Review

Reactive oxygen species and the mitochondrial signaling pathway of cell death

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Summary. Reactive oxygen species (ROS) are produced as a by-product of cellular metabolic pathways and function as a critical second messenger in a variety of intracellular signaling pathways. Thus, a defect or deficiency in the anti-oxidant defense system on the one hand and/or the excessive intracellular generation of ROS on the other renders a cell oxidatively stressed. As a consequence, direct or indirect involvement of ROS in numerous diseases has been documented. In most of these cases, the deleterious effect of ROS is a function of activation of intracellular cell-death circuitry. To that end, involvement of ROS at different phases of the apoptotic pathway, such as induction of mitochondrial permeability transition and release of mitochondrial death amplification factors, activation of intracellular caspases and DNA damage, has been clearly established. For instance, the ROS-induced alteration of constitutive mitochondrial proteins, such as the voltage-dependent anion channel (VDAC) and/or the adenine nucleotide translocase (ANT) can induce the pro-apoptotic mitochondrial membrane permabilization. Not only do these observations provide insight into the intricate mechanisms underlying a variety of disease states, but they also present novel opportunities for the design and development of more effective therapeutic strategies.

Key words: Apoptosis, Bcl-2, Mitochondria, Reactive oxygen species, Permeability transition pore complex

Reactive oxygen species

Definition

Reactive oxygen species (ROS) is a collective term that includes not only oxygen radicals (superoxide and hydroxyl) but also some non-radical derivatives of molecular oxygen (O_2) such as hydrogen peroxide (H_2O_2) (Halliwell, 1999).

Intracellular sources of ROS

ROS are produced in all mammalian cells, partly as a result of normal cellular metabolism, and partly due to activation of membrane-bound enzyme systems, such as the NADPH oxidase complex, also known as Nox proteins, in response to exogenous stimuli.

The mitochondrial electron-transport chain

In many aerobic cells, electron-transport chains are probably the most important *in vivo* source of ROS, in particular superoxide anion ($O_2^{\bullet-}$) (Fig. 1). In the eukaryotic system these electron-transport chains are specifically located in the mitochondria and endoplasmic reticulum. The mitochondrial electron-transport chain is a multi-component system involved in a series of oxidation-reduction reactions between redox couples or pairs; transfer of electrons from a suitable donor (reductant) to a suitable electron acceptor (oxidant). These oxidation-reduction reactions involve either the transport of electrons only as in the case of the cytochromes, or electrons and protons together, as occurs between NADH and FAD. The part of the electron-transport chain that actually uses O_2 is the terminal oxidase enzyme, cytochrome oxidase (Halliwell, 1999). Cytochrome oxidase releases no detectable oxygen radicals into free solution. However,

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during the transfer of electrons through earlier components of the transport chain a few electrons do leak out directly on to O_2, resulting in the generation of $O_2^{\bullet-}$. It follows that damage to mitochondrial organization that severely affects the smooth flow of electrons through the electron-transport chain could favor leakage of electrons and increase $O_2^{\bullet-}$ production (Halliwell, 1999). The supposition that mitochondria are the principal source of ROS during oxidative tissue injury derives from the observations that isolated mitochondria produce $O_2^{\bullet-}$ through either auto-oxidation of the flavin component of complex I (NADH hydrogenase), and/or by auto-oxidation of the ubisemiquinone at complex III.

Proteins of the Nox family

A second potent source of intracellular ROS, best characterized in neutrophils and cells of the phagocytic lineage are the membrane-localized NADPH oxidases (Nox) (Bokoch and Knaus, 2003). The Nox system of stimulated phagocytic leukocytes consists of a membrane heterodimeric flavocytochrome (cytochrome b559) comprising of two subunits, gp91phox (Nox2) and p22phox, and four cytosolic proteins, p47phox, p67phox, p40phox and the small guanosine triphosphate (GTP)-binding protein Rac (1 and 2) (for review see: Henderson and Chappel, 1996). The primordial oxygen radical generated by this complex is $O_2^{\bullet-}$, produced by the NADPH-derived one-electron reduction of O_2. Elicitation of $O_2^{\bullet-}$ production *in vivo* involves the stimulus-dependent translocation of some or all components of this cytosolic complex to the plasma membrane and their assembly with cytochrome b559. Over the past three years, multiple mammalian Nox2 homologs (Nox1, Nox3, Nox 4 and Nox5) have been identified in various tissues; all Nox proteins show structural homology to Nox2 and produce intracellular $O_2^{\bullet-}$ (for review see Lambeth, 2002).

Cell antioxidant defenses

ROS are conventionally described as cytotoxic. This idea is supported by the fact that levels of ROS are tightly regulated by multiple defense mechanisms involving small anti-oxidant molecules and ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) (Halliwell and Gutteridge, 1999). Deficiencies or alterations in the cell inherent anti-oxidant defenses modulate the fate of ROS in the cell, hence its redox status.

Scavenging $O_2^{\bullet-}$

In eukaryotic cells the intracellular concentration of $O_2^{\bullet-}$ is tightly regulated by the activities of the two principal scavenger enzymes, namely Cu/Zn SOD and Mn SOD.

Cu/Zn SOD. The Cu/Zn SOD enzyme, found in virtually all eukaryotic cells, has a relative molecular mass of about 32 kD and contains two protein subunits each of which bears an active site containing one Cu and one Zn cation. Primarily located in the cytosol, but also present in lysosomes, nucleus, and the mitochondrial inter-membranous space, Cu/Zn SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Halliwell and Gutteridge, 1999).

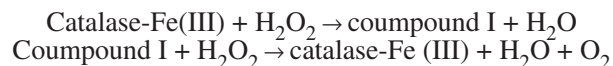$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (ground-state)}$$

MnSOD. First isolated from *Escherichia coli*, MnSOD in eukaryotic cells is expressed mainly in the mitochondria. Mn SOD is not inhibited by cyanide or diethyldithiocarbamate, has a relative mass of 40 kD, is destroyed by treatment with chloroform plus ethanol and contains manganese at its active site. Despite these differences MnSOD essentially catalyzes the same reaction as Cu/Zn SOD (Halliwell and Gutteridge, 1999).

Scavenging H_2O_2

As mentioned previously, dismutation of $O_2^{\bullet-}$ generates H_2O_2, which is usually removed in cells by two types of enzymes, catalase and peroxidases. Catalase directly catalyzes the decomposition of H_2O_2 to ground state O_2 and is unique in that H_2O_2 serves as both a donor and acceptor, while the peroxidases remove H_2O_2 by using it to oxidize other substrates, such as NADH and glutathione (GSH).

