Resuscitation Science

Hydrogen Inhalation During Normoxic Resuscitation Improves Neurological Outcome in a Rat Model of Cardiac Arrest Independently of Targeted Temperature Management

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Background—We have previously shown that hydrogen (H2) inhalation, begun at the start of hyperoxic cardiopulmonary resuscitation, significantly improves brain and cardiac function in a rat model of cardiac arrest. Here, we examine the effectiveness of this therapeutic approach when H2 inhalation is begun on the return of spontaneous circulation (ROSC) under normoxic conditions, either alone or in combination with targeted temperature management (TTM).

Methods and Results—Rats were subjected to 6 minutes of ventricular fibrillation cardiac arrest followed by cardiopulmonary resuscitation. Five minutes after achieving ROSC, post–cardiac arrest rats were randomized into 4 groups: mechanically ventilated with 26% O2 and normothermia (control); mechanically ventilated with 26% O2, 1.3% H2, and normothermia (H2); mechanically ventilated with 26% O2 and TTM (TTM); and mechanically ventilated with 26% O2, 1.3% H2, and TTM (TTM+H2). Animal survival rate at 7 days after ROSC was 38.4% in the control group, 71.4% in the H2 and TTM groups, and 85.7% in the TTM+H2 group. Combined therapy of TTM and H2 inhalation was superior to TTM alone in terms of neurological deficit scores at 24, 48, and 72 hours after ROSC, and motor activity at 7 days after ROSC. Neuronal degeneration and microglial activation in a vulnerable brain region was suppressed by both TTM alone and H2 inhalation alone, with the combined therapy of TTM and H2 inhalation being most effective.

Conclusions—H2 inhalation was beneficial when begun after ROSC, even when delivered in the absence of hyperoxia. Combined TTM and H2 inhalation was more effective than TTM alone. (Circulation. 2014;130:2173-2180.)

Key Words: antioxidants • cardiopulmonary resuscitation • heart arrest • ischemia • reperfusion injury

Editorial see p 2133
Clinical Perspective on p 2180

We previously demonstrated that inhalation of 2% H2 started at the beginning of hyperoxic (98% O2) cardiopulmonary resuscitation (CPR) and lasting until 2 hours after the return of spontaneous circulation (ROSC) improved survival and neurological deficit score (NDS) in a rat model of CA.12 This effect was comparable to that of targeted temperature management (TTM) alone (33°C).12 Several issues require further investigation before clinical application of these findings becomes feasible. First, there was no direct evidence for preservation of brain function at a histological level. A brief episode of global brain ischemia produces selective and often extensive neuronal loss in vulnerable brain structures in humans and rodents such as the hippocampal CA1 pyramidal neurons. In addition, cell death does not occur immediately but is delayed for days (delayed neuronal death). Accordingly, previous reports assessed CA1 pyramidal neuron necrosis at 10 and 30 days13 or at 7 days14 after ischemia. In our previous study, although H2 inhalation improved neurological outcome (based on the NDS) at 24 hours after ROSC, this phenomenon was not associated with a reduction in either brain edema or hippocampal CA1 pyramidal neurons at this early time point. Second, in our previous study,
inhalation of H₂ was started at the beginning of CPR, but in a clinical setting, hypothermia is applied after ROSC. Therefore, this present study investigated whether the benefit of H₂ inhalation is similar when begun after ROSC. Third, because all animal groups in our previous study were ventilated with 98% O₂, H₂ may protect against only the harmful effects of hyperoxia. We wish to investigate therapy benefit under normoxic conditions. We therefore investigated whether H₂ inhalation without hyperoxia improves neurological outcome in a rat model after resuscitation from CA independently of TTM.

Methods

Animal Preparation

Seventeen-week-old male Wistar ST rats weighing an average of 408 g were used in this study after institutional approval was obtained from the Animal Ethics Committee. The rats were housed in a rodent facility under 12-hour light/dark cycle conditions.

