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Targeting antioxidants to mitochondria and cardiovascular diseases: The effects of mitoquinone

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Summary

Mitochondria have long been known to play a critical role in maintaining the bioenergetic status of cells under physiological conditions. Mitochondria produce large amounts of free radicals, and mitochondrial oxidative damage can contribute to a range of degenerative conditions including cardiovascular diseases (CVDs). Although the molecular mechanisms responsible for mitochondrion-mediated disease processes are not correctly understood, oxidative stress seems to play an important role. Consequently, the selective inhibition of mitocondrial oxidative damage is an obvious therapeutic strategy. This review considers the process of CVD from a mitochondrial perspective and provides a summary of the following areas: reactive oxygen species (ROS) production and its role in pathophysiological processes such as CVD, currently available antioxidants and possible reasons for their efficacy and inefficacy in ameliorating oxidative stress-mediated diseases, and recent developments in mitochondria-targeted antioxidants that concentrate on the matrix-facing surface of the inner mitochondrial membrane. These mitochondrion-targeted antioxidants have been developed by conjugating the lipophilic triphenylphosphonium cation to antioxidant moieties such as ubiquinol. These compounds pass easily through biological membranes and, due to their positive charge, they accumulate several-hundred-fold within mitochondria. In this way they protect against mitochondrial oxidative damage and show potential as a future therapy for CVDs.

key words: **cardiovascular disease • endothelium • mitochondria • nitric oxide • oxidative stress • reactive oxygen species**

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BACKGROUND

In physiological conditions a homeostatic balance exists between the formation of reactive oxygen species (ROS) and their elimination by endogenous antioxidant-scavenging compounds and enzymes [1]. Oxidative stress occurs when this balance is disrupted by excessive production of ROS, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}HO$), peroxynitrite ($ONOO^-$), and/or inadequate antioxidant defenses [2], including those caused by superoxide dismutase (SOD), catalase, vitamins C and E, and reduced glutathione (GSH). Both processes can lead to cardiovascular diseases (CVDs). The free-radical hypothesis for vascular dysfunction postulates that ROS can lead to a modification of lipids, proteins, and nucleic acids which, in turn, contributes to the etiology of the disease [3]. However, this perspective has been challenged in recent years by the recognition that these molecules can play a role in signal transduction [4]. This process has come to be known as "redox cell signaling", which refers to how ROS and reactive nitrogen species (RNS) can lead to the activation of pathways that control cell differentiation and apoptosis [5].

Over the last decade, the management of CVDs in experimental animals has improved remarkably, without a similar achievement being noted in humans. Thus there is a continuing search for more effective antioxidants that can counteract the oxidative stress induced in CVD, such as atherosclerosis and hypertension. The mitochondrial respiratory chain in the inner mitochondrial membrane is a major intracellular source of ROS [6], indicating that a specific action of antioxidants on the mitochondrial respiratory chain may constitute an important mechanism of cardiovascular protection.

ROS can cause nonspecific damage to different macromolecules leading to alteration or loss of cellular function. Therefore it is conceivable that mitochondria are more vulnerable to oxidative damage than other cellular organelles. In fact, mitochondria are continuously exposed to ROS and accumulate oxidative damage more rapidly than the rest of the cell, in particular because ROS are highly reactive and short-lived [7]. For these reasons, mitochondrial dysfunction disrupts the function of cells, tissues, and organs and contributes to a wide range of diseases. The recognition of mitochondria as an arbiter in the life and death of cells has highlighted the need to develop antioxidants and other cytoprotective agents that are targeted to mitochondria.

Mitochondrial oxidative damage and dysfunction contribute to a number of cell pathologies and have particular relevance to CVDs such an atherosclerosis and hypertension. Studies seeking to counteract the deleterious effects of ROS have shown antioxidants such as α-tocopherol, ubiquinol, and N-acetylcysteine to decrease mitochondrial oxidative damage in different models [8–11]. However, as these compounds do not significantly accumulate within mitochondria, their effectiveness remains limited [12], which makes increasing the antioxidant capacity of the mitochondrial compartment a therapeutic objective. Yet, to be pharmaceutically viable, a small-molecule antioxidant is required, and so there has been a recent impulse in research efforts to develop mitochondrion-targeted antioxidants. One approach to addressing these challenges is to target antioxidants to mitochondria through conjugation to a lipophilic cation, such as triphenylphosphonium (TPP), which is cell-permeable and considerably potent in reducing intracellular ROS and thereby preventing cell death [13,14].

MECHANISMS OF ROS PRODUCTION

ROS are important secondary messengers that are generated in response to different types of environmental stress. At lower levels of ROS/RNS, damage to key targets in the mitochondria, such as mtDNA, is prevented by intramitochondrial antioxidant defenses. Oxidative stress is a state in which excess ROS overwhelm endogenous antioxidant systems. ROS have distinct functional effects on each cell type in the vasculature and exert both physiological and pathophysiological influence.

In nearly all diseases in which mitochondrial dysfunction has a contribution, a major cause of the damage it causes are the ROS that it produces, either directly or as a secondary consequence of other malfunctions [15–17]. The proximal ROS is O_2^-, produced by the respiratory chain, probably at complexes I and III [15–17], although it does have other sources within the mitochondria. $O_2^{\cdot-}$ itself is not particularly reactive [18] and it can react with aconitase to release ferrous iron [19]. O_2^- can increase the rate of formation of H_2O_2, which react with ferrous iron to form the highly reactive $^{\cdot}HO$. Mitochondrial ROS cause damage to different cellular structures, thereby disrupting mitochondrial function [18–20]. There are a series of mitochondrial antioxidant defenses for intercepting ROS and minimizing oxidative damage, but excessive production of ROS or disruption of the antioxidant defenses leads to extensive oxidative damage to the mitochondria [16]. As mitochondrial oxidative damage is either a primary or significant secondary cause of cell damage and death in degenerative diseases, a general therapeutic approach to decrease mitochondrial oxidative damage could be applied in a range of clinical situations [16,21].

ROS generated in the vasculature include $O_2^{\cdot-}$, H_2O_2, hypochlorous acid (HOCl), OH, and singlet oxygen ($^1O_2$). One of the most important ROS in the vasculature is $O_2^{\cdot-}$ [22]. In addition, the formation of $ONOO^-$ has been implicated in CVDs. Multiple enzymatic systems produce $O_2^{\cdot-}$ and its derivatives in the vasculature, including NADPH oxidases, xanthine oxidase (XO), nitric oxide synthases (NOS), myeloperoxidase (MPO), and mitochondria. Indeed, leakage of electrons from the mitochondrial electron transport chain (ETC) is the main source of ROS, particularly $O_2^{\cdot-}$ [23].

RNS have also been implicated in cell damage and death. NOS catalyses the synthesis of NO, which may react with $O_2^{\cdot-}$ to produce $ONOO^-$. In fact, $ONOO^-$ has been implicated in CVD-inducing mitochondrial damage and, by association, apoptosis [24]. Therefore, with respect to CVD, the regulation of mitochondrial functions by NO acquires further relevance when taking into account the effects of this molecule on electron transfer, energy-transducing processes, and oxyradical production in mitochondria [25].

