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Ubiquinol and a Coenzyme Q Reducing System Protect Platelet Mitochondrial Function of Transfusional Buffy Coats from Oxidative Stress

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The conditions under which Coenzyme Q (CoQ) may protect platelet mitochondrial function of transfusional buffy coats from aging and from induced oxidative stress were investigated.

The Pasteur effect, i.e. the enhancement of lactate production after inhibition of mitochondrial respiratory chain, was exploited as a marker of mitochondrial function as it allows to calculate the ratio of mitochondrial ATP to glycolytic ATP.

Reduced CoQ_{10} improves platelet mitochondrial function of transfusional buffy coats and protects the cells from induced oxidative stress. Oxidized CoQ is usually less effective, despite the presence, shown for the first time in this study, of quinone reductase activities in the platelet plasma membranes. The addition of a CoQ reducing system to platelets is effective in enhancing the protection of platelet mitochondrial function from the oxidative stress. The results support on one hand a possibility of protection of mitochondrial function in aging by exogenous CoQ intake, on the other a possible application in protection of transfusional buffy coats from storage conditions and oxidative deterioration.

Keywords: Coenzyme Q; DT-diaphorase; Mitochondria; Oxidative stress; Pasteur effect; Platelets

INTRODUCTION

It is widely accepted that the electron transfer system of the mitochondrial inner membrane is the main source of superoxide radical, $O_2^{\cdot -}$, although the electron transfer components of the respiratory chain that are responsible for its generation are still controversial.[1–3] The rate of mitochondrial $O_2^{\cdot -}$ generation varies greatly, even in the same type of tissue, among different mammalian species and is inversely related to the maximum life span potential of the species.[4] Dismutation of the superoxide radical results in the formation of hydrogen peroxide (H_2O_2), which can further react to form the hydroxyl radical ($HO^{\cdot}$), to an extent estimated to be 1–2% of the total cellular oxygen consumption.[5]

Reactive oxygen species (ROS), incessantly generated by mitochondria or by other sites, can cause damage to mitochondrial components (DNA, RNA and proteins) and initiate degradation processes. Consequently, several theories have been proposed that envisage mitochondria as the driving force in the aging process. The postulate of the "Free radical theory of aging"[6] is the imbalance between ROS production and the antioxidant defences, resulting in the accrual of steady-state levels of oxidative molecular damage. Direct evidence in support of this hypothesis is that the increase of antioxidant defences by simultaneous overexpression of Cu/Zn superoxide dismutase, which converts superoxide anion radicals into H_2O_2, and catalase, which removes H_2O_2, retards the age-associated increase

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in the levels of molecular oxidative damage and extends the life span of *Drosophila melanogaster* by one-third.[7] The "Mitochondrial theory of aging"[8,9] postulates that random mitochondrial DNA (mtDNA) alterations (point mutations and deletions) in somatic cells are responsible for the energetic decline accompanying senescence.

In recent years several groups provided experimental support to this hypothesis detecting age-related deletions[10–12] and point mutations[13,14] that accumulate during life in human heart and brain tissue by more than a factor of 1000,[15] particularly in the D-loop region involved in mitochondrial replication and transcription.[16] Another line of evidence comes from histochemical studies showing structural and functional defects of the complexes of the respiratory chain and a decline of ATP synthesis capacity with age.[17] Also in line with this theory is the finding that Complex I (NADH Coenzyme Q reductase), of which seven subunits are encoded by mtDNA, is the most deeply affected by aging.[18–21] Postmitotic cell types should be very susceptible to a deterioration of their population of mitochondria, whereas rapidly dividing cells should be less affected because they dilute mitochondrial membrane damage and rejuvenate the mitochondrial population since healthy mitochondria outgrow defective ones during the growth spurt after mitosis.[22]