The rats were fasted overnight except for free access to water and then were anesthetized by intraperitoneal injection of pentobarbital sodium (45 mg/kg). The trachea was intubated via a tracheostomy with a 14-gauge cannula and mechanically ventilated with a tidal volume of 0.65 mL/100 g body weight, a respiratory rate of 100 breaths per minute, and fraction of inspired oxygen (FiO₂) of 0.21 (ventilator: SN-480-7, Shimano). Polyethylene catheters (PE50, Natsume) were inserted into the left femoral arteries and veins and flushed intermittently with saline solution containing 2.5 IU/mL bovine heparin. Arterial blood pressure was measured, and an ECG was recorded by subcutaneous needle electrodes. Core temperature was monitored by a rectal temperature probe (BAT-10, Physitemp Instruments Inc) and maintained with a heating plate (SCP-85, AsOne) throughout the experiment as appropriate temperature management.

Ventricular Fibrillation and CPR Model

Ventricular fibrillation (VF) was induced by electrical stimulation via a transthoracic epicardium electrode, as previously described. This stimulator (Isostim, World Precision Instruments Inc) was used to perform direct and constant electric stimulation of the epicardium with crude current, continuous single stimulation, a delay of 10 milliseconds, a wave width of 1 millisecond, a frequency of 50 Hz, an intensity of 1 mA, and a stimulation duration of 3 minutes. The mechanical ventilation was stopped and disconnected from the tracheal tube after the onset of VF. CA was defined by an abrupt blood pressure drop, disappearance of the pulse wave signal, and VF on the ECG recording. After 6 minutes of VF induction, advanced cardiac life support was started in which the rats were ventilated (0.65 mL/100 g, 100 breaths per minute) and chest compressions (200 per minute) were started by the investigator’s finger paced by a metronome. Chest compressions were adjusted to a uniform rate and a target aortic diastolic pressure of >20 mm Hg. Adrenalin (2 μg/100 g) and 0.1 mL sodium bicarbonate (8.4%) were immediately administered to the rats at the beginning of CPR and repeated at 3-minute intervals as needed. The administration of intravenous fluid during CPR was limited to ≤2 mL. Three minutes after chest compressions were started, defibrillation was performed with direct-current single-phase waves of 2 J when the ECG displayed VF. If the defibrillation failed, CPR was repeated and defibrillations were again performed 1 minute after CPR. ROSC was defined as the return of supraventricular rhythm with a mean aortic pressure >50 mm Hg for a minimum of 5 minutes. If spontaneous circulation was not restored in the rats after 6 minutes, CPR was considered to be a failure. The concentration of H₂ in the gas mixture was monitored with a Breath Gas Analyzer model TGA-2000 (TERAMECS, Kyoto, Japan). Sham-operated controls were subjected to the same operative procedure without electric stimulation.

Premixed Gas Comprising H₂ Gas and Oxygen

The maximal concentration of flammable H₂ is 1.3% in mixed gas formulations comprising flammable gas and oxygen at greater than atmospheric concentration. The High Pressure Gas Safety Act in Japan states that flammable gas contained in the mixed gas cannot exceed one third of the lower explosion limit (4%). Our preliminary experiments revealed that although 2% inhaled H₂ is superior to 1% inhaled H₂ for reducing reactive oxygen metabolites in blood, both are essentially equivalent in suppressing systemic inflammatory activation, and both equally improved the survival and functional outcomes in our rat model of CA with VF (data not shown). Therefore, we decided to use premixed gas comprising 1.3% H₂ and 26% O₂ in the following experiments.

Experimental Protocol

Five minutes after achieving ROSC, animals were randomized into 4 groups (2:1:1:1 ratio): mechanical ventilation with 26% O₂ and normothermia (control group, n=13); mechanical ventilation with 26% O₂, 1.3% H₂, and normothermia (H₂ group, n=7); mechanical ventilation with 26% O₂ and TTM (TTM group, n=7); and mechanical ventilation with 26% O₂, 1.3% H₂, and TTM (TTM+H₂ group, n=7).

Gas inhalation was continued for 2 hours (Figure 1). In animals of the TTM groups, rapid cooling was started after randomization and was induced with ice packs and an electric fan. Body temperature reached 33°C within 15 minutes. Once the target temperature was reached, it was maintained for 2 hours, followed by a slow rewarming period at a rate of 1.5°C/h, after which the animals were maintained at 37°C until the end of the experiment. Normothermic animals were maintained at 37°C. Arterial blood pressure, ECG recordings, intrathoracic pressure, and rectal temperature were monitored for 4 hours. No inotropic agent was administered. After a recovery period of 4 hours, rats were weaned from the ventilator, all vascular catheters and tracheal tubes were removed, and surgical wounds were sutured. After each experimental period, rats were returned to their cages with easily accessible food and water and were observed in a rodent facility with a controlled room temperature of 22°C. Buprenorphine (0.01 mg/kg) was injected intramuscularly daily to ensure surgical pain relief during the recovery period for all groups.

