Systems biology of antioxidants

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ABSTRACT

Understanding the role of oxidative injury will allow for therapy with agents that scavenge ROS (reactive oxygen species) and antioxidants in the management of several diseases related to free radical damage. The majority of free radicals are generated by mitochondria as a consequence of the mitochondrial cycle, whereas free radical accumulation is limited by the action of a variety of antioxidant processes that reside in every cell. In the present review, we provide an overview of the mitochondrial generation of ROS and discuss the role of ROS in the regulation of endothelial and adipocyte function. Moreover, we also discuss recent findings on the role of ROS in sepsis, cerebral ataxia and stroke. These results provide avenues for the therapeutic potential of antioxidants in a variety of diseases.

INTRODUCTION

ROS (reactive oxygen species), such as O$_2^-$ (superoxide anion) and H$_2$O$_2$, are traditionally thought to be toxic by-products of cellular metabolism and pathogenic agents of many diseases and aging. These molecules react with other cell molecules, such as lipids, proteins and nucleic acids, leading to alterations in a variety of metabolic

Key words: antioxidant, ataxia, diabetes, free radical, mitochondrion, sepsis, stroke.

Abbreviations: ADCA, autosomal-dominant cerebellar ataxia; AGE, advanced glycation end-product; AngII, angiotensin II; BH$_4$, tetrahydrobipterin; C/EBP, CCAAT/enhancer-binding protein; CHOP-10, C/EBP-homologous protein-10; CoQ, co-enzyme Q; DHAA, dehydroascorbic acid; eNOS, endothelial NO synthase; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; GPDH, glycerol-3-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, glutathione; HIF-1, hypoxia-inducing factor-1; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible NO synthase; JNK, c-Jun N-terminal kinase; α-KGDH, α-ketoglutarate dehydrogenase; LPS, lipopolysaccharide; MDA, malondialdehyde; MitoQ10, mitochondrial-targeted co-enzyme Q; MPTP, mitochondrial permeability transition pore; NEFA, non-esterified ‘free’ fatty acid; NF-κB, nuclear factor-κB; 3-NO$_2$-Tyr, 3-nitrotyrosine; O$_2^-$, superoxide anion; ONOO$^-$, peroxynitrite; PAI-1, plasminogen activator inhibitor-1; PARP, poly(ADP-ribose) polymerase; PDH, pyruvate dehydrogenase; PG1$_2$, prostacyclin; PKC, protein kinase C; PP2A, serine/threonine protein phosphatase 2A; PUFA, polyunsaturated fatty acid; QUH$_2$, ubiquinol; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCA, spinocerebellar ataxia; SHR, spontaneously hypertensive rats; SHR-SP, stroke-prone SHR; SOD, superoxide dismutase; Cu/ZnSOD, copper/zinc SOD; Mn-SOD, manganese SOD; SP1, stimulating protein 1; SVCT2, Na$^+$-dependent vitamin C transporter 2; TAC, total antioxidant capacity; TGF-β1, transforming growth factor-β1; TNFα, tumour necrosis factor α; TPP$^+$, triphenylphosphonium cation; α-TTP, α-tocopherol transfer protein; Trx, thioredoxin; UCP, uncoupling protein.

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pathways and abnormal function of cellular structures and macromolecules. Although low-to-moderate levels of ROS have physiological roles as signalling molecules in various cellular and developmental processes, they are better known for their harmful effects. Different cellular sources of ROS, such as NADPH oxidases, participate in cell signalling, with the mitochondrial production of ROS being a tightly controlled process involved in redox signalling. Several clinical studies have demonstrated an association between redox imbalance and both cardiovascular and metabolic diseases [1].

ROS are transient, unstable and largely localized to cellular compartments, making their direct measurement difficult. As a result, the role of free radicals in pathological conditions has been inferred from the measurement of indirect markers of oxidative stress, such as lipid oxidation and the activities of free-radical-scavenging enzymes. Proteins exposed to ROS exhibit altered structures, undergo spontaneous fragmentation, and manifest increased proteolytic susceptibility and loss of function.

Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways, including removal of O₂, scavenging ROS or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation. The natural antioxidant system can be classified into two major groups: enzymatic and non-enzymatic antioxidants.

The non-enzymatic antioxidants can be classified further into directly acting antioxidants (e.g. scavengers and chain-breaking antioxidants) and indirectly acting antioxidants (e.g. chelating agents). The former serve important roles in defence mechanisms against oxidative stress. This subgroup currently contains several hundred compounds. Most of them, including ascorbic and lipoic acids, polyphenols and carotenoids, are derived from dietary sources. The cell itself synthesizes a minority of these molecules, such as GSH (glutathione) and NADPH.

Antioxidant vitamins are some of the main defence mechanisms of the body’s non-enzymatic antioxidant systems. Ascorbic acid (vitamin C), α-tocopherol (the principal constituent of vitamin E) and β-carotene (provitamin A) are the best-studied natural antioxidants. Large-scale clinical trials have been conducted to determine whether vitamin supplements with antioxidant action decrease the risk of cardiovascular diseases. Some experimental and epidemiological studies have indicated the beneficial effects of antioxidant vitamin supplementation on the development and progression of atherosclerotic plaques, resulting in a reduction of cardiovascular events. However, a recent study with well-defined primary and secondary prevention endpoints does not support this hypothesis [2].

Vitamin A, the first fat-soluble vitamin to be identified, has three active forms: retinol, retinal and retinoic acid; collectively called retinoid. The carotenoids have an important antioxidant role in quenching free radical reactions, particularly those involving singlet oxygen. This prevents damaging chain reactions that cause lipid peroxidation and damage to DNA, both of which are postulated precursors of atherosclerotic processes. The most important of these is β-carotene, which has a high antioxidant effect.

Vitamin C as an electron donor is a potent water-soluble antioxidant in humans and is among the most abundant antioxidants. Dietary vitamin C sources are largely from fresh fruits and vegetables. Its biological role is related to its reducing capability; ascorbate is readily reduced to DHAA (dehydroascorbic acid).

Vitamin E is among the most important fat-soluble antioxidants, playing an essential protective role against free radical damage. The form that accounts for 90% of the vitamin activity in tissues is α-tocopherol. The chemical structure of tocopherols and tocotrienols (which have a hydroxy group on the ring structure) makes them effective hydrogen donors. In donating hydrogen, vitamin E becomes oxidized, preventing the oxidation of metabolically more important substances, for example PUFA (polyunsaturated fatty acid) in cell membranes. This is important when free radicals are present, as these highly reactive substances can attack double bonds, initiating chain reactions that generate more free radicals. When fatty acids are damaged, lipid peroxides are produced that can alter the function of the cell membrane and cause irreversible damage to metabolic pathways.

**MITOCHONDRIAL ROS AND ITS REGULATION**

It is estimated that between 0.15 and 2% of cellular O₂ consumption results in O₂⁻ formation [3]. The role played by mitochondrial ROS is of importance, although our knowledge about the site of production, the regulation of ‘good’ compared with the ‘bad’ ROS and their targets are far from complete. Although endothelial cells and adipocytes are not especially rich in mitochondria, they are both nonetheless linked to oxidative stress when they are stressed.

Mitochondria are present in every eukaryotic cell (from hundreds to thousands) with the exception of peripheral red blood cells. Although mitochondria have a limited role in energy production in vascular endothelial cells, ROS originating from mitochondria are thought to be important in physiological and pathological processes. Similarly, mitochondrial ROS in adipocytes modulates adipocyte differentiation and function. It is believed that human cells can produce ~10 million ROS/mitochondrion per day. Thus an understanding of the cellular and molecular mechanisms will facilitate the development of therapeutic interventions targeting mitochondrial ROS.
A variety of cellular systems, including NADPH oxidase, xanthine oxidase, uncoupled cNOS (endothelial NO synthase) and cytochrome P450 enzymes, can generate ROS, but, in most mammalian cells, mitochondria are the principal organelles for ROS production. Mitochondrial ROS are produced during oxidative metabolism through the one-electron reduction of O$_2$ to form O$_2^-$; the mitochondrial electron transport chain generates O$_2^-$ predominantly at complexes I and III [4] (Figure 1).

