

Review

Mitochondria, oxidative stress, and antioxidant defences*

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Mitochondria are strongly involved in production of reactive oxygen species, considered today as the main pathogenic agent of many diseases. A vicious circle of oxidative stress and damage to cellular structures can lead to either cell death by apoptosis or to a cellular energetic decline and ageing. The early involvement of mitochondria in apoptosis includes expression of pro-apoptotic factors, release of cytochrome *c* from the inter-membrane space and opening of the permeability transition pore: cytochrome *c* release appears to precede pore opening. The mitochondrial theory of ageing considers somatic mutations (deletions) of mitochondrial DNA induced by oxygen radicals as the primary cause of energy decline; experimentally, Complex I appears to be mostly affected. We have developed the Pasteur effect (enhancement of lactate production by mitochondrial inhibition) as a bio-marker of mitochondrial bioenergetics in human platelets, and found it to be decreased in aged individuals. Cells counteract oxidative stress by antioxidants; among lipophilic antioxidants coenzyme Q is the only one of endogenous biosynthesis; exogenous coenzyme Q, however, may protect cells from oxidative stress *in vivo*.

The occurrence of oxidative stress, caused by reactive oxygen species (ROS), is considered today as the main etiological and/or patho-

genic agent of most diseases (Diplock, 1994). Moreover, it is currently believed that ROS are involved in the progressive deterioration

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Abbreviations: AAPH, 2,2'-azobis-2-amidopropane; CoQ, coenzyme Q; mtDNA, mitochondrial DNA; ROS, reactive oxygen species.

of cell structures accompanying ageing (Harman, 1988; Shigenaga *et al.*, 1994; Lenaz, 1998a). Thus, the first energy source of aerobic organisms, oxygen, is also the basis of ageing and death.

Mitochondria are major producers of ROS by the respiratory chain (Chance *et al.*, 1979), and at the same time they are very vulnerable in their sophisticated machinery for oxidative phosphorylation (Ernster, 1993); thus, research has largely focused on mitochondria in order to understand ROS biochemistry and pathology. In addition, two major developments opened breakthroughs in the involvement of mitochondria in pathology, i.e. the discovery that *mitochondrial DNA* (mtDNA) mutations are at the basis of human diseases (Schapira, 1997), and the role, previously unexpected, of mitochondria in the mechanisms of cell death (Bernardi, 1998).

Among the cellular sources of ROS, the mitochondrial respiratory chain is perhaps the most powerful (Boveris *et al.*, 1976): it was calculated that 1–4% of oxygen reacting with the respiratory chain is incompletely reduced to superoxide anion and hence to hydrogen peroxide (Richter, 1988). Production of ROS may increase in respiratory State 4 with respect to State 3 (Boveris & Chance, 1973), or also when cytochrome oxidase activity is lowered (Sohal, 1993), because in both instances oxygen concentration increases and the level of reduced one-electron donors in the chain is concomitantly increased, enhancing their reaction with oxygen (Skulachev, 1996; Papa & Skulachev, 1997). According to Skulachev (1996), uncoupling of electron transfer chains, by raising the rate of oxygen consumption, may represent a device for decreasing ROS production in mitochondria. ROS production by the respiratory chain is also increased after anoxia and re-perfusion (Downey, 1990; Downey *et al.*, 1991); one additional reason may be that the anoxic period, by depleting ATP, induces damage and release of catalytic metals (Hershko, 1992), such as iron and copper, which are abundant

in the inner mitochondrial membrane and are known to enhance the effect of ROS.

All biological molecules may be modified by ROS; their aggressive behaviour is bound to damage first those molecules which are nearest to the site of production: mitochondrial structures, therefore, are also believed to represent major targets of oxidative stress (Ernster, 1993). Lipid peroxidation (Ernster, 1993; Gardner, 1989), protein oxidation (Stadtman, 1993), and DNA damage (Richter *et al.*, 1988; Dizdaroglu, 1991) are all consequences of oxidative stress at the level of mitochondria. Whatever the initial damage, be it directly in protein, or indirectly in the lipid environment (Lenaz & Degli Esposti, 1994), or in the encoding sequence of DNA, it will be anyway a functional protein alteration to ensue, with failure of enzymatic, transport, or receptor systems.

A vicious circle of oxidative stress and damage of cellular structures (Ozawa, 1995; 1997) has been postulated to occur: since any damage to the respiratory chain may induce enhancement of ROS production, whatever the initial alteration a progressive and continuous perpetuation of the damage will be the inevitable consequence, with amplification of the original defect through somatic mutations of mtDNA, and defective mtDNA encoded proteins. It is plausible that an acute stress would trigger a mechanism inducing cell death, whereas a milder stress may slowly induce impairment of cell function, as in ageing (Lenaz, 1998a) (Fig. 1).

This review will be divided into three parts. In the first part we will consider the major role of mitochondria and oxidative stress in apoptotic cell death; the second part will deal with evidence about the involvement of mitochondria in the ageing process; finally, the battery of instruments the cells contain to counteract oxidative stress will be considered.

We will restrict our discussion largely to work performed in our own laboratory and discuss it in the light of recent literature; this paper, therefore, is not intended to represent

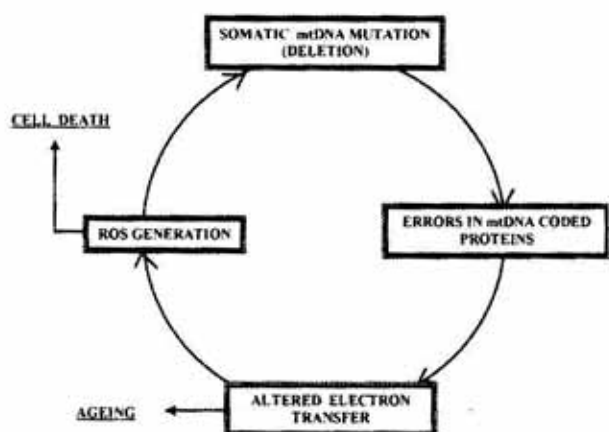


Figure 1. A vicious circle of mtDNA mutations and oxidative stress.

a complete survey of the literature in the field.

