
REVIEW

Oxidative Stress in Immature Brain Following Experimentally-Induced Seizures

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Summary

The existing data indicate that status epilepticus (SE) induced in immature animals is associated with oxidative stress and mitochondrial dysfunction. This has been demonstrated using two models of SE, induced by substances with a different mechanism of action (DL-homocysteic acid and 4-aminopyridine) which suggests that the findings are not model-dependent but they reflect more general phenomenon. Oxidative stress occurring in immature brain during and following seizures is apparently due to both the increased free radicals production and the limited antioxidant defense. Pronounced inhibition of mitochondrial complex I in immature brain was demonstrated not only during the acute phase of SE, but it persisted during long periods of survival, corresponding to the development of spontaneous seizures (epileptogenesis). The findings suggest that oxidative modification is most likely responsible for the sustained deficiency of complex I activity. It can be assumed that the substances with antioxidant properties combined with conventional therapies might provide a beneficial effect in treatment of epilepsy.

Key words

Immature rats • Experimentally-induced seizures • Oxidative stress • Mitochondrial dysfunction • Antioxidant defense

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Introduction

There is a large body of evidence that oxidative stress and mitochondrial dysfunction are implicated as an important factor in the pathogenesis of many neurological disorders, including epilepsy (Patel 2004, Lin and Beal 2006, Waldbaum and Patel 2010, Ikonomidou and Kaindl 2011, Folbergrová and Kunz 2012). Oxidative stress can be defined as an imbalance between reactive oxygen and/or reactive nitrogen species (ROS and/or RNS) production and antioxidant defense against these species. It may arise from an over-production of ROS and/or RNS, a reduction in the tissue's intrinsic antioxidant defense mechanisms or a combination of both (Albers and Beal 2000, Hardingham and Lipton 2011).

Free radicals are normal intermediates in aerobic metabolism of all cells and they can be produced by a number of pathways. The major source of ROS is considered mitochondria (Turrens 2003, Murphy 2009). It is becoming recognized that ROS and RNS have a dual biological role: in low or moderate concentrations they play an important role in normal physiological functions influencing several signaling pathways (Dröge 2002), but at excessive levels they are harmful to living systems. There exist various endogenous antioxidant defense mechanisms, both enzymatic and non-enzymatic, which within certain limits can counteract increased ROS/RNS production. However, under conditions of aberrant formation of free radicals the endogenous antioxidant capacity can become overwhelmed leading to oxidative stress and potential oxidative damage to cellular proteins, lipids, nucleic acids (with consequent accumulation of dysfunctional proteins, lipid peroxidative

products and damaged nuclear or mitochondrial DNA) and to disruption of redox signaling and control (Halliwell 2006, Jones 2006, Sayre *et al.* 2008).

The consequence of mitochondrial dysfunction is not only decreased ATP production, but also other potentially detrimental events, such as impaired intracellular calcium homeostasis, induction of permeability transition, increased generation of free radicals and oxidative stress, all of which predispose cells to necrosis or apoptosis (Folbergrová and Kunz 2012).

Oxidative stress in immature brain following seizures

The widely-held opinion was that oxidative stress during seizures is age-dependent and it does not occur in immature brain. This assumption was based on the negative findings concerning ROS formation and various markers of oxidative stress in immature rats during seizures induced by kainate (Bruce and Baudry 1995, Patel and Li 2003, Sullivan *et al.* 2003). Findings of recent studies using other models of seizures clearly demonstrate that oxidative stress may also occur in immature brain during seizures. Increased free radical formation was reported in one-day-old chicken during prolonged seizures induced by Li-pilocarpine (Tsai *et al.* 2010). When seizures, having a character of status epilepticus (SE), were induced in immature 12-day-old rats by homocysteic acid (an endogenous excitatory amino acid with a potent activity on NMDA receptor), massive neuronal degeneration has been demonstrated in many brain regions (Folbergrová *et al.* 2005). The increased free radical production was suggested by the fact that the treatment with the spin trapping agent N-tert-butyl- α -phenylnitron (PBN) provided a clear-cut protective effect, evident as the improved recovery of brain energy status and as a partial, but significant attenuation of neuronal degeneration associated with this model (Folbergrová *et al.* 2006). The presence of oxidative stress in this model of SE (HCA) was detected by several indirect markers, such as increased lipid peroxidation, decreased activity of aconitase, increased H₂O₂ production in isolated mitochondria (Folbergrová *et al.* 2007) and significant increases of three mitochondrial markers of oxidative damage, namely 3-nitrotyrosine (3-NT), 4-hydroxynonenal (4-HNE) and protein carbonyls (Folbergrová *et al.* 2010). Furthermore, a marked decrease of mitochondrial complex I activity was observed (Folbergrová *et al.* 2007, 2010). The presence

of oxidative stress was confirmed by a direct approach, measuring superoxide anions in situ (using hydroethidine method). Increased O₂⁻ formation was detected in all the studied brain regions, namely CA1, CA3 and dentate gyrus of the hippocampus, cerebral cortex and thalamus following 60 min lasting HCA-induced seizures (Folbergrová *et al.* 2012). Similar results were also obtained using another model of SE, induced in immature rats by 4-aminopyridine (4-AP) (a potassium channel blocker) (Folbergrová *et al.* 2011). The increased formation of superoxide anion in both models was completely prevented by the treatment with superoxide dismutase (SOD) mimetics, (Mn(III)tetrakis (1-methyl-4-pyridyl) porphyrine pentachloride (MnTMPYP) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol).

Mitochondrial dysfunction in immature brain

The role of mitochondrial function/dysfunction in relation to epilepsy has been studied in humans (Kunz *et al.* 2000, Lee *et al.* 2008) and in several experimental models of epilepsy in adult animals (Cock *et al.* 2002, Kudin *et al.* 2002, Chuang *et al.* 2004, Slevin *et al.* 2006). It has been shown recently that similar signs of mitochondrial dysfunction also occur in immature animals with seizures.

Complex I (NADH-ubiquinone oxidoreductase) of the respiratory chain contains in mammals at least 45-46 subunits, flavin mononucleotide (FMN), about eight iron-sulfur clusters and one or more ubiquinone binding sites (Walker 1992). Its primary function is transfer of electrons from NADH to ubiquinone that is coupled to translocation of protons across the inner mitochondrial membrane. The accumulating evidence indicates an extreme sensitivity of complex I to both oxidative and nitrosative stress (Robinson 1998, Sriram *et al.* 1998, Bautista *et al.* 2000, Brown and Borutaite 2004, Kudin *et al.* 2004, Papa *et al.* 2008). Complex I deficiency is the frequent cause of mitochondrial human diseases and it was also observed in various neurodegenerative disorders, particularly in Parkinson's disease (Shapira *et al.* 1990, Tretter *et al.* 2004). Severe impairment of complex I was also detected in hippocampal subfields from patients with chronic temporal lobe epilepsy (Kunz *et al.* 2000) and in hippocampal samples from adult rats after pilocarpine-induced (Kudin *et al.* 2002) or kainic acid-induced (Chuang *et al.* 2004) status epilepticus (SE).

In HCA model of SE, induced in immature rats,

marked decrease (~60 %) of complex I activity was determined in cerebral cortex mitochondria during the acute phase of seizures (Folbergrová *et al.* 2007) and this decrease persisted during long periods of survival (up to 5 weeks), i.e. periods corresponding to the development of spontaneous seizures (epileptogenesis) in this model (Folbergrová *et al.* 2010). The decrease was selective for complex I and it was not associated with changes in the size of the assembled complex I or with changes in mitochondrial content of complex I. The decrease of complex I activity was substantially reduced by treatment with selected free radical scavengers, namely SOD mimetics MnTMPYP and Tempol and a selective peroxynitrite scavenger and decomposition catalyst 5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinate Iron (III) (FeTPPS) (Folbergrová *et al.* 2007, 2010). Furthermore, as already mentioned above, concurrently with the decreased activities of complex I significant increases in three markers of mitochondrial oxidative damage (3-NT, 4-HNE and protein carbonyls) were detected (Folbergrová *et al.* 2010). All these findings strongly suggest that oxidative modification (inactivation) of complex I is very likely responsible for the sustained deficiency of complex I activity, in accordance with extreme sensitivity of this enzyme to ROS and RNS.

It was postulated that oxidative modification might be localized on some critical subunit of complex I and thus lead to the decreased activity. This possibility has been recently confirmed by Ryan *et al.* (2012). These authors, using kainate model of SE in adult rats, demonstrated during both the acute and chronic phases of epileptogenesis decreased activity of complex I. Mass spectrometry analysis identified specific metal catalyzed carbonylation to arginin 76 within the 75 kDa subunit of complex I. Computational-based molecular modeling studies predicted that carbonylation at this site within the 75 kDa subunit can induce substantial structural alterations to the protein complex leading to the impaired function. It remains to be determined whether similar specific and irreversible oxidative modification may also occur on complex I of immature rats during and following SE when increases of mitochondrial protein carbonyls concomitant with the persistent inhibition of complex I activity were observed (Folbergrová *et al.* 2010).

Most likely also other posttranslational oxidative modifications of complex I can occur. Thus modification leading to complex I inhibition is nitration of tyrosine

(and/or tryptophane) residues within the complex, apparently due to peroxynitrite or peroxynitrite-derived radicals (Riobó *et al.* 2001, Radi *et al.* 2002, Yamamoto *et al.* 2002, Murray *et al.* 2003, Singh *et al.* 2007). Murray *et al.* (2003) reported that exposure of enriched bovine heart complex I preparations to peroxynitrite resulted in a significant inhibition of complex I activity and at the same time five complex I subunits were found to be nitrated (contained 3-nitrotyrosine). Similar findings were reported by Pearce *et al.* (2005) demonstrating that irreversibly inhibited activity of complex I was accompanied by formation of 3-NT in 3 out of 46 subunits. Based on significant increases of 3-NT in brain mitochondria of immature rats during and following HCA-induced seizures (Folbergrová *et al.* 2010), it is tempting to postulate that similar mechanism might contribute to the sustained inhibition of complex I activity.

Complex I has been shown to be also inhibited by S-nitrosation of some of its protein thiols (mostly cysteine residues) to form S-nitrosothiols (Burwell *et al.* 2006, Dahm *et al.* 2006). However, other potential mechanisms can be considered, e.g. ROS-induced oxidation of cardiolipin, a phospholipid required for optimal activity of complex I (see Petrosillo *et al.* 2008) or iron-sulfur clusters can be a potential target similarly as proposed for inactivation of aconitase. It remains to be clarified by future experiments employing appropriate methodologies which of the above mentioned oxidative mechanisms may be involved in inactivation of complex I in immature brain following SE.

It can be expected that such a marked inhibition of complex I will have significant implications for mitochondrial function. It has been shown that pronounced inhibition of complex I was not accompanied by impaired ATP production, apparently due to excess capacity of complex I demonstrated by energy thresholds, i.e. the extent to which mitochondrial complex activities can be inhibited before significant changes in oxidative phosphorylation occur (Folbergrová *et al.* 2007, 2010 and references therein). There are, however, other potential consequences of inhibited complex I. It is well established that complex I is not only a target for ROS and RNS, but it is also the important source of ROS and/or RNS production, especially when partially inhibited (Sipos *et al.* 2003, Kudin *et al.* 2004, Kussmaul and Hirst 2006, Fato *et al.* 2008, Parihar *et al.* 2008). It can thus be assumed that increased ROS and/or RNS production may contribute to neuronal injury

demonstrated in HCA model of SE (Folbergrová *et al.* 2005, 2006, 2008).

In addition to complex I, also other sensitive targets can be attacked by ROS and/or RNS. Tyrosine nitration has been e.g. detected on manganese-superoxide dismutase with concomitant enzyme inactivation (Yamakura *et al.* 1998, MacMillan-Crow *et al.* 1998, Bayir *et al.* 2007) or on glutamine synthetase (Bidmon *et al.* 2008), accompanied by the reduction of enzyme activity. Since MnSOD is an important enzyme for removing superoxide anions in mitochondria, its inhibition can result in increased oxidative stress.

It has been shown that high-affinity astroglial and neuronal glutamate transporters, which are important for the maintaining low levels of synaptic glutamate, are extremely sensitive to oxidative damage (Trotti *et al.* 1998). Their inhibition (similarly as inhibition of glutamine synthetase) can potentially contribute to the increased excitability seen during development of spontaneous seizure, i.e. to be a potential contributor to epileptogenesis. Interestingly, increased glial glutamate transporter EAAT2 expression was shown recently to reduce epileptogenic process following pilocarpine-induced SE (Kong *et al.* 2012).

Another target sensitive to oxidative damage is DNA, particularly mitochondrial (mtDNA), as demonstrated in experimental models of epilepsy in adult rats (e.g. Kudin *et al.* 2002, Jarrett *et al.* 2008). There is a lack of knowledge at present concerning SE induced in immature animals.

Furthermore, polyunsaturated fatty acids present in phospholipids of biological membranes are highly susceptible to oxidation by ROS. Oxidative damage to lipids, evident as an increase of several markers of lipid peroxidation (increase of F(2)isoprostanes, isofuranes, thiobarbituric acid reactive substances, 4-hydroxynonenal) has been detected following SE induced both in adult (Dal-Pizzol *et al.* 2000, Patel *et al.* 2001, 2008) and immature animals (Folbergrová *et al.* 2007, 2010).

Antioxidant defense in immature brain

There exist several endogenous enzymatic and non-enzymatic antioxidant defense mechanisms against free radicals. The superoxide anion ($O_2^{\cdot-}$) is one of the most reactive ROS and it is the precursor of other reactive species. These reactive species are controlled *via* multiple enzyme systems, comprising superoxide

dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GR) and thioredoxin/peroxiredoxin systems (Fridovich 1995, Chen *et al.* 2006, Day 2009, Toppo *et al.* 2009, Cox *et al.* 2010).

There are many studies concerning antioxidant enzymes under seizures induced by various convulsant in adult animals (for references see Folbergrová *et al.* 2013). There are, however, limited data concerning immature animals following seizures. Bruce and Baudry (1995), using kainate (KA) model of seizures induced in rats of PD12 and PD 17, did not observe any change in the activities of SOD, GPX and catalase at various periods studied (at 8, 16, 48 h and 5 days after KA administration). However, in their study no signs of oxidative stress could be detected. A recent study of Tsai *et al.* (2010) demonstrates that prolonged seizures in one-day-old chicken induced by Li-pilocarpine resulted in the increased free radical production and the decreased activities of CuZnSOD and catalase. Recent study (Folbergrová *et al.* 2013) demonstrates activities of major antioxidant enzymes in cerebral cortex of immature rats during the acute phase of seizures induced at PD 12 by DL-HCA and during several periods of survival following these seizures. In HCA-treated animals the activity of total SOD, SOD1 (CuZnSOD) and particularly SOD2 (MnSOD) significantly increased at 20 h and 6 days of survival. Upregulation of SOD2 was also confirmed in mitochondria at the protein level by immunoblotting. The detected increase of both the activity and content of SOD2 is apparently a reaction to the increased mitochondrial production of superoxide anion. Literary data suggest that the generation of superoxide anion at the level of complex I is directed into the mitochondrial matrix (Turrens 2003, Murphy 2009). Importantly, MnSOD that was shown to be upregulated is localized also in the matrix. It should be emphasized that the upregulation of SOD2 was not accompanied by similar changes in the activity of catalase and/or GPX as might be expected as a compensatory mechanism in response to the increased H_2O_2 . The activities of other antioxidant enzymes including catalase and GPX did not significantly differ upon HCA treatment from the appropriate controls at any of the studied time intervals (Folbergrová *et al.* 2013). It is worthwhile to note that in the studies on adult animals with seizures, the increased SOD activity was always accompanied by an increased activity of catalase and/or GPX provided that activity of these enzymes were analyzed (Bruce and Baudry 1995,

Freitas *et al.* 2004, Tejada *et al.* 2006, 2007, Freitas 2009, Peternel *et al.* 2009). These findings thus suggest that antioxidant defense in brain of adult animals may be apparently well developed. On contrary, the existing data suggest that the antioxidant defense in brain of immature rats during and following seizures is apparently limited. The upregulation of MnSOD as the response to the increased O_2^- production may not be beneficial in view that the increased H_2O_2 (as a consequence of increased O_2^- dismutation) may not be sufficiently removed. The increased levels of H_2O_2 in the presence of reduced transition metals can lead *via* Fenton reaction to formation of very toxic hydroxyl radicals. It can thus be assumed that insufficient antioxidant defense in immature brain can have potentially serious consequences.

As to the non-enzymatic antioxidant defense mechanisms, several substances such as vitamin C, vitamin E, DL- α -lipoic acid have been shown to provide a partial antioxidant effect in various models of seizures in adult animals (Waldbaum and Patel 2010). However, clinical trials have been so far controversial.

The most abundant intracellular non-enzymatic antioxidant defense is reduced glutathione (GSH) which is oxidized to its disulfide redox partner GSSG. GSH/GSSG ratio is commonly used biomarker of redox status and oxidative stress. The time-dependent decrease of this ratio was reported in KA and LiPilo model of SE in adult animals (Liang and Patel 2006, Waldbaum *et al.* 2010). Similar results were obtained using alternative redox couple, reduced coenzyme A (CoASH) and its disulfide with GSH (CoASSG). Ratios of hippocampal GSH/GSSG and CoASH/CoASSG following Li-Pilo-induced SE in adult rats decreased by 24 h and the decrease persisted throughout the epileptogenesis (Waldbaum *et al.* 2010). The altered redox status provides further evidence for seizure-induced mitochondrial oxidative stress. Whether SE induced in immature animals is associated with changes in GSH and GSSG and /or CoASH/CoASSG levels remains to be determined by future experiments.

Antioxidant therapy

As mentioned above, existing data suggest the association of seizures and SE with oxidative stress, both in adult and immature animals. It is thus conceivable that efforts have been aimed at developing the substances capable of detoxifying ROS and/or RNS and thus potentially providing a protective effect. Synthetic

metalloporphyrin catalytic antioxidants (small molecule mimics of SOD and/or catalase and some of them also potent detoxifiers of lipid peroxides and peroxynitrite) (Patel and Day 1999, Liang *et al.* 2012) appear to be a promise as a novel neuroprotective agents. Some of these compounds have been shown to be effective in animal models of epilepsy, lessening oxidative stress and neuronal damage induced by SE in adult animals (e.g. Rong *et al.* 1999, Liang *et al.* 2000, 2012). As already mentioned above, they can be also effective in immature brain. The increased formation of O_2^- associated with SE induced by HCA (Folbergrová *et al.* 2012) or 4-AP (Folbergrová *et al.* 2011) was completely blocked by two employed SOD mimetics MnTMPYP and Tempol. These antioxidants and also selective peroxynitrite scavenger FeTPPS provided significant attenuation of complex I inhibition, both during the acute phase of seizures (Folbergrová *et al.* 2007) and during the period of epileptogenesis (Folbergrová *et al.* 2010). In addition, treatment with MnTMPYP and Tempol resulted in a partial amelioration of neuronal degeneration associated with HCA model of SE (Folbergrová *et al.* 2011, 2012). It can be assumed that the efficient SOD and/or catalase mimetics should possess not only SOD activity but also sufficient catalase activity in order to detoxify the increased H_2O_2 levels, since the later (as mentioned above) can lead *via* Fenton reaction to very toxic hydroxyl radicals.

Improvement of neuroprotective effect can be expected when redox-modulating metalloporphyrin catalytic antioxidants with markedly enhanced lipophilicity (Sheng *et al.* 2011, Rajic *et al.* 2012) will be available commercially. It can also be assumed that antioxidants with multiple effects, interfering with several signaling pathways, like transcription of endogenous antioxidant genes and other (Kroon *et al.* 2010, Sahebkar *et al.* 2010) can have better chance for beneficial effect. Promising substance appears to be e.g. resveratrol, a natural polyphenol present in red wine whose neuroprotective effect has been reported in various models of neurological disorders in adult animals.

It should be noted that the existing data suggest that administration of various antioxidant compounds, in spite of their marked neuroprotective effect, do not alter behavioral seizure intensity or frequency, in experimental models of epilepsy, both in adult and immature animals (e.g. Rong *et al.* 1999, Liang *et al.* 2000, Frantseva *et al.* 2000, Folbergrová *et al.* 2006, 2010, 2012). This suggests that they are lacking an obvious anticonvulsant effect. It

remains to be determined whether or not they could influence the development of spontaneous seizures, i.e. to be antiepileptogenic. To achieve potential beneficial effect the sustained treatment during the long period will be most likely required (Sheng *et al.* 2009). Since ROS and RNS in low concentration serve important physiological functions, the chronic administration of antioxidant compounds should be performed with a caution to avoid potential unwanted consequences.

In conclusion

The existing data clearly indicate that SE induced in immature animals is associated with oxidative stress and mitochondrial dysfunction. This has been demonstrated in two models of SE, induced by substances with a different mechanism of action (HCA and 4-AP) which suggests that the findings are not model-dependent but they reflect more general phenomenon. Oxidative stress occurring in immature brain during and following seizures is apparently due not only to the increased free radicals production but also due to the limited antioxidant defense. Pronounced inhibition of mitochondrial complex I in immature brain was

demonstrated not only during the acute phase of SE, but it persisted during long periods of survival, corresponding to the development of spontaneous seizures (epileptogenesis). The findings suggest that oxidative modification may be most likely responsible for the sustained deficiency of complex I activity.

The up to now findings suggest that the substances with antioxidant properties combined with conventional therapies might provide a beneficial effect in treatment epilepsy.

Conflict of Interest

There is no conflict of interest.

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