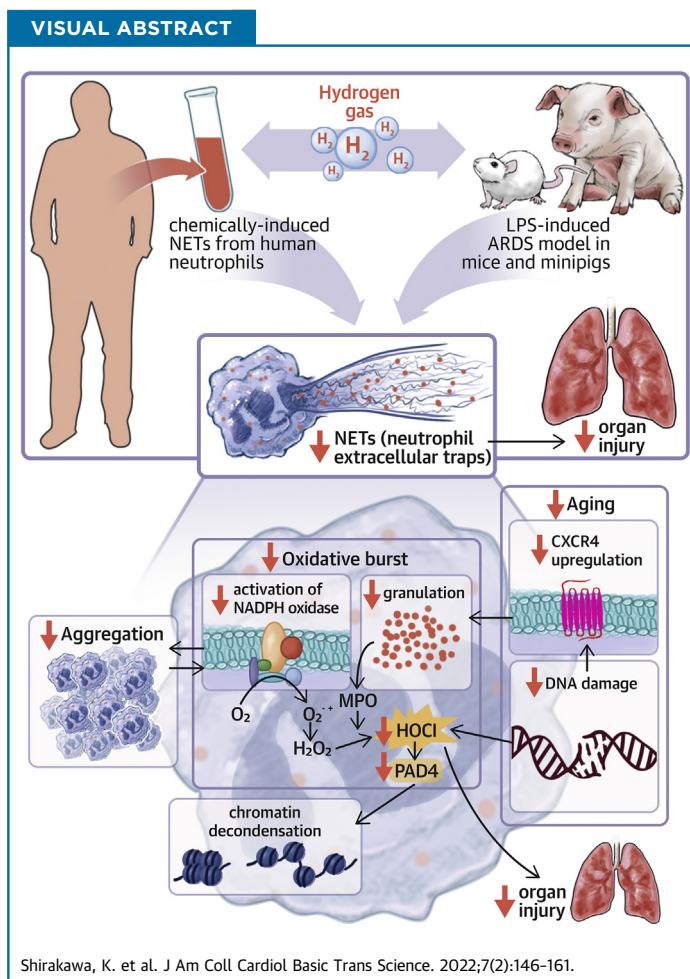


PRECLINICAL RESEARCH

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.

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the [Author Center](#).

Manuscript received July 6, 2021; revised manuscript received November 5, 2021, accepted November 6, 2021.

SUMMARY

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. CXCR4^{high} 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-acetylcysteine 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 Science 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/>).

ABBREVIATIONS AND ACRONYMS

- BAL** = bronchoalveolar lavage
- CitrH3** = citrullinated histone H3
- CVD** = cardiovascular disease
- dsDNA** = double-stranded DNA
- HOCl** = hypochlorous acid
- LPS** = lipopolysaccharide
- MI** = myocardial infarction
- MPO** = myeloperoxidase
- NAC** = N-acetyl-L-cysteine
- NET** = neutrophil extracellular trap
- PA** = pulmonary artery
- PADI4** = peptidyl arginine deiminase 4
- PMA** = phorbol-12-myristate-13-acetate
- ROS** = reactive oxygen species

Neutrophil extracellular traps (NETs) are innate immune responses that protect against infections by actively spreading neutrophil nuclear DNA in a spider web pattern around the periphery of the infection site. NETs trap pathogenic micro-organisms, including bacteria and viruses, and sterilize them with antimicrobial proteins that are attached to the DNA, such as histones, neutrophil elastase, and myeloperoxidase (MPO).¹ Recent studies show that NETs are responsible for immunothrombosis, a process of thrombus formation involving immune cells that enhances the innate immune response to pathogens.^{2,3} In contrast, NETs also contribute to severe pathologies, such as acute respiratory distress syndrome associated with acute lung injury, and disseminated intravascular coagulation associated with sepsis.³⁻⁵ Dysregulation of NETs, either by overproduction or inadequate degradation, leads to tissue damage, hypercoagulability, and thrombosis.^{2,6} Recently, NETs have received much attention because of their involvement in the severity of COVID-19-related lung injury.^{3,7,8} In addition to infectious diseases, NETs are involved in the pathogenesis of several cardiovascular diseases (CVDs), including acute coronary syndrome, stable coronary artery disease, ischemia-reperfusion injury, pulmonary embolism, and atherosclerosis.^{9,10} Therefore, it is not surprising that NETs have been implicated as therapeutic targets

to address inflammation and thrombotic tissue damage.

