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□ ORIGINAL ARTICLE □

Hydrogen May Inhibit Collagen-Induced Platelet Aggregation: An *ex vivo* and *in vivo* Study

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Abstract

Objective Hydrogen selectively reduces hydroxyl radicals and peroxynitrite, and numerous experimental and clinical studies suggest that hydrogen can exert potent cellular protective effects against a wide variety of diseases. Furthermore, there is increasing evidence that antioxidants can modulate platelet activation. The aim of the present study was to investigate the relationship between hydrogen and collagen-induced platelet aggregation.

Methods For human *ex vivo* studies, we collected blood samples from six healthy humans and added normal saline or hydrogen-rich saline to blood and platelet-rich plasma. We found that collagen (1 μg/mL)-induced platelet aggregation was significantly inhibited by hydrogen-rich saline compared with a normal saline group (p=0.044). For rat *in vivo* studies, animals (n=17) were exposed to either nitrogen-based mixed gas with hydrogen (H_2 gas group; n=9) or without hydrogen (non-H_2 gas group; n=8). Additionally, another animals (n=13) administered either normal (NS group; n=7) or hydrogen-rich saline (HS group; n=6) (5 ml/kg) via intravenous infusion. Blood samples were drawn from the vena cava before treatment and from the right ventricle after treatment. Collagen (12 μg/mL)-induced platelet aggregation was then measured.

Results Collagen-induced platelet aggregation was significantly decreased in H_2 gas and HS group rats (p=0.042, 0.018, respectively), while there was no difference in non-H_2 gas and NS group rats before and after treatment.

Conclusion In summary, these data suggest that hydrogen may inhibit collagen-induced platelet aggregation.

Key words: hydrogen, hydroxyl radical, platelet aggregation, collagen

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Introduction

Hydrogen is the simplest and most abundant natural chemical element, constituting approximately 75% of the universal elemental mass (1). Hydrogen can selectively reduce the levels of hydroxyl radicals (˙OH) and peroxynitrite ($ONOO^-$), which are strong oxidative reactive oxygen species (ROS) that react indiscriminately with nucleic acids, lipids, and proteins, thus resulting in DNA fragmentation, lipid peroxidation, and protein inactivation (2). Since Ohsawa et al. discovered that hydrogen gas has antioxidant and anti-apoptotic properties which act against ischemia-reperfusion injury in the brain (3), hydrogen has come to the forefront of therapeutic medical gas research (2). Several other studies have also demonstrated similar protective effects of hydrogen in ischemia-reperfusion injuries caused by oxidative stress in the brain, liver, heart, and intestines (4-8). Nevertheless, research on the biological use of hydrogen is just beginning, and therefore, the precise mechanism(s) underlying how hydrogen exerts its cytoprotective effects remain unclear.

Platelets exert a crucial function in hemostasis, wound repair, and the formation of vascular plugs, and there is increasing evidence that antioxidants modulate platelet activation (9, 10). Several publications have suggested that ROS

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can modulate platelet activity (9, 11-13). The release of several ROS, including superoxide anion (O_2^-), ˙OH, and hydrogen peroxide (H_2O_2), from both unstimulated (resting) and collagen or thrombin-stimulated platelets, has been reported (9). Caccese et al., showed that collagen-induced platelet aggregation was associated with ˙OH and O_2^- formation which activate arachidonic acid metabolism (13). In fact, several antioxidant agents, such as vitamin E and salicylic acid, have been shown to inhibit collagen-induced platelet aggregation (13, 14).

We therefore hypothesized that hydrogen may act as an inhibitor of collagen-induced platelet aggregation via the reduction of ˙OH. The aim of the present study was to investigate the relationship between hydrogen and collagen-induced platelet aggregation.

Materials and Methods

Production of hydrogen-rich saline

Hydrogen-rich saline (HS) was produced with a non-destructive hydrogen adding apparatus (Miz Co., Fujisawa, Japan; Patent No.4486157, Patent Gazette of Japan 2010). Bags of physiological saline solution (500 mL; Terumo Co., Tokyo, Japan) were immersed, without opening or altering the bag, in a water tank in which water is electrolyzed periodically to produce hydrogen-rich water of up to concentrations of 1.6 ppm. The hydrogen concentration increased in the bag by diffusion through the wall of the bag to more than 1.0 ppm, until saturated. Further information can be found using the following link: http://www.e-miz.co.jp/english/technology.html#non_destructive. Before performing experiments, the pH and oxygen pressure in normal saline (NS) and HS were measured using a pH meter (Denver Instrument Co., Denver, CO, USA) and oxygen sensor (Bioresearch, Nagoya, Japan). The results were obtained from four different samples.