MITOCHONDRIA, OXIDATIVE STRESS, AND APOPTOSIS

Apoptosis is a form of cell death requiring a well-defined chain of enzymatic events which are genetically programmed (Kroemer *et al.*, 1995); in this requirement, apoptosis differs from necrosis, although both types of cell death can derive from the same exogenous and endogenous insults (Wyllie *et al.*, 1980; Shimizu *et al.*, 1996). A recent, exciting, de-

velopment has been the discovery that mitochondria are deeply and early involved in the apoptotic cascade (Susin *et al.*, 1998, for review) (Fig. 2). The release of cytochrome *c* from the inter-membrane space (Krippner *et al.*, 1996; Liu *et al.*, 1996; Yang *et al.*, 1997; Kluck *et al.*, 1997; Cai *et al.*, 1998) activates *caspase-3*, a protease whose activation also requires a protein called Apaf-1, and is inhibited by Bcl-2 and similar proteins. The proteins of the Bcl-2 family (Hockenbery *et al.*, 1990; Reed *et al.*, 1998) are localised in the outer membrane, where pro-apoptotic proteins such as Bax and Bad, structurally related to the former, are also present. Also opening of the so-called *permeability transition pore* (Bernardi, 1996), a mega-channel of the inner mitochondrial membrane of still uncertain identification, is a fundamental step in apoptosis (Marchetti *et al.*, 1996). Its opening induces mitochondrial depolarisation and swelling (Bernardi & Petronilli, 1996). The relation between cytochrome *c* release and pore opening is still controversial (Cai *et al.*, 1998): whether the latter phenomenon, with swelling and rupture of the outer membrane, is the cause of cytochrome *c* release, whether cytochrome *c* loss and consequent decrease of respiration induce depolarisation and pore opening, or whether they are two independent phenomena. Oxidative stress may be an intrinsic component of the apoptotic cascade (France-

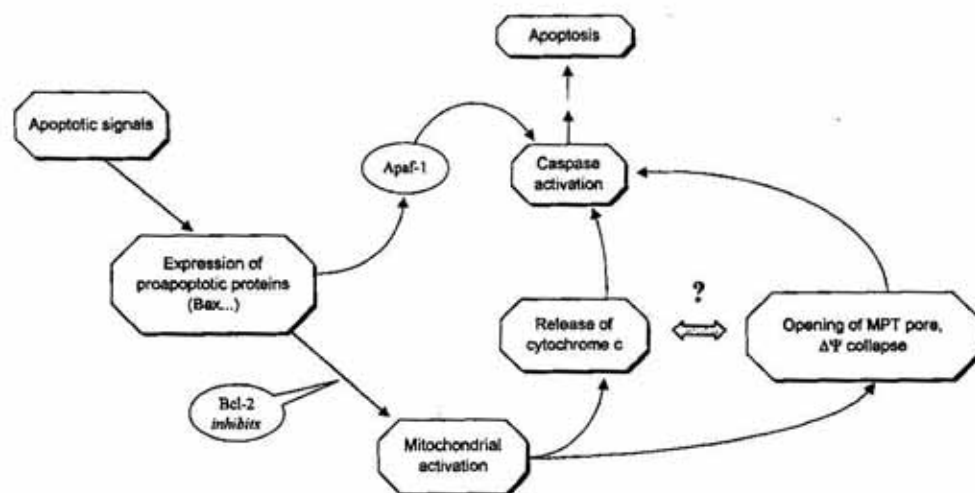


Figure 2. Involvement of mitochondria in the apoptotic cascade.

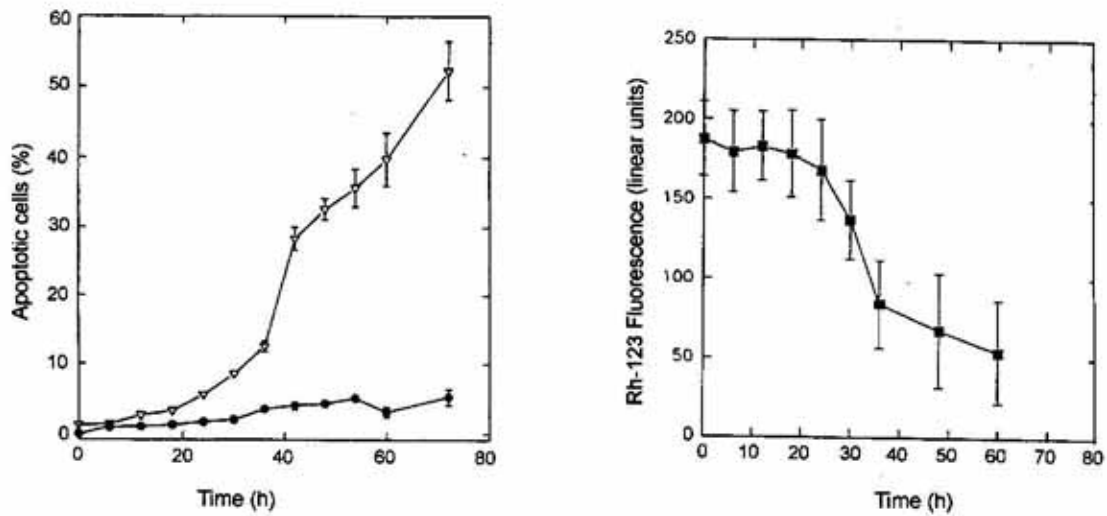


Figure 3. Time course of apoptosis (left panel) and of mitochondrial membrane potential (right panel) in SH-SY5Y cells subjected to an oxidative stress.

Cells, differentiated with 10 μM retinoic acid, were treated with 200 μM AAPH. Apoptotic cells were detected by TUNEL and $\Delta\Psi_{\text{mit}}$ by Rhodamine 123 incorporation and flow cytometry (Lenaz *et al.*, 1998).

Lanord *et al.*, 1997; Susin *et al.*, 1998; Nicholls & Budd, 1998) and is known to cause apoptosis (Petit *et al.*, 1996).

To better understand the steps involved in apoptosis at the mitochondrial level, we have investigated apoptosis induced by a free radical initiator in neuroblastoma cultured cells; in our hands this system has proven to act slowly, thus allowing to establish the time course of different events (Lenaz *et al.*, 1998).

After addition of 200 μM 2,2'-azobis-2-amidinopropane (AAPH), an increase of ROS occurred very early with a peak followed by a steady-state period after about 6 h from the initiator; apoptotic death started after 30 h, preceded by a fall of mitochondrial membrane potential ($\Delta\Psi_{\text{mit}}$) after 24 h (Figs. 3 and 4). Nevertheless, release of cytochrome *c* from mitochondria to cytoplasm was maximal already at 12 h from the oxidative stress (Table 1). Thus, in the above system, cytochrome *c* release is an earlier event than the opening of the permeability transition pore, that is the likely cause of mitochondrial potential collapse.

The mechanism by which cytochrome *c* is released from the inter-membrane space is unknown; it has been suggested that pro-

apoptotic proteins, such as Bax, can form large channels in the outer membrane (Reed *et al.*, 1998); alternatively, the outer membrane itself might be ruptured allowing loss of soluble proteins from the inter-membrane space (Vander Heiden *et al.*, 1997; Schendel *et al.*, 1997).