The search for a sensitive marker of aging 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 good system in that they are easy to collect from blood specimens. Moreover platelets have some similarities with neurones and could be a possible marker of acquired neurological diseases.[23] Platelets rely on glycolysis as well as mitochondrial oxidative phosphorylation for their energy supply. A quantitative determination of glycolytic and mitochondrial ATP can be provided by the Pasteur effect,[24] based on stimulation of glycolysis by a decreased mitochondrial oxidative phosphorylation in order to maintain a constant ATP synthesis, and meanwhile on pyruvate reduction to lactate to regenerate oxidized pyridine nucleotides. Since in the absence of mitochondrial function the stoichiometric ratio for glucose breakdown in the glycolytic pathway is two lactate molecules per glucose, it follows that lactate production stimulated by inhibition of respiration is equivalent to the correspondingly inhibited production of ATP by the mitochondria.[24] Thus, the delta-lactate (Δ-lactate) in presence and absence of antimycin A (AA) represents the amount of ATP synthesized via oxidative phosphorylation in physiological conditions, whereas the basal lactate production represents ATP produced by glucose breakdown exclusively by glycolysis.

The Pasteur effect in platelets was used as a biomarker of mitochondrial lesions[21] resting on the assumption that alterations occurring in senescence and in age-related diseases be present in all cells, so that platelets may signal generalized bioenergetic deficiencies.[25] An alteration of Complex I activity in platelet mitochondria in aged individuals was indicated by an altered inhibitor sensitivity[20] that may represent an indication of altered energy conservation.

The deep involvement of ROS in pathology has prompted the suggestion that antioxidant intake may prevent or retard the arousal of aging and disease. Among antioxidants, ubiquinone or CoQ holds a special position, in that it is not a significant dietary constituent but is synthesized by man: its biosynthesis, however, becomes limiting under pathological conditions.[26] CoQ occurs ubiquitously and in high levels among different tissues and has long been considered to have both pro- and antioxidant action, in addition to its conventional role in mediating electron transport between both NADH and succinate dehydrogenase and the cytochrome system of mitochondria. It is found largely in the reduced hydroquinone form (ubiquinol) which exerts a powerful antioxidant role either directly on superoxide or indirectly on lipid radicals, both singly and in combination with vitamin E.[27] The antioxidant action of ubiquinol normally yields the ubisemiquinone radical, that is converted back to ubiquinol by re-reduction by means of various electron transfer systems, including the mitochondrial respiratory chain, a plasma membrane oxidoreductase (PMOR),[28] a cytosolic NADPH quinone oxidoreductase[29] and DT-diaphorase.[30]

NAD(P)H: quinone acceptor oxidoreductase (also referred to as DT-diaphorase or NQO1-EC 1.6.99.2)[31] is an inducible enzyme deputed to the maintenance of the reduced antioxidant form of CoQ in membrane systems. It is located in the cytosolic fraction of the cell and may interact with the membrane-cytosolic interface. It is supposed that DT-diaphorase was selected during evolution to act as CoQ reductase to protect cellular membrane components from free radical damage.[30]

Besides the natural homologue of man, CoQ_{10}, short chain ubiquinone homologues and analogues also have antioxidant properties and, being water soluble, might be considered to be good candidates as protective agents: some of them, however, are strong inhibitors of Complex I,[32] while some have been found to open the permeability transition pore of the inner mitochondrial membrane, thus inducing mitochondrial depolarisation.[33]

It is known that oral intake of CoQ_{10} rises plasma levels of the quinone to large extents;[34] however the effect of an increased plasma level of the quinone on its uptake by cells and on its protecting action on mitochondrial function are scarcely known.

Transfusional buffy coats of concentrated platelets obtained by centrifugation of whole blood units and washed platelets have been utilized as models for investigations of mitochondrial function.[21,35] The present study aims to test conditions under which incubation with CoQ may protect platelets from aging *in vitro* and from an induced oxidative stress, as a model of prevention of oxidation-induced pathological events by enhanced plasma levels of the quinone. In addition, hints may be derived from this study on practical purposes of protecting buffy coats from storage deterioration.

