may pass through blood-brain barrier and improve the metabolic condition in the brain in our patient. We have proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm [8], (b) pyruvate can activate the pyruvate dehydrogenase complex (PDHC) by inhibiting the pyruvate dehydrogenase kinase [8,9], and (c) pyruvate can scavenge the hydrogen peroxide by a non-enzymatic reaction [15]. Pyruvate improved the hemodynamic condition by intracoronary infusion in patients with congestive heart failure [16,17], or the neurological recovery following cardiopulmonary arrest and resuscitation [18]. In our patient, we determined the daily supplement of pyruvate by the presence of diarrhea as adverse effects or by the capacity of amount of oral administration. In our patient, daily administration of sodium pyruvate resulted in 0.5d/kg/day TID. The exact pharmacological mechanisms why serum pyruvate is also decreased after the pyruvate therapy, have to be clarified in future study, by using proteome analysis or comprehensive multiple analysis of total cell metabolism.

Considering the progressive nature of LS, pyruvate may prevent the neurodegeneration and lactic acidosis in our patient. Though the efficacy of pyruvate on LS will be evaluated by randomized double-blind placebo-controlled study design in future, pyruvate therapy is a possible candidata for therapeutic choice for currently incurable mitochondrial disorders such as LS.

Acknowledgements

Authors thank to the Prof. Shinichi Hirose for treating the initial therapy and for providing the initial laboratory data. This work was supported in part by Grants #13670853 (Y.K.), and #16390308 (Y.K.) from the Ministry of Culture and Education in Japan, and #CCT-B-1803 (Y.K.) from Evidence-based Medicine, Ministry of Health, Labor and Welfare in Japan. N.P. is a recipient of grant in aid for young investigator from Heiwa Research Foundation in Japan. This work was supported in part by a grants from the program Grants-in-Aid for Scientific Research [A-22240072, B-21390459, and C-21590411 to M.T.]; by Grant 20B-13 from the program Research Grants for Nervous and Mental Disorders of the Ministry of Health, Labour, and Welfare (to Y. K. and M.T.); and by grants for scientific research from the Takeda Science Foundation (to M.T.)

References

[1] Leigh D. Subacute necrotizing encephalomyelopathy in an infant. J Neurol Neurosurg Psychiatry 1951;14:216–21.
[2] Finsterer J. Leigh and Leigh-like syndrome in children and adults. Pediatr Neurol 2008;39:223–35.
[3] Robinson BH. Lactic acidemia and mitochondrial disease. Mol Genet Met 2006;89:3–13.
[4] Rahman S, Blok RB, Dahl HH, Danks DN, Kirby DM, Chow CW, et al. Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 1996;39:343–51.
[5] Morris AA, Leonard JV, Brown GK, Bidouki SK, Bindoff LA, Woodward CE, et al. Deficiency of respiratory chain complex I is a common cause of Leigh disease. Ann Neurol 1996;40:25–30.
[6] Naito E, Ito M, Yokota I, Saijo T, Ogawa Y, Kuroda Y. Diagnosis and molecular analysis of three male patients with thiamine-responsive pyruvate dehydrogenase complex deficiency. J Neurol Sci 2002;201:33–7.
[7] Quinzii CM, Hirano M, DiMauro S. CoQ10 deficiency diseases in adults. Mitochondrion 2007;7:S122–6.
[8] Tanaka M, Nishigaki Y, Fuku N, Ibi T, Sahashi K, Koga Y. Therapeutic potential of pyruvate therapy for mitochondrial diseases. Mitochondrion 2007;7:399–401.
[9] Komaki H, Nishigaki Y, Fuku N, Hosoya H, Murayama K, Ohtake A, et al. Pyruvate therapy on Leigh syndrome due to cytochrome *c* oxidase deficiency. Biochim Biophys Acta 2010;1800:313–5.
[10] Naito E, Ito M, Takeda E, Yokota I, Yoshijima S, Kuroda Y. Molecular analysis of abnormal pyruvate dehydrogenase in a patient with thiamine-responsive congenital lactic acidemia. Pediatr Res 1994;36:340–6.
[11] Matsuda J, Ito M, Naito E, Yokota I, Kuroda Y. DNA diagnosis of pyruvate dehydrogenase deficiency in female patients with congenital lactic acidemia. J Inherit Metab Dis 1995;18:534–46.
[12] Koga Y, Akita Y, Junko N, Yatsuga S, Povalko N, Fukiyama R, et al. Endothelial dysfunction in MELAS was improved by L-arginine supplementation. Neurology 2006;66:1766–9.
[13] Kaufmann P, Engelstad K, Wei Y, Jhung S, Sano MC, Shungu DC, et al. Dichloroacetate causes toxic neuropathy in MELAS: a randomized, controlled clinical trial. Neurology 2006;66:324–30.
[14] Miller JP, Oldendof WH. Regional kinetic constants for blood–brain barrier pyruvic acid transport in conscious rats by the monocarboxylic acid carrier. J Neurochem 1986;46:1412–6.
[15] Long LH, Halliwell B. Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. Biochem Biophys Res Commun 2009;388:700–4.
[16] Hermann HP, Pieske B, Schwarzmuller E, Keul J, Just H, Hasenfuss G. Haemodynamic effects of intracoronary pyruvate in patients with congestive heart failure: an open study. Lancet 1999;353:1321–3.
[17] Mallet RT, Sun J, Knott EM, Sharma AB, Olivencia-Yurvati AH. Metabolic cardioprotection by pyruvate: recent progress. Exp Biol Med 2005;230:435–43.
[18] Sharma AB, Barlow MA, Yang S-H, Simpkins JW, Mallet RT. Pyruvate enhances neurological recovery following cardiopulmonary arrest and resuscitation. Resuscitation 2008;76:108–19.

Biochimica et Biophysica Acta 1820 (2012) 551–552

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Preface

Biochemistry of mitochondria, life and intervention 2010

Mitochondrial research and medicine have been continuously expanded for the last 40 years. Since mitochondria play a central role in the metabolism of carbohydrates, lipids, and amino acids, alterations of mitochondrial functions have been implicated in various human disorders, such as mitochondrial myopathy, diabetes mellitus, aging-process, Alzheimer's disease, Parkinsonism, cancer, atherosclerosis, obesity, and metabolic syndrome.

Recent developments of clinical research and medicine indicate that the many human disorders have a link to mitochondrial function and possible to indicate the therapeutic application for cure of the disorders. ROS production from the respiratory chain plays pivotal roles not only in the control of proliferation and differentiation of cells but also in the regulation of mitochondrial mass in the cell. ROS is also related with aging and cartinogenesis. Molecular phathophysiology of maintenance of mitohondria is also discovered as fission and fusion mechanism, which are related to the quality control of mitochondria (mitophagy) seen in Parkinsonism. Many animal models are created by KO mice and are investigated the pathophysiology of disorders. Therapeutic clinical approaches are also investigated such as L-arginine on MELAS, sodium pyruvate for lactic acidosis, and hydrogen water for mitochondrial disorders. Assisted reproductive technology for mitochondrial disease patients is well developed in the fields to apply the clinical application. Such fundamental studies of mitochondrial bioenergetics could apply the new therapeutic indication for mitochondrial disorders.

In this special issue of BBA-general on " Biochemistry of Mitochondria, Life and Intervention 2010" which contains selected papers from 7th annual meeting of Asian Society for Mitochondrial Research and Medicine and 10th J-mit (Japanese Society of Mitochondrial Research and Medicine), we discuss the new aspect of mitochondrial functions relating to human disorders, and possible and on-going therapeutic approach of human disorders. This issue is organized in five chapters as follows: (i) Update mitochondrial research field, (ii) Mitophagy (fission and fusion), (iii) Animal model of mitochondrial disorders, (iv) Therapeutic approach of mitochondrial disorders, and (v) Mitochondrial pathophysiology in atherosclerosis, cancer, and aging.

Dr. Yasutoshi Koga is a professor of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Japan. After he completed the MD and PhD, he joined the Mitochondrial Research Group in 1990 as a post doctoral research fellow granted by Muscular Dystrophy Association at the Department of Neurology, College of Physicians and Surgeons of Columbia University (Profs. DiMauro and Schon EA), where he directed his research to mitochondrial genetics especially pathogenic mechanism of MELAS. This led to the development of rho-zero cybrid system in mitochondrial research in 1992. He is the vice-president of Asian Society of Mitochondrial Research and Medicine and is organizing the Joint Symposium of 7th Asian Society of Mitochondrial Research and Medicine, and 10th Japanese Society for Mitochondrial Research and Medicine in 2010 at Fukuoka, Japan. He pioneered the development of a novel therapeutic procedure for MELAS and has completed the investigator-mediated clinical trial of L-arginine on MELAS. He received the Kelsey Wright Award from United Mitochondrial Disease Association (USA) in 2008. He now become a core committee member of International Mitochondrial Research and Medicine especially therapeutic division.

Shigeo Ohta *PhD.*
(Born on June 2, 1951, 58 year-old)
(male and married)
Present Status:
Professor and Chairman of Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School
Education
1974: Graduated from Faculty of Science, the University of Tokyo
1979: PhD degree, Graduate School of Pharmaceutical Sciences, the University of Tokyo
Professional history:
1979–1980: Research associate of School of Medicine Gunma University
1980–1982: Assistant Professor of School of Medicine Gunma University
1981–1985: Research associate of Biocenter, Basel University, Switzerland
1985–1991: Assistant Professor of Jichi Medical School
1991–1994: Associate Professor of Jichi Medical School
1994–present: Professor and Chairman of Department of Biochemistry, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School
Editors
Associate Editor of *Mitochondrion* (2005–present)
Associate Editor of *Journal of Alzheimer's Disease* (2005–2007)
Associate Editor of *Medical Gas Research* (2010–)
Committee
The president of The Japanese Society of Mitochondrial Research and Medicine
The president of The Japanese Society for Cell Death Research
A committee member of The Japanese Society of Biochemistry

0304-4165/$ – see front matter
doi:10.1016/j.bbagen.2012.01.008

Main Scientific fields:
Molecular and Cellular Biology
Mitochondriology
Molecular Biology on Oxidative stress
Hydrogen medicine
On November 18, 2011

Dr. Yau-Huei Wei graduated in June 1974 from the Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan. He joined the laboratory of the late Professor Tsoo E. King in 1976 and earned his PhD degree in 1980 from the Department of Chemistry, State University of New York at Albany, New York, USA. He returned to Taiwan after one year of postdoctoral training at the Departments of Chemistry and Physics, SUNY-Albany. He was appointed as an associate professor during 1981–1985 at the Department of Biochemistry, National Yang-Ming Medical College, Taipei, Taiwan. He was promoted to full professor in 1985 and served as the chairman of the department until 1991. He also served as the Director, Common Instrumentation Center (1986–1989) and the Dean of Student Affairs (1989–1991) of the College. Dr. Wei was appointed as the Director General, Department of Life Sciences, National Science Council of Taiwan, 2001–2005. He served as the Dean of Academic Affairs (2006–2008) and was a Distinguished Professor (2007–2009), National Yang-Ming University. In August 2009, Dr. Wei was appointed the founding President of Mackay Medical College. He has actively participated in the promotion of international collaboration in biomedical research and mitochondrial medicine. Dr. Wei was one of the founding members of Asian Society for Mitochondrial Research and Medicine, and was the Vice-President (2002–2005) and President (2005–2008) of the Society. He has been the President of Taiwan Society for Mitochondrial Research and Medicine (2006–2012). Since 2006, Dr. Wei has served on the editorial board of Biochimica et Biophysica Acta-General Subjects. Dr. Wei's major research has focused on "Molecular and cellular biology studies of mitochondrial diseases, cancer and age-related diseases" and "The cross-talk between mitochondria and the nucleus and metabolic shift in the differentiation of stem cells". He was among the few investigators to show that mitochondrial function decline and mitochondrial DNA mutations are important contributory factors of human aging. His research team was one of the earliest groups to demonstrate that oxidative stress and oxidative damages elicited by mitochondrial DNA mutations contributes to the pathophysiology of many mitochondrial disorders. In the past few years, Dr. Wei and his students have established that mitochondrial biogenesis and respiratory function as well as antioxidant enzymes are upregulated in a coordinate manner in the process of differentiation of stem cells. Dr. Wei and his students have published in SCI journals ~300 research papers and ~30 review articles and book chapters in the fields of bioenergetics, mitochondrial medicine, free radical biology and medicine, molecular and cellular biology, male infertility, and aging research.

