Study of intracellular signaling pathways triggered by natural antioxidants in human endothelial cells

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CHAPTER 1
INTRODUCTION


**Reactive Oxygen Species (ROS)**

§ 1.1 Chemistry of ROS

Reactive Oxygen Species (ROS) are a variety of molecules and free radicals derived from molecular oxygen. A radical is a clusters of atoms one of which contains an unpaired electron in its outermost shell of electrons. This is an extremely unstable configuration, and radicals quickly react with other molecules or radicals to achieve the stable configuration of 4 pairs of electrons in their outermost shell (one pair for hydrogen).

Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (also known as a triplet state). Since the two single electrons have the same spin, oxygen can only react with one electron at a time and therefore it is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as singlet oxygen) becomes a powerful oxidant as the two electrons with opposing spins can quickly react with other pairs of electrons, especially double bonds.

Oxygen is a crucial molecule to higher organisms life, acting as final acceptor for the electron released during biologic oxidations, under aerobic conditions. The superior role of aerobic versus anaerobic metabolism depends on the higher energy yield; so the aerobic respiration is the normal procedure for energy production in animal cells. Only a few mammalian tissues (notably erythrocytes and skeletal muscle) have developed major ability to obtain energy under anaerobic conditions from carbohydrate metabolism, through the glycolytic pathway.
The reduction-oxidation reactions occur during normal metabolic processes: during normal respiration, molecular oxygen is sequentially reduced by the addition of four electrons to generate water.

The reduction of oxygen by one electron at a time produces several different oxygen metabolites, which are collectively called ROS.

Superoxide anion ($O_2^-$), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of $O_2^-$ (either spontaneously or through a reaction catalysed by superoxide dismutases) produces hydrogen peroxide ($H_2O_2$), which in turn may be fully reduced to water or partially reduced to hydroxyl radical ($•OH$), one of the strongest oxidants in nature. The formation of $•OH$ is catalysed by reduced transition metals, which in turn may be re-reduced by $O_2^-$, propagating this process [1]. In addition, $O_2^-$ may react with other radicals including nitric oxide (NO•) in a reaction controlled by the rate of diffusion of both radicals. The product, peroxynitrite (ONOO•), is also a very powerful oxidant [2]. The oxidants derived from NO• have been recently called reactive nitrogen species (RNS).

The structure of ROS is shown in the figure below, along with the notation used to denote them.

Another radical derived from oxygen is singlet oxygen, designated as $^1O_2•$. This is an excited form of oxygen in which one of the electrons jumps to a superior orbital following absorption of energy.
§ 1.2 Sources of ROS

As mentioned above, the reactive oxygen species are the main byproducts formed in the cells of aerobic organisms, and can initiate autocatalytic reactions so that molecules to which they react are themselves converted into free radicals to propagate the chain of damage. The amount of free radical production is determined by the balance of many factors, and ROS are produced both endogenously and exogenously.

In mammalian cells the endogenous sources of ROS include:

- *mitochondria* (mainly complex I & III, but also monoamino oxidase, \(\alpha\)-ketoglutarate dehydrogenase, glycerol phosphate dehydrogenase, p66\text{shc} [3]);
- *endoplasmic reticulum* (mainly cytochrome P-450 and b5 enzymes, diamine oxidase, Ero1 [4]);
- *peroxisomes* (mainly fatty acid oxidation, D-amino acid oxidase, L-2-hydroxyacid oxidase and urate oxidase [5]);
- *cytosol* (NO synthases, lipoxygenases and PGH synthase [6-8])
- *plasma membrane* (NADPH oxidases, lipoxygenase [9; 10]) and extracellular space (xanthine oxidase [11]).

*Mitochondria* seem to be (quantitatively) the most important subcellular site of \(O_2^-\) and \(H_2O_2\) production in mammalian organs and the steady state concentration of \(O_2^-\) in the mitochondrial matrix is about 5- to 10-fold higher than that in the cytosolic and nuclear spaces [12]. Approximately 98% of the oxygen we metabolize undergoes a concerted tetravalent reduction to produce water in a reaction catalyzed by cytochrome
oxidase (cytochrome c-oxygen oxidoreductase) of complex IV in the mitochondrial electron transport chain ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$).

The Electron Transport Chain (ETC) is an essential mechanism for generation of cellular energy and it is localized to the mitochondrial inner membrane. The ETC consists of four multi-subunit enzyme complexes and the mobile electron carriers, coenzyme Q (inner membrane) and cytochrome c (intermembrane space), that pass electrons sequentially from high (NADH or FADH$_2$) to low (molecular oxygen) redox potential.

Cytochrome oxidase is the terminal electron acceptor in the chain and must give up its reducing equivalents to allow continued electron transport: if electrons stop flowing through the chain, the protonmotive force dissipates and ATP production cannot continue.

During normal respiration, approximately 2–4% of electron flow through the ETC results in only partial reduction of $O_2$, generating superoxide ($O_2^-$) which dismutates to form hydrogen peroxide ($H_2O_2$), which can further react to form the hydroxyl radical (HO•).

Although the mitochondrial electron transport chain is a very efficient system, the very nature of the alternating one-electron oxidation-reduction reactions it catalyzes (generating a constantly alternating series of “caged” radicals), predispose each electron carrier to side reactions with molecular oxygen. Thus, for example, as ubiquinone within the electron transport chain cycles between the quinone (fully oxidized) to semiquinone (one-electron reduction product) to quinol (fully reduced by two electrons) states, there is a tendency for an electron to pass to oxygen directly (generating $O_2^-$) instead of to the next electron carrier in the chain.

There are two specific sites where electrons may leak out of the chain to partially reduce oxygen. One is the NADH dehydrogenase in complex I and the other is the ubiquinone–cytochrome c reductase in complex III [13; 14]. In contrast, although complex IV contains intermediates corresponding to the

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three oxyradicals, $O_2^-$, $H_2O_2$ and •OH, these intermediates are tightly bound and chemically “disguised”, preventing production of reactive oxygen species by complex IV [15].

Several iron-sulfur clusters within the respiratory chain are also subject to such toxic, $O_2^-$ generating, side reactions with oxygen. Thus it is commonly held that mitochondrial generation of $O_2^-$ represents the major intracellular source of oxygen radicals under physiological conditions.

In addition to these toxic electron transport chain reactions of the inner mitochondrial membrane, also the mitochondrial outer membrane enzyme, monoamine oxidase, is a quantitatively large source of $H_2O_2$ that contributes to an increase in the steady state concentrations of reactive species within both the mitochondrial matrix and cytosol [12]. Monoamine oxidase is a flavoprotein ubiquitously expressed in higher eukaryotic organisms and it catalyzes the oxidative deamination of primary aromatic amines along with long-chain diamines and tertiary cyclic amines. The enzyme-catalyzed oxidative deamination of biogenic amines is a quantitatively large source of $H_2O_2$ that contributes to an increase in the steady state concentrations of reactive species within both the mitochondrial matrix and cytosol.

Reactive oxygen species cause damage to mitochondrial components and initiate degradative processes. The sites of “electron leak” during normal respiration provide insight into the likely sources of increased oxyradical production in pathologic states.
The endoplasmic reticulum (ER) is a membrane-bound intracellular organelle that is primarily involved in lipid and protein biosynthesis. Smooth ER (lacking bound ribosomes) contains enzymes that catalyze a series of reactions to detoxify lipid-soluble drugs and other harmful metabolic products. The most extensively studied of these are the cytochrome P-450.

Cytochrome P450s comprise a superfamily of heme-thiolate proteins named for the spectral absorbance peak of their carbon-monoxide-bound species at 450 nm. They support the oxidative, peroxidative and reductive metabolism of such endogenous and xenobiotic substrates as environmental pollutants, agrochemicals, plant allelochemicals, steroids, prostaglandins and fatty acids. Cytochrome P450s have traditionally been referred to as hydroxylases, mixed function oxidases and monooxygenases [16]. Their main function is to activate molecular oxygen to yield a reactive species that can attack relatively inert chemical sites in order to introduce hydroxyl groups into structures unreactive as hydrocarbon chains and aromatic rings. This serves to facilitate the biotransformation of compounds that would otherwise lack functional groups suitable for conjugation. In addition to hydroxylations, however, cytochrome P450s also catalyze a broad variety of other chemical reactions.
including deaminations, desulfurations, dehalogenations, epoxidations, N-, S-, and O-dealkylations, N-oxidations, peroxidations and sulfoxidations. Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle [17]. In fact the underlying concept of its activity is a multi step transfer of 2 electrons to a substrate while binding one oxygen atom to it, the second being reduced to water. Part of the oxygen involved is inevitably reduced to superoxide.


Also peroxisomes are an important source of cellular H₂O₂ production [5]. They contain a number of H₂O₂-generating enzymes including glycolate oxidase, D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase and fatty acyl-CoA oxidase. Peroxisomal catalase utilizes H₂O₂ produced by these enzymes to oxidize a variety of other substrates in “peroxidative” reactions.
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 [19]. Specific signaling roles have not been ascribed to peroxisome-derived oxidants, and only a small fraction of $\text{H}_2\text{O}_2$ generated in these intracellular organelles appears to escape peroxisomal catalase [18].

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 degree takes place in practically every cell. Enzymes participating in the process are cyclooxygenase, lipooxygenase and cytochrome P-450 [20]. Arachidonic acid may be a source of ROS even by a non-enzymatic process.

Instead the best characterized of the plasma membrane oxidases in general is the NADPH oxidase. It is present in both professional phagocytic cells (macrophages, neutrophils and eosinophils) and nonphagocytic cells (such as endothelia or smooth muscle cells) [21]. This multicomponent enzyme catalyzes the one-electron reduction of $\text{O}_2$ to $\text{O}_2^{-}$, with NADPH as the electron donor through the transmembrane protein cytochrome b558 (a heterodimeric complex of gp91$^{\text{phox}}$ and p22$^{\text{phox}}$ protein subunits). The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to $\text{O}_2$ on the outside. During phagocytosis, the plasma membrane is internalized as the wall of the phagocytic vesicle, with what was once the outer membrane surface now
facing the interior of the vesicle. This targets the delivery of $\text{O}_2^{-•}$ and its reactive metabolites internally for localized microbicidal activity [22].