### Production of ROS from complex I
The largest protein complex of mitochondrial electron transport is NADH:ubiquinone oxidoreductase or complex I, which couples the oxidation of NADH to the reduction of ubiquinone [5]. During this process, electrons escape from complex I and react with ambient O$_2$ to produce O$_2^-$, which is released only in the mitochondrial matrix. The presence of two separate sites of O$_2^-$ production in complex I is generally accepted. The first is the non-covalent-bound flavin mononucleotide [6], and O$_2^-$ is formed by the transfer of one electron from fully reduced flavin to O$_2$ and depends on the NADH/NAD$^+$ ratio. Thus, when NADH concentrations increase, such as during ischaemia, damage to the mitochondrial electron transport or complex I deficit leads to ROS formation [7].

The second site of O$_2^-$ production is the quinone-binding site in complex I [8]. The electron chain operates at near-equilibrium [9], and the association of both a high proton-driving force and a reduced CoQ (coenzyme Q) pool forces the return of electrons from QH$_2$ (ubiquinol) into complex I. During this reverse electron transfer, the majority of ROS originates from the quinone-binding site whereas its contribution is negligible during forward electron transport. However, the physiological importance of O$_2^-$ generation by reverse electron transfer in vivo is unclear.

### Production of ROS from complex III
QH$_2$: cytochrome c oxidoreductase or complex III couples the oxidation of QH$_2$ to the reduction of cytochrome c. Because cytochrome c is only capable of reduction by a single electron, the electron flux occurs through a bifurcated process called the Q-cycle [10], which involves semi-ubiquinone intermediates and two quinone-binding sites Qo and Q$_i$. The generation of ROS in complex III results from the reaction of O$_2$ with the Qo site and semi-ubiquinone, and other conditions that stabilize the O$_2$ molecule in the Qo site increases O$_2^-$ production [11]. The production of ROS by complex III is probably lower than that...
by complex I under physiological conditions. However, during ischaemia/reperfusion conditions, several factors including the loss of cytochrome c and changes in the redox state of CoQ or cytochrome c pool occur.

**Others sites of ROS production in mitochondria**

Several observations have implicated other sites within mitochondria as capable of producing ROS. In the tricarboxylic acid cycle, α-KGDH (α-ketoglutarate dehydrogenase) catalyses the oxidation of α-ketoglutarate in succinyl-CoA to produce NADH. One component of this enzyme, the dihydrolipoamide dehydrogenase (E3), contains a flavin that can produce ROS when the concentrations of the NADH+ are decreased. Because the E3 subunit is also common to the PDH (pyruvate dehydrogenase) complex, this enzyme can also generate ROS [12]. Moreover, in the β-oxidation pathway, fatty acid oxidation reduces the electron transfer protein flavoprotein, which transfers its electrons to the ubiquinone pool via the electron transfer protein flavoprotein CoQ oxidoreductase present in the inner mitochondrial membrane. This enzyme as well as others connected to the CoQ pool [such as the GPDH (glycerol-3-phosphate dehydrogenase) or DHODH (dihydrolipoamide dehydrogenase)] can also generate ROS [13]. Although the physiological consequences and the quantitative importance of this production of ROS have not been established, the link between ROS generation and fatty acid metabolism probably has physiological relevance.

In addition to the sites discussed above, electron transfer between cytochrome c and the adaptor protein p66shc can generate H2O2 in mitochondria under conditions of cellular stress. This production of H2O2 was originally described as a factor promoting the apoptotic response [14] and is now also implicated in the pathophysiological mechanisms linking oxidative stress, insulin resistance and cardiovascular diseases [15].

**ROS production**

Excessive ROS production from mitochondria can diffuse into the cytosol and react with other free radicals including NO, a ubiquitous intracellular messenger that regulates many aspects of neural and cardiovascular activities. Although low concentrations of NO and O2− are not toxic in a physiological environment, an imbalance between the production of these two radicals can be partially responsible for alterations in the molecular mechanisms regulating cell life. NO and O2− react by enzyme-independent mechanisms to form ONOO− (peroxynitrite), a strong oxidant that reacts with most biological molecules to cause cell damage. ONOO− affects the activity of several proteins and enzymes through post-translational modifications, including tyrosine nitration, which is a two-step process in which the first reaction is the generation of tyrosyl radicals by oxidation of tyrosine residues by reactive species generated from ONOO−. In the second step, tyrosyl radicals react with NO2 to form 3-NO2-Tyr (3-nitrotyrosine). Tyrosine nitration can alter protein activity, and increased circulating levels of 3-NO2-Tyr occur in cardiovascular diseases such as coronary artery disease [16]. In addition to tyrosine nitration, ONOO− participates in hydroxylation and oxidation reactions [17,18].

**Regulation of mitochondrial ROS production**

Mitochondrial production of ROS is largely determined by the concentration of reduced electron donors in the respiratory chain, the O2 concentration [19] and by the accessibility to O2 of the electron donor. Modification of any of these parameters has a profound impact on ROS production. The redox level of the electron transport chain is thermokinetically controlled and the forces associated with respiratory chain activity (the NADH/NAD+ ratio and the proton-driving force) are powerful regulators of the proportion of the electron carriers present in a redox form.

**Dependence of ROS production on membrane potential**

The strong dependence of mitochondrial ROS production on the membrane potential, the major component of the proton-driving force, is illustrated by substantial decreases in ROS production when the membrane potential is dissipated by chemical uncouplers [20]. All modes of ROS production are sensitive to uncoupling, and this is particularly the case for ROS produced during reverse electron transport. The membrane potential can be dissipated under physiological conditions by mitochondrial UCPs (uncoupling proteins), a family of anion carriers present in the inner mitochondrial membrane [21]. One function of these proteins is to modulate ROS production by two possible means: UCP activity that can be regulated either by endogenous matrix O2− [22] or by products of lipid peroxidation [23].

**Dependence of ROS production on NADH/NAD+ ratio**

A high NADH/NAD+ ratio greatly influences mitochondrial ROS formation. As discussed below, this ratio regulates the production of ROS by both complex I and α-KGDH by means that are not strictly coupled to a high membrane potential. Thus the rate of mitochondrial NADH oxidation can influence ROS production in mitochondrial chain disorders such as deficits in complex I [24]. The cellular NADH concentration is modulated by several factors, including the need of ATP and the rate...
of electron transport in the respiratory chain. Resting mitochondria (not making ATP) are characterized by low rates of O₂ consumption, high levels of membrane potential and reduced electron carriers leading to high ROS production. When mitochondria are synthesizing ATP, both the NADH/NAD⁺ ratio and the membrane potential are lowered, resulting in lower ROS production. By regulating the rate of electron transport in the respiratory chain, endogenous modulators such as Ca²⁺ [25], ATP [26] and NO [27] also control this ratio. For example, reversible inhibition of cytochrome oxidase by NO leads to the accumulation of NADH and increases in ROS production. Unlike NO, a moderate increase in mitochondrial Ca²⁺ stimulates the rate of oxidative phosphorylation and thus tends to a decrease in ROS. Conversely, mitochondrial Ca²⁺ overload observed in pathological situations, such as ischaemia, stimulates intramitochondrial ROS production, but independently of the NADH concentration. Low levels of cytochrome c, secondary to its mitochondrial release induced by excess Ca²⁺, slows the electron transfer from complex III to complex IV and causes ROS generation mainly at the Q-cycle [28].

**Dependence of ROS production on the O₂ concentration**

The intramitochondrial O₂ availability is also an important parameter in regulating ROS production. Indeed, the in vitro production of ROS increases when the concentration of O₂ is raised above normal atmospheric levels. The effects of decreasing the O₂ concentration on ROS production are not completely understood. In particular, during hypoxia, some studies paradoxically report increased ROS production at the level of complex III [29]. Hypoxia-induced ROS production does not appear to be toxic for the cell [30], and may play a critical role in hypoxic signalling pathways even if their roles in stabilizing HIF-1 (hypoxia-inducing factor-1) are unclear [31].