MATERIALS AND METHODS

Chemicals and Reagents

guttaQuinon™, a novel CoQ_{10} formulation consisting of CoQ_{10}-nanoparticles dispersed in aqueous media, was a kind gift from Dr F. Enzmann of MSE Pharmazeutika GmbH, Bad Homburg, Germany. DT-diaphorase, purified from rat liver, was a kind gift from Dr J. Segura-Aguilar. The reagents used in the study were obtained either from Sigma Chemical Corporation (St Louis, MO, USA) or from Merck (Darmstad, Germany) except solvents for HPLC that were obtained from Mallinckrodt Baker (Deventer, Holland).

Platelet Purification

Concentrated blood platelets from transfusional buffy coats treated with anticoagulant (Na citrate–citric acid) were diluted 1:1 with 0.12 M NaCl, 0.03 M Tris HCl, 3 mM Na EDTA, 5 mM glucose pH 7.4 and centrifuged at 300*g* for 10 min. The upper phase platelet-rich plasma (PRP) was washed with the same buffer and centrifuged at 1000*g* for 10 min; the platelet pellet was re-suspended with the previous buffer at a final concentration of 200×10^6 cells/ml for lactate test, 5×10^9 for Thiobarbituric acid reactive substances (TBARS) determination, 50×10^6 cells for HPLC quantitation and 400×10^6 cells for polarographic oxygen uptake determinations. Aliquots of platelet suspension were collected for protein measurement following the method of Lowry.[36]

Lactate Determination

Aliquots of washed platelets were pre-incubated for 1 h (unless otherwise indicated) with or without addition of different CoQ suspensions (either oxidized or reduced CoQ in liposomes or guttaQuinon™) at room temperature with stirring. Aliquots of 0.5 ml were then incubated for 3 h at 37°C in presence/absence of 50 mM 2,2′-azobis-2-aminopropane (AAPH), a free radical generator, with or without addition of 5 μM AA, a strong inhibitor of respiratory chain Complex III.[21] After the incubation the samples were centrifuged at 1500*g* for 10 min and the lactate containing supernatant was frozen; aliquots were assayed using the method of Everse.[37]

HPLC Quantitative Determination

The same incubation procedure was used to prepare aliquots for HPLC quantitation of CoQ_{10},[38] the pellet of platelets was washed three times then stored at −80°C up to extraction. CoQ was extracted using the method of Tsai.[39]

DT-diaphorase CoQ Reducing System

Platelets isolated as earlier were re-suspended with the same buffer at a final concentration of 200×10^6 cells/ml then pre-incubated for 2 h with or without 20 μM guttaQuinon™, 150 μM β-NADPH and 1000 units DT-diaphorase. Aliquots of 0.5 ml of platelets were then treated in the earlier described way for lactate determination.

TBARS Determination

At difference with most other determinations, for TBARS determination higher amounts of platelets (5×10^9 cells) were used in order to obtain considerable levels of protein concentration (1 mg).

Accordingly, the concentration of AAPH was raised from 50 to 200 mM to induce a detectable oxidative stress and, likewise, reduced CoQ_{10} concentration was raised to 200 μM. The reaction is usually described as measuring malondialdehyde or malondialdehyde-like substances; even if other substances react with thiobarbituric acid, under normal conditions their concentrations are very low.[40] 50 μl of 50 mM sodium dodecyl sulphate, 10 μl of 10 mM butylated hydroxy toluene, 500 μl of 10% trichloroacetic acid were added to samples of platelet suspensions (0.5 ml).

The mixtures were cooled on ice and then 500 μl of thiobarbituric acid (0.75% w/v in HCl 0.1 M) were added. The samples were boiled at 95°C for 20 min, cooled on ice and then centrifuged at 1075*g* for 15 min at 0°C. The supernatant (1 ml) was collected and absorbance was measured at 532–580 nm ($\varepsilon = 1.56 \times 10^{-5}$/M/cm).

