Mitochondria-Targeted Vitamin E Antioxidant: An Agent for Cardiovascular Protection

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Abstract: Mitochondria play an important role in controlling the bioenergetic status of cells in physiological conditions. Consequently, mitochondrial dysfunction leads to a range of human vascular diseases in humans. Although the molecular mechanisms responsible for mitochondria-mediated disease processes are not altogether clear, oxidative stress appears to be closely linked to the subsequent increase in the amount of free radicals. Accordingly, strategies for the targeted delivery of antioxidants to mitochondria are being developed. In this review, we shall briefly discuss the metabolism of cellular reactive oxygen species and its role in pathophysiology, currently available antioxidants and possible reasons for their ineffectiveness in ameliorating oxidative stress-mediated diseases, and recent developments in mitochondria-targeted antioxidants (in particular mitochondria-targeted vitamin E) and their potential for the treatment of cardiovascular disease.

INTRODUCTION

Oxidative stress defines an imbalance in the production of oxidizing chemical species and their effective removal by protecting antioxidants and scavenger enzymes. 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]. The free radical hypothesis for vascular dysfunction postulates that ROS leads to a modification of lipids, proteins and nucleic acids, which in turn contributes to the etiology of the disease [2].

Cardiovascular diseases (CVD) - ischemia-reperfusion injury, sepsis, hypertension, coronary artery disease, congestive heart failure, diabetes, and atherosclerosis - are the leading cause of death and disability in the Western world. Over the last decade, the management of CVD has improved remarkably in experimental animals but not in humans, and although much of the existing published research suggests that enhancing oxidant scavenging protects against some of the cardiomyocyte disturbances during CVD, there is also data that suggests that the pro-oxidant activity of some antioxidants also exerts powerful biological effects. Thus, there

is an ongoing search for more effective antioxidants that counteract the oxidative stress induced in CVD, such as that seen in atherosclerosis and hypertension [3].

Mitochondria are organelles that play a vital role in both life and death, and are the primary sources of cellular energy. However, the mitochondrial respiratory chain in the inner mitochondrial membrane is a major intracellular source of ROS [4], and is therefore also central to processes that induce apoptosis, indicating that a specific action of antioxidants on the mitochondrial respiratory chain may constitute an important mechanism of cardiovascular protection.

The excess of ROS can damage cellular lipids, proteins and DNA, thereby disrupting their normal function. Owing to this, oxidative stress has been implicated in a number of human diseases. The delicate balance between beneficial and harmful effects of ROS is a crucial aspect in living organisms and is achieved by mechanisms referred to as "redox regulation". This process protects living organisms from different forms of oxidative stress and maintains "redox homeostasis" by controlling the redox status *in vivo* [5]. It is therefore 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 [6]. 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

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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 contributes to a number of cell pathologies, and are particularly relevant to CVDs such an atherosclerosis, hypertension and cancer. Studies in which the deleterious effects of ROS have been counteracted have shown antioxidants such as α-tocopherol, ubiquinol and N-acetylcysteine to decrease mitochondrial oxidative damage in different models [7-9]. However, as these compounds do not significantly accumulate within mitochondria, their effectiveness remains limited [10]. Thus, increasing the antioxidant capacity of the mitochondrial compartment is a therapeutic objective.

Mitochondria have emerged as a novel target for disease treatment, and targeting mitochondria may provide the means for establishing an effective treatment of the disesase, a long sought-after goal. One approach to 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 [11].

ROS PRODUCTION

Oxygen metabolism continuously generates small amounts of ROS [1]. ROS are normally produced during physiologic processes such as cellular respiration and inflammatory defense mechanisms, and are important secondary messengers. When levels of ROS/reactive nitrogen species (RNS) are low, damage to key targets in the mitochondria, such as mtDNA, is prevented by intramitochondrial antioxidant defenses. ROS have distinct functional effects on each cell type in the vasculature and exert both a physiological and pathophysiological influence. In nearly all diseases to which mitochondrial dysfunction contributes, a major cause of the damage it causes are the ROS that it produces, either directly or as a secondary consequence of other malfunctions [12,13].

