Therapeutic potential of targeting hydrogen peroxide metabolism in the treatment of brain ischaemia

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For many years after its discovery, hydrogen peroxide (H₂O₂) was viewed as a toxic molecule to human tissues; however, in light of recent findings, it is being recognized as an ubiquitous endogenous molecule of life as its biological role has been better elucidated. Indeed, increasing evidence suggests that H₂O₂ may act as a second messenger with a pro-survival role in several physiological processes. In addition, our group has recently demonstrated neuroprotective effects of H₂O₂ on in vitro and in vivo ischaemic models through a catalase (CAT) enzyme-mediated mechanism. Therefore, the present review summarizes experimental data supporting a neuroprotective potential of H₂O₂ in ischaemic stroke that has been principally achieved by means of pharmacological and genetic strategies that modify either the activity or the expression of the superoxide dismutase (SOD), glutathione peroxidase (GPx) and CAT enzymes, which are key regulators of H₂O₂ metabolism. It also critically discusses a translational impact concerning the role played by H₂O₂ in ischaemic stroke. Based on these data, we hope that further research will be done in order to better understand the mechanisms underlying H₂O₂ functions and to promote successful H₂O₂ signalling based therapy in ischaemic stroke.

Abbreviations
3-AT, 3-amino-1,2,4-triazole; ACSF, artificial cerebral spinal fluid; BSO, buthionine sulfoximine; CAT, catalase; DA, dopamine; DHE, dihydroethidium; fEPSP, field excitatory postsynaptic potential; GPx, glutathione peroxidase; HIF, hypoxia-inducible factor; IPC, ischaemia preconditioning; IR, ischaemia-reperfusion; K_ATP, ATP-sensitive K⁺ channel; MCAo, middle cerebral artery occlusion; MCS, mercaptosuccinate; mTOR, mammalian target of rapamycin; NF, nuclear factor; NOS, nitric oxide synthase; O₂, molecular oxygen; O₂⁻, superoxide anion; OGD, oxygen/glucose-deprivation; •OH, hydroxyl radical; PGC1α, PPARγ coactivator1α; PI3K, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor; Prx, peroxiredoxin; ROS, reactive oxygen species; SNC, substantia nigra pars compacta; SOD, superoxide dismutase; TDP, thiolate-dependent phosphatase; Tg(CAT), transgenic mouse over-expressing catalase; TRP, transient receptor potential; WT, wild-type

Nomenclature
The drug/molecular target nomenclature used in this review conforms to the British Journal of Pharmacology’s Guide to Receptors and Channels (Alexander et al., 2011), where applicable.

Historical notes
The history of H₂O₂ began in 1818 when it was discovered by Thénard who named it eau oxygénée (Thénard, 1818). Since the mid-1800s, H₂O₂ has been marketed for a wide variety of uses, including non-polluting bleaching, oxidizing agent,
disinfectant in food processing and even fuel for rockets. The presence of H$_2$O$_2$ in living systems was identified in 1856 (Schoenbein, 1856). However, it was only in 1894 that 100% pure H$_2$O$_2$ was first extracted from H$_2$O by Wolffenstein through vacuum distillation (Wolffenstein, 1894). In 1888, the first medical use of H$_2$O$_2$ was described by Love as efficacious in treating numerous diseases, including scarlet fever, diphtheria, nasal catarrh, acute coryza, whooping cough, asthma hay fever and tonsillitis (Love, 1888). Similarly, Oliver and collaborators reported that intravenous injection of H$_2$O$_2$ was efficacious in treating influenza pneumonia in the epidemic following World War I (Oliver et al., 1920). Despite its beneficial effects, in the 1940s medical interest in further research on H$_2$O$_2$ was slowed down by the emerging development of new prescription medicines. In the early 1960s, Urschel, and later Finney and co-workers, conducted several studies on myocardial ischaemia demonstrating a rescue afforded by H$_2$O$_2$, thereby suggesting an important protective action of H$_2$O$_2$ against ischaemia-reperfusion (IR) injury (Finney et al., 1967; Urschel, 1967). Notably, Farr is generally considered to be the pioneer of ‘oxidative therapy’ by proposing intravenous infusion of H$_2$O$_2$ to treat a wide variety of diseases (Farr, 1988). Later, Willhelm promoted the therapeutic use of H$_2$O$_2$ to treat cancer, skin diseases, polio and bacteria-related mental illness. He defined H$_2$O$_2$ as ‘God’s given immune system’ (Willhelm, 1989; Green, 1998). Another player in the H$_2$O$_2$ story was Grotz, who obtained pain relief by testing H$_2$O$_2$ on himself to treat his arthritis pain (Green, 1998).

**Metabolism of H$_2$O$_2$**

H$_2$O$_2$ is mainly generated as a by-product of aerobic metabolism in the mitochondria (Fridovich, 1995), where formation of the superoxide anion (O$_2^-$) results from partial reduction of molecular oxygen (O$_2$) in the electron transport chain. A smaller amount of O$_2^-$ is also produced by enzymatic activities including NOS, xanthine oxidase, NADPH oxidase, dehydrogenases and peroxidases (Boveris and Chance, 1973; Rhee, 2006; Zhao et al., 2009; Finkel, 2011). In addition, enzymes such as superoxide dismutase (SOD), in its three isoforms (cytosolic, extracellular Cu/Zn-SOD, and mitochondrial Mn-SOD), are also responsible for H$_2$O$_2$ production from O$_2^-$ (Graham et al., 1978; Fridovich, 1995). The dismutation reaction catalyzed by SOD is as follows: 2O$_2^-$ + 2H$^+$ $\rightarrow$ H$_2$O$_2$ + O$_2$. H$_2$O$_2$ is also generated as a direct by-product of MAO enzyme activity. In fact, the oxidative deamination reaction catalyzed by MAO requires O$_2$ to degrade bioamines and produces H$_2$O$_2$, the corresponding aldehyde and ammonia according to the overall equation:

