UNIVERSITÀ DEGLI STUDI DI ROMA "TOR VERGATA"

FACOLTÀ DI SCIENZE MATEMATICHE FISICHE E NATURALI

DOTTORATO DI RICERCA IN
BIOLOGIA CELLULARE E MOLECOLARE

XXII CICLO

Pro-apoptotic role of AMP-activated protein kinase under oxidative conditions linked to bioenergetic impairment: implications for cancer treatment and neurodegeneration

Simone Cardaci

A.A. 2009/2010

Docente Guida
Prof. Maria Rosa Ciriolo

Coordinatore
Prof. Giovanni Cesareni

INDEX

4

ABSTRACT

The imbalance between ROS production and clearance leads to oxidative stress, a condition implicated in the pathogenesis of several cell disorders such as neurodegeneration and cancer. In these settings, pro-oxidant conditions activate many redox-sensitive proteins involved in the regulation of the apoptotic program. Among them, the tumor suppressor p53 and the mitogen-activated protein kinases (MAPK) are implicated both in the pathogenesis of neurodegenerative disorders and in many chemotherapeutic strategies aimed to the elimination of cancer-prone cells from the replicative pool. Recently, AMP-activated protein kinase (AMPK) has been identified as a component of the signaling cascade able to sense bioenergetic and oxidative challenges. Moreover, its capability to trigger apoptosis by activating p53 or some members of the MAPK family (e.g. $p38^{MAPK}$), makes it a putative player both in neurodegenerative disease and in cancer management.

On the basis of this knowledge, this PhD thesis is aimed to elucidate the pro-apoptotic properties of AMPK in SH-SY5Y neuronal cell line under pro-oxidant conditions associated to bioenergetic impairment. This research has been performed by using three compounds known to affect cellular redox homeostas: Bis[(2-oxindol-3-ylimino)-2-(2-aminoethyl)pyridine-N,N']copper(II) perchlorate ($Cu(isaepy)_2$), tetrahydrobiopterin (BH4) and sodium nitroprusside (SNP). Our experiments demonstrated that $Cu(isaepy)_2$, by acting as a DLC-like molecule, was able to induce cell death by activating the AMPK/ $p38^{MAPK}$/p53 signaling axis, in response to a mitochondrial impairment. The cross-talk between these proteins was found to be operative also in SH-SY5Y cell death induced by BH4, an obligatory cofactor for tyrosine hydroxylase in dopamine synthesis, and to be sensitive to the alteration of glycolytic metabolism. Finally the observation that the iron-nytrosil complex SNP was unable to affect cellular energetics, explains on the one hand the uneffectivenes of AMPK in mediating SNP-induced apoptosis and, on the other, suggests a possible role for this energy-responsive kinase in apoptosis engagement only under condition of bioenergetic stress.

INTRODUCTION

Redox Biology

Reactive oxygen species

Aerobic organisms require molecular oxygen (O_2) for vital cellular processes. As consequence of respiration and enzymatic activities, cells can generate partially reduced forms of O_2 collectively referred to as "reactive oxygen species" (ROS). They include two types of reactive molecules:

1) free radicals, which contain one unpaired electron in their outer molecular orbitals, such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$);

2) non-radical ROS, which do not have unpaired electron(s) but are chemically reactive and can be converted to radical species, such as hydrogen peroxide (H_2O_2).

In living systems, ROS production can occur both under physiological conditions and in response to several noxious stimuli in different subcellular compartments such as mitochondria, endoplasmic reticulum, cytosol and cell membrane. The major intracellular source of ROS is represented by the mitochondrial respiratory chain, the principal site of energy production. Within the electron transport chain, electrons are passed through a series of proteins (mitochondrial complexes) via redox reactions to the cytochrome *c* oxidase at the level of which the tetravalent reduction of O_2 to H_2O_2 occurs. During this metabolic process 1–3% of all electrons "leak" prematurely, generating superoxide *via* one-electron reduction of oxygen, mainly at level of complex I and ubisemiquinone site of complex III. Although not particularly reactive towards organic molecules and diffusible across biologic membranes, $O_2^{\cdot-}$ can be spontaneously or enzymatically dismutated to form H_2O_2, that, in the presence of divalent metal catalysts (*e.g.,* iron), can generate the high reactive and harmful $OH^{\cdot}$ which is able to oxidize irreversibly all the biological macromolecules (Valko et al., 2007).

In addition to mitochondrial production, ROS can arise as the product of enzymatic reactions. NADPH oxidases, also known as NOX proteins, catalyze the one-electron reduction of O_2 to produce $O_2^{\cdot-}$, which is rapidly converted to H_2O_2. Although it mainly localizes at the plasmalemma of phagocytic cells, such as macrophages, to produce ROS as part of the innate immune response, a family of NADPH oxidases has now been identified in non-phagocytic cells, including vascular tissue, which produce ROS in a regulated manner at lower levels than in phagocytes, presumably for

signaling responses to physiological stimuli. Moreover, ROS can also arise as by-product of several metabolic processes, such as during β-oxidation within the peroxisomes, prostaglandin and leukotrienes synthesis by cyclooxygenase and 5-lipoxygenase respectively or detoxification reactions of xenobiotics and drugs, mainly catalyzed by cytochrome P450 in endoplasmic reticulum (Nordberg and Arnér, 2001; Kontos *et al.*, 1985).

Besides ROS, cells are also continuosly exposed to reactive nitrogen species (RNS), such as peroxynitrite (ONOO–), generated by the oxidation of nitric oxide (NO), a gas synthesized from L-arginine in mammals by a family of enzymes known as nitric oxide synthases (NOSs).

Oxidative damages to biomelecules

When produced at elevated concentrations, ROS can cause the progressive modification of cellular biochemicals, as direct consequence of their chemical reactivity, impairing cell functions and eventually induce cell death (Valko et al., 2006).

Damages to membranes. Lipid peroxidation, the most relevant oxidative damage affecting cell membranes, refers to the addition of oxygen to unsaturated fatty acids to form, organic hydroperoxides (ROOH) *via* a free radical chain reaction mechanism. Besides leading biophysical changes that disrupt membrane integrity and organelles function, lipid peroxides can in turn give rise to toxic metabolites such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), able to stimulate cellular signaling pathways generally associated with the promotion of cell death (Wang et al., 1996; Siems at al., 1995).

Damages to proteins. ROS can carbonylate aminoacid residues side chains, produce protein-protein cross-links, and oxidize protein backbones, resulting in protein fragmentation. Protein carbonylation is considered the main ROS-mediated irreversible protein oxidation and a widespread indicator of severe oxidative damage and disease-derived protein dysfunction. In fact, whereas moderately carbonylated proteins are degraded by the proteasomal system, heavily carbonylated proteins are prone to generate high-molecular-weight aggregates. It has been indicated that such aggregates are resistant to degradation and accumulate as damaged or unfolded proteins, thus eliciting pro-apoptotic pathways (Stadtman, 2004).

Damages to nucleic acids. ROS, mainly OH·, are known to react with all components of DNA, damaging both the purine and pyrimidine bases and the deoxyribose backbone as well (Halliwell and Gutteridge, 1999), thus irreversibly inhibiting DNA replication and transcription. The major oxidative DNA lesion is the formation of 8-oxo-2'deoxyguanosine (8-OH-G), which is found to be recurring in several neurodegenerative disease such as Parkinson's Disease and Amiotrofic Lateral Sclerosis, where down-regulation of its repairing enzyme 8-oxoguanine glycosylase 1 (OGG1) occurs.

Although its oxidation and the derived biological effects are less studied, RNA may be more susceptible to oxidative insults than DNA. In fact the RNA single-strand structure, not protected by hydrogen bonding and less surrounded by specific proteins, exposes more sites to ROS than DNA; moreover RNA is less compartmentalized than nuclear or mitochondrial genome, but distributed broadly in cell. For these reasons, oxidative modifications have been detected not only in protein-coding RNAs but also in structural and regulatory RNAs causing on the errors in proteins synthesis, as well as alterations of gene expression (Valko et al., 2007).

Likewise ROS, RNS produced at high concentrations, can cause cellular damages through a phenomenon known as nitrosative stress. In particular, NO has been shown to modify protein structure and function by nitrosylation and nitrotyrosination. The former is a reversible reaction of NO with the sulfur moiety of cysteine to form nitrosothiols on interacting proteins; the latter is the irreversible reaction of tyrosine residues with ONOO⁻ to form 3-nitrotyrosine. Moreover, RNS contribute to glutamate excitotoxicity, participate in organelle fragmentation, mobilize iron from internal stores and exacerbate ROS-mediated cellular damages and ultimately induce cell death (Knott and Bossy-Wetzel, 2009).

Mechanisms of antioxidant defence

Exposure to ROS leds organisms to develop a series of defence mechanisms aimed at preventing oxidative damages and at repairing oxidatively modified cellular structures.

The first level of defence is represented by enzymatic antioxidants, which include highly efficient radical scavenging proteins such as superoxide dismutase, catalase and glutathione peroxidase.

Superoxide dismutase. Superoxide dismutase (SOD), the first identified antioxidant enzyme, efficiently catalyze superoxide radical dismutation (K_{cat} ~ 10^9 sec^{-1}M^{-1}) producing hydrogen peroxide and molecular oxygen:

$$2 \, O_2^{\bullet -} + 2 \, H^+ \rightarrow H_2O_2 + O_2$$
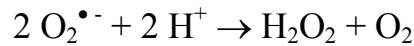
This metalloenzyme is present in all eukaryotic cells in tree isoforms: (i) copper/zinc containing isoform (Cu/Zn-SOD or SOD1), localized in cytosol and mitochondrial intermembrane space (Liou *et al*, 1993); (ii) manganese-dependent enzyme (Mn-SOD or SOD2), exclusively localized in mitochondrial matrix (Barra *et al*, 1984); (iii) extracellular SOD (EC-SOD or SOD3), expressed on cell surface only by some cell types such as fibroblasts and endothelial cells (Marklund, 1982).

Catalase. Catalase is a tetrameric eme-containing oxidoreductase which catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen:

$$2 \, H_2O_2 \rightarrow 2 \, H_2O + O_2$$
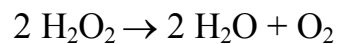
This enzyme is mainly localized in peroxisomes in which the breakdown of fatty acid molecules (β-oxidation) occurs, producing H_2O_2 as byproduct (Kirkman et al., 1997).

Glutathione peroxidase. Glutathione peroxidase (GPx) is the general name of the selenium-containing enzyme family with peroxidase activity whose main biological role is to reduce lipid hydroperoxides and hydrogen peroxide to their corresponding alcohols:

$$ROOH + 2 \, GSH \rightarrow ROH + GSSG + H_2O$$

This family comprises several isoenzymes encoded by different genes, which vary in celullar location and substrate specificity, but share reduced glutathione (GSH) as the same electron donor (Saito and Takashi, 2002). Therefore, GPx activity depend on intracellular GSH availability, whose oxidized form, glutathione disulfide (GSSG), is reduced back to the sulfhydryl form by the NADPH-dependent glutathione reductase (GR):

$$GSSG + NADPH + H^+ \rightarrow 2 \, GSH + NADP^+$$

Although peroxide detoxification is efficiently achieved by GPx/GR systems, cells also control peroxide levels by means of peroxiredoxins (Prxs), a family of redundant and abundant redox-active cysteine containing oxidoreductases (Hall A, 2009).

Besides being protected against oxidative insults by an interacting network of antioxidant enzymes, cells are equipped with several non-enzimatic antioxidants that scavenge oxyradical species or neutralize their reactivity. According to their solubility, they are classified in hydrophobic and hydrophilic antioxidants. In general, lipid-soluble antioxidants, such as tocopherols and tocotrienols, protect cell membranes from lipid peroxidation (Urano et al., 1992), by removing the free radical intermediates and preventing the propagation chain reaction. Converserly, water-soluble antioxidants, such as ascorbic acid and glutathione, react with oxidants, mainly H_2O_2, in the cytosol and nucleus. Moreover, according to their structure and chemical reactivity, non-enzymatic antioxidants are divided into two classes: (i) low-molecular weight compounds with an aromatic structure or conjugated dienes, able to quench radical reactions thus becoming stable and unreactive radicals (i.e. tocopherol, tocotrienols, polifenols and carotenoids) (Fukuzawa et al., 1998); (ii) thiol compounds, such as glutathione and thioredoxin (Trx), able to act as donors of reducing equivalents, either directly or in concert with specific enzymes, forming disulfides. Among them, GSH plays a pivotal role as the main thiol antioxidant and redox buffer of the cell (Schafer and Buettner, 2001). It is synthesized in the cytosol by the sequential action of glutamate-cysteine ligase (γ-GCL) and glutathione synthetase (GS) and it is present in the reduced (GSH) and two possible oxidized forms: "classic disulfide", formed by the joining of two GSH molecules by means of disulfide bridge or mixed-disulfide, between GSH and protein cysteines. Both oxidized forms can be reduced back to GSH by glutatione reductase and the tioredoxin/tioredoxin reductase enzymatic system respectively. By means of these efficient enzymes, the reduced form of glutathione is phisiologycally 10-1000 fold higher than the oxidized one. Differently, under pro-oxidant conditions, oxidized glutathione accumulates inside the cells and the ratio of GSH/GSSG decreases. In response to these conditions, cells are able to maintain GSH concentration by several mechanisms: (i) stimulating GR activity; (ii) increasing GSH synthesis; (iii) forming mixed disulfides; (iv) extruding GSSG (Filomeni et al., 2005).

Cellular "redox status"

The presence of several efficient systems aimed to maintain GSH redox status highlights the pivotal role of this compound in cell homeostasis. In fact, besides detoxifying oxyradicals species, xenobiotic compounds and regenerating some antioxidants back to their active forms (i.e. vitamins C and E), glutathione is the major determinant of the redox state of a cell. Free radicals and reactive diamagnetic species derived from radicals operate at low, but measurable concentrations in the cells. Their "steady state" concentrations are determined by the balance between their rates of production and their rates of removal by various antioxidants. Thus each cell is characterized by a particular concentration of electrons ("redox status") stored in an electronic network, resulting from the contribution of the redox state of each redox couple linked together (Filomeni et al., 2005). Glutathione (GSSG/GSH), nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH), and thioredoxin [TrxSS/Trx(SH)$_2$] redox couples are among the most important in maintaining an intracellular reducing environment. Since the GSH concentration is 100- to 10000-fold higher than the reduced form of the other couples (1-100 mM), the glutathione redox state usually determines the steady-state value of the intracellular redox potential. Changes in the cellular redox environment can alter signal transduction, DNA, RNA and protein synthesis, enzyme activation, and even regulation of the cell cycle. Indeed a high reducing environment has been shown to stimulate cell proliferation, a moderately oxidising state initiates cell differentiation, whereas further shifts towards more oxidising conditions leads to cell death. For example, pro-oxidants such as certain arachidonic acid metabolites, lipid hydroperoxides, redox cycling compounds (i.e. quinones and reactive aldehydes), increase the intracellular concentration of ROS that can induce apoptosis. Antioxidants that serve as reducing agents, such as N-acetyl-cysteine, GSH, and thiol-containing proteins (e.g., thioredoxin), have been shown to prevent apoptosis. Thus, the redox environment of the cell and its fluctuations strongly operates in determining cellular functioning (Schafer and Buettner, 2001).

Role of protein sulfhydryls in the cellular redox status

Several proteins contain free sulfhydryl groups (PSH) and their concentration, in cells and tissues, is much greater than that of GSH. These

groups can be present as thiols (-SH), disulfides (PS-SP), or as mixed disulfides, for example PS-SG when conjugated with GSH. PSH can bind GSH, cysteine, homocysteine, and γ-glutamylcysteine to form mixed disulfides, but GSH is the dominant ligand (Seres et al., 1996). The oxidation of the thiol form or the reduction of the disulfide form of an enzyme can result in the modulation of the enzyme function (Watanabe et al., 1972; Ernest and Kim, 1973). Protein S-thiolation/dethiolation is a dynamic process that occurs under physiological conditions in cells. It is reversible, but occurs at different rates depending on the protein and the nature of its thiol groups. The protein S-glutathiolation status should be, in some way, a reflection of the redox state of the GSH buffer-system in the cell. Indeed, the oxidation of protein sulfhydryls to mixed disulfides is an early cellular response to oxidative stress (Thomas *et al.*, 1995), which can take place in a number of different ways:

i) two-electron oxidation of a protein-thiol, followed by reaction with GSH

$$PSOH + GSH \rightarrow PS\text{-}SG + H_2O$$
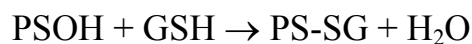
ii) one-electron oxidation of PSH or GSH followed by the formation of a disulfide

$$PS^\bullet + GS^- + O_2 \rightarrow PS\text{-}SG + O_2^{\bullet-}$$

$$PS^- + GS^\bullet + O_2 \rightarrow PS\text{-}SG + O_2^{\bullet-}$$

iii) thiol/ disulfide exchange reactions

$$PSH + GSSG \rightarrow PS\text{-}SG + GSH$$

$$P_1SH + P_2S\text{-}SG \rightarrow P_1S\text{-}SP_2 + GSH$$

$$P_1SH + P_2S\text{-}SG \rightarrow P_1S\text{-}SG + P_2SH$$
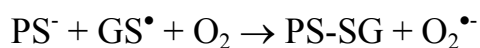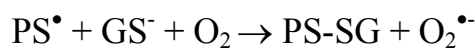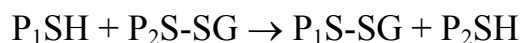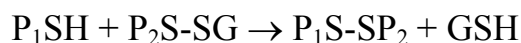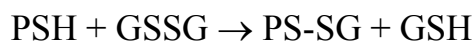
These reactions demonstrate that protein thiols can play a pivotal role in antioxidant defence and, in such a way, modulate the intracellular redox environment. Indeed, as demonstrated by the first two thiol/disulfide exchange reactions, these chemical processes are able both to restore intracellular GSH content and to remove GSSG. Moreover, thiol/disulfide exchange prevents further oxidative reactions and allows GSH content to be

preserved, maintaining the reducing power of its redox system unchanged. Once the oxidative insults have been met and the restoration of an appropriate GSH/GSSG ratio is ongoing, protein sulfhydryls can be returned to the cellular thiol pool by the reaction of GSH with PS-SG:

$$PS\text{-}SG + GSH \rightarrow PSH + GSSG$$
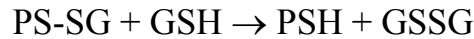
and GSH will be regenerated from GSSG through the GR.