The nicotinamide adenine dinucleotide phosphate oxidase-mediated production of superoxide ions, a reactive oxygen species (ROS), plays a major role in the formation of NETs.¹¹ Superoxide is converted to H₂O₂, which serves as a substrate for the MPO-catalyzed hypochlorous acid (HOCl) production.¹² HOCl is a potent oxidant that acts as a disinfecting agent after it is released from the cell. However, intracellular HOCl plays a major role in NET formation.¹³

Hydrogen scavenges the hydroxyl radical (•OH), one of the most powerful oxidizing agents, and intervenes in lipid radical chain reactions in the cell membrane to modify lipid mediators.^{14,15} In addition, it is proven, both in clinical and animal studies, that it has therapeutic effects on acute lung injury, sepsis, and ischemia-reperfusion injury.¹⁶⁻²⁰ It was also reported recently that H₂ and O₂ were used in combination during an outbreak of COVID-19 in Wuhan, China, to inhibit the severity of COVID-19-related lung injury.²¹ However, the potential link or direct correlation between H₂ and NETs has not yet been established.

Therefore, in this study, we tested the hypothesis that hydrogen may inhibit NET formation associated with excessive neutrophil activation. We investigated the effect of H₂ gas on NET formation using

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.²² 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 H2JI1 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,²³ with modifications. The animals received continuous intravenous infusion of *Escherichia coli* LPS (serotype O55: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,²⁴ 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⁺. The cells were then 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 × 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 Gell. 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).

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

H₂ INHIBITS PMA-INDUCED NEUTROPHIL AGGREGATION AND SUBSEQUENT NET RELEASE. To determine the effect of H₂ on NET formation, 1 × 10⁷ 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 H₂-treated medium for 3 hours. Roswell Park Memorial Institute 1640 medium containing 15 mmol/L N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.2) was treated with H₂ 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

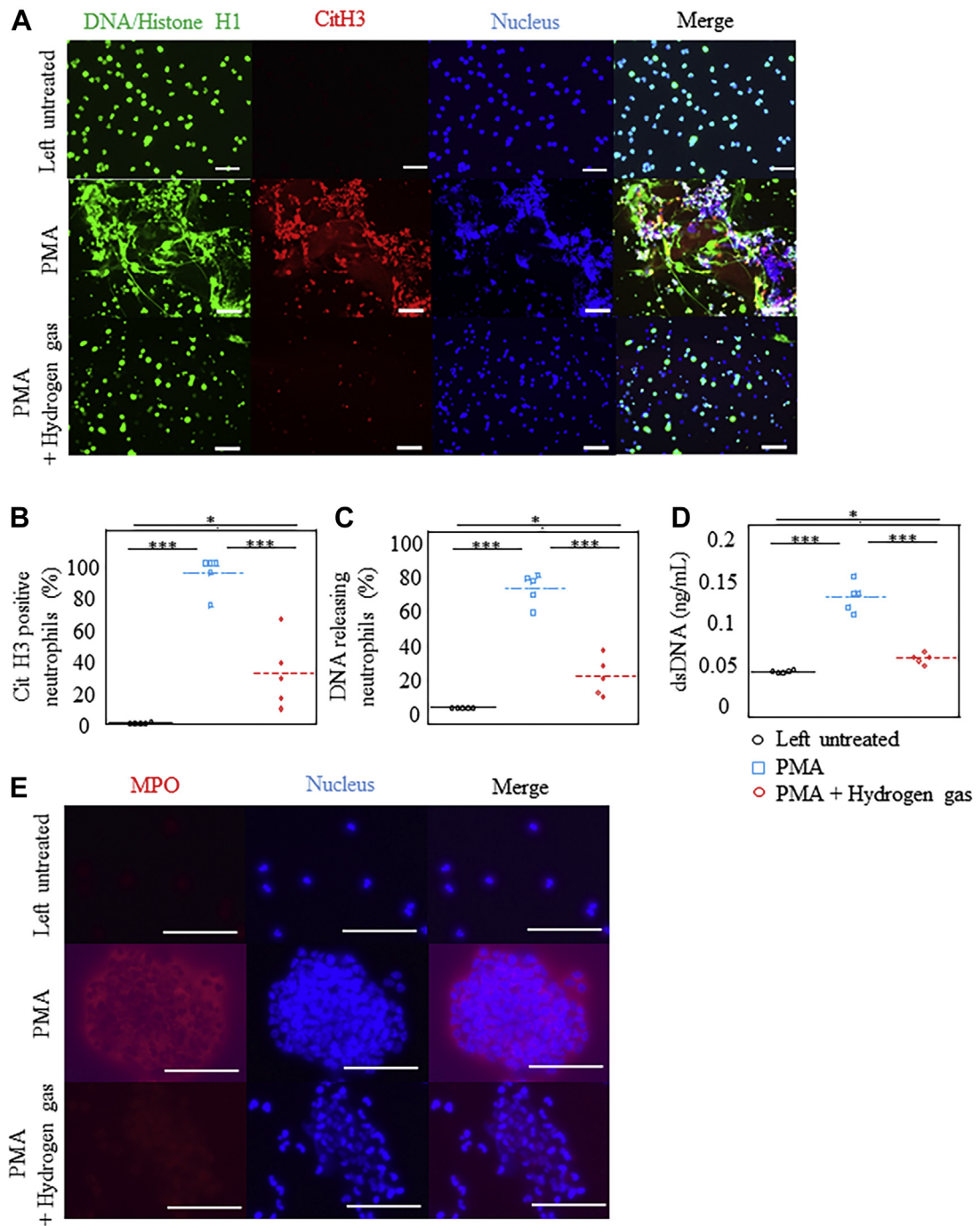
suppressed in H₂-treated cultures compared with those in control cultures ([Figures 1A to 1C](#), [Supplemental Figure 2C](#)). Furthermore, the release of dsDNA from neutrophils was markedly inhibited in the H₂-treated medium ([Figure 1D](#)).

