ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 2, 2000
Mary Ann Liebert, Inc.

Forum Review Article

Plasma Membrane Redox System in the Control of Stress-Induced Apoptosis

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ABSTRACT

The plasma membrane of animal cells contains an electron transport system based on coenzyme Q (CoQ) reductases. Cytochrome b_5 reductase is NADH-specific and reduces CoQ through a one-electron reaction mechanism. DT-diaphorase also reduces CoQ, although through a two-electron reaction mechanism using both NADH and NADPH, which may be particularly important under oxidative stress conditions. Because reduced CoQ protects membranes against peroxidations, and also maintains the reduced forms of exogenous antioxidants such as α-tocopherol and ascorbate, this molecule can be considered a central component of the plasma membrane antioxidant system. Stress-induced apoptosis is mediated by the activation of plasma membrane-bound neutral sphingomyelinase, which releases ceramide to the cytosol. Ceramide-dependent caspase activation is part of the apoptosis pathway. The reduced components of the plasma membrane antioxidant system, mainly CoQ, prevent both lipid peroxidation and sphingomyelinase activation. This results in the prevention of ceramide accumulation and caspase 3 activation and, as consequence, apoptosis is inhibited. We propose the hypothesis that antioxidant protective function of the plasma membrane redox system can be enough to protect cells against the externally induced mild oxidative stress. If this system is overwhelmed, intracellular mechanisms of protection are required to avoid activation of the apoptosis pathway. Antiox. Redox Signal. 2, 213–230.

INTRODUCTION: TRANSPLASMA MEMBRANE REDOX ACTIVITY AND CELL GROWTH CONTROL

In 1985, Frederick L. Crane and co-workers (Crane *et al.*, 1985) compiled available evidence supporting the participation of electron transfer reactions at the plasma membrane in the control of cell growth and development. The pioneering studies of Ellem and Kay (1983) and Sun *et al.* (1984a,b) had reported that the impermeable electron acceptor ferricyanide was able to stimulate the growth of cultured cells in serum-deficient media. The activity of the transmembrane dehydrogenase was shown to vary according to the transformation state of cells, and it was suggested that cytotoxic effects of several antiproliferative drugs were related to the inhibition of transplasma membrane electron transport (Sun *et al.*, 1983, 1984c, 1986a,b; Sun and Crane, 1984; McLoughlin *et al.*, 1984). In addition, the activity of the transmembrane dehydrogenase was integrated into the cascade of signaling processes that regulate cell growth because it was modulated by growth factors (Sun *et al.*, 1985, 1990; Crane *et al.*, 1991).

As ferriciyanide is an artificial electron ac-

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ceptor, a physiological role for transplasma membrane electron transport in growth control remained to be generally accepted until the natural acceptors and/or intermediate redox carriers and the components of the signaling machinery were identified. In addition to oxygen, the semioxidized form of ascorbate, the ascorbate free radical (AFR), and iron-containing compounds such as diferric transferrin were proposed as natural acceptors for the transmembrane dehydrogenase (Sun *et al.*, 1987; Alcain *et al.*, 1990). In addition, transplasma membrane redox activity measured as ascorbate stabilization was shown to be modulated by growth factors (Navas *et al.*, 1992) and cAMP (Rodríguez-Aguilera *et al.*, 1993).

Plasma (and also Golgi apparatus) membranes contain the lipophilic molecule coenzyme Q (CoQ, ubiquinone) at levels comparable to those of mitochondria in rat liver (Kalén *et al.*, 1987; Takahashi *et al.*, 1993). Similar to its function in the inner mitochondrial membrane, it has been shown that CoQ also plays a role as an intermediate carrier at the plasma membrane by shuttling electrons from intracellular reduced pyridine nucleotides to external impermeable oxidants (Sun *et al.*, 1990, 1992; Crane *et al.*, 1991, 1993; Villalba *et al.*, 1995). Consistent with this function, addition of CoQ_{10} (or various agents that affect its redox state) can maintain growth in serum-limiting conditions (Alcaín *et al.*, 1990; Sun *et al.*, 1992, 1995; Crane *et al.*, 1993; Martinus *et al.*, 1993; Villalba *et al.*, 1996a) and conversely, inhibitors that compete for CoQ at the plasma membrane redox system, such as capsaicin, inhibit cell growth (Wolvetang *et al.*, 1996; Macho *et al.*, 1998).

Stimulatory effects of CoQ_{10} and ferricyanide have been shown to be additive (Sun *et al.*, 1992). Flow cytometry analysis of cells grown in the absence of serum, but in the presence of either ferricyanide or CoQ_{10}, have shown that growth stimulation by ferricyanide produces a large increase in G_1 phase protein, whereas CoQ_{10} increases DNA synthesis and transition to the G_2 phase of the cell cycle (Crane *et al.*, 1993). From these data, we can conclude that CoQ_{10} and ferricyanide can stimulate growth by acting on different regulatory steps. Both CoQ and ferricyanide can accept electrons from the plasma membrane dehydrogenase (Sun *et al.*, 1992; Villalba *et al.*, 1995), but a substantial difference between these two molecules has to be noted. While ferricyanide only acts as an external impermeable "electron sink," CoQ will be integrated into the plasma membrane redox chain and, in its reduced hydroquinone form ($CoQH_2$, ubiquinol), this compound is also a powerful lipophilic antioxidant that protects lipids from oxidative damage (Frei *et al.*, 1990; Stocker *et al.*, 1991), and maintains other antioxidants in their reduced active forms (Beyer, 1994; Kagan *et al.*, 1990a,b, 1996; Gómez-Diaz, 1997a; Santos-Ocaña *et al.*, 1998). Because serum contains CoQ, part of the mitogenic action of serum has been attributed to this molecule (Crane *et al.*, 1993).

Various mechanisms were initially proposed to explain how transmembrane electron flux to impermeable oxidants translates into signaling. These included proton release from cells during transmembrane electron transport (Sun *et al.*, 1984b, 1986a) and the activation of Na^+/H^+ antiport (García-Cañero *et al.*, 1987), which resulted in the increase of intracellular pH. A basis for the growth stimulation by CoQ could be its contribution to plasma membrane NADH oxidation, or the modification of the membrane quinone redox balance (Sun *et al.*, 1992). In addition, it is now firmly established that both oxidants and antioxidants can modulate the activity of a number of transcription factors controlling cell growth, adhesion, differentiation, transformation, and cell death (Palmer and Paulson, 1997; Suzuki *et al.*, 1997; Li *et al.*, 1998; Ricciarelli *et al.*, 1998; Klatt *et al.*, 1999). These processes in which exchange of reducing equivalents serves as an integral component of the signal transduction pathway are referred to as redox signaling.