The balance between these sources of ROS depends on the physiological and pathophysiological states of the organism and it is often difficult to identify the source of ROS gen-

eration. It is known, however, that ROS have an important regulatory function [5]. In fact, ROS/RNS can lead to the activation of pathways that control cell differentiation and apoptosis, both of which are mechanisms of particular relevance to CVDs. Hence, a basal or total concentration of ROS, especially at the level of the mitochondria, is essential for basic cell signaling processes. In other words, all ROS are not created equal, with compartmentalization and concentration gradients being fundamentally important.

ROS production in mitochondria occurs at several sites of the electron-transport chain. Importantly, it is increasingly clear that each of the sites can be regulated. Thus, in the light of redox cell signaling, the identification of physiological regulators of mitochondrial ROS production is of great importance. Several molecules/factors have been put forward as regulators of mitochondrial ROS production, including an elevated inner membrane potential, Ca^{2+}, and NO [26]. Beyond the electron-transport chain, the TCA dehydrogenases, specifically 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase, have been shown to generate ROS when the $NADH/NAD^+$ ratio is high. Complex III of the electron transport chain is another site of high ROS production.

OXIDATIVE STRESS: DAMAGE TO MITOCHONDRIA

Inherited dysfunction of the mitochondrial oxidative phosphorylation system is increasingly recognized as a CVD in humans, and mitochondrial diseases have been associated with mutations of mitochondrial DNA [27], including mitochondrial ATP synthase [28]. Mitochondrial ROS can cause damage to mitochondrial proteins, lipids, and DNA, thereby disrupting mitochondrial function and causing ROS to flow into the cytosol [18-20]. Mitochondrial DNA (mtDNA) is particularly susceptible to modification by ROS/RNS [29] because a) mtDNA is in close proximity to the site of ROS/RNS production, b) mtDNA lacks histone proteins, which can protect it from oxidative damage, and c) mitochondrial polymerases lack specificity for base excision and are themselves modified by ROS, which can lead to changes in polymerase function and increased mutation rates in mtDNA.

The diseases resulting of familial mitochondrial DNA deletions and mutations are not as common as those from nuclear DNA defects. This may be because mitochondria contain several copies of their genome; hence, continuous fusion of mitochondria mixes modified genes with normal genes. Most isolated defects of ATP synthase are associated with alterations in the biosynthesis of the enzyme and can be caused by mutations in subunit genes or in ancillary proteins essential for assembly of the enzyme.

The recent discovery that acute and chronic stress in the cells leads to structural and functional impairments of mitochondria has redefined the role of mitochondria in disease etiology [13,30] and is of a special relevance to CVDs. In fact, the accumulation of mtDNA damage over a lifetime may increase one's susceptibility to the development of a pathology. Mitochondrial dysfunction triggers signaling cascades for cell necrosis and apoptosis and leads to organ failure and diseases. Although the list of mitochondrion-related diseases is growing rapidly and includes cancer, heart failure, diabetes, obesity, stroke, neurodegenerative diseases, and aging, they all share the common features of disturbances of mitochondrial Ca^{2+}, ATP, or ROS metabolism [30]. Some studies have shown that mitochondrial dysfunction contributes to the progression of neurodegenerative diseases such as Parkinson's and to strokes [31]. Myocardial ischemia-reperfusion injury also leads to mitochondrial Ca^{2+} overload, which subsequently leads to uncontrollable ROS generation and opening of the mitochondrial permeability transition pore [30], which ultimately induces apoptosis. Therefore, compounds that minimize mitochondrial Ca^{2+} overload, decrease mitochondrial ROS accumulation, and promote mitochondrial energy production are all potentially useful in the treatment of the above-mentioned conditions.

As mitochondria represent the major site for the generation of cellular oxidative stress and play a key role in mediating programmed cell death (apoptosis), damage to mtDNA may be an important contributor to human aging, cancer, and CVD by leading to alterations in membrane permeability, modification of protein structure, and functional changes.

Whereas ROS and RNS are capable of targeting a variety of subcellular components, mitochondria are continually exposed to ROS and accumulate oxidative damage more rapidly than the rest of the cell. In fact, the mitochondrial membrane, proteins, and mtDNA appear to be particularly sensitive to oxidative and nitrosative damage [32]. Mitochondrial oxidative damage has been implicated in clinically relevant situations, including CVDs such as ischemia-reperfusion injury and neurodegenenerative diseases, having been found to contribute to their pathophysiology by disrupting mitochondrial function. Although the extent of mitochondrial oxidative stress *in vivo* remains unclear, its importance has been demonstrated in mice lacking Mn-SOD, which die shortly after birth [33], while mice lacking Cu Zn-SOD, the cytosolic form of the enzyme, survive [34].

Vascular pathologies are multifactorial, but it is clear that mitochondrial dysfunction contributes to the pathophysiology of these diseases. Studies have shown that ROS induce a variety of effects, including preferential and sustained mtDNA damage, altered mitochondrial transcript levels and mitochondrial protein synthesis, and lowered mitochondrial redox potentials in vascular cells [35]. ROS and RNS have been implicated in atherogenesis, and increased oxidative stress is a shared feature among many CVD risk factors, mediating post-translational modifications of mitochondrial proteins that can result in their activation (e.g. cytochrome c, aconitase) [36]. It is important to point out that exogenous and endogenous sources of $^{\cdot}NO$ inhibit mitochondrial respiration (O_2 consumption), resulting in greater $O_2^{\cdot-}$ production but also promoting O_2 diffusion into tissues, thereby decreasing the "steepness" of the O_2 gradient from the vascular lumen as well as controlling different transcription factors such as hypoxia-inducible factor (HIFα) [37].

Mitochondrial damage can alter the capacity of the cell to generate energy, redox signaling, and a variety of important functions regulated by mitochondrial oxidant generation and response, thus mediating changes in cell function that may not be directly related to cellular energy. For example,

it has been shown that cardiac mitochondria that sustain ischemic injury are more sensitive (complex IV activity) to changes in NO concentration than controls [38]. ROS generated in the mitochondrial respiratory chain have been proposed as intermediate, secondary messengers to the activation of NF-κB [39]. Hence the relative balance between the stimuli of mitochondrial ROS production and the concomitant accumulation of organelle damage can ultimately influence cellular response and function, and the cellular response to increased oxidative stress may in turn initiate CVD. Consequently, excessive production of mitochondrial ROS or disruption to the antioxidant defenses leads to extensive oxidative damage to mitochondria [16]. As mitochondrial oxidative damage is either a primary cause or a significant secondary factor leading to cell damage and death, a general therapy for decreasing mitochondrial oxidative damage should be effective in a range of clinical situations [16,21]. Therefore, mitochondrial damage may serve as a general, yet direct, index or predictor of mitochondrial dysfunction.

ANTIOXIDANTS AND MECHANISMS

The term antioxidant is not clearly defined and, according to its use in the literature, may refer to an array of compounds with a range of mechanisms of action. Halliwell [40] has proposed the following practical definition: "an antioxidant is any substance that, when present at concentrations lower than those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". There is an abundance of oxidizable substrates in the cell, including proteins, lipids, carbohydrates and DNA. Thus, antioxidants may work to prevent the formation of ROS or to detoxify them, in addition to scavenging ROS or their precursors.

