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Hydrogen sulfide donor NaHS alters antibody structure and function via sulfhydration

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ABSTRACT

Hydrogen sulfide (H_2S) has emerged as an important biological mediator with numerous pathophysiological roles. One of the well-documented actions of H_2S is to inhibit immunity, especially cellular immunity. Currently, limited information is available regarding its effects on humoral immunity. Given that H_2S has reducing activity and that the effector molecules in humoral immunity, such as antibody and complement, contain abundant disulfide bonds that are indispensable for their functions, we speculated that H_2S might regulate antibody activity via modification of disulfide bonds. Here we addressed this possibility. Exposure of antibodies to H_2S donors resulted in cleavage of the disulfide bonds between the heavy and light chains of antibodies, which was associated with antibody sulfhydration. Further analysis revealed that H_2S-treated antibodies exhibited a marked reduction in antigen binding ability. It potently prevented the antibody-mediated agglutination of red blood cells and interrupted aggregation of antibody-coated microspheres. H_2S also greatly inhibited antibody-induced and complement-mediated cell lysis in glomerular mesangial cells, as well as anti-CD95 IgM antibody-initiated cell apoptosis in Jurkat cells. Moreover, it significantly suppressed the alternative complement activation pathway. Collectively, our results revealed, for the first time, that pharmacologic levels of H_2S inhibit humoral immune responses via direct sulfhydration of the effector molecules. Our study thus provides novel mechanistic insights into the immunoregulatory actions of H_2S and suggests that H_2S may have potential to treat certain humoral immune diseases.

1. Introduction

Hydrogen sulfide (H_2S) is an endogenous gaseous mediator, which is produced by many tissues and organs. It has a wide range of physiological functions, including acting as a neurotransmitter, relaxing the blood vessels, regulating blood pressure, scavenging reactive oxygen species and protecting cells against oxidative stress. Most of these biological actions of H_2S are ascrible to regulation on intracellular redox status and posttranslational modification of protein Cys residues [1–4].

H_2S also regulates immune responses. It has been implicated in many types of inflammatory diseases [2,5,6]. Supplementation of H_2S prevents and alleviates many immune-related diseases [2]. On the other hand, H_2S deficiency contributes to the initiation and progression of some autoimmune diseases [5]. H_2S regulates many molecular events related to inflammatory and immune responses. For examples, it suppresses NLRP3 inflammasome formation [7], inhibits NF-κB activation [8], and reduces the production of a variety of inflammatory mediators [9]. Furthermore, H_2S also modulates the function of many immune-potent cells. It inhibits monocyte adhesion to endothelial cells [10] and regulates Treg-cell-associated immune homeostasis [11]. The mechanisms underlying these regulatory actions of H_2S are related to the sulfhydration of key functional molecules [4].

Most of the reported effects of H_2S on immunity have been in the field of cellular immunity, concerning its modification on intracellular molecules, such as NFκB, NFYB, Keap, etc. [4,11,12]. Studies about its direct actions on immune-regulatory humoral factors are still scarce. Given that there exists a certain physiological level of H_2S in serum and intestinal lumen [6], and that therapeutic administration of pharmacological H_2S donors could lead to a marked increase in local H_2S

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concentration, it is conceivable that the effector molecules in humoral immunity, such as antibody and complements, could also be exposed to a relatively high concentration of H_2S. Recently, we have reported that disulfide bonds in antibody and complements are indispensable for its normal structure and function [13]. Also, we have shown that H_2S has reducing activity. It directly cleaves the disulfide bonds in TGFβ and thioredoxin [14,15]. These findings prompted us to speculate that H_2S may directly modulate humoral immunity through disruption of the disulfide bonds in the effector molecules. This study aimed to address this hypothesis.

Using an in vitro model of antibody-mediated immune responses, we studied the influences of H_2S on antibody-initiated immune responses and explored the potential mechanisms. Our results showed that H_2S donor NaHS altered antibody structure and function through a mechanism involving antibody sulfhydration. Our study thus indicates that the direct modification of humoral effector molecules could be an important pharmacological mechanism by which H_2S regulates immune responses.

2. Materials and methods

2.1. Materials

Dynabeads protein A and protein G Mag Sepharose were from Novex in life technologies (Life Technologies-Novex, Carlsbad, CA) and GE Healthcare (Buckinghamshire, UK), respectively. HRP-conjugated anti-rabbit or mouse IgG, anti-β-actin and anti-caspase-3 antibodies were purchased from Cell Signaling, Inc. (Beverly, MA). Protein A-purified rabbit IgG was obtained from Innovative Research (Novi, MI; Catalog No. IR-RB-GF). Anti-Thy-1 monoclonal antibody 1-22-3 was kindly gifted by Dr. Kawachi (Institute of Nephrology, Niigata University, Japan). Rhodamine-conjugated anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse red blood cell (RBC) antibody was purchased from Abgent (San Diego, CA). Anti-Human CD95 antibody was purchased from Affymetrix (San Diego, CA; Catalog Number16-0958). Sodium sulfide (Na_2S) and Hoechst 33342 solution were purchased from Dojindo (Kumamoto, Japan). Alexa 680 C2 maleimide and The Easy-Titer IgG Assay Kit were from Thermo Scientific (Rockford, IL). NaHS and all other reagents were bought from Sigma (Tokyo, Japan).

2.2. Rabbit serum

Rabbit blood was drawn from the ear auricular artery of the Japanese White Strain Rabbit. The serum was obