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RESEARCH PAPER

Neuroprotective effect of hydrogen peroxide on an *in vitro* model of brain ischaemia

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Background and purpose: Reactive oxygen species (ROS) have been postulated to play a crucial role in the pathogenesis of ischaemia-reperfusion injury. Among these, hydrogen peroxide (H_2O_2) is known to be a toxic compound responsible for free-radical-dependent neuronal damage. In recent years, however, the 'bad reputation' of H_2O_2 and other ROS molecules has changed. The aim of this study was to assess the protective role of H_2O_2 and modification in its endogenous production on the electrophysiological and morphological changes induced by oxygen/glucose deprivation (OGD) on CA1 hippocampal neurons.
Experimental approach: Neuroprotective effects of exogenous and endogenous H_2O_2 were determined using extracellular electrophysiological recordings of field excitatory post synaptic potentials (fEPSPs) and morphological studies in a hippocampal slice preparation. *In vitro* OGD was delivered by switching to an artificial cerebrospinal fluid solution with no glucose and with oxygen replaced by nitrogen.
Key results: Neuroprotection against *in vitro* OGD was observed in slices treated with H_2O_2 (3 mM). The rescuing action of H_2O_2 was mediated by catalase as pre-treatment with the catalase inhibitor 3-amino-1,2,4-triazole blocked this effect. More interestingly, we showed that an increase of the endogenous levels of H_2O_2, due to a combination of an inhibitor of the glutathione peroxidase enzyme and addition of Cu,Zn-superoxide dismutase in the tissue bath, prevented the OGD-induced irreversible depression of fEPSPs.
Conclusions and implications: Taken together, our results suggest new possible strategies to lessen the damage produced by a transient brain ischaemia by increasing the endogenous tissue level of H_2O_2.
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Keywords: hydrogen peroxide; ischaemia; electrophysiology; hippocampus; neuroprotection

Abbreviations: ACSF, artificial cerebral spinal fluid; 3-AT, 3-amino-1,2,4-triazole; fEPSP, field excitatory postsynaptic potentials; GD, glucose deprivation; GPXs, glutathione peroxidases; MPS, mercaptosuccinate; O_2^-, superoxide anion; OGD, oxygen/glucose deprivation; OH^-, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase

Introduction

The brain requires a continuous supply of oxygen and glucose to maintain function. Deprivation of energy sources following stroke, global ischaemia or respiratory failure, can lead rapidly to transient or permanent injury of neurons by affecting the cells' energy requirements, pump function or membrane integrity (Lo *et al*., 2003). The selective vulnerability of pyramidal neurons in the hippocampal subregion CA1 is one of the hallmarks of the rodent models of global cerebral ischaemia (Kirino, 1982; Pulsinelli *et al*., 1982). In humans, a similar pattern, with selective and delayed degeneration of CA1 neurons, has been documented in autopsy studies on survivors after a cardiac arrest (Petito *et al*., 1987; Horn and Schlote, 1992). Although reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and/or hydroxyl radical (OH^-), have been shown to cause damage to protein, lipids and nucleic acids and may contribute to the harmful side effect of hypoxia/reoxygenation (Traystman *et al*., 1991), there is also evidence that ROS have normal regulatory actions and play a major role in the modulation of cellular signal transduction

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cascades in a variety of physiological processes (Sen and Packer, 1996; Finkel, 2003). Hydrogen peroxide is a diffusible molecule that can be synthesized and destroyed rapidly in response to external stimuli, and, only recently, it has become clear that it meets all the important criteria for an intracellular messenger for signal transduction and signal amplification (Rhee *et al*., 2005; Stone and Yang, 2006) and acts as an ubiquitous second messenger under subtoxic conditions (Finkel, 1998; Rhee *et al*., 2000). Moreover, H_2O_2 can modulate synaptic transmission (Pellmar, 1987; Chen *et al*., 2001) and synaptic plasticity (Auerbach and Segal, 1997; Klann and Thiels, 1999).

Several antioxidant mechanisms serve to counterbalance the potential deleterious effects of ROS. Among these, the enzymatic scavengers superoxide dismutase (SOD), catalase and glutathione peroxidases (GPXs) (Brigelius-Flohe, 1999) are responsible for the balance of formation and conversion of H_2O_2, maintaining the intracellular concentration of H_2O_2 at a constant level (Halliwell, 1999). When these downstream enzymatic pathways are impaired and H_2O_2 is not degraded, cellular metabolism may be affected. However, in conditions of low oxygen supply, such that occurring during hypoxia or ischaemia, metabolic degradation of H_2O_2 through catalase results in production of H_2O and O_2, therefore providing an alternative source for O_2 (Auerbach and Segal, 1997; Klann and Thiels, 1999). Indeed, previous observations *in vitro* have shown that H_2O_2 exerts a protective role by restoring synaptic transmission after an episode of hypoxia in the hippocampus (Fowler, 1997), and that it may act as a supplementary source of O_2 in the spinal cord (Walton and Fulton, 1983). More recently, it has been suggested that endogenous H_2O_2 plays an important cardioprotective (Yaguchi *et al*., 2003) and neuroprotective role in several *in vitro* models of ischaemic preconditioning (Furuichi *et al*., 2005; Xiao-Qing *et al*., 2005). We also have recently shown a protective role of H_2O_2 in oxygen-deprived dopaminergic neurons of the rat substantia nigra (Geracitano *et al*., 2005). The goal of the present study was to evaluate the neuroprotective role of exogenous H_2O_2 and the potential of modifying the activity of key enzymes (Cu,Zn-SOD, catalase and GPX) involved in the endogenous production and degradation of H_2O_2. To this aim, we used both electrophysiological and morphological techniques, in an *in vitro* model of hippocampal ischaemia.

