H₂ Inhibits the Formation of Neutrophil Extracellular Traps

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HIGHLIGHTS

- NETs have been implicated as therapeutic targets to address inflammation and thrombotic tissue damage in conditions such as sepsis, acute respiratory disease syndrome, COVID-19, and CVDs.
- H₂ has been clinically and experimentally proven to ameliorate inflammation; however, the underlying molecular mechanisms remain elusive.
- Compared with control neutrophils, PMA-stimulated human neutrophils exposed to H₂ exhibited reduced citrullination of histones and release of NET components; mechanistically, H₂-mediated neutralization of HOCl produced during oxidative bursts suppresses DNA damage.
- Inhalation of H₂ inhibited the formation and release of NET components in the blood and BAL of the LPS-induced sepsis in mice and aged mini pigs.
- H₂ therapy is potentially a new therapeutic strategy for inflammatory diseases involving NETs associated with excessive neutrophil activation.
Neutrophil extracellular traps (NETs) contribute to inflammatory pathogenesis in numerous conditions, including infectious and cardiovascular diseases, and have attracted attention as potential therapeutic targets. H2 acts as an antioxidant and has been clinically and experimentally proven to ameliorate inflammation. This study was performed to investigate whether H2 could inhibit NET formation and excessive neutrophil activation. Neutrophils isolated from the blood of healthy volunteers were stimulated with phorbol-12-myristate-13-acetate (PMA) or the calcium ionophore A23187 in H2-exposed or control media. Compared with control neutrophils, PMA- or A23187-stimulated human neutrophils exposed to H2 exhibited reduced neutrophil aggregation, citrullination of histones, membrane disruption by chromatin complexes, and release of NET components. CXCR4high neutrophils are highly prone to NETs, and H2 suppressed Ser-139 phosphorylation in H2AX, a marker of DNA damage, thereby suppressing the induction of CXCR4 expression. H2 suppressed both myeloperoxidase chlorination activity and production of reactive oxygen species to the same degree as N-acetylcyesteine and ascorbic acid, while showing a more potent ability to inhibit NET formation than these antioxidants do in PMA-stimulated neutrophils. Although A23187 formed NETs in a reactive oxygen species-independent manner, H2 inhibited A23187-induced NET formation, probably via direct inhibition of peptidyl arginine deiminase 4-mediated histone citrullination. Inhalation of H2 inhibited the formation and release of NET components in the blood and bronchoalveolar lavage fluid in animal models of lipopolysaccharide-induced sepsis (mice and aged mini pigs). Thus, H2 therapy can be a novel therapeutic strategy for NETs associated with excessive neutrophil activation. (J Am Coll Cardiol Basic Trans Sci 2022;7:146–161) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
neutrophils from healthy volunteers. In addition, we studied the effects of H₂ inhalation on NET formation in mice and micro mini pigs.

**METHODS**

**ANIMALS AND CARE.** The present study was designed according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The experiments were performed in accordance with our institutional guidelines and the Japanese law on the protection and management of animals. Ethical approval was granted by the Research Council and Animal Care and Use Committee of Keio University (approval nos: 12094-(8), 20008-(0)) and HAMRI Co, Ltd (approval no: 20-H055).

Male BALB/c mice (10 weeks old, weighing 25-30 g) were purchased from CLEA Japan Inc. The mice were housed in a temperature-controlled room with a 12-hour day/night cycle at 25 °C and had free access to food and water.

Female micro mini pigs (263-308 weeks old, weighing 23-37 kg) were purchased from Fuji Micra Inc. The pigs were housed in separate cages in a temperature-controlled room with a 12-hour day/night cycle at 25 °C and had free access to food and water. Before surgery, the pigs were fasted for 12 hours with free access to water.

**H₂ INHALATION VIA NASAL CANNULA BY H₂ GENERATOR.** We adjusted the nostril inserts of the nasal cannula (Nakamura Medical Industry Co, Ltd) to fit the shape of the pig’s nose. This study used the H2J11 H2 inhaler, manufactured by Doctors Man Co, Ltd. The inhaler uses a proton-exchange membrane water electrolysis system that can continuously generate high-purity (>99.999%) H₂. The nasal cannula was inserted deep into the nasal cavity of the pig under spontaneous breathing, and H₂ produced from the H₂ generator was administered at a flow rate of 250 mL/min. A veterinary anesthesia mask (Shinano Manufacturing Co, Ltd) was placed over the nasal cannula, and an ADS 1000 (model 2000) veterinary anesthesia delivery system (Tokushima Iryoki Co, Ltd) was used to deliver a mixture of O₂ and isoflurane. The flow rate of the O₂/isoflurane gas mixture was maintained above 6 L/min to avoid reinhalation of exhaled CO₂ that would remain in the mask.

**LPS INJECTION INTO MICRO MINI PIGS.** A lipopolysaccharide (LPS) was administered as previously described, with modifications. The animals received continuous intravenous infusion of *Escherichia coli* LPS (serotype 055:B5, 20 µg/kg/h; Sigma-Aldrich) for 1 hour. LPS was dissolved in saline and delivered by a syringe pump (Supplemental Figure 1A).

Hemodynamic and respiratory parameters were measured every 30 minutes during an experimental period of 180 minutes. The chest was opened through a midthoracic incision, the pulmonary artery was clamped, and pulmonary artery blood was collected from the distal part of the clamp with an 18-G needle (Supplemental Figure 1B). Plasma was collected from a container with 3.2% sodium citrate, and the cell pellets from a container with EDTA-2K were smeared onto glass slides using Smear Gell (Geno Staff) after centrifugation.