During aerobic metabolism, the oxidoreduction energy of mitochondrial electron transport is converted into the high-energy phosphate bond of ATP *via* a multicomponent NADH dehydrogenase complex. Molecular O_2 is the final electron acceptor for cytrocrome-c-oxidase (complex IV, the terminal component of the respiratory chain) and is ultimately reduced to H_2O. However, a small quantity of O_2 may be incompletely reduced, as a leakage of single electrons causes the reduction of oxygen to superoxide (O_2^-). The rate of ROS production depends on mitochondrial inner membrane potential, which can be depolarized by mitochondrial ATP-sensitive potassium channel openers. Most O_2^- and some hydroperoxy radicals (OH) are generated in Complex I (NADH coenzyme Q reductase) and Complex III (Ubiquinone cytochrome c reductase) of the respiratory chain. Moreover, it has been shown that electrons derived from FADH2 (Complex II substrate) can undergo reverse transport into Complex I, which may constitute the primary source of O_2^- production in mitochondria. Thus, a variety of mitochondrial sites of O_2^- production have been identified, including several respiratory complexes and individual enzymes [14].

In the mitochondria, peroxynitrite, NO, and other RNS interact with complexes I, III, and IV of the respiratory chain [3]. In particular, the key site for the interaction of NO with mitochondria is at cytochrome-C-oxidase, which is competitively inhibited following the reversible inhibition of O_2 consumption and ATP synthesis [15]. The interaction between RNS and mitochondria could also have significant effects on both mitochondrial and cellular signaling events implicated in apoptosis and redox regulation of gene expressions [16]. Furthermore, mtDNA damage by ROS as a consequence of the RNS-induced impairment in oxidative phosphorilation may play a significant role in the pathogenesis of hypertension.

As mitochondrial oxidative damage is either a primary or significant secondary cause of cell damage and death in degenerative diseases, a general therapeutical approach would be to reduce mitochondrial oxidative damage in a range of clinical situations [17]. In the vasculature, there are different types of ROS, including O_2^-, H_2O_2, hypochlorous acid (HOCl), OH and singlet oxygen ($^1O_2$), for whose production several enzymes are thought to be responsible. These include NAD(P)H oxidase, NO synthase (NOS), lipoxygenases, cycloxigenases, xanthine oxidase, cytochrome P450 enzymes (CYP 450) and mitochondria. In addition, the formation of $ONOO^-$ has been implicated in CVD.

The balance between these sources of ROS depends on the physiologic and pathophysiologic states of the organism. Moreover, the source of ROS generation is often difficult to indentify. It is clear, however, that ROS have an important regulatory function [5]. In fact, ROS/RNS can activate pathways that control cell differentiation and apoptosis, both of which are mechanisms of particular relevance to CVD. Hence, a basal or total concentration of ROS, especially at the level of the mitochondria, is essential for basic cell signalling processes. In other words, all ROS are not created equally, with compartmentalisation and concentration gradients being fundamentally important.

Mitochondria represent both a major site of ROS production and a possible target of ROS action [3]. Moreover, the ROS-induced instability of critical non-lipid molecules, such as mitochondrial DNA (mtDNA), may alter oxidative phosphorilation and produce a further increase in ROS production (the "ROS-induced ROS release"). In the light of redox cell signalling, the identification of physiological regulators of mitochondrial ROS production is of great relevance. Several molecules/factors have been proposed as regulators of mitochondrial ROS production, including an elevated inner membrane potential, Ca^{2+} and NO.

OXIDATIVE STRESS AND MITOCHONDRIA

An excessive production of ROS in mitochondria may damage mtDNA, which is located close to the inner membrane and is not protected by histone proteins, which is also the case with nuclear DNA [18]. Under normal conditions in mitochondria, there is a balance between ROS formation and antioxidants. In some pathological circumstances, however, antioxidant defences and mtDNA repairing enzymes are rendered insufficient, resulting in oxidative stress and leading to mtDNA damage. This may be of relevance as, although 95% of mitochondrial proteins are encoded by nuclear DNA,

mtDNA contains gene coding for 13 components of the respiratory chain, including subunits of NADH dehydrogenase and cytochrome oxidase and cytochrome b [19].