Methods

Brain slice preparation

All animal procedures were in compliance with the European Council Directive (86/609/EEC). Male Wistar rats, (140–180 g body weight) under anaesthesia with halothane, were killed by decapitation. Their hippocampi were rapidly removed and placed in an ice-cold, oxygenated (95% O_2 to 5% CO_2), artificial cerebral spinal fluid (ACSF) of the following composition (mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 24 $NaHCO_3$ and 10 glucose. Parasagittal slices (400 μm thickness) were cut using a vibratome and kept in oxygenated ACSF for at least 1 h at room temperature.

Extracellular recordings

A single slice was placed on a nylon mesh, completely submerged in a small chamber (0.5 ml) and superfused with oxygenated ACSF (~30 °C) at a constant flow rate of 3 ml min^{-1}. Test pulses (80 ms, 0.06 Hz) were delivered through a bipolar nichrome electrode positioned in the stratum radiatum. Evoked extracellular potentials were recorded with glass microelectrodes (2–10 MΩ), filled with 3 M NaCl, placed in the CA1 region of the stratum radiatum. *In vitro* oxygen/glucose deprivation (OGD) was obtained by perfusing the slice with ACSF without glucose and gassed with nitrogen (95% N_2 to 5% CO_2). At the end of the ischaemic period, the slice was again superfused with normal (glucose-containing) oxygenated ACSF. Hypoglycaemic solutions were obtained by omitting glucose from standard ACSF.

Morphological studies

After the recording session, slices were stained with Cresyl Fast Violet solution to evaluate the number of cells irreversibly damaged either by the handling procedures alone (control) or by the putative ischaemic insult (untreated and treated with 3 mM H_2O_2). Immediately after each treatment, slices were collected from the nylon mesh and placed for 30 min in normal ACSF warmed to 35 °C, and saturated with 95% O_2, 5% CO_2. This time window was chosen for all groups to allow sufficient time for recovery after each treatment. Then, they were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, washed and dehydrated in 30% sucrose solution for 3 h at room temperature. Finally, the slices were frozen in liquid nitrogen and cut (20 μm thickness) with a cryostat in serial sections. Sections were subsequently mounted on slides, stained with Cresyl Fast Violet and analysed under light microscopy (Zeiss-Axioplan 2). Neuronal cells count was performed blind and concerned the CA1 pyramidal cell layer of the hippocampal formation. A 25-μm^2 grid was positioned on identical areas of the hippocampus in each section ($\times 40$ objective) and the viable cells were counted in two fields of each brain section.

Statistical analysis

Data are expressed as mean ± s.e.mean and were tested for statistical significance with a paired, two-tailed Student's *t*-test or by one-way ANOVA followed by Tukey–Kramer multiple comparisons test, as appropriate. When significant differences were observed, Newman–Keuls multiple comparison test was inferred. A value of $P<0.01$ was considered significant.

Drugs

All the compounds were obtained from Sigma-Aldrich (Milan, Italy). H_2O_2 was diluted daily from a 30% stock solution. The concentration of the stock solution was 8.8 M.

Results

The effect of different periods of OGD on hippocampal synaptic transmission

The ability of hippocampal slices to recover synaptic function on return of normal oxygenation depends on the duration of the OGD; the longer the OGD lasted, the less synaptic function could recover. Figure 1 illustrates the time course of the field excitatory postsynaptic potential (fEPSP) depression caused by OGD applied for a period of 9 and 14 min. OGD for 9 min caused a transient fEPSP depression that was always reversible after returning to normal oxygenated ACSF (101 ± 1%, $n=9$, paired Student's *t*-test $P<0.0001$). During OGD, the amplitude of the field potential rapidly decreased and in 5 min was almost abolished. Prolonging the OGD to 11 min caused a loss of the field potentials in 50% of the experiments recorded (6/12, data not shown). Therefore, 14 min of OGD, which always provided an irreversible loss of the field potentials (2 ± 0.09%, $n=15$, paired Student's *t*-test $P<0.0001$), was chosen as the standard ischaemic insult to test, in subsequent experiments, the neuroprotective potential of various compounds. After 14 min of OGD, no recovery of the field potentials was observed during reoxygenation up to 180-min perfusion in normal ACSF (data not shown). Towards the end of the OGD, a transient reappearance of the fEPSP was often observed, being ascribed to the increase in extracellular potassium concentration (~8 mM) caused by the anoxic episode (Sick *et al*., 1987).