$$R-	ext{CH}_2\text{-NH}_2 + O_2 + H_2O \rightarrow H_2O_2 + R-\text{CHO} + NH_3$$

where R stands for alkyl group (Tipton, 1968; Tipton et al., 2004). Moreover, H$_2$O$_2$ generation can be the result of p66$^{shc}$ enzyme activity (Giorgio et al., 2007). H$_2$O$_2$ is subsequently converted to H$_2$O by scavenger enzymes such as cytosolic and mitochondrial glutathione peroxidase (GPx) which catalyzes the reaction: H$_2$O$_2$ + 2GSH $\rightarrow$ 2H$_2$O + GSSG, or decomposed in peroxisomes to H$_2$O and O$_2$ by catalase (CAT) according to the equation: 2H$_2$O$_2$ $\rightarrow$ 2H$_2$O + O$_2$; the latter has been observed to be more effective than GPx in detoxifying neurons from H$_2$O$_2$ (Halliwell, 1999; Dringen et al., 2005). A smaller contribution to regulate H$_2$O$_2$ levels also comes from thioredoxins as well as peroxiredoxins (Prx) (Rhee, 2006; Mishina et al., 2011). H$_2$O$_2$ metabolism is highly dynamic: its intracellular concentration reflects the balance between processes of generation and removal (Halliwell, 1999). Actually, there are no certain measurements of either intracellular or extracellular H$_2$O$_2$ concentration. Many attempts to address this point have failed due to high cellular peroxidase-mediated depletion and technical limitations of H$_2$O$_2$-sensitive fluorescent dyes (Rice, 2011). However, *in vivo* microdialysis has been used to try to determine the extracellular production of H$_2$O$_2$ in the brain during IR. A fourfold rise in H$_2$O$_2$ from basal levels has been detected in dialysates from the rat anterior lateral striatum during reperfusion after 30 min of global forebrain ischaemia (approximately 100 µM at the peak during reperfusion phase) (Hyslop et al., 1995). Similarly, fluorometry of 2',7'-dichlorofluorescin oxidation coupled with *in vivo* microdialysis have been applied in the gerbil hippocampal CA1 region in order to monitor changes in H$_2$O$_2$ concentration during IR. A marked and rapid increase in H$_2$O$_2$ level was recorded in the reperfusion phase, although to a lesser degree (range 1–3 µM), which continued to increase in dialysates until 30 min of reperfusion after transient ischaemia (5 min) (Lei et al., 1998). By means of mathematical models, upper limits (100 nM to 1 µM) have been recently estimated to be 10 to 100-fold lower than exogenously applied concentrations (Antunes and Cadenas, 2000), indicating a signalling action of H$_2$O$_2$ at 15–150 µM without any oxidative damage (Rice, 2011). Noteworthy, concentrations of H$_2$O$_2$ that can be reached in rat vascular smooth muscle cells exposed to IR insult are likely to be higher than 1 mM (Sundaresan et al., 1995). In spite of this, we are still searching for effective tools to detect the real concentration of H$_2$O$_2$ in both the intracellular and extracellular compartments of the brain. Of note, 1–3 mM H$_2$O$_2$ has been used for investigations of synaptic function and intracellular Ca$^{2+}$ changes in the hippocampus (Pellmar, 1987; Nistico et al., 2008; Gerich et al., 2009). These exogenous concentrations appear to have pathophysiological relevance. Conversely, the other important question related to the toxicity of relatively high extracellular concentrations of H$_2$O$_2$ has not really been solved yet. In fact, it has been reported that hippocampal neurons from primary culture tolerate 300 µM H$_2$O$_2$ for at least 30 min (Miller et al., 2005). However, it has to be considered that the concentration of H$_2$O$_2$ that reaches the intracellular milieu could be significantly lower than that superfused on tissue. Firstly, H$_2$O$_2$ transport might be limited by lipid membrane composition and diffusion rate (Antunes and Cadenas, 2000); secondly, it might be differently transported by aquaporins and other channels (Bienert et al., 2007). Therefore, the high concentrations used in *in vitro* experiments may not reflect the content reached in the intracellular compartment that could be markedly lower.

