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REVIEW

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 (H_2O_2) 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 metabolism. It also critically discusses a translational impact concerning the role played by H_2O_2 in ischaemic stroke. Based on these data, we hope that further research will be done in order to better understand the mechanisms underlying H_2O_2 functions and to promote successful H_2O_2 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, ischaemic 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; O_2, molecular oxygen; $^{\cdot}O_2^-$, superoxide anion; OGD, oxygen/glucose-deprivation; •OH, hydroxyl radical; PGC1α, PPARγ coactivator1α; PI3K, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptorγ; 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 H_2O_2 began in 1818 when it was discovered by Thénard who named it *eau oxygénée* (Thénard, 1818). Since the mid-1800s, H_2O_2 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_2O_2 in living systems was identified in 1856 (Schoenbein, 1856). However, it was only in 1894 that 100% pure H_2O_2 was first extracted from H_2O by Wolffenstein through vacuum distillation (Wolffenstein, 1894). In 1888, the first medical use of H_2O_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_2O_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_2O_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_2O_2, thereby suggesting an important protective action of H_2O_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_2O_2 to treat a wide variety of diseases (Farr, 1988). Later, Willhelm promoted the therapeutic use of H_2O_2 to treat cancer, skin diseases, polio and bacteria-related mental illness. He defined H_2O_2 as 'God's given immune system' (Willhelm, 1989; Green, 1998). Another player in the H_2O_2 story was Grotz, who obtained pain relief by testing H_2O_2 on himself to treat his arthritis pain (Green, 1998).

Metabolism of H_2O_2

H_2O_2 is mainly generated as a by-product of aerobic metabolism in the mitochondria (Fridovich, 1995), where formation of the superoxide anion ($^{\cdot}O_2^-$) results from partial reduction of molecular oxygen (O_2) in the electron transport chain. A smaller amount of $^{\cdot}O_2^-$ is also produced by enzymatic activities including NOS, xanthine oxidase, NADPH oxidase, dehydrogenases and peroxidases (Boveris and Chance, 1973; Rhee, 2006; Bao *et al.*, 2009; Finkel, 2011). In addition, enzymes such as superoxide dismutase (SOD), in its three isoforms (cytosolic, extracellular Cu,Zn-SOD, and mitochondrial Mg-SOD), are also responsible for H_2O_2 production from $^{\cdot}O_2^-$ (Graham *et al.*, 1978; Fridovich, 1995). The dismutation reaction catalyzed by SOD is as follows: $2\,^{\cdot}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. H_2O_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 degradate bioamines and produces H_2O_2, the corresponding aldehyde and ammonia according to the overall equation: $R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \rightarrow H_2O_2 + R\text{-}CHO + NH_3$, where R stands for alkyl group (Tipton, 1968; Tipton *et al.*, 2004). Moreover, H_2O_2 generation can be the result of $p66^{Shc}$ enzyme activity (Giorgio *et al.*, 2007). H_2O_2 is subsequently converted to H_2O by scavenger enzymes such as cytosolic and mitochondrial glutathione peroxidase (GPx) which catalyzes the reaction: $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$, or decomposed in peroxisomes to H_2O and O_2 by catalase (CAT) according to the equation: $2H_2O_2 \rightarrow 2H_2O + O_2$; the latter has been observed to be more effective than GPx in detoxifying neurons from H_2O_2 (Halliwell, 1999; Dringen *et al.*, 2005). A smaller contribution to regulate H_2O_2 levels also comes from thioredoxins as well as peroxiredoxins (Prx) (Rhee, 2006; Mishina *et al.*, 2011). H_2O_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_2O_2 concentration. Many attempts to address this point have failed due to high cellular peroxidase-mediated depletion and technical limitations of H_2O_2-sensitive fluorescent dyes (Rice, 2011). However, *in vivo* microdialysis has been used to try to determine the extracellular production of H_2O_2 in the brain during IR. A fourfold rise in H_2O_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_2O_2 concentration during IR. A marked and rapid increase in H_2O_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_2O_2 at 15–150 μM without any oxidative damage (Rice, 2011). Noteworthy, concentrations of H_2O_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_2O_2 in both the intracellular and extracellular compartments of the brain. Of note, 1–3 mM H_2O_2 has been used for investigations of synaptic function and intracellular Ca^{2+} changes in the hippocampus (Pellmar, 1987; Nisticò *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_2O_2 has not really been solved yet. In fact, it has been reported that hippocampal neurons from primary culture tolerate 300 μM H_2O_2 for at least 30 min (Miller *et al.*, 2005). However, it has to be considered that the concentration of H_2O_2 that reaches the intracellular *milieu* could be significantly lower than that superfused on tissue. Firstly, H_2O_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_2O_2: a paradox player