Catalase. Catalase consists of four subunits, each of which contains a ferric heme group bound to its active site. Catalase activity in cells is largely found in sub-cellular organelles bound to a single membrane and known as peroxisomes. The catalase reaction is essentially a dismutation reaction similar to SOD; one H_2O_2 is reduced to H_2O and the other is oxidized to O_2.

$$Catalase\text{-}Fe(III) + H_2O_2 \rightarrow coumpound\ I + H_2O$$
$$Coumpound\ I + H_2O_2 \rightarrow catalase\text{-}Fe\ (III) + H_2O + O_2$$

The exact structure of compound I is uncertain (Halliwell, 1999).

Glutahione peroxidase. GPX consists of four protein subunits, each of which contains one atom of the element selenium at its active site (Halliwell, 1999). GPX removes H_2O_2 by coupling its reduction to H_2O_2 with the oxidation (GSSG) of GSH.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$
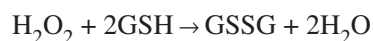
Most cells contain substantial activities of both GPX and catalase. Usually, subcellular compartmentalization influences H_2O_2 removal. H_2O_2 produced by peroxisomal enzymes such as glycollate oxidase and

urate oxidase is largely disposed of by catalase, whereas H_2O_2 arising from the mitochondria, the endoplasmic reticulum or by the action of cytosolic CuZn SOD is acted upon by GPX.

Reactive oxygen species and oxidative stress

Methodology for the identification/quantification of ROS

Since the emergence of an interest in ROS and their implication in various cellular processes, there has been a heightened interest in the development of specific and sensitive methods for the detection and quantification of ROS. Three criteria have been particularly evaluated: (i) sensitivity and linear range of the assay; (ii) specificity of identification between various species, and (iii) versatility to measure ROS in a cellular context or in an enzymatic assay.

The main approaches for identifying reactive species formed in biological systems are the 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay, and the luminol- and lucigenin-amplified chemiluminescence assays (Table 1).

DCFH-DA is a cell-permeable non-fluorescent molecule, very sensitive to intracellular redox change. Esterases cleave DCFH-DA into CDFH2 which, in turn, can be oxidized by peroxidases, cytochrome c or Fe^{2+} into the fluorescent dichlorofluorescein (DCF) in the presence of H_2O_2. However, the use of a cell-free system has shown increased formation of DCF by peroxynitrite (PN) (linear detection: 50-500 nM); (Kooy et al., 1997) but not by $O_2^{\bullet-}$ and nitric oxide (NO) alone (from 1 to 10 microM) (Kooy, et al., 1997; Myhre, et al., 2003). Under conditions where NO and $O_2^{\bullet-}$ are produced simultaneously, oxidation of DCF may be mediated by the formation of PN (Kooy et al., 1997). The levels of intracellular fluorescence can be measured either by flow cytometry (Bass et al., 1983), fluorescence microscopy, or confocal scanning microscopy. Once optimized, the DCF assay is highly sensitive, linear and specific for measuring oxidative stress in irradiated cells (Wan et al., 2003).

The oxidation of luminol or the reduction of lucigenin leads to chemiluminescence that can be used as a measure of ROS generation (Table 1). However, there have been several contradictory reports about the specificity of these reagents (Faulkner and Fridovich, 1993). For instance, one study showed that H_2O_2, $O_2^{\bullet-}$ and hypochlorite (HOCl) alone are capable of oxidizing luminol. More precisely, H_2O and HOCl would spontaneously react at physiologic al pH to produce luminol-dependent chemiluminescence 100 times the sum of the chemiluminescence of either reagent alone. This enhancement would be due to a co-oxidation by HOCl and H_2O_2, or to a novel oxidant generated by the interaction of HOCl and H_2O_2. Evidence has been presented for a case against the participation of hydroxyl radical, superoxide anion or singlet oxygen in the oxidation of luminol by HOCl and HO_2 (Brestel, 1985). Lucigenin (bis-N-methylacridinium nitrate) is widely used as a chemiluminescent probe for detecting $O_2^{\bullet-}$ formation in biological systems. Before photon emission, lucigenin (Luc2+) must first be reduced by one electron to produce a cationic radical (Luc+). Its reaction with $O_2^{\bullet-}$ produces an unstable dioxetane, which decomposes into two molecules of N-methylacridone, one of which is in an electronically excited state and returns to the ground state upon the release of a photon (Minkenberg and Ferber, 1984; Gyllenhammar, 1987; Li et al., 1998).

ROS production: a balance between life and death

A disturbance in the intracellular balance between ROS production and the activities of the anti-oxidant defense systems in favor of the former renders an oxidatively stressed cell. Although the exact measurement of the overall redox state in biological samples is difficult to achieve, representative redox couples such as PSSP/PSH (thiols), NADP+/NADPH,

Table 1. Methodology for the identification and the quantification of ROS.

PROBE	SUPEROXIDE	HYDROGEN PEROXIDE	PEROXY- NITRITE	ADVANTAGES	DRAWBACKS	REFERENCE
DCFH-DA (2'-7'-dichlorodihydro-fluorescein diacetate)	No	Yes	No	Cell-permeable. Non-fluorescent molecule, must be cleaved by esterases and oxidized in the presence of H_2O_2. Very sensitive.	Need of SOD inhibitors to eliminate this source of peroxide production.	Degli Esposti, 2001; Kooy, 1997; Black, 1974
Luminol	No/Yes	Yes	Yes	Cell-permeable. New assay with oxidation by HOCl specific of nM H_2O_2.	Interference of various ROS.	Faulkner, 1993; Brestel, 1985
Lucigenin (bis-N-methyl-acridinium)	Yes	Weak	No	Cell-permeable. Use at low concentrations.	May cause extra superoxide production	Li, 1998; Gyllenhammar, 1987; Minkenberg, 1984

GSSG/GSH, TrxSS/Trx(SH)2 (thioredoxin), and ascorbate/dehydroascorbate represent the most commonly used markers of redox state. Although enormous strides have been made in the understanding of the effect of redox state on cell physiology, the exact quantitative importance of redox regulation is still not clear. In addition, the response of cells to oxidative stress varies depending on the magnitude of the stress and could be either upregulated or downregulated. Thus, the level of oxidative stress determines whether the end result is proliferation, differentiation, senescence or cell death.