TABLE I Lactate production in platelets

	Control (μmol/min/10^{11} cells)	Control + AAPH (μmol/min/10^{11} cells)
Basal lactate (glycolytic ATP)	1.82 ± 0.44	2.43 ± 0.23†
With AA*	3.81 ± 0.41	3.41 ± 0.29
Δ-lactate (AA* minus basal) (oxidative ATP)	2.00 ± 0.28	0.98 ± 0.36‡
Oxidative ATP/glycolytic ATP	1.10 ± 0.40	0.40 ± 0.20†
Glucose flux through anaerobic glycolysis		
ATP yield/glucose in anaerobic glycolysis	0.91 (94.3%)	1.21 (97.8%)
Mean ATP yield/glucose in oxidative phosphorylation	0.055 (5.7%)	0.027 (2.2%)

* AA: Antimycin A. Values are means ± SD of four experiments. See text for experimental details. †Significantly different from control $p < 0.05$. ‡Significantly different from control $p < 0.01$.

Plasma Membrane Oxidoreductase (PMOR) Activity

Total membranes were isolated from intact platelets by ultrasonic irradiation in a Labsonic sonifier at low frequency for 5 cycles of 10 s sonication and 50 s rest. Subsequently samples were sedimentated by centrifugating at 500*g* and the obtained supernatant was centrifugated at 200,000*g*. The pellets were re-suspended in a phosphate buffered saline medium (0.15 M NaCl, 20 mM Na phosphate pH 7.4). PMOR activity was evaluated both in intact platelets and in fractions obtained from centrifugation.

Activity was measured by oxidizing endogenous pyridine nucleotides, using the dye dichlorophenol–indophenol (DCIP) as acceptor, and following the absorbance of DCIP reduction at 600 minus 700 nm in a Sigma-Biochem ZWS dual wavelength spectrophotometer at 30°C, using an extinction coefficient of 21/mM/cm. PMOR was differentiated from endogenous DT-diaphorase by performing each assay in absence and presence of 10 μM dicoumarol, an inhibitor of DT-diaphorase, and subtracting the dicoumarol-sensitive activity (DT-diaphorase) from the total activity. Each assay also contained a battery of respiratory inhibitors to prevent interference with mitochondrial oxidation of NADH (2 μM rotenone, 2 μM AA, 10 μM myxothiazol and 2 mM KCN).

Oxygen Consumption by Intact Platelets

Platelets were suspended in 2 ml of the same buffer used for washing with the addition of 5% of anticlotting. Cellular respiration was recorded in an Oroboros Oxygraph (Innsbruck, Austria).

RESULTS

Effect of Pre-incubation With CoQ_{10} on Δ-lactate

Incubation of washed platelets with the free radical generator AAPH (50 mM) greatly reduces the Pasteur effect, mostly by enhancing basal lactate production (Table I). The decrease of Δ-lactate (ATP obtained by oxidative phosphorylation) is paralleled by a decrease of the mitochondrial ATP/glycolytic ATP ratio. Assuming a glycolytic stoichiometry of two ATP per glucose in absence of mitochondrial function and an oxidative stoichiometry of 36 ATP molecules per glucose allows to calculate the total metabolic flux of glucose through anaerobic glycolysis and oxidative phosphorylation; the oxidative flux was only 5.7% in normal platelets, but decreased to 2.2% after AAPH.

Pre-incubation of the platelets with reduced CoQ_{10} is shown in Fig. 1. To assess the optimal concentration of reduced CoQ, different concentrations were pre-incubated for 1 h at room temperature with washed platelet suspensions, then incubated for 3 h at 37°C in presence and absence of AAPH: an enhancement of mitochondrial function occurred in the treated platelets with respect to the control; moreover a protection of platelets occurred from oxidative damage (AAPH), particularly at 100 μM reduced CoQ_{10} (Fig. 1).