Emerging role of H_2O_2 in the physiological control of cell functioning

H_2O_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, H_2O_2 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). H_2O_2 is also implicated in intracellular Ca^{2+} signalling and organelle function modulation in rat hippocampus (Gerich *et al.*, 2009). Additional evidence has indicated a dynamic modulation exerted by H_2O_2 in the nigrostriatal dopaminergic (DAergic) system. In fact, it inhibits substantia nigra DAergic neurons and striatal DA release by activating ATP-sensitive K^+ channels (K_{ATP}) (Chen *et al.*, 2001; Avshalumov *et al.*, 2003; 2005; 2008). Of note, H_2O_2 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 H_2O_2 signalling: concentration as a determining factor

H_2O_2 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, H_2O_2 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 H_2O_2 target sulfhydryl 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 H_2O_2 (Rhee *et al.*, 2000; Giorgio *et al.*, 2007) (Figure 1). In fact, H_2O_2 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, H_2O_2 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, H_2O_2 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, H_2O_2 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 H_2O_2 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 H_2O_2-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 H_2O_2 is also implicated in several growth factor-triggered signals (Stone and Yang, 2006; Valko *et al.*, 2007). Further evidence has shown that H_2O_2 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 H_2O_2 (Giorgio *et al.*, 2007; Oliveira-Marques *et al.*, 2009). H_2O_2 may induce apoptosis in neuronal cells by inhibiting the mammalian target of rapamycin signalling (Chen *et al.*, 2010). In endothelial cells, an excess of H_2O_2 activates Fas and JNK pathways in apoptosis (Cai, 2005). H_2O_2 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 H_2O_2 interaction with free transition metals (mostly reduced Fe^{2+} and Cu^+ ions) (Halliwell, 1992; Cohen, 1994). In its dual role as an indispensable signal molecule and a potential threat for biological components, H_2O_2 plays the double-faced role of 'Dr. Jekyll and Mr. Hyde' (Gough and Cotter, 2011). Indeed, a real H_2O_2 paradox exists: on one hand, in low amounts H_2O_2 has a physiological role in the homeostatic maintenance of normal cell functioning; on the other hand, high amounts of H_2O_2 can be harmful for cells (Figure 1).

H_2O_2 sensing during ischaemic injury: implications for neuroprotection

In order to maintain H_2O_2 physiological signalling function, both intracellular and extracellular concentrations of H_2O_2 need to be constantly maintained at a level below toxicity threshold via an accurate and complex metabolic regulation (Halliwell, 1999; Góth, 2006). In mammalian cells, redox sensor function has been suggested for Prx-1, thiol peroxidases and thiolate-dependent phosphatases, which may affect H_2O_2 signalling fluxes (Stone and Yang, 2006; D'Autréaux and Toledano, 2007). More importantly, specific responses to H_2O_2-induced oxidative stress are regulated in eukaryotic cells by acetylation or deacetylation of 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 H_2O_2 stimulate p53 tumour suppressor-mediated antioxidant response by activating antioxidant genes (e.g. GPx, SOD, sestrins), while high levels of it induce p53-dependent apoptosis (Veal *et al.*, 2007). The peroxisome proliferator-activated receptorγ (PPARγ) coactivator1α (PGC1α) also establishes a crucial link between mitochondrial production of ROS and anti-ROS programmes by regulating H_2O_2-inducible antioxidant enzymes (SOD, CAT, GPx) (St-Pierre *et al.*, 2006; D'Autréaux and Toledano, 2007). Strong evidence now suggests PPARγ agonists as new therapeutic targets for the treatment of IR injury (Giaginis *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 H_2O_2,

