別紙１

厚生労働科学研究費補助金

長寿科学総合研究事業

トランスジェニックマウスを用いた ミトコンドリア酸化ストレスの抑制による アルツハイマー病予防・治療法の開発 に関する研究

平成１７年度～１８年度　総合研究報告書

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別紙３

厚生労働科学研究費補助金（長寿総合研究事業）
（総合）研究報告書

「トランスジェニックマウスを用いたミトコンドリア酸化ストレスの抑制による アルツハイマー病予防・治療法の開発」に関する研究

主任研究者　太田成男（日本医科大学大学院医学研究科　教授）

研究要旨：ミトコンドリア　アルデヒド脱水素酵素２（ALDH2）酵素欠損マウス(DALマウス)を作製した。DALマウスは、酸化ストレスに対し脆弱で、加齢にともなって、タウ蛋白質のリン酸化が顕著で神経変性と空間認知・学習記憶能力が低下した。さらに、アルツハイマー病と動脈硬化の危険因子であるAPOE欠損とALDH2欠損が脳の機能障害進行に相乗効果を示した。以上の結果、DALマウスは、酸化ストレスを軽減させ加齢に伴う認知症を予防する還元剤の評価に用いることが有効であることが示唆された。この酸化ストレスを軽減させるための還元剤を探索した。水素分子は有害な活性酸素を選択的に消去し、細胞を酸化ストレスから保護することを見出した。水素分子は特に急激な酸化ストレスに対して、培養細胞と実験動物に対し、極めて有効な保護作用を示した。水素分子には、副作用がないので長期服用によってアルツハイマー病を予防する可能性が示された。

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Ａ．研究目的

アルツハイマー病患者脳では、酸化ストレスが亢進されていること、ミトコンドリアから発せられる活性酸素が酸化ストレスの主な発生源となっていることは、周知の事実となっている。ミトコンドリアとアルツハイマー病の関連について、多くの研究者から関心がもたれているのは、本年のJournal of Alzhemer's Diseaseにミトコンドリア特集が組まれたことなどからも明らかである。ミトコンドリアとアルツハイマー病の関連が今まで以上に注目されるようになったのは、アルツハイマー病の直接的原因とされるアミロイドβペプチド（Aβ）がミトコンドリア内にも存在し、Aβがミトコンドリアから発せされる活性酸素の原因となっているからという報告があいついだからである。ミトコンドリア内に存在するAβ結合性アルコール脱水素酵素（ABAD）がAβの結合と活性酸素の発生を担うとされた。

本研究の目的は、脳の酸化ストレスを軽減し、神経を保護することによって、アルツハイマー病の発症を遅延させること、すなわち予防すること、あるいは治療できるようにすることである。そのために、(A)アルツハイマー病を予防するに足る理想的な抗酸化物質を見いだすこと、(B)その抗酸化物質を評価できるモデル動物を作製することである。そのために、加齢に伴い認知障害を示すモデル動物を作製する。そして、(C)抗酸化剤による還元による効果を評価する。同時に、(D)酸化ストレスとAβの関連をより明確にすることが、この２年間の本研究の具体的目的である。

酸化ストレスは活性酸素の発生により亢進されるが、アルツハイマー病を予防するために活性酸素を還元して消去するには以下の条件が必要である。

①脳血液関門を通過できることが必要である。既知の多くの抗酸化物質は脳血液関門を通過できない。

②ミトコンドリアなどの活性酸素を発生させる場所に到達できることが必要である。抗酸化物質が目的の場所へ到達できなければ、効果的にはならない。

③活性酸素の中でも、superoxide(O_2^-・)、過酸化水素(H_2O_2)、一酸化窒素(NO・)は、生体内で重要な役割を果たす。特に、神経では、NOは神経伝達物質であり、血管拡張の働きをするので、抗酸化物質によって消去されすぎてはならない。ヒドロキシルラジカル(・OH)とペルオキシナイトライト($ONOO^-$)が細胞障害生を示す有害物質であるので、この有害な活性酸素のみを消去することが重要である。
④・OHを効果的に消去して、脳と細胞を実際の保護でいること。
⑤Fe^{2+}とCu^+は、H_2O_2に触媒として作用し、有害な・OHを生成する。Fe^{3+}やCu^{2+}を還元するとFe^{2+}とCu^+になる。アミロイドが沈着した老人斑などでは、鉄を含むヘムが結合していることが知られているので、アルツハイマー病では、Fe^{3+}とCu^{2+}を還元しない抗酸化物質がアルツハイマー病予防には必要である。

以上の条件を満たす抗酸化物質として、水素(H_2)が以上の条件をすべて満たす抗酸化物質であることを証明した。

B．研究方法

(A) 水素（H2）がアルツハイマー病を予防できるに足る抗酸化物質であることの証明の実験法。
①試験管内で各々の種類の活性酸素を発生させ、水溶液に溶かし込んだ水素と反応させ、活性酸素の濃度を測定した。
②培養細胞PC12にミトコンドリアの呼吸鎖阻害剤を加え、活性酸素を発生させ、蛍光色素で検出した。水素がどの活性酸素を消去するかを明らかにした。
③培養細胞をCu^{2+}とビタミンCで処理し、Cu+に還元して、OHを発生させ、H2がその細胞毒性を軽減するかどうかを調べた。
④初代培養細胞に虚血再灌流状態にし、活性酸素を発生させ、H2が神経細胞死を軽減するかどうかを調べた。
⑤ラット脳の脳中大動脈を梗塞し、脳梗塞に陥らせ、水素ガスを吸引させることによって、脳梗塞障害を軽減するかどうかを調べた。

(B)酸化ストレス亢進マウスの作製とその評価
(1) ALDH2はミトコンドリア内に存在する酵素であり、酸化ストレス防御機構として働いていることを私たちは以前示した。ラットALDH2遺伝子の一塩基置換により活性が消失するALDH2*2遺伝子を作製し、マウスの受精卵に導入することにより、ALDH2酵素活性欠損マウスを作製した。不活型ALDH2*2は活性型ALDH2*1と結合し、不活化するので、不活型遺伝子を導入することによって、不活型マウスを作製した。筋肉のALDH2の活性を失った系統（DAL02）と脳のALDH2の活性を失った系統(DAL110)を作製した。DALはdominant negative ALDH2の意である。
(2) 酸化ストレスの指標として、４-ヒドロキシ-２-ノネナール(4-HNE)を測定した。4-HNEは過酸化脂質より生成される有毒な物質でアルデヒド基をもち、ALDH2が分解に寄与していることを私たちが以前明らかにしている。そのため、DALマウス（ALDH2不活型マウス）では4-HNEが蓄積し、酸化ストレスの指標を明確にできる。
(3) DAL02マウスでは筋肉、DAL101では脳の組化学的標本を作製し、加齢に伴う変化を評価した。アルツハイマー病の指標であるタウのリン酸化を調べた。
(4) DAL101マウスとapoE(-/-)マウスを掛け合わせ、ALDH2酵素欠損とapoEが同時に欠損したDAL(+/+)apoE(-/-)を作製した。
(5) DAL101とDAL(+/+)apoE(-/-)を水迷路実験により、空間認知機能を評価した。
(6) さらに、WaterMaizeにより認知機能を評価した。