**ISOLATION OF NEUTROPHILS.** Human neutrophils were isolated from the peripheral blood of healthy volunteers, who provided written informed consent, using Lympholyte-poly (CEDARLANE Laboratories) according to the manufacturer’s instructions, after approval from the Ethics Review Committee of Keio University (#20200183). Polynuclear cells containing neutrophils were collected, washed, and resuspended in 5 mL of red blood cell lysis buffer (BioLegend), then washed twice in Hank’s buffered salt solution without Ca²⁺/Mg²⁺ (Wako Pure Chemicals), and finally resuspended in the Roswell Park Memorial Institute 1640 medium (Wako).

Neutrophils from micro mini pigs were isolated using previously described methods, with modifications. Blood from the pulmonary artery of micro mini pigs was collected in a container coated with EDTA-2K and treated with red blood cell lysis buffer for 10 minutes at 4 °C. Neutrophils were separated from mononuclear cells by layering 5 mL of the cell suspension on 5 mL of Percoll 1.081 (GE Healthcare), which was layered under 5 mL of Percoll 1.087, followed by centrifugation at 1,000g for 20 minutes at 25 °C. The middle layer, enriched with neutrophils, was collected and washed twice in Hank’s buffered salt solution without Ca²⁺/Mg²⁺ (Wako Pure Chemicals), and finally resuspended in red blood cell lysis buffer and washed twice with Hank’s buffered salt solution without Ca²⁺/Mg²⁺.

**INDUCTION OF NETs EX VIVO IN H₂-EXPOSED CULTURES OF HUMAN NEUTROPHILS.** Cell cultures of neutrophils in Roswell Park Memorial Institute 1640 medium containing 15 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid were exposed to H₂ using a hydrogen-filling device with a built-in hydrogen-absorbing alloy (Doctors Man Co, Ltd). The control medium was left untreated. Aliquots containing 1 x 10⁷ isolated human neutrophils were stimulated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or A23187 (Sigma-Aldrich) in the control or H₂-exposed medium at 37 °C for 3 hours in 15-mL Falcon tubes.
(Thermo Fisher Scientific). After incubation, the cells were centrifuged at 4 °C for 10 minutes at 500g to collect the supernatant. The cell pellets were then smeared to the glass slides using Smear Gel. The percentage of dead cells after stimulation is shown in Supplemental Figures 2A and 2B.

**QUANTIFICATION OF HOCl PRODUCTION IN NEUTROPHILS.** The concentration of HOCl was analyzed using aliquots of human neutrophils with a colorimetric hypochlorite assay kit according to the manufacturer’s instructions (Cayman Chemical Company).

**ENZYME-LINKED IMMUNOSORBENT ASSAY.** Levels of citrullinated histone H3 (CitH3) (Clone 11D3) in culture supernatants or plasma were determined using enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Cayman Chemical Company).

**QUANTIFICATION OF NETs.** Culture supernatants and plasma were collected and stored at −80 °C. DNA content was measured using the Quant-it Picogreen double-stranded DNA (dsDNA) Assay Kit (Invitrogen), according to the manufacturer’s instructions.

**STATISTICAL ANALYSIS.** Differences between groups were analyzed by a paired or unpaired Student’s t-test in case of normal distribution or by the Wilcoxon signed-rank or Mann-Whitney U test, as appropriate, in case of non-normal distribution. Differences among multiple groups were compared using analysis of variance followed by Tukey’s post hoc test for multiple pairwise comparisons. Data are presented by dot plots with reference line at mean. A value of \( P < 0.05 \) was considered statistically significant. Statistical analysis was performed by IBM SPSS Statistics (version 26) software.

**RESULTS**

**H\(_2\) INHIBITS PMA-INDUCED NEUTROPHIL AGGREGATION AND SUBSEQUENT NET RELEASE.** To determine the effect of \( H_2 \) on NET formation, \( 1 \times 10^7 \) neutrophils isolated from the peripheral blood of healthy volunteers (n = 5) were stimulated with 100 nmol/L PMA, a protein kinase C activator, in an \( H_2 \)-treated medium for 3 hours. Roswell Park Memorial Institute 1640 medium containing 15 mmol/L N2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (pH 7.2) was treated with \( H_2 \) using a filling device with a built-in hydrogen-absorbing alloy; untreated medium was used as a control.

Immunostaining analysis revealed that citrullination of histones (CitH3), membrane disruption by chromatin complexes, and release of NET components (DNA/Histone H1, SYTOX Green) were suppressed in \( H_2 \)-treated cultures compared with those in control cultures (Figures 1A to 1C, Supplemental Figure 2C). Furthermore, the release of dsDNA from neutrophils was markedly inhibited in the \( H_2 \)-treated medium (Figure 1D).

After 1 hour of PMA stimulation, preceding NET release, aggregation of neutrophils was observed in the control medium, which was significantly suppressed in the \( H_2 \)-treated medium (Figure 1E, Supplemental Figure 2D, Video 1). Aggregation of neutrophils promotes an oxidative burst and the formation of NETs. These results indicate that \( H_2 \) inhibits neutrophil aggregation and, possibly, the subsequent release of NETs.

**H\(_2\) HAD A MINOR EFFECT ON GENE EXPRESSION IN PMA-STIMULATED NEUTROPHILS.** To the best of our knowledge, this is the first study to demonstrate the bioactive effects of \( H_2 \) at the cellular level. To elucidate the mechanism of the inhibitory effect of \( H_2 \) on neutrophil priming leading to NET formation and release, neutrophils isolated from 3 healthy volunteers were either left unstimulated or stimulated with PMA in either the control or \( H_2 \)-treated medium for 1 hour, and global transcriptome changes were compared by RNA sequencing.

