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ABSTRACT PAGE

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FREE RADICALS ACCELERATE THE DECAY OF LONG-TERM POTENTIATION IN FIELD CA1 OF GUINEA-PIG HIPPOCAMPUS

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Abstract—Free radicals have been implicated in a number of pathological conditions. To evaluate the neurophysiological consequences of free radical exposure, slices of hippocampus isolated from guinea-pigs were exposed to hydrogen peroxide which reacts with tissue iron to generate hydroxyl free radicals. Long-term potentiation, a sustained increase in synaptic responses, was elicited in field CA1 by high frequency stimulation of an afferent pathway. We found that 0.002% peroxide did not directly affect the responses evoked by stimulation of the afferent pathway but did prevent maintenance of long-term potentiation. Short-term potentiation and paired-pulse facilitation were not affected by peroxide treatment. Peroxide was less effective if removed following high frequency stimulation and was ineffective if applied only after high frequency stimulation. Input/output analysis showed that the increase in synaptic efficacy was reduced with peroxide treatment. Changes in the enhanced ability of the synaptic potential to generate a spike were less apparent.

These data show that the interference of free radicals with long-term potentiation may contribute to pathological deficits. It is possible that intracellular calcium regulation is disrupted by peroxide treatment. A number of second messenger systems involved with long-term potentiation are potential targets for free radical attack.

Long-term potentiation (LTP) is a persistent increase in monosynaptic efficacy following a high frequency train. Because the potentiation can last for hours or even days *in vivo*,¹¹ this electrophysiological phenomenon has been considered to be a correlate of memory and learning. The biochemical changes that underlie LTP are complex, possibly involving a number of second messenger systems.^{17,20,22,30,32}

Free radicals and active oxygen compounds (e.g. peroxide, superoxide and hydroxyl radicals) are normally generated with cellular metabolism but are well controlled by intrinsic enzyme systems and antioxidants.^{7,10,12,13} Under certain pathological conditions, this delicate balance can be disrupted. Free radicals are thought to contribute to a number of diseases such as ischemic injury, aluminum toxicity, Alzheimer's disease and Down's syndrome,^{7,9,12,16,23,35,41} all of which affect cognitive processes.

Previous studies have shown that free radicals can interfere with neuronal electrophysiology.^{27,29} Hydroxyl radicals can be generated *in vitro* through the Fenton reaction, peroxide reacts with iron intrinsic to the tissue to produce this very

reactive free radical. Hydroxyl radicals attack membrane lipids and cellular proteins, which disrupts cell function. Exposure of a hippocampal slice to peroxide ($\geq 0.005\%$) decreases synaptic responses, decreases orthodromic spike generation²⁷ and increases spike frequency adaptation.²⁹ Free radical scavengers (dimethylsulfoxide, Trolox-C) and an iron chelator (deferoxamine) prevent most of the peroxide damage,²⁹ suggesting that hydroxyl radicals, and not the peroxide itself, are the reactive oxygen species. Colton *et al.*⁶ used a similar model and found that peroxide reduced the potentiation occurring 15 min after high frequency stimulation.

This paper examines the actions of free radicals on LTP.

EXPERIMENTAL PROCEDURES

Male Hartley (Harlan Sprague-Dawley, Inc., Indianapolis, IN) guinea-pigs (250-300 g) were anesthetized with isoflurane and euthanized by cervical dislocation. The brain was removed and chilled by submersion in ice cold artificial cerebrospinal fluid (aCSF: NaCl 124 mM, KCl 3 mM, CaCl₂ 2.4 mM, MgSO₄ 1.3 mM, K₂HPO₄ 1.24 mM, NaHCO₃ 26 mM, glucose 10 mM, equilibrated with 95% O₂/5% CO₂). Hippocampi were dissected out, sliced on a Mcllwain tissue chopper to a nominal thickness of 415 μ m and incubated in a holding chamber at room temperature for at least 90 min.

