ROS

REVIEW ARTICLES

The Role of Reactive Oxygen Species in Epilepsy

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ABSTRACT | Excess of reactive oxygen species (ROS) is increasingly recognized as a key factor in seizure-induced neuronal damage. Thus, targeting ROS is a priority to prevent seizures and epilepsy comorbidities such as cognitive decline, which are largely driven by neuronal damage. One drawback of this approach is that moderate levels of ROS are important in physiological cellular function and adaptation. This may also explain why antioxidant strategies targeting the brain have largely been unsuccessful. To overcome this difficulty, more fine-tuned ROS suppression in epilepsy is warranted. To achieve this goal, it is necessary to identify the key producers of ROS in seizures and epilepsy. Recent studies show that NADPH oxidase and xanthine oxidase are enzymes generating ROS in settings where energy metabolism is high, such as seen during seizure activity. An alternative approach is by targeting inducible networks of cellular antioxidant defences such as the Keap1–Nrf2 system. Nrf2 has emerged as a powerful regulator of endogenous antioxidant defences, and one drug (dimethyl fumarate) activating the Nrf2 pathway is already in use in inflammatory central nervous system (CNS) diseases. Both targeting key producers of ROS during seizure activity and Nrf2-enhancing strategies represent exciting new avenues of drug discovery in epilepsy.

KEYWORDS | Mitochondria; NADPH oxidase; Nrf2; Reactive oxygen species; Seizures; Xanthine oxidase

ABBREVIATIONS | ARE, antioxidant response element; ATP, adenosine triphosphate; CNS, central nervous system; GSH, reduced glutathione; Keap1, Kelch-like ECH-associated protein 1; MDA, malondialdehyde; MPTP, mitochondrial permeability transition pore; NMDA, *N*-methyl-*D*-aspartate; NMDAR, NMDA receptor; NOX, NADPH oxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; SERCA, sarcoendoplasmic reticulum calcium transport ATPase; SOD, superoxide dismutase; XO, xanthine oxidase

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1. INTRODUCTION

The term reactive oxygen species (ROS) is used to summarize oxygen radicals such as superoxide and hydroxyl radical, as well as H_2O_2, which readily forms oxygen radicals [1]. ROS are by-products of many biological reactions and generated at a low level during normal cellular activity. Initial interest in ROS was centred around the role of these species in cell damage as a result of ageing [2]. Later, the importance of ROS in physiological signalling was discovered, and the impact of ROS on cell damage in different acute and chronic diseases was recognized [3–5]. It is important to note that the reactivity of ROS varies from highly reactive species, such as the hydroxyl radical, to much less reactive species, such as hydrogen peroxide. Conversion of hydrogen peroxide into hydroxyl radicals is promoted by reduced transition metals, such as ferrous or cuprous ions, which is known as Fenton reaction [6]. This plays a role in iron and copper rich tissues such as the brain.

One important aspect to note is that ROS are produced constantly within the cell. This background ROS production is not only a by-product of enzymatic reactions such as at complexes I and II of the mitochondria, but plays a role in cellular adaptive mechanisms and signalling [7, 8]. It is only when this physiological ROS load is exceeded that the above mentioned mechanisms of cellular damage apply which are important in human disease.

But how do ROS cause cellular damage and cell death? First, ROS can lead to protein oxidation and thus dysfunction of enzymes and also to oxidative DNA damage. Damage to DNA can result in activation of repair enzymes, such as poly(ADP-ribose) polymerase (PARP), leading to rapid ATP depletion, thereby stimulating cell death cascades [9, 10]. Second, ROS contribute to mitochondrial permeability transition pore (MPTP) opening by increasing Ca^{2+} load. MPTP opening indicates a point of no return, resulting in a cascade leading to cell death. Ca^{2+} overload is a powerful trigger of MPTP, and ROS contribute to such overload via stimulating IP_3/ryanodine receptors and inhibiting SERCA pumps and plasma membrane Ca^{2+} channels (PMCA) [11]. Besides the indirect effect of ROS on MPTP opening, ROS have an immediate, direct effect on MPTP opening, and MPTP opening can be triggered by substances that increase ROS such as *tert*-butyl hydroperoxide (*t*-BHP) or phenylarsine oxide (PAO) [12–14]. Third, ROS can react with lipids in cell membranes, a process termed lipid peroxidation. ROS degrade polyunsaturated lipids, forming malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and thereby increase instability of the cell membranes.

2. ROS PRODUCERS AND ROS SCAVENGERS FORM A FINE-TUNED SYSTEM DETERMINING CELLULAR ROS ENVIRONMENT

The level of ROS within the cell depends on the activity of ROS producers and scavengers. A balance exists with extreme ends of the spectrum represented by excessive ROS production leading to cell death on the one side, and loss of physiological ROS signalling on the other side (**Figure 1**).