Redox status in cell death process

Necrosis and Apoptosis

In the last 30 years, two major distinct types of cell death, necrosis and apoptosis, have been delineated according to cellular, morphological, and biochemical characteristics. Necrosis, an extensive degenerative process resulting from acute and non physiological injury, is associated with membrane damage and leakage of cell constituents into the extracellular space, that may lead to local inflammation and damage to the surrounding tissues (Searle et al., 1982). On the other hand, apoptosis represents a form of cell death that requires a regulated or programmed sequence of events which include: (i) appearance of an appropriate stimulus; (ii) activation of specific proteases (caspases) and nucleases responsible for cell shrinkage and DNA fragmentation; (iii) phagocytic removal of membrane-bound apoptotic bodies (Parone et al., 2002; Wolf et al., 1999). These tight regulations make apoptosis a critical regulator of development and tissue homeostasis of multicellular organism and allow to understand how its alteration can lead to uncontrolled proliferative conditions, such as cancer, or enhanced cell demise, as observed in neurodegenerative disorders.

Cells can initiate the apoptotic process by three known different but interconnected pathways. The first, is activated by cytotoxic T Lymphocytes, when exposed to infected/dysfunctional somatic cells. In this case, the apoptotic program is engaged by the perforin-dependent plasmamembrane alterations allowing granzymes B, a type of serine proteases, to enter the target cell and activate effector caspases (caspase-3, -6, and -7) (Shresta et al., 1998).

The second apoptotic program, called "extrinsic" pathway, refers to the initiation and propagation of apoptotic signaling in response to activation of

cell surface receptors, such as members of the tumor-necrosis factor receptor (TNF-R) superfamily of death receptors. This pathway initiates when a death ligand, such as the Fas ligand (FasL) or tumor necrosis factor alpha (TNF-α), interacts with its corresponding cell surface receptor such as Fas/APO-1/CD95, or the tumor necrosis factor receptor (TNF-R1/2). Receptor activation triggers its oligomerization, and the rapid recruitment of adaptors such as FADD (Fas-associated death domain protein) or TRADD (TNF-R1-associated death domain), and pro-caspase-8 to its cytoplasmic death domain to form a death-inducing signal complex (DISC). The recruitment of pro-caspase-8 to the DISC followed by its activation elicits a series of downstream events, leading to the subsequent cleavage of caspase-3 and multiple caspase substrates, responsible for cell death (Nagata, 1999). The extrinsic pathway can be amplified by the caspase-8 cleavage of Bid, a pro-apoptotic member of Bcl-2 familiy, into truncated Bid which facilitates Bax activation leading to mitochondrial damage and release of apoptogenic factors (Brustovetsky et al., 2003).

The third apoptotic program, called "intrinsic" or "mitochondrial" pathway, is engaged in response to a broad spectrum of environmental and chemical stress agents including ionizing and UV radiations, hypoxic and oxidative conditions. It initiates with the mitochondrial translocation of proapoptotic Bcl-2 family proteins (*i.e.,* Bax/Bak) in response to proapoptotic stimuli. Upon incorporation into the outher mitochondrial membrane by their carboxyl-terminus signal-anchor, Bax/Bak oligomerize to form pores that alter mitochondrial membrane permeability (MMP) facilitating the release of cytochrome *c* and several additional apoptogenic factors from the mitochondrial inner membrane space. Once released, cytochrome-*c* binds to apoptotic protease activating factor-1 (Apaf-1) and dATP in the cytosol to form the so called "apoptosome" complex, which binds and activates pro-caspase-9 through mutual caspase recruitment domain (CARD) interactions. The activated initiator caspase-9 in turn activates downstream effector caspases-3/-7, which act upon multiple substrates, whose degradation can account for apoptotic changes (Yang et al., 1997). Besides cytochrome-c, the opening of the mitochondrial permeability transition (MPT) pore, allows two groups of pro-apoptotic factors, normally sequestered from the intermembrane space, to be released into the cytosol. The first group consists of, Smac/DIABLO, and the serine protease HtrA2/Omi, which are reported to promote apoptosis by inhibiting IAP (inhibitors of apoptosis proteins) activity. The second group includes AIF, endonuclease G and CAD, involved in frangmentation of DNA and chromatin condensation. The
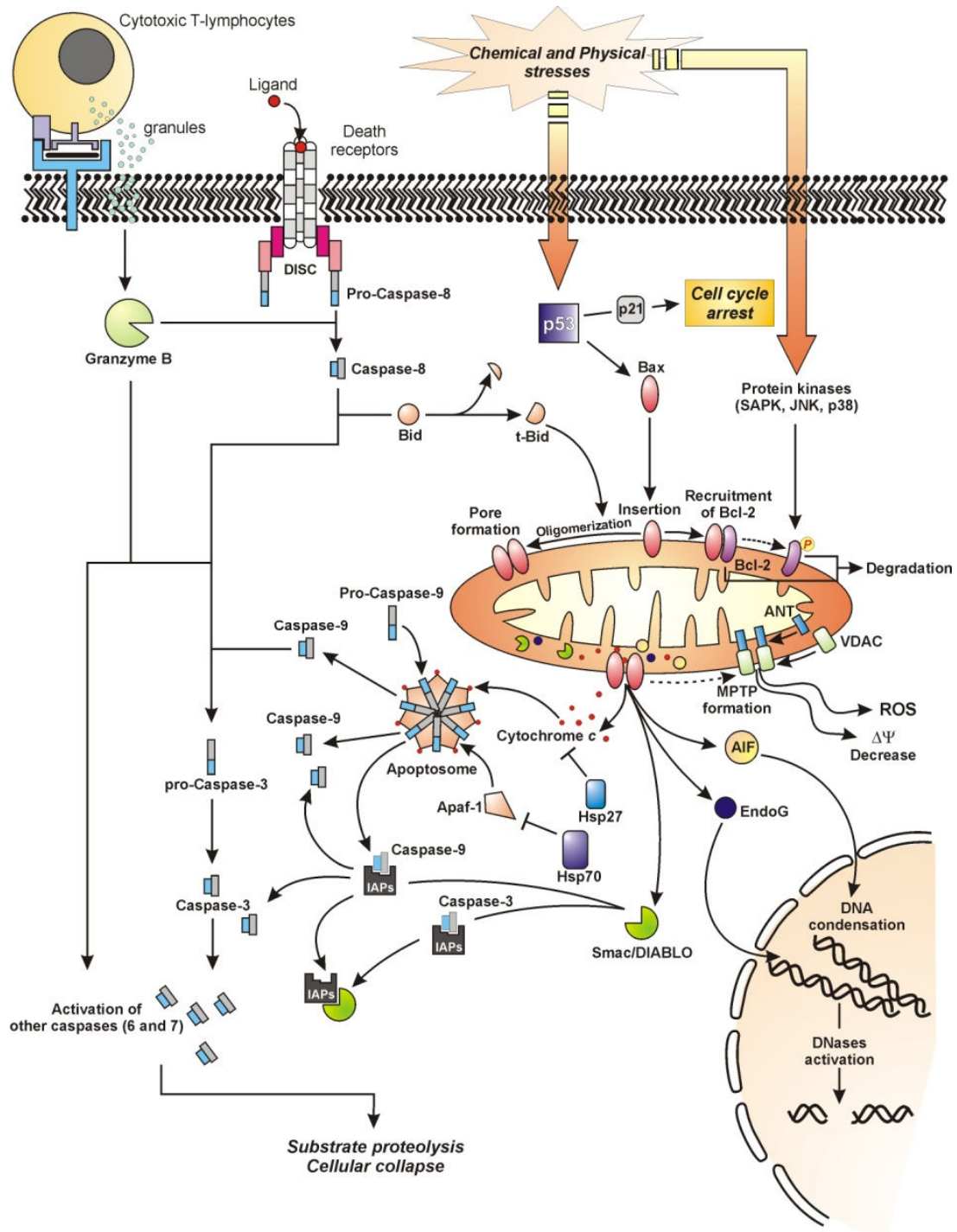
Figure 1
Principal apoptotic pathways (modified from Rossi et al., 2004)

extrinsic and intrinsic pathways both converge at the point of the execution phase, considered the final stage of apoptosis. It involves the activation of the executioner caspases, such as caspase-3, caspase-6, and caspase-7, which activate endonucleases, responsible for DNA fragmentation, and proteases that degrade the nuclear and cytoskeletal proteins. Among the executioner caspases, caspase-3 is considered to be the most important and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). It specifically activates the endonuclease CAD. In proliferating cells, CAD is bound to its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD which then degrades chromosomal DNA and causes chromatin condensation, which is a distinctive feature of apoptotic morphology (Elmore S., 2007).

The involvement of redox status in cell death process has been widely demonstrated. In fact, if reducing environment are known to stimulate cell proliferation and differentiation, oxidizing conditions can result in apoptosis or necrosis. In particular whereas apoptosis may occur with lethal but moderate oxidative stimuli, necrosis would result from severe oxidative challenges (Lee et al., 1999). This is in line with the different energy requirements of these processes. In fact, apoptosis requires energy in the form of ATP to carry out the organized program of cell death (Richter et al., 1996), therefore, a severe oxidative stress would deplete energy stores and damage the ATP generating machinery needed to implement the structural changes associated with apoptosis. However, besides being a stimulus for apoptotic induction, growing evidences demonstrate that ROS may actively participate in apoptotic signaling by modulating the activity of specific redox-sensitive proteins, such as transcription factors, or by acting as "second messengers", eliciting phopshorylative signaling, such as MAPK pathway engagement. Both mechanisms are referred to "redox signaling" because the signal is delivered through redox chemistry.

Redox-regulated transcription factors: role of p53 in apoptotic outcome

A way through which ROS can modulate signal transduction is the oxidation of specific cysteine residues, called "reactive cysteines", because, at neutral pH, they exist as thiolate anions due to charge interactions with neighboring aminoacid residues. Depending on the type of oxidant and the chemical microenvironment where these residues are localized, they can be oxidized to sulfenic acids, disulfide as well as more highly oxidized states,

such as sulfinic and sulfonic acid. These oxidative modifications act as nano-switches, influencing the structure of the proteins leading to a "gain" or "loss" of their function (Schafer and Buettner, 2001).

Several pieces of evidence demonstrate that many transcription factors regulate gene expression only in their reduced status, and their nuclear translocation is often dependent on redox changes of their reactive cysteines. OxyR is the first redox-sensitive transcription factors identified in prokaryotes. Pioneering studies by Storz and co-workers show that, upon exposure to H_2O_2, the activation of OxyR involves the formation of a disulfide bond between Cys199 and Cys208, two residues that are separated by 17Å in the reduced – and inactive – form of the protein. The net outcome of this reaction is a conformational change of protein structure responsible for the enhanced DNA binding specificity and the recruitment of RNA polymerase (Zheng et al., 1998). Following studies from the Stamler laboratory pointed out that, besides this 'on–off' modulation, a finer regulation for OxyR binding to DNA can occur. Only Cys199 is critical for OxyR activity and this residue can be differently oxidized depending on the extent of redox unbalance and the nature of the oxidizing molecules. Cysteine modification by *S*-nitrosylation (S-NO), *S*-glutathiolation (S-SG), intramolecular disulfide bond (S-S) formation, or oxidation to sulfenic acid (S-OH, *S*-hydroxylation) may be alternatively exploited to control gene expression (Kim et al., 2002).

While the response of prokaryotes to oxidative stress depends essentially on the "duration" or of exposure, eukaryotes have evolved a spatial regulation of transcription factors activity. A good example of redox-dependent spatial regulation of enzymatic activity is represented by the nuclear factor erythroid 2-related factor 2 (Nrf2). It is a transcription factor responsible for the transcriptional activation of the antioxidant responsive element (ARE)-driven genes, which codify for phase II detoxification enzymes and antioxidant proteins such as, glutathione S-transferases, and glutamate-cysteine ligase (Eggler et al., 2008). In resting conditions, Nrf2 is retained in the cytoplasm through its interaction with Keap1 which prevents its nuclear translocation and rules its turn-over, as well. During redox umbalance, Nrf2 is released from its cytoplasmatic anchoring by oxidative modifications on either proteins, allowing, in such a way, its nuclear localization and recruitment to ARE *cis* elements.

Another example of redox-active transcription factor is p53, one of the most characterized tumor suppressor proteins acting in response to different forms of cellular insults that lead to genomic instability (i.e. oxidative

stress). Essentially, it mediates a variety of antiproliferative processes, among which is the control of cell cycle and apoptosis are the most representative (Vousden and Lane, 2007). In resting condition, p53 has a short half-life and is normally maintained at low levels by continuous ubiquitylation catalyzed by Mdm2, COP1 (constitutively photomorphogenic 1), and Pirh2 (p53-induced protein with a RING-H2 domain) and subsequent degradation by 26S proteasome. Physiological p53 levels are important for redox homeostasis since maintain ROS at non-toxic levels through transactivation of antioxidant genes, such as the mammalian sestrin homologues SESN1, SESN2, and glutathione peroxidase-1 (Sablina et al., 2005). Under stress conditions, phosphorylation of p53 at various sites, induced by several protein kinases, including the redox-sensitive MAPK family, increases its stabilization and accumulation in nuclei in which it coordinates different responses to stress signals. In response to DNA genomic instability, p53 promotes cell cycle arrest at G_1 or G_2 phase to allow DNA to be repaired before proceeding into mitosis, by inducing the expression of cyclin-dependent kinases inhibitors such as p21. When the damage is too severe to be repaired, p53 also regulates the expression of several genes (e.g., Bax and Puma) able to engage the mitochondrial apoptotic pathway (Fridman and Lowe, 2003; Yu and Zhang, 2005). The involvement of p53 in cell death process is also highlited by its ability to oxidize intracellular environment, facilitating the engagement of apoptotic machinery. In fact, several studies demonstrated that hyperphysiological levels of p53 transactivate two ROS-generating enzymes, NQO1 (NAD(P)H:quinone oxidoreductase) and POX (proline oxidase) and down-regulates the expression of antioxidant genes, such as MnSOD leading to oxidative stress and consequently to apoptosis (Drane et al., 2001; Polyak et al, 1997; Rivera and Maxwell, 2005).

Besides modutaling redox homeostasis and being activated by oxidative damages, p53 may also be affected by ROS directly. In fact it has been demonstrated that p53 itself is redox active due to the presence of several reactive cysteines the oxidation of which abolishes p53 DNA-binding activity. Moreover, the oxidation of specific cysteine residues seems to determine the selective transactivation of certain categories of p53 target genes. For example, Buzek et al. reported that oxidation of Cys277 decreases p53 binding to GADD45 but not to p21. Although the structural nature of this redox modification is still unknown and GADD45 and p21 are both involved in DNA repair and cell growth arrest, this discovery reveals a fascinating connection between ROS and p53 function, suggesting that redox

modification can be a potential mechanism for target gene selection (Buzek et al., 2002).

MAPK pathway: crossroad between ROS and phopshorylation

A compelling mechanism of redox signaling is the cross-talk between redox modifications and phosphorylative cascades. Mitogen activated protein kinases (MAPK)-mediated transduction pathways show this synergism where the upstream control of the system is managed by the specificity of reactive cysteines oxidation, and the downstream propagation of the signal is handled by phosphorylative chain reactions (Filomeni et al., 2005). MAPKs belong to an evolutionary conserved and ubiquitous signal transduction superfamily of Ser/Thr protein kinases that regulate apoptosis, and other cellular programs such as growth, motility, differentiation in responses to environmental stimuli. The MAPK superfamily comprises three primary signaling cascades named after their terminal MAPKs: the extracellular signal regulated kinases (ERK1/2) pathway, the *c*-Jun NH2-terminal kinases or stress-activated kinases (JNK/SAPK) pathway, and the p38^{MAPK} pathway. Each pathway consists of a multi-tiered hierarchy of kinases that sequentially phosphorylate and activate their downstream target kinases. Thus, MAPKs are phosphorylated by the mitogen activated protein kinase kinases (MKK or MEK) which are in turn regulated by the MEK kinases (MEKK or MAPKKK), such as the apoptosis signal regulating kinase 1 (ASK1). Because each of these groups consists of many functionally-related kinases, this diversity generates a large repertoire of distinct signaling cascades (Kyriakis and Avruch, 2001). The terminal, activated MAPKs ultimately phosphorylate a number of target proteins, including multiple transcription factors involved in gene regulation, such as p53. Whereas ERK1/2 preferentially respond to stimulation by growth-related signals, playing pivotal roles in the regulation of cell growth, proliferation, and differentiation, JNK and p38^{MAPK} are preferentially activated in response to a variety of stresses and pro-apoptotic signals, such as TNFα, FasL, H_2O_2 and growth factor deprivation (Kumar et al., 2003).

The direct link between ROS and MAPK signaling has been evidenced for the first time in Saitoh laboratory by using ASK1 as bait in the yeast two-hybrid assay. This kinase was found to be inhibited by the formation of a dimer with the small redox-sensitive protein tioredoxin. Moreover, this interaction was found only in non-stressed cells and seemed to be modulated

by the intracellular redox state. In particular, a more oxidizing environment was suggested to cause disulfide bridge formation between Cys32 and Cys35 on the Trx moiety thus destabilizing the dimer. As a result, ASK1 dissociates from Trx, forms an oligomeric complex, autophosphorylates at Thr845 and phospho-activates p38^{MAPK} and JNK-upstream kinases, MKK 3/6 and MKK 4/7, respectively (Saitoh et al., 1998).