MITOCHONDRIA AND AGEING

The progressive decline of cell functions accompanying senescence has been attributed to either a genetic program inborn in all organisms or to stochastic errors occurring at random in somatic cells and leading to a final collapse of their functions (Medvedev, 1990). Among the stochastic theories, *the free radical theory of ageing* (Harman, 1956; 1988) was based on the idea that cells, continuously exposed to ROS, are progressively damaged in their most vital macromolecules. The implication of mitochondria both as producers and as targets of ROS has been the basis for *the mitochondrial theory of ageing* (Miquel *et al.*, 1980; Linnane *et al.*, 1989); the theory postulates that random mtDNA alterations in somatic cells are responsible for the energetic decline

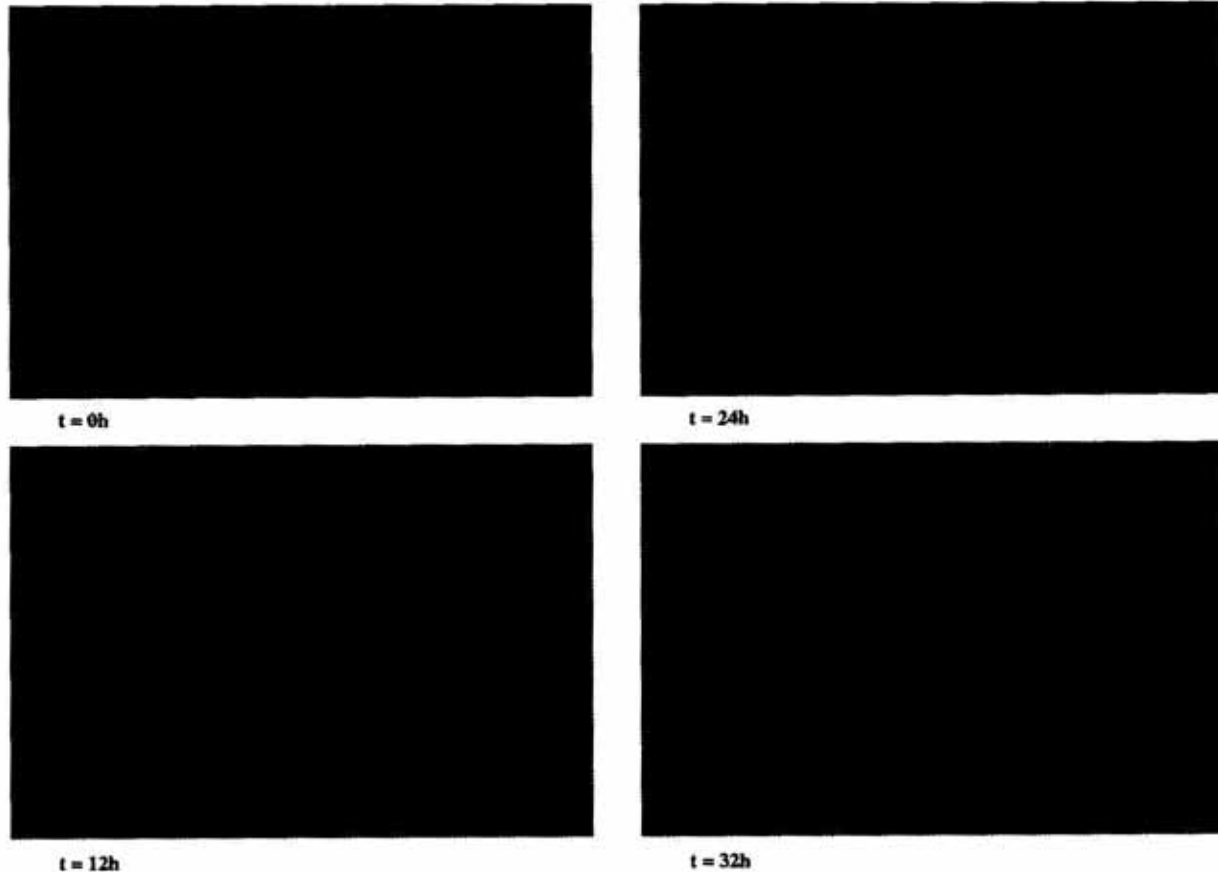


Figure 4. Mitochondrial membrane potential *in situ* in SH-SY5Y cells subjected to oxidative stress, as in Fig. 3.

$\Delta\Psi_{mit}$ was detected by JC1 fluorescence and confocal microscopy. Orange fluorescence indicates probe incorporation in mitochondria and energization; green fluorescence indicates monomeric extramitochondrial probe and mitochondrial depolarization (M. Cavazzoni and G. Lenaz, unpublished).

accompanying senescence. It is proposed that accumulation of somatic mutations of mtDNA, induced by exposure to ROS, leads to errors in the mtDNA-encoded polypeptides; these errors are stochastic and randomly transmitted during mitochondrial division and cell division. The consequence of these alterations, which affect exclusively the four mitochondrial complexes involved in energy conservation (Table 2), would be defective electron transfer and oxidative phosphorylation. Respiratory chain defects may become associated with increased ROS production, thus establishing a vicious circle (Ozawa, 1995), described in Fig. 1.

A proof of the existence of a vicious circle of oxidation and mtDNA damage requires demonstration that ROS production and their effects increase in ageing.

The inverse relation existing of both ROS production and metabolic rate with normal life-span of different animal species (Cutler, 1985; Sohal *et al.*, 1995; Agarwal & Sohal, 1994) is in line with this postulate, suggesting that mitochondria, as the sites of oxygen consumption, must be deeply involved in the ageing process. Moreover, it was indeed shown that hydrogen peroxide generation increases with age (Sohal *et al.*, 1995; Sastre *et al.*, 1996; Hagen *et al.*, 1997). In isolated rat hepatocytes we have shown that hydrogen peroxide production is higher and the effect of an oxidative stress by adriamycin is much stronger in cells isolated from older rats than from young animals (Fig. 5).