The prevailing view strongly favors the participation of ROS in apoptosis induction (Buttke and Sandstrom, 1994). Indeed, numerous agents that induce apoptosis stimulate intracellular production of ROS, most frequently leading to an accumulation of H_2O_2. Moreover, many inhibitors of apoptosis have antioxidant properties, or enhance the cellular antioxidant defense mechanism. These observations have led to the suggestion that ROS are effectors for a variety of triggers of apoptosis, including TNF-α, C2 ceramide, anti-IgM antibody, dexamethasone, irradiation and numerous anticancer drugs (Buttke and Sandstrom, 1994). On the other hand, the observation that hypoxia can induce apoptosis supports the argument that ROS are not necessary for mediating apoptotic cell death (Jacobson and Raff, 1995; Shimizu et al., 1995). In addition, we and others, have shown that an increase in intracellular ROS, in particular $O_2^{\bullet-}$, can inhibit apoptotic signaling in tumor cells, irrespective of the trigger (Clement and Stamenkovic, 1996; Clement et al., 1998; Pervaiz and Clement, 2002; Pervaiz et al., 1999). Thus, an important effect of survival signals may be to maintain critical cellular redox equilibrium, based, at least in part, on adequate intracellular ROS production. Therefore, an alteration in the cellular redox state may be critical for directly inducing or regulating cellular response to apoptotic stimuli. Whereas a high concentration of intracellular ROS (oxidative stress) provides a direct effector mechanism for necrotic cell death, a mild increase in ROS, either H_2O_2 or $O_2^{\bullet-}$ (prooxidant state), could provide protection against apoptosis. By contrast, an $H_2O_2/O_2^{\bullet-}$ ratio that favors reduction of the intracellular milieu (reduced state) sensitizes cells to apoptotic triggers that could eventually lead to spontaneous apoptosis. Thus, the divergent signaling by ROS is a function of their absolute intracellular concentrations and the critical balance between $O_2^{\bullet-}$ and H_2O_2. At the extreme end of the spectrum, an overwhelming production of $O_2^{\bullet-}$ and H_2O_2 induces an oxidative stress that can lead to inhibition of caspases and necrotic cell death. This divergent effect of ROS on cell-death pathways has stimulated enormous interest in deciphering underlying mechanism(s) that allow ROS to function as pro-life in one setting and pro-death in another. We have demonstrated the existence of this phenomenon with drug-induced apoptosis in tumor cells whereby in a multiple-drug regimen, a dose of one

drug that generates a slight pro-oxidant intracellular milieu, endows cells the ability to resist death. Incidentally, a significantly higher concentration of the same compound resulted in efficient apoptosis of tumor cells.

Pathophysiological implications of redox regulation

Following the discovery that ROS may be formed as by-products of intracellular enzymatic reactions, the realization set in that free radicals accounted for gross cellular damage, triggered DNA damage and mutagenesis, accelerated the biological aging process and were somehow involved in disease states, such as neuro-degenerative disorders, inflammatory conditions, and cancer. Although over the years it has become apparent that mild increases in ROS may have roles/effects other than death and demolition, a sustained/chronic oxidative stress nevertheless has invariably been associated with a variety of pathological conditions. This could either be a direct effect of ROS on biological molecules, such as proteins, lipids, and nucleic acids, or be brought about indirectly through dysregulation of signal transduction and/or gene expression.

Age-related increase in ROS and the free radical: Theory of aging

There is strong evidence to suggest a causal relationship between intracellular ROS and aging, yet current data fail to distinguish whether a chronic oxidative stress is a result of age-related increase in ROS production or whether the increase in ROS production hastens the aging process. However, observations linking mitochondrial DNA damage and gene mutations induced by age-related oxidative stress to skeletal muscle atrophy in rats (Wanagat et al., 2001), and age-related manifestations of oxidative damage in rhesus monkey skeletal muscles (Zainal et al., 2000) provide strong evidence linking ROS and aging-related atrophic changes. In addition, age-related changes in stress-response genes (Lee et al., 1999), inflammatory mediators (Lee et al., 2000), ROS scavenging enzymes (Ji et al., 1990), and most importantly a pro-oxidative shift in the thiol/disulfide redox state (Hack et al., 1998), link the aging process to oxidative tissue damage.

According to the free radical theory of aging, age-related degenerative changes are largely a consequence of free radical damage. This has been substantiated by experimental evidence in animals which directly links oxidative stress to the aging process. For instance, mitochondrial MnSOD overexpressing mutation (daf-2) in Caenorhabditis elegans causes longevity (Honda and Honda, 1999), whereas a mutation (*mev-1/cyt-1*) that renders succinate dehydrogenase cytochrome ß inactive results in premature aging (Ishii, 2000). Similarly, in the fruit fly Drosophila a specific mutation (mth) and overexpression of SOD significantly prolongs life span

and increases resistance to free-radical generation (Lin et al., 1998; Orr and Sohal, 1994). This effect has also been documented in mice, where mutations in the $p66^{shc}$ provide protection from oxidative damage and increase life span in the mutant animals (Migliaccio et al., 1999).

Further evidence linking pro-oxidative changes and the aging process comes from data demonstrating the involvement of ROS in age-related cellular senescence. This has been attributed partly to dysregulation of signaling networks involved in cell proliferation (Riabowo et al., 1992) and/or differentiation (Smith et al., 2000). To that end, oxidative induction of telomere shortening in aging human fibroblasts (Harley et al., 1990; Sitte et al., 1998) has led to the realization that telomere shortening may be a major cause of cellular senescence.

Taken together, these data are convincing evidence to suggest that a chronic oxidative stress could result in changes consistent with the aging process or that age-related accumulation of ROS may trigger the consequential changes observed in the elderly.

Oxidative stress and neurodegenerative disease

The involvement of oxidative stress in the pathophysiology of neurodegenerative disorders, such as Down's syndrome, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and spongiform encephalopathies (SE) has been documented. Down's syndrome is commonly associated with the development of AD and is the most frequent cause of mental retardation. Cortical neurons from fetal Down's cases exhibit a 3-4 fold higher intracellular ROS levels than age-matched normal brain cells, and exposure of Down's syndrome neurons in culture to radical scavengers prevents degeneration (Busciglio and Yankner, 1995). The reason for this increased oxidative stress in Down's cases is linked to an increased expression of the Cu/Zn SOD resulting in abnormally higher intracellular H_2O_2, which contributes to cellular senescence (de Haan et al., 1996) and peroxidative damage (Elroy-Stein et al., 1986). These data have been corroborated in Cu/Zn SOD transgenic mice, which exhibit signs of premature aging and neuromuscular dysfunction (Avraham et al., 1988, 1991). Mutations in the Cu/Zn SOD gene have also been identified in ~20% of patients with ALS, thus pointing to a role of an altered redox status in neuronal apoptosis and degeneration in these patients (Pasinelli et al., 2000).

Lipid peroxidation downstream of an increase in ROS has also been detected in the brains of AD patients (Pratico et al., 1998) and amyloid ß protein damage has been shown to be mediated by increased intracellular ROS (Multhaup et al., 1997). These findings provide a strong association between progressive decline in cognitive function and ROS-mediated neuronal loss in AD.

Breakdown of cellular defense against ROS has also been implicated in neuronal damage observed in the prion disease bovine spongiform encephalopathy. In this regard, the normal cellular prion protein (PrP^C) has been proposed to play a role in the control of intracellular redox status (Brown and Besinger, 1998). Prion-infected neurons exhibit a marked increase in sensitivity to oxidative stress compared to their normal counterparts with a significant increase in lipid peroxidation and a dramatic decrease in the levels of anti-oxidant enzymes (Milhavet et al., 2000).