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 [20,21], and is of a special relevance to CVD. In fact, the accumulation of mtDNA damage over a lifetime may increase one's susceptibility to developing pathology. Mitochondrial dysfunction triggers signalling cascades for cell necrosis and apoptosis, and leads to organ failure and diseases. The list of mitochondria-related conditions is growing rapidly and includes cancer, heart failure, diabetes, obesity, stroke, neurodegenerative diseases and aging, but disturbances of mitochondrial Ca^{2+}, ATP, or ROS metabolism are common features of all [20]. The critical role of intracellular Ca^{2+} overload in the genesis of myocyte dysfunction is well established [22]. In general, Ca^{2+}-overload can be induced by a direct effect of ROS on Ca^{2+}-handling proteins, or indirectly, by inducing membrane lipid peroxidation. Intracellular Ca^{2+} overload seems to be a common denominator for stimulation of neointimal hyperplasia and, in turn, atherosclerosis, as well as vasoconstriction for the development of hypertension, myocardial cell damage observed in ischemia-reperfusion, and cardiac hypertrophy in heart failure.

NO damages mtDNA to a greater impact than nuclear DNA [23], and ROS and RNS are capable of targeting a variety of subcellular components. In addition, 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 [24]. Mitochondrial oxidative damage has been implicated in clinically relevant situations, including CVDs such as ischaemia-reperfusion injury and neurodegenenerative processes, as they have been found to contribute to the pathophysiology of said conditions 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 [25], while mice lacking Cu Zn-SOD, the cytosolic form of the enzyme, survive [26].

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 an undermined mitochondrial redox potential in vascular cells [27]. ROS may reduce the effect of NO by directly inactivating it, but the mechanism by which this occurs is unclear. In addition, ROS affect NO responses by oxidizing sites within proteins with which NO reacts. It appears that this mode of ROS action contributes to cardiac pathophysiology, thereby mediating post-translational modifications of mitochondrial proteins that lead to their activation (e.g., cytochrome c, aconitase) [28]. Another important factor is the significant association of hypertension with mitochondrial uncoupling proteins (UCPs), which has been reported in both experimental and human hypertensive states. In particular, mice with doxy-

cline expression of UCP1 in arterial walls develop hypertension and dietary atherosclerosis [29].

Mitochondrial damage can alter the capacity of the cell to generate energy, redox signalling 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 to changes in NO concentration than controls [30]. ROS generated in the mitochondrial respiratory chain have been proposed as intermediate, secondary messengers to the activation of NF-κB [31]. Hence, the relative balance between the stimuli of mitochondrial ROS production and the concomitant accumulation of organelle damage may 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 of antioxidant defenses leads to extensive oxidative damage to mitochondria [32]. As mitochondrial oxidative damage is either a primary cause or a significant secondary factor of cell damage and death, a general therapy for decreasing mitochondrial oxidative damage should be effective in a range of clinical situations [3]. 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, can refer to an array of compounds with varying mechanisms of action. Halliwell [33] 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". Exposure to ROS from a variety of sources prompts organisms to develop a series of defence mechanisms [34]. Defence mechanisms against free radical-induced oxidative stress involve (a) preventative mechanisms, (b) repair mechanisms, (c) physical defences and (d) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Nonenzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids and other antioxidants [3].

Antioxidants are central to the redox balance in the human body. They do not act in isolation, but synergistically. Primary endogenous antioxidant defences prevent ROS formation, either by removing ROS precursors or by inhibiting catalysts such as GPx and CAT. Secondary antioxidants react with ROS that have already formed - for example, vitamins C and E - either removing or inhibiting them [3].

One of the most important antioxidants is GSH, the major thiol antioxidant and redox buffer of the cell. This highly abundant substance is the major soluble antioxidant in these cell compartments. As GSH is synthesized in the cytosol by the sequential action of glutamate–cysteine ligase and glutathione synthetase, its mitochondrial presence requires inner membrane transport. Two mitochondrial electroneutral antiport carrier proteins have been shown to transport GSH; these are the dicarboxylate carrier protein and the 2-

oxoglutarate carrier protein. Recently, it has been shown that externally added GSH is readily taken up by mitochondria [35].