After 1 hour of PMA stimulation, preceding NET release, aggregation of neutrophils was observed in the control medium, which was significantly suppressed in the H₂-treated medium ([Figure 1E](#), [Supplemental Figure 2D](#), [Video 1](#)). Aggregation of neutrophils promotes an oxidative burst and the formation of NETs.²⁵ These results indicate that H₂ inhibits neutrophil aggregation and, possibly, the subsequent release of NETs.

H₂ 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 H₂ at the cellular level. To elucidate the mechanism of the inhibitory effect of H₂ 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 H₂-treated medium for 1 hour, and global transcriptome changes were compared by RNA sequencing.

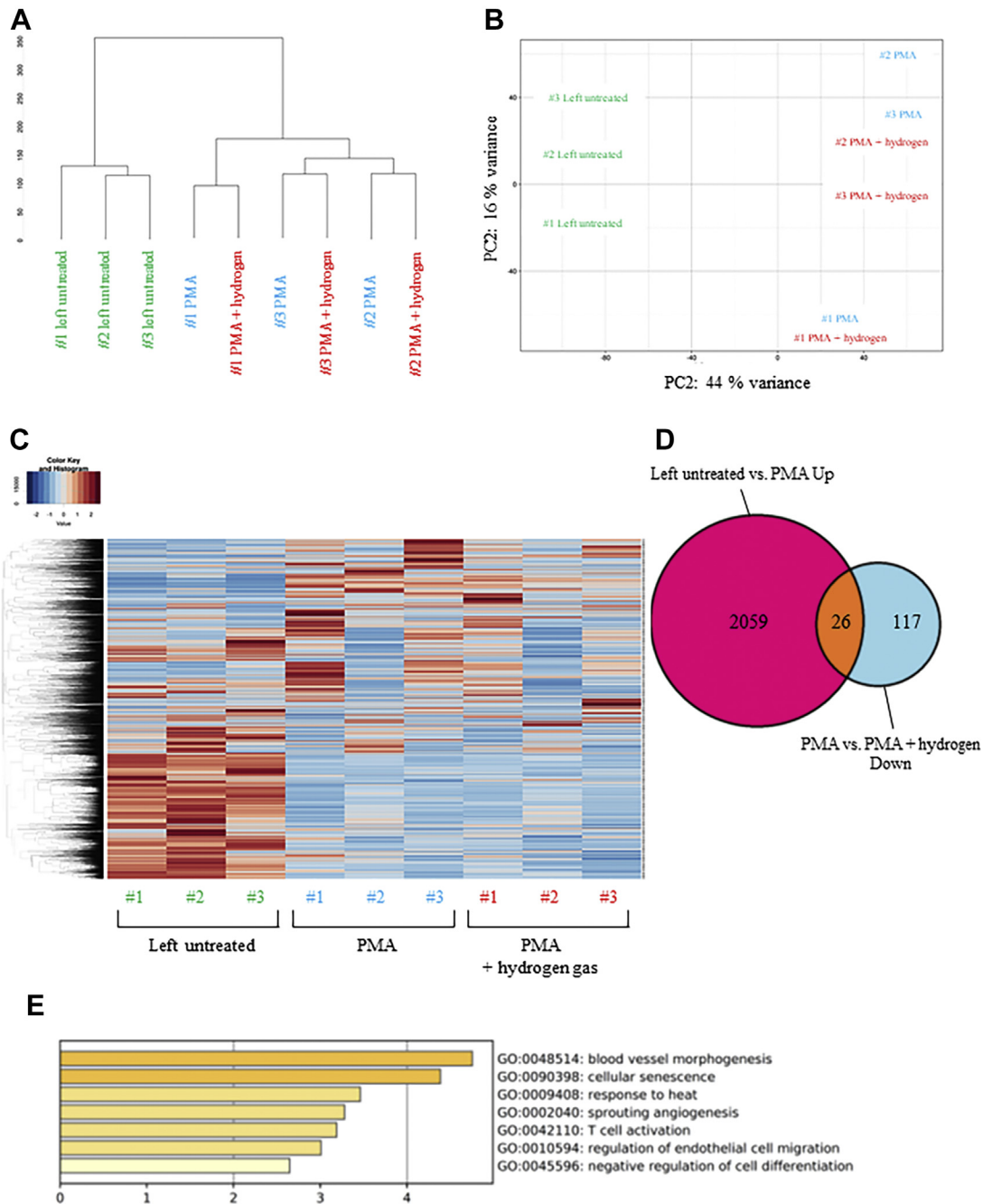
Hierarchical clustering of the RNA sequencing data showed that the untreated population and the PMA-stimulated population formed separate clusters; however, the presence or absence of H₂ had no effect on cluster formation ([Figure 2A](#)). Principal component analysis showed similar results ([Figure 2B](#)). Furthermore, the heat maps showed that H₂ 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 H₂ after PMA stimulation in human neutrophils.