The most accepted redox signaling molecule is nitric oxide (NO), although a role as secondary messengers has been also recognized for several reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, and for lipid peroxidation products such as lipid hydroperoxides and 4-hydroxynonenal (Suzuki *et al.*, 1997; Kreuzer *et al.*, 1998). Nuclear factor kappa B (NF-κB) transcription factor has been implicated in the regulation of cell proliferation, transformation,

and tumor development (Hinz *et al.*, 1999). It has been recently demonstrated that ferricyanide at low concentrations (from nanomolar to the low micromolar range) activates NF-κB in macrophages under conditions where no net oxidation of NAD(P)H or glutathione are observed, indicating that a redox reaction at the plasma membrane is sufficient to initiate redox signaling independent of major shifts in the redox state of the cell (Kaul *et al.*, 1998). NAD(P)H depletion observed when cells are exposed to higher concentrations of ferricyanide (Kaul *et al.*, 1998) could explain the toxic effect on cell growth observed when ferricyanide is used at concentrations above 30–100 μ*M* (Ellem and Kay, 1983; Sun *et al.*, 1985). Major changes in the redox equilibrium of cells, such as alterations in the ratio of reduced-to-oxidized glutathione (which is tightly coupled to the $NADP^+$/NADPH balance through glutathione reductase), also play an important role in redox signaling processes (Klatt *et al.*, 1999). On the other hand, antioxidants may also influence cellular signaling by two general mechanism: (1) the quenching of ROS acting as secondary messengers, and (2) the modification of redox-sensitive sites on molecules involved in the regulation of gene expression (Palmer and Paulson, 1997).

The maintenance of a cell population is based on the equilibrium among proliferation, differentiation, and cell death (apoptosis). Redox activity at the plasma membrane is also altered during differentiation processes, although the observed changes have been diverse. For instance, a decrease of redox activity in plasma membrane has been reported to occur during differentiation induced by retinoic acid (Crane *et al.*, 1990). However, plasma membrane redox activity increase during differentiation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in HL-60 cells, stimulating ferricyanide reductase activity at the first 24 hr of differentiation and changing the NAD^+/NADH ratio (Burón *et al.*, 1993; López-Lluch *et al.*, 1995). Also, TPA-induced differentiation of K-562 cells increases ascorbate stabilization in whole cells and NADH-AFR reductase at the plasma membrane (Gómez-Díaz *et al.*, 1997a). Although the observed increase in CoQ_{10} levels at the plasma membrane could account for the stimulated transplasma membrane redox activity in TPA-differentiated K-562 cells (Gómex-Díaz *et al.*, 1997a), an increased expression of CoQ-reductases could also contribute to the overall reduction of extracellular acceptors. Accordingly, TPA is known to increase expression of several eukaryotic genes containing recognition sequences for the transcription factor AP1 and related protein(s) in their promoter regions. This transcription factor is known to play a role in the expression of the NAD(P)H:quinone oxidoreductase (NQO_1, DT-diaphorase) (Li and Jaiswal, 1992; Kepa and Ross, 1999), an enzyme characterized as a CoQ-reductase in the plasma membrane (see below).

Because oxidative stress is one condition involved in the development of cell death (Slater *et al.*, 1996), the participation of antioxidant CoQ in transplasma membrane electron transport has opened new perspectives for understanding how redox reactions at the plasma membrane can control cell growth. In this way, the potential of plasma membrane dehydrogenases to prevent cell death through the maintenance of antioxidant molecules has to be considered as a very interesting function that can be involved in the maintenance of cell populations (Barroso *et al.*, 1997a,b; Villalba *et al.*, 1998). This review focuses on the role of CoQ and other antioxidants related to its redox state, as a central component of the plasma membrane electron transport system, in the control of cell population by the prevention of serum withdrawal-induced cell death, a model for stress-mediated apoptosis (Raff, 1992; Ishizaki *et al.*, 1995). The activation of a plasma membrane neutral sphingomyelinase (n-SMase), which results in an increase of ceramide levels, has been recognized as one of the initial steps in the cascade of events leading to cell death induced by serum-withdrawal (Jayadev *et al.*, 1995; Liu *et al.*, 1997). Because of its plasma membrane localization (Chatterjee, 1993), and its sensitivity to antioxidants such as glutathione (Liu and Hannun, 1997; Liu *et al.*, 1998a), n-SMase is a good candidate to be a target enzyme for regulation by the CoQ-dependent redox system at the plasma membrane. Novel data obtained by our group on the regulation of n-SMase and apoptosis by CoQ and other antioxidants will be discussed.

CoQ AS A CENTRAL MOLECULE OF PLASMA MEMBRANE REDOX SYSTEM

Incubation of plasma membranes with NADH results in a reduction of CoQ_{10}, which can be followed by direct absorbance reading at 410 nm (Sun *et al.*, 1992; Villalba *et al.*, 1995). By measuring the reduced and oxidized forms of CoQ after separation of membrane lipid extracts by high-performance liquid chromatography (HPLC), Arroyo *et al.* (1998) have shown that incubation of liver plasma membranes with NADH produces a decrease in the levels of the oxidized form with a concomintant increase in the reduced form, without change in the total amount of CoQ. These result indicate that $CoQH_2$ regeneration can be accomplished by enzymatic mechanisms at the plasma membrane.

Several enzymes have been reported to catalyze CoQ reduction in extramitochondrial membranes. At the plasma membrane, the NADH-cytochrome b_5 reductase can reduce CoQ through a one-electron reaction mechanism (Nakamura and Hayashi, 1994), and it is likely that this enzyme could be involved in the maintenance of basal levels of reduced CoQ under normal conditions (Constantinescu *et al.*, 1993, 1994; Navarro *et al.*, 1995; Arroyo *et al.*, 1998). In addition, the soluble enzyme DT-diaphorase can reduce quinones through a two-electron reaction mechanism (Lind *et al.*, 1990; Li *et al.*, 1995) and has been also reported to maintain the reduced state of hydrophobic CoQ homologues in phospholipid liposomes. It has been suggested that this could represent its actual role *in vivo* (Beyer *et al.*, 1996; Lindi *et al.*, 1997). DT-diaphorase is an inducible enzyme whose expression is increased by oxidative challenge (Rushmore *et al.*, 1991). Thus, the participation of this enzyme in CoQ reduction at the plasma membrane might be of particular importance under conditions of enhanced oxidative stress.