Increased production of ROS in ageing is also inferred from the increase of oxidised cellular components with age (Rice-Evans &

Table 1. Effect of oxidative stress on SH-SY5Y differentiated cells (Lenaz *et al.*, 1998)

Time (hours)	Peroxide content ^a		Bax protein content ^b		Cytochrome <i>c</i> released ^c		$\Delta\Psi_{mit}$ ^d		Apoptotic death ^e	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
	(pmol/10 ⁶ cells)						Fluorescence arbitrary units		(%)	
0	0.7 ± 0.2	0.8 ± 0.2	+	+	-	-	234 ± 17	228 ± 31	0.9 ± 0.2	1.1 ± 0.1
6	3.5 ± 0.3	52.3 ± 5.8	+	+	-	-	221 ± 21	216 ± 44	1.0 ± 0.3	1.3 ± 0.6
9	2.7 ± 0.4	46.2 ± 4.3	+	++++	-	-	225 ± 35	218 ± 22	1.2 ± 0.4	2.6 ± 0.4
12	4.1 ± 0.5	38.1 ± 4.1	+	+++	-	+++	205 ± 27	213 ± 33	1.6 ± 0.3	3.2 ± 0.6
24	2.6 ± 0.4	25.8 ± 2.9	+	+++	-	+++	211 ± 44	134 ± 23	2.0 ± 0.4	7.1 ± 0.6

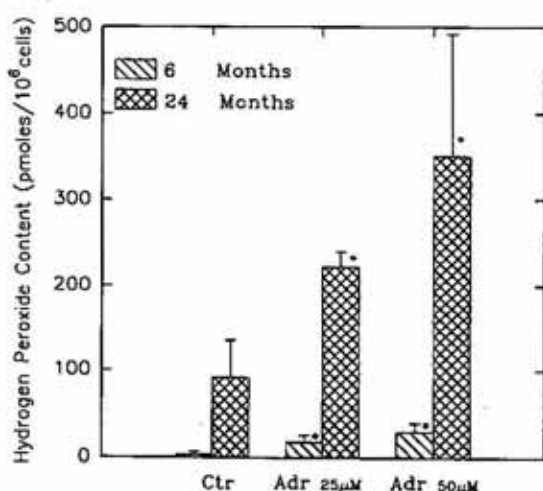
^aFree-radical content was determined as dichlorofluorescin-derived green fluorescence and quantitatively converted through a calibration curve. ^bBax protein was determined by immunoblotting using the monoclonal antibody sc-1716 (Santa Cruz Biotechnology). ^cCytosolic cytochrome *c* was measured by immunoblotting using the monoclonal antibody 7H8.2C12 (Pharmingen). ^d $\Delta\Psi_{mit}$ was measured as Rhodamine-123 uptake. ^eApoptotic cells were quantified by Hoechst-33342 staining or TUNEL.

Diplock, 1993; Diplock, 1994; Stadtman, 1993; Ozawa, 1997). Among the damaged molecules, mtDNA is most important for the mitochondrial theory. The search for point mutations in mtDNA in ageing has led to controversial results (Schon *et al.*, 1996; Pallotti *et al.*, 1996); on the other hand, large dele-

tions have been consistently found in aged individuals (Schon *et al.*, 1996). Considering the total number of deletions, it is suggested that they can account for such an amount to overcome the threshold required for decreasing the respiratory chain activity. Ageing is accompanied by progressive fragmentation of mtDNA, formation of mini-circles and progressive cell death by apoptosis (Yoneda *et al.*, 1995; Ozawa, 1995; 1997).

The finding of extensive deletions in ageing and the notion that oxidative stress can induce deletions (Shigenaga *et al.*, 1994; Yoneda *et al.*, 1995; Yakes & Van Houten, 1997) is no proof *per se* that oxidative stress is involved: in fact mtDNA deletions can arise from altered nuclear-mitochondrial interactions, as in an autosomal dominant form of PEO (paralysis of extra-ocular muscles) (Zeviani *et al.*, 1989), indicating that a nuclear gene product affects the proclivity of mtDNA to suffer deletions.

The observation that oxidative phosphorylation defects in cultured cells from aged donors could be reversed by constructing cybrids (cytoplasmic hybrids), where the nucleus from the old donor was substituted by a nucleus from an immortalised cell (Hayashi *et al.*, 1994), suggested that a nuclear mutation

**Figure 5. Increase of oxidative stress in ageing.**

The figure shows peroxide production in hepatocytes isolated from adult (6 months) and old (24 months) rats and the effect of an oxidative stress induced by adriamycin. Peroxide production was determined by the fluorescent probe dichlorofluorescin diacetate (unpublished data from our laboratory). Ctr, control; Adr, adriamycin treated.

Table 2. Polypeptides encoded by mtDNA

Mitochondrial enzyme	H ⁺ pumping	Subunits encoded by mtDNA
Complex I (NADH-CoQ reductase)	+	ND1, ND2, ND3, ND4, ND4L, ND5, ND6
Complex II (succinate CoQ reductase)	-	-
Complex III (ubiquinol cytochrome c reductase)	+	cytochrome <i>b</i>
Complex IV (cytochrome c oxidase)	+	CO I, CO II, CO III
ATP synthase	+	ATPase-6, ATPase-8

might be responsible for the mitochondrial deficit; although a subsequent study demonstrated formation of respiratory-deficient clones having decreased mtDNA by fusing mitochondria from fibroblasts of aged donors with ρ^0 cells (Laderman *et al.*, 1996), the intervention of nuclear recessive mutations responsible for decreased mitochondrial translation in ageing was documented by demonstrating that nuclear hybrids of mtDNA-less HeLa cells and fibroblasts from old donors, formerly deficient in mitochondrial protein synthesis, resumed normal translation activity (Isobe *et al.*, 1998). Thus a unique role of mtDNA in ageing has yet to be established.

The search for age-dependent changes in mitochondrial bioenergetics has produced a plethora of conflicting results, reporting either significant changes or no differences (cf. Hansford, 1983; Lenaz, 1998). There may be several reasons for explaining such conflicting data (Lenaz, 1998a).

First, it has been theorised that mtDNA mutations are expected to accumulate and to lead to damage mainly in post-mitotic cells (Miquel, 1992), where oxidative metabolism is very elevated as in neurones or is subjected to bursts as in skeletal muscle; moreover, in such cells, the lesions could be conserved, at difference with mitotic cells where division leads to selection and washing away of deficient cells (Byrne *et al.*, 1991). Mitochondria, when isolated, are obtained from tissues containing both differentiated non-dividing cells and relatively non-ageing dividing cells; thus small changes in one population only may become undetectable; furthermore energy-

deficient cells may undergo elimination by apoptosis (Lawen *et al.*, 1994); the continuous cell loss when mitochondria become deficient would prevent observing important energetic changes in the remaining population. The best demonstration of bioenergetic alteration in mtDNA-encoded proteins has been the histochemical detection of a loss of cytochrome *c* oxidase activity (but not of succinate dehydrogenase) in muscle mitochondria from old individuals (Muller-Höcker *et al.*, 1992); the mosaic distribution of the cytochrome oxidase deficient fibres agrees with the stochastic distribution of mitochondrial damage expected from the mitochondrial theory (Linnane *et al.*, 1989).

If the energetic impairment derives from a stochastic damage to the mitochondrial genes, then it is important to select the mitochondrial activity which is more likely to be affected. Since 7 out of the 13 polypeptides encoded by mtDNA belong to Complex I (NADH-coenzyme Q reductase), it is expected that this enzyme should be mostly affected by ageing (Lenaz *et al.*, 1997). The so-called ND sub-units, encoded by mtDNA, are involved in binding of the electron acceptor, i.e. coenzyme Q (CoQ, ubiquinone) and of several inhibitors, such as rotenone, and most probably in the mechanism of energy conservation by proton translocation (Degli Esposti & Ghelli, 1994; Brandt, 1997).