The genes whose expression was increased by PMA and repressed by H₂ 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 H₂-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 H₂ pretreatment ([Figure 2D](#)). Furthermore, gene ontology analysis of these 26 genes showed that H₂ administration repressed the genes involved in cellular senescence among the

FIGURE 1 H₂ Inhibited the Formation of NETs by Human Neutrophils

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 $\times 20$. Bars = 100 μ m. **(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 $\times 120$. Bars = 50 μ m. * $P < 0.05$; *** $P < 0.001$. NET = neutrophil extracellular trap.

FIGURE 2 Bioinformatic Analysis of RNA-Seq Data From Human Neutrophils



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.

genes induced by PMA (Figure 2E, Table 1). These 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). CXCR4^{hi} 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, CXCR4^{hi} neutrophils were also detected to be SYTOX⁺ (Figures 3D and 3E). In the H₂-treated medium, the increase in the population of CXCR4^{hi} neutrophils seen in the control medium was suppressed, and the population of SYTOX⁺ CXCR4^{hi} 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 CXCR4^{hi} 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

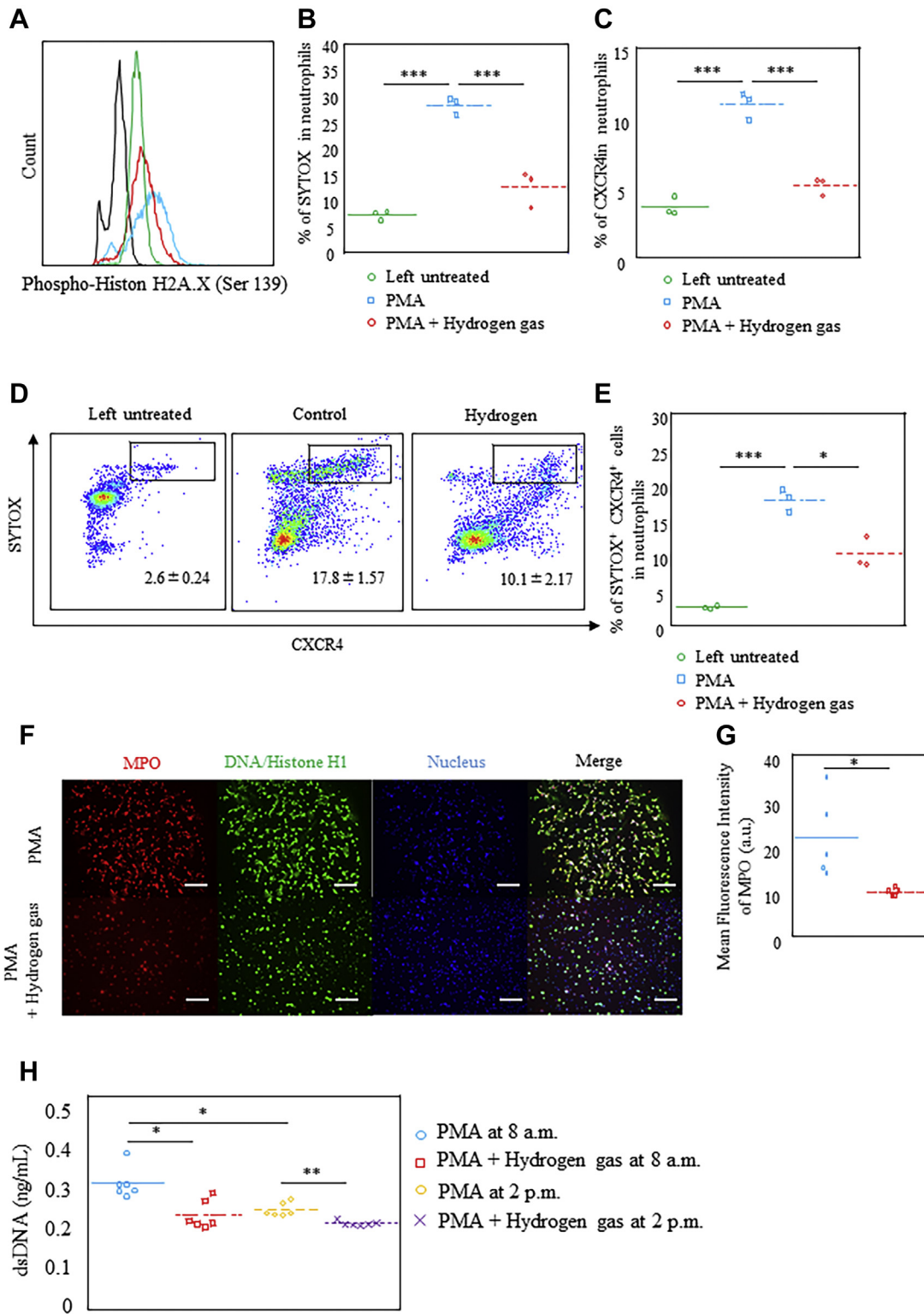
TABLE 1 List of Genes Common Between Those Up-Regulated on PMA Treatment and Those Down-Regulated on Subsequent H₂ Administration