**Antioxidant defence against mitochondrial ROS formation**

Mitochondrial and cell cytosolic antioxidant systems can neutralize excess mitochondrial ROS under most conditions. With the exception of generation at complex III, ROS production in mitochondria is exclusively directed towards the matrix where Mn-SOD (manganese SOD) hastily catalyses dismutation to H₂O₂ [32], which is then reduced to H₂O by catalase, GSH and Trx (thioredoxin) systems (Trx2) [33]. As the regeneration of GSH and reduced Trx2 depends on the NADPH/NADP⁺ redox state, an efficient mitochondrial bioenergetic function is required to maintain antioxidant activity. Complex III generates ROS on both sides of the mitochondrial inner membrane and in the intermembrane space, where Cu/Zn-SOD (copper/zinc SOD) converts O₂⁻ into H₂O₂, which diffuses in the cytosol. Thus the efflux of H₂O₂ from the mitochondria is relatively modest, but may be modulated by either mitochondrial ROS themselves or changes in antioxidant defences.

**MITOCHONDRIAL ROS AND ENDOTHELIAL CELL PATHOPHYSIOLOGY**

Vascular endothelial cells are highly glycolytic and, compared with other cells, consume relatively low amounts of O₂ [34]. However, even if the endothelial mitochondrial electron transport chain plays a limited role in energy production, it can be an important source of ROS in some pathologies. Enhanced formation of mitochondrial ROS restricts vasodilation, while, at the same time, also inhibiting other pathways essential in preserving normal endothelial function, for example during hyperglycaemia (Figure 2) and hypertension.

**Mitochondrial ROS and hyperglycaemia**

Increased mitochondrial ROS production during hyperglycaemia is central to the general pathology of diabetes [35]. Emerging evidence supports the hypothesis that some features of Type 2 diabetes are caused by mitochondrial dysfunction and ROS production. High glucose induces increased O₂⁻
generation in endothelial cells in vitro [36], with a major contribution from mitochondrial sources [37] and a smaller contribution from NADPH oxidase. Overexpression of the gene responsible for Mn-SOD activity in cultured endothelial cells decreases O$_2^-$ levels [37]. Increased glycolysis generates O$_2^-$ by enhancing the proton electrochemical gradient generated by the mitochondrial electron transport chain. Once generated, O$_2^-$ inhibits enzymatic activity of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), diverting fructose 6-phosphate into the hexosamine pathway. These events lead to enhanced SP1 (stimulating protein 1) transactivation, as well as SP1-dependent gene expression of mediators implicated in vascular risk associated with insulin resistance, such as TGF-$\beta$1 (transforming growth factor-$\beta$1) and PAI-1 (plasminogen activator inhibitor-1) [35]. Moreover, hyperglycaemia-induced GAPDH inhibition is a consequence of poly(ADP-ribosylation) of GAPDH by PARP (poly(ADP-ribose) polymerase), which is activated by DNA strand breaks produced by mitochondrial O$_2^-$ overproduction [38]. In addition, inhibition of GAPDH activity activates the pro-inflammatory transcription factor NF-$\kappa$B (nuclear factor-$\kappa$B), which, in endothelial cells, is PKC (protein kinase C)-dependent. When PKC is activated by incubation with high glucose, a variety of effects on gene expression are observed: eNOS synthase activity is decreased, resulting in decreased NO production [35], while there are increases in ET-1 (endothelin-1) production [39] and in the levels of TGF-$\beta$1 and PAI-1 [40]. Hyperglycaemia-induced activation of PKC (in vitro and in vivo) is prevented by UCP1 and Mn-SOD.

The adaptor protein p66Shc is also implicated in mitochondrial ROS generation and the translation of oxidative signals into apoptosis. Findings suggest that oxidative stress, apoptosis, endothelial dysfunction and vascular inflammation induced by high glucose are triggered by a PKC/p66Shc-dependent mechanism [14]. Glucose-induced activation of the $\beta$ isoform of PKC leads to Ser$^{36}$ phosphorylation of p66Shc, allowing transfer of the protein from the cytosol to the mitochondrion. In the mitochondrion, p66Shc binds to a complex that includes members of the TIM-TOM import complex. Pro-apoptotic stimuli destabilize the p66Shc–mtHsp70 (mitochondrial heat-shock protein 70) complex, leading to the release of p66Shc in its monomeric form. Once activated, p66Shc oxidizes cytochrome $c$ and catalyses the reduction of O$_2$ to H$_2$O$_2$. The latter induces the opening of the MPTP (mitochondrial permeability transition pore), with the subsequent increase in the mitochondrial membrane permeability to ions, solutes and water, the swelling and disruption of the organelle, and consequent release of pro-apoptotic factors into the cytosol [14].

Finally, oxidative stress occurring during hyperglycaemia promotes ONOO$^-$ production and the consequent tyrosine nitrination of proteins [41–43]. Indeed, hyperglycaemia induces ONOO$^-$ production through a PKC-dependent activation of NADPH oxidase [41], and the subsequent nitrination and inhibition of PGI$_2$ (prostacyclin) synthase [42,43]. Further investigations have revealed that the nitrination occurred at Tyr$^{435}$ of PGI$_2$ synthase [44]. This PGI$_2$ synthase inhibition contributes to the PGI$_2$ deficiency and the increased vasoconstriction, endothelial cell apoptosis and inflammation observed under hyperglycaemic conditions.

**Mitochondrial ROS and hypertension**

Hypertension is associated with increased ROS production by vascular endothelial cells. The molecular mechanisms involved in AngII (angiotensin II)-mediated mitochondrial dysfunction are via increased mitochondrial H$_2$O$_2$ production and decreased mitochondrial membrane potential, respiratory control ratio and mitochondrial GSH. These deleterious effects of AngII on mitochondrial function are dependent on activation of vascular NADPH oxidases via PKC-dependent pathways. O$_2^-$ generated by NADPH oxidases can then directly activate the mitoK$_{ATP}$ (mitochondrial K$_{ATP}$) channels or react with NO to form ONOO$^-$, which can nitrate protein tyrosine residues and damage respiratory complexes, leading to mitochondrial dysfunction. Through a positive-feedback loop, increased mitochondrial H$_2$O$_2$ production can lead to the further activation of cellular NADPH oxidase, resulting in even more intracellular O$_2^-$ production and decreases in NO bioavailability [1]. BH$_4$ (tetrahydrobiopterin), an essential cofactor for the activity of eNOS, represents an easily modified target of ONOO$^-$; this oxidative modification converts BH$_4$ into biologically inactive BH$_2$ (dihydrobiopterin) to cause the ‘uncoupling’ of eNOS activity, with consequent O$_2^-$ and H$_2$O$_2$ formation instead of NO [45].

Moreover, transgenic mice overexpressing the mitochondrial antioxidant Trx2 are resistant to AngII-induced hypertension and endothelial dysfunction [46]. Scavenging mitochondrial O$_2^-$ (by either the mitochondrial-targeted SOD mimic Mito-TEMPO or by overexpression of Mn-SOD) inhibits oxidative stress and prevents endothelial NO deficiency caused by AngII, showing that mitochondrial O$_2^-$ stimulates extra-mitochondrial NADPH oxidase activity in a feed-forward manner [47]. The first phase of NADPH oxidase activation by AngII is followed by a sustained activation by H$_2$O$_2$ [48]; it is likely that mitochondria generate H$_2$O$_2$ since Mito-TEMPO (by inhibiting mitochondrial H$_2$O$_2$) reduces NADPH activity [47]. These findings suggest that mitochondrial ROS contributes to the development of hypertension and indicates the interplay between mitochondrial and extra-mitochondrial sources of ROS.
The regulation of adipocyte function by mitochondrial ROS

Diverse stimuli, such as hyperglycaemia, inflammation, electron transfer chain inhibitors or a decrease in the expression of UCPs, can induce the overproduction of mitochondrial ROS in adipocytes. An increase in ROS leads to an inhibition of adipocyte differentiation associated with the promotion of ectopic accumulation of fatty acids (FA), an alteration in pro-inflammatory cytokines (IL-6) and adipokines (increase in resistin and decrease of adiponectin) levels, as well as an insulin-resistance state which consequently enhance NEFA (FFA) release. These mechanisms participate in the development of peripheral insulin resistance, which is the major component of the metabolic syndrome and Type 2 diabetes.