Peroxide solutions were made fresh daily from 50% concentrate (Fisher). Most experiments used a peroxide concentration of 0.002% (720 μ M). Previous studies^{27,29} on hippocampal slices used concentrations between 0.005% (1.8 mM) and 0.01% (3.6 mM). The 0.002% concentration was chosen for the present study because at this level peroxide had no direct effects on electrophysiological potentials in the brain slice. These concentrations of peroxide

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Abbreviations: aCSF, artificial cerebrospinal fluid; HFS, high frequency stimulation; I/O, input/output; LTP, long-term potentiation; PSP, postsynaptic potential; STP, short-term potentiation.

are comparable to those used as a model of free radical damage to electrophysiological events in cardiac tissue ($100 \mu\text{M}$ – 10 mM)^{11,14}

For electrophysiological recording, a slice was placed in a laminar flow submersion chamber (Zbrzez design)¹⁰ and perfused with aCSF at 30°C. A bipolar stimulating electrode was positioned in the stratum radiatum of field CA1 to stimulate afferent pathways. Glass microelectrodes (2N NaCl) were placed in the stratum radiatum of field CA1 to record the afferent volley and the population postsynaptic potential (population PSP) and in the stratum pyramidale to record the population spike. The magnitude of the population spike was calculated from the mean of the early and late positivities minus the maximal negativity. The population PSP was quantified from the maximum negative slope early in the synaptic response. Signals from the microelectrodes were recorded with WPI high gain d.c. amplifiers and then digitized, stored and analysed on an LSI 11/23 minicomputer.

For 30 min, baseline recordings were made to ensure stability of the tissue. If the recordings deviated substantially from the initial values or developed secondary population spikes, the experiment was terminated. The slice was stimulated (0.2 Hz) at an intensity sufficient to produce a half maximal response. Averages of four responses were stored at 5-min intervals.

In most experiments, perfusion of peroxide was started after this equilibration period and continued for the duration of the experiment. After 30 min, a high frequency stimulus (HFS, 100 Hz, 1 s) was delivered at the half maximal stimulus amplitude. Following HFS, data collection continued for another 60 min. In most experiments, data continued to be stored every 5 min. In experiments used for the exponential curve fitting, data were collected more frequently and were not averaged, in order to allow accurate representation of the time course. In some experiments, the perfusion of peroxide was either delayed to follow HFS or terminated immediately after HFS. Some slices were untreated (i.e. never exposed to peroxide). In control experiments, no HFS was delivered and the actions of peroxide alone were followed. The timing of the experiments was identical in all other respects. In two of the 78 slices, HFS failed to cause even short term potentiation (STP, i.e. potentiation that develops immediately and decays within 15 min), these two experiments were not included in the analysis.

Input/output (I/O) curves were generated before and after HFS in peroxide-treated and untreated slices using a range of stimulus intensities (0.0–0.5 mA, 200 μs). Three relationships were examined using the averages from eight experiments: population spike vs afferent volley, population PSP vs afferent volley and population spike vs population PSP. The third relationship (population spike vs population PSP) was also evaluated for each individual experiment. I/O curves were generated 30 min prior to HFS and 60 min following HFS. The timing was designed to prevent any influence of STP, sometimes produced by generating the I/O curve, on the LTP evoked by HFS. Since the pre-HFS time point coincided with the application of peroxide, the peroxide concentration was not at its peak. This is not a concern since peroxide at the concentration used does not change the I/O curves (data not shown). Data for the I/O curves were analysed as previously described.¹⁰ In brief, best-fit sigmoid curves were determined for both pre- and post-HFS data. For each curve, a parameter was computed from the maximal y -value divided by the x -value at half maximal y . Changes in this parameter have been effective in evaluating changes in I/O curves.

Paired pulse facilitation was studied by using two identical stimuli (200 μs) separated by intervals from 10 to 200 ms. The stimulus strength was adjusted to produce approximately a half maximal population spike. Amplitude of the second population spike was expressed as a percent-

age of the first population spike. Facilitation was evaluated in normal aCSF and 30 min after perfusion with 0.01% peroxide.

Data are expressed as mean values $\pm$ standard errors. Student's t -test was used for comparisons of two predetermined sets of data. Analysis of variance was used to evaluate differences among more than two treatment groups. For all statistical analyses, a probability level of $P < 0.05$ was considered to reflect significance.