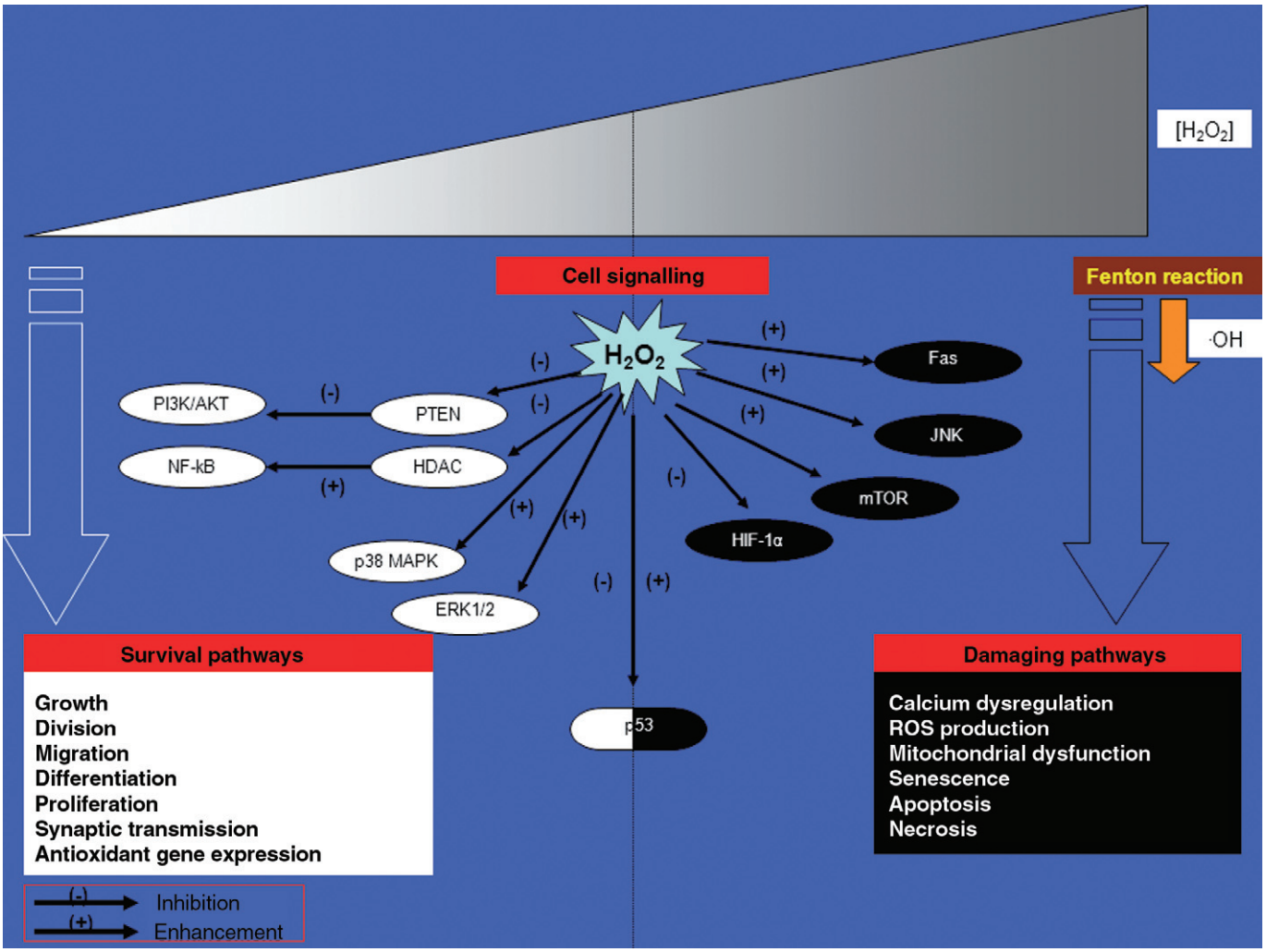


Figure 1

The H_2O_2 paradox in the regulation of cell signalling transduction cascade. The H_2O_2 biological functions depend on the concentration of H_2O_2 within the cell. At low concentrations, H_2O_2 acts as a messenger in a great variety of biological processes contributing to cell survival. In high concentrations, H_2O_2 can cause deleterious effects, mainly via •OH-derived radicals, by inducing a severe oxidative stress and cell death. Accordingly, a crucial target of H_2O_2 double-faced action is represented by the tumour suppressor protein p53 which can be either activated by low levels of H_2O_2, thus triggering an antioxidant response (anti-apoptotic programme), or inhibited by high levels of H_2O_2 leading to programmed cell death (pro-apoptotic programme) respectively.

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 H_2O_2 elimination by CAT may provide an alternative source for O_2, causing neuroprotection in hypoxic conditions (Topper *et al.*, 1996; Auerbach and Segal, 1997; Klann and Thiels, 1999).

Effects of H_2O_2 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% N_2 to 5% CO_2). At the end of the insult, the slice was again perfused with normal oxygenated (95% O_2

to 5% CO_2) 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 eletrophysiological 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 K_{ATP} 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 H_2O_2 may act as a supplementary source of O_2 in an isolated neonatal rat spinal cord preparation *in vitro* (Walton and Fulton, 1983) and a recovery of the synaptic function by H_2O_2 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 H_2O_2 in DAergic neurons of the rat SNc exposed to hypoxic insult (Geracitano *et al.*, 2005). Notably, H_2O_2 reversed membrane hyperpolarization and blocked spontaneous firing associated with oxygen-deprivation in DAergic neurons (Figure 2A,B). In contrast, in normoxic conditions, H_2O_2 (3 mM) blocked the spontaneous activity of the DAergic cells by inducing a K_{ATP} channels-dependent outward current