We used 43 consecutive rats for the survival study. Among them, 9 rats were excluded from further analyses (ROSC was not achieved in 5 rats; CA was not induced because of a technical failure in 4 rats), and randomization did not begin until ROSC. The survival time after CPR was recorded up to 7 days.

Neurological Deficit Evaluation

A single investigator who was unaware of each animal’s group assignment performed all NDS evaluations. Consciousness and breathing, cranial nerve reflexes, motor function, sensory function, and coordination were scored according to an NDS system (0–500 scale; 0=normal, 500=death or brain death), as described previously.

Y-Maze Test

The Y-maze test, a gross test for spatial memory, uses a Y-maze apparatus composed of 3 equally spaced arms (120°, 80 cm long × 30 cm high × 15 cm wide). This ethologically relevant test is based on the rodents’ innate curiosity to explore novel areas. Briefly, rats were placed into one of the arms of the maze (start arm) and allowed to explore the maze for 8 minutes (test trial). A rat is considered to enter an arm if the rat’s head enters the arm. The Y-maze test was cleaned with dilute alcohol and dried with paper towels.

Histopathological Analysis

At 7 days after ROSC, rats were decapitated. The left sides of the brains were quickly removed and fixed with Zamboni solution. The
brains were histologically compared with those of sham-operated rats. Coronal tissue slices (6-μm thickness) of paraffin-embedded brain tissue (at the level of the hippocampus) were stained for histological evaluation. To examine changes in the neurons, astrocytes, and microglia after global cerebral ischemia/reperfusion, we performed immunohistochemical staining with an anti–neuronal nuclei antibody (NeuN; catalog No. MAB377, Millipore, Temecula, CA) for neurons, anti-ionized calcium-binding adapter molecule 1 antibody (Iba-1; catalog No. 019–19741, Wako Pure Chemical Industries, Ltd, Osaka, Japan) for microglia/macrophages, and biotin-conjugated microtubule-associated protein 2 antibody (MAP2; catalog No. M9942, Sigma-Aldrich, St. Louis, MO) for axonal damage, Fluoro-Jade C (FJC) labeling23 (Fluoro-Jade C Ready-to-Dilute Staining Kit; catalog No. TR-100-FJ, Biosensis, Thebarton, South Australia, Australia) for degenerative neuronal cells, and glial fibrillary acidic protein (GFAP) staining (anti-GFAP antibody; catalog No. AB5804, Millipore, Temecula, CA) for activated astrocytes.

In each NeuN-, Iba1-, and MAP2-stained section, 2 slide fields were randomly examined using a defined rectangular field area (0.14 mm²). MAP2 staining is reported as the relative intensity of staining (percent) of the area, calculated with automated counting software (Image J 1.46r, National Institutes of Health, Bethesda, MD). Fluorescence microscopic examination of FJC and GFAP staining was performed on 2 slide fields of a defined field area (0.05 mm²). Images were analyzed with Image J. Automated counting data for each stained section were reported as degenerating cells in blue and activated astrocytes in red.

**Statistical Analysis**

Measurements are reported as mean±SEM. For single comparisons, we performed an unpaired 2-tailed Student t test; for multiple comparisons, we used an ANOVA followed by the Tukey correction for post hoc comparisons. Physiological data (rectal temperature, mean arterial pressure, heart rate) were examined by a mixed-effects model for repeated measures analyses comprising treatment group, time, and treatment-by-time interaction as factors and random intercept for each subject. NDSs were on an ordinal scale and were analyzed by the Kruskal–Wallis with Mann–Whitney U analyses between multiple groups. Kaplan–Meier analysis and the log-rank test were used to calculate survival rates. Statistical significance was considered at a 2-sided value of P<0.05. Statistical analyses were performed with SPSS software (SPSS Inc, Chicago, IL).