Once reduced, CoQ can donate electrons to drive transplasma membrane electron transport (Sun *et al.*, 1992; Villalba *et al.*, 1995). Solvent extraction of the membranes to remove CoQ, or treatment with quinone antagonists or nonfunctional CoQ analogues that compete for the quinone site, inhibit several activities related to the transmembrane flux of electrons to extracellular acceptors, such as NADH-AFR oxidoreductase, NADH-oxygen and diferric transferrin reductases, and part of the NADH-ferricyanide oxidoreductase. These activities can be recovered after restoration of CoQ_{10} (Sun *et al.*, 1992; Villalba *et al.*, 1995). Also, short-chain CoQ analogues increase redox activity at the plasma membrane (Vaillant *et al.*, 1996).

In addition to its role as electron carrier, $CoQH_2$ can also act as an antioxidant protecting the plasma membrane against peroxidations (Villalba *et al.*, 1996b, 1998). The CoQ-dependent plasma membrane oxidoreductase may be also important for regeneration of the reduced forms of other antioxidants, thus contributing to enhanced general protection. Plasma membrane CoQ_{10} levels of some leukemic cell lines cultured in 10% fetal calf serum-supplemented media, such as HL-60, ρ^0HL-60, Daudi, and K562 cells, range from about 50 to 100 pmol/mg protein with the following order: K562 < HL-60 < ρ^0HL-60 < Daudi. Interestingly, an inverse correlation between CoQ concentrations at the plasma and lipid peroxidation rates among these cell lines has been observed (López-Lluch *et al.*, 1999), which indicates that CoQ may be one of the major determinants for plasma membrane resistance against peroxidation.

The regeneration of α-tocopherol from the reduction of its phenoxyl (α-tocopheroxyl) radical by $CoQH_2$ or ubisemiquinone is well documented (Kagan *et al.*, 1990a,b, 1996; Beyer, 1994; Quinn *et al.*, 1999). This mechanism results in enhanced antioxidant protection that may also be operative at the plasma membrane (Constantinescu *et al.*, 1993, 1994). Kagan *et al.* (1998) reported that the NADH-cytochrome b_5 reductase can regenerate Trolox, a soluble analogue of α-tocopherol, by reducing its phenoxyl radical in a process that requires NADH and CoQ_0, and partially involves superoxide anions. Semiquinone radicals generated by reaction of superoxide with CoQ might be responsible for part of the regeneration activity observed (Stoyanovky *et al.*, 1995). Transplasma membrane NADH-AFR reductase may also have an antioxidant role through regeneration of extracellular ascorbate. A role for $CoQH_2$ in the regeneration of extracellular

ascorbate by reducing AFR outside the cells has been deduced from experimental evidence showing a requirement for CoQ in transmembrane NADH-AFR reductase, and in the ability of cells to decrease ascorbate oxidation rates and to scavenge AFR (Gómez-Díaz, 1997a,b; Santos-Ocaña *et al.*, 1998; Villalba *et al.*, 1998). In addition, ascorbate can also regenerate α-tocopherol in membranes, liposomes, and serum lipoproteins (Kagan *et al.*, 1990b; Thomas *et al.*, 1992; Sharma and Buettner, 1993), although this relationship has not been confirmed *in vivo* (Liebler, 1993).

Several lines of evidence support the idea that one of the functions of the plasma membrane redox system is the maintenance of antioxidant molecules, and a central regulatory role for CoQ in such a system could be postulated (Villalba *et al.*, 1996b, 1998). (1) The biosynthesis of CoQ has been demonstrated in all cells. In fact, CoQ is the only lipophilic antioxidant that can be synthesized *de novo* in all organisms tested (Ernster and Dallner, 1995). (2) CoQ biosynthesis is regulated according to the prevailing oxidative status, and levels of CoQ can be elevated as a cellular adaptation to oxidative stress conditions (Ernster and Dallner, 1995). According to Turunen *et al.* (1999), breakdown products of CoQ that can be formed during lipid peroxidation may play a role in the enhancement of CoQ biosynthesis under oxidative conditions. Besides CoQ, enzymes involved in its reduction at the plasma membrane are also upregulated under oxidative stress. Liver plasma membranes obtained from oxidatively stressed rats that had been fed with a diet deficient in vitamin E and selenium contained significantly increased levels of CoQ, NADH-cytochrome b_5 reductase, and membrane-bound DT-diaphorase. Thus, endogenous CoQ-reductases could compensate for the loss in antioxidant protection due to deficiency in those antioxidants whose levels are dependent on dietary supplementation (Navarro *et al.*, 1998, 1999). (3) reduced $CoQH_2$ or semireduced ubisemiquinone ($CoQ^{\cdot -}$) mediate the regeneration of other exogenous antioxidants and this may result in a sparing effect that decreases nutritional requirements. (4) CoQ participation in membrane-based electron-transporting systems couples antioxidant protection to the cell metabolism, via the oxidation of reduced pyridine nucleotides by the CoQ-reductases (Kagan and Tyurina, 1998).

The interactions between CoQ and other constituents of the plasma membrane redox systems have been summarized in Fig. 1.

PLASMA MEMBRANE ANTIOXIDANTS AND THE REGULATION OF CELL DEATH

Cell death can occur through different mechanisms which can be distinguished on the basis of unique morphological and biochemical features. Among these, necrosis and apoptosis are the most widely described forms of cell death. Necrosis is the consequence of a dramatic disturbance in cell homeostasis caused by severe physical or chemical harm to the cells, resulting in the swelling of cytoplasm, nucleus, and organelles, and the rupture of the plasma membrane. Leakage of lysosomal and cytosolic enzymes causes further tissue destruction and inflammatory reactions (Lee and Shacter, 1999). On the other hand, apoptosis is a regulated and active form of cell death in which the cell machinery participates to achieve its self-destruction. Early rupture of the plasma membrane is not observed during apoptosis. Instead, cells shrink and the nuclei condense and fragment. During the final stages of apoptosis, the cell is fragmented into small apoptotic bodies that are removed by phagocytic cells. Because no release of cytoplasmic components to the surrounding medium occurs, the apoptosis is a noninflammatory form of cell death. Apoptosis can be considered as a programmed cell death in the selective removal of cells during development (Clarke, 1990; Oppenheim, 1991).