Human ex vivo study

These studies were approved by the Ethics Committee of the National Defense Medical College. Written informed consent was obtained from all study participants.

Blood collection

Six healthy volunteers participated in this experiment (four males, two females; age range from 30 to 57 years, mean 38.2 years). We confirmed that none had a history of any platelet defects and none had ingested any drugs known to interfere with platelet function for at least 15 days. Blood samples were drawn without stasis from an antecubital vein with a 21-gauge needle. Two samples of 2.7 mL of blood mixed with 0.3 mL of 3.8% sodium citrate (9 : 1) were collected from each volunteer.

Platelet aggregation

NS (0.5 mL) or HS (0.5 mL) was added to each set of blood and incubated for 10 minutes. Platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood at 1,000 rpm for 10 minutes and the platelet count in the PRP was measured by a Bürker-Turk cell counter (Erma, Tokyo, Japan). Platelet-poor plasma (PPP) was obtained by centrifugation of citrated blood at 3,000 rpm for 10 minutes. The PRP platelet count was adjusted to 300,000 platelets/μL with PPP. NS (0.1 mL) or HS (0.1 mL) was added to 0.9 mL of PRP and incubated for a further 10 minutes. Collagen (mainly type I, obtained from a horse tendon; LMS Co., Tokyo, Japan) (1 μg/mL) was added to PRP and platelet aggregation was measured with a platelet coagulation measuring system (platelet aggregometer; PA-20; Kowa Company Ltd., Tokyo, Japan). Results were calculated using maximum aggregation ratios.

Rat in vivo study

These studies were approved by the Animal Care and Use Committee of the National Defense Medical College.

Animals

Thirty Sprague-Dawley rats (male, 320-390 g, 9-10 weeks of age) were used. The rats were housed in individual cages under controlled environmental conditions (12/12 h light/dark cycle, 20-22℃) with food and water freely available, for 1 week before experimental procedure.

Blood collection

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Pretreatment blood samples were drawn without stasis from the vena cava with a 22-gauge needle. Posttreatment blood samples were drawn from the right ventricle. Blood (1.8 mL) mixed with 0.2 mL of 3.8% sodium citrate (9 : 1) was collected from each rat.

Platelet aggregation

From blood samples obtained before and after treatments, PRP and PPP was obtained as described above. The PRP platelet count was adjusted to 500,000 platelets/μL with PPP. Collagen (12 μg/mL) was added to PRP and platelet aggregation was measured with a platelet aggregometer. Measurements were performed within 30 minutes of blood collection. Results were calculated using maximum aggregation ratios.

Hydrogen gas treatment

Seventeen rats were randomly divided into two groups: non-H_2 gas group (n=9) or H_2 gas group (n=8). Following the first blood collection rats were placed individually in a sealed Plexiglas box with inflow and outflow outlets. H_2 gas group rats were exposed to nitrogen-based standard mixed gas with 1.3% hydrogen and 30% oxygen (Saisan Co., Saitama, Japan) for 3 hours, while the non-H_2 gas group rats were exposed to nitrogen-based high pressure mixed gas with 30% oxygen (without hydrogen) (Saisan Co., Saitama, Japan) for 3 hours. We applied a hydrogen concentration of

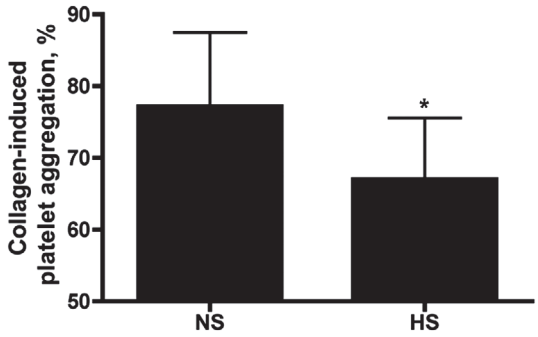

Figure 1. **Collagen-induced platelet aggregation in human *ex vivo* samples (n = 6) with normal saline (NS) or hydrogen-rich saline (HS). Compared with NS, HS significantly inhibited collagen-induced platelet aggregation (77.1 ± 21.3% in NS group vs. 67.0 ± 17.7 % in HS group; p = 0.044).**