Hierarchical clustering of the RNA sequencing data showed that the untreated population and the PMA-stimulated population formed separate clusters; however, the presence or absence of \( H_2 \) had no effect on cluster formation (Figure 2A). Principal component analysis showed similar results (Figure 2B). Furthermore, the heat maps showed that \( H_2 \) administration did not cause drastic changes in the gene expression of PMA-stimulated human neutrophils (Figure 2C). These results suggest that the variations in gene expression that are induced by PMA stimulation alone were greater than those induced by the effect of \( H_2 \) after PMA stimulation in human neutrophils.

The genes whose expression was increased by PMA and repressed by \( H_2 \) administration were investigated. Our results showed that, compared with the group left untreated, 2,059 differentially expressed genes were up-regulated in the PMA-stimulated group. PMA-stimulation with \( H_2 \)-exposed medium down-regulated 117 differentially expressed genes compared with those in the control medium. To further examine the differences in gene expression in these groups, Venn diagrams were plotted for the overlap of the genes. Among the 2,059 genes up-regulated on PMA stimulation, 26 genes were down-regulated by \( H_2 \) pretreatment (Figure 2D). Furthermore, gene ontology analysis of these 26 genes showed that \( H_2 \) administration repressed the genes involved in cellular senescence among the
Neutrophils purified from the blood of healthy volunteers were left untreated or stimulated with phorbol-12-myristate-13-acetate (PMA) in either a H2-exposed medium or control medium for 1 hour. (A) Microscopic images depicting the fluorescence of DNA/histone H1 (Alexa Fluor 488, green), citrullination of histone 3 (CitH3) (citrulline R2 + R8 + R17; Alexa Fluor 594, red), and 4′,6-diamidino-2-phenylindole (blue) in cultured neutrophils. Original magnification ×20. Bars = 100 μm. (B,C) Percentage of CitH3-positive or DNA-releasing neutrophils (n = 5). (D) Quantification of double-stranded DNA (dsDNA) released by activated neutrophils into the culture medium (n = 6). (E) Microscopic images depicting the fluorescence of myeloperoxidase (MPO) (Alexa Fluor 594, red) and 4′,6-diamidino-2-phenylindole (blue) in cultured neutrophils. Original magnification ×120. Bars = 50 μm. *P < 0.05; ***P < 0.001. NET = neutrophil extracellular trap.
Neutrophils purified from the blood of healthy volunteers were stimulated with phorbol-12-myristate-13-acetate (PMA) in either a H₂-exposed medium or control medium for 1 hour. (A) The dendrogram depicting hierarchical clustering of all RNA-sequencing (RNA-seq) samples. (B) Principal component (PC) analysis plot of all RNA-seq samples. Replicates of the same cell type are indicated by the same color, as shown in the legend. (C) Heat map of gene expression profiles shown with gene-wise (row) and sample-wise (column) clustering dendrograms. The color corresponds to the square root of standardized transcripts per million values by row, as shown in the legends on the upper left. (D) Venn diagram showing the overlap between genes up-regulated on PMA treatment (untreated vs PMA) with those down-regulated on subsequent H₂ administration (PMA vs PMA + H₂). (E) Gene Ontology (GO) enrichment analysis of biological processes for the 26 overlapping genes.
The results indicate that although H2 has a drastic effect on PMA-induced neutrophil aggregation and NET formation and release, its effect on the gene expression profile of neutrophils during the first hour of PMA stimulation is very limited. The few changes in gene expression that were observed suggest that H2 may affect the process of neutrophil aging.

**H2 Suppressed PMA-Induced DNA Damage in Neutrophils.** H2AX is a variant of H2A, one of the core histones that make up nucleosomes. Formation of γ-H2AX via phosphorylation of the Ser-139 residue of H2AX is an early cellular response to the induction of DNA double-strand breaks and a molecular marker of DNA damage.26,27 The expression of γ-H2AX was markedly increased in neutrophils stimulated with PMA for 1 hour in the control medium. In contrast, this change was significantly suppressed in hydrogen-containing medium (Figure 3A). Flow cytometry analysis demonstrated that SYTOX+ NET-forming neutrophils increased 1 hour after PMA stimulation in the control medium. However, subsequent H2 administration decreased the SYTOX+ neutrophils to a level comparable to that of the control neutrophils (Figure 3B). CXCR4hi aged neutrophils are known to be prone to NET formation.28,29 Interestingly, the subset of neutrophils expressing CXCR4 on their cell surface increased 1 hour after PMA stimulation (Figure 3C). Furthermore, CXCR4hi neutrophils were also detected to be SYTOX+ (Figures 3D and 3E).

In the H2-treated medium, the increase in the population of CXCR4hi neutrophils seen in the control medium was suppressed, and the population of SYTOX− CXCR4hi neutrophils was also lower than that in the control medium (Figures 3C to 3E). The ability of neutrophils to form NETs is also regulated by the content of their intracellular granules.28,30 We hypothesized that CXCR4hi neutrophils are prone to NET formation because CXCR2-mediated homeostatic degranulation is impaired and granules containing NET-forming proteins accumulate in the cells. Interestingly, the intracellular MPO content in the H2-treated medium was lower than that in the control medium, which is consistent with the suppression of CXCR4 expression on the cell surface (Figures 3F and 3G). These results suggest that H2 may inhibit PMA-stimulated CXCR4 expression in neutrophils and maintain homeostatic degranulation, thereby suppressing NET formation.

