at 100 μM, that at the same concentration had shown lower c-myc, ODC gene expression.

A growing body of literature indicates that RES, a plant constituent enriched in the skin of blue grapes, is one of the promising agents for the prevention of heart disease, cancer and inflammatory diseases. We demonstrated that RES modulates biphasically the viability in HUVECs, inducing an apoptotic phenomenon.

In MTT assay, low concentrations of RES induced an increase in cell viability within 24 hours of treatment, with a peak absorbance at 10 μM significantly. And the only 10 μM concentration of RES gave a significant increase in DNA synthesis. From these two experiments, we confirm a biphasic effect of RES. So the suitable concentration of RES for cell proliferation could be 10 μM. And to find out, to better understand the suitable dose for cell apoptosis, we measured the degree of DNA fragmentation, using a radioisotopic technique with [³H] thymidine on RES-treated HUVECs with the concentrations 25, 40, 50 μM. As shown in Figure 4.4, the concentration 25 μM decreases slowly the DNA fragmentation compared to the control, and at the concentration of 50 μM, we saw the significantly higher DNA fragmentation. This result has encouraged us to hypothesize that the concentration of RES 50 μM could be the point to start an apoptotic program. For this result, we evaluated the expression of some genes involved in this pathway. Using a semi-quantitative RT-PCR, we studied Bcl-2, Bax gene. In study of Bcl-2 and Bax gene, the levels of Bcl-2

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mRNA decreased and of Bax gene increased significantly with the augmentation of RES concentration up to 50 µM and this result agrees with previous experiments. About the experiments on expressions of c-myc, ODC gene and Cyclin D1, they had a strong decrease after 50 µM treatment. That means from the concentrations of RES up to 50 µM, HUVECs start to withdraw from cell cycle. Therefore, the two concentrations of RES related to cell proliferation and apoptosis may be 10 and 50 µM.

In the study on bladder cancer cells of Stocco B., there were same employed concentrations of RES like ours. To evaluate if RES has any effect on cancer cells, these cells were treated with different concentrations of RES (0.1-100 µM). Low concentrations of RES (0.1-25 µM) were not able to inhibit cell growth of cancer cells. However, cancer cells incubated with higher RES concentrations (50 -100 µM) have shown statistically significant decrease in their cell growth. Briefly, we have observed a biphasic effect of RES on cell viability of cancer cells, since at high concentrations (>20 µM) it has induced death cell, whereas at low concentrations (0.1-20 µM) it has not and also protected them from oxidative stress [128].

Our data show that, at low concentration (0.1-25 µM), RES decreases neither cell viability nor DNA synthesis and does not induce a significantly cell permeability. On the other hand, at highest concentrations tested (>25 µM), it was able to decrease the cell growth such as DNA synthesis and to increase the percentage of cells that present permeability, signals of death cell. This biphasic effect of RES already has been demonstrated to other kind of cells, including normal and cancer cells. For example, in prostate cancer cells, Kuwajerwala showed that the effect of RES on DNA synthesis varied dramatically, depending on the concentration and the duration of treatment and using an extended treatment it was observed a dual effect of RES on DNA synthesis. At 5-10 µM it caused a 2- to 3-fold increase in DNA synthesis, and at >15 µM, it inhibited DNA synthesis [99].

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From above results, we demonstrated that RES induced apoptosis in the same concentration range that inhibited cell proliferation. The induction of apoptosis is a possible explanation for the anti-proliferative effect of RES.