ROS scavengers, or antioxidants, are specialized molecules within the cell. ROS scavengers are either enzymes, such as catalase and superoxide dismutase (SOD), or non-enzymatic substances, including glutathione (**Figure 1**). Glutathione is certainly one of the most important antioxidants in the cell, which is typically found in millimolar concentrations. The maintenance of glutathione in the reduced state is dependent on NADPH, which is provided via the pentose phosphate pathway, an important pathway of glucose metabolism.

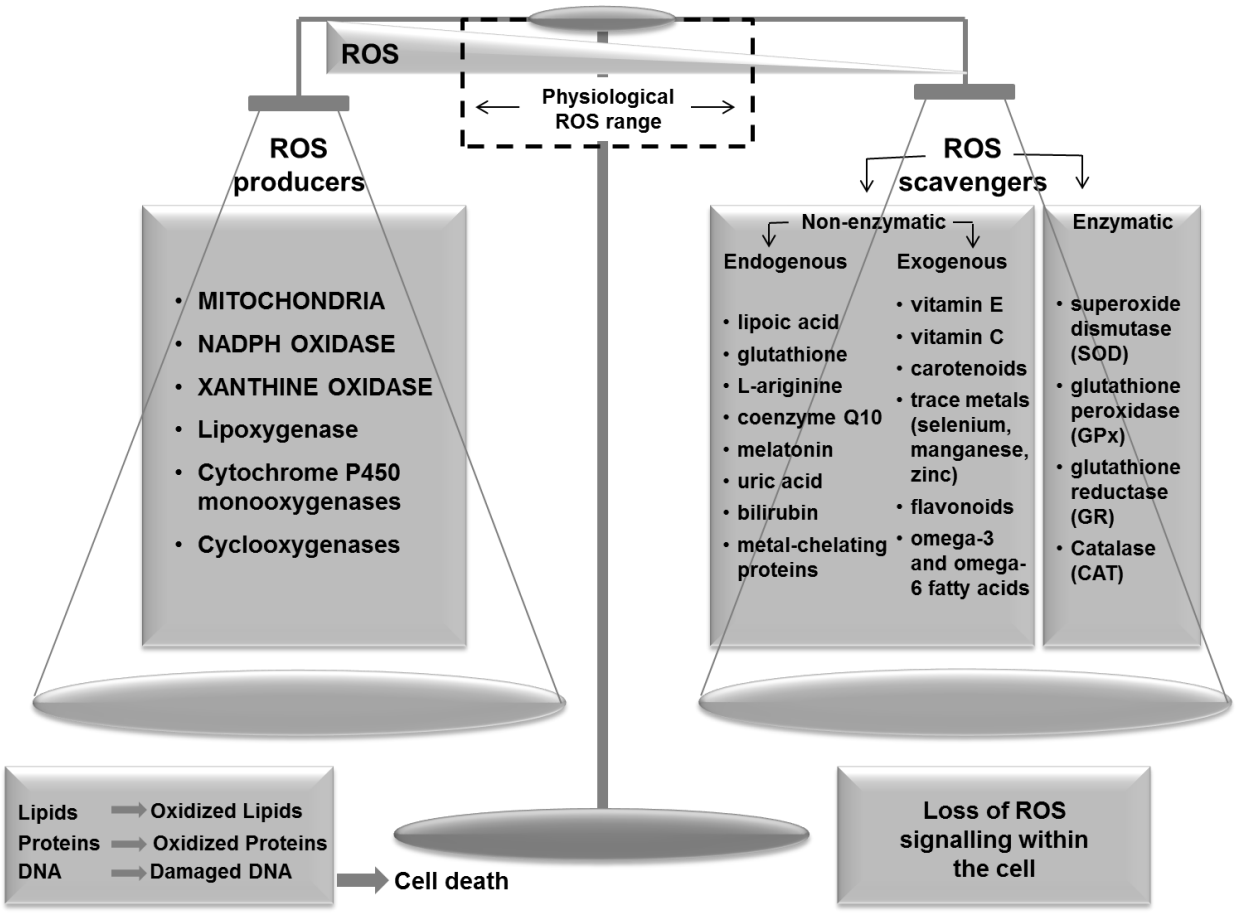

FIGURE 1. Cellular ROS homeostasis. See text (Section 2) for detailed description.

Different sources of ROS exist within the cell. These can be classified according to the (1) enzymatic and non-enzymatic reactions which lead to ROS generation [15], (2) compartments within the cell generating ROS, and (3) types of ROS generated. Interestingly, although it is likely that the type of ROS and the compartment generating ROS will have an impact on their toxicity to adjacent structures, not much attention has been paid to these factors. It is very difficult to determine the relative contribution of a particular ROS source to the net ROS burden and even more difficult to estimate the burden of ROS induced cell damage within the cell [16]. This is due to the fact that often in sub-compartments of the cell where ROS are produced in large amounts, scavengers are also readily available as exemplified by peroxisomes, where catalase, as an ROS scavenger, is abundant. This highlights that estimations of net ROS production are highly dependent on methods measuring ROS in the whole cells (i.e., in a physiological setting) [17]. Highly specialized antioxidants, such as catalase, are an important tool of self-defence within the cell whereby small concentrations of enzymes provide high efficiency buffering of ROS. Another form of ROS buffering is provided by non-enzymatic reactions (**Figure 1**).