Antioxidants are central to the redox balance in the human body. They do not act in isolation, but synergistically. Primary endogenous antioxidant defenses prevent ROS formation, whether by removing ROS precursors or by inhibiting catalysts such as GSH peroxidase and catalase. Secondary antioxidants react with ROS that have already formed, either removing or inhibiting them; for example, vitamins C and E. Endogenous antioxidant defenses exist in a number of locations, both intracellularly (on the cell membrane) and extracellularly [10,11,41]. Types of antioxidants include, for example, antioxidant vitamins (e.g. ascorbic acid, α-tocopherol, β-carotene), inorganic antioxidants (e.g. selenium), synthetic antioxidants (e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), prolyl gallate, 2,4,6-trimethylphenol), and a range of plant-derived polyphenols.

Intracellular antioxidants

The SOD enzymes are a family of metalloenzymes which promote the rapid conversion of $O_2^{\cdot -}$ to H_2O_2. Three forms of SOD are recognized to be important: copper-zinc SOD (cytoplasm), manganese SOD (mitochondria), and extracellular SOD (extracellular matrix). Catalase and GSH peroxidase, an enzyme which requires the presence of reduced GSH in order to act, catalyse the conversion of H_2O_2 to H_2O. GSH contains thiol (sulfydryl) groups and also exerts a direct antioxidant activity through the donation of hydrogen ions, by which it repairs damaged DNA. Oxidative stress on and modulation of GSH/GSSG (GSSG, glutathione disulfide) levels also up-regulate gene expression of several other antioxidant proteins, such as manganese SOD, GSH peroxidase, thioredoxine, and methallothionein.

Membrane antioxidants

An alternative spectrum of antioxidants is necessary for the hydrophobic lipid interior of membranes. Fat-soluble vitamin E (α-tocopherol) is the most important antioxidant in this environment [42]. β-carotene, lycopene, and co-enzyme Q have also been implicated as membrane antioxidants. Lipid-soluble antioxidants are important in preventing membrane polyunsaturated fatty acids from undergoing lipid peroxidation, which leads to loss of membrane integrity.

Extracellular antioxidants

ROS may also be present in the extracellular compartment, especially as a result of neutrophil activation. The plasma and red-cell components of blood both act as antioxidants; red cells have a copper-zinc, SOD-dependent pathway for the inactivation of $O_2^{\cdot -}$, and catalase and GSH peroxidase for dealing with H_2O_2, while a number of metal-binding plasma proteins, including apotransferrin, lactoferrin, and ceruloplasmin, have an important function as antioxidants. Albumin is also effective via its oxidizable thiol group, which permits the scavenging of ROS and non-radical molecules and the binding of reactive transition metal ions. A number of important smaller molecules that are present in the plasma act as secondary antioxidants. These include vitamin E, vitamin C, uric acid, and bilirubin.

Mechanisms of antioxidant action are best understood in the context of their clinical and biochemical effects on ROS [10,11,43]. Three processes are involved in the formation of lipid-derived ROS: initiation, propagation, and termination. Chain-breaking antioxidants interrupt this process by preventing propagation on the radical chain or by terminating it. α-tocopherol is a classic example of a chain-breaking antioxidant. The antioxidant enzyme SOD catalyses the conversion of $O_2^{\cdot -}$ to H_2O_2. H_2O_2 can be detoxified by the Se-dependent GSH (GPx) and by catalase. The detoxification of H_2O_2 is particularly important because the reaction of H_2O_2 with transition metals can lead to the formation of OH.

In summary, increasing the antioxidant capacity of the mitochondrial compartment is a potential therapy, but depends on a small-molecule antioxidant if it is to be pharmaceutically viable [44]. However, small-molecule antioxidants will spread around the body, with only a small fraction being consumed by the mitochondria, which is the case of the antioxidant vitamin E, which produces no benefits in the treatment of Parkinson's diseases [45]. An explanation for this disappointing result may be a combination of poor uptake into the body and limited delivery to the mitochondria [44,46].

Mitochondrial antioxidant defenses

Mitochondria may limit the effects of ROS *via* small-molecule antioxidants, including GSH, ascorbic acid, and vitamin E [47]. The antioxidant enzyme MnSOD converts $O_2^{\cdot -}$

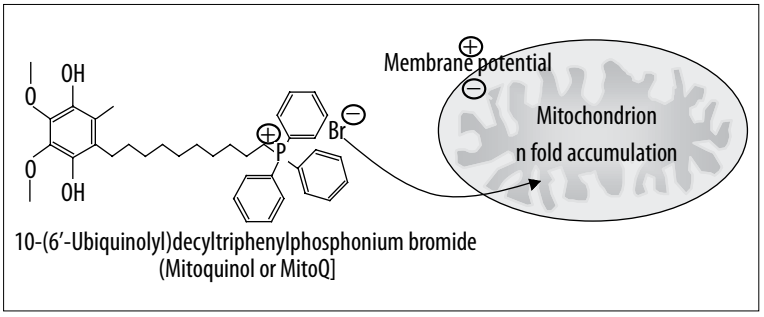

Figure 1. Using lipophilic cations to target compounds to mitochondria enables reagents to be delivered selectively to mitochondria within cells. Lipophilic cations accumulate within the mitochondria are driven by the membrane potential and can pass through lipid bilayers easily as their positive charge is delocalized over an extended area.

to H_2O_2. The mitochondrial isoform of GSH peroxidase and the thioredoxine-dependent enzyme peroxiredoxin III both detoxify H_2O_2. Alternatively, H_2O_2 is capable of diffusing from the mitochondria into the cytoplasm. The mitochondrial GSH pool is different from that of the cytosol and is maintained in its reduced state by a mitochondrial isoform of GSH reductase. This enzyme requires NADPH, which is produced within the mitochondria by the NADP-dependent isocitrate dehydrogenase and through a $\Delta\mu_{H^+}$-dependent transhydrogenase. Within the mitochondrial phospholipid bilayer, the fat-soluble antioxidants vitamin E and coenzyme Q both prevent lipid peroxidation, while coenzyme Q also recycles vitamin E and is itself regenerated by the respiratory chain. The mitochondrial isoform of phospholipid hydroperoxide GPx degrades lipid peroxides within the mitochondrial inner membrane.

MITOCHONDRIAL-TARGETED ANTIOXIDANTS

Mitochondria have an important role in cell signaling and so contribute to both adaptation to oxidative stress and the development of cardiovascular conditions such as ischemia-reperfusion injury, neurodegenerative diseases, diabetes, and atherosclerosis [48]. However, available antioxidants have not proven to be especially effective against this range of disorders. It is possible that they do not reach the relevant sites of ROS generation, especially if mitochondria are the primary source of ROS.

Mitochondria have long been known to play a critical role in maintaining the bioenergetic status of cells under physiological conditions, and are therefore relevant targets for drug-delivery strategies. Hence there is considerable interest in developing strategies to target molecules with therapeutic potential for mitochondria [49,50]. One approach to addressing these challenges is to target antioxidants to mitochondria by conjugation to a lipophilic cation, such as triphenylphosphonium (TPP) [14,51]. This procedure leads to orally bioavailable molecules which accumulate in the cell, are driven by the plasma membrane potential, and accumulate further into the mitochondria where the antioxidant moiety protects from oxidative stress. The electron transport generates a proton gradient that drives the production of ATP by ATP synthase. Thus, a negative potential of 150-180 mV is generated across the inner mitochondrial membrane. The mitochondrial outer membrane is permeable to small molecules; the inner membrane thus represents the major barrier for drug delivery to the mitochondria. This high potential gradient across the mitochondrial inner membrane can be exploited to deliver lipophilic cations to the mitochondria.