From midnight to early morning, CXCR4 expression in neutrophils is high and they are prone to form NETs. In the daytime, neutrophils have low CXCR4 expression and low intracellular granule content, suggesting that their NET-forming capacity is reduced.28 The release of dsDNA from neutrophils was significantly higher in neutrophils collected at 8:00 AM than in those collected at 2:00 PM (Supplemental Figure 3H). The inhibitory effect of H2 on the release of dsDNA was confirmed regardless of the time of day, but it was more pronounced in neutrophils collected at 8 AM (Supplemental Figure 3H). These results indicate that the expression level of CXCR4 regulates NET-forming capacity in humans as well as in mice. In addition, in relation to the diurnal variation of homeostatic degranulation, it was suggested that the inhibitory effect of H2 on NET formation may also vary depending on the time of day.

**H2 Suppressed HOCl Generation.** MPO catalyzes the reaction between H2O2 and chloride anion (Cl−), leading to the production of HOCl, which has strong oxidizing abilities and mediates inflammatory cellular damage.12,31,32 We tested whether H2 inhibits the ability of PMA-stimulated neutrophils to generate HOCl. The HOCl generation ability of isolated human neutrophils, evaluated by MPO chlorination activity, was notably suppressed in the hydrogen-exposed medium compared with that in the control medium (Figures 4A and 4B). However, H2 did not suppress phagocytosis or chemotaxis (Figures 4C and 4D).

| Gene Symbol | Log2 Fold Change | P Value |
|-------------|-----------------|---------|
| CA13        | −4.18           | 0.019   |
| LMP2        | −3.77           | 0.016   |
| DLL4        | −2.63           | 0.020   |
| MIR6514     | −2.47           | 0.008   |
| TAD2        | −2.23           | 0.025   |
| RH1         | −2.08           | 0.030   |
| NDC1        | −1.62           | 0.005   |
| NARAP       | −1.48           | 0.007   |
| DNAJA4      | −1.48           | 3.00 × 10−5 |
| CD160       | −1.36           | 0.033   |
| EIF3C       | −1.35           | 0.039   |
| RASD1       | −1.33           | 0.040   |
| GPA33       | −1.33           | 0.038   |
| MCR1        | −1.29           | 0.035   |
| RRA1        | −1.27           | 0.036   |
| WDR61       | −1.22           | 0.001   |
| HAVCR2      | −1.17           | 0.015   |
| H2AFX       | −1.13           | 2.10 × 10−5 |
| ID2         | −1.10           | 6.23 × 10−5 |
| TECR        | −1.10           | 0.009   |
| SNHG1       | −1.07           | 3.42 × 10−6 |
| BAG3        | −1.07           | 0.009   |
| POU1F1      | −1.06           | 0.026   |
| PLX2        | −1.06           | 0.001   |
| CCDC85B     | −1.06           | 0.012   |
FIGURE 3  H₂ Ameliorated the DNA Damage in Human Neutrophils

A

Phospho-Histon H2A.X (Ser 139)

Count

B

% of SYTOX in neutrophils

C

% of CXCR4 in neutrophils

D

SYTOX

CXCR4

2.6 ± 0.24

17.8 ± 1.57

10.1 ± 2.17

E

% of SYTOX+ CXCR4+ cells in neutrophils

F

MPO  DNA/Histone H1  Nucleus  Merge

PMAPMA + Hydrogen gas

PMAPMA + Hydrogen gas

G

Mean Fluorescence Intensity of MPO (a.u.)

H

dsDNA (ng/mL)

• PMA at 8 a.m.
• PMA + Hydrogen gas at 8 a.m.
• PMA at 2 p.m.
• PMA + Hydrogen gas at 2 p.m.

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These results indicate that the quenching of HOCl by H₂ is the key mechanism for the suppression of NET formation.

**H₂ SUPPRESSED A23187-INDUCED NETs FORMATION.** We examined whether hydrogen has an inhibitory effect on NET formation induced by other than PMA. The calcium ionophore A23187 is known to form NETs in a reactive oxygen species (ROS)-independent manner by increasing the intracellular Ca²⁺ concentration. A 23187-induced CitH3, membrane disruption by chromatin complexes, release of NET components (DNA/histone H1, SYTOX Green), and the release of dsDNA from neutrophils were suppressed in H₂-treated cultures compared with those in control cultures (Supplemental Figures 3A and 3B). However, A23187 stimulation, compared with PMA stimulation, resulted in lower ROS production in neutrophils, and H₂ had little effect on ROS production (Supplemental Figure 3C). There was also no significant difference in MPO chlorination activity between the control medium and the H₂-treated medium (Supplemental Figure 3D). Nevertheless, the expression of γ-H2AX was markedly increased in neutrophils stimulated with A23187 in the control medium. In contrast, this change was significantly suppressed in H₂-treated medium (Supplemental Figure 3E). Flow cytometric analysis demonstrated that CXCR4 and SYTOX expression was significantly suppressed in the H₂-treated medium compared with those in the control medium (Supplemental Figures 3F and 3G).

It has been reported that A23184-induced NETs formation is more dependent on the activity of peptidyl arginine deiminase 4 (PADI4) than PMA-induced NETs formation is. Indeed, the PADI4-specific inhibitor GSK484 was able to completely inhibit A23187-induced NETs formation, whereas it only partially inhibited PMA-induced NET formation (Supplemental Figures 4A to 4C). In neutrophils stimulated with A23187, the release of dsDNA from stimulated neutrophils with GSK484 and H₂ was comparable, and there was no additive effect of GSK484 and H₂ (Supplemental Figure 4C). These results suggest that in A23184-induced NETs formation, H₂ suppresses NET formation by inhibiting Ca²⁺-dependent PADI4 activation, not by suppressing ROS.