**H$_2$O$_2$: a paradox player**

*Emerging role of H$_2$O$_2$ in the physiological control of cell functioning*

H$_2$O$_2$ is often considered a toxic molecule for a wide range of living systems. It has also been reported to be implicated in...
severe pathological conditions such as cancer, ischaemia and neurodegenerative diseases (Halliwell and Gutteridge, 1999; Halliwell et al., 2000). However, robust evidence has led to re-evaluation of its role as an important regulatory signal in a variety of biological processes (Sundaresan et al., 1995; Sen and Packer, 1996; Rhee, 2006; Stone and Yang, 2006; D’Autréaux and Toledano, 2007; Miller et al., 2007; Veal et al., 2007; Gerich et al., 2009; Groeger et al., 2009; Rice, 2011), thus suggesting that the deleterious role of this oxidant has been overestimated. In particular, H2O2 can modulate synaptic transmission (Pellmar, 1987; Katsuki et al., 1997; Chen et al., 2001; Avshalumov et al., 2003; 2008) and plasticity in the rodent brain (Colton et al., 1989; Auerbach and Segal, 1997; Klann and Thiels, 1999; Kamsler and Segal, 2003). H2O2 is also implicated in intracellular Ca2+ signalling and organelle function modulation in rat hippocampus (Gerich et al., 2009). Additional evidence has indicated a dynamic modulation exerted by H2O2 in the nigrostriatal dopaminergic (DAergic) system. In fact, it inhibits substantia nigra DAergic neurons and striatal DA release by activating ATP-sensitive K+ channels (KATP) (Chen et al., 2001; Avshalumov et al., 2003; 2005; 2008). Of note, H2O2 may also act as an excitatory agent on non-DAergic neurons by inducing transient receptor potential (TRP) channel (subgroup melastatin type TRPM2) activation (Rice, 2011).

Mechanisms, targets and outcomes of H2O2 signalling: concentration as a determining factor

H2O2 is a chemical messenger able to spread locally in and out of the cell. It passes across cell membranes through specific aquaporin 3 channels or freely, like other diffusible messengers (such as NO, carbon monoxide and hydrogen sulphide) (Bienert et al., 2006; 2007; Miller et al., 2010). In chemical terms, H2O2 is poorly reactive and is more stable than other reactive oxygen species (ROS) because it is not itself a free radical. Therefore, it is able to survive long enough to act distant from its place of generation. It is widely accepted that low levels of H2O2 target sulphydryl groups of protein cysteine residues by oxidizing them and consequently, affecting the activity of key signal transduction kinases and phosphatases, thus representing the ‘signalling face’ of H2O2 (Rhee et al., 2000; Giorgio et al., 2007) (Figure 1). In fact, H2O2 may affect cell-signalling survival pathways by reversibly inhibiting many proteins (i.e. phosphatases). Phosphatases are potent negative regulators of the survival pathways that transduce their signal through phosphorylation of key proteins (Groeger et al., 2009). For instance, H2O2 promotes the cell survival signalling cascade [e.g. phosphatidylinositol 3-kinase (PI3K)/AKT] by inactivating Tyr and Ser/Thr phosphatases (e.g. PTEN, FAK, SHP2, CDC25, PTP1B) (Giorgio et al., 2007). On the other hand, H2O2 is also responsible for the activation of MAPKs (e.g. ERK1/2, p38 MAPK) and for the modulation of transcription factors involved in cellular response to stress stimuli such as hypoxia and oxidative stress (Giorgio et al., 2007; Oliveira-Marques et al., 2009). Under hypoxia, H2O2 may affect the activity of the transcription factor hypoxia-inducible factor (HIF)-1α by inhibiting HIF-1α DNA-binding activity and accumulation (Groeger et al., 2009). The regulatory role played by H2O2 on the nuclear factor (NF)-κB pathway is still controversial: among the described actions, an important one is the increase of DNA-binding activity of NF-κB through the H2O2-induced inactivation of the enzyme histone deacetylase which is implicated in chromatin remodelling (Groeger et al., 2009; Oliveira-Marques et al., 2009). Recently, it has also been demonstrated that H2O2 is also implicated in several growth factor-triggered signals (Stone and Yang, 2006; Valko et al., 2007). Further evidence has shown that H2O2 stimulates the renal epithelial Na+ channel through a PI3K pathway, thus suggesting its involvement in systemic blood pressure homeostasis (Ma, 2011). Notably, cell damage, death (either by necrosis or apoptosis) and senescence appear to be induced only by high levels of H2O2 (Giorgio et al., 2007; Oliveira-Marques et al., 2009). H2O2 may induce apoptosis in neuronal cells by inhibiting the mammalian target of rapamycin signalling (Chen et al., 2010). In endothelial cells, an excess of H2O2 activates Fas and JNK pathways in apoptosis (Cai, 2005). H2O2 oxidizes, in a manner to cause cytotoxic damage to biological macromolecules (such as lipids, proteins and DNA), only if converted to the highly reactive hydroxyl radical (•OH) (Winterbourn, 2008; Oliveira-Marques et al., 2009). The latter is generated via Fenton reaction by H2O2 interaction with free transition metals (mostly reduced Fe2+ and Cu+ ions) (Halliwell, 1992; Cohen, 1994). In its dual role as an indispensable signal molecule and a potential threat for biological components, H2O2 plays the double-faced role of ‘Dr. Jekyll and Mr. Hyde’ (Gough and Cotter, 2011). Indeed, a real H2O2 paradox exists: on one hand, in low amounts H2O2 has a physiological role in the homeostatic maintenance of normal cell functioning; on the other hand, high amounts of H2O2 can be harmful for cells (Figure 1).

