NMDA Receptor Activation Mediates Hydrogen Peroxide–Induced Pathophysiology in Rat Hippocampal Slices

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Avshalumov, Marat V. and Margaret E. Rice. NMDA receptor activation mediates hydrogen peroxide–induced pathophysiology in rat hippocampal slices. J Neurophysiol 87: 2896–2903, 2002; 10.1152/jn.01011.2001. Endogenous reactive oxygen species (ROS) can act as modulators of neuronal activity, including synaptic transmission. Inherent in this process, however, is the potential for oxidative damage if the balance between ROS production and regulation becomes disrupted. Here we report that inhibition of synaptic transmission in rat hippocampal slices by H2 O2 can be followed by electrical hyperexcitability when transmission returns during H2 O2 washout. As in previous studies, H2 O2 exposure (15 min) reversibly depressed the extracellular population spike (PS) evoked by Schaffer collateral stimulation. Recovery of PS amplitude, however, was typically accompanied by mild epileptiform activity. Inclusion of ascorbate (400 μM) during H2 O2 washout prevented this pathophysiology. No protection was seen with isoascorbate, which is a poor substrate for the stereoselective ascorbate transporter and thus remains primarily extracellular. Epileptiform activity was also prevented by the N-methyl-D-aspartate (NMDA) receptor antagonist, dl-2-amino-5-phosphonopentanoic acid (AP5) during H2 O2 washout. Once hyperexcitability was induced, however, AP5 did not reverse it. When present during H2 O2 exposure, AP5 did not alter PS depression by H2 O2 but did inhibit the recovery of PS amplitude seen during pulse-train stimulation (10 Hz, 5 s) in H2 O2. Inhibition of glutamate uptake by l-trans-2,4-pyrrolidine dicarboxylate (PDC; 50 μM) during H2 O2 washout markedly enhanced epileptiform activity; coapplication of ascorbate with PDC prevented this. These data indicate that H2 O2 exposure can cause activation of normally silent NMDA receptors, possibly via inhibition of redox-sensitive glutamate uptake. When synaptic transmission returns during H2 O2 washout, enhanced NMDA receptor activity leads to ROS generation and consequent oxidative damage. These data reveal a pathological cycle that could contribute to progressive degeneration in neurological disorders that involve oxidative stress, including cerebral ischemia.

INTRODUCTION

The reactive oxygen species (ROS), H2 O2, has been shown previously to modulate synaptic transmission (e.g., Frantseva et al. 1998; Pellmar 1986). Most studies of this modulation have used the hippocampal slice preparation, in which H2 O2 reversibly depresses the population spike (PS) evoked by stimulation of the Schaffer collaterals and recorded in CA1 (Avshalumov et al. 2000; Fowler 1997; Pellmar 1986, 1987). Both pre- and postsynaptic sites of action may be involved, since H2 O2 can inhibit neurotransmitter release (Chen et al. 2001) and can cause hyperpolarization of CA1 pyramidal neurons (Seutin et al. 1995). Additional actions of H2 O2 (and other ROS) include modulation of synaptic plasticity (Auerbach and Segal 1997; Klann et al. 1998), possibly mediated by alterations in intracellular signaling pathways, including activation of certain kinases (Klann and Thiels 1999).

The reversibility of the effects of H2 O2 on synaptic transmission and the demonstration that similar effects are seen with endogenously generated, as well as exogenously added H2 O2 (e.g., Auerbach and Segal 1997; Chen et al. 2001) implicate H2 O2 as an endogenous neuromodulator. This ROS is generated during normal mitochondrial respiration from dismutation of the superoxide radical (·O2–) (Boveris and Chance 1973; Fridovich 1979; Halliwell 1992). Pathological consequences of H2 O2 elevation are normally prevented by the endogenous antioxidant network, which includes the H2 O2-scavenging enzymes glutathione (GSH) peroxidase and catalase (Cohen 1994; Desagher et al. 1996; Sokolova et al. 2001). It should be noted, however, that modulatory, as well as pathological consequences of H2 O2 are often mediated by the hydroxyl radical (·OH) rather than by H2 O2 per se (Avshalumov et al. 2000; Halliwell 1992; Pellmar et al. 1989; Sah and Schwartz-Bloom 1999; Ying et al. 1999). Whereas the enzymes superoxide dismutase (SOD), GSH peroxidase, and catalase regulate cellular levels of ·O2– and H2 O2, there are no analogous enzymes for the highly reactive ·OH. Instead, ·OH management depends on the endogenous antioxidants ascorbate and GSH (Cohen 1994; Rice 2000). Consistent with this, normal extracellular concentrations of ascorbate can prevent PS depression in the presence of exogenous H2 O2 (Avshalumov et al. 2000).