The NADPH oxidase of nonphagocytic cells is similar in structure and it is activated by various hormones and cytokines. It is permanently in a fully preassembled state and constantly producing low amounts of $\text{O}_2^{-•}$, most likely with a regulatory function [23]. NADPH oxidase superoxide production is directed mainly intracellularly as opposed to NADPH oxidase generated superoxide in neutrophils, where it serves as a defence mechanism outside the cell.

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 ($\text{O}_2^{-•}$) and hydrogen peroxide ($\text{H}_2\text{O}_2$). 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 [1].
§ 1.3 Role of ROS

It has been established that ROS can be both harmful and beneficial in biological systems depending on the environment [24; 25]. Beneficial effects of ROS involve, for example, the physiological roles in cellular responses to noxia 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 [26]. 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 [27].
§ 1.4 Antioxidants

Humans have evolved highly complex antioxidant systems (enzymatic and nonenzymatic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. An ideal antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels.

Excessive production of ROS, outstripping endogenous antioxidant defense mechanisms, determines the degree of oxidative stress.

The most efficient enzymatic antioxidants involve superoxide dismutase (SOD), catalase and glutathione peroxidase [22]. SOD speeds the conversion of superoxide to hydrogen peroxide, whereas catalase and glutathione peroxidase convert hydrogen peroxide to water.

First in the line of enzymatic ROS degradation is superoxide dismutase (SOD). This enzyme exists in 3 forms: a) Cu/Zn SOD present mainly in cytosolic matrix, b) MnSOD localized preferentially in mitochondria and c) extracellular SOD.

The SODs catalyze the reaction: $\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$

This dismutation, or disproportionation reaction, makes use of the fact that superoxide is both an oxidant and a reductant, eager to get rid of its extra electron or to take on another. The enzyme uses one superoxide radical to oxidize another.

Catalase works in much the same way, because hydrogen peroxide can be a weak reductant as well as a fairly strong oxidant; it catalyzes the reaction: $\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$

This enzyme is present in the peroxisome of aerobic cells and it is very efficient in promoting the conversion of hydrogen peroxide to water and
molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute [1].

Glutathione peroxidase acts in association with tripeptide glutathione (GSH), which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also competes with catalase for hydrogen peroxide as a substrate and it is the major source of protection against low levels of oxidative stress [1]. This enzyme uses NADPH as the reducing species for hydrogen peroxide:

\[ \text{NADPH} + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{NADP}^+. \]

Glutathione peroxidase can reduce lipid peroxides as well as hydrogen peroxide and it is a very important enzyme in the prevention of lipid peroxidation to maintain the structure and function of biologic membranes.

The non-enzymatic antioxidants are mostly “scavengers” of free radicals, such as vitamin C, vitamin E (inhibits oxidation of membrane lipids), uric acid (efficient scavenger of peroxynitrite, present in plasma and airway lining fluid), albumin, bilirubin, glutathione or N-acetylcysteine (NAC). NAC is a potent drug which acts directly by reacting with ROS (forming NAC disulfides in the end) and indirectly, serving as a GSH precursor [28]. However, as with many antioxidant substances, NAC in high doses can exhibit prooxidative effects [29].

Although the metal chelating plasma proteins (transferrin, ceruloplasmin, albumin, haptoglobin, and hemopexin) do not interact directly with and decompose ROS, they are considered antioxidants because they bind redox active metals and limit the production of metal-catalyzed free radicals. So the maintenance of intracellular redox homeostasis is dependent on a complex web of antioxidant molecules.
The antioxidants can be endogenous or obtained exogenously, eg as a part of a diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. However, under conditions which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions.

Polyphenols are the most abundant antioxidants in our diet and are widespread constituents of fruits, vegetables, cereals, olive, dry legumes, chocolate and beverages, such as tea, coffee and wine [30]. Despite their wide distribution, the healthy effects of dietary polyphenols have come to the attention of nutritionists only in the last years. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure. Polyphenols comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another.

The main groups of polyphenols are: flavonoids, phenolic acids, phenolic alcohols, stilbenes and lignans. The flavonoids, which share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins). In addition to this diversity, polyphenols
may be associated with various carbohydrates and organic acids and with one another [30].

The chemical structures of polyphenols and flavonoids are shown in the figure below.

The chemical structures of polyphenols.
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The chemical structures of flavonoids
As antioxidants, polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated to oxidative stress. Experimental studies, in fact, strongly support a role of polyphenols in the prevention of cardiovascular diseases, cancer, osteoporosis, diabetes mellitus and neurodegenerative diseases [31]. In particular, it has been shown that the consumption of polyphenols limits the development of atheromatous lesions, inhibiting the oxidation of low density lipoprotein [32 - 35], which is considered a key mechanism in the endothelial lesions occurring in atherosclerosis. However, many controlled clinical trials have failed to demonstrate that increased antioxidants consumption has a protective action against cardiovascular diseases [36].

For many years, polyphenols and other antioxidants were thought to protect cell constituents against oxidative damage through scavenging of free radicals. However, this concept now appears to be an oversimplified view of their mode of action [37]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [38 - 40]. Both antioxidant and prooxidant [41] effects of polyphenols have been described, with contrasting effects on cell physiologic processes: if as antioxidants they improve cell survival, as pro-oxidant they may induce apoptosis and block cell proliferation [42]. On the other hand, accumulating evidence indicates that polyphenols might exert several other specific biological effects such as the inhibition or reduction of different enzymes, among which telomerase [43], cyclooxygenase [44; 45], lipoxygenase [46; 47] and the interaction with signal transduction pathways and cell receptors [48 - 50]. Moreover polyphenols can affect caspase-dependent pathways [51; 52], cell cycle regulation [53] and platelet functions [54].
For many years they received more and more attention as potential therapeutic agents against several chronic degenerative diseases [55; 56], but the complex relationships between antioxidant status and disease are still poorly understood and have been studied intensively.

*Resveratrol* is a naturally occurring phytoalexin; its chemical name is trans-3,5,4’-trihydroxy stilbene. It occurs in two isoforms cis– and trans–resveratrol, but trans–resveratrol is more biologically active than its cis–isoform. Resveratrol 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. Grape skin is the main source of resveratrol. Apart from these naturally occurring substances, red wine and white wine also contain resveratrol. It is also synthesized in response to environmental stressors that include water deprivation, UV irradiation and especially fungal infections [57].

Resveratrol attracted interest during early nineties in the context of “French paradox”; the phenomena wherein certain population of France, in spite of eating a regular high fat diet, was less susceptible to heart diseases [58]. The apparent cardioprotection was attributed to the regular consumption, in their diet, of moderate doses of red wine rich in resveratrol [59]. In last few decades, resveratrol 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 [60], vasorelaxing [61] activity and it has been demonstrated to inhibit lipid peroxidation [63; 64] and platelet aggregation, which is a major contributor in the process of atherosclerosis [62], ex vivo.
Moreover resveratrol has been shown to possess potential anticancer activity in various cancer cells at the initiation, promotion, and progression stages [65 - 67]. It is also well known to possess anti cancer properties in animal model [68].

In the vast majority of cases, resveratrol displays inhibitory/stimulatory effects in the micromolar range, which is potentially attainable pharmacologically. It appears that resveratrol, as a pharmacological agent, has a wide spectrum of targets[69].

Coumaric acids are organic compounds that are hydroxy derivatives of cinnamic acid. There are three isomers, \( o \)-coumaric acid, \( m \)-coumaric acid, and \( p \)-coumaric acid, that differ by the position of the hydroxy substitution of the phenyl group. \( p \)-Coumaric acid is the most abundant isomer in nature. Together with sinapyl alcohol and coniferyl alcohols, \( p \)-coumaric acid is a major component of lignocellulose. It is biosynthesized from cinnamic acid by the action of the P450-dependent enzyme 4-cinnamic acid hydroxylase. \( p \)-Coumaric acid can be found in a wide variety of edible plants such as peanuts, tomatoes, carrots, and garlic. It is a crystalline solid that is slightly soluble in water, but well soluble in ethanol and diethyl ether. \( p \)-Coumaric acid has antioxidant properties and is believed to reduce the risk of stomach cancer [70] by reducing the formation of carcinogenic nitrosamines [71].

\textit{§ 1.5 ROS and cell signalling}

Cells communicate with each other and respond to extracellular stimuli through biological mechanisms called cell signalling or signal transduction [18; 72]. Signal transduction is a process enabling information to be transmitted from the outside of a cell to various functional elements inside the cell. Signal transduction is triggered by extracellular signals such as hormones, growth factors, cytokines and neurotransmitters [73].
Signals sent to the transcription machinery responsible for expression of certain genes are normally transmitted to the cell nucleus by a class of proteins called transcription factors. By binding to specific DNA sequences, these factors regulate the activity of RNA polymerase II. These signal transduction processes can induce various biological activities, such as gene expression, cell growth, muscle contraction and nerve transmission [74].

Although the endogenous generation of ROS are a consequence of metabolic activities, many environmental stimuli including cytokines, ultraviolet (UV) 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, 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 [75]. A number of stress response mechanisms have evolved to help the cell and organism adapt to acute stress, and acting in either a cooperative or antagonistic fashion they serve to coordinate the acute cellular stress response and ultimately determine the outcome. Many of these pathways have been faithfully preserved throughout evolution. Among the main stress signalling pathways and/or central mediators activated in response to oxidant injury are the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signalling cascades, the phosphoinositide 3-kinase (PI(3)K)/Akt pathway, the nuclear factor (NF)-κB signalling system, p53 activation, and the heat shock response [75].

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.