Complex I activity is investigated using low relative molecular mass quinones (Lenaz, 1998b), which are homologues or analogues of the natural acceptor, a long isoprenoid chain ubiquinone, which is CoQ₁₀ in man and

most mammals, and largely CoQ₉ in rodents (Ramasarma, 1985). The overall rate of the respiratory chain is hyperbolically related to the rate of reduction of the CoQ pool and to the rate of its re-oxidation by the *pool equation* (Kröger & Klingenberg, 1973): since problems exist in the correct evaluation of Complex I activity with the artificial acceptors (Estornell *et al.*, 1993; Fato *et al.*, 1996), we have preferred, when possible, to use the pool equation to calculate Complex I activity in liver and heart of aged rats (Genova *et al.*, 1995) (Table 3): the experimental rates of NADH-CoQ₁ reductase activity were underestimated, whereas the rates calculated from the pool equation showed clear decreases of activity in the aged animals.

in an individual step: values approaching 1 indicate that the step is rate-limiting, values approaching zero indicate that the step is not rate-limiting. Following NADH oxidation in our system we found a very similar rotenone inhibition curve in NADH oxidase and NADH-CoQ reductase activity, with a flux control coefficient approaching 1 in both mitochondria from young and old rats (Fig. 6), indicating that Complex I is rate-limiting over the entire respiratory chain in both types of animals, and therefore the NADH oxidase activity is a reflection of Complex I activity alone (Lenaz *et al.*, 1998).

In non-synaptic mitochondria from brain cortex of old rats, in addition to the decrease of NADH oxidation rate, we also found a de-

Table 3. Experimental and calculated Complex I activities of liver and heart mitochondria from 6- and 24-month-old rats (Genova *et al.*, 1995)

Activity	Liver		Heart	
	6-month	24-month	6-month	24-month
	$(\mu\text{mol}/\text{min} \times \text{mg protein})$		$(\mu\text{mol}/\text{min} \times \text{mg protein})$	
NADH-O ₂	0.157 ± 0.037	0.101 ± 0.043	0.983 ± 0.203	0.700 ± 0.162
NADH-DB	0.154 ± 0.021	0.120 ± 0.041	0.292 ± 0.081	0.256 ± 0.059
NADH-CoQ (calculated ^a)	0.226 ± 0.065	0.134 ± 0.081	2.258 ± 0.691	1.288 ± 0.497

^aFrom the pool equation, see text.

In non-synaptic mitochondrial particles from rat brain cortex (Genova *et al.*, 1997), we found that the aerobic NADH oxidation is significantly decreased, suggesting a Complex I defect; the relevance of NADH oxidation to Complex I activity, however, requires that the activity of Complex I is rate-limiting over the whole respiratory chain, otherwise threshold effects would be present (Davey & Clark, 1996; Kuznetsov *et al.*, 1997).

The metabolic control theory (Kacser & Burns, 1979) determines the control that various steps in a pathway have over the global flux of the same pathway; the flux control coefficient is defined as the fractional change in pathway flux induced by a fractional change

crease of its rotenone sensitivity (Genova *et al.*, 1997), evidenced as an increase of the I₅₀, the inhibitor concentration eliciting 50% inhibition of activity (Table 4). Since rotenone, as well as most Complex I inhibitors, bind the hydrophobic ND subunits (Degli Esposti, 1998), this finding would be in line with the mitochondrial theory. No major changes were found in synaptic mitochondria (Genova *et al.*, 1997), an unexpected difference whose explanation may be that they have a lower respiration rate and would therefore be less exposed to ROS attack; another explanation could be that only healthy non-damaged mitochondria migrate by axonal flow from the cell body to the synapse.

HUMAN PLATELETS AS BIOMARKERS OF AGEING

The search for a sensitive marker of ageing in man, representing an individual index of biological age and predisposition to age-related diseases, must consider mitochondrial function; since such a function may only be investigated in cells containing mitochondria, blood platelets may represent a unique system (Holmsen, 1987), in that they possess mitochondria and may be easily collected by non-invasive procedures. Moreover, platelets were proposed as a possible marker of acquired neurological diseases, bearing some biological similarities with neurones (Da Prada *et al.*, 1988). The rational use of platelets as a bio-marker of mitochondrial lesions (Schapira, 1998a, b) rests on the assumption that alterations occurring in senescence and in age-related diseases be present in all cells, and that platelets may signal generalised bioenergetic deficiencies (Lenaz *et al.*, 1998).

It is known that platelets yield energy for their functions by both glycolysis and mitochondrial oxidative phosphorylation (Holmsen, 1987): the main function of platelets is their energy-dependent aggregation process (De Gaetano, 1981), a part of the mechanism of blood clotting, physiologically elicited *via* exocytosis of their secretory granules.

An alteration of Complex I activity in platelet mitochondria in aged individuals is indi-

cated by the increase of I_{50} of rotenone inhibition (Merlo Pich *et al.*, 1996), similar to that detected in rat brain cortex mitochondria. Since, as already mentioned, rotenone binds subunits of the enzyme involved in proton translocation, the altered inhibitor sensitivity may represent an indication of altered energy conservation. Accordingly, the mitochondrial contribution to energy-dependent platelet aggregation decreased in the aged (Lenaz *et al.*, 1998). The rate of the irreversible phase of platelet aggregation induced by ADP was taken as an index of bioenergetic competence of the cells.

Platelet aggregation can be partially inhibited by the respiratory chain inhibitor, antimycin A: the sensitivity of aggregation to antimycin was significantly decreased in old individuals with respect to young controls, suggesting that energy for aggregation is provided more and more by glycolysis in the old population.

This interesting result is however not likely to quantitatively indicate the loss of mitochondrial function, since inhibition of aggregation would depend upon the extent to which total cellular ATP is rate-limiting for aggregation, and how much glycolytic substrate-level phosphorylation compensates the decrease of oxidative phosphorylation (cf. Holmsen *et al.*, 1974). A quantitative determination of glycolytic and mitochondrial ATP can be provided by the Pasteur effect (Holmsen & Robkin,

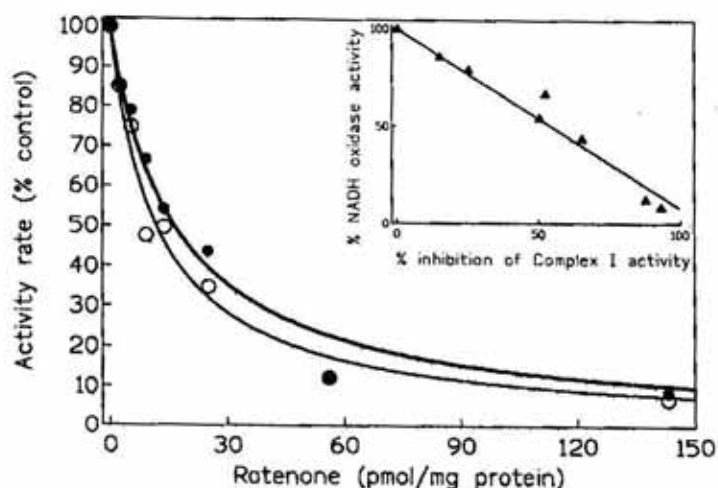


Figure 6. Flux control of NADH oxidation in rat brain cortex non-synaptic mitochondria (cf. Lenaz *et al.*, 1998).