GSH in the nucleus maintains the redox state of critical protein sulphydryls vital for DNA repair and expression. Oxidised GSH (GSSG) is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism. Too high a concentration of GSSG may produce oxidative damage in many enzymes. The following constitute the primary protective roles of GSH against oxidative stress [36]: (i) it is a cofactor of several detoxifying enzymes against oxidative stress, e.g. (GPx), glutathionetransferase and others; (ii) it participates in amino acid transport through the plasma membrane; (iii) it scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of GPx; (iv) it is capable of regenerating the most important antioxidants, Vitamins C and E, back to their active forms, reducing the tocopherol radical of Vitamin E directly or indirectly *via* reduction of semidehydroascorbate to ascorbate. The capacity of GSH for regenerating the most important antioxidants is linked with the redox state of the GSH disulphide-glutathione couple (GSSG/2GSH) [37]. The various roles of enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidants (Vitamin C, Vitamin E, carotenoids, lipoic acid and others) in protection against oxidative stress are related in numerous reviews and original papers [3].

Herein, we briefly describe the role of vitamin E (α-tocopherol), an important lipid-soluble antioxidant that defends the integrity of long-chain polyunsaturated fatty acids in the membranes of the cell, thus maintaining their bioactivity [38]. Vitamin E possesses antiinflammatory properties, as stimulated macrophages and epithelial cells treated with this antioxidant *in vitro* display a decreased cyclooxygenase-2 activity and lower levels of PGE2 synthesis [39]. In relation to transcription factors, there are 2 types of nuclear receptors that respond to modulation by vitamin E: pregnane X receptor (PXR) and the peroxisome proliferator-activated receptors (PPARs).

Mechanisms of antioxidant action are best understood in the context of their clinical and biochemical effects on ROS [3,9]. Three processes are involved in the formation of lipid-derived ROS: initiation, propagation and termination. Chain-breaking antioxidants interrupt this process by preventing or terminating propagation on the radical chain. α-tocopherol is a classic example of a chain-breaking antioxidant. The antioxidant enzyme SOD catalyses the conversion of O_2^- to H_2O_2, the latter of which is 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 the OH.

In summary, increasing the antioxidant capacity of the mitochondrial compartment represents a potential therapy, but its pharmaceutical viabilty depends on it being a small-molecule antioxidant [40]. However, small-molecule antioxidants spread all over the body, with only a small fraction being consumed by the mitochondria. This occurs in the case of the antioxidant vitamin E, which produces no benefits in the treatment of Parkinson's diseases [41]. An explanation for this disappointing result may be a combination of poor uptake into the body and limited delivery to the mitochondria [40,42].

Mitochondrial Antioxidant Defences

Mitochondria may limit the effects of ROS *via* small molecular antioxidants, including GSH, ascorbic acid and vitamin E [3]. The antioxidant enzyme MnSOD converts O_2^- 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.

MITOCHONDRIA-TARGETED ANTIOXIDANTS

Mitochondria have an important role in cell signalling, contributing to both adaptation to oxidative stress and the development of cardiovascular conditions such as ischemia-reperfusion injury, neurodegenerative diseases, diabetes and atherosclerosis [43]. However, available antioxidants are not 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 traditionally been considered to be a major source of damaging free radicals in ischemic-reperfusion brain injury, including hypoxia–ischemia [44]. That said, there is considerable uncertainty about the nature and significance of mitochondrially derived oxygen free radicals in cell dysfunction and death [45]. Since oxygen free radicals do not easily cross biological membranes, they must be detoxified primarily in the compartment within which they are produced [46].

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 for targeting molecules with therapeutic potential [47,48].

One approach to addressing these challenges is to target antioxidants to mitochondria by conjugation to a lipophilic cation, such as triphenylphosphonium (TPP) [11,49]. This procedure leads to orally bioavailable molecules, which accumulate in the cell, are driven by the plasma membrane potential and accumulate further inside 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 foremost barrier to the delivery of drugs 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 delivers of 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 as the activation energy for movement of lipophilic cations through the hydrophobic barrier of a biological membrane is far lower than for other cations [40].