Hussain studied on induction of apoptosis in malignant cells which is a very important mechanism of action of anticancer drugs [129]. The exact mechanism of action of RES is not fully understood, however, it has been proposed that RES causes its pro-apoptotic effects via generation of ROS in various cancers. They confirm these findings by clearly demonstrating by H_2DCFDA based experiments that RES treatment causes release of ROS in cells. The mitochondrial apoptotic pathway plays an important role on inducing apoptosis via activation of pro-apoptotic molecules such as Bax leading to release of cytochrome c into cytosol and activation of caspases [41]. RES mediated its apoptotic effects in cells via in-activation of Bad leading to conformational changes in Bax protein and its translocation into the mitochondrial membrane. Once Bax is translocated to the mitochondrial membrane, it renders the membrane leaky thereby causing changes in the mitochondrial membrane potential. Loss of mitochondrial membrane potential is one of the main mechanisms responsible for cytochrome c release in response to different cytotoxic stimuli. In cytosol, cytochrome c plays a key role by activating pro-caspase 9 in the presence of ATP. This in turn causes cleavage of caspase-9 that propagates the death signal by activating caspase-3 and causing cleavage of Poly ADP-ribose Polymerase (PARP). Activation and cleavage of PARP is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death. Our study gave the same results as this study, suggesting that RES treatment generates ROS in endothelial cells which mediated its apoptotic effects.

RES protects human health through chemoprevention to cardio-protection. RES provides protection against cancer, cardiovascular diseases, aging related diseases and also possess chemo-protective, neuroprotective,

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anti-inflammatory properties. In case of myocardial injury and aging related diseases, RES protects cells from apoptosis thereby working as an anti-apoptotic agent whereas in cancer prevention, RES kills the cancer cell by inducing apoptosis, thus working as a pro-apoptotic agent. So RES can function both as pro-apoptotic and anti-apoptotic agents. But the question is how RES can function as both pro- and anti-apoptotic agents?

In our study, to investigate potential intracellular signals involved in proliferative and apoptotic effect of RES in HUVECs and whether RES, at a concentration able to affect cell function, we studied p-MEK, p-ERK1/2 and p-38. These signals are a chain of proteins in the cell that communicates a signal from a receptor on the surface of the cell to the DNA in the nucleus of the cell. Cultured HUVECs were stimulated for 0 hour, 16 hours and 24 hours. At the time point of 0 hour and 16 hours, after developing the film, we obtained no bands, that means RES effect is time-depending. The figure 4.10 showed the bands from treatment in 24 hours. In this figure, there are any significant differences between the control and the treated cells, meaning that RES does not influence on these studied protein kinases. That suggests the other protein kinases participated in the pathway related to expression of Cyclin D1, Bcl-2, Bax, c-myc and ODC gene. Unlike one study of Klinge, it reported that RES activates extracellular signal regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family and stimulates endothelial nitric oxide synthase at low concentration (50 nM) in endothelial cells [130,131]. The primary goal of this study was to determine whether RES rapidly activated MAPK and eNOS via estrogen receptor activation in endothelial cells, indicating that the MAPK pathway is involved in eNOS activation. This is different from our results due to the treatment time of RES on cells and the completely own goals.

To investigate the mechanisms underlying the signaling pathways related

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to proliferative and apoptotic endothelial cells, we study on other protein kinases, protein kinase C. PKC is a family of phospholipid-dependent serine/threonine kinases, categorized on the basis of their structure and response to phosphatidylserine, calcium and diacyl-glycerol.

Recent studies have indicated that the effects of RES on the processes of cytokine production, endothelial cell growth and proliferation and also on changes in cell morphology, likely result from an impact on elements of the signaling networks that regulate them. In particular, it has been shown that RES can interfere with signaling cascades by modulating kinase and other enzyme activities. In this study of the mechanism of the effects of RES on the activites of PKC isozymes induced by association with membranes was investigated. In particular, by virtue of the centrality of PKC in signaling cascades that regulate the functioning of endothelial cells, the inhibitory effects of RES on PKC activities are likely to be important determinants in the mechanisms by which RES exerts its beneficial effects on cellular and cardiovascular function [132].

Figure 4.11 showed the PKC activity on HUVECs treated with RES for 40 minutes. Higher concentrations (50; 100 µM) lead to significant decrease of PKC activity, with a minimum at 100 µM, that at the same concentration showed lower c-myc, ODC gene and Cyclin D1 expression in nucleus.