that was sensitive to tolbutamide (1 mM) (a non-selective blocker of K_{ATP} channels) (Figure 2C). Of note, H_2O_2 decreased the hypoxia-mediated outward current in a concentration-dependent manner. Conversely, H_2O_2 did not counteract membrane hyperpolarization associated with hypoglycaemia. The superfusion of H_2O_2 (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), H_2O_2 was unable to decrease hypoxia-mediated outward current and thus restore the spontaneous firing rate (Figure 2D). The protective effects of H_2O_2 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 H_2O_2 in hypoxic DAergic neurons by serving as a supplementary source of O_2, through its degradation by CAT and thus, interfering with K_{ATP} channels opening consequent to O_2 deprivation (Figure 2E). On the other hand, under normoxic conditions, H_2O_2 (3 mM) induced by itself a tolbutamide-sensitive outward current in DAergic neurons. This outward response is due to the opening of K_{ATP} channels by a direct action of H_2O_2 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 H_2O_2 and of the modification in its endogenous levels by the pharmacological modulation of H_2O_2 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 H_2O_2 (3 mM, 30 min) during OGD exposure (30 min) in CA1 region (Nisticò *et al.*, 2008) (Figure 3A). Importantly, the neuroprotective effects of H_2O_2 were still maintained even when applied 7 min after the ischaemic conditions had already been imposed (Figure 3B). Again, the rescuing action of H_2O_2 (3 mM) was mediated by the CAT-induced formation of O_2. 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 H_2O_2, 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 $U \cdot mL^{-1}$), which augments H_2O_2 production, limited the OGD-induced irreversible depression of fEPSPs. These results were in line with previous observation of neuroprotection afforded by H_2O_2 against hypoxic insult on DAergic cells (Geracitano *et al.*, 2005) and propose novel therapeutic strategies based on increasing the endogenous tissue levels of H_2O_2 in the ischaemic brain.

