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STUDY OF INTRACELLULAR SIGNALS IMPACTED BY A GREEN TEA STANDARDIZED EXTRACT (POLYPHENON E) IN CANCER CELLS

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LIST OF ABBREVIATION

BSA	Bovine serum albumin
CAT	Catalase
CTRL	Control
DNPH	Dinitrophenylhydrazine
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ERK	Extracellular signal-regulated kinase
ETC	Mitochondrial electron transport chain
FBS	Fetal bovine serum
GC	Gallocatechin
GCG	Gallocatechin-3-gallate
GPx	Glutathione peroxidase
GSH	Glutathione
GTCs	Green tea catechins
H_2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
JNK	c-Jun amino-terminal kinase
MAPK	Mitogen-activated protein kinase
MMP	Mitochondrial membrane potential
mtROS	Mitochondria reactive oxygen species
NADH	Nicotine adenine dinucleotide
Nox	NADPH oxidase

PCa	Prostate cancer
PI3K	Phosphoinositide3-kinase
Poly E	Polyphenon E
ROS	Reactive oxygen species
RTKs	Phosphorylates receptor tyrosine kinases
SAPKs	Stress-activated protein kinases
SDS	Dodecylsulfate
SOD	Superoxide dismutase
TRAMP	Transgenic adenocarcinoma of the mouse prostate

ABSTRACT

Purpose - Green tea consumption has been shown to exhibit cancer-preventive activities in preclinical studies. Polyphenon E (Poly E) is a green tea standardized extract. This study was undertaken to examine the effects of Poly E on PC3 prostate cancer cells and identify the potential signals involved in the anti-proliferative effect of Poly E.

Experimental Design and results - PC3 prostate cancer cells were used as model system**.** Treatment of PC3 cells with 30 and 100 μg/ml Poly E significantly decreased cell viability and proliferation, while increasing apoptosis. At all the tested concentrations, Poly E did not exert any antioxidant effect, eliciting instead a pro-oxidant effect at concentrations 30 and 100 μg/ml**.** The pro-oxidant effect of Poly E is consistent with the observed cytotoxicity, thus establishing a correlation between pro-oxidant activity and the anti-proliferative effect of Poly E in PC3 cells. Furthermore, Poly E-induced cell death was associated with mitochondrial dysfunction in PC3 cells. Exposure of PC3 cells to Poly E caused phospho-Akt inhibition while induced phospho-ERK activation, implicating these two signaling pathways in the observed anti-proliferative effect.

Conclusion - Our data indicated a correlation between pro-oxidant activity and anti-proliferative effect Poly E in PC3 cells and suggest ERK and AKT pathways as potential molecular machinery involved in observed phenomena.

CHAPTER 1

INTRODUCTION

1.1. Chemistry of ROS

Reactive oxygen species (ROS) are the collective term for all highly reactive oxygen (O_2) derivatives.

There are two types of ROS, those of free radicals, which contain unpaired valence shell electrons, and non-radical ROS, which do not have unpaired electron(s) but are chemically reactive and prone to become radical ROS. Examples of radical ROS are superoxide ($O_2^{-\bullet}$), hydroxyl (•OH), peroxyl RO_2•) and alkoxyl (RO•). Non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) [14], all of these oxidants are derived from molecular oxygen [85].

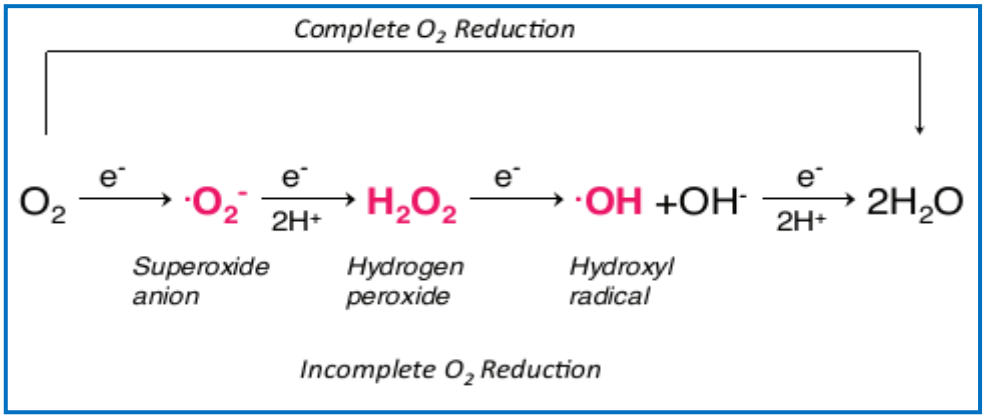


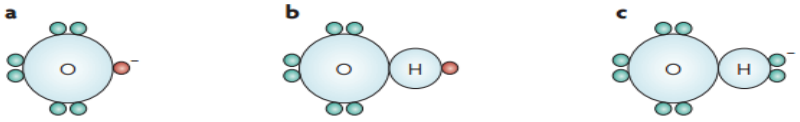
Figure 1.1. Complete and incomplete reduction of molecular oxygen [13].

Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical because it contains two unpaired electrons with parallel spin in different π-antibonding orbitals. This spin restriction is responsible for its relative stability and paramagnetic properties. O_2 possesses the ability to accept electrons to its antibonding orbitals, become "reduced" in the process, and, therefore, function as a strong oxidizing agent. A one-electron reduction of O_2 results in the formation of superoxide anion ($O_2^{-\bullet}$), which is the

precursor of most ROS and a mediator in oxidative chain reactions [78, 122, 127].

During mitochondrial oxidative metabolism, the majority of oxygen consumed is reduced to water; however, a small proportion of molecular oxygen is converted to reactive oxygen species, primarily the superoxide anion $O_2^{-\bullet}$ [69]. Superoxide anion is considered the ‘‘primary’’ ROS because it can further interact with molecules in the cell and form ‘‘secondary’’ ROS via enzyme or metal-catalyzed processes [128]. Superoxide rapidly dismutates to hydrogen peroxide either spontaneously, particularly at low pH or catalyzed by superoxide dismutase. Hydrogen peroxide (H_2O_2) generated in this reaction can be converted to hydroxyl free radicals via the Fenton reaction [14].

Hydroxyl radicals and superoxide anions can further react with other molecules in biological systems, leading to the formation of other free radicals [69]. Similarly, reactive nitrogen species (RNS) include radical species such as primary nitric oxide NO•. NO• and H_2O_2 are membrane permeable, diffusible molecules, which are less reactive and longer-lived than •OH, thus being best suited for intra and even intercellular signalling [106].



The figure shows the electrons in the outer orbital of superoxide (a), hydroxyl radical (b) and hydroxide (c). The paired electrons are indicated in green and the unpaired electrons are in red [125].

1.2. Sources of ROS

ROS can be generated exogenously or produced intracellularly from different sources.

Potential endogenous sources include mitochondria (mtROS), plasma membrane, endoplasmic reticulum and peroxisomes [40, 69, 122, 125].

Mitochondria is considered as the most important subcellular site of $O_2^{-\bullet}$and H_2O_2 production in mammalian organs and the steady state concentration of $O_2^{-\bullet}$in the mitochondrial matrix is about 5- to 10-fold higher than that in the cytosolic and nuclear spaces [23].

Generation of mtROS mainly occurs in the mitochondrial electron transport chain (ETC) located on the inner mitochondrial membrane during the process of oxidative phosphorylation. Oxidative phosphorylation is involved to in energy production in mitochondria. Five big protein complexes are responsible for this process. These ETC complexes are named complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinol-cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase). Electrons donated from nicotine adenine dinucleotide (NADH) at complex I and flavin adenine dinucleotide (FADH2) at complex II pass through ETC and ultimately reduce O_2 to water at complex IV.

The transfer of electrons through the ETC leads to pumping of protons across the mitochondrial inner membrane at complexes I, III, and IV, creating mitochondrial membrane potential.

This proton-motive force allows complex V - ATP synthase to generate ATP from ADP and inorganic phosphate when protons re-enter the mitochondrial matrix through the complex V enzyme [77]. However, under the conditions of normal metabolism, the process of ETC is not perfect. Leakage of electrons at complex I and complex III leads to partial reduction of oxygen to form superoxide $O_2^{-\bullet}$[18, 42, 44, 117], in which complex III is the main site of ROS production [44].

It is estimated that 1% to 3% of O_2 reduced in mitochondria is in the form of $O_2^{-\bullet}$[77, 128]. The complex I leaks $O_2^{-\bullet}$towards the mitochondrial matrix, while complex III leaks $O_2^{-\bullet}$towards both the intermembrane space and mitochondrial matrix. Subsequently, $O_2^{-\bullet}$is quickly dismutated to hydrogen peroxide (H_2O_2) by two dismutases including superoxide dismutase 2 (SOD2) in mitochondrial matrix and superoxide dismutase 1 (SOD1) in mitochondrial intermembrane space. Collectively, both $O_2^{-\bullet}$and H_2O_2 generated in this process are considered as mtROS. These two mtROS have different fates however. Given its electrophilic property and short half-life, $O_2^{-\bullet}$can hardly pass through mitochondrial outer membrane and unlikely to become the candidate of signaling transduction molecule in the cell. Instead, $O_2^{-\bullet}$can undergo radical-radical reaction with nitric oxide (NO) to form peroxynitrite ($ONOO_2^{-\bullet}$) within mitochondria, a detrimental oxidant capable of induction of DNA damage, disruption of mitochondrial integrity, and irreversible modification of proteins. In contrast, H_2O_2 is electrophobic and more stable than $O_2^{-\bullet}$. Indeed, the concentrations of H_2O_2 in mitochondria are 100 times greater than that of $O_2^{-\bullet}$.These properties render mitochondrial H_2O_2 an ideal signaling molecule in the cells [74].

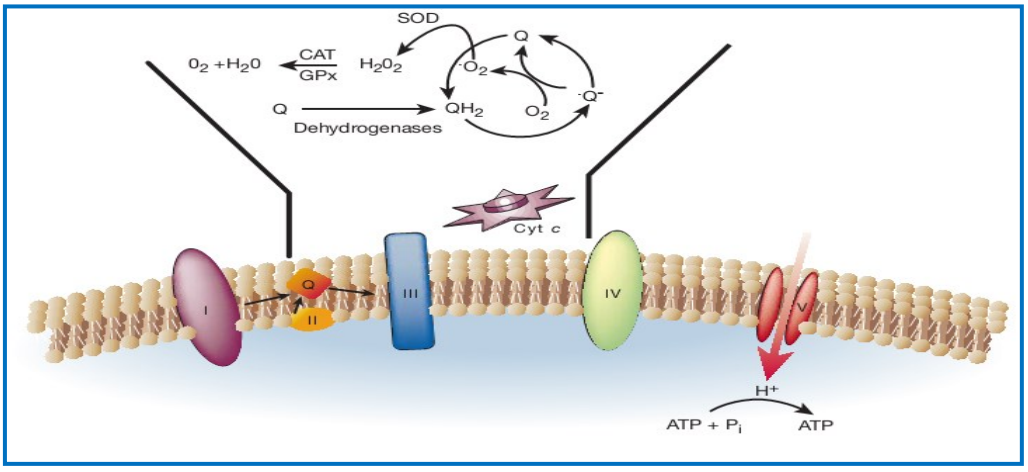


Figure 1.2. Complex III is the major source of mitochondrial ROS production [44].

Additional endogenous sources of cellular ROS are plasma membrane-associated oxidases. One of the best characterized is the NADPH oxidase [122]. It is found in polymorphonuclear leukocytes, monocytes, and macrophages [16]. NADPH oxidase is considered as a "professional" ROS producer [14, 72], whereas the other enzymes produce ROS only as by-products along with their specific catalytic pathways [62]. This multicomponent enzyme catalyzes the one-electron reduction of O_2 to $O_2^{-\bullet}$, with NADPH as the electron donor through the trans-membrane protein cytochrome b558 (a heterodimeric complex of gp91phox and p22 phox protein subunits) [5, 122]. The transfer of electrons occurs from NADPH on the inner aspect of the plasma membrane to 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 $O_2^{-\bullet}$and its reactive metabolites internally for localized microbicidal activity [122].

Based on sequence homology with gp91phox (Nox2), other members of the NADPH oxidase (Nox) family were identified and their expression characterized in nonphagocytic cells such as vascular smooth muscle and endothelial cells, and fibroblasts [5]. Unlike Nox2 in phagocytic cells, the subcellular localization of other Nox isoforms in nonphagocytic cells is not restricted to the cell membrane. Moreover, their dependence on cytosolic regulatory subunits for ROS generation also varies among different Nox members [14].

Several studies have suggested that NADPH oxidase play an important role in the modulation of multiple redox-sensitive intracellular signaling pathways by generating ROS molecules in mammalian cells. This function include inhibition of protein tyrosine phosphatases, activation of certain

redox-sensitive transcription factors, and modulation of the functions of some ion channels [14]. In addition, it is noted that at the cellular level, NADPH oxidase can be activated by a large group of chemical, physical, environmental and biological factors. These stimuli are considered as cellular stresses. NADPH oxidase/ROS-mediated signaling might modulate the cells to be adapted to the stress or to undergo apoptosis [62].