Gene Symbol	Log ₂ Fold Change	P Value
CA13	-4.18	0.019
LY6E	-3.77	0.016
DLL4	-2.63	0.020
MIR6514	-2.47	0.008
TEAD2	-2.23	0.025
RNH1	-2.08	0.030
NDC1	-1.62	0.005
NRARP	-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
MCUR1	-1.29	0.035
RRAD	-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
PLK2	-1.06	0.001
CCDC85B	-1.06	0.012

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).

FIGURE 3 H₂ Ameliorated the DNA Damage in Human Neutrophils



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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.³³ A23187-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.³³ 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

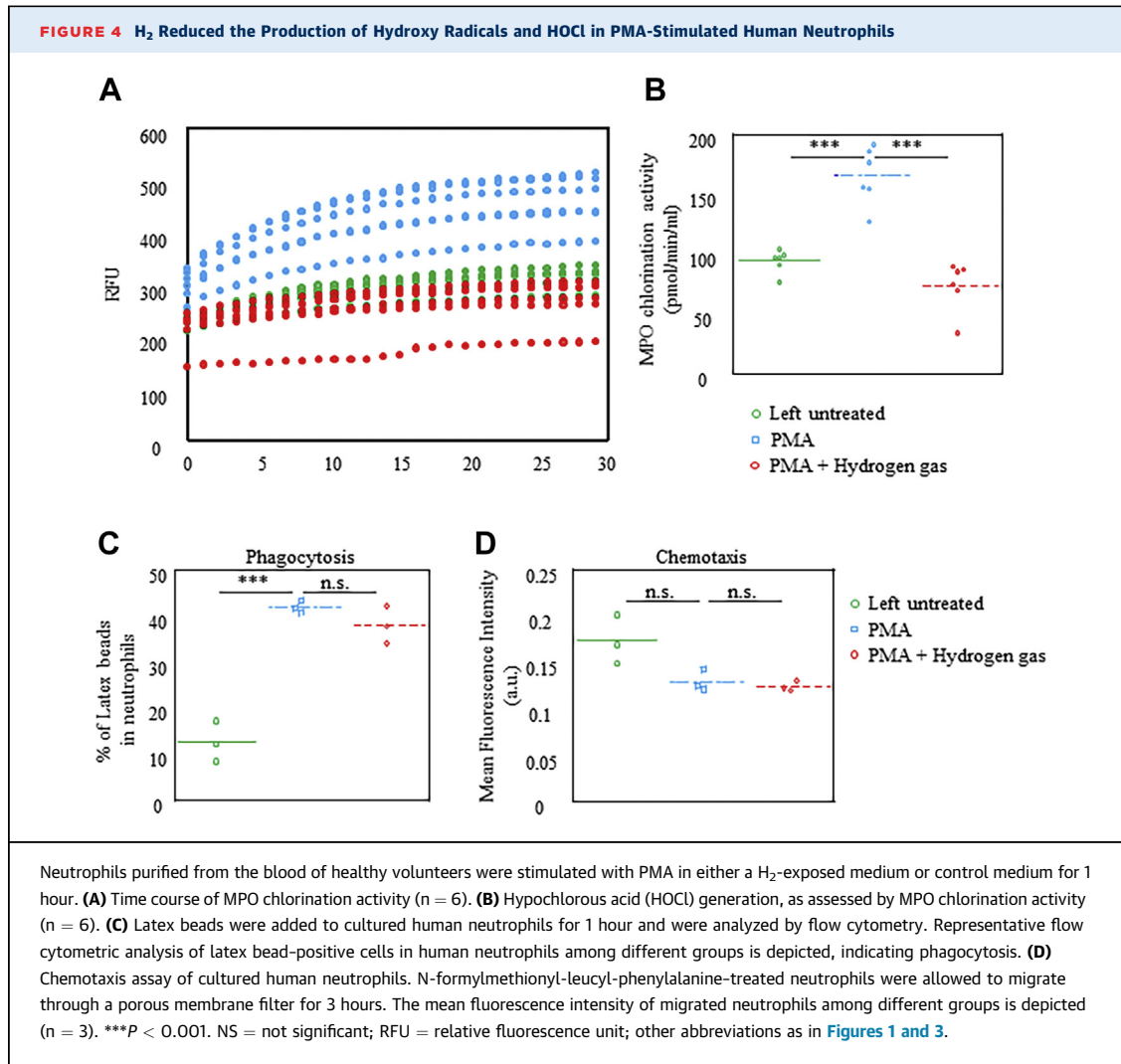
results suggest that in A23184-induced NETs formation, H₂ suppresses NET formation by inhibiting Ca²⁺-dependent PADI4 activation, not by suppressing ROS.