In addition to modulatory actions when H2 O2 levels are controlled, prolonged exposure to this ROS can also cause cell death of both neurons and glia in vitro (Desagher et al. 1996; Hoyt et al. 1997; Mailly et al. 1999; Sokolova et al. 2001). Importantly, Mailly and colleagues (1999) reported that H2 O2–induced neuronal apoptosis was due to increased extracellular glutamate concentration, N-methyl-D-aspartate (NMDA) receptor activation, and increased ROS production that occurred during washout of H2 O2. Transient exposure to H2 O2 also caused an increase in glutamate overflow from hippocampal neurons, including cerebral ischemia.
slices, which contributed to long-lasting damage to inhibitory transmission (Sah and Schwarz-Bloom 1999). Increased glutamate levels in both of these studies suggests that an immediate consequence of H$_2$O$_2$ exposure might be inhibition of redox-sensitive glutamate transporters (for review, see Tronti et al. 1998), with further pathological consequences including hyperexcitability and even cell death.

Here we report that recovery of the PS in rat hippocampal slices after H$_2$O$_2$ exposure can be accompanied by pathological electrical activity, indicated by an additional PS in response to Schaffer-collateral stimulation in CA1. As in cultured neurons (Mailly et al. 1999), this epileptiform activity appears to be a consequence of enhanced NMDA receptor activation during H$_2$O$_2$ washout and involves secondary NMDA receptor–mediated ROS production. Additional experiments tested the hypothesis that glutamate transporter inhibition might contribute to this pathophysiology.

METHODS

Hippocampal slice preparation

Hippocampal slices (400-μm thickness) were prepared from young adult (50- to 60-day-old) male Long-Evans rats. All animal experimentation was conducted following National Institutes of Health guidelines and with approval by the NYU School of Medicine Institutional Animal Care and Use Committee. Before decapitation, rats were deeply anesthetized with 50 mg/kg pentobarbital sodium. After decapitation, the brain was rapidly removed and placed in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) for about 1 min. The brain was then bisected, blocked, and mounted on the stage of a Vibratome (Ted Pella, St. Louis, MO), and transverse hippocampal slices were cut in ice-cold ACSF, as described previously (Avshalumov et al. 2000). Normal ACSF contained (in mM) 120 NaCl, 5 KCl, 35 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 1.5 CaCl$_2$, and 1.3 MgCl$_2$, equilibrated with 95% O$_2$-5% CO$_2$. Freshly prepared slices were maintained in ACSF at room temperature for at least 1 h before recording.

Extracellular recording

For measurement of the evoked PS, slices were transferred to a submersion-recording chamber (Warner Instrument, Hamden, CT), where they were continuously superfused with ACSF at 1.5 ml/min at 31°C. Extracellular PS evoked by Schaffer-collateral stimulation were recorded in the stratum pyramidale of CA1 with a standard glass electrode (2- to 7-MΩ tip resistance, backfilled with 1 M NaCl) connected to an Axoprobe 1A amplifier (Axon Instruments, Foster City, CA). Stimulation-pulse duration was 100 μs, with stimulus intensity adjusted to the lowest potential (0.9–1.8 mV) required to evoke a PS of maximal amplitude (Avshalumov et al. 2000). The stimulating electrode was a twisted bipolar electrode, made from Teflon-insulated platinum-iridium wire. The evoked PS was elicited at 5-s intervals using an external timing circuit (Master-8, A.M.P.I., Jerusalem, Israel) and was monitored on a digital oscilloscope. Data acquisition was controlled by Clampex 7.0 software (Axon Instruments, Foster City, CA), which imported PS records to a PC via a DigiData 1200B D/A board (Axon Instruments) for averaging and analysis. In some experiments, the behavior of the PS elicited by pulse-train stimulation (10 Hz, 5 s) was also recorded; train stimulation was controlled by the Master-8, with the same pulse duration and amplitude as in single-pulse stimulations.