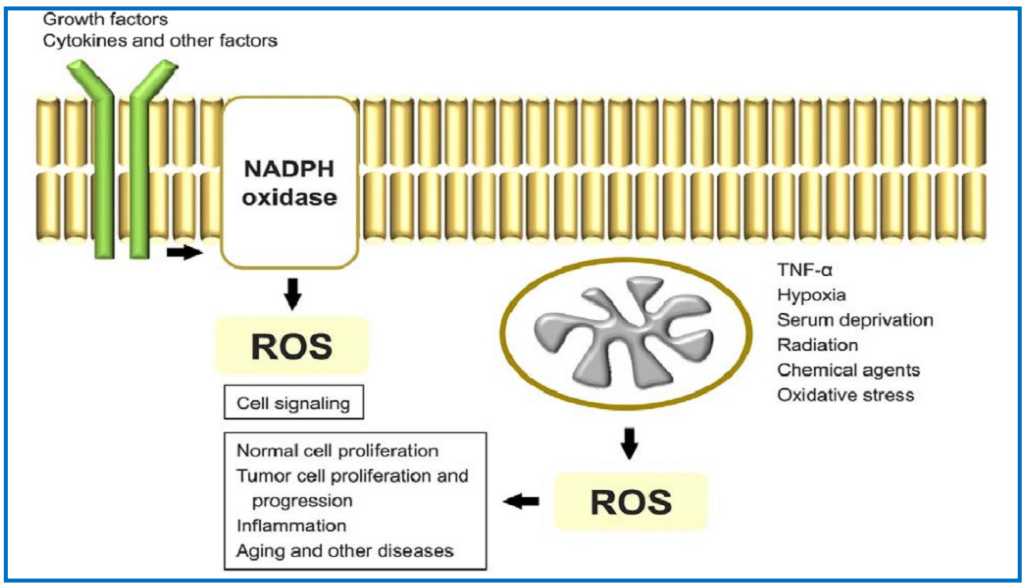


Figure 1.3. Mitochondrial ROS accumulation in response to various stimuli [12].

Endoplasmic reticulum contains the cytochrome P450 is also one of the main sources of ROS. Cytochrome P450 (CYPs) belongs to a multi-gene family of heme proteins and its name derive from the spectral absorbance peak of their carbon monoxide-bound species at 450 nm [12, 57]. Most of the P450 enzymes, particularly those involved in foreign compound metabolism, are located on the cytoplasmic side of the endoplasmic reticulum. P450 enzymes catalyze the metabolism of a wide variety of endogenous and exogenous substrates including lipids, lipophilic drugs, and xenobiotic chemicals [10, 101]. They are responsible for modulating the pharmacokinetic and pharmacogenetic parameters of drugs and pharmaceutical products. These

enzymes are also involved in the metabolism of toxic chemicals, as well as physiological substrates such as arachidonic acid, eicosanoids, cholesterol and steroids, bile-acids, vitamin D3, and retinoic acid [10].

It is possible that the production of ROS, in particular, superoxide anion and hydrogen peroxide emerges following the breakdown or uncoupling of the P450 catalytic cycle owing to the induction of cytochrome P450 enzymes [129].

Uncoupling process can take place during NAD(P)H/O_2-dependent P450 monooxygenase reactions. The expenditure of electrons, derived from NAD(P)H, into unoccupied P450 molecules results in uncoupling of the substrate monooxygenase function and concomitant production of $O_2^{-\bullet}$and/or H_2O_2 rather than monooxygenated product [57, 140]. P450 generation of ROS is tightly controlled by regulation of gene transcription as well as by modulation of interactions between protein constituents of the monooxygenase that affects its activity, coupling, and stability [140]. ROS produced by CYP systems can potentially cause lipid peroxidation, cell toxicity, and death [12, 139].

Peroxisomes are known to generate cellular H_2O_2 under physiologic conditions. The liver is the primary organ where peroxisomes create an significant H_2O_2 source. Besides, other organs that contain peroxisomes are also exposed to these H_2O_2-generating mechanisms [129].

In mammalian peroxisomes, the potential sources of H_2O_2 formation are the oxidases that transfer hydrogen from their respective substrates to molecular oxygen. These enzymes are mainly glycolate oxidase, D-amino acid oxidase, urate oxidase, L-a-hydroxyacid oxidase, and fatty acyl-CoA oxidase. The β-oxidation of fatty acids has been recognized as most important metabolic process in peroxisomes contributing to the production of H_2O_2 [46, 106]. On

the other hand, mammalian peroxisomes have various ROS metabolizing enzymes, particularly, catalase is one of the most abundant peroxisomal proteins. Its predominant role is most likely to prevent the accumulation of toxic levels of H_2O_2. This may in turn prevent the formation of hydroxyl radicals by the Fenton reaction. The oxidative and peroxidative activities of catalase may serve to metabolize and/or detoxify a variety of toxic molecules that enter the circulation [46, 122].

ROS can be produced by a number of exogenous processes. Environmental agents such as non-genotoxic carcinogens can directly generate or indirectly induce ROS in cells. The induction of oxidative stress and damage occurs after exposure to various xenobiotics, such as chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates [129]. Some peroxisome-proliferating compounds are among the classes of compounds that induce oxidative stress and damage in vitro and in vivo [69].

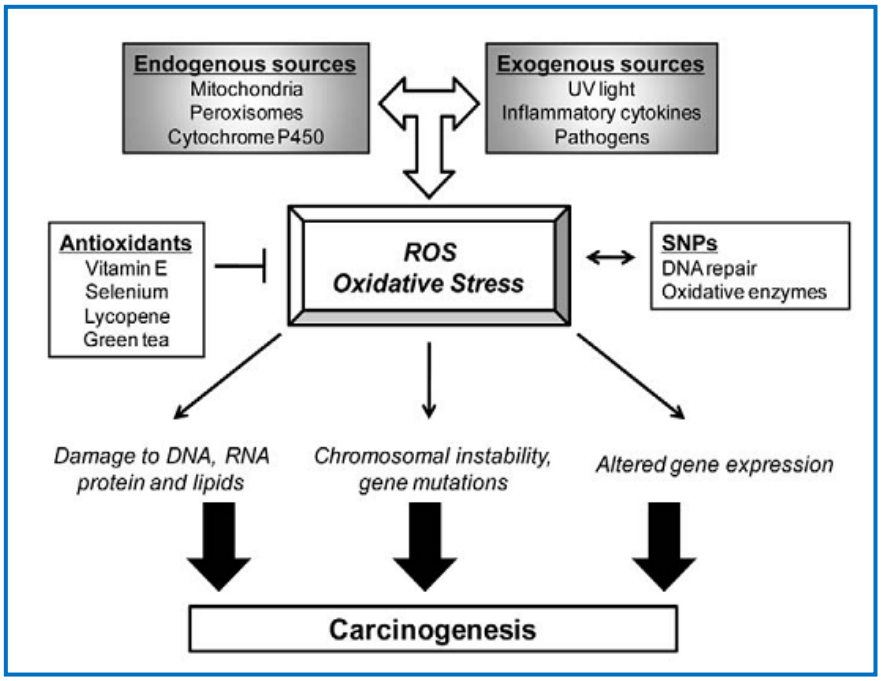


Figure 1.4. Reactive oxygen species (ROS) and their role in the development of human cancer [69].

1.3. Function of ROS

ROS are well known for playing both deleterious and beneficial role in biological systems [21, 83, 129]. Beneficial effects of ROS are shown in the modulation of several physiologic responses, since ROS are part of a signaling network regulating cell function. Cellular ROS are involved in signals controlling cell growth and differentiation, regulating the activity of enzymes (such as ribonucleotide reductase), mediating inflammation by stimulating cytokine production, and eliminating pathogens and foreign particles [125]. In contrast, overproduction of ROS can damage cell structures, including lipids and membranes, proteins and nucleic acids, which is termed oxidative stress. This contributes to many pathological conditions, including cancer, neurological disorders, atherosclerosis, hypertension, ischemia, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma [16, 129].

1.4. ROS and cancer

Compared with normal cells, cancer cells have a persistent pro-oxidative characteristic that can lead to intrinsic oxidative stress. Increased ROS production has been reported in several in vitro and in vivo cancer models [94, 119, 124, 125]. In cancer cells, there is increasing evidence of (a) elevated ROS generation (b) enhanced formation of ROS-mediated reaction/products and their detection in the plasma and urine, (c) over-expression of antioxidant enzymes in response to the elevated oxidative stress [94]. For example, cell lines from melanoma, colon, pancreatic carcinoma, breast and ovarian cancer, and neuroblastoma produce more H_2O_2 than non-transformed cells [119].

The mechanism and the pathways responsible for increased ROS in cancer cells is still unknown, several mechanisms have been proposed for

explain this enhanced oxidative state in cancer development. First assumption is that, oncogenic signals cause increased ROS generation [94, 125]. The oncogene c-myc, for example, increases ROS generation, induces DNA damage, and mitigates p53 function. Another possible mechanism is malfunction of the mitochondrial respiratory chain with mtDNA mutations. This leads to increased "leakage" of electrons from the respiratory complex and more free radical production [94]. Loss of functional p53 is also considered as mechanism of high ROS production in cancer cells. It is associated with redox imbalance, increased ROS stress, high mutagenesis and aggressive tumour growth [125].

Increase in ROS levels can cause various biological responses, including adaptation, increase in cellular proliferation and adaptation to cell apoptosis [38].

Under physiological conditions, redox homeostasis of normal cells is maintained with a low level of basal ROS by balancing ROS generation (pro-oxidants) and elimination (antioxidant capacity). Normal cells can tolerate a certain level of exogenous ROS owing to their 'reserve' antioxidant capacity, which can be mobilized to prevent the intracellular ROS level from reaching the cell-death threshold [125].

Under mild oxidative stress, cells develop an adaptation system to face oxidative stress. The redox buffering systems and various antioxidant enzymes are employed by the cell to counteract the potential toxic effects of elevated ROS. The glutathione system (GSSG/2GSH) is the most abundant redox couple in maintaining cellular redox [94, 105].

A moderate increase of ROS may promote cell proliferation and survival [94, 125]. The mechanisms responsible for stimulation of cell proliferation may involve the direct ROS interaction with specific receptors and modulation of

the redox states of signaling molecules such as protein kinases and transcription factors [94].

However, when ROS reach a certain level (the toxic threshold), it may overwhelm the antioxidant capacity of the cell causing its death [125]. Excessive ROS levels can disrupt the normal redox homeostasis and affect the cellular fate by damaging cells [50], thus providing cue for therapeutic strategies to selectively kill cancer cells using ROS-mediated mechanisms [125].

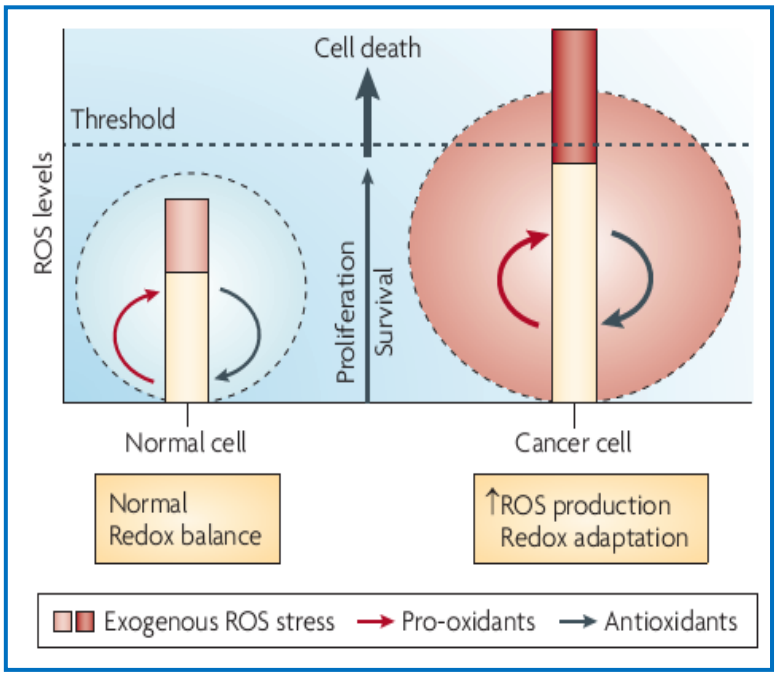


Figure 1.5. Redox balance in normal and cancer cell [125].

1.5. ROS and cells signaling

All multicellular organisms depend on highly complex networks of both extracellular and intracellular signals which modulate cell-cell communication in different physiological processes such as developmental organogenesis, maintenance of normal tissue homeostasis and repair responses to tissue injury. Extracellular signals are composed of growth factors, cytokines, hormones and neurotransmitters that bind to specific cell

surface receptors [122]. Signals sent to the transcription machinery responsible for expression of genes are transmitted to the cell nucleus by a class of proteins called transcription factors, which binding to specific DNA sequences regulate the activity of RNA polymerase II. These transduction processes can induce various biological activities, such as muscle contraction, gene expression, cell growth and differentiation [129].

