

Review

The Nervous System Cytoskeleton under Oxidative Stress

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Received: 10 September 2013; in revised form: 30 September 2013/ Accepted: 12 October 2013 / Published: 21 October 2013

Abstract: Oxidative stress is a key mechanism causing protein aggregation, cell death and neurodegeneration in the nervous system. The neuronal cytoskeleton, that is, microtubules, actin filaments and neurofilaments, plays a key role in defending the nervous system against oxidative stress-induced damage and is also a target for this damage itself. Microtubules appear particularly susceptible to damage, with oxidative stress downregulating key microtubule-associated proteins [MAPs] and affecting tubulin through aberrant post-translational modifications. Actin filaments utilise oxidative stress for their reorganisation and thus may be less susceptible to deleterious effects. However, because cytoskeletal components are interconnected through crosslinking proteins, damage to one component affects the entire cytoskeletal network. Neurofilaments are phosphorylated under oxidative stress, leading to the formation of protein aggregates reminiscent of those seen in neurodegenerative diseases. Drugs that target the cytoskeleton may thus be of great use in treating various neurodegenerative diseases caused by oxidative stress.

Keywords: microtubule; actin; neurofilament; posttranslational modification

Abbreviations

Abl: Abelson murine leukaemia viral oncogene homolog 1; ADF: actin depolymerising factor; ADNP: activity-dependent neuroprotective protein; APP: amyloid precursor protein; Arg: Abl-related gene; BPAG: bullous pemphigoid antigen; Cdk5: cell division kinase 5; CRMP: collapsin response-mediator

proteins; DAT: dopamine transporter; EB1: end binding 1; GSH: glutathione; GSK: glycogen synthase kinase; HDAC6: histone deacetylase 6; HNE: 4-hydroxy-2-nonenal; LKE: lanthionine ketimine; L-NAME: N omega-nitro-L-arginine methyl ester; MAP: microtubule-associated protein; NADPH: nicotinamide adenine dinucleotide phosphate; NAP: NAPVSIPQ; NF: neurofilament; NMDA: N-methyl-D-aspartic acid; PCOOHs: phosphatidylcholine hydroperoxides; Shot: short stop; UCH-L1: ubiquitin C-terminal hydrolase L1.

1. Introduction

Oxidative stress represents an imbalance between the production of reactive oxygen species and the ability of the biological system to detoxify the reactive intermediates or to repair the resultant damage. Oxidative stress leads to the formation of peroxides and free radicals, which damage proteins, lipids and DNA. Oxidative stress is implicated in various neurodegenerative disorders, largely due to the abnormal accumulation of oxidatively damaged macromolecules, including β -amyloid and Tau [1].

The neuronal cytoskeleton plays a key role in both protecting cells against oxidative stress and is itself the target of oxidative stress-induced damage. The neuronal cytoskeleton consists of microtubules, actin filaments and neurofilaments (intermediate filament proteins). All three are linked through crosslinking proteins to form a unified whole. These cytoskeletal components are regulated through changes in expression levels, post-translational modifications and the effects of the binding of partner proteins. Oxidative stress affects the regulation of the neuronal cytoskeleton through these regulatory mechanisms, with some changes being protective and others pathogenic. Thus, the cytoskeleton plays a key role in cellular responses to oxidative stress and the onset of oxidative stress-induced neurodegeneration.

2. Microtubules and Oxidative Stress in the Nervous System

2.1. Drug Studies Indicate a Role for Microtubules in Oxidative Stress

The stabilisation of microtubules with taxol or destabilisation with vinblastine causes concentration-dependent cell death with apoptotic features in neurons. Treatment with taxol increases the amount of reactive oxygen species, and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or suppression of gp91 (phox) attenuates taxol-induced neuronal death, although the use of NADPH oxidase inhibitors does not decrease vinblastine-induced cell death [2]. The microtubule depolymerising agent, colchicine, upregulates genes related to oxidative stress, glutathione peroxidase 1 and catalase, in rat cerebellar granule neurons [3]. Colchicine causes loss of cholinergic neurons and cognitive dysfunction that is associated with excessive production of free radicals, including malondialdehyde and nitrite, and a decrease in reduced glutathione [4]. Treatment of primary mesencephalic neurons with microtubule-depolymerising agent stimulates dopamine transporter (DAT) activity, leading to an increased rate of dopamine uptake and increased levels of dopamine-induced oxidative stress along with accelerated cell death. DAT co-immunoprecipitates with α - and β -tubulin, suggesting a functional link between the microtubule cytoskeleton and DAT [5].