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 PMA-induced 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 described³⁴ 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

FIGURE 3 Continued

Neutrophils purified from the blood of healthy volunteers were stimulated with PMA in either a H₂-exposed medium or control medium for 1 hour. (A) Representative flow-cytometric analysis of phospho-histone H2A.X (Ser-139) indicating neutrophils. (B) Percentage of SYTOX⁺ neutrophils (n = 3). (C) Flow cytometric analysis of CXCR4⁺ cells in neutrophils (n = 3). (D,E) Flow cytometric analysis of SYTOX⁺ CXCR4⁺ cells in neutrophils (n = 3). (F) Microscopic images depicting the fluorescence of MPO (Alexa Fluor 594, red), DNA/histone H1 (Alexa Fluor 488, green), and 4',6'-diamidino-2-phenylindole (blue) in cultured neutrophils. Original magnification \times 20. Bars = 100 μ m. (G) Mean fluorescence intensity of MPO in neutrophils (n = 5) measured using the particle analysis function of the BZ-H1C software (KEYENCE). (H) Quantification of dsDNA released by activated neutrophils into the culture medium at the indicated time points (n = 6). Flow cytometric analysis was performed in n \geq 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. AU = arbitrary unit; other abbreviations as in Figure 1.



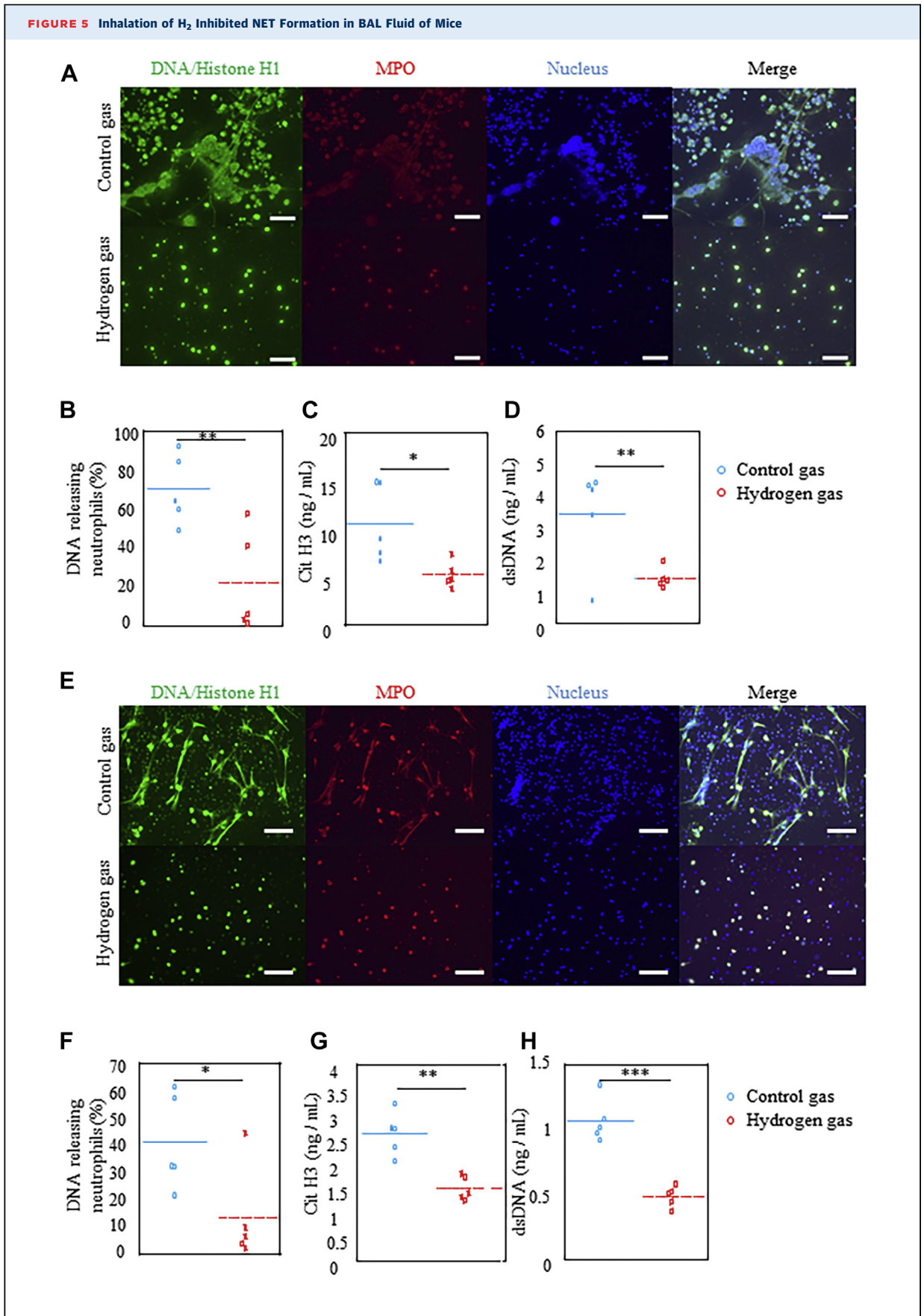
the pulmonary arteries (PAs) of the LPS-induced acute lung injury model mice ([Supplemental Figures 7A and 7B](#)); however, inhalation of H₂, compared with control gas, significantly suppressed this change ([Supplemental Figures 7C and 7D](#)).