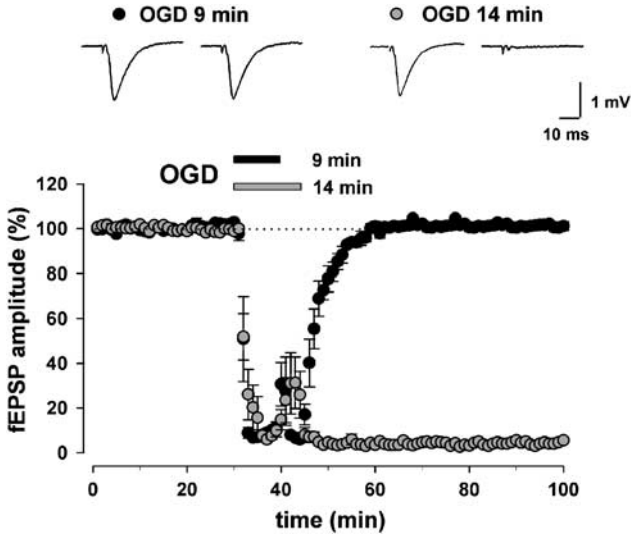

Figure 1 Effects of oxygen/glucose deprivation (OGD) on field excitatory postsynaptic potentials (fEPSPs) evoked by electrical stimulation of the stratum radiatum in the CA1 hippocampal region. The recovery of fEPSP amplitude following 9 min ($n=9$) or 14 min ($n=15$) of OGD. Exposure to 9 min of OGD (black circles) causes a transient fEPSP depression that was always reversible after returning to normal oxygenated artificial cerebral spinal fluid (ACSF), whereas exposure to 14 min of OGD (grey circles) caused an fEPSP depression that was always irreversible even after a prolonged re-exposure to normal oxygenated ACSF. Each point represents fEPSP amplitude expressed as percentage of the mean baseline responses recorded before OGD application. Bars indicate the time duration of OGD. Insets display representative traces immediately before the OGD and at the end of the experiment.

Neurotoxic effect vs neuroprotective effect of exogenous H_2O_2 on fEPSPs

In the present study, we examined the effect of H_2O_2 added to the bathing solution on the synaptic responses recorded from rat hippocampal slices in control conditions and during OGD. In the CA1 region, the sensitivity of fEPSPs to H_2O_2 was assessed by applying increasing concentrations of this compound. H_2O_2 irreversibly depressed the synaptic AMPA receptor-mediated response with an IC_{50} value of 5.8 ± 0.4 mM ($n=4$; Figures 2a and b), suggesting that H_2O_2 is a neurotoxic agent in normoxic conditions. Conversely, as illustrated in Figure 2c, exposure to combined OGD and H_2O_2 (3 mM) resulted in a transient suppression of the fEPSP to 6 ± 3%, and a subsequent return to 101 ± 1% ($n=6$, paired Student's *t*-test $P<0.0001$) after 60 min reoxygenation. Notably, H_2O_2 was able to confer neuroprotection even when the duration of OGD was extended up to 30 min. Surprisingly, H_2O_2 was able to rescue the irreversible loss of fEPSP, even when applied 7 min after OGD had started, highlighting its potent neuroprotective effect once the ischaemic conditions had been imposed. In fact, fEPSP fully recovered to 97.4 ± 6% ($n=6$, paired Student's *t*-test $P<0.0001$, Figure 2d). The EC_{50} value of H_2O_2-mediated fEPSPs recovery in OGD was 0.67 ± 0.1 mM ($n=4$; Figure 2e). For our experiments we used 3 mM H_2O_2, a concentration that always resulted in neuroprotection.

H_2O_2 specifically counteracted hypoxia and did not oppose the effects of hypoglycaemia

The CA1 pyramidal neurons are among the most vulnerable in the brain to hypoglycaemic stress. Although studies on brain slices have demonstrated that exposure to glucose-free medium enhances glutamate and aspartate release (Burke and Nadler, 1989), synaptic transmission inevitably fails during hypoglycaemia (Fowler, 1993). Our next step was to test whether H_2O_2 may also prevent the loss of synaptic function induced by a period of glucose deprivation (GD). As shown in Figure 4, perfusion of slices in a glucose-free ACSF caused a continually decaying loss of the field potentials that was complete after 30 min (6 ± 1%, $n=6$, paired Student's *t*-test $P<0.0001$, Figure 2f). However, in contrast to what we found during OGD, no recovery was observed following perfusion with H_2O_2 (3 mM) in aglycaemic ACSF (2 ± 0.5%, $n=5$, paired Student's *t*-test $P<0.0001$, Figure 2g), suggesting that hydrogen peroxide exerts its protective role against OGD by compensating for the lack of oxygen. The loss of fEPSP observed in the presence of H_2O_2 occurred even more rapidly, confirming that hydrogen peroxide may paradoxically result a toxic compound in a normoxic environment. In both conditions, the GD-mediated loss of fEPSPs was irreversible following reperfusion with normoglycaemic ACSF (data not shown).

