

Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals

Ikuroh Ohsawa¹, Masahiro Ishikawa¹, Kumiko Takahashi¹, Megumi Watanabe^{1,2}, Kiyomi Nishimaki¹, Kumi Yamagata¹, Ken-ichiro Katsura², Yasuo Katayama², Sadamitsu Asoh¹ & Shigeo Ohta¹

Acute oxidative stress induced by ischemia-reperfusion or inflammation causes serious damage to tissues, and persistent oxidative stress is accepted as one of the causes of many common diseases including cancer. We show here that hydrogen (H_2) has potential as an antioxidant in preventive and therapeutic applications. We induced acute oxidative stress in cultured cells by three independent methods. H_2 selectively reduced the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protected cells; however, H_2 did not react with other ROS, which possess physiological roles. We used an acute rat model in which oxidative stress damage was induced in the brain by focal ischemia and reperfusion. The inhalation of H_2 gas markedly suppressed brain injury by buffering the effects of oxidative stress. Thus H_2 can be used as an effective antioxidant therapy; owing to its ability to rapidly diffuse across membranes, it can reach and react with cytotoxic ROS and thus protect against oxidative damage.

Oxidative stress arises from the strong cellular oxidizing potential of excess reactive oxygen species (ROS), or free radicals^{1–5}. Most of the superoxide anion radical (O_2^{\bullet}) produced is generated in mitochondria by electron leakage from the electron transport chain and the Krebs cycle⁶. O_2^{\bullet} is also produced by metabolic oxidases, including NADPH oxidase and xanthine oxidase⁷. Superoxide dismutase converts O_2^{\bullet} into hydrogen peroxide (H_2O_2)⁸, which is detoxified into H_2O by either glutathione peroxidase or catalase. Excess O_2^{\bullet} reduces transition metal ions such as Fe^{3+} and Cu^{2+} (ref. 2), the reduced forms of which in turn can react with H_2O_2 to produce hydroxyl radicals ($\bullet OH$) by the Fenton reaction. $\bullet OH$ is the strongest of the oxidant species and reacts indiscriminately with nucleic acids, lipids and proteins. There is no known detoxification system for $\bullet OH$; therefore, scavenging $\bullet OH$ is a critical antioxidant process⁹.

Despite their cytotoxic effects, O_2^{\bullet} and H_2O_2 play important physiological roles at low concentrations: they function as regulatory signaling molecules that are involved in numerous signal transduction cascades and also regulate biological processes such as apoptosis, cell proliferation and differentiation^{7,10}. At higher concentrations, H_2O_2 is converted into hypochlorous acid by myeloperoxidase; hypochlorous acid defends against bacterial invasion⁵. Nitric oxide (NO^{\bullet}), another ROS, functions as a neurotransmitter and is essential for the dilation of blood vessels¹¹. Thus, cytotoxic radicals such as $\bullet OH$ must be neutralized without compromising the essential biological activities of other, physiologically beneficial, ROS. Here we demonstrate that molecular hydrogen (dihydrogen, H_2) can alleviate $\bullet OH$ -induced cytotoxicity without affecting the other ROS, and propose that H_2 has potential as an antioxidant for preventive and therapeutic applications.

RESULTS

H_2 selectively reduces $\bullet OH$ in cultured cells

H_2 reduces the $\bullet OH$ that is produced by radiolysis or photolysis of water¹²; however, whether H_2 can effectively neutralize $\bullet OH$ in living cells has not been directly investigated. As the cellular damage produced by spontaneous generation of $\bullet OH$ is not sufficient to be detectable, we induced O_2^{\bullet} production in PC12 cultured cells. To do this, we treated the cells with a mitochondrial respiratory complex III inhibitor, antimycin A (ref. 13); following such treatment, O_2^{\bullet} in these cells is rapidly converted into H_2O_2 . The addition of antimycin A increased levels of O_2^{\bullet} and H_2O_2 , as judged by the fluorescence signals emitted by the oxidized forms of MitoSOX (Fig. 1a) and 2',7'-dichlorodihydrofluorescein (H_2DCF) (Supplementary Fig. 1 online), respectively. We dissolved H_2 and O_2 into medium as described in the Methods, and confirmed the prolonged (24 h long) maintenance of H_2 levels (Supplementary Fig. 2 online). H_2 dissolved in culture medium did not decrease MitoSOX and DCF signals in the cells (Fig. 1a,b and Supplementary Fig. 1). Additionally, H_2 did not decrease the steady-state level of NO^{\bullet} (Supplementary Fig. 1). In contrast, H_2 treatment significantly decreased levels of $\bullet OH$, as assessed by the fluorescence signal emitted by the oxidized form of 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) (refs. 14,15 and Fig. 1c,d). When we exposed the cells to antimycin A (30 $\mu g/ml$) in the absence of H_2 , the HPF signals increased in both the nuclear region and the cytoplasm, probably because H_2O_2 diffused from the mitochondria to produce $\bullet OH$. Notably, H_2 decreased $\bullet OH$ levels even in the nuclear region (Fig. 1c).

¹Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, 1-396 Kosugi-cho, Nakahara-ku, Kawasaki City 211-8533, Japan. ²Department of Internal Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. Correspondence should be addressed to S.O. (ohta@nms.ac.jp).

Received 25 September 2006; accepted 15 March 2007; published online 7 May 2007; doi:10.1038/nm1577

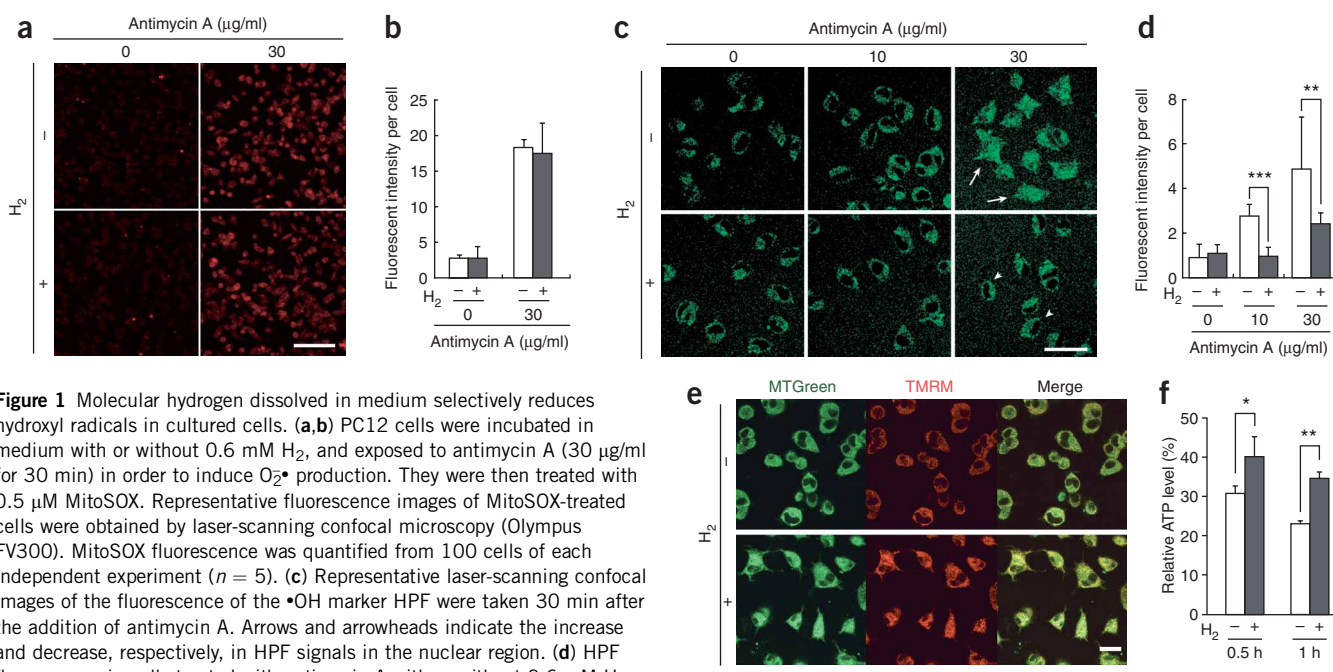


Figure 1 Molecular hydrogen dissolved in medium selectively reduces hydroxyl radicals in cultured cells. **(a,b)** PC12 cells were incubated in medium with or without 0.6 mM H₂, and exposed to antimycin A (30 µg/ml for 30 min) in order to induce O₂• production. They were then treated with 0.5 µM MitoSOX. Representative fluorescence images of MitoSOX-treated cells were obtained by laser-scanning confocal microscopy (Olympus FV300). MitoSOX fluorescence was quantified from 100 cells of each independent experiment (*n* = 5). **(c)** Representative laser-scanning confocal images of the fluorescence of the •OH marker HPF were taken 30 min after the addition of antimycin A. Arrows and arrowheads indicate the increase and decrease, respectively, in HPF signals in the nuclear region. **(d)** HPF fluorescence in cells treated with antimycin A with or without 0.6 mM H₂ was quantified from 100 cells (*n* = 4). ***P* < 0.01, ****P* < 0.001. **(e)** At 30 min after adding antimycin A (10 µg/ml) with or without H₂ (0.6 mM), cells were incubated with 1 µM MTGreen and 100 nM TMRM for 10 min and then imaged. The two images were superimposed (merge). **(f)** Cells were pretreated with 4.5 g/l 2-deoxyl-D-glucose (an inhibitor of glycolysis) and 1 mM pyruvate, and relative cellular ATP levels were quantified after exposure to 30 µg/ml antimycin A. ATP levels of cells not treated with antimycin A were set at 100% (*n* = 3). **P* < 0.05, ***P* < 0.01. Scale bars: 100 µm in **a**; 50 µm in **c**; 20 µm in **e**. Histograms show mean ± s.d.

After antimycin A treatment, H₂ prevented the decline of the mitochondrial membrane potential, as detected by fluorescence of tetramethylrhodamine methyl ester (TMRM), which depends upon the mitochondrial membrane potential, whereas fluorescence levels of MitoTracker Green (MTGreen), which are independent of the membrane potential, were unchanged (**Fig. 1e**). This suggested that H₂ protected mitochondria from •OH. H₂-treated cells looked normal, whereas H₂-untreated cells were shrunken and had abnormal round shapes (**Fig. 1e**). Along with this protective effect, H₂ also prevented a decrease in the cellular levels of ATP synthesized in mitochondria (**Fig. 1f**). The fact that H₂ protected mitochondria and nuclear DNA provided evidence that H₂ penetrated most membranes and diffused into organelles.