(C) 水素によるDALマウス等の酸化ストレス軽減による評価
①DAL02に水素を溶かし込んだ水（以下水素水）を飲用させた。
②動脈硬化モデルとなるapoE(-/-)マウスに水素水を飲ませ、大動脈をオイルレッド染色により、動脈硬化軽減への効果を評価した。
③DAL(+/+)apoE(-/-)に水素ガス（2%）を吸引させ、水迷路実験による空間認知機能が改善するかどうかを調べた。
④apoE(-/-)マウスに水素ガスを吸わせる、あるいは水素水を飲ませてその表現型を調べた。

(D) 酸化ストレスとアミロイドβペプチド(Aβ)の関連の研究方法
①Aβ結合アルコール脱水素酵素(ABAD)を培養細胞に導入し、ABADがミトコンドリアに局在

していることを確認した。
②ABADによって4-HNEへの耐性が上昇すること、ABADによって4-HNEが分解されることを確認した。
③ABADの4-HNE分解能がAβによって阻害することを調べた。

CとD．研究成果と考察
(A)水素の効果
①水素は、試験管内実験においても、培養細胞中においても、水素は•OHと$ONOO^-$のみを選択的に還元消去した。O2-•、H_2O_2、NO•を、水素は還元しなかったので、水素は有害な活性酸素のみを消去し、生体にとって必要な活性酸素は消去しないことが明らかとなった。
②水素は、活性酸素をミトコンドリアから発生させたとき、ミトコンドリアの膜電位を低下させず、ATP合成能も低下させなかった。また、核で生じた•OHを消去し、核酸の酸化を低下させた。したがって、水素は、細胞の至る所に拡散し、•OHを消去して、細胞を護ることが示された。
③Fe3+やCu2+をはじめとする生体内で酸化還元反応を司る酸化物を還元せず、生体内反応を撹乱せず、•OH産生を引き起こすCu^+やFe^{2+}を生成しないことを明らかにした。
H_2O_2から発生させた時、
④脳虚血モデルラットの脳傷害を劇的に軽減した。脳梗塞巣を小さくし、予後の運動機能、体温、体重の改善が見られた。
以上の結果より、水素は脳神経を護り、副作用のない理想的な抗酸化物質でることが示唆された。
なお、この研究結果は、Nature Medicineno 2007年5月号に掲載される。

(B)モデル動物
① DAL101マウスでは脳に、DAL02マウスでは筋肉に、加齢依存的に酸化ストレスマーカーの4-HNEが増加した。DAL02マウスでは加齢に伴い骨格筋にミトコンドリアの蓄積と異常が観察された。これは、軽度認知機能障害(MCI)に見られる現象である。
②DAL101の初代神経培養細胞は、4-HNEへの耐性が低下していた。
③DAL101では、加齢に伴って、海馬の神経変性とグリオーシスが見られた。
④DAL101では、Tau蛋白質のリン酸化が見られた。
⑤DAL101では、水迷路試験で加齢に伴う空間認知機能が明らかに低下した。
⑥以上の現象は、apoE(-/-)と掛け会わせることによって、表現型が生じるまでの時間が短縮された。
以上の結果は、ALDH2欠損により酸化ストレスが亢進し、加齢に伴う認知機能が低下したことを示す。さらにTau蛋白質のリン酸化が促進されたのでアルツハイマー病に類似の現象が生じていた。

(C) 水素によるDALマウス等の酸化ストレス軽減による評価
①DAL02に水素水を飲ませると血液中、筋肉中の4-HNEが減少し、酸化ストレスが軽減していることが示された。
②apoE(-/-)に水素水を飲ませると、動脈硬化が抑制された。
③水素の投与による認知機能の改善については、実験進行中である。

(D) (D) 酸化ストレスとアミロイドβペプチド(Aβ)の関連の研究方法
①ABADはミトコンドリアに存在し、4-HNEを解毒する作用があり、Aβによってその解毒作用が阻害される。結果的にAβによって、酸化ストレスが亢進することになることを明らかにした。

E．結論
水素のOHラジカル消去能は顕著であり、かつ目的の項で示した
酸化ストレスの最終産物である4-HNEを蓄積させるマウスを確立し、加齢に伴う認知症モデル動物を作製した、同時にタウ蛋白のリン酸化、

なお、水素の選択的還元作用とその臨床への可能性についての論文は、Nature Medicineの2007年5月号に掲載される。また、ABADに関する論文は投稿中である。ALDH2欠損マウスに関しては、J. Alzheimer's Diseaseから総説の執筆を依頼されすでに掲載された。詳しい論文は、投稿準備中である。

F．健康危険情報
本研究において、健康危険情報はない。

G．研究発表

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H．知的財産の出願・登録状況

１．特許出願

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別紙４

研究成果の刊行に関する一覧表

雑誌

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研究成果の刊行物・別刷

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Hydrogen acts as a therapeutic antioxidant by the selective reduction of cytotoxic oxygen radicals

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Acute oxidative stress induced by ischemia-reperfusion or inflammation causes serious damage to tissues, while perpetual oxidative stress is accepted as one of the causes of many common diseases including cancer. We show here that hydrogen (H_2) has great potential as an antioxidant in preventive and therapeutic applications. Acute oxidative stress was induced in cultured cells by three independent methods. H_2 selectively reduced the hydroxyl radical, which is the most cytotoxic reactive oxygen species (ROS), and effectively protected cells, but did not react the other ROSs that possess physiological roles. As an acute animal model, oxidative stress damage was induced in the rat 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 could be used as effective antioxidant therapy as it protects against oxidative damage and easily penetrates tissue and cellular structures by gaseous rapid diffusion.

Oxidative stress arises from the strong cellular oxidizing potential of excess reactive oxygen species (ROS) or free radicals[1–5]. The majority of superoxide anion radical (O_2^-•) is generated in mitochondria by electron leakage from the electron transport chain and via the Krebs cycle[6]. O_2^-• is also produced by metabolic oxidases, including NADPH oxidase and xanthine oxidase[7]. O_2^-• may be converted by superoxide dismutase into hydrogen peroxide (H_2O_2)[8], which is detoxified into H_2O by either glutathione peroxidase or catalase. Excess O_2^-• may reduce transition metal ions such as iron and copper[2], which in turn react with H_2O_2 to produce hydroxyl radicals (•OH) by the Fenton reaction. •OH is the strongest of the oxidant species and indiscriminately reacts with nucleic acids, lipids, and proteins. There is no known detoxification system for •OH; therefore, scavenging •OH is a critical antioxidant process[9].