For each slice, the evoked PS was monitored for 25–30 min in normal ACSF to ascertain that the response was stable. Typical PS amplitude in these submerged slices was 2 mV, as we have reported previously (Avshalumov et al. 2000). Slices with PS amplitude lower than 1 mV were not tested further. For each experimental condition, three evoked PS records were stored and averaged. The amplitude (in mV) of PS records was measured from the mean of the positive peak preceding and the positive peak following the negative PS.

Chemicals and solutions

ACS grade hydrogen peroxide solution, sodium ascorbate (L-ascorbate), ascorbate (D-ascorbate), DL-2-amino-5-phosphonopentanoic acid (AP5), and all components of ACSF were obtained from Sigma (St. Louis, MO); L-trans-2,4-pyridoline dicarboxylate (PDC) was from Tocris (Ballwin, MO). Test agents were added to ACSF immediately before use (Avshalumov et al. 2000).

Statistical analysis

Data are given as means ± SE, normalized to percent of control PS amplitude (100%) recorded before H$_2$O$_2$ exposure or pulse train stimulation for a given slice. For statistical analysis, either Student’s t-test or one-way ANOVA followed by Kruskal-Wallis post hoc analysis test was used as appropriate. The statistical significance was P < 0.05. All illustrated electrophysiological records represent the averaged responses from 5–14 slices (as indicated).

RESULTS

Epileptiform activity after H$_2$O$_2$ washout

The mean amplitude of the evoked PS in submerged hippocampal slices under control conditions was 2.1 ± 0.2 mV (n = 14; Fig. 1A). Within this population, the minimum amplitude was 1.6 mV and the maximum was 2.3 mV, such that the illustrated average responses well represent typical PS behavior. We have shown previously that the effect of exogenous H$_2$O$_2$ on the evoked PS in rat hippocampal slices has a sharp dependence on H$_2$O$_2$ concentration: 1.2 mM H$_2$O$_2$ does not alter PS amplitude significantly, whereas a maximal effect is seen at 1.5 mM H$_2$O$_2$ with no further depression at 2.0 mM H$_2$O$_2$ (Avshalumov et al. 2000). As before, 15-min exposure to 1.5 mM H$_2$O$_2$ caused a decrease in PS amplitude to 19 ± 2% of control (n = 14; P < 0.001), with recovery to 83 ± 11% (n = 14; P > 0.05 vs. control) after 30 min H$_2$O$_2$ washout (Fig. 1A). Additionally, however, we report here that the recovery of the PS after H$_2$O$_2$ washout can be accompanied by mild epileptiform activity, indicated by an additional peak following the primary PS (Fig. 1A; arrow).