A variety of stimuli have been reported to trigger cell death by apoptosis but as a rule, it occurs under those conditions where cells are unable to maintain their normal physiology. A few examples include glucocorticoids, exposure of cells to radiation, DNA-damaging agents such as etoposide, oxidation of cellular components, or absence of hormones or growth factors (Ishizaki *et al.*, 1995; Slater *et al.*, 1996). An increase in the cellular accumulation of ox-

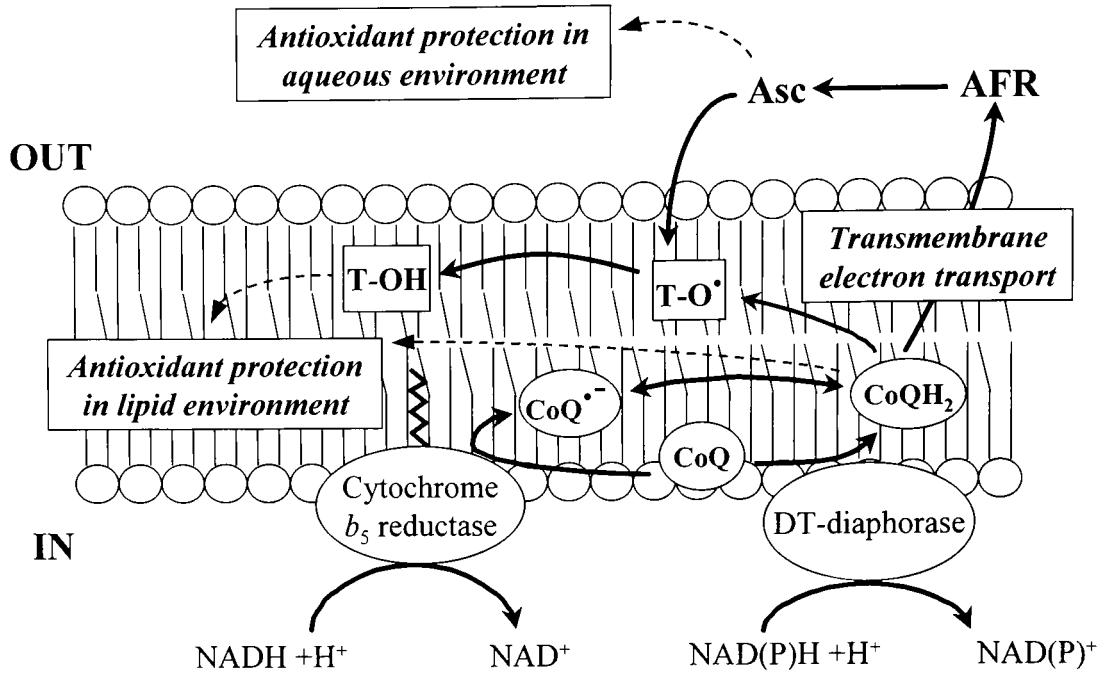

FIG. 1. The function of CoQ as a central molecule in plasma membrane redox system. CoQ links transplasma membrane electron transport and antioxidant protection to the major metabolic routes via the enzymatic recycling of $CoQH_2$. Enzymatic reduction of CoQ at the plasma membrane can be accomplished by several CoQ-reductases. The NADH-cytochrome b_5 reductase is a one-electron quinone reductase intrinsic to the membrane that catalyzes the reduction of CoQ to the semiquinone ($CoQ^{\cdot -}$ using NADH. $CoQ^{\cdot -}$ can either accept a second electron to generate $CoQH_2$ or dismutate to one CoQ and one $CoQH_2$. The cytosolic enzyme DT-diaphorase is a two-electron quinone reductase that reduces CoQ directly to $CoQH_2$ using both NADH and NADPH. $CoQH_2$ (or perhaps also the semiquinone) can donate electrons to extracellular acceptors, such as the ascorbate free radical (AFR), to mediate transmembrane electron transport. Reduction of extracellular AFR can contribute to the maintenance of ascorbate (Asc). Inside the membrane, $CoQH_2$ and $CoQ^{\cdot -}$ can react with α-tocopheroxyl radicals ($T\text{-}O^{\cdot}$) to regenerate α-tocopherol (T-OH). Both $CoQH_2$ and T-OH are lipophilic antioxidants that protect membranes against oxidations. Ascorbate also contributes to antioxidant protection in the aqueous environment, and enhances protection in the membrane by reducing $T\text{-}O^{\cdot}$ at the water–lipid interphase. Inducible responses to oxidative stress include the increase of plasma membrane CoQ and CoQ-reductases. A substantial increase of DT-diaphorase at the plasma membrane is one of the major observed changes, that can avoid pro-oxidant reactions of $CoQ^{\cdot -}$ in the absence of α-tocopherol.

idized species has been recognized as a common feature of apoptosis, and antioxidants have been reported to exert a general protective role against apoptosis induced by very different stimuli (Slater *et al.*, 1996). In most cases, low concentrations of oxidants promote apoptosis, whereas high concentrations result in cell death by necrosis. For instance, exposure of cells to low amounts of hydrogen peroxide promotes apoptosis, whereas necrosis occurs at millimolar concentrations of peroxide (Lennon *et al.*, 1991; Gardner *et al.*, 1997). Similarly, increasing doses of redox-cycling quinones progressively result in cell proliferation, apoptosis, and necrosis (Dypbukt *et al.*, 1994). In fact, high levels of oxidative stress can inhibit the apoptotic machinery by depleting cellular ATP thus leading to necrotic cell death (Lee and Shacter, 1999).

Apoptosis induced by serum-withdrawal: a model for stress-mediated cell death

Serum provides to cells those factors required to survive; its depletion activates an intrinsic cell death program (Raff, 1992; Raff *et al.*, 1993). Serum withdrawal causes cell cycle arrest and apoptosis in several cell types (Ishizaki *et al.*, 1995). Evidence exists for an indirect generation of oxidative stress by growth factor withdrawal. Removal of serum increases lipid peroxidation rates that arise in parallel to the induction of apoptosis (Slater *et al.*, 1996). Accordingly, lipid hydroperoxides can induce

apoptosis in antioxidant-defective cells (Sandstrom *et al.*, 1994), and apoptosis induced by serum-withdrawal can be prevented by expression of intracellular antioxidants such as catalase and superoxide dismutase (Hockenbery *et al.*, 1993; Greenlund *et al.*, 1995; Tilly and Tilly, 1995). Oxidative stress triggered by growth factors withdrawal is apparently related to a down-regulation of enzymes playing important roles in antioxidant defense, which results in the increased sensitivity to endogenous oxidative agents generated by the cell metabolism. It has been shown that nerve growth factor increases the expression of a number of antioxidant enzymes, such as catalase, glutathione peroxidase, and γ-glutamylcysteine synthetase in neuronal PC12 cells (Pan and Pérez-Polo, 1993; Sampath *et al.*, 1994). Also, insulin and platelet-derived growth factor (PDGF) in combination are required to maintain elevated levels of the quinone reductase DT-diaphorase in high-density growth-arrested BALB/c 3T3T cells (Schlager *et al.*, 1993).