H2O2 sensing during ischaemic injury: implications for neuroprotection

In order to maintain H2O2 physiological signalling function, both intracellular and extracellular concentrations of H2O2 need to be constantly maintained at a level below toxicity threshold via an accurate and complex metabolic regulation (Halliwell, 1999; Göth, 2006). In mammalian cells, redox sensor function has been suggested for Prx-1, thiol peroxidases and thiolate-dependent phosphatases, which may affect H2O2 signalling fluxes (Stone and Yang, 2006; D’Autréaux and Toledano, 2007). More importantly, specific responses to H2O2-induced oxidative stress are regulated in eukaryotic cells by acetylation or deacetylation of several factor-transcription factors of the class O forkhead box family, which could lead to either cell death or a quiescent cellular state (Brunet et al., 2004; van der Horst et al., 2004). Moreover, low levels of H2O2 stimulate p53 tumour suppressor-mediated antioxidative response by activating antioxidiant genes (e.g. Gpx, SOD, sestrins), while high levels of it induce p53-dependent apoptosis (Veal et al., 2007). The peroxisome proliferator-activated receptor (PPARα) coactivator1α (PGC1α) also establishes a crucial link between mitochondrial production of ROS and anti-ROS programmes by regulating H2O2-inducible antioxidant enzymes (SOD, CAT, GPx) (St-Pierre et al., 2006; D’Autréaux and Toledano, 2007). Strong evidence now suggests PPARα agonists as new therapeutic targets for the treatment of IR injury (Giagnini et al., 2008; Kaundal and Sharma, 2010), similarly, PGC1α could be a candidate for drug action as well. Interestingly, a dramatic accumulation of ROS has been reported as an additional side effect of IR (Flamm et al., 1978; Traystman et al., 1991). Indeed, ROS, including H2O2,
are considered prime mediators of neuronal injury. During an IR episode, the oxidative stress either could result from increased ROS production or decreased activity of cellular defence systems (White et al., 2000; Valko et al., 2007). On the other hand, with regard to the ischaemia, experimental evidence also suggests that H₂O₂ elimination by CAT may provide an alternative source for O₂, causing neuroprotection in hypoxic conditions (Topper et al., 1996; Auerbach and Segal, 1997; Klann and Thiels, 1999).

**Effects of H₂O₂ on rodent in vitro and in vivo models of brain ischaemia**

Valid experimental approaches are required for the development of a successful therapy for ischaemic stroke. Although models of brain ischaemia have been the main source of a plethora of information on stroke, these models often fail to mimic the complex scenario of stroke as observed clinically. Such limitations should be carefully considered when designing experiments to ensure translation of preclinical data to the clinic (Lipsanen and Jolkkonen, 2011). In our studies, we chose three different widely accepted experimental models of brain ischaemia: hypoxia and oxygen/glucose deprivation (OGD) as in vitro brain ischaemia models, and middle cerebral artery occlusion (MCAo) as in vivo brain ischaemia model (Lipton, 1999). In hypoxia and OGD in vitro models, ischaemic stroke was mimicked by applying oxygen-deprived and oxygen/glucose-deprived artificial cerebral spinal fluid (ACSF) media, respectively, over a brain slice and gassing it with nitrogen (95% N₂ to 5% CO₂). At the end of the insult, the slice was again perfused with normal oxygenated (95% O₂

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**Figure 1**

The H₂O₂ paradox in the regulation of cell signalling transduction cascade. The H₂O₂ biological functions depend on the concentration of H₂O₂ within the cell. At low concentrations, H₂O₂ acts as a messenger in a great variety of biological processes contributing to cell survival. In high concentrations, H₂O₂ can cause deleterious effects, mainly via •OH-derived radicals, by inducing a severe oxidative stress and cell death. Accordingly, a crucial target of H₂O₂ double-faced action is represented by the tumour suppressor protein p53 which can be either activated by low levels of H₂O₂, thus triggering an antioxidant response (anti-apoptotic programme), or inhibited by high levels of H₂O₂ leading to programmed cell death (pro-apoptotic programme) respectively.
to 5% CO₂) ACSF medium. Both models allowed us to rapidly screen bath-applied compounds by determining their effect as well as their mechanism of action against the acute damage during electrophysiological recording. Among the in vivo models currently used in stroke research, the transient focal ischaemia model (whole animal) represented by MCAo has been extensively used (Durukan and Tatlisumak, 2009). MCAo requires microsurgery to perform the filamentous intraluminal occlusion of the middle cerebral artery. This technique presents several advantages: it models focal infarction in a large vascular territory of the brain, where it is possible to distinguish core and penumbra regions; it is relatively less invasive because it does not require craniotomy; and it allows investigations after reperfusion.

**Substantia nigra**

DAergic neurons of the substantia nigra pars compacta (SNc) are highly sensitive to metabolic stress. Very likely, as a safety mechanism to preserve energy consumption, these neurons typically respond to energy deprivation with membrane hyperpolarization, mainly through opening of KATP channels (Mercuri et al., 1994). After a prolonged hypoxia, this early hyperpolarization is followed by a profound and irreversible depolarization, due to opening of cationic conductance and failure of Na⁺/K⁺-ATPase pump (Mercuri et al., 1994; Lees and Leong, 1995). Moreover, previous observations have shown that H₂O₂ may act as a supplementary source of O₂ in an isolated neonatal rat spinal cord preparation in vitro (Walton and Fulton, 1983) and a recovery of the synaptic function by H₂O₂ during hypoxic insult in rat hippocampal slices (Fowler, 1997). In the past years, we have used both electrophysiological and morphological techniques to investigate a possible protective role of H₂O₂ in DAergic neurons of the rat SNc exposed to hypoxic insult (Geracitano et al., 2005). Notably, H₂O₂ reversed membrane hyperpolarization and blocked spontaneous firing associated with oxygen-deprivation in DAergic neurons (Figure 2A,B). In contrast, in normoxic conditions, H₂O₂ (3 mM) blocked the spontaneous activity of the DAergic cells by inducing a KATP-channels-dependent outward current that was sensitive to tolbutamide (1 mM) (a non-selective blocker of KATP channels) (Figure 2C). Of note, H₂O₂ decreased the hypoxia-mediated outward current in a concentration-dependent manner. Conversely, H₂O₂ did not counteract membrane hyperpolarization associated with hypoglycaemia. The superfusion of H₂O₂ (3 mM) during prolonged hypoxia (40 min) rescued most of the DAergic neurons from irreversible firing inhibition. Noteworthy, in the presence of 3-amino-1,2,4-triazole (3-AT, 30 mM), a specific inhibitor of CAT activity (Appleman et al., 1956; Margoliash and Novogrodsky, 1958), H₂O₂ was unable to decrease hypoxia-mediated outward current and thus restore the spontaneous firing rate (Figure 2D). The protective effects of H₂O₂ have been confirmed by inhibition of the hypoxia-induced release of cytochrome c, a well-known early indicator of apoptotic pathway activation (Fujimura et al., 2000; Sims and Anderson, 2002). These findings suggest a protective action of H₂O₂ in hypoxic DAergic neurons by serving as a supplementary source of O₂ through its degradation by CAT and thus, interfering with KATP channels opening consequent to O₂ deprivation (Figure 2E). On the other hand, under normoxic conditions, H₂O₂ (3 mM) induced by itself a tolbutamide-sensitive outward current in DAergic neurons. This outward response is due to the opening of KATP channels by a direct action of H₂O₂ on the channels (Avshalumov et al., 2005) (Figure 2E).