In our experience, slices that have a stable, single PS during the first 10–15 min of recording do not develop additional peaks (hyperexcitability) during normal recording for up to 3 h (not illustrated). This indicates that repetitive stimulation at 5-s intervals alone does not induce such hyperexcitability. To examine whether stimulation during H$_2$O$_2$ exposure or washout might contribute to the H$_2$O$_2$-induced pathophysiology, however, H$_2$O$_2$ was applied and washed out in the absence of Schaffer-collateral stimulation (Fig. 1B). For this study, control records were obtained with normal stimulation parameters, then the stimulus turned off. Slices were then exposed to H$_2$O$_2$ for 15 min followed by 30-min washout, as usual, after which the stimulus was turned on again to indicate PS behavior. The same post-H$_2$O$_2$ hyperexcitability was seen under these conditions (Fig. 1B; n = 6) as when the stimulus was applied at 5-s intervals during H$_2$O$_2$ exposure and withdrawal (Fig. 1A). Thus evoked electrical activity and transmitter release did not contribute to the induction of this pathophysiology.
neuronal ascorbate transporter, whereas isoascorbate is not (Tsukaguchi et al. 1999), such that ascorbate is taken up into the intracellular compartment, while isoascorbate remains predominantly extracellular (Brahma et al. 2000). Inclusion of ascorbate in the washout medium at its normal physiological concentration of 400 μM (see Rice 2000) completely prevented the appearance of an additional PS (Fig. 2A), which implicated ROS involvement. By contrast, isoascorbate (400 μM), did not prevent H₂O₂-induced pathology (Fig. 2B; arrow), suggesting that the site of ROS generation or action was intracellular.

As noted above, PS amplitude of slices after H₂O₂ exposure and washout was slightly, but not significantly different from control levels (Figs. 1A and 2C). The additional PS was on average, 33 ± 3% of the control PS amplitude (n = 14; P < 0.001; Fig. 2C). When ascorbate was present in the washout medium, PS amplitude after H₂O₂ exposure also recovered to control levels (93 ± 3%; n = 8; P > 0.05 vs. control) with no detectable second PS (Fig. 1C). By contrast, when H₂O₂ was washed out with isoascorbate, the amplitude of the primary PS did not fully recover (78 ± 1%; n = 6; P < 0.05 vs. control) and was accompanied by an additional PS that was 27 ± 2% of control (n = 6; Fig. 2C).

Protective effect of NMDA receptor antagonism

It was recently reported that neuronal toxicity following H₂O₂ exposure could be prevented by NMDA receptor antag-

![Fig. 1](http://jn.physiology.org/)

![Fig. 2](http://jn.physiology.org/)

We then examined whether hyperexcitability could occur following cessation of synaptic transmission alone. For this experiment, slices were superfused for 15 min with nominally Ca²⁺-free ACSF (no added Ca²⁺; n = 3) or normal ACSF (1.5 mM Ca²⁺ plus 0.5 mM Mn²⁺; n = 3), both caused complete suppression of the evoked PS so that the data were pooled (Mn²⁺/30 mM Ca²⁺). No additional peak was seen in the recovered PS after 30-min washout with normal ACSF (wash). Illustrated PS records are the averaged responses for each condition from 5–14 slices, as indicated.

Involvement of intracellular ROS

We showed previously that the inhibitory effect of H₂O₂ on the hippocampal PS can be prevented when H₂O₂ is applied in the presence of the ROS scavenger, ascorbate, or its nonphysiological steroisomer, isoascorbate (D-ascorbate) (Avshalumov et al. 2000). When this primary inhibitory effect of H₂O₂ was prevented, no epileptiform activity was seen on H₂O₂ washout (not illustrated). To investigate ROS involvement in the development of the pathophysiology, we examined the effect of ascorbate and isoascorbate when either was present only during washout. Ascorbate is a good substrate for the stereospecific activity. After control PS records were taken, either normal artificial cerebrospinal fluid (ACSF) plus 0.5 mM Mn²⁺ (n = 3) or ACSF with 0 mM Ca²⁺ (n = 2) were superfused for 15 min; both caused complete suppression of the evoked PS so that the data were pooled (Mn²⁺/30 mM Ca²⁺). No additional peak was seen in the recovered PS after 30-min washout with normal ACSF (wash). Illustrated PS records are the averaged responses for each condition from 5–14 slices, as indicated.

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No additional peaks developed during AP5 washout periods of up to 1.5 h (not illustrated), which indicated that the continued presence of AP5 was not required to “suppress” epileptiform activity. Moreover, once epileptiform activity was present, application of AP5 did not alter it (not illustrated). This further suggests that the increased excitability was not caused by persistent activation of NMDA receptors. Together, these data implicate transient NMDA receptor activation during H2O2 washout as a cause of irreversible pathophysiology.