The figure shows the stepwise inhibition by rotenone of NADH-CoQ reductase (○) and of NADH oxidase (●). The inset is a plot of NADH oxidase rates against inhibition of Complex I activity after rotenone titration. Flux control coefficients were calculated as described elsewhere (Lenaz *et al.*, 1998). The curves were almost identical for adult and old animals.

Table 4. Biochemical parameters in nonsynaptic mitochondria from rat brain cortex (cf. Genova *et al.*, 1997)

Age	Mitochondrial yield	NADH oxidase activity		NADH-ferricyanide reductase		I ₅₀ of rotenone corrected ^a
		nmol/min × mg protein	nmol/min × g tissue	μmol/min × mg protein	μmol/min × g tissue	
Months	mg protein/g tissue					
4	4.63 ± 1.06 [13]	206 ± 74 [13]	926 ± 318 [13]	4.29 ± 1.26 [13]	19.2 ± 5.2 [13]	6.8 ± 1.7 [11]
24	3.40 ± 1.82 [10]	130 ± 51 [13]	444 ± 356 [10]	3.49 ± 0.73 [13]	11.8 ± 6.0 [10]	10.8 ± 4.8 [11]
	n.s.	P = 0.006	P = 0.003	P = 0.062	P = 0.004	P = 0.025

Free nonsynaptic mitochondria were purified from brain cortex of single animals and assayed for NADH oxidation according to Genova *et al.* (1995). The I₅₀, i.e. the inhibitor concentration eliciting half-inhibition, was taken as an indicator of Complex I sensitivity to rotenone. The specific activity of ferricyanide reduction was used as a parameter proportional to the content of active Complex I in the membrane. ^a(pmol rotenone/mg protein)/(NADH ferricyanide reductase).

1980): a decreased mitochondrial function stimulates glycolysis in order to maintain a constant ATP synthesis, and meanwhile pyruvate is reduced to lactate to regenerate oxidised pyridine nucleotides. Since glycolysis in the absence of mitochondrial function yields two ATP and two lactate molecules per glucose broken down, it follows that stimulation of lactate production by inhibition of respiration is equivalent to the correspondingly inhibited mitochondrial ATP production.

Lactate production by washed platelets is strongly enhanced by antimycin A inhibition of mitochondrial respiration; the Δ-lactate production in presence and absence of antimycin A represents the amount of ATP produced *via* oxidative phosphorylation, whereas the basal lactate production represents glycolytic ATP (Holmsen, 1987) (Table 5). Both the Δ-lactate and the ratio of oxidative ATP over glycolytic ATP are significantly decreased in aged individuals (Table 6).

The site of the mitochondrial bioenergetic lesion is probably respiratory Complex I, as shown by the decreased rotenone sensitivity of this enzyme in the aged (Merlo Pich *et al.*, 1996). The fact that the "common" deletion of mtDNA (Schon *et al.*, 1996), detected in all post-mitotic tissues investigated so far, is absent in platelets (Biagini *et al.*, 1998), would both agree and contrast the mitochondrial theory; in fact the theory expects mtDNA to be affected in post-mitotic tissues but not in

mitotic or short-lived cells (Miquel, 1992); on the other hand a Complex I defect has been detected: such a defect, following this line of reasoning, should not result from mtDNA deletions. Thus mtDNA cannot be the sole cause of bioenergetic defects detected in ageing (Lenaz, 1998a); a decreased stability of Complex I, leading to decreased rotenone sensitivity and to decreased energy conservation, could be alternatively due to direct damage of the enzymatic proteins or of the lipid environment of the enzyme (Lenaz, 1998a): to this respect, the reported decrease of cardiolipin

Table 5. Lactate production in human platelets.

Typical example of platelets from a young individual.

Treatment	Lactate
	(μmol / 10 ¹¹ cells × min)
Control (glycolytic ATP)	0.18
+ Antimycin A	4.57
Δ-Lactate (mitochondrial ATP)	4.39
Mitochondrial ATP	4.39
Glycolytic ATP	0.18
= $\frac{4.39}{0.18} = 24.4$	

Glucose flux through anaerobic glycolysis

0.18:2* = 0.09 μmol / min per 10¹¹ cells (43%)

4.39:36** = 0.12 μmol / min per 10¹¹ cells (57%)

*ATP yield/glucose in anaerobic glycolysis.

**Mean ATP yield/glucose in oxidative phosphorylation.

content in the aged (Paradies *et al.*, 1992) was accompanied by decreased cytochrome oxidase activity (Paradies *et al.*, 1997a,b).

The use of the Pasteur effect as a bio-marker is independent of the primary site of the mitochondrial change in platelets from aged individuals and is able to recognise any mitochondrial alteration inducing decreased ATP synthesis.

ANTIOXIDANT DEFENCES: ROLE OF COENZYME Q

Cells contain enzymatic systems capable to convert ROS into less toxic or non-toxic species (Diplock, 1994); the co-ordinate action of superoxide dismutase and peroxidases (glutathione peroxidase being of particular importance) detoxifies superoxide to water; if the action of superoxide dismutase is however not accompanied by that of peroxidases, the accumulation of hydrogen peroxide would rather induce production of the damaging hydroxyl radical by means of the Fenton reaction (Ceballos-Picot *et al.*, 1991; Croft *et al.*, 1992).

Other defence systems include metal-binding proteins, preventing the pro-oxidant

Among antioxidants, a special position is held by coenzyme Q (CoQ₁₀ in man (Ramasarma, 1985)), the only lipid-soluble antioxidant which is normally synthesised by the organism (Elmberger *et al.*, 1987; Appelkvist *et al.*, 1994). Its strong hydrophobicity, due to the long isoprenoid chain at the 6-position, makes it inserted in the membrane phospholipid bilayer (Fato *et al.*, 1986; Lenaz, 1988; Samori *et al.*, 1992; Lenaz *et al.*, 1992; 1994), probably in a folded conformation, as suggested by computer simulation and molecular modelling (Di Bernardo *et al.*, 1998); such a folded structure makes the CoQ molecule more readily diffusible in the phospholipid bilayer (Lenaz *et al.*, 1999) (Fig. 7), where its lateral diffusion is required for electron transfer between flavoprotein enzymatic complexes and Complex III of the mitochondrial respiratory chain (Lenaz, 1988).