**H₂ INHIBITS PMA-INDUCED NETs FORMATION MORE EFFECTIVELY THAN ANTIOXIDANTS.** It is known that ROS is deeply involved in PMA-induced NETs formation. N-acetyl-L-cysteine (NAC) inhibited ROS and HOCl production to the same level as H₂ did (Supplemental Figures 5A and 5B). The antioxidant capacity of L-ascorbic acid was weaker than that of NAC (Supplemental Figures 5A and 5B). Interestingly, in PMA-stimulated neutrophils, the inhibitory effects of hydrogen on H2AX phosphorylation and CXCR4 expression and on neutrophil aggregation were stronger than those of NAC (Supplemental Figures 5C to 5G). As a result, PMA-induced CitH3, membrane disruption by chromatin complexes, release of NET components (DNA/histone H1, SYTOX Green), and the release of dsDNA from neutrophils were inhibited more strongly by hydrogen than by antioxidants such as NAC and L-ascorbic acid (Supplemental Figures 6A and 6B). These results suggest that not only removal of ROS, but also other mechanisms are involved in the inhibition of neutrophil aggregation and protection from DNA damage by hydrogen.

**INHALATION OF H₂ INHIBITED RELEASE OF NETs IN MICE AND AGED MICRO MINI PIGS.** Next, we tested the inhibitory effect of H₂ inhalation on NET formation in mice and micro mini pigs. An acute lung injury model was generated in 10-week-old male BALB/c mice by intratracheal administration of 8 mg/kg LPS. The mice were reared and exposed to H₂ as previously described and sacrificed 24 hours later (n = 5). H₂ inhalation inhibited the aggregation of neutrophils and formation of NETs (Figures 5A and 5B) in bronchoalveolar lavage (BAL). The concentrations of CitH3 and dsDNA in BAL were also lower in the H₂-exposed group than in the control group (Figures 5C and 5D). Similarly, in neutrophils isolated from mouse blood, inhalation of H₂ suppressed NET formation (Figures 5E to 5H). CitH₃⁺ cells appeared in...
the pulmonary arteries (PAs) of the LPS-induced acute lung injury model mice (Supplemental Figures 7A and 7B); however, inhalation of H₂, compared with control gas, significantly suppressed this change (Supplemental Figures 7C and 7D).

Next, 5-year-old mini pigs were randomly assigned to the H₂ or control groups, and LPS (20 μg/kg) was administered through the internal jugular vein continuously for 1 hour using an infusion pump (Supplemental Figure 1A). In the H₂-exposed group, 100% hydrogen was administered via nasal cannula at a rate of 250 mL/min with spontaneous breathing, as previously reported. 35 We confirmed that the H₂ concentration in the carotid blood during H₂ inhalation was maintained at approximately 5%. After 3 hours of LPS administration, the main trunk of the PAs was exposed through a midthoracic incision, and blood was collected from the distal part after clamping the proximal part of the PAs (Supplemental Figure 1B). In the PAs, LPS administration resulted in a high degree of NET formation in the control group (Figure 6A), whereas H₂ inhalation significantly inhibited NET formation and reduced plasma dsDNA levels (Figures 6B and 6C). H₂ inhalation also reduced CitH3⁺ cells in the lung compared with the number of cells in the control gas group (Figures 6D and 6E). These results indicate that inhalation of hydrogen can inhibit the formation and release of NETs in the blood, BAL, and lung in sepsis models of mice and aged pigs.

**DISCUSSION**

In the present study, we found that H₂ inhibited the PMA- and A23187-induced NET formation and release
FIGURE 5 Inhalation of H₂ Inhibited NET Formation in BAL Fluid of Mice

A. DNA/Histone H1, MPO, Nucleus, Merge

Control gas
Hydrogen gas

B. DNA releasing neutrophils (%)

C. CH H3 (ng/mL)

D. dS/dNA (ng/mL)

Control gas
Hydrogen gas

E. DNA/Histone H1, MPO, Nucleus, Merge

Control gas
Hydrogen gas

F. DNA releasing neutrophils (%)

G. CH H3 (ng/mL)

H. dS/dNA (ng/mL)

Control gas
Hydrogen gas

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in human neutrophils. Furthermore, we confirmed that H2 inhalation therapy suppressed NET formation and release in mice and aged micro mini pig models of LPS-induced sepsis.

PMA directly activates protein kinase C, resulting in the potent induction of both extracellular and intracellular ROS and triggers the formation of NETs in a ROS-dependent manner. The nicotinamide adenine dinucleotide phosphate oxidase activation can convert oxygen to superoxide radicals (O2−), and superoxide can be converted to H2O2 spontaneously or by superoxide dismutase. In neutrophils, MPO can catalyze the production of HOCl from H2O2 and chloride ions. This process is called oxidative burst. Oxidative bursts induce hypercitrullination of histones, decrease nucleosome binding, and cause overall chromatin decondensation. Following intracellular NET formation, the cell envelope is broken, and NETs are released into the extracellular space.

Ca2+ ionophore A23187 increases intracellular Ca2+ concentration by directly facilitating the transport of Ca2+ across the plasma membrane, which induces a rapid citrullination of multiple cellular proteins. Therefore, PADI4-mediated histone citrullination contributes greatly to NET formation in A23187-stimulated human neutrophils. It has been reported that A23187-induced NETs are not suppressed by administration of protein kinase C inhibitor. A23187 leads to a lower level of ROS compared with PMA or suppresses oxidative bursts in human neutrophils. In fact, ROS scavenger or MPO inhibitor has no inhibitory effect on NET formation. Thus, PMA and A23187 have different mechanisms leading to NET formation.