ROS involvement in Diabetes Mellitus and vascular disease

Increased intracellular production of ROS has also been linked to increased serum levels of glucose (hyperglycemia) and to the pathogenesis of atherosclerosis and hypertension. Elevated glucose levels, a hallmark of diabetes mellitus, are associated with increased ROS production through several different mechanisms, including mitochondrial complex II (Baynes, 1991; Nishikawa et al., 2000). Oxidative stress in diabetes mellitus has also been associated with a pro-oxidative shift of the glutathione redox state in the blood (De Mattia et al., 1998), and strategies to ameliorate mitochondrial ROS production have been shown to prevent the development of some of the complications of the disease (Nishikawa et al., 2000). Along the same lines, an increase in plasma levels of glycated proteins in diabetic patients stimulates intracellular $O_2^{\bullet-}$ production through interaction with corresponding cell-surface receptors (Yan et al., 1994). In addition, complications of hyperglycemia and diabetes mellitus, such as increased peroxidation of low-density lipoproteins (LDL) in endothelial cells and atherosclerosis (Maziere et al., 1995; Aragno et al., 2000) are attributed to oxidative shift in the cellular redox state.

The role of ROS in the pathogenesis of atherosclerosis and hypertension has been documented. Oxidative stress leads to the activation of monocytes and macrophages via ligation of oxidized LDL to its receptor on these scavenger cells, which stimulates intracellular production of H_2O_2 by upregulating the expression of mitochondrial MnSOD (Kinscherf et al., 1997). This results in massive apoptosis of macrophages and sets the stage for the development of the atherosclerotic lesions (Kinscherf et al., 1997). In addition, upregulation of cell-adhesion molecules, such as ICAM-1, increased secretion of cytokines such as TNF, angiotensin II, and interferon-γ, NADPH oxidase-dependent production of $O_2^{\bullet-}$ from endothelial cells (Ohara et al., 1993; Griendling et al., 1994; De Keulenaer et al., 1998) further facilitates the atherosclerotic process. The involvement of ROS in the pathogenesis of atherosclerosis is further corroborated by the observations that atherogenesis and lipid peroxidation could be prevented by vitamin E and that induction of vitamin E deficiency in atherosclerosis-susceptible (APO-Lipoprotein E knock-out) mice significantly hastened and increased the severity of the atherosclerotic lesions in the proximal aorta (Terasawa et al., 2000).

ROS-mediated vascular events have also been implicated in the inflammatory response and tissue damage observed during ischemia-reperfusion injury that follows organ transplantation, and in myocardial infarction and stroke (Downey, 1990; Chan, 1996; Cordis et al., 1998). To that effect, increased activity of redox-sensitive transcription factors, such as NF-κB and AP-1, has been demonstrated in a murine model of ischemia-reperfusion (Clerk et al., 1998).

Role of ROS in cell transformation and carcinogenesis

Normal homeostasis of cell numbers results from a tight control between rates of cell proliferation, differentiation and cell death. Therefore, abnormal accumulation of cells in cancer can be viewed as the difference between the rates of cell proliferation and apoptosis. Indeed, not only do normal cells undergo increased proliferation upon exposure to low concentrations of $O_2^{\bullet-}$ or H_2O_2, but also certain types of cancer cells produce substantial amounts of ROS (Burdon et al., 1989; Burdon, 1996). Along the same lines, treatment with the anti-oxidant N-acetylcysteine resulted in a significant decrease in the proliferative index of colon carcinoma cells (Esteve et al., 1999). Further support for a role of active oxygen in carcinogenesis is the data demonstrating the ability of $O_2^{\bullet-}$ and other oxygen radicals to promote the process of cellular transformation, and the findings that most anti-oxidants inhibit chemically-induced transformation. In addition, a large body of work clearly highlights the tumor suppressor activity of the mitochondrial MnSOD (Cullen et al., 2003; Weydert et al., 2003). In this regard, tumor cells in general not only express lower levels of MnSOD, but are also defective in their ability to induce MnSOD activity in response to oxidative challenge. In addition, the reduced expression of MnSOD has been shown to enhance invasiveness in cancers and the re-expression of MnSOD in a number of cancer types suppresses the malignant phenotype (Darby et al., 2003; Weydert et al., 2003). One obvious consequence of the anti-oxidant enzyme deficiency/imbalance is alteration in cellular redox status. This has, indeed, been documented in a variety of cancer types that exhibit abnormally high levels of DNA oxidation. To provide further impetus to the hypothesis that a pro-oxidant state favors carcinogenesis or that it may be a feature of neoplastic lesions are the observations that colon adenomas show a much lower level of oxidative damage than colon carcinomas (Oberley, 2002).

More convincing evidence linking a pro-oxidant intracellular milieu with tumor promotion comes from the studies linking cell survival pathways, such as the PI3Kinase-Akt circuit, with an increase in intracellular ROS generation (Oberley, 2002). Along the same lines, a direct involvement of intracellular $O_2^{\bullet-}$ in proliferation induced by the oncoprotein p21Ras was demonstrated in a lung fibroblast model (Irani et al., 1997). This was shown to be a function of Ras-dependent activation of the small GTP-binding protein Rac1 that initiates NADPH oxidase assembly. Indeed, cancer cells constitutively expressing the activated form of Ras (RasV12) showed significantly higher intracellular levels of $O_2^{\bullet-}$, which could be suppressed by dominant-negative Rac1 (Pervaiz et al., 2001). More recently, the identification of the non-phagocytic mitogenic oxidase Nox1 and its ability to stimulate growth via $O_2^{\bullet-}$ generation provides support for the pro-oxidant theory of carcinogenesis (Suh et al., 1999). In addition, an alternative mechanism by which a slight pro-oxidant intracellular milieu could promote carcinogenesis is by inhibiting the effector components of the apoptotic pathway (Clement and Stamenkovic, 1996; Pervaiz et al., 1999), thus tipping the scale in favor of proliferation and cell accumulation.

Mitochondrial targets of ROS

Pro-oxidant radicals or pharmacological agents can trigger an increase in mitochondrial membrane permeability, better known as mitochondrial permeability transition (MPT); MPT is controlled by the mitochondrial permeability transition pore complex (PTPC) (Halestrap and Brenner, 2003). More precisely, MPT is currently defined by a collapse of the membrane potential ($\Delta\Psi m$), an uptake of H^+ ions, the progressive acceleration of respiration and large swelling of the matrix accompanied by Ca^{2+} release, oxidation of pyridine nucleotides and the accumulation of lysophospholipids (Beatrice et al., 1980). Although numerous issues regarding the nature of PTPC remain unresolved, recent advances provide strong evidence for direct targeting of PTPC component(s) and its regulation by intracellular ROS.