2.2. The Proteasome, Oxidative Stress and Microtubules

Oxidative stress causes the aggregation of a number of proteins implicated in neurodegenerative disorders [1]. Mutations that affect the dynein motor machinery are sufficient to cause motor neuron disease. Indeed, decreased dynein function impairs autophagic clearance of aggregate-prone proteins [6], losing its function in aggresome formation and failing to return autophagosomes and lysosomes to the centre of the cell for degradation [7]. An intact microtubule cytoskeleton is essential for aggresome formation, and cytoplasmic dynein/dynactin is responsible for the directed transport of misfolded protein into aggresomes [8]. Thus, breakdown of the microtubule cytoskeleton under oxidative stress will, in turn, increase the load of aggregated protein in a cell, potentially leading to overload and cell death.

3. Role of Post-Translational Modifications of Tubulin

3.1. Acetylation

HDAC6 is responsible for the deacetylation of α -tubulin in the brain. An inhibitor of HDAC6, tubastatin A, induces elevated levels of α -tubulin, but not histone, in primary cortical neuron cultures. Tubastatin A also confers dose-dependent protection of neurons against glutathione depletion-induced oxidative stress [9]. Microtubule acetylation is a post-translational modification that promotes the binding and transport of kinesin-1 in neurons [10]. Multiple organelles are transported along microtubules by kinesins, and the breakdown of this process can lead to increased oxidative stress in neurons.

Oxidative stress caused by hydrogen peroxide leads to axonal swelling and beading, consistent with disruption of microtubules by oxidative stress and subsequent hold-up of axonal transport [11]. HDAC6 plays an important role in the modulation of mitochondrial transport. When inhibited with the specific HDAC6 inhibitor, tubacin, more kinesin-1 associates with mitochondria. HDAC6 activity is regulated by GSK3 β via phosphorylation [12]. Mitochondria in Alzheimer's disease brains are perinuclear, with few organelles in distal processes, where they are normally located in healthy cells and are needed for exocytosis, ion channel pumps, synaptic function and other activities. Alzheimer's disease neurons thus have an increased amount of reactive oxidative species and decreased metabolic capability [13]. Axonal transport of mitochondria and mitochondrial dynamics may also be disrupted in amyotrophic lateral sclerosis [14] and in retinal ganglion cell neurodegeneration [15], where oxidative stress is again a factor. Indeed, mitochondrial dysfunction is thought to play a key role in disease pathogenesis, because neurons are among the most energy-consuming cell types and have a highly developed cell shape [16].

Tau protein inhibits kinesin-dependent transport of peroxisomes into neurites, and loss of peroxisomes makes cells vulnerable to oxidative stress [17]. Here, again, tubulin acetylation may play a key role in promoting peroxisomal transport to distal processes, and the oxidative stress-induced breakdown of microtubules may lead to increased oxidative stress in these processes. Finally, Parkinson's disease toxins, such as rotenone, disrupt vesicle transport along microtubules, leading to increased accumulation of vesicles in the cell body and increased cytosolic concentration of dopamine, due to leakage from the vesicles. Elevated oxidative stress induced by dopamine oxidation may trigger apoptosis of dopaminergic neurons [18].

Loss of α -tubulin acetylation and impaired motor-protein transport are implicated in a number of neurological disorders, including Huntington's disease [19], familial dysautonomia [20] and amyotrophic lateral sclerosis, all of which occur through disruption of the α -tubulin acetylase activity of the Elongator complex [21]. These diseases are also characterised by oxidative stress [22]. It seems likely that the breakdown in transport of cargoes along microtubules due to defects in tubulin acetylation may play a key role in promoting oxidative stress and subsequent neurodegeneration, whereas increased α -tubulin acetylation and microtubule-based transport may act to alleviate oxidative stress. Transcription factor trafficking is also affected in oxidatively-stressed neurons [23].