Morphological studies of the protective effect of H_2O_2

The protective effect of H_2O_2 observed with the electrophysiological *in vitro* model of cerebral ischaemia was also confirmed by histological experiments. We analysed viable CA1 cells in hippocampal slices subdivided into three

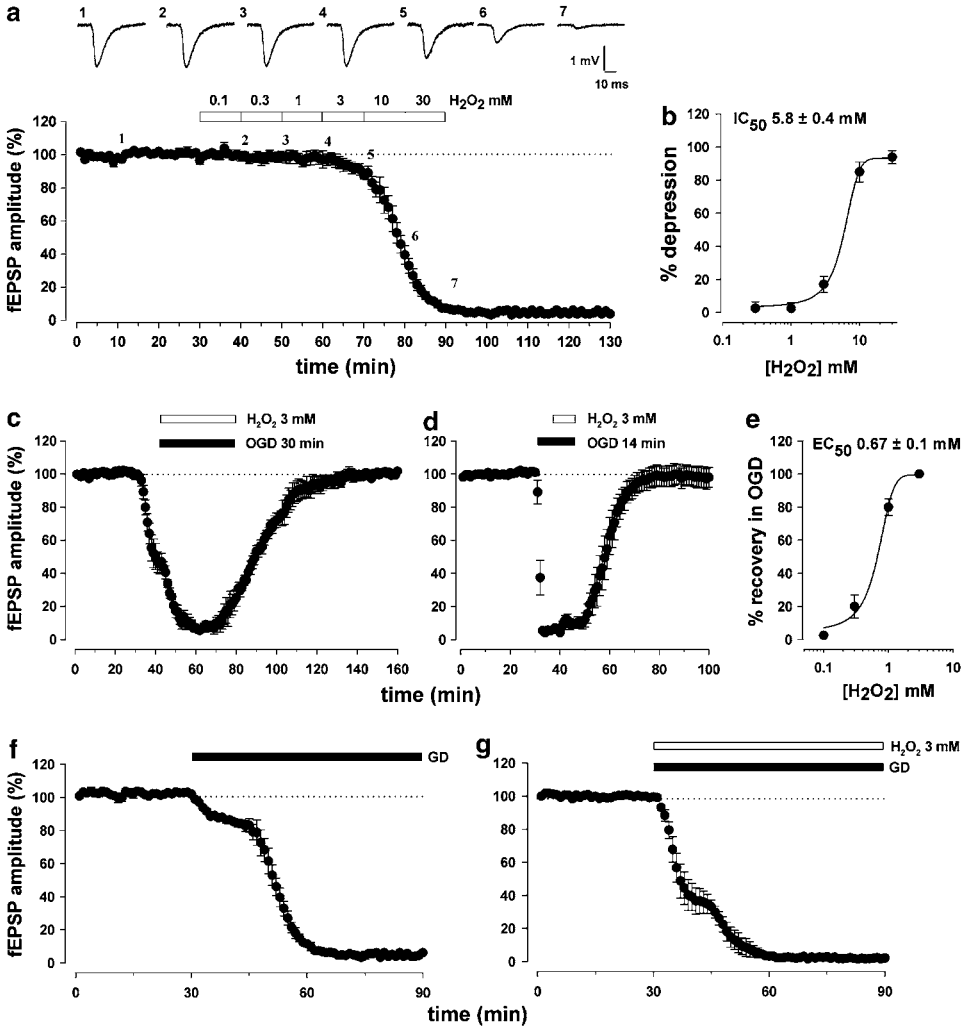

Figure 2 The effects of hydrogen peroxide (3 mM) on field excitatory postsynaptic potentials (fEPSPs) in the CA1 region of the hippocampus. (**a**) Pooled data from four experiments showing the effects of increasing concentrations of H_2O_2 on the synaptic depression of fEPSPs. (**b**) Concentration–response curve ($n=4$) showing the peak synaptic fEPSP depression vs [H_2O_2]. (**c**–**d**) Pooled data from six experiments showing the protective effect of H_2O_2 (3 mM) over a 30-min oxygen/glucose deprivation (OGD) exposure and its ability to rescue synaptic transmission even, when applied after OGD administration. (**e**) Concentration–response curve ($n=4$) showing the peak synaptic fEPSP recovery vs [H_2O_2] during OGD. (**f**) Pooled data from six experiments showing the complete loss of fEPSPs as a consequence of perfusing the slices in a glucose-deprived (GD) medium. (**g**) The same effect was insensitive to treatment with H_2O_2 (3 mM).

experimental groups ($n=6$): control slices, slices exposed to ischaemic ACSF and slices exposed to ischaemic ACSF treated with H_2O_2 (3 mM). In the control group, the viable neurons in the pyramidal cell layer of the CA1 hippocampal area show a good preservation of cell morphology (Figures 3a and b). In contrast, in the ischaemic group, the damaged neurons could be easily detected as round cell bodies with no distinction between cytoplasm and nucleus (Figures 3c and d). Interestingly, treatment with H_2O_2 in ischaemic conditions minimized neuronal damage caused by OGD alone (Figures 3e and f). Quantitative statistical analysis carried out by counting viable cells showed that the CA1 region of slices exposed to 14 min of OGD exhibited a marked decline in viable neurons when compared to control animals (Figure 3g). However, histological examinations revealed that H_2O_2 was able to rescue neurons that were otherwise destined to degenerate during OGD (Figure 3g).