The second system, by which a redox signal switches to a phosphorylative event is represented by the glutathione S-transferase π-1 (GST)/JNK complex. JNK activity is physiologically maintained at low levels within the cell, even in the presence of high concentrations of growth factors, and seems to be inhibited in unstressed cells. Adler and co-workers demonstrated that under resting conditions the monomeric form of GSTπ binds to the C-terminal of JNK suppressing its activity, while under H$_2$O$_2$ or UV treatment, the GST/JNK complex dissociates leading to the formation of GST dimer and/or multimers allowing JNK phospho-activation (Adler et al., 1999).
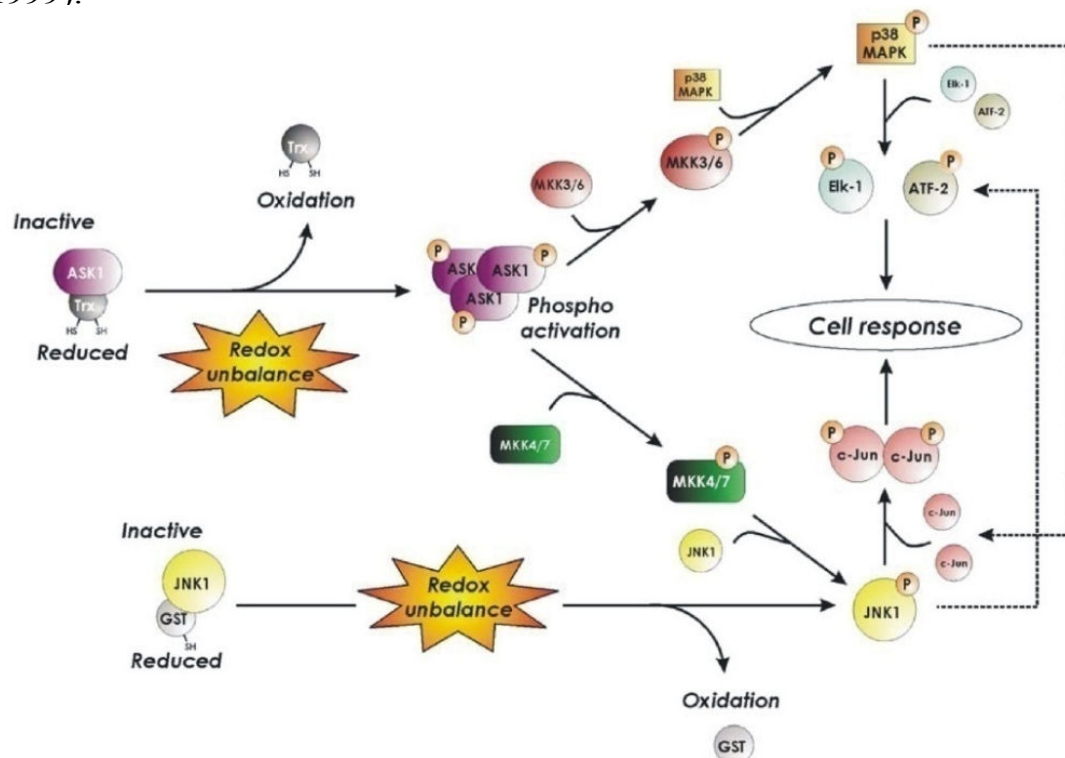

Figure 2
Redox regulation of MAPK (from Filomeni et al., 2005)

Pathological implications of altered redox regulation

The redox state of a cell and its modulation controls the most important cellular functions, ranging from proliferation to death. Therefore, it is kept within a narrow range under normal conditions, similar to the manner in which a biological system regulates its pH. This control is achieved by balancing pro-oxidants species generation with their elimination by ROS-scavenging systems. Disruption of redox homeostasis, due either to an elevation of ROS production or to a decline of redox buffer capacity, determines the onset of a condition known as "oxidative stress", characterized by the accumulation of oxidative damages to macromolecules and the impairment of cellular functions. Therefore, the maintenance of the redox homeostasis is critical for cell survival and its alteration is involved in pathophysiology of different diseases, including cancer and neurodegenative disordes (Dalle- Donne et al., 2006; Dhalla et al., 2000; Sayre et al., 2001).

Oxidative stress in cancer

Role and mechanisms of oxidative stress

One of the first evidence supporting the role of ROS in the etiology of cancer arose from clinical and epidemiological investigations, which highlighted the ability of tumor promoting factors such as solar UV exposure, chemical carcinogens and chronic inflammation, to increase the production of free radicals in living organisms. Afterwards, on the basis of their ability to induce DNA mutational rates to comparable levels as those promoted by other well known carcinogens, it became evident that ROS could be considered powerful cancer initiators. In fact, compelling evidence suggests that, compared with their normal counterparts, many cancer hystotypes have increased levels of ROS. For example, cells isolated from blood samples of patients with different forms of leukaemia showed increased ROS production compared with normal lymphocytes (Zhou et al., 2003; Kamiguti et al., 2005). Moreover, several studies have shown increased levels of oxidative damage products, such as DNA base oxidation (8-OH-G) and lipid peroxidation products in clinical tumour specimens, plasma and cancer cell lines (Patel et al., 2007; Tsao et al., 2007; Kumar et al., 2008).

Although oxidative umbalance promotes all steps of malignant transformation, other than genomic instability, such as cancer cells proliferation, migration and invasive behaviours, the precise pathways leading to oxidative stress in cancer cells remain unclear. Several intrinsic and extrinsic mechanisms are thought to cause oxidative stress during cancer development and disease progression. Activation of oncogenes, aberrant metabolism and mitochondrial dysfunction are intrinsic factors known to cause increased ROS production in cancer cells. The expression of genes that are associated with tumour transformation, such as *Ras, Bcr-Abl* and *c-Myc*, were found to induce ROS production. For instance, in *H-Ras*-transformed fibroblast cells, a large amount of superoxide was generated through the activation of the membrane-associated ROS-producing enzyme NOX, contributing to maintain transforming cells in a proliferative status (Pelicano *et al.,* 2004; Irani *et al.,* 1997). Besides oncogenic transformation, mitochondrial DNA (mtDNA) mutations have also been shown to be correlated with increased ROS levels in certain types of cancer cells, including solid tumours and leukaemia. Several protein components of the electron transport chain are encoded by mtDNA. Thus, mutations of mtDNA are likely to cause impairment in electron transfer, leading to leakage of electrons and the generation of superoxide (Carew *et al.,* 2003). In addition to the internal stimuli, extrinsic factors such as inflammatory cytokines, could also affect intracellular redox homeostasis. In fact pro-inflammatory molecules attract and activate macrophages and neutrophyls able to induce an oxidative burst damaging cellular structures, including genomic and mitochondrial DNA.

Adaptation to oxidative stress

As cancer cells actively produce high levels of ROS and are consistently exposed to such endogenous oxidants, the intrinsic oxidative stress exerts selective pressure to enrich the population of cells that are capable of stress adaptation (Schneider et al., 2004). These surviving cells acquire adaptive mechanisms to counteract the potential toxic effects of elevated ROS and to promote cell-survival pathways. For example, oncogenic *H-Ras*-transformed cells, which exhibited increased superoxide and hydrogen peroxide levels, were shown to express higher levels of antioxidants such as peroxiredoxin-3 and thioredoxin peroxidase compared with their non-tumorigenic parental cells, likely as a key mechanism to evade ROS-induced apoptosis (Young et

al., 2004). Likewise, studies using inducible c-Myc in melanoma cells showed that this protein controlled the expression of the GSH synthesis enzymes allowing cells to overcome oxidative stress by upregulating intracellular GSH content (Benassi et al., 2006).

The mechanisms of redox adaptation may involve multiple pathways to activate redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB), Nrf2, c-jun and HIF-1, which lead to the increased expression of antioxidant enzymes and cell-survival molecules such as members of Bcl-2 family and Akt survival pathway.

Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach

As excessive levels of oxidants can be detrimental for cell functioning, cancer cells with increased oxidative stress are more vulnerable to damage by further pro-oxidant insults induced by exogenous agents (Pelicano et al., 2004). Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal counterparts. Basically, therapeutic approaches aimed to alter redox balance in cancer cells involve the use of chemical compounds able to: (i) inhibits antioxidants defences, such as the SOD inhibitor 2-methoxyestradiol (2-Me) or the Trx-1 inhibitor 1-methylpropyl 2-imidazolyl disulphide (PX-12); (ii) promote ROS production, such as the redox cyclers daunorubicin and doxorubicin. To maximally exploit the ROS-mediated cell-death mechanism as a therapeutic strategy, it is possible to combine drugs that induce ROS generation with compounds that suppress the cellular antioxidant capacity. This approach might be particularly useful in cancer cells that have become adapted to stress and resistant to anticancer agents (Trachootham et al. 2009).

Oxidative stress in neurodegeneration

Oxidative stress increases with age and therefore it can be considered as an important concurring factor in several neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer disease (AD) and Amiotrofic Lateral Sclerosis (ALS). Because of its high metabolic rate and relatively reduced capacity for cellular regeneration compared with other organs, the brain is

believed to be particularly susceptible to the damaging effects of ROS. Moreover, the high content of oxidable polyunsaturated fatty acids, the presence of redox-active metals such as iron and copper and the generation of oxyradicals as metabolic by-products of some neuronal metabolites, such as dopamine and neuromelanine, contribute to the enhanced vulnerability of neurons to oxidative damages (Fasano et al., 2006; Bharath et al., 2002). In cases of PD, AD and ALS, various markers of oxidative damage have been reported within the specific brain region that undergoes selective neurodegeneration. For example, lipid peroxidation products, including 4-hydroxynonenal and malondialdehyde, have been identified in the cortex and hippocampus of patients with AD, in *substantia nigra* of post-mortem PD brains the and in spinal fluid from patients with ALS. Although cells display several mechanisms to counteract ROS production, in these neurological discorders, the activities of various antioxidant defense molecules are reduced and the magnitude of depletion parallels the severity of the disease. For example, it has been reported that GSH is decreased by 40% in the substantia nigra of patients with PD or in presymptomatic stages (Sian et al., 1994), and several studies point out that GSH depletion enhances the toxicity of several PD inducing neurotoxins such as MPP^+, 6-OHDA and MPTP (Nakamura et al., 1997; Pileblad et al., 1989). Likewise, the antioxidant enzymes SOD, catalase, GPx and methionine sulfoxide reductase, which reverses oxidation at protein methionine residues, display reduced activities in AD affected brain regions (Zemlan et al., 1989; Pappolla et al., 1992).

Increased oxidative alterations to proteins, such as α-synuclein in PD, β-amyloid in AD and SOD1 in ALS might result in increased protein misfolding and impaired degradation. This, in turn, might cause the toxic accumulation of soluble protofibrils or insoluble aggregates within the affected brain regions that can contribute to neurodegeneration. For instance, dopamine metabolism yields oxidative by-products such as $O_2^{\cdot-}$, H_2O_2 and dopaminergic quinones, which might be involved, along with iron, in the oxidation of α-synuclein, a protein found to be accumulated in the Lewy bodies both in PD and other synucleopathies. Oxidized α-synuclein might, in turn, not be properly recognized by the ubiquitin-proteasome protein degradation system, resulting in its buildup in the cell (Conway et al., 2001; Volles and Lansbury, 2002; Lotharius and Brundin, 2002). Moreover, lipid peroxidation products such as 4-HNE, whose accumulation can be enhanced by GSH depletion in PD, can further enhance cross-linking of damaged proteins, inhibit proteasomal function, thus promoting cytosolic accumulation of misfolded proteins (Okada et al., 1999).

The involvement of oxidative stress in the pathogenesis of neurodegeneration is strengthened by the engagement of many redox-activated intracellular signaling pathways that lead to the apoptotic cellular demise. For example, the accumulation of phospho-active c-Jun-N-terminal kinase (JNK) and p38^{MAPK} has been detected in *post-mortem* PD brains, and their pharmacological inhibition induces neuroprotection (Gomez-Lazaro et al., 2008; Ferrer et al., 2001; Wang et al., 2004). Moreover, the insensitivity of p53-null mice to MPTP, as well as resistance observed in mice treated with p53 inhibitor pfithrin, demonstrate the involvement of p53 in PD pathogenesis (Trimmer et al., 1996; Duan et al., 2002). Despite its implication in neurodegeneration, it is still unclear whether oxidative stress is the primary initiating event or a as consequence of pathogenetic mechanisms underlying neuronal demise. However, a growing body of evidence implicates oxidative stress as being involved in at least the propagation of cellular injury that leads to neuropathology in these various conditions. It is intimately linked with an integrated series of cellular phenomena, which all seem to contribute to neuronal demise. In fact, the interaction between these various components is not necessarily a cascade but might be a cycle of events, of which oxidative stress is a major component. Inhibition of oxidative stress therapeutically might act to "break the cycle" of cell death. Therefore, many researchers in the neurodegenerative field are concentrating on modulating or emulating the protective effects of key enzymatic components that regulate oxidative stress, with the aim of developing antioxidant rational drugs. For example, the clinical evidences that neurodegenerations can be ameliorated upon dietary or supplementary intake of natural antioxidants (Valko et al., 2006) and the evidence that genetically or pharmacologically chelated iron prevents degeneration of dopaminergic midbrain neurons (Kaur et al., 2003), suggested that antioxidant and metal chelation therapy could be a feasible approach to prevent or modulate the redox-umbalance which contribute to neuronal demise.

Cellular Bioenergetics

Overview of cellular respiration

Cells require a constant supply of energy to sustain fundamental biological processes such as growth, proliferation, differentiation and also death. In heterotrophic organisms this energy derives from the oxidation of organic molecules or nutrients (e.g. glucose, aminoacids and fatty acids), to produce adenosin triphosphate (ATP), which serves as versatile bioenergetic "currency" to drive energy-requiring processes, such as biosynthetic reactions. The set of catabolic reactions that takes place to convert biochemical energy from nutrients into ATP is called cellular respiration. It is the results of three main catabolic processes: (i) *glycolysis*, the anaerobic cytosolic process that converts one molecule of glucose into two net molecules of pyruvate and ATP; (ii) *Krebs cycle*, called also tricarboxylic acid cycle (TCA cycle), an 8-step process during which one mole of acetyl-Coa, formed through oxidative decarboxylation of glycolytic pyruvate, leads to the formation of 1 ATP, 1 FADH$_2$ and 3 NADH; (iii) *oxidative phosphorylation (OXPHOS)*, a complex metabolic process that rely on the transfer of electrons from NADH and FADH$_2$ to molecular oxygen through the mitochondrial electron transport chain, in order to generate a chemiosmotic potential used to drive the phopshorylation of ADP.
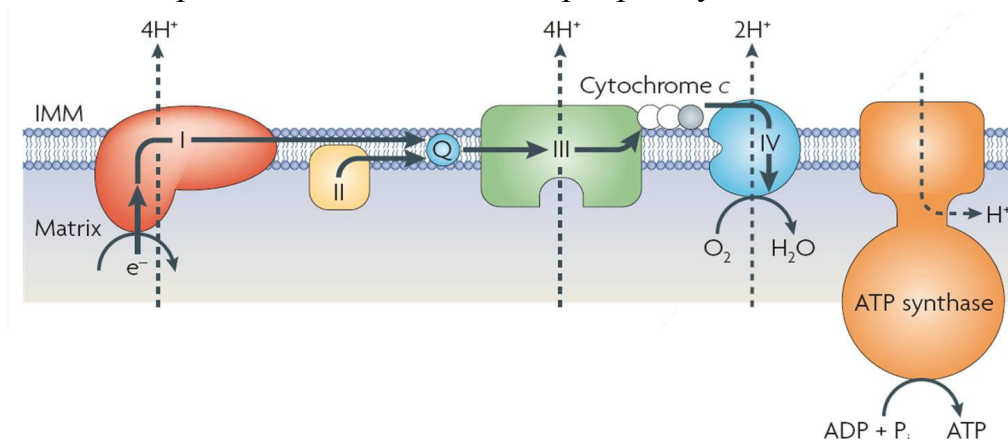

Figure 3
Oxidative phosphorylation (modified from Ow et al., 2008)

Without oxygen, pyruvate is not metabolized by cellular respiration but undergoes lactic acid fermentation. This process allows glycolysis to continue, by cycling NADH back to NAD^+, but results in minimal ATP production when compared to the 36 moles of ATP per glucose produced by aerobic respiration. As aerobic catabolism is 18-fold more efficient than the anaerobic one, mitochondria, the cellular organelles where the post glycolytic reactions takes place, can be considered the cellular "powerhouses".

Pathological alteration of cellular bioenergetics

Tumor cell metabolism: Achilles' heel of cancer

Cancer cells differ from diffentiated ones due to a plethora of molecular changes, many of which may be linked into a "metabolic and bioenergetic reprogramming" aimed to sustain faster rate of growth and proliferation. The main biochemical reorganization, called "Warburg phenomenon", was discovered by the Nobel Prize winner Otto Warburg in the 1920s. It consists of an increase in glycolysis, maintained in conditions of high oxygen tension (''aerobic glycolysis''), which gives rise to enhanced lactate production (Brahimi-Horn et al., 2007; Warburg et al., 1924). Although the mechanisms responsible of this metabolic switch are not completely understood, the enhanced glucose uptake for glycolytic ATP generation or anabolic reactions constitutes an advantage for tumor growth. In fact, tumor cells live in conditions of fluctuating oxygen tension (due to inconstant hemodynamics of distant blood vessels) that would be lethal for cells that rely only on oxidative phosphorylation to generate ATP. Moreover the huge amount of glycolytic lactate released by cancer cells, besides making more acidic the environment favoring, thus, tumor invasion and suppressing anticancer immune effectors, can be taken up by stromal cells to regenerate pyruvate that can be extruded to refuel tumor cells (Koukourakis et al., 2006). By this way, anerobic (cancer cells) and aerobic components (nontransformed stromal cells) generate a micro-system engaging complementary metabolic pathways aimed to buffer and recycle products of anaerobic metabolism to sustain cancer cell bioenergetics.

In keeping with this features, ATP depleting molecules, such as the glycolytic or glucose uptake inhibitors and several oxidative phosphorylation (OXPHOS) inhibiting compounds, have been exploited as cancer cell death inducers. Indeed, in a variety of cancer cells, inhibition of glycolysis with a
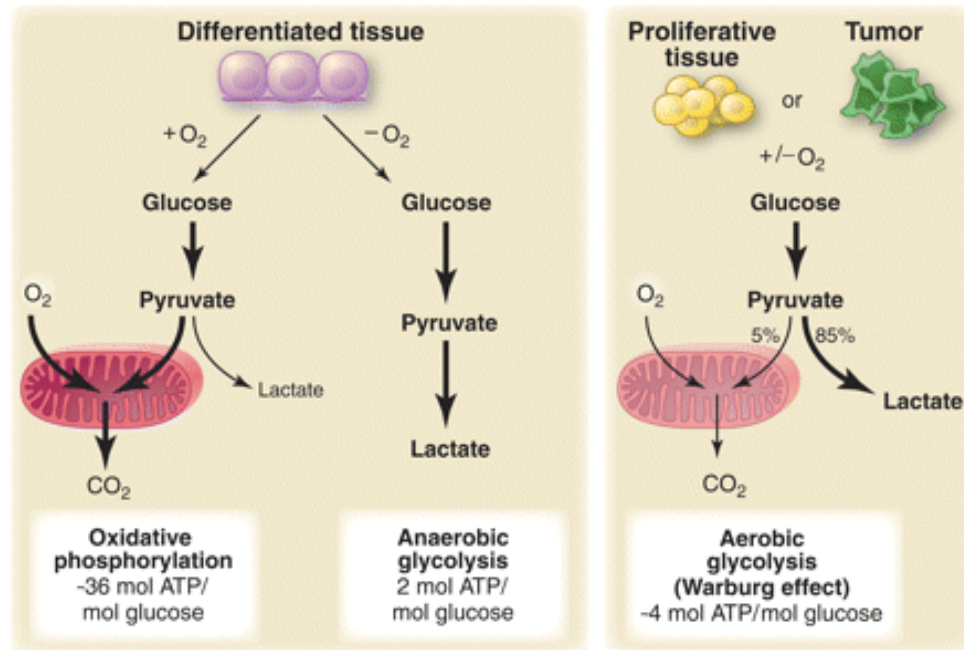
Figure 4
Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis and Warburg effect. (modified from Vander Heiden et al., 2009)

non-metabolizable glucose analog, 2-deoxyglucose (2-DG), or 3-bromopyruvate, caused a marked decrease in ATP level, especially in clones where mitochondrial ATP supply was compromised. ATP depletion led also to rapid dephosphorylation of the pro-apoptotic Bcl-2 family protein Bad which induces migration of Bax to the mitochondria and subsequent massive cell death (Xu et al., 2005).