The activation of a Mg^{2+}-dependent neutral sphingomyelinase (n-SMase) located at the plasma membrane has been recognized as one of the initial signaling events that take place during apoptosis induced by growth factors-withdrawal (Jayadev *et al.*, 1995; Liu *et al.*, 1997). Ceramide accumulated by hydrolysis of a distinct pool of signaling sphingomyelin (Zhang *et al.*, 1997) has been proposed to function as a biostat, in such a way that the increase in ceramide levels initiates cellular responses mediating cell growth, differentiation, stress response, and apoptosis (Hannun, 1996). Ceramide can modulate protein kinases, nuclear factors, enzymes of the eicosanoids pathway, and gene expression (Kim *et al.*, 1991; Ballou *et al.*, 1992; Raines *et al.*, 1993; Hannun, 1994). Serum deprivation induces a progressive increase of ceramide levels, which is then able to induce cell death after its intracellular accumulation (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Jayadev *et al.*, 1995; Barroso *et al.*, 1997a). This compound activates then proteases of the interleukin-converting enzyme (ICE) family (caspases), the general executioners of apoptosis (Miura *et al.*, 1993; Kumar, 1995; Martin *et al.*, 1995; Nicholson *et al.*, 1995; Mizushima *et al.*, 1996; Smyth *et al.*, 1996; Wolf and Green, 1999).

The gene product Bcl-2, first identified in human B-cell lymphomas, has been recognized as a general regulator of apoptosis (Reed, 1994; Smyth *et al.*, 1996; Zhang *et al.*, 1999). In fact, there is evidence to suggest that Bcl-2 suppress apoptosis by an antioxidant mechanism. Bcl-2 protects against lipid peroxidation induced by hydrogen peroxide (H_2O_2) and β-amyloid peptide in both isolated plasma and mitochondrial membranes (Bruce-Keller *et al.*, 1998). It also suppresses lipid peroxidation developed after the apoptotic signal (Hockenbery *et al.*, 1993; Kane *et al.*, 1993), and prevents the release of cytochrome *c* from mitochondria (Yang *et al.*, 1997). As mild oxidative stress has been established as one of the most reproducible inducers of cell death (Slater *et al.*, 1996), the potential of CoQ to prevent the initiation phase of apoptosis, upstream of the Bcl-2-dependent phase, appears as a very interesting function to maintain cell population (Barroso *et al.*, 1997a,b).

Electron-transporting membranes and stress-induced apoptosis: the role of mitochondria and the plasma membrane

Intracellular generation of ROS by electron-transporting membranes has been related to signaling processes controlling cell growth and apoptosis (Palmer and Paulson, 1997). For instance, it has been reported that ceramide generated by treatment of cells with tumor necrosis factor-α (TNF-α) increases intracellular H_2O_2 production by mitochondria. As a result, NF-κB activation and cell death induced by TNF-α can be blocked by inhibitors of mitochondrial electron transport, such as rotenone (Schulze-Osthoff *et al.*, 1993; García-Ruíz *et al.*, 1997). The increase in ROS production has been also related to NADPH-oxidase activity at the plasma membrane (Lo and Cruz, 1995). In this case, specific inhibition of the plasma membrane NADPH-oxidase, rather than inhibition of the mitochondrial electron transport chain, blocks the generation of signaling ROS (for review, see Palmer and Paulson, 1997). Recently, inhibition of quinone reductases by dicumarol has been shown to block the stress-activated protein kinase (SAPK/JNK) cascade, together with inhibition of NK-κB activation, resulting in increased apoptosis of TNF-α-stimulated

cells (Cross *et al.*, 1999). The location of the target reductase was not determined.

Quinone-dependent electron transport at the plasma membrane has been also related to ROS generation during the apoptotic signaling. Drugs that compete for CoQ_{10}, such as the vanilloids capsaicin dihydrocapsaicin, and resiniferatoxin (Vaillant *et al.*, 1996), and previously identified inhibitors of the plasma membrane oxidoreductase, such as chloroquine and retinoic acid, prevented cell growth; the pro-apoptotic effect of these substances was partially reversed by CoQ_{10}. Apoptosis induced by CoQ-antagonists involved calcineurin activation and could be inhibited downstream by Bcl-2 (Wolvetang *et al.*, 1996). Although CoQ_{10}-antagonists could impair both the plasma membrane and the mitochondrial electron transport chain, inhibition of the plasma membrane redox system was proposed as the cause triggering apoptosis. Cell death was decreased by preincubating cells with differic transferrin, a compound known to stimulate the plasma membrane NADH-oxidase (Sun *et al.*, 1987). In addition, apoptosis induced by vanilloids and inhibitors of the plasma membrane NADH-oxidase activity was distinguished from apoptosis induced by the mitochondrial inhibitor rotenone because the latter did not involve calcineurin (Wolvetang *et al.*, 1996). Calcium influx into the cells appears to mediate apoptosis induced by vanilloids and other inhibitors of the plasma membrane oxidoreductase. Accordingly, redox activity at the plasma membrane seems to modulate transplasma membrane fluxes of calcium in synaptosomes (Buillard *et al.*, 1990; Buillard and Dreyer, 1991), and ROS have been demonstrated to regulate gene expression by stimulating calcium-signaling (Suzuki *et al.*, 1997).

Thus, inhibition of the plasma membrane redox system through interference with a CoQ-binding site may redirect the normal electron flow and generate ROS. Interestingly, the consequent rise in ROS occurred before dissipation of the mitochondrial membrane potential was observed, further supporting the participation of the plasma membrane and not mitochondria (Macho *et al.*, 1998, 1999). Similar results have been observed with the anticancer drug adriamycin, a well-known inhibitor of transplasma membrane redox system (Sun and Crane, 1990). This compound induces the accumulation of oxygen radicals and also triggers the apoptotic process (Lenaz *et al.*, 1998).