Catalase mediates the protective effect of H_2O_2

We then investigated whether the protective effect of H_2O_2 was secondary to intracellular generation of O_2 through the catalase pathway (Llinas and Sugimori, 1980; Walton and Fulton, 1983). To this aim, we evaluated the consequence of H_2O_2 administration on 14 min of OGD in the continuous presence of the irreversible catalase inhibitor 3-amino-1,2,4-triazole (3-AT; 20 mM). Slices were preincubated with 3-AT (20 mM) for ~60 min, and were continuously perfused with this inhibitor during recordings. Under these conditions, the recovery of synaptic transmission usually provided by the presence of H_2O_2 (3 mM) during exposure to 14 min of OGD was completely abolished (with 3-AT, 1 ± 1%; without 3-AT, 101 ± 1%, $n=6$, paired Student's *t*-test $P<0.0001$; Figure 4a), suggesting that catalase mediated the protective effect of H_2O_2. Next, we wanted to ascertain that 3-AT did not accentuate *per se*

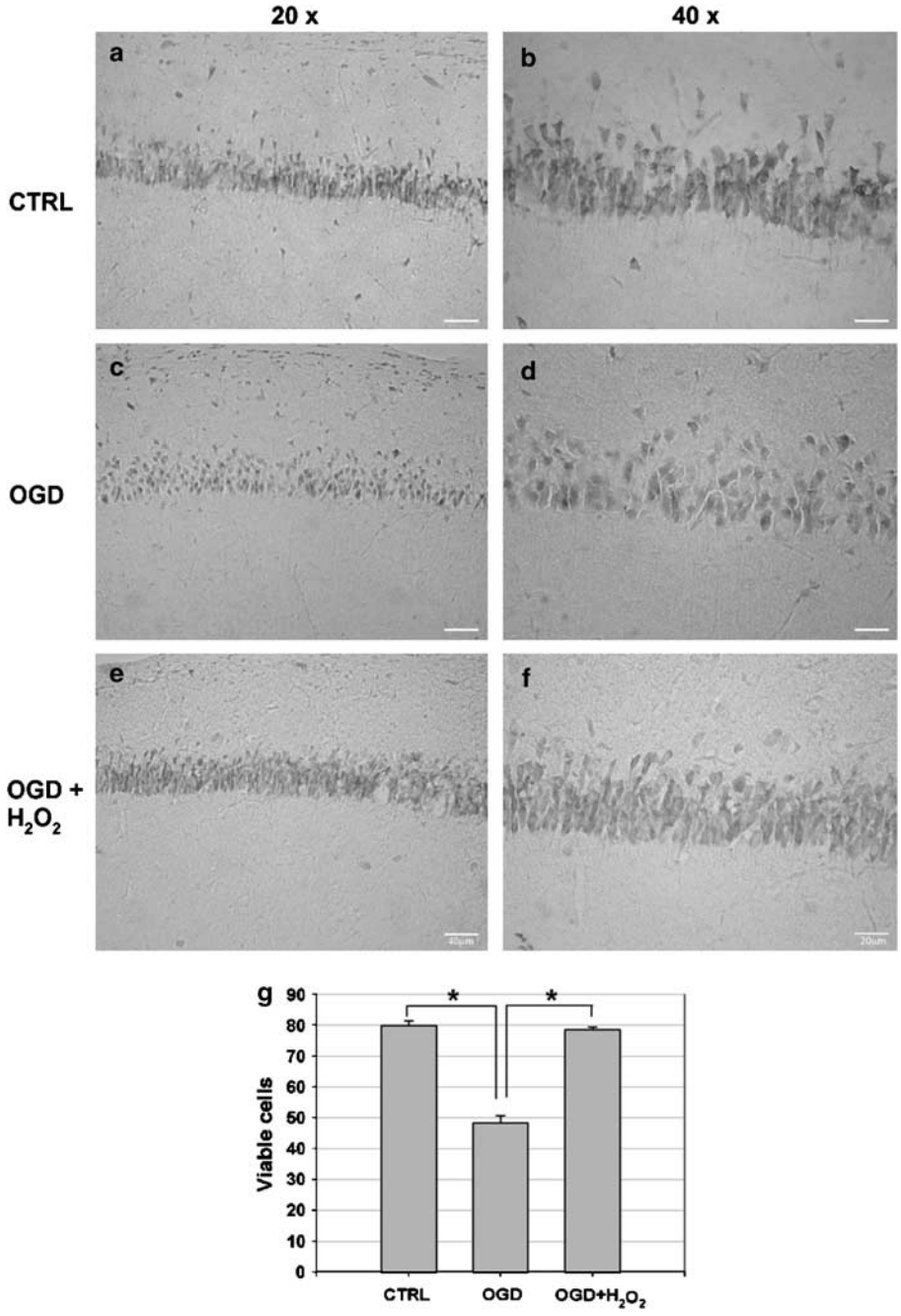

Figure 3 Representative photomicrographs showing the protective effect of H_2O_2 (3 mM) against 14 min of oxygen/glucose deprivation (OGD). (**a**–**f**) Photomicrographs of rat hippocampal sections stained with Cresyl Violet; (**a**, **c** and **e**) low magnification (× 20); (**b**, **d** and **f**) high magnification (× 40). (**a** and **b**) Refer to slices taken from a control animal; (**c** and **d**) refer to slices treated with 14 min of OGD; finally, (**e** and **f**) show the effect of pretreatment with H_2O_2 (3 mM). Slices were post-fixed 30 min after each treatment. (**g**) Density of Cresyl Violet viable cells per unit test area (25 μm^2) in the CA1 hippocampal area. Slices exposed for 14 min to OGD contained more damaged cells than control slices (OGD-treated vs untreated, $^*P<0.001$, $n=6$). A pretreatment with H_2O_2 (3 mM) significantly reduced the severity of cell damage after OGD compared with the untreated ischaemic slices (H_2O_2-treated vs OGD-treated, $^*P<0.001$, $n=6$).