RESULTS

Peroxide at a concentration of 0.002% had no direct effect on the amplitude of the population spike, even after a 90 min exposure (Fig. 1) ($n = 4$). Yet, this concentration of peroxide significantly impaired the ability of HFS to produce LTP. In untreated slices ($n = 17$), HFS caused an immediate increase in the population spike to $285 \pm 14\%$ of control amplitude. Within 15 min, this amplitude fell to $238 \pm 13\%$ of control and sustained that level for the remainder of the experiment. All 17 slices showed potentiation of at least 130% of control 60 min following HFS. In slices treated with peroxide ($n = 16$), there was also an early enhancement of the amplitude ($270 \pm 15\%$) which was not significantly different from potentiation in untreated slices (t -test, $P > 0.05$). In contrast to untreated tissue, however, by 30 min the amplitude was only $185 \pm 13\%$ and by 60 min only $124 \pm 14\%$. The amplitude of population spikes in peroxide-treated slices did not establish a plateau but slowly fell throughout the measurement period (Fig. 1A). Only five of 16 slices showed population spike amplitudes of 130% of control or greater at 60 min post-HFS. At 60 min post-HFS, the population spike amplitudes of treated and untreated slices were significantly different from one another (t -test, $P < 0.05$). The inset in Fig. 1A shows sample traces from treated and untreated slices. The population spike in untreated slices showed a substantial increase in amplitude that was sustained for 60 min. Potentiation was not sustained in peroxide-treated slices and the population spikes before and 60 min after HFS are nearly the same size.

A similar pattern was evident with the population PSP (Fig. 1B). In untreated slices the magnitude of the early slope of the population PSP was increased to $284 \pm 26\%$ of control with a decline to $203 \pm 20\%$ of control within about 15 min. The increase was sustained for the remainder of the experiment ($202 \pm 28\%$ at 60 min post-HFS). At 60 min after HFS, in 13 of 17 slices the population PSP was at least 130% of control amplitude. Sample traces before and 60 min following HFS in untreated slices show a sustained enhancement of the synaptic response. Treatment with 0.002% peroxide prevented the maintained increase in the PSP. By 60 min post-HFS the population PSP was nearly the same as during the control period. The initial increase was $261 \pm 30\%$ and declined to $129 \pm 17\%$ after 60 min. Only five of 16 slices showed population PSPs that

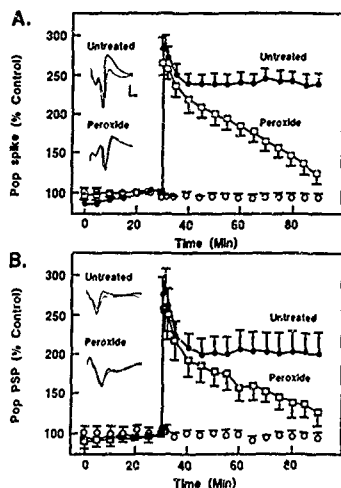


Fig. 1. Hydrogen peroxide (0.002%) prevents the maintenance of LTP. (A) Amplitude of population spike expressed as percentage of control plotted vs time. Error bars show standard error of the mean for each time point. Open circles population spike in tissue treated with peroxide but not stimulated with HFS ($n = 4$). Open squares peroxide present, HFS applied at 30 min time point ($n = 16$). Closed circles untreated tissue, HFS at $t = 30$ min ($n = 17$). Insets show sample traces from individual experiments. Light trace is from before HFS while bold trace occurred 60 min after HFS ($t = 90$ min). Calibration: 1 mV, 2 ms. (B) Changes in population PSP with time. Symbols same as in A. Insets show sample population PSPs from same experiments as in A: light trace from before HFS, bold trace from 60 min post-HFS. Same calibration as in A.

were at least 130% of control. Sample traces show very little difference between synaptic responses recorded prior to HFS and responses 60 min following HFS in peroxide-treated slices. At 60 min post-HFS, treated and untreated population PSPs were statistically different from one another (t -test, $P < 0.05$).

A higher concentration of peroxide (0.005%) was tested with HFS on three slices. As previously observed,²⁹ this concentration had a direct effect to decrease both the synaptic response and the population spike. HFS elicited early potentiation of both population spike amplitude and population PSP slope. Within about 30 min, however, the responses were back to control level and continued to decline. Population spike potentiation fell to only $105 \pm 28\%$ of control within 30 min. At 45 min post-HFS, the average population spike was $58 \pm 33\%$ of control. The synaptic response was similarly reduced very quickly.

Paired-pulse facilitation was evaluated with 0.01% peroxide ($n = 3$; data not shown). Facilitation was

maximal at an interstimulus interval of 30 ms, $302 \pm 50\%$ in untreated slices and $297 \pm 28\%$ in peroxide-treated slices. In agreement with Colton *et al.*,⁶ facilitation was unaffected by peroxide at all interstimulus intervals tested (10–200 ms).