Striatum

Little information is as yet available as to whether H_2O_2 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 O_2 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 H_2O_2 intravenously, our aim was to examine the effect of increasing the endogenous levels of H_2O_2 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 $mg \cdot kg^{-1}$) 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

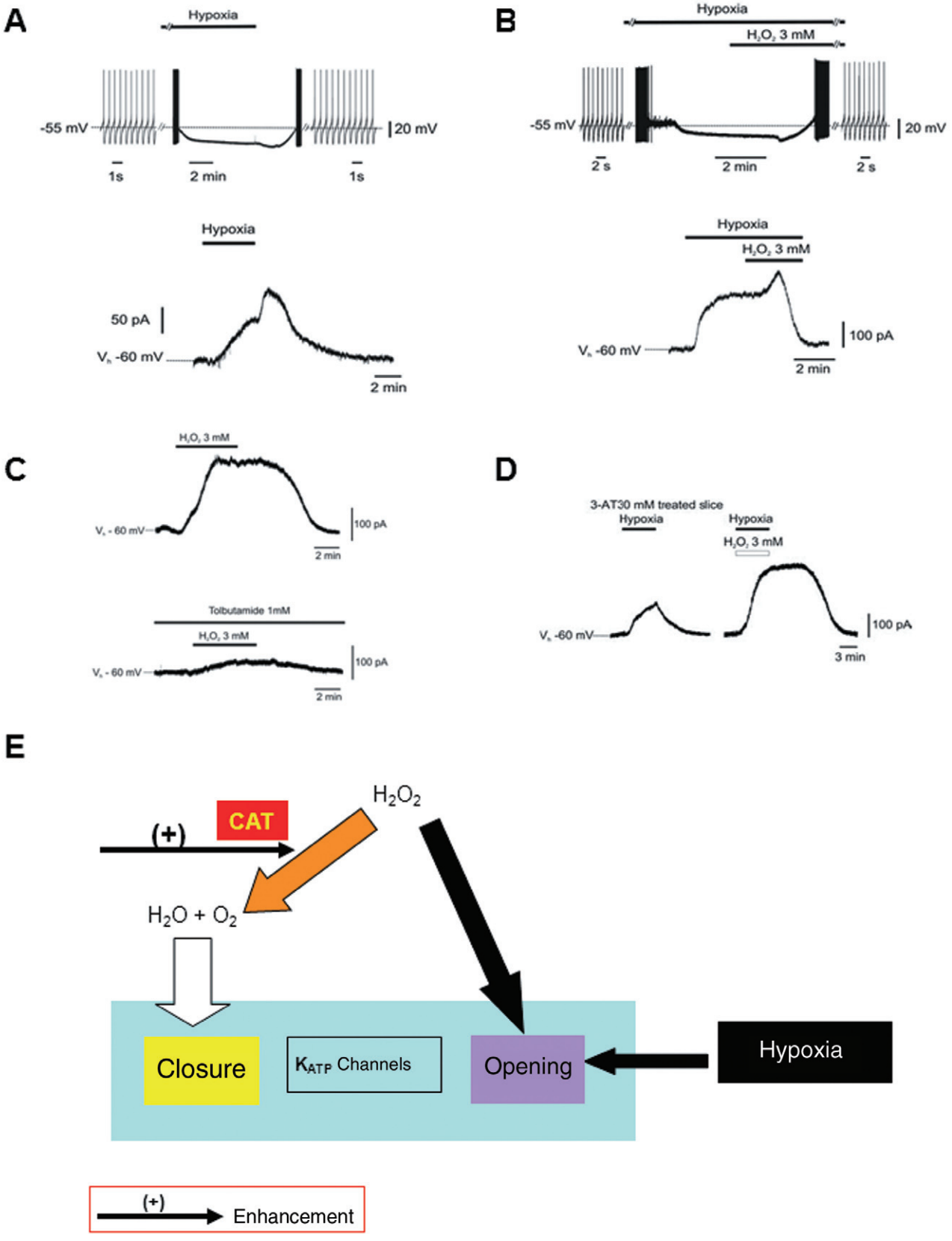

A
Hypoxia
-55 mV
20 mV
1s
2 min
1s
Hypoxia
50 pA
V_h -60 mV
2 min
B
Hypoxia
H2O2 3 mM
-55 mV
20 mV
2 s
2 min
2 s
Hypoxia
H2O2 3 mM
V_h -60 mV
100 pA
2 min
C
H2O2 3 mM
V_h - 60 mV
100 pA
2 min
Tolbutamide 1mM
H2O2 3 mM
V_h - 60 mV
100 pA
2 min
D
3-AT30 mM treated slice
Hypoxia
Hypoxia
H2O2 3 mM
V_h -60 mV
100 pA
3 min
E
H2O2
(+)
CAT
H2O + O2
Closure
KATP Channels
Opening
Hypoxia
(+)
Enhancement