Yasutoshi Koga
Guest editor
Kurume University Graduate School of Medicine, Japan
Corresponding author. Tel.: +81 942 31 7565; fax: +81 942 38 1792.
E-mail address: yasukoga@med.kurume-u.ac.jp

Masashi Tanaka
Co-editor
Tokyo Metropolitan Institute of Gerontology, Japan

Shigeo Ohta
Co-editor
Nippon Medical School, Japan

Yau-Huei Wei
Co-editor
National Yang-Ming University, Taiwan

ORIGINAL RESEARCH

American Heart Association | American Stroke Association

H_2 Gas Improves Functional Outcome After Cardiac Arrest to an Extent Comparable to Therapeutic Hypothermia in a Rat Model

Kei Hayashida, MD; Motoaki Sano, MD, PhD; Naomi Kamimura, PhD; Takashi Yokota, PhD; Masaru Suzuki, MD, PhD; Yuichiro Maekawa, MD, PhD; Akio Kawamura, MD, PhD; Takayuki Abe, PhD; Shigeo Ohta, PhD; Keiichi Fukuda, MD, PhD; Shingo Hori, MD, PhD

Background—All clinical and biological manifestations related to postcardiac arrest (CA) syndrome are attributed to ischemia–reperfusion injury in various organs including brain and heart. Molecular hydrogen (H_2) has potential as a novel antioxidant. This study tested the hypothesis that inhalation of H_2 gas starting at the beginning of cardiopulmonary resuscitation (CPR) could improve the outcome of CA.

Methods and Results—Ventricular fibrillation was induced by transcutaneous electrical epicardial stimulation in rats. After 5 minutes of the subsequent CA, rats were randomly assigned to 1 of 4 experimental groups at the beginning of CPR: mechanical ventilation (MV) with 2% N_2 and 98% O_2 under normothermia (37°C), the control group; MV with 2% H_2 and 98% O_2 under normothermia; MV with 2% N_2 and 98% O_2 under therapeutic hypothermia (TH), 33°C; and MV with 2% H_2 and 98% O_2 under TH. Mixed gas inhalation and TH continued until 2 hours after the return of spontaneous circulation (ROSC). H_2 gas inhalation yielded better improvement in survival and neurological deficit score (NDS) after ROSC to an extent comparable to TH. H_2 gas inhalation, but not TH, prevented a rise in left ventricular end-diastolic pressure and increase in serum IL-6 level after ROSC. The salutary impact of H_2 gas was at least partially attributed to the radical-scavenging effects of H_2 gas, because both 8-OHdG- and 4-HNE-positive cardiomyocytes were markedly suppressed by H_2 gas inhalation after ROSC.

Conclusions—Inhalation of H_2 gas is a favorable strategy to mitigate mortality and functional outcome of post-CA syndrome in a rat model, either alone or in combination with TH. **(*J Am Heart Assoc.* 2012;1:e003459 doi: 10.1161/JAHA.112.003459)**

Key Words: cardiac arrest • cardiopulmonary resuscitation • hydrogen gas • therapeutic hypothermia • ventricular fibrillation

Despite advances in the management of patients who suffer a nontraumatic cardiac arrest (CA), survival rates remain low, and many survivors are left with neurological and cardiac sequelae.[1] Post-CA syndrome, including neurological dysfunction, cardiac damage, and "sepsis-like" systemic inflammation, is likely to contribute to the multisystem organ dysfunction and ultimate demise of many CA victims.[2] Therapeutic hypothermia (TH) is widely accepted as the gold-standard method to improve survival and limit neurological outcomes in patients who achieve return of spontaneous circulation (ROSC) after CA. Despite that, it is still underutilized.[3] Thus, the development of alternative approaches with or without TH is an unmet medical need in ameliorating the prognosis of post-CA patients.

Molecular hydrogen (H_2) has many potential therapeutic applications as a novel antioxidant.[4,5] Since the first article reporting H_2 effects, in *Nature Medicine* in 2007,[6] the protective effects of H_2 have been confirmed in different animal models, including limiting the infarct volume of brain[6] and heart[7] by reducing ischemia–reperfusion injury without altering hemodynamic parameters and providing protection against multiple-organ damage elicited by generalized inflammation.[8] There are also some preliminary clinical data on this topic.[9–17]

All clinical and biological manifestations related to post-CA syndrome are attributed to ischemia–reperfusion injury in various organs including brain and heart. This study tested the hypothesis that inhalation of H_2 gas during hyperoxic resuscitation can improve CA outcome. TH was chosen as the gold standard endorsed by professional societies and backed up by a significant body of evidence.[18–25] We subjected rats to 5 minutes of ventricular fibrillation (VF) cardiac arrest (CA), followed by therapeutic hypothermia (TH), H_2 treatment, or a

From the Departments of Emergency and Critical Care Medicine (K.H., M. Suzuki, S.H.) and Cardiology (M. Sano, Y.M., A.K., K.F.), Center for Clinical Research (T.A.), School of Medicine, Keio University, Tokyo, Japan; Department of Biochemistry and Cell Biology, Institute of Development and Aging Science, Graduate School of Medicine, Nippon Medical School, Kanagawa, Japan (N.K., T.Y., S.O.).

Correspondence to: Motoaki Sano, MD, PhD, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan. E-mail: msano@a8.keio.jp

Received June 7, 2012; accepted August 20, 2012.

combination of both. Controls were subjected to normothermic conditions. All groups were ventilated with 98% O_2.

Materials and Methods

Animal Preparation

Fifteen-week-old male Wistar ST rats weighing an average of 373 g were used according to institutional approval by the Animal Ethics Committee. Rats were housed in a rodent facility under a 12-hour light–dark cycle during this study.

For experiments, rats were fasted overnight except for free access to water and then anesthetized with an intraperitoneal injection of pentobarbital sodium (45 mg/kg). The surgical procedures were carried out as previously described.[19,20] The tracheas of the animals were intubated through a tracheostomy with a 14-gauge cannula and mechanically ventilated with a tidal volume (TV) of 0.65 mL/100 g, a respiratory rate (RR) of 100/min, and an FiO_2 of 0.21 (Ventilator: SN-480-7, Shinano, Japan). Polyethylene catheters (PE50, Natsume, Japan) were inserted into the left femoral artery and vein and flushed intermittently with saline solution containing 2.5 IU/mL bovine heparin. Arterial blood pressure was measured, and an electrocardiogram was recorded by subcutaneous needle electrodes. Core temperature was monitored by a rectal temperature probe (BAT-10, Physitemp Instruments Inc) and maintained by a heating plate (SCP-85, AsOne, Japan) throughout the experiment to ensure appropriate temperature management.

Ventricular Fibrillation and CPR

Ventricular fibrillation (VF) was induced by electrical stimulation via a transthoracic epicardium electrode, as previously described.[26] The stimulator (Isostim, World Precision Instrument Inc) was used to perform direct and constant electrical stimulation of the epicardium with crude current, continuous single stimulation, a delay of 100 ms, a wave width of 1 ms, a frequency of 50 Hz, an intensity of 1 mA, and a stimulation duration of 3 minutes. Five minutes after initiation of VF, advanced cardiac life support was started; the rats were ventilated (0.65 mL/100 g, 100 breaths/min), and then chest compressions (200/min) were started by a finger of the same investigator using a metronome assistant. Adrenalin (2 μg/100 g) and 0.1 mL sodium bicarbonate (8.4%) were immediately administered to the rats at the beginning of CPR. Repeated doses were administered at 3-minute intervals as needed. Defibrillation (Nihon Koden, Tokyo, Japan) was performed with direct-current single-phase wave 3 J if the electrocardiogram displayed VF 1 minute after CPR. If the defibrillation failed, CPR was repeated, and defibrillations were again performed 1 minute after CPR. If the spontaneous circulation of the rats was not restored after 10 minutes with the above treatment, CPR was considered a failure.

After ROSC, rats were mechanically ventilated and invasively monitored for 2 hours in maintaining the target temperature. Rats were continuously given 1 mL/h isotonic saline for 2 hours after ROSC. After a recovery period of 2 hours, telemetry probes (Mini mitter, Respironics Inc) were implanted into the inguinal cavities to monitor activity. Rats were then weaned from the ventilator, extubated, and returned to their cages with easily accessible food and water. The survival time after CPR was recorded up to 72 hours.

Experimental Protocol

Rats were randomly assigned to 1 of 4 experimental groups when mechanical ventilation (MV) was resumed at the beginning of CPR: MV with 2% N_2 and 98% O_2 at normothermia (the control group), MV with 2% H_2 and 98% O_2 at normothermia (H_2 group), MV with 2% N_2 and 98% O_2 in targeting therapeutic hypothermia (the TH group), and MV with 2% H_2 and 98% O_2 in targeting TH (the H_2+TH group) (Figure 1). The concentration of H_2 in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERAMECS, Kyoto, Japan).

Baseline variables (weight, blood pressure, heart rate, body temperature, and preparation time) were the same in the 4 groups (Table 1).

The concentration of 2% H_2 was determined on the basis of previous observations[7] as an optimal and safe concentration. Rats under ventilation received the respective gases via the tracheal tube until 2 hours after ROSC. In animals assigned to TH, body cooling was initiated coincident with the start of CPR. Rectal temperature was measured with a digital thermometer (BAT-10, Physitemp Instruments Inc) and taken as the body temperature, which was reduced to 33°C within 15 minutes with the aid of ice packs and a cooling plate (SCP-85, AsOne, Japan). Once reached, the target temperature was maintained for 2 hours after CPR and then returned to 37°C over a rewarming period of 30 to 60 minutes. For those animals not subjected to cooling, rectal temperature was maintained at 37°C using a hot plate (SCP-85, AsOne, Japan) for 1 hour after resuscitation. The analytical methods comprised 3 parts. In part 1, rats were divided randomly into 4 groups as described above; their neurological deficits were evaluated 24 and 48 hours after ROSC and their survival rate monitored up to 72 hours after ROSC. Arterial blood for blood gas analysis and peripheral venous blood was obtained at baseline and 10, 60, 90, and 120 minutes after ROSC. In part 2, myocardial functional recovery was monitored during the first 2 hours after ROSC, and then serum samples were obtained to measure cytokines level. In part 3, the sham-operated animals and the resuscitated animals, with or

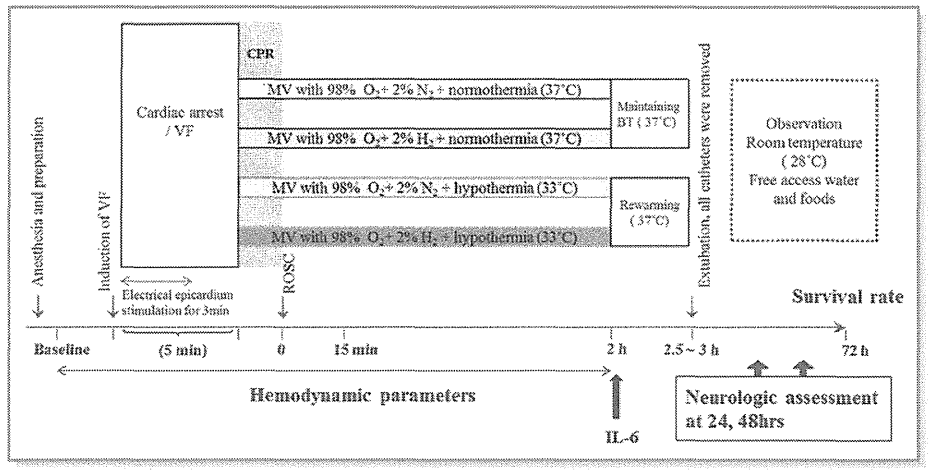

Figure 1. Experimental protocol of CPR and postresuscitation care in VF-induced cardiac arrest model. When mechanical ventilation was resumed (start CPR), rats were assigned to 1 of 4 experimental groups: mechanical ventilation (MV) with 98% O_2+2% nitrogen (N_2), the control group; MV with 98% O_2+2% hydrogen (H_2), the H_2 group; MV with 98% O_2+2% N_2+therapeutic hypothermia (TH), the TH group; and MV with 98% O_2+2% H_2+TH, the H_2+TH group. VF indicates ventricular fibrillation; MV, mechanical ventilation; BT, body temperature; and ROSC, return of spontaneous circulation.