Several features of lipophilic cations make them effective at delivering antioxidants to the mitochondria: They can pass directly through phospholipid bilayers without requiring a specific uptake mechanism, and they accumulate substantially within the mitochondria owing to their high membrane potential. Lipophilic cations move without difficulty through phospholipid bilayers because the activation energy for moving lipohilic cations through the hydrophobic barrier of a biological membrane is far lower than for other cations. Hence, by attaching a compound to a lipophilic cation, it is possible to deliver it selectively to mitochondria within cells [52,53]. The aim is to make hybrid molecules in which the lipophilic cation delivers a bioactive "passenger" to mitochondria within cells, thereby enabling the rational manipulation of mitochondrial function *in vivo* [53,54]. The ability of mitochondria to accumulate modified lipophilic cations has been exploited as a potential therapy by which cancer cells are killed selectively [55,56], as said cells have a higher mitochondrial membrane potential than non-transformed cells, leading to the greater accumulation of lipophilic cations [57,58]. Lipophilic cations have been modified in various ways in order to promote cancer cell death through their linking to toxic compounds [58–61].

Mitochondria are a site of significant oxidative damage in clinical circumstances [62], and several antioxidants, such as vitamin E, ubiquinol, N-acetylcysteine, SOD mimetics, and spin traps, have been shown to protect them against such damage [62,63]. Therefore, targeting an antioxidant to the mitochondrial matrix should increase its protection against oxidative damage [53,55]. By covalently attaching a TPP cation to the antioxidant tocopherol moiety of vitamin E, the antioxidant is delivered selectively to mitochondria [53] and protects the mitochondria from oxidative damage more effectively than vitamin E (α-tocopherol) itself.

TPP-based, mitochondrially targeted antioxidants: mitoquinone

In principle, a wide range of antioxidants could be targeted to mitochondria by conjugation to the TPP moiety, and antioxidants targeted to all components of the intramitochondrial ROS cascade have been developed (Figure 1). Antioxidants have the potential to block oxidative damage and redox signaling, and exogenous ubiquinones have been widely used for this purpose in mitochondrial studies. As lipid peroxidation is important in many forms of mitochondrial oxidative damage, and because the alkylTPP conjugates are strongly associated with the mitochondrial inner membrane, focus until now has been on antioxidants that are effective against lipid peroxidation.

These molecules are based on the predominant human form of endogenous ubiquinone, coenzyme Q_{10} (CoQ_{10}), which is synthesized in the mitochondrial inner membrane and comprises a ubiquinone head group attached to a tail of 10 five-carbon isoprenoid units. The ubiquinone moiety is redoxactive, accepting two electrons and two protons in its reduction to a ubiquinol, while the extremely hydrophobic tail ensures that within the cell it is almost exclusively associated with phospholipid bilayers. The redox activity of the ubiquinone moiety enables it to act as a mobile electron carrier in the mitochondrial inner membrane, where it is reduced to a ubiquinol by several membrane- bound dehydrogenases and oxidized back to a ubiquinone by complex III. Furthermore, the reduced ubiquinol form of CoQ_{10} has an important protective function as a chain-breaking antioxidant, by which it terminates lipid peroxidation in phospholipid bilayers. Therefore, ubiquinone supplementation is a promising therapeutic strategy in human pathology, as it may both stimulate oxidative phosphorylation, by complementing any defects in respiration, and protect against oxidative damage. However, this duality complicates experimental interpretation, as all effects of CoQ_{10} can be attributed to its interaction with oxidative phosphorylation, oxidative damage, or redox signaling pathways.

Given the known antioxidant properties of ubiquinone, several studies have been undertaken to prepare and test an analogous to endogenous ubiquinone. MitoQ has been used to prevent mitochondrial oxidative damage and to demonstrate the involvement of mitochondrial ROS in signaling pathways. MitoQ is taken up by energized mitochondria and is absorbed into the matrix face of the inner mitochondrial membrane. Further distribution studies have shown that about one half of the MitoQ taken up by cells is localized in the mitochondrial fraction. In the respiratory chain, MitoQ is reduced to the active ubiquinol antioxidant by complex II, but it is not a good substrate for complex I or electron transfer flavoprotein-ubiquinone oxidoreductase.

$MitoQ_{10}$ cannot restore respiration in mitochondria lacking coenzyme Q, as its reduced form is poorly oxidized by complex III. Consequently, all the effects of $MitoQ_{10}$ are likely to be due the accumulation of the antioxidant ubiquinol form [53]. Furthermore, when the ubiquinol form of $MitoQ_{10}$ acts as an antioxidant, it is oxidized to the ubiquinone form, which is then rapidly reduced by complex II, thereby restoring antioxidant efficacy [53]. This is significant, because the recycling of an antioxidant back to its active form after it has neutralized an ROS is a critical factor in the efficacy of many antioxidants [64,65].

The uptake of $MitoQ_{10}$ is faster than that of TPMP, presumably as a result of its greater hydrophobicity and, consequently, lower activation energy for passage through the plasma membrane [66]. MitoQ is better tolerated than MitoVitE and in fact shows no toxicity at 750 nmol. However, such toxicity is evident at 1000 nmol. No toxic effects have been shown for TPMP up to 43 days, for MitoVit E up to 14 days, or for MitoQ up to 14 days [61]. Efficacy depends on chain length by creating and examining a series of MitoQ molecules with different numbers of carbons (3, 5, 10, 15) in their linker chains.

$MitoQ_{10}$ and other mitochondrial-targeted antioxidants such as MitoE2 decrease levels of ROS, which is evident when fluorescence is measured [67,68]. For example, MitoQ prevents H_2O_2-induced oxidation and the formation of malondyaldehyde [60]. MitoQ (1 µM) blocks H_2O_2-induced caspase activation and apoptotic cell death in Jurkat cells, but has no effect on staurosporine-induced apoptosis in Jurkat cells or TNFα-induced apoptosis in cells in general. MitoQ regulates important processes such as the accumulation of HIF in Hep3B cells in response to hypoxia [69], as well as the AIF factor in a model of apoptosis [70]. In several studies in which nitroglycerin produced an increase in ROS [71,72], MitoQ was shown to prevent such an increase.

Several studies have been conducted to determine the role of hydrophobicity in the uptake of MitoQ analogues by mitochondrial membranes [54]. In these studies, analogues were prepared so that the length of the hydrocarbon chain separating the TPP and ubiquinol moieties was 3, 5, 10, and 15 methylene units. As expected, the octan-1-ol/saline partition coefficient increased as the chain length was increased. Although antioxidant effects were observed with all analogues, $MitoQ_{10}$ and $MitoQ_{15}$ showed the highest efficacy in blocking ferrous iron-induced lipid peroxidation. In fact, the main antioxidant action of $MitoQ_{10}$ is its prevention of lipid peroxidation. It remains to be seen if this is the foremost mechanism by which $MitoQ_{10}$ acts as a protective agent in all cell types and forms of oxidative stress. Studies on chain-length analogues of MitoQ have been extended to investigate their interactions with the mitochondrial respiratory chain and ROS [52]. The reduction of $MitoQ_{10}$ affords antioxidant protection against lipid peroxidation, $ONOO^-$, and $O_2^{\cdot -}$.