Figure 2

Electrophysiological effects of H_2O_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_2O_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_2O_2 ($n = 6$) (lower trace). (C) In normoxia, H_2O_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_2O_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 *J Physiol*, used with permission]. (E) Hypothesized mechanism of neuroprotection afforded by H_2O_2 against hypoxic insult in SNc DAergic neurons. In normoxic conditions, H_2O_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_2O_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.

act by decreasing the activity of GPx and thus augmenting the endogenous level of H_2O_2. Consistent with these findings, superfusion of striatal slices with MCS (1 mM) limited the irreversible cortico-striatal 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 vivo* and *in vitro* ischaemic conditions, through a mechanism which, by blocking GPx, very likely increases endogenous levels of H_2O_2 and its consequent conversion to O_2 and H_2O by CAT.

Can the pharmacological modulation of enzymatic pathways leading to an enhanced H_2O_2 conversion to O_2 and H_2O be therapeutic?

The therapeutic potential of modulating the enzymatic pathways leading to H_2O_2 production and its conversion to O_2 and H_2O (SOD, GPx, CAT) has been previously investigated via either transgenic or pharmacological intervention tools in both *in vitro* and *in vivo* ischaemic models. The modification of H_2O_2 signalling might have a key aspect in the expression of neuronal damage during an episode of IR.

Targeting SOD enzyme

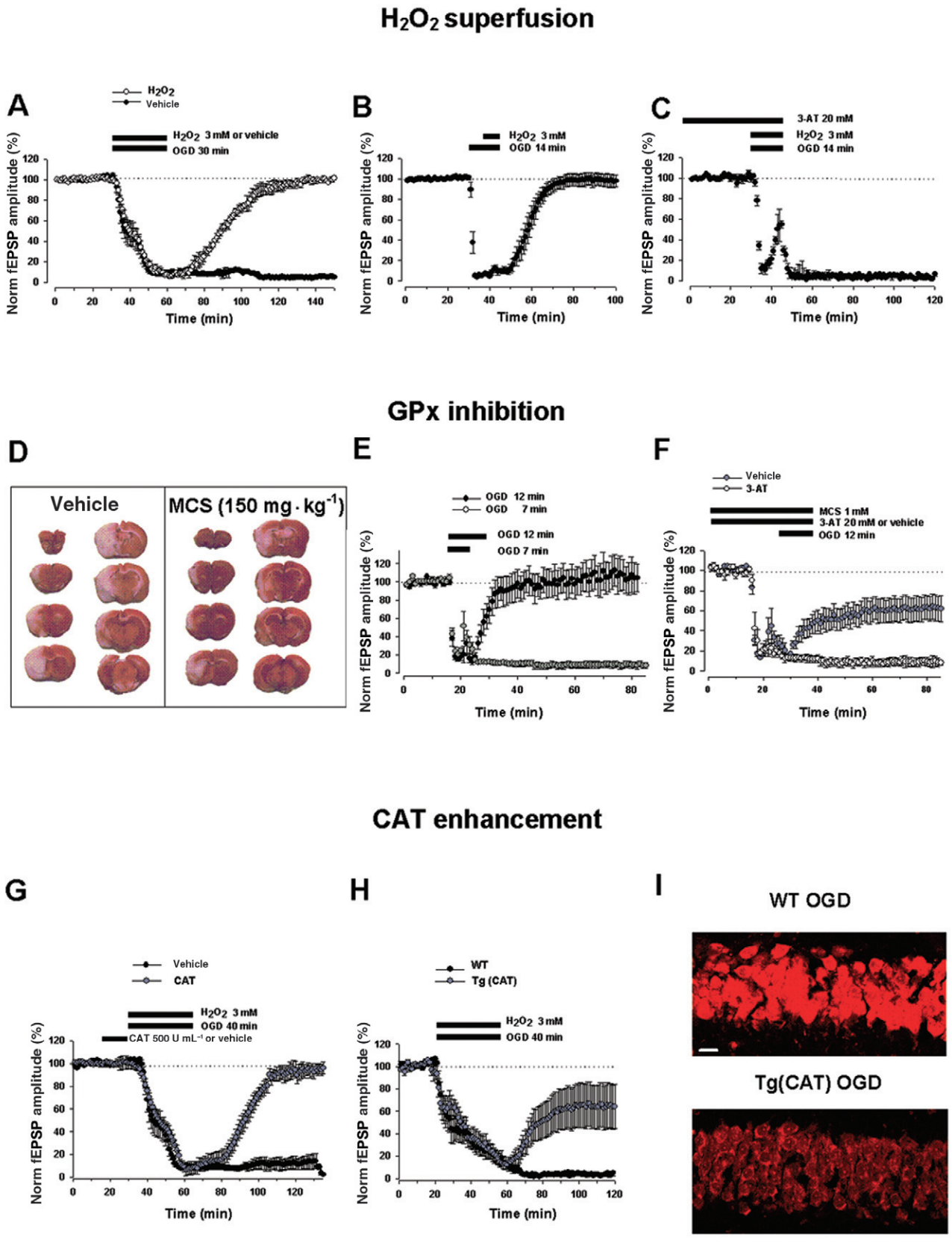
Transgenic mice overexpressing Cu,Zn-SOD enzyme are more resistant to focal brain ischaemia (Yang *et al.*, 1994). However, neither selective deletion nor overexpression of Cu,Zn-SOD affect the outcome of permanent focal brain ischaemia (Chan *et al.*, 1993; Fujimura *et al.*, 2001). On the contrary, 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 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 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_2O_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_2O_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; Ushitora *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_2O_2 in oxygen-deprived brain slices of the