H₂ dissolved in medium protects cultured cells against •OH

We placed PC12 cells in culture medium containing H₂ and O₂, and, at the same time, induced oxidative stress by adding antimycin A. At 24 h after the induction of ROS with antimycin A, we observed that H₂ seemed to protect nuclear DNA from oxidation, as shown by decreased levels of oxidized guanine (8-OH-G) (**Fig. 2a,b** and ref. 16). Moreover, H₂ also decreased levels of 4-hydroxyl-2-nonenal (HNE), an end-product of lipid peroxides (**Fig. 2c,d** and ref. 17), indicating that it protected lipids from peroxidation. Further, H₂ dissolved in medium protected cells from cell death in a dose-dependent manner (**Fig. 2e,f**). When we removed H₂ from medium that had been saturated with H₂, the protective effect disappeared (**Fig. 2f**), suggesting that the observed effect was not due to a reaction of H₂ with the medium. Moreover, we confirmed that H₂ protected cellular viability by using two methods: a modified MTT assay (WST-1 assay) and measurement of cellular lactate dehydrogenase (LDH) leakage from damaged cells (**Supplementary Fig. 3** online). To exclude the possibility that the protective effect of H₂ was due to a reaction with

antimycin A, we induced ROS by adding menadione, an inhibitor that acts on mitochondrial complex I, and observed that H₂ protected cells in this system as well (**Supplementary Fig. 3**).

To verify that H₂ protects against •OH, we pretreated cells with Cu²⁺ and then exposed them to ascorbate, in order to reduce intracellular Cu²⁺ to Cu⁺, which in turn catalyzes the production of •OH from cellular H₂O₂ that is endogenously produced. This treatment primarily induced •OH inside the cells (by the Fenton reaction), thus directly confirming that H₂ protects cells against cellular •OH (**Fig. 2g,h**).

Spin-trapping identifies a free radical that is reduced by H₂

To identify the free radical species that H₂ reduces, we studied the effects of H₂ on electron spin resonance (ESR) signals of spin-trapping reagents. We produced •OH by the cellular Fenton reaction, and semiquantified the cellular levels of •OH by spin-trapping using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Measurements of ESR indicated that H₂ treatment did indeed decrease signals of •DMPO-OH derived from •OH (**Fig. 3a–c**).

Moreover, when we induced O₂• production by treating cells with antimycin A in the presence of DMPO, we observed multiple ESR signals¹⁸. These signals seemed to consist of those from the •DMPO-OH and •DMPO-H radicals (**Fig. 3d–f**). The •DMPO-H radical is derived from the hydrogen radical (H•), which can be induced by porphyrins. To visualize the signals decreased by H₂, we obtained the differential spectrum. We found that only •OH-derived signals were decreased by H₂ treatment (**Fig. 3e**). These results strongly suggest the selective reduction of cellular •OH by H₂ treatment.

H₂ selectively reduces •OH and ONOO⁻ in cell-free systems

Next, we confirmed in a pure solution that HPF fluorescence can be used to monitor the reduction of •OH by H₂ during continuous •OH

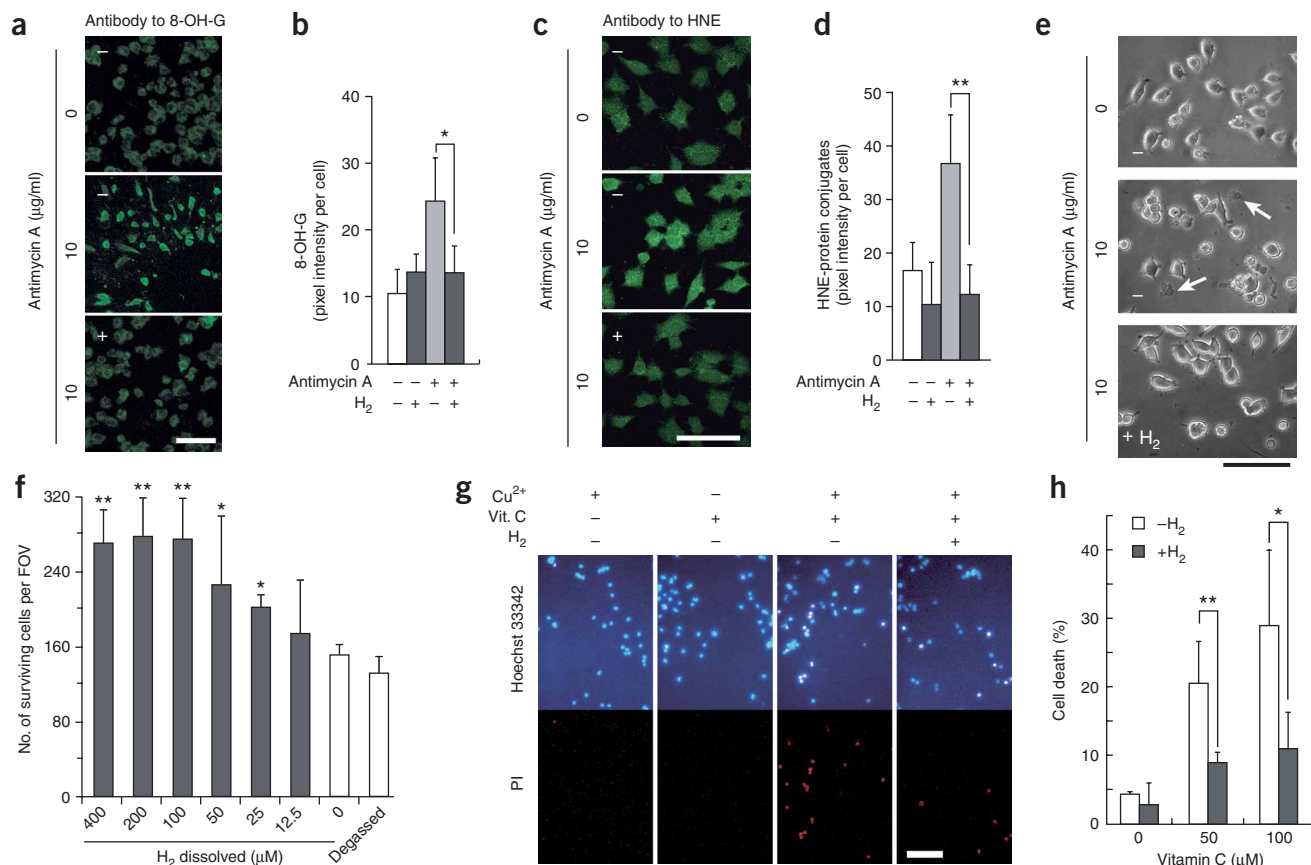


Figure 2 Molecular hydrogen protects cultured PC12 cells by scavenging hydroxyl radicals. (**a–d**) PC12 cells were maintained with 10 $\mu\text{g/ml}$ antimycin A, with (+) or without (–) 0.6 mM H_2 , for 24 h in a closed flask, and immunostained with antibodies to 8-OH-G or HNE. Fluorescence signals in response to 8-OH-G and HNE were quantified using 100 cells from each independent experiment ($n = 4$). $*P < 0.05$, $**P < 0.01$. (**e**) Phase-contrast pictures of PC12 cells 24 h after the exposure to antimycin A, with (+) or without (–) 0.6 mM H_2 . Arrows indicate dead cells. (**f**) Cell survival was assessed by manually counting the cells (Methods; $n = 4$). $*P < 0.05$, $**P < 0.01$ (compared with 0 μM H_2). (**g**) PC12 cells were exposed to intracellular $\bullet\text{OH}$ produced by the Fenton reaction, with or without 0.6 mM H_2 . Cells were preincubated with 1 mM CuSO_4 , washed, and exposed for 1 h to 0.1 mM ascorbate (Vit. C) in order to reduce intracellular Cu^{2+} to Cu^+ (Supplementary Methods). The cells were costained with propidium iodide (PI) (for dead cells) and Hoechst 33342 to visualize the nuclei. (**h**) Cell survival was assessed by manually counting the cells as in **f** ($n = 5$). $*P < 0.05$, $**P < 0.01$. Scale bars: 50 μm in **a,c,e**; 100 μm in **g**. Histograms represent mean \pm s.d.

production by the Fenton reaction. In this condition, H_2 suppressed increases in HPF signals in a dose-dependent manner (Fig. 4a–c). But when we mixed a solution containing H_2 with HPF preoxidized with $\bullet\text{OH}$, fluorescence signals from oxidized HPF did not decrease (data not shown), supporting the idea that H_2 directly reacts with $\bullet\text{OH}$.

Next, we examined the reactivity of H_2 with other ROS or reactive nitrogen species (RNS). We prepared H_2O_2 and peroxynitrite (ONOO^-) by dilution of the respective stock solutions, $\text{O}_2\bullet$ by the enzymatic reaction of xanthine oxidase with xanthine, and $\text{NO}\bullet$ by the spontaneous reaction of 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) in cell-free systems (Supplementary Methods online). H_2 reduced ONOO^- (Fig. 4d) somewhat, but did not reduce H_2O_2 , $\text{NO}\bullet$ and $\text{O}_2\bullet$ (Fig. 4e–g). In cell-free experiments, we examined whether H_2 reduced the oxidized forms of biomolecules involved in metabolic oxidation-reduction reactions. At room temperature and neutral pH, solutions saturated with H_2 did not reduce the oxidized form of nicotinamide adenine dinucleotide (NAD^+), the oxidized form of flavin adenine dinucleotide (FAD) or the oxidized form of cytochrome C (data not shown). Thus we infer that H_2 does not affect the metabolism involved in oxidation-reduction

reactions or the levels of $\text{O}_2\bullet$, H_2O_2 , and $\text{NO}\bullet$, all of which play essential roles in signal transduction.

H_2 protects neurons from *in vitro* ischemia and reperfusion

We also induced oxidative stress in a primary culture of neocortical cells¹⁹ under more physiological conditions. It is known that rapid transition from an ischemic condition to reperfusion results in oxidative stress damage²⁰. To mimic ischemia, we subjected neocortical cells to oxygen glucose deprivation (OGD) under nitrogen or hydrogen gas for 60 min, followed by reperfusion with medium containing O_2 and glucose.

HPF fluorescence showed that 10 min after the completion of OGD followed by reperfusion, $\bullet\text{OH}$ levels notably increased when H_2 was absent, but diminished when H_2 was present (Supplementary Fig. 4 online). At 24 h after OGD and reperfusion, H_2 increased neuron survival and vitality (Supplementary Fig. 4), indicating that H_2 protects neurons against oxidative stress-induced cell death.