Akt, also known as protein kinase B (PKB), is an evolutionarily conserved serine/threonine kinase. Mammalian cells express three Akt isoforms (Akt1–3) encoded by three separate genes. The amino acid sequences of the three isoforms are almost identical; relative expression of these isoforms, however, differs in various mammalian tissues. Akt is activated by extracellular signals that activate PI3K. For instance, upon
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Activation, growth factor receptors activate p110 (the catalytic subunit of PI3K) either by recruiting p85 (the regulatory subunit of PI3K) or by activating Ras, which can directly activate p110. Upon activation, p110 phosphorylates phosphoinositides (PI) at the D3 position of the inositol ring to generate PI (3,4,5) P3 (PIP3). The rate-limiting step in Akt activation is the binding of PIP3 to the pleckstrin homology (PH) domain of Akt and subsequent translocation of Akt to the plasma membrane, where it is phosphorylated on a threonine in the catalytic domain and a serine in the C-terminal regulatory domain. These modifications are required for full activation. The kinase that phosphorylates the threonine is PDK1, while the kinase that phosphorylates the serine was recently identified as mTORC2, which is a complex containing the mammalian target of rapamycin (mTOR) and Rictor [76]. Antagonizing PI3K activity can negatively regulate the activity of Akt. PTEN (for “phosphatase and tensin homolog deleted from chromosome 10”) is a 3 phosphoinositide phosphatase that negatively regulates Akt activity by reducing the intracellular level of PIP3 produced by PI3K [77]. Akt activity is also downregulated by activation of its downstream effector mTORC1, which in turn induces a negative feedback mechanism that inhibits Akt activity [78]. Hyperactivated Akt both provides protection from apoptosis and promotes uncontrolled cell-cycle progression [79], two major prerequisites for cancer susceptibility and this may explain, at least in part, its frequent activation in human cancers [78]. However, the principal role of Akt is to facilitate growth factor-mediated cell survival and to block apoptotic cell death, which is achieved by phosphorylating and deactivating proapoptotic factors such as BAD, caspase-9, and murine double minute-2 (MDM2) [80]. Akt also phosphorylates and inactivates glycogen synthase kinase-3β (GSK-3β), the inactivation of which prompts upregulation of cyclin D and enhances cell cycling. Akt is regulated by oxidative stress for cell survival, and phosphorylates IkB kinase-a/b. Activated IkB kinase-a/b, in turn, causes
activation and nuclear translocation of NF-kB-dependent prosurvival genes [80].

The MAPKs, instead, comprise a family of ubiquitous prolinedirected, protein-serine/threonine kinases, which play an essential role in sequential transduction of biological signals from the cell membrane to the nucleus [81]. In mammalian cells, there are three well-defined subgroups of MAPKs: the extracellular signal regulated kinases (ERKs, including ERK-1 and ERK-2 isoforms), the c-Jun N-terminal kinases (JNKs, including JNK-1, JNK-2, and JNK-3 isoforms), and the p38 MAPKs (including p38-α, p38-β, p38-γ, and p38-δ isoforms). Each subgroup of MAPKs is activated through a cascade of sequential phosphorylation events, beginning with the activation of MAPK kinase kinases (MAP3Ks). The MAP3Ks phosphorylate and activate a downstream dualspecificity MAPK kinases (MAP2Ks), which in turn...
stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues within a conserved tripeptide motif [81; 82]. The three subgroups of MAPKs (i.e., ERKs, JNKs, and p38 MAPKs) are involved in both cell growth and cell death, and the tight regulation of these pathways is paramount in determining cell fate [83]. The deleterious consequences of sustained activation of MAPK pathways may include excessive production of MAPK-regulated genes, uncontrolled proliferation, and unscheduled cell death.

Studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathways [84]. A number of cellular stimuli that induce ROS production also in parallel can activate MAPK pathways in multiple cell types. The prevention of ROS accumulation by antioxidants blocks MAPK activation after cell stimulation with cellular stimuli [84; 85], indicating the involvement of ROS in activation of MAPK pathways. Moreover, direct exposure of cells to exogenous H$_2$O$_2$, to mimic oxidative stress, leads to activation of MAPK pathways [86].


The initiating events leading to activation of pathways in response to oxidants are incompletely understood. Although a large number of signaling
Introduction

pathways appear to be regulated by ROS, the signaling molecules targeted by ROS are less clear. There is growing evidence, however, that redox regulation might occur at multiple levels in the signaling 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 autophosphorylates 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 [18]. Oxidants seem to activate the ERK and the PI(3)K/Akt pathways largely through stimulation of growth-factor receptors, mimicking the actions of natural ligands. Many growth-factor receptors have been shown to undergo enhanced phosphorylation in response to direct treatment with oxidants, and agents or conditions that prevent receptor phosphorylation likewise inhibit the activation of ERK and Akt by oxidants [75]. One mechanism proposed to explain this effect is oxidant-mediated inactivation of critical phosphatases necessary for dephosphorylation (turning off) of the growth-factor receptors. Support for such a mechanism has come from the finding that hydrogen peroxide, either derived exogenously or produced endogenously after growth-factor stimulation, can reversibly inactivate protein-tyrosine phosphatase 1B in cells. The activation of growth-factor-receptor signalling pathways by oxidants is consistent with the demonstration that low concentrations of exogenous hydrogen peroxide are mitogenic [75]. Oxidative stress might induce activation of the JNK and p38 kinase pathways by an additional mechanism. The redox regulatory protein thioredoxin (Trx) has been shown to bind to apoptosis signal-regulating kinase (ASK1), an upstream activator of both JNK and p38, and under normal conditions inhibit its activity. However, oxidative stress causes dissociation of the Trx–ASK1 complex and subsequent activation of the downstream JNK and p38 kinase.
Similarly, biochemical evidence indicates that under non-stressed conditions glutathione S-transferase binds to JNK to inhibit its activation, but that this interaction is also disrupted by oxidative stress [75]. These results show an intimate coupling between alterations in the intracellular redox state and the activity of downstream stress-activated pathways. The observation that multiple pathways are sensitive to a rise in ROS levels indicates that these pathways may have evolved, in part, to allow organisms to survive within an aerobic environment. In addition, it suggests that a rise in ROS might represent a common, if not universal, signal of cellular stress.

§ 1.6 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 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 [87]. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide). Upon receiving specific signals instructing the cells to undergo apoptosis a number of distinctive changes occur in the cell. A family of proteins known as caspases are typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as...
DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus [88]. Apoptotic cells display distinctive morphology during the apoptotic process. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance. Cells continue to shrink, packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterised by the appearance of membrane blebs or blisters process. Small vesicles called apoptotic bodies are also sometimes observed [88]. In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors called death receptors. These ligands can either be soluble factors or can be expressed on the surface of cells such as cytotoxic T lymphocytes. The latter occurs when T-cells recognise damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection. Apoptosis can also be induced by cytotoxic T-lymphocytes using the enzyme granzyme.

In other cases apoptosis can be initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation or chemicals or to viral infection. It might also be a consequence of growth factor deprivation or oxidative stress caused by free
radicals. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria. Besides serving as the major energy producer, mitochondria also play a critical role in the regulation of apoptosis, and act as a major switch to initiate apoptosis in mammalian cells [89]. They contain many pro-apoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome c. Although apoptosis can occur in the absence of cytochrome c release, cytochrome c directly activates downstream effectors when injected into the cytosol in the absence of upstream signals. Release may occur secondary to the onset of mitochondrial permeability transition (MPT) pore, which is permeable to solutes of less than 1200 Da [90]. The onset of MPT pore leads to loss of mitochondrial membrane potential and to swelling of the matrix space with eventual disruption of mitochondrial membranes and release of cytochrome c [91]. This catastrophic event is caused by oxidative damage to mitochondria in concert with mitochondrial calcium overload. Therefore, an increased mitochondrial formation of ROS triggers the intrinsic pathway by increasing the permeability of the outer mitochondrial membrane through the opening of transition pores. The opening of the permeability transition pore is favoured by oxidative stress through oxidation of intracellular glutathione and other critical sulfhydryl groups [87]. As a result of this process, cytochrome c moves from the intermembrane space into the cell’s cytoplasm where it joins another factor (Apaf-1). In the presence of dATP this complex polymerizes into an oligomer known as ‘apoptosome’. The apoptosome activates a protease (caspase-9), which in turn activates caspase-3. The cascade of proteolytic reactions also activates DNAses and in the end the process results in cell death. Under normal conditions, various anti-apoptotic factors (including Bcl-xL) prevent the mitochondrial permeability transition as long as they remain bound to the outer membrane [87]. This factor is eliminated when another factor, Bax, is translocated to
mitochondria, starting apoptosis. The gradual loss of cytochrome c from the intermembrane space during apoptosis favours the mitochondrial formation of $O_2^{-\cdot}$ in two ways: (1) cytochrome c is a scavenger of $O_2^{-\cdot}$ and (2) as cytochrome c is released, the respiratory chain becomes more reduced because electron flow between Complex III and Complex IV slows down [87].

ROS appear to be mitochondria derived and responsible for later mitochondrial events leading to full activation of the caspase cascade. How ROS acts in this scenario is not entirely understood. Oxidation 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 postulated, initial released ROS could directly or indirectly (via ceramide generation) increase the gating potential of the pore. Taken together, it seems that mitochondria are both source and target of ROS.
CHAPTER 2

AIM OF THE WORK
The deleterious effects of free radicals have been known for almost 50 years. More recently, however, an essential role for free radicals in physiologic control of several aspects of cell function has been demonstrated. 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 [30]. A variety of pathogenic stimuli can increase ROS production within the endothelial cell (EC) triggering biochemical and cellular processes, such as apoptosis and proliferation that eventually result in endothelial dysfunction. This phenomenon represents the initial step in the progression toward pathological syndromes, such as atherosclerosis and hypertension [92]. In this context, it has been hypothesized that Natural Antioxidants (NA) consumption, can counteract the effects of ROS by preventing ROS-induced oxidative damage and preserve endothelial function reducing the occurrence of cardiovascular events [93]. In fact their consumption has been associated with a reduced incidence of risk factors for cardiovascular diseases (CVD) [36].

CVD is of multifactorial etiology associated generally to a variety of risk factors for its development including hypercholesterolaemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity among others. During the last few decades, research data has prompted a passionate debate as to whether oxidation, or specifically, oxidative stress mediated by free radicals/ROS/RNS, is a primary or secondary cause of many chronic diseases. As a result, scientific resources have focused to a large extent on the role that antioxidants could play to delay or prevent oxidative stress and consequently the incidence of chronic disorders.