An ideal pre-incubation time of 60–90 min at room temperature was found in different incubation experiments. Occasional determination of TBARS confirmed some protection of platelets by $CoQ_{10}H_2$ from AAPH and a good protection from oxidative damage during the incubation procedures. Also oxygen consumption by the intact cells (which was

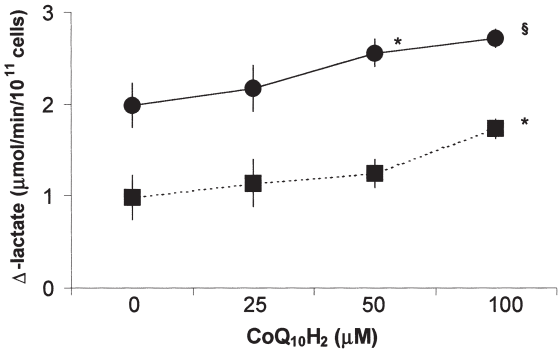

FIGURE 1 Δ-lactate values in platelets pre-incubated for 1 h in presence of different concentrations of $CoQ_{10}H_2$. The data are means ± SD of four experiments. *Significantly different from corresponding 0 point $p < 0.05$. §Significantly different from corresponding 0 point $p < 0.01$.

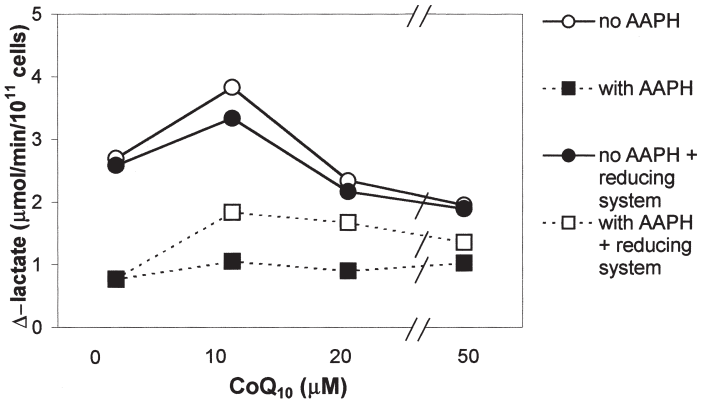

FIGURE 2 Δ-lactate values in platelets pre-incubated for 2 h in presence and absence of a reducing system: DT-diaphorase 1000 units, 150 μM NADPH, 10–50 μM guttaquinon™. The data are the overall result of five experiments. Statistical analysis was not performed because of considerable differences observed in the basal amount of lactate in different buffy coats.

fully KCN-sensitive) was higher after pre-incubation with reduced CoQ_{10}: a mean of 7.3 vs. 5.0 pmol of oxygen consumed per second per 10^8 platelets was found in two closely agreeing determinations. It was not possible to measure cellular oxygen uptake after AAPH addition due to the high oxygen consumption by the AAPH chemical reaction.

Having assessed that addition of reduced ubiquinone to platelets ameliorates their mitochondrial function and protects them from the deleterious effect of an oxidative stress, we have decided to find out whether addition of oxidized CoQ_{10} to either platelet suspensions or the parent buffy coats is also able to maintain mitochondrial bioenergetics. In some experiments it was found that overnight pre-incubations with either water-soluble CoQ_{10} (guttaQuinon™) or liposome-dispersed quinone protected the platelets from aging (increased storage time) and from the AAPH-induced oxidative stress, as shown by the increased Pasteur effect in comparison with the control (not shown). Unfortunately, both with guttaQuinon™ and with liposomal CoQ_{10} the results were non-reproducible in all experiments. Since reduced CoQ_{10} was always effective, the variability of results should be ascribed to variable efficiency of the CoQ reducing systems in the cells.

In order to create better conditions of CoQ_{10} reduction, we added DT-diaphorase (1000 units) and NADPH (150 μM) to washed platelets and guttaQuinon™ (10–50 μM) and pre-incubated for 2 h at room temperature with stirring. The "reducing system" protects from oxidative damage with respect to the control (Fig. 2), presumably by reducing the added oxidized CoQ in the extracellular environment before its cellular uptake. Concentrations of CoQ higher than 10 μM were found to exert lower protection or even to be inhibitory to the