Hence, by attaching a compound to a lipophilic cation, it is possible to deliver it selectively to mitochondria within cells [11]. 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* [50]. It has been shown that the TPMP cation is taken up into energized mitochondria within the perfused heart [51], liver [52] and skeletal muscle [53].

Mitochondria are a site of significant oxidative damage in clinical circumstances [54], and several antioxidants, such as vitamin E, ubiquinol, N-acetyl cysteine, SOD mimetics and spin traps, have been shown to protect it against such damage [54,55]. Therefore, targeting an antioxidant to the mitochondrial matrix should increase its protection against oxidative damage [56]. By covalently attaching a TPP cation to the antioxidant tocopherol moiety of Vitamin E, the antioxidant is delivered selectively to mitochondria [56] and protects the mitochondria from oxidative damage more effectively than vitamin E (α-tocopherol) itself. Importantly, TPP-derived compounds are orally bioavailable to mice when fed TPMP, MitoE2, or MitoQ *via* their drinking water, as uptake into the plasma and from there into the heart, brain, liver, kidney and muscle is consequentially observed [57].

TPP-Based, Mitochondrially Targeted Antioxidants: MitoE$_2$

A wide range of antioxidants could be targeted to mitochondria by conjugation to the TPP moiety (Fig. **1**). Antioxidants have the potential to block oxidative damage and redox signalling, and exogenous ubiquinones have been widely used for this purpose in mitochondrial studies. There are many forms of mitochondrial oxidative stress, of which lipid peroxidation is one of the most important. For this reason, until now studies have focused mainly on antioxidants that are effective against lipid peroxidation.

MitoVitE was the first mitochondria-targeted antioxidant to be identified, and consists of the α-tocopherol moiety of vitamin E to a TPP cation by a 2-carbon chain. The α-tocopherol moiety is an effective chain-breaking antioxidant that prevents lipid peroxidation, with the resulting α-tocopheroxyl radical then being recycled by the endogenous mitochondrial coenzyme Q pool [58]. Studies performed to determine the bioavailability of MitoVitE have shown that it accumulates in the brain when orally administered [59]. In addition, the cationic moiety of mitoVit E crosses the blood-placenta barrier of pregnant and is delivered to foetuses [60]. MitoVitE appears to promote cell survival at lower concentrations than vit E and scavenges endogenous oxidants more effectively [44]. Furthermore, Siler et al have described that MitoVitE protects GSHPx/GSSG functions [59].

It is well known that TPP and TPMP are taken up into cells through the plasma membrane [61]. The uptake by MitoQ10 is faster than that of TPMP, and rapid cell subfractionation in 143B cells indicates that at least 90% of MitoE2 is situated in the mitochondria [62].

MITOCHONDRIAL DAMAGE AND CARDIOVASCULAR DISEASES

CVD-coronary artery disease (CAD), hypertension and stroke are the leading causes of death and disability in the developed world [63]. An early prognosis and improved therapies for preventing and curing these diseases depends on an understanding of the basic pathophysiologic mecha-
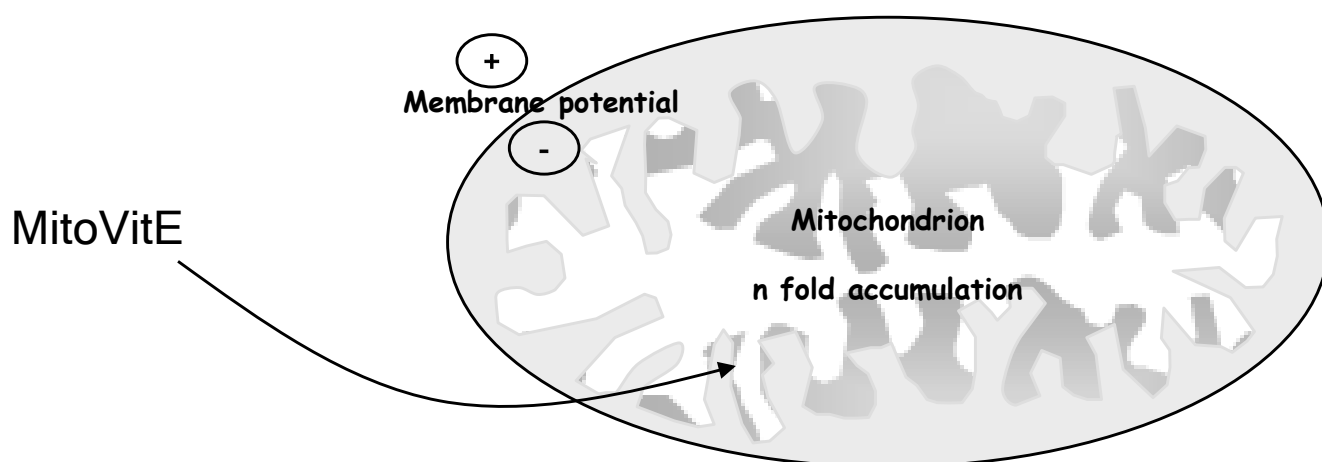