Table 1. Baseline Physiological Variables

	Control (n=14)	H_2 (n=14)	TH (n=14)	H_2+TH (n=13)
Weight, g	373.6±6.1	367.5±7.9	376.4±8.6	375.9±4.8
Preparation time, minutes	42.4±1.2	41.7±0.8	40.1±0.8	40.1±1.2
HR at baseline, bpm	398±9	377±10	399±6	361±25
MBP at baseline, mm Hg	133±3	124±5	122±5	120±5
Temperature at baseline, °C	36.0±0.0	37.0±0.0	36.9±0.0	36.9±0.0

Values expressed as mean±SEM. TH indicates therapeutic hypothermia; HR, heart rate; MBP, mean blood pressure; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation.

without H_2 inhalation, were decapitated 24 hours after ROSC. The same surgical procedures, monitoring, and control of body temperature except for the induction of VF were done for the sham animals. Wet-to-dry weight ratios were measured for lung. Histopathological analyses of heart were performed to evaluate the favorable effect of H_2 gas inhalation.

Neurological Deficit Evaluation

Neurological deficit score (NDS) evaluations were performed by a single investigator who was unaware of group assignment. Consciousness and breathing, cranial nerve reflexes, motor function, sensory function, and coordination were scored according to an NDS system (0% to 100% scale; 0 normal, 100 brain death), as described previously.[27]

Evaluation of Postresuscitation Myocardial Function

A Millar transducer catheter (SPR-320) was placed in the left ventricle (LV) cavity via the right internal carotid artery to monitor LV pressure using the polygraph system (Power Lab, ADInstrument, Castle Hill, Australia) before the induction of VF. LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and peak positive dP/dt and negative dP/dt were monitored during the first 2 hours after ROSC.

Measurement of Serum IL-6 Level

Serum concentrations of IL-6 were measured 2 hours after ROSC in the 4 experimental groups and a sham-operated control group without VF with an ELISA Kit (OptEIA, BD Biosciences) according to the manufacturer's instructions.

Lung Water Content Determined by Wet–Dry Method

Twenty-four hours after ROSC, the right lower lobes of the lungs were removed in a standard fashion, weighed immediately after removal, and then placed in a laboratory oven (60°C) for slow evaporation over 72 hours. The dried samples were weighed, and the water content (%) was calculated as (wet weight−dry weight)/(wet weight)×100%.

Histopathological Analysis

Twenty-four hours after ROSC, the rats were decapitated. The hearts were quickly removed and fixed with Zamboni's solution. Coronal tissue slices (6-μm thickness) of the hearts (at the level of the left ventricle papillary muscle) were stained with hematoxylin and eosin and/or Azan–Mallory for histological evaluation.

For immunohistochemistry, the fixed sections were immunostained overnight at 4°C using a mouse monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE; Japan Institute for the Control of Aging, NIKKEN SEIL Co, Ltd) to assess lipid peroxidation or a mouse monoclonal antibody against 8-hydroxy-deoxyguanosine (8-OHdG; Japan Institute for the Control of Aging, NIKKEN SEIL Co, Ltd) to detect the extent of nucleic acid oxidation.

In each Azan–Mallory-, 4-HNE-, and 8-OHdG-stained section, 4 slide fields were randomly examined using a defined rectangular field area (0.14 mm^2). Images of Azan and 4-HNE staining were analyzed using Adobe Photoshop CS 5.1, and data of each staining section are reported as fibrotic tissue area stained blue (%) and 4-HNE relative intensity area (%) using automated counting software (Image J 1.46r, National Institute of Health). The 8-OHdG-positive cells were counted using automated counting software (Image J 1.46r, National Institute of Health), and the data were represented as the number of 8-OHdG-positive cells per field. A fluorescence microscope (Biorevo BZ-9000, Keyence, Osaka, Japan) was used for imaging.

Statistical Analysis

Continuous variables are expressed as mean±SEM. Normally distributed data were analyzed by 1-way analysis of variance (ANOVA) with Bonferroni correction for post hoc comparisons between multiple experimental groups. NDSs were analyzed by Kruskal–Wallis with Mann–Whitney *U* analyses between multiple groups because the values are categorical variables. Kaplan–Meier analysis and the log-rank test were used to calculate survival rates. Hemodynamic and laboratory data were examined by a mixed-effects model for repeated-measures analyses, followed by ANOVA with Bonferroni correction for post hoc comparisons. The mixed-effects model for repeated-measures analysis contained treatment group, time, and treatment-by-time interaction as factors and random intercept for each subject. Significance was considered at the level of $P<0.05$. The Bonferroni-adjusted *P* value was defined such that the raw *P* value multiplies the number of comparisons. Statistical analyses were performed using SPSS software (SPSS Inc, Chicago, IL).

Results

Inhalation of H_2 Gas Improved Early Post-ROSC Survival After Cardiac Arrest With Ventricular Fibrillation

First, we compared inhaled H_2 gas with TH for the effect on early post-ROSC survival after VF-induced CA. Post-CA rats were assigned to the control group, H_2 group, TH group, or H_2+TH group at the time of CPR. There was no significant difference in terms of procedures for performing CPR, including CPR time to ROSC, dose of epinephrine, and number of defibrillations (Table 2). The resuscitation rates were 92.4% (13 survivors of 14 rats) in the control group, 92.4% (13 survivors of 14 rats) in the H_2 group, 92.4% (13 survivors of 14 rats) in the TH group, and 100% (13 survivors of 13 rats) in the H_2+TH group, respectively.

Arterial oxygen partial pressure (PaO_2) was higher in the TH group compared with the controlled normothermia group, whereas serum potassium levels 60 and 120 minutes after ROSC were lower in the TH group compared with the controlled normothermia group. These results were consistent with evidence that hypothermia reduces oxygen consumption by 6% to 10% per degree Celsius[23] and could potentially induce electrolyte loss.[23] The groups did not differ in terms of

Table 2. Physiological Variables and Therapies During Cardiopulmonary Resuscitation

	Control (n=14)	H_2 (n=14)	TH (n=14)	H_2+TH (n=13)
CPR time to ROSC, seconds	117±20	99±18	109±11	106±18
Total dose of epinephrine, μg	8.5±1.0	8.0±0.8	8.0±0.0	8.5±0.5
Total administration of defibrillation	1.0±0.2	0.9±0.2	1.2±0.2	1.0±0.1
ROSC rate, n (%)	13 (92.9)	13 (92.9)	13 (92.9)	13 (100)
Temperature at the end of 2-hour protocol, °C	36.9±0.0	36.9±0.0	33.0±0.0*†	32.8±0.0*†

Values expressed as mean±SEM. TH indicates therapeutic hypothermia; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation.
*$P<0.001$ compared with the control group.
†$P<0.001$ compared with the H_2 group.

Table 3. Group Arterial Blood Gas Analyses and Lactate Concentration Before Cardiac Arrest and in 2 Hours After Return of Spontaneous Circulation

		After ROSC		
	Baseline	10 Minutes	60 Minutes	120 Minutes
pH				
Control	7.48±0.03	7.18±0.02	7.37±0.02	7.36±0.03
H_2	7.53±0.02	7.20±0.01	7.38±0.01	7.38±0.01
TH	7.51±0.01	7.19±0.01	7.35±0.01	7.31±0.01
H_2+TH	7.53±0.01	7.18±0.01	7.36±0.00	7.34±0.00
PaO_2, mm Hg**				
Control	86±3	314±51	438±35	480±19
H_2	92±7	319±41	364±27	481±21
TH	84±3	513±38$^{\#\dagger}$	575±19$^{\#\#\dagger\dagger}$	582±21$^{\#\#\dagger\dagger}$
H_2+TH	92±4	433±41	534±22††	571±19$^{\#\dagger}$
$PaCO_2$, mm Hg				
Control	29.9±1.3	41.3±2.6	38.7±2.7	38.8±3.9
H_2	28.9±1.7	42.5±1.8	36.9±2.2	38.1±1.9
TH	30.9±1.3	43.1±3.1	39.5±2.2	42.8±2.5
H_2+TH	28.9±0.7	46.8±2.1	38.0±1.1	42.3±1.2
Na, mmol/L				
Control	139.8±0.5	140.7±1.0	140.3±0.6	139.5±0.5
H_2	139.4±0.6	141.2±0.7	139.3±0.7	139.6±0.6
TH	140.8±0.6	141.3±0.7	140.2±0.8	141.3±0.7
H_2+TH	139.6±0.4	142.8±0.6	141.9±0.8	140.2±0.7
K, mmol/L*				
Control	3.8±0.0	3.9±0.2	3.8±0.1	4.2±0.1
H_2	3.9±0.0	3.8±0.2	3.8±0.1	4.2±0.1
TH	3.9±0.0	3.4±0.2	3.4±0.0$^{\#\dagger}$	3.7±0.1$^{\#\dagger}$
H_2+TH	4.0±0.0	3.4±0.1	3.2±0.5$^{\#\dagger}$	3.5±0.0$^{\#\dagger}$
SaO_2, %				
Control	97.5±0.3	99.0±0.6	99.9±0.0	100±0.0
H_2	97.4±0.4	99.6±0.1	100±0.0	100±0.0
TH	97.2±0.3	99.9±0.0	100±0.0	100±0.0
H_2+TH	97.6±0.4	99.9±0.0	100±0.0	100±0.0
Glucose, mg/dL**				
Control	196±8	238±22	293±15	269±20
H_2	185±11	264±25	328±22	319±20
TH	193±16	258±24	339±21	338 ±38
H_2+TH	178±10	205±18	304±10	337±17
Hematocrit, %				
Control	48.6±0.9	52.5±1.3	51.3±1.1	49.5±1.2
H_2	48.1±1.1	54.0±0.9	50.3±0.8	49.0±0.8
TH	49.8±0.9	53.3±1.1	51.3±0.7	51.3±0.9
H_2+TH	49.2±0.8	54.2±0.6	52.0±0.6	50.0±0.6

Continued

Table 3. Continued

	Baseline	After ROSC		
		10 Minutes	60 Minutes	120 Minutes
Lactate, mg/dL				
Control	1.4±0.1	8.9±0.7	2.3±0.3	2.0±0.2
H_2	1.3±0.1	8.5±0.7	2.4±0.2	1.9±0.1
TH	1.7±0.2	8.9±0.7	3.1±0.6	2.8±0.9
H_2+TH	1.4±0.1	8.4±0.4	3.0±0.2	1.9±0.2
Base excess, mmol/L				
Control	1.1±0.6	−12.7±1.2	−2.8±0.9	−3.5±0.9
H_2	1.3±0.7	−11.1±0.8	−3.0±0.7	−3.1±0.8
TH	1.6±0.6	−11.7±1.0	−3.8±1.0	−4.1±1.4
H_2+TH	1.6±0.6	−8.4±2.0	−3.7±0.7	−2.3±0.7

Values expressed as mean±SEM. Resuscitated animal were analyzed (n=13 for each group). TH indicates therapeutic hypothermia; ROSC, return of spontaneous circulation.
*P<0.05; **P<0.01, statistically significant differences for treatment by time between 4 groups by mixed-effects model for repeated-measures analyses.
#P<0.05; ##P<0.01 vs the control group.
†P<0.05; ††P<0.01 vs the H_2 group.