**Results**

**H₂ Therapy Begun After ROSC Under Normoxic Conditions Improved Animal Survival and Neurological Recovery in Post-CA Rats**

There were no differences among the 4 experimental groups in hemodynamics, blood gases, or chemistries at baseline, during CPR, or during post–CA care after ROSC (Tables I and II in the online-only Data Supplement and Figure 2B and 2C). There were also no significant differences in the number of gasps during CA, diastolic pressure during CPR, or the number of defibrillations required to establish ROSC among the 4 experimental groups (Table I in the online-only Data Supplement).

Rectal temperature was rapidly reduced from 37.0±0.0°C to 32.8±0.2°C at 15 minutes after ROSC in hypothermic animals, and the normothermic animals showed no significant changes in rectal temperature during the experiments (Figure 2A).

The survival rate at 7 days after ROSC was 5 of 13 rats (38.4%) in the control group; 5 of 7 rats (71.4%) survived...
in the H₂ group; 5 of 7 rats (71.4%) survived in the TTM group; and, 6 of 7 rats (85.7%) survived in the TTM+H₂ group (P<0.05 versus control group; Figure 3).

The NDS was evaluated at 24, 48, at 72 hours and 7 days after ROSC (Table). NDS values at 72 hours after ROSC were better in the H₂ group (185±82; P<0.05), the TTM group (217±74; P<0.05), and the TTM+H₂ group (90±68; P<0.01) than in the control group (395±40), whereas scores in the TTM+H₂ group were better than in the TTM group (P<0.05). As a practical example, rats with an NDS of 395 were unresponsive and immobile, reacted minimally to stimuli, and were associated with high mortality. Rats with an NDS of 217 generally had an increased respiratory rate, sluggish responses to pain, and disordered coordination, but they retained some mobility, whereas rats with a NDS of 90 appeared alert, reacted briskly to stimuli, and had mild respiratory impairment. NDS values at 7 days were also better in the H₂ group (166±87; P<0.05), the TTM group (185±84; P<0.05), and the TTM+H₂ group (88±69; P<0.01) than in the control group (389±43; Table). Improved neurological outcome after H₂ and hypothermia treatment was consistent when NDS values were evaluated separately for survivors only (Table III in the online-only Data Supplement). To confirm the prognostic value of NDS, we examined the relationship between NDS category at 24 hours after ROSC and mortality. A higher NDS at 24 hours after ROSC was associated with a poorer survival (Figure 4).

Rats were also subjected to a Y-maze test to assess motor activity and spatial memory. Motor activity at 7 days after ROSC was significantly lower in the control (P<0.01 versus sham) and TTM (P<0.05 versus sham) groups than in the sham group (Figure 5A), whereas motor activity at 7 days after ROSC was nonsignificant in the H₂ and TTM+H₂ groups compared with the sham group. The control group appeared deficient in spatial working memory as tested by spontaneous alternation in the Y-maze compared with the sham group (P<0.05), whereas spatial working memory at 7 days after ROSC became nonsignificant in the other groups compared with the sham group (Figure 5B).

**H₂ Inhalation Rescued Neuronal Death and Suppressed Microglia Activation in the Hippocampus and Cerebral Cortex**

A transient period of global cerebral ischemia and reperfusion after CA is associated with delayed neuronal death and activation of astrocytes and microglia in selectively vulnerable areas such as the hippocampus and cerebral cortex in both experimental animals and humans. The number of surviving NeuN-positive cells in the hippocampus CA1 from the control group was significantly lower than in sham-operated post-CA rats. The number of NeuN-positive cells in the hippocampus CA1 was significantly preserved in the H₂, TTM, and TTM+H₂ groups compared with the control group (Figure 6A). MAP2, abundant in neuronal dendrites, functions in maintaining the cytoskeletal integrity of dendrites. Extensive loss of MAP2-immunoreactive dendrites was apparent in the hippocampus CA1 in the control group. The intensity of MAP2-immunoreactive dendrites was significantly higher in the H₂, TTM, and TTM+H₂ groups compared with the control group, suggesting maintenance of normal structural integrity of CA1 dendrites (Figure 6B). There were significantly more Iba1-positive activated microglia/macrophages in the CA1 in the control and TTM groups compared with the other groups (Figure 6C).