Fig (1). Using lipophilic cations to target compounds to mitochondria, enables reagents to be delivered selectively to mitochondria within cells. Lipophilic cations accumulate within the mitochondria driven by the membrane potential, and can pass through lipid bilayers easily as their positive charge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

nisms of CVD. The oxidative hypothesis for atherosclerosis has been critical in the obtaining of knowledge about the molecular mechanism of the disease. In fact, ROS influence many physiological processes, including host defence, hormone biosynthesis, fertilization and cellular signaling. Oxidative stress has been implicated in various pathologies, including hypertension, atherosclerosis and diabetes.

Vascular pathologies are multifactorial, but it is clear that oxidative stress and mitochondrial dysfunction contribute to their pathophysiology. These processes appear to involve not only damage to the organelle and loss of bioenergetic function but also disruption of mitochondrial-dependent redox signalling pathways.

It has been shown that CVD are associated with a deterioration of mitochondrial energy production [64]. Moreover, in experimental hypertension, mitochondrial energy deficiency and a decreased activity of Complex IV have been observed in the hypertrophied myocardium [65] with an associated decrease in ATP production [66].

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 [67]. 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 [68].

Because almost all of the mtDNA codifies specific proteins of the electron transport chain, it is plausible that any mutations or deletions in mtDNA can result in decreased energy production and additional generation of ROS, thus enhancing the cellular signals that initiate hypertension, atherosclerosis, apoptosis and necrosis [69]. In this way, the role of intramitochondrially 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 [70]. 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 [71]. A lower level of vascular SOD2-specific activity has been associated with increased exposure to several risk factors [67]. 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 [72]. Likewise, overexpression of mitochondrial antioxidants and/or UCPs has been shown to protect against the effects of ischemia/reperfusion and oxidative stress [71].

CVD Risk Factors also Cause Mitochondrial Damage and Dysfunction

There is increasing evidence that damage to the vascular environment due to oxidative stress plays a major role in the pathogenesis of atherosclerosis, in addition to classical risk factors such as age, arterial hypertension, diabetes, dyslipidemia, smoking, vascular wall inflammation and genetic predisposition. Accumulating evidence indicates that oxidative stress is crucial to the initiation and progression of CVD [73]. In addition, it is surely more than a coincidence that several of the aforementioned factors also appear to cause cardiovascular mitochondrial damage and/or dysfunction [74].

Atherosclerosis

Increased production of ROS in mitochondria, accumulation of mitochondrial DNA damage and progressive respiratory chain dysfunction are associated with atherosclerosis or cardiomyopathy in human and animal models of oxidative stress. Atherosclerosis, the primary cause of 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.

Atherosclerotic vascular disease and its clinical sequelae are the leading causes of morbidity and mortality in the developed world. An elevated level of low-density lipoprotein (LDL) is associated with an increased risk of CAD. Recent reviews highlight the role of an activated endothelium and inflammatory cell recruitment in the initiation of and progression of early atherosclerosis. 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 while Ox-LDL can damage endothelial cells, thereby inducing the expression of adhesion molecules [75]. ROS limit the bioavailability of NO and induce inflammatory gene expression, cell growth/apoptosis, migration and matrix reorganization, all of which are mechanisms that are central to the initiation and progression of atherosclerosis. NO signalling 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 [76]. MtDNA damage not only correlates with the extent of atherosclerotic lesions in apolipoprotein E (apoE) knockout mice but precedes atherogenesis in young apoE knockout mice. Mitochondrial dysfunction results from manganese superoxide dismutase (SOD2) deficiency, increased mtDNA damage and accelerated atherosclerosis in apoE knockout mice, which is consistent with the notion that increased ROS production and DNA damage in mitochondria are early events in the initiation of atherosclerosis. Multivariate analysis reveals that DNA-adduct levels are a significant predictor of the stage of atherosclerosis, even after adjustment for age, smoking, obesity and other CVD risk factors [77]. Evidence suggests that