Despite their cytotoxic effects, O_2^-• and H_2O_2 at low concentrations play important physiological roles; 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 to defend against bacterial invasion[5]. Additionally, nitric oxide (NO•) functions as a neurotransmitter and is essential for the dilation of blood vessels[11]. Thus, cytotoxic radicals such as •OH must be neutralized without compromising the essential biological activities of other ROSs including NO•. Here, we demonstrate that molecular hydrogen (dihydrogen: H_2) can alleviate •OH-induced cytotoxicity without affecting the other ROSs and propose that H_2 has a great deal of potential as an antioxidant for preventive and therapeutic applications.

RESULTS

Molecular hydrogen selectively reduces hydroxyl radicals in cultured cells

H_2 reduces •OH that is produced by radiolysis or photolysis of water[12]; however, it has not been directly examine whether H_2 can effectively neutralize •OH in living cells. Since spontaneous generation of ROS would not produce sufficient quantities of cellular damage to be detectable, we induced O_2^-• in PC12 cultured cells by treatment with a mitochondrial respiratory complex III inhibitor, antimycin A[13], where O_2^-• would be rapidly converted into H_2O_2. The addition of antimycin A actually increased levels of O_2^-• and H_2O_2, as judged by the fluorescent signals from MitoSOX (**Fig. 1a**) and 2',7'-dichlorodihydrofluorescein (H_2DCF) (**Supplementary Fig. 1**), which are oxidized mainly with O_2^-• and H_2O_2 to exhibit fluorescence, respectively. We dissolved H_2 and O_2 into medium as described in **Methods** and confirmed the prolonged maintenance of H_2 for 24 h (**Supplementary Fig. 2**). 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• (**Supplementary Fig. 1**). In contrast, H_2 treatment significantly decreased levels of •OH, which was monitored by the fluorescent signal of 2-[6-(4'-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl)benzoate (HPF)[14,15] (**Fig. 1c**, **d**). When we exposed the cells to antimycin A (30 μg/ml) in the absence of H_2, the HPF signals increased even in the nuclear region as well as cytoplasm probably because H_2O_2 diffused from mitochondria to produce •OH (**Fig. 1c** as indicated by arrows). Interestingly, H_2 decreased the •OH levels even in the nuclear region (**Fig. 1c**, as indicated by arrowheads in the right panel).

Moreover, after the treatment with antimycin A, H_2 prevented a decline of the mitochondrial membrane potential as detected by fluorescent signals of tetramethylrhodamine methyl ester (TMRM), which depend upon the mitochondrial membrane potential, while fluorescent levels of MitoTracker Green

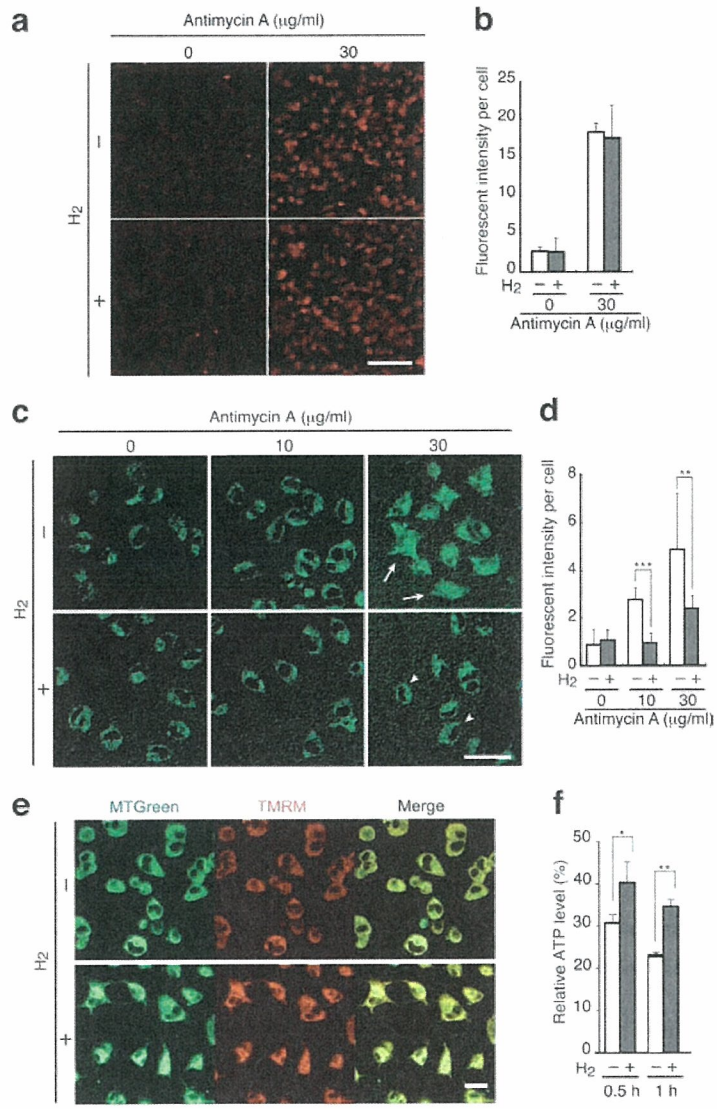

Figure 1 Molecular hydrogen dissolved in medium selectively reduces hydroxyl radicals in cultured cells. (**a**, **b**) PC12 cells were held in medium with or without 0.6 mM H_2, and exposed to antimycin A (30 μg/ml) to induce O_2^-• for 30 min and then treated with 0.5 μM MitoSOX. (**a**) Representative fluorescence images of MitoSOX-treated cells were obtained by laser-scanning confocal microscopy (Olympus FV300). Scale bar: 100 μm. (**b**) MitoSOX fluorescence was quantified from 100 cells of each independent experiment using NIH Image software (mean ± SD, $n = 5$). (**c**) Representative laser-scanning confocal images of the fluorescence of •OH marker HPF were taken 30 min after the addition of antimycin A. Scale bar: 50 μm. (**d**) HPF fluorescence in cells treated with antimycin A with or without 0.6 mM H_2 was quantified from 100 cells as described above (mean ± SD, $n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$. (**e**) At 30 min after adding antimycin A (10 μg/ml) with or without H_2 (0.6 mM), 1 μM MTGreen and 100 nM TMRM were incubated for 10 min, and cells were imaged. Scale bar: 20 μm. The two images were superimposed (merge). (**f**) Relative cellular ATP levels were quantified using a cellular ATP measurement kit (purchased from TOYO B-Net. Co.) after exposure to 30 μg/ml antimycin A. ATP levels of antimycin A-untreated cells were taken as 100% (mean ± SD, $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

(MTGreen), which are independent of the membrane potential, were unchanged (**Fig. 1e**), suggesting that H_2 protected mitochondria from ROS. H_2-treated cells looked normal, while H_2-untreated cells were shrunken to give round shapes (**Fig. 1e**). The protective effect was accompanied with preventing a decrease in the cellular ATP level (**Fig. 1f**). Thus, H_2 penetrated most membranes to diffuse into organelles including the nucleus and mitochondria.