3.2. 3-Nitrotyrosination

The conjugated aromatic ring of tyrosine is a target for free-radical attack, leading to the formation of 3-nitrotyrosine under conditions of oxidative stress [24]. α -Tubulin is tyrosinated at its C-terminus by the action of a tubulin-tyrosine ligase, and the C-terminal tyrosine can then be removed by a tubulin carboxypeptidase. This post-translational modification prevents the binding of kinesin-1 to microtubules in dendrites, thus navigating it to axonal microtubules [25]. High glucose and hyperglycemia can result in the production of high concentrations of reactive oxygen species with subsequent damage to the central nervous system. Indeed, in neuroblastoma and glioma cells exposed to D-glucose, there is a downregulation of tubulin isoforms and an increase in 3-nitrotyrosine modification of tubulin [26]. There is some controversy over the effect of 3-nitrotyrosine incorporation into tubulin on microtubule function. One study found that 3-nitrotyrosine incorporation into mammalian cells by tubulin-tyrosine ligase was irreversible and caused changes in microtubule organisation, cell morphology and the redistribution of the motor protein, dynein [27]. However, another study found that the incorporation of 3-nitrotyrosine into microtubules was reversible and not detrimental to dividing cells [28]. Therefore, the effect of 3-nitrotyrosine incorporation into microtubules is still an open question, but it may interfere with the correct localisation of neuronal motor proteins and MAPs.

3.3. Glutathionylation

Friedreich's ataxia is caused by mutations in the gene for frataxin, a mitochondrial protein implicated in iron metabolism. Loss of frataxin causes iron overload in tissues and an increase in free-radical production and oxidation. There is a significant rise in the dynamic pool of tubulin, possibly caused by increased protein glutathionylation [29]. Loss of glutathionylation of cytoskeletal proteins may give rise to oxidative stress, as this is a way that cells regulate microtubule cytoskeletal stability [29]. It has been recently suggested that microtubule glutathionylation, an increased intracellular level of oxidized glutathione, may cause an alteration of the cytoskeleton organisation and function, leading to axon degeneration [30].

4. Peroxidation of Lipids Damages Microtubules

Lipid peroxidation is regarded as a major cause of brain damage by oxygen radicals. The antioxidant, melatonin, prevents lipid peroxidation caused by okadaic acid in neuroblastoma cells and

prevents the disruption of the microtubule cytoskeleton [31]. Phosphatidylcholine hydroperoxides (PCOOHs) disrupt both the formation of neurites and neuronal microtubules. Differentiated cells are less susceptible to this form of attack than undifferentiated cells [32]. Another product of lipid peroxidation, acrolein, disrupts microtubules in PC12 cells, as well as sympathetic ganglion cells *in vitro* [33], and this may be due in part to increased phosphorylation of Tau protein [34]. In a model system of microtubule degradation by lipid-derived free radicals, it was shown that the kinetics of this process is determined by the level of lipid saturation and the presence of free radical scavengers [35]. Oxidised very-low-density lipoprotein and hydrogen peroxide reduced phosphorylation of the microtubule-destabilising protein, stathmin, whereas the lipid peroxidation product, 4-hydroxy-2-nonenal (HNE; an aldehyde), increased it, suggesting that free radicals, but not aldehydes, may play a key role in neuronal cell death caused by lipoprotein oxidation [36]. However, HNE disrupts microtubules and modifies cellular tubulin via Michael addition [37], as well as inhibiting dephosphorylation of Tau protein [38].

5. Oxidative Stress and MAPs

Oxidative stress leads to changes in the suite of MAPs found in neurons through changes in expression levels, post-translational modification, oxidative damage and breakdown of proteins by proteolysis.

5.1. Changes in MAP Expression Levels under Oxidative Stress

Expression levels of various MAPs change under conditions of oxidative stress in neurons. Some of these MAPs—MAP2, Tau and doublecortin, are microtubule-stabilising proteins, and the reduction in their expression levels may serve to destabilise microtubules. A proteomic analysis of proteins differentially regulated under acute oxidative stress showed that a number of MAPs changed their levels of expression. Doublecortin was downregulated. Collapsin response-mediator proteins (CRMPs) were upregulated. α -tubulin 6 was downregulated, and Tau protein and MAP2 were downregulated [39]. Other studies support these findings. Chronic ozone exposure causes an increase of reactive oxygen species and oxidative stress. Ozone causes cell swelling in neurons and a decrease in the MAP doublecortin and, also, chronic loss of brain repair in the hippocampus in adult rats [40].