Inhalation of H_2 gas protects brain injury by reperfusion

To examine the therapeutic applicability of H_2 as an antioxidant, we used a rat model of ischemia. ROS are generated during cerebral

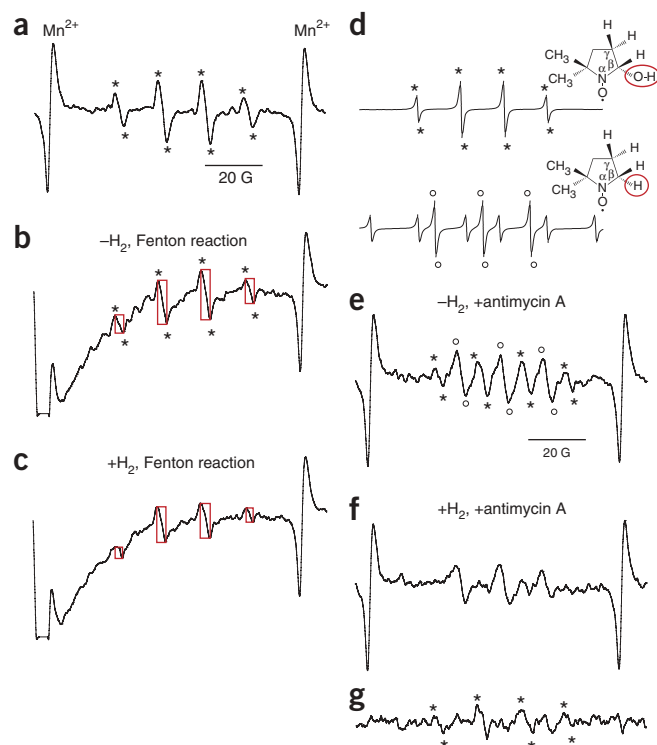


Figure 3 Spin-trapping identifies the free radical species that H_2 reduces. (a) Standard electron spin resonance (ESR) signals of the \bullet DMPO-OH radical were obtained by trapping \bullet OH with a spin-trapping reagent (DMPO; details in **Supplementary Methods**). (b,c) PC12 cells were preincubated with 0.1 M DMPO and 2 mM $CuSO_4$ for 30 min at 37 °C with or without 0.6 mM H_2 . After removal of this medium, the cells were treated with 0.2 mM ascorbate and 0.1 mM H_2O_2 for 5 min at 23 °C to produce \bullet OH and then scraped into a flat cuvette for ESR measurement. Rectangle height reflects signal intensity. (d) The \bullet DMPO-OH and \bullet DMPO-H radicals¹⁸ and their corresponding ESR signals are illustrated. (e,f) PC12 cells were incubated in PBS containing 0.1 M DMPO and 30 μ g/ml antimycin A for 7 min at 23 °C, with or without 0.6 mM H_2 , then scraped into a flat cuvette for ESR measurement. (g) A differential spectrum was obtained by subtracting the spectrum in f from that in e, in order to visualize the signals decreased by H_2 treatment. * indicates \bullet DMPO-OH signals derived from \bullet OH. * and o indicate \bullet DMPO-OH and \bullet DMPO-H signals, respectively (a, b, d, e and g).

ischemia and reperfusion, and are one of the major causes of brain damage^{21,22}. We produced focal ischemia in rats by occluding the middle cerebral artery (MCA) for 90 min, and then performed reperfusion for 30 min (ref. 23). In three of four conditions, rats inhaled H_2 gas, mixed with nitrous oxide (N_2O) for anesthesia, during the entire 120 min process (proportions of H_2 , O_2 and N_2O (vol/vol/vol) were 1%:30%:69%, 2%:30%:68%, and 4%:30%:66%); in the fourth condition, H_2 was absent, (H_2 : O_2 : N_2O (vol/vol/vol) was 0%:30%:70%). We carefully monitored physiological parameters during the experiments (Methods) and found no significant changes resulting from the inhalation of H_2 (**Supplementary Table 1** online). Additionally, there was no significant influence on cerebral blood flow,

as measured by the Doppler effect (ref. 24 and **Supplementary Fig. 5** online). H_2 dissolved in arterial blood was increased by the inhalation of H_2 in proportion to the concentration inhaled; the amount of H_2 dissolved in venous blood was less than that in artery blood, suggesting that H_2 had been incorporated into tissues (**Fig. 5a**).

At 1 d after MCA occlusion, we sectioned and stained brains with 2,3,5-triphenyltetrazolium chloride (TTC), a substrate for mitochondrial respiration (**Fig. 5b**). We estimated infarct volumes by assessing the staining of brain areas (white indicates infarct, **Fig. 5b,c**), and found a clear H_2 -dependent decrease in infarct volume, with 2–4% of H_2 providing the most substantial effect (**Fig. 5c**). We also noted that H_2 exerted its effect only when it was inhaled during reperfusion; when H_2 was inhaled during ischemia, infarct volume was not significantly decreased (**Fig. 5d,e**). For comparison, we tested two other compounds: edaravone (approved in Japan as an ROS scavenger for the treatment of cerebral infarction²⁵) and FK506 (in clinical trials for cerebral infarction in the United States²⁶). H_2 was more effective than edaravone and as effective as FK506 in alleviating oxidative injury (**Fig. 5c**). These results indicate the potential of H_2 for therapy.

Inhalation of H_2 gas suppresses the progression of damage

At 1 week after MCA occlusion, the difference in infarct volume between untreated and H_2 -treated rats increased, compared to 1d after

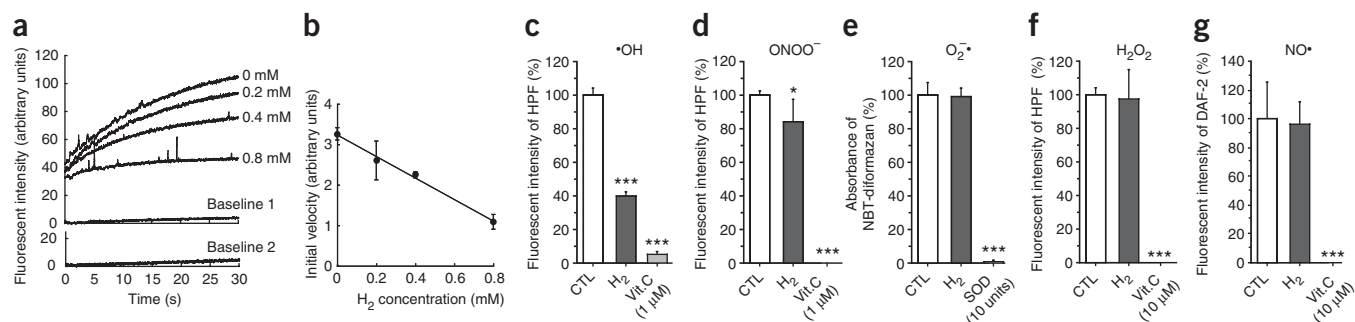


Figure 4 Molecular hydrogen dissolved in solution scavenges hydroxyl radicals at 23 °C and pH 7.4 in cell-free systems. (a,b) The Fenton reaction, which generates hydroxyl radicals, was initiated by adding H_2O_2 (to a final concentration of 5 μ M) in a closed cuvette at 23 °C with gentle stirring (**Supplementary Methods**). Levels of \bullet OH in the presence of various concentrations of H_2 dissolved in the solution were assessed for HPF fluorescence. (a) Representative time course traces of HPF fluorescence at each concentration of H_2 . Baselines 1 and 2 show HPF fluorescence (in the presence of 0.8 mM H_2) in the absence of H_2O_2 (baseline 1) and in the absence of ferrous perchlorate (baseline 2). (b) Mean \pm s.d. of initial rates of increase in HPF fluorescence (four independent experiments). (c–g) Levels of \bullet OH and two reactive nitrogen species (RNS: $NO\bullet$ and peroxynitrite ($ONOO\bullet$)) remaining after incubation with 0.6 mM of H_2 at 23 °C (details in the **Supplementary Methods**). Vitamin C (Vit. C) and superoxide dismutase (SOD) were used as positive controls. Signals generated in the absence of H_2 (CTL) were set at 100%. Data represent mean \pm s.d. ($n = 6$). * $P < 0.05$, *** $P < 0.001$. NBT-diformazan: oxidized form of nitroblue tetrazolium (NBT, a detector of $O_2\bullet^-$). DAF-2: diaminofluorescein-2 (a detector of $NO\bullet$).

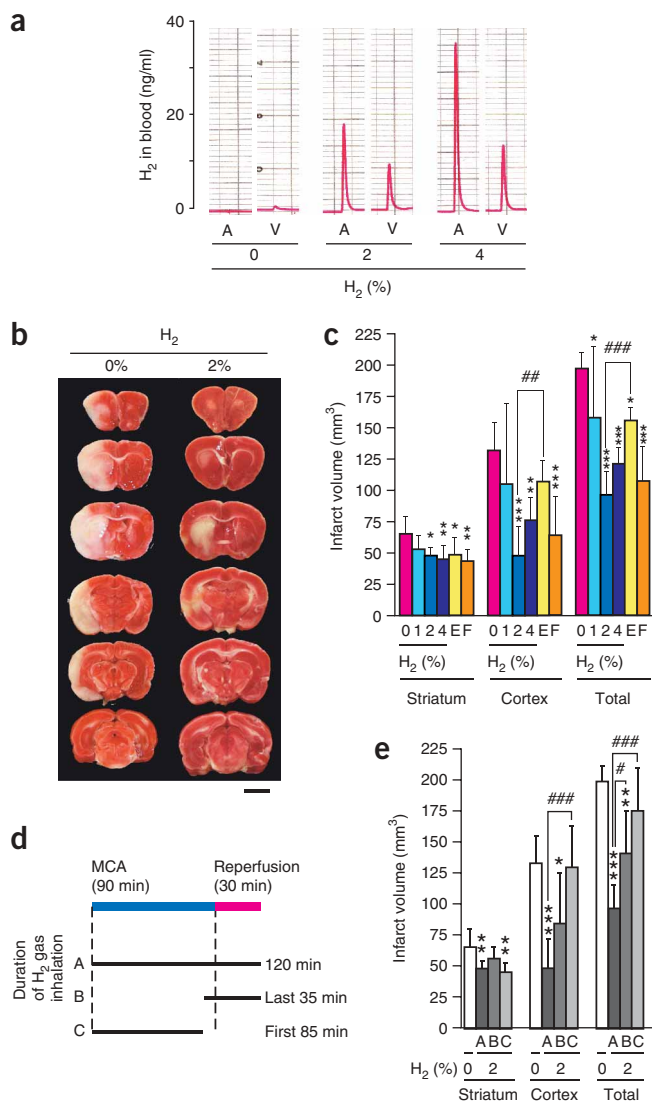


Figure 5 Inhalation of hydrogen gas protects against ischemia-reperfusion injury. **(a)** Rats inhaled H_2 and 30% O_2 for 1 h under the anesthetics N_2O and halothane. Arterial (A) and venous (V) blood were collected, and the amount of H_2 was examined by gas chromatography. **(b)** Rats underwent middle cerebral artery (MCA) occlusion. During the 120-min procedure, the indicated concentration of mixed gas was inhaled. One day after MCA occlusion, the forebrain was sliced into six coronal sequential sections and stained with the mitochondrial respiratory substrate TTC. Scale bar, 5 mm. **(c)** Infarct volumes of the brain were calculated in the brain slices. E and F, treatment with edaravone and FK506 ($n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with 0% of H_2 . $##P < 0.01$, $###P < 0.001$ compared with 2% of H_2 . **(d)** Schematic of experiment with three different durations of hydrogen gas (2%) inhalation. **(e)** Infarct volumes of the brain for different durations of inhalation (calculated as in **c**) ($n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with 0% of H_2 . $\#P < 0.05$, $###P < 0.001$ compared with 120 min of treatment. A, B and C represent the different durations of H_2 gas inhalation (shown in **d**). Histograms represent mean \pm s.d.