ROS are not only predominantly implicated in causing cell damage, but also play a major role in several aspects of intracellular signaling and regulation [41]. It has been shown that cells are capable of generating endogenously and constitutively ROS used in the induction and maintenance of redox-regulated signal transduction pathways involved in cell growth and differentiation [128].

As mentioned earlier, overproduction of ROS can break the normal redox balance and cause oxidative stress. Cell survival depends on the ability of the cell to adapt to the stress, and to repair the damaged molecules. Cells may also respond to the stress by undergoing apoptosis. Several mechanisms help cells and organisms adapt to acute stress. They act in either a cooperative or antagonistic fashion. Many of these pathways have been preserved throughout evolution. Among the main stress signaling 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) signaling cascades, the phosphoinositide3-kinase (PI(3)K)/Akt pathway, the nuclear factor (NF)-kB signaling system, p53 activation, and the heat shock response. However, 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 [44].

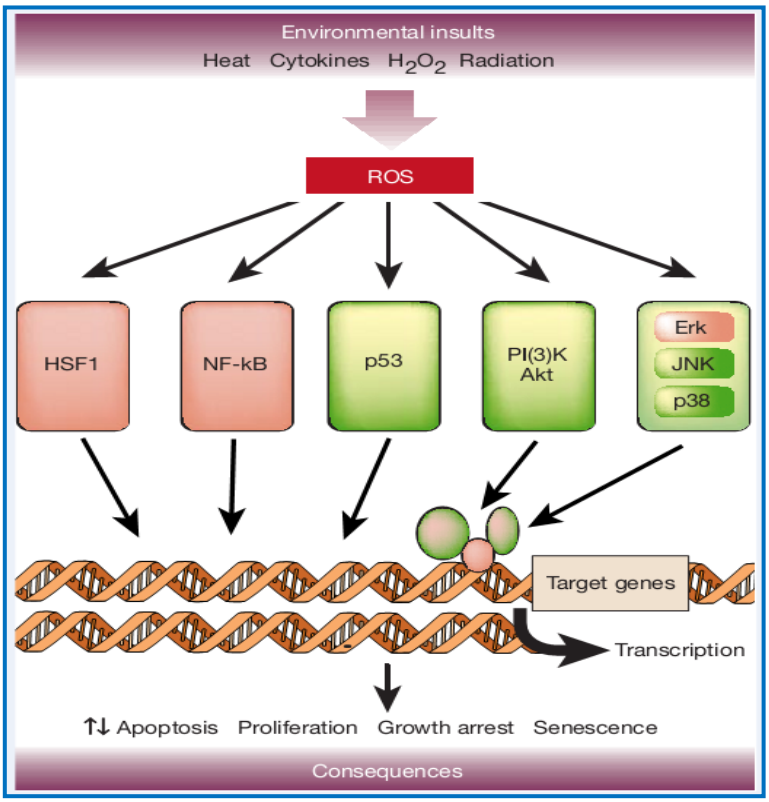


Figure 1.6. Major signaling pathways activated in response to oxidative stress [44].

Controlling production of ROS is essential for the activity of signal transduction pathways, and one broad class of signal transduction molecules on which ROS influence function is the mitogen-activated protein kinases (MAPKs) [108].

MAPKs are a family of serine/threonine kinases involved in regulating a wide array of cellular processes such as proliferation, differentiation, stress adaptation, and apoptosis. There are three multimember subfamilies: the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases. The ERK, JNK, and p38 subfamilies are activated via independent (though sometimes overlapping) signaling cascades involving a MAPK kinase (MAPKK) that is responsible for phosphorylation of the

MAPK, and a MAPK kinase kinase (MAPKKK) that phosphorylates and activates MAPKK. MAPKs mediate their effects through phosphorylation of a wide range of effector proteins, most notably transcription factors, which in turn lead to changes in the pattern of gene expression [28, 81, 131]. Studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathways [48, 86]. The prevention of ROS by antioxidants blocks MAPK activation after cell stimulation, indicating the involvement of ROS in activation of MAPK pathways [86].

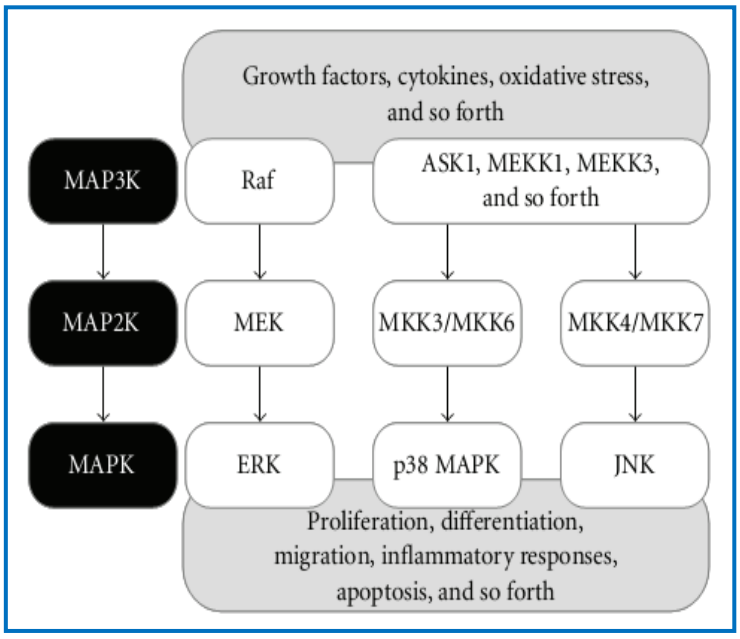


Figure 1.7. MAPK cascades [115].

The ERK pathway is a member of MAPKs family, which lying at the heart of many signal transduction processes. It constitutes a major pathway through which growth factor receptors transduce proliferative signals to the nucleus [70, 81].

The ERK has two isoforms ERK2/ERK1 (also known as p42/p44MAPK). These enzymes are activated through a sequential phosphorylation cascade that amplifies and transduces signals from the cell membrane to the nucleus [24]. Upon receptor activation, membrane-bound

GTP-loaded Ras recruits one of the Raf kinases, A-Raf, B-Raf and C-Raf (or Raf1), into a complex where it becomes activated. Then, Raf phosphorylates two serine residues on the kinase mitogen protein kinase kinase 1 and 2 (MEK1⁄2; also known as MAP2K1 and MAP2K2, respectively), which in turn activate ERK1⁄2 by tandem phosphorylation of threonine and tyrosine residues on the dual-specificity motif. Finally, active ERKs regulate through phosphorylation many cytoplasmic and nuclear targets that perform important biological functions [24, 99]. Growth factor receptors are most commonly activated by ligand-induced dimerization or oligomerization that phosphorylates receptor tyrosine kinases (RTKs) [9, 115]. Ligand-independent clustering and activation of growth factor receptors in response to ROS have also been well demonstrated [89, 115].

ERK activation generally promotes survival of cells in response to ROS [44, 108] but also can promote apoptosis under certain conditions and in certain cell types. For instance, ERK facilitates hypoxia-induced apoptosis of macrophages and cisplatin-induced apoptosis of HeLa cells [81]. Collectively, a growing number of studies have shown that activation of ERK by chemopreventive compounds (i.e. resveratrol and quercetin) results in anti-proliferative effects such as apoptosis, senescence, or autophagy in cancer cells [68, 100, 109, 110].

The other two MAPK families, the JNK and p38 families, sometimes are known as the stress-activated protein kinases (SAPKs) for their role in cellular response to different stresses, including cytokines, radiation, osmotic shock, mechanical injury, heat shock and oxidative damage [81, 108].

Akt or protein kinase B (Akt/ PKB) is a serine/threonine kinase, like ERK, plays an important role in integrating cellular responses to growth factors and other extracellular signals. It is a crucial regulator of human

physiology, and it controls an impressive array of diverse cellular functions including the modulation of growth, survival, proliferation and metabolism. The Akt family comprises three homologous isoforms, Akt1 (PKBa), Akt2 (PKBb) and Akt3 (PKBg), which have a highly conserved domain structure formed by an N-terminal pleckstrin homology (PH) domain [80]. Akt modulated downstream of phosphatidylinositol-3-kinase (PI3K) in response to extracellular stimuli following a multistep process. Many growth factors and cytokines stimulate an activity in the lipid enzyme PI3K, resulting in a subsequent increase in PI(3,4)P2 and PI(3,4,5)P3 on the plasma membrane. Akt is phosphorylated at two sites: the Thr308 residue within the T-loop of the catalytic domain, is phosphorylated by the phosphoinositide-dependent kinase 1 (PDK1); the Ser473 residue within the carboxyl terminal hydrophobic domain by the mammalian target of rapamycin complex 2 (mTORC2) [27]. Akt plays crucial roles in mammalian cell survival and has been shown to be activated in various cancers. Activated Akt promotes cell survival through the NF-kB signaling pathway and inhibits apoptosis through inactivation of several proapoptotic factors including Bad, Forkhead transcription factors, and caspase-9 [2]. It also enhances energy production by stimulating nutrient transporters, which in turn supports mTOR-dependent protein translation. Thus, by preventing apoptosis and increasing oxidative metabolism, Akt lies at the center of complex signaling pathways that incorporate a multitude of potentially oncogenic signals [76].

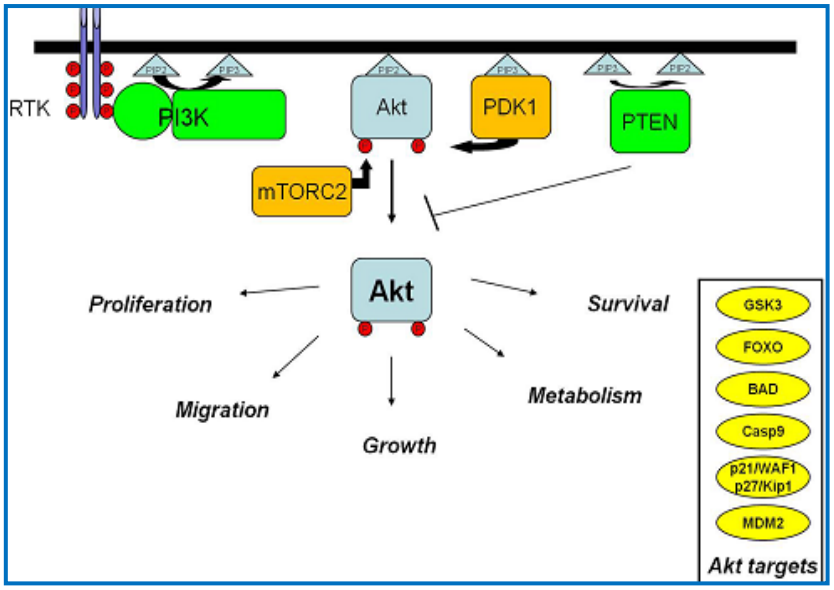


Figure 1.8. Schematic regulation of the Akt pathway [27].

At the cellular level, oxidant exposure can cause a wide spectrum of phenotypic responses from proliferation, to growth arrest, to senescence, or cell death. Besides the major signaling pathways such as ERK, JNK and p38, Akt is known to be activated in response to oxidant injury. These pathways exert phenotypic effects largely by modulating the activities of transcription factors which lead to alternations of gene expression [55].

In recent years, this kinase has been reported to be a pivotal player of the antioxidant-induced pro-oxidant effects. For instance, coumaric acid and resveratrol, two common antioxidants can causes endothelial cells damage by down-modulating Akt phosphorylation [93]. It has also been considered an attractive target for cancer prevention and treatment. Several phytochemicals including genistein, indole-3-carbinol, diosgenin, curcuminoids, EGCG, and black raspberries are known to suppress the activation of Akt [2].

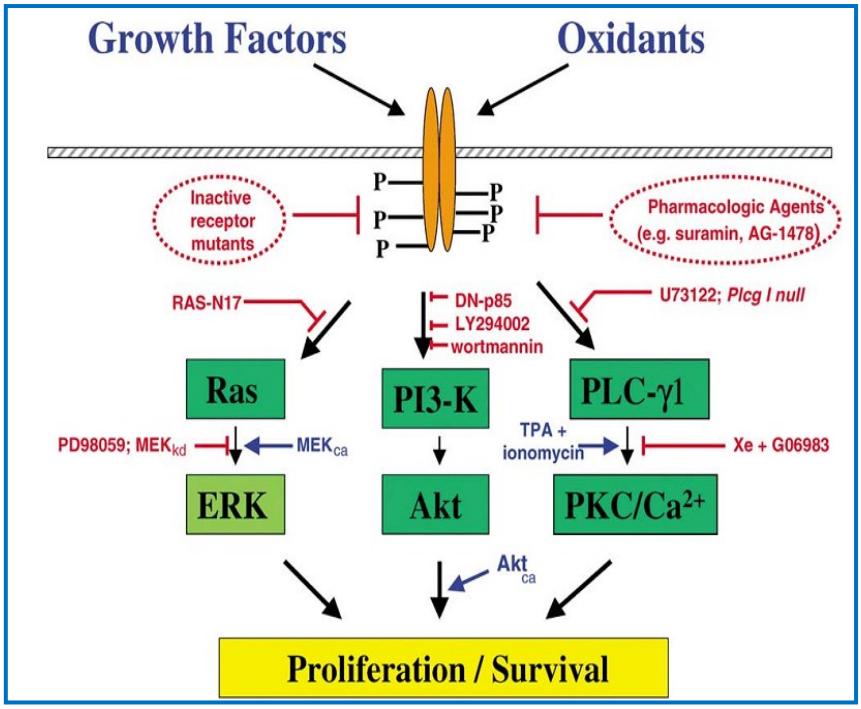


Figure 1.9. Proliferation - associated pathways activated by oxidants and important for cell survival [55].