Furthermore, MitoQ has been used to investigate nucleotide-sensitive mitochondrial proton transport through uncoupling proteins (UCP) [57]. In fact, $O_2^{\cdot -}$-induced uncoupling was prevented by MitoQ (and MitoVit E), but not by the untargeted antioxidants ubiquinone, decylubiquinone, vitamin E, or trolox.

Due to the role of oxidative damage in telomere shortening and, consequently, in senescence and aging, the effect of MitoQ on telomere shortening has been investigated [58]. Indeed, incubation of cells under hyperoxic conditions (40% O_2) resulted in an increase in ROS production and was reduced by MitoQ. Incubation of cells with MitoQ lengthened the replicate life-span by approximately 40% and reduced hyperoxia-induced telomere shortening, indicating that reduction of oxidative stress slows telomere shortening and prolongs the replicate life-span of cells.

To function as a form of therapy, mitochondrial-targeted antioxidants must be delivered to the mitochondria within patients' cells, preferably following oral administration. TPP cations pass easily through phospholipid bilayers, so they should pass from the gut to the bloodstream and from there to most tissues. For example, the effect of MitoQ on cardiac ischemia-reperfusion injury, which is associated with mitochondrial oxidative damage, has been investigated in a rat model [59]. Rats drank water containing 500 µM MitoQ for 14 days, and at the end of the treatment period their hearts were removed and perfused in order to assess ventricular contractile function and left ventricular developed pressure (LVDP). MitoQ afforded significant protection against ischemia-reperfusion-induced decreases in

LVDP, whereas little or no benefit was provided by the non-targeted ubiquinone (Q_3OH) or TPP bromide. When mitochondrial function was assessed in heart mitochondria from control and MitoQ-treated rats, MitoQ was shown to protect against the ischemia-reperfusion-induced decreases in the respiratory control ratio (RCR), damage to complex I, and decreases in aconitase activity. The amount of MitoQ in the hearts of treated rats was approximately 20 pmol MitoQ/g wet weight, which corresponds with a concentration of 100–200 nM MitoQ.

MitoQ is currently being developed as a pharmaceutical product, and in phase I trials has shown to be effective when administered orally (1 mg/kg), while phase II trials have provided encouraging results with respect to Parkinson's disease and Friedreich's ataxia [73]. This work with MitoQ has established a satisfactory pharmaceutical profile for the TPP moiety and will undoubtedly be the basis for future pharmaceutical formulations of other mitochondrial-targeted antioxidants. For this reason, we believe that MitoQ is a potentially effective compound to be used in the prevention of CVD.

Mitochondrial Damage and Cardiovascular Diseases

The CVDs coronary artery disease, hypertension, congestive heart failure, and stroke are the leading causes of death and disability in the developed world [74,75]. An early prognosis and improved therapies for preventing and curing these diseases depends on an understanding of the basic pathophysiological mechanisms of CVD. The oxidative hypothesis for atherosclerosis has been critical in the development of our knowledge about the molecular mechanism of the disease. Vascular pathologies are multifactorial, but it is clear that mitochondrial dysfunction can contribute to the pathophysiology of these diseases. This appears to involve not only damage to the organelle and loss of bioenergetic function, but also disruption of mitochondrion-dependent redox signaling pathways.

Several lines of evidence suggest that an association exists between CVD development and mitochondrial function and damage. CVD patients present more marked mtDNA damage in both the heart and the aorta than healthy controls [76,77]. Atherosclerotic lesions in brain microvessels from Alzheimer's patients and rodent Alzheimer's models show a significant presence of mtDNA deletions and abnormalities (as do their endothelium and perivascular cells), suggesting that the mitochondria within the vascular wall are a central target for oxidative stress-induced damage [78]. Accumulated mtDNA damage will hinder the replacement of respiratory chain proteins damaged by ROS production. These damaged proteins are more likely to generate ROS in an uncontrolled manner, thereby accelerating bioenergetic dysfunction. In this way, the role of intramitochondrial antioxidants is critical, as they may alter the progression of the disease and prevent damage to existing proteins.

In a mouse model it has been found that previous myocardial infarction is associated with increased ROS and decreased mtDNA copy number, mitochondrial-encoded gene transcripts, and related enzymatic activities (complexes I, III, and IV). However, nuclear-encoded genes (complex II) and citrate synthase are unaffected in said mice [79]. Cardiotoxic ROS generators increase mtDNA deletions and lipid peroxidation in the myocardial mitochondria, and overexpression of mitochondrial antioxidants reverses these effects and increases cardiac tolerance to ischemia [80]. Decreased vascular SOD2-specific activities have been associated with increased exposure to several risk factors [76], increased susceptibility to ischemia/reperfusion-mediated cardiac damage and resistance to cardiac preconditioning [81]. Moreover, deficiencies in mitochondrial antioxidants and/or regulatory proteins that modulate mitochondrial oxidant production have been shown to promote the onset of CVD *in vivo*, which endorses the theory that mitochondrial-generated oxidants contribute to atherogenesis [82]. Likewise, overexpression of mitochondrial antioxidants and/or UCPs has been shown to protect against the effects of ischemia/reperfusion and oxidative stress [80,83].

CVD risk factors also cause mitochondrial damage and dysfunction

Accumulating evidence indicates that oxidative stress plays a major role in the initiation and progression of CVD [84]. Numerous factors, such as atherosclerosis, hypercholesterolemia, diabetes, tobacco exposure, age, and ischemia-reperfusion injury, increase the risk of CVD, and while it is not yet clear whether or not they alter cellular function in a similar fashion, a common feature is that they increase oxidative stress [85-86]. Moreover, it is surely more than a coincidence that several of said factors also appear to cause cardiovascular mitochondrial damage and/or dysfunction [76,85].

Atherosclerosis

Atherosclerosis, the primary cause of coronary artery disease (CAD), is a multifactorial pathology whose molecular etiology involves the interaction of many genes and environmental factors. The majority of CVDs are a result of complications caused by atherosclerosis. Atherosclerosis is a chronic inflammatory disease characterized by a loss of endothelial cell function [87], recruitment of monocytes into the arterial wall [88], and formation of lipid-foam cells. The endothelium plays a key role in the initial stages of atherosclerosis, especially in inducing adherence of monocytes and downstream signaling events. Many risk factors for atherosclerosis, such as increased levels of modified LDL, a smoking habit, increased ROS, and diabetes, have been shown to damage the endothelium, and it has been hypothesized that dysfunction of the endothelium initiates atherosclerotic lesion formation.

Endothelial cells, smooth muscle cells, and macrophages are sources of ROS for the oxidative modification of phospholipids, and Ox-LDL can damage endothelial cells, thereby inducing the expression of adhesion molecules [89]. ROS limit the bioavailability of NO and induce inflammatory gene expression, cell growth/apoptosis, migration, and matrix reorganization, all of which are central mechanisms for the initiation and progression of atherosclerosis. NO signaling in the endothelium can be affected by a number of factors during atherosclerosis.