The particular role of the plasma membrane redox system in the regulation of apoptosis, in comparison to that of the mitochondria, has been further studied with the use of mitochondria-defective ρ^0 cells that are depleted of a functional mitochondrial respiratory chain. These cells can be obtained by several weeks of treatment with ethidium bromide that results in a depletion of mitochondrial DNA (mtDNA), becoming thus totally dependent on the addition of substrates, such as pyruvate, for reoxidation of excess reduced pyridine nucleotides (King and Attardi, 1989). Some of these cells, such as the fibroblast-derived $\rho^0$701.2a cell line, are much more sensitive to serum withdrawal than parental cells and can be protected from apoptosis by the overexpression of Bcl-2 (Jacobson *et al.*, 1993). However, it has been shown that ρ^0 cells derived from the Namalwa cell line up-regulate the plasma membrane-associated redox activity during depletion of mtDNA, and can maintain growth with substrates for the plasma membrane redox system, such as ferricyanide or CoQ_{10} (Larm *et al.*, 1994; Lawen *et al.*, 1994). It has been also shown that ρ^0 cells derived from the HL-60 cell line up-regulate plasma membrane redox activity as a consequence of a significant increase in CoQ_{10} levels at this membrane (Gómez-Díaz *et al.*, 1997b). Both HL-60 and Namalwa-derived ρ^0 cells are more resistant to serum removal than parental cells (Larm *et al.*, 1994; Barroso *et al.*, 1997a).

Interestingly, ρ^0HL-60 cells produce a dramatic increase in their CoQ_{10} levels at the plasma membrane upon serum removal, and lipid peroxidation is significantly inhibited (Barroso *et al.*, 1997a). As stated above, CoQ_{10} addition reverses the pro-apoptotic effect of vanilloids and other inhibitors of the plasma membrane oxidoreductase activity. Thus, the ability of ρ^0HL-60 cells to increase CoQ_{10} at the plasma membrane could be related to less generation of ROS after serum-withdrawal resulting in a protection against cell death. On the other hand, a role for mitochondrial CoQ in promoting apoptosis has been also demonstrated, which is apparently in contrast with

the protective role of plasma membrane CoQ shown here. The pro-apoptotic effect is based on a generation of free radicals due to the improper function of the inner membrane electron chain (Quillet-Mary *et al.*, 1997), likely after cytochrome *c* release from mitochondria. In this way, we could speculate that the increase in plasma membrane CoQ, which correlates with improved survival of cells, is a method for cellular protection against free radical generation by damaged mitochondria.

Prevention of cell death by plasma membrane antioxidants

Mild oxidative stress is one of the conditions involved in the development of cell death induced by a variety of stimuli. Although its has been shown that several forms of induced apoptosis are independent of ROS, indirect generation of oxidative stress in developing apoptosis after growth factor withdrawal is generally accepted and, as a consequence, this mechanism can be inhibited by intracellular antioxidants (Greenlund *et al.*, 1995; Tilly and Tilly, 1995; Slater *et al.*, 1996; see above). In our group, we have studied the anti-apoptotic effect of those antioxidants related to the plasma membrane redox system, namely ascorbate, α-tocopherol, and CoQ, and its relationship to the antiapoptotic protein Bcl-2. As stated above, Bcl-2 suppress apoptosis by an antioxidant mechanism (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Bruce-Keller *et al.*, 1998) and cells containing Bcl-2, such as HL-60, are partially protected against both lipid peroxidation and apoptosis. However, Daudi, a cell line that lacks Bcl-2, is very sensitive to apoptosis induced by serum-withdrawal and transient expression of Bcl-2 decreases lipid peroxidation and protects cells against apoptosis. The addition of ascorbate or α-tocopherol to these serum-free cultures produces a significant decrese in lipid peroxidation and inhibits apoptosis induced by serum-withdrawal, regardless of the cellular content of Bcl-2 (Barroso *et al.*, 1997b; López-Lluch *et al.*, 1999). Ascorbate has been also shown to protect against apoptosis induced by removal of follicle-stimulating hormone in cultured ovarian follicles (Tilly and Tilly, 1995). Its protective role is not restricted to apoptosis induced by withdrawal of growth factors, but it has been also observed in apoptosis induced by etoposide (Maellaro *et al.*, 1996).

The action of ascorbate on cell death and growth has been reported to be highly dependent on ascorbate concentration. Ascorbate protects the cells against oxidative stress-induced apoptosis at concentrations lower than 1 m*M* (Sato *et al.*, 1993; Tilly and Tilly, 1995; Maellaro *et al.*, 1996; Barroso *et al.*, 1997b) and stimulates the growth of different cell lines in serum-limiting conditions (Alcaín *et al.*, 1990; Alcaín and Burón, 1994). Ascorbate also protects against cell death induced by UV irradiation (Savini *et al.*, 1999). On the other hand, ascorbate can induce cell death at concentrations higher than 1 m*M*, most likely due to its pro-oxidant activity (De Laurenzi *et al.*, 1995; Sakagami *et al.*, 1996). Pro-apoptotic effect of ascorbate has been shown to be dependent on the AFR intensity, and cytotoxicity appeared only when the radical intensity exceeded a certain threshold level (Sakagami *et al.*, 1996). Because AFR can accept electrons from the CoQ-dependent transplasma membrane dehydrogenase outside the cells (Villalba *et al.*, 1995) this effect could be similar to that of ferricyanide, which stimulates growth at low concentrations, but inhibits growth when used at concentrations above 30–100 μM (see above). As observed for those agents that interfere with the normal electron flow at the plasma membrane (Wolvetang *et al.*, 1996), apoptosis induced by exposure of cells to high concentrations of ascorbate also involved calcium signaling (Sakagami *et al.*, 1996). However, this pro-oxidant toxic effect of ascorbate is likely to occur only *in vitro*, whereas ascorbate acts mainly as an antioxidant under physiological conditions *in vivo* (Carr and Frei, 1999).