Molecular hydrogen dissolved in medium protects cultured cells against oxidative stress

We placed PC12 cells in culture media containing H_2 and O_2, and at the same time induced oxidative stress by adding antimycin A. At 24 h after the induction of ROS with antimycin A, H_2 was revealed to protect nuclear DNA from oxidation, as shown by decreased levels of oxidized guanine (8-OH-G) (**Fig. 2a, b**)[16]. Moreover, H_2 also decreased levels of 4-hydroxyl-2-nonenal (HNE), an end-product of lipid peroxides (**Fig. 2c, d**)[17], indicating that it protected lipids from peroxidation. Consequently, cells were protected against cell death by H_2 dissolved in medium in a dose-dependent manner (**Fig. 2e, f**). When we removed H_2 from medium that had been saturated with H_2, the protective effect disappeared (**Fig. 2f**, column "degassed"), suggesting that H_2 did not affect the medium to exhibit the protective effect. Moreover, we confirmed cellular viability protected by H_2 using alternative methods: a modified MTT assay (WST-1 assay) and measurement of cellular lactate dehydrogenase (LDH)-leakage from damaged cells (**Supplementary Fig. 3**). To exclude the possibility that H_2 reacts with antimycin A to exhibit a protective effect, we induced ROS by adding menadione, which is an inhibitor acting on mitochondrial complex I. H_2 also protected cells in this ROS generating system (**Supplementary Fig. 3**).

To ensure that the protective effects of H_2 against •OH, we pretreated cells with Cu^{2+} and then exposed to ascorbate to reduce intracellular Cu^{2+} to Cu^{+}, which in turn catalyzes the production of •OH from cellular H_2O_2. Since this treatment primarily induces •OH inside the cells by the Fenton reaction, we confirmed

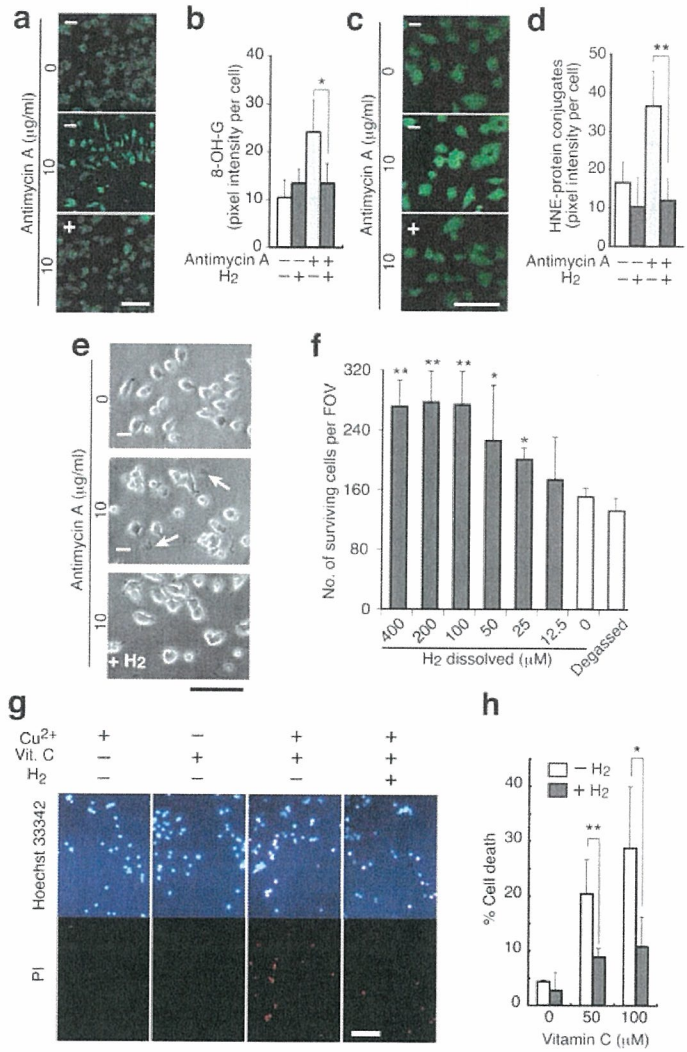

Figure 2 Molecular hydrogen protects cultured PC12 cells by scavenging hydroxyl radicals. **(a–d)** PC12 cells were maintained with 10 μg/ml antimycin A with (+) or without (–) 0.6 mM H_2 for 24 h in a closed flask as described in **Methods**, and immunostained with antibodies to 8-OH-G (**a**) or HNE (**c**). Fluorescent signals to 8-OH-G (**b**) or HNE (**d**) were quantified with NIH Image software using 100 cells from each independent experiment (mean ± SD, $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$. (**e**) Phase-contrast pictures of PC12 are shown 24 h after the exposure to antimycin A with (+) or without (–) 0.6 mM H_2, where arrows indicate dead cells. Scale bar: 50 μm (**a**, **c**, **e**). (**f**) Cell survival was assessed by manual counting of cells double-stained with 1 μM propidium iodide (pink for dead cells) and 5 μM Hoechst 33342 (blue for dead and living cells) (mean ± SD, $n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$. (**g**) PC12 was exposed to intracellular •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 to 0.1 mM ascorbate to reduce intracellular Cu^{2+} into Cu^{+} for 1 h as described in **Supplementary methods**. The cells were co-stained with propidium iodide and Hoechst 33342 to visualize the nuclei (**g**). Scale bar: 100 μm. Cell survival was assessed as above (**h**) (mean ± SD, $n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$.

that H_2 protected cells more directly against •OH (**Fig. 2g, h**).