There are numerous reports of a correlation existing between DNA damage and atherosclerosis [90]. Multivariate analysis reveals that DNA-adduct levels are a significant pre-

dictor of the stage of atherosclerosis, even after adjustment for age, smoking, obesity, and other CVD risk factors [91]. In other studies, higher levels of immunoreactivity against 8-oxoG have been observed in plaques of the human carotid artery than in the adjacent inner media and non-atherosclerotic mammary arteries. 8-oxoG immunostaining is observed in all cell types of the plaque, including macrophages, smooth muscle cells, and endothelium. Evidence suggests that there are more DNA repair mechanisms in the atherosclerotic plaques than in control tissues [92]. In line with these studies is the observation that mtDNA damage is increased in cardiovascular tissues in CVD patients [78]. However, whether this damage is an effect or initiator of CVD remains unclear. Animal studies have shown that vascular mtDNA damage is greater in animal models of atherosclerosis and that this damage occurs prior to, or simultaneously with, the development of the disease [78].

Defects in oxidative phosphorylation from heart mitochondria have been identified in strains of pigeons susceptible to atherosclerosis, in which the dissociation of NADH transhydrogenation from ATP regulation enhanced lipid biosynthesis [93]. Mitochondrial respiratory dysfunction has also been demonstrated in myocardial mitochondria of atherosclerotic swine.

Ballinger et al. 2002 [76] showed that oxidative mitochondrial DNA damage correlated positively with the extent of atherosclerotic lesions in arteries of humans and apoE knockout mice, and that this damage preceded the onset of the disease in these mice. Besides, LDL receptor knockout cells are more exposed to oxidative stress and are more susceptible to cell death due to an undermined mitochondrial antioxidant defense system and a higher susceptibility to mitochondrial pore transition. Thus, the LDL receptor defect has two important pro-atherogenic consequences that sometimes manifest themselves before the disease initiates, namely, increased extracellular levels of oxidizable substrate (LDL) and an imbalance of cell redox processes, which can occur in the vascular wall where local oxidative stress takes place, subsequently triggering lipoprotein oxidation, cell death, and atherogenesis.

In another study, Ballinger et al. demonstrated that SOD2 mutant ($SOD2^{-/-}$) mice exhibited inhibition of the respiratory chain enzymes NADH-dehydrogenase (complex I) and succinate dehydrogenase (complex II) as well as inactivation of redox-sensitive enzymes such as aconitase, accompanied by accumulation of oxidative mitochondrial DNA damage. $SOD2^{+/-}$ mice also exhibit a rise in $O_2^{\cdot-}$ levels concomitant with a 50% decrease in MnSOD activity in mitochondria (compared with $SOD2^{+/+}$ mice). In this way, these studies underline the value of mitochondrion-targeted antioxidants as a therapeutic tool for counteracting the development of atherosclerosis.

Atherosclerotic disease remains a leading cause of death in developed societies, and ROS play a pivotal role in atherogenesis. Oliveira et al. [94] have shown that mitochondria from atherosclerosis-prone, hypercholesterolemic LDL receptor knockout mice have an oxidative phosphorylation efficiency similar to that of control mice, while their net production of ROS and susceptibility to developing membrane permeability transition are both higher.

Oxidized lipids are capable of initiating diverse cellular responses through both receptor-mediated mechanisms and direct post-translational modification of proteins. Typically, exposure of cells to low concentrations of oxidized lipids induces cytoprotective pathways, whereas high concentrations lead to apoptosis. In fact, incubation of endothelial cells with concentrations of oxLDL characteristic of cytoprotection induce a mitochondrial complex I activity that seems to depend on the induction of oxidative stress [95]. Reports of oxLDL inducing the transcription and expression of SOD2 protein in human macrophage endorse the hypothesis that oxLDL increases mitochondrial oxidative stress [96]. Increased mitochondrial oxidant generation and decreased mitochondrial membrane potential have also been studied in human macrophage cells treated with oxLDL, in which scavengers of peroxides prevented said effects [97]. A standard consequence of increased oxidative stress due to exposure to ox-LDL is the breaking of the nuclear DNA strand, which is thought to be a signal of an increase in p53 protein levels. Inhibition of macrophage lysis in atherosclerotic lesions, without affecting macrophage apoptosis, is likely to prevent lesion progression and permit lesional cellularity, lesion remodeling, and regression to be controlled, which highlights mitochondrially targeted antioxidants as a therapeutic tool in the control of hypercholesterolemia and, therefore, atherosclerosis.

Treatment of mouse peritoneal macrophages with free cholesterol (FC) causes a marked decrease in mitochondrial membrane potential, cytochrome c release, activation of caspase-9 and effector caspases, and elevated levels of bax [85]. While SOD2 activity and GSH concentration are significantly higher in the atherosclerotic intima than in the media of the aorta of hyperlipidemic rabbits, SOD2 activity and GSH concentration are also found to be inversely related to age and plaque size, suggesting that both are directly correlated and may be related to the early stages of atherosclerotic lesion formation [96]. Hence the effects of hypercholesterolemia on SOD2 expression and activity may be age related and represent an early "protective" response to the increased mitochondrial oxidation that occurs during the initial events of CVD risk factor exposure and/or atherogenesis. In general, free cholesterol, oxidized low-density lipoprotein, and glycated high-density lipoprotein are further causes of mitochondrial dysfunction and/or apoptosis. High-fat diets reduce the expressions of genes involved in free-radical scavenging (SOD1, GPX, and SOD2) and increase the expressions of stress-response (Hsp 70) and signal-transduction genes (Ras, MAPK1) in rats. However, while UCP-2 levels increase, UCP-3 levels remain unaffected. Antioxidant supplementation of a high-fat diet reduces the magnitude of these differences [98].

Apoptosis can be induced by a number of different stress factors in the cardiovascular system and has been implicated in a number of chronic disorders, including atherosclerosis [99,101].

Diabetes

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes. In fact, recently it has been suggested that increased mitochondrial ROS production during hyperglycemia is central to the general

pathology of diabetes [102–105]. Therefore, mitochondrial ROS production and oxidative damage may contribute to the onset, progression, and pathological consequences of both type 1 and type 2 diabetes. Emerging evidence supports the hypothesis that both of these prominent features of type 2 diabetes are caused by mitochondrial dysfunction and ROS production

Hyperglycemia induces increased $O_2^{\cdot-}$ generation in endothelial cells *in vitro*, and studies suggest that the majority of this $O_2^{\cdot-}$ is produced by mitochondria [103] with some contribution from NADPH oxidase. In this respect it has been speculated that hyperglycemia increases the inner membrane proton gradient as a result of overproduction of electron donors (e.g. NADH and FADH2) by the TCA cycle, which is manifested in an increased production of $O_2^{\cdot-}$ and increased activity of antioxidant enzymes [106]. These same factors also prevent glucose-induced activation of phosphokinase C and NF-κB activation in endothelial cells [107]. Similarly, overexpression of SOD2 significantly reduces IL-1, TNFα, IFNγ activation of NF-κB, and the induction of iNOS in insulin-producing cells. In this sense, some studies have addressed the role of mitochondrial ROS and oxidative damage in TNF-induced apoptosis using mitochondrial-targeted derivatives of vitamin E (MitoVitE), ubiquinol (MitoQ), and PBN (MitoPBN). These targeted antioxidants, MitoVit E, MitoQ, and MitoPBN, accumulate selectively in the mitochondrial matrix, protecting the mitochondria against oxidative damage. MitoQ and MitoVit E protect cells from a variety of apoptotic stimuli, including 5-fluorouracil, growth factor deprivation, and GSH depletion in frataxin-depleted cells, and also inhibit H_2O_2-induced growth factor receptor signaling [108]. These results confirmed the role of mitochondrial ROS in said processes and demonstrate that mitochondrial-targeted antioxidants are useful tools for determining the role of mitochondrial ROS in signal transduction. Therefore, mitochondrial ROS are critical modulators of TNF-induced apoptosis, which is mediated, at least in part, by a delay in the activation of NF-κB. This suggests that mitochondrial ROS are produced in response to TNF treatment and that they impede a full apoptotic response to TNF by enhancing NF-κB-mediated expression of antiapoptotic proteins.