Addition of the lipophilic antioxidants CoQ_{10} (oxidized or reduced) or α-tocopherol to serum-free cultures also protected cells against apoptosis independently of Bcl-2 expression (Barroso *et al.*, 1997a; López-Lluch *et al.*, 1999). Similar to ascorbate, addition of CoQ_{10} to culture media also stimulates cell growth in the absence of serum in several cell lines including HeLa, BALB/3T3, HL-60, and K562 (Alcaín *et al.*, 1990; Sun *et al.*, 1992; Crane *et al.*, 1993; Mar-

tinus *et al.*, 1993; Sun *et al.*, 1995; Villalba *et al.*, 1996a). It is clear that α-tocopherol (or its hydrophilic analogue Trolox) can protect cells from apoptosis induced by a variety of stimuli such as serum-withdrawal (Barroso *et al.*, 1997b) and radiations (McClain *et al.*, 1995). It can also cooperate with ascorbate to protect cells against apoptosis induced by an oxidative attack (Sato *et al.*, 1993; Glascott *et al.*, 1996). Furthermore, addition of α-tocopherol to serum-free medium is sufficient to restore growth and, in the case of transformed cells, yields of cells can be even greater than those obtained in the presence of 10% serum (Burdon *et al.*, 1990). However, both α-tocopherol and Trolox can also regulate negatively the growth of a number of cell lines, such as U937 monoblastoid and smooth muscle cells, likely through a mechanism that is not accounted by its antioxidant properties (Tasinato *et al.*, 1995; del Bello *et al.*, 1999). α-Tocopherol but not β-tocopherol, an analogue of α-tocopherol with similar antioxidant properties, inhibits the proliferation of smooth muscle cells by inactivating protein kinase C-α (PKCα) through the activation of a protein phosphatase, which, in turn, dephosphorylates PKCα (Boscoboinik *et al.*, 1991; Azzi *et al.*, 1995, 1997; Tasinato *et al.*, 1995; Ricchiarelli *et al.*, 1998). On the other hand, activators of PKC can inhibit apoptosis induced by diverse mechanisms (Song *et al.*, 1992; Delia *et al.*, 1995) and Ras-specific cell death is dependent upon suppression of PKC activity (Chen and Faller, 1995). Whether or not prevention of stress-induced apoptosis by α-tocopherol is based on the regulation of PKC remains to be determined.

Effect of CoQ on the signaling pathway of serum withdrawal-induced apoptosis

How is the signaling pathway of serum withdrawal-induced apoptosis affected by CoQ? Barroso *et al.* (1997a) have observed that long-term accumulation of ceramide in HL-60 cells after serum withdrawal can be prevented by incubating cells with 40 μ*M* CoQ(H_2). Under these conditions, lipid peroxidation rates and the number of apoptotic cells are considerably decreased compared to serum-free cultures in the absence of added CoQ_{10}. Interestingly, ρ^0HL-60 cells, which contain elevated levels of CoQ_{10} at the plasma membrane, do not accumulate ceramide after withdrawal of serum, even in the absence of exogenous CoQ_{10}. These observations could indicate that a higher CoQ content at the plasma membrane determines a lack of ceramide accumulation after serum withdrawal and protection against cell death.

Regulation of serum withdrawal-induced apoptosis by plasma membrane CoQ apparently occurs at a very early step of death signaling because early events related to the development of the apoptotic program, such as the activation of neutral sphingomyelinase and short-term accumulation of ceramide, are also prevented by CoQ_{10} (Fernandez-Ayala, personal communication). Although it is clear that prevention of apoptosis by exogenous antioxidants including CoQ_{10} does not require Bcl-2 (Barroso *et al.*, 1997b; López-Lluch *et al.*, 1999), this antiapoptotic factor is indeed required in such systems where protection through the up-regulation of endogenous CoQ_{10} has been reported. The increase in CoQ associated to the plasma membrane has been observed for those cell lines expressing Bcl-2, such as HL-60 and ρ^0HL-60 (Barroso *et al.*, 1997a). A subline of CEM-C7H2 which overexpress transgenic Bcl-2 (CEM-9F3; Geley *et al.*, 1997; Susín *et al.*, 1997) also increases levels of plasma membrane CoQ_{10} after removal of serum. However, no change or even a decrease of plasma membrane CoQ_{10} was observed in those cell lines, which do not contain Bcl-2 or show a low expression of this protein, such as Daudi and CEM-C7H2 (Barroso, 1998). Thus, the possibility exists that Bcl-2 is required to ensure survival of cells after serum withdrawal and to allow for up-regulation of CoQ at the plasma membrane. However, whether or not Bcl-2 regulates the ability of cells to increase CoQ at the plasma membrane in response to oxidative stimuli remains to be elucidated.

Because growth factors withdrawal triggers apoptosis through the specific activation of the n-SMase (Jayadev *et al.*, 1995; Liu *et al.*, 1997), prevention of ceramide accumulation and protection against cell death by CoQ_{10} and other lipophilic antioxidants that affect its redox state could be interpreted on the basis of an inhibitory action on the n-SMase, similar to the

role proposed for glutathione (Liu and Hannun, 1997; Liu *et al.*, 1998b). However, hydroperoxides in plasma membrane are able to activate both phospholipase A_2 (PLA_2) (Hashizume *et al.*, 1991; Salgo *et al.*, 1993), and the n-SMase (Jayadev *et al.*, 1994, 1997). Thus, it is possible that CoQ and other antioxidants could indirectly inhibit the n-SMase by avoiding PLA_2 activation due to a decrease in lipid peroxidation rates. Finally, an alternative explanation to the lack of accumulation of ceramide after serum withdrawal in the presence of lipophilic antioxidants would be an unspecific protection, not directly related to the n-SMase, which decreases cell suffering and enhances survival, resulting in less accumulation of ceramide only as a secondary effect.

To address this problem, we have tested the putative inhibitory role of CoQ (oxidized and reduced) and other lipophilic antioxidants (α-tocopherol, α-tocopherylquinone, and α-tocopherylhydroquinone) on the n-SMase assayed with isolated plasma membranes or purified preparations of solubilized n-SMase (S.F. Martin, P. Navas, and J.M. Villalba, unpublished observations). A detailed description of our results will be published elsewhere. Briefly, we have found that several $CoQH_2$ homologues with varying isoprenoid side-chains inhibited very efficiently the n-SMase activity of both isolated plasma membranes and purified enzyme preparations, and $CoQH_2$ was much more active that the other tested lipophilic antioxidants at the same concentration. These results support a direct inhibitory action of $CoQH_2$ on the n-SMase. However, an indirect inhibition of the n-SMase, through the decrease in lipid peroxidation rates as an additional mechanism for n-SMase regulation, cannot be discarded because inhibition of the enzyme by $CoQH_2$ was much more pronounced in plasma membranes than in purified enzyme.

Thus, protection afforded by antioxidants related to the plasma membrane redox system is apparently located upstream Bcl-2 into the signaling pathway of apoptosis induced by serum-withdrawal, and may be represented by inhibition of the n-SMase which determines less ceramide accumulation. Bcl-2 can be found in different endomembranes, but is not apparently present at the plasma membrane (Hockenbery *et al.*, 1993). Intracellularly located Bcl-2 is essential to provide antioxidant protection against apoptosis (Hockenbery *et al.*, 1993; Kane *et al.*, 1993) and to prevent the release of cytochrome *c* from mitochondria (Yang *et al.*, 1997; Kluck *et al.*, 1997). At the plasma membrane, the antioxidant system sustained by the transmembrane oxidoreductase could then protect against oxidative stress generated at the extracellular space and regulate the activity of signaling n-SMase without the requirement for additional intracellular antioxidants (Fig. 2).