MITochondrial ROS AND ADIPOCYTE PATHOPHYSIOLOGY

The role of mitochondria in adipocyte function is not well known, but several studies have suggested that mitochondrial dysfunction, particularly by overproduction of ROS, accompanies adipocyte function in diseases such as the metabolic syndrome, Type 2 diabetes and obesity. Many stimuli, such as hypoxia [49], hyperglycaemia [50], inflammation [51] or decreased expression of UCPs [52], can also increase mitochondrial ROS production. Here we consider the mechanisms associated with mitochondrial ROS alterations leading to adipocyte dysfunction (Figure 3).

Adipogenesis is a key feature of metabolic diseases. Carrière et al. [53] studied the role of mitochondrial ROS in the regulation of pre-adipocyte proliferation and differentiation, and demonstrated that an increase in mitochondrial ROS production caused by inhibition of the electron transport chain (complex I and V) prevented pre-adipocyte proliferation. Their study also indicated that increases in ROS production (by inhibition of complex III of the electron transport chain) reduced adipocyte differentiation by enhancing the expression of transcription factor CHOP-10 [C/EBP (CCAAT/enhancer-binding protein)-homologous protein-10], an inactive analogue of C/EBP proteins. Moreover, mitochondrial ROS may be at the origin of hypoxia-dependent inhibition of adipocyte differentiation [49].

In a cellular model of insulin resistance induced by TNFα (tumour necrosis factor α), Houssis et al. [51] analysed genome-wide expression in treated and untreated adipocytes. ROS-related genes were the highest scoring, highlighting the importance of ROS in insulin resistance. In addition, increased levels of ROS preceded the onset of detectable insulin resistance. As the expression of mitochondrial-targeted catalase abolished the insulin resistance induced by TNFα, it is likely that ROS was of mitochondrial origin. Moreover, ROS induces JNK (c-Jun N-terminal kinase) activation, which may be responsible for the decrease in insulin receptor substrate activity observed in insulin resistance. Other studies report that TNFα down-regulates UCP2 expression in adipocytes [54], resulting in increased mitochondrial ROS production that can activate the JNK pathway, phosphorylate IRS (insulin receptor substrate) and decrease insulin signalling [55]. Insulin resistance in adipocytes also results in excessive lipolysis, which contributes to the high circulating levels of NEFAs (non-esterified ‘free’ fatty acids) and the development of lipotoxicity and insulin resistance in peripheral tissues [56].

Adipose tissues secrete large amounts of adipokines, including, among others, cytokines, adiponectin, leptin and resistin. Hyperglycaemia increases mitochondrial ROS production and enhances inflammatory responses by the overproduction of IL (interleukin)-6 [50]. This cytokine can act at both a local (autocrine and/or paracrine) and systemic (endocrine) level, and participates in the development of insulin resistance. Recent studies have shown that mitochondrial O$_2^-$ overproduction induced by high glucose concentrations decreases the production of adiponectin and increases the production of resistin in cultured adipocytes, with CHOP-10 (probably the target of mitochondrial ROS) being responsible for the down-regulation of adiponectin [57]. UCPs are also involved in the regulation of adipokine expression, as shown by Chevillotte et al. [52] in UCP2-null mice, where circulating levels of adiponectin and its cellular expression were decreased in adipose tissue. Likewise, in cultured 3T3-L1 adipocytes, the decrease in mitochondrial ROS production by overexpression of UCP2 is associated with an increase in adiponectin mRNA levels and secretion. Taken together, these results show that mitochondrial-derived ROS may be a key signal in the deregulation of adipokine expression, an important component of peripheral insulin resistance.

UCPs are also involved in the regulation of lipid metabolism. Increased expression of UCP1 leads to decreased intracellular lipid content via a reduction in fat synthesis in adipocytes [58,59]. Other studies have found that mitochondrial uncoupling induced by FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone)
inhibits fatty acid synthase activity and increases \([\text{Ca}^{2+}]\), (intracellular \([\text{Ca}^{2+}]\)) and therefore lipolysis inhibition \([60]\). Consequently, under conditions that decrease UCPs expression, such as inflammation \([54]\), an increase in lipolysis and thus circulating NEFAs are responsible for ectopic deposition of fat in non-adipose tissue associated with insulin resistance.

It is important to also note that, despite the pathological role of mitochondrial ROS in adipocyte function, many studies have suggested that ROS has physiological roles at low-to-moderate concentrations. Insulin induces the production of mitochondrial-derived \(\text{H}_2\text{O}_2\), leading to inhibition of PTP1B (protein tyrosine phosphatase 1B) and sensitization of insulin signalling \([61]\). Another example is the recently reported requirement of mitochondrial complex III ROS for the activation of adipogenic transcriptional machinery in human mesenchymal stem cells undergoing differentiation into adipocytes \([61a]\).

TARGETING MITOCNDRIAL ROS THERAPY

Currently available antioxidants have limited usefulness in combating the complications associated with diabetes, the metabolic syndrome, aging, hypertension and other clinical conditions \([62]\). Although there may be several reasons that need to be considered, one probable suggestion is that these antioxidants may not be targeting the mitochondrial generation of free radicals. In the following section we describe several strategies to control mitochondrial ROS production.

The first step is to target the delivery of therapeutics to mitochondria. A number of approaches have been used to target molecules to mitochondria and these can be divided into two principal approaches: (i) mitochondrial-potential-dependent methods, such as \(\text{TPP}^+\) (triphenylphosphonium cation) conjugated to lipophilic antioxidants, for example mitochondrial-targeted co-enzyme Q (MitoQ\(_{10}\)), which, depending on their charge, allow lipophilic cations to accumulate within the mitochondrial matrix; (ii) mitochondrial-potential-independent methods, such as cell-permeant small peptides that selectively accumulate in the inner membrane and possess intrinsic mitoprotective activities \([63]\).

\(\text{CoQ}_{10}\) (co-enzyme \(\text{Q}_{10}\)), an endogenous compound found in the inner mitochondrial membrane, is essential to electron transport and ATP production via the respiratory chain. In addition to its role in bioenergetics, supplementation with \(\text{CoQ}_{10}\) inhibits thrombus formation and reduces ROS production. Both clinical and rodent studies have reported moderately beneficial actions of \(\text{CoQ}_{10}\) in reducing blood pressure, decreasing blood glucose, forestalling myocardial damage secondary to chemotherapeutic administration, limiting tumour growth, enhancing endothelial function and improving cognitive function in both Alzheimer’s and Parkinson’s disease patients \([64]\). However, one of the major limiting factors in the use of \(\text{CoQ}_{10}\) as a supplement is its bioavailability and delivery to the source of ROS generation. The first study using a mitochondrial-targeted lipophilic \(\text{TPP}^+\) covalently bound to \(\text{QH}_2\), MitoQ\(_{10}\), has been reported in an animal model of cardiac ischaemia/reperfusion injury \([65]\). In that study, MitoQ\(_{10}\) was given to rats in their drinking water for 2 weeks and the hearts were then isolated and exposed to ischaemia/reperfusion injury using a Langendorff perfusion system; under these conditions, MitoQ\(_{10}\) protected against heart dysfunction, tissue damage and mitochondrial function compared with methylTPP or short-chain quinol as independent controls of the two different functional groups in MitoQ\(_{10}\). In another study, MitoQ\(_{10}\) improved endothelial dysfunction and attenuated cardiac hypertrophy in SHR (spontaneously hypertensive rats) \([66]\). Several other studies have used MitoQ\(_{10}\) in a variety of animal models of disease \([67]\), and the results indicate that MitoQ\(_{10}\) protects against liver damage in an animal model of sepsis \([68]\), contributes to the aetiology of the metabolic syndrome and atherosclerosis in a mouse model \([69]\), protects pancreatic \(\beta\)-cells against oxidative stress and improves insulin secretion in glucotoxicity and glucolipotoxicity \([70]\), and even protects against oxidative stress and cell death in the brain of rats exposed to the insecticide dichlorvos \([71]\). Importantly, the first clinical evidence of a potential benefit of MitoQ\(_{10}\) in humans comes from a study that MitoQ\(_{10}\) reduces liver damage induced by hepatitis virus infection \([72]\).

Another strategy to target antioxidants to mitochondria comes from Szeto \([63]\), who designed mitochondrial-targeted small peptide molecules (‘SS peptides’) capable of scavenging \(\text{H}_2\text{O}_2\) and \(\text{ONOO}^-\) and inhibiting lipid peroxidation. By reducing mitochondrial ROS production, these molecules inhibit MPTP and cytochrome \(c\) release and so prevent oxidant-induced cell death \([73]\). Using these peptides in an animal model of ischaemia/reperfusion injury improves cardiac function \([74]\), whereas treatment with SS peptides attenuated mitochondrial \(\text{H}_2\text{O}_2\) release induced by a high-fat diet and preserved insulin sensitivity in skeletal muscle \([75]\). Importantly, pre-clinical studies support the use of these peptides during ischaemia/reperfusion injury and neurodegenerative disorders \([76]\).

Finally, another potent mitochondrial antioxidant is melatonin, a major secretory product of the pineal gland that is secreted each night. Besides its role in signalling of the nycthemeral process, melatonin also acts as an antioxidant and as a regulator of mitochondrial bioenergetic function. Melatonin is effective for preventing oxidative stress/nitrosative stress-induced mitochondrial dysfunction that is seen in
experimental models of neurodegenerative diseases. In addition, melatonin retards aging and inhibits the lethal effects of septic shock or ischaemia/reperfusion lesions through mechanisms that maintain respiratory complex activities, electron transport chain integrity and ATP production in mitochondria. Unlike MitoQ or SS peptides, melatonin is endogenously produced and selectively taken up by mitochondrial membranes, a function not shared by other antioxidants. Melatonin thus emerges as a potentially important therapeutic tool for treating neurodegenerative diseases and also for preventing the lethal effects of septic shock or ischaemia/reperfusion [77,78].

**Sepsis**

Sepsis syndromes are major causes of morbidity and death; this condition encompasses the systemic inflammatory response syndrome, sepsis (infection plus systemic manifestations of infections), severe sepsis (acute organ dysfunction secondary to infection) and septic shock (severe sepsis plus hypotension not reversed with fluid resuscitation) [79]. Oxidative stress is manifested in these patients and is caused by increased production of ROS and ONOO\(^-\); these can deplete antioxidant molecules and alter redox-sensitive signalling pathways [80]. Many symptoms are attributable to impairments in microvascular function that lead to plasma extravasation, tissue hypoxia and mitochondrial dysfunction which precipitate organ failure and shock [81]. Treatment mainstays are antibiotic therapy and source control to remove the sepsis-inducing insult, fluid resuscitation to normalize the mean circulating filling pressure, and vasopressor or combined inotropic–vasopressor therapy to prevent shock [79]. Some therapeutic measures used in critically ill patients are currently aimed at improving O\(_2\) delivery to parenchymal tissues, but may be ineffective due to the mitochondrial impairment condition known as cytopathic hypoxia [82]. Because of its characteristic decreases in ATP biosynthesis, energy generation and use, cytopathic hypoxia is a condition of cellular inability to use oxygen productively. However, as patients may benefit from adjuvant therapy that modulates oxidative stress, it is timely to discuss a potential therapeutic role for administration of the antioxidant ascorbate.

In patients with sepsis, pathogenic bacteria, bacterial products [e.g. LPS (lipopolysaccharide)] and inflammatory cytokines from the host [e.g. IFN-\(\gamma\) (interferon-\(\gamma\))] elicit a systemic inflammatory response that changes microvascular function [80]. The present review will focus on three components of microvascular dysfunction during sepsis that may be modified by ascorbate, namely maldistribution of capillary blood flow, decreased arteriolar reactivity to vasoconstrictors and increased microvascular permeability to macromolecules (i.e. endothelial barrier dysfunction).

First, a sepsis-inducing insult causes a maldistribution of capillary blood flow that impairs the microvascular perfusion of tissues [83]. The number of perfused capillaries decreases and the number of stopped-flow capillaries increases, which causes tissue hypoxia due to an increased diffusion distance for oxygen transfer from blood to parenchymal cells. In capillaries of mouse skeletal muscles during sepsis, the stoppage of blood flow arises from increased ROS production, platelet adhesion, fibrin deposition and a propensity for thrombosis in capillaries.

Remarkably, intravenous injection of ascorbate prevents and reverses the maldistribution of capillary blood flow [83]. Reversal of flow stoppage by ascorbate is mediated by eNOS. Ascorbate maintains the eNOS cofactor BH\(_4\) in its reduced form, which enables eNOS to synthesize NO that dislodges platelets from the capillary wall [84].

Secondly, a sepsis-inducing insult causes a loss of arteriolar responsiveness to vasoconstrictors (angiotensin, noradrenaline and vasopressin) and this hyporeactivity contributes to hypotension [85,86]. In microvascular endothelial cells, a sepsis-inducing insult increases the protein expression of the NOX1 and \(\text{p47}^{\text{phox}}\) subunits of NADPH oxidase, resulting in increased NADPH oxidase activity that becomes the principal source of O\(_2^-\) in these cells [87]. NADPH-oxidase-dependent redox signalling then mediates the expression of iNOS (inducible NO synthase), which can produce abundant amounts of the vasodilator NO [88]. Ascorbate protects against arteriolar hyporeactivity and arterial hypotension in experimental sepsis by inhibiting the expression of iNOS in microvascular endothelial cells [83,86,89]. The blockade of iNOS induction is a consequence of intracellular ascorbate inhibiting NADPH-oxidase-dependent redox signalling in these cells [88].

Thirdly, endothelial barrier dysfunction induced by sepsis increases the permeability of capillaries and venules to plasma proteins, such as albumin, and this can lead to plasma extravasation, oedema and shock [81]. Experiments with animal models of sepsis show that administration of ascorbate prevents oedema formation and hypotensive shock [90]. These beneficial effects of ascorbate are attributable, in part, to preservation of the endothelial permeability barrier.

Studies in microvascular endothelial cell monolayer cultures have elucidated how sepsis and ascorbate alter paracellular permeability. A sepsis-inducing insult (e.g. incubation with LPS and IFN-\(\gamma\)) causes contraction of the cytoskeleton and dissociation of intercellular junctions in the endothelium, leading to a widened intercellular space that facilitates transendothelial flux of macromolecules [91]. Pharmacological inhibitors or gene knockout of NADPH oxidase and iNOS [92] can prevent this increase in paracellular permeability. Endothelial barrier dysfunction induced by sepsis also depends on the
Intracellular ascorbate protects the microvascular endothelial barrier from a sepsis-inducing insult

The largest rectangle represents a microvascular endothelial cell, arrows with solid lines show stimulation and those with broken lines show inhibition. A sepsis-inducing insult increases NADPH oxidase (Nox) and iNOS activities, which raise ONOO\(^-\) concentrations to levels that activate PP2A and thereby induce endothelial barrier dysfunction. Ascorbate and DHAA enter the cell through SVCT2 and GLUT1 respectively, and DHAA is then reduced to ascorbate. Intracellular ascorbate rapidly scavenges ONOO\(^-\) and inhibits the induction of NADPH oxidase subunits and iNOS.

The amount of total vitamin C (i.e. ascorbate and DHAA) provided in standard parenteral nutrition multivitamin preparations (nominally 200 mg of ascorbate/day) is insufficient to normalize plasma ascorbate levels in patients with sepsis [98]. Consequently, it is not surprising that parenteral administration of higher doses of ascorbate may improve outcome. Indeed, a randomized controlled trial found that a combination of high-dose parenteral ascorbate (3 g/day for up to 28 days) and vitamin E decreased the incidence of organ failure and duration of intensive care unit stay in critically ill patients who started treatment within 24 h after traumatic injury [99]. Administering ascorbate alone may also be beneficial, because a randomized trial of patients who received high-dose parenteral ascorbate (1584 mg/kg of body weight every 24 h) in the first 24 h after burn injury found that this treatment decreased resuscitation fluid volume requirements, oedema formation and organ failure [100].