It is even more difficult to estimate the relative contribution of different sources of ROS to the overall ROS production in the brain in vivo. The estimates that are available are based on non-physiological models such as isolated mitochondria. In addition, studies have used succinate as a substrate to stimulate ROS production [18]. Succinate is a non-physiological substrate which stimulates maxi-

mally mitochondrial ROS production, thereby limiting conclusions which can be drawn under physiological conditions [16]. These methodological limitations have been pointed out previously, and the cytosolic contribution to ROS production in homogenates is very likely underestimated [18].

3. ROS AND THE BRAIN

Whereas ROS contribute to disease in many organs, this is particularly true for the brain. The brain contributes to ~2% to the total body weight, yet consumes 20% of the oxygen through oxidative phosphorylation [19, 20]. Not only is there an abundance of oxygen in the brain which fuels production of ROS, but downstream ROS induced damage is also more likely given the high levels of oxidizable polyunsaturated fatty acids (PUFAs) in the brain [21]. In addition, redox active metals such as copper and iron are present throughout the brain with particularly high levels in the basal ganglia [22].

Detrimental effects of ROS have been shown in neurodegenerative disorders [23, 24] and also in acute neurological diseases, such as stroke [25]. Besides cell damage in ischemia, excessive neuronal activity such as that seen in seizures and epilepsy has been shown to be mediated by ROS [26, 27].

Epilepsy is one of the most common neurological diseases with a high incidence and prevalence [28]. Epilepsy has devastating consequences, and antiepileptic medication fails to control seizures in a third of patients warranting new treatment strategies [29]. Epilepsy is a chronic condition which has a significant impact on patient's life. This is not only due to the debilitating nature of seizures, but also largely driven by co-morbidities including depression and cognitive decline [30, 31].

Epilepsy is defined as recurrent unprovoked seizures with seizures reflecting excessive neuronal activity [32]. The excessive neuronal activity can lead to cell death, and it is believed that such cell death contributes to epileptogenesis itself as well as co-morbidities seen in epilepsy [33]. Cell death in epilepsy is triggered by a cascade of events with calcium overload through NMDA receptors and subsequent excess ATP consumption to restore calcium homeostasis, representing the major initial hits to cell homeostasis. Both events are linked to an increase in enzymatic ROS production, with NMDA receptor opening triggering ROS production through NADPH oxidase [34] and ATP depletion feeding ROS production through xanthine oxidase given that breakdown of ATP in situations of high energy demand leads to increases of ATP metabolites which are substrates of xanthine oxidase [35]. ROS are produced during seizure activity and lead to cell death in prolonged seizures and epilepsy [26, 27]. ROS therefore are promising targets to address seizure-induced cell death. However, we and others have also shown that signalling cascades in the brain are fine-tuned by ROS [8, 36, 37]. Brain protection from ROS-induced injury, therefore, has to take this into account. How to effectively target ROS remains elusive. In the past, antioxidant strategies have mainly relied on antioxidants, scavengers of ROS. However, these have largely failed [38–40] warranting development of new treatment strategies.

Strategies aimed at more fine-tuned ROS homeostasis may solve this problem. Such strategies may aim at inhibiting sources of ROS rather than dampening overall ROS content within cells via antioxidants. Another option is to target endogenous key players of antioxidant defences. Such a key player is the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is a key transcription factor mediating protection against electrophiles and oxidants, thereby enhancing cell survival [41].

4. EPILEPSY AND REACTIVE OXYGEN SPECIES

Oxidative stress has been implicated in the pathology associated with acute seizures, status epilepticus and epilepsy. Evidence for increased ROS production in in vivo epilepsy models was initially collected in brain homogenates [42] and subsequently confirmed with fluorescent dyes in vitro [26, 43]. Prolonged seizures in rats lead to decreases in brain glutathione levels [44]. Interestingly, the ketogenic diet, an effective treatment for pharmacoresistant epilepsy, boosts glutathione levels in the brain [45]. There is a consensus about the fact that seizure activity induces ROS production and that this contributes to seizure induced cell death. That these ROS affect cell integrity has been demonstrated as well. MDA, a product of lipid peroxidation induced by ROS, has been shown to increase following chronic epilepsy [42, 46, 47]. However, the sources of ROS involved in sei-

zure-induced cell damage remain a matter of debate. Traditionally, mitochondria have been proposed to be the main site of ROS production in seizures and epilepsy, but recent reports highlight that sources other than mitochondria are also involved in ROS generation during seizures, and may even be more important.