DISCUSSION

This study shows that molecular hydrogen can selectively reduce ROS *in vitro*. As $\bullet OH$ and $ONOO^-$ are much more reactive than other ROS (ref. 14), it stands to reason that H_2 will react with only the strongest oxidants. This is advantageous for medical procedures, as it means that the use of H_2 should not have serious unwanted side effects. It is likely that H_2 is mild enough not to disturb metabolic oxidation-reduction reactions or to disrupt ROS involved in cell signaling—unlike some antioxidant supplements with strong reductive reactivity, which increase mortality, possibly by affecting essential defensive mechanisms²⁹.

H_2 has a number of advantages as a potential antioxidant: it effectively neutralizes $\bullet OH$ in living cells, and, unlike most known antioxidants, which are unable to successfully target organelles³⁰, it has favorable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus. Despite the moderate reduction activity of H_2 , its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. Its ability to protect nuclear DNA and mitochondria suggests that it could reduce the risk of life style-related diseases and cancer.

H_2 markedly decreased oxidative stress and suppressed brain injury caused by ischemia and reperfusion. Inhalation of H_2 gas was more efficacious than a treatment currently approved for cerebral infarction and, furthermore, mitigated hepatic injury caused by ischemia and reperfusion (K. Fukuda, S.A., M.I., Y. Yamamoto, I.O. and S.O., unpublished data). This finding indicates that the beneficial effects of H_2 are not specific to cerebral injury but can be used for injuries in other organs.

This study suggests that H_2 protects cells and tissues against strong oxidative stress by scavenging $\bullet OH$. However, it remains possible that H_2 also protects from stress by directly or indirectly reducing other strong oxidant species in living cells. For instance, H_2 may induce cytoprotective factors; however, we found no H_2 -induced change in the expression of several genes involved in cytoprotection or reduction (K.N., M.I., I.O. and S.O., unpublished data). Further studies will reveal the mechanisms by which H_2 protects cells and tissues against oxidative stress.

Acute oxidative stress may be caused by several factors, including inflammation, intense exercise, cardiac infarction, cessation of blood flow and organ transplantation. For treatment, H_2 dissolved in saline could easily be delivered intravascularly. For prevention, H_2 saturated in water could be administered. Inhalation of H_2 has already been used in the prevention of decompression sickness in divers and has shown a

occlusion (Fig. 6a,b). The behavior of each rat, graded according to a neurological score²⁷, revealed that the inhalation of H_2 during ischemia and reperfusion improved movement (Fig. 6c). Moreover, although body weight and body temperature of H_2 -untreated rats gradually declined, those in H_2 -treated rats eventually recovered (Fig. 6d,e). Thus H_2 suppressed not only the initial brain injury, but also its progressive damage.

We examined H_2 -mediated molecular changes at 12 h, 3 d or 7 d after occlusion, by staining brain sections with antibodies to 8-OH-G in order to assess the extent of nucleic acid oxidation (Supplementary Fig. 6 online), and with antibodies to HNE to assess lipid peroxidation (Supplementary Fig. 6). For both of these oxidative markers, staining was substantially reduced in H_2 -treated rats as compared to untreated rats. We also stained identical regions of the brain with antibodies to Iba1 (ref. 28) and antibodies to GFAP, which are specific to activated microglia and to astrocytes, respectively (Fig. 6f,g and Supplementary Fig. 6). We found a distinct H_2 -dependent decrease in the accumulation of microglia, indicative of inflammation and remodeling. Taken together, these results indicate that H_2 can markedly decrease oxidative stress and suppress brain injury.

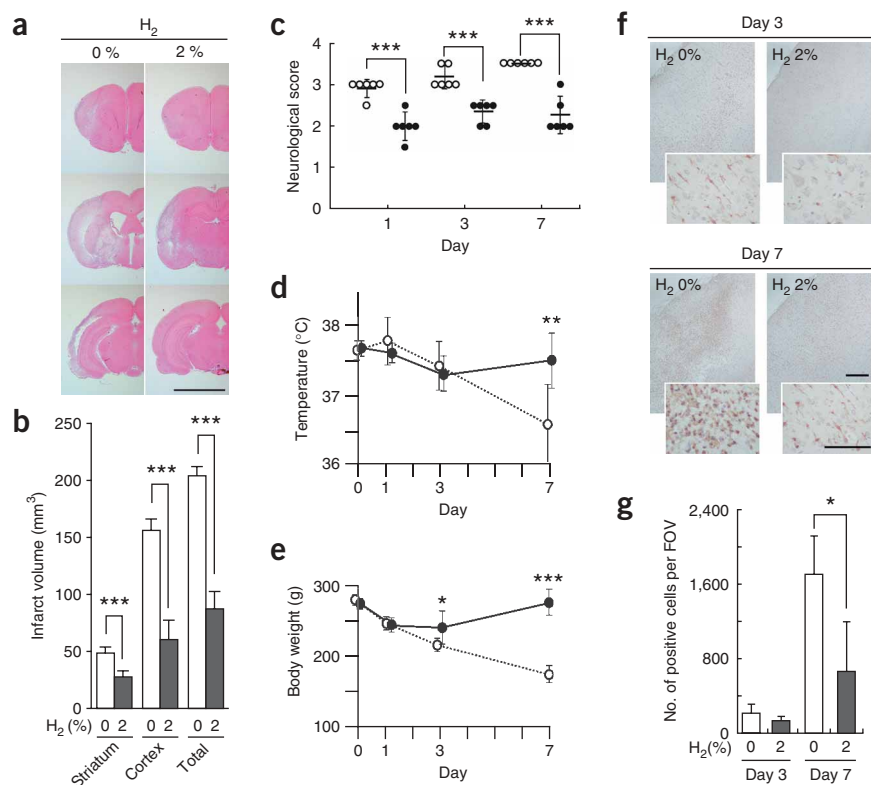


Figure 6 Inhalation of H_2 gas improved brain injury after 1 week. Rats inhaled 2% of hydrogen gas during the 120-min ischemia and reperfusion procedure and were maintained for 1, 3 or 7 d. **(a)** One week after MCA occlusion, the brains were sliced and stained with hematoxylin and eosin. Three representative slices are shown. Scale bar, 5 mm. **(b)** Infarct volumes (light-pink regions in **a**) were calculated ($n = 6$). $***P < 0.001$. **(c)** Neurological scores were graded on a scale of 0 to 5, as described previously²⁷; score 0, no neurological deficit; 1, failure to fully extend the right forepaw; 2, circling to the right; 3, falling to the right; 4, unable to walk spontaneously; and 5, dead. When a rat's neurological score was judged to be between 1 and 2, 2 and 3, or 3 and 4, the score was set at 1.5, 2.5 and 3.5, respectively. Closed and open circles represent treatment with or without H_2 ($n = 6$). $***P < 0.001$. **(d,e)** Body weights and temperature were monitored with (closed circles) or without (open circles) inhalation of 2% hydrogen gas ($n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **(f)** On days 3 or 7 after MCA occlusion, coronal 6- μ m sections from the ischemic core area in the temporal cortex were stained with antibody to Iba1 (a microglial marker). Scale bar, 200 μ m (100 μ m in the inset). **(g)** Cells positive for the Iba1 antibody²⁸, per field of view (FOV), were counted in the ischemic core area, as indicated in **f** ($n = 6$). $*P < 0.05$. Data represent mean \pm s.d.

good safety profile³¹. Notably, H_2 has no risk of flammability or explosion at a concentration of less than 4.7% in air. We propose that H_2 , one of the most well-known molecules, could be widely used in medical applications as a safe and effective antioxidant with minimal side effects.

METHODS

Hydrogen and oxygen measurements. We measured molecular hydrogen (H_2) and oxygen (dioxygen, O_2) dissolved in solution by using a hydrogen electrode (ABLE) and an oxygen electrode (Strathkelvin Instruments), respectively. We determined hydrogen gas concentration by gas chromatography (Teramecs). For measuring H_2 levels in blood, we pretreated rats with heparin to avoid blood clotting, collected arterial and venous blood (5 ml each) in test tubes, and then immediately injected the blood samples into closed aluminum bags containing 30 ml of air. After complete transfer of the H_2 gas from the blood to the air in the closed bag, we subjected 20 ml of the air to gas chromatography using standard H_2 gas, in order to quantify the amount of H_2 .

Hydrogen treatment of cultured cells. Over a 2-h period, we dissolved H_2 beyond saturation levels into DMEM medium under 0.4 MPa pressure. We dissolved O_2 into a second medium by bubbling O_2 gas at the saturated level (42.5 mg/l), and CO_2 into a third medium by bubbling CO_2 gas. All three media were maintained at atmospheric pressure. We then combined the three media (H_2 medium: O_2 medium: CO_2 medium) in the proportion 75%:20%:5% (vol/vol/vol) and added fetal bovine serum (FBS) to achieve a final concentration of 1%. For culture, we put the combined medium into a culture flask and immediately examined H_2 or O_2 concentration with an H_2 or O_2 electrode. Then we filled the culture flask with mixed gas consisting of 75% H_2 , 20% O_2 and 5% CO_2 (vol/vol/vol) and cultured cells in the closed culture flask. We prepared degassed medium lacking H_2 by stirring the medium, which had been saturated with H_2 , in an open vessel for 4 h, and checked the concentration of H_2 with a hydrogen electrode. In the experiments on the dose dependence of H_2 (results shown in Fig. 2f), we diluted the combined medium with a fourth medium containing 1% FBS equilibrated with air containing 5% CO_2 , in order

to obtain the desired concentration of H_2 ; we then filled the culture flasks with the mixed gas diluted with air containing 5% CO_2 .