Our results suggest that the central mechanism that H2 suppresses NET formation is the strong reduction of ROS production like MPO-induced HOCl in PMA-stimulated human neutrophils; however, H2 mainly targets other than ROS in A23187-stimulated human neutrophils.

In fact, H2 significantly suppressed histone citrullination and NET formation despite little effect on ROS production and MPO chlorination activity in A23187-stimulated human neutrophils. GSK484, a PADI4-specific inhibitor, showed no additional effect of suppressing A23187-induced NET formation compared to H2 alone. These results suggest that H2 may have an inhibitory effect of Ca2+-dependent PADI4 activation. Furthermore, PMA-induced NET formation was also suppressed by antioxidants such as NAC and ascorbic acid, which is consistent with previous reports. NAC suppressed ROS and MPO chlorination activity to the same degree as H2 did; nevertheless, the effect of suppressing NET formation was stronger with H2 than with NAC. These results also support that H2 suppresses NET formation by its inhibitory effect of not only ROS but also other mechanisms.

Neutrophils from patients with chronic granulomatous disease without functional nicotinamide adenine dinucleotide phosphate oxidase lack the ability to release NETs in response to classical stimuli. To promote NET formation, ROS must be processed internally by MPO. Neutrophils derived from patients deficient in MPO fail to form NETs when exposed to PMA. These lines of evidence confirm the ability of HOCl to stimulate NET release. Recently, it has been reported that CXCR2-CXCL2 signaling induces degranulation of neutrophils and mitigates their potential histotoxicity through a “disarming” mechanism. In other words, CXCR2-CXCL2 signaling reduces the intracellular granule content, thereby reducing NET formation capacity. As neutrophils age, CXCR4, a negative regulator of CXCR2 signaling, on the cell surface is up-regulated. This change facilitates the clearance of senescent neutrophils in the bone marrow and CXCR4 neutrophils are known to be prone to NET formation. Furthermore, it has been shown that NET formation in response to PMA was strongly reduced in MRP8CRK Cxcr4−/− mice, which lacked CXCR4 specifically in neutrophils.

**FIGURE 5 Continued**

(A) Microscopic images depicting DNA/histone H1 (Alexa Fluor 488, green), MPO (Alexa Fluor 594, red), and nuclei (4′,6-diamidino-2-phenylindole, blue) in the bronchoalveolar lavage (BAL) fluid from mice that were intratracheally administered with lipopolysaccharide (E. coli serotype O55:B5) and kept in cages perfused with either H2 or control gas. Original magnification ×20. Bar = 100 µm. (B) Quantification of the percentage of DNA-releasing neutrophils in the BAL fluid of each group (n = 5). (C) Quantification of CitH3 in the BAL fluid of each group (n = 5). (D) Quantification of dsDNA released in the BAL fluid of each group (n = 5). (E) Microscopic images depicting DNA/histone H1 (Alexa Fluor 488, green), MPO (Alexa Fluor 594, red), and nuclei (4′,6-diamidino-2-phenylindole, blue) in the blood of mice that were intratracheally administered with lipopolysaccharide and kept in cages perfused with either H2 or control gas. Original magnification ×20. Bar = 100 µm. (F) Quantification of the percentage of DNA-releasing neutrophils in the blood of each group (n = 5). (G) Quantification of CitH3 in the plasma of each group (n = 5). (H) Quantification of dsDNA released in the plasma of each group (n = 5). *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations as in Figure 1.
**FIGURE 6** Inhalation of H₂ Inhibited NET Formation in the PAs of Micro Mini Pigs

**(A)** Microscopic images depicting DNA/histone H1 (Alexa Fluor 488, green) and nuclei (4',6-diamidino-2-phenylindole, blue) in purified neutrophils isolated from the pulmonary artery (PA) of micro mini pigs administered with 20 μg/kg lipopolysaccharide intravenously with the inhalation of H₂ or control gas. Original magnification ×20. Bars = 100 μm. **(B)** Quantification of the percentage of DNA-releasing neutrophils (n = 3). **(C)** Quantification of dsDNA released in the blood from the PA of each group (n = 3). **(D)** Representative immunofluorescence images of lung sections from micro mini pigs administered with 20 μg/kg lipopolysaccharide intravenously with the inhalation of H₂ or control gas. Microscopic images depicting the fluorescence of CitH3 (Alexa Fluor 594, red), DNA/histone H1 (Alexa Fluor 488, green), and 4',6-diamidino-2-phenylindole (blue). Original magnifications ×40 and ×120, respectively. Bars = 100 μm. **(E)** Number of CitH3-positive cells in low power field (LPF) (n = 5). **P < 0.01; ***P < 0.001. Abbreviations as in Figure 1.
Oxidative burst also induces DNA damage. DNA damage is the primary cause of cellular aging, and extensive DNA damage and the subsequent DNA repair pathway induce NETosis, leading to chromatin decondensation. We detected oxidative burst-induced phosphorylation of the Ser-139 residue of H2AX, a marker of double-strand breaks in DNA, and up-regulation of CXCR4 in PMA- or A23187-stimulated human neutrophils. This up-regulation of CXCR4 is also a response to DNA damage. In fact, NETs forming neutrophils expressed CXCR4. The process of NET release is thought to be driven by the amount of HOCl as HOCl production exceeds a certain threshold, NETs are released. These results suggest that H2 reduces DNA damage associated with oxidative burst and suppresses CXCR4 expression, thereby keeping intracellular MPO content low and preventing intracellular production of HOCl from reaching the threshold where the process of NET release begins.