H2O2 superfusion
A
H2O2
Vehicle
H2O2 3 mM or vehicle
OGD 30 min
Norm fEPSP amplitude (%)
Time (min)
B
H2O2 3 mM
OGD 14 min
Norm fEPSP amplitude (%)
Time (min)
C
3-AT 20 mM
H2O2 3 mM
OGD 14 min
Norm fEPSP amplitude (%)
Time (min)
GPx inhibition
D
Vehicle
MCS (150 mg · kg-1)
E
OGD 12 min
OGD 7 min
OGD 12 min
OGD 7 min
Norm fEPSP amplitude (%)
Time (min)
F
Vehicle
3-AT
MCS 1 mM
3-AT 20 mM or vehicle
OGD 12 min
Norm fEPSP amplitude (%)
Time (min)
CAT enhancement
G
Vehicle
CAT
H2O2 3 mM
OGD 40 min
CAT 500 U mL-1 or vehicle
Norm fEPSP amplitude (%)
Time (min)
H
WT
Tg (CAT)
H2O2 3 mM
OGD 40 min
Norm fEPSP amplitude (%)
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I
WT OGD
Tg(CAT) OGD

Figure 3

Protective effects of H_2O_2 superfusion and pharmacological modulation of enzymatic pathways leading to H_2O_2 production and degradation. (A) In hippocampal slices, H_2O_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_2O_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_2O_2-mediated neuroprotection: the CAT inhibitor 3-AT (20 mM) prevented H_2O_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_2O_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_2O_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 $^{\cdot}O_2^-$ 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 *IJIP*, 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 also 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_2O_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_2O_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_2O_2 in normoxic conditions. In fact, it decreased fEPSPs depression evoked by repeated applications of H_2O_2. Notably, a lower sensitivity to H_2O_2-mediated field depression, very likely due to a better functioning of CAT, was indicated by the rightward shift of the H_2O_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_2O_2 as a preconditioning factor in neuroprotection

Another important aspect to consider is the implication of H_2O_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_2O_2 have been observed in several *in vitro* models of IPC (Furuichi *et al.*, 2005; Xiao-Qing *et al.*, 2005). In fact, it has been demonstrated that the generation of H_2O_2 during brief OGD (10 min) induces IPC in rat primary cultured cortex neurons (Furuichi *et al.*, 2005). In addition, H_2O_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_2O_2 (2 mM) decreased rat cerebral infarct size (cortical area) 24 h after MCAo (1 h), suggesting an involvement of H_2O_2 during the induction

