Hydrogen Gas Protects H9c2 Cardiomyocytes from H₂O₂ - induced Oxidative Stress

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Abstract
The purpose of our study was to investigate basic mechanisms of hydrogen (H₂) gas – induced cardioprotection during oxidative stress in a cardiomyocytes cell culture model. H₂ gas has been demonstrated to reduce the hydroxyl radical (·OH), the most cytotoxic of reactive oxygen species (ROS), and effectively protected cells. Here, we developed a new model for evaluating that H₂ gas has the potential to act as antioxidant on H₂O₂-induced death of H9c2 cell. Treatment with H₂O₂ (500µM) induced death of H9c2 cells. However, co-treatment with H₂ gas increases viability of cells. These results suggest that method is a useful alternative animal model to clarify the mechanism which H₂ gas protects H9c2 cardiomyocytes from oxidative stress.

Key words: hydrogen gas; oxidative stress; cardioprotection; H9c2

Introduction
Opening of the infarct-related coronary artery is a valued therapeutic goal in acute myocardial infarction (AMI). However, cardiomyocytes continue to die during reperfusion. During ischemia and reperfusion, the mechanisms of cell death during this phase of AMI have been subject to debate (Saraste et al., 1997). Although restoration of blood flow is critical, the re-introduction of molecular oxygen triggers a cytotoxic cascade during which reactive oxygen species (ROS) are generated by the mitochondria.

The central role of ROS in reperfusion injury has been well demonstrated in recent studies showing that inhibitors of mitochondrial respiratory complexes I and III prevent reperfusion ROS generation and improve cellular viability (Chen et al., 2006, Burwell et al., 2006). However, therapy to reduce free radicals during early reperfusion failed to relieve this pathological cascade of oxidative damage after reperfusion injury (Penna et al., 2006).

A recent article demonstrated that molecular hydrogen (H₂) is a novel antioxidant agent which confers protection in focal brain ischemia reperfusion injury (Ohsawa et al., 2007). This observation was echoed by others in different organs such as in ischemia and reperfusion injury in liver and heart through the inhibition of oxidant stress using mice or rat models (Fukuda et al., 2006, Hayashida et al., 2008).

Evaluating of therapeutic antioxidant by reducing cytotoxic oxygen radicals, mammalian models are used. However, alternative testing, originating from the viewpoint of animal welfare, replaces animal testing with experiments that use non-animal systems for the purpose of research, education, toxicity testing, or production, and includes reductions in the use of animals and lessening or eliminating pain or distress to animals.
Therefore, the aim of our investigations was to study in vitro model, which is easy to use, whether 
H2 gas protects cardiomyocytes from H2O2 induced oxidative stress and cell death in H9c2.

Materials and methods

Cell culture and Treatment

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the American Type Culture Collection (ATCC CRL-1446™). H9c2 cardiomyocytes were maintained in Dulbecco’s modified Eagle’s medium DMEM with 10% fetal bovine serum (FBS), 1% penicillin /streptomycin in a humidified atmosphere containing 5% CO2. Cells in the exponential phase of growth were used. For all experiments, the cells were plated at an appropriate density according to the experimental design and were grown for 36 h before experimentation.

H2 gas treatment of H9c2

Cells used for this experiment were seeded at 1.2 \times 10^4 cells on 96-well flat-bottom plates. Before experiments, Cultures were exposed to H2 gas by placing them into Modular Incubator Chamber (MIC-101, Billups Rothenberg Inc) through 2 % H2, 71 % N2, 22 % O2 and 5 % CO2 gas. H2 concentrations were measured by an H2 sensor (XP-3160; New Cosmos Electric, Osaka, Japan). The chamber was then sealed and placed at 37 °C in conventional cell incubator.

Assay of cell viability in H9c2

Cell viability was determined using a WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulphophenyl]-2H-tetrazolium, monosodium salt) assay according to the manufacturer’s protocol (Nacalai Tesque, Kyoto, Japan) (Ishiyama et al., 1997). H9c2 (1.2 \times 10^4 per well) cells were cultured in 96-well flat-bottom plates and treated with H2O2. They were divided into different groups with H2O2 concentrations (500 μM) for 1 hour. Then WST (10 μL per well) was added to each well. The wells were lysed after incubation at 37 °C for 2 hours. The absorbance at 450 nm was measured by a spectrophotometer microplate reader. The percent viability was defined as the relative absorbance of the treated cells versus the untreated control cells. Three independent experiments were performed for each experimental condition.