**Hippocampus**

The CA1 hippocampal pyramidal neurons are known to be very vulnerable to IR insult (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984). Recently, we have evaluated the neuroprotective role of exogenous H₂O₂ and of the modification in its endogenous levels by the pharmacological modulation of H₂O₂ producing (Cu,Zn-SOD) and degrading enzymes (CAT and GPx) against in vitro OGD damage in hippocampal slices. Similar to what has been observed in a previous report (Fowler, 1997), we found that the irreversible depression of fEPSPs caused by in vitro OGD was abolished when slices were treated with H₂O₂ (3 mM, 30 min) during OGD exposure (30 min) in CA1 region (Nisticò et al., 2008) (Figure 3A). Importantly, the neuroprotective effects of H₂O₂ were still maintained even when applied 7 min after the ischaemic conditions had already been imposed (Figure 3B). Again, the rescuing action of H₂O₂ (3 mM) was mediated by the CAT-induced formation of O₂. In fact, a pretreatment of the slices with the CAT inhibitor (3-AT, 20 mM) blocked this protective effect (Figure 3C). Moreover, we have shown that an increase of the endogenous levels of H₂O₂, due to a combined bath-application of mercaptosuccinate (MCS, 1 mM) (a potent and specific inhibitor of selenium-dependent GPx) (Chaudiere et al., 1984), and Cu,Zn-SOD (120 U·mL⁻¹) which augments H₂O₂ production, limited the OGD-induced irreversible depression of fEPSPs. These results were in line with previous observation of neuroprotection afforded by H₂O₂ against hypoxic insult on DAergic cells (Geracitano et al., 2005) and propose novel therapeutic strategies based on increasing the endogenous tissue levels of H₂O₂ in the ischaemic brain.

**Striatum**

Little information is as yet available as to whether H₂O₂ may contribute to neuroprotection in in vivo brain ischaemia because its systemic infusion induces gas embolism which can cause additional occlusions of the vessels. As a matter of fact, there is O₂ formation in the vessels due to the ubiquitous localization of the CAT enzyme (Watt et al., 2004; French et al., 2010). As it was not feasible to inject H₂O₂ intravenously, our aim was to examine the effect of increasing the endogenous levels of H₂O₂ in an in vivo model of brain ischaemia. This increase has been accomplished through inhibition of GPx by systemic intraperitoneal administration of MCS. We observed that MCS (1.5–150 mg·kg⁻¹) dose dependently decreased brain infarct damage produced by transient (2 h) MCAo in rat (Amantea et al., 2009) (Figure 3D). Interestingly, neuroprotection was observed when MCS was administered 15 min before the ischaemic insult, and no protection was detected when the drug was injected 1 h before MCAo or upon reperfusion. Such results were in accordance with another study showing a prolongation of survival time of rats following 20 min brain ischaemia when pretreated with buthionine sulfoximine (BSO), a drug that is a glutathione depletor (Vanella et al., 1993). BSO could...
H$_2$O$_2$ as a therapeutic target in the ischaemic brain

Figure 2

Electrophysiological effects of H$_2$O$_2$ on SNC DAergic neurons. (A) Hypoxia caused firing discharge inhibition in current-clamp sharp electrode intracellular recordings (n = 11) (upper trace), and an outward current followed by a transient post-hypoxic outward current in voltage-clamp (V$_{holding}$ = −60 mV) intracellular recordings (n = 8) (lower trace). (B) During hypoxia, after H$_2$O$_2$ (3 mM) perfusion, a transient hyperpolarization followed by complete firing recovery was observed (n = 6) (upper trace); the hypoxia-induced outward current was reverted by H$_2$O$_2$ (n = 6) (lower trace). (C) In normoxia, H$_2$O$_2$ (3 mM) induced a reversible outward current (n = 4) (upper trace); the K$_{ATP}$ channel antagonist tolbutamide (1 mM) inhibited such current (lower trace). (D) Hypoxia induced outward current in the presence of CAT inhibitor 3-AT (30 mM); in the same neuron, such current is increased, not prevented in the presence of H$_2$O$_2$ (n = 8) in whole-cell patch-clamp voltage-clamp recordings (P < 0.01). Data in the graphs are expressed as means ± SEM. Bars indicate the exposure time to compounds and OGD [A–D modified from Geracitano et al. (2005); copyright / Physiol, used with permission]. (E) Hypothesized mechanism of neuroprotection afforded by H$_2$O$_2$ against hypoxic insult in SNC DAergic neurons. In normoxic conditions, H$_2$O$_2$ causes direct K$_{ATP}$ channel opening (we believe that this is a generic cellular defensive response to insults). Also, hypoxia induces K$_{ATP}$ channel opening in DAergic cells. However, H$_2$O$_2$ exerts neuroprotection by serving as an alternative source of O$_2$ through the enhancement of its degradation via the CAT enzyme-mediated pathway. Thus, it counteracts the K$_{ATP}$ channels opening.