As oxidative damage forms part of the pathophysiology of diabetes, there is interest in determining whether or not antioxidants reduce this damage [109]. Too few large-scale, double-blind trials on the use of antioxidants in diabetes have been carried out to come to any reliable conclusions [109,110]. However, a few small-scale trials have pointed towards the efficacy of the natural antioxidants α-tocopherol, ascorbate, coenzyme Q, and α-lipoic acid, although other trials have produced somewhat ambiguous results regarding the efficacy of ascorbate and α-tocopherol [110]. These natural antioxidants can be administered at high doses and have shown some efficacy in other degenerative diseases, which provides a strong rationale for testing them in diabetes [46], though it must be said that the uptake and distribution to tissues of hydrophobic natural antioxidants such as coenzyme Q is often poor [111]. In addition, many other artificial antioxidants are presently in development, such as mimetics of SOD or peroxidase, and may be more potent than natural antioxidants and possess an improved bioavailability, pharmacokinetics, and stability [112]. However, these artificial antioxidants are novel drugs and will need to be submitted to clinical trials. Both natural and artificial antioxidants are distributed throughout the body, with only a small proportion reaching the mitochondria, where much of the oxidative damage associated with hyperglycemia would seem to occur.

In relation to the use of mitochondrial-targeted antioxidants, and because mitochondrial oxidative damage is thought to be critical to the pathophysiology of diabetes, antioxidants that accumulate within mitochondria may offer more protection than untargeted antioxidants. As a first step toward testing this hypothesis, a strategy has been developed to deliver antioxidants to mitochondria by covalent attachment to the TPP cation through an alkyl chain. Importantly, the accumulation of these antioxidants by mitochondria protects them from oxidative damage far more effectively than untargeted antioxidants, suggesting that the accumulation of antioxidants within mitochondria does increase their efficacy. Most interestingly, the compounds in question were effective in preventing cell death in fibroblasts from Friedreich's ataxia patients. As cell death in this model is due to endogenous mitochondrial oxidative damage [113], it has been suggested that the accumulation of antioxidants by mitochondria within cells reverses mitochondrial oxidative damage, and that their uptake into mitochondria makes them far more effective than untargeted antioxidants.

If these mitochondrial-targeted molecules are to have therapeutic potential in the treatment of diabetes, then it is essential that they are taken up selectively by mitochondria *in vivo*. Given that alkylTPP cations pass easily through lipid bilayers by means of carrier-unmediated transport, they should be taken up by the mitochondria in all tissues, in contrast to hydrophilic compounds, which rely on the tissue-specific expression of carriers for uptake [46]. Several studies in mice fed for several weeks with mitochondrion-targeted antioxidants have shown stable steady-state concentrations in all the tissues assessed, including brain, heart, liver, and kidneys [57]. These data are consistent with the following pharmacokinetic model: following absorption from the gut into the bloodstream, orally administered mitochondrial-targeted antioxidants are taken up into all the tissues through an unmediated movement through the lipid bilayer of the plasma membrane, and are assisted by the plasma membrane potential. From the cytosol, most of the lipophilic cations are driven by the large membrane potential and taken up into the mitochondria. After several days of feeding, the cation concentration within mitochondria achieves a steady-state distribution with circulating blood levels. At this point, the mitochondrial concentration is several hundred-fold higher than that in the bloodstream. As the mitochondrial pool of compound is in dynamic equilibrium, once feeding stops the accumulated cations re-equilibrate back into the bloodstream and are excreted relatively rapidly.

Given that these compounds accumulate within mitochondria, the intramitochondrial concentration is approximately millimolar. These concentrations are likely to fall within a therapeutically effective range, because mitochondrial-targeted antioxidants prevent oxidative damage to isolated mitochondria at 1–2.5 mmol/l [60]. As these compounds are further accumulated in cells, similar protective effects are found when cultured cells are incubated with

500 nmol/l to 1 µmol/l mitochondrial-targeted antioxidants [114]. Therefore, oral delivery of well-tolerated doses of mitochondrial-targeted antioxidants can deliver potentially therapeutic concentrations to mitochondria *in vivo*. Their efficacy in preventing oxidative damage to mitochondria *in vivo* could be tested in mouse models of mitochondrial oxidative damage.

Tobacco smoke exposure

Active and passive exposure to tobacco smoke is an important cause of morbidity and mortality [114]. Multiple studies have shown that chronic smokers are at more risk of diseases related to atherosclerosis, such as coronary obstruction and acute myocardial infarction, and of sudden death. In this sense, chronic smoking causes endothelium dysfunction, increased oxidation of LDL-cholesterol, reduction of blood levels of HDL-cholesterol, and increased blood levels of adhesion molecules and fibrinogen, joint factors that can lead to platelet aggregation and, eventually, vascular spasm [115].

Tobacco smoke exposure has also been related to other cardiac conditions. Greenspan et al. [116] demonstrated the acute effects of nicotine on hemodynamic and functional cardiac variables. Rats exposed to chronic carbon monoxide, another component found in the vapor phase of mainstream cigarette smoke, showed an increase in their endothelin-1 gene expression, which induced myocardial hypertrophy [117]. In addition, impairment of the left ventricular function, evaluated by means of transthoracic echocardiography, has been demonstrated in rats exposed to cigarette smoke for 30 days and 4 months [118]. These findings suggest that exposure to nicotine/tobacco smoke is associated with alterations of both functional and morphological cardiac variables.

Cigarette smoke exposure is a principal cardiovascular risk factor that contributes to atherosclerosis and hypertension. The majority of individuals chronically exposed to tobacco smoke will succumb to CVD. Smoking reduces arterial O_2 carrying capacity through increased serum carboxy-hemoglobin levels and causes mitochondrial respiration dysfunction in cardiac cells [119]. When combined with other CVD risk factors (e.g. hypercholesterolemia), second-hand tobacco smoke exposure synergistically accelerates both mitochondrial damage and atherogenesis [77]. Further illustration of the link between accumulated mtDNA defects and susceptibility to CVD is the impact of exposure to environmental tobacco smoke in utero. Gestational exposure to tobacco smoke in mice increases the rate of development of atherosclerosis as the animals mature [120].