**Activation of NMDA receptors during H2O2 exposure**

The question then arises of whether enhanced NMDA receptor activation occurs during H2O2 exposure. We previously reported that by the end of pulse-train stimulation (10 Hz, 5 s), the amplitude of the depressed PS in the presence of H2O2 recovers to about 75% of that seen during train stimulation in ACSF alone (Avshalumov et al. 2000). We therefore tested the hypothesis that increased NMDA receptor activation contributed to the recovery of the PS during prolonged stimulation in the presence of H2O2. As before, PS amplitude during pulse-train stimulation increased twofold during the first few stimulus pulses under control conditions, with an average increase to 227 ± 17% of the single pulse control by the end of the train (n = 10; P < 0.001 vs. single-pulse control). This pattern was unaffected by AP5 (50 μM; Fig. 4).

In the presence of H2O2, PS amplitude was initially depressed during pulse-train stimulation, but began to return after typically 25 pulses at 10 Hz, with an amplitude that was 157 ± 10% of single-pulse control by the end of the train (n = 10; P < 0.05 vs. single-pulse control or the last pulse of the train in ACSF alone). Recovery of PS amplitude during pulse-train stimulation in H2O2 was inhibited by AP5. By the end of the train in H2O2 plus AP5, PS amplitude had increased to only 94 ± 7% of the single pulse control (n = 10; P > 0.05), which

![Graph](image-url)
was significantly lower than the last pulse in ACSF ($P < 0.001$) or in H$_2$O$_2$ alone ($P < 0.05$; Fig. 4). These data show that PS recovery during pulse-train stimulation in H$_2$O$_2$ involves activation of normally silent NMDA receptors.

Glutamate transport inhibition increased H$_2$O$_2$-dependent pathology

Elevated glutamate spillover in the presence of H$_2$O$_2$ has been proposed to reflect decreased glutamate uptake (Mailly et al. 1999; Sah and Schwartz-Bloom 1999) caused by oxidative inhibition of redox-sensitive glutamate transport proteins (Trotti et al. 1998; Volterra et al. 1994). To investigate whether this might contribute to the pathophysiological consequences of H$_2$O$_2$ in the present studies, we first examined the effect of the nonselective transport inhibitor, PDC (50–100 $\mu$M) on the evoked PS. Superfusion with PDC alone did not induce epileptiform activity. Indeed, PDC caused a progressive decrease in PS amplitude, especially at 100 $\mu$M (not illustrated), as has been reported previously for other glutamate transport inhibitors (el-Sherif et al. 1999). When PDC (50 $\mu$M) was included in the H$_2$O$_2$ washout medium, however, a marked enhancement in epileptiform activity was seen after PDC was also washed out (Fig. 5A). This exacerbation of the H$_2$O$_2$-dependent pathophysiology shows that inhibition of glutamate transporters could be a contributing factor. Additionally, we examined whether the PDC-enhanced effect could also be prevented by ascorbate. Strikingly, when ascorbate (400 $\mu$M) was included with PDC, the enhanced epileptiform activity was completely inhibited (Fig. 5B), which further implicates secondary, glutamate-dependent ROS production during H$_2$O$_2$ washout.

**DISCUSSION**

The present results indicate that transient elevation of H$_2$O$_2$ in rat hippocampal slices not only can suppress synaptic transmission, but also can lead to secondary pathophysiology as synaptic transmission returns during H$_2$O$_2$ withdrawal. The induction of hippocampal hyperexcitability on H$_2$O$_2$ washout could be prevented by ascorbate or AP5, but not by isoascorbate. These data implicate NMDA receptor activation with subsequent intracellular ROS generation as the cause of irreversible damage when glutamatergic transmission returns after transient H$_2$O$_2$ exposure.