The endothelium is a complex organ system that controls the homeostasis of the vasculature by integrating signals between the vascular wall and the vessel lumen. Under physiological conditions, it maintains a
normal vascular tone and blood fluidity by elaborating a variety of factors, such as nitric oxide, prostacyclin and endothelin. However, in pathological situations the endothelium can also modify its phenotype facilitating vasoconstriction, inflammation and thrombotic events. These abnormal responses manifest in different clinical settings, such as hypercholesterolemia, hypertension, diabetes mellitus, and occur in the absence of any morphological change of the vessel.

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 reactive oxygen species and subsequent oxidative stress.

The widely accepted notion that consumption of antioxidants 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 exogenous antioxidant substances has generally failed to live up to their early promise; in fact many controlled clinical trials have failed to demonstrate that increased NA consumption has a protective action against CVD [36].

The reason for these disappointing findings is unclear, but some possible explanations have been proposed. In the endothelium, ROS are not only involved in pathological processes but, by modulating redox-regulated intracellular signals, they also finely tune EC physiology [94].

It has been proposed that ROS might function as dual effectors modulating both prosurvival and antisurvival signals [95]. Strategies aimed at the suppression of all ROS signaling could have the unexpected consequences of negatively impacting endothelial function. In addition, NA can have a pro-oxidant effect under particular conditions, paradoxically increasing ROS production and resulting in cell damage [96].
In this light, antioxidant-based strategies based on the suppression of all intracellular ROS, to prevent endothelial damage, may have the untoward effect of leading to EC loss.

While there is a consistent body of literature on the protective effects of NA against diseases or toxic drugs, there are relatively few reports on their possible toxicity. Indeed, NA-associated cardiovascular effects appear to be concentration dependent [97] 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 natural antioxidant, such as resveratrol and coumaric acid, affect differently the (patho)physiology of endothelial cells extracted from umbilical cord (HUVEC).

We initially evaluated the change of intracellular levels of ROS in presence of NA. Furthermore, using inhibitors of specific enzyme responsible of ROS production, the potential source of these molecules has been investigated.

To further elucidate the molecular basis of NA-induced oxidative stress, we sought to identify signaling transduction pathways responsible for the observed changes in the cellular response; so we studied specific pathways that regulate proliferation, apoptosis and cell cycle. In particular, we tried to assess whether AKT pathway could regulates cell survival in response to NA-induced oxidative stress.

Finally we investigated the molecular mechanism underlying the observed phenomena, considering the involvement of a possible mitochondrial damage in response to intracellular ROS produced after cells treatment with NA.
CHAPTER 3
MATERIALS AND METHODS
§ 3.1. Reagents.
Coumaric acid, resveratrol, rotenone, Angiotensin II, insulin, and N-acetyl cysteine (NAC) were supplied by Sigma (St Louis, MO). Diphenyleneiodonium (DPI), Wortmannin, LY-294002, and Staurosporine were from Calbiochem (San Diego, CA).

§ 3.2 Cell culture and treatments.
Human umbilical vein ECs (Cell Applications, San Diego, CA) were cultured in EC basal medium (Cell Applications) supplemented with Endothelial Cell Growth Supplement (Cell Applications). When confluent, ECs were subcultured at a split ratio of 1:2 and used within three passages. In order to mimic physiological vessel wall conditions, before experimentation, cells were grown until confluence to reach contact-dependent growth inhibition. Unless not specified in the text, cells were plated in 96-well black plates (BD Falcon, Franklin Lakes, NJ) and processed for experiments in EC-defined medium (European Collection of Cell Cultures, Salisbury, UK). Intracellular ROS measurements, immunoblotting, and protein carbonylation assays were performed after 80 min, while cell viability, metabolic assays, and apoptosis were done after 4 h. Dose- and time-dependent immunoblot experiments were performed as indicated in figure legends, after culturing ECs for 12 h under serum-free condition.

In selected experiment, cells were preincubated for 30 min with the Akt activator insulin and the specificity of insulin-mediated Akt activation was demonstrated by using the selective phosphoinositide-3 kinase (PI3K) inhibitors Wortmannin (20nM) and LY-294002 (10µM) [98].

To study the contribution to protein carbonylation and ROS levels of flavin-containing oxidases, we employed the flavoproteins inhibitor DPI (10µM) [99]. Staurosporine treatment (1µM) was used to induce EC apoptosis [100].
As an index of NADH- and NADPH-dependent oxidase activity, we assessed treatment-induced variations of NADH and NADPH consumption [101]. DPI-sensitive flavin oxidase activity was assessed as previously reported [102].

§ 3.3 Measurements of intracellular ROS.
Intracellular ROS levels were determined by using the ROS molecular probe 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) (Molecular Probe, Eugene, OR) as previously described with minor modification [103]. 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 min with Hanks’ Balanced Salt Solution (HBSS) containing 5µM H$_2$DCF-DA, then washed twice with HBSS and fluorescence was measured by using a GENios plus microplate reader (Tecan, Männedorf, 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 prooxidant outcome was evaluated by comparison of five measurements and expressed as a percentage of untreated controls.

§ 3.4 Measurements of protein carbonylation.
Proteins are one of the major targets of oxygen free radicals and other reactive species. Oxidative modification of proteins modifies the side chains of methionine, histidine, and tyrosine and forms cysteine disulfide bonds. Metal catalyzed oxidation of proteins introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues in a site-specific manner.
Protein carbonyl groups were measured with the OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA), following the protocol provided by the manufacturer.

In brief, proteins (20 µg) were denatured with 12% sodium dodecylsulfate (SDS), derivatized to 2,4-dinitrophenylhydrazone (DNPhydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH) then mixed with neutralization solution and β–mercaptoethanol. To evaluate the selectivity of carbonyl measurements, some protein samples underwent the protein carbonyl detection procedure without the derivatization step (negative control).

DNP-derivatized proteins were electrophoresed through a reducing 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane.

The membrane was blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated overnight at 4°C with rabbit anti-DNP antibody (1:500). The levels of carbonylated proteins were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) for 1 h at room temperature.

Blots were developed by an enhanced chemiluminescence System (Amersham, Buckinghamshire, UK) and densitometric analyzed by using the Versadoc Imaging System (Bio-Rad, Hercules, CA). Individual densitometric results were normalized to β-actin immunoreactivity and results were expressed as a percentage of untreated controls.

§ 3.5 Measurement of NADH and NADPH consumption.

The rate of oxidation of NADH and NADPH in the presence of oxygen (oxidase activity) was measured spectrophotometrically using methods similar to those previously described [101].

After treatments, confluent human umbilical vein endothelial cells (HUVEC) were scraped off from the flasks in 300 µl of 50mM phosphate buffer, pH 7.4,
plus protease and phosphatase inhibitors. The cell suspension was sonicated for three cycles of 20 s on ice; the homogenate was assessed for protein content and stored on ice until use. The assay was performed in 200 µl of 40mM Tris-Mes buffer (pH 7.4) containing either 250 µM NADH or 250 µM NADPH.

The reaction was started by adding 50 µl of each sample in the reaction mix and the rate of NADH or NADPH consumption was monitored during 30 min by reading the decrease in absorbance at 340 nm using a GENios plus microplate reader (Tecan). The extinction coefficient used to calculate the amount of NADH/NADPH consumed was 3.732 * 10^{-3} ml/nmol, which results from the microplate reader path length for a reaction volume of 200 µl in a 96-well plate.

For measurements of specific flavin oxidase activity, the rate of NADH or NADPH consumption inhibitable by DPI, a flavoproteins inhibitor, was used [99]. This was done by adding DPI (10µM) 15 min prior to treatments. The “DPI-inhibitable” NADH/NADPH consumption was used as a measure of flavin-containing NADH or NADPH oxidase activity [102]. All measurements were corrected for protein content, and results were expressed as nanomoles per minute per milligram of protein.

§ 3.6 Cell viability assay.

Cell viability was assessed by using propidium iodide (PI), Hoechst 33342 double fluorescent staining. PI can only enter cells with disrupted membrane integrity and therefore stains nonviable cells. Thus, all cell nuclei could be recognized by the blue fluorescence of Hoechst, while nuclei of damaged cells fluoresced red due to the accumulated PI. After treatments, cells were stained with PI and Hoechst (10 µg/ml) (Invitrogen, Carlsbad, CA), washed with PBS, and fluorescence was measured by using a GENios plus microplate reader (Tecan). Excitation and emission wavelengths used for fluorescence
quantification were, respectively, 340 and 485 nm for Hoechst and 485 and 612 nm for PI. Results were expressed as a percent of untreated control cells.

§ 3.7 Cell metabolic 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 takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) 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 an increase in the amount of MTT formazan formed and an increase in absorbance.

So after treatments, cells were added with 20 µl MTT solution (5 mg/ml) in medium M199 and incubated at 37°C in a cell incubator for 60 min. 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 (0.04N HCl in absolute isopropanol), and plates were analyzed at 570 nm using a GENios plus microplate reader (Tecan) with background subtraction at 650 nm.

Results were expressed as a percent of untreated control cells.
§ 3.8 Cell apoptosis assay.

Cell apoptosis was assessed after treatments by using the fluorimetric kit APOPcentage (Biocolor Ltd, Carrickfergus, UK), following the protocol provided by the manufacturer. The assay has been used with several adherent cell lines including HUVEC [104]. The assay uses the dye 3,4,5,6-tetrachloro-2’,4’,5’,7’-tetraiodofluorescein that is selectively imported by cells that are under going apoptosis. Maintaining the asymmetric composition is an energy dependant process involving the activity of enzymes, termed ‘flippases’. In apoptotic committed cells flippase regulation is either overwhelmed, or is inactivated by the activity of the enzyme ‘scramblase’ (floppase). Exposure of phosphatidylserine to the exterior surface of the membrane has been linked to the onset of the execution phase of apoptosis. The transfer of phosphatidylserine to the outside of the membrane permits the transport of the APOPcentage dye into the cell. The uptake of the dye is uni-directional, leading to dye accumulation within the cell. As the cell shrinks in volume, during the apoptotic process, the cell dye content becomes more concentrated. Confluent cells, plated in 96-well black plates (BD Falcon), were treated as described in § 3.2 of the “Materials and Methods” section. At the end of treatments, the APOPcentage dye was added to each well (dilution 1:10) and cells incubated for 30 more min at at 37°C in a 5% CO₂ incubator. After thoroughly washing, 100 µl of APOPcentage dye release reagent was added to each well, and the cell-bound dye recovered into solution was measured using a GENios plus microplate reader (Tecan) with excitation and emission of 530 and 580 nm, respectively. Results were expressed as a percent of untreated control cells.
§ 3.9 Immunoblotting analysis.