Can the pharmacological modulation of enzymatic pathways leading to an enhanced H$_2$O$_2$ conversion to O$_2$ and H$_2$O be therapeutic?

The therapeutic potential of modulating the enzymatic pathways leading to H$_2$O$_2$ production and its conversion to O$_2$ and H$_2$O (SOD, GPx, CAT) has been previously investigated via either transgenic or pharmacological intervention tools in both in vitro and in vivo ischaemic conditions. The modification of H$_2$O$_2$ signalling might have a key aspect in the expression of neuronal damage during an episode of IR.

Targeting GPx enzyme

Also, mice overexpressing GPx are more resistant to ischaemic insult (Weisbrot-Lefkowitz et al., 1998; Furling et al., 2000; Ishibashi et al., 2002). An increased infarct size has been observed in GPx knockout mice (Crack et al., 2001), more likely due to excessive H$_2$O$_2$ accumulation in the brain during reperfusion, whereas the cerebroventricular infusion of exogenous GPx was not able to improve the outcome of global IR (Yano et al., 1998). On the other hand, the non-selective GPx mimetic ebselen has protective effects in several ischaemia models (Warner et al., 2004).

Targeting CAT enzyme

Interestingly, the manipulation of CAT, the other crucial enzyme involved in H$_2$O$_2$ degradation, has given more homogeneous results against the ischaemic insult. In fact, CAT overexpression in the heart of transgenic mice has been shown to provide myocardial protection against IR injury (Mele et al., 2006). Additional strategies based on exogenously administered CAT enzyme (Forsman et al., 1988; Castillo et al., 1990) or on CAT overexpression by viral vector have been used to examine its protective role in IR injury both in in vitro and in vivo systems (Wang et al., 2003; Gu et al., 2004; Gáspár et al., 2009; Kim et al., 2009; Zemlyak et al., 2009; Ushitara et al., 2010; Chen and Tang, 2011). Of note, systemic infusion of CAT failed to improve neurological deficits after complete ischaemia (Forsman et al., 1988), but resulted in a decrease of myocardial injury following coronary ischaemia (Gardner et al., 1983). In the light of our recent findings demonstrating a CAT-mediated neuroprotective effect of H$_2$O$_2$ in oxygen-deprived brain slices of the

act by decreasing the activity of GPx and thus augmenting the endogenous level of H$_2$O$_2$. Consistent with these findings, superfusion of striatal slices with MCS (1 mM) limited the irreversible cortico-strial regional field potential depression caused by OGD (12 min) (Figure 3E,F). Once again, the protective effect of MCS superfusion was lost by concomitant bath-application of 3-AT (20 mM), confirming the involvement of CAT in mediating the functional rescue at the synaptic level (Figure 3F). Thus, MCS resulted in neuroprotection both on in vitro and in vivo ischaemic conditions, through a mechanism which, by blocking GPx, very likely increases endogenous levels of H$_2$O$_2$ and its consequent conversion to O$_2$ and H$_2$O by CAT.

show an increased tolerance to both focal and global brain ischaemia (Sheng et al., 1999a; 2000), whereas extracellular Cu,Zn-SOD knockout mice show greater damage (Sheng et al., 1999b). In agreement with the data obtained in transgenic animals, it has been shown that polyethylene glycol-conjugated SOD has a potential therapeutic effect in ischaemia (Liu et al., 1989). Moreover, nonpeptidyl SOD-mimetics have proven effective in hypoxia-ischaemia injury in immature rats (Shimizu et al., 2003). However, the short half-life, the reduced capability to penetrate the blood–brain barrier and the antigenicity of SOD have limited its pharmacological use.

Targeting SOD enzyme

Transgenic mice overexpressing Cu,Zn-SOD enzyme are more resistant to focal brain ischaemia (Yang et al., 1994). However, neither selective deletion nor overexpression of Cu,Zn-SOD affect the outcome of permanent focal brain ischaemia (Chan et al., 1993; Fujimura et al., 2001). On the contrary, Mn-SOD selective deletion worsens the outcome of both transient and permanent MCAo (Murakami et al., 1998; Kim et al., 2002). From these studies it appears that there is the need of a ROS productive reperfusion phase for SOD enzymes to change the fate of the ischaemic tissue (Warner et al., 2004). To our knowledge, no published study has evaluated yet the long-term effects of SOD overexpression on IR outcome and the stability of the achieved protection (Warner et al., 2004). It is known that mice overexpressing Cu,Zn-SOD extracellularly...
**Figure 3**