We also evaluated neuronal degeneration and astrocyte activation in the cerebral cortex by FJC and GFAP...
immunostaining. FJC+ degenerating neurons and GFAP+ reactive astrocytes were conspicuous in the control group, whereas the TTM+H₂ group showed significantly fewer degenerating neurons than the control group (Figure 7).

**Discussion**

Inhalation of H₂ begun before ROSC has been shown to improve animal survival and NDSs in a rat model of CA to an extent comparable to that of TTM (33°C). In this study, we demonstrated a beneficial effect of H₂ inhalation begun after ROSC, even in the absence of hyperoxia. H₂-treated animals exhibited improved NDS scores, with correlative reductions in neuronal degeneration and microglial activation in regions typically vulnerable to ischemic injury.

To date, therapeutic hypothermia is the only approach proven to improve outcome in patient with PCAS when applied after ROSC. However, Nielsen et al. recently reported that therapeutic hypothermia at a target temperature of 33°C did not confer a benefit compared with a target temperature of 36°C in patients with PCAS. One explanation for the lack of benefit at the lower temperature is that improvement in patient intensive care over the past decade reduces any potential incremental benefit of a single intervention. From the viewpoint of translating the present results into clinical benefit, it is particularly important that the combined therapy of TTM and H₂ inhalation was more effective than TTM alone. The NDS at 24, 48, and 72 hours after ROSC demonstrated improved neurological outcome in the TTM+H₂ group compared with the TTM group.

Motor activity was also better in the TTM+H₂ group than in the TTM group. Consistent with this, degenerative neuronal changes in the cerebral cortex and survival rate at 7 days after ROSC were both significantly improved by the TTM+H₂ therapy compared with the control group but not by TTM alone.

The patients in the Hypothermia After Cardiac Arrest trial were allowed to passively cool to a temperature of 32°C to 34°C and were maintained as such for 24 hours, with passive rewarming occurring over a median period of 8 hours. In the Bernard et al. study, patients were treated with TTM of 33°C for 12 hours and active rewarming over a period of 6 hours. In an animal study, Ye et al. have demonstrated that a shorter duration of mild hypothermia, induced rapidly and early after ROSC, improved postresuscitation microcirculation, myocardial and cerebral functions, and survival as well as, or better than, prolonged hypothermia in a rat model of CPR. Taking into account the high metabolic rate of rats compared with humans, we chose a 2-hour duration for therapeutic hypothermia, followed by 2 hours of rewarming.

Previously, we demonstrated that post-CA rats die of systemic ischemia/reperfusion injury. In that study, the left ventricular end-diastolic pressure gradually increased to ≥20 mmHg at 2 hours after ROSC in the control group. Histological analyses at 24 hours after ROSC exhibited contraction band necrosis, coagulation necrosis with cytoplasmic eosinophilia, loss of nuclei, and vacuolar degeneration surrounded by inflammatory cell infiltration in the myocardium of the post-CA control rats, all effects that could be associated with increased water content in the lung. The early death in these post-CA rats was also associated with...
a systemic inflammatory response as shown by the increased serum interleukin-6 levels at 2 hours after ROSC and impaired neurological function based on the NDS at 24 hours after ROSC. The early mortality of control group post-CA rats was comparable to other recently published findings. Using a rat model of 6-minute VF CA, Ma et al\textsuperscript{34} showed that 4 of 10 animals survived >72 hours, whereas Janata et al\textsuperscript{18} showed better outcomes than ours. However, the experimental protocol used by Janata et al was different from ours in that the VF CA was induced by a 1-minute impulse, and if spontaneous defibrillation occurred, an additional 15-second impulse was delivered. In addition, after ROSC, sodium bicarbonate was given to treat metabolic acidosis and crystalloids, whereas boluses of vasopressin 0.005 IU/kg and epinephrine 5 $\mu$g/kg were given intravenously to keep MAP >60 mm Hg. In contrast, we performed constant electric stimulation of the epicardium for 3 minutes, and there was no spontaneous reversion of VF.