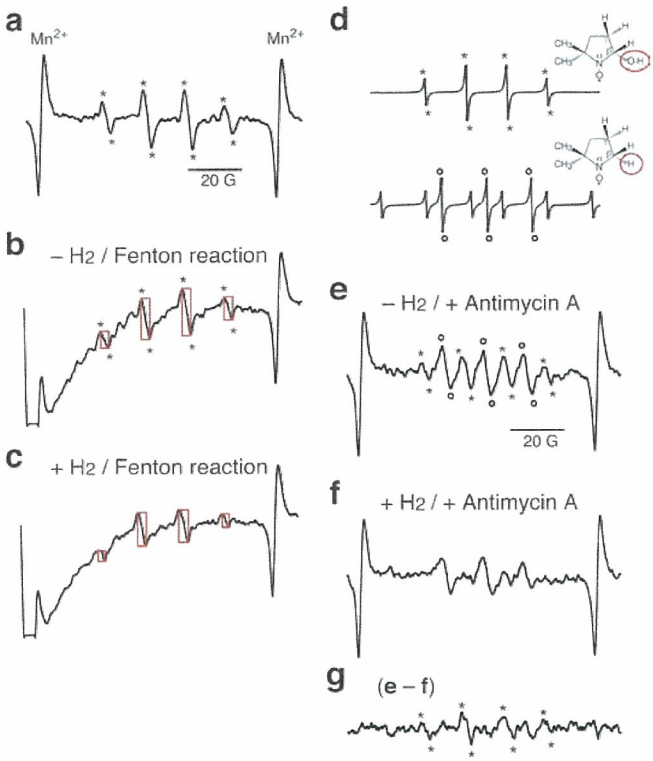

Figure 3 Spin trapping method identifies the free radical species that H_2 reduces. (**a**) Standard ESR signals of the •DMPO-OH radical were obtained by trapping •OH with a spin trapping reagent DMPO as described in **Supplementary methods**. (**b**, **c**) PC12 cells were preincubated with 0.1 M DMPO and 2 mM $CuSO_4$ for 30 min at 37 °C without (**b**) or with (**c**) 0.6 mM H_2. After the removal of this medium, the cells were treated with 0.2 mM ascorbate and 0.1 mM H_2O_2 for 5 min at room temperature to produce •OH and then scraped into a flat cuvette for electron spin resonance (ESR) measurement. (**d**) The •DMPO-OH and •DMPO-H radicals and their corresponding ESR-signal are illustrated. The •DMPO-H radical may be derived from the porphyrin-mediated hydrogen radical[18]. (**e**, **f**) PC12 cells were incubated in PBS containing 0.1 M DMPO and 30 μg/ml antimycin A for 7 min at room temperature with or without 0.6 mM H_2, then scraped into a flat cuvette for ESR measurement. Star (*) and circle (o) indicate the positions corresponding to signals of •DMPO-OH and •DMPO-H, respectively. (**g**) A differential spectrum was obtained by subtracting (**f**) from (**e**) to visualize the signals decreased by H_2 treatment. Star (*) indicates the position of signals corresponding to •DMPO-OH derived from •OH.

Spin trapping method identifies a free radical that hydrogen reduces

To identify the free radical species that H_2 reduces, we produced •OH by the Fenton reaction and semi-quantified cellular levels of •OH by spin trapping method using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Electron spin resonance (ESR) measurement indicated that H_2 treatment decreased signals of •DMPO-OH derived from •OH (**Fig. 3a–c**). Moreover, when we treated cells with antimycin A to induce O_2^-• in the presence of DMPO, multiple ESR signals[18] were observed; these signals seemed to consist of those from the •DMPO-OH and •DMPO-H radicals (**Fig. 3d–f**). However, a differential spectrum visualized that only signals derived from •OH were decreased by H_2 treatment (**Fig. 3e**). These results strongly suggest the selective reduction of cellular •OH by H_2 treatment.

Molecular hydrogen selectively reduced •OH and $ONOO^-$ in cell-free systems

Next, we confirmed in a pure solution that HPF fluorescence could monitor the reduction of •OH by H_2 during continuous •OH production by the Fenton reaction. H_2 actually suppressed increases in HPF signals in a dose-dependent manner (**Fig. 4a–c**). When we mixed a solution containing H_2 with HPF pre-oxidized with •OH, fluorescent signals from oxidized HPF did not decrease (data not shown), supporting that H_2 directly reacts with •OH. Next, we produced H_2O_2, NO•, O_2^-• and peroxynitrite ($ONOO^-$) by each specific method in cell-free systems as described in **Supplementary methods**. H_2 somewhat reduced $ONOO^-$ (**Fig. 4d**), but failed to reduce H_2O_2, NO• and O_2^-• (**Fig. 4e–g**). Moreover, we examined whether H_2 reduced the oxidized forms of biomolecules involved in metabolic oxidation-reduction reactions in cell-free experiments. At room temperature and neutral pH, solutions saturated with H_2 did not reduce NAD^+, FAD and the oxidized form of cytochrome *c* (data not shown). Thus we surmise that H_2 would not affect the metabolism involved in oxidation-reduction reactions and would not affect O_2^-•, H_2O_2, and NO• that play essential roles in signal transduction.

Hydrogen protects neurons from *in vitro* ischemia/reperfusion

We also induced oxidative stress in a primary culture of neocortical cells[19] under more physiological conditions. It is known that rapid transition from an ischemic condition to reperfusion results in oxidative stress damage[20], so to mimic ischemia, we subjected

neocortical cells to oxygen-glucose deprivation (OGD) under nitrogen or hydrogen gas for 60 min. Ten min after the completion of OGD, •OH levels notably increased in the absence of H_2, while in the presence of H_2, •OH was markedly diminished, as shown by HPF fluorescence (**Supplementary Fig. 4**). Twenty-four h after OGD, H_2 increased neuron survival (**Supplementary Fig. 4**) and increased vitality vs. control cells (**Supplementary Fig. 4**), indicating that H_2 protected neurons against oxidative stress-induced cell death.

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Inhalation of hydrogen gas protects brain injury caused by ischemia and reperfusion

Then, to examine the medical applicability of H_2 as an antioxidant, we used a rat model of ischemia. ROS is generated during cerebral ischemia although multiple causes affect injury by a variety of mechanisms[21, 22]. We produced focal ischemia in rats by occlusion of the middle cerebral artery (MCA) for 90 min with subsequent reperfusion for 30 min[23]. H_2 gas was inhaled during the entire 120 min process unless otherwise indicated; rats inhaled H_2 in a mix of nitrous oxide (N_2O) (for anesthesia), O_2, and H_2 gases (70–67% / 30–29% / 0–4%, v/v, respectively). We carefully monitored physiological parameters during the experiments as described in **Methods** and found no significant change by the inhalation of H_2 (**Supplementary Table**). Additionally, no significant influence on cerebral blood flow was seen as measured by the Doppler effect[24] (**Supplementary Fig. 5**). The inhalation of H_2 actually increased H_2 dissolved in artery blood, and H_2-levels decreased in venous blood, suggesting that H_2 is incorporated into tissues (**Fig. 5a**). One 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 brain areas displaying distinct white coloration (**Fig. 5b, c**) and found a clear H_2-dependent decrease in infarct volume, with 2–4%of H2 providing the most notable effect (**Fig. 5c**). H_2 exerted its effect during