the ischaemic damage, and hence, block the protective effect of H_2O_2. For this, we added 3-AT (20 mM) alone to slices exposed to 9 min of OGD, an insult from which the fEPSPs always recover (see Figure 1). As shown in Figure 4b, synaptic transmission still fully recovered to baseline following this procedure with 3-AT (101 ± 1%, $n=6$, paired Student's *t*-test $P<0.0001$).

Pharmacological strategies aimed to increase the endogenous levels of H_2O_2 prevent OGD-induced irreversible depression of synaptic transmission

Given that H_2O_2 exhibited a strong neuroprotective effect against OGD, in a subsequent set of experiments, we aimed to pharmacologically enhance the endogenous levels of H_2O_2, thus favouring the catalase pathway. We achieved this

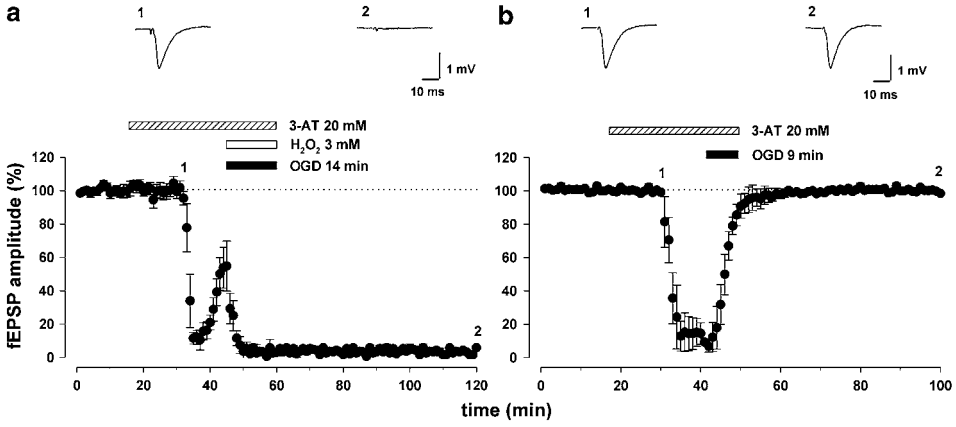

Figure 4 Catalase is involved in the protective effect of H_2O_2. (**a**) Pooled data from six experiments showing that the protective effect of H_2O_2 (3 mM) was prevented by superfusion, both in pretreatment and during oxygen/glucose deprivation (OGD), with the catalase inhibitor 3-amino-1,2,4-triazole (3-AT; 20 mM), suggesting that the protective role of H_2O_2 was mediated by catalase. The field excitatory postsynaptic potential (fEPSP) traces were taken at the time points indicated by numbers in the corresponding graph. (**b**) Treatment of slices with 3-AT alone was not able to alter fEPSPs recorded from slices exposed to 9 min of OGD compared to control conditions ($n = 6$).