CONCLUSIONS

Compelling evidence is now available about the participation of the plasma membrane redox system in the control of cell growth, differentiation, and cell death, and both oxidants and antioxidants may be involved in the regulation of these processes. Oxidants, such as ferricyanide and AFR, can stimulate cell growth in low-serum media by modulating the signaling machinery that controls growth and proliferation. The activation of the transcription factor NF-κB in cells treated with low concentrations of ferricyanide has been demonstrated. Growth stimulation by CoQ may be also the result of increased CoQ-dependent transmembrane flux of electrons. Higher activities, which cannot be compensated by homeostatic intracellular mechanisms, may result in growth arrest due to severe alterations in the intracellular redox balance. On the other hand, reduced $CoQH_2$ is a powerful antioxidant that also regenerates other antioxidants such as ascorbate and α-tocopherol, and antioxidants can regulate several forms of stress-induced apoptosis. The participation of CoQ as a central molecule in the maintenance of a plasma membrane redox system involved in the regeneration of antioxidant molecules opens new perspectives to understand the role of redox reactions associated with the plasma membrane in the control of cell populations. Serum withdrawal-induced apoptosis is a model of stress-induced cell death that can be inhibited by antioxidants, and activation of the n-SMase is one of the earliest events recognized in the signaling pathway of this form of cell death. Although an indirect in-

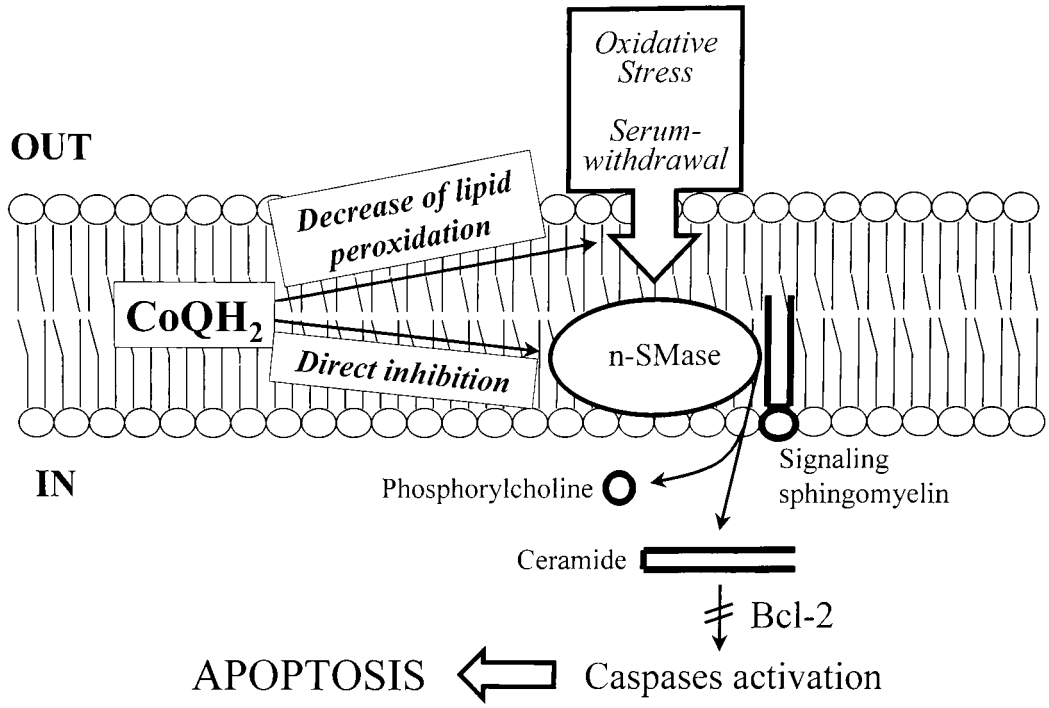

FIG. 2. A model for the role of $CoQH_2$ in the prevention of the initiation phase of serum withdrawal-induced apoptosis. Oxidative stimuli, such as serum withdrawal, activate a Mg^{2+}-dependent, neutral sphingomyelinase (SMase) which hydrolyzes a distinct signaling pool of sphingomyelin, apparently located in the inner leaflet of the plasma membrane, to phosphorylcholine and ceramide. Ceramide acts as an intracellular secondary messenger that activates the caspase cascade, leading to the irreversible death by apoptosis. The antiapoptotic protein Bcl-2 inhibits caspase activation and cytochrome *c* release from mitochondria. $CoQH_2$ and other lipophilic antioxidants maintained by the plasma membrane redox system can inhibit the initiation phase of apoptosis upstream Bcl-2. The protective role of $CoQH_2$ relies on (1) the direct inhibition of the n-SMase by lipophilic antioxidants and (2) an indirect effect due to a decrease of lipid peroxidation rates, which, in turn, avoids n-SMase activation.

hibition of the n-SMase by the CoQ-dependent antioxidant system due to a decrease in lipid peroxidation rates at the plasma membrane cannot be excluded, it is clear that $CoQH_2$ is also a direct inhibitor of this enzyme. Thus, the antioxidant system sustained by plasma membrane oxidoreductases could protect against cell death by regulating the activity of signaling n-SMase without the requirement for additional intracellular antioxidants. If this system is overwhelmed, then the apoptosis pathway can be activated and intracellular mechanisms of protection would be required.

ABBREVIATIONS

AFR, Ascorbate free radical; CoQn, coenzyme Qn (oxidized); CoQnH2, Ubiquinol, Coenzyme Qn (reduced); CoQnH, ubisemiquinone, Coenzyme Qn (semireduced); H_2O_2, hydrogen peroxide; HPLCs, high-performance liquid chromatography; ICE, interleukin-converting enzyme; mtDNA, mitochondrial DNA; n-SMase, neutral sphingomyelinase; NF-κB, nuclear factor kappa B; NO, nitric oxide; NQO_1, NAD(P)H:quinone oxidoreductase; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLA_2, phospholipase A_2; ROS, reactive oxygen species; SAPK/JNK, stress-activated protein kinase; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

ACKNOWLEDGMENTS

The work of the authors has been supported by the Spanish Ministerio de Educación y Cultura, Grants PB98-0329-CO2-01 and PB98-0329-CO2-02.

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Received for publication December 7, 1999; accepted February 22, 2000.

This article has been cited by:

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