Therefore, we here aim to review the potential sources and mechanisms of ROS production in epilepsy and how targeting these sources can prevent seizures, epilepsy, and seizure-induced cell death. By summarizing these, we want to highlight potential future research pathways and translational aspects. Besides targeting sources of ROS production, we wish to highlight how regulation of key players of antioxidant defences may be used to protect cells from ROS-induced damage. Nrf2, which is increasingly recognized as the master regulator of cellular redox homeostasis, is one such key player.

5. MITOCHONDRIA–SOURCE AND TARGET OF ROS IN EPILEPSY

Where and how do mitochondria generate ROS? Pioneering work in the area of mitochondrial ROS production was done by Britton Chance and coworkers in the 1970s who discovered hydrogen peroxide production in mitochondria upon oxygen exposure [48, 49]. The sites at which mitochondria generate ROS have mostly been studied by providing electrons for specific complexes from appropriate substrates and using specific inhibitors. This way, one can pharmacologically isolate the maximum capacity of the mitochondrial site in producing ROS [50]. Via this approach complexes I and III have traditionally been assumed as the sites of ROS production in mitochondria [51]. Recent studies have partly challenged these results and have shown that the site of mitochondrial ROS production is highly dependent on the substrate given in the experiment [52, 53].

Multiple studies and reviews have highlighted a role of mitochondria in seizure-induced ROS production [43, 54]. ROS production in mitochondria has been shown during seizures and this ROS production has been linked to complex III-dependent superoxide production [55]. However, there are also some conflicting results, and we have not found prominent ROS production of mitochondrial origin during seizure-like activity [26]. It is difficult to target mitochondria as a source of ROS production in seizures, given that mitochondria are involved in ATP production. Thus non-mitochondrial sources of ROS are increasingly recognized as better targets to address seizure-induced cell damage. Recent reports highlight that NADPH oxidase, an enzyme complex located at cellular membranes is involved in ROS production during seizures.

6. NADPH OXIDASE-INDUCED ROS GENERATION AND EPILEPSY

Even before the discovery of NADPH oxidase, it was known that certain cells in the immune system respire rapidly to generate a burst of respiratory activity, which was later discovered to be superoxide [56, 57]. It was a clinical observation which fuelled the discovery of the enzyme involved in generating this burst of ROS. Berendes and colleagues described a clinical syndrome in males with recurrent granulomatous infections, lymphadenopathy, and hypergammaglobulinemia, and the syndrome is referred to as chronic granulomatous disease [58]. It was found that patients suffering from this syndrome were lacking the respiratory phagocyte burst. Subsequent studies in these patients led to the discovery of the proteins involved in this respiratory burst including the catalytic key subunit of NADPH oxidase, $gp91^{phox}$ (NOX2) [59]. To date, different subtypes of the NADPH oxidase have been identified and named after the catalytic subunit as NOX1-5 enzymes in addition to two protein complexes which share similarities to the NOX proteins but have an additional extracellular peroxidase domain (DUOX 1 and 2 [60]). Importantly, the NADPH oxidase is a protein complex which is not constitutively active but requires assembly of a number of proteins to function [61] (**Figure 2A** and **2B**). Upon activation, electrons can be transferred from the NADPH, which is used as a substrate, to an FAD binding site, then to two heme proteins, which ultimately react with oxygen to form superoxide [60, 62] (**Figure 2C**).

Whereas at the time of discovery of NADPH oxidase NOX proteins had been thought to represent a specific feature of the phagocyte machinery, over the past decades NOX protein expression has been shown in multiple tissues. NOX expression has been demonstrated in all types of brain cells except for oligodendrocytes [63]. The main NOX proteins