Protective effects of H$_2$O$_2$ superfusion and pharmacological modulation of enzymatic pathways leading to H$_2$O$_2$ production and degradation. (A) In hippocampal slices, H$_2$O$_2$ (3 mM) exogenously bath-applied during OGD (30 min) exposure-induced irreversible loss of fEPSPs (black circles, $n = 6$) caused a complete recovery of synaptic function (white circles, $n = 6$, $P < 0.0001$) in extracellular recordings. (B) Ability of H$_2$O$_2$ to rescue synaptic transmission even when applied 7 min after OGD had started (black circles, $n = 6$, $P < 0.0001$). (C) CAT enzyme is involved in H$_2$O$_2$-mediated neuroprotection; the CAT inhibitor 3-AT (20 mM) prevented H$_2$O$_2$-induced recovery of fEPSPs (black circles, $n = 6$, $P < 0.0001$) [A–C modified from Nisticò et al. (2008); copyright *BJP*, used with permission]. (D) Representative brain coronal sections (2 mm thick), stained with 2,3,5-triphenyltetrazolium chloride (TTC), showing the infarct area (unstained) in rats treated with the GPx inhibitor MCS (150 mg·kg$^{-1}$) or vehicle (PBS, 1 ml·kg$^{-1}$), i.p., 15 min before transient (2 h) MCAo followed by 22 h reperfusion. Compared with vehicle-treated animals, systemic administration of MCS significantly decreases brain infarct damage produced by transient MCAo in penumbral areas ($n = 4–6$ rats per experimental group, $P < 0.05$). (E) At the cortico-striatal synaptic transmission, exposure to OGD (7 min) (white circles, $n = 4$) caused a reversible fEPSPs depression, whereas OGD (12 min) caused an irreversible loss of the fEPSPs in extracellular recordings (black circles, $n = 11$). (F) Treatment with MCS (1 mM) 15 min before and during OGD protected synaptic responses from fEPSPs loss (grey circles, $n = 11$, $P < 0.05$). Administration of 3-AT (20 mM, white circles, $n = 5$) reversed the neuroprotection by MCS indicating a CAT-mediated effect [D–F modified from Amantea et al. (2009); copyright *Int Rev Neurobiol*, used with permission]. (G) Pretreatment with CAT (500 U·mL$^{-1}$, 15 min) in the presence of OGD (40 min) plus H$_2$O$_2$ (3 mM) (grey circles, $n = 6$) induced a complete recovery of fEPSPs against the irreversible loss caused by OGD (40 min) alone (black circles, $n = 6$, $P < 0.005$). (H) CAT overexpression in the transgenic mice Tg (CAT) (grey circles, $n = 13$) in the presence of H$_2$O$_2$ (3 mM) induced a partial recovery of synaptic response from OGD (40 min) which was significantly different compared with WT mice (black circles, $n = 10$, $P < 0.05$). (I) The figure shows O$_2$ radical formation decrease measured in the CA1 hippocampal region by using fluorescent probe DHE after 1 h of superfusion, in OGD (40 min) exposed-Tg(CAT) slices (lower) as compared with WT group (upper) ($n = 3$ mice per experimental group, $P < 0.05$). Scale bar: 25 µm [G–I modified from Armogida et al. (2011); copyright *BJP*, used with permission]. In all graphs, data are expressed as means ± SEM; bars indicate the exposure time to compounds and OGD.

**Substantia nigra, hippocampus, striatum and in an in vivo model of transient focal brain ischaemia** (Geracitano et al., 2005; Nisticò et al., 2008; Amantea et al., 2009), we have investigated whether either the exogenous administration or the overexpression of CAT is protective in *in vitro* and *in vivo* brain ischaemic models (Armogida et al., 2011). Along with previous studies, our findings indicate that hippocampal synaptic transmission was restored only when CAT (500 U·mL$^{-1}$, 15 min) was bath-applied before a relative long period of OGD (40 min, that in control condition kills the neurons) in combination with H$_2$O$_2$ (3 mM) (Figure 3G). The CAT-induced neuroprotection was also confirmed in a transgenic mouse overexpressing the enzyme CAT [Tg(CAT)]. In fact, an increased resistance of hippocampal slices against OGD compared with wild-type (WT) animals was observed in the presence of H$_2$O$_2$ (Figure 3H). Furthermore, Tg(CAT) mice showed a decreased infarct size after MCAo compared with WT mice. By using DHE detection, we also observed lower levels of ROS likely reflecting increased ROS metabolism in the Tg(CAT) compared with WT mice 1 h after OGD (40 min) condition (Figure 3I).

Interestingly, CAT pretreatment blunted the damaging effect of H$_2$O$_2$ in normoxic conditions. In fact, it decreased fEPSPs depression evoked by repeated applications of H$_2$O$_2$. Notably, a lower sensitivity to H$_2$O$_2$-mediated field depression, very likely due to a better functioning of CAT, was indicated by the rightward shift of the H$_2$O$_2$-induced concentration-response curve in Tg(CAT) compared with WT mice.

**Targeting SOD and CAT enzymes simultaneously**

Conjugation with macromolecules such as liposome-entrapped SOD and CAT (Yusa et al., 1984), polyethylene glycol derivatives (Liu et al., 1989; Armstead et al., 1992; Yabe et al., 1999) or synthetic SOD–CAT mimetics (such as salen-manganese complexes and manganese porphyrins) exhibiting both SOD and CAT activities (Baker et al., 1998; Doctrow et al., 2002; Zhou et al., 2007; Zhou and Baudry, 2009) has been carried out to facilitate antioxidant compounds delivery to the brain tissue and to increase enzymatic bioavailability and half-life. Either exogenous SOD or CAT delivered into living cells through transduction-mediated cell-penetrating peptide PEP-1 fusion proteins protected myocardium from IR damage in rats. Furthermore, the combined transduction of PEP-1-SOD1 and PEP-1-CAT enhanced their protective effect (Huang et al., 2011). Interestingly, targeted cell-penetrating CAT derivative with enhanced peroxisome targeting efficiency (CAT-SKL) delivery also protected neonatal rat myocytes from IR injury (Undyala et al., 2011). Moreover, the administration of SOD/CAT mimetics before ischaemia has been reported to be neuroprotective in animal model of brain ischaemia (Sharma and Gupta, 2007).