Whereas, the antitumor effect of glycolytic inhibitors is based almost exclusively on ATP depletion, pro-apoptotic effects of OXPHOS affecting molecules, besides relying on a deeper impairment of cellular bioenergetics, induce a severe redox unbalance by enhancing mitochondrial ROS production. Among them, delocalized lipophilic cations (DLCs) are considered an efficient class of chemotherapeutics. These compounds, penetrate the hydrophobic barriers of the plasma and mitochondrial membranes and, by exploiting their positive charge, accumulate in mitochondria in response to the transmembrane potentials ($\Delta\psi_m$) (Modica-Napolitano and Aprille, 2001). Although they share a common mechanism

for mitochondrial internalization, their mechanisms of mitochondriotoxicity are quite varied. For example, the prototype DLC, rhodamine 123 (Rh123), and the thiopyrylium AA-1, compromise mitochondrial bioenergetic function by inhibition of F_0F_1-ATPase (Modica-Napolitano and Aprille, 1987; Sun et al., 1994). In contrast, the quaternary ammonium compound dequalinium chloride (DECA) and certain DLC thiocarbocyanines inhibit mitochondrial respiration through inhibition of NADH-ubiquinone reductase activity in the respiratory complex I (Anderson et al., 1989; Anderson et al., 1993).

The chemotherapeutic success of these molecules depends on their ability to selectively target tumor cells, even when they are administered systemically. Fortunately, selectivity is already possible for carcinoma cell killing since these cells have higher $\Delta\psi_m$ and, thus, accumulate lipophilic cations into mitochondria to much higher concentrations than normal epithelial cells.

Bioenergetic alteration in neurodegeneration

If metabolic alterations in cancer cells represent an advantageous way to sustain their growth and proliferation, and the main goal of mitochondrial targeting is to kill malignant cells, bioenergetic impairment is a detrimental hallmark contributing to the pathogenesis of almost all neurodegenerative disorders. Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. Evidence of this involvement first emerged following the observation that accidental exposure of drug abusers to MPTP, an inhibitor of complex I (NADH/ubiquinone oxidoreductase) of the mitochondrial electron transport chain, resulted in an acute and irreversible parkinsonian syndrome almost indistinguishable from PD (Langston et al., 1983). Since the discovery that MPTP kills dopaminergic neurons of the substantia nigra pars compacta (SNpc) in both human and non-human primates, as well as in various other mammalian species, this neurotoxin has been used extensively as an experimental animal model of PD (Dauer and Przedborski, 2003). Besides inducing an ATP collapse, the impairment of mitochondrial respiration by complex I blockade in dopaminergic neurons arises and /or contributes to increased production of ROS and subsequent oxidative damage to proteins, lipids and DNA, deleterious events both detected in post-mortem PD brain and playing a critical role in experimental models of this disorder.

In contrast to PD, the main mitochondrial target in Alzheimer's disease is not complex I, but cytochrome *c* oxidase (Complex IV). Biochemical analyses, in platelet mitochondria isolated from patients with AD and in post mortem AD brains, revealed a striking reduction (30-40%) of complex IV activity, whereas other respiratory chain activities were not significantly different from control values (Cottrell et al., 2001; Maurer et al., 2000; Mutisya et al., 1994; Parker et al 1994). This alteration, that leads to a hypoenergetic metabolism and enhanced ROS production, seems to depend on the accumulation of Aβ in AD brain mitochondria. In particular, it has been pointed out that Aβ binding to heme triggers its functional deficiency responsible, in turn, for the impairment of complex IV and the increased ROS release from mitochondria (Atamna and Frey, 2004).

Besides mitochondrial dinsfunction, a large number of reports documents alteration in glucose metabolism both in AD and in PD. Positron emission tomography (PET) imaging combined with 18F-2-deoxy-2-fluoro-d-glucose have shown a decline of 21–28% in regional glucose uptake and utilization in AD brain relative to elderly normal controls (de Leon et al., 1983a and de Leon et al., 1983b). The decline in glucose metabolism, which occurs in the areas that are known to be affected early in AD, such as *posterior cingulate*, parietal, and temporal cortex (Reiman et al., 1996 and Small et al., 2000), appears before the onset of memory deficits and seems to sensitize the neurons to energy deficiency (Arias et al., 2002). Biochemical studies pointed out that glucose transport is impaired by Aβ by a mechanism involving membrane lipid peroxidation. In particular exposure of cultured rat hippocampal and cortical neurons to Aβ induces conjugation of 4-hydroxynonenal to the neuronal glucose transport protein GLUT3 affecting in such a way glucose uptake and intracellular ATP generation (Mark et al., 1997).

Using several different methods, including PET analysis, it has been shown that glucose uptake is impaired in several regions of PD brains as well (Ma and Eidelberg et al., 2007). *In vitro* studies demonstrate that glucose starvation is able to induce PD-like pathological changes in dopaminergic cells, such as a selective α-synuclein increase and aggregation into intracytoplasmic inclusions (Bellucci et al., 2008). Moreover, in a genetic model of autosomal recessive PD, it has been recently demonstrated that ROS production inhibits GLUTs functionality, thus reducing substrate delivery, causing impaired respiration and rendering neurons vulnerable to cell death (Gandhi et al., 2009). Overall, these data demonstrate that, whatever the mechanism may be for his engagement, bioenergetic

impairment affects diverse cellular processes that culminate in cell demise. Therefore, therapeutic strategies targeted at preventing, delaying, or treating energetic dysfunctions should contribute to the prevention and/or treatment of neurodegenerative disorders

Regulation of bioenergetics and apoptosis: role of AMPK

Cells have evolved an homeostatic system that enables them to respond to changing in bioenergetic demands. This dynamically regulated system is programmed to fit the requirements for cell proliferation or meet the specific needs of each differentiated tissue as appropriate. For instance, when energy status is compromised, the system activates catabolic pathways and switches off protein, carbohydrate and lipid biosynthesis, as well as cell growth and proliferative process in order to maintain an high ratio of ATP to ADP. One of the major players in maintaining this balance is the AMP-activated protein kinase (AMPK), a functionally conserved bioenergetic sensor, which promote the activation of energy restorative metabolic pathways and inhibit energy consumptive processes (Hardie, 2007). AMPK is a serine/threonine kinase composed of a catalytic α subunit and regulatory β and γ subunits. In mammals, several alternative genes have been identified for each of these subunits (e.g. $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$) which show differential tissue-specific expression, making, thus, various heterotrimers possible with different substrates preferences and regulative properties. AMPK is allosterically activated by the binding of AMP to four conserved cystathionine β-synthase motifs (CBS1–4) of the γ-subunit. This binding promotes the phosphorylation of a threonine residue (Thr172) in the kinase domain of α subunit by three known protein kinases, LKB1, CaMKKβ (Ca2+/calmodulin-dependent protein kinase β) and TAK1 (TGFB-activated kinase 1) and inhibits its dephosphorylation by protein phosphatase-2Cα (Ronnett et al., 2009). Enzyme phosphor-activation is therefore dependent on the cellular energy status. In fact, under unstressed conditions, catabolism maintains a high ratio of ATP:ADP. This drives the adenylate kinase reaction (2 ADP $\Leftrightarrow$ ATP + AMP) in favour of ADP synthesis maintaining, consequently, the cellular AMP:ATP ratio low and the AMP-activated protein kinase (AMPK) inactive.
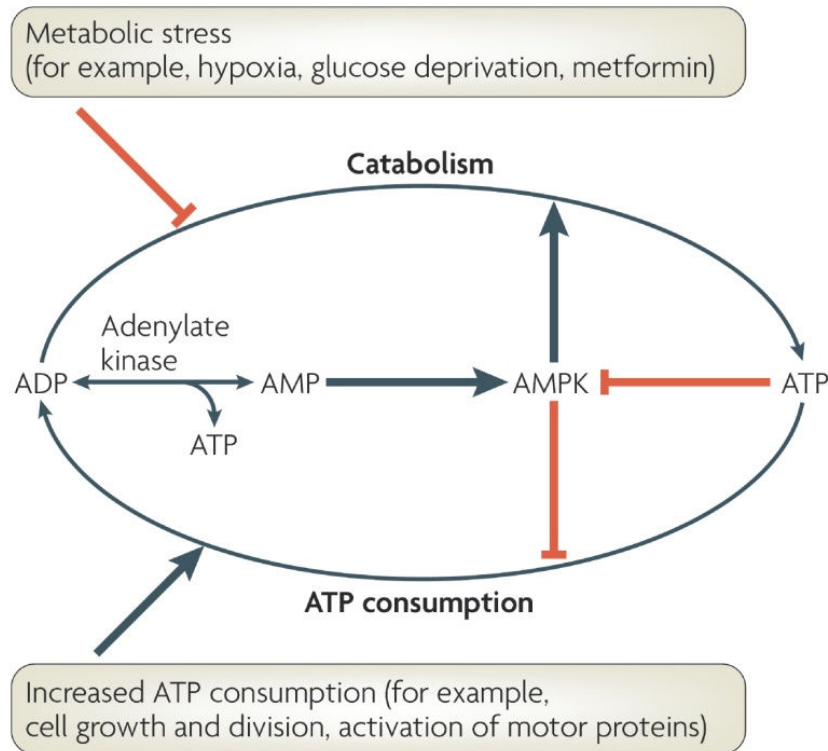
Figure 5
Regulation of energy homeostasis by AMPK system (from Hardie et al., 2007)

Upon metabolic stress that interferes with ATP synthesis (e.g., hypoxia, glucose deprivation, nutrient starvation) or conditions that accelerates ATP consumption (for example, activation of motor proteins, ion pumps or channels, or biosynthetic pathways), the ADP:ATP ratio tends to increase. This is amplified by adenylate kinase into a much larger increase in the AMP:ATP ratio that switches on AMPK activation. In turn, AMPK restores energy homeostasis by promoting catabolism and inhibiting ATP-consuming processes. This is accomplished by phosphorylation and modification of numerous target proteins including biosynthetic enzymes, transporters transcription factors, ion channels and cell-cycle/signaling proteins. For instance, AMPK inactivates ACC (acetyl-CoA carboxylase) and HMG-CoA

(3-hydroxy-3-methylglutaryl-coenzyme A) reductase, thus reducing fatty acid and cholesterol synthesis when energy is limiting (Carling et al. 1987;Winder and Hardie 1996) or stimulates catabolism by promoting glucose uptake (Glut4 translocation) and glycolysis activating 6-phosphofructo-2-kinase kinase (PFK-2) (Winder and Hardie, 1996; Merrill et al., 1997; Kurth-Kraczek et al., 1999). Besides regulating directly key enzymes of energy metabolism, AMPK modulates energy-demanding processes, such as cell growth, proliferation and apoptosis, by regulating

Figure 6
Overview of AMPK signaling
(from CST web-site: http://www.cellsignal.com/reference/pathway/AMPK.html)

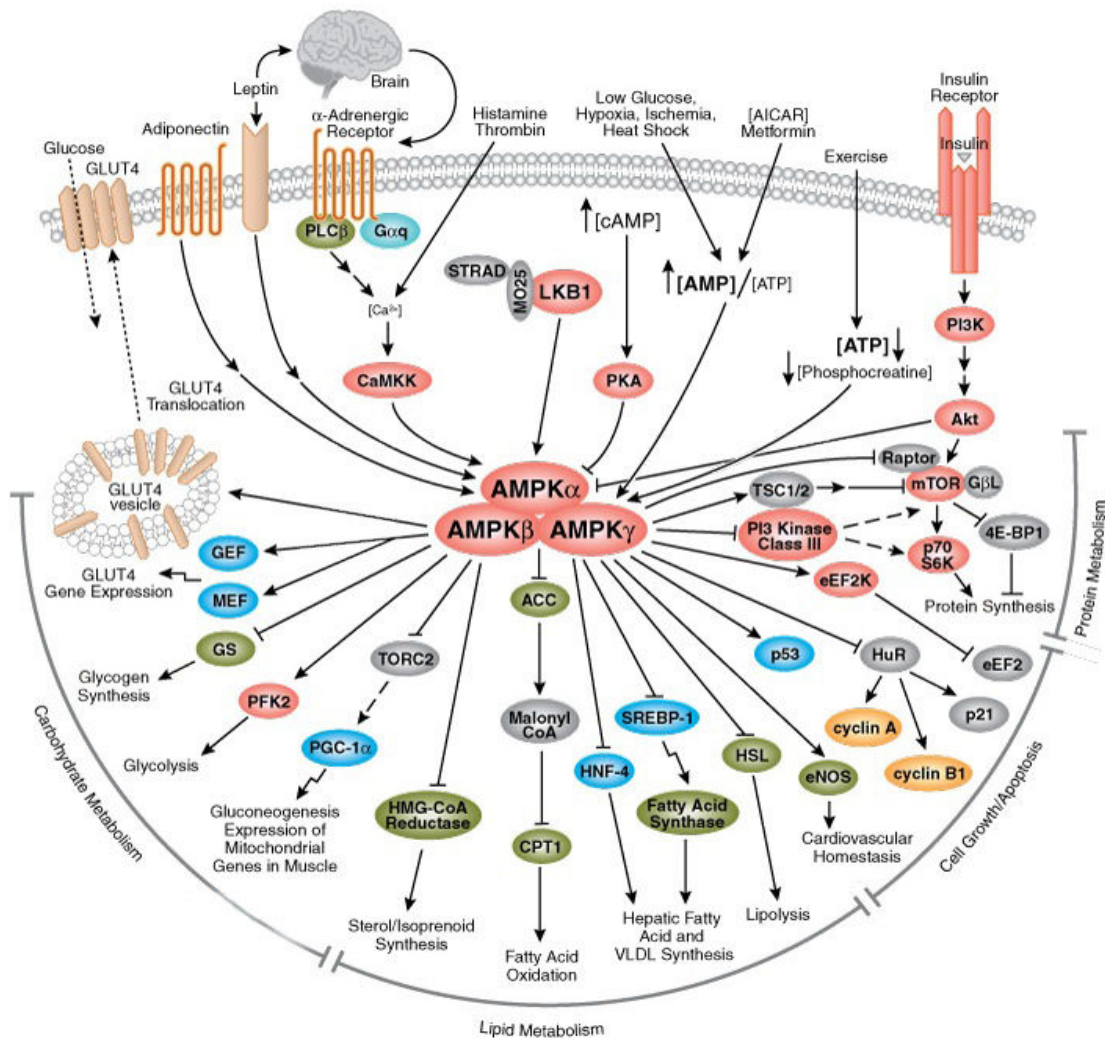signaling proteins and affecting changes in gene expression. Whereas AMPK-mediated cell growth inhibition mainly depend on the suppression of the mammalian target-of-rapamycin (mTOR) activity to limit protein synthesis and activate autophagy (Steinberg and Kemp, 2009), cell cycle arrest and apoptosis induction seem to rely on p53 activation and MAPK pathway engagement. In particular, AMPK-mediated p53 phospho-activation play a crucial role both in the regulation of a G_1/S metabolic checkpoint, that responds to glucose availability (Jones et al., 2005), and in apoptosis induction elicited by severe carbon source depletion and genotoxic stresses (Okoshi et al., 2007; Cao et al., 2008). Among MAPK, the antiproliferative effect of AMPK seem to be mainly induced by the activation of $p38^{MAPK}$ and JNK. The former mediates pro-apoptotic activity of AMPK upon UV and H_2O_2 exposure (Cao et al., 2008); the latter is phospho-activated in condition of sustained AMPK activation and upon cannabinoid antagonists exposure (Lee et al., 2008; Kefas et al., 2003, Meisse et al., 2002). Overall these data suggest that AMPK can be considered as a molecular target of a feasible therapeutic strategy for diseases based on cell proliferation such as cancer. Indeed, in keeping with the pivotal role of AMPK in the regulation of proliferative processes, in the last years novel chemotherapeutics have been developed to affect selectively tumor cells growth and viability by promoting AMPK activation. For instance, it has been pointed out that several antidiabetic drugs such as metformin, and the thienopyridone A769662 can delay the growth of spontaneous tumors in AMPK responsive-manner (Fruman et al., 2008, Hwang et al., 2006). Moreover, the evidence that the phosphatidylinositol ether lipid analogues (PIAs) and some natural products such as curcumin and selenium are able to elicit death in various cancer cell lines (Memmott et al., 2008, Pan et al., 2008) by engaging AMPK-dependent pro-apoptotic signaling pathways, straightens the feature of this kinase as a promising target both for anti-cancer and chemopreventive drugs.

Although AMPK activation is an adaptive response to stress in numerous systems, and an attractive therapeutic approach for proliferative diseases, consequences of persistent AMPK activation in non renewing tissues, such as neurons, could not be beneficial. In fact, several in vitro studies point out that AMPK over-activation is harmful: AICAR, one of the pharmacological activators of this kinase, was found to be pro-apoptotic in human neuroblastoma cells (Garcia-Gil et al., 2003), rat hippocampal HN9 cells, and mouse MMIN cells (Pesi et al., 2000). Moreover, it has been recently demonstrated *in vivo* that the biguanide pharmacological AMPK activator metformin, significantly increases the generation of both intracellular and extracellular *β*-amyloid (A*β*) peptides which are pivotal in AD pathogenesis (Chen et al., 2008). Moreover *in vivo* studies suggest that AMPK activation

occurring after cerebral ischemia is detrimental to neuronal survival: in fact, genetic and pharmacological inhibition of AMPK under these conditions *reduced* stroke damage (Li et al., 2007; McCullough et al., 2005). Collectively these data suggest that *excessive* neuronal AMPK activation when oxygen and glucose substrates are lacking may induce a detrimental 'metabolic failure-like' state. Under these conditions, such as in the context of neurodegenerative disorders, pharmacological AMPK inhibition could be beneficial to neuronal survival.

EXPERIMENTAL PROCEDURES

Materials

Isatin-diimine copper(II) complex Bis[(2-oxindol-3-ylimino)-2-(2-aminoethyl)pyridine-N,N']copper(II) perchlorate, [Cu(isaepy)$_2$](ClO$_4$)$_2$, named *Cu(isaepy)$_2$*, was synthesized as previously described (15). The analogous isatin-imine zinc(II) complex [Zn(isaepy)Cl$_2$], designated as *Zn(isaepy)*, was prepared similarly using zinc chloride to metallate *in situ* the isaepy ligand. Tetrahydrobiopterin (BH4) and Sodium Nitroprusside (SNP) were from Alexis (Lausen, Switzerland). Buthionine sulfoximine (BSO), catalase, EDTA, EGTA, N-acetylcysteine (NAC), paraformaldehyde, propidium iodide, Triton X-100 Antimycin A, catalase, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), glucose, propidium iodide, rotenone, sodium pyruvate, methyl succinate, sodium nitrite and Griess reagent were from Sigma (St. Louis, MO). Goat anti-mouse and anti-rabbit IgG (H+L)-horseradish peroxidase conjugate was from Bio-Rad Lab. (Hercules, CA). N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) was from US Biological (Cleveland, OH). Protease inhibitor cocktail was from Roche Applied Science (Monza, Italy). All other chemicals were from Merck (Darmstadt, Germany).

Cell cultures

Human neuroblastoma SH-SY5Y cell line was purchased from the European Collection of Cell Culture and grown in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% glutamine. The cells were maintained at 37° C in a 5% CO_2 atmosphere in air and routinely trypsinized, plated at 4×10^4/ cm^2 flasks. Cell viability was assessed by Trypan blue exclusion. Glucose-free culture conditions were achieved according to Bellucci et al (Bellucci et al., 2008) with minor modifications. Briefly, SH-SY5Y cell line was incubated in 15 mM Hepes-buffered HBSS pH 7.45, supplemented with 10% FCS, 0.01 mM nonessential amino acids, 1% penicillin/streptomycin and 1% glutamine without glucose. As control, cells were grown in the same medium supplemented with 16 mM glucose, which correspond to the concentration present in DMEM/F12 medium.

Transfections

For siRNAs, Twenty-four hours after plating, 50% confluent SH-SY5Y cells were transfected, with a 21-nucleotide siRNA duplex directed against the p53 mRNA target sequence (sip53) (MWG Biotech, Ebersberg, Germany) or with a SignalSilence® Pool p38^{MAPK} siRNA (sip38^{MAPK}) (Cell Signaling Technology, Beverly, MA). Control cells were transfected with a scramble siRNA duplex, which does not present homology with any other human mRNAs (siScr). For plasmids, transfections were performed with a pcDNA3 empty vector or with a pcDNA3 vector containing: *i*) the myc-tagged coding sequence for the α2 subunit of AMPK carrying the T→A substitution at the residue 172 (kindly provided by Prof. David Carling from the Clinical Sciences Centre, Imperial College, Hammersmith Hospital, Du Cane Road, London, UK); *ii*) the flag-tagged coding sequence for the kinase inactive α1 subunit of p38^{MAPK}(kindly provided by Prof. Jiahuai Han, from The Scripps Research Institute, Department of Immunology, North Torrex Pines Road, La Jolla, CA, USA). After transfection, cells were immediately seeded into fresh medium and used after 48 h, since this time was sufficient to significantly increase the expression of these dominant/negative forms of the proteins. Cells were transfected by electroporation using a Gene Pulser Xcell system (Bio-Rad) according to the manufacturer's instructions and directly seeded into fresh medium. Transfection efficiency was estimated by co-transfecting siRNA or plasmids with non-specific rhodamine-conjugated oligonucleotides and found to be > 80 %.

Treatments

A 10 mM solution of BH4 or a 0.5 M solution of SNP were prepared just before the experiments by dissolving the powders in water. Treatments were performed at final concentrations ranging from 50 to 200 μM for BH4 and 0.5 to 2 mM for SNP, in medium supplemented with serum. As control, equal volumes of water were added to untreated cells.

A 5 mM solution of Cu(isaepy)$_2$ or Zn(isaepy) were prepared just before the experiments by dissolving the lyophilized compounds in DMSO. Treatments were performed in serum-supplemented media with Cu(isaepy)$_2$ or Zn(isaepy) at the final concentration of 50 μM. As control, equal volumes of water were added to untreated cells. Glucose sodium pyruvate and methyl-succinate were added to the medium to reach the final concentration

of 30, 10 and 50 mM, respectively and maintained throughout the experimental time. The pan-caspase inhibitor zVAD-*fmk* (Alexis) was used at a final concentration of 20 µM, pre-incubated for 1 h before the addition of BH4 or SNP, and maintained throughout the experimental time. Buthionine sulfoximine (BSO) was used at the final concentration of 1 mM, added 12 h before BH4 addition and maintained in the medium throughout the experimental time. NAC was used at the final concentration of 5 mM. After 12 h-incubation, NAC was left out and replaced with fresh NAC-free medium. Conversely, 1 µM catalase was added 1 h before BH4 addition and maintained in the medium throughout the experimental time. The Jnk inhibitors I and II (Calbiochem) were used at the concentration of 10 µM. The MEK3/6 inhibitor SB203580 (Calbiochem) was used at the concentration of 15 µM. All MAPK inhibitors were added 1 h before BH4 addition and maintained in the medium throughout the experimental time.

Analysis of cell viability and apoptosis

Cell viability was estimated by direct count upon Trypan blue exclusion by optic microscopy. For the evaluation of apoptosis, adherent (after trypsinization) and detached cells were combined, washed with PBS and stained with 50 µg/ml propidium iodide prior to analysis by a FACScalibur instrument (Becton Dickinson, San Josè, CA). The percentages of apoptotic cells were evaluated according to Nicoletti et al. (Nicoletti et al., 1991) by calculating peak area of hypodiploid nuclei (SubG1).

Protein determination

Proteins were determined by the method of Lowry et al (Lowry et al., 1951). This assy is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, in which the phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color with a absorbance peack at 695 nm. Protein concentration is the calculed on the basis of standard curve obtained measuring the absorbance at 695 nm of various concentrations of bovine serum albumin (BSA).

Western blot analyses

Total protein extracts were obtained by rupturing cells with 30 min of incubation on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM sodium ortovanadate) and protease inhibitor cocktail (Roche Applied Science, Monza, Italy) followed by centrifugation at $22,300 \times g$ for 20 min at 4°C. Protein extracts were then separated by SDS-PAGE and blotted onto nitrocellulose membrane (Bio-Rad). Monoclonal anti-p53 (clone BP5312), anti-actin (Sigma); anti-Hsp90 (BD-Transduction Laboratories, Franklin Lakes, NJ); anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-phospho JNK1, anti-phospho c-Jun, anti-poly-ADP ribose polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-subunit II of cytochrome *c* oxidase, anti-39 kDa subunit of Complex I (Invitrogen-Molecular Probes), polyclonal anti-phospho-thr172 of AMPKα-subunits, anti-Bax, anti-JNK, anti-Hsp60 (Santa Cruz Biotechnology); anti-caspase-3 (clone3G2), anti-caspase-9 (Cell Signaling Technology); anti phospho-thr180/tyr182 p38[MAPK] (Invitrogen, San Giuliano Milanese, Italy) were used as primary antibodies. Densitometry was performed using Quantity One Software (Bio-Rad).

Cell fractionation and oxygen consumption

Mitochondria from mouse liver or SH-SY5Y cells were obtained by mincing the hepatic tissue with 3 volumes (w/v) of 10 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 10 mM KCl, and protease inhibitor cocktail. After 15 min on ice, equal volumes of 400 mM sucrose, 10 mM TES, 0.1 mM EGTA, and 2 μM DTT, pH 7.2 were added, and tissue disrupted by 40 strokes in a glass Dounce. Mitochondria-containing supernatants, obtained after centrifugation of lysates at $900 \times g$ for 10 min, were further centrifuged at $12,000 \times g$ for 15 min to finally separate mitochondria (pellet) from the cytosol (super).

Oxygen consumption was determined at 25°C using a Clark-type oxygen electrode equipped with thermostatic control and magnetic stirring. Mitochondria were re-suspended in 1.5 ml of "experimental" buffer (125 mM KCl, 10 mM Tris-MOPS, pH 7.4, 10 μM EGTA-Tris, pH 7.4, 5 mM glutamate, 2.5 mM malate, 1 mM K_2HPO_4). NADH was added at final concentration of 1 mM in the presence or absence of 50 μM Cu(isaepy)$_2$. Rotenone and antimycin A were used at the final concentration of 5 μM. whereas KCN at 1 mM. Catalase was added to the experimental buffer at the

concentration of 0.5 U. Before each analysis, as well as in the evaluation of P:O ratio upon incubations with Cu(isaepy)$_2$, state III/state IV ratio was measured by adding ADP (0.2 μmol) to check the goodness of mitochondrial fraction.

Fluorescence microscopy analyses

Cells were plated on chamber slides at $6 \times 10^4/cm^2$, fixed with 4% paraformaldehyde and permeabilized. For the determination of DNA damage, they were washed exhaustively with PBS, blocked with PBS containing 10% FCS, incubated with a monoclonal anti-ser-139-phosphorylated histone H2A.X antibody (clone JBW301 – Upstate Biotechnology, Lake Placid, NY), and further probed with Alexa fluor®-488 secondary antibody (Invitrogen-Molecular Probes). Alternatively, for the determination of p38^{MAPK}, p53 and Hsp60 activation/localization, cells were stained with specific primary antibodies and further probed with Alexa fluor®-488 or Alexa fluor®-568 secondary antibodies. To visualize nuclei, cells were also incubated with the cell permeable DNA dye Hoechst 33342 (Calbiochem-Novabiochem), washed with PBS and analyzed by fluorescence microscopy. Images of cells were acquired and digitized with a Delta Vision Restoration Microscopy System (Applied Precision Inc., Issaquah, WA) equipped with an Olympus IX70 fluorescence microscope.

ΔΨ$_m$ measurement

$\Delta\Psi_m$ was analysed taking advantage of the $\Delta\Psi_m$-sensitive probe MitoTracker Red® (Invitrogen-Molecular Probes, San Giuliano Milanese, Italy) by fluorescence microscopy or cytofluorometrically. In the first case, cells were seeded on chamber slides at $6 \times 10^4/cm^2$ and treated for 6 h with Cu(isaepy)$_2$. At the end of treatment, they were stained with 50 nM of MitoTracker Red® in combination with an anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed and fixed with 4% paraformaldehyde. Images of cells were acquired and digitized with a Delta Vision Restoration Microscopy System (Applied Precision Inc., Issaquah, WA) equipped with an Olympus IX70 fluorescence microscope. Alternatively, after 6h of treatment with Cu(isaepy)$_2$ or 12 h of SNP incubation, cells were stained with 50 nM of MitoTracker Red® for further

30 min, washed, trypsinised, re-suspended in PBS and cytofluorometrically analysed.

ATP measurement

Cells were lysed and incubated in 100 mM Tris-Hcl, 4 mM EDTA, pH 7.75 for 2 min at 100° C. ATP levels were then measured by the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science, Milan, Italy) using a microplate luminometer (Perkin Elmer, Milan, Italy) after incubation with the luciferin/luciferase reagents.

Measurement of ROS levels and carbonylated proteins

Intracellular ROS content was determined by flow cytometry using the cell permeable ROS-specific probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen-Molecular Probes) according to the manufacturer's instructions. Briefly, cells were incubated with 50 μM DCFH-DA for 30 minutes at 37°C, after which they were washed, resuspended in PBS, scraped and immediately analyzed by flow cytometry using a 488-nm excitation beam and a 538-nm band-pass filter.

Carbonylated proteins were detected using the Oxyblot Kit (Intergen Purchase, NY) after reaction with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25 °C. Samples were then resolved by 10% SDS-PAGE and DNP-derivatized proteins were identified by immunoblot using an anti-DNP antibody.

Measurement of glutathione by HPLC

Intracellular reduced (GSH) and oxidized (GSSG) forms of the tripeptide glutathione were assayed upon formation of *S*-carboxymethyl derivatives of free thiols with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene as described by Reed et al. (Reed et al., 1980). Briefly, cells were washed three times with PBS, resuspended in a reducing buffer composed by PBS:HCl 0.01M (1:1 v/v) and lysed by repeated cycles of freezing and thawing under liquid nitrogen. Proteins were then precipitated by adding 5% meta-phosphoric

acid (MPA). After centrifugation at 22300 g for 15 min, low molecular weight thiols were derivatized by adding 10% iodoacetic acid (IAA) and $NaHCO_3$. After 1h incubation in the dark, samples were incubated for 12h with 1.5% (v/v) 1-fluoro-2,4-dinitrobenzene (Sanger reagent) and resolved by HPLC on μBondpack NH_2, 3.9 × 300 mm (Waters) coloumn. Data are expressed as nanomoles of GSH and GSSG per milligrams of protein.

Measurement of glucose uptake

Cells were incubated for 15, 30 and 60 min with 100 μM of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent analogue of the non-metabolizable 2-deoxy-D-glucose, in the presence or absence of 1 μM catalase. Cells were exhaustively washed with PBS to stop 2-NBDG uptake, collected and fluorescence analyzed by flow cytometry using a 488-nm excitation beam and a 538-nm band-pass filter. Data are expressed as percentage of 2-NBDG uptake decrease with respect to untreated cells.

Extracellular lactate assay

500 μl of medium was collected, proteins precipitated by the addition of 250 μl of 30% ice-cold trichloroacetic acid and centrifugatied at 5,000 × g for 5 minutes. 20 μl of each sample was incubated for 30 min at room temperature in 580 μl of a 0.2 M glycine/hydrazine buffer, pH 9.2, containing 0.6 mg/ml NAD^+ and 17 U/ml lactate dehydrogenase. NAD^+ reduction was spectrophotometrically followed at 340 nm and nmoles of NADH formed were considered stoichiometrically equivalent to extracellular lactate.

Detection of external plasma-membrane thiols

Cells were incubated for 1 h with 100 μM BH4, washed, and incubated with 10 μM Alexa fluor-488® C5-maleimide (Invitrogen) for 15 min at 37 °C in PBS. Cells were detached, pelletted, washed and resuspended in PBS. Labelled thiols were detected by cytofluorimetric analyses; alternatively, cells were grown on chamberslides and visualized by fluorescence microscopy after being exhaustively washed with PBS. Images of cells were

rapidly digitized with a Cool Snap video camera connected to Nikon Eclipse TE200 epifluorescence microscopy. All images were captured under constant exposure time, gain and offset.

Measurement of nitrite and nitrate concentration

NO released from SNP was indirectly quantified by measuring the oxidation by-products nitrites and nitrates (NO_x). Analyses of NO_x concentration were done either in water or cell media by the reaction with the Griess reagent according to Kotsonis *et al.* (Kotsonis et al. 1999). Total NO_x were measured upon nitrate reductase-mediated reduction of nitrates. The concentration of NO_x was determined by a standard curve obtained with known amount of sodium nitrite and expressed as μM.

Data presentation

All experiments were done at least three different times unless otherwise indicated. Data are expressed as means ± S.D. and significance was assessed by Student's *t* test corrected by Bonferroni's method. Differences with *p* values < 0.05 were considered significant.

AIM OF THE WORK

Disruption of redox homeostasis plays a pivotal role in several pathological conditions such as neurodegeneration and cancer. In these settings, oxidative stress is a primary initiating event or a direct consequence of a bioenergetic failure that results in the impairment of many cellular functions, leading to cell death. On the basis of the tight relation between redox state and cellular bioenergetics, growing interest has been achieving by the molecular mechanisms responsible for the control of cell fate upon oxidative stress-dependent modification of energy metabolism. A pivotal role in the modulation of bioenergetic demands is played by AMPK, which regulate energy-consumptive processes, such as cell growth and apoptosis by modulating several signaling proteins, including p53 and the MAPK pathway. In keeping with its pivotal role of bioenergetic sensor, AMPK can be considered as a molecular target of a feasible therapeutic strategy for diseases based on cell proliferation such as cancer. Despite its attractive implication in this context, several studies suggest that consequences of persistent AMPK activation in non renewing tissues, such as neurons, could not be beneficial to cell survival, due to the induction of a detrimental 'metabolic failure-like' state.

On the basis of this background, the aim of this PhD work is to elucidate the role of AMPK in neuronal apoptosis under pro-oxidant conditions linked to bioenergetic impairment. The investigations have been performed by treating the dopaminergic SH-SY5Y neuroblastoma cell line with three compounds known to influence cellular redox homeostas: Bis[(2-oxindol-3-ylimino)-2-(2-aminoethyl)pyridine-N,N']copper(II) perchlorate (Cu(isaepy)$_2$), tetrahydrobiopterin (BH4) and sodium nitroprusside (SNP). The different action of these chemicals on redox state and cellular metabolism was used to investigate the contribution of mitochondrial functionality and glucose uptake efficiency on AMPK activation and to study its role in the engagement of stress-activated pro-apoptotic signaling pathways.

RESULTS

PART I

Previous studies carried out in our laboratory characterized the pro-apoptotic activity of the Bis[(2-oxindol-3-ylimino)-2-(2-aminoethyl)pyridine N,N']copper(II), named Cu(isaepy)$_2$, an isatin-Schiff base copper(II) complex, and demonstrated that it is highly efficient in the induction of death in SH-SY5Y neuroblastoma cells via the mitochondrial route (Filomeni et al., 2007). These investigations provided clear-cut evidence that this copper complex induces intracellular ROS accumulation and damage to specific cellular compartments such as nuclei and mitochondria. Moreover, the positive charge of the complex and the preferential accumulation of copper within the mitochondrial compartment, allowed to suggest a role for Cu(isaepy)$_2$ as DLC-like molecule. On the basis of this evidence, the first part of my PhD work was aimed to clarify the anticancer properties of Cu(isaepy)$_2$, investigating its mitochondriotoxic action and the molecular mechanisms responsible for the final induction of apoptosis.

Cu(isaepy)$_2$ increases NADH-dependent oxygen consumption of isolated mitochondria independently on ADP

To characterize the site of action of Cu(isaepy)$_2$ in mitochondrial compartment we purified mitochondria from mouse liver and measured NADH-dependent oxygen consumption upon incubation with 50 μM of Cu(isaepy)$_2$, a concentration previously found to be pro-apoptotic in SH-SY5Y cells (Filomeni et al., 2007). Moreover we compared the effects of this complex with an equimolar concentration of the Zn(isaepy), an analogous isatin-Schiff base complex, in which copper is replaced by the non-redox active zinc ion (Fig. 1), that has been previously demonstrated to be only slightly effective in inducing citotoxicity (Filomeni et al., 2007). As shown in Fig. 2A only Cu(isaepy)$_2$ significantly increased NADH-dependent
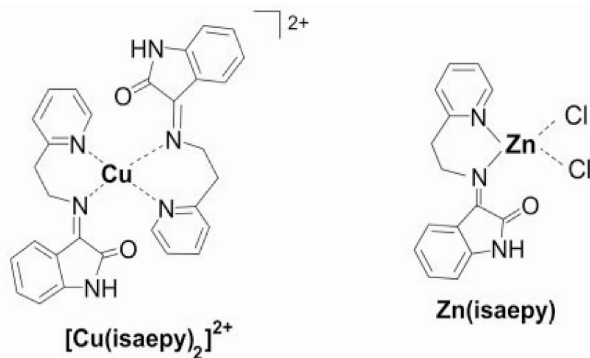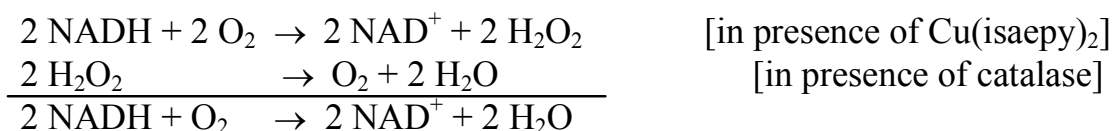

Figure 1
Chemical structures of Cu(isaepy)$_2$ and Zn(isaepy)

oxygen consumption of about 60% with respect to mitochondria incubated with vehicle alone; conversely, succinate-dependent respiration was completely unaffected (data not shown). To assess whether this increase in oxygen consumption was a phenomenon still responsive to ADP we measured state III/state IV ratio. Fig. 2B shows that, in the presence of Cu(isaepy)$_2$, oxygen consumption was insensitive to ADP addition. In particular, P:O decreased to values close to 1, indicating that Cu(isaepy)$_2$ behaved as an uncoupling molecule.

Previous investigations performed in our laboratory pointed out the pro-oxidant ability of Cu(isaepy)$_2$. Therefore, to assess whether Cu(isaepy)$_2$ complex dissipated NADH-deriving reducing equivalent for the partial reduction of oxygen to form ROS, mitochondria were incubated with Cu(isaepy)$_2$ in experimental buffer containing glutamate/malate and NADH in the presence of 0.5 U of catalase. By means of its ability in H$_2$O$_2$ disproportionation, the presence of catalase should have increased oxygen tension within the experimental buffer. In particular, if the reactions are:

$$2\ NADH + 2\ O_2\ \rightarrow\ 2\ NAD^+ + 2\ H_2O_2 \qquad [\text{in presence of Cu(isaepy)}_2]$$
$$2\ H_2O_2 \qquad\qquad \rightarrow\ O_2 + 2\ H_2O \qquad\qquad [\text{in presence of catalase}]$$
$$\overline{2\ NADH + O_2\quad\ \rightarrow\ 2\ NAD^+ + 2\ H_2O}$$

a net consumption of only 1 mol of oxygen per 2 moles of NADH occurred. Fig. 2C shows that the addition of catalase halved oxygen consumption, indicating that Cu(isaepy)$_2$-mediated NADH oxidation was responsible for O$_2^{\cdot-}$/H$_2$O$_2$ generation. We then evaluated which mitochondrial complex was involved in the increased oxygen consumption by Cu(isaepy)$_2$. To this aim, isolated mitochondria were incubated with Cu(isaepy)$_2$ in the presence of rotenone, antimycin A or KCN, which inhibit irreversibly complex I, III and IV, respectively. Fig. 2D shows that rotenone was the sole inhibitor able to influence the increase of oxygen consumption induced by Cu(isaepy)$_2$ (-18%), implying a direct, although not exclusive, involvement of complex I in this phenomenon.

Cu(isaepy)$_2$ oxidatively impairs mitochondrial functionality and induces bioenergetic failure in SH-SY5Y cells

The data obtained by isolated mitochondria indicated that Cu(isaepy)$_2$ behaved as an uncoupler by oxidizing NADH to produce oxy-radical
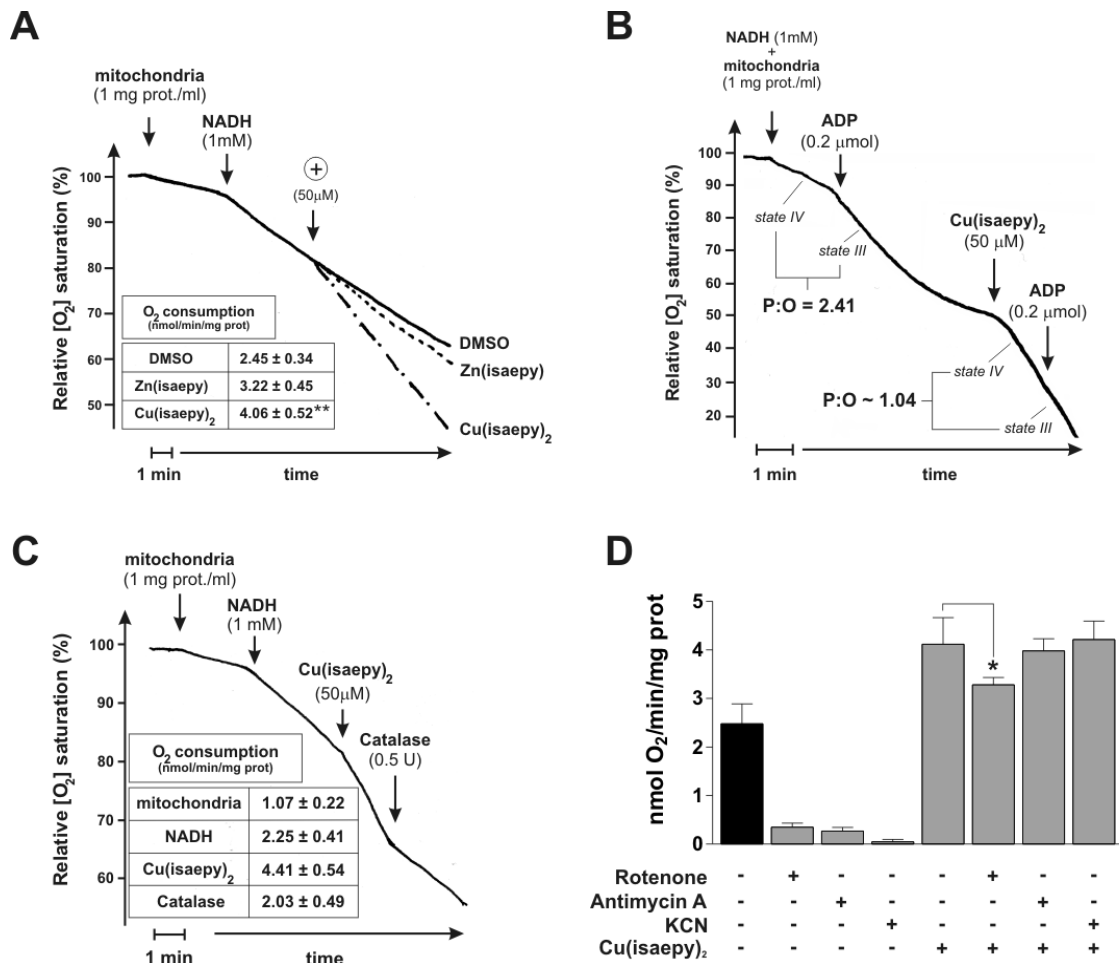
Figure 2
Effect of Cu(isaepy)₂ on isolated mitochondria

Purified mitochondria from mouse liver were used to measure oxygen consumption by a Clark-type oxygen electrode maintained at 25°C. *A. Cu(isaepy)₂ affects oxygen consumption.* 1 mM NADH, 50 mM Cu(isaepy)2, 50 mM Zn(isaepy) or DMSO (equal volumes) were added to mitochondria sequentially, as indicated. Traces shown are from one representative experiment out of ten that gave similar results. Data are expressed in the bottom table as nmoles of O_2 consumed·min^{-1}·mg protein^{-1} and represent the mean ± SD of n = 8 independent experiments; **$p < 0.01$. *B. Cu(isaepy)₂ affects state III/state IV ratio.* Trace shown and P:O values are from one representative experiment out of five that gave similar results. *C. Cu(isaepy)₂ induces ROS production.* 0.5 U catalase were added to mitochondria. Trace shown is from one representative experiment out of seven that gave similar results. Data are expressed in the bottom table as nmoles of O_2 consumed·min^{-1}·mg protein^{-1} and represent the mean ± SD of n = 5 independent experiments. *D. Cu(isaepy)₂ affects Complex I activity.* 5 mM rotenone, 5 mM antimycin A and 1 mM KCN were added to mitochondria. Data are expressed as nmoles of O_2 consumed·min^{-1}·mg protein^{-1} and represent the mean ± SD of n = 5 independent experiments; *$p < 0.05$.

63

species. To confirm its mitochondriotoxic properties in a cell system, we treated SH-SY5Y cells with 50 µM Cu(isaepy)$_2$ for 6 h and evaluated mitochondrial network by fluorescence microscopy. Fig. 3 shows images obtained by means of double staining with MitoTracker Red and a specific antibody against the mitochondrial specific chaperone Hsp60. Upon treatment with Cu(isaepy)$_2$ mitochondria appeared isolated and organized as dotted or fragmented, features predictive of mitochondrial fission and removal (Benard and Rossignol, 2008). Moreover, MitoTracker Red staining indicated that only few mitochondria maintained unaltered their
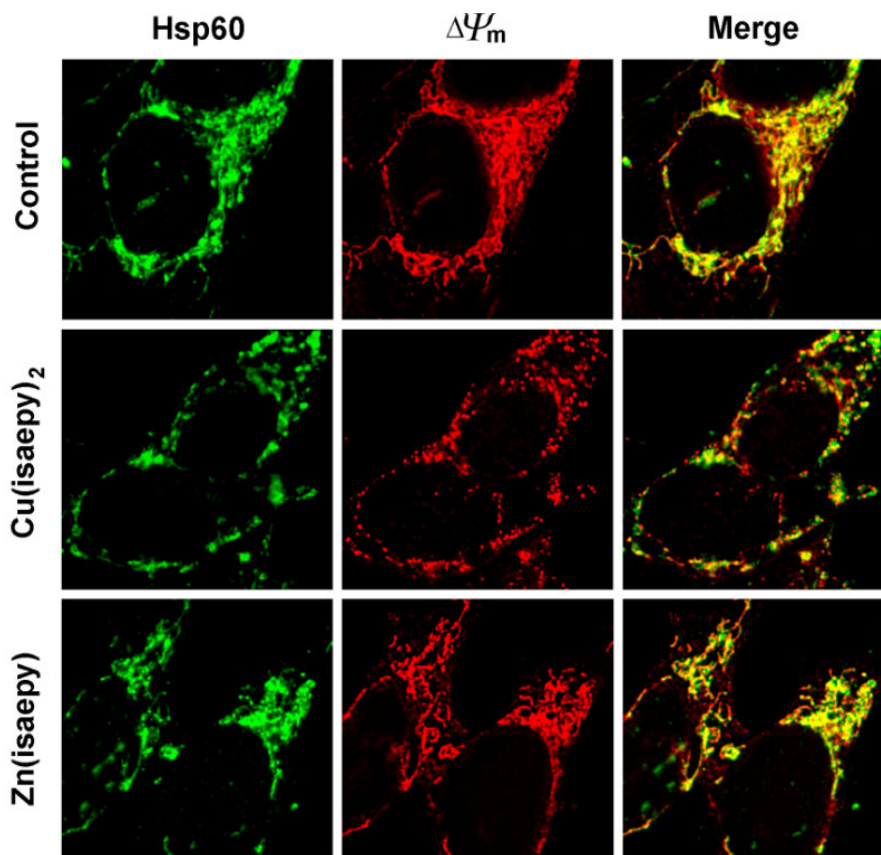
Figure 3
Effect of Cu(isaepy)$_2$ on mitochondrial network

SH-SY5Y cells were grown on chamber-slides and treated for 6 h with 50 µM Cu(isaepy)$_2$ or Zn(isaepy). Before fixation, cells were incubated for 30 min with 50 nM MitoTracker Red® and subsequently probed with an anti-Hsp60 specific antibody. Images of cells were digitized with a Delta Vision Restoration Microscopy System equipped with an Olympus IX70 fluorescence microscope.

transmembrane potential ($\Delta\Psi$m). By contrast, only slight effects were visible when the cells were treated with Zn(isaepy). In this case mitochondria maintained their reticular organization and $\Delta\Psi$m, confirming that copper was indispensable for Cu(isaepy)$_2$-induced mitocondriotoxicity. To investigate whether $\Delta\Psi$m loss was an effect downstream of mitochondrial electron transfer chain impairment we analysed, by Western blot, the intracellular content of complex I and IV. Fig. 4A shows that
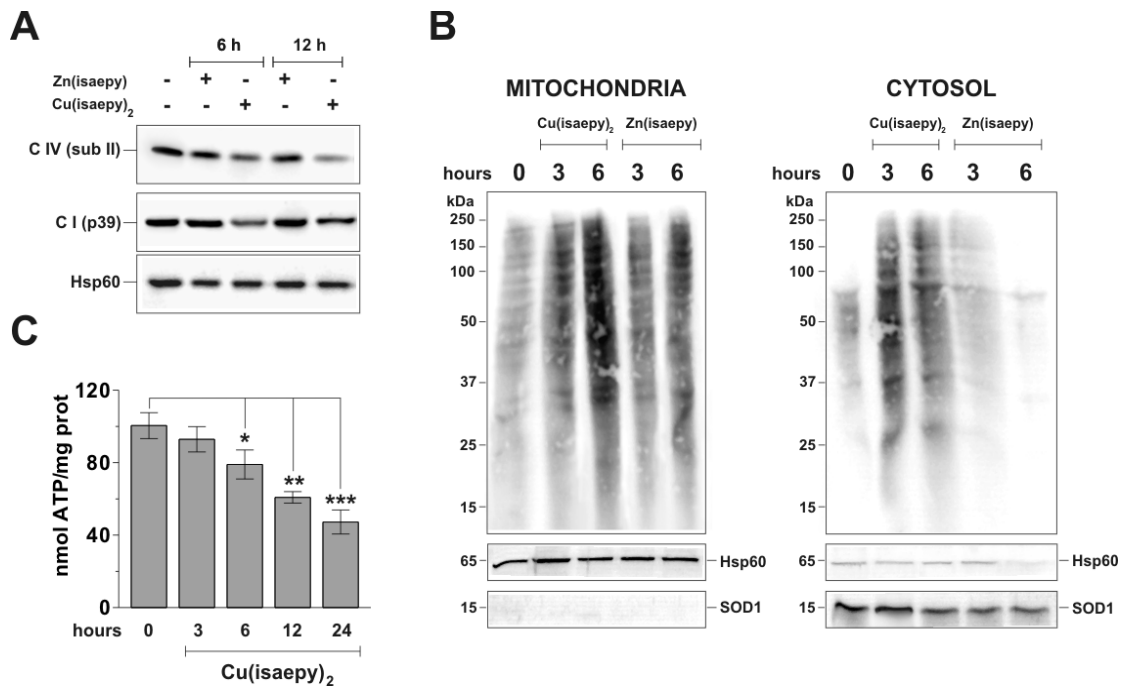

Figure 4
Structural/functional alterations in mitochondria of Cu(isaepy)$_2$-treated SHSY5Y cells

A. SH-SY5Y cells were treated for 6 and 12 h with 50 μM Cu(isaepy)$_2$ or Zn(isaepy). Fifteen μg of mitochondrial protein extracts was loaded onto each lane for detection of subunit II of cytochrome *c* oxidase, *C IV (sub II)*, and 39 kDa subunit of Complex I, *C I (p39)*. Hsp60 was used as loading/purity control. Immunoblots are from one experiment representative of three that gave similar results. *B.* SH-SY5Y cells were treated for 3 and 6 h with 50 μM Cu(isaepy)$_2$ or Zn(isaepy). Ten μg of mitochondrial proteins and 25 μg of cytosolic proteins were reacted with dinitrophenylhydrazine (DNP), resolved on 10% SDS-PAGE and DNP-derivatized proteins identified by Western blot using an anti-DNP antibody. SOD1 and Hsp60 were used as loading/purity controls. Immunoblots are from one experiment representative of three that gave similar results. *C.* SH-SY5Y cells were treated with 50 μM Cu(isaepy)$_2$. At indicated times, cells were harvested and used for ATP measurement. Data are expressed as % of control and represent the mean ± SD of n = 12 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

subunit II of cytochrome *c* oxidase was significantly reduced already after 6 h of treatment. The 39 kDa subunit of complex I was also affected by Cu(isaepy)$_2$ addition, although to a lesser extent, whereas no change in the levels of both proteins were observed upon treatment with Zn(isaepy). To examine the possibility that Cu(isaepy)$_2$-produced ROS at the mitochondrial level could be responsible for the decrease of complex I and IV, we measured the level of protein carbonyls, as by-products of ROS-mediated damage, both on cytosolic and mitochondrial-enriched fractions. Fig. 4B shows that Cu(isaepy)$_2$ yielded an earlier and higher increase in carbonyl levels than Zn(isaepy), in both fractions. Moreover, the trend of accumulation of the oxidised proteins was completely different in the two compartments. In particular, they increased time-dependently in the mitochondrial extracts, while were efficiently buffered in the cytosol, where proteasome-dependent degradation takes place efficiently. To assess if oxidative damages could impair mitochondrial function, ATP content was measured in SH-SY5Y cells. Fig 4C shows that ATP decreased time-dependently, starting from 6 h and reaching more than 50% decrement after 24 h of treatment with Cu(isaepy)$_2$, demonstrating that this complex was efficient in inducing both structural and functional mitochondrial defects.

To evaluate whether bioenergetic impairment could be responsible for apoptotic engagement, we increased glycolytic rate by supplementating SH-SY5Y cell medium with glucose up to a concentration of 30 mM. Alternatively, we added 10 mM sodium pyruvate or 50 mM methyl-succinate to supply the electron transfer chain with reducing equivalent, avoiding glycolysis. Fig 5 shows that, although at different extent, these

Figure 5
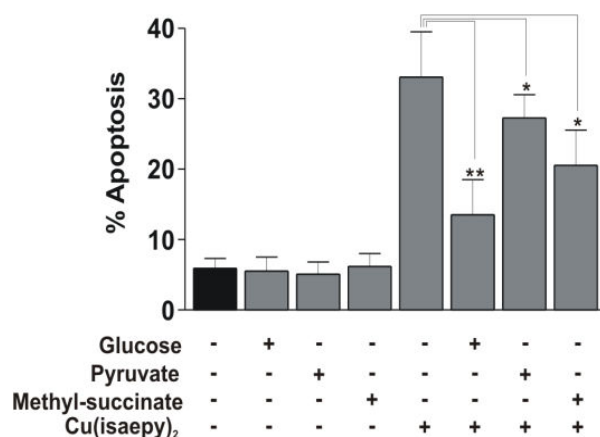*Effect of fuel supplies on Cu(isaepy)$_2$ induced apoptosis.*

SHSY5Y cells were treated for 24 h with 50 μM Cu(isaepy)$_2$ in the presence of 30 mM glucose, 50 mM methyl-succinate or 10 mM sodium pyruvate and cytofluorometrically analysed for the extent of apoptotic cells. Data are expressed as % of apoptosis and represent the mean ± SD of n = 4 independent experiments. *****, $p < 0.05$, ******, $p < 0.01$.