1.6. ROS and apoptosis

Apoptosis is characterized by controlled autodigestion of the cell. This differs from necrosis by distinct morphological and biochemical features, such as chromatin condensation, membrane surface blebbing, oligonucleosomal DNA fragmentation and finally, the breakdown of the cell into a series of smaller units (membrane-bound fragments). These are called apoptotic bodies and in most tissues are phagocytosed by adjacent cells. Such events are associated with activation of specific proteases termed caspases and loss of membrane phospholipid asymmetry resulting in phosphatidylserine externalization [82]. Apoptosis can be initiated by a variety of stimuli, including hyperthermia, growth-factor or hormone withdrawal, glucocorticoids, oxidants, ionizing radiation and multiple classes of chemotherapeutic agents [17]. In the apoptotic process, initial stress-

induced damage does not kill cells directly, rather it causes an apoptotic signaling programme that leads to cell death [47].

Apoptotic cell death may be triggered through the extrinsic (receptor-mediated) or the intrinsic (mitochondria-mediated) pathway [39]. The extrinsic pathway of apoptosis is mediated by death receptors in that ligand-receptor binding initiates protein-protein interactions at cell membranes that activate initiator caspases. Major known receptors include Fas, TNF receptor 1, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1, and TRAIL receptor 2 [8]. The death receptor is consist of three functional extracellular ligand-binding, transmembrane and intracellular domains. Apoptotic signaling is initiated by the association of death-domain-containing adaptor proteins [33].

The intrinsic pathway can be triggered by many stimuli including ROS. Mitochondria are the major site of ROS production and accumulation of ROS may lead to the initiation of apoptosis [39]. Release of cytochrome c is considered as the central event, since it is important for aggregation of the adapter molecule (Apaf). The Bax, a proapoptotic member of the Bcl-2 family can directly induce mitochondria to release cytochrome c. The damage of the mitochondrial pores by ROS may contribute to cytochrome c release due to disruption of the mitochondrial membrane potential. In contrast, it is unclear how the initial ROS is released from mitochondria. If a sequential event is assumed, initial released ROS could directly or indirectly (via ceramide generation) increase the gating potential of the pore. Collectively, it seems that mitochondria are both source and target of ROS [114].

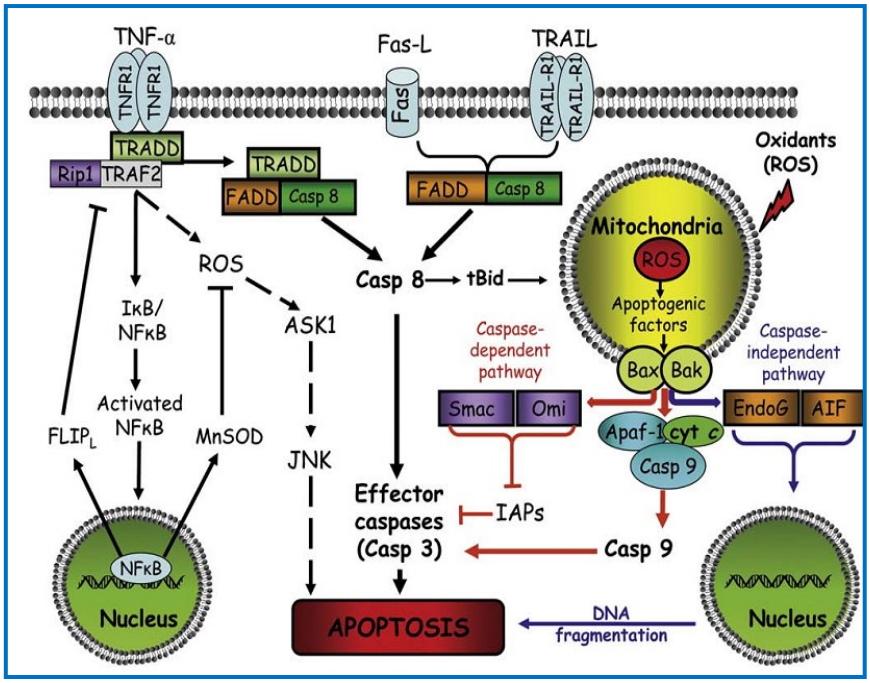


Figure 1.10. Death - receptor - mediated and mitochdrial pathways of cells apoptosis [33].

A complete understanding of the redox control of apoptotic initiation and execution could support the development of therapeutic interventions targeted at oxidative stress-associated disorders [33].

1.7. Antioxidants

The term "antioxidant" refers to "any substance that prevents oxidation of biomolecules either directly by scavenging reactive oxygen species or indirectly by upregulating the antioxidant defense or DNA repair systems" [87].

Human have endogenous defenses that play crucial role in maintaining a balanced intracellular redox status. The antioxidant activity can be useful through different ways. It functions as inhibitors of free radical oxidation reactions (preventive oxidants) by inhibiting formation of free lipid radicals; by interrupting the propagation of the autoxidation chain reaction (chain

breaking antioxidants). Besides, it acts as singlet oxygen quenchers; through synergism with other antioxidants. It can also reduce agents that convert hydroperoxides into stable compounds. Finally, as inhibitors, it can inhibit pro-oxidative enzymes lipooxygenases [26].

It has been described that there are two major groups of antioxidant systems in human, enzymatic antioxidants and non-enzymatic oxidants that work synergistically in maintaining the intracellular redox homeostasis [98]. An effective antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels.

Regarding enzymatic antioxidants, three important enzymes that prevent the formation or neutralize free radicals are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

The enzyme superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{-\bullet}$ to H_2O_2, thereby preventing the accumulation of this free radical. Subsequently, H_2O_2 can be converted into H_2O and O_2 by catalase and/or glutathione peroxidase. Three isoforms of SOD have been identified, including a cytoplasmic Cu/Zn-SOD, a mitochondrial Mn-SOD and an extracellularly localized SOD [16, 53, 60, 74]. Overall, CuZn-SOD and Mn-SOD are generally thought to act as bulk scavengers of superoxide radicals [16]. Especially, Mn-SOD is one of the most effective antioxidant enzymes that has anti-tumour activity [129].

Catalase is a heme-containing tetramer of four polypeptide chains that is located in the peroxisome of aerobic cells. It is highly efficient at reducing hydrogen peroxide to water and molecular oxygen. Catalase has one of the biggest turnover rates for all enzymes, allowing just one molecule of catalase to convert 6 million molecules of hydrogen peroxide each minute [26, 98,

129]. The significantly decreased capacity of detoxifying hydrogen peroxide in some kind of tumours is related to a decreased level of catalase [129].

Glutathione peroxidase (GPx) are a family of tetrameric enzymes that contain the unique amino acid selenocysteine within the active sites [16, 19]. It functions in combination with tripeptide glutathione (GSH), which exists in cells in high concentration. This enzyme catalyzes the conversion of hydrogen peroxide or organic peroxide to water or alcohol while simultaneously oxidizing GSH. It also emulates catalase for hydrogen peroxide as a substrate and it is the potential source of protection against low levels of oxidative stress [98, 129].

In addition to enzymatic antioxidants, some enzymes are considered as secondary enzymatic defense, including glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase reduces glutathione (antioxidant) from its oxidized to its reduced form, thus recycling it to continue neutralizing more free radicals. Glucose-6-phosphate produces NADPH, creating a reducing environment. These two enzymes do not neutralize free radicals directly, but have capacity of supporting to the other endogenous antioxidants.

With regard to the non-enzymatic endogenous defense, there are some important antioxidants such as glutathione (GSH), vitamins C, Vitamin E [26].

Glutathione is an endogenous tripeptide that protects the cells against free radicals either by donating a hydrogen atom or an electron. It is also very important in the regeneration of other antioxidants like ascorbate [26]. Glutathione is considerable in cytosol, nuclei, mitochondria and is the major soluble antioxidant in cell compartments [98]. GSH/GSSG ratio is a good

measure of oxidative stress of an organism. GSH transfers its electron to H_2O_2 to reduce it into H_2O and O_2 [16, 98].

Vitamin C (ascorbic acid) is a very essential antioxidant that works in aqueous environments of the body. It can convert vitamin E free radicals back to vitamin E and acts as scavenger of oxygen free radicals [26, 98, 129]. Vitamin E donates electron to peroxyl radical, which is produced during lipid peroxidation. It induces apoptosis of cancer cells and inhibits free radical generation [16].

Uric acid and Coenzyme Q10 have been also considered as non-enzymatic endogenous antioxidants [26].

The endogenous antioxidant system has remarkable efficiency. However, under condition where ROS are produced in excess, this system may not be sufficient, and humans may require exogenous antioxidants (e.g. from diet) to maintain a balanced redox status [26]. Besides exogenous antioxidants such as vitamin C, vitamin E, flavonoids are considered as a powerful antioxidants [49].

Flavonoids constitute the most important single group of polyphenols [129]. They are present in fruits, vegetables, nuts, plant-derived beverages such as tea, wine and traditional Eastern medicines [49]. The flavonoids family includes flavonols, anthocyanins, isoflavonoids, flavanones and flavones. All these sub-groups of compounds share the same diphenylpropane ($C_6C_3C_6$) skeleton [95]. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes, while flavones and flavonols do not share this phenomenon and are rarely found together [26].

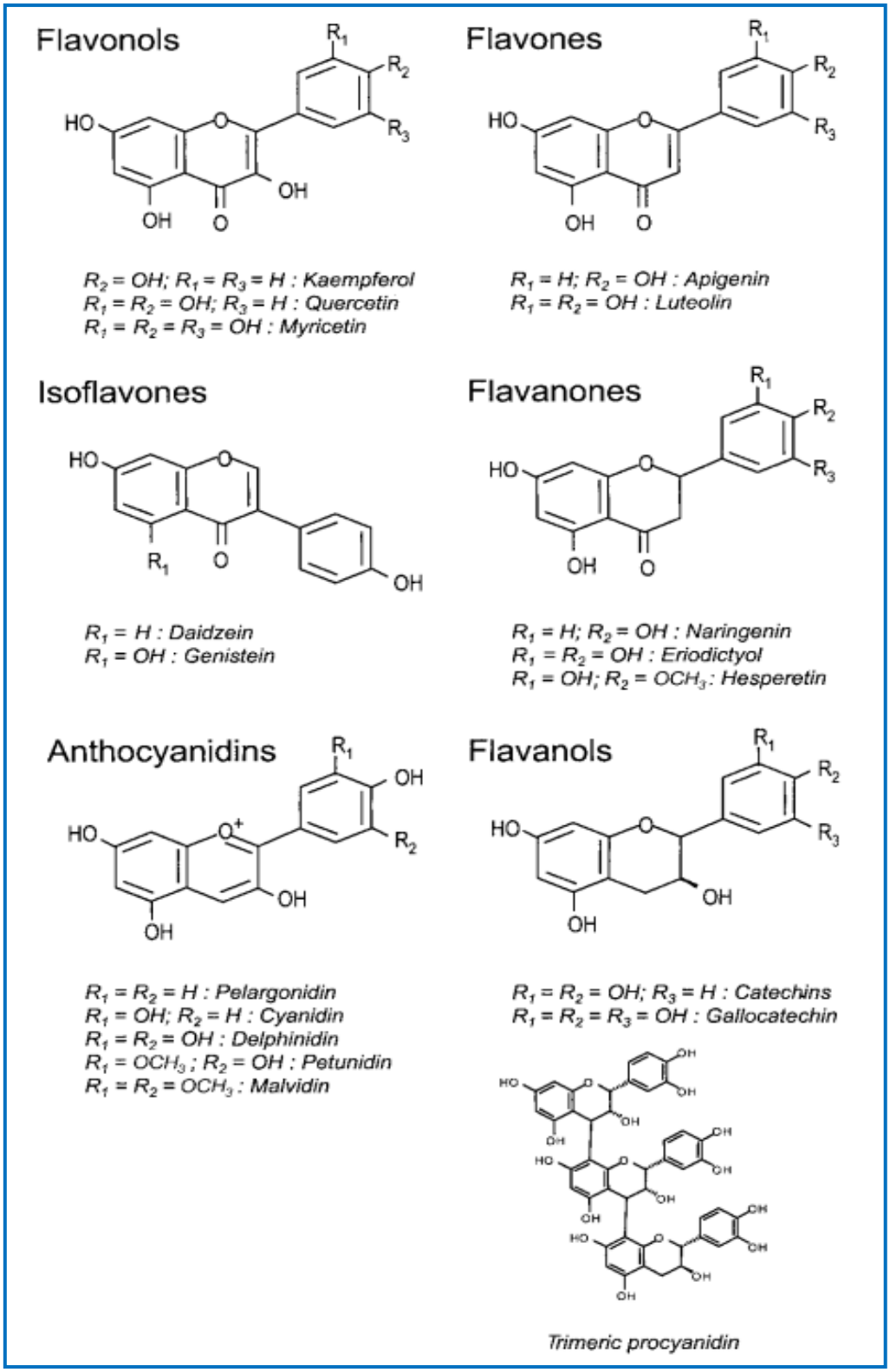


Figure 1.11. Chemical structures of flavonoids [79].