Peroxide might prevent LTP by interfering with its induction or its expression. To distinguish between these possibilities, peroxide was applied at different times during the process. Table 1 illustrates the change in population spike 60 min after HFS with four experimental treatments. (1) untreated ($n = 17$), (2) treated with peroxide throughout the experimental period ($n = 16$), (3) treated with peroxide before and up to 10 min after HFS ($n = 16$) and (4) treated with peroxide only after HFS (5–60 min post-HFS, $n = 9$). Analysis of variance showed that potentiation in slices treated with peroxide throughout (treatment 2) was significantly less than in untreated slices (treatment 1, $P < 0.05$). When peroxide was removed after HFS (treatment 3), population spike amplitude was not maintained at the level of untreated slices but decayed more slowly than in slices with continued exposure to peroxide (treatment 2), potentiation was not statistically different from that in either of the first two treatments. When peroxide was applied only after HFS (treatment 4), potentiation of the population spike was sustained as in untreated slices. Analysis of synaptic potentials at 60 min post-HFS showed a similar pattern (data not shown): peroxide following HFS was ineffective while peroxide removed after HFS was only partly effective in reducing LTP.

In the previous series of experiments, the potentiation following HFS was evaluated at only one stimulus intensity, kept constant throughout the experiment. In an effort to evaluate the change in the response to a range of stimulus strengths, I/O curves were generated 30 min prior to HFS and 60 min after HFS. As seen in Fig. 2, in untreated slices ($n = 8$), HFS increased the ability of the afferent volley to elicit a population spike throughout the

Table 1. Timing of peroxide application affects amount of potentiation resulting from high frequency stimulation

Treatment	Percentage increase in population spike ($\pm$ SEM)
(1) Untreated	137.5 ± 14.5
(2) Peroxide throughout	$24.0 \pm 13.6^*$
(3) Peroxide before and during HFS	85.5 ± 16.8
(4) Peroxide after HFS	136.1 ± 16.4

Measurements show percentage increase in population spike 60 min after HFS compared to control. The population spike from untreated tissue shows significant potentiation. Treatment with peroxide (0.002%) throughout the experiment prevented sustained potentiation.

*Analysis of variance, $P < 0.05$. Application of peroxide before and during HFS but washed out within 5 min prevented some but not all potentiation ($P < 0.05$). Application of peroxide only after HFS did not affect potentiation ($P < 0.05$).

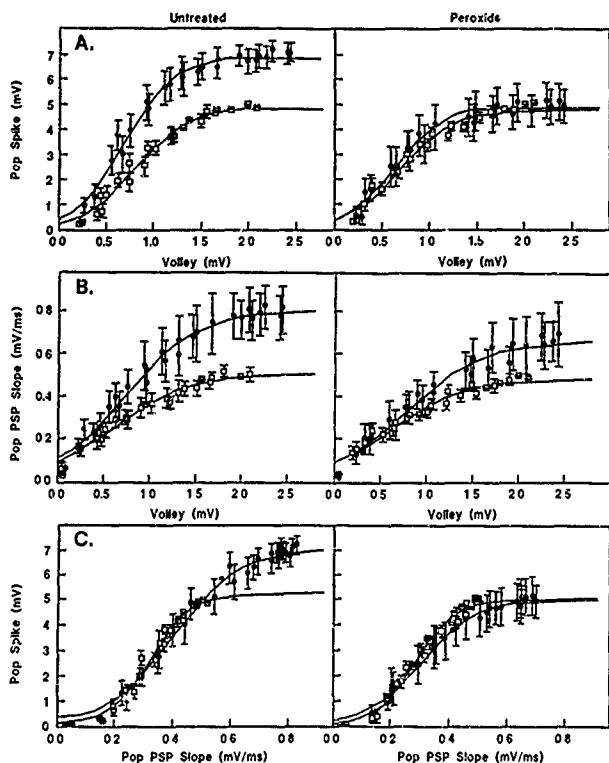


Fig 2 I/O curves obtained from tissue treated with 0.002% peroxide ($n = 8$, right) and untreated ($n = 8$, left). Curves obtained 30 min prior to HFS (open squares) and 60 min after HFS (closed circles) in both conditions (A) Plot of population spike vs volley shows significant enhancement of population spike following HFS in untreated tissue which is not evident in peroxide-treated tissue (B) Plot of population PSP vs volley shows enhancement of synaptic response in untreated tissue which is less dramatic with peroxide treatment. (C) Plot of population spike vs population PSP shows only minimal changes in E/S coupling both in treated and untreated tissue