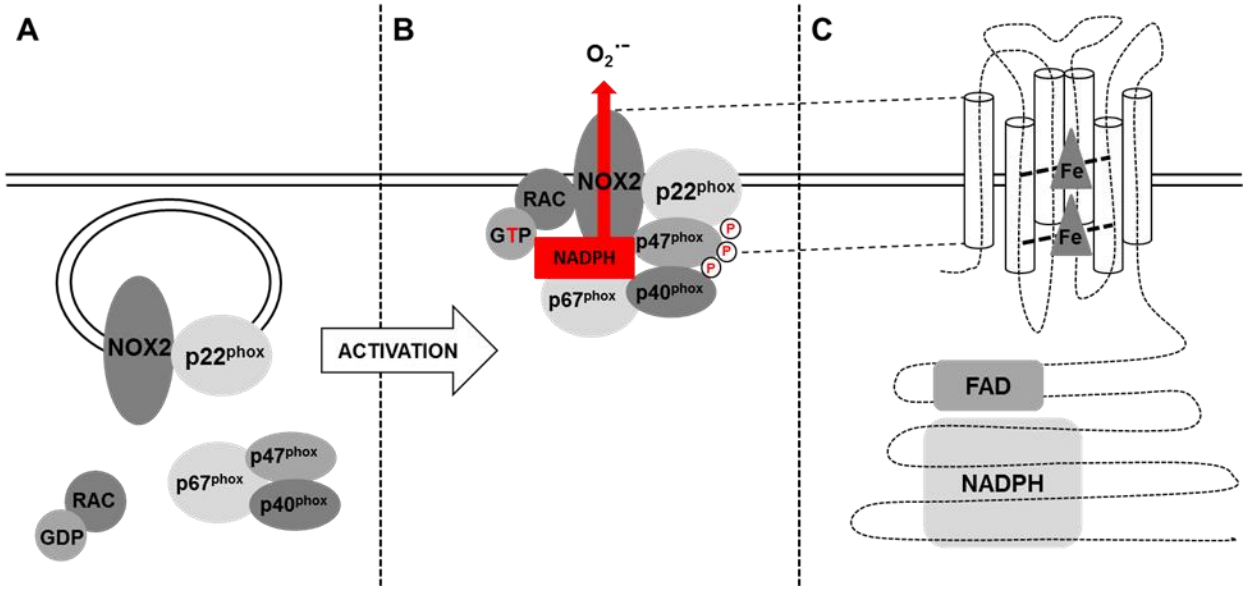

FIGURE 2. NADPH oxidase assembly. Subunits of the NADPH oxidase complex are present in the cytoplasm (A). Upon activation NADPH oxidase subunits assemble to form the functional NADPH oxidase, a transmembrane enzyme capable of generating superoxide (B). The functional subunit of this enzyme represents single electron transporters that pass electrons from NADPH to FAD to the first heme to the second heme that finally provides electrons to oxygen, thereby generating superoxide (C); figure modified after [60].

reported in the brain are NOX1, NOX2, and NOX4. Interestingly, different NOX isoforms have been implicated in different diseases. Brain ischemia for example has been linked to the NOX4 isoform, and NMDA receptor activation to the NOX2 isoform [34]. Another important feature of the NADPH oxidase system is its subcellular distribution. NOX enzymes are transmembrane proteins. Subunits of NOX2-protein complex are present in the cytoplasm and translocate to assemble with membrane bound NOX2 (gp91) to form the fully functional NADPH oxidase (**Figure 2B**). This is important since it highlights two characteristics of NADPH oxidases: First, NOX2–NADPH oxidase is a proteins complex, which is not constitutively active, but activated after assembly. Second, NOX2, the functional subunit, is bound to a membrane. NADPH oxidase can be bound to the plasma membrane and also to intracellular membranes. It is also possible that in different cell types different locations of NADPH oxidase are found. In neurons, for example, NOX2 has been found to be located at the synaptic membrane [64]. For the NOX4 isoform, studies have suggested its binding to intracellular membranes, particularly the endoplasmic reticulum [65].

The NMDA receptor plays an important role in excitation and thus also in seizures which can be seen as the maximum expression of hyperexcitability. We have recently demonstrated NMDA receptor-dependant activation of NADPH oxidase during seizure-like activity [26], and we have shown that this leads to cell death (**Figure 3**). In vivo evidence confirms this, and seizure induced cell death has been ameliorated in the pilocarpine model of epilepsy by suppressing ROS generation via NADPH oxidase [66, 67]. This role of NADPH oxidase has been supported by a recent study showing NOX2 expression in both neurons and glial cells of human epileptic hippocampi obtained from epilepsy surgery for pharmacoresistant epilepsy [68].

But how to target ROS production via NADPH oxidase? Unfortunately, a chemical isoform-specific targeting of NADPH oxidase is not possible since NADPH oxidase inhibitors are largely unspecific. It also seems that this is a difficult strategy since NADPH oxidase isoforms are not tissue specific; for

example, NOX2 is present both in neurons and in leukocytes. Systemic suppression of NADPH oxidase, therefore, is likely to disrupt the immune response. One possibility to circumvent this is to indirectly block NADPH oxidase. NADPH oxidase is a transmembrane enzyme which generates superoxide upon activation. By generating a charged molecule, it also requires charge compensation to fully function. Such charge compensation in phagocytes is provided by Hv1 proton channel, and superoxide production is substantially reduced in the absence of Hv1 [69]. Interestingly, in rat brain slices charge compensation and thus function of NADPH oxidase are not dependent on a proton current [70]. Up until now, the mechanism of charge compensation after NADPH oxidase activation in the brain remains unknown. It is likely that this charge compensatory mechanism is an attractive target for CNS drug development and for new antiepileptic strategies.