Recent interest in phenolic compounds in general, and flavonoids in particular, has increased considerably due to their possible beneficial implications in human health [107]. They possess a high number of protective biologic properties: antioxidant, anti-carcinogenic, anti-inflammatory [90]. In relation to cancer, several studies from in vitro, epidemiological investigations in vivo, and human clinical trials have been reported that flavonoids have good effects on cancer chemoprevention and therapy. They may protect DNA from oxidative damage, inhibit carcinogen activation, and activate carcinogen-detoxifying systems [49].

Flavonoids have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom [107, 126]. More frequently, they function as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers) [107]. Chain-breakers donate an electron to the free radical, neutralizing the radicals and themselves becoming stable (less reactive) radicals, thus inhibiting the chain reactions [126].

In addition to radical scavenging, flavonoids act as metal chelators. Chelation of transition metals such as Fe^{2+}can directly decrease the rate of Fenton reaction, thus preventing oxidation caused by strongly reactive hydroxyl radicals [26, 107, 126]. Flavonoids also activate antioxidant enzymes, reduce α-tocopherol radicals (tocopheroxyls), inhibit oxidases, and increase levels of uric acid and low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin and kaempferol [26].

Other antioxidant actions of flavonoid have also been mentioned such as inhibition of xanthine oxidase and elevation of endogenous antioxidants. Flavonoids can enhance the expression of antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose

hydroperoxides, hydrogen peroxide and superoxide anions, respectively, and inhibit the expression of xanthine oxidase [58, 126].

As well as many of others so-called antioxidants, under certain reaction conditions, flavonoids can display a pro-oxidants activity and hence, promote the oxidation of other compounds [26, 49, 97]. In relation to the chemopreventive properties of flavonoids, their pro-oxidant effect has been proposed as an important mechanism related to their anticancer and apoptosis-inducing properties. Another mechanism for these properties also suggested is that their pro-oxidant phenoxyl radicals can induce mitochondrial toxicity by collapsing the mitochondrial membrane potential [49]. Cumulatively, flavonoids might modulate actions in cells through actions at protein kinase and lipid kinase signaling pathways [132].

Green tea contains many polyphenols, which include flavanols, flavandiols, flavonoids and phenolic acids. Most of the green tea polyphenols are flavanols, commonly known as catechins [11]. Green tea derived from the plant (*Camellia sinensis*), is a popular beverage in some parts of the world. It is characterized by the presence of large amounts of flavan-3-ols also known as catechins. A typical cup of brewed green tea is made using 2 g of tea leaves in 200 mL of hot water and contains approximately 600-900 mg water extractable solids. Of these solids, approximately 30-40% by weight are the tea catechins [71].The green tea catechins mainly consist of (-)-gallocatechin (GC), (+) catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), and (-)-gallocatechin-3-gallate (GCG) [45], of which EGCG is the most abundant by weight [45, 71] and accounts for 50-75% of the total amount of catechins [65]. EGCG is the most effective in reacting with the majority of ROS. It

plays a role as chelators of metal ions, prevents the formation of ROS from the auto-oxidation of many compounds [136].

Several studies in vivo and in vitro have been shown that green tea have preventive effects against chronic diseases such as diabetes, heart diseases, neurodegenerative diseases and cancer [71, 113]. In this context, using laboratory animal models, many mechanisms have been suggested for the cancer preventive effects of green tea and EGCG. This includes inhibition of growth factor signaling, inhibition of key cellular enzymes, inhibition of gene transcription, and induction of tumor suppressor genes [66, 135]. While many studies have focused on the effects and mechanism of EGCG on various cell types, the effects of Polyphenon E (Poly E) on tumor cells, as well as its mechanism of action, have to be elucidated yet. Polyphenon E is a well-defined pharmaceutical-grade mixture of polyphenols that contain about 50% EGCG and 30% other catechins [29]. Since the formulation is highly reproducible and easily prepared, Poly E is an attractive derivative of green tea for clinical chemoprevention trials [116].

Interestingly, the antioxidant activity of green tea polyphenols and, more recently, the pro-oxidant effects of these compounds, have also been suggested as potential mechanisms for cancer prevention [71].

1.8. Green Tea and prostate cancer

Prostate cancer (PCa) is one of the most frequently diagnosed male cancer in the Western countries and continues to represent a major cause of cancer-related mortality, despite medical advances. The reasons of this high incidence are unknown, but there is significant difference in PCa incidence and mortality among ethnic groups, with African-American men being at the greatest risk for diagnosis, followed by Caucasian and Hispanic men [88]. Asian-Americans seem to be at the lowest risk for PCa [88, 130].

About less than 10% of PCa has been shown to be inherited suggesting that a variety of genetic and environmental factors may be important contributions to PCa development [118]. The Asians appear to have the lowest risk of developing PCa which may be due to consuming specific dietary constituents daily over many years. Over the last two decades many epidemiological studies, both cohort and case-control studies, have suggested that green tea consumption correlates with a lower risk of certain cancers such as breast, colon, and prostate [63]. It is suggested that these compounds affect enzyme activities and signal transduction pathways, resulting in cell proliferation suppression, apoptosis enhancement, as well as angiogenesis and cell invasion inhibition [37].

Because of its low toxicity, green tea is a good potential candidate as a prostate cancer chemopreventive agent [31], also because of its specificity to transformed and malignant cells [4, 34]. Recently, a case-control study conducted in China showed that green tea consumption is etiologically associated with PCa, suggesting the protective effect of green tea against this disease [61]. This evidence has proposed that administration of biologically active compounds from green tea might be effective in lowering the incidence of PCa [15].

CHAPTER 2

RESEARCH OBJECTIVES

A dramatic increases of prostate cancer (PCa) in the Western countries has been widely recognized [63, 67]. The increasing frequency and mortality associated with PCa emphasizes the importance for new approaches to reduce its incidence and prevent progression to advanced metastatic cancer. Many dietary polyphenols are being examined as potential PCa chemopreventive agents. Among all dietary agents proposed for PCa chemoprevention, green tea polyphenols have received much attention. It is important to emphasize that green tea polyphenols possess both cancer chemopreventive and chemotherapeutic effects [67]. EGCG is the major catechin in green tea, which possesses antioxidant, anti-mutagenic, anti-proteolytic and anti-proliferative activity [103]. Polyphenon E (Poly E), is a standardized and well-characterized decaffeinated extract of green tea that contains 50% EGCG and lesser amounts of the catechins EGC, ECG, EC, GCG. This compound is of interest because it is readily prepared and its components may exert synergistic or additive effects with respect to anti-tumor activity [111] .

The induction of apoptosis has been proposed as one of the principal mechanisms for the EGCG anticancer. Although it is widely accepted that the compounds antioxidant properties are at the basis of their anticancer effect, their pro-oxidant action has been lately postulated being the trigger of the induction of cancer cells apoptosis. ROS, which include superoxide, singled O_2, hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical are formed, in most cases by chemical and physical carcinogens or by inflammatory cytokines. High concentration of ROS (oxidative stress) leads to damage of proteins, lipids and nucleic acid components, as a consequence, cells will either undergo apoptosis or survive due to activated repair and/or diverse antioxidant mechanisms [43], which ultimately may leads to neoplastic cell transformation.

A recent study shown that some natural antioxidants, such as coumaric acid and resveratrol, can dose dependently induce both antioxidant and pro-oxidant effects and ultimately modulates cell fate [93]. In this context, whether anti- or pro-oxidant effect of Poly E is involved in its anticancer activity is not clear. Moreover, the intracellular signals modulated by Poly E in cancer cells and their potential relationships with Poly E anti- or pro-oxidant activity are largely unknown.

Therefore, our study has been carried out with the following intents:

1. To examine the effect of Poly E in cultured PCa cells
2. To examine anti- and pro-oxidant effect of Poly E in cultured PCa cells and its potential correlation with an anti-proliferation activity
3. To identify potential intracellular signals involved in the anti-proliferative effect of Poly E in PCa cells

CHAPTER 3

MATERIALS AND METHODS

3.1. Reagents.

Polyphenon E (Poly E), a green tea standardized extract was manufactured by Mitsui Norin Co. Ltd. (Shizuoka, Japan) and was kindly provided by Prof. Saverio Bettuzzi (University of PARMA, Italy).
Primary antibodies (anti- phospho-ERK1/2, anti-ERK, anti- phospho-Akt and anti-Akt) were obtained from Cell Signaling-Technology (Beverly, MA, USA); β-actin antibody from Sigma-Aldrich (Saint-Louis, MO, USA); the HRP-conjugated secondary antibodies were from Pierce Biotechnology-Thermo Scientific (Rockford, USA).

3.2. Cell culture and treatments.

PC3 human prostate cancer cells from ATCC (Rockville, MD) were cultured in F_{k12} nutrient mixture 1X (Invitrogen, Carlsbad, CA) respectively, supplemented with 7% fetal bovine serum (FBS) and penicillin G (100 U/ml), streptomycin (100 μg/ml) and 0,25 μg/ml amphotericin B . The cells were maintained at 37°C and 5% CO_2 in a humid environment.

Poly E was dissolved in PBS plus for experiments using fluorescence. For the others, Poly E was solubilised in cell medium with 2,5% FBS. The PC3 (70-80% confluent) were treated with Poly E (10, 30, and 100 μg/ml) with different time points depending on experiments. Cells used as controls were incubated with the vehicle only.

3.3. Cell viability assay (ATP assay)

Cell viability assay was assessed by using the CellTiter-Glo®Luminescent Cell Viability Assay. This is a homogeneous method to determine the number of viable cells in culture based on quantitation of the produced ATP, which signals the presence of metabolically active cells. When cells lose membrane integrity, they lose in the ability to synthesize

ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm.

The homogeneous “add-mix-measure” format results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells.

Briefly, after treatment for 24 h, plates were removed from the incubator and allowed to equilibrate at room temperature for 30 minutes and equal volume of CellTiter-Glo reagent was added directly to the wells. These contents were mixed for 2 minutes on an orbital shaker to induce cell lysis. Plates were incubated at room temperature for 10 minutes to stabilize luminescent signal. The luminescence was measured on GENios plus microplate reader (Tecan, Männedorf, CH). The results are expressed as percent of control.

3.4. Cell metabolic assay (MTT assay)

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

Yellow MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product.
This reduction occurs only when mitochondrial reductase enzymes are active, 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 a large amount of MTT formazan formed and an increase in absorbance.

So after treatments for 24 h, 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 control.

3.5. Cell proliferation assay (BrdU incorporation Assay)

Cell proliferation was assessed by using chemiluminescent immunoassay, which based on the measurement of BrdU incorporation during DNA synthesis. When cells are pulsed with BrdU, it is incorporated into newly synthesized DNA strands of actively proliferating cells. The incorporation of BrdU into cellular DNA may then be detected using anti-BrdU antibodies, allowing assessment of the population of cells which are synthesizing DNA.

Confluent cell was treated with different concentrations of Poly E and the proliferation was evaluated at different time points (24 h and 48 h). BrdU is added to cells cultured in microplates, followed by incubation for 10 hours. After the culture supernatant is removed, the cells are fixed by Fix-Denat solution for 30 min. Fix-Denat was discarded and cells was incubated with an anti-BrdU antibody conjugated to peroxidase (anti-BrdU-POD) for 90 min. After rinsing three times with washing buffer, substrate solution was added and allowed to react for 6 min at room temperature. Finally, light emission was

read by using a GENios plus microplate reader (Tecan, Männedorf, CH). Results were expressed as means ± SD.

3.6. Cell apoptosis assay

Cell apoptosis was assessed after treatments by using the fluorimetric kit APOPercentage (Biocolor Ltd, Carrickfergus, UK), following the protocol provided by the manufacturer. The assay has been used with several adherent cell lines including prostate cancer cell lines [7].

The assay uses the dye 3,4,5,6,-tetrachloro-2',4',5',7'-tetraiodofluorescein that is selectively imported by cells that are undergoing 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 APOPercentage 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 with various concentrations of Poly E. The apoptosis was examined after 24 h and 48 h. At the end of treatments, the APOPercentage dye was added to each well (dilution 1:10) and cells incubated for 30 more min at 37°C in a 5% CO_2 incubator. After thoroughly washing, 100 µl of APOPercentage 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 means ± SD.

3.7. Measurements of intracellular ROS.

Intracellular ROS levels were determined by using the ROS molecular probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probe, Eugene, OR) as previously described with minor modification [93]. Within the cell, esterases cleave the acetate groups on H_2DCF-DA, thus trapping the reduced form of the probe (H_2DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF.