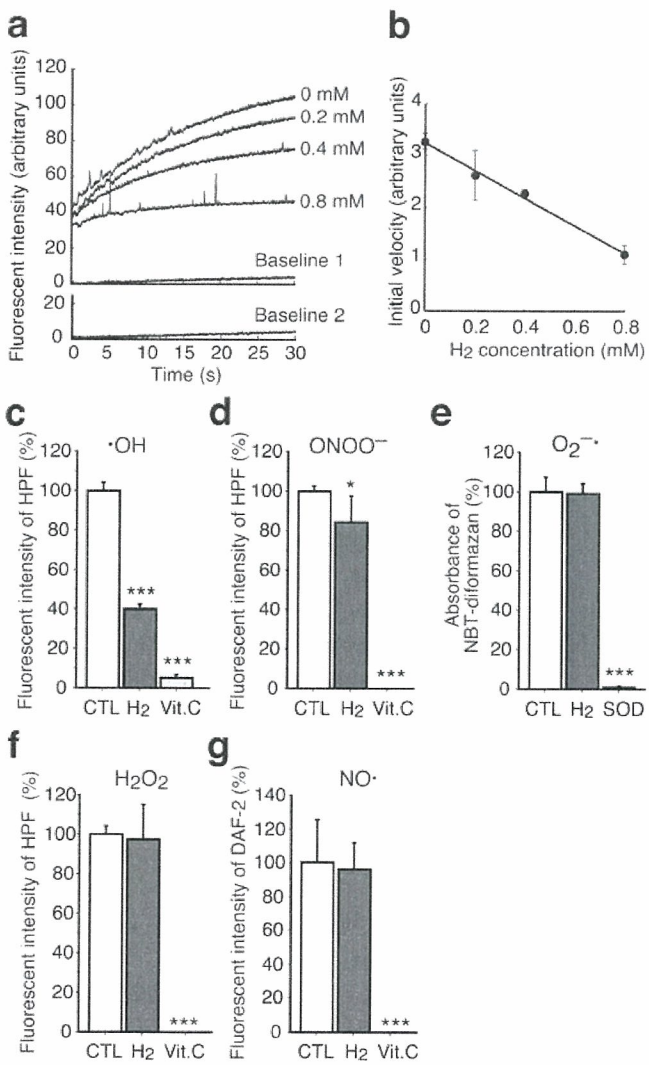

Figure 4 Molecular hydrogen dissolved in solutions scavenges hydroxyl radicals at room temperature and neutral pH in cell-free systems. The Fenton reaction, which generates hydroxyl radicals, was initiated by adding H_2O_2 to 5 μM in a closed cuvette at 23 °C with gentle stirring as described in **Supplementary methods**. In the presence of various concentrations of H_2 dissolved in the solution, •OH was monitored by the HPF fluorescence with a fluorescence spectrometer. (**a**) Representative time course traces show the resultant 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), respectively. (**b**) Mean values and the standard deviations of initial rates were obtained from four independent experiments. (**c–g**) Each ROS or reactive nitrogen species [RNS: NO• and peroxynitrite ($ONOO^-$)] was produced by each specific method as described in **Supplementary methods**. After incubation with 0.6 mM of H_2 at 23 °C, remaining ROS or RNS was detected with each probe as described in **Supplementary methods.** As a positive control, vitamin C (Vit. C) (1 μM for **c** and **d**, and 10 μM for **f** and **g**) or superoxide dismutase (10 units of SOD for **e**) was used. (**c**): •OH, (**d**): $ONOO^-$, (**e**): O_2^{-}•, (**f**) H_2O_2 and (**g**) NO•. Signals without H_2 were taken as 100% (mean ± SD, $n = 6$). $^*P < 0.05$ and $^{***}P < 0.001$.