Lanthionine ketimine (LK) is a member of a class of thioethers that are present in the mammalian central nervous system. LK interacts with CRMP2 and decreases CRMP2 coprecipitation with β -tubulin. A cell-permeable LK-ester, LKE, protects against oxidative challenge with hydrogen peroxide [41]. CRMP2 is a GTPase-activating protein, and this activity is important for microtubule assembly and neurite formation [42]. However, the fact that CRMPs are upregulated under oxidative stress and tubulin-associated CRMP decreases following treatment of neurons with neuroprotective LK indicates that CRMP has a role in promoting oxidative stress alterations of the microtubule cytoskeleton.

5.2. Oxidative Stress Modifies MAPs and Tubulin

Soluble oligomeric forms of amyloid beta-peptide induce oxidative stress in neurons, and this oxidative stress leads to the proteolysis of MAP1A, MAP1B and MAP2 by caspase-3 and calpain [43]. Quinones modify tubulin, leading to its aggregation into dimers and other oligomers [44], which

disrupt microtubule formation. The I93M mutation in ubiquitin C-terminal hydrolase L1 (UCH-L1) is associated with familial Parkinson's disease. UCH-L1 is a major target of oxidative/carbonyl damage associated with sporadic Parkinson's disease. Familial Parkinson's disease-associated and carbonyl-modified UCH-L1 in sporadic Parkinson's disease share aberrant properties, with both modified forms modulating tubulin polymerisation, and this gain of function may be relevant to both familial and sporadic Parkinson's disease [45]. M sulfoxides map to neuron-enriched β -tubulin in Alzheimer's disease brains, suggesting oxidative modification of β -III tubulin as a contributor to the neuronal cytoskeletal disruption characteristic of Alzheimer's disease [46].

Tau protein is implicated in neurodegenerative diseases, including Alzheimer's disease. Peroxynitrite induces the oxidative crosslinking and site-selective nitration of Tau monomers. This causes the formation of aggregates that are crosslinked by inter-filament bridges, inhibiting the ability of monomeric Tau to promote tubulin assembly [47]. Arg, a member of the Abl family of tyrosine kinases, phosphorylates Tau protein at Y394. Arg plays a key role in both oxidative stress response and neural development. Paired helical filaments that comprise neurofibrillary tangles in Alzheimer's disease contain an abnormally hyperphosphorylated form of Tau [48]. During mitochondrial inhibition and oxidative stress, actin-depolymerising factor (ADF)/cofilin assemble into rods along the processes of cultured neurons and recruit phosphorylated Tau protein. Microtubules are disrupted in processes that possess rods [49], suggesting that neurofibrillary tangles may be initiated by F-actin bundling caused by oxidative stress [50]. Oxidised products of dopamine (dopamine quinone) also promote Tau polymerisation, again suggesting a link between oxidative damage and the onset of tauopathies [51]. The peptidyl-prolyl cis/trans isomerase Pin1 regulates the function and processing of both Tau proteins and amyloid precursor protein (APP), both of which are implicated in Alzheimer's disease. In humans, the Alzheimer's disease brain Pin1 is downregulated by oxidative modification and/or genetic changes [52].

5.3. MAPs May Act to Protect Neurons against Oxidative Stress

Some MAPs appear to function to decrease oxidative stress in neurons. NAPVSIPQ (NAP), an 8-amino acid peptide derived from activity-dependent neuroprotective protein (ADNP), interacts with microtubules and protects microtubules from degradation under oxidative stress, probably inhibiting an early event of apoptosis [53]. NAP specifically binds β III-tubulin, and taxol reduces NAP-tubulin binding [54]. Parkin is a MAP that is mutated in familial Parkinson's disease. Elevating Parkin in cells reduces markers of oxidative stress, whereas blocking its expression increases oxidative stress. Parkin also acts as a ubiquitin ligase in the ubiquitin-proteasome system, and mitochondrial function is deficient in mouse and fly Parkin knockdown models. It is unclear which of Parkin's functions is neuroprotective [55]. Hydrogen peroxide treatment of neuronal and non-neuronal mixed rat retinal cell cultures causes the loss of MAP2 in the neuronal soma, and downregulation of MAP2 increases the vulnerability of retinal neurons to oxidative stress [56].

6. Tensegrity: Interactions between Microtubules, Actin and Neurofilaments in the Nervous System

Tensional integrity (tensegrity) architecture has been proposed to be a means by which cells stabilise their shape and sense mechanical signals from the nanoscale to the macroscale [57].

Importantly, these networks are in a state of isometric tension (*i.e.*, they experience a tensile prestress), ensuring that molecular-scale mechanochemical transduction mechanisms proceed simultaneously [58]. In cells, it is suggested that microtubules provide compression-resistant components and actin-filaments, tensional elements, in a unified tensegrity architecture whole. There is evidence to support this theory, with living cells behaving like discrete structures composed of an interconnected network of actin filaments and microtubules when mechanical stresses are applied to cell surface integrin receptors [59].

Short Stop (Shot) encodes a neuronally expressed family of proteins required for sensory and motor axon extension in *Drosophila melanogaster*. Shot isoforms contain an N-terminal F-actin and C-terminal microtubule-binding domains that crosslink F-actin and microtubules in cultured cells. Both the F-actin and microtubule-binding domains are required in the same molecule for axon extension [60]. Shots also play a key role in dendrite formation and axonal terminal arborisation at the neuromuscular junction, forming a complex with the microtubule plus-end-binding (+TIP) protein, EB1 [61]. Thus, there is evidence for the necessity of a linkage between microtubules and actin filaments in neuronal development, possibly due to the reliance of neurons upon cytoskeletal tensegrity architecture to maintain prestress.

Actin and neurofilaments are also crosslinked by dystonin, a member of the bullous pemphigoid antigen (BPAG) family of plakins [62]. Disruption of dystonin causes sensory neuropathy in mice and disorganisation of neurofilaments and microtubules [63]. Neurofilaments undergo strain stiffening suggesting that, as well as microtubules, they act as compression-resistant components of neuronal cytoskeletal tensegrity architecture [64]. Any perturbation of actin filaments, microtubules or neurofilaments, for example, by oxidative stress, will lead to a loss of prestress and the ability of the cytoskeleton to function as an integrated whole.

7. Actin and Oxidative Stress in the Nervous System

7.1. Actin and Actin-Binding Proteins Respond to Oxidative Stress

Interestingly, although actin and actin-binding proteins respond to oxidative stress, they appear less susceptible than microtubules. This may be in part due to the fact that the actin cytoskeleton actually utilises oxidative stress as a developmental mechanism.

Oxidative stress increases cellular actin aggregation and the binding of PINK1 to Parkin, both of which proteins are implicated in Parkinson's disease. Parkin also associates with actin, suggesting that oxidative stress may disrupt actin organisation through changing the binding substrates of Parkin [65]. Cu^{2+} -induced stress causes glutathione (GSH) adducts of cysteines of synaptosomal actin followed by its deglutathionylation and dimerisation. Torpedo cyclophilin B is able to sustain peroxiredoxin-1 activity and, thus, might be involved in presynaptic defence against oxidative stress affecting G-actin [66]. Oxidative stress results in a rapid dephosphorylation of cellular cofilin (an actin-binding protein) prior to its assembly into rod-shaped inclusions. The ATP-sensitive interaction of cofilin phosphatase chronophin with chaperone hsp90 mediates the formation of cofilin/actin rods [67].

TNF alpha and II-1beta stimulate a redox-dependent reorganisation of the actin cytoskeleton into lamellipodia under the regulation of Rac1 and a neuronal NADPH oxidase as the source of reactive

oxygen species. However, the persistent presence of reactive oxygen species provoked carbonylation of actin, which coincided with the loss of lamellipodia and the arrest of cellular plasticity [68]. Carbonylation of β -actin was also detected following the production of reactive oxygen species following ischemia-reperfusion in the hippocampus of the macaque monkey [69].

7.2. Glial and Astrocyte Actin Cytoskeletons

Astrocytes and glia modify their actin cytoskeletons to protect neurons against oxidative stress. Homocysteine is an excitatory amino acid that enhances the vulnerability of neuronal cells to oxidative injury. In astrocytes exposed to homocysteine, there is a dramatic change in the actin cytoskeleton and a change in cell morphology to fusiform and/or flattened cells with retracted cytoplasm. Neurons do not appear to change their actin cytoskeletal organisation in response to homocysteine. Thus, astrocytes reorganise their actin cytoskeleton in response to oxidative stress in order to protect neurons from its effects [70]. The oxidant, paraquat, induces rapid differentiation and proliferation of glial cells, including the expression of smooth muscle actin. In pure neuronal cultures, paraquat caused apoptosis of photoreceptors and amacrine neurons, and coculturing neurons with glial cells completely prevented this apoptosis. Thus, neuronal oxidative stress is ameliorated by the reorganisation of the actin cytoskeleton in glial cells [71].

8. Neurofilaments and Oxidative Stress

8.1. Neurofilament Phosphorylation and Oxidative Stress

Aggregation of neurofilament (NF) protein because of phosphorylation is a key process in neurodegenerative diseases, including Charcot-Marie-Tooth disease [72], Alzheimer's disease and amyotrophic lateral sclerosis [73]. Injection of quinolinic acid increases phosphorylation of the low molecular weight NF subunit in neurons, whereas N-methyl-D-aspartic acid (NMDA) antagonist MK-801 and the antioxidant, N omega-nitro-L-arginine methyl ester (L-NAME), prevent this hyperphosphorylation. The hyperphosphorylation is targeted at the Ser55 phosphorylating site on the NFL head domain, a regulatory site for NF assembly *in vivo* [74]. In the spinal cord in Friedreich's ataxia, there is an abnormal distribution of phosphorylated forms of NFs [75]. Pin1 modulates oxidative stress-induced phosphorylation of high molecular weight NF protein. Cyclin-dependent kinase 5 (cdk5) phosphorylates NF protein, and overexpression of cdk5 inhibits NF axonal transport and induces accumulation of disordered phospho-NF cables. Oxidative stress recapitulates this phenotype in a cdk5-dependent manner [76].

8.2. Oxidative Stress Modifies Neurofilaments

The lipid peroxidation product, acrolein, leads to the generation of protein carbonyl compounds and the formation of NF aggregates by dityrosine crosslinking. These aggregates display thioflavin T reactivity, reminiscent of amyloid [77]. In Alzheimer's disease brains, there is an increase in N-malondialdehyde-lysine formation targeting NFL proteins, suggesting an important role for lipid-peroxidation-derived protein modification in Alzheimer's disease pathogenesis [78]. Neuroaxonal spheroid aggregates in leukoencephalopathy contain amyloid and NF proteins, and there

is an increase in markers for oxidative stress [79]. The lipid peroxidation product, HNE, causes the formation of adducts in NF proteins, but here, it is suggested that HNE may play a protective role against toxic aldehydes resulting from oxidative damage, due to its consistency from birth to senility and its dependence on phosphorylation [80]. Modification of NFs by oxidative advanced glycation end-products in diabetic neuropathy leads to axonal degeneration [81]. Oxidative stress also affects the neuronal cytoskeleton through proteolysis and/or abnormal structural changes in NFs [82], consistent with antioxidant treatment of dogs, which leads to a decrease in protein carbonyl levels of NF triplet L protein [83].

9. Conclusions

We have demonstrated that the nervous system cytoskeleton plays a key role in oxidative stress responses and the onset of oxidative stress-induced neurodegeneration. Drugs that target the cytoskeleton, for example, the HDAC6 inhibitor, tubastatin, may thus be of great use in treating various neurodegenerative diseases caused by oxidative stress. Diet, too, may be important, with zinc availability, for example, causing alterations in tubulin thiol redox status [84]. The development of a polypeptide inhibitor of cdk5, potentially useful in reducing protein aggregation caused by the phosphorylation of NF and Tau proteins, is undergoing tests in mice [85]. The development of an inhibitor against the Elongator complex, α -tubulin acetylase, would provide key insights into the role of tubulin acetylation in oxidative stress responses, while a proteomic approach to the identification of cytoskeleton-associated proteins under oxidative stress conditions would be of great interest.

Conflicts of Interest

The authors declare no conflict of interest.

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