Induction of oxidative stress by antimycin A and menadione. We maintained PC12 cells at 37 °C in DMEM medium containing 1% FBS with or without 0.6 mM H_2 in a closed flask filled with mixed gases as described above. We treated the cells with menadione or antimycin A, which inhibit complex I or complex III, respectively, of the mitochondrial electron transport chain, and thus produce $O_2^{\bullet-}$ (by accelerating the leakage of electrons). After exposure to antimycin A for 24 h, we assessed cell survival by manually counting the cells double-stained with 1 μ M propidium iodide (dead cells labeled pink) and 5 μ M Hoechst 33342 (dead and living cells labeled blue) under a fluorescent microscope. To examine the protective effect by H_2 on mitochondria, we pretreated cells with 4.5 g/l 2-deoxy-D-glucose (an inhibitor of glycolysis) and 1 mM pyruvate (a substrate of oxidative phosphorylation) for 30 min, exposed them to antimycin A with or without 0.6 mM H_2 and then quantified cellular ATP levels using a cellular ATP measurement kit (TOYO B-Net.).

Cerebral infarction model. Animal protocols were approved by the Animal Care and Use Committee of Nippon Medical School. We anesthetized male Sprague-Dawley rats (body weight: 250–300 g) with halothane (4% for induction, 1% for maintenance) in a mixture of nitrous oxide and oxygen (70%:30%, vol/vol). We maintained temperature (37.5 ± 0.5 °C) using a thermostatically controlled heating blanket connected to a thermometer probe in the rectum, and, at the same time, monitored physiological parameters (using a cannula in the tail artery), including blood gases (pCO_2 and pO_2), pH, glucose level and blood pressure. We attempted to maintain constant levels of pH and pO_2 by regulating the amount of halothane and the $N_2O:O_2$ ratio. We produced focal ischemia by performing intraluminal occlusion of the left middle cerebral artery (MCA), using a nylon monofilament with a rounded tip and a distal silicon rubber cylinder as previously described²³. The rats underwent MCA occlusion for 90 min and then reperfusion for 30 min; they inhaled H_2 gas during the entire process except in the experiments corresponding to Figure 5d,e. We treated rats with edaravone and FK506 using the most effective concentrations (refs. 25, 23 and Fig. 5c). After the rats recovered from anesthesia, they were maintained at 23 °C.

At 24 h after MCA occlusion, we removed brains under anesthesia and sliced them into six coronal sequential sections (2 mm thick). We stained the sections with 2,3,5-triphenyltetrazolium chloride (TTC) (3%), and then measured infarct and noninfarct areas using an optical disector image analysis system (Mac SCOPE, Mitsuya Shoji). We outlined the border between infarct and noninfarct tissues, and obtained the area of infarction by subtracting the nonlesioned area of the ipsilateral hemisphere from that of the contralateral side. We calculated the volume of infarction as infarct area \times thickness. At 12 h, 3 d or 7 d after MCA occlusion, we quickly removed brains under anesthesia, and fixed them with 10% formalin. We sliced paraffin-embedded brains into a series of 6- μ m sections, and stained sections with hematoxylin and eosin (H&E). We then quantified the pink areas with a graphic analyzer system (Mac Scope). For immunostaining, we stained the sections with antibodies by using VECTASTAIN ABC reagents according to the supplier's instructions.

Statistical analysis. We used StatView software (SAS Institute) for the statistical analyses. For single comparisons, we performed an unpaired two-tailed Student's *t*-test; for multiple comparisons, we used an analysis of variance (ANOVA) followed by Fisher's exact test. We performed experiments for quantification in a blinded fashion.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by grants to S.O. from the Ministry of Health, Labor and Welfare (H17-Chouju-009, longevity science; and 17A-10, nervous and mental disorders) and the Ministry of Education, Culture, Sports, Science and Technology (16390257).

AUTHOR CONTRIBUTIONS

S.O. conceived the experiments. S.O., I.O., K.K. and Y.K. designed the experiments. I.O., S.A. and S.O. performed data analysis. I.O., M.I., K.T., M.W., K.N., K.Y., S.A. and S.O. performed the experiments. S.O. and I.O. wrote the paper.

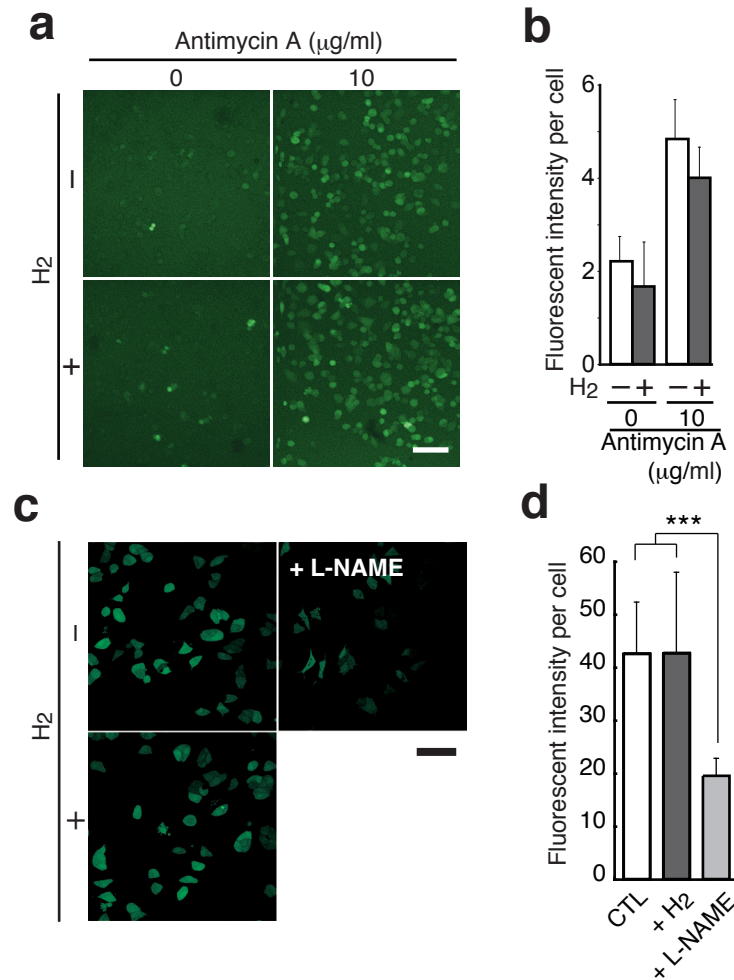
COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturemedicine>

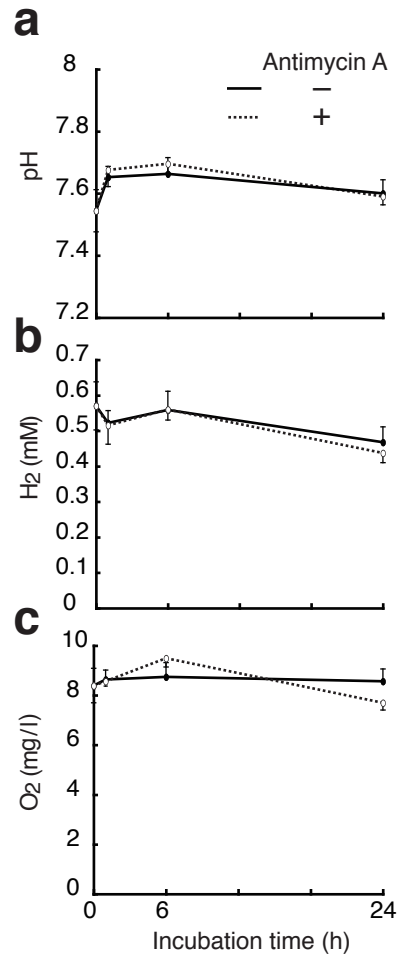
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

- Wallace, D.C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**, 359–407 (2005).
- Reddy, P.H. Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. *J. Neurochem.* **96**, 1–13 (2006).
- Ohta, S. A multi-functional organelle mitochondrion is involved in cell death, proliferation and disease. *Curr. Med. Chem.* **10**, 2485–2494 (2003).
- Wright, E., Jr., Scism-Bacon, J.L. & Glass, L.C. Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *Int. J. Clin. Pract.* **60**, 308–314 (2006).
- Winterbourn, C.C. Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid. *Toxicology* **181**, 223–227 (2002).
- Chinopoulos, C. & Adam-Vizi, V. Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. *FEBS J.* **273**, 433–450 (2006).
- Sauer, H., Wartenberg, M. & Hescheler, J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cell. Physiol. Biochem.* **11**, 173–186 (2001).
- Turrens, J.F. Mitochondrial formation of reactive oxygen species. *J. Physiol. (Lond.)* **552**, 335–344 (2003).
- Sheu, S.S., Nauduri, D. & Anders, M.W. Targeting antioxidants to mitochondria: a new therapeutic direction. *Biochim. Biophys. Acta* **1762**, 256–265 (2006).
- Liu, H., Colavitti, R., Rovira, I.I. & Finkel, T. Redox-dependent transcriptional regulation. *Circ. Res.* **97**, 967–974 (2005).
- Murad, F. Discovery of some of the biological effects of nitric oxide and its role in cell signaling. *Biosci. Rep.* **24**, 452–474 (2004).
- Buxton, G.V., Greenstock, C.L., Helman, W.P. & Ross, A.B. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($\bullet\text{OH}/\text{O}^\bullet$) in aqueous solution. *J. Phys. Chem. Ref. Data* **17**, 513–886 (1988).
- Ohsawa, I., Nishimaki, K., Yasuda, C., Kamino, K. & Ohta, S. Deficiency in a mitochondrial aldehyde dehydrogenase increases vulnerability to oxidative stress in PC12 cells. *J. Neurochem.* **84**, 1110–1117 (2003).
- Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H.J. & Nagano, T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J. Biol. Chem.* **278**, 3170–3175 (2003).
- Tomizawa, S. *et al.* The detection and quantification of highly reactive oxygen species using the novel HPF fluorescence probe in a rat model of focal cerebral ischemia. *Neurosci. Res.* **53**, 304–313 (2005).
- Kamiya, H. Mutagenicities of 8-hydroxyguanine and 2-hydroxyadenine produced by reactive oxygen species. *Biol. Pharm. Bull.* **27**, 475–479 (2004).
- Petersen, D.R. & Doorn, J.A. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic. Biol. Med.* **37**, 937–945 (2004).
- Falick, A.M., Mahan, B.H. & Myers, R.J. Paramagnetic resonance spectrum of the $^1\Delta_g$ oxygen molecule. *J. Chem. Phys.* **42**, 1837–1838 (1965).
- Asoh, S. *et al.* Protection against ischemic brain injury by protein therapeutics. *Proc. Natl. Acad. Sci. USA* **99**, 17107–17112 (2002).
- Halestrap, A.P. Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem. Soc. Trans.* **34**, 232–237 (2006).
- Lipton, P. Ischemic cell death in brain neurons. *Physiol. Rev.* **79**, 1431–1568 (1999).
- Ferrari, R. *et al.* Oxidative stress during myocardial ischemia and heart failure. *Curr. Pharm. Des.* **10**, 1699–1711 (2004).
- Nito, C., Kamiya, T., Ueda, M., Arai, T. & Katayama, Y. Mild hypothermia enhances the neuroprotective effects of FK506 and expands its therapeutic window following transient focal ischemia in rats. *Brain Res.* **1008**, 179–185 (2004).
- Takada, J. *et al.* Adenovirus-mediated gene transfer to ischemic brain is augmented in aged rats. *Exp. Gerontol.* **38**, 423–429 (2003).
- Zhang, N. *et al.* Edaravone reduces early accumulation of oxidative products and sequential inflammatory responses after transient focal ischemia in mice brain. *Stroke* **36**, 2220–2225 (2005).
- Labiche, L.A. & Grotta, J.C. Clinical trials for cytoprotection in stroke. *NeuroRx* **1**, 46–70 (2004).
- Murakami, K. *et al.* Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J. Neurosci.* **18**, 205–213 (1998).
- Ito, D. *et al.* Microglia-specific localisation of a novel calcium binding protein, Iba1. *Brain Res. Mol. Brain Res.* **57**, 1–9 (1998).
- Bjelakovic, G., Nikolova, D., Gluud, L.L., Simonetti, R.G. & Gluud, C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *J. Am. Med. Assoc.* **297**, 842–857 (2007).
- James, A.M., Cocheme, H.M. & Murphy, M.P. Mitochondria-targeted redox probes as tools in the study of oxidative damage and ageing. *Mech. Ageing Dev.* **126**, 982–986 (2005).
- Fontanari, P. *et al.* Changes in maximal performance of inspiratory and skeletal muscles during and after the 7.1-MPa Hydra 10 record human dive. *Eur. J. Appl. Physiol.* **81**, 325–328 (2000).