One hour after PMA stimulation, neutrophils exhibited oxidative burst and aggregation, and H2 strongly inhibited these changes. However, RNA-sequencing analysis showed that its effect on gene expression was insignificant. This means that H2 does not directly affect signaling pathways and gene expression involved in PMA-induced neutrophil priming. In fact, H2 had no effect on phagocytosis or chemotaxis in PMA-stimulated neutrophils. HOCl is an order of magnitude more reactive with biomolecules than peroxynitrite (ONOO·) and H2O2 are and causes damage to biomacromolecules such as nucleic acids, proteins, and lipids. H2 is thought to mitigate DNA damage by reducing HOCl, a potent oxidant.

A recent multicenter randomized clinical trial showed that H2 inhalation improved the severity and symptoms in patients with COVID-19 pneumonia. Neutrophilia in patients with COVID-19 pneumonia is indicative of a poor prognosis, and NET hyperplasia has been observed in the blood, BAL fluid, and PAs of patients with severe COVID-19 pneumonia. The inhibitory effect of H2 on excessive neutrophil activation and NET formation may lead to improved prognosis in patients with COVID-19 pneumonia. Furthermore, HOCl has been shown to play a pivotal role in the pathogenesis of various inflammatory diseases and tissue injuries through protein modification. Modification of low-density lipoprotein by HOCl has been implicated in human atherosclerosis. HOCl-modified low-density lipoprotein leads to unregulated uptake of oxidized low-density lipoprotein into macrophages, followed by their differentiation into foamy macrophages. HOCl oxidizes cardiac myoglobin, which may cause cardiac inflammation and dysfunction in mice after myocardial infarction (MI). HOCl is also shown to have a profound adverse effect on left ventricular remodeling and function after MI.

**STUDY LIMITATIONS.** First, we indirectly demonstrated the possibility that H2 suppresses PADI4 activity by using GSK484, a PADI4-specific inhibitor, but the exact target of H2 remains unclear because of its difficulty of depletion of PADI4 by using small, interfering RNA in terminally differentiated human neutrophils.

Next, we showed that H2 suppresses NET formation more effectively than other antioxidants, such as NAC or L-ascorbic acid, do in vitro, but we did not directly compare H2 with other antioxidants in vivo. However, in the present study, it was confirmed that inhalation of low concentrations of H2 effectively suppressed NET formation.

Finally, it has not been directly proven that H2 inhibits NET formation in patients with MI or COVID-19. H2 has been already shown to be safe for inhalation and effective in patients with MI and COVID-19. Thus, elucidating the inhibitory mechanism of NET formation in humans would provide a mechanistic framework for its use in treatment of sepsis, acute respiratory disease syndrome, COVID-19, and CVDs.

**CONCLUSIONS**

In this study, we show for the first time that hydrogen inhibits the formation and release of NETs in human neutrophils induced by PMA stimulation in vitro and in mouse and aged mini-pig models of LPS-induced sepsis in vivo. The key mechanism is thought to be that H2 neutralizes HOCl produced during oxidative bursts and suppresses DNA damage. In addition, H2 may suppress NET formation even by a mechanism independent of ROS scavenging. Hydrogen therapy targeting NETs or extracellular HOCl is expected to be effective in acute conditions such as excessive neutrophil activation and NET formation associated with sepsis, as well as in chronic conditions such as atherosclerosis and myocardial remodeling after MI.

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**REFERENCES**

1. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532–1535.
2. Grover SP, Mackman N. Neutrophils, NETs, and immunothrombosis. Blood. 2018;132(13):1360–1361.
3. Middleton EA, He XY, Denorme F, et al. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. Blood. 2020;136(10):1169–1179.
4. Song C, Li H, Li Y, et al. NETs promote ALI/ARDS inflammation by regulating alveolar macrophage polarization. Exp Cell Res. 2019;382(2):111486.
5. Stiel L, Mayeur-Rousse C, Helms J, Meziani F, Mauveux L. First visualization of circulating neutrophil extracellular traps using cell fluorescence during human septic shock-induced disseminated intravascular coagulation. Thromb Res. 2019;183:153–158.
6. Yang J, Wu Z, Long Q, et al. Insights into immunothrombosis: the interplay among neutrophil extracellular trap, von Willebrand Factor, and ADAMTS13. Front Immunol. 2020;11:610696.
7. Leppkes M, Knopf J, Naschberger E, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. EBioMedicine. 2020;58:102925.
8. Barnes BJ, Aderover JM, Baxter-Stoltzfus A, et al. Targeting potential drivers of COVID-19 neutrophil extracellular traps. J Exp Med. 2020;217(6):e20200652.
9. Döring Y, Libichert LI. The effects of neutrophil extracellular traps participate in cardiovascular diseases: recent experimental and clinical insights. Curr Res. 2020;126(9):1228–1241.
10. Laridan E, Martind K, De Meyer SF. Neutrophil extracellular traps in arterial and venous thrombosis. Semin Thromb Hemost. 2019;45(3):86–93.
11. Ravindran M, Khan MA, Palanijay N. Neutrophil extracellular trap formation: physiology, pathology, and pharmacology. Biomolecules. 2019;9(8):365.
12. Ufığ A, Leichtli L. The effects of neutrophil-generated hypochlorous acid and other hypohalous acids on host and pathogens. Cell Mol Life Sci. 2021;78(2):385–414.
13. Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple IL. Hypochlorous acid regulates neutrophil extracellular trap release in humans. Clin Exp Immunol. 2012;167(2):261–268.
14. Iuchi K, Imoto A, Kamimura N, et al. Molecular mechanisms of hydrogen regulate gene expression by modifying the free radical chain reaction-dependent generation of oxidized phospholipid mediators. Sci Rep. 2016;6:18971.
15. Ohkawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med. 2007;13(6):688–694.
16. Moon DH, Kang DY, Haam SJ, et al. Hydrogen gas inhalation ameliorates lung injury after hemorrhagic shock and resuscitation. J Thorac Dis. 2019;11(4):1519–1527.
17. Xie K, Liu L, Yu Y, Wang G. Hydrogen gas presents a promising therapeutic strategy for sepsis. Biom Res Int. 2014;2014:807635.