Thus circulating levels of ascorbate are depressed in sepsis. Parenteral administration of high doses of ascorbate decreases morbidity and improves survival and microvascular function (i.e. capillary blood flow, arteriolar reactivity and the endothelial permeability barrier). The underlying mechanisms involve ascorbate’s preservation of eNOS activity and inhibition of NADPH oxidase and iNOS expression. Taken together, these observations are consistent with the hypothesis that high-dose parenteral ascorbate should be considered for development as an adjuvant therapy for sepsis. An important caveat is that, to minimize the known...
Stroke

Although tremendous achievements have been made in the clinical and technological diagnosis of stroke in the past decade, the therapeutic outcome remains unsatisfactory. Stroke remains the primary cause of adult disability in the world, with ischaemic stroke being the most frequently occurring type of stroke, accounting for 80–85% of all strokes in Western countries. Apart from death, the greatest burden of stroke on the healthcare system is cost of care for long-term physical and mental disability. Strategies to reduce the incidence of stroke include prevention strategies, such as lifestyle choices, treating hypertension and lowering cholesterol, while pharmacological treatment of patients with acute stroke are designed to reduce death and disability. However, once an attack has occurred, effective treatments are limited; thus prevention is considered the most effective strategy to curb the stroke pandemic.

Stroke emerges as a condition associated with a combination of multiple risk factors, including hypertension, atherosclerosis, diabetes and smoking, of which hypertension is the most prevalent and powerful cause, across age, sex and geographic regions. The goal of stroke prevention is to identify high-risk patients and to target the modifiable risk factors through the use of appropriate pharmacological and non-pharmacological interventions.

Individual susceptibility to stroke varies greatly among hypertensive patients. Interestingly, a similar variation in susceptibility to stroke occurs in SHR, and thus a substrain of SHR was developed in the 1970s based on their susceptibility to stroke and named SHR-SP (stroke-prone SHR). SHR-SP are a useful and unique animal model for studying the pathogenesis of spontaneous stroke. These rats experience a lethal stroke between 10 months (for males) and 14 months (for females) [102]. The differences between SHR-SP and SHR could be exploited to provide some insights into the mechanisms underlying the pathogenesis and development of stroke, as both SHR and SHR-SP are hypertensive. Thus a comparison of SHR-SP with SHR could provide new insights into the pathogenesis and development of stroke beyond hypertension. One approach to this is to examine differentially expressed protein profiles in the whole brain of SHR-SP and SHR by using two-dimensional fluorescent difference gel electrophoresis, a hypothesis-free high-throughput strategy capable of resolving several thousands of individual protein spots on a single gel. Such experiments indicate that some antioxidative proteins were significantly down-regulated in SHR-SP when compared with SHR.

The generation of free radicals leading to oxidative stress plays an important role in the pathogenesis of ischaemic brain injury [103]. Oxidative stress, caused by the imbalance between the generation and detoxification of ROS/RNS, plays an important role in brain aging, neurodegenerative diseases and other related adverse conditions, such as stroke. O$_2^-$ is produced in mitochondria, and some enzymes, such as xanthine oxidase, cyclo-oxygenase 2 and NADPH oxidase catalyse the reduction of O$_2$ to O$_2^-$. SOD degrades O$_2^-$ to H$_2$O$_2$. In the mitochondrial matrix, H$_2$O$_2$ is detoxified to H$_2$O by GPx and catalase (CAT). Metal ions such as Fe$^{2+}$/Fe$^{3+}$ catalyse O$_2^-$ and H$_2$O$_2$ to OH$^-$ (hydroxyl radical), which leads to dysfunction of nucleic acids, protein and lipids in the cell. Vitamin C (VC) and vitamin A (VA), as free radical scavengers, can neutralize those toxic reactants and protect the DNA and protein from damage. Vitamin E (VE), as an effective hydrogen donor, prevents the oxidation of important metabolic substances, for example PUFAs in cell membranes.

![Figure 5](image)

**Figure 5 Effects of oxidative damage and antioxidants on stroke**

Oxidative stress, caused by the imbalance between the generation and detoxification of ROS/RNS, plays an important role in brain aging, neurodegenerative diseases and other related adverse conditions, such as stroke. Oxidative stress plays an important role in brain aging, neurodegenerative diseases and other related adverse conditions, such as ischaemia. Although ROS/RNS serve as signalling molecules at physiological levels, an excessive amount of these molecules leads to oxidative modification and, therefore, dysfunction of proteins, nucleic acids and lipids (Figure 5). The brain is particularly vulnerable to oxidative-stress-induced damage due to the high density of mitochondria, increased levels of PUFAs and metals, high levels of oxygen consumption and reduced antioxidant capacity compared with other tissues; this constellation makes oxidative stress an important contributor to ischaemic damage in the brain.

Oxidative damage to proteins can be estimated based on carbonyl content and has been found to be high.
in specific brain regions and to be elevated during aging and neurodegenerative disorders. PUFAs present in phospholipids of biological membranes are highly susceptible to oxidation by ROS. Non-specific oxidation of PUFAs, known as lipid peroxidation, is a free-radical-mediated pathway and is used as an index of irreversible neuronal damage of cell membrane phospholipids. Oxidative damage to DNA and its consequences such as gene mutation and deletions have long been implicated in the pathogenesis of a variety of human disorders associated with mitochondrial dysfunction and aging. Oxidative damage to DNA can be variable, producing structural damage such as strand breaks, protein–DNA cross-links and/or modification of base pairs.

As the final common pathway of these processes is the generation of excessive ROS/RNS, attempts to ameliorate neural damage resulting from stroke have included the use of antioxidants and free radical scavengers that neutralize these toxic reactants. Enzymic antioxidants include SOD, catalase, peroxidase and some supporting enzymes. A number of therapeutic interventions have been tested in a variety of animals in an attempt to reduce the neuronal loss and neurophysiological deficits associated with experimental stroke. Some of these experimental treatments include using inhibitors of NO synthesis, inhibitors of NADPH oxidase (NOX) and providing either enzymic or non-enzymic antioxidants [104]. The intent for using these treatments is to limit free radical damage in the affected brain region. Some of the naturally occurring and synthetic antioxidants that have been used include SOD, SOD mimics and ebselen [GPx (glutathione peroxidase) mimic]; however, the findings have met with mixed success mainly because of the large size (which limits cell permeability), short circulating half-lives and antigenicity associated with such treatments [105].

A selection of major studies on antioxidants and stroke appears in Table 1. For example, in a large long-term trial of male physicians [105a], neither vitamin E nor vitamin C had a significant effect on total mortality, but vitamin E was associated with an increased risk of haemorrhagic stroke. It may seem contradictory that randomized controlled trials have failed to show beneficial effects of antioxidant supplementation on stroke risk. Consumption of antioxidant-rich foods may reduce the risk of stroke by inhibition of oxidative stress and inflammation. TAC (total antioxidant capacity) takes into account all antioxidants and the synergistic effects between them. In a cohort of women with cardiovascular disease history [105b], TAC of the diet may be of importance for the prevention of total stroke among cardiovascular-disease-free women and haemorrhagic stroke among women with a cardiovascular disease history.

As mentioned above, vitamins have also been widely tested as to their ability to reduce molecular and cellular damage in the CNS (central nervous system) during ischaemic injury. Both animal experiments and human trials have demonstrated that oxidative damage to membrane lipids and proteins is, indeed, increased during cerebral ischaemia [106]. In a study of hypertensive rats, the TAC level and GPx activity in SHR-SP were significantly lower than those in SHR, indicating that the antioxidant defence system was damaged in SHR-SP, whereas MDA (malondialdehyde) levels in SHR-SP were significantly higher than that in SHR, suggesting that the potential damage to membrane lipids was more severe in SHR-SP than that in SHR [106a]. MDA is a lipid peroxidation product and correlates with the magnitude of the ischaemic stroke and the clinical outcome, whereas TAC and GPx have been shown to be useful tools for estimating the antioxidant activity in clinical settings [107]. The increased lipid peroxidation products/free radical formation, together with a reduced antioxidant defence system, indicated the increased oxidative stress level in SHR-SP that may play a pivotal role in the pathogenesis of stroke-associated neuronal injury.