range of stimulus intensities ($166 \pm 9\%$). In tissue treated with 0.002% peroxide ($n = 8$), this enhancement is greatly reduced ($112 \pm 7\%$). HFS also increased the ability of the afferent volley to evoke a synaptic potential in untreated slices ($134 \pm 13\%$) (Fig 2B). Peroxide decreased this potentiation ($105 \pm 11\%$) (Fig. 2B). The third set of graphs (plot of population spike vs population PSP) provides an indication of the ability of the synaptic potential to evoke a spike, also called E/S coupling. Other authors^{1,2,37} have shown that E/S coupling is sometimes enhanced with LTP. Andersen *et al.*² reported that only 50% of their slices showed this phenomenon. In the present experiment, there was no obvi-

ous change in this relationship following LTP in the average response of eight untreated slices ($109 \pm 5\%$), yet four of eight of these slices did show enhanced E/S coupling (i.e. greater than 20% increase) in agreement with Andersen *et al.*² In peroxide-treated slices, there is also no E/S enhancement in the averaged curves ($94 \pm 4\%$) and only two of eight treated slices showed enhancement.

Potentiation following HFS can be resolved into components by evaluating the time constants of decay of the response back to baseline.²⁴ In each of the eight treated and eight untreated slices in the experiment above, data at additional time points were collected to provide a more accurate represen-

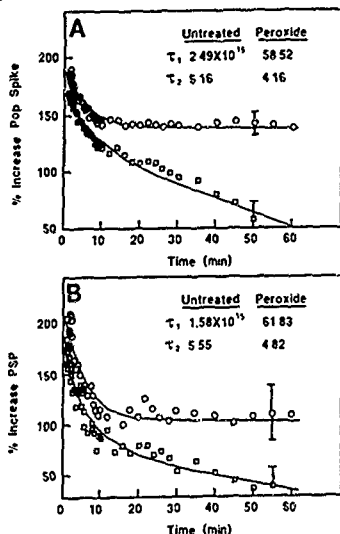


Fig. 3 Curves describing the sum of two exponential processes were fitted to the data for the decay of potentiation of the population spike (A) and the population PSP (B) both with (open squares) and without (open circles) peroxide treatment. HFS given at time = 0. Calculated time constants (min) shown in insets. Details of curve fitting in text. Points represent averages from eight experiments. One representative error bar is shown for each curve.

tation of changes in the amplitude of the population spike and the population PSP with time. The percentage increase in response was plotted vs time, with time = 0 at the time of HFS (Fig. 3). The four resultant curves (population spike and population PSP, with and without peroxide treatment) are described by the equation for the sum of two exponentials

$$\% \text{ change} = a \cdot \exp(-t/\tau_1) + b \cdot \exp(-t/\tau_2),$$

where a , b , τ_1 , and τ_2 are evaluated for the best fit to the experimental data with a routine (FITFUNCTION) in the analytical package RS/1 (BBN Software Product, Cambridge, MA). τ_1 and τ_2 are the time constants for the slow and fast components of the curve, respectively. a and b are weighting constants for the two exponentials and were insensitive to peroxide. While the computed values seem to fit the data very well, we do not assume that they are necessarily unique solutions to the equation. The time constants of decay of potentiation of the population spikes of the best fit curves were 2.49×10^{15} and 6.16 min in untreated tissue and 58.52

and 4.16 min in peroxide-treated slices. It is obvious that the slower time constant is decreased by peroxide treatment, whereas the faster time constant is not much affected. The time constants for the synaptic response showed a similar effect. In untreated slices the calculated time constants were 1.58×10^{15} and 5.55 min and in peroxide-treated slices they were 61.83 and 4.82 min. Again, the slow time constant is greatly decreased by treatment with peroxide while the faster time constant is not. Thus, the late phase of LTP appears to be selectively altered by peroxide.

DISCUSSION

The electrophysiological effects of peroxide on field potentials in hippocampal slices have been shown to be mediated by free radicals.²⁹ In the present study, we demonstrate that peroxide interferes with LTP at concentrations that do not affect unpotentiated synaptic transmission. The I/O curves show that free radicals predominantly impair potentiation of the synaptic response and have much less effect on spike generation (E/S coupling). This contrasts with higher concentrations of peroxide, which significantly reduced both synaptic efficacy and E/S coupling.²⁷ The decreased synaptic potentials in previous studies were hypothesized to be a consequence of reduced transmitter release.²⁸ Free radical effects on LTP, a complex neuronal process, may involve other mechanisms.