For ROS measurements cultured cells were pre-incubated for 30 minutes with PBS plus containing 1 μM H_2DCFDA, then washed with PBS and treated as described. Fluorescence was measured by using a Tecan GENios plus micro-plate reader (Tecan, Männedorf, Switzerland) in a light-protected condition. Excitation and emission wavelengths used for fluorescence quantification were 485 nm and 535 nm respectively. Treatments-induced variation of fluorescence was measured for 2 h, 6 h, 12 h. All fluorescence measurements were corrected for background fluorescence and protein concentration. Using untreated cells as a reference the anti- and pro-oxidant outcome was evaluated by comparison of five measurements and expressed as percent of controls.

3.8. Measurements of protein carbonylation.

Protein carbonylation, one of the most harmful irreversible oxidative protein modifications, is considered as a major marker of oxidative stress-related disorders. Oxidative modification of proteins alters 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 measurements are often performed to assess the extent of oxidative stress in the context of cellular damage. The OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA) were used to measure protein carbonyl groups with 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 arbitrary units.

3.9. MMP assay

Mitochondrial membrane potential (MMP) 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'-tetraethyl-benzimidazolyl-carbocyanine iodide) to signal the loss of mitochondrial membrane potential [59].

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 for 12 h. 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. The results were expressed as ratio green and red.

3.10. Protein extraction

Cells were cultured in T25 culture flasks (BD Falcon) and reach confluent. Following Poly E treatments, the cells were harvested at 15 min, 30 min, 1 h, 2 h post-treatment and then washed with cold PBS. The cells were then incubated in ice-cold lysis buffer (CytoBuster protein extraction reagent; Novagen, Darmstadt, Germany) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2,3; Sigma, St Louis, MO) over ice for 10 min. The cell debris were scraped, the lysate was collected in a microfuge tube and cleared by centrifugation at 16,000g for 5 min at 4^0C.

The supernatant (total cell lysate) was collected, aliquoted, and stored at - 80^0C. The protein content in the lysates was measured by Bradford assay following the manufacturer's protocol (Sigma, St Louis, MO).

3.11. Western blot analysis

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 apparatus (100V for 1h). The non-specific sites were blocked by incubating the blot with TBS containing 5% BSA and 0.1% Tween-20 (blocking buffer) for 2 hours on the shaker, at room temperature.

The blot was washed with wash buffer (TBS containing 0.1% Tween-20) for 3 x 5 min and then 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 (Cell Signaling, Danvers, MA) and β-actin (Sigma-Aldrich, Saint-Louis, MO, USA).

The next day, after thoroughly washing in TBS/Tween, 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.

3.12. 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 $p < 0.05$. 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 Poly E on cell viability and metabolic activity of PC3

Cell viability was evaluated by using the ATP assay as described in "Materials and Methods". Cells were stimulated with increasing concentrations (10, 30, 100 μg/ml) of Poly E for 24 h, while untreated cells were used as control (CTRL). Although a reduction of cell viability was observed at a concentration of 10 μg/ml Poly E, it is not significant compared to the control. In contrast, the treatment of cells with the higher concentrations (30, 100 μg/ml) of Poly E, significantly lowered the viability of cells in comparison to the untreated ones (figure 4.1).

Similar to the observed cell death, a significant decrease in cell metabolic activity was induced by both 30 and 100 μg/ml of Poly E, as depicted by the data reported in figure 4.1 obtained with the MTT assay. Moreover, a correlation was evident between MTT and ATP data. Results from five pooled measurements are shown, and expressed as percent of untreated controls.

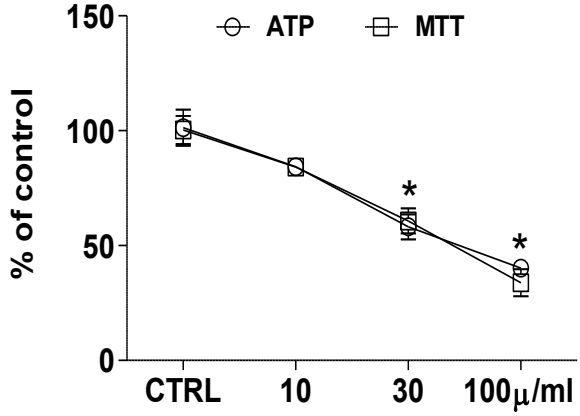


Figure 4.1. Confluent PC3 cells were stimulated by Poly E for 24 h with various concentrations. Cell viability and cell metabolic activity were assessed after treatments by ATP assay and MTT assay as described in "Materials and Methods". The results are expressed as percent of control. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

4.2 .Dose-dependent effect of Poly E on PC3 cell proliferation

Further investigation on the cytotoxicity of Poly-E on PC3 was conducted by using the BrdU assay. This method is based on the measurement of BrdU incorporation during DNA synthesis. Cells were treated with different concentrations of Poly E and cell proliferation was assessed after 24 h. As reported in figure 4.2, 24 hrs treatment of Poly E induced a dose-dependent decrease in the DNA synthesis of PC3. Consistent with data of previous experiments, this result demonstrated that Poly E is inhibiting the proliferation and inducing cell death in PC3 at both concentrations 30 and 100 μg/ml. Results are expressed as means ± SD.

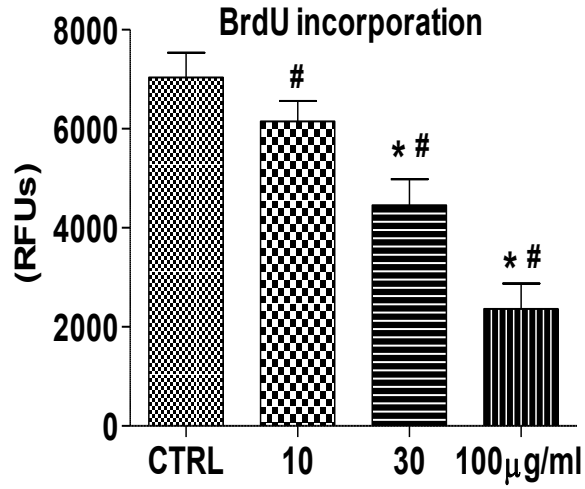


Figure 4.2. Confluents PC3 were treated with different concentrations of Poly E for 24 h. Cell proliferation was evaluated by using BrdU assay as reported as "Materials and Methods". Quantification of cell proliferation in cultured PC3 in the absence (CTRL) or presence of the indicated treatments. Poly E caused dose-dependent inhibition of proliferation of PC3. *Significantly different from the control, #significantly different from each other ($p < 0.05$).

4.3. Poly E induce apoptosis in prostate cancer cells

To determine whether the inhibition of PC3 cell proliferation by Poly E was due to the induction of apoptosis, we evaluated the rate of apoptotic cell

death by using the fluorimetric kit APOPercentage (Biocolor Ltd, Carrickfergus, UK).

PC3 cells were treated with different concentrations of Poly E for 24 h and then assessed for apoptosis. The figure 4.3 shown that Poly E was able to induce an increase in cellular apoptosis at concentrations of 30 and 100 μg/ml, while having no apoptotic effect at the concentration of 10 μg/ml, which is in agreement with the previous experiments on cell viability, cell metabolic and cell proliferation. The results are expressed as means ± SD.

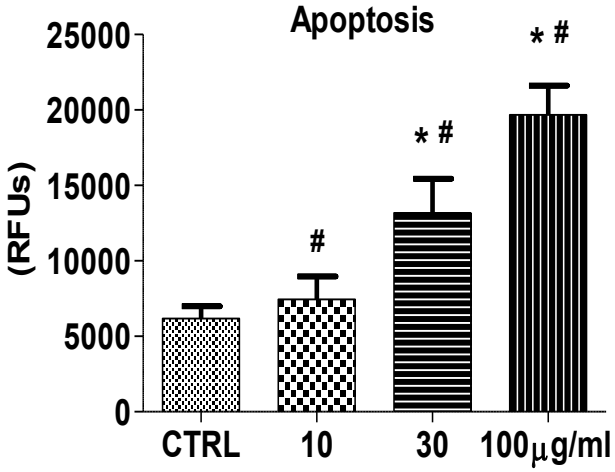


Figure 4.3. Confluent PC3 cells were stimulated for 24 h with the indicated concentrations. Apoptosis were assessed after treatments as reported in the ‘‘Materials and Methods’’. Quantification of apoptosis in cultured PC3 cells in the absence (CTRL) or presence of the indicated treatments. *Significantly different from the control, #significantly different from each other ($p < 0.05$).

4.4. Time-dependent effect of Poly E on PC3 cell proliferation and apoptosis

To further investigating the anti-proliferative effect of Poly E, we ran a set of time-dependent experiment a the concentration 30 μg/ml of Poly, whose among all the concentrations previously used, was the one that first

showed cytotoxic effects. Interestingly, Poly E induced a statistically significant decrease of proliferation and along with a concomitant increase in apoptosis at 48 h, as compared to 24 h (figure 4.4). The results are expressed as ratio treated and control.

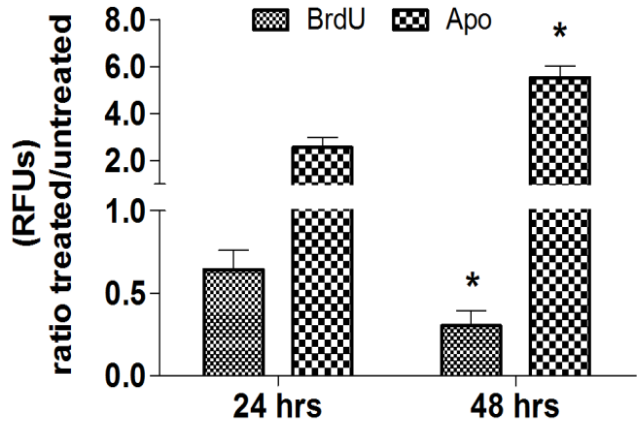


Figure 4.4. Confluent PC3 cells were stimulated with 30 µg/ml of Poly E for 24 h and 48 h. Cell proliferation was assessed by using BrdU assay, the apoptosis by the kit APO percentage as described in "Materials and Methods". The graphs represent the time-dependent effect of Poly E on PC3 cell proliferation and apoptosis. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

4.5. Dose and time-dependent effects of Poly E on PC3 ROS levels

To gain further mechanistic insight on the effect Poly E upon PC3 cell we assessed the potential variation of intracellular ROS levels. Indeed, ROS have been shown to be involved in regulation of both cell death and survival [123]. Intracellular ROS generation was examined in PC3 in response to Poly-E using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). This probe enters the cells and is oxidized in the presence of ROS, generating the fluorescent compound, DCF. To determine the effects of Poly E on PC3, cells were treated with the previously indicated concentrations and intracellular ROS levels were assessed after 2 h of stimulation, by a fluorescence detector. Figure 4.5 shown that ROS levels were significantly increased by the Poly E

treatment at dose of 30 and 100 μg/ml, while no antioxidant effect was observed at all the used concentrations. The observed pro-oxidant effect is consistent with the previously reported cytotoxicity results, suggesting the Poly-E-induced pro-oxidant effect as responsible for the reported PC3 death.

With the dose of 30 μg/ml, we next assess ROS generation with the intent to investigate a potential time-dependent effect of Poly E at 2 h, 6 h, 12 h. Figure 4.6 shown that ROS were increasingly generated after 12 h of Poly E treatment in PC3.

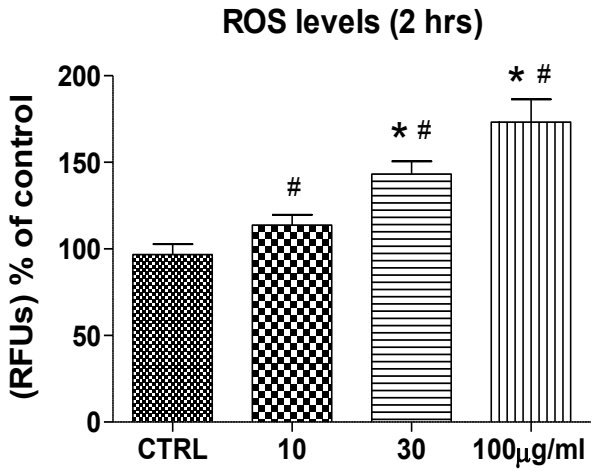


Figure 4.5. Confluent PC3 cells were stimulated with various concentrations of Poly E. ROS levels were assessed 2 h after treatment as described in the "Materials and Methods". Quantification of ROS levels in cultured PC3 in the absence (CTRL) or presence of the indicated treatments. The results are expressed as percent of control. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

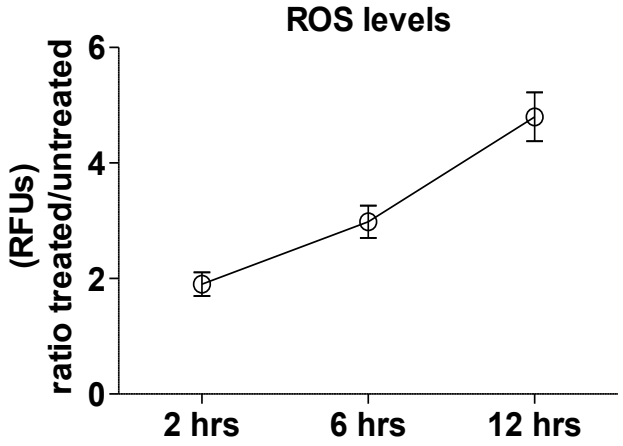


Figure 4.6. ROS leves were assessed after 2 h, 6 h and 12 h of treatment with 30 µg/ml of Poly E. The results are expressed as ratio treated and control.