In order to confirm the association between high oxidative stress and increased infarct area in SHR-SP, hypertensive rats were treated with a combination of vitamins C and E to improve the antioxidant defence system. After 4 weeks of treatment, the combination of vitamins significantly decreased MDA levels and increased TAC levels and GPx activity in both the brain and serum of SHR-SP [106a]. Further investigation showed that the infarct area of SHR-SP was significantly reduced by the treatment with the combination of vitamins [106a]. These results confirm the hypothesis that, on the basis of hypertension, a high level of oxidative stress is responsible for the high susceptibility to stroke. In fact, only approximately 2 % of stroke patients benefit from access to early thrombolysis [108]. As the onset of reperfusion cannot be predicted in those patients, the early use of antioxidants could contribute to protecting the brain from further damage caused by a marked burst of ROS production. Thus the majority of patients could benefit from an alternative treatment, such as the down-regulation of ROS production.

**Ataxias**

ADCAs (autosomal-dominant cerebellar ataxias) are a clinically, pathologically and genetically heterogeneous group of neurodegenerative disorders caused by degeneration of cerebellum and its afferent and efferent connections. The degenerative process may additionally involve the pontomedullar systems, pyramidal tracts, basal ganglia, cerebral cortex, peripheral nerves (ADCA I) and the retina (ADCA II) or can be limited to the cerebellum (ADCA III) [109,110]. The most common of these dominantly inherited autosomal ataxias, ADCA I, includes many SCA (spinocerebellar ataxia) subtypes, some of which are caused by pathological
Table 1  Use of antioxidants in human and animal stroke

<table>
<thead>
<tr>
<th>Model</th>
<th>Methods</th>
<th>Antioxidants</th>
<th>Primary finding(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Patients with thrombotic cerebrovascular stroke</td>
<td>Endogenous antioxidant levels</td>
<td>Elevation of homocyst(e)ine, lipid peroxide and NO oxide plasma levels in stroke patients. Plasma levels of the antioxidant ascorbic acid were lower in stroke patients, whereas plasma levels of SOD were similar in stroke and control groups. There was a significant and strong positive correlation between homocyst(e)ine and lipid peroxides. Plasma levels of ascorbic acid were negatively correlated with both homocyst(e)ine and lipid peroxide, whereas NO levels were positively correlated with SOD.</td>
<td>[107]</td>
</tr>
<tr>
<td>Human</td>
<td>Women’s Health Study (1992–2004); 39876 apparently healthy American women aged at least 45 years were randomly assigned to receive vitamin E or placebo and aspirin or placebo, using a 2 × 2 factorial design, and were followed up for an average of 10.1 years</td>
<td>Administration of 600 units of natural-source vitamin E on alternate days</td>
<td>No significant effects on the incidences of ischaemic or haemorrhagic stroke.</td>
<td>[2]</td>
</tr>
<tr>
<td>Rat</td>
<td>Male Wistar Kyoto rats subjected to 90 min of transient MCAO. Animals were divided into three groups: vehicle, azelnidipine and amlodipine groups. Rats were treated with azelnidipine (1 mg/kg of body weight) and amlodipine (1 mg/kg of body weight) by gastric gavage for 2 weeks before MCAO. Rats were killed 24 h after MCAO.</td>
<td>Azelnidipine and amlodipine (CCBs)</td>
<td>Azelnidipine and amlodipine reduced infarct volume and brain oedema. The azelnidipine-treated group showed a greater reduction in infarct volume and cerebral oedema than the amlodipine group. There was no attenuation of cerebral blood flow in the CCB groups. The number of HEL-, 4-HNE-, AGE- and 8-OhdG- (oxidative stress markers for early lipid, late lipid, protein and DNA peroxidations respectively) positive cells were decreased in the CCB-treated groups. These molecules were again lower in the azelnidipine group than in the amlodipine group.</td>
<td>[129]</td>
</tr>
<tr>
<td>Human</td>
<td>The Physicians’ Health Study II; a randomized double blind placebo-controlled factorial trial of vitamin E and vitamin C that started in 1997 and continued until its scheduled completion on 31 August 2007. There were 14641 American male physicians enrolled, who were initially 50 years of age or older, including 754 men (5.1 %) with prevalent CVD at randomization.</td>
<td>Vitamin E and vitamin C</td>
<td>Compared with placebo, vitamin E and vitamin C had no effect on the incidence of total stroke. Neither vitamin E nor vitamin C had a significant effect on total mortality, but vitamin E was associated with an increased risk of haemorrhagic stroke.</td>
<td>[105a]</td>
</tr>
<tr>
<td>Rat</td>
<td>SHR-SP were randomly divided into untreated and treated (vitamins C and E) groups. After treatment for 4 weeks, half of the animals were killed for detection of TAC, Gpx and MDA. The remaining rats underwent MCAO and the infarct areas were measured.</td>
<td>Vitamin C (200 mg/kg of body weight per day) and vitamin E (100 mg/kg of body weight per day)</td>
<td>Compared with SHR, the infarct area of SHR-SP was larger, and antioxidant proteins GSTPi2 and GSTAS were lower; TAC and GPx activities were decreased, and MDA levels were elevated. Treatment with vitamins C and E decreased MDA, and increased TAC and GPx activity significantly in SHR-SP, while also decreasing the infarct area.</td>
<td>[106a]</td>
</tr>
<tr>
<td>Human</td>
<td>Women (31035 CVD-free and 5680 with CVD history at baseline), 49–83 years of age, from the Swedish Mammography Cohort. Diet was assessed with a food frequency questionnaire. Dietary TAC was calculated using oxygen radical absorbance capacity values. Stroke cases were ascertained by linkage with the Swedish Hospital Discharge Registry.</td>
<td></td>
<td>The multivariable hazard ratio of total stroke comparing the highest with the lowest quintile of dietary TAC was 0.83 in CVD-free women. Among women with a CVD history, the hazard ratios for the highest compared with the lowest quartile of TAC were 0.90 for total stroke and 0.55 for haemorrhagic stroke.</td>
<td>[105b]</td>
</tr>
</tbody>
</table>
Among the different variants of autosomal-dominant SCAs, SCA2 is among the three most frequent types, together with SCA3 and SCA6. It is estimated that nearly 87% of SCA patients are affected with SCA2. The highest concentration of SCA2 families in the world is in Holguín, Cuba. Within the geographic extension of this founder effect, the prevalence rate is already 40.18 cases per 100 000 inhabitants.

CAG trinucleotide repeat expansion in the coding region on the mutated gene, as is the case in SCA1, SCA2, SCA3/MJD (Machado-Joseph Disease), SCA17 and DRPLA (dentatorubral-pallidolusian atrophy) [109].

Epidemiology of SCA2
Most of the epidemiological studies of hereditary ataxias have been performed in isolated geographical regions in families and as such are not large enough for linkage analysis. The collective worldwide prevalence is estimated to be approximately 5–7 cases per 100 000 people, although higher values have been reported in particular populations because of founder effects (Figure 6). In Cuba, molecular analyses of genes SCA1–SCA3, SCA6, SCA17 and DRPLA identified 753 patients with SCA and 7173 asymptomatic relatives, belonging to 200 unrelated families; of the SCA patients, 86.79% were affected with SCA2 [110]. The highest concentration of SCA2 families in the world are in the region of Holguín, Cuba, where the prevalence rate is 40.18 cases per 100 000 inhabitants. Within this region, the most affected area is the municipality of Báguanos, with a remarkable rate of 134 cases per 100 000 inhabitants. The high prevalence of the SCA2 mutation in Holguín most probably reflects a founder effect [111]. The existence of a genetic anticipation phenomenon in 80% of ataxia cases in Cuba is an alert for the possibility of the appearance of the disease in childhood, where the course is more severe and dramatic. The existence of a pre-symptomatic and pre-natal diagnostic programme poses the necessity of developing some therapeutic procedures to modify the course and severity of the disease during the pre-symptomatic stages [112].