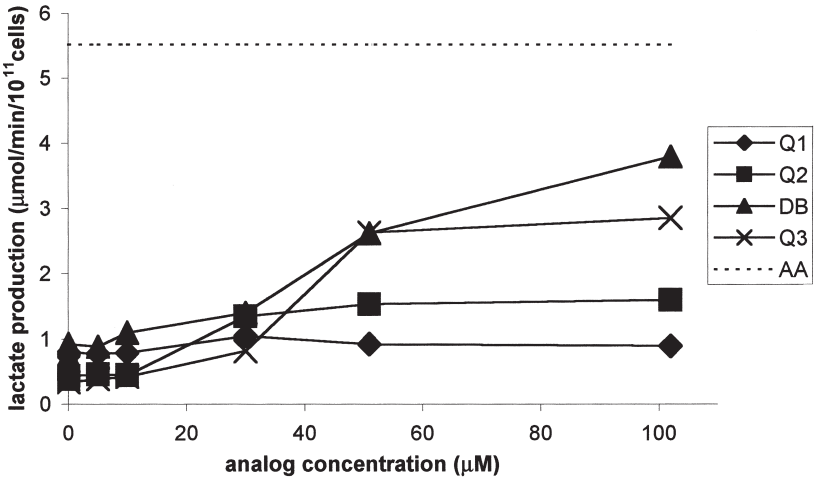

FIGURE 3 Effect of CoQ homologues and analogues on lactate production in platelets: typical example of platelets from transfusional buffy coats (1–4 experiments). Statistical analysis was not performed because of considerable differences observed in the basal amount of lactate in different buffy coats. AA: antimycin A. DB: decylubiquinone. The dashed line represents lactate production in presence of AA (mitochondrial chain completely inhibited by AA).

TABLE II Quinone reductase activities in platelet fractions

	Total quinone reductase activity	With dicoumarol
Intact platelets (pmol/min/10^6 cells)	34.00 ± 1.41	34.00 ± 5.66
Pellet 500 g (nmol/min/mg)	21.42 ± 5.35	17.16 ± 8.13
Pellet 200,000 g (nmol/min/mg)	53.35 ± 18.84	42.00 ± 21.23
Supernatant (nmol/min/mg)	4.80 ± 0.61	4.36 ± 1.23

Values are means ± SD of two experiments. See text for experimental details.

system, possibly as a result of the vehicle for guttaQuinon™ solubilization.

Effect of CoQ Homologs and Analogues on Mitochondrial Function

CoQ homologues and analogues having short chains at the 6 position of the benzoquinone ring are commonly used as substrates for respiratory activities in place of the extremely hydrophobic CoQ_{10}.

These quinones share with CoQ_{10} its antioxidant activity, thus they could be preferred to the physiological homologue as antioxidants for their easier handling *in vitro*: however some of them are inhibitors of respiratory chain enzymes, in particular of Complex I and their effect in intact cells is not known. For this reason we have investigated the effect of some of the most commonly used quinones on mitochondrial bioenergetics in intact platelets by testing their action on lactate production. Fig. 3 shows the effect of the quinones compared with that of AA (total inhibition of mitochondrial function that corresponds to the maximum production of lactate). If we except CoQ_1, that has no inhibitory effect even at 100 μM concentration, all other quinones tested increase lactate production (consequently decreasing Δ-lactate) and are therefore inhibitors of mitochondrial respiratory chain in the intact cell.

CoQ_{10} Incorporation in Platelets

In order to evaluate the extent of CoQ incorporation into the platelets after pre-incubation, we carefully washed the platelets three times after incubation with reduced CoQ_{10}. The CoQ_{10} content in the last supernatant was 1% of the CoQ added to the cells. Compared with a normal content of CoQ_{10} of 71 ± 29 pmol/mg protein, pre-incubation results in an increase of up to 200-fold, suggesting that large amounts of quinone remain adherent to the cell membrane during incubation and are not removed by the washing procedure. It is worth noting that after several hours large part of the incorporated CoQ is still in the reduced state.