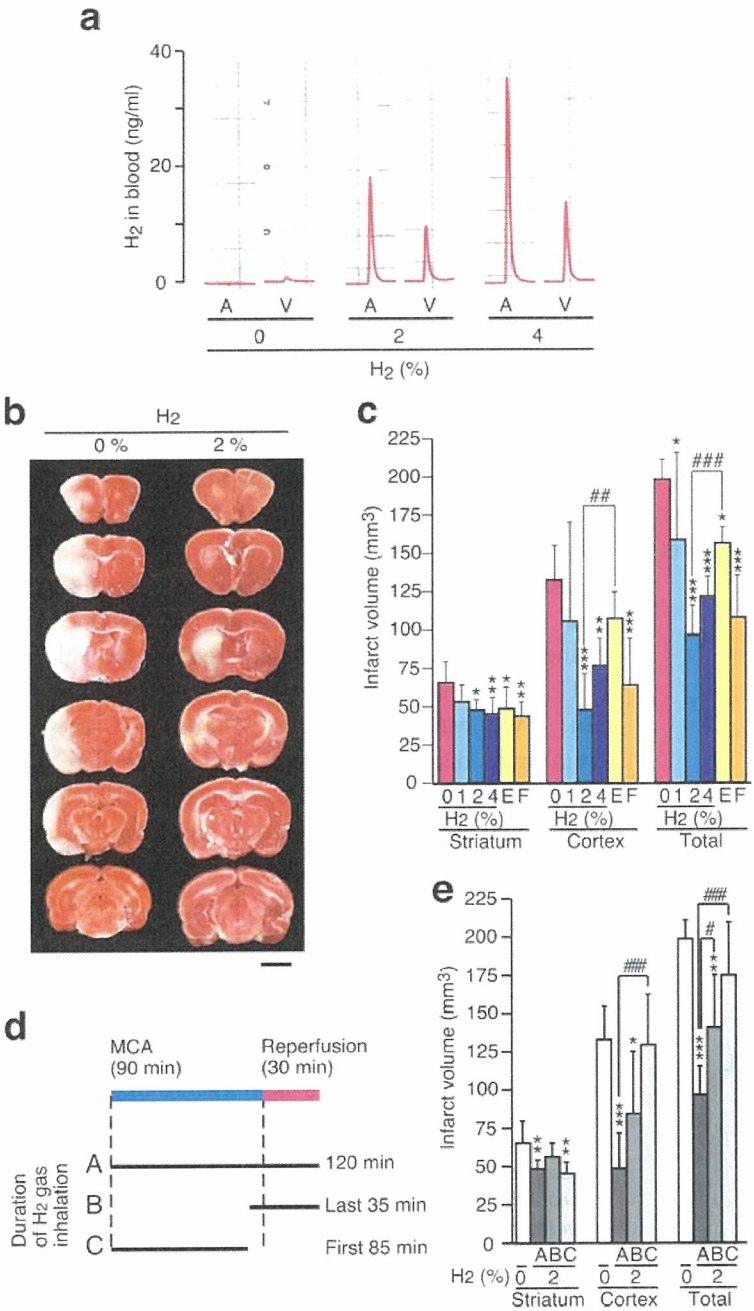

Figure 5 Inhalation of hydrogen gas protects against ischemia reperfusion injury. (**a**) Rats inhaled H_2 and 30% O_2 for 1 h under anesthetic N_2O and halothane. Arterial and venous blood was collected and the amount of H_2 was examined by gas chromatography as described in **Methods**. Profiles of gas chromatography are shown, where A and V indicate arterial and venous blood, respectively. The vertical scale indicates amounts of blood H_2 after calculations. (**b**) Rats underwent MCA occlusion as described in **Methods**. During the 120-min procedure, the indicated concentration of mixed gas was inhaled. One d 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" indicate the results of infarct volumes treated with edaravone and FK506 under their optimum conditions[25,26], respectively (mean ± SD, n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with 0% of H_2. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with 2% of H_2. (**d**) Schematic representation of three different durations of hydrogen gas (2%) inhalation is represented. (**e**) Infarct volumes of the brain were calculated as described above (mean ± SD, n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with 0% of H_2. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with A. A – C represent different durations of H_2 gas inhalation as shown in (**d**).

reperfusion; when H_2 was inhaled during ischemia, but not during reperfusion, infarct volume was not significantly decreased (**Fig. 5d, e**). For comparison, we tested two other compounds: edaravone is approved for the treatment of cerebral infarction as an ROS scavenger in Japan[25] and FK506 is in clinical trials for cerebral infarction in the United States[26]. H_2 was more efficacious in alleviating oxidative injury than decreased (**Fig. 5d, e**). For comparison, we tested two other compounds: edaravone is approved for the treatment of cerebral infarction as an ROS scavenger in Japan[25] and FK506 is in clinical trials for cerebral infarction in the United States[26]. H_2 was more efficacious in alleviating oxidative injury than edaravone and was comparable with FK506 (**Fig. 5c**), ensuring the potential for the therapeutic efficacy of H_2.

Inhalation of hydrogen gas suppresses the progression of brain injury by decreasing oxidative stress

One week after MCA occlusion, the difference in

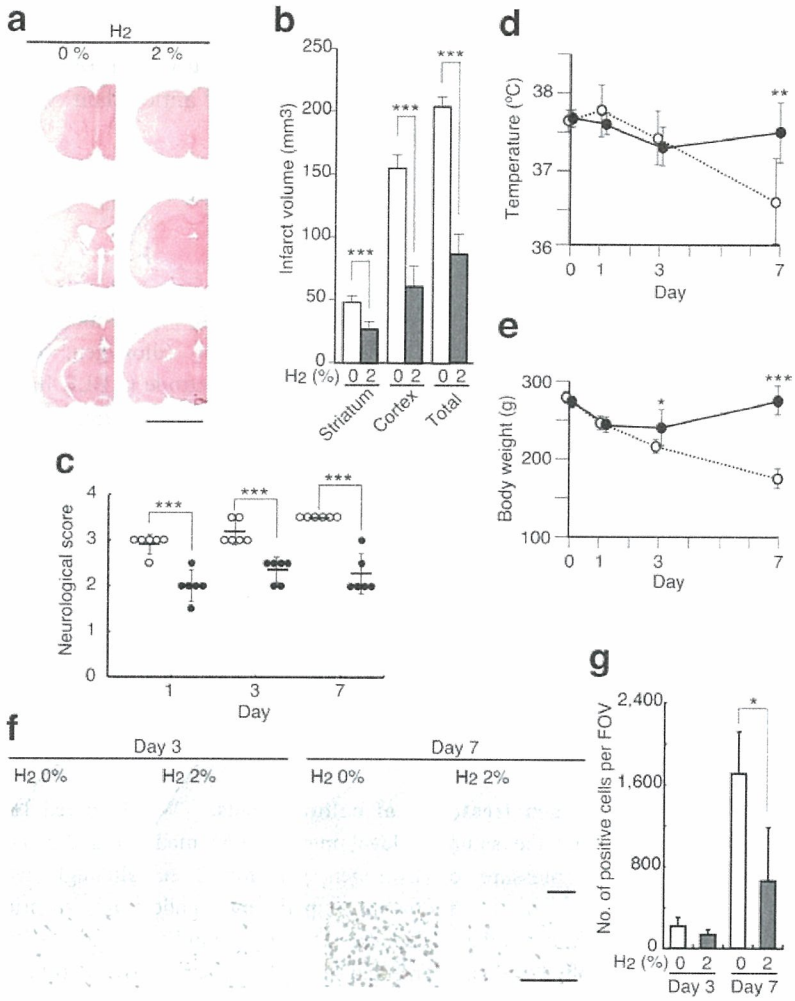

Figure 6 Inhalation of H_2 gas improved brain injury after 1 week. Rats inhaled 2% of hydrogen gas during the 120-min ischemia reperfusion procedure and were maintained for indicated periods. (**a**) One week after MCA occlusion, the brains were sliced and stained with hematoxylin and eosin. Three representative slices are shown. Scale bar; 5 mm. (**b**) Infarct volumes (light-pink regions in **a**) were calculated (mean ± SD, $n = 6$). $^{***}P < 0.001$. (**c**) Neurological scores were graded from 0 to 5 as described[27]: 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 was judged between score 2 and 3, the score was determined as 2.5. H_2-treated rats (closed circle) and H_2-untreated rats (open circle) (mean ± SD, $n = 6$). $^{***}P < 0.001$. (**d**, **e**) Body weights and temperature were monitored with (closed circle) or without (open circle) inhalation of 2% hydrogen gas (mean ± SD, $n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (**f**) On 3 or 7 d after MCA occlusion, coronal 6 μm-sections were stained with anti-Iba1 as a microglial marker at the ischemic core area in the temporal cortex. Scale bar: 200 μm or 100 μm (inset). (**g**) Positive cells with anti-Iba1 antibody[24] per field of view (FOV) were counted in exactly the same regions (mean ± SD, $n = 6$). $^{*}P < 0.05$.

6a, **b**). We carefully observe the behavior of each rat and graded it as a neurological score[27], revealing the improvement of functional assessment by the inhalation of H_2 during ischemia reperfusion (**Fig. 6c**). Moreover, H_2-treated rats also showed improvements in body weight and temperature vs. untreated rats (**Fig. 6d**, **e**). Thus, H_2 suppressed not only the initial brain injury, but also the progression of injury. To assess H_2-mediated molecular changes at 12 h, 3 or 7 d after occlusion, we stained brain sections with anti-8-OH-G to assess the extent of nucleic acid oxidation (**Supplementary Fig. 6**) and with anti-HNE to assess lipid peroxidation (**Supplementary Fig. 6**), and found a substantial decrease in the staining of both of these oxidative markers in H_2-treated rats. Moreover, we also stained identical regions of the brain with anti-Iba1[28] and anti-GFAP (specific to activated microglia and to astrocytes, respectively) (**Fig. 6f**, **g** and **Supplementary Fig. 6**), and found a distinct H_2- dependent decrease in the accumulation of microglia, which is an indicator of inflammation and remodeling. Taken together, H_2 has great potential to markedly decrease oxidative stress and suppress brain injury.