The permeability transition pore complex (PTPC)

PTPC, also called the mitochondrial megachannel, is a polyprotein complex (MW≈ 600kD) involved in the regulation of mitochondrial matrix homeostasis. Its opening as a large unspecific channel leads to MPT and results in an increase in the permeability of the inner membrane (IM), permitting the influx or efflux of any molecule of MW≈1500 Da (Zoratti and Szabo, 1995). Under stress conditions, MPT is deleterious to the mitochondrial physiology and leads to the activation of a multi-step process initiating apoptosis, necrosis or autophagy (Lemasters et al., 1998). Thus, as a consequence of the opening of PTPC, the mitochondrial matrix swells, the $\Delta\Psi m$ dissipates and the outer membrane (OM) ruptures locally, triggering the release of apoptogenic proteins into the cytosol, including cytochrome c (Cyt c), apoptosis-inducing factor (AIF), Smac/Diablo, pro-caspases and Endo G (Kroemer and Reed, 2000; Belzacq et al., 2002). This leads to mitochondrial membrane permeabilization (MMP) which orchestrates the triggering of the degradation

phase of apoptosis (Kroemer and Reed, 2000). In addition, PTPC opening could mediate autophagy, a process involved in the degradation of damaged and depolarized mitochondria in autophagosomes (Elmore et al., 2001). The physiological function of the PTPC pore is regulated by a variety of signals, including the $\Delta\Psi$m, matrix pH, the concentration of divalent cations (Ca^{2+}, Mg^{2+}), the intracellular redox equilibrium and the matrix volume (Halestrap and Brenner, 2003). As measured by electrophysiology, mammalian PTPC exhibits multiple conductance states, suggesting that the channel is composed of several cooperating subunits (Zoratti and Szabo, 1994).

Upon apoptosis induction, PTPC opening can precede or occur concomitantly with Bax translocation from cytosol to mitochondria (De Giorgi et al., 2002), but usually before the release of Cyt c and AIF from mitochondria (for review: Belzacq et al., 2002). As a possibility, Bax can cooperate with ANT forming a new class of channels to induce a lethal MPT (Marzo et al., 1998a,b; Brenner et al., 2000). These channels are inhibited by classical MPT inhibitors such as bongkrekic acid (BA) and cyclosporin A (CsA), Bcl-2, ADP, and ATP, while they cannot be induced by mutants of Bax (Marzo et al., 1998a,b; Brenner et al., 2000). Orrenius et al. found that the induction of MPT in association with oxidative stress was the most efficient stimulus for the release of Cyt c in comparison to Bax alone, Bax +ROS, or Ca^{2+}+Pi (Ott et al., 2002). This supports an active role for ROS in the pro-apoptotic MMP.

The composition of PTPC is dynamic and may evolve in response to the energetic demand of the cell and to apoptosis induction (Brdiczka et al., 1998; Scorrano et al., 2002; Verrier et al., 2004). This may result from the remodeling of mitochondrial cristae and contact sites during the process of apoptosis induction. Biochemical and functional studies using purified proteins reconstituted in artificial biomembrane (e.g. liposomes or black lipid membranes) have provided two models of structure/function relationship of the PTPC. One model proposes that the PTPC is composed of three proteins, the adenine nucleotide translocator (ANT, in the IM), the voltage-dependent anion channel (VDAC, in the OM) and cyclophilin D (CypD, in the matrix) (Crompton et al., 2002; Halestrap et al., 2002), whereas a second model suggests a more complex composition depending on tissue, differentiation and development state. As shown in Fig. 1, this model supports the association of the PBR (in the OM), hexokinase (HK, in the cytosol), and creatine kinase (CK, in the intermembrane space) with the ANT, VDAC and CypD (Beutner et al., 1996; Marzo et al., 1998a).

It is important to note that in both models some proteins are anti-apoptotic (HK, CK) whereas other are pro-apoptotic (ANT, VDAC, PBR), favoring the closed or the open state of PTPC, respectively. Thus, PTPC function could be modulated by physical interaction of some of its members with onco- and anti-oncoproteins of the Bax/Bcl-2 family. ANT, VDAC and HK (glucokinase in liver) were identified as ligands of Bax, Bcl-2, Bid, Bcl-xL, Bad and Bak (Marzo et al., 1998b; Shimizu et al., 2000; Cao et al., 2001; Capano and Crompton, 2002; Cheng et al., 2003; Danial et al., 2003). Among these proteins, some members of the Bax/Bcl-2 family were shown to be regulators of channel activity of ANT and VDAC (Brenner et al., 2000; Shimizu et al., 2000), as well as modulators of the ADP/ATP translocase activity of ANT (Belzacq et al., 2003) (Fig. 2). Moreover, other PTPC members such as HK or CK can prevent lethal opening of the PTPC in the presence
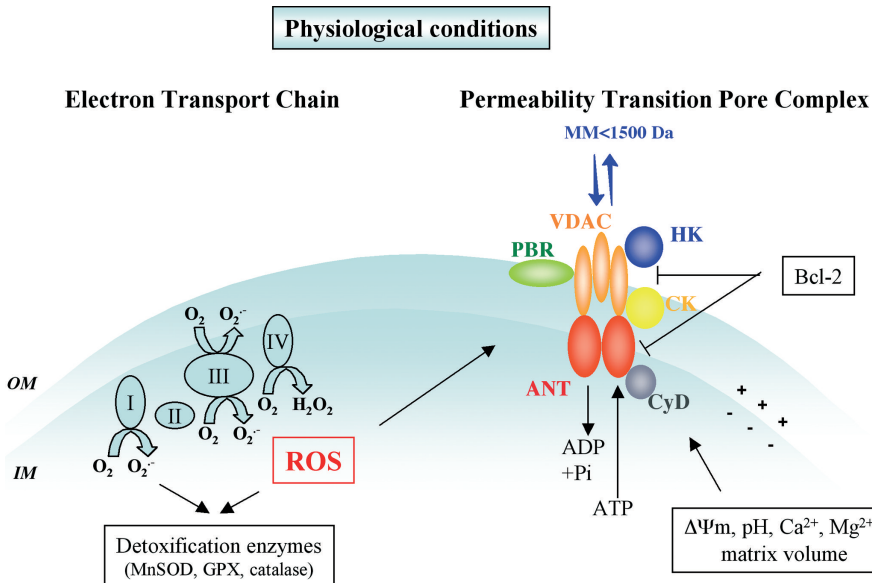

Fig. 1. ROS generation in physiological conditions. The respiratory chain is represented showing the sites of production of reactive oxygen species (ROS) directed toward the intermembrane space (from complex III) or toward the matrix (from complexes I, III and IV), where they can be detoxified by enzymes such as manganese superoxide dismutase (MnSOD), glutathione peroxidase (GPX) or catalase. The permeability transition pore complex (PTPC) is a polyproteic channel involved in the control of matrix homeostasis. It plays a role in ADP/ATP translocation and in flux of any molecule of MW≈1500Da. It is regulated by Bcl-2, membrane parameters ($\Delta\Psi$m), and matrix parameters (pH, Ca^{2+}, Mg^{2+}, volume and redox state).

of their substrates (O'Gorman et al., 1997; Azoulay-Zohar et al., 2004).