**H$_2$O$_2$ as a preconditioning factor in neuroprotection**

Another important aspect to consider is the implication of H$_2$O$_2$ in the ischaemic preconditioning (IPC) phenomenon by which a brief sub-lethal ischaemic episode induces tolerance against subsequent prolonged ischaemia that usually induces lethal damage. Cardioprotective (Yaguchi et al., 2003) and neuroprotective effects of H$_2$O$_2$ have been observed in several *in vitro* models of IPC (Furuchi et al., 2005; Xiao-Qing et al., 2005). In fact, it has been demonstrated that the generation of H$_2$O$_2$ during brief OGD (10 min) induces IPC in rat primary cultured cortex neurons (Furuchi et al., 2005). In addition, H$_2$O$_2$, at low concentration (10 μM), can protect PC12 cell line against DA-induced apoptosis most likely by restoring mitochondrial function (Xiao-Qing et al., 2005). Accordingly, in a study conducted by Simerabet et al. (2008), the stereotactic *in situ* infusion of H$_2$O$_2$ (2 mM) decreased rat cerebral infarct size (cortical area) 24 h after MCAo (1 h), suggesting an involvement of H$_2$O$_2$ during the induction
phase of IPC (Simerabet et al., 2008). Moreover, in another study carried out by Chang et al. (2008), exogenous low concentration of H\textsubscript{2}O\textsubscript{2} (15 \textmu{}M) may contribute to IPC against OGD (24 h) in rat primary neurons by increasing HIF-1\alpha protein expression.

Conclusions and future directions

At present, ischaemic stroke is the second-leading cause of death worldwide and represents a serious unmet medical need. Although therapeutic interventions such as thrombolytic therapy with recombinant tissue plasminogen activator have been shown to be effective (Wechsler, 2011), a ‘neuroprotective strategy’, preventing or lessening the damaging components of the ischaemic cascade, has not been unequivocally demonstrated in clinical trials (Fisher, 2011). Failure of such programmes could be attributable to both insufficient preclinical models and clinical designs (Fisher, 2011; Macrae, 2011). The data discussed in the present review suggest exploiting possible neuroprotective strategies based on targeting H\textsubscript{2}O\textsubscript{2} metabolism in stroke. In spite of the fact that no clinical studies have been conducted evaluating the therapeutic usage of drugs targeting H\textsubscript{2}O\textsubscript{2} metabolism in stroke, the experimental observations obtained so far by our and other groups support the idea that H\textsubscript{2}O\textsubscript{2} might represent an attractive target for the development of novel therapies to diminish the burden of brain ischaemia. Thus, according to our hypothesis, neuroprotection in ischaemia could be principally obtained by two mechanisms when the H\textsubscript{2}O\textsubscript{2} metabolism is pharmacologically manipulated to boost CAT pathway: (i) one mechanism produces a supplementary source of O\textsubscript{2} to partially compensate for the lack of O\textsubscript{2} that occurs in the ischaemic cerebral tissue (this role could be more prominent in the ischaemic phase); and (ii) the other is characterized by enhanced CAT which detoxifies more easily brain tissue from ROS, thus decreasing the accumulation of H\textsubscript{2}O\textsubscript{2} and the radical •OH derived from H\textsubscript{2}O\textsubscript{2} excess (this role could be more prominent during the reperfusion phase when there is an increased generation of ROS) (Figure 4). Therefore, pharmacological agents effective in the treatment of brain ischaemia should obtain an increase in the level of H\textsubscript{2}O\textsubscript{2} by blocking GPx preferably associated to an increased enzymatic activity of SOD and CAT. Indeed, in the study conducted by Avshalumov et al. (2004), either GPx or CAT inhibition enhanced H\textsubscript{2}O\textsubscript{2} toxicity in rat hippocampal slices, confirming the importance of the integrity of glial antioxidant network and supporting further CAT pathway enhancement rather than GPx inhibition in the prevention of pathophysiological consequences.

In addition, of paramount importance for the therapeutic potential of such treatment is the decrease of the damaging effects of H\textsubscript{2}O\textsubscript{2} in normoxic conditions (e.g. by using potent antioxidant agents) and the rapid boost of H\textsubscript{2}O\textsubscript{2} enzymatic degradation to O\textsubscript{2} through the CAT pathway (e.g. by using efficient SOD-CAT mimetics). We believe that the therapeutic potential of drugs targeting H\textsubscript{2}O\textsubscript{2} metabolism needs to be explored in depth at a preclinical level in order to transform their theoretical use in brain ischaemia in a true clinical application. A future challenge in the hands of neuroscientists is to validate an H\textsubscript{2}O\textsubscript{2} signalling-mediated pharmacological treatment of stroke.

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Conflict of interest

The authors state no conflict of interest with respect to the authorship and/or publication of this article.

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H2O2 as a therapeutic target in the ischaemic brain