The results reported in this thesis have been published or submitted to 4 peer-reviewed journals:

1) **Cardaci, S**.*, Filomeni, G.*, Rotilio, G., and Ciriolo, M.R. (2008). Reactive oxygen species mediate p53 activation and apoptosis induced by sodium nitroprusside in SH-SY5Y cells. Mol. Pharmacol. *74*, 1234-1245.

2) Filomeni, G, Piccirillo, S, Graziani, I, **Cardaci, S**., Da Costa Ferreira, A.M., Rotilio, G., and Ciriolo, M.R. (2009). The isatin-Schiff base copper(II) complex Cu(isaepy)$_2$ acts as delocalized lipophilic cation, yields widespread mitochondrial oxidative damage and induces AMP-activated protein kinase-dependent apoptosis. Carcinogenesis [In press].

3) **Cardaci, S**.*, Filomeni, G.*, Rotilio, G., and Ciriolo, M.R. (2009). Activation of AMPK/p38^{MAPK}/p53 signaling axis mediates apoptosis in response to the isatin-Schiff base copper(II) complex Cu(isaepy)$_2$-induced bioenergetic stress. Biochem. J. [under review].

4) **Cardaci, S**.*, Filomeni, G.*, Rotilio, G., and Ciriolo, M.R. (2009). Tetrahydrobiopterin oxidatively affects glucose uptake in SH-SY5Y cells and elicits apoptosis through the activation of AMP-activated protein kinase/p38^{MAPK}/p53 signaling axis. Mol. Pharmacol. [under review].

* Equal contribution to the work

REFERENCES

Adler, V., Yin, Z., Fuchs, S.Y., Benezra, M., Rosario, L., Tew, K.D., Pincus, M.R., Sardana, M., Henderson, C.J., Wolf, C.R. et al. (1999). Regulation of JNK signaling by GSTp. EMBO J. *18*, 1321–1334.

Anderson, W.M., Delinck, D.L., Benninger, L., Wood, J.M., Smiley, S.T., and Chen, L.B. (1993). Cytotoxic effect of thiacarbocyanine dyes on human colon carcinoma cells and inhibition of bovine heart mitochondrial NADH-ubiquinone reductase activity via a rotenone-type mechanism by two of the dyes. Biochem. Pharmacol. *45*, 691-696.

Anderson, W.M., Patheja, H.S., Delinck, D.L., Baldwin, W.W., Smiley, S.T., and Chen, L.B. (1989). Inhibition of bovine heart mitochondrial and Paracoccus denitrificans NADH-ubiquinone reductase by dequalinium chloride and three structurally related quinolinium compounds. Biochem. Int. *19*, 673-685.

Arias, C., Montiel, T., Quiroz-Baez, R., and Massieu, L. (2002). beta-Amyloid neurotoxicity is exacerbated during glycolysis inhibition and mitochondrial impairment in the rat hippocampus in vivo and in isolated nerve terminals: implications for Alzheimer's disease. Exp. Neurol. *176*, 163–174.

Atamna, H., and Frey 2nd, W.H. (2004). A role for heme in Alzheimer's disease: heme binds amyloid beta and has altered metabolism. Proc. Natl. Acad. Sci. USA *101*, 11153–11158.

Barra, D., Schinina, M.E., Simmaco, M., Bannister, J.V., Bannister, W.H., Rotilio, G., and Bossa F. (1984). The primary structure of human liver manganese superoxide dismutase. J. Biol. Chem. *259*, 12595-12601.

Bellucci, A., Collo, G., Sarnico, I., Battistin, L., Missale, C., and Spano, P. (2008). Alpha-synuclein aggregation and cell death triggered by energy deprivation and dopamine overload are counteracted by D2/D3 receptor activation. J. Neurochem. *106*, 560-577.

Benassi, B., Fanciulli, M., Fiorentino, F., Porrello, A., Chiorino, G., Loda, M., Zupi, G., Biroccio, A. (2006)c-Myc phosphorylation is required for cellular response to oxidative stress. Mol. Cell. *21*, 509-19.

Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., Andersen, J.K. (2002). Glutathione, iron and Parkinson's disease. Biochem. Pharmacol. *64*, 1037-1048.

Brahimi-Horn, M.C., Chiche, J., and Pouyssegur, J. (2007). Hypoxia signaling controls metabolic demand. Curr. Opin. Cell Biol. *19*, 223–229.

Brustovetsky, N., Dubinsky, J.M., Antonsson, B., and Jemmerson, R. (2003). Two pathways for tBID-induced cytochrome c release from rat brain mitochondria: BAK- versus BAX-dependence. J. Neurochem. *84*, 196–207.

Bulavin, D.V., Saito, S., Hollander, M.C., Sakaguchi, K., Anderson, C.W., Appella, E., and Fornace, A.J. Jr. (1999). Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J. *18*, 6845-6854

Buzek, J., Latonen, L., Kurki, S., Peltonen, K., Laiho, M. (2002). Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. Nucleic. Acids. Res. *30*, 2340-2348.

Cao, C., Lu, S., Kivlin, R., Wallin, B., Card, E., Bagdasarian, A., Tamakloe, T., Chu, W.M., Guan, K.L., and Wan, Y. (2008). AMP-activated protein kinase contributes to UV- and H_2O_2-induced apoptosis in human skin keratinocytes. J. Biol. Chem. *283*, 28897-28908.

Carew, J.S., Zhou, Y., Albitar, M., Carew, J.D., Keating, M.J., and Huang P. (2003). Mitochondrial DNA mutations in primary leukemia cells after chemotherapy: clinical significance and therapeutic implications. Leukemia. *17*, 1437-1447.

Carling, D., Zammit, V. A., and Hardie D. G. (1987). A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett. *223*, 217–222.

Chen, J., Chang, B., Williams, M., and Murad, F. (1991). Sodium nitroprusside degenerates cultured rat striatal neurons. Neuroreport *2*,121–123.

Chen, Y., Zhou, K., Wang, R., Liu, Y., Kwak, Y.D., Ma, T., Thompson, R.C., Zhao, Y., Smith, L., Gasparini, L., Luo, Z., Xu, H., and Liao, F.F. (2008). Antidiabetic drug metformin (GlucophageR) increases biogenesis of Alzheimer's amyloid peptides via up-regulating BACE1 transcription. Proc. Natl. Acad. Sci. U S A. *106*, 3907-3912.

Cho, S., Volpe, B.T., Bae, Y., Hwang, O., Choi, H.J., Gal, J., Park, L.C., Chu, C.K., Du, J., and Joh, T.H. (1999). Blockade of tetrahydrobiopterin synthesis protects neurons after transient forebrain ischemia in rat: a novel role for the cofactor. J. Neurosci. *19*, 878–889.

Choi, H.J., Jang, Y.J., Kim, H.J., and Hwang, O. (2000). Tetrahydrobiopterin is released from and causes preferential death of catecholaminergic cells by oxidative stress. Mol. Pharmacol. *58*, 633-640.

Choi, H.J., Kim, S.W., Lee, S.Y., and Hwang, O. (2003). Dopamine-dependent cytotoxicity of tetrahydrobiopterin: a possible mechanism for selective neurodegeneration in Parkinson's disease. J. Neurochem. *86*, 143-152.

Chongthammakun, V., Sanvarinda, Y., and Chongthammakun, S. (2009). Reactive oxygen species production and MAPK activation are implicated in tetrahydrobiopterin-induced SH-SY5Y cell death. Neurosci. Lett. *449*, 178-182.

Ciriolo, M.R., De Martino, A., Lafavia, E., Rossi, L., Carrì, M.T., and Rotilio, G. (2000). Cu, Zn superoxide dismutase-dependent apoptosis induced by nitric oxide in neuronal cells. J. Biol. Chem. *275*, 5065–5072.

Conway, K.A., Rochet, J.C., Bieganski, R.M., Lansbury, P.T. Jr. (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science. *294*,1346-1349.

Cottrell, D.A., Blakely, E.L., Johnson, M.A., Ince, P.G., and Turnbull, D.M. (2001). Mitochondrial enzyme-deficient hippocampal neurons and choroidal cells in AD. Neurology *57*, 260–264.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., and Milzani, A. (2006). Biomarkers of oxidative damage in human disease. Clin. Chem. *52*, 601-623.

Dauer, W., and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. Neuron *39*, 889-909.

de Leon, M.J., Ferris, S.H., George, A.E., Christman, D.R., Fowler, J.S., Gentes, C., Reisberg, B., Gee, B., Emmerich, M., Yonekura, Y., et al. (1983a). Positron emission tomographic studies of aging and Alzheimer disease. AJNR Am. J. Neuroradiol. *4*, 568–571.

de Leon, M.J., George, A.E., Ferris, S.H., Rosenbloom, S., Christman, D.R., Gentes, C.I., Reisberg, B., Kricheff II, and Wolf, A.P. (1983b). Regional correlation of PET and CT in senile dementia of the Alzheimer type. AJNR Am. J. Neuroradiol. *4*, 553–556.

Dhalla, N.S., Temsah, R.M., and Netticadan, T. (2000). Role of oxidative stress in cardiovascular diseases. J. Hypertens. 18, 655-673.

Drane, P., Bravard, A., Bouvard, V., and May, E. (2001). Reciprocal down-regulation of p53 and SOD2 gene expression-implication in p53 mediated apoptosis. Oncogene. *20*, 430-439.

Duan, W., Zhu, X., Ladenheim, B., Yu, Q.S., Guo, Z., Oyler, J., Cutler, R.G., Cadet, J.L., Greig, N.H., and Mattson, M.P. (2002). p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. Ann. Neurol. *52*, 597-606.

Duan, W., Zhu, X., Ladenheim, B., Yu, Q.S., Guo, Z., Oyler, J., Cutler, R.G., Cadet, J.L., Greig, N.H., and Mattson, M.P. (2002). p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. Ann. Neurol. *52*, 597-606.

Eggler, A.L., Gay, K.A., and Mesecar, A.D. (2008). Molecular mechanisms of natural products in chemoprevention: induction of cytoprotective enzymes by Nrf2. Mol. Nutr. Food Res. *1*, 84-94.

Ernest, M.J., and Kim, K.H. (1973). Regulation of rat liver glycogen synthetase. Reversible inactivation of glycogen synthetase D by sulfhydryl-disulfide exchange. J. Biol. Chem. *248*, 1550-1555.

Fasano, M., Bergamasco, B., and Lopiano, L. (2006). Modifications of the iron-neuromelanin system in Parkinson's disease. J. Neurochem. *96*, 909-916.

Feng, Z., Li, L., Ng, P.Y., and Porter, A.G. (2002), Neuronal differentiation and protection from nitric oxide-induced apoptosis require c-Jun-dependent expression of NCAM140. Mol. Cell Biol. *22*, 5357–5366.

Ferrer, I., Blanco, R., Carmona, M., Puig, B., Barrachina, M., Gomez, C., and Ambrosio, S. (2001). Active phosphorylation-dependent mitogen-activated protein kinase (MAPK/ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 kinase expression in Parkinson's disease and Dementia with Lewy bodies. J. Neural. Transm. *108,* 1383–1396.

Filomeni, G., Cerchiaro, G., Da Costa Ferreira, A.M., De Martino, A., Pedersen, J.Z., Rotilio, G., and Ciriolo, M.R. (2007). Pro-apoptotic activity of novel Isatin-Schiff base copper(II) complexes depends on oxidative stress induction and organelle-selective damage. J. Biol. Chem. *282*, 12010-12021.

Filomeni, G., Rotilio, G., and Ciriolo, M.R. Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. (2005). Cell Death Differ *12*, 1555-1563.

Fridman, J.S and Lowe, S.W. (2003). Control of apoptosis by p53. Oncogene *22*, 9030-9040.

Fruman, D.A., and Edinger, A.L. Cancer therapy: staying current with AMPK. (2008). Biochem J. *412*, 3-5.

Fukuzawa, K., Inokami, Y., Tokumura, A., Terao, J., and Suzuki A. (1998). Rate constants for quenching singlet oxygen and activities for inhibiting lipidperoxidation of carotenoids and alpha-tocopherol in liposomes. *Lipids. 33*, 751-756.

Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., Downward, J., Latchman, D.S., Tabrizi, S.J., Wood, N.W. et al. (2009). PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. Mol. Cell. *33*, 627-638.

Garcia-Gil, M., Pesi, R., Perna, S., Allegrini, S., Giannecchini, M., Camici, M. and Tozzi, M. G. (2003). 5'-aminoimidazole-4-carboxamide riboside induces apoptosis in human neuroblastoma cells. Neuroscience *117*, 811–820.

Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J., and Morrison, R.S. (2000). p38 MAP kinase mediates bax

translocation in nitric oxide-induced apoptosis in neurons. J. Cell Biol. *150*, 335–347.

Gomez-Lazaro, M., Galindo, M.F., Concannon, C.G., Segura, M.F., Fernandez-Gomez, F.J., Llecha, N., Comella, J.X., Prehn, J.H., and Jordan, J. (2008). 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. J. Neurochem. *104*, 1599-1612.

Hall, A., Karplus, P.A., and Poole, L.B. (2009). Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. FEBS J. *276*, 2469-2477.

Halliwell, B., and Gutteridge, J.M.C. (1999). Free radicals in biology and medicine (3rd ed.) (Oxford University Press).

Han, J.H., Ahn, Y.H., Choi, K.Y., and Hong, S.H. (2009). Involvement of AMP-activated protein kinase and p38 mitogen-activated protein kinase in 8-Cl-cAMP-induced growth inhibition. J. Cell Physiol. *218*, 104-112.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat. Rev. Mol. Cell Biol. *8*, 774-785.

Huang, C., Ma, W.Y., Maxiner, A., Sun, Y., and Dong, Z. (1999). p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. J. Biol. Chem. *274*, 12229-12235.

Hwang, O., Choi, H.J., Park, S.Y. (1999). Up-regulation of GTP cyclohydrolase I and tetrahydrobiopterin by calcium influx. NeuroReport *10*, 3611-3614.

Hwang, J.T., Kim, Y.M., Surh, Y.J., Baik, H.W., Lee, S.K., Ha, J., and Park, O.J. (2006). Selenium regulates cyclooxygenase-2 and extracellular signal-regulated kinase signaling pathways by activating AMP-activated protein kinase in colon cancer cells. Cancer Res. *66*, 10057-10063.

Irani, K., Xia, Y., Zweier, J.L., Sollott, S.J., Der, C.J., Fearon, E.R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont P.J. (1997). Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science. *275*, 1649-1652.

Jones, R.G., Plas, D.R., Kubek, S, Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J., and Thompson, C.B. (2005). AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol. Cell *18*, 283–293.

Kamiguti, A.S., Serrander, L., Lin, K., Harris, R.J., Cawley, J.C., Allsup, D.J., Slupsky, J.R., Krause, K.H., and Zuzel, M. (2005). Expression and activity of NOX5 in the circulating malignant B cells of hairy cell leukemia. Immunol. 175, 8424-8430.

Karunakaran, S., Saeed, U., Mishra, M., Valli, R.K., Joshi, S.D., Meka, D.P., Seth, P., and Ravindranath, V. (2008). Selective activation of p38 mitogen-activated protein kinase in dopaminergic neurons of substantia nigra leads to

nuclear translocation of p53 in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice. Neurosci. *28,* 12500-12509.

Kaur, D., Yantiri, F., Rajagopalan, S., Kumar, J., Mo, J.Q., Boonplueang, R., Viswanath, V., Jacobs, R., Yang, L., Beal, M.F. et al. (2003). Genetic or pharmacological iron chelation prevents MPTP-induced neurotoxicity in vivo: a novel therapy for Parkinson's disease. Neuron. *37,* 899-909.

Kefas, B.A., Cai, Y., Ling, Z., Heimberg, H., Hue, L., Pipeleers, D., and Van de Casteele, M. (2003). AMP-activated protein kinase can induce apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase. J. Mol. Endocrinol. *30,* 151-161.

Kim, S.O., Merchant, K., Nudelman, R., Beyer, Jr. W.F., Keng, T., DeAngelo, J., Hausladen, A. and Stamler J.S. (2002). OxyR: a molecular code for redox-related signaling. Cell *10*9, 383–396.

Kim, S.T., Chang, J.W., Choi, J.H., Kim, S.W., and Hwang, O. (2005). Immobilization stress causes increases in tetrahydrobiopterin, dopamine, and neuromelanin and oxidative damage in the nigrostriatal system. J. Neurochem. *95,* 89-98.

Kim, S.T., Chang, J.W., Hong, H.N., and Hwang, O. (2004). Loss of striatal dopaminergic fibers after intraventricular injection of tetrahydrobiopterin in rat brain. Neurosci. Lett. *359*: 69-72.

Kim, S.W., Jang, Y.J., Chang, J.W., and Hwang, O. (2003). Degeneration of the nigrostriatal pathway and induction of motor deficit bytetrahydrobiopterin: an in vivo model relevant to Parkinson's disease. Neurobiol. Dis. *13,* 167-176.

Kirkman, H.N., Galiano, S., and Gaetani G.F. (1987). The function of catalase-bound NADPH. J. Biol. Chem. *15,* 660-666.

Kirsch, M., Korth, H.G., Stenert, V., Sustmann, R., and de Groot, H. (2003). The autoxidation of tetrahydrobiopterin revisited. Proof of superoxide formation from reaction of tetrahydrobiopterin with molecular oxygen. J. Biol. Chem. *278,* 24481-24490.

Klintworth, H., Newhouse, K., Li, T., Choi, W.S., Faigle, R., and Xia, Z. (2007). Activation of c-Jun N-terminal protein kinase is a common mechanism underlying paraquat- and rotenone-induced dopaminergic cell apoptosis. Toxicol. Sci. *97,* 149-162.

Knott, A.B., and Bossy-Wetzel, E. (2008). Nitric Oxide in health and disease of the nervous system. Antioxid. Redox. Signal. (In Press).