**NMDA receptor involvement in H$_2$O$_2$-induced pathophysiology**

A primary effect of H$_2$O$_2$ on synaptic transmission in hippocampal slices appears to be decreased synaptic release of glutamate (Pellmar 1987). In support of inhibitory effects of H$_2$O$_2$ on transmitter release, we have shown that H$_2$O$_2$ can reversibly depress synaptic release of dopamine, which can readily be monitored in brain tissue using voltammetric microelectrodes (Chen et al. 2001). Opposing the action of decreased glutamate release, however, H$_2$O$_2$ can also reversibly inhibit glutamate uptake by acting as an oxidant at sulfhydryl groups in glutamate transport proteins (Trotti et al. 1997, 1998; Volterra et al. 1994). Increased glutamate spillover following H$_2$O$_2$ exposure in both neuron-enriched cultures and hippocampal slices has been attributed to oxidative inhibition of glutamate transport (Mailly et al. 1999; Sah and Schwartz-Bloom 1999). Enhanced glutamate levels and subsequent NMDA receptor activation was found to be the initiating event in H$_2$O$_2$-induced neuronal apoptosis (Mailly et al. 1999). Similarly, Sah and Schwartz-Bloom (1999) reported that H$_2$O$_2$ exposure can induce long-term disruption of Cl$^-$ homeostasis in hippocampal slices, which can lead to a long-term decrease in the efficacy of inhibitory transmission; significantly, the changes in ion homeostasis in that study could be inhibited by glutamate receptor antagonists (Sah and Schwartz-Bloom 1999).

Loss of inhibitory transmission following transient H$_2$O$_2$ exposure is presumably an underlying factor in the irreversible hyperexcitability seen in the present studies. The prevention of this H$_2$O$_2$-induced pathophysiology by AP5 further implicates similar initiating events, including decreased glutamate uptake and increased NMDA receptor activation. Previous studies have shown that uptake inhibition can increase the NMDA receptor–mediated component of the evoked PS in hippocampal slices, even when glutamate release is depressed (el-Sherif et al. 1999). Thus inhibition of glutamate transport during H$_2$O$_2$ exposure could also contribute to the NMDA receptor–dependent recovery of the evoked PS during pulse-train stimulation in the presence of H$_2$O$_2$ seen here (Fig. 4). If involved, however, transport inhibition by H$_2$O$_2$ must be only partial, since the effect could be amplified by pharmacological blockade using PDC (Fig. 5A). Additional actions of H$_2$O$_2$ that might activate normally silent NMDA receptors include ROS-dependent stimulation of tyrosine kinase activity (Klann and Thieils 1999). Stimulation of this pathway can increase NMDA receptor phosphorylation (Zheng et al. 1998), which potentiates NMDA receptor–mediated Ca$^{2+}$ entry (Soderling and Derckach 2000). It should be noted that post-H$_2$O$_2$ hyperexcitability is not a consequence of permanently activated NMDA receptors, however, since AP5 could not inhibit epileptiform activity once it had appeared.

In addition, the actions of H$_2$O$_2$ in the present studies appear to be independent of the redox modulatory site of NMDA receptors. Sulphydryl oxidation at this redox-sensitive site causes a decrease in NMDA receptor activity (Aizenman et al. 1989, 1990); indeed, this may be an important site in regulating

![Figure 5](http://jn.physiology.org/)

**Figure 5.** Glutamate transporter inhibition enhances H$_2$O$_2$-induced pathophysiology. *A:* evoked PS before H$_2$O$_2$ application (control), after 15-min superfusion with H$_2$O$_2$, and after 20-min washout of H$_2$O$_2$ in the presence of 1-trans-2,4-pyrrolidine dicarboxylate (PDC; 50 $\mu$M) then 30-min PDC wash with ACSF alone (wash post-PDC; $n = 8$). Note multiple additional peaks after H$_2$O$_2$ washout with PDC (arrows). *B:* evoked PS under the same conditions as in *A,* except that ascorbate (400 $\mu$M) was included with PDC during the first 20 min of H$_2$O$_2$ washout, followed by 30-min wash of PDC + Asc (wash post-PDC + Asc). Each record is the average response for each condition from 8 slices.
the severity of epileptiform activity under conditions in which NMDA receptor involvement is enhanced (e.g., low Mg2+). This inhibitory effect contrasts sharply to the H2O2-induced increase in NMDA receptor activity seen here (e.g., Fig. 4) and in previous studies (Mailly et al. 1999). Thus if H2O2 did have an inhibitory effect at the redox-modulatory site in the present experimental paradigm, it was overshadowed by this predominant activating action.