4.6. Time-dependent effect of Poly E on PC3 protein carbonylation

To confirm the pro-oxidant effect of Poly E, we assessed variations of the protein carbonyl content in response to Poly E treatment. To this end, cells were treated with 30 µg/ml at different time points 2 h, 6 h and 12 h, then protein carbonylation was assessed. Protein carbonylation is the most widely used biomarker for proteins oxidative damage and reflects cellular damage induced by multiple forms of ROS [35].

Protein carbonyl groups were measured with the OxyBlot protein oxidation detection kit (Chemicon, Temecula, CA), following the protocol provided by the manufacturer.

Representative western blottings of protein carbonylation experiments are reported in figure 4.7. As shown in figures 4.8, the protein carbonylation pattern elicited by Poly E strictly overlapped that of ROS, strongly confirming the pro-oxidant effect exerted by Poly E at a concentration 30 µg/ml. This established the correlation between pro-oxidant activity and the anti-proliferative effect of Poly E in prostate cancer cells.

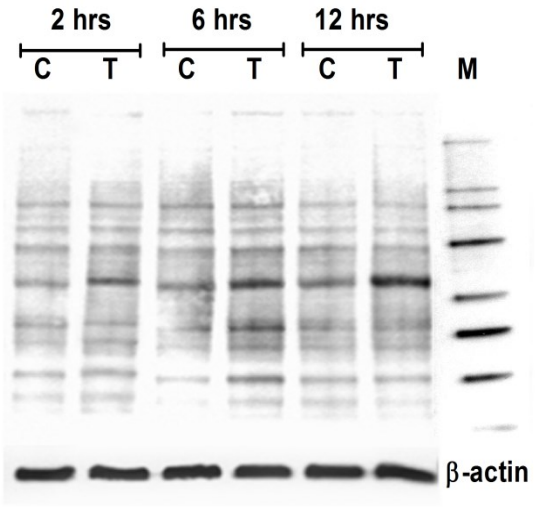


Figure 4.7. Representative western blottings of protein carbonylation experiments

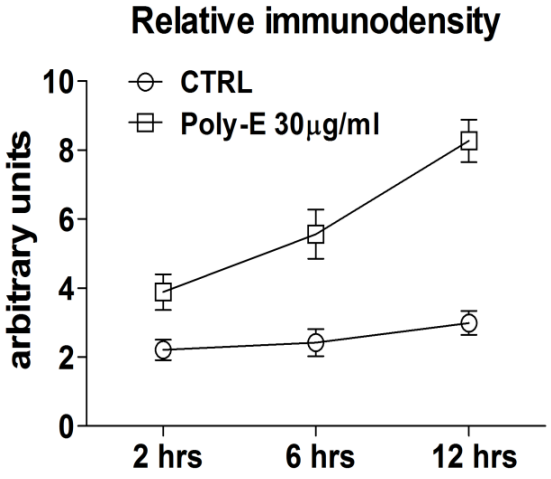


Figure 4.8. Measurement of protein carbonylation in cultured PC3 cells in the absence of Poly E (CTRL) or presence of Poly E 30 μg/ml. Protein carbonylation was assessed after treatments as reported in the ‘‘Materials and Methods’’. The graphs represent the immunodensity quantitative analysis of immunoblot experiment.

4.7. Poly E induced mitochondrial dysfunction-mediated cell death

Mitochondrial membrane potential (ΔΨm) is crucial for maintaining the physiological function of the respiratory chain to generate ATP. A significant reduction of ΔΨm induces cells depleted of energy with

consequently death. ROS are important signalling molecules, but excessive accumulation in pathological conditions leads to oxidative stress [64]. A pathology-associated rise of ROS can trigger mitochondrial membrane permeability promoting the dissipation of mitochondrial membrane potential (MMP) and ultimately cell death [96].

For this reason, we studied ΔΨm variation by evaluating the changes in fluorescence intensity of cells, stained with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). This is a fluorescent dye that accumulates in mitochondria in a MMP-dependent manner, showing red fluorescent JC-1 aggregates at higher MMP. When MMP decreases, JC-1 aggregates depart from mitochondria and change to green fluorescent JC-1 monomers. Therefore, the ratio of the red signal to the green can be used to detect the occurrence of MMP depolarization in the early stages of cell death due to mitochondrial damage. After treatment of Poly E for 12 h, PC3 were incubated with JC1 and analysed at the fluorometer. Results were calculated as the ratio (green/red) of fluorescence of sample, averaged after the fluorescence values had been corrected for the back-ground and protein content.

The figure 4.9 shown the relationship between red and green fluorescence emitted, demonstrating that Poly E induced mitochondrial dysfunction-mediated cells death. The decreasing of mitochondrial membrane potential suggests that Poly E-induced oxidative stress may cause mitochondrial damage and dysfunction in prostate cancer cells.

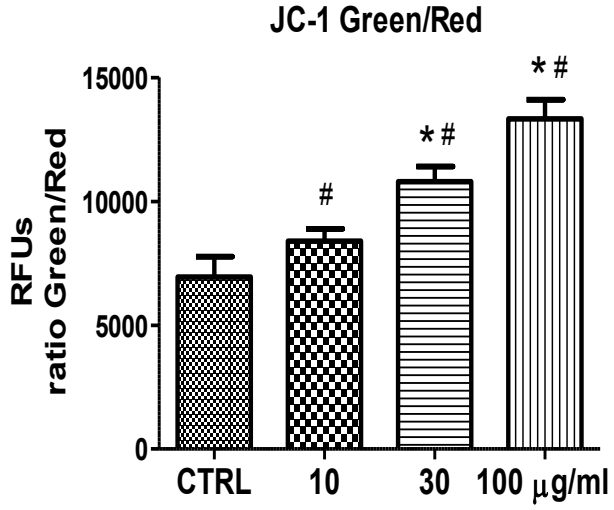


Figure 4.9. Mitochondrial dysfunction mediates Poly E-induced cell death. PC3 cells were stimulated for 12 h as indicated in figure, and MMP was assessed after treatments. The results are expressed as the ratio (green/red) of fluorescence. *Significantly different from the control, #significantly different from each other ($p< 0.05$).

4.8. Poly E downregulates Akt phosphorylation and upregulates ERK phosphorylation

To investigate potential intracellular signals involved in anti-proliferative effect of Poly E in PCa cells, we studied Akt and ERK p42/44. These signals are considered as central mediators in response to oxidant injury [44] and have critical role in the development of cancer including PCa [113].

In this study we examine whether Poly E, at a concentration able to affect ROS accumulation and cell function, could differentially influence ERK p42/44 and Akt.

Cultured cells were stimulated for 15 min, 30 min, 1 h, and 2 h with a Poly E concentration of 30 μg/ml and processed for Western blotting as described in Materials and Methods. We evaluated the effect of Poly E on the constitutive levels of protein expression of phospho-Akt, total Akt, phospho-

ERK, total ERK in PC3. As a result, a decrease of Akt phosphorylation was observed after 2 h of treatment, an effect consistent with the observed pro-oxidant damage. Meanwhile, protein expression levels for Akt were unchanged at all time points tested (figure 4.10).

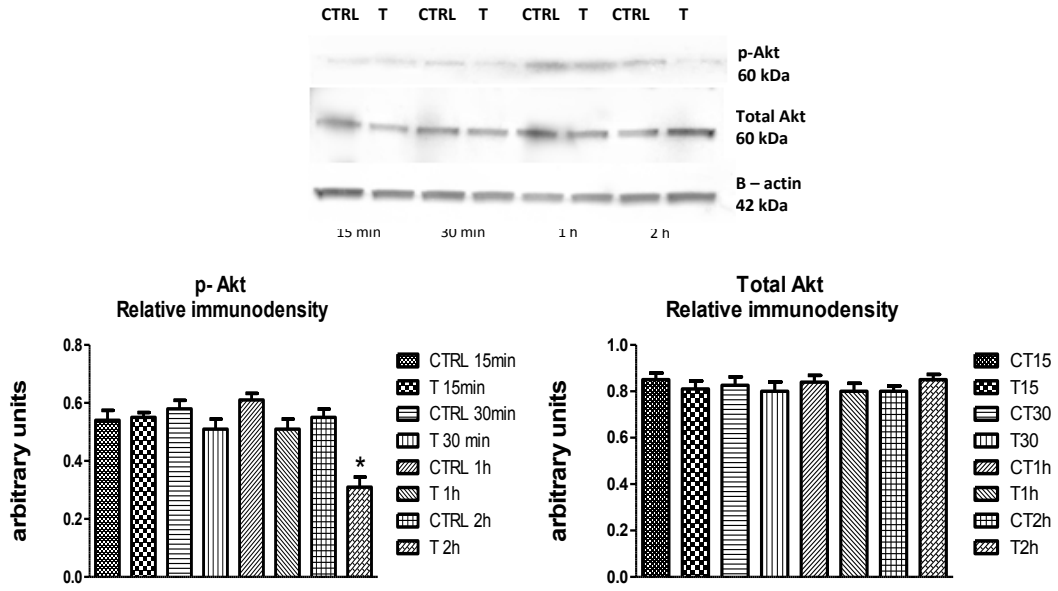


Figure 4.10. Confluent PC3 cells were stimulated with concentration 30 µg/ml of Poly E as indicated in figure and then processed for immunoblotting as described in the ‘‘Materials and Methods’’. Representative immunoblot of total and phospho-Akt (Ser473). The graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Poly E. Data are expressed as arbitrary units.*Significantly different from its own control.

Surprising, at the dose 30 µg/ ml, Poly E elicited a significant increase of ERK (p42/44MAPK) phosphorylation, which was evident at all time points observed (figure 4.11). Similar to total Akt, the protein expression levels for total ERK were unchanged statistically (figure 4.11).

Our data shown significant change of both Akt and ERK phosphorylation in response to Poly E, and suggest that Akt and ERK could be involved in the anti-cancer effects of Poly E.

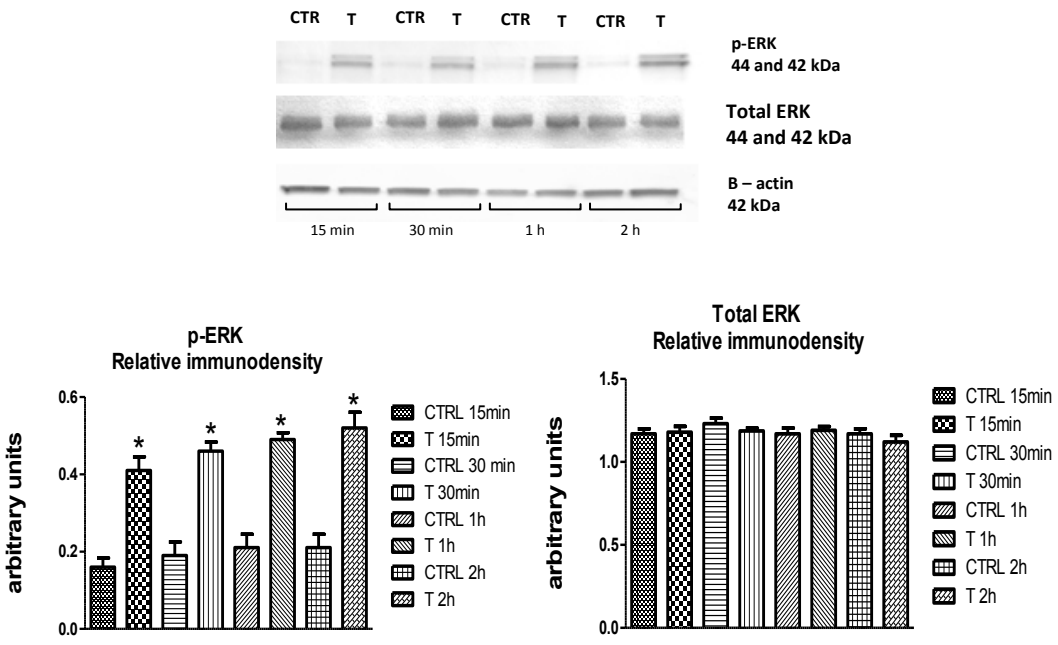


Figure 4.11. Confluent PC3 cells were stimulated with concentration 30 μg/ml of Poly E as indicated in figure and then processed for immunoblotting as described in the ‘‘Materials and Methods’’. Representative immunoblot of total and phosphop42/44ERK (Thr202/Tyr204). The graphs represent the immunodensity quantitative analysis of three different immunoblot experiments using Poly E. Data are expressed as arbitrary units.*Significantly different from its own control.

CHAPTER 5

DISCUSSION

Tea, next to water, is the most widely consumed beverage in the world. The tea plant *Camellia sinensis* has been cultivated in Asia for thousands of years, and Green tea has been used for centuries in China, Japan, and Thailand as a traditional medicine with a variety of applications [92]. Green tea possesses anti-carcinogenic effects, such as inhibition of growth proliferation, induction of apoptosis, induction of phase II detoxifying enzymes, and reduction of oxidative damage to DNA [92, 102]. Several studies have more specifically shown that consumption of green tea polyphenols is associated with decreased risk and/or slower progression of prostate cancer [63, 92].