Cells were cultured in T25 culture flasks (BD Falcon), treated as indicated in § 3.2 of the “Materials and Methods” section and then processed for immunoblotting as previously reported [105]. Sample proteins were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 45 minutes. Then they were transferred onto nitrocellulose membrane using electroblotting (100V for 1h). After protein immobilization the membrane was blocked with TBS containing 5% non-fat powdered milk and 0.1% Tween-20 (blocking buffer) for 1 hour, on the shaker, at room temperature. So the nitrocellulose membrane was incubated with primary antibody in blocking buffer overnight at 4°C. We used specific antibodies against the total and phosphorylated form of the protein kinase Akt, the mitogen-activated protein kinases (MAPKs) p42/44MAPK and stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK)MAPK, and the cleaved and uncleaved form of caspase-3 (Cell Signaling, Danvers, MA).

The next day, after thoroughly washing in TBS containing 0.1% Tween-20, the nitrocellulose membrane was incubated with the secondary antibody solution in blocking buffer for 1 hour, on the shaker, at room temperature. Densitometric analysis was performed by using the Versadoc Imaging System (Bio-Rad) to scan the signals. Results were expressed as arbitrary units, and ratios of individual densitometric results were normalized to β-actin immunoreactivity.

§ 3.10 MMP assay

Mitochondrial membrane potential was assessed after treatments by using the fluorimetric kit JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium, Inc. USA), following the protocol provided by the manufacturer.
JC-1 Assay Kit uses a cationic dye (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide) to signal the loss of mitochondrial membrane potential [106].

The loss of mitochondrial membrane potential ($\Delta \Psi$) is a hallmark for apoptosis. It is an early event preceding phosphatidylserine externalization and coinciding with caspase activation.

In healthy cells, the dye stains the mitochondria bright red. The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form, which become fluorescent red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence.

Confluent cells, plated in 96-well black plates (BD Falcon), were treated as described in § 3.2 of the “Materials and Methods” section.

At the end of treatments, the JC-1 dye was added to each well and cell incubated for 15 min at 37°C in a 5% CO$_2$ incubator.

After thoroughly washing, 100 µl of PBS was added to each well, and red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) were measured using a GENios plus microplate reader (Tecan) with excitation and emission of 530 and 580 nm, respectively.
§ 3.11 Statistical analysis.

Data are expressed as means ± SDs of four or five 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 Bonferroni 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 5.00 for Windows (GraphPad Software, San Diego, CA).
CHAPTER 4
RESULTS
§ 4.1 Dose-dependent effect of NA on EC ROS levels and protein carbonylation

The study began with the quantification of ROS levels in endothelial cells (ECs) treated with various concentrations of two NA (Resveratrol and Coumaric Acid) and untreated cells (CTRL) used as control. Intracellular ROS generation was examined in ECs in response to NA using 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA). This probe enters the cells and can be oxidized in the presence of ROS, generating the fluorescent compound, DCF.

First of all we validated this ROS assay treating ECs with increasing doses of H₂O₂ and Angiotensin II to test their capability to detect variations of intracellular ROS levels in response to these well-known pro-oxidants in the used cellular model [107; 108]. Data reported in figure 1A show that the probe has a significant dynamic range and responds linearly to increasing doses of H₂O₂ and Angiotensin II, confirming the test validity.

Then to determine the effects of NA on ECs, 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 five pooled measurements are shown below and results are expressed as percentage of untreated controls. Treatment of ECs with 0.5µM resveratrol exerted a significant antioxidant effect confirming the protective role of NA previously described and further validating our experimental system [109; 110]. However, the exposure of cell cultures to higher concentrations of resveratrol 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 10 and 25µM (fig. 1B).
Coumaric acid treatment resulted in a similar pattern with a marked prooxidant effect starting at 10µM and persisting at 25µM (fig. 1C).

We next investigated whether the combination of low doses of the two tested compounds had a synergistic or additive effect on intracellular ROS levels. Using the two NA together (fig. 1D) no differences, on intracellular ROS levels, were detected versus the same concentration (0.5 and 5µM) of an antioxidant alone.

To gain further information on the antioxidant and pro-oxidant effect of these two compounds, we assessed variations of the protein carbonyl content in response to NA treatment. In fact carbonylation of proteins is considered a widespread indicator of oxidative damage and disease-derived protein dysfunction [111]. Protein carbonyl groups were measured with the OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA), following the protocol provided by the manufacturer as described in Material and Methods. Figures 1E and 1F show as the protein carbonylation pattern elicited by both NA strictly overlapped that of ROS, strongly confirming the antioxidant and pro-oxidant effect exerted by the tested compounds. Representative western blottings of protein carbonylation experiments are reported in figure 2.
Results

Fig. 1. Confluent ECs were stimulated for 80 min as indicated in figure. Intracellular ROS levels were assessed after treatments as described in the ‘‘Materials and Methods’’ section. (A) Dose-response effect of H2O2 and Angiotensin II on intracellular ROS levels. (B and C) Intracellular ROS levels in cultured ECs in the absence (CTRL) or presence of the indicated concentration of (B) resveratrol and (C) coumaric acid. (D) Effect of single or combined dose of NA on intracellular ROS generation. (E and F) Measurement of protein carbonylation in cultured ECs in the absence (CTRL) or presence of the indicated concentration of (E) resveratrol and (F) coumaric acid. Protein carbonylation was assessed after treatments as reported in the ‘‘Materials and Methods’’ section. Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments. (Individual densities from the different bands were added up to generate one single value.)

CTRL, untreated cells; R, resveratrol; C, coumaric acid; H2O2, hydrogen peroxide; and Ang II, Angiotensin II. Data are expressed as percent of control. (A–F)

*Significantly different from the control, §significantly different from each other, and # significantly different from each other (p < 0.05).
§ 4.2 NA dose dependently induce ECs impairment

To investigate how the ROS induced by NA, affect the physiology of human endothelial cells, we measured cell viability, metabolic activity and apoptosis.

Cell viability was assessed by using propidium iodide (PI), Hoechst 33342 double fluorescent staining as described in Material and Methods. Exposure of cell cultures to increasing concentrations of resveratrol and coumaric acid decreased cell survival (figs. 3A and 3B), an effect consistent with the increase in ROS levels and protein carbonyl content. It was observed that treatment of EC with 0.5 and 5 μM of NA did not induce obvious changes in cell viability compared to untreated cells. In contrast, treatment of cells with higher concentrations (10, 25 μM) of resveratrol and coumaric acid induced a statistically significant reduction in cell viability compared to the controls.
Then we evaluated apoptosis using the fluorimetric kit APOPercentage (Biocolor Ltd, Carrickfergus, UK), following the protocol provided by the manufacturer as described in Material and Methods. The cells were treated with different concentrations of NA and we used, as positive control, cells treated with staurosporine (1µM), a known inducer of apoptosis. As can be seen in fig. 3B, the NA induced an increase in apoptosis at higher concentrations, but they had no effect at lower concentrations; these results confirm those already obtained in the previous experiments on the measurement of ROS and cell viability.

However, while viability decreased dose dependently (fig. 3A), apoptosis did not increase in a dose-dependent fashion (fig. 3B), suggesting a potential shift toward a necrotic mechanism at high NA concentrations. However, the involvement of apoptosis was confirmed by increased levels of cleaved (active form) caspase-3 immunoreactivity (fig. 4).

Consistent with the observed cell damage, a significant decrease in cell metabolic activity, was induced by both 10 and 25µM of NA (fig. 3C). Importantly, the treatment of ECs with 0.5 and 5µM of NA failed to induce any evident variation in the Hoechst/PI fluorescence ratio or apoptotic rate as compared to untreated cells (figs. 3A and 3B) excluding the possibility of a general toxic effect of NA in our experimental system. Rather, consistent with the reported antioxidant effect, an increase of metabolic activity and a significant antiapoptotic effect were detected at the dose of 0.5µM (figs. 3B and 3C).

We next ascertained the causative role of ROS in NA-induced protein carbonylation and ECs damage by using the ROS scavenger N-acetyl-cysteine (NAC). Pretreatment of cell cultures with 5mM NAC prevented both NA-induced protein carbonylation and ECs damage (figs. 5A–C).
Fig. 3. Confluent ECs were stimulated for 4 h as indicated in figure. Cell viability, metabolic activity, and apoptosis were assessed after treatments as reported in the ‘‘Materials and Methods’’ section. Staurosporine at concentration of 1µM was used to induce ECs apoptosis. (A) Quantification of Hoechst/PI ratio, (B) apoptosis, and (C) metabolic activity in cultured ECs in the absence (CTRL) or presence of the indicated NA concentration. CTRL, untreated cells; R, resveratrol; C, coumaric acid; and Stauro, staurosporine. Data are expressed as percent of maximum. (A–C) *Significantly different from the control, §significantly different from each other, #significantly different from each other, and •significantly different from all values (p < 0.05).
Fig. 4. Confluent ECs were stimulated for 80 minutes as indicated in figure and then processed for immunoblotting as described in material and methods. The upper part of the figure shows the immunoreactivity of cleaved (active form) and uncleaved (non-active form) caspase-3, while the lower part reports the immunodensity quantitative analysis. Immunodensity values (cleaved and uncleaved) are represented on different graphs sharing the same y-axis. CTRL; untreated cells. R; resveratrol. C; coumaric acid. Stauro; staurosporine. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. *; significantly different from the control (p < 0.05).
Results

Fig. 5. (A) Protein carbonylation. Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments. (Individual densities from the different bands were added up to generate one single value). Confluent ECs were stimulated for 80 min as indicated in figure. In selected experiment, before NA treatment, ECs were preincubated for 30 min with 5mM of the ROS scavenger NAC. (B) and (C) Confluent ECs were stimulated for 4 h as indicated in figure. Hoechst/PI ratio (B) and apoptosis quantification (C) were assessed after treatments as reported in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were preincubated for 30 min with 5mM of the ROS scavenger NAC. CTRL, untreated cells; R, resveratrol; C, coumaric acid; and Stauro, staurosporine. (A and B) Data are expressed as percent of control.*Significantly different from the control, §significantly different from each other, #significantly different from each other, and •significantly different from all values (p < 0.05).
§ 4.3 *Flavin-containing oxidases mediate NA-induced intracellular ROS production and protein carbonylation*

Since flavin oxidases are an important source of intracellular ROS [112], we planned to investigate their potential involvement in the NA-induced pro-oxidant effect. To this end, we pretreated cells cultures with the broad flavoproteins inhibitor, diphenylene iodonium (DPI) and then assessed intracellular ROS generation and protein carbonyl levels in response to NA treatment. As reported in figures 6A and 6B, DPI significantly blunted ROS production and protein carbonylation in both resveratrol- and coumaric acid-treated cells, clearly indicating the involvement of DPI-sensitive flavin-containing systems in the observed NA-induced pro-oxidant effect.