enhancement by blocking the GPX pathway with mercaptosuccinate (MPS, 1 mM) or by applying exogenous Cu,Zn-SOD (120 U ml^{-1}), the generator of H_2O_2. Various degrees of success and failure were obtained in neuroprotection when either of these treatments was used alone. In our experiments, exogenous Cu,Zn-SOD (120 U ml^{-1}), when added to the bathing solution of hippocampal slices, was able to prevent only four times out of seven (data not shown) the irreversible loss of synaptic transmission typically obtained following 14 min of OGD exposure. A comparable variability of response was seen when applying MPS (1 mM) alone (protection occurring in five out of seven slices). The reasons for such failures are not clear, but could be related to various factors including the difficulty of these molecules to cross the plasmalemmal membrane. Interestingly, when Cu,Zn-SOD (120 U ml^{-1}) was applied together with MPS (1 mM), both in pretreatment and during the 14-min OGD, the irreversible loss of synaptic transmission usually obtained with 14-min OGD was always prevented (103 ± 0,5 %, $n = 6$, paired Student's *t*-test $P < 0.0001$; Figure 5a). Furthermore, full recovery of synaptic transmission after 14-min OGD was also observed when applying MPS (1 mM), with micromolar doses of hydrogen peroxide (H_2O_2, 10 μM), both in pretreatment and during OGD (96 ± 3%, $n = 6$, paired Student's *t*-test $P < 0.0001$; Figure 5b). This may suggest that the required amount of endogenous H_2O_2, supposedly protective during OGD, could not be reached by applying either MPS or Cu,Zn-SOD alone, but only by their combined interaction. However, H_2O_2 alone, even when applied at a 10-fold higher concentration (100 μM), failed to restore synaptic transmission after 14 min of OGD (5 ± 2%, $n = 6$, paired Student's *t*-test $P < 0.0001$; Figure 5c).

Discussion and conclusions

Since its discovery, H_2O_2 has been known as a toxic agent for human tissues. However, in the light of recent findings, its role should now be re-evaluated. In fact, this molecule, despite its bad reputation, is now being recognized as an endogenous molecule of life, which is essential for the proper development and proliferation of cells. Therefore, a low concentration of H_2O_2 is vital for some physiological processes, while a high concentration is toxic for human cells. The enzyme catalase is the main regulator of hydrogen peroxide metabolism and, due to its structure and function, is very effective in degrading toxic concentrations of hydrogen peroxide, producing H_2O and O_2, without changing its low, physiological concentration. Therefore, activation of catalase occurring during conditions where H_2O_2 accumulates in the brain (Cino and Del Maestro, 1989; Simonson *et al*., 1993), in the heart (Shlafer *et al*., 1987; Vandeplassche *et al*., 1989; Chen and Lesnefsky, 2006), in the kidney (Kunduzova *et al*., 2002) and during perinatal hypoxia ischaemia (Fullerton *et al*., 1998) may represent an early endogenous defence mechanism aimed to compensate for the lack of oxygen supply. Indeed, it was shown that a higher activity of catalase occurs in the substantia nigra pars compacta (Hung and Lee, 1998; Avshalumov *et al*., 2005), and that H_2O_2 degradation through catalase is able to overcome the metabolic stress typically associated with episodes of energy deprivation (Geracitano *et al*., 2005).

The aim of this study was to assess the protective role of H_2O_2 and the potential of manipulating its endogenous production on the electrophysiological and morphological changes induced by OGD on CA1 hippocampal neurons.

We used field potential recordings in the hippocampus as it represents an ideal way to monitor the activity of a large population of neurons, hence to detect the functional changes that occur during a period of energy failure (Whittingham *et al*., 1984; Lobner and Lipton, 1993; Fowler, 1997). Accordingly, we have found that a sustained short period of OGD induced an irreversible functional impairment of synaptic neurotransmission that represents an index of neuronal injury. This is also confirmed by the morphological observation of the tissue using Cresyl Violet staining. Thus, the irreversible electrophysiological changes that closely correlate with the histologically defined neuronal damage, confirmed that H_2O_2 exerts a strong neuroprotective effect by compensating for the lowered levels of O_2