The biosynthesis of CoQ is particularly complex (Andersson *et al.*, 1994); the benzoquinone ring is synthesised from the essential amino acid phenylalanine up to 4-hydroxybenzoate, whereas the isoprenoid chain is formed by a pathway common to that of cholesterol and dolichol biosynthesis (Ernster & Dallner, 1995). CoQ biosynthesis requires the dietary intake of several vitamin cofactors: it

Table 6. Lactate production in platelets from young and aged individuals

	Ctl	AA	Δ lactate (AA - Ctl) = oxidative ATP	oxidative ATP/ glycolytic ATP
	$\mu\text{mol lactate /min} \times 10^{11}$ cells			
Young [26]	0.45 \pm 0.28	4.18 \pm 1.04	3.73 \pm 1.19	26.46 \pm 57.94
Aged [26]	0.85 \pm 0.78	3.47 \pm 0.85	2.62 \pm 0.89	5.41 \pm 4.94
<i>P</i>	0.021	0.009	0.0002	0.089

action of heavy metals, metabolic intermediates acting as free radical scavengers, and antioxidants largely taken in the organism by nutrition, such as ascorbic acid, vitamin E, carotenes, polyphenols, flavonoids etc. (Diplock, 1994).

is therefore conceivable that one or more such factors may become limiting under physiological or pathological conditions (Appelkvist *et al.*, 1994), thus slowing down ubiquinone biosynthesis and inducing a ubiquinone deficiency state.

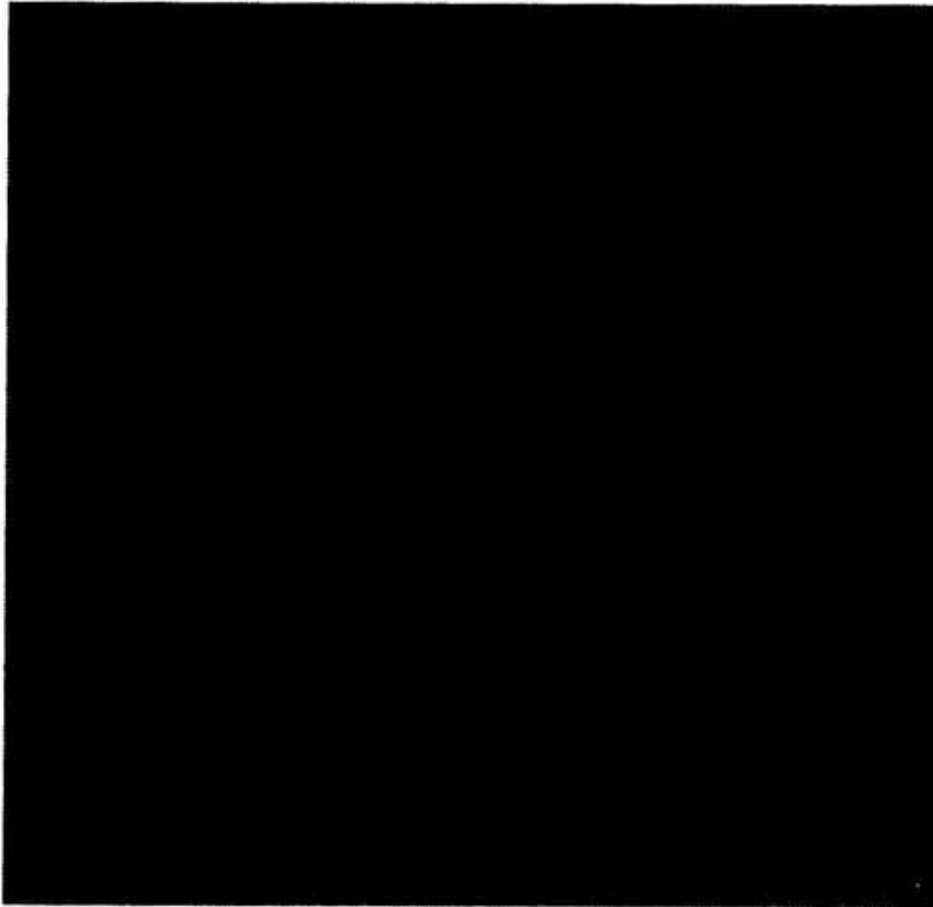


Figure 7. A folded structure for CoQ₁₀ in a lipid bilayer (see text for explanations; cf. Di Bernardo *et al.*, 1998).

Besides its bioenergetic role, as a component of the mitochondrial respiratory chain, CoQ is also a component of extra-mitochondrial redox chains (Crane & Navas, 1997), whose function, among others, would be to remove excess of reducing power formed by glycolysis when mitochondrial respiration is decreased (Lawen *et al.*, 1994; Larm *et al.*, 1994).

The role of CoQ in relation with ROS is intriguing: in spite of the CoQ intermediates in the function of Complexes I and III being considered as the source of one-electron oxygen reduction (Skulachev, 1996), ubiquinone in its reduced form is a powerful antioxidant. As an antioxidant, the reduced form of CoQ is exploited either directly upon superoxide or indirectly on lipid radicals (Beyer & Ernster, 1990; Beyer, 1994; Ernster & Dallner, 1995); ubiquinol can also act together with vitamin E (α -tocopherol) by re-generating the active

form from the tocopheroxyl radical (Kagan *et al.*, 1990; Ernster *et al.*, 1992).

The antioxidant action of ubiquinol yields the ubisemiquinone radical; this species is converted back to its antioxidant form by re-reduction, which occurs through the electron transfer chain in mitochondria, and is operated by various quinone reductases present in different cell fractions (Navarro *et al.*, 1995; Villalba *et al.*, 1995; Beyer *et al.*, 1996; 1997; Takahashi *et al.*, 1995; 1996; Arroyo *et al.*, 1999).

Ubiquinone may be transformed from a safe electron carrier to a superoxide generator when the ubisemiquinone anion, arising from one-electron oxidation of ubiquinol, becomes accessible to protons (Nohl *et al.*, 1996): such a condition may originate from changes in membrane structure.