Kontos, H.A., Wei, E.P., Ellis, E.F., Jenkins, L.W., Povlishock, J.T., Rowe, G.T., and Hess, M.L. (1985). Appearance of superoxide anion radical in

cerebral extracellular space during increased prostaglandin synthesis in cats. Circ. Res. *57*,142-151.

Kotsonis, P., Frey, A., Fröhlich, L.G., Hofmann, H., Reif, A., Wink, D.A., Feelisch, M. and Schmidt, H.H. (1999). Autoinhibition of neuronal nitric oxide synthase: distinct effects of reactive nitrogen and oxygen species on enzyme activity. Biochem. J. *340*, 745-752.

Koukourakis, M.I., Giatromanolaki, A., Harris, A.L., and Sivridis, E. (2006). Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. Cancer Res. *66*, 632–637.

Kyriakis, J.M. and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. *81*, 807–869.

Kumar, B., Koul, S., Khandrika, L., Meacham, R. B., and Koul, H. K. (2008). Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. Cancer Res. *68*, 1777–1785.

Kumar, S., Boehm, J., and Lee, J.C. (2003). p38 MAP kinases: key signaling molecules as therapeutic targets for inflammatory diseases. Nat. Rev. Drug. Discov. *2*, 717–726.

Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W.W. (1999). 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes *48*, 1667–1671.

Langston, J.W., and Ballard, P.A. Jr. (1983). Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N. Engl. J. Med. *309*, 310.

Lee, Y., and Shacter, E. (1999). Oxidative stress inhibits apoptosis in human lymphoma cells. J. Biol. Chem. *274*, 19792–19798.

Lee, S.Y., Moon, Y., Hee Choi, D., Jin Choi, H., and Hwang, O. (2007). Particular vulnerability of rat mesencephalic dopaminergic neurons to tetrahydrobiopterin: Relevance to Parkinson's disease. Neurobiol. Dis. *25,* 112-120.

Lee, Y.M., Uhm, K.O., Lee, E.S., Kwon, J., Park, S.H., Kim, H.S. (2008). Biochem. Biophys. Res. Commun. *370*, 641-645. AM251 suppresses the viability of HepG2 cells through the AMPK (AMP-activated protein kinase)-JNK (c-Jun N-terminal kinase)-ATF3 (activating transcription factor 3) pathway.

Li, J., Zeng, Z., Viollet, B., Ronnett, G.V., and McCullough, L.D. (2007). Neuroprotective effects of adenosine monophosphate-activated protein kinase inhibition and gene deletion in stroke. Stroke. *38*, 2992-2999.

Li, L., Feng, Z., and Porter, A.G. (2004). JNK-dependent phosphorylation of c-Jun on serine 63 mediates nitric oxide-induced apoptosis of neuroblastoma cells. J. Biol. Chem. *279*,4058–4065.

Liou, W., Chang, L.Y., Geuze, H.J., Strous, G.J., Crapo, J.D., and Slot J.W. (1993). Distribution of CuZn superoxide dismutase in rat liver. Free Radic. Biol. Med. *14*, 201-217.

Lotharius, J., and Brundin, P. (2002). Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease. Hum. Mol. Genet. *11*, 2395-2407.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Ma, Y., and Eidelberg, D. (2007). Functional imaging of cerebral blood flow and glucose metabolism in Parkinson's disease and Huntington's disease. Mol. Imaging. Biol. *9*, 223-233.

Mark, R.J., Pang, Z., Geddes, J.W., and Uchida, K. (1997). Mattson, M.P. Amyloid beta-peptide impairs glucose transport in hippocampal and cortical

Marklund, S.L. (1982). Human copper-containing superoxide dismutase of high molecular weight. Proc. Natl. Acad. Sci. U S A. *79*, 7634-7638.

Maurer, I., Zierz, S., and Moller, H.J. (2000). A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiol. Aging *21*, 455–462.

McCullough, L.D., Zeng, Z., Li, H., Landree, L.E., McFadden, J., and Ronnett, G.V. (2005). Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. J. Biol. Chem. *280*, 20493-20502.

Meisse, D., Van de Casteele, M., Beauloye, C., Hainault, I., Kefas, B.A., Rider, M.H., Foufelle, F., and Hue, L. (2002). Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. FEBS Lett. *526*, 38-42.

Memmott, R.M., Gills, J.J., Hollingshead, M., Powers, M.C., Chen, Z., Kemp, B., Kozikowski, A., and Dennis, P.A. (2008). Phosphatidylinositol ether lipid analogues induce AMP-activated protein kinase-dependent death in LKB1-mutant non small cell lung cancer cells. Cancer Res. *68*, 580-588.

Merrill, G. F., Kurth, E. J., Hardie, D. G. and Winder, W.W. (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. *273*, E1107–E1112.

Modica-Napolitano, J.S., and Aprille, J.R. (1987). Basis for the selective cytotoxicity of rhodamine 123. Cancer Res. *47*, 4361-4365.

Modica-Napolitano, J.S., and Aprille, J.R. Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. (2001). Adv. Drug. Deliv. Rev. *49*, 63-70.

Mutisya, E.M., Bowling, A.C., and Beal, M.F. (1994). Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J. Neurochem. *63*, 2179–2184.

Nagata, S. (1999). Fas ligand-induced apoptosis. Annu. Rev. Genet. *33*, 29–55.

Nakamura, K., Wang, W., and Kang, U.J. (1997). The role of glutathione in dopaminergic neuronal survival. J. Neurochem. *69*, 1850-1858.

Newhouse, K., Hsuan, S.L., Chang, S.H., Cai, B., Wang, Y., and Xia, Z. (2004). Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. Toxicol. Sci. *79*, 137-146.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Meth. *139*, 271-279.

Nordberg, J., and Arnér E.S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic. Biol. Med. *31*, 1287-1312. of cytochrome c from mitochondria blocked. *Science 275*, 1129–1132.

Okada, K., Wangpoengtrakul, C., Osawa, T., Toyokuni, S., Tanaka, K., and Uchida, K. (1999). 4-Hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress. Identification of proteasomes as target molecules. J. Biol. Chem. *274*, 23787-23793.

Okoshi, R., Ozaki, T., Yamamoto, H., Ando, K., Koida, N., Ono, S., Koda, T., Kamijo, T., Nakagawara, A., and Kizaki, H. (2008) Activation of AMP-activated protein kinase induces p53 dependent apoptotic cell death in response to energetic stress. J. Biol. Chem. *283*, 3979-3987.

Ow, Y.P., Green, D.R., Hao, Z., and Mak, T.W. (2008). Cytochrome c: functions beyond respiration. Nat. Rev. Mol. Cell Biol. *9*, 532-542.

Pan, W., Yang, H., Cao, C., Song, X., Wallin, B., Kivlin, R., Lu, S., Hu, G., Di, W., and Wan, Y. (2008). AMPK mediates curcumin-induced cell death in CaOV3 ovarian cancer cells. Oncol. Rep. *20*, 1553-1559.

Pappolla, M.A., Omar, R.A., Kim, K.S., and Robakis, N.K. (1992). Immunohistochemical evidence of oxidative stress in Alzheimer's disease. Am. J. Pathol. *140*, 621-628.

Parker, Jr. W.D., Parks, J., Filley, C.M., and Kleinschmidt-DeMasters, B.K. (1994). Electron transport chain defects in Alzheimer's disease brain. Neurology *44*, 1090–1096.

Parone, P.A., James, D., and Martinou, J.C. (2002). Mitochondria: regulating the inevitable. Biochimie *84*,105-111.

Patel, B.P., Rawal, U.M., Dave, T.K., Rawal, R.M., Shukla, S.N., Shah, P.M., Patel, P.S. (2007). Lipid peroxidation, total antioxidant status, and total thiol levels predict overall survival in patients with oral squamous cell carcinoma. Integr. Cancer Ther. 6, 365-372.

Pelicano, H., Carney, D., and Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. Drug Resist. Updat. *7*, 97–110.

Perier, C., Bové, J., Wu, D.C., Dehay, B., Choi, D.K., Jackson-Lewis, V., Rathke-Hartlieb, S., Bouillet, P., Strasser, A. et al. (2007). Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. Proc. Natl. Acad. Sci. USA. *104***, 8161–8166.

Pesi, R., Micheli, V., Jacomelli, G., Peruzzi, L., Camici, M., Garcia-Gil, M., Allegrini, S. and Tozzi, and M. G. (2000). Cytosolic 5'-nucleotidase hyperactivity in erythrocytes of Lesch-Nyhan syndrome patients. Neuroreport *11*, 1827–1831.

Piccirillo, S., Filomeni, G., Brune, B., Rotilio, G., and Ciriolo, MR. (2009). Redox mechanisms involved in the selective activation of Nrf2-mediated resistance versus p53-dependent apoptosis in adenocarcinoma gastric cells. J. Biol. (In Press).

Pileblad, E., Magnusson, T., Fornstedt, B. (1989). Reduction of brain glutathione by L-buthionine sulfoximine potentiates the dopamine-depleting action of 6-hydroxydopamine in rat striatum. J. Neurochem. *52*, 978-980.

Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. Nature. *389*, 300-305.

Ramakrishna Rao, D.N., and Cederbaum, A.I. (1996). Generation of reactive oxygen species by the redox cycling of nitroprusside. Biochim. Biophys. Acta *1289***, 195–202.

Rauhala, P., Khaldi, A., Mohanakumar, K.P., and Chiueh, C.C. (1998). Apparent role of hydroxyl radicals in oxidative brain injury induced by sodium nitroprusside. Free Radic Biol Med *24***, 1065–1073.

Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., and Potter, D.W. (1980). High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. Anal. Biochem. *106*, 55-62.

Reiman, E.M., Caselli, R.J., Yun, L.S., Chen, K., Bandy, D., Minoshima, S., Thibodeau, S.N., Osborne, D. (1996). Preclinical evidence of Alzheimer's

disease in persons homozygous for the epsilon 4 allele for apolipoprotein E. N. Engl. J. Med. *334*, 752–758.

Richter, C., Schweizer, M., Cossarizza, A., and Franceschi, C. (1996). Control of apoptosis by cellular ATP level. FEBS Lett. *378*, 107–110. 1996.

Rivera, A., and Maxwell, S.A. (2005). The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway. J. Biol. Chem. *280*, 29346-29354.

Rossi, L., Aquilano, K., Filomeni, G., Lombardo, M.F., Rotilio, G., and Ciriolo, M.R. (2004). Putative Mechanisms of Apoptosis Related to Redox Unbalance. In: *"Frontiers in Neurodegenerative Disorders and Aging: Fundamental Aspects, Clinical Perspectives and New Insights."* pp. 207-250. NATO Sciences Series, IOS Press (Ozben K. and Chevion M. Eds.)

Ronnett, G.V., Ramamurthy, S., Kleman, A.M., Landree, L.E., and Aja, S. (2009). AMPK in the brain: its roles in energy balance and neuroprotection. J. Neurochem. *109*, 17-23.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). Nat Med. *11*, 1306-1313.

Saito, Y., and Takahashi, K. (2002). Characterization of selenoprotein P as a selenium supply protein. Eur. J. Biochem. *269*, 5746-5751.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K. and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. 17, 2596-2606.

Saporito, M.S., Thomas, B.A., and Scott, R.W. (2000). MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. J. Neurochem. *75*, 1200-1208.

Sayre, L.M., Smith, M.A., and Perry, G. (2001). Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr Med Chem. 8, 721-738.

Schafer, F.Q., and Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 30, 1191-1212.

Schneider, B. L. and Kulesz-Martin, M. (2004). Destructive cycles: the role of genomic instability and adaptation in carcinogenesis. Carcinogenesis *25*, 2033–2044.

Searle, J., Kerr, F. J., and Bishop, C. J. (1982). Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol. Annu. *17*, 229–259.

Seres, T., Ravichandran, V., Moriguchi, T., Rokutan, K., Thomas, J.A., and Johnston, R.B. Jr. (1996). Protein S-thiolation and dethiolation during the

respiratory burst in human monocytes. A reversible post-translational modification with potential for buffering the effects of oxidant stress. J Immunol. *156*, 1973-1980.

Shresta, S., Pham, C.T., Thomas, D.A., Graubert, T.A., and Ley, T.J. (1998). How do cytotoxic lymphocytes kill their targets? Curr. Opin. Immunol. *10*, 581-587.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., and Marsden, C.D. (1994). Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann. Neurol. *36, 348*-355.

Siems, W. G., Grune, T., and Esterbauer, H. (1995). 4-Hydroxynonenal formation during ischemia and reperfusion of rat small-intestine. Life Sci. *57*, 785–789.

Simpson, I.A., Dwyer, D., Malide, D., Moley, K.H., Travis, A., and Vannucci, S.J. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. Am. J. Physiol. Endocrinol. Metab. *295*, 242-253.

Small, G.W., Ercoli, L.M., Silverman, D.H., Huang, S.C., Komo, S., Bookheimer, S.Y., Lavretsky, H., Miller, K., Siddarth, P., Rasgon, N.L. et al. (2000). Cerebral metabolic and cognitive decline in persons at genetic risk for Alzheimer's disease. Proc. Natl. Acad. Sci. USA *97*, 6037–6042.

Stadtman, E. R. (2004). Role of oxidant species in aging. Curr. Med. Chem. *11*, 1105–1112.

Steinberg, G.R, and Kemp, B.E. (2009). AMPK in Health and Disease. Physiol Rev. *89*, 1025-1078.

Sun, X., Wong, J.R., Song, K., Hu, J., Garlid, K.D., and Chen, L.B. (1994). AA1, a newly synthesized monovalent lipophilic cation, expresses potent in vivo antitumor activity. Cancer Res. *54*, 1465-1471.

Thomas, J.A., Poland, B., and Honzatko, R. (1995). Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. *319*, 1-9.

Toulouse, A., and Sullivan, A.M. (2008). Progress in Parkinson's disease-where do we stand? Prog. Neurobiol. *85*, 376-392.

Trachootham, D., Alexandre, J., and Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug. Discov. *8*, 579-591.

Trimmer, P.A., Smith, T.S., Jung, A.B., Bennett, J.P. Jr. (1996). Dopamine neurons from transgenic mice with a knockout of the p53 gene resist MPTP neurotoxicity. Neurodegeneration *5*, 233-239.

Tsao, S. M., Yin, M. C. and Liu, W. H. (2007). Oxidant stress and B vitamins status in patients with non-small cell lung cancer. Nutr. Cancer *59*, 8–13.

Urano, S., Inomori, Y., Sugawara, T., Kato, Y., Kitahara, M., Hasegawa, Y., Matsuo, M., and Mukai K. (1992). Vitamin E: inhibition of retinol-induced hemolysis and membrane-stabilizing behavior. J. Biol. Chem*. 267*, 18365-70.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell. Biol. 39, 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. *160*, 1-40.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. *324*, 1029-1033.

Volles, M.J., and Lansbury, P.T. Jr. (2002). Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. Biochemistry. *41*, 4595-4602.

Vousden, K.H., and Lane, D.P. (2007). p53 in health and disease. Nat. Rev. Mol. Cell. Biol. *8*, 275–283.

Wang, M. Y., Dhingra, K., Hittelman, W. N., Liehr, J. G., deAndrade, M.,&Li, D. H. (1996). Lipid peroxidation-induced putative malondialdehyde–DNA adducts in human breast tissues. Cancer Epidemiol. Biomark. Prev. *5*, 705–710.

Wang, W., Shi, L., Xie, Y., Ma, C., Li, W., Su, X., Huang, S., Chen, R., Zhu, Z., Mao, Z. et al. (2004). SP600125, a new JNK inhibitor, protects dopaminergic neurons in the MPTP model of Parkinson's disease. Neurosci. Res. *48*, 195-202.

Warburg, O., Posener, K., and Negelein, E. (1924). U¨ ber den Stoffwechsel derTumoren. Biochem. Z. *152*, 319–344.

Watanabe, A., Tabeta, K., and Kosaka, K. (1972). Glutathione-dependent interconversion of microheterogeneous forms of glucose-6-phosphate dehydrogenase in rat liver. J. Biochem. *72*, 695-701.

Winder, W.W., and Hardie D.G. (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am. J. Physiol. *270*, E299–E304.

Wolf, B.B., Schuler, M., Echeverri, F., and Green, D.R. (1999). Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation

factor-45/inhibitor of caspase-activated DNase inactivatio. J. Biol. Chem. *274*, 30651-30656.

Wüllner, U., Löschmann, P.A., Schulz, J.B., Schmid, A., Dringen, R., Eblen, F., Turski, L., and Klockgether, T. (1996). Glutathione depletion potentiates MPTP and MPP+ toxicity in nigral dopaminergic neurones. Neuroreport. *22*, 921-923.

Xu, R.H., Pelicano, H., Zhou, Y., Carew, J.S., Feng, L., Bhalla, K.N., Keating, M.J., and Huang, P. (2005). Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. *65*, 613-621.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science 275*, 1129–1132.

Young, T.W., Mei, F.C., Yang, G., Thompson-Lanza, J.A., Liu, J., Cheng, X. (2004). Activation of antioxidant pathways in ras-mediated oncogenic transformation of human surface ovarian epithelial cells revealed by functional proteomics and mass spectrometry. Cancer Res. *64*,4577-4584.

Yu, J., and Zhang, L. (2005). The transcriptional targets of p53 in apoptosis control. Biochem. Biophys. Res. Commun. *331*, 851–858.

Zemlan, F.P., Thienhaus, O.J., and Bosmann, H.B. (1989). Superoxide dismutase activity in Alzheimer's disease: possible mechanism for paired helical filament formation. Brain Res. *476*, 160-162.

Zhang, J., Xie, Z., Dong, Y., Wang, S., Liu, C., and Zou, M.H. (2008). Identification of nitric oxide as an endogenous activator of the AMP activated protein kinase in vascular endothelial cells. J. Biol. Chem. *283*, 27452-27461.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. Science *279*, 1718–1721.

Zhou, Y., Hileman, E. O., Plunkett, W., Keating, M. J. and Huang, P. (2003). Free radical stress in chronic lymphocytic leukemia cells and its role in cellular sensitivity to ROSgenerating anticancer agents. *Blood 101*, 4098–4104.

Zhu, X., Lee, H.G., Raina, A.K., Perry, G., and Smith, M.A. (2002). The role of mitogen-activated protein kinase pathways in Alzheimer's disease. Neurosignals *11*, 270-281.

Zou, M.H., Kirkpatrick, S.S., Davis, B.J., Nelson, J.S., Wiles, W.G. 4th, Schlattner, U., Neumann, D., Brownlee, M., Freeman, M.B., Goldman, M.H. (2004). Activation of the AMP-activated protein kinase by the anti-diabetic

drug metformin in vivo. Role of mitochondrial reactive nitrogen species. J. Biol. Chem. *279*,43940-43951.