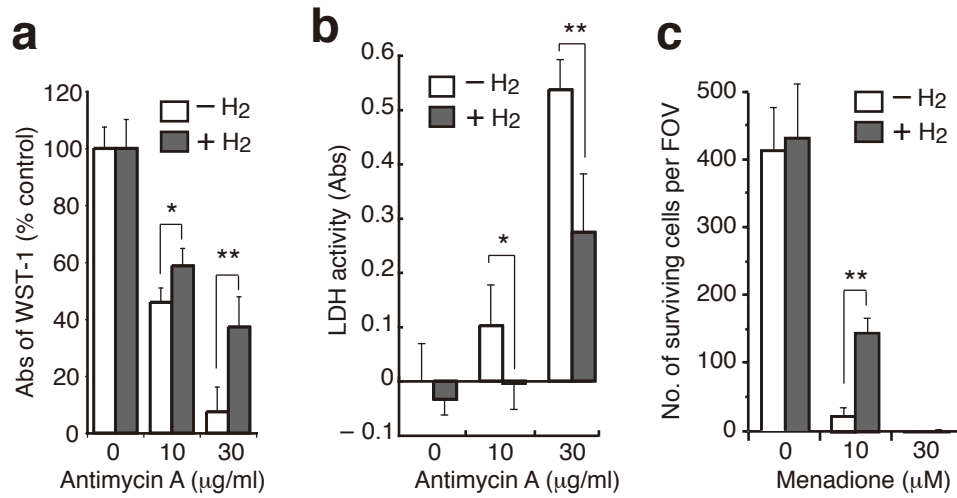
Supplementary Figure 1 Molecular hydrogen dissolved in culture medium does not reduce cellular hydrogen peroxide and nitric oxide.

(a) PC12 cells were held in medium with or without 0.6 mM H₂, and antimycin A (10 μg/ml) was added to the medium to induce O₂^{-•}, which was rapidly converted into H₂O₂. Representative laser-scanning confocal images of the fluorescence of H₂O₂ marker 2',7'-dichlorodihydrofluorescein (H₂DCF) were taken 1 h after the addition of antimycin A. Scale bar: 100 μm. (b) DCF fluorescence in cells treated with antimycin A in the presence or absence of 0.6 mM H₂ was quantified from 100 cells from each independent experiment using NIH Image software (mean ± SD, *n* = 4). (c, d) Cellular NO• was detected with a cellular NO•-specific fluorescent probe, DAF-2 DA (diaminofluorescein-2 diacetate, purchased from Daiichi Pure Chemicals Co.) by laser-scanning confocal microscopy using excitation and emission filters of 488 and 510 nm, respectively. As a negative control, an inhibitor of NOS (L-NAME: N^G-Nitro-L-arginine methyl ester, purchased from Sigma) was added so as not to generate NO•. Scale bar: 50 μm. (d) DAF-2 DA fluorescence was quantified as described in (b) (mean ± SD, *n* = 5). ****P* < 0.001.



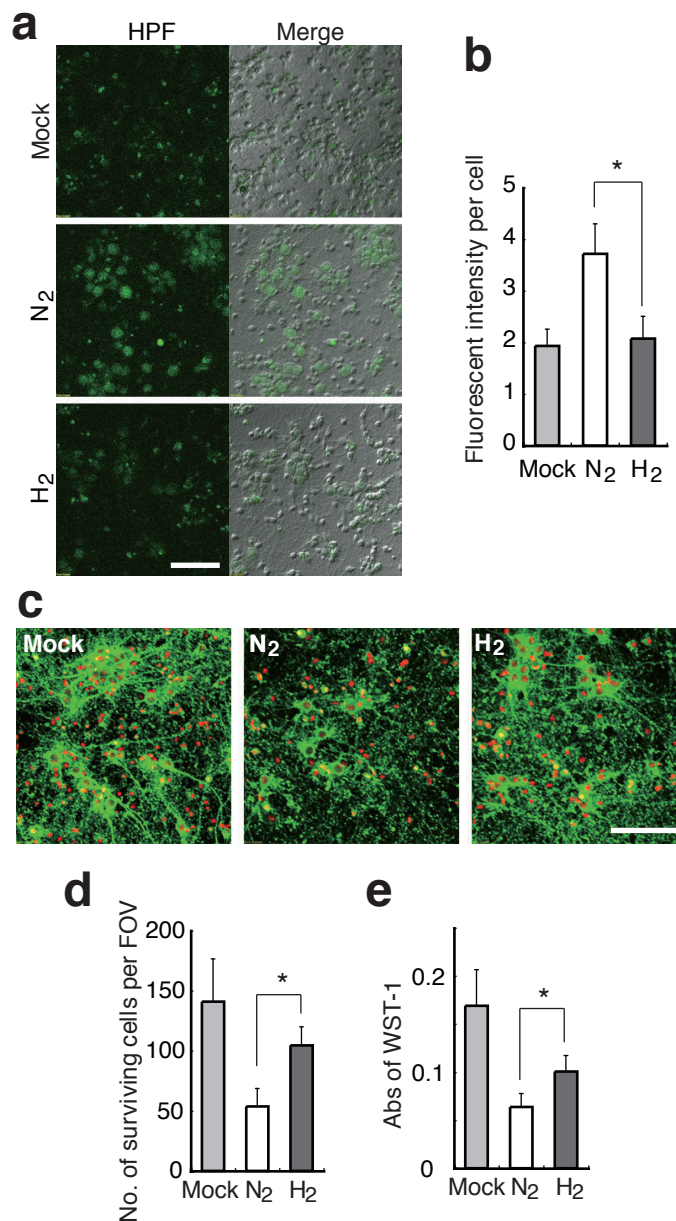
Supplementary Figure 2 pH, H₂ and O₂ maintain constant in culture medium in a closed flask filled with a mixed gas.

DMEM culture medium with dissolved H₂ and O₂ was prepared as described in **Methods**. PC12 cells (5×10^5) were cultured in medium with or without antimycin A (10 μ g/ml) in a closed culture flask (25 cm²) filled with a mixed gas composed of 75% of H₂, 20% of O₂ and 5% of CO₂. At the indicated time, pH, H₂ or O₂ in the medium was monitored with a pH meter, H₂ or O₂ electrode. One flask was used for one measurement. Data show the mean \pm SD ($n = 4$).



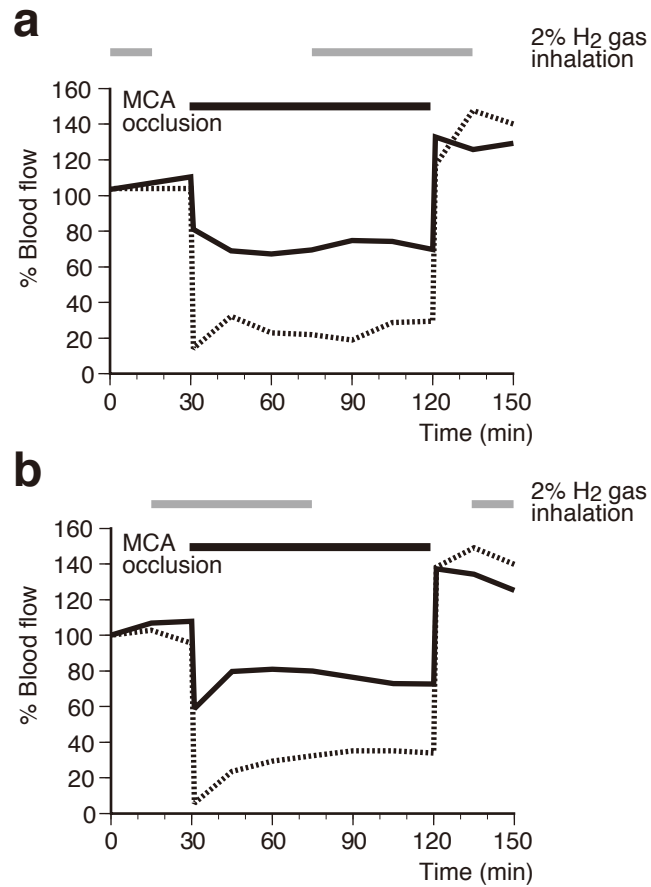
Supplementary Figure 3 Several methods confirm protection of cells by H₂ against oxidative stress.