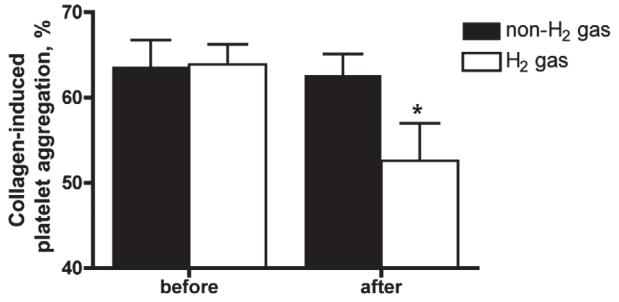

Figure 2. **Collagen-induced platelet aggregation in rat *in vivo* samples in a non-H_2 gas group (n = 6) or H_2 gas group (n = 7). Collagen-induced platelet aggregation was significantly decreased in the H_2 gas group (63.9 ± 7.1% before inhalation vs. 52.6 ± 13.3% after inhalation; p = 0.042), while there were no differences in collagen-induced platelet aggregation in the non-H_2 gas group (63.6 ± 8.8% before inhalation vs. 62.6 ± 7.0% after inhalation; p = 0.805).**

1.3% because it was safe when a high pressure mixed gas was used with 30% oxygen.

Hydrogen-rich saline treatment

Thirteen rats were randomly divided into two groups; NS group (n=6) or HS group (n=7). NS or HS (5 ml/kg) was administered via intravenous infusion into the vena cava after the first blood collection. Posttreatment blood samples were obtained 3 hours after infusion.

Statistical methods

All data are presented as mean ± SD. Comparisons of pH and partial oxygen pressure between NS and HS were analyzed by a Student's t test for unpaired data. Other analyses were performed using a Student's t test for paired data. A value of $p<0.05$ was considered statistically significant. All statistical analyses were performed with SPSS version 11.0 (SPSS, Inc., Chicago, IL, USA). Graphpad Prism 4.0 (GraphPad, San Diego, CA, USA) was used to make graphs.

Results

pH and oxygen pressure measurement

Following the generation of HS, the pH and oxygen partial pressure was compared with NS. There were no pH differences between the two salines (6.74±0.03 in NS vs. 6.70± 0.03 in HS; p=0.18). However, HS had a lower oxygen partial pressure than NS (150.6±1.0 mmHg in NS vs. 131.8 ± 5.1 mmHg in HS; p<0.01).

Human ex vivo study

HS significantly inhibited collagen-induced platelet aggregation compared with NS (77.1±21.3% in the NS group vs. 67.0±17.7% in the HS group; p=0.044) (Fig. 1). The mean rate of inhibition of platelet aggregation in the HS group was 12.8%.

Rat in vivo study

We first confirmed that there were no differences in collagen-induced platelet aggregation between each group before treatment.

Effect of hydrogen gas on aggregation

Collagen-induced platelet aggregation was significantly decreased in H_2 gas group rats (63.9±7.1% before inhalation vs. 52.6±13.3% after inhalation; p=0.042), while there were no differences in collagen-induced platelet aggregation in the non-H_2 gas group rats (63.6±8.8% before inhalation vs. 62.6 ± 7.0% after inhalation; p=0.805) (Fig. 2). The mean rate of inhibition of platelet aggregation in H_2 gas group rats was 17.7%.

Effect of hydrogen-rich saline on aggregation

Collagen-induced platelet aggregation was significantly decreased in HS group rats (63.7±4.7% before infusion vs. 43.4±19.9% after infusion; p=0.018), while there were no differences in collagen-induced platelet aggregation in NS group rats (62.8±10.8% before infusion vs. 60.0±13.5% after infusion; p=0.086) (Fig. 3). The mean rate of inhibition of platelet aggregation in HS group rats was 31.8%.