Given that ROS generation in NA-treated cells was inhibited by DPI, we hypothesized that these ROS were being generated, at least in part, by increased activity of flavin-containing oxidases. Since DPI is a general flavoproteins inhibitor, we first sought to demonstrate that NA activated DPI-inhibitable oxidases activity at the same time of ROS measurement.

Using an established assay [92; 102], we measured the rate of NADH and NADPH consumption in ECs as an index of cellular oxidase activity in response to NA treatment. As shown in figure 6C, the rate of NADPH consumption in NA-treated cells was about 2-fold that of control cells, while there was no significant change in the rate of NADH consumption. Subsequently, the effect of DPI on NADH and NADPH consumption was examined. As observed for NA-induced ROS generation (fig. 6A), DPI was able to prevent NA-induced NADPH utilization (fig. 6C), clearly implicating DPI-sensitive flavin oxidases in the cellular response to moderately high NA concentration. DPI-inhibitable NADPH consumption was also noted in NA-unstimulated cells; however, this effect was about one-third of the increase in NA treated cells. DPI-sensible NADH consumption was observed in control cells but not in NA-treated cells.
Fig. 6. Confluent ECs were stimulated for 80 min as indicated in figure. In selected experiment, before NA treatment, ECs were preincubated for 15 min with DPI (10 μM). (A) Intracellular ROS levels and (B) protein carbonylation. Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments. (Individual densities from the different bands were added up to generate one single value.) (C) NADH and NADPH oxidation. CTRL, untreated cells; R, resveratrol; C, coumaric acid; and DPI, diphenyleneiodonium. (A and B) Data are expressed as percent of control. (A–C) *significantly different from the control, §significantly different from each other, and #significantly different from each other (p < 0.05).
§ 4.4 NA dose dependently downregulate Akt phosphorylation

To further investigate the molecular mechanism underlying the effects of the antioxidants, we tried to identify the signal transduction pathway responsible for cellular responses previously observed. For this purpose we studied the protein kinase Akt, the MAPK p42/44MAPK and SAPK/JNKMAPK because their function may be influenced by ROS and they are important regulators of death and survival in different cell types, including endothelial cells [80; 95].

We therefore investigated whether NA, at doses able to affect ROS generation and cell function, could differentially modulate MAPKs and Akt activation.

The cultured cells were stimulated for 15, 30, 60, and 80 minutes with different concentrations of NA and processed for Western blotting as described in Materials and Methods. Immunoblot analysis from dose-response experiments revealed that Akt activation mirrored NA-induced antioxidant and pro-oxidant effects. Indeed, a dose-dependent decrease of Akt phosphorylation was observed at 10 and 25µM (fig. 7), an effect consistent with the observed dose-related pro-oxidant damage (Figs. 1 and 3). The time-course analysis shows that NA-induced Akt dephosphorylation started at 15 min and remained persistently evident until 80 min.

On the other hand, the lower dose of NA (0.5µM) elicited a transient increase of both p42/44MAPK and Akt activation (fig. 7), which is compatible with the decrease pro-oxidant effect and the improvement of cell function observed at the same NA concentration (figs. 1 and 3). Protein expression levels for p42/44MAPK and Akt were unchanged at all time points tested. Additionally, no changes in both the levels of protein expression and phosphorylation were observed for SAPK/JNKMAPK in response to NA treatment. Data presented in figure 8 show no change in both expression and phosphorylation of p42/44MAPK and SAPK/JNKMAPK in
response to long-time (2–4 h) treatment with the highest dose of NA. Interestingly, while Akt protein levels remained constant over the time course, a significant decrease in Akt phosphorylation was evident at 2 and 3 h after NA treatment, returning to control level at 4 h (fig. 8).

Fig. 7. Confluent ECs were stimulated as indicated in figure and then processed for immunoblotting as described in the "Materials and Methods" section. (A) Representative immunoblot of total and phospho-Akt (Ser473), total and phosphop42/44MAPK (Thr202/Tyr204), and total and phospho-SAPK/JNKMAPK (Thr183/Tyr185) for ECs treated with resveratrol. (B) Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Resveratrol. (C) Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Coumaric acid. (Individual densities from the different bands were added up to generate one single value.) Data are expressed as arbitrary units. R: resveratrol. C: coumaric acid. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. (B and C) *significantly different from its own control, #significantly different from the control and significantly different from each other within the same time point, §significantly different from the same concentration within all the experimental time points, and ◦significantly different from the same concentration at the 15-min time point (p < 0.05).
Results

Fig. 8. Confluent ECs were stimulated for the indicated time points (in hours) with 25 µM NA and then processed for immunoblotting as described in material and methods. A: Representative immunoblot of total and phospho-p42/44MAPK (Thr202/Tyr204), total and phospho-Akt (Ser473) and total and phospho-SAPK/JNKMAPK (Thr183/Tyr185) for ECs treated with 25 µM Resveratrol. B: Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Resveratrol (25 µM). C: Graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Coumaric acid (25 µM). Numbers on the X-axis represent: (1) total and (2) phospho-p42/44MAPK (Thr202/Tyr204), (3) total and (4) phospho-Akt (Ser473), (5) total and (6) phospho-SAPK/JNKMAPK (Thr183/Tyr185). (individual densities from the different bands were added up to generate one single value). CTRL; untreated cells. R; resveratrol. C; coumaric acid. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. (B-C) *; significantly different from its own control (p < 0.05).
§ 4.5 Akt dephosphorylation and EC damage are mediated by flavin oxidases

We next wanted to understand if inhibition of flavin oxidases could restore Akt function and prevent NA-induced adverse responses. To this end, both Akt levels and activation (as determined by the phosphorylation status) were assessed in antioxidant-treated cells in the presence or absence of DPI. As shown in figure 9A, DPI pretreatment preserved Akt phosphorylation, suggesting a pivotal role for flavin oxidases in the downregulation of prosurvival pathways. The rescue of Akt signals was accompanied by a similar reduction in cell damage confirming the biological relevance of this mechanism in our vascular model (figs. 9B and C). Treatment with the only DPI did not elicit any significant adverse effects, which rules out the possibility of a general DPI toxicity under the current experimental conditions (figs. 9B and C).
Fig. 9. (A) Confluent ECs were stimulated for 80 min as indicated in figure and then processed for immunoblotting as described in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were preincubated for 15 min with the flavoprotein inhibitor DPI (10µM). The upper and lower part of panel A show, respectively, the Akt and phospho-Akt immunoreactivity (Ser 473) and the quantitative immunodensity. Immunodensity values (total and phosphorylated) are represented on different graphs sharing the same y-axis. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. (B and C) Confluent ECs were stimulated for 4 h as indicated in figure. Hoechst/PI ratio (B) and apoptosis quantification (C) were assessed after treatments as reported in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were preincubated for 15 min with the flavoproteins inhibitor DPI (10µM). CTRL, untreated cells, R, resveratrol, C, coumaric acid; and DPI, diphenyleneiodonium. Data are expressed as percent of maximum. (A–C) *significantly different from the control (p < 0.05).
§ 4.6 Akt activation rescues ECs from oxidative stress damage

Whereas dephosphorylation of Akt plays a central role in driving NA-induced cellular damage, we assumed that the restoration of its activity would counteract the NA effects. To test this hypothesis, we preincubated ECs for 30 min with the Akt activator insulin [98] and then, after exposure to NA, we assessed Akt levels and activation. As reported in figures 10A and 10B, treatment of ECs with 10µM insulin significantly increased the levels of Akt phosphorylation as compared to untreated cells. Pretreatment with insulin prevented Akt dephosphorylation induced by NA, confirming that Akt activation could be restored. Insulin has been shown to stimulate Akt via activation of PI3K, an effect that can be blocked by the PI3K inhibitors Wortmannin and LY-294002 (Hermann et al., 2000). Preincubation of ECs with 20nM Wortmannin (figs. 10A and 10B) or 10µM LY-294002 (fig. 11) abolished the protective effect of insulin on Akt dephosphorylation demonstrating that Akt signaling could be activated specifically via a PI3K-mediated mechanism.