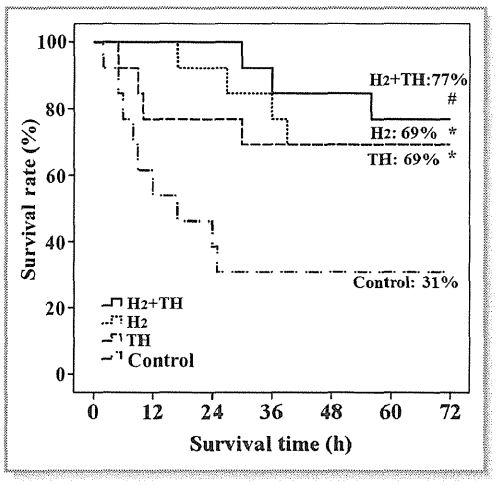

Figure 2. Kaplan–Meier analyses of cumulative survival at 72 hours. Statistically significant differences: #P<0.01 compared with the control group; *P<0.05 compared with the control group. TH indicates therapeutic hypothermia.

partial pressure of carbon dioxide ($PaCO_2$), pH, base excess, hematocrit, or lactate (Table 3).

The survival rate 24 hours after ROSC was 43% (6/13) in the control group, 92% (12/13) in the H_2 group, 77% (10/13) in the TH group, and 100% (13/13) in the H_2+TH group, whereas 72-hour survival rates were 31% (4/13), 69% (9/13), 69% (9/13), and 77% (10/13), respectively (Figure 2). These results indicated that inhalation of H_2 gas during CPR and during the first 2 hours after ROSC significantly improved survival rate, comparable to those of hypothermia.

Inhalation of H_2 Gas Improved NDS in Postarrest State

Interestingly, appearance was the most obvious difference between these groups of post-CA animals (Figure 3A). The typical sick-rat appearance of a hunched back and unkempt hair was often seen in post-CA control rats, whereas post-CA rats treated with H_2 gas appeared healthy. All animals were evaluated for neurological function based on their NDS (100=worst NDS; 0=best NDS) 24 and 48 hours after ROSC. NDS 24 hours after ROSC was significantly lower in the H_2 group (25.7±7.7%), TH group (38.0±10.7%), and H_2+TH group (8.0±2.5%) compared with the control group (77.1±8.6%) (Figure 3B). The H_2+TH group showed significantly better NDS 24 hours after ROSC compared with the H_2 and TH groups. NDS 48 hours after ROSC was significantly better in the H_2 group (41.9±11.7%), TH group (36.0±12.4%), and H_2+TH group (18.5±10.0%) than in the control group (82.6±9.2%), with the H_2+TH group showing a significantly better NDS 48 hours after ROSC than the H_2 group (Figure 3C). These results indicated that functional outcome was improved by inhalation of H_2 gas during CPR to an extent comparable to TH in post-CA rats. Inhalation of H_2 gas plus TH had additive effect on NDS 24 hours after ROSC, but not 48 hours after ROSC, in the rat model of CA with ventricular fibrillation.

Inhalation of H_2 Gas Significantly Suppressed Elevations of IL-6 After Cardiac Arrest and Cardiopulmonary Resuscitation

Serum IL-6 levels 2 hours after ROSC markedly increased in post-CA rats of the controlled normothermia group

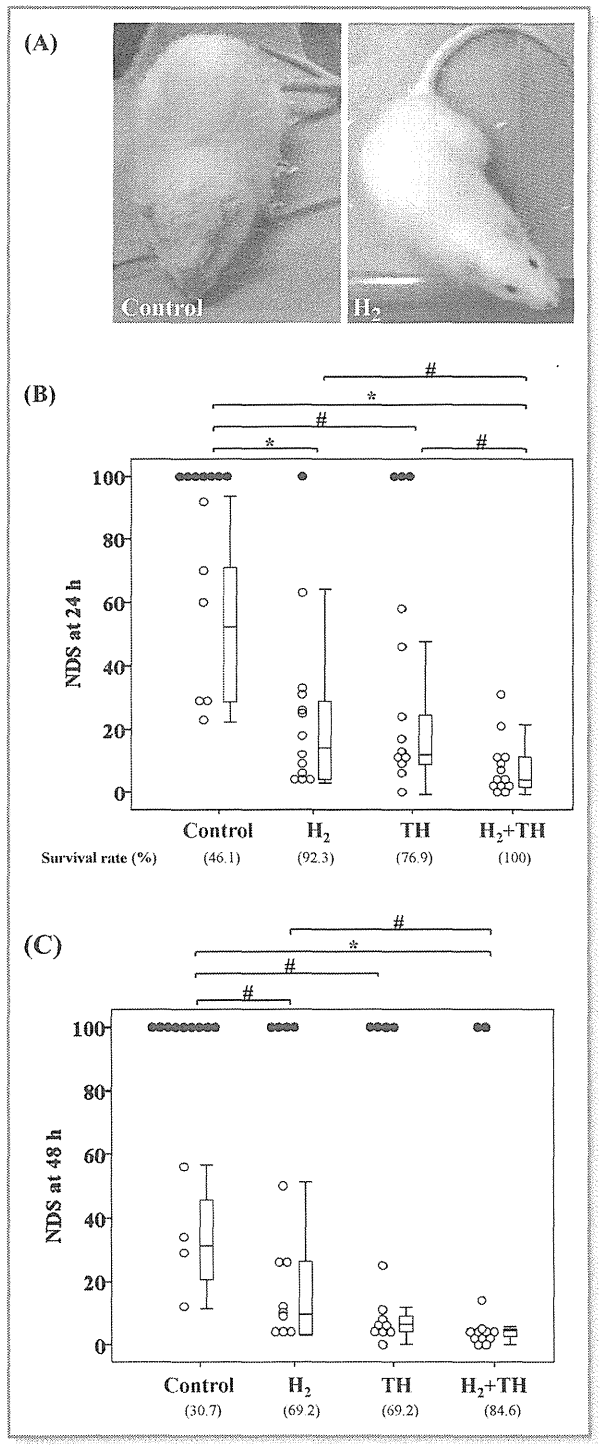

Figure 3. A, Representative photographs of rat appearance 24 hours after ROSC in the control group (left) and in the H_2 group (right). Survival rate and neurological deficit scores (NDSs) 24 hours (B) and 48 hours (C) after ROSC. Dead rats (indicated by score=100) are indicated by closed circles. Box plots indicate NDS of survivors 24 hours and 48 hours after ROSC. TH indicates therapeutic hypothermia. Statistically significant differences: #P<0.05 between groups; *P<0.001 between groups.

(2239.9±440.8 pg/mL) compared with sham-operated rats (27.4±13.3 pg/mL), but were almost equal in post-CA rats of the TH (2431.2±634.7 pg/mL) and controlled normothermia groups, indicating that TH has little effect on the systemic inflammatory response at this point.

Serum IL-6 levels 2 hours after ROSC were not significantly increased in post-CA rats of the H_2 group (848.9±257.0 pg/mL) or H_2+TH group (933.3±339.0 pg/mL) compared with sham-operated rats, but in the H_2+TH group, serum IL-6 levels were suppressed to the same degree as that of post-CA rats receiving only H_2 gas. These results indicated that the elevation of IL-6 2 hours after ROSC was markedly suppressed by H_2 gas inhalation (Figure 4).

Inhalation of H_2 Gas Kept LVEDP Low During the First 2 Hours After ROSC

We examined the impact of H_2 gas inhalation on left ventricular functional recovery during the post-CA reperfusion period in comparison with TH. Hemodynamic parameters prior to induction of CA did not differ among the control, H_2, TH, and H_2+TH groups. Mean blood pressure was also not different among the 4 groups during the first 2 hours after ROSC; however, heart rate was significantly lower in the TH group compared with the control and H_2 groups.

Recovery of dP/dt max, an indicator of left ventricular systolic function, after 10 minutes of ROSC was significantly higher in the H_2 and H_2+TH groups than in the control and TH groups. Recovery of negative dP/dt max, an indicator of left ventricular diastolic function, after 10 minutes of ROSC was significantly superior in the H_2 group compared with the other groups. This superiority conferred by H_2 gas inhalation tended to continue during the first 2 hours after ROSC, and left

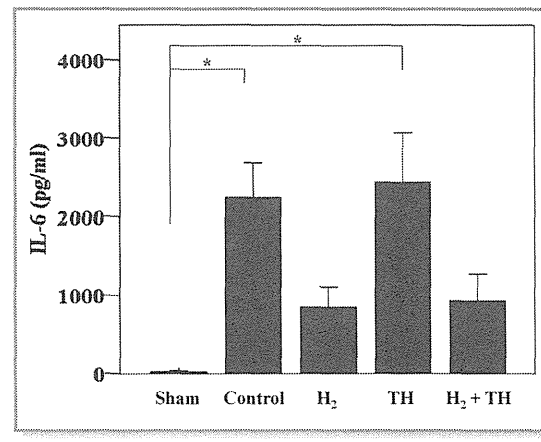

Figure 4. Serum IL-6 concentrations 2 hours after ROSC in the experimental and sham-operated groups. Bars represent the mean and standard error. *P<0.01 compared with the sham-operated group. ROSC indicates return of spontaneous circulation; TH, therapeutic hypothermia.

DOI: 10.1161/JAHA.112.003459

ventricular systolic pressure was recovered after CA and ROSC to pre-CA levels. There was no difference in terms of left ventricular systolic pressure among the 4 groups during the first 2 hours after ROSC. By contrast, there was a striking difference with respect to left ventricular end-diastolic pressure (LVEDP), which gradually increased to ≥20 mm Hg 2 hours after ROSC in the control group. Remarkably, H_2 gas inhalation kept LVEDP at pre-CA levels during the first 2 hours after ROSC (Figure 5), and this protective effect was observed both under controlled normothermia and under TH conditions.

Inhaled Hydrogen Gas Attenuated Cardiomyocyte Degeneration and Necrosis, Inflammatory Cell Infiltration, Reactive Fibrosis, and Oxidative Stress in the Postcardiac Arrest Heart

A favorable effect of inhaled H_2 gas during CPR and during the first 2 hours after ROSC on functional recovery of the heart in rats after CA with VF prompted us to examine the pathohistological changes 24 hours after ROSC between control and H_2 gas–treated rats. Consistent with the continuous elevation of LVEDP in post-CA rats of the control group, water content of the lung, an indicator of lung edema, tended to be higher in controls than in sham-operated rats, although lung water content was similar between post-CA H_2 and sham rats (Figure 6).

Azan–Mallory-stained gross sections of whole heart also revealed that perivascular and interstitial fibrosis on the endocardial side of the myocardium emerged in the post-CA control rats 24 hours after ROSC but that this reactive fibrosis was less severe in the post-CA rats administered H_2 gas (Figure 7).

Histological analysis with hematoxylin/eosin staining 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 rats of the control group.