The mode of action of H\textsubscript{2} therapy is primarily suppression of oxidative stress. Cellular redox homeostasis is maintained by a delicate balance between ROS production and antioxidant defences.\textsuperscript{35} When ROS are produced excessively or endogenous antioxidant capacity is diminished, indiscriminate oxidation leads to potentially damaging oxidative stress. The hydroxyl radical (•OH) is a major trigger of the chain reaction forming free radicals,\textsuperscript{36} and once occurring on biomembranes, it continues and expands, causing serious cellular damage. In contrast, H\textsubscript{2}O\textsubscript{2} and •NO function as signaling molecules that induce antioxidant enzymes. Notably, H\textsubscript{2} reacts with strong oxidants such as •OH in cells but remains mild enough to neither disturb metabolic redox reactions nor affect signaling ROS. In addition, H\textsubscript{2} rapidly diffuses into tissues and cells. Thus, H\textsubscript{2} can be used as an effective antioxidant therapy.\textsuperscript{37} Inhaled H\textsubscript{2} acts rapidly; therefore, it may also be suitable as a defense against acute oxidative stress elicited by ischemia/reperfusion.\textsuperscript{1,4,11} Inhaled H\textsubscript{2} at therapeutic doses has no adverse effects on the saturation level of arterial oxygen ($S_{\text{O}}2$) or hemodynamic parameters, including blood pressure, heart rate, and left ventricular pressure.\textsuperscript{4} H\textsubscript{2} may accumulate in the lipid phase more than in the aqueous phase, especially in unsaturated lipid regions, which are the major target of the radical chain reaction. Thus, H\textsubscript{2} may confer an advantage in suppressing the radical chain reaction, avoiding lipid peroxide production and the consequent generation of oxidative stress markers such as 4-hydroxyl-2-nonenal and malondialdehyde. Additionally, OH• can modify deoxy-guanine (dG) to 8-OHdG. Indeed, we showed that inhalation of H\textsubscript{2} gas decreased these oxidative markers in rat models of cardiac ischemia/reperfusion injury and PCAS.\textsuperscript{4,12} H\textsubscript{2} inhalation was protective and, in some aspects, superior to the use of hypothermia in other studies.\textsuperscript{4,12} For example, H\textsubscript{2} gas inhalation, but not therapeutic hypothermia, prevented

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7.png}
\caption{Effect of H\textsubscript{2} and targeted temperature management (TTM) on neuronal death and astrocyte activation in the cerebral cortex at 7 days after cardiac arrest. Representative photomicrographs of the immunohistochemical localization of DAPI, Fluoro-Jade C (FJC), and glial fibrillary acidic protein (GFAP) staining in cerebral cortex. A, E, I, M, and Q, Nuclear DNA was labeled with DAPI (blue); B, F, J, N, and R, degenerating neurons were labeled with FJC (green); C, G, K, O, and S, reactive astrocytes were labeled with GFAP (red); and D, H, L, P, and T, merged images. n=3 to 6. Bar, 50 μm. *P<0.01 vs the sham-operated group; †P<0.01 GFAP vs the control (CTL) group. The bars represent the mean and SEM.}
\end{figure}
PCAS-associated increases in left ventricular end-diastolic pressure and serum interleukin-6 at 2 hours after ROSC. These results are surprising because therapeutic hypothermia is believed to confer protection against reperfusion injury by multiple mechanisms, including suppression of free radicals, enzymes, and excitatory and inflammatory reactions, in addition to the direct physical protection of membranes. In contrast, the cornerstone of H₂ therapy is selective ROS attenuation.

The reduction of oxidative stress by H₂ may lead to various effects, including anti-inflammatory and antiapoptotic responses via changes in gene expression, signal transduction, and mitochondrial membrane potential, although H₂ has a mode of action independent of its antioxidative properties. In a rat model of hyperoxic lung injury, H₂ reduced mRNA levels encoding several proinflammatory cytokines, including interleukin-1β, interleukin-6, tumor necrosis factor-α, and intercellular adhesion molecule-1, in the lung. Inhalation significantly improved the survival rate and organ damage in a mouse model of cecal ligation and puncture–induced sepsis, as well as zymosan-induced generalized inflammation. H₂ also has antiapoptotic properties such as mitigating hyperoxia-induced lung epithelial cell apoptosis via the induction of Bcl-2 protein and suppressing hyperoxia-mediated induction of Bax protein. Inhalation of H₂ also reduced infarct size in a canine model of cardiac ischemia/reperfusion injury via opening of the mitochondrial Kₐ₅₆ channels followed by inhibition of mitochondrial permeability transition pores.