Treatment of human monocytes and vascular smooth muscle cells (VSMCs) with tobacco smoke filtrate results in loss of mitochondrial membrane potential, apoptosis, and necrosis, effects that are prevented by N-acetylcysteine (NAC) treatment [121]. More recently it has been shown that rats treated with benzo(a)pyrene in order to induce an atherogenic phenotype have an upregulated expression of mtDNA transcripts. Cultures acquired from these rats had increased growth rates and shows marked enhancement of proliferation to serum mitogens [122]. Endothelial adaptive responses that accompany the chronic manifestations of smoke exposure include hypertension, increased endothelial adhesiveness, increased vascular permeability, and vascular remodeling.

Studies have been developed in order to counteract the negative effects of tobacco in CVD. NAC prevented tobacco smoke-induced deltapsim disruption and apoptosis, while the caspase inhibitor Z-VAD.Fmk had no affect on deltapsim, though it did prevent apoptosis, and SOD had no effect [123], underlining mitochondria as the main target for ROS-mediated effects of tobacco smoke exposure.

Age

There is emerging evidence that cellular senescence contributes to the pathogenesis of human atherosclerosis. Senescent vascular cells accumulate in human atheroma tissues and exhibit various features of dysfunction. Some epidemiological studies have demonstrated that, even in the absence of other risk factors (e.g. diabetes, hypertension, hypercholesterolemia), vascular aging significantly increases cardiovascular morbidity [124]. Previous reports have revealed that vascular aging is characterized by an age-dependent decline in endothelial function due to a reduced bioavailability of NO and increased production of ROS. Yet the mechanisms underlying the process of vascular aging are still poorly understood. There is evidence that aging is associated with an increase in mtDNA damage and a decline in the expression/activity of mitochondrial enzymes in various organs [125]. Numerous mitochondrial functions decline with age and are generally accompanied by a concomitant reduction in OXPHOS efficiency and increased mtDNA damage. Whereas studies have shown that aging is accompanied by both decreased energetic capacity and increased mitochondrial ROS generation, the effects of age on mitochondrial redox signaling properties have not been studied in great detail. It is likely that, as happened with its energetic functions, the efficiency of the redox functions of the mitochondrial decline with age. It has also been shown that CVD risk factors such as hypercholesterolemia and tobacco smoke accelerate mitochondrial damage [76]. Hence many CVD risk factors may act on the cardiovasculature by inducing mitochondrial damage and dysfunction, thus mediating a "premature" aging process. In this respect, an individual's age during CVD risk factor exposure may influence disease development.

As the natural antioxidants vitamin E and CoQ are thought to protect mitochondria from oxidative damage *in vivo*, mitochondrial-targeted derivatives of these molecules were initially developed to counteract oxidative stress. *In vitro* experiments show that MitoVitE and MitoQ are rapidly and selectively accumulated by isolated mitochondria and by mitochondria within isolated cells [57]. It is significant that the accumulation of these antioxidants by mitochondria protect them from oxidative damage far more effectively than untargeted antioxidants [60]. This suggests that the accumulation of antioxidants within mitochondria increases their efficacy. Furthermore, mitochondrial-targeted antioxidants have also been shown to modulate the role of mitochondrial ROS production through putative redox signaling pathways [108] and to prevent telomerase shortening [58].

Aging is a slow process best studied in intact organisms. Therefore, for mitochondrial-targeted molecules to be used

as probes of mitochondrial ROS production in aging, they must be accumulated by mitochondria *in vivo*. TPP cations accumulate in the mitochondria of all tissue, as they can cross phospholipid bilayers with ease by means of carrier-unmediated transport. This contrasts with hydrophilic compounds, which often depend on tissue-specific transport pathways for uptake. When mice were fed mitochondrial-targeted antioxidants for several weeks, stable-state concentrations were observed in all the tissues assessed, including the brain, heart, liver, and kidneys [55].

Ischemia-reperfusion injury

Stroke is the second most common cause of death in developed countries and one of the major causes of death and disability, and thus puts serious weight on public health systems, being the neurological disease which accounts for the largest number of hospitalizations. Stroke causes cellular damage related to the sharp reduction in available O_2 and to deficiencies in energy-supplying substances, which leads to a sharp increase in lactate production and, therefore, acidosis. These events reflect a marked imbalance between energy use and production. This oxidative stress induces changes in Ca^{2+} that subsequently activate enzymes such as phospholypases, proteases, and nucleases, thereby contributing to the cell damage that produces ROS. In this way, during ischemia there is a decrease in mitochondrial oxidative phosphorylation that induces a prompt drop in ATP levels and an increase in the levels of ADP and AMP. Lactates therefore accumulate, causing extensive tissue acidosis. The increase in adenine nucleotide and the decrease in pH block the mitochondrial pore.

When ischemia is followed by reperfusion, mitochondrial respiratory functions are rapidly and completely restored. However, a decline soon occurs in mitochondrial O_2 consumption in tissue subregions containing damaged cells. Thus, post-ischemia reperfusion induces a cascade of alterations that increase the severity of damage; in fact, mitochondria hydrolyze ATP when O_2 is lacking in order to maintain the mitochondrial membrane potential. This activity destroys any available ATP, favors Ca^{2+} accumulation and increases the generation of ROS, which damages the mitochondria.

The first consequence of a decrease in oxidative phosphorylation is an increase in state-4 O_2 consumption. During reoxygenation, cytochrome c and cardiolipin are simultaneously and rapidly released, allowing the release of cytochrome c from the mitochondria, which leads to cell death. This is evidence of the potential of mitochondria as a source of oxidative damage after a stroke, while ROS that burst during reperfusion may also damage the mitochondrial DNA (mtDNA). Studies have revealed specific mtDNA deletion patterns following transient global ischemia, traumatic brain injury, and focal ischemia.

Therefore, mitochondrial protection may play a key role in strategies for treating ischemia-reperfusion injury [125] with the following objectives: the inhibition of ROS generation by respiratory complexes I and III, the protection of mitochondrial lipids, proteins, and DNA from ROS-induced damage in order to prevent mitochondrial membrane alterations, and the release of the proapoptotic signal that induces cell death. Other studies describe a significant role for antioxidants such as taurine in ischemia-reperfusion injury, with an apparent clinical function emerging in human trials of taurine administered prior to coronary artery bypass grafting and heart valve surgery [126].

CONCLUSIONS

The loss of control of ROS formation in the mitochondria contributes to the pathology of CVD. In fact, mitochondria play a critical role in apoptotic cell death and cell apoptosis, the latter being implicated in the development of CVD. Accumulated knowledge regarding these mechanisms has led to the development of a range of strategies for developing mitochondria-targeted antioxidants that prevent ROS-induced mitochondrial oxidative damage. The use of selective antioxidants to attenuate apoptotic signaling may shed light on the role of generalized mitochondrial redox alterations during cell death. Mitochondrially targeted antioxidants represent a potential therapy for the many diseases that involve mitochondrial oxidative damage. The development of transgenic or inbred mouse models for mitochondria-associated diseases constitutes an important tool for the testing and further development of mitochondria-targeted therapies.

MitoQ has proved to be active in a rat model of cardiac ischemia-reperfusion injury and is successfully delivered orally to humans. However, pre-clinical studies in intact rodent models and in other mammals are necessary in order to evaluate the effectiveness and toxicity of mitochondrially targeted antioxidants. These compounds could confirm the validity of this research pathway, which may bear fruit in clinically effective therapeutic agents. Finally, there is considerable scope for finely tuning the chemical biology of these compounds to target specific ROS and other mitochondrial genes.

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