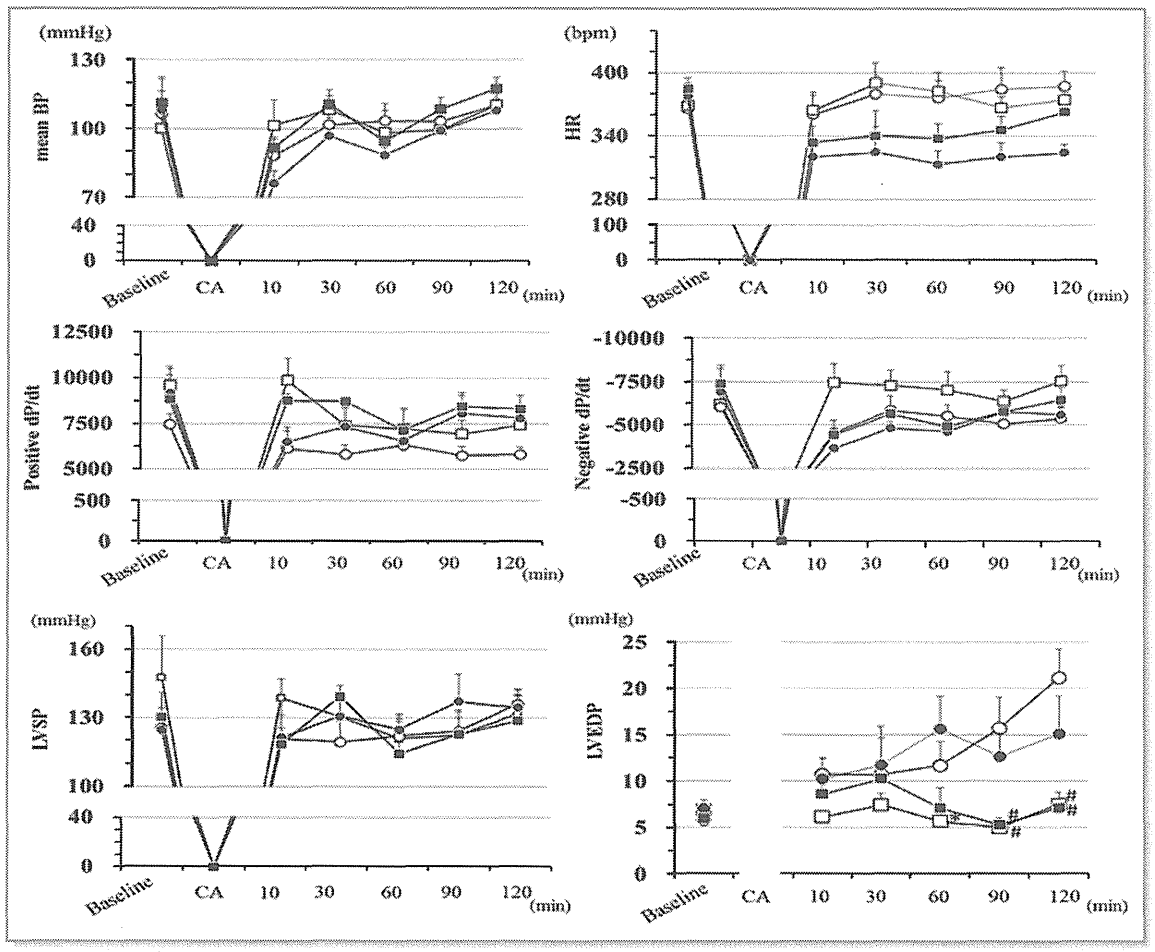

Figure 5. Changes in mean blood pressure (MBP), heart rate (HR), and LV peak positive, negative dP/dt and LV systolic pressure (LVSP), LV diastolic pressure (LVEDP), n=5 to 6; $^{\#}P<0.05$ compared with the control group; $^{*}P<0.05$ compared with the TH group.

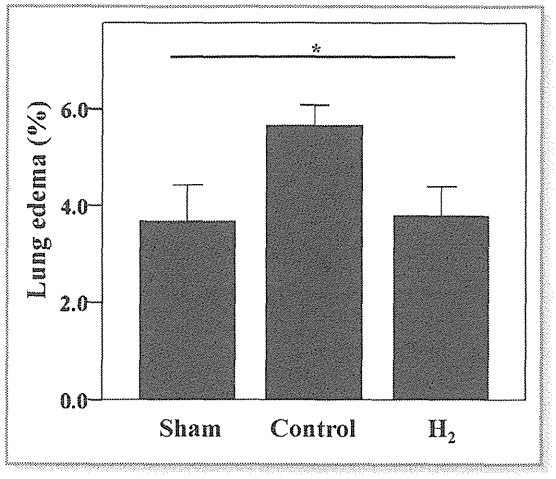

Figure 6. Effects of hydrogen on lung edema. Lung edema 24 hours after ROSC (n=3 to 6); *P<0.05 for analysis of variance between groups. Bars represent the standard error. ROSC indicates return of spontaneous circulation.

These pathohistological changes were less severe in rats administered H_2 gas (Figure 8).

Immunohistochemistry for 8-OH-dG (which is an index of oxidative DNA damage) and 4-HNE (which is the end product of lipid peroxidation) was carried out to investigate oxidative stress in myocardium obtained from the post-CA rats 24 hours after ROSC. The 8-OHdG-positive cells and 4-HNE-positive cardiomyocytes were distributed throughout the myocardium, particularly on the endocardial side of the myocardium. Notably, there were fewer 8-OHdG- positive cells and 4-HNE-positive cardiomyocytes obtained from the post-CA rats administered H_2 gas (Figure 9). These results indicated that inhalation of H_2 gas ameliorated oxidative myocardial injury during CPA and after ROSC.

Discussion

Molecular hydrogen (H_2) is a novel antioxidant with the following unique properties: (1) H_2 is permeable to cell

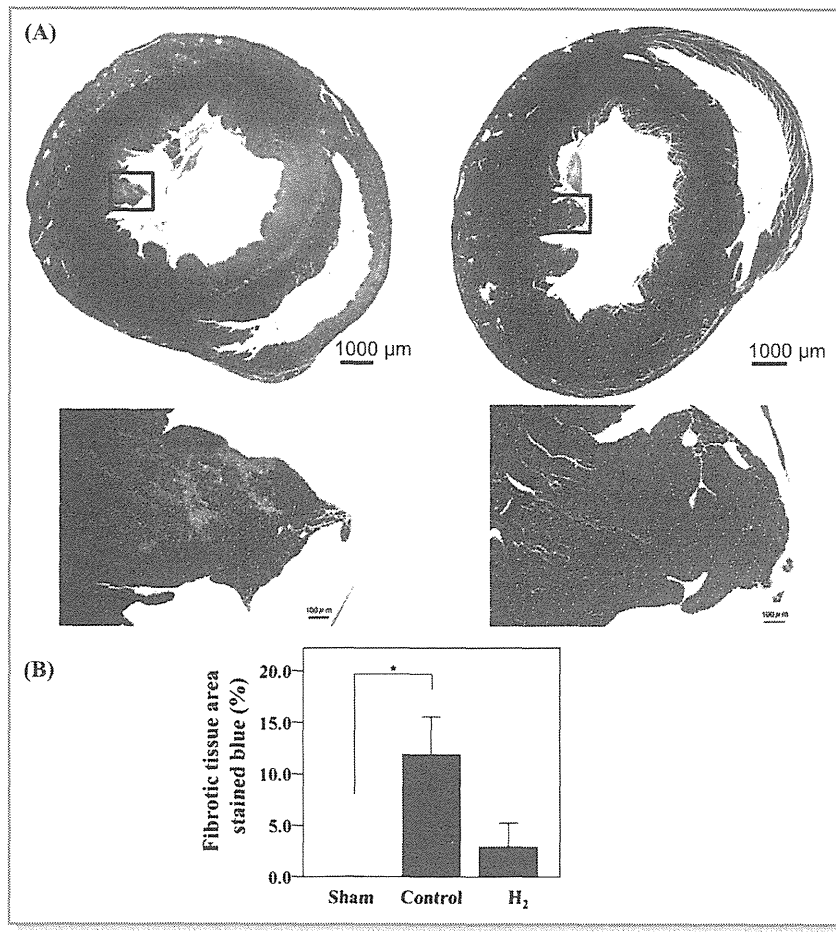

Figure 7. A, Representative photographs of Azan–Mallory staining of heart sections obtained 24 hours after ROSC in the absence (right) or presence (left) of H_2 inhalation. B, Fibrotic tissue area stained blue (%) in sham and rats 24 hours after ROSC with or without H_2 inhalation. Bars represent the standard error (n=4 to 6); *P<0.05 between groups. ROSC indicates return of spontaneous circulation.

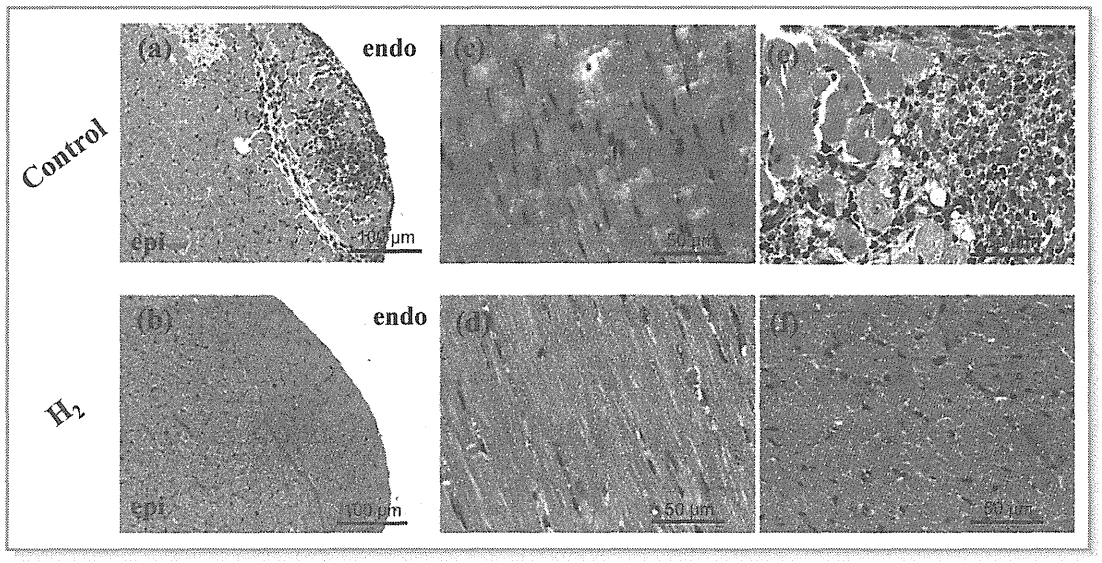

Figure 8. Hematoxylin and eosin staining of transverse sections of rat heart in the presence or absence of H_2 inhalation. Shown is a microscopic view of inflammatory cell infiltration (a), contraction band necrosis (c), cytoplasmic eosinophilia (e), loss of nuclei (e), and vacuolar degeneration (e) in the left ventricle. These observations were reduced by inhalation of H_2 (b, d, f).

membranes and can target organelles, including mitochondria and nuclei; (2) H_2 specifically quenches detrimental ROS such as •OH and peroxynitrite (ONOO−) while maintaining the metabolic oxidation–reduction reaction and other less potent ROS, such as O_2^-•, H_2O_2, and nitric oxide (NO•)[4,5]; and (3) inhaled H_2 gas is rapidly transported and thus can reach "at-risk" ischemic organs including the brain in a timely fashion.[5]

We hypothesized that H_2 therapy may improve outcomes of post-CA syndrome characterized by systemic ischemia–reperfusion injury and "sepsis-like" systemic inflammation because H_2 treatment by oral, intravenous administration, or inhalation has demonstrated extended effectiveness in various situations, such as in brain ischemia–reperfusion injury,[6] myocardial ischemia–reperfusion injury,[7] sepsis,[28] diabetes,[9,29] intestinal grafts,[30] hemodialysis,[11] liver injury,[31] and spinal cord injury[32] and in animal models of Parkinson's disease[33] and Alzheimer's disease.[34] The present study demonstrated for the first time that inhalation of 2% H_2 gas starting at the beginning of CPR and given for 2 hours after ROSC significantly improves the functional status of the brain (based on NDS) and heart and suppresses the systemic inflammatory response, thereby improving survival rate in a rat model of CA with VF. The salutary impact of H_2 gas could be at least partially attributed to its radical-scavenging effect in the heart, because both 8-OHdG- and 4-HNE-positive cardiomyocytes obtained from the post-CA rats 24 hours after ROSC were markedly suppressed by H_2 gas inhalation. The improved appearance in the H_2 group relative to control animals could be at least in part a consequence of better cardiovascular performance, because lung edema tended to been diminished in the H_2 group compared with the controls. Because the neurological assessment was based only on the NDS in this study, we do not have compelling evidence that the benefits were mediated via attenuation of brain injury in the present study.