PC12 cells incubated in the presence of or absence of 0.6 mM H₂ were treated with the indicated concentration of antimycin A (**a**, **b**) or menadione (**c**), and maintained with each H₂ concentration for 24 h as described in **Methods**. (**a**) As another method, a modified MTT assay (WST-1 assay) was applied to the cell system according to a Cell Counting Kit (purchased from Wako) to ensure the protective effect by H₂ against oxidative stress (mean ± SD, $n = 4$). * $P < 0.05$, ** $P < 0.01$. (**b**) Lactate dehydrogenase (LDH) activities were measured to estimate cellular LDH leakage from damaged cells according to an LDH-Cytotoxic Test kit (Wako). LDH activity in medium of antimycin A- and H₂-untreated cells was taken as the background (mean ± SD, $n = 4$). * $P < 0.05$, ** $P < 0.01$. (**c**) Instead of antimycin A, menadione was used to induce oxidative stress for 24 h and living cells were enumerated as described in **Fig. 2f** (mean ± SD, $n = 4$). ** $P < 0.01$.



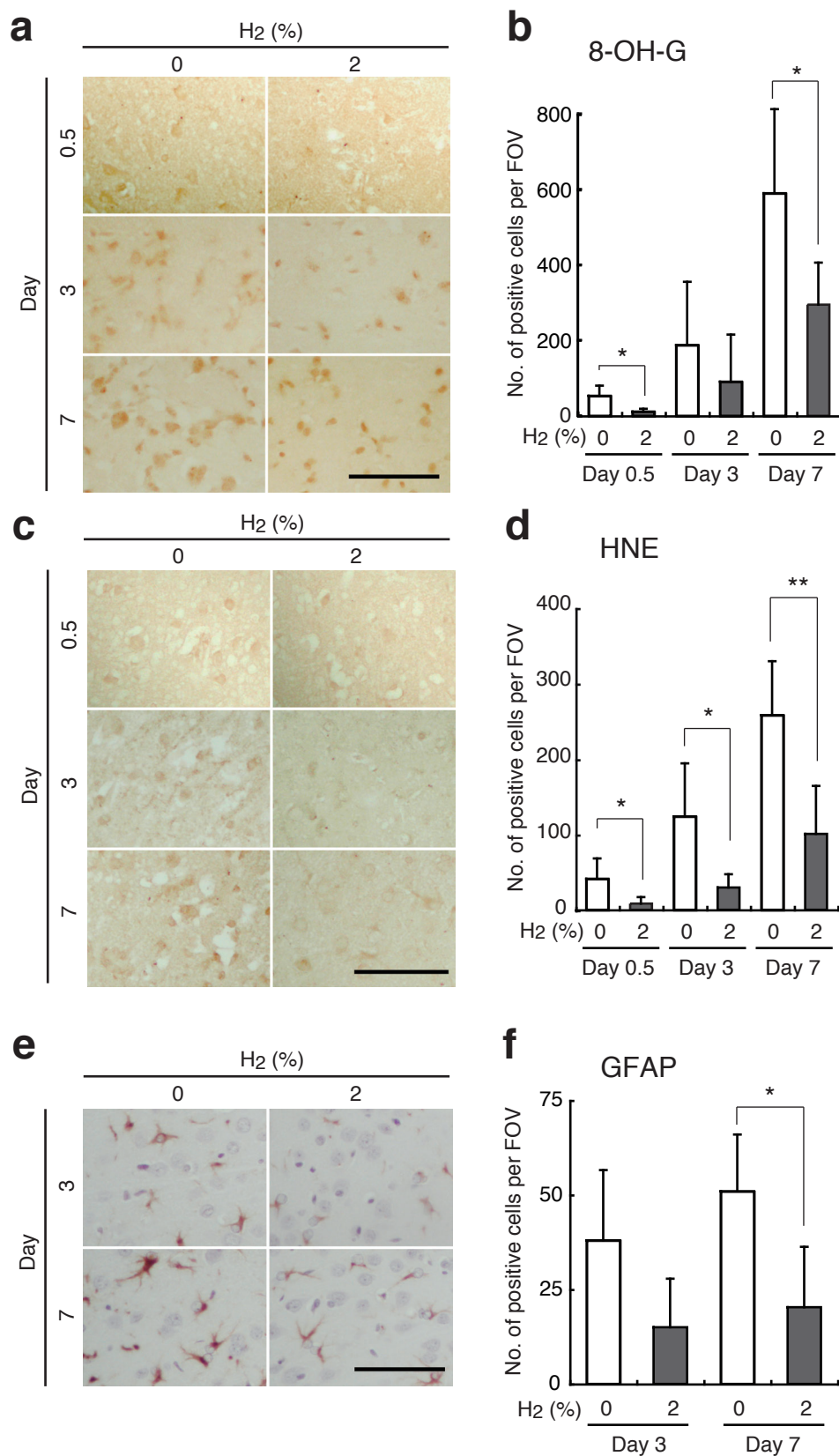
Supplementary Figure 4 Molecular hydrogen protects cultured neurons from ischemia and reperfusion *in vitro*.

A primary culture of rat neocortical cells was prepared and subjected to OGD (oxygen glucose deprivation) as described in **Supplementary methods**. (a) Ten min after reperfusion, cells were stained with HPF (left, fluorescent images; right, superimposition of the fluorescent HPF images with Nomarski differential interference contrast images). “Mock” indicates that cells were treated with DMEM medium containing glucose and oxygen instead of being subjected to OGD. Scale bar: 100 μ m. (b) The average fluorescent intensity of HPF was measured in 100 cells (mean \pm SD, $n = 4$). $*P < 0.05$. (c) Twenty hours after OGD, surviving neurons were fixed and immunostained with the neuron-specific antibody to TUJ-1 (green) and with PI (red). Scale bar: 100 μ m. (d) Dead cells were washed out in the staining procedure and living cells were enumerated under a fluorescent microscope in four fields of view (FOV) per well (mean \pm SD, $n = 4$). $*P < 0.05$. (e) Twenty hours after OGD, viability in a well was estimated by a modified MTT viability assay according to a Cell Counting Kit (WST-1 assay) (mean \pm SD, $n = 4$). $*P < 0.05$.



Supplementary Figure 5 Cerebral blood flow is not influenced by H₂ inhalation.

Middle cerebral artery occlusion was produced as described in **Methods**. Cerebral blood flow was measured by laser Doppler flowmetry using an ALF21 (ADVANCE Co.) at 2 mm lateral to the bregma for penumbra (solid line) and 5 mm lateral to the bregma for ischemic core (dotted line). Periods of 2% H₂ inhalation and middle cerebral artery (MCA) occlusion are shown by grey and solid thick lines, respectively.



Supplementary Figure 6 The brain after induction of ischemia reperfusion injury with or without H₂ treatment was immunostained.

Twelve h (0.5 d), 3 or 7 d after MCA occlusion, the brains were fixed and embedded in paraffin. Coronal 6- μ m-sections were stained with antibody to 8-OH-G in the ischemic penumbra area in the temporal cortex (**a**), with antibody to HNE in the ischemic penumbra area in the temporal cortex (**c**), and with antibody to GFAP in the ischemic penumbra area in the occipital cortex (**e**). Scale bar: 100 μ m. Positive cells with antibodies to 8-OH-G (**b**), HNE (**d**) and GFAP (**f**) per field of view (FOV) were counted in exactly the same regions in a blinded manner (mean \pm SD, $n = 6$). * $P < 0.05$, ** $P < 0.01$.

Supplementary Table 1

Physiological parameters during cerebral ischemia reperfusion

preischemia							ischemia						
0% H ₂							0% H ₂						
No.	temp. (°C)	pH	pCO ₂	pO ₂	glucose (mg/dl)	pressure (mmHg)	No.	temp. (°C)	pH	pCO ₂	pO ₂	glucose (mg/dl)	pressure (mmHg)
1	37.4	7.47	39	107	120	110	1	37.4	7.42	44	89	130	145
2	37.5	7.39	51	113	114	95	2	37.1	7.40	51	98	117	120
3	37.5	7.47	43	109	119	108	3	37.4	7.44	47	115	115	130
4	37.4	7.46	43	134	120	110	4	37.0	7.42	48	119	117	150
5	37.1	7.44	40	109	103	110	5	37.5	7.42	42	113	105	150
6	37.2	7.45	39	125	110	120	6	37.5	7.44	41	112	105	153
Average	37.4	7.45	43	116	114	109	Average	37.3	7.42	46	108	115	141
S.D.	0.2	0.03	5	11	7	8	S.D.	0.2	0.02	4	12	9	13
2% H ₂							2% H ₂						
1	37.1	7.45	46	130	109	105	1	37.3	7.41	48	111	120	120
2	37.4	7.44	50	118	104	87	2	37.6	7.43	43	99	97	135
3	37.7	7.40	46	105	114	103	3	37.8	7.42	45	104	100	150
4	36.9	7.45	47	121	107	100	4	37.0	7.39	52	97	105	150
5	37.5	7.46	41	120	109	100	5	37.3	7.41	45	109	107	145
6	37.0	7.46	45	114	107	115	6	37.5	7.42	47	108	113	160
Average	37.3	7.44	46	118	108	102	Average	37.4	7.41	47	105	107	143
S.D.	0.3	0.02	3	8	3	9	S.D.	0.3	0.01	3	6	8	14
4% H ₂							4% H ₂						
1	37.6	7.48	36	118	113	120	1	37.0	7.40	48	110	105	145
2	37.2	7.45	40	134	96	112	2	36.8	7.40	46	107	94	120
3	37.6	7.46	43	119	90	125	3	37.0	7.41	47	83	91	130
4	36.7	7.46	39	128	103	120	4	37.6	7.43	43	111	97	145
5	36.8	7.43	45	111	97	120	5	37.4	7.45	44	105	100	140
6	37.5	7.49	34	127	103	100	6	37.4	7.44	46	110	105	150
Average	37.2	7.46	40	123	100	116	Average	37.2	7.42	46	104	99	138
S.D.	0.4	0.02	4	8	8	9	S.D.	0.3	0.02	0	11	6	11