ROS regulation of MMP

Pharmacological evidence indicates that MPT can be induced directly by pro-oxidant agents, such as t-butyl hydroperoxide (tBHP), phenylarsine oxide (PAO), N-ethylmaleimide (NEM), diazenedicarboxylic acid bis 5N, N-dimethylamide (diamide) or in response to a Ca^{2+}-stimulated production of ROS by the respiratory chain, notably at the level of the coenzyme Q (Kowaltowski et al., 1997) (Fig. 3). Thus, ROS may modify membrane protein thiols to produce cross-linking reactions and to open membrane pores upon Ca^{2+} binding. As one possibility, the binding of Ca^{2+} to submitochondrial particles may induce conformational changes within the PTPC, as well as lipid lateral-phase separation leading to a disorganization of respiratory chain components, favoring ROS production and consequent protein and lipid oxidation (Kowaltowski et al., 1997). In turn, the opening of PTPC can itself induce a second burst of ROS generation, which is dependent on intrinsic cellular ROS-scavenging redox mechanisms, particularly glutathione, as shown in cardiomocytes (Zorov et al., 2000). Moreover, neurotoxins that induce Parkinsonian neuropathology, such as MPP(+) and rotenone, can stimulate $O2^{\bullet-}$ production at complex I of the electron-transport chain and also free radical production at proximal redox sites, including mitochondrial matrix dehydrogenases (Fiskum et al.,

2003). Consequently, these ROS induce a pro-apoptotic mitochondrial dysfunction and a lethal MMP, resulting either from cytosolic Bax translocation to the OM and activation, or from a Ca^{2+}-dependent MPT followed by OM rupture (Fiskum et al., 2003).

ROS-induced MPT has also been implicated in pathological hepatic and cardiac ischemia/reperfusion processes. Thus, reperfusion after a period of ischemia is associated with the generation of ROS and mitochondrial Ca^{2+} overload favoring the opening of PTPC. Levels of endogenous antioxidants are usually not high enough to prevent reperfusion injury alterations, and the use of exogenous supplement of antioxidants, especially after acute ischemia, can be a way to inhibit PTPC opening and to prevent irreversible damage (Rajesh et al., 2003).

ANT, an inner mitochondrial membrane target of ROS

In mammals, ANT represents almost 10% of mitochondrial membrane proteins located in the IM. It belongs to the mitochondrial carrier family (Kuan and Saier, 1993) and in physiological conditions promotes the exchange of intracytosolic ADP against matricial ATP in response to cytosolic ATP demand (Pfaff and Klingenberg, 1968; Pfaff et al.,1969). The crystallographic structure of ANT indicates that the monomeric form of the protein is folded in 6 transmembrane helices linked by protruding hydrophilic loops, the N- and C-terminal end facing the intermembrane space (Pebay-Peyroula et al., 2003). This
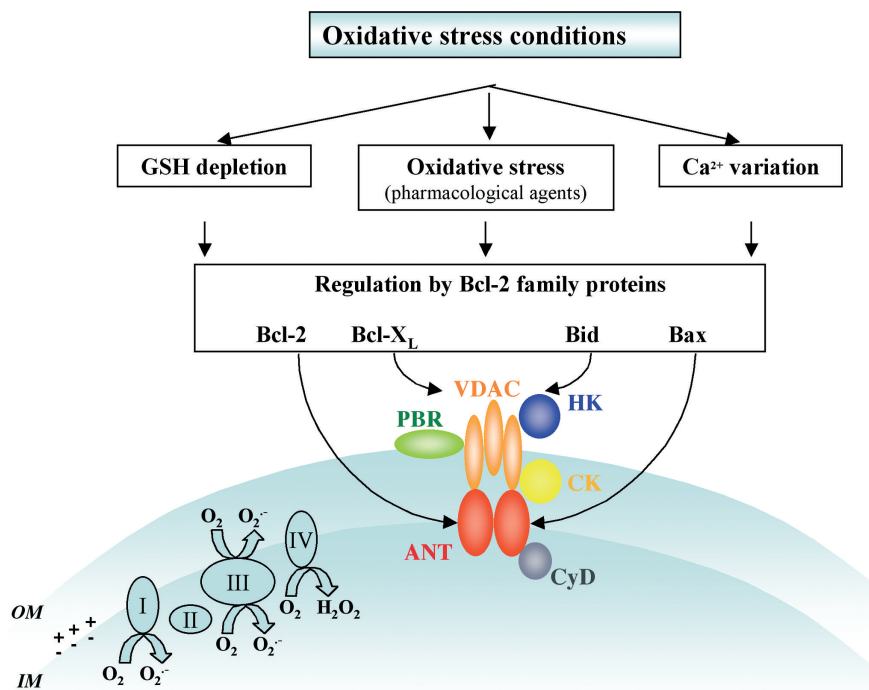

Fig. 2. Oxidative stress and Bax/Bcl-2 family regulation. GSH depletion, calcium variations or oxidative modifications can induce a multi-step mechanism leading to cell death (see text). Then, the ROS produced by drugs, the cellular context or the respiratory chain can modify VDAC, ANT, CK or CypD proteins. This process can be regulated by Bcl-2 family proteins, which are able to directly target several members of the PTPC (see arrows).

structure is compatible with the exposure of several residues, such as cysteine, to ROS attack, as proposed by previously predicted secondary structure methods (Fiore et al., 1998) .

Thus, oxidative stress induced by reagents such as tBHP, diamide and also by thiol reagents such as PAO greatly sensitize the PTPC to Ca^{2+} by two mechanisms (Halestrap et al., 1997). First, CyP-D binding to ANT is increased and second, the binding of adenine nucleotides to ANT is greatly impaired. Thus, PAO can covalently bind to vicinal thiol groups on the ANT. PAO also shifts the voltage dependence of the MPT, allowing the pore to open at more negative potentials. This thiol modification would inhibit adenine nucleotide binding to the matrix surface of the ANT whilst $\Delta\Psi m$ would enhance this binding. Indeed, three cysteine residues (Cys_{56}; Cys_{159}; Cys_{256}) in ANT exhibit differential reactivity to various thiol reagents and oxidizing agents in a conformation-dependent manner (Fig. 3) (Majima et al., 1993-1995). These residues have been proposed to be the major candidates as the thiol groups that regulate both CyP-D binding and the inhibitory effects of ADP and $\Delta\Psi m$ on the PTPC (Halestrap et al., 1997; McStay et al., 2002). Specific cross-linking of Cys_{159} and Cys_{256} (which are located within the adenine nucleotide-binding site of the ANT) with NEM not only inhibits adenine nucleotide binding and ADP/ATP translocation by the ANT, but also almost totally prevents the inhibition of PTPC opening by ADP. Furthermore, diamide causes a disulphide bond to form between these two cysteine residues through the mediation of oxidized glutathione (Halestrap et al., 1997). This latter process is inhibited by low concentrations of NEM that alkylate glutathione within the matrix. PAO and diamide treatment of mitochondria also reduced the ability of copper phenanthroline to link two ANT monomers through their Cys_{56} residues. This cross-linking would induce a

conformational transition in the ANT, reducing the reactivity of Cys_{56}. Moreover, as Cys_{56} is closed to Pro_{61}, the putative binding site for CyP-D, this conformational change may provide an explanation for the increased CyP-D binding to the ANT induced by PAO and oxidative stress (Halestrap et al., 1997).