We next investigated whether restoring Akt activation could rescue NA-induced ECs damage. To test this hypothesis, we activated Akt using insulin and then we assessed apoptosis and cell viability upon NA treatment.Akt activation was able to abolish completely the apoptotic response and to rescue cell viability (figs. 12A and 12B). Interestingly, Akt activation could reduce basal levels of apoptosis in cultured cells, an important validation of our experimental model. Blocking Akt function by using either the PI3K inhibitor Wortmannin (Figs. 12A and 12B) completely abrogated the protective effect, further validating Akt as the mediator of the insulin protective effect. These findings clearly indicate Akt as a crucial mechanism of cell survival in response to NA-induced oxidative stress.
Fig. 10. (A and B) Confluent ECs were stimulated for 80 min as indicated in figure and then processed for immunoblotting as described in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were pretreated for 30 min with 10μM insulin in either the absence or the presence of a further 15 min preincubation with the selective PI3K inhibitor Wortmannin (20nM). The left part of the panel shows the immunoreactivity of total and phospho-Akt (Ser473), while the right part reports the immunodensity quantitative analysis. Immunodensity values (total and phosphorylated) are represented on different graphs sharing the same y-axis. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. CTRL, untreated cells; R, resveratrol; C, coumaric acid; I, insulin; W, Wortmannin. Data are expressed as percent of control. (A and B) *significantly different from the control (p < 0.05).
Fig. 11. Confluent ECs were stimulated for 80 minutes as indicated in figure and then processed for immunoblotting as described in the "Materials and Methods" section. In selected experiment, before NA treatment, ECs were pre-treated for 30 minutes with 10 µM insulin in either the absence or presence of a further 15 minutes pre-incubation with the selective PI3K inhibitors LY-294002 (10 µM). The left part of the panel shows the immunoreactivity of total and phospho-Akt (Ser473), while the right part reports the immunodensity quantitative analysis. Immunodensity values (total and phosphorylated) are represented on different graphs sharing the same y-axis. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. CTRL; untreated cells, R; resveratrol, C; coumaric acid, I; insulin, LY; LY-294002. Data are expressed as percent of control. (A-B) *; significantly different from the control (p < 0.05).
Fig. 12. Confluent ECs were stimulated for 4 h as indicated in figure. Cell viability and apoptosis were assessed after treatments as reported in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were pretreated for 30 min with 10µM insulin in either the absence or the presence of a further 15 min preincubation with the selective PI3K inhibitor Wortmannin (20nM).

(A) Quantification of Hoechst/PI ratio and (B) apoptosis in cultured ECs in the absence (CTRL) or presence of the indicated treatments. CTRL, untreated cells; R, resveratrol; C, coumaric acid; I, insulin; W, Wortmannin; and Stauro, staurosporine. Data are expressed as percent of control. (A and B) *significantly different from the control, #significantly different from each other, §significantly different from each other, □significantly different from each other, ◦significantly different from each other, and •significantly different from all values (p < 0.05).
§ 4.7 CYP2C9 mediate NA-induced ECs damage

Although liver is the most critical tissue in drug metabolism, CYP 2C9, an isoform of cytochrome P450, is also constitutively present in the endothelium and is reported to be a significant source of ROS in coronary arteries [113; 114].

We then investigated whether the CYP2C9 was involved in the induction of cell death triggered by natural antioxidants.

To this end, we preincubated ECs for 30 min with a specific inhibitor of CYP2C9, sulfaphenazole (SPZ) (6 µM) and then, after exposure to NA, we evaluated apoptosis using the fluorimetric kit APOPercentage (Biocolor Ltd, Carrickfergus, UK). As shown in fig. 13A, SPZ pre-treated cells didn’t show apoptosis in contrast to no pre-treated cells, indicating that the SPZ neutralizes NA-toxic effects.

The loss of mitochondrial membrane potential (ΔΨm) is a hallmark for apoptosis death and mitochondrial function. It is an early event preceding phosphatidylserine externalization and coinciding with caspase activation. For this reason, we studied also ΔΨm variation by evaluating the changes in fluorescence intensity of cells, stained with lipophilic cationic dye 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 has advantages over other cationic dyes because it can selectively enter into mitochondria and reversibly change colour from green to red as the membrane potential increases. In healthy cells, with high mitochondrial ΔΨm, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells, with low ΔΨm, JC-1 remains in the monomeric form, which shows only green fluorescence.

After NA treatment, ECs were incubated with JC1 and analyzed at the fluorometer.
As can be seen from fig. 13B, that shows the relationship between red and green fluorescence emitted, pretreatment with SPZ annuls the NA toxic effect on mitochondria, which instead can be observed in cells subjected to treatment with only NA. The decreasing of mitochondrial membrane potential suggests that NA-induced oxidative stress may cause mitochondrial damage and dysfunction in ECs; furthermore this data suggest a role for Cytochrome P450 (CYP) 2C9 in NA-induced toxicity.
Fig. 13. Confluent ECs were stimulated for 4 h as indicated in figure. Apoptosis and MMP assay were assessed after treatments as reported in the “Materials and Methods” section.

In selected experiment, before NA treatment, ECs were pre-treated for 15 minutes with 5 µM of the CYP2C9 inhibitor sulfaphenazole. (A) Apoptosis and (B) Red/Green JC-1 ratios in cultured ECs in the absence (CTRL) or presence of the indicated treatments.

CTRL: untreated cells; R: resveratrol; C: coumaric acid; SPZ: sulfaphenazole; and Stauro, staurosporine. Data are expressed as arbitrary units. * significantly different from CTRL.
§ 4.8 SPZ prevents NA-induced p-Akt down-regulation.

Then we evaluated if SPZ also prevented NA-induced p-Akt down-regulation. As shown in fig. 14 SPZ pretreatment preserved Akt phosphorylation, suggesting that this one could work downstream of CYP2C9 in mediating cellular responses to NA.

Fig. 14. Confluent ECs were stimulated for 80 min as indicated in figure and then processed for immunoblotting as described in the “Materials and Methods” section. In selected experiment, before NA treatment, ECs were pre-treated for 15 minutes with 5 µM of the CYP2C9 inhibitor sulfaphenazole. The upper part of the figure shows the immunoreactivity of total and phospho-Akt, while the lower part reports the immunodensity quantitative analysis. CTRL: untreated cells; R: resveratrol; C: coumaric acid; SPZ: sulfaphenazole. Ratios of individual densitometric results were normalized to β-actin immunoreactivity. Data are expressed as arbitrary units. * significantly different from CTRL.
§ 4.9 Cyclosporine A prevents NA-induced ECs impairment.

Finally we investigated whether the mitochondria were involved in modulating NA-induced cellular impairment. This is why we treated ECs with the mitochondrial permeability transition pore (MPTP) inhibitor cyclosporine A (CsA) and evaluated apoptosis using the fluorimetric kit APOPercentage (Biocolor Ltd, Carrickfergus, UK).

In fact CsA affects mitochondria by preventing the mitochondrial permeability transition pore from opening, thus inhibiting cytochrome c release.

Ciclosporin is believed to elicit its effects by directly binding to the cyclophilin D protein (CypD) that constitutes part of the mitochondrial permeability transition pore (MPTP) [115; 116], and by inhibiting the calcineurin phosphatase pathway.[117; 118].

As shown in fig 15 CSA pre-treatment completely prevented oxidative cell damage strongly indicating mitochondrial involvement in NA-induced ECs impairment.
Fig. 15. Quantification of Apoptosis in cultured ECs in the absence (CTRL) or presence of the indicated treatments. In selected experiment, before NA treatment, ECs were pre-treated for 30 minutes with 2 µM Cyclosporine A a specific MPTP inhibitor. CTRL: untreated cells; R: resveratrol; C: coumaric acid; CsA: cyclosporine A; and Stauro, staurosporine. Data are expressed as percent of maximum. * significantly different from CTRL.
CHAPTER 5

DISCUSSION
The change of intracellular levels of ROS and the consequent activation of specific signaling pathways induce a coordinated set of integrated physiological responses in cardiovascular tissue. These include growth of smooth muscle cells, induction of inflammatory response, impairment of endothelium-dependent relaxation, and cardiac hypertrophy. Each of these responses, when uncontrolled, contributes to vascular diseases.

The potential value of antioxidants in treating conditions associated with oxidative stress is well known to scientists and clinicians and it is of immense interest to patients.

Oxidative stress is a term used to describe an imbalance between the production and destruction of reactive oxygen species (ROS), thereby leading to cellular and tissue injury. The basic properties of oxygen are responsible for the destructive power of free radicals, in particular, their high reactivity. Humans consume ~ 250 g of oxygen every day, and of this ~ 3–5% is converted to $O_2^{•−}$ and other reactive species [119]. The damage inflicted by ROS on cellular and extracellular targets such as membrane lipids, proteins, and DNA clearly contributes to tissue and organ dysfunction in many pathological states.

The implication of oxidative stress in the etiology of several chronic and acute degenerative disorders suggests that antioxidant therapy represents a promising avenue for treatment. Strategies for the intervention and prevention of cardiovascular disease require an understanding of the basic molecular mechanism(s) by prophylactic agents (synthetic antioxidants, dietary antioxidant factors from food plants and medicinal plants) that may potentially prevent or reverse the promotion or progression of the disease. Administration of exogenous antioxidants has been extensively investigated as a means to attenuate myocardial ischemia-reperfusion injury and to treat or prevent chronic cardiovascular diseases. Investigations in a variety of animal
models have shown beneficial effects of several drugs. However, clinical trials have furnished inconsistent results.

Given the central role that the endothelium plays in cardiovascular homeostasis and the involvement of EC dysfunction in CVD pathogenesis [92], the EC represent an excellent model to investigate the impact of NA on vascular (patho)physiology.

Polyphenols are the most abundant antioxidants in the diet. Their total dietary intake could be as high as 1 g/d, which is much higher than that of all other classes of phytochemicals and known dietary antioxidants. For perspective, this is ~ 10 times higher than the intake of vitamin C and 100 times higher that the intakes of vitamin E and carotenoids [31]. Their main dietary sources are fruits and plant-derived beverages such as fruit juices, tea, coffee, and red wine. Vegetables, cereals, chocolate, and dry legumes also contribute to the total polyphenol intake.

Our results show that for NA such as the polyphenols coumaric acid and resveratrol, the concentration will determine the overall effects on EC (Figs. 3A–C). While low doses of NA showed an antioxidant effect, surprisingly, a modest increase in concentration induced a completely opposite result (Figs. 1B and 1C), resulting in cell damage (Figs. 5A–C). A similar behavior has been reported with other NA in vitro and in vivo experimental models [97; 120], suggesting a dual role for NA in regulating EC biology.

A significant number of reports exist in the literature indicating that resveratrol can function both as pro-apoptotic and anti-apoptotic agents. Careful review of the studies on cancer prevention with resveratrol reveals that in each case, resveratrol was used at high concentration/dose [10-40 mM] [121 - 125]. In contrast resveratrol protects hearts in a relatively low dose [5-20 μM] [126 - 129]. This would tend to indicate that resveratrol provides diverse health benefits in a
dose response manner. There are quite a few studies to describe the dose dependency of resveratrol towards health benefit. However, since antioxidant effects can vary depending on cell types and molecules used, the mechanisms involved in antioxidant-mediated cellular responses are still largely unknown.