Epidemiological evidence proposed that consuming 1-3 cups or more of green tea daily for greater than a year may decline the risk for prostate cancer incidence by nearly half [61]. Recent studies have indicated the presence of tea polyphenols in prostate tissue after consumption of this beverage [54].

Green tea polyphenols have shown also promise in inhibiting prostate cancer growth in several vivo studies. It has demonstrated that administration of green tea polyphenols (0.1% in drinking fluid) to transgenic adenocarcinoma of the mouse prostate (TRAMP) mice for 24 weeks markedly inhibits prostate cancer development and distant site metastases [1, 52].

EGCG, the most abundant catechin in green tea, has been shown to be the main effector of the anti-carcinogenic properties. For this reason, EGCG is the most commonly studied green tea catechins (GTCS). However, whole mixtures of GTCs may more accurately reflect the human consumption of green tea, possibly due to the fact that tea constituents other than catechins may also have anti-carcinogenic activity [34]. Poly E, a decaffeinated pharmaceutical preparation of tea catechins that contains approximately 50%

EGCG and 30% other catechins may be preferable to EGCG. The catechins in this mixture may exert synergistic effects [111].

In addition, the effects of Poly E on tumor cells and its mechanisms of action are poorly known [116]. In the present study, we examine the effects of Poly E on PC3 prostate cancer cells. Cell viability, cell metabolic activity and cell proliferation were evaluated by ATP, MTT and BrdU assay, respectively. The results demonstrate that Poly E is able to induce loss of both viability and inhibition of PC3 DNA synthesis in dose-dependent manner (figure 4.1 - 4.2). At concentration of 30 and 100 μg/ml, a cytotoxic effect is shown as judged by the low viability. These results are consistent with the observation that the concentrations of 30 and 100 μg/ml of Poly E caused apoptotic cell death (figure 4.3). This suggests that cell proliferation inhibitory effects can be due to apoptosis. Moreover, both cell proliferation and apoptosis, induced by Poly E were also evaluated in time-dependent manner. The cytotoxicity effect of Poly E is clearly evidenced by the strong decrease of cell proliferation and the dramatic increase in apoptosis for 48 h compare to 24 h at dose 30 μg/ml (figure 4.4). This proposes that apoptosis is a major mechanism for cell killing in the presence of Poly E. Similar effects of this compound were seen in studying the colon cancer, where Poly E preferentially inhibited growth of the Caco2, HCT116, HT29, SW480, and SW837 colon cancer cells compared to the FHC normal human fetal colon cell line [111]. It has been also demonstrated that Poly E inhibited proliferation of immortalized Barrett's cells as well as various adenocarcinoma cells by suppressing cyclin D1 expression through both transcriptional and post-translational mechanisms [116]. In others studies the anti-proliferative effects of green tea polyphenols, especially EGCG have been shown to be mediated by apoptosis [4, 51]. In the study of Gupta et al. [51] on LNCaP and DU145 prostate cancer cells, EGCG

was found to promote apoptosis as evidenced by DNA fragmentation at 40 μg/ml and 80 μg/ml. Chung et al. have also demonstrated that the induction of apoptosis by green tea catechins in DU145 prostate cancer cells [32].

While tea and other plant polyphenols are generally considered as antioxidants [91, 134], it is known that tea polyphenols also have pro-oxidant properties [11, 45, 71]. Commonly, green tea polyphenols have been shown to have strong antioxidant activity in vitro by their ability to quench free radical species and chelate transition metals. Studies in animal models and in human subjects have been less conclusive regarding the direct antioxidant effects of the tea polyphenols [71]. In this study, we examined the antioxidant effect of Poly E by measurement ROS levels. As a result, at all the tested concentration, Poly E did not exert any antioxidant effect. It may be possible that, only under certain circumstances, the direct antioxidant effects of tea polyphenols play a role in counteracting cancer. For example, in conditions of high oxidative stress (e.g., ulcerative colitis, hepatitis, etc.), tea polyphenols may be able to directly react with and scavenge free radicals [71]. Our data are similar to a previous study where a green tea extract and the EGCG exert oxidation and lack antioxidant activities on HL60 and RAW 264.7 cells [43]. The antioxidant effect of green tea polyphenols may also depend on certain of cell types.

The pro-oxidant effect of green tea polyphenols has been described in vitro. Under normal physiologic pH condition, EGCG may undergo auto-oxidation to form dimers, accompanying with the generation of ROS intermediates [56, 104]. It has also been reported that EGCG may induce the production of H_2O_2 in the culture media [138].

Inhibition of cancer cell viability and induction of apoptosis by green tea polyphenols in vitro appear to be, in part, due to the production of ROS

[43, 137]. One of these studies found that both a green tea extract and EGCG induced apoptosis in HL60 and RAW264.7 cells, and that in general tea extract was more effective [43]. Treatment of HL-60 cells with 50 μM EGCG caused the generation of ROS and a concomitant increase in apoptosis [43].

In our experiments, the pro-oxidant effect of Poly E was assessed by ROS generation. The results show that Poly E causes a rapid and significant ROS generation in PC3 cells at 2 h (figure 4.5) and ROS reached a maximum level at 12 h (figure 4.6). Interestingly, at apoptosis-inducing concentrations, Poly E causes ROS formation. ROS includes free radicals such as superoxide, hydroxyl radical, and non radical derivatives of oxygen such as hydrogen peroxide [84]. ROS are essential for normal cell function where they play key roles in regulating signal transduction events, enzyme activity, and cytokine production [128]. However, deregulation of redox homeostasis can cause cell damage, cancer initiation and therapy resistance [128, 133]. Cancer cells become more dependent on increased ROS levels and a highly functional antioxidant system than healthy cells, and as a consequence, they are more sensitive to agents that deteriorate antioxidant capacity or induce further oxidative stress levels [94]. When ROS reach a toxic threshold, they can trigger cancer cell death [125]. In our study, ROS level are increasingly generated and this is the evidence that Poly E elicited potential pro-oxidant effect at concentrations 30 and 100 μg/ml on PC3 cells.

Proteins are major targets for ROS and carbonyl groups are introduced into proteins by a variety of oxidative pathways. ROS can react directly with the protein or indirectly with molecules such as sugars and lipids, generating products (reactive carbonyl species, RCS) that react with the protein. Protein carbonylation is the biomarker for oxidative damage to proteins and reflects cellular damage induced by multiple forms of ROS [35]. For this reason, we

investigate protein carbonylation caused by Poly E. The results demonstrate that protein carbonylation exerted by Poly E overlap that of ROS (figure 4.7 - 4.8), strongly confirm the pro-oxidant effect of Poly E. This activity is consistent with observed cytotoxicity, thus establishing a correlation between pro-oxidant effect and the anti-proliferative activity of Poly E in prostate cancer cells. Our data support the fact that oxidative stress plays a role as a common mediator of apoptosis [22].

Apoptotic cell death may be triggered through the extrinsic (receptor-mediated) or the intrinsic (mitochondria-mediated) pathway. The intrinsic pathway can be triggered by many stimuli including ROS. Mitochondria are the major site of ROS production and accumulation of ROS may lead to the initiation of apoptosis [39]. There is growing evidence that altered mitochondrial function is associated with apoptosis induced by various stimuli [91, 93]. Decrease in the mitochondrial membrane potential, impairment of the mitochondrial respiratory chain, and depletion of ATP are characteristic consequences of oxidative stress [20]. Although the ways to promote apoptosis may be varied for different agents, the roles of ROS and mitochondria in many cases are thought to be crucial [25, 32, 120]. Thus, to elucidate the possible involvement of mitochondrial damage in Poly E-induced apoptosis, we evaluated mitochondrial transmembrane potential by using MMP assay with JC-1. Our results indicate that the decrease in mitochondrial transmembrane potential occurs 12 h after treatment of Poly E (figure 4.9). This suggests that Poly E-induced oxidative stress may cause mitochondrial damage and dysfunction in prostate cancer cells. These findings can comparable with other previous results, for example it has been proposed that mitochondrial dysfunction is the main mechanism involved in apoptotic cell death induced by EGCG treatment in B lymphoblastoid Ramos

cells [91]. Mitochondrial depolarization in DU145 cells might be related to green tea catechins-induced apoptosis [32].

In this study, a close correlation was observed for the levels of growth suppression, apoptosis induction, ROS formation and mitochondrial dysfunction in PC3cells treated with Poly E.

It has been suggested that the IGF (Insulin-Like Growth Factor) axis plays a relevant role in prostate cancer onset and development. Binding of IGF1 to its cognate receptor activates the intracellular tyrosine kinase domain, that produces phosphorylation of many protein substrates, including members of the MAPkinase cascade and PI3K/AKT. MAP kinases and the PI3K/AKT pathway are both involved in the complex modulation of signaling pathways, which regulates cellular processes like proliferation, survival/death, and motility, usually altered in carcinogenesis [37, 73].

Here, we also investigate the potential signals involved in the anti-proliferative effect of Poly E in prostate cancer cells.

Akt plays important roles in mammalian cell survival signaling and it has been shown to be activated in various tumors. Activated Akt promotes cell survival through the NF-kB signaling pathway and inhibits apoptosis through inactivation of several proapoptotic factors including Bad, Forkhead transcription factors, and caspase-9 [2]. Akt lies at the center of complex signalling pathways that incorporate a multitude of potentially oncogenic signals [76]. This kinase has been considered an attractive target for cancer prevention and treatment [2].

Here, we investigate whether activation of Akt protein is altered during Poly E-induced apoptosis. PC3 cells were stimulated with 30 µg/ml of Poly E at different point times 15 min, 30 min, 1 h, 2 h. The western blot analysis shows no change in the expression of total Akt in Poly E-treated PC3 cells.

However, Poly E decreased the level of phosphorylated Akt at 2 h post-treatment at the concentration that induce the cytotoxicity (30 µg/ml) (figure 4.10). The downregulation of Akt phosphorylation can be attributed to the inactivation of cell survival pathways, resulting in the induction of apoptosis in PC3 cells treated with Poly E. This observation is in accordance with other studies on PC3 cells. The study of Li et al. have shown the inhibition of phosphorylated Akt in genistein-treated PC3 cells [75]. Chinni et al. have also demonstrated that Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells [30]. In addition, several phytochemicals including diosgenin [112], green tea catechins [121] and curcumin [3] are known to suppress the activation of Akt [2].

It has been proposed that Akt may be an attractive therapeutic target in cancer therapy [36]. Our results indicated that Akt is a potential signal by which Poly E suppresses cell growth and triggers apoptosis in PC3 cells.

The ERK pathway is a member of family of MAPKs, which lying at the heart of many signal transduction processes. It constitutes a major pathway through which growth factor receptors transduce proliferative signals to the nucleus [70, 81]. ERK activation generally promotes survival of cells in response to ROS [44, 108]. Paradoxically, several studies using different model systems have produced findings to suggest that ERK activation can contribute to apoptosis in response to oxidant injury [81]. Once activated, ERK can initiate apoptotic enzymes or phosphorylate transcription factors that regulate the expression of pro-apoptotic genes [24].

In our study, Poly E leads to rapid elevation of the levels of phospho-ERK l. The upregulation of ERK phosphorylation was evident at all the tested time points 15 min, 30 min, 1 h, 2 h (figure 4.11). Our data are consistent with growing number of studies that have shown activation of ERK by

chemopreventive compounds. This activity results in anti-proliferative effects such as apoptosis, senescence, or autophagy in cancer cells [68, 100, 109, 110].

Our data indicated that Poly E inhibits phospho-Akt and activates phospho-ERK in PC3 cells. These results are in accordance with several studies of EGCG-modulated signalings in prostate cancer cells. Siddiqui et al. [113] found that EGCG is able to decrease PI3K and phospho-Akt levels and increase ERK1/2 level in both DU145 and LNCaP cells. Our study suggests a similar potential mechanism at the basis of the anti-proliferative effects of Poly E in prostate cancer cells. Similarly, treatment of PC3 cells with EGCG induced activation of the ERK1/2 pathway, suggesting that ERK1/2 activation is partially responsible for the anti-proliferative EGCG effects in PC3 cells [6].

CONCLUSION

Summary these data clearly demonstrate that treatment of PCa cells with 30 and 100 μg/ml Poly E significantly decreased cell viability and proliferation, while increasing apoptosis.

At all the tested concentrations, Poly E did not exert any antioxidant effect, eliciting instead a pro-oxidant effect at concentrations 30 and 100 μg/ml in PCa cells.

The pro-oxidant effect of Poly E is consistent with the observed cytotoxicity, thus establishing a correlation between pro-oxidant activity and the anti-proliferative effect of Poly E in PCa cells.

Poly E induced mitochondrial dysfunction-mediated cells death in PCa cells.

Akt and ERK are potential intracellular signals involved in the anti-proliferative effect of Poly E in PCa cells.

Collectively, our data show the anti-proliferative effect of Poly E in PCa cells and delineated the potential signaling pattern involved in this activity.

However, further investigation will be necessary to better elucidate the molecular mechanisms at the basis of the anticancer effect of Poly E.

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