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



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.³³ 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.³⁶ In neutrophils, MPO can catalyze the production of HOCl from H₂O₂ and chloride ions.³⁰ This process is called oxidative burst.³⁴ Oxidative bursts induce hypercitrullination of histones, decrease nucleosome binding, and cause overall chromatin decondensation.³³ 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,³⁷ which induces a rapid citrullination of multiple cellular proteins.^{38,39} Therefore, PADI4-mediated histone citrullination contributes greatly to NET formation in A23187-stimulated human neutrophils.³³ It has been reported that A23187-induced NETs are not suppressed by administration of protein kinase C inhibitor,⁴⁰ A23187 leads to a lower level of ROS compared with PMA or suppresses oxidative bursts in human neutrophils.⁴¹ In fact, ROS scavenger or MPO inhibitor has no inhibitory effect on NET formation.⁴⁰ Thus, PMA and A23187 have different mechanisms leading to NET formation.

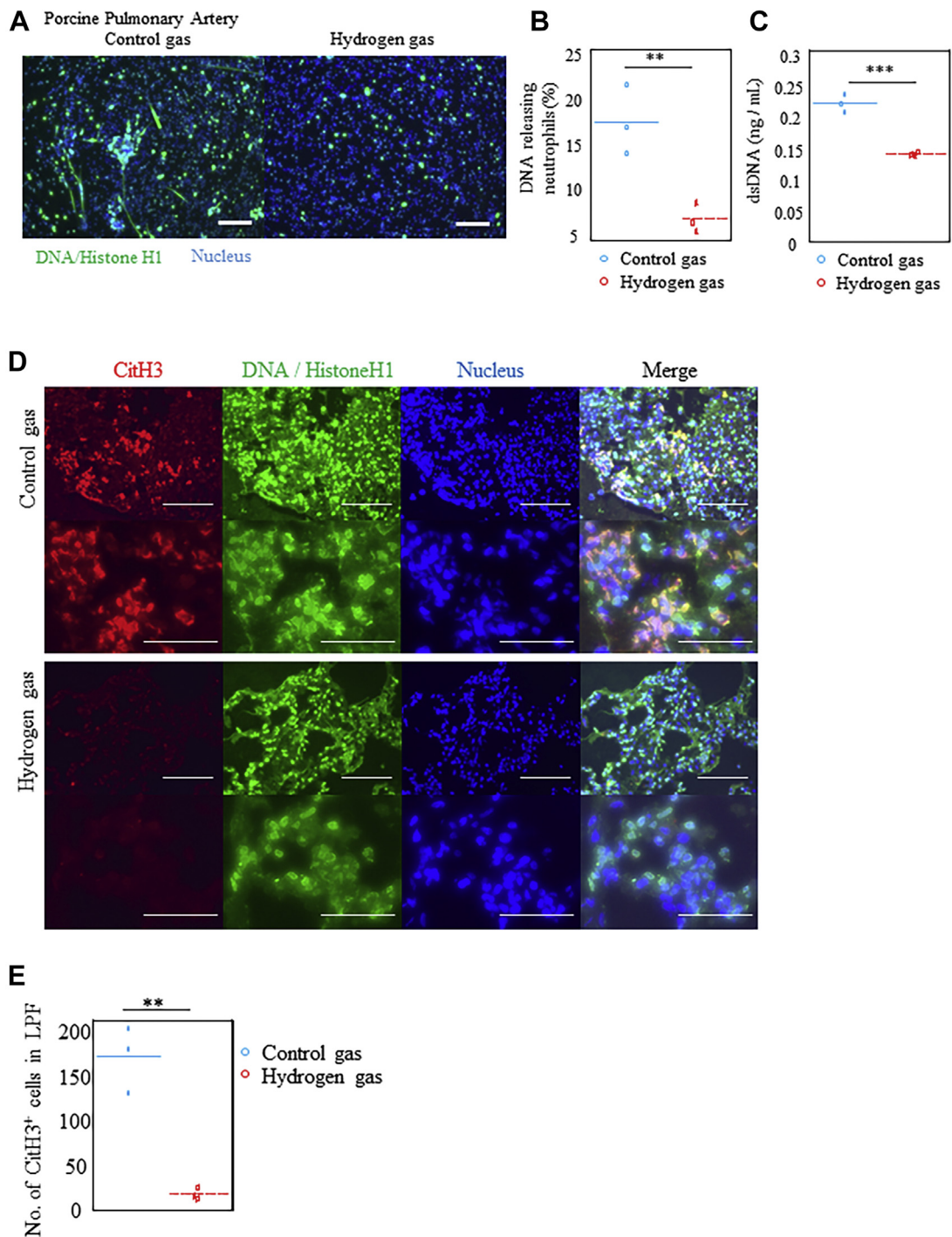
Our results suggest that the central mechanism that H₂ suppresses NET formation is the strong reduction of ROS production like MPO-induced HOCl in PMA-stimulated human neutrophils; however, H₂ mainly targets other than ROS in A23187-stimulated human neutrophils.