**COMPETENCY IN MEDICAL KNOWLEDGE:** NETs contribute to severe pathologies, such as acute respiratory distress syndrome associated with and disseminated intravascular coagulation associated with sepsis or COVID-19. NETs are also involved in the pathogenesis of several CVDs, including acute coronary syndrome, stable coronary artery disease, ischemia-reperfusion injury, pulmonary embolism, and atherosclerosis. H2 has been clinically and experimentally proven to ameliorate inflammation in these disorders; however, the underlying molecular mechanisms remain elusive. Our study shows that inhalation of H2 may offer an attractive option for ameliorating inflammation by inhibiting the formation and release of NETs and excessive neutrophil activation.

**TRANSLATIONAL OUTLOOK:** Our findings revealed the inhibitory effect of H2 on NET formation in human neutrophils, mice, and micro mini-pigs. H2 has been clinically proven to be safe and to ameliorate inflammation in patients with MI. A multicenter randomized clinical trial demonstrated that H2 inhalation improved severity and symptoms in patients with COVID-19 pneumonia. These findings support clinical translation of hydrogen therapy as ameliorating inflammatory diseases involving NETs associated with excessive neutrophil activation.
18. Sano M, Suzuki M, Homma K, et al. Promising novel therapy with hydrogen gas for emergency and critical care medicine. Acta Med Surg. 2017;52:113-118.

19. Hayashida K, Sano M, Ohsawa I, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun. 2008;373(1):30-35.

20. Katsumata Y, Sano F, Abe T, et al. The effects of hydrogen gas inhalation on adverse left ventricular remodeling after percutaneous coronary intervention for ST-elevated myocardial infarction—first pilot study in humans. Circ J. 2017;81(7):940-947.

21. Guan WJ, Wei CH, Chen AL, et al. Hydrogen/oxygen mixed gas inhalation improves disease severity and dyspnea in patients with Coronavirus disease 2019 in a recent multicenter, open-label clinical trial. J Thorac Dis. 2020;12(6):3448-3452.

22. Kilkenny C, Browne WJ, Cuthill I, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. Vet Pathol. 2012;49(1):27-30.

23. Albertini M, Clement MG, Lafortuna CL, et al. Programmed cell death receptors involved in antigen uptake by dendritic cells and macrophages. PLoS One. 2015;10:e0123293.

24. Kirchner T, Hermann E, Möller S, et al. FLAPs and 5-aminosalicylic acid inhibit the formation of neutrophil extracellular traps. J Clin Invest. 2017;133(3):357-368.

25. Zhou Y, Di Pucchio T, Sims GP, Mittereder N, Di Cunto F, Papayannopoulos V. Neutrophil extracellular traps in COVID-19. Cell Death Discov. 2020;7(1):113.

26. Djukanovic R, Kellett R, Zhang H, et al. H2, neutrophil extracellular traps, and critical care medicine. JACC: Basic to Translational Science. 2022;7(2):253-275.

27. Jaffe SL, Ponzetta A, Di Mitri D, Santoni A, Bonecchi R, Mantovani A. Neutrophil diversity and plasticity in tumour progression and therapy. Nat Rev Cancer. 2020;20(9):485-503.

28. Barbizán A, Yamamoto K. DNA damage responses to oxidative stress. DNA Repair. 2004;3(8-9):1109-1115.

29. Schumacher B, Pothof J, Vijd J, Hoeijmakers JHJ. The central role of DNA damage in the ageing process. Nature. 2021;592(7856):695-703.

30. Zou Z, Khan MA, Palaniyar N. ROS induces NETosis by oxidizing DNA and initiating DNA repair. Cell Death Discov. 2021;7(1):113.

31. Nair RR, Madivala SV, Saini DK. Clampdown of inflammation in aging and anticancer therapies by limiting upregulation and activation of GCPRs, CCR4, and NKP44. NPJ Aging Mech Dis. 2018;4:9.

32. Guan WJ, Ni ZY, Hu Y, et al. China Medical Treatment Expert Group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020;382(17):1708-1720.

33. Zuo Y, Yahavarti S, Shi H, et al. Neutrophil extracellular traps in COVID-19. JCI Insight. 2020;5(11):e138999.

34. Ackermann M, Verleden SE, Kuehnel M, et al. Pulmonary vascular endotheliolysis, thrombosis, and angiogenesis in Covid-19. N Engl J Med. 2020;383(2):120-128.

35. Radhika A, Sudhakaran PR. Upregulation of macrophage-specific functions by oxidized LDL: lysosomal degradation-dependent-and-independent pathways. Mol Cell Biochem. 2013;372(1-2):181-190.

36. Wang XS, Kim HB, Szuchman-Sapir A, McMahon A, Dennis JM, Witting PK. Neutrophils recruited to the myocardium after acute experimental myocardial infarct generate hypochlorous acid that oxidizes cardiac myoglobin. Arch Biochem Biophys. 2016;612:103-114.

37. Vasiliev N, Williams T, Brennan ML, et al. Myeloperoxidase-generated oxidants modulate left ventricular remodeling but not infarct size after myocardial infarction. Circulation. 2005;112(18):2812-2820.

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