**Protective role of ascorbate; involvement of secondary ROS production**

Increased NMDA receptor activation, regardless of mechanism, can initiate a neurotoxic cascade involving elevated intracellular Ca2+ (Lipton and Rosenberg 1994; Urunishi et al. 2001; Versun et al. 2001) and increased mitochondrial ROS production (Bindokas et al. 1996; Dugan et al. 1995; Dykens 1994; Lafon-Cazal et al. 1993; Reynolds and Hastings 1995; Urunishi et al. 2001; Versun et al. 2001). Increased ROS levels can lead to mitochondrial damage (Hoyt et al. 1997), which can initiate a further cycle of increased intracellular Ca2+, increased ROS production, and cytotoxicity (Brouillet et al. 1995).

Ascorbate, by acting as an ROS scavenger to decrease levels of intracellular ROS generated downstream from NMDA receptor activation, can break this pathological cycle and decrease NMDA-mediated excitotoxicity (Atlante et al. 1997; Ciani et al. 1996). Prevention of irreversible hyperexcitability in the present studies by both AP5 and ascorbate implicates NMDA receptor–dependent ROS generation as the initiating event in secondary, H2O2-dependent pathology. This is further supported by the ability of ascorbate to prevent the enhanced pathophysiology caused by pharmacological inhibition of glutamate uptake by PDC during H2O2 withdrawal (Fig. 5B).

Significantly, isoascorbate was not protective against this secondary pathology (Fig. 2B), indicating that the site of ROS generation was intracellular. Isoascorbate has similar electrochemical properties to ascorbate (Iheanacho et al. 1995), but is a poorly transported substrate for the stereoselective, neuronal ascorbate transporter, SVCT2 (Spector and Lorenzo 1974; Tsukaguchi et al. 1999). Thus isoascorbate does not readily enter brain cells, whereas ascorbate does (Brahma et al. 2000; Rice et al. 1994). We have previously used these isomers to distinguish between intra- versus extracellular sites of action for ascorbate neuroprotection (Avshalumov et al. 2000; Brahma et al. 2000). Once in the intracellular compartment, ascorbate is dynamically maintained in the reduced state by endogenous thiols, including glutathione (GSH), by a GSH-dependent dehydroascorbate reductase, and indirectly by other components of the antioxidant network (Buettner 1993; Fornai et al. 1999; Meister 1994; Rose 1993; Winkler et al. 1994). Isoascorbate has limited access to this intracellular antioxidant network and thus is more susceptible to oxidation in brain tissue. Here, the incomplete recovery of PS amplitude during H2O2 washout with isoascorbate (Fig. 2C) might reflect a slight pro-oxidant effect of this agent in the extracellular compartment, as we have seen in other studies (Brahma et al. 2000).

Ascorbate has been suggested to be neuroprotective under some conditions by decreasing NMDA receptor activity via the redox modulatory site (Majewska and Bell 1990). As noted above, NMDA receptor activity is decreased by oxidation, which can be reversed by thiol reducing agents (Aizenman et al. 1989, 1990). Thermodynamically, ascorbate cannot reduce oxidized thiols (Buettner 1993), so that a “protective” effect of ascorbate linked to redox modulation of NMDA receptors must be the result of pro-oxidant action, as ascorbate itself oxidizes (Rice 2000). The lack of equal “protection” by ascorbate and isoascorbate in the present studies argues against this mechanism of action here; rather, the data are consistent with ROS scavenging by ascorbate in the intracellular compartment.