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.^{42,43} 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.⁴⁴ To promote NET formation, ROS must be processed internally by MPO.^{30,45} Neutrophils derived from patients deficient in MPO fail to form NETs when exposed to PMA.⁴⁵ 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.²⁸ 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,⁴⁶ on the cell surface is up-regulated.^{47,48} This change facilitates the clearance of senescent neutrophils in the bone marrow,^{47,48} and CXCR4^{hi} neutrophils are known to be prone to NET formation.^{28,29} Furthermore, it has been shown that NET formation in response to PMA was strongly reduced in *MRP8^{CRE} Cxcr4^{fl/fl}* mice, which lacked CXCR4 specifically in neutrophils.⁴⁹

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 $\mu\text{g}/\text{kg}$ lipopolysaccharide intravenously with the inhalation of H₂ or control gas. Original magnification $\times 20$. 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 $\mu\text{g}/\text{kg}$ 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 $\times 40$ and $\times 120$, respectively. Bars = 100 μm . **(E)** Number of CitH3⁺ 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.⁵⁰ DNA damage is the primary cause of cellular aging,⁵¹ and extensive DNA damage and the subsequent DNA repair pathway induce NETosis, leading to chromatin decondensation.⁵² We detected oxidative burst-induced phosphorylation of the Ser-139 residue of H2AX, a marker of double-strand breaks in DNA,^{26,27} and up-regulation of CXCR4 in PMA- or A23187-stimulated human neutrophils. This up-regulation of CXCR4 is also a response to DNA damage.⁵³ 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.^{12,31} 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.²¹ Neutrophilia in patients with COVID-19 pneumonia is indicative of a poor prognosis,⁵⁴ and NET hyperplasia has been observed in the blood, BAL fluid, and PAs of patients with severe COVID-19 pneumonia.^{3,55,56} 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.^{31,32} 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.⁵⁷ HOCl oxidizes cardiac myoglobin, which may cause cardiac inflammation and dysfunction in mice after myocardial infarction (MI).⁵⁸ HOCl is also shown to have a profound adverse effect on left ventricular remodeling and function after MI.⁵⁹

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.

ACKNOWLEDGMENTS The authors are grateful to Nagahiro Minato and Yoko Hamazaki (Kyoto University, Japan) for technical assistance. The authors are

also grateful to Dr R. Nakaki, Dr Y. Kondo, and M. Kawamura (Rhelixa, Inc) for analyzing the RNA-sequencing data.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

This research was funded by Japanese Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) grant 18H02812 (2018-2020) and grants from Doctors Man Co, Ltd (to MS); and JSPS Grant-in Aid for Young Scientists grant JP18K15197 (2018-2020), Grant in-Aid for JSPS Fellows grant JP19J00583H (2018-2020). The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication. Drs Kobayashi and Sano have received advisory fees and research fees from Doctors Man Co, Ltd. Dr Sano has received advisory fees and research fees from Taiyo Nippon Sanso; is the registered inventor of the following patents jointly filed by Keio University and Taiyo Nippon Sanso: hydrogen mixed gas supply device for medical purposes (patent number: 5631524), medicinal composition for improving prognosis after restart of patient's own heartbeat, and medicinal composition for improving and/or stabilizing circulatory dynamics after onset of hemorrhagic shock; and 3 other patents (whose names are translated into English): pharmaceutical compositions for reducing weight loss after organ harvesting (joint application with Keio University and Taiyo Nippon Sanso), method for generating organ preservation solution containing hydrogen and organ preservation solution containing hydrogen (joint application with Keio University and Doctors Man, application number PCT/JP2019/045790). All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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PERSPECTIVES

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.

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KEY WORDS H₂, neutrophil extracellular traps, phorbol-12-myristate-13-acetate

APPENDIX For supplemental methods, figures, video, and references, please see the online version of this paper.