Discussion

In the present study we found that hydrogen inhibited collagen-induced platelet aggregation in both human *ex vivo* and rat *in vivo* studies, with inhibition rates of approximately 13-32%. The biological significance of these inhibition rates remains unclear as platelet aggregation was partially, not completely, inhibited.

Hydrogen can exert potent cytoprotective effects under a variety of ischemia-reperfusion conditions, including cerebral ischemia, myocardial infarction, and ischemic bowel disease, by selectively reducing $^{\cdot}OH$ and $ONOO^{-}$ (1-8). Hydrogen has a number of advantages as a potential antioxi-

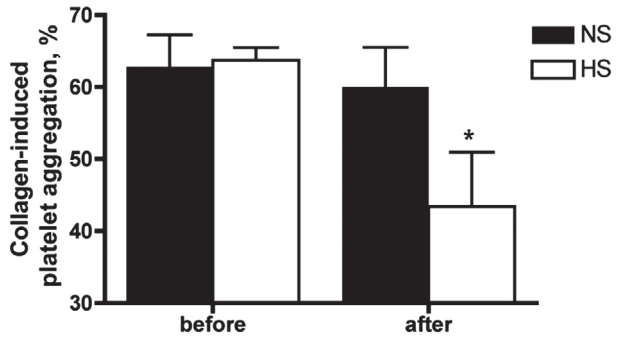

Figure 3. Collagen-induced platelet aggregation in rat *in vivo* samples in a NS group (n = 8) or HS group (n = 9). Collagen-induced platelet aggregation was significantly decreased in the HS group (63.7 ± 4.7% before infusion vs. 43.4 ± 19.9% after infusion; p = 0.018), while there were no differences in collagen-induced platelet aggregation in the NS group (62.8 ± 10.8% before infusion vs. 60.0 ± 13.5% after infusion; p = 0.086).

dant. First, hydrogen is highly diffusible and can potentially reach subcellular compartments, such as the mitochondria and nuclei, which are the primary sites of ROS generation and DNA damage (1-3). Second, hydrogen selectively reduces ˙OH and $ONOO^-$, and does not interact with O_2^- and H_2O_2, both of which have physiological roles (1-3), and therefore, hydrogen therapy is considered to be safe, as there have been no reported major adverse effects (1, 2). Third, hydrogen is easy to use and economical. All of these factors taken together thus make hydrogen a good candidate for protecting organs against ischemia-reperfusion injuries.

It is well appreciated from clinical and experimental studies that platelets are activated following ischemia-reperfusion injury, and that they exacerbate organ injuries (15-20). Furthermore, Leo et al. reported that platelets exposed to anoxia-reoxygenation were significantly activated via an increase in ˙OH (21). Based on these results, we suggest that inhibition of platelet aggregation may be an additional factor that contributes to the beneficial effects of hydrogen in several ischemia-reperfusion conditions.

We found that the oxygen partial pressure of HS was slightly lower than NS. However, hypoxia itself has been shown to have no effect on collagen-induced platelet aggregation (22), and we therefore believe that the lower oxygen partial pressure in HS had no effect on the present results.

Based on these results, we hypothesized that hydrogen reduces platelet-derived ˙OH, which was observed to increase following the addition of collagen, leading to changes in arachidonic acid metabolism, which resulted in an inhibition of platelet aggregation. Additionally, the partial inhibition of platelet aggregation by hydrogen may contribute to the selective reduction of ˙OH and $ONOO^-$, because hydrogen has no effect on other ROS (O_2^- and H_2O_2).

However, further investigations into whether hydrogen can normalize the abnormal activation of platelets in ischemia-reperfusion injury models are required. Additionally, further *in vivo* investigations in humans will be necessary to examine the optimal applications of hydrogen therapy.

Conclusion

The present study suggests that hydrogen may inhibit collagen-induced platelet aggregation. Hydrogen may be useful for the normalization of abnormally activated platelets under ischemia-reperfusion conditions. Further investigations are thus necessary to elucidate the detailed mechanisms underlying how hydrogen exerts its cytoprotective effects, and to determine the optimal applications of hydrogen therapy.

The authors state that they have no Conflict of Interest (COI).

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