HFS induces at least two phases of potentiation, the later one being LTP. The early phase has been referred to as STP.^{24,33,36} Our results in untreated tissue suggest that LTP does not decrement measurably. While the calculated time constant may not be an accurate assessment of the extended time course, it does make the point that in untreated tissue, LTP is a sustained process. In contrast, in peroxide-treated tissue, decay of LTP has a time constant of only about 1 h. On the other hand, the earlier component of potentiation, with a time constant of 5–6 min, is not very sensitive to peroxide. This component is likely to correspond to what McNaughton²⁴ called potentiation, which has a time constant around 1.5 min *in vivo*. Temperature sensitivity can account for much of the quantitative difference in the time constant between his experiments and the present results.

Analysis of the time constants of decay revealed that only the late phase of potentiation was affected by peroxide while the early decay was unchanged. Previous studies have shown that STP and LTP have very different mechanisms. Several authors^{19,24,42} have suggested that STP reflects an increase in the probability of transmitter release, most likely due to an increase in presynaptic calcium. Similarly, paired-pulse facilitation is caused by an increase in the probability of transmitter release. In contrast, it has been suggested that enhanced calcium entry at presynaptic terminals is not the mechanism for

LTP,^{24,25} LTP does not appear to entail an increase in the probability of transmitter release, instead McNaughton²⁴ hypothesized that the presynaptic component of synaptic enhancement in LTP could reflect an increase in the number of quanta available for release or an increase in the number of binding sites for vesicles. Since STP and paired-pulse facilitation are unaffected by peroxide exposure, it is unlikely that free radicals are interfering with presynaptic calcium entry. In fact, voltage clamp studies in hippocampal pyramidal cells have shown that sustained high threshold calcium currents²⁷ and transient low threshold calcium currents (unpublished data) are insensitive to peroxide. Other steps in the release process need to be considered as possible peroxide-sensitive sites.

Induction of LTP also requires calcium postsynaptically,^{4,21,26} but only within 5 min of HFS.²⁴ It is possible that peroxide is interfering with a postsynaptic calcium-dependent process. Remote calcium spikes in hippocampal neurons show an increased threshold with exposure to peroxide²⁷ and calcium-dependent processes in a number of cell types are reportedly sensitive to free radical damage.^{4,5,14,15}

Peroxide must be present during induction of LTP to be effective. Yet its action is to prevent maintenance of the potentiation. This suggests that peroxide is interfering with some process during the induction phase of LTP required to fully express the potentiation. Recent reports^{17,20,22,30} show that a number of second messenger systems must similarly be available during HFS for LTP to occur. Blocking postsynaptic protein kinase C or calmodulin II kinase prevents the induction of LTP, STP is evident, but by 30 min post-HFS the responses are back to control levels.^{20,22} As with peroxide, the time of application of kinase inhibitors is critical. Intracellular injection of the blockers in the postsynaptic cell after HFS has no effect. In these experiments it is not possible to

remove the blockers after injection, which complicates comparison with our experiments in which removal of peroxide was less effective than continued exposure. It is possible that peroxide is interfering with one of the several second messenger systems thought to be involved with LTP.

Recent studies^{14,28} suggest that the oxidation/reduction state of the *N*-methyl-D-aspartate affects its electrophysiological response. In a number of neuronal preparations, dithiothreitol, a sulfhydryl reducing agent, caused long-term enhancement of the response to *N*-methyl-D-aspartate.¹⁴ This effect could be reversed by oxidation with dithio-bis-nitrobenzoic acid. Tauck and Ashbeck²⁸ reported that dithiothreitol, at a concentration that had no direct effects on the synaptic potential, was able to enhance LTP. It is possible that the free radicals formed in the present study oxidize the *N*-methyl-D-aspartate receptor, decrease its contribution to the synaptic response even with HFS and thereby reduce the expression of LTP.

Hydrogen peroxide reacts with tissue iron to generate hydroxyl free radicals. While free radicals are constantly formed in healthy tissue, the intrinsic antioxidant systems keep them in check. However, under pathological conditions, free radical generation can exceed the tissue's ability to control them. Our study suggests that under such conditions, LTP, and perhaps memory processes, can be disrupted.

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