A study on isolated rat hepatocytes clearly showed the antioxidant effect of exogenously

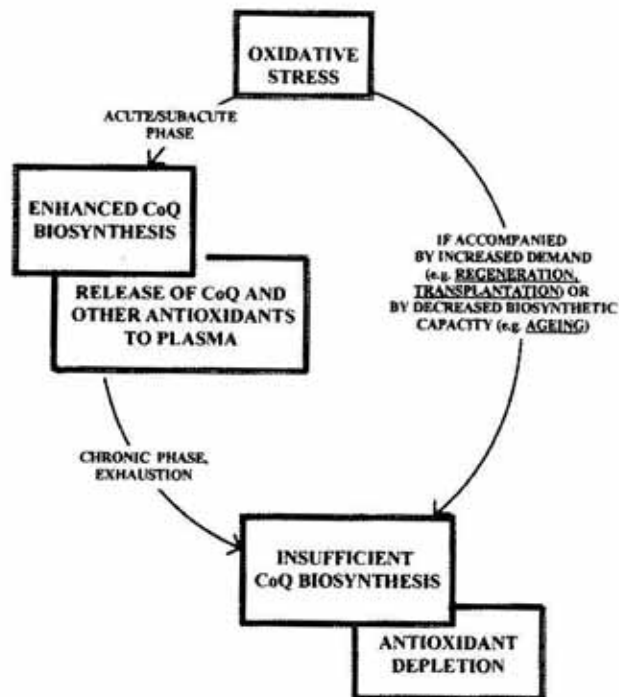


Figure 8. Reactions of endogenous CoQ to oxidative stress.

added CoQ₁₀ (Beyer *et al.*, 1996). The anti-cancer quinone glycoside, adriamycin, induces an oxidative stress by enhancing ROS production in mitochondria and endoplasmic reticulum. In hepatocytes, adriamycin enhances ROS production: concomitantly endogenous CoQ is re-oxidised and the mitochondrial membrane potential falls. Incubation of the cells with exogenous CoQ₁₀ prevents ROS formation and protects both reduced CoQ and $\Delta\Psi_{mit}$ (Table 7). The cytosolic enzyme DT-diaphorase seems to be responsible for reduction of both endogenous and exogenous CoQ, as shown by the effect of dicoumarol, an inhibitor of DT-diaphorase, preventing the protective action of exogenous CoQ addition (Beyer *et al.*, 1996; 1997).

If ageing is the result of prolonged oxidative stress, an adequate antioxidant supply might contrast the process. The content of vitamin antioxidants depends on dietary intake, and may be subjected to decreases due to intestinal absorption defects and bad dietary habits of the aged. CoQ, being synthesised, is a special case. Some studies have shown a CoQ decrease with age (Beyer *et al.*, 1985; Kalen *et al.*, 1990; Genova *et al.*, 1995); however, this is not true for brain, where high levels are

maintained throughout ageing (Söderberg *et al.*, 1990; Battino *et al.*, 1995) in accordance with the steady level of nonaprenyl-4-hydroxybenzoate transferase (Andersson *et al.*, 1995). Nevertheless, even if the levels of CoQ and other antioxidants do not dramatically fall with ageing, we must consider that the antioxidant defences should actually strongly *increase* to cope with the enhanced oxidative stress. In some instances of acute or sub-acute stress this is actually the case: the CoQ plasma level in the rat doubles by simple sham operation (Formiggini *et al.*, 1996), indicating that surgical stress can induce increased ubiquinone release from tissues and/or increased biosynthesis. On the other hand, a stronger metabolic stress, such as liver resection (Formiggini *et al.*, 1996; Genova *et al.*, 1999), can exhaust the CoQ biosynthesis capability, yielding lowered plasma CoQ levels (Fig. 8).

Senescence is associated with increased incidence of degenerative diseases, such as Parkinson's and Alzheimer's diseases and age-linked macular degeneration; these diseases often have a strong genetic component, which is however associated with exogenous factors, among which oxidative stress and mi-

Table 7. Exogenous coenzyme Q₁₀ protects isolated rat hepatocytes from adriamycin-induced oxidative stress (Beyer *et al.*, 1996)

	Hepatocyte treatment		
	Control	Adriamycin	Adriamycin + CoQ ₁₀
$\Delta\Psi_{mit}$ ^a	233.1 ± 25.2	55.8 ± 6.0	163.8 ± 18.7
Peroxide production ^b	17.8 ± 3.2	207.9 ± 28.4	28.2 ± 6.1
CoQ ₉ ^c	181.9 ± 22.9	460.2 ± 37.8	281.9 ± 22.6
CoQ ₉ H ₂ ^c	545.7 ± 32.2	261.8 ± 25.7	445.7 ± 28.2
Reduction (%)	75	36	61
CoQ ₁₀ ^c	30.6 ± 11.7	145.6 ± 32.1	750.4 ± 72.2
CoQ ₁₀ H ₂ ^c	94.6 ± 21.3	trace	403.6 ± 34.3
Reduction (%)	76	none	35

Hepatocytes were isolated by a collagenase liver-perfusion technique. Hepatocytes (10⁶ cells per ml) were suspended in Krebs-Henseleit medium (pH 7.4) supplemented with 5 mM glucose under an atmosphere of 95% O₂ + 5% CO₂ in a shaking bath at 37°C and incubated with 50 μM adriamycin for 2 h. The incorporation of CoQ₁₀ was achieved by coinubation of isolated hepatocytes and CoQ₁₀-enriched liposomes. Flow cytometry analyses were performed with EPICS C cell sorter (Coulter). Fluorochromes were excited at 488 nm with an argon laser at 200 mW. Fluorescence was collected through a 488-blocking filter, a 560-nm-long-pass dichroic mirror and a 520-nm-short-pass filter. Values are means ± S.D. (n = 8). ^aMitochondrial membrane potential was estimated by incubating hepatocytes (3 × 10⁵ cells per ml) with Rhodamine-123 (50 ng/ml) for 10 min. ^bHydrogen peroxide, released during treatment with adriamycin, was assayed by incubation at 37°C with dichlorofluorescein-diacetate (2 μM) for 15 min and fluorescence units were converted to concentration units (pmol/10⁶ cells) using a calibration curve. ^cpmol/10⁶ cells.

tochondrial involvement may be major triggering factors (Lenaz, 1998a; Beal, 1992, 1998; Götz *et al.*, 1994; Schapira, 1998a,b; Zerbini, 1999). A decrease of CoQ₁₀ and of antioxidant defences in plasma was found in patients affected by age-related macular degeneration of the retina (unpublished data from our laboratory).

The proposal of an antioxidant therapy, as an attempt to reduce or retard the damaging effect of ROS in ageing and age-related degenerative diseases, is strongly supported by the excellent results obtained in Alzheimer disease and Huntington disease (Beal, 1998, 1999). The use of CoQ as a dietary supplement requires its distribution to deficient tissues. Dietary CoQ is quickly taken into blood, where it is mainly transported by LDL (Karls-son *et al.*, 1993), and is rapidly incorporated in liver and reticulo-endothelial cells. The uptake of CoQ₁₀ by other tissues has not been shown in nutritional experiments in the rat (Zhang *et al.*, 1996), although there are indirect observations suggesting its incorpora-

tion in tissues when the endogenous levels are lowered (Mortensen, 1993).

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