We recognize several limitations of our study. First, the small rodent brain has metabolic and rheological properties that are different from those in the complex human brain. Findings analogous to those from the rat CPR model are yet to be demonstrated in large-animal and clinical studies. Second, pentobarbital used for anesthesia may adversely affect basic cardiac function. Although there was no difference among experimental groups in the pentobarbital dose or the time from premedication dosing to onset of CA, hypothermia might reduce the systemic clearance of pentobarbital and thus affect myocardial contractility. Third, the rats in the TTM group were subjected to hypothermia for only 2 hours. Such a short duration of hypothermia has not been replicated in larger animals or preclinical work. Further studies are required before clinical application, especially if such a short therapeutic window is being proposed. Fourth, the experimental design does not allow conclusions to be drawn about the most appropriate concentrations of H₂ or whether increased duration of H₂ ventilation would provide a greater degree of protection. Finally, it is possible that greater differences between TTM and H₂ therapy may have been revealed with a larger number of animals or with a longer duration of therapy.

Conclusions
We have demonstrated for the first time that H₂ inhalation begun after normoxic resuscitation improves neurological outcome independently of TTM in a rat model of CA. Our findings suggest a potentially novel and easily applicable solution to PCAS. Although further investigation is required, H₂ is expected to be an innovative therapeutic tool for unmet medical needs that currently constitute a considerable health burden, particularly for critically ill patients. This study may pave the way for successful translation of H₂ inhalation therapy into clinical practice.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL
**Supplementary Table 1. Variables at baseline and during cardiopulmonary resuscitation**

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<th>Variable</th>
<th>Ctl (n = 13)</th>
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<th>TTM (n = 7)</th>
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<td>Total administration of defibrillation, n</td>
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<td>1.3 ± 0.3</td>
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</table>

Ctl, control; TTM, targeted temperature management; BL, baseline; HR, heart rate; MAP, mean arterial blood pressure; B.E., base excess; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. CA, cardiac arrest; DBP, diastolic blood pressure; s, second. Values expressed as mean ± SEM.
**Supplementary Table 2.** Arterial blood gas and chemistries analyses at baseline and during post-CA care after ROSC

<table>
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<td>7.35 ± 0.02</td>
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<td>7.37 ± 0.01</td>
<td>7.39 ± 0.02</td>
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<tr>
<td>Ctl</td>
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<td>103 ± 7</td>
<td>95 ± 5</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>H₂</td>
<td>74 ± 5</td>
<td>99 ± 7</td>
<td>93 ± 4</td>
<td>97 ± 4</td>
</tr>
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<td>TTM</td>
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<td>112 ± 8</td>
<td>111 ± 5</td>
<td>116 ± 6</td>
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<td>114 ± 5</td>
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<td>36 ± 2</td>
<td>36 ± 3</td>
<td>32 ± 2</td>
</tr>
<tr>
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<td>33 ± 1</td>
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<td>36 ± 2</td>
<td>39 ± 2</td>
</tr>
<tr>
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<td>39 ± 3</td>
<td>36 ± 3</td>
<td>37 ± 3</td>
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<td>-6.6 ± 1.4</td>
<td>-7.4 ± 1.2</td>
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<td>-4.6 ± 0.8</td>
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<td>2.2 ± 0.2</td>
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<td>2.1 ± 0.6</td>
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</tr>
</tbody>
</table>

Ctl, control; TTM, targeted temperature management. Values expressed as mean ± SEM. There were no significant differences for treatment by time among the four groups by mixed-effects model for repeated-measures analyses.
**Supplementary Table 3.** Neurological Deficit Score of survivors at 7 d after cardiac arrest

<table>
<thead>
<tr>
<th></th>
<th>Ctl</th>
<th>H₂</th>
<th>TTM</th>
<th>TTM + H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of survivors</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>NDS at 7d</td>
<td>193 ± 56</td>
<td>33 ± 19*</td>
<td>59 ± 35*</td>
<td>20 ± 16**</td>
</tr>
</tbody>
</table>

Ctl, control; TTM, targeted temperature management. Values expressed as mean ± SEM.

Significant differences: *P < 0.05, **P = 0.01 compared to the Ctl group.