Multiple lines of evidence presented in this work implicate flavin oxidases as key players in the response to high levels of NA. First, the flavoproteins inhibitor DPI completely blunted the increased oxidases activity elicited by NA (Fig. 6C), while DPI-sensitive oxidase activity in control cells was minimal, clearly indicating that NA activated DPI-sensitive flavin oxidases over the basal level (Fig. 6C). Second, DPI also significantly counteracted the increased ROS production and protein carbonylation elicited by NA treatment, thus implicating DPI-sensitive flavin containing systems in the observed NA-induced pro-oxidant effect (Figs. 6A and 6B). Lastly, flavin oxidase involvement was further confirmed by DPI’s ability to rescue the cellular phenotype induced by NA treatment, preventing apoptosis and cell death (Figs. 9B and 9C).

Here, we also suggest a role for Akt as a key regulator of EC responses to NA treatment. Indeed, coumaric acid and resveratrol downregulated Akt phosphorylation in a dose dependent fashion (Fig. 7), an effect superimposable with dose response effects observed for ROS generation, protein carbonylation, and cell function (Figs. 1 and 3). This effect was mirrored by the increased Akt activation at low NA doses (Fig. 7), which was consistent with the decreased ROS levels and the improvement of cells function observed at the same NA concentration (Figs. 1 and 3). In addition, the ability of DPI to restore NA-induced Akt dephosphorylation and cell damage indicates that this kinase works downstream of the flavin oxidases, integrating ROS signals into general cellular responses (Figs. 9A–C). Indeed, Akt is a central signaling molecule in regulating survival and death of
different types of cells including EC, and its activity can be redox regulated [95; 80]. Rescue of Akt phosphorylation and recovery of cell function after insulin pretreatment indicate that this kinase is pivotal in mediating NA-induced effects (Figs. 10 and 12). In fact, its inhibition completely abrogated cell protection, further validating Akt as the mediator of the insulin protective effect (Fig. 10). Given the complete lack of insulin-mediated protective effects of Akt on NA action, Akt-independent signals, potentially activated by insulin, are unlikely to be involved in the investigated phenomena.

Furthermore in the present study we found that treatment of ECs with the mitochondrial permeability transition pore (MPTP) inhibitor cyclosporine A (CsA), completely prevents oxidative cell damage strongly indicating mitochondrial involvement in NA-induced ECs impairment (Fig. 15).

Finally NA-induced pro-oxidant effects were counteracted by sulfaphenazole (SPZ), suggesting a role for Cytochrome P450 (CYP) 2C9 in NA-induced toxicity (Fig. 13). SPZ also prevented NA-induced p-Akt down-regulation and mitochondrial membrane potential (MMP) impairment (Figs 13, 14), indicating that Akt can work downstream of CYP2C9 in mediating cellular responses to NA.

Our study is the first to show in a human vascular model that moderately high-doses of NA can induce mitochondrial-dependent cell damage mediated by CYP2C9- and the Akt pathway. We believe the present results are of particular importance in light of the popularity of antioxidant rich diets and therapeutic approaches aimed at reducing cardiovascular risk.

In fact the toxicology of NA has become a controversial area of debate. Our results support the idea that high doses of NA rather than producing protective antioxidant effects may prompt pro-oxidant-induced damage. Confirming previous observations [109; 110], we also reported that low concentrations of NA can prompt a significant antioxidant effect. Such a phenomena may be explained by the interaction of NA with lipid rafts/caveolae, specialized
plasma membrane microdomains involved in ROS compartmentalization, and redox-regulated signal transduction [162]. Indeed, recent data indicate that nanomolar concentration of resveratrol can enhance endothelial NO production through a caveolae-dependent mechanism involving p42/44MAPK activation [110]. Resveratrol at concentrations attainable with moderate wine consumption (0.1–0.5µM) has also been reported to activate Akt, increase NO production, and inhibit NADPH oxidase-dependent ROS generation in human platelets [109]. Our results, showing reduced ROS levels in association with p42/44MAPK and Akt activation in response to low dose of NA, suggest that a similar mechanism may be conceivable. Indeed in addition to resveratrol, improved endothelial function via a caveolae-mediated antioxidant effect has been also reported for other NA, such as quercetin and red wine polyphenols [131; 132]. Surprisingly, the effects of NA 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, resveratrol at a concentration of 50µM, well above the concentrations currently used in this work, protects HUVEC from oxidized low-density lipoprotein–induced oxidative damage [133]. However, it has been suggested that resveratrol can work as a pro-oxidant under low oxidative conditions, while it becomes antioxidant under strong oxidative conditions [134]. Thus, the interaction of NA 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. In vivo, there is evidence suggesting that NA can accumulate in specific compartments at relatively high concentrations. For example, after chronic consumption, resveratrol has been shown to be detectable in plasma up to 1 week after washout [135], and plasma peak concentrations of 32 and 8.1µM have been reported in rodents [136; 137]. Because of the lipophilic nature of most NA, their tissue levels may provide a better indicator of the in
vivo biologically active concentrations outlasting their presence in the plasma. Indeed, in accordance with the reported ability of ECs to uptake NA [138], in rats fed dietary relevant doses, concentrations of resveratrol in tissues such as the heart, liver, and kidney were higher (~10 to 30µM) than in plasma [139; 140]. Also, it has been suggested that plasmatic proteins may be natural NA reservoirs in vivo, modulating their plasma concentration and tissue delivery [138; 141]. Moreover, interactions between different NA may also influence their kinetics and metabolism in the liver increasing NA circulating levels [142]. Interestingly, several studies consistently indicate that resveratrol metabolites half-life, and concentration in plasma is 10 times higher compared to that of the native compound [143] and whether these metabolites may serve as pool from which free resveratrol can be released locally in various tissues cannot be excluded at the moment. Such an aspect seems to be relevant also in clinical practice. Although some studies demonstrate a significant inverse correlation between NA-rich foods consumption and cardiovascular risk, others indicate that NA fail to protect against CVD or may accelerate their development [36]. Evidence suggests that the oxidant and antioxidant status of people should be probably checked before undergoing high antioxidant intake or supplementation [144; 145]. The human population is heterogeneous regarding the ROS level. Screening the human population regarding innate or acquired ROS levels can provide the necessary information about individual oxidative status. High doses of antioxidants can reduce the ROS level in people who over produce ROS and protect them against cancer, cardiovascular diseases and other ROS-dependent morbid conditions. For people with a low ROS level, high doses of antioxidants can be deleterious, suppressing the already low rate of ROS generation and the ROS-dependent cancer preventive apoptosis. Screening and monitoring the human population regarding the ROS level can transform antioxidants into safe and powerful disease-preventive tools.
To this regard is worth noting that NA are not only part of the human diet but are often ingested as an additional dietary supplement at high dosage or used in clinical trial at pharmacological doses. For example, commercial dietary supplements contain on average between 50 and 325 mg resveratrol, with some high-potency varieties containing up to 500 mg. Supplement doses range from a daily dose of 50 to as high as 2000 mg/day (0.8–33 mg/kg body weight/day for a 60 kg human). Despite present diffuse contradictions, it appears that at least populations with insufficient or unbalanced nutritional levels may benefit from an increased intake of dietary antioxidants or supplements [146]. Thus antioxidant supplementation could potentially be harmful to those tissues that are not subjected to substantial oxidative stress. Conversely, for those disorders that are associated with marked increases in ROS production the temporal and spatial characteristics of oxidant production pose great challenges in regard to delivering effective antioxidant therapy. Instead, the methods currently available to assess the degree of oxidative stress, and the efficacy of antioxidant therapy, in vivo are quite limited. To our knowledge, organs and tissue levels of NA in people under pharmacological treatment or supplementation with high doses of NA are so far unknown. Ideally, antioxidant therapies should be judged on the basis of their therapeutic efficacy. Unfortunately, determination of the efficacy of antioxidant therapy is hampered by the lack of available methodology to quantify ROS in tissues and blood vessels in vivo. Surrogate end points, such as assessment of endothelial function or lipid peroxidation products in the plasma, do not adequately reflect the capacity of antioxidants to protect the deeper layers of the blood vessel wall from oxidative injury. Negative results of clinical trials must be interpreted cautiously in the absence of verification that antioxidant therapy successfully reduces vascular oxidant stress. Moreover, ROS may participate only in certain subsets of vascular diseases and/or in specific patient subpopulations. The aforementioned potential
pitfalls of antioxidant therapy might be considered theoretical rather than pragmatic. Although antioxidants are typically given in constant amounts and dosing intervals, oxidative stress is not a continuous, uniform process. For example, marked intensification of oxidative stress occurs transiently after vascular balloon injury, and, most likely, during periods of increased inflammatory activity in atherosclerotic lesions [147]. The oxidants may activate signaling cascades and gene expression that, once set in motion, no longer require the presence of ROS.

Although further studies are required to better characterize the molecular mechanism of the NA-induced cell toxicity, our findings support recent observations suggesting that NA can have a potent nonspecific toxicity towards normal cells [148]. It remains to be elucidated whether NA-induced EC toxicity could help to explain some of the mixed results obtained with NA-based strategies in the prevention or treatment of cardiovascular pathologies.

Much remains to be learned concerning the signaling pathways and genes that are regulated by ROS. Because redox-sensitive responses appear at times to be cell specific, it will be important to identify the sources of oxidant stress in each cell, the mechanism of regulation of antioxidant enzymes and the effect of ROS on signaling pathways specific to the function of that particular cell and to gain further insight into the physiological responses affected by oxidant stress. An understanding of these events will enable us to devise therapeutic strategies to target specific cellular events contributing to vascular disease. It would also be necessary to establish optimal doses of antioxidants capable of coping with high and low levels of ROS.

However, since there is a substantial body of published work that shows NA can reach in vivo concentrations comparable to the ones we used in vitro, we suggest that our results could be representative of a physiologically relevant in vivo mechanism.
CHAPTER 6

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