It should be noted that previous reports of the effects of H2O2 on hippocampal physiology did not describe epileptiform activity after H2O2 exposure. Many of these studies, especially those by Pellmar and colleagues (Pellmar 1986, 1987; Pellmar et al. 1989) were made in guinea pig hippocampal slices. In preliminary comparisons using the same experimental conditions as in the present studies with rat slices, we found that guinea pig tissue is apparently resistant to H2O2–washout pathology, with no epileptiform activity after H2O2 washout (unpublished data). This may be a consequence of the higher GSH content of guinea pig brain tissue compared with rat (Rice et al. 1995), since Pellmar reported that the recovery of the PS after H2O2 washout in guinea pig hippocampal slices is GSH dependent and is significantly decreased by GSH-synthesis inhibition (Pellmar et al. 1989).

**Relevance for neuropathology**

Together, these data confirm the importance of the antioxidant network, including ascorbate, in preventing oxidative damage during normal cell signaling by ROS. Under conditions in which the antioxidant network is compromised, however, the sequence of events described here could contribute to secondary pathology. For example, increased levels of H2O2, -OH, and other ROS have been detected during reperfusion in vivo after ischemic periods as brief as 5–30 min (Cao et al. 1988; Delbarre et al. 1992; Hyslop et al. 1995; Lei et al. 1997), when the antioxidant network is relatively intact (Lyser et al. 1991; Uemura et al. 1991). Increased ROS generation also occurs in the penumbra surrounding the infarct (Solenski et al. 1997), which can contribute to progressive damage in penumbral tissue (Chan 1996; Love 1999).

During reperfusion after brief ischemia, extracellular H2O2 levels can reach 200 μM (Hyslop et al. 1995; Lei et al. 1997). Assuming that the primary site of generation is the mitochondria, as during normal metabolism (Boveris and Chance 1973), intracellular levels may be even higher. It should be noted that under normal conditions, even low densities of neurons and glia in culture have sufficient peroxidase activity to rapidly metabolize 200-μM exogenous H2O2 to below detectable levels (Desagher et al. 1996; Sokolova et al. 2001). Consequently, actual tissue levels of H2O2 during exogenous application in the present brain slice studies in are also expected to be substantially lower than the applied concentration (1.5 mM) required to depress the evoked PS.

Consistent with the cycle of progressive pathology proposed here, glutamate receptor activation is an important contributing factor to ischemia-induced ROS generation (Lipton 1999; Love 1999). Extracellular glutamate levels increase 6- to 30-fold during ischemia, resulting in concentrations that can exceed 1 mM (Benveniste et al. 1984; Benveniste and Hüttemeier 1990; Shimizu et al. 1993; Takagi et al. 1993). Moreover, ROS
production during reperfusion is proportional to the increase in extracellular glutamate during the ischemic period (Morimoto et al. 1996).

Summary and implications

On the basis of the present findings and earlier reports in the literature, we propose that the following sequence of events leads to irreversible pathophysiology after oxidative stress, here caused by exogenous H$_2$O$_2$. Initially, H$_2$O$_2$ causes a depression of synaptic transmission mediated by glutamate (as well as by other transmitters, including GABA) (Frantseva et al. 1998). At the same time, H$_2$O$_2$ exposure has other consequences, including possible oxidative inhibition of glutamate transporters and/or modulation of the activity state of NMDA receptors. When glutamatergic transmission returns during H$_2$O$_2$ washout, enhanced NMDA receptor activation leads to increased ROS production and irreversible pathology, apparently mediated by secondary oxidative damage. Thus the present studies reveal a neuropathological cycle, initiated by oxidative stress, involving NMDA receptor recruitment, and secondary generation of ROS, which could reinitiate the cycle. In addition to ischemia/reperfusion, this sequence of events might contribute to progressive pathology in other neurological disorders in which oxidative stress is an underlying cause, including traumatic brain injury, amyotrophic lateral sclerosis, Parkinson’s disease, and Alzheimer’s disease (Globus et al. 1995; Harris et al. 1996; Olanow and Tatton 1999; Trotti et al. 1998, 1999).

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