DISCUSSION

This study shows the selective reduction of ROS *in vitro* by molecular hydrogen. As •OH has much stronger reactivity than other ROSs[14], and $ONOO^-$ is subsequent to •OH[14], it is reasonable for H_2 to react only with the strongest oxidants; this is advantageous for medical procedures using H_2 without serious unwanted side effects. H_2 could be moderate enough not to disturb metabolic oxidation-reduction reactions

or not to disrupt ROSs involved in cell signaling. Recent publications have warned that mortality is increased by consumption of some antioxidant supplements with strong reduction reactivity, perhaps because some essential defensive mechanisms are affected[29].

H_2 has a number of advantages as a potential antioxidant; it effectively neutralizes •OH in living cells, and has favorable distribution characteristics in its own physical ability to penetrate biomembranes and diffuse into cytosol, mitochondria and the nucleus, unlike most known antioxidants which are unable to successfully target organelles[30]. Despite moderate reduction activity of H_2, the gaseous rapid diffusion of H_2 may provide potential for highly effective reduction of cytotoxic radicals. The protection of nuclear DNA and mitochondria suggests preventive effects on life style-related diseases, cancer and the aging process.

Indeed, H_2 markedly decreased oxidative stress and suppressed brain injury caused by ischemia and reperfusion. The inhalation of H_2 gas was more efficacious than the treatment approved for cerebral infarction. Moreover, inhalation of H_2 mitigated hepatic injury caused by ischemia and reperfusion (manuscript in preparation), indicating that H_2 is not specific to the cerebral injury but applicable to other organs.

Although this study suggests scavenging •OH by H_2, it remains possible to protect cells and tissues against strong oxidative stress not only by scavenging •OH, but also by reducing other strong oxidant species directly or indirectly in living cells. As an alternative explanation, H_2 may induce some cytoprotective factors; however, we found no change by H_2 in several gene expressions involved in the cytoprotection or reduction reaction (data not shown). Further studies will reveal the mechanisms how H_2 protects cells and tissues against oxidative stress.

Acute oxidative stress may arise from a variety of different situations: inflammation, heavy exercise, cardiac infarction, cessation of operative bleeding, organ transplantation, and others. As a therapeutic use, H_2 dissolved in saline could easily be delivered intravascularly. As a preventive use, H_2 could be taken in as water saturated with H_2. Inhalation of H_2 has already been used in the prevention of decompression sickness in divers and has shown a good safety profile[31]. Importantly, H_2 has no risk of flammability or explosion at a concentration 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 with a hydrogen electrode (ABLE Inc.) and an oxygen electrode (Strathkelvin Instruments Ltd), respectively, while we determined hydrogen gas concentration by gas chromatography (Teramecs Co.). For measuring H_2 levels in blood, we pretreated a rat with heparin to avoid clotting blood, and collected arterial and venous blood (5 ml each) into test tubes, and then immediately injected 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 to quantify the amount of H_2 using standard H_2 gas.

Hydrogen treatment of cultured cells. We dissolved H_2 beyond the saturated level into DMEM medium under 0.4 MPa pressure of hydrogen gas for 2 h, although we performed the following experiments under atmospheric pressure. We dissolved O_2 into another medium by bubbling and supplemented fetal bovine serum to 1% to both media (H_2 media and O_2 media) and the third medium containing CO_2, and then combined the three media at various ratios to obtain the desired concentration of H_2 and 8.5 mg/l of O_2 at 25 °C. 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, and in turn filled gas composed of the desired ratio of H_2 and N_2 ($H_2 + N_2 = 75\%$), 20% of O_2 and 5% of CO_2 into the culture flask; for example, when the medium contained 0.6 mM H_2, we adjusted the H_2 gas to 75%. We cultured cells in the combined medium in a closed culture flask filled with the mixed gas of CO_2, N_2, H_2 and O_2. 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.

Induction of oxidative stress by antimycin A and menadione. We maintained PC12 cells at 37 °C in DMEM media supplemented with 1% fetal bovine serum in a closed flask filled with H_2 and O_2 gases as described above, and treated with menadione or antimycin A, which is an inhibitor

to complex I or complex III of the mitochondrial electron transport chain, respectively, to accelerate the leakage of electrons to produce $O_2^{-}\bullet$.

Cerebral infarction model. Animal protocols were approved by the Animal Care and Use Committee of Nippon Medical School. We anesthetized male Sprague Dawley rats (250–300 g) with halothane (4% for induction, 1% for maintenance) in nitrous oxide/oxygen (70% / 30%, v/v). 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, cannulated the tail artery to monitor physiological parameters, including blood gases (pCO_2 and pO_2), pH, glucose level and blood pressure, and attempted to maintain the pH and pO_2 constant by regulating halothane and the gas ratio of N_2O to O_2. We produced focal ischemia by intraluminal occlusion of the left middle cerebral artery (MCA) with a nylon monofilament with a rounded tip and a distal silicon rubber cylinder as described[23]. The animals underwent MCA occlusion for 90 min and then reperfusion for 30 min; H_2 gas was inhaled during the entire process unless otherwise indicated. After recovering from anesthesia, we maintained the animals at 23 °C.

At 24 h after MCA occlusion, we removed brains under anesthesia and sliced into 6 coronal sequential sections (2 mm thick), and then stained sections with 2,3,5-triphenyltetrazolium chloride (TTC) (3%), followed by analyses using an optical dissector image analysis system (Mac SCOPE, Mitsuya Shoji). We outlined the border between infarct and non-infarct tissues, and estimated the area of infarction by subtracting the non-lesioned area of the ipsilateral hemisphere from that of the contralateral side. We obtained the infarct area by subtracting the area of the non-lesioned ipsilateral hemisphere from that of the contralateral side, and calculated the volume of infarction by (infarct areas × thickness). At 12 h, 3 or 7 d after MCA occlusion, we quickly removed brains under anesthesia, and fixed with 10% formalin. We sliced paraffin-embedded brains into a series of 6-μm sections, and stained sections with hematoxylin and eosin (H&E), followed by quantifying 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.

Statistical analysis. We performed statistical analyses using StatView software (SAS Institute) by applying an unpaired two-tailed Student's *t*-test and ANOVA followed by Fisher's exact test for single and multiple comparisons, respectively. We performed experiments for quantification in a blinded fashion.

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

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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, and S.O. and I.O. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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