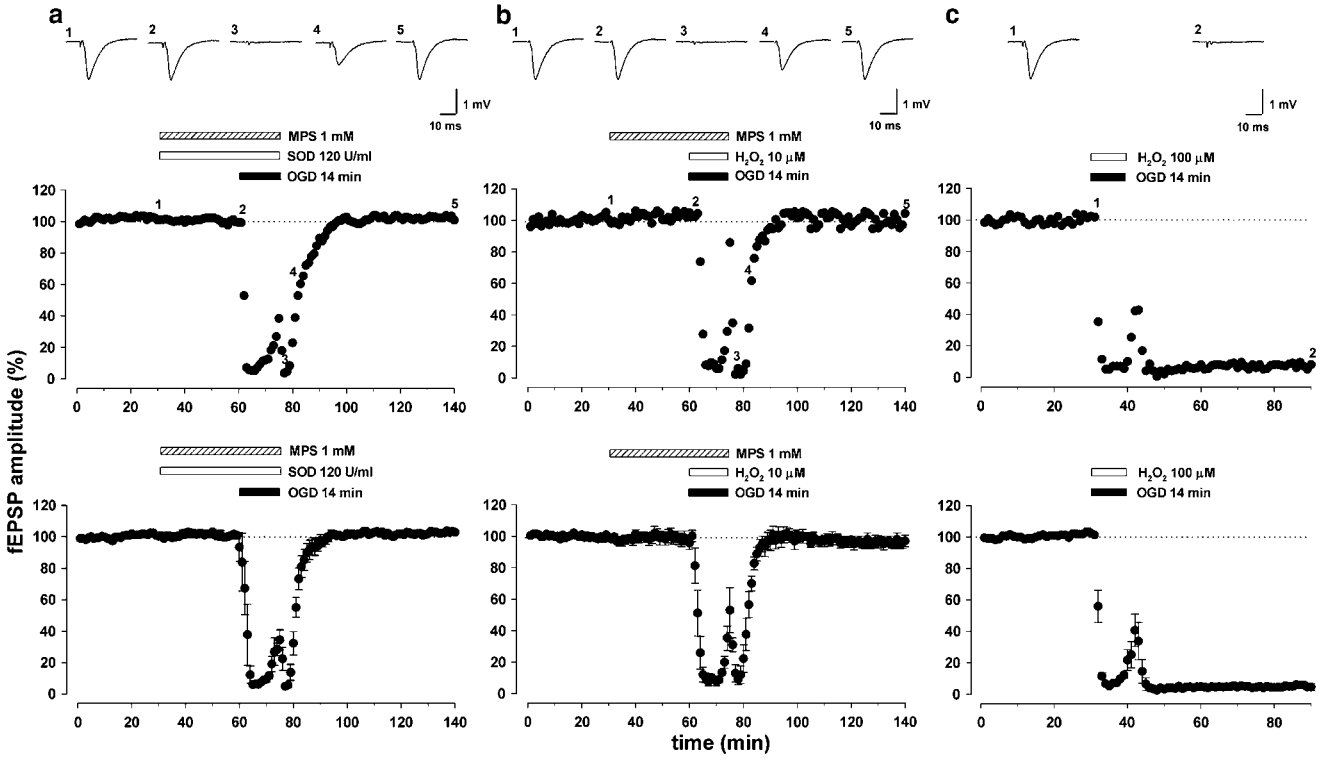

Figure 5 Pharmacological strategies aimed to increase the endogenous production of H_2O_2. (**a**) A single example (upper graph) and pooled data from six experiments (lower graph) showing the protective effect of mercaptosuccinate (MPS; 1 mM) and superoxide dismutase (SOD; 120 U ml) on the irreversible field excitatory postsynaptic potential (fEPSP) depression induced by 14 min of oxygen/glucose deprivation (OGD). Bars indicate the duration of exposure to the compounds and the OGD. The fEPSP traces were taken at the time points indicated by numbers in the corresponding graph. Each point represents fEPSP amplitude expressed as percentage of the mean baseline responses recorded before drug application. Data in the lower graph, expressed as percentage of baseline values, are means ± s.e.mean. (**b**) A single example (upper graph) and pooled data from six experiments (lower graph) showing the recovery of synaptic transmission after 14 min of OGD when applying a combination of MPS (1 mM) and a low concentration of H_2O_2 (10 μM), both in pretreatment and during OGD. (**c**) A single example (upper graph) and pooled data from six experiments (lower graph) indicating how a higher concentration of H_2O_2 (100 μM) was unable to prevent the irreversible loss of synaptic transmission following 14 min of OGD.

during OGD. This is demonstrated by the lack of neuroprotection when the production of O_2 from H_2O_2 was blocked by the catalase inhibitor 3-AT and by the failure of H_2O_2 to oppose hypoglycaemia-induced loss of synaptic function. Nevertheless, we adopted a model of combined oxygen and GD to mimic ischaemic conditions *in vivo*. We were also able to show clear protection against the ischaemic insult by increasing the endogenous content of H_2O_2 in the tissue. Therefore, we have concluded that manipulation of the enzymic production of H_2O_2 was neuroprotective in the slice preparations, probably due to a supplementary source of O_2 generated by breakdown of H_2O_2 during the ischaemic episode. In fact, increasing the endogenous level of H_2O_2 by the simultaneous reduction of the activity of GPX and by exogenously applying Cu,Zn-SOD also allowed the hippocampal tissue to be rescued from the damage caused by OGD. Inhibition of GPX alone appeared not to raise endogenous H_2O_2 to a level that was high enough to rescue the neurons. We therefore had to add Cu,Zn-SOD, further boosting the production of H_2O_2 in the hippocampal tissue and, under these conditions, a considerable protection was achieved.

Our observation that we could rescue neurons from injury caused by OGD through manipulation of the cellular content of H_2O_2 suggests that a valuable protective strategy could be to raise the endogenous level of this compound in the ischaemic tissue. In fact, H_2O_2 is highly diffusible and rapidly metabolized through the action of catalase into free O_2, thus acting as a donor of molecular O_2 that we think could be vital in a pathological condition such as ischaemia.

Therefore, we would propose that H_2O_2 can act in a protective role, as the application of exogenous H_2O_2, as well as the pharmacological manipulation of the enzymes involved in its production and degradation, conferred neuroprotection in an *in vitro* model of OGD. Of course, at this stage, we would not suggest therapeutic use of exogenous H_2O_2, which would be potentially toxic if directly administered in humans. However, the combination of the blockade of the GPX pathway together with Cu,Zn-SOD, by transiently increasing the endogenous concentrations of H_2O_2 to below toxic levels, would seem to be a protective strategy in conditions of energy deprivation.

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Conflict of interest

The authors state no conflict of interest.

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