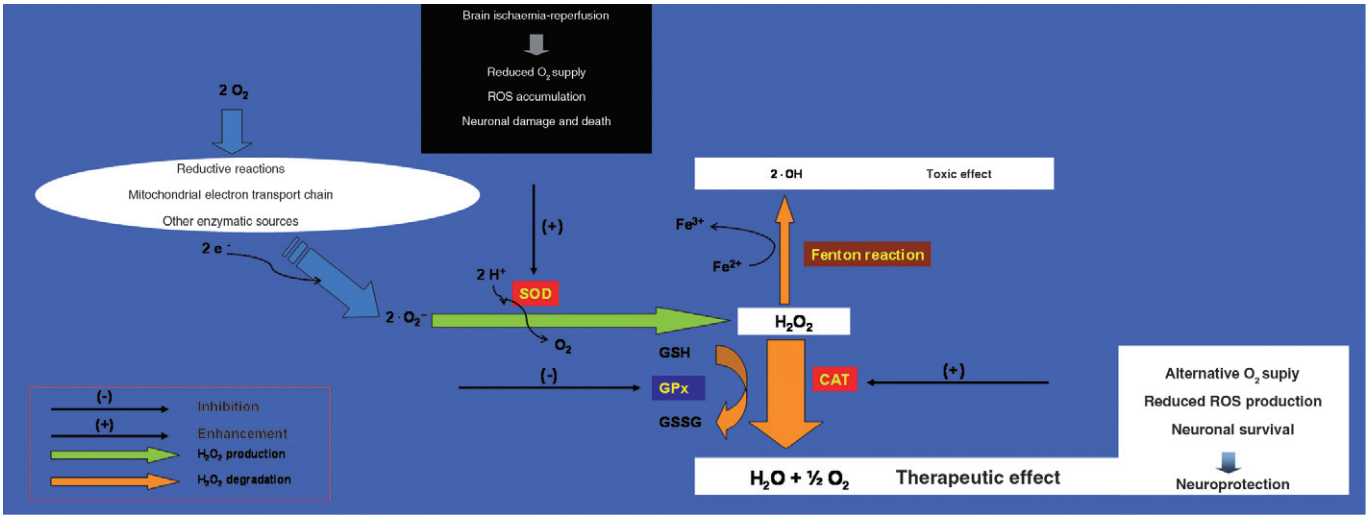

Figure 4

Working model of proposed enzymatic targets of H_2O_2 metabolism for the treatment of brain ischaemia. A therapeutic effect against brain ischaemia could be achieved by the pharmacological modulation of H_2O_2 producing (SOD) and degrading enzymes (CAT and GPx). In *in vitro* and *in vivo* brain ischaemia models, neuroprotection is afforded via CAT pathway activation mainly through two mechanisms: supplementary production of O_2 to compensate for the lack of O_2 and detoxification from H_2O_2 derived •OH radical associated-oxidative stress.

phase of IPC (Simerabet *et al.*, 2008). Moreover, in another study carried out by Chang *et al.* (2008), exogenous low concentration of H_2O_2 (15 μM) may contribute to IPC against OGD (24 h) in rat primary neurons by increasing HIF-1α 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_2O_2 metabolism in stroke. In spite of the fact that no clinical studies have been conducted evaluating the therapeutic usage of drugs targeting H_2O_2 metabolism in stroke, the experimental observations obtained so far by our and other groups support the idea that H_2O_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_2O_2 metabolism is pharmacologically manipulated to boost CAT pathway: (i) one mechanism produces a supplementary source of O_2 to partially compensate for the lack of O_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_2O_2 and the radical •OH derived from H_2O_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_2O_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_2O_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_2O_2 in normoxic conditions (e.g. by using potent antioxidant agents) and the rapid boost of H_2O_2 enzymatic degradation to O_2 through the CAT pathway (e.g. by using efficient SOD-CAT mimetics). We believe that the therapeutic potential of drugs targeting H_2O_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_2O_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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