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Review

Oxidative stress and mitochondrial dysfunction in neurodegeneration; cardiolipin a critical target?

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ABSTRACT

Oxidative stress and subsequent impairment of mitochondrial function is implicated in the neurodegenerative process and hence in diseases such as Parkinson's and Alzheimer's disease. Within the brain, neuronal and astroglial cells can display a differential susceptibility to oxidant exposure. Thus, astrocytes can up regulate glutathione availability and, in response to mitochondrial damage, glycolytic flux. Whilst neuronal cells do not appear to possess such mechanisms, neuronal glutathione status may be enhanced due to the trafficking of glutathione precursors from the astrocyte. However, when antioxidants reserves are not sufficient or the degree of oxidative stress is particularly great, mitochondrial damage occurs, particularly at the level of complex IV (cytochrome oxidase). Whilst the exact mechanism for the loss of activity of this enzyme complex is not know, it is possible that loss and/or oxidative modification of the phospholipid, cardiolipin is a critical factor. Consequently, in this short article, we also consider (a) cardiolipin metabolism and function, (b) the susceptibility of this molecule to undergo oxidative modification following exposure to oxidants such as peroxynitrite, (c) loss of mitochondrial cardiolipin in neurodegenerative disorders, (d) methods of detecting cardiolipin and (e) possible therapeutic strategies that may protect cardiolipin from oxidative degradation.

1. Introduction

Oxidative stress, arising from reactive oxygen or nitrogen species (ROS or RNS), has been implicated in the pathogenesis of neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's disease (AD) (Reviewed in [1]). Furthermore, loss of mitochondrial function, at the level of the electron transport chain (ETC) is also associated with the neurodegenerative process [1]. Consequently, in view of the susceptibility of the ETC to oxidative damage, loss of ETC activity, due to oxidant exposure, has been proposed to be a plausible mechanism for the neuronal cell death associated with PD, AD and other neurodegenerative conditions [1]. In view of this, a number of therapeutic strategies have been proposed, largely involving the use of antioxidants, in an attempt to prevent the oxidative inactivation of the ETC and thus limit neuronal cell death [1]. Unfortunately, to date, clinical trials reveal limited efficacy and so this may suggest the need for better delivery of antioxidant molecules to brain cells and/or their mitochondria. Alternatively, new vulnerable molecules, essential for mitochondrial function, may need to be identified that could provide new therapeutic targets. One such molecule is cardiolipin (CL) and this short article will review brain oxidative stress/mitochondrial dysfunction (within the context of neurodegeneration) and how potential alterations in functional CL may contribute to an impairment of the ETC.

2. Oxidative stress and energy metabolism in neurones and astrocytes

The brain is particularly dependent on mitochondrial energy supply to maintain normal function. Whist the individual components of the electron transport chain exhibit varying degrees of sensitivity towards ROS and RNS, it appears that neuronal and astrocytic cells can display a differential response to an ETC that has a compromised ability to generate ATP [2].

With regards to astrocytes, inhibition of mitochondrial ATP generation results in a preservation of cellular energy status due to a stimulation of glycolysis. Such an up regulation of glycolysis appears to be mediated through the AMP protein kinase and 6-phosphofructo-2-kinase pathway (PFK2) [3]. In direct contrast, neuronal cells have a virtual absence of PFK2 and hence are particularly sensitive to failure of mitochondrial oxidative phosphorylation [3].

In addition to exhibiting a differential response to mitochondrial dysfunction, antioxidant status, in particular reduced glutathione (GSH) metabolism, appears different between astrocytes and neuronal cell. Data derived largely from cell culture studies reveal that the GSH

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status of astrocytes is superior, by approximately two fold, when compared to neuronal cells [2]. Such a discrepancy can be attributed to a greater specific activity, within astrocytes, of the rate limiting enzyme for GSH biosynthesis, glutamate cysteine-ligase (GCL) [4].

When compared to astrocytes, neurones can display marked sensitivity to RNS. However, vulnerability appears to be diminished when neurones are co-cultured with astrocytes [5]. Such protection appears to arise as a result of an increase of neuronal GSH availability [5]. This increase in GSH content occurs as a consequence of the trafficking of GSH precursors from astrocytes to neurones. Prevention of this trafficking results in the failure of the neuronal cells to up-regulate their GSH status and renders the ETC susceptible to damage by RNS [6].

Under conditions where the activity of the ETC is irreversibly compromised, complex IV (cytochrome oxidase) appears, with regards to neural cells, to be a particularly susceptible to RNS exposure and/or GSH depletion [6–8]. Whilst the exact mechanism for this loss of complex IV activity is not known, it is possible, in view of the particular requirement of this enzyme for cardiolipin (see below), that oxidative modification of this phospholipid is a contributing factor. Support for this suggestion may come from our previous studies that demonstrated that Trolox, a vitamin E analogue and hence potential inhibitor of lipid peroxidation, was superior, when compared to other antioxidants, at preventing complex IV damage following RNS exposure [9].

3. Cardiolipin

Cardiolipin (CL) is a dimeric phospholipid with an unusual structure consisting of four acyl side chains in comparison to more typical lipids which have two acyl chains [10] (Fig. 1). In eukaryotes, CL is found almost exclusively in the mitochodrial inner membrane, where it accounts for ~25% of all mitochondrial lipids [11]. In the inner membrane, CL is distributed approximately 60:40 between the matrix and intermembrane space [12,13].

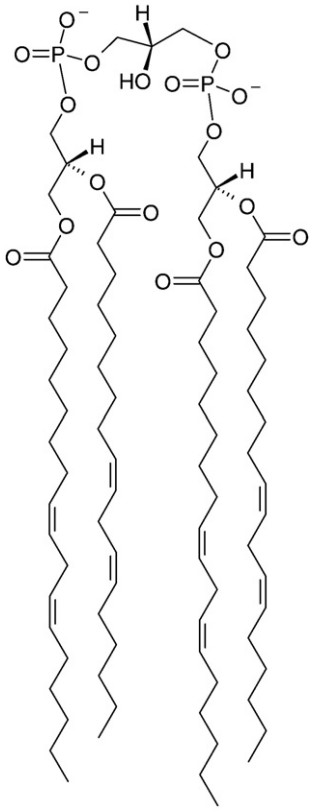

Fig. 1. Structure of cardiolipin with four linoleic acid side chains. This form is typically designated as CL(18:2)$_4$ indicating the length and number of double bonds in each carbon side chain.

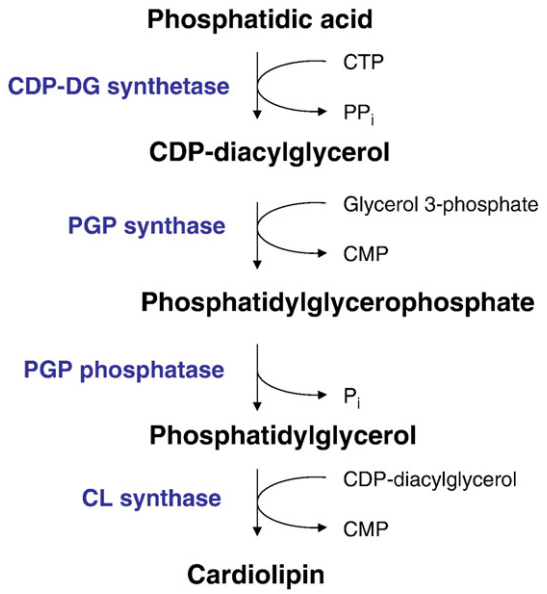

Fig. 2. Synthesis of cardiolipin in eukaryotes. After synthesis of cytidinediphosphate-diacylglycerol (CDP-DAG) from phosphatidic acid, CL synthesis consist of three steps, which all take place in the inner mitochondrial membrane. The first step is the formation of phosphatidylglycerophosphate (PGP) from CDP-diacylglyceol and glycerol 3-phosphate by PGP synthase. In the second step, PGP is dephosphorylated by PGP phosphatase to produce phosphatidylglycerol (PG). In the final step, catalysed by CL synthase, PG is combined with CDP-diacyglycerol to form CL.

CL is synthesised from phosphatidic acid via a number of enzymatic steps (Fig. 2) [10]. Whilst the initial steps of CL synthesis are comparable to that of other phospholipids, the final reaction, catalysed by CL synthase is unique [10]. The mitochondrial inner membrane itself contains all the enzymes of the CL pathway downstream of phosphatidic acid, the latter being synthesised in the outer mitochondrial membrane [10]. CL biosynthesis, though not fully characterised, appears to be highly regulated. For instance, there is an apparent strong dependence on the intracellular concentration of cytidine triphosphate and ATP [10]. In addition, the CL pathway is integrally related to mitochondrial development and complex IV assembly [10]. Although the fatty acid side chains of CL precursors may vary considerably, in mature CL the fatty acid side chains are usually symmetrical and contain a single type of fatty acid [14]. In most mammalian tissue (heart, skeletal muscle, liver and kidney) the predominant form of CL is tetralinoleoyl CL — CL(18:2)$_4$ [14,15]. However, there is some variation in side chain composition of CL, with testes containing high levels C16:0 [15] and brain containing high levels of polyunsaturated side chains such as C20:4 and C22:6 [16–18]. The remodelling of the acyl side chains is not fully understood, but the structural conformity and symmetry of the resulting CL molecules is postulated to be important for different biological functions [14].

4. CL and mitochondria

CL is an integral part of the mitochondrial inner membrane and is associated with many mitochondrial proteins. Therefore it has always been assumed to have an important role in mitochondrial function. However, it is only relatively recently that defined functions of CL have been proposed. Studies of mutant cells deficient in CL provided some of the first insights into the role of CL in mitochondria. In yeast mutants, lacking functional CL synthase, no CL is present in the mitochondrial membranes and these cells lose viability at elevated temperatures. When grown on glucose, these mutants show reduced respiratory rate, deficient ATPase and complex IV activities plus impairment of the ADP–ATP translocator [19].

CL deficiency has also been studied in mammalian cells. The first studies of CL deficiency in mammalian cells were performed on Chinese hamster ovary cells lacking a functional PGP synthase, which synthesises a direct precursor to CL (Fig. 2). As a consequence, these cells display reduced levels of CL, altered mitochondrial morphology, reduced ATP concentrations, diminished oxygen consumption and defective ETC activity [20].

Further insights into the role of CL in human cells have been obtained from the study of patients with Barth syndrome, in which CL metabolism is affected. Barth syndrome is an X-linked disease characterised by cardiomyopathy, neutropenia, muscle weakness and a loss of mitochondrial function [21]. This disorder arises from a defect in the TAZ gene, which codes for a group of proteins called the tafazzins, produced by alternative splicing [22]. The tafazzins are phospholipid acyltransferases involved in CL remodelling [23]. Patients with Barth's syndrome exhibit a loss of functional CL, in particular the tetralinoleoyl form, in a variety of tissues [24]. They also exhibit an increase in monolysocardiolipins (CL missing an acyl chain) [24].

A direct role of tafazzin in CL metabolism has also been shown in yeast, where yeast expressing the TAZ mutation exhibit a decrease in normal CL species containing C18:1 and C16:1 fatty acids, an increase in monolysocardiolipin [25] and destabilisation of respiratory chain complexes [26]. Incubation of fibroblasts from patients with Barth syndrome with linoleic acid leads to an increase in tetralinoleoyl CL content [27], but the effects of this treatment in patients await further investigation.

Of the associations between mitochondrial enzymes and CL, the interaction between CL and complex IV is perhaps the best documented. Thus, each monomer of the complex is known to bind two molecules of CL [28] and loss of CL results in low activity. However, activity of purified enzyme can be restored with $CL(18:2)_4$ but not variants such as dilysocardiolipin (CL with only two acyl groups) [29], CL containing two C6 chains [29] or, in the case of heart mitochondria, peroxidised CL [30]. A number of models have been proposed in order to explain the effect of CL on complex IV activity. These include; (a). CL acts to increase the surface concentration of cytochrome *c*, (b). CL increases the surface concentration of protons, (c). CL brings about a conformational change of the enzyme that enhances catalytic activity [31].

The effects of CL upon ETC function are not confined to complex IV, i.e. complexes I and III also bind CL and display low activity in its absence. Furthermore, CL facilitates the association of complex III and IV into "supercomplexes". Such supercomplex formation is thought to improve the efficiency of oxidative phosphorylation by eliminating the need for diffusion of substrates and products between the individual ETC components [32].

In addition to effects upon the ETC, CL interacts with the ADP–ATP translocator. Purified ADP–ATP carrier from bovine heart contains six molecules of tightly bound CL per protein dimer and these CLs can only be removed under denaturing conditions [33]. These CLs appear to act as a prosthetic group for the protein and removal of these CLs results in the loss of carrier function [34].

5. The interaction of CL with cytochrome *C* and its role in apoptosis

Recent findings have demonstrated that CL and its metabolites have key roles in the apoptotic cell death program [35]. At the early stages of apoptosis, CL is redistributed to the outer monolayer of the mitochondrial inner membrane and the outer mitochondrial membrane [13,36,37]. Redistribution of CL is likely to occur via interactions with apoptosis-associated proteins such as Bid/tBid and Bax at contact sites between the inner and outer membranes [38,39]. tBid, the truncated form of Bid, displays a high affinity for monolysocardiolipins, formed during the remodelling of CL. Since acyl chain remodelling of CL occurs outside the mitochondria, Bid and tBid have been proposed to have a role in the normal metabolic cycle of CL [40].

Redistribution enables significant amounts of CL to interact with cytochrome *C* in the intermembrane space. The cytochrome *C*–CL complex formed can act as a potent peroxidase in the presence of hydrogen peroxide leading to the formation of CL hydroperoxides and release of cytochrome *C* [13]. Release of cytochrome *C* from the mitochondria is a key feature of apoptosis [41] and decreases in CL content [42] or increases in CL peroxidation [43–45] have been associated with liberation of cytochrome *C* from the mitochondrion and subsequent tissue degeneration. However, release of cytochrome *C* is not the only role CL hydroperoxides have in apoptotis, since the addition of exogenous CL hydroperoxides to cytochrome *C* deficient cells is sufficient for the release of other pro-apoptotic factors such as Smac/Diablo [13].

Oxidation of CL may also trigger the remodelling pathway involving Bid [35]. It has been hypothesised that oxidised side chains of CL are hydrolysed in the inner membrane by phospholipase A_2 and monolysocardiolipin produced is subsequently transported by Bid to the ER for remodelling [35].

6. CL in the brain

Unlike the majority of mammalian tissues, tetralinoleoyl CL is not the predominant form of CL in the brain. In mouse brain, stearic (18:0), oleic (18:1), cervonic (22:6) and arachidonic acid (20:4) each contribute to about 20% of acyl side chains of CL [46]. In contrast, linoleic acid (18:2) contributes to less than 5% of acyl side chains [16,17]. The reason for this difference in CL side chain between brain and other tissue is not known. However, this difference in fatty acid composition could render brain CL more susceptible to peroxidation, i.e. due to the higher double bond content of the acyl side chains. Support for this suggestion comes from studies using HL60 cells that have been engineered to contain more

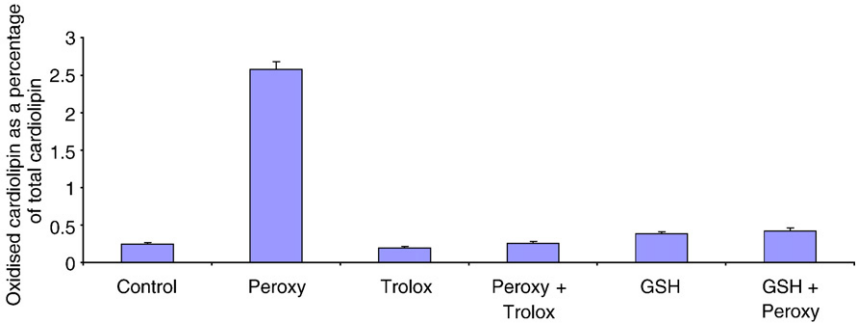

Fig. 3. The effect of peroxynitrite and antioxidants on oxidised cardiolipin levels in muscle homogenates. Pooled muscle homogenates were incubated for 3 h in the presence or absence of peroxynitrite (Peroxy) (1 mM)±Trolox (2.5 mM) or GSH (2 mM). After incubation, lipids were extracted and unoxidised and oxidised cardiolipin was measured by LC-MS (n=3–6). Lipid extraction and LC-MS analysis were carried out as previously described by Valianpour et al., 2002 [55]. Unoxidised cardiolipin was measured in multiple reaction monitoring (MRM) mode as a transition from 723>279. Oxidised cardiolipin was measured as a transition from 731>279. (Mass spectrometer settings:Negative ion mode −3.3 kV; Collision 30; Cone 25 V.). $(C14:0)_4$-CL was used as an internal standard.

C22:6 CL species. These cells display increased sensitivity to apoptotic stimuli, in comparison to wild-type cells that contain predominantly C16:0 and C18:2 CL species [13].

As is the case for GSH metabolism and the ability to invoke a glycolytic response following ETC damage (see above), brain CL status also appears to be heterogeneous. Thus, synaptic and non-synaptic mitochondria vary in their CL content and display a differential effect with regards to the effects of ageing [47].

With regards to oxidative stress and neurodegeneration a number of experimental models support the concept of loss of functional brain CL. Thus, studies upon aged rat brain report CL depletion, loss of complex IV activity and mitochondrial membrane potential. Similarly, models of traumatic brain injury, familial amytrophic lateral sclerosis (mice over expressing superoxide dismutase) and Parkinson's disease (mice lacking alpha synuclein) report CL deficiency, increased lipid peroxidation and loss of ETC activity [16,46,48].

7. CL oxidation

As indicated above, CL is particularly susceptible to oxidation because (1) it contains easily oxidised unsaturated side chains, such as linoleic acid or docosahexanoic acid (cervonic acid) and (2) it is

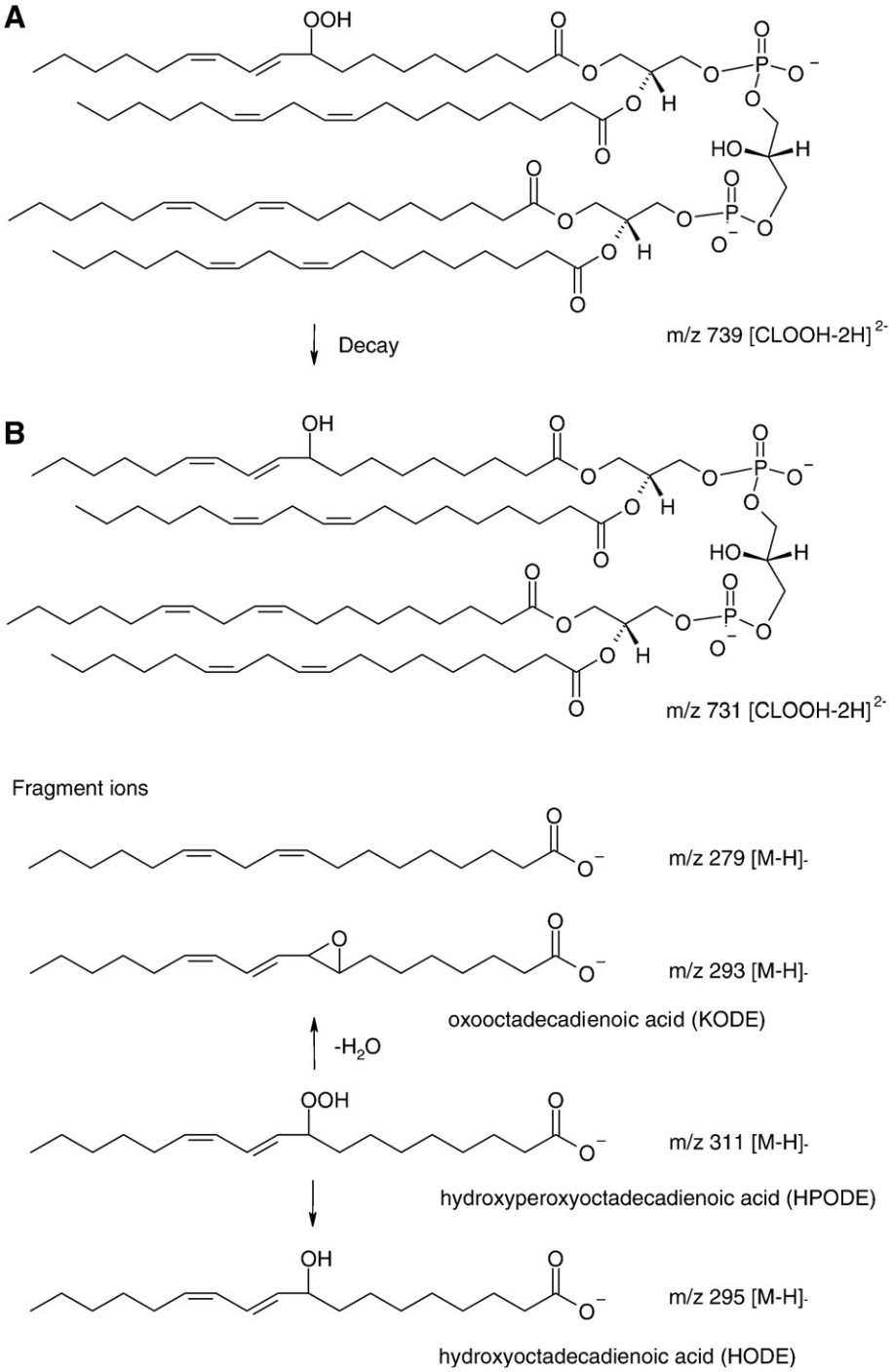

Fig. 4. A. Cardiolipin hydroperoxide. B. The main oxidised cardiolipin specie observed in the experiment detailed in Fig. 3 had a molecular ion of m/z 731 and fragment ions of 279 and 293. This oxidised product was probably the result of decay of cardiolipin hydroperoxide, with loss of water, during analysis.

associated with the ETC, a major site of reactive oxygen species. Therefore there is currently growing interest in identifying and measuring oxidised CL species.

Classical lipid peroxidation proceeds via the following steps; (1) abstraction of a hydrogen, forming an alkyl radical, (2) addition of oxygen, to form a peroxy radical, (3) formation of a hydroperoxide via abstraction of a hydrogen from another lipid chain (thereby continuing lipid peroxidation) or from an antioxidant such α-tocopherol/trolox. CL oxidation is considered to proceed in a similar manner so the main products are cardiolipin hydroperoxides (CLOOH), containing varying numbers of oxygens.

When considering CL oxidation, preliminary data from our own laboratory (Figs. 3 and 4) and that of others [16,49–51] reveal that numerous oxidised products may be produced. The nature of these species may depend upon the site and extent of oxidation, as well as on the breakdown of the oxidised species to secondary oxidation products (Fig. 4). The complexity and range of products formed, following exposure of CL to oxidants such as $ONOO^-$, illustrates the need to define exactly which oxidised species are being generated. This may be of particular importance as different CL oxidation products could potentially exert differing biological effects.

8. Measurement of CL

CL and oxidised CL have been measured by a variety of techniques. These include HPLC [52], thin layer chromatography (TLC), nonyl acridine orange (NAO) binding and liquid chromatography with mass spectrometry (LC-MS) [53–57]. These methods vary in sensitivity and specificity, e.g. NAO in addition to reflecting CL status also responds to the mitochondrial membrane potential [57]. For simple chemical systems assessing CL oxidation, UV detection of conjugated dienes or TLC may be sufficient. However, for complicated biological samples, a sensitive technique such as LC-MS, which is able to distinguish the various oxidised forms of CL from other lipids, would be the method of choice.

9. Potential therapeutic strategies to prevent CL oxidation

In view of the sensitivity of CL to RNS and ROS, antioxidants may have a role in preventing CL oxidation. Preliminary data from our laboratory support this suggestion by demonstrating that Trolox and GSH are both capable of retarding ONOO-mediated oxidation [Fig. 3]. However, such findings were performed upon tissue homogenates and whilst providing a useful proof of principle, targeting to the mitochondria may be required for intact cellular systems and *in vivo*. Thus, molecules such as MitoQ and MitoE (ubiquinone and vitamin E homologues respectively), which target and accumulate within mitochondrial membranes [58], could prove to be particularly effective at preventing CL oxidation in appropriate cellular and *in vivo* model systems.

10. Conclusion

There is now a growing body of evidence to implicate oxidative stress and mitochondrial dysfunction in the pathogenesis of neurodegenerative disorders. However, it also now clear that different brain cell types, namely astrocytes and neurones, can have differing antioxidant reserves and metabolic responses to a compromised ETC. Appreciation of such differences may be important when considering new therapeutic strategies to combat the neurodegenerative process. Similarly, new targets susceptible to oxidation will continue to be identified that if their degradation could be prevented may form the basis of new treatment regimes. One such molecule appears to be CL and it now becoming clear that this phospholipid plays a critical role in maintaining optimal ETC function and controlling apoptosis. However, the nature of the acyl side chains can render this molecule vulnerable to oxidative damage with a consequential loss of biological function. Further work is therefore required to identify CL status and the nature of any oxidised degradation products in a range of appropriate model systems and human biopsy material. Such data may then provide the basis for drugs that can specifically prevent loss of functional CL from the mitochondrial inner membrane.

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