Through figure 4.12, PKC activity for 24 hours are expressed highest at 25 µM, lowest at 100 µM, that at the same concentration showed highest Bcl-2 gene expression and lowest Bax expression. From these results, RES modulates PKC activity in HUVEC proliferation, apoptosis.

PKC is known to generate ROS [133] and ROS activate signal transduction cascade and transcription factors leading to up-regulation of genes and proteins involved in proliferation and apoptosis pathway [5,8]. On the other hand, effects of RES on PKC activity are studied. We, therefore,

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hypothesized that dose-dependently RES-induced PKC activation in HUVECs may stimulate cellular ROS and ROS thus generated may in turn activate PKC and provide signal amplification in these pathways. Our hypothesis is based on a study of Lee HB which indicated that high glucose generates cellular ROS in HPMC through activation of PKC, NADPH oxidase, and mitochondrial metabolism [134].

The toxicology of RES has become a controversial area of debate. Our results support the idea that high doses of RES rather than producing protective antioxidant effects may prompt pro-oxidant-induced damage. Confirming previous observations, we also reported that low concentrations of RES can prompt a significant antioxidant effect. Such a phenomena may be explained by the interaction of RES with lipid rafts/caveolae, specialized plasma membrane micro-domains involved in ROS compartmentalization, and redox-regulated signal transduction [135]. Indeed, recent data indicate that nanomolar concentration of RES can enhance endothelial NO production through a caveolae-dependent mechanism involving p42/44 MAPK activation [136]. But in our study, there was no significant difference in this mechanism between the control and treated cells. This suggests another mechanism involving in proliferative and apoptotic pathway is supposed to PKC activity. We are wondering whether there are any relationships between ROS levels and PKC activity due to dose-dependently responses.

Surprisingly, the effects of RES have been mainly tested against oxidative-induced damage or other toxic insults, whereas relatively little effort has been put in assessing their potential effects under normal conditions. For example, RES at a concentration of 50 μM, well above the antioxidant concentrations currently used in this work, protects HUVEC from oxidized low-density lipoprotein-induced oxidative damage [137]. However, it has been suggested that RES can work as a pro-oxidant under low oxidative

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conditions [138]. Thus, the interaction of RES with the cellular redox state could be of primary importance, especially when precise redox modulation is needed to allow normal cell function or to promote cell death.

From above reasons, further studies are required to better characterize the molecular mechanism of the RES-induced cell toxicity. Our findings support recent observations suggesting that RES can have a potent nonspecific toxicity towards normal cells [139]. It remains to be elucidated whether RES-induced endothelial cell toxicity could help explain some of the mixed results obtained with RES-based strategies in the prevention or treatment of cardiovascular pathologies and cancers.

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CONCLUSION

The effects of RES on HUVEC functions

- The RES effect on ECs is biphasic. At low concentrations increase cell growth, while at high concentrations cause the opposite effect.

- Treatment of HUVECs with RES exerted a significant antioxidant effect confirming the protective role of RES previously described. The exposure of HUVECs to higher RES concentrations increased intracellular ROS levels in a dose-dependent manner.

- The results of cell viability and DNA synthesis may identify cell proliferation in HUVECs by using low RES concentrations.

- The result of DNA fragmentation experiments may indicate cell death by apoptosis in HUVECs related to the expression of Bax and the decrease of the expression of Bcl-2, using RES 50 μM, is consistent with the significant increase in DNA fragmentation, a typical marker of apoptosis. And at this concentration, RES makes cell cycle arrest by reducing Cyclin D1 and ODC, c-myc expression.

- PKC appears to be the potential mediator of the observed RES effect on HUVEC proliferation and apoptosis and whether or not a link with ROS levels.

- However, further investigation will be necessary to better elucidate the molecular mechanisms at the basis of the proliferative and apoptotic effect of RES, and whether ROS interacts the PKC activity in the pathway related to HUVEC functions.

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