Statistical analyses

Data are presented as the mean ± SEM. Differences were assessed by ANOVA followed by Fisher's Protected Least Significant Difference for multiple comparisons. The results were considered statistically significant at level of P<0.05.

Results

Effects of single treatment of H2O2 on H9c2 cells viability

We first determined the dose at which cytotoxicity develops in a period of 1 h upon H2O2 exposure in exposure in H9c2 cells through the WST-8 assay. Cells were treated with increasing doses of H2O2 for 1 h. As shown in Figure 1, H2O2 impaired cell viability in a concentration-dependent manner over the tested concentration range (200 or 500 μM). A maximum reduction was 44.3±3.6% of the control group and it was observed at 500 μM of H2O2. We chose the level of H2O2 500 μM for use in our experiments based on this result. In order to evaluate whether H2 gas is cytotoxic to H9c2 cells, we determined the viability of cells treated with H2 gas (2 %) for 18 h using the WST-8 assay.

Effects of H2 gas on H2O2-induction cell death in H9c2 cell

In order to determine the effects of H2 gas, on H2O2-induction cell death, the H9c2 cells were pretreated with H2 gas for 18 h, after which
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co-incubated with 500 µM of H₂O₂ for additional 1 h. As shown in Figure 2, co-treatment of H₂ gas increases viability of cells at 191.6±5.7 %.

Discussion

In the present study, we demonstrate that a long period of H₂ gas provides protection against H₂O₂ induced oxidative stress in H9c2 cardiomyocytes in vitro. In addition, we established a new method which can be simply performed in evaluating the therapeutic effects of H₂ gas.

Treatment of acute myocardial infarction (AMI) has advanced considerably in terms of early reperfusion therapy, due to the development of catheter interventions and thrombolytic procedures. However, reperfusion therapy after brief ischemia not only triggers lethal arrhythmias and myocardial stunning but also paradoxically aggravates the area of infarction. During ischemia and reperfusion, cell death of cardiomyocytes causes direct cardiomyocytes from cytotoxic stimuli such as oxidative stress. Therefore, many studies have focused on ways to protect cardiomyocytes from cytotoxic stimuli such as oxidative stress. Due to its important in heart function, cardiomyocytes seem to have mechanisms protecting itself against cytotoxic stimuli (Ohta, 2014).

ROS are produced on reperfusion of ischemic myocardium, and are considered a major cause of lethal reperfusion injury (Zweier et al., 1987, Bolli et al., 1988, Vanden Hoek et al., 2000). Radical oxygen species O₂⁻ and H₂O₂ are detoxified by antioxidant defense enzymes, unlike •OH and ONOO⁻, which so far no enzyme could detoxify. Recently, Ohsawa et al. demonstrated that molecular H₂ gas has the oxidative stress, which is caused by the accumulation of intracellular ROS is one of the leading factors triggering cardiomyocytes apoptosis (Ohsawa et al., 2007). The degree of cardioprotection against ischemia-reperfusion injury was evaluated by measuring oxidative damage and infarct size 30 min after left anterior descending coronary artery occlusion and reperfusion in vivo. Inhalation of an incombustible level of H₂ gas (2%) before reperfusion significantly reduced oxidative stress-induced myocardial injury and infarct size (Hayashida et al., 2008).

Similarly, in our study, we found reduced cell viability in H₂O₂ damaged H9c2 cells, whereas H9c2 cell pretreated with H₂ gas (2%) was significantly increased cell viability in vitro. Therefore, we consider that one possible mechanism of H₂ gas preserving cell survival pathways could be by reducing •OH in vitro.

In conclusion, we propose the usefulness of in vitro model for evaluating the therapeutic effects of H₂ gas in H9c2 cell. Further understanding of the mechanisms underlying the signaling pathways involved in H₂-mediated anti-oxidant activity in vitro.

References


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