there are more DNA repair mechanisms in the atherosclerotic plaques than in control tissues [78]. In accordance with these findings, increased levels of mtDNA damage have been observed in the cardiovascular tissue of CVD patients [68]. However, whether this damage is an effect or initiator of CVD remains unclear. Animal studies have shown that vascular mtDNA damage is more pronounced in animal models of atherosclerosis, and that this damage occurs prior to, or simultaneously with, the development of the disease [68].

Defects in oxidative phosphorylation in heart mitochondria have been identified in strains of pigeons susceptible to atherosclerosis, in which lipid biosynthesis was enhanced by the dissociation of NADH transhydrogenation from ATP regulation [28].

Ballinger *et al*. 2002 [67] demonstrated that oxidative mitochondrial DNA damage correlated positively with the extent of atherosclerotic lesions in arteries from human and apoE knockout mice, and that this damage preceded the onset of the disease in these mice. Indeed, LDL receptor knockout cells are more exposed to oxidative stress, and are more susceptible to cell death due to an undermined mitochondrial antioxidant defence system and a higher susceptibility to mitochondrial pore transition. Thus, the LDL receptor defect leads to two important pro-atherogenic consequences that sometimes manifest themselves prior to the intiation of the disease; 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 with SOD2 mutant ($SOD2^{-/-}$) mice, inhibition of mitochondrial complex I and complex II, and inactivation of redox-sensitive enzymes such as aconitase, accompanied by accumulation of oxidative mitochondrial DNA damage, were observed. These studies highlight the value of mitochondrial-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.* [79] have shown that mitochondria from atherosclerosis-prone, hypercholesterolemic LDL receptor knockout mice have an oxidative phosphorylation efficiency similar to that of control mice, while both their net production of ROS and susceptibility to developing membrane permeability transition are higher.

Oxidized lipids are capable of initiating diverse cellular responses through both receptor-mediated mechanisms and direct post-translational modification of proteins. In fact, incubation of endothelial cells with concentrations of oxLDL characteristic of cytoprotection induces a mitochondrial complex I activity that seems to depend on the induction of oxidative stress [80-83]. 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 [84]. 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 remodelling and regression which endorse mitochondrially-targeted antioxidants as a therapeutic tool in the control of hypercholesterolemia and, therefore, atherosclerosis [3].

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 elevated levels of bax [74]. While SOD2 activity and GSH concentration are significantly higher in the atherosclerotic intima than in the media of the aorta of hyperlipidemic rabbits, both are found to be inversely related to age and plaque size, suggesting that they are directly correlated and related to the early stages of atherosclerotic lesion formation [85].

High-fat diets reduce the expression of genes involved in free radical scavenging (SOD1, GPX, and SOD2) and increase the expression 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 [86].

Oxidative stress results in lipid peroxidation, damage of mitochondrial components (including mitochondrial DNA), mitochondrial dysfunction, damage of endothelial cells, vascular smooth muscle cells and erythrocytes, and, ultimately, apoptosis *via* either the receptor-mediated pathway or the mitochondria-mediated pathway and activation of the caspase cascade. Apoptosis can be induced by a number of different stress factors in the cardiovascular system and has been implicated in several chronic disorders, including atherosclerosis [87].