CoQ Reductase in Platelets

Preliminary experiments show the presence of quinone reductase activity in platelets both on the membrane surface and in the membranous and soluble fraction after cell rupture by mild sonication (Table II), as shown by DCIP reduction almost completely insensitive to dicoumarol (thus excluding high levels of endogenous DT-diaphorase activity). The efficiency of such systems in reducing CoQ_{10} in platelets *in vivo* is not yet proved.

DISCUSSION

The Pasteur effect, i.e. the enhancement of lactate production after inhibition of mitochondrial respiration, allows easy calculation of the relative relevance of glycolytic and mitochondrial ATP production and may be used as a marker of mitochondrial function in suitable cells. In this study we have exploited the above marker for testing the effect of CoQ as a protective antioxidant in platelets obtained from buffy coats, in an *in vitro* model of the aging process. In agreement with its known antioxidant effect, reduced CoQ_{10} (ubiquinol) both improved platelet mitochondrial function and protected the cells from an oxidative stress. On the other hand, incubation with oxidized CoQ_{10} (both in form of water-soluble guttaQuinon™ and of liposomal suspension) produced variable results, probably due to lack of sufficient reduction of the quinone by the cells. In this study, for the first time we showed that platelets contain quinone reductase activities, such as PMOR[28] and DT-diaphorase,[30] well characterized in other cell types; the activity is however rather low, and it is doubtful whether it is intrinsically sufficient to reduce exogenous CoQ_{10}. A possible way to increase the efficiency of CoQ_{10} reduction for platelet protection was to add a CoQ reducing system (DT-diaphorase) to the platelet incubation system: indeed the ubiquinol-regenerating system was effective in enhancing the protection of platelet mitochondrial function from the oxidative stress.

There are some intriguing relations of PMOR activity and oxidative stress. De Grey suggested[41] that PMOR may increase survival of cells deficient in mitochondrial function through the regeneration of

oxidized pyridine nucleotide (NAD^+/NADH homeostasis) required to sustain glycolytic ATP production in the presence of diminished respiratory chain activity. There is new evidence for a plasma-membrane-associated hydroquinone oxidase designed as CNOX (constitutive plasma membrane NADH oxidase (NOX)) functioning as a terminal oxidase for PMOR.[42] The hyperactivity of the PMOR system results in an NOX activity capable of generating ROS at the cell surface. This would serve to propagate the aging cascade both to adjacent cells and to circulating blood components. The generation of superoxide by NOX is however inhibited by CoQ exogenous addition. To this purpose it must be borne in mind that CoQ content decreases with age in many tissues[18,43,44] except in brain[45,46] where high levels of CoQ are maintained throughout aging. Most important are the circulating levels of CoQ that could come into contact with an overactive or aberrant cell surface PMOR system or with circulating NOX isoforms that may play roles in aging related to oxidative stress.

In a previous study we investigated the production of Δ-lactate in washed blood platelets from young (19–30 years) and old donors (67–87 years): both Δ-lactate and the ratio of oxidative over glycolytic ATP were significantly decreased in aged individuals.[35] The results of our *in vitro* study on the protective action of CoQ raise interesting possibilities about protection of mitochondrial function in aging by exogenous CoQ_{10} intake.

The pharmacological use of water soluble CoQ analogues and homologues would be very useful, since CoQ_{10} appears to be absorbed and incorporated in tissues with low efficiency: however our survey of some of the most commonly used quinones has prompted some doubts on their possible use, since they appear to be toxic for mitochondrial function in intact cells. The toxicity of such homologues as CoQ_2 and CoQ_3 may be related to their inhibitory action on Complex I. However the toxic effect of decyl-ubiquinone, a good respiratory chain substrate[32] that exerts no inhibition on Complex I and is an inhibitor of the mitochondrial permeability transition pore,[33] is at present unexplained. Much caution is therefore necessary in transposing mitochondrial effects to actions in the whole cell.

The results of this study may also find application in protection of transfusional buffy coats from oxidative deterioration. Up to now the effect of antioxidants was limited to ascorbate;[46] since CoQ is involved in regeneration of ascorbate from the ascorbate free radical through the PMOR system,[47] its effect on platelet maintenance is worth of investigation.

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