In parallel, Kroemer's group showed that thiol cross-linking agents including diamide, dithiodipyridine (DTDP), or bis-maleimido-hexane (BMH) could act directly on ANT (Costantini et al., 2000). Thus, ANT alone reconstituted into artificial lipid bilayers confers a membrane permeabilization response to thiol cross-linking agents (Costantini et al., 2000). Diamide, DTDP, and BMH, but not t-BHP or arsenite (as a negative control) cause the oxidation of a critical cysteine residue (Cys_{56}) of ANT. Thiol modification within ANT is observed in intact cells, isolated mitochondria, and purified ANT. Interestingly, recombinant Bcl-2 fails to prevent thiol modification of ANT. Concomitantly, a series of different thiol cross-linking agents (diamide, DTDP, and BMH, PAO), but not t-BHP or arsenite induce MMP and cell death irrespective of the expression level of Bcl-2 (Costantini et al., 2000). These data indicate that thiol cross-linkers cause a covalent modification of ANT, which, beyond any control by Bcl-2, leads to MMP and cell death.

Recent studies also suggest an important role for 4-hydroxynonenal (HNE), an aldehydic product of lipid peroxidation, as well as other lipid peroxidation products (see below), in stress-mediated signaling for apoptosis (Vieira et al., 2001). Thus, 4-HNE, in comparison to nitric oxide (NO), and peroxynitrite (PN), has been demonstrated to be involved in the pathological demise of cells via apoptosis in various cell lines. The cell death mediated by these agents is inhibited by Bcl-2, thus suggesting the involvement of mitochondria in this death pathway (Vieira et al., 2001). In vitro, HNE, NO, and PN
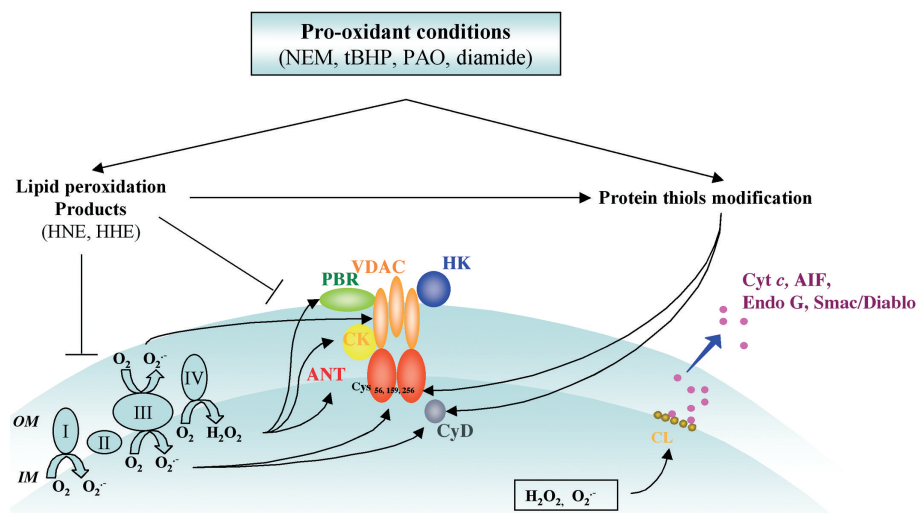

Fig. 3. Pro-oxidant conditions targeting the PTPC and lipids. ANT ($Cys_{56, 159, 256}$), VDAC, CK, PBR, and CypD are the main oxidative targets within the PTPC. Modifications occur directly or indirectly, from $O_2^{.-}$ and H_2O_2, lipid oxidation products (HNE, HHE) or drug effect, and affect PTPC proteins or cardiolipin (CL).

can cause a direct permeabilization of mitochondrial membranes, and this effect is inhibited by CsA, indicating involvement of the PTPC in the permeabilization event. HNE, NO, and PN also permeabilize proteoliposomes containing the ANT, but not protein-free control liposomes. ANT-dependent HNE-induced permeabilization is partially inhibited by the antioxidants trolox and butylated hydroxytoluene. In vitro, HNE adduction or nitrotyrosylation of ANT was detected, suggesting that ANT preferentially undergoes thiol oxidation/derivatization (Vieira et al., 2001). HNE also caused significant lipid peroxidation, which is antagonized by butylated hydroxytoluene, but not by recombinant Bcl-2 (Vieira et al., 2001). Taken together these data suggest that HNE, as well as NO and PN, may directly act on ANT to induce MMP and apoptosis. Thus, these data contribute to emphasize the role of ANT as a central target for oxidative stress within the PTPC.

VDAC, a target for intracellular $O_2^{\bullet-}$

Another putative target of ROS within PTPC is VDAC (Fig. 3). Porin represents almost 10% of proteins in the OM, and its physiological role is to allow the diffusion of solutes of MW<6kD between the cytosol and the intermembrane space (Rostovtseva and Colombini, 1996). VDAC can also serve as a receptor for cytolosolic HK, an enzyme involved in the cytosolic phosphorylation of glucose (for review: Cesar Md Mde and Wilson, 2004). During apoptosis, VDAC opening has been reported to be modulated positively (Vander Heiden et al., 2001) or negatively (Kusano et al., 2000) by Bcl-XL and Bid (Rostovtseva et al., 2004), but not Bax (Rostovtseva et al., 2004). More precisely, VDAC2, an isoform restricted to mammals, inhibits BAK activation and mitochondrial apoptosis, providing a connection between mitochondrial physiology and the core apoptotic pathway (Cheng et al., 2003).

With the aim of studying the mechanisms underlying activation of the apoptotic machinery by ROS, Madesh and Hajnoczky (2001) characterized a mechanism of VDAC-dependent permeabilization of the OM by $O_2^{\bullet-}$. Thus, exposure of permeabilized hepatocytes from the prototypic HepG2 cell line to $O_2^{\bullet-}$ elicited rapid and massive Cyt c release, whereas H_2O_2 failed to induce any release. Both $O_2^{\bullet-}$ and H2O2 promoted activation of the PTPC by Ca^{2+}, but Ca^{2+}-dependent pore opening was not required for $O_2^{\bullet-}$-induced Cyt c release. Pretreatment of the cells with drugs or an antibody, which block the VDAC, prevented $O_2^{\bullet-}$-induced Cyt c release, whereas BA or CsA, were inefficient (Madesh and Hajnoczky, 2001). Furthermore, after $O_2^{\bullet-}$ exposure, encapsulated Cyt c can be released from VDAC-containing liposomes, a process inhibited by VDAC inhibitors such as 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and Konigs polyanion. $O_2^{\bullet-}$-induced Cyt c release did not depend on Bax translocation to mitochondria or on Bak, which was not

detected in HepG2 cells. $O_2^{\bullet-}$-induced Cyt c release was followed by caspase activation and execution of apoptosis (Madesh and Hajnoczky, 2001). Thus, $O_2^{\bullet-}$ triggers apoptosis via VDAC-dependent permeabilization of the mitochondrial OM without apparent contribution of proapoptotic Bcl-2 family proteins.