Diabetes

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes. It is now widely accepted, given the current weight of experimental evidence, that ROS contribute to cell and tissue dysfunction and damage caused by glucolipotoxicity in diabetes. The source of ROS in the insulin-secreting pancreatic beta-cells and in the cells that are targets for insulin action is thought to be the mitochondrial electron transport chain [88]. 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^- generation in endothelial cells *in vitro*, and studies suggest that the majority of this O_2^- is produced by mitochondria [88], with some contribution from NADPH oxidase. In this respect, it is 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^- and a higher activity of antioxidant enzymes. These factors also prevent glucose-induced activation of phosphokinase C and NF-κB activation in endothelial cells [89]. Similarly, overexpression of SOD2 significantly reduces IL-1, TNFα, IFNγ activation of NF-κB

and the induction of iNOS in insulin-producing cells [90]. In this sense, some studies have addressed the role of mitochondrial ROS and oxidative damage in TNF-induced apoptosis using mitochondria-targeted derivatives of vitamin E (MitoVitE), ubiquinol (MitoQ) and PBN (MitoPBN) [44]. 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 signalling [44]. These results confirm the role of mitochondrial ROS in said processes, and demonstrate that mitochondria-targeted antioxidants are useful tools for determining the role of mitochondrial ROS in signal transduction.

As oxidative damage forms part of the pathophysiology of diabetes, there is interest in determining whether or not antioxidants reduce this damage [91]. Too few large-scale, double-blind trials of antioxidants in diabetes have been carried out in order to draw any reliable conclusions [91]. 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 [92]. These natural antioxidants can be administered at high doses and have shown some efficacy in other degenerative diseases, which provides the basis for testing them in diabetes [42], though it must be said that the uptake and distribution to tissues of hydrophobic natural antioxidants such as Coenzyme Q is often poor [93]. In addition, many other artificial antioxidants, such as mimetics of SOD or peroxidase, are presently under development. They may be more potent than natural antioxidants and possess an improved bioavailability, pharmacokinetics and stability [94]. 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 seems 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. Most interestingly, the compounds in question would seem to prevent cell death in fibroblasts from Friedich Ataxia patients. As cell death in this model is caused by endogenous mitochondrial oxidative damage [95], 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 mitochondria-targeted molecules are to be effective in the treatment of diabetes, it is essential that they are taken up selectively by mitochondria *in vivo*. Given that alkylTPP cations pass easily through lipd bilayers by means of non-carrier-mediated 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 [42]. Various studies in mice fed for several weeks with mitochondria-targeted antioxidants have shown stable steady-state concentrations in all the tissues assessed, including brain, heart, liver and kidneys [96]. These data are consistent with the pharmacokinetic model by which, following absorption from the gut into the bloodstream, orally administered mitochondria-targeted antioxidants are taken up into all the tissues via a non-mediated movement through the lipid bilayer of the plasma membrane, during which they 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 becomes a steady-state distribution with circulating blood levels. At this point, the mitochondrial concentration is several hundred-fold higher than that in the bloodstream [11]. Once feeding ceases, due to the dynamic equilibrium of the mitochondrial pool, the accumulated cations re-equilibrate back into the bloodstream and are excreted relatively rapidly [11].

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 [97]. As these compounds are further accumulated into cells, similar protective effects are found when cultured cells are incubated with 500 nmol/L to 1 μmol/L mitochondria-targeted antioxidants [11]. In this way, oral delivery of well-tolerated doses of mitochondrial-targeted antioxidants 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.

CONCLUSIONS AND PERSPECTIVES

Based on experimental evidence and clinical studies, oxidative stress is thought to be involved in the pathogenesis of CVD. Mitochondria may be both a source and target of ROS, and damage to mtDNA induced by ROS has emerged as an important etiological factor in a number of CVD.

Accumulated knowledge regarding these mechanisms has led to the development of a range of strategies for developing mitochondrially-targeted antioxidants that prevent ROS-induced mitochondrial oxidative damage. Mitochondrially-targeted antioxidants represent a potential therapy for the many diseases that involve mitochondrial oxidative damage. Research over the coming years will no doubt indicate in which organs these compounds are effective, whether or not they reduce mitochondrial oxidative damage in diseases, and whether or not such as effect ultimately leads to a positive outcome for the patient.

MitoVitE has proved to be active in different models and is succesfully 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. Finally, there is considerable scope for finely tuning the chemical biology of these compounds in order to target specific ROS and other mitochondrial genes.

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