We found that H_2 inhalation had protective and, in some aspects, superior effects, comparable to those of hypothermia. The results are surprising, because TH is believed to confer protection against reperfusion injury by multiple mechanisms,[23] including the suppression of free radicals, enzymes, and excitatory and inflammatory reactions, in addition to the direct physical protection of membranes, whereas the cornerstone of H_2 therapy is selective ROS attenuation, which is only 1 facet of TH therapy. All groups were ventilated with 98% O_2, and increased reperfusion damage due to hyperoxia could have improved the chance of detecting the H_2 protective effect. If H_2 protects only from the harmful effects of hyperoxia, then avoiding hyperoxia/targeting normoxia might have the same benefit. Slow rewarming is also considered important in avoiding harmful systemic responses, including vasodilation, hypotension, and rebound cerebral edema. The optimal rate of warming is not known, but the consensus is currently about 0.25°C to 0.5°C of warming per hour.[35] Rewarming speed after hypothermia in a rodent model of CA was variable among different TH protocols in previous research.[19,24,36] Here, we chose 60 minutes of rewarming based on the high metabolic rate of rats compared with humans. However, a rapid rewarming speed after hypothermia might reduce the protective effects of cooling. Consistent with previous observations,[37] blood lactate increased while the base excess decreased in post-CA rats,

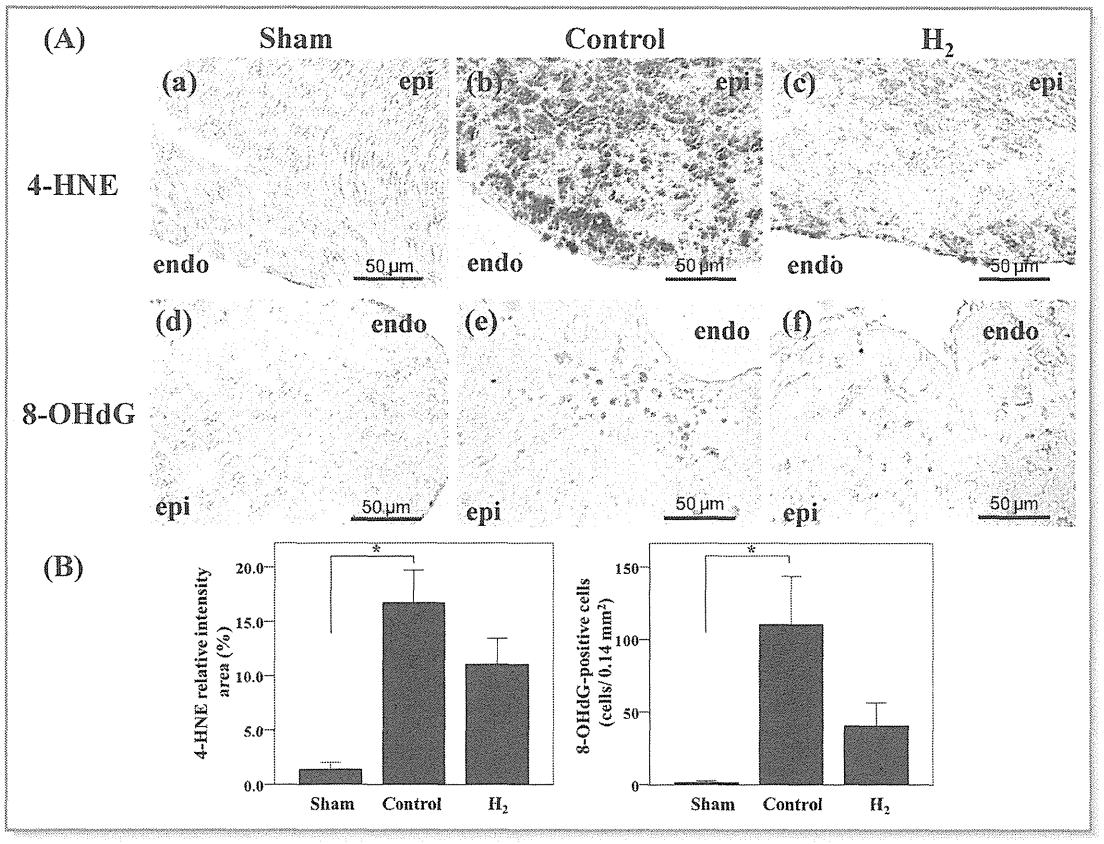

Figure 9. A, Immunohistochemical staining with antibodies against 4-HNE and 8-OHdG in transverse sections of sham and rats 24 hours after ROSC with or without H_2 inhalation. H_2 decreased levels of 4-HNE (b, c) and 8-OHdG (e, f). B, 4-HNE relative intensity area (%) and 8-OHdG-positive cells (cells/0.14) in sham and rats 24 hours after ROSC with or without H_2 inhalation. Bars represent the standard error (n=4 to 6); $^*P<0.05$ between groups.

peaking 10 minutes after ROSC and returning to near baseline levels 60 minutes after ROSC. Although the prognostic value of these measures[38] after CA have been reported in clinical studies, we could not detect any difference in the time course of blood lactate levels and base excess after ROSC among the control, H_2, TH, and H_2+TH groups.

Finally, we observed that TH did not affect IL-6 levels 2 hours after ROSC, whereas inhaled H_2 gas did, although we did not aim to refute the anti-inflammatory effect of TH. Rather, we should emphasize the anti-inflammatory effect of H_2. Previous in vitro studies and observations in patients with traumatic brain injury also demonstrated the effects of hypothermia on IL-6 levels.[39–41] In addition, H_2 gas inhalation significantly improved the survival rate and organ damage of septic mice with cecal ligation and puncture, and that favorable effect was accompanied by a reduction in serum and tissue proinflammatory cytokine levels.[28]

Conclusions

This study provided novel evidence that H_2 inhalation administered at the beginning of CPR and continued for 2 hours after ROSC markedly improves NDS, myocardial outcome, and 72-hour survival rate in rats after CA to an extent comparable to TH.

Limitation of the Study

We could not conclude that whether H_2 inhalation has the same beneficial outcome as TH because of the small sample size and low statistical power. Because neurological outcome was assessed on the basis of the NDS only, further studies are clearly needed to determine whether H_2 directly confers neuroprotection in post-CA status. Whether H_2 gas must be applied at the beginning of CPR or if a delayed application could have similar effect was also not tested in this study, and indeed, hypothermia could be applied in a delayed fashion. The use of H_2 remains in its infancy, and further studies are necessary to delineate the enigmatic effect of H_2 beyond that of a simple antioxidant.

Acknowledgments

The authors thank Y. Miyake (Keio University School of Medicine) and S. Kotoda (Bioresearch Center) for technical assistance. Dr Sano is a

core member of the Global Center of Excellence (GCOE) for Human Metabolomics Systems Biology at MEXT.

Sources of Funding

This work was supported by a Grant-in-Aid for Scientific Research (KAKENHI; to S.H.), the Marumo Research Foundation of the Japanese Society of Acute Medicine (to K.H.), the Research Foundation of the General Insurance Association of Japan (to K.H.), and Graduate School Doctoral Student Aid Program, Keio University (to K.H.).

Disclosures

None.

References

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'Donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, Hong Y. Heart disease and stroke statistics – 2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* 2009;119:e21–e181.
2. Neumar RW, Nolan JP, Adrie C, Aibiki M, Berg RA, Bottiger BW, Callaway C, Clark RS, Geocadin RG, Jauch EC, Kern KB, Laurent I, Longstreth WT Jr, Merchant RM, Morley P, Morrison LJ, Nadkarni V, Peberdy MA, Rivers EP, Rodriguez-Nunez A, Sellke FW, Spaulding C, Sunde K, Vanden Hoek T. Post-cardiac arrest syndrome: epidemiology, pathophysiology, treatment, and prognostication. A consensus statement from the International Liaison Committee on Resuscitation (American Heart Association, Australian and New Zealand Council on Resuscitation, European Resuscitation Council, Heart and Stroke Foundation of Canada, InterAmerican Heart Foundation, Resuscitation Council of Asia, and the Resuscitation Council of Southern Africa); the American Heart Association Emergency Cardiovascular Care Committee; the Council on Cardiovascular Surgery and Anesthesia; the Council on Cardiopulmonary, Perioperative, and Critical Care; the Council on Clinical Cardiology; and the Stroke Council. *Circulation.* 2008;118:2452–2483.
3. Merchant RM, Soar J, Skrifvars MB, Silfvast T, Edelson DP, Ahmad F, Huang KN, Khan M, Vanden Hoek TL, Becker LB, Abella BS. Therapeutic hypothermia utilization among physicians after resuscitation from cardiac arrest. *Crit Care Med.* 2006;34:1935–1940.
4. Ohta S. Recent progress toward hydrogen medicine: potential of molecular hydrogen for preventive and therapeutic applications. *Curr Pharm Des.* 2011;17:2241–2252.
5. Ohta S. Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochim Biophys Acta.* 2012;1820:586–594.
6. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med.* 2007;13:688–694.
7. Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, Endo J, Katayama T, Kawamura A, Kohsaka S, Makino S, Ohta S, Ogawa S, Fukuda K. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun.* 2008;373:30–35.
8. Xie K, Yu Y, Zhang Z, Liu W, Pei Y, Xiong L, Hou L, Wang G. Hydrogen gas improves survival rate and organ damage in zymosan-induced generalized inflammation model. *Shock.* 2010;34:495–501.
9. Kajiyama S, Hasegawa G, Asano M, Hosoda H, Fukui M, Nakamura N, Kitawaki J, Imai S, Nakano K, Ohta M, Adachi T, Obayashi H, Yoshikawa T. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutr Res.* 2008;28:137–143.
10. Nakao A, Toyoda Y, Sharma P, Evans M, Guthrie N. Effectiveness of hydrogen rich water on antioxidant status of subjects with potential metabolic syndrome – an open label pilot study. *J Clin Biochem Nutr.* 2010;46:140–149.
11. Nakayama M, Nakano H, Hamada H, Itami N, Nakazawa R, Ito S. A novel bioactive haemodialysis system using dissolved dihydrogen (H2) produced by water electrolysis: a clinical trial. *Nephrol Dial Transplant.* 2010;25:3026–3033.
12. Shimouchi A, Nose K, Shirai M, Kondo T. Estimation of molecular hydrogen consumption in the human whole body after the ingestion of hydrogen-rich water. *Adv Exp Med Biol.* 2012;737:245–250.
13. Ono H, Nishijima Y, Adachi N, Sakamoto M, Kudo Y, Nakazawa J, Kaneko K. Hydrogen(H2) treatment for acute erythymatous skin diseases. A report of 4 patients with safety data and a non-controlled feasibility study with H2 concentration measurement on two volunteers. *Med Gas Res.* 2012;2:14.
14. Aoki K, Nakao A, Adachi T, Matsui Y, Miyakawa S. Pilot study: effects of drinking hydrogen-rich water on muscle fatigue caused by acute exercise in elite athletes. *Med Gas Res.* 2012;2:12.
15. Ito M, Ibi T, Sahashi K, Ichihara M, Ohno K. Open-label trial and randomized, double-blind, placebo-controlled, crossover trial of hydrogen-enriched water for mitochondrial and inflammatory myopathies. *Med Gas Res.* 2011;1:24.
16. Ono H, Nishijima Y, Adachi N, Tachibana S, Chitoku S, Mukaihara S, Sakamoto M, Kudo Y, Nakazawa J, Kaneko K, Nawashiro H. Improved brain MRI indices in the acute brain stem infarct sites treated with hydroxyl radical scavengers, Edaravone and hydrogen, as compared to Edaravone alone. A non-controlled study. *Med Gas Res.* 2011;1:12.
17. Kang KM, Kang YN, Choi IB, Gu Y, Kawamura T, Toyoda Y, Nakao A. Effects of drinking hydrogen-rich water on the quality of life of patients treated with radiotherapy for liver tumors. *Med Gas Res.* 2011;1:11.
18. Colbourne F, Corbett D. Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. *Brain Res.* 1994;654:265–272.
19. Ye S, Weng Y, Sun S, Chen W, Wu X, Li Z, Weil MH, Tang W. Comparison of the durations of mild therapeutic hypothermia on outcome after cardiopulmonary resuscitation in the rat. *Circulation.* 2012;125:123–129.
20. Schneider A, Teschendorf P, Vogel P, Russ N, Knapp J, Bottiger BW, Popp E. Facilitation of hypothermia by quinpirole and 8-OH-DPAT in a rat model of cardiac arrest. *Resuscitation.* 2012;83:232–237.
21. Hickey RW, Ferimer H, Alexander HL, Garman RH, Callaway CW, Hicks S, Safar P, Graham SH, Kochanek PM. Delayed, spontaneous hypothermia reduces neuronal damage after asphyxial cardiac arrest in rats. *Crit Care Med.* 2000;28:3511–3516.
22. Callaway CW, Rittenberger JC, Logue ES, McMichael MJ. Hypothermia after cardiac arrest does not alter serum inflammatory markers. *Crit Care Med.* 2008;36:2607–2612.
23. Polderman KH. Mechanisms of action, physiological effects, and complications of hypothermia. *Crit Care Med.* 2009;37:S186–S202.
24. Abella BS, Zhao D, Alvarado J, Hamann K, Vanden Hoek TL, Becker LB. Intra-arrest cooling improves outcomes in a murine cardiac arrest model. *Circulation.* 2004;109:2786–2791.
25. Peberdy MA, Callaway CW, Neumar RW, Geocadin RG, Zimmerman JL, Donnino M, Gabrielli A, Silvers SM, Zaritsky AL, Merchant R, Vanden Hoek TL, Kronick SL. Part 9: Post-cardiac arrest care: 2010 American Heart Association guidelines for cardiopulmonary resuscitation and emergency cardiovascular care. *Circulation.* 2010;122:S768–S786.
26. Lin JY, Liao XX, Li H, Wei HY, Liu R, Hu CL, Huang GQ, Dai G, Li X. Model of cardiac arrest in rats by transcutaneous electrical epicardium stimulation. *Resuscitation.* 2010;81:1197–1204.
27. Neumar RW, Bircher NG, Sim KM, Xiao F, Zadach KS, Radovsky A, Katz L, Ebmeyer E, Safar P. Epinephrine and sodium bicarbonate during CPR following asphyxial cardiac arrest in rats. *Resuscitation.* 1995;29:249–263.
28. Xie K, Yu Y, Pei Y, Hou L, Chen S, Xiong L, Wang G. Protective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and HMGB1 release. *Shock.* 2010;34:90–97.
29. Kamimura N, Nishimaki K, Ohsawa I, Ohta S. Molecular hydrogen improves obesity and diabetes by inducing hepatic FGF21 and stimulating energy metabolism in db/db mice. *Obesity (Silver Spring).* 2011;19:1396–1403.
30. Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, McCurry KR, Bauer AJ, Nakao A. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. *Am J Transplant.* 2008;8:2015–2024.
31. Liu Q, Shen WF, Sun HY, Fan DF, Nakao A, Cai JM, Yan G, Zhou WP, Shen RX, Yang JM, Sun XJ. Hydrogen-rich saline protects against liver injury in rats with obstructive jaundice. *Liver Int.* 2010;30:958–968.
32. Chen C, Chen Q, Mao Y, Xu S, Xia C, Shi X, Zhang JH, Yuan H, Sun X. Hydrogen-rich saline protects against spinal cord injury in rats. *Neurochem Res.* 2010;35:1111–1118.
33. Fujita K, Seike T, Yutsudo N, Ohno M, Yamada H, Yamaguchi H, Sakumi K, Yamakawa Y, Kido MA, Takaki A, Katafuchi T, Tanaka Y, Nakabeppu Y, Noda M.

Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *PLoS One.* 2009;4:e7247.

34. Li J, Wang C, Zhang JH, Cai JM, Cao YP, Sun XJ. Hydrogen-rich saline improves memory function in a rat model of amyloid-beta-induced Alzheimer's disease by reduction of oxidative stress. *Brain Res.* 2010;1328:152–161.
35. Che D, Li L, Kopil CM, Liu Z, Guo W, Neumar RW. Impact of therapeutic hypothermia onset and duration on survival, neurologic function, and neurodegeneration after cardiac arrest. *Crit Care Med.* 2011;39:1423–1430.
36. Walters JH, Morley PT, Nolan JP. The role of hypothermia in post-cardiac arrest patients with return of spontaneous circulation: a systematic review. *Resuscitation.* 2011;82:508–516.
37. von Planta I, Weil MH, von Planta M, Bisera J, Bruno S, Gazmuri RJ, Rackow EC. Cardiopulmonary resuscitation in the rat. *J Appl Physiol.* 1988;65:2641–2647.
38. Carden DL, Martin GB, Nowak RM, Foreback CC, Tomlanovich MC. Lactic acidosis during closed-chest CPR in dogs. *Ann Emerg Med.* 1987;16:1317–1320.
39. Aibiki M, Maekawa S, Ogura S, Kinoshita Y, Kawai N, Yokono S. Effect of moderate hypothermia on systemic and internal jugular plasma IL-6 levels after traumatic brain injury in humans. *J Neurotrauma.* 1999;16:225–232.
40. Kimura A, Sakurada S, Ohkuni H, Todome Y, Kurata K. Moderate hypothermia delays proinflammatory cytokine production of human peripheral blood mononuclear cells. *Crit Care Med.* 2002;30:1499–1502.
41. Suehiro E, Fujisawa H, Akimura T, Ishihara H, Kajiwara K, Kato S, Fujii M, Yamashita S, Maekawa T, Suzuki M. Increased matrix metalloproteinase-9 in blood in association with activation of interleukin-6 after traumatic brain injury: influence of hypothermic therapy. *J Neurotrauma.* 2004;21:1706–1711.

Original Paper

European Neurology

Eur Neurol 2012;67:232–237
DOI: 10.1159/000336568

Received: August 8, 2011
Accepted: January 14, 2012
Published online: March 14, 2012

Evaluation of Systemic Redox States in Patients Carrying the MELAS A3243G Mutation in Mitochondrial DNA

Masamichi Ikawa[a] Kenichiro Arakawa[b] Tadanori Hamano[a] Miwako Nagata[c]
Yasunari Nakamoto[a] Masaru Kuriyama[a] Yasutoshi Koga[d] Makoto Yoneda[a]

[a]Second Department of Internal Medicine, and [b]Department of Cardiology, Faculty of Medical Sciences, University of Fukui, and [c]Department of Neurology, Nakamura Hospital, Fukui, and [d]Department of Pediatrics and Child Health, Kurume University School of Medicine, Fukuoka, Japan

Key Words
MELAS · A3243G mutation · Mitochondrial DNA · Oxidative stress · Antioxidant activity · Redox states · d-ROMs test · BAP test

Abstract
Background/Aims: To clarify the change of systemic redox states in patients carrying the A3243G mutation in mitochondrial DNA (A3243G), we evaluated oxidative stress and antioxidant activity in the serum of patients. ***Methods:*** Oxidative stress and antioxidant activity in the serum samples obtained from 14 patients carrying A3243G and from 34 healthy controls were analyzed using the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests, respectively. ***Results:*** The mean d-ROMs level of all patients was significantly greater than that of the controls ($p < 0.005$), and the mean BAP/d-ROMs ratio of all patients was significantly lower than that of the controls ($p < 0.02$). In the patients with a history of stroke-like episodes ($n = 10$), both mean d-ROMs and BAP levels were increased compared with those of the controls (both $p < 0.01$). The mean BAP level of the patients without a history of stroke-like episodes ($n = 4$) was significantly decreased compared with that of the controls ($p < 0.001$), but the mean d-ROMs levels were not significantly different. ***Conclusion:*** d-ROMs and BAP tests indicated that patients carrying A3243G are always exposed to underlying oxidative stress, even at a remission state of stroke-like episodes.

Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome is the most common type of mitochondrial disease, and is mainly caused by an A-to-G transition mutation at nucleotide position 3243 (A3243G) in mitochondrial DNA (mtDNA) [1]. MELAS is characterized by stroke-like episodes that occur repeatedly and provoke neurological symptoms (e.g. headache, epilepsy, hemiparesis, and dementia) due to 'stroke-like' brain lesions [2]. In other words, stroke-like episodes are diagnostic symptoms of MELAS, and are crucial factors determining the prognosis of patients with this syndrome [2].

In addition, A3243G is responsible for not only stroke-like episodes but also mitochondrial cardiomyopathy or diabetes mellitus (DM) [1, 3–6]. Conversely, some patients carrying A3243G present with typical MELAS syn-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0014–3022/12/0674–0232$38.00/0

Accessible online at:
www.karger.com/ene

Makoto Yoneda, MD, PhD
Second Department of Internal Medicine
Faculty of Medical Sciences, University of Fukui
23-3 Shimoaiduki, Matsuoka, Eiheiji-cho, Fukui 910-1193 (Japan)
Tel. +81 776 61 8351, E-Mail myoneda@u-fukui.ac.jp

Table 1. Demographic characteristics of patients and controls

Subject	Patients			Normal controls
	all	'stroke type'	'non-stroke type'	
Number	14	10	4	34
Gender (males/females)	7/7	5/5	2/2	20/14
Mean age at examination, years	32.1 ± 14.7	27.8 ± 12.5	42.8 ± 15.8	34.6 ± 7.4
Clinical features				
Stroke-like episodes, n	10	10	0	0
Cardiomyopathy, n	6	2	4	0
Diabetes, n	3	0	3	0
Under antioxidant therapy, n	11	10	1	0

Values are mean ± SD.

dromes with stroke-like episodes, and others present with only cardiomyopathy or DM without stroke-like episodes. However, the pathophysiological difference of phenotypes between the presence and absence of stroke-like episodes in patients carrying A3243G remains obscure.

Recent studies using cells cultured in vitro demonstrated increased oxidative stress in cells with impaired mitochondria due to A3243G [7–10]. Oxidative stress is provoked by reactive oxygen species (ROS) generation exceeding antioxidant defenses, such as manganese superoxide dismutase and glutathione peroxidase, and damages nucleic acids, proteins and lipids, which leads to cellular dysfunction. Indeed, previous pathological or imaging studies demonstrated enhanced regional oxidative stress in lesions of both stroke-like episodes and cardiomyopathy in patients carrying A3243G [11–13]. Therefore, there is a high possibility that oxidative stress participates in the pathogenesis caused by A3243G, and influences the phenotypic diversity. In other words, redox (reduction-oxidation) states should be evaluated in patients carrying A3243G both with and without a history of stroke-like episodes to clarify the role of oxidative stress in the emergence of stroke-like episodes.

To perform such an investigation, a rapid and reliable method of evaluating redox states in patients carrying A3243G is needed. Direct measurement of oxidative stress and antioxidant activity in living humans has been difficult; redox states have thus not been clearly evaluated in patients carrying A3243G to date. Recently, the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests have been used to evaluate redox states in serum. The d-ROMs level reflects the intensity of oxidative stress, and the BAP level indicates the activity of endogenous antioxidants [14, 15]. Their effectiveness as clinical markers has been reported in various diseases [16–22]. We evaluated redox states in fresh serum of both patients carrying A3243G and healthy volunteers using d-ROMs and BAP tests, and clarified the change of redox states due to A3243G and the pathophysiological difference in phenotypes with or without stroke-like episodes.