More recently, in addition to be a target, VDAC also appeared to control the release of $O_2^{\bullet-}$ from mitochondria to cytosol (Han et al., 2003). This could lead to a protection of cytosolic proteins from mitochondrial $O_2^{\bullet-}$ via a better accessibility to the cytosolic SOD and an enhanced scavenging of $O_2^{\bullet-}$. However, the pathophysiological significance of this discovery remains to be explored.

Mitochondrial CK, a target for H_2O_2

Mitochondrial creatine kinase (Mi-CK) catalyzes the reversible transphosphorylation between ATP and creatine to ADP and phosphocreatine. This enzyme of energy metabolism occurs in the intermembrane space in dimer and octamer, both forms being interconvertible (Stachowiak et al., 1998a). The octameric form seems to be the functional unit in vivo (Soboll et al., 1999) when associated with ANT and VDAC in the contact sites between the mitochondrial OM and IM (Beutner et al., 1996, 1998). Several studies showed that Mi-CK can be a main target of ROS, notably H_2O_2 and PN (Fig. 3). These two agents affect the CK enzymatic activity via modification of aromatic amino acid side chains and sulfhydryl groups, as well as affecting the octamer/dimer ratio (Stachowiak et al., 1998b). Importantly, Walliman et al. confirmed the impact of CK targeting by PN, and induction of octamer/dimer transition in vivo in ischemia-reperfusion animal models (Soboll et al., 1999).

The antioxidant role of peripheral benzodiazepin receptor, PBR

The PBR is an OM protein involved in the formation of PTPC. PBRs are located in many tissues and notably, are strongly expressed in the superficial layers of the human epidermis. PBRs play a role in protection against free radical damage, stimulation of steroidogenesis, immunomodulation, porphyrin transport, heme biosynthesis, anion transport and regulation of mitochondrial functions and apoptosis modulation (Beurdeley-Thomas, et al., 2000; Galiegue et al., 2003). PBR is located at the surface of the mitochondria where it is physically associated with VDAC (Fig. 3).

Recently, PBR has been implicated in auto-immune diseases such as human lupus erythematosus (Bribes and Casellas, 2003; Bribes et al., 2003). Using Mrl/Lpr mice, which develop inflammatory pathologies similar to human lupus erythematosus (LE), they showed that PBR ligands are able to regulate PBR expression and to protect mice against the inflammatory process. These

results suggest new applications for PBR ligands and photodynamic therapy as potential therapeutic modalities against auto-immune disorders (Bribes and Casellas, 2003; Bribes et al., 2003).

Moreover, PBR at least affords a transient protection during the early events of ultraviolet (UV) light-induced apoptosis. Thus, when the effects of UVB, which generates singlet-oxygen damage, in PBR-transfected T lymphoma Jurkat cell line were compared to those of the wild type counterpart devoid of any PBR expression, PBR-transfected cells were more resistant to apoptosis and exhibited a delayed mitochondrial DYm drop, a diminished $O_2^{\bullet-}$ production, and a reduced caspase-3 activation. These findings suggest that PBR may regulate early death signals leading to UV-induced apoptosis via $O_2^{\bullet-}$ (Stoebner et al., 2001). This correlates with the previous work of Carayon et al. (1996) suggesting that hematopoietic cell lines resist to H_2O_2 cytotoxicity proportionally to their level of PBR expression. Accordingly, stable transfection of Jurkat cells with the human PBR cDNA leads to an increased resistance of the cells to H_2O_2 (Carayon et al., 1996). These data provide strong evidence that PBRs may play an anto-oxidant role and protect against oxidant-induced damage.

PTPC targeting via lipid oxidation

Reportedly, aldehydic lipid peroxidation products could modify the physicochemical status of the membrane via interaction with mitochondrial membrane lipids and/or proteins. Thus, the ADP/ATP transport activity of ANT in intact mitochondria or in proteoliposomes containing purified ANT can be inhibited by two unsaturated aldehydes, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE) (Fig. 3). This decreased activity has been attributed to a modification of sulfhydryl groups, which are essential for ANT activity, as it has been described above. In contrast, pretreatment of the proteoliposomes with HNE also caused a decrease in the reconstituted ANT activity by indirectly altering the physiochemical status of the lipid environment in which ANT was inserted. These results support the notion that reactive aldehydes derived from mitochondrial lipid peroxidation can impair the membrane function by either interacting directly with the protein and indirectly with the lipid moieties in the membrane (Chen et al., 1995). However, consequences of the alteration of ANT translocation activity on the capacity of ANT to mediate the pro-apoptotic MMP remain to be determined.

Cardiolipin is a particular lipid that is exclusively found in the inner mitochondrial membrane. Cardiolipin is associated with several mitochondrial proteins from the IM, as well as proteins from the intermembrane space such as ANT and Cyt *c* respectively. Release of Cyt *c* from mitochondria is a central event in apoptosis induction and appears to be mediated by ROS. In conditions of succinate-mediated ROS production by the respiratory chain (H_2O_2 and $O_2^{\bullet-}$) and cardiolipin oxidation, Cyt *c* release from mitochondria occurs by a two-step process, consisting of its dissociation from cardiolipin and the permeabilization of the OM (Fig. 3). All these events can be prevented by ADP inhibition of ROS production (Petrosillo et al., 2003).

Concluding remarks

ROS are produced as a by-product of cellular metabolic pathways and their intracellular concentration is kept in check by the cell anti-oxidant defense systems. A slight increase in intracellular ROS that does not overwhelm the anti-oxidant defenses has also been shown to function as a critical second messenger involved in a variety of intracellular signaling pathways. However, a defect or deficiency in the anti-oxidant defense system on the one hand and/or the excessive intracellular generation of ROS on the other renders a cell oxidatively stressed. There is enough evidence to implicate eleveated intracellular ROS with damage to cellular macromolecules, such as proteins, lipids and nucleic acids. As a consequence, direct or indirect involvement of ROS in a host of disease states has been documented. In most of these cases, the deleterious effect of ROS is a function of activation of intracellular cell-death circuitry. To that end, involvement of ROS at different phases of the apoptotic pathway, such as induction of mitochondrial permeability transition and release of mitochondrial death amplification factors, activation of intracellular caspases and DNA damage, has been clearly established. Not only do these observations provide insight into the intricate mechanisms underlying a variety of disease states, but they also present novel opportunities for the design and development of more effective therapeutic strategies.

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