reperfusion for 15 min							reperfusion for 30 min						
0% H ₂							0% H ₂						
No.	temp. (°C)	pH	pCO ₂	pO ₂	glucose (mg/dl)	pressure (mmHg)	No.	temp. (°C)	pH	pCO ₂	pO ₂	glucose (mg/dl)	pressure (mmHg)
1	37.3	7.39	45	101	132	155	1	37.5	7.41	41	110	135	140
2	37.2	7.40	52	94	108	135	2	37.4	7.40	49	97	111	130
3	37.3	7.46	44	105	113	135	3	37.0	7.40	51	109	115	118
4	37.5	7.43	46	119	116	153	4	37.5	7.42	46	99	118	135
5	37.2	7.40	44	122	104	155	5	37.1	7.43	40	134	105	130
6	37.7	7.41	43	107	105	140	6	37.7	7.35	50	93	97	110
Average	37.4	7.42	46	108	113	146	Average	37.4	7.40	46	107	114	127
S.D.	0.2	0.03	3	11	10	10	S.D.	0.3	0.03	5	15	13	11
2% H ₂							2% H ₂						
1	37.5	7.42	42	107	120	120	1	37.4	7.39	45	116	115	100
2	37.5	7.41	45	98	100	95	2	37.4	7.43	42	97	103	90
3	37.2	7.40	46	109	111	150	3	37.0	7.38	48	117	112	150
4	37.4	7.39	49	100	110	108	4	37.3	7.36	53	109	110	110
5	37.3	7.40	45	108	107	130	5	37.5	7.37	46	119	107	95
6	37.1	7.39	49	113	105	130	6	37.2	7.38	51	115	109	125
Average	37.3	7.40	46	106	109	122	Average	37.3	7.39	48	112	109	112
S.D.	0.2	0.01	3	6	7	19	S.D.	0.18	0.02	4	8	4	23
4% H ₂							4% H ₂						
1	37.4	7.39	49	103	111	140	1	37.1	7.43	37	142	107	125
2	37.3	7.36	49	93	96	120	2	37.4	7.29	41	133	96	112
3	37.4	7.39	46	90	92	135	3	37.5	7.39	47	93	90	135
4	37.4	7.41	45	113	96	145	4	37.4	7.39	45	134	100	130
5	37.1	7.43	45	107	98	140	5	37.1	7.40	44	138	100	125
6	37.3	7.42	44	120	97	150	6	37.1	7.40	47	143	94	140
Average	37.3	7.40	46	104	98	138	Average	37.3	7.38	44	131	98	128
S.D.	0.12	0.03	2	12	7	10	S.D.	0.19	0.05	4	19	6	10

Supplementary methods

Detection of ROS by fluorescent indicators. We treated PC12 cells with 10 μ M of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) (purchased from Molecular Probes), 5 μ M diaminofluorescein-2 diacetate (DAF-2 DA) (purchased from Daiichi Pure Chemicals Co.), or 5 μ M of 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl]benzoate (HPF) (Daiichi Pure Chemicals Co.) for 30 min to detect cellular H₂O₂, NO• or •OH, respectively. We took fluorescent images with a laser-scanning confocal microscope (Olympus FV300) using excitation and emission filters of 488 nm and 510 nm, respectively. HPF can be specifically oxidized by •OH, peroxynitrite (ONOO⁻) and lipid peroxides, but neither H₂O₂, NO• nor O₂⁻• (ref. 14). For the detection of cellular O₂⁻•, we used 0.5 μ M MitoSOX (purchased from Molecular Probes), and took images using excitation and emission filters of 543 nm and 565 nm, respectively. Fluorescent signals were quantified from 100 cells of each experiment using US National Institutes of Health Image software.

Staining of mitochondria. For staining of mitochondria, we co-stained with MitoTracker Green (MTGreen) (1 μ M; Molecular Probes) and tetramethylrhodamine methyl ester (TMRM) (100 nM; Molecular Probes). Fluorescence from MTGreen is independent of the membrane potential, whereas that from TMRM is sensitive to the membrane potential. MTGreen and TMRM were detected using excitation at 488 and 543 nm, and emission filters of 510 and 565 nm, respectively.

Immunostaining. We purchased antibodies against HNE and 8-OH-G from Nikken Seil Co, and antibodies against TUJ-1 and GFAP from COVANCE and ThermoImmunon, respectively. We used BODIPY FL goat anti-mouse IgG (Molecular Probe) as a secondary antibody and visualized signals with a laser-scanning confocal microscope. Fluorescence signals in response to 8-OH-G and HNE were quantified with NIH Image software.

Intracellular Fenton reaction. We preincubated PC12 cells with 1 mM CuSO₄ for 30 min in medium containing 1% FCS, washed once with phosphate-buffered saline (PBS) containing CaCl₂ (0.1 g/l), MgCl₂.6H₂O (0.1 g/l), glucose (1g/l) and sodium pyruvate

(0.036 g/l) (pH 7.2), and then exposed to the indicated concentration of ascorbate (vitamin C) for 1 h in phosphate-buffered saline as described above. As negative controls, CuSO₄ or ascorbate was omitted. Note that Cu⁺² is reduced by ascorbate to Cu⁺, which catalyzes the Fenton reaction to produce •OH from H₂O₂ that is being spontaneously produced in the cells.

Electron spin resonance measurement. We used 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a free radical trapper, and detected electron spin resonance (ESR) signals with a KEYCOM ESR spectrometer type ESR-X01. As a standard of the reactant of •OH with DMPO, we produced •OH by the Fenton reaction in the mixture of 0.1 mM H₂O₂ and 1 mM FeCl₂ in the presence of 0.1 mM DMPO and subjected the whole solution to ESR measurement. For the measurements, we normalized the sensitivity of each experiment with the strength of the internal ESR signal derived from Mn²⁺. To obtain a spectrum, ESR was scanned for 2 min, accumulated 10 times, and all signals were averaged.

For H₂ treatment, we prepared media containing 0.6 mM H₂ and 8.5 mg/l O₂, and filled a closed culture flask with 75% H₂, 20% O₂ and 5% CO₂ gases. We pretreated PC12 cells (2 × 10⁶ cells in a 25 cm² flask) with 0.1 M DMPO and 2 mM CuSO₄ in DMEM containing 1% FCS for 30 min at 37 °C in the presence or absence of 0.6 mM H₂. After the removal of this medium, we exposed the cells to 0.2 mM ascorbate and 0.1 mM H₂O₂ in 0.3 ml of PBS in the presence or absence of 0.6 mM H₂ for 5 min at room temperature to produce •OH by the Fenton reaction, and scraped the cells into a flat cuvette for ESR measurement. In the other method, we preincubated PC12 cells (2 × 10⁶ cells in a 25 cm² flask) in 0.3 ml of PBS containing 0.1 M DMPO and 30 µg/ml antimycin A for 7 min at room temperature in the presence or absence of 0.6 mM H₂, and then scraped the cells into a flat cuvette for ESR measurement. A differential spectrum was obtained by digitally subtracting one spectrum from the other to visualize the signals decreased by H₂ treatment.

Primary culture. We prepared primary cultures of neocortical neurons from 16-day rat embryos by the method described previously¹⁹. In brief, neocortical tissues were cleaned of meninges, minced, and treated with a protease cocktail (SUMILON). After mechanical dissociation by pipetting, we resuspended cells in nerve-cell culture medium

(SUMILON), and then plated onto poly-L-lysine-coated plates at a density of 5×10^4 cells / cm², changed to Neurobasal Medium (Invitrogen) with B-27 (Invitrogen) once every three days and then used neurons at day 11. One day before OGD, we changed the medium to Neurobasal Medium with B-27 minus AO (Invitrogen), and confirmed neuronal identity by immunostaining with antibodies to neuron marker TUJ-1, and astrocyte marker GFAP. We used preparations only containing over 90% neurons for experiments.

Oxygen-glucose deprivation. To initiate OGD, we replaced the culture medium with a glucose-deficient DMEM from which O₂ had been removed by bubbling in a mixed gas of either N₂ (95%):CO₂ (5%) or H₂ (95%):CO₂ (5%) and maintained the culture for 60 min at 30 °C under an atmosphere of either N₂ (95%):CO₂ (5%) or H₂ (95%):CO₂ (5%). Treatment was terminated by exchanging the experimental medium with stocked culture medium and further incubation at 37 °C with air including 5% CO₂.

Reaction of H₂ in cell-free systems. We performed fluorescence spectroscopic studies with a Shimadzu RF-5300PC. For solution studies, we dissolved H₂ in water beyond the saturated level under 0.4 MPa of hydrogen pressure for 2 h and then used it under atmospheric pressure. We determined H₂ concentrations with a hydrogen electrode in each experiment.

To detect the reaction of H₂ with the oxidized form of cytochrome *c*, FAD, or NAD⁺, we incubated solutions containing 10 μM cytochrome *c*, 1 mM FAD or 1mM NAD⁺ with or without 0.8 mM H₂ in a closed cuvette at 23 °C for 30 min, and observed no reaction by absorbance at 415, 400 and 340 nm, respectively.

We monitored the reactivity of H₂ with various ROS by HPF, DAF-2, or nitroblue tetrazolium (NBT). We measured fluorescent signals of HPF and DAF-2 at 515 nm with excitation at 490 and 495 nm, respectively, and the reduction of NBT to NBT-diformazan by absorbance at 550 nm.

To detect the reaction of H₂ with •OH, we mixed hydrogen solution, phosphate buffer (10 mM at pH 7.4), ferrous perchlorate (0.1 mM), and HPF (0.4 μM). We initiated the Fenton reaction by adding H₂O₂ to 5 μM in a closed cuvette at 23 °C with gentle stirring and monitored fluorescence for 30 s.

To detect the reaction of H₂ with O₂⁻•, we mixed solutions containing xanthine and

NBT (supplied by TREVIGEN) with or without 0.8 mM H₂ in a closed cuvette, initiated the reaction by adding xanthine oxidase at 23 °C and monitored for 5 min.

To detect the reaction of H₂ with H₂O₂, we incubated solutions including phosphate buffer (10 mM at pH 7.4) and H₂O₂ (10 μM) with or without H₂ (0.8 mM) in a closed glass tube at 23 °C for 30 min. We converted the remaining H₂O₂ to •OH by 0.2 μM horseradish peroxidase and then incubated with 10 μM HPF for 5 min.

To detect the reaction of H₂ with NO•, we incubated solutions containing phosphate buffer (10 mM at pH 7.4) and 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7, 0.1 μM, purchased from Dojin Chemicals Co.) with or without 0.8 mM H₂ in a closed cuvette at 23 °C for 30 min, and monitored the remaining NO• by incubation with 5 μM DAF-2 for 10 min.

To detect the reaction of H₂ with peroxynitrite (ONOO⁻), we diluted a stock solution of 1 μM ONOO⁻ in alkali 200-fold into 10 mM phosphate buffer with 0.4 μM HPF in the presence or absence of 0.8 mM H₂, and then examined HPF signals after 23 °C for 1 min.