Subjects and Methods

Subjects

Fourteen Japanese patients (7 men and 7 women; mean age 32.1 ± 14.7 years) carrying A3243G were recruited at the University of Fukui Hospital, Fukui, and at the Kurume University Hospital, Fukuoka, Japan (table 1). Patients were classified by the presence or absence of stroke-like episodes into 'stroke type' and 'non-stroke type'. Ten patients with a history of stroke-like episodes were categorized as 'stroke type', and the other 4 patients who presented with mainly cardiomyopathy without a history of stroke-like episodes were categorized as 'non-stroke type'. Eleven patients were treated by antioxidant therapy such as coenzyme Q_{10} (CoQ_{10}; daily dose 30–90 mg) and/or vitamin E (daily dose 100 mg) administration; 10 of these patients were 'stroke type', and the other patient was 'non-stroke type'. Eight patients categorized as 'stroke type' were also treated with an oral administration of L-arginine (daily dose 14–21 g). All patients were in remission, free from exacerbation of symptoms or acute stroke-like episodes, when they were examined. Functional status was evaluated using the performance status rating (mean rating 1.3 ± 1.0). In 'stroke type' patients, the mean age of the first stroke-like episode was 21.1 ± 15.2 years, and the mean duration between the examination and the last stroke-like episode was 14.2 ± 9.2 months. 'Stroke type' patients had headaches and/or vomiting on average twice a month, but almost none had convulsions. Thirty-four Jap-

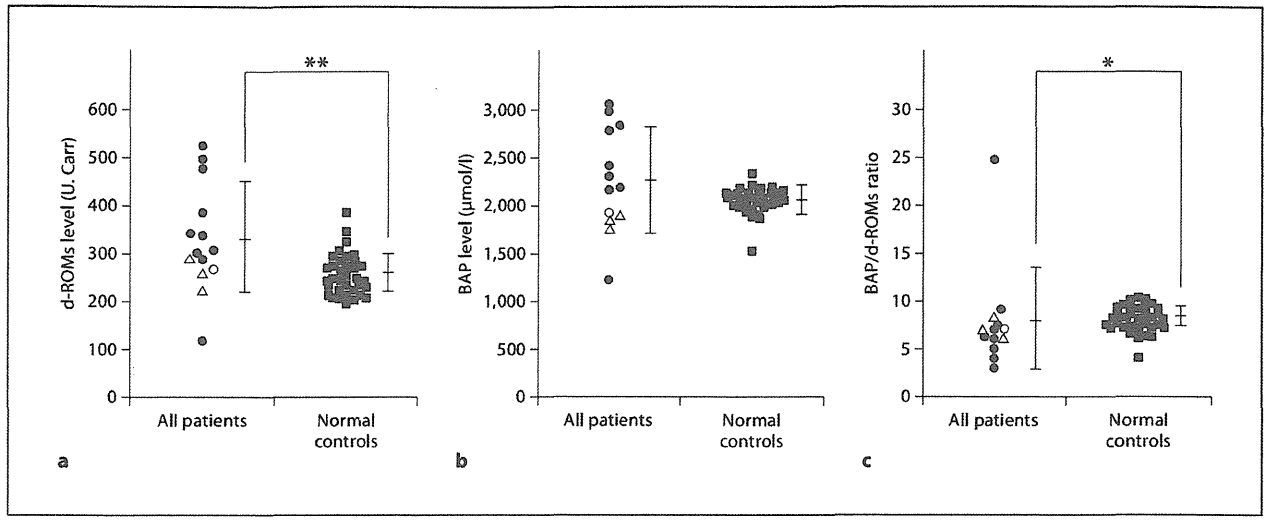

Fig. 1. Scatter plots portraying the levels of d-ROMs (**a**) and BAP (**b**) and BAP/d-ROMs ratios (**c**) in all the patients and controls. Circles and triangles correspond to the patients with and without antioxidant administration, respectively. In addition, closed and open diagrams correspond to the 'stroke type' patients and 'non-stroke type' patients, respectively. * $p < 0.02$, ** $p < 0.005$, according to the two-tailed Mann-Whitney U test. Bars indicate mean ± SD.

anese healthy volunteers (20 men and 14 women; mean age 34.6 ± 7.4 years) were also recruited as normal controls from the local community (table 1). This study was approved by the Ethics Committee of the University of Fukui. All subjects provided written informed consent to participate in the study.

Measurement of Oxidative Stress Levels

The oxidative stress levels were evaluated by measuring the quantity of hydroperoxides (R-OOH) in fresh serum samples using the d-ROMs test with the Free Radical Analytical System 4 (FRAS4^R; H&D srl, Parma, Italy) automatically [14]. Blood sampling was performed at fasting and at rest. Hydroperoxides consist of dehydrogenized and peroxidized proteins, lipids and fatty acids produced by ROS. In the d-ROMs test, hydroperoxides are turned to radicals by the Fenton reaction in an acid medium, and these generated radicals oxidize *N,N*-diethyl-paraphenylenediamine (DEPPD). Oxidized DEPPD quantity is determined by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and oxidative stress levels can be evaluated easily and quickly. The values are expressed as U. Carr, where 1 U. Carr corresponds to 0.8 mg/l H_2O_2.

Measurement of Antioxidant Activity Levels

The antioxidant activity levels were evaluated by measuring the quantity of molecules with antioxidative potency in fresh serum samples using the BAP test in the FRAS4^R automatically [15, 17]. Blood sampling was performed at fasting and at rest. In the BAP test, serum molecules with antioxidative potency reduce and decompose compounds of ferric chloride ($FeCl_3$) and thiocyanate derivative (AT) to $FeCl_2$ and free AT. Free AT is achromatized and dissociates from compounds, and is quantified by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and antioxidant activity levels can be evaluated easily and quickly. The results are expressed as μmol/l.

Statistical Analysis

The BAP-to-d-ROMs ratio (BAP/d-ROMs ratio) was calculated from the ratio of the BAP levels and d-ROMs levels for each subject. Data are presented as means ± standard deviations (SD). The resultant differences between normal controls and all patients were analyzed by means of a two-tailed Mann-Whitney U test. Since the subject number of each group was small, a non-parametric Kruskal-Wallis test was used for multiple data comparison and a post hoc Dunn test was performed to evaluate differences among normal controls, 'stroke type' patients and 'non-stroke type' patients. All statistical analyses were performed in SPSS Statistics Version 17.0 (SPSS Japan Inc., Tokyo, Japan), and $p < 0.05$ was considered significant.

Results

The levels of serum d-ROMs and BAP, and BAP/d-ROMs ratios of all the patients and controls are shown in figure 1, and those of the 'stroke type' patients, 'non-stroke type' patients and controls are shown in figure 2. The mean age of each group demonstrated no significant differences.

The mean d-ROMs level of all patients (332.6 ± 110.7 U. Carr) was significantly higher than that of the controls

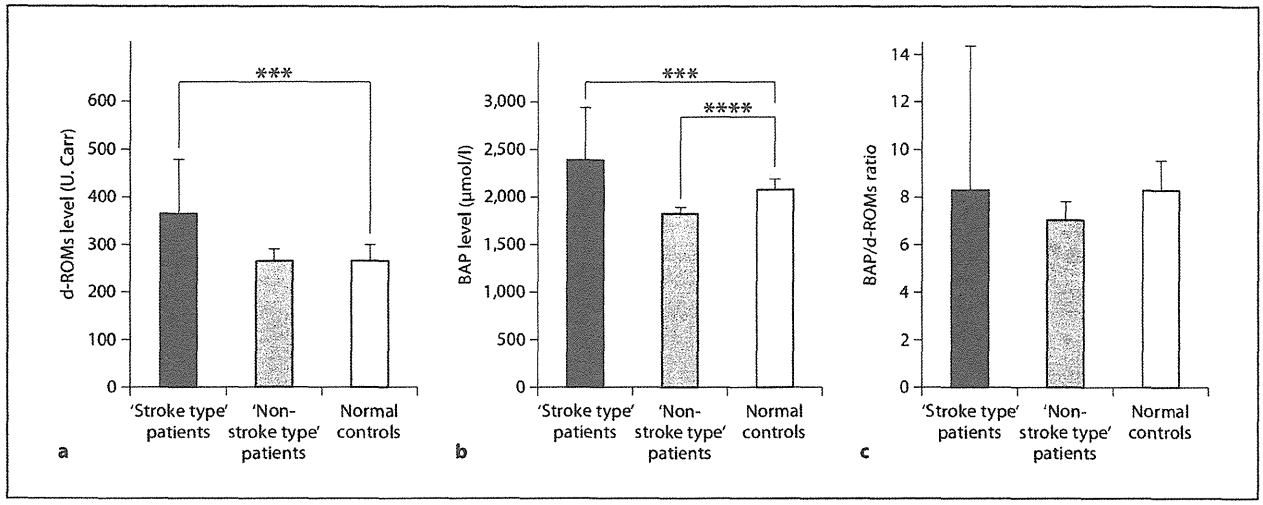

Fig. 2. The mean d-ROMs (**a**) and BAP (**b**) levels and mean BAP/d-ROMs ratio (**c**) in the 'stroke type' patients (black), 'non-stroke type' patients (grey) and controls (white). *** $p < 0.01$, **** $p < 0.001$, according to the Dunn test. Bars indicate mean ± SD.

(259.1 ± 42.0 U. Carr; $p < 0.005$) (fig. 1a). In particular, the mean d-ROMs level of the 'stroke type' patients (361.0 ± 119.6 U. Carr) was significantly greater than that of the controls ($p < 0.01$) (fig. 2a). Meanwhile, the mean d-ROMs level of 'non-stroke type' patients (261.5 ± 28.0 U.Carr) demonstrated no significant differences compared with those of the controls and 'stroke type' patients (fig. 2a).

The mean BAP level of all patients (2,258.9 ± 517.7 μmol/l) was not significantly different compared with that of the controls (2,057.6 ± 149.5 μmol/l) (fig. 1b). However, compared with the controls, 'stroke type' patients (2,428.9 ± 523.1 μmol/l) demonstrated significantly high BAP levels ($p < 0.01$), and 'non-stroke type' patients (1,834.0 ± 59.2 μmol/l) demonstrated significantly low BAP levels ($p < 0.001$) (fig. 2b). There was no significant difference between 'stroke type' patients and 'non-stroke type' patients in terms of the mean BAP levels.

The mean BAP/d-ROMs ratio of all patients (7.87 ± 5.05) was significantly lower than that of the controls (8.13 ± 1.30; $p < 0.02$) (fig. 1c). However, there were no significant differences among the controls and patient groups (fig. 2c).

There was no relationship between the functional status evaluated by performance status rating and the d-ROMs level or BAP level or BAP/d-ROMs ratio.

Discussion

In the present study, the d-ROMs and BAP tests were applied to evaluate the redox states in serum of patients carrying A3243G. These tests demonstrated that oxidative stress represented by the d-ROMs levels was increased and redox balance represented by the BAP/d-ROMs ratios was decreased (tendency for oxidation) in the patients compared with those of the controls (fig. 1). These findings suggested that an imbalance of redox states due to mitochondrial dysfunction affects the pathogenesis in patients carrying A3243G.

In the 'stroke type' patients in particular, both d-ROMs levels (oxidative stress) and BAP levels (antioxidant activity) were increased compared with those of the controls (fig. 2a, b). In vitro studies previously demonstrated that A3243G enhances ROS generation leading to oxidative stress [7–10], and enhanced oxidative stress is proportional to mitochondrial dysfunction [7, 23]. In the present study, all of the 'stroke type' patients have been treated with antioxidants, and 8 out of 10 patients were also treated with an oral administration of L-arginine. Although serum antioxidant activity may be increased by antioxidants and L-arginine therapy, serum oxidative stress was still increased in 'stroke type' patients. Increased oxidative stress even with increased antioxidant activity suggested a severe deterioration of mitochondrial function in patients with a history of stroke-like epi-