SCA2 features
SCA2 is characterized by progressive gait and limb ataxia, cerebellar dysarthria (difficulty in pronunciation), dysmetria (disturbed control of range and timing of movements), dysdiadochokinesia (inability to perform rapid movements), tremor and slowing of horizontal saccadic eye movements [112]. This neurodegenerative disease affects not only the cerebellum and its fibre connections, but also the peripheral nervous system and extracerebellar central pathways. Some of these abnormalities can also appear during the pre-symptomatic stage.

The gene for SCA2 is located on the long arm of chromosome 12 (12q23–24.1) and encodes a cytoplasmic protein (ataxin 2) found in many body tissues and neurons [113–115] (Figure 7). The peripheral lesion of the nerves, mainly at the axonal level with signs of demyelination, is interpreted as a secondary axonal disorder. The reduction in the amplitude of the sensory potential is more marked in patients with a longer duration of the disease, indicating
Molecular characteristics of SCA2, structure of the SCA2 gene and ataxin-2 protein

The gene responsible for SCA2 was localized to chromosome 12q24.1 and then independently identified by three groups and shown to contain unstable triplet-repeat expansions as the pathogenic mutation, coding for a polyglutamine domain in the N-terminal region of the protein ataxin-2. This triplet-repeat reads \((\text{CAG})_{8-31}\text{-CAA-(CAG)_{4-31}-(CAG)_{8-31}}\) in the DNA and mRNA of most normal individuals, but can vary in length in the human population. The presence of more than 31 triplets in the SCA2 gene may cause clinically manifested neurodegeneration. The SCA2 gene contains 25 exons, encompassing approximately 130 kb of genomic DNA.

a progressive increase in the number of affected fibres with time [110–112]. Measurements of motor and sensory nerve conduction in a large sample of SCA2 gene carriers over a 20-year span was conducted and the progressive changes observed in this longitudinal study suggest the identification of three electrophysiological stages in the course of this disease [111].

First stage: pre-clinical sensory axonal neuropathy

During this stage, electrophysiological abnormalities appear even before the clinical disease onset (SCA2 stage 0). These alterations consist of a decrease in sensory potential amplitudes without definitive clinical manifestations of peripheral neuropathy, which could be classified as the earliest subclinical alterations in SCA2. The results of normal motor nerve conduction studies suggest the sparing of the motor nerve fibres during pre-symptomatic stages [111].

Second stage: sensory axonal neuropathy

This stage corresponds to the initial clinical manifestations of the cerebellar syndrome. It is usually associated with SCA2 stage I and is characterized by an increase in the sensory electrophysiological abnormalities and the emergence of other more variable sensory alterations, such as latencies increase and nerve conduction slowing [111].

Third stage: sensory-motor neuropathy

This stage is characterized by a mixed (sensory and motor) peripheral neuropathy with decreased motor potential amplitudes and accentuated sensory involvement [111]. There is a growing awareness that oxidative stress occurs in several clinical conditions [116].

ROS and neurotoxicity in ataxias

The inherited ataxias are a large heterogeneous group of neurodegenerative disorders caused by a variety of gene mutations, the effects of which are exerted through different pathogenic mechanisms. Despite this diversity, oxidative stress appears to be a common factor in the pathogenesis of these disorders, indicating that antioxidants might be potential therapeutics for these currently incurable conditions. Some inherited ataxias, such as ataxia with vitamin E deficiency, are directly caused by defects in small-molecule antioxidants and could well be treated by supplying the defective molecule. In most ataxias, however, oxidative stress has more complex disease-specific causes and consequences, which
needs to be better investigated to enable more effective treatments to be developed [117].

Oxidative stress is known to occur in cerebral ataxia; thus we will review the status of an extensive array of oxidative stress markers that could be useful in the clinical setting when assessing the damage to biomolecules in patients with SCA2. To control the accumulation of ROS, aerobic cells have developed antioxidant systems that include antioxidants such as GSH, SOD, GPx and catalase [118].

Lipid peroxidation causes oxidative conversion of PUFAs into cytotoxic products such as MDA, which is a measure of oxidative stress in addition to other markers that better estimate unfinished peroxidation products, such as hydroperoxides [119]. Importantly, ROS also directly oxidize and thus damage DNA, proteins and lipids, and in the process can produce new metabolites capable of functioning as signalling molecules that can subsequently activate a number of cellular-stress-sensitive pathways, such as NF-κB, p38, MAPK (mitogen-activated protein kinase), JNK, PKC and AGE (advanced glycation end-product), that can mediate tissue and cellular damage [116,120–122].

We have an increased understanding of the molecular basis of the neurotoxicity associated with SCA2. There are emerging roles for microelements in the regulation of the human nervous system, for example zinc and copper are thought to mediate neural function in health and disease [113,114]. Zinc is the second most abundant (after iron) microelement in nervous tissue, and a decrease in zinc levels occur in Parkinson's disease, Alzheimer's disease and some types of ataxia [123]. Exogenous zinc exerts neuroprotective actions through the antagonism of cerebellar NMDA (N-methyl-D-aspartate) receptors, an area of the brain area that is the most affected in SCA2, suggesting that some microelements could directly or indirectly regulate oxidative reactions during the onset and/or clinical outcomes of SCA2.

Oxidative stress probably plays an important role in several aspects of different neurodegenerative complications. The concentration of vitamin E is regulated by α-TTP (α-tocopherol transfer protein) that facilitates α-tocopherol export from the liver. Mutations of the TTPA gene (encoding α-TTP) are linked to an ataxia type with acute vitamin E deficiency, making the Tppa−/− mouse a useful model to study age-related neuronal degeneration arising from chronic oxidative stress [124]. An initial characterization of the brains from the Tppa−/+ mouse indicated accumulations of lipofuscin (a yellow autofluorescent coarse granule that is formed by reaction of protein and carbonyl compounds) and MDA (the end product of lipid peroxidation); importantly, supplementation with α-tocopherol was able to reverse these changes in Tppa−/+ mice. Lipofuscin accumulation was greatest in areas of neurodegeneration. Although there are numerous animal models that show rapid neuronal cell death caused by oxidative damage, the Tppa−/− mouse is the first to show a slowly progressing late-onset neuronal degeneration that is caused by chronic oxidative stress [124].

The methodology for evaluating oxidative stress in clinical settings is poorly described [125,126]. An efficient clinical diagnostic test of redox status could be important in the setting of degenerative damage associated with oxidative stress, or to follow the outcomes of a nutritional or therapeutic regimen [122]. Although MDA concentrations are unchanged in ataxia patients, there are significant differences in total peroxides. These products constitute intermediates in lipid peroxidation processes and are thus as toxic and reactive as the final products. Some reports show beneficial changes in total peroxides during antioxidant supplementation that are related to reduced levels of total peroxides in ataxia patients [117]. This could be due to ROS generation that overwhelms natural defence mechanisms, so that lipid peroxidation products are produced, but apparently not terminal products such as MDA. Accumulation of peroxides leads to the uncontrolled progression of oxidative damage to other biomolecules such as cell membranes [127]. In support of this, studies of oxidative stress markers in neurodegenerative diseases report altered redox metabolism in ataxia patients [128], with increases in oxidative damage and modifications of the antioxidant background [118].

CONCLUSIONS

Mitochondrial ROS are not just by-products of mitochondrial respiration, but also play a key role in cell signalling. The mitochondrial electron transport chain represents an important source of ROS in cells, and the production of mitochondrial ROS is controlled by a number of mechanisms. Mitochondrial ROS signalling is implicated in the regulation of many functions in endothelial cells and adipocytes, and there is support for a role of ROS in a variety of diseases including hypertension, stroke, Type 2 diabetes, sepsis and cerebral ataxia. This overview has discussed approaches for therapeutic interventions aimed at decreasing ROS levels in a variety of diseases and has summarized the strengths and weaknesses of strategies reliant on currently available antioxidants.

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