ROS generated through membrane-bound NADPH oxidase are likely a consequence of excessive synaptic activity and thus NMDA receptor activation, such as that seen in seizures. Yet, there are other mechanisms that might lead to ROS production in seizures. Seizure activity produces large ionic currents with the subsequent demand for energy to restore ionic transmembrane homeostasis. This involves breakdown of energetic substrates such as ATP, which leads to an increase in adenosine as a metabolite of ATP. Adenosine is metabolized amongst others by xanthine oxidase which, as noted below, is an important producer of ROS.

7. XANTHINE OXIDASE AND EPILEPSY

Xanthine oxidase (XO), a ubiquitously expressed flavoprotein, is widely distributed in mammalian tissues and is a key enzyme of purine catabolism [35]. It is located in the cytoplasm [71] and on the cell membrane [72]. XO catalyses both the transformation of hypoxanthine to xanthine and the conversion of xanthine to uric acid. In this process, H_2O_2 is generated. XO expression is high in the liver and intestine though this enzyme has been shown to play a role in brain injury, and notably, ROS generated by XO were shown to play a role in stroke [73–75]. It is important to note that XO-catalysed metabolism is indirectly linked to energetic substrates since the purine analogue adenosine, a catabolite of ATP, fuels xanthine metabolism. Under conditions of ATP depletion, adenosine is likely to increase with an increase of ROS produced by XO. We have previously shown that seizure-like activity leads to ATP depletion [76]. It is likely that increased XO-catalysed metabolism and thus ROS generated by this enzyme play a role in sustained seizure activity, such as that seen in status epilepticus, when ATP levels are depleted. We have shown that XO inhibition can reduce seizure-induced cell death in vitro [26]. Nevertheless, inhibition of XO has shown conflicting results in the treatment of seizures with some studies reporting a benefit [77–80], whereas other studies failed to show an effect [81, 82]. XO inhibition is certainly an appealing strategy since inhibitors of this enzyme are readily available and can easily cross the blood brain barrier [83].

Targeting sources of ROS during seizure activity is an appealing strategy to decrease the ROS burden and thus ROS-induced cellular damage in seizures. Besides this, transcriptional regulation of antioxidant genes is emerging as a powerful tool in controlling cellular ROS load. Such transcriptional regulator is Nrf2 (**Figure 3**).

8. OTHER SOURCES OF ROS IN EPILEPSY

Cyclooxygenase and lipoxygenase metabolize arachidonic acid after its release from membrane phospholipids through the action of phospholipase A. Both lipoxygenase and cyclooxygenase are powerful mediators of inflammation, and non-steroidal anti-inflammatory drugs largely work through inhibition of cyclooxygenase. Cyclooxygenase- and lipoxygenase-catalysed oxidation reactions involve generation of ROS. There is evidence that lipoxygenase may be active during kainic acid-induced seizures in rats [84], and inhibition of lipoxygenase and cyclooxygenase protects rats against kainic acid-induced seizures and neurotoxicity [85], an effect which may in part be mediated via ROS inhibition.

The cytochrome P450 system is another family of enzymes, which generate ROS. Cytochrome P450 enzymes have mainly been linked to antiepileptic drug metabolism and are expressed in high levels in the liver. Expression levels of these enzymes in brain tissues are low [86]. In addition, chemical induction of brain cytochrome P450 enzymes did not lead to any relevant lipid peroxidation, suggesting that the

ROS generated by cytochrome P450 enzymes are efficiently scavenged by antioxidant systems [87].

9. NRF2 AND EPILEPSY

Nrf2 is a powerful regulator of endogenous antioxidant defenses [41]. One of the main negative regulators of Nrf2 is cytoplasmic Kelch-like ECH-associated protein 1 (Keap1) [88], which continuously targets the transcription factor for ubiquitination and proteosomal degradation [89–91]. The Keap1–Nrf2 system controls an important inducible network of cellular antioxidant defences. Small molecule inducers activate Nrf2 by chemically modifying reactive cysteine sensors of Keap1 [92, 93] or by disrupting the Keap1–Nrf2 binding interface [94, 95]. This leads to Nrf2 stabilization, which then translocates to the nucleus and binds (as a heterodimer with a small Maf transcription factor) to the antioxidant response elements (AREs), specific sequences present in the promoter regions of its target genes, ultimately stimulating the transcription of genes that encode antioxidant proteins [96]. These include enzymes involved in ROS scavenging or inhibition of ROS formation, such as glutathione S-transferases (GSTs) and NAD(P)H:quinone oxidoreductase 1 (NQO1). Nrf2 also regulates the gene expression of both the catalytic (GCLC) and the regulatory (GCLM) subunits of γ-glutamylcysteine ligase (GCL), the enzyme that catalyses the rate-limiting step in the biosynthesis of reduced glutathione (GSH) [97], as well as of the xCT subunit of system x_c^-, which imports cystine into cells [98], in turn providing the cysteine precursor for GSH biosynthesis. In addition to its role in GSH biosynthesis, Nrf2 is critical for the conversion of oxidized glutathione (GSSG) to GSH by regulating the transcription of the glutathione reductase 1 (GR1) gene [99], and the genes encoding four principal NADPH-generating enzymes, i.e., malic enzyme 1 (ME1), isocitrate dehydrogenase 1 (IDH1), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (PGD) [100–103]. Thioredoxin (TXN), thioredoxin reductase 1 (TXNR1), and sulfiredoxin (SRX), which are needed for the reduction of oxidized protein thiols, are also direct transcriptional targets of Nrf2 [104–107].

Our groups have highlighted an important role of Nrf2 in boosting substrate availability for mitochondrial respiration [108, 109], thereby also inevitably influencing mitochondrial ROS production. As would be predicted with high substrate availability fuelling hyperpolarization of the mitochondrial membrane potential, we found that ROS of mitochondrial origin are increased when Nrf2 is constitutively active as, for example, in Keap1-deficient cell lines [110]. More recently, we found that not only mitochondria are affected by Nrf2 induction, but also NOX type expression, with different expression patterns of NOX subtypes in cell lines with constitutively active Nrf2 compared to controls [110]. This highlights a fine-tuned NOX regulation, which may allow prevention of negative side effects on brain signalling due to excessive NOX suppression.

Attempts at targeting Nrf2 activation as a key regulator of the cellular redox metabolism for cytoprotection have been promising [111], and a potent naturally occurring inducer of Nrf2, sulforaphane, has entered clinical trials using well characterized and highly standardized plant extracts as sources of sulforaphane [112, 113]. Nrf2 involvement in epilepsy has been studied by Mazzuferi and colleagues [114]. They started by screening biosets from epilepsy-related studies. Using this bottom-up approach they were able to identify Nrf2 as a key transcription factor in epileptic tissues. They showed that Nrf2 is activated in a short window after status epilepticus, and they were able to demonstrate that overexpression of Nrf2 through an adeno-associated virus vector (AAV) reduced seizure-induced neuronal cell death in vivo. Pharmacological activation of Nrf2 by sulforaphane was found to suppress the progression of amygdala kindling in rats [115]. Dimethyl fumarate, a drug which has been licensed for the treatment of relapsing multiple sclerosis, is another Nrf2 inducer which is already in clinical use [116].

10. CONCLUSIONS

ROS have been convincingly shown to contribute to seizures, epilepsy, and epilepsy comorbidities. Combating ROS, which are also by-products of seizure activity, might reduce epileptogenesis (i.e., the development of epilepsy itself), lower seizure frequency, and prevent cell death in epilepsy. Preventing cell death in epilepsy is a priority since it likely contributes to co-morbidities in epilepsy, such as depression and impaired memory function.

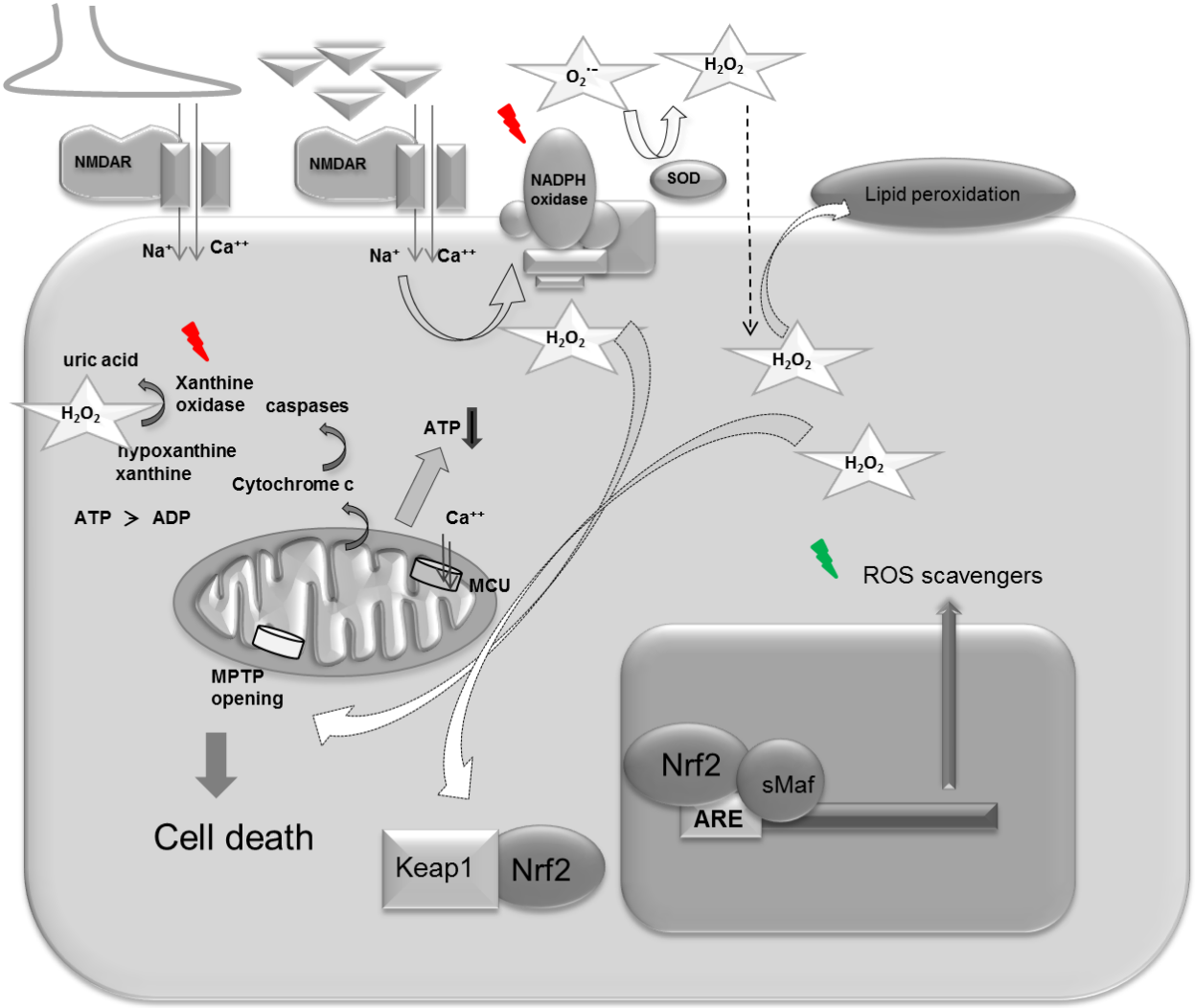

FIGURE 3. ROS producers, targets of ROS, and ROS scavengers in epilepsy. NADPH oxidase and xanthine oxidase are key players of ROS production during seizure-like activity. NADPH oxidase is activated via NMDA receptors during excessive hyperexcitability such as is seen during seizure activity. Targets of ROS-induced damage are not only mitochondria, but also DNA, proteins, and lipids. Nrf2, a key regulator of antioxidant defenses is activated in situations where the cellular ROS burden is high. MCU denotes mitochondrial calcium uniporter.

Research related to ROS in epilepsy–like in other neurological diseases–has focussed on restoring redox balance by scavenging ROS. This approach dismisses the fine-tuned ROS system, which is also implicated in brain signalling. In addition scavenging ROS is difficult to achieve in vivo, and antioxidant trials aimed at scavenging ROS in brain disease have largely failed [38]. Such failure might be explained by pharmacokinetic and pharmacodynamic properties of antioxidants, as well as by their ability in certain cases to behave as pro-oxidants, which hamper their exogenous application. In contrast to the relatively inefficient and short-lived nature of small molecule exogenous antioxidants, the endogenous

antioxidant system regulated by the Keap1–Nrf2 pathway provides a highly efficient and long-lasting means to counteract the deleterious effects of ROS as the ultimate antioxidants are proteins with long half-lives. In order to boost the discovery of therapies targeting ROS-induced damage in epilepsy, the spatial distribution of ROS generators within the cell and their importance in different temporal phases of the seizure and epilepsy need to be unravelled.

Recently, the focus has shifted to NADPH oxidase as an important ROS producer in epilepsy (**Figure 3**). This membrane bound enzyme complex is located in proximity of synapses and its activity has been linked to NMDA receptor opening [34], thus being at the hotspot of excitatory transmission, which is the core feature of seizure activity. Targeting NADPH oxidase is difficult, and unselective NADPH oxidase inhibitors are likely to fail due to side effects such as bacterial infections given the importance of NADPH oxidase in mediating the immune response through phagocytes. One way to circumvent this is to unravel the mechanism which is involved to compensate for the charge that is created upon activation of NADPH oxidase. Interfering with this mechanism is likely to reduce NADPH oxidase function. So far it remains unclear what this mechanism is in the brain, but it seems to be tissue specific and is different from the charge compensatory mechanism in phagocytes, which is mediated by proton channels [69, 70]. We believe that targeting this mechanism will be an exciting avenue for drug discovery. Another approach which may be complementary to this is the interference with XO.

Very little research has been dedicated to XO and its role in seizure-induced ROS generation. It is likely that this enzyme's contribution to overall cellular ROS production increases as seizures progress given its strong link to ATP metabolism. There is experimental evidence that ATP levels decline ~40 min after prolonged seizures [117], leading to raised purine metabolites such as hypoxanthine and xanthine. These are metabolized by XO, which produces ROS. Targeting this enzyme is attractive since blood brain barrier-permeable inhibitors are readily available. Given that NADPH oxidase and XO represent different mechanisms of ROS production possibly acting at different stages of seizure activity, it is likely that a combination approach might be the most efficient way to combat ROS production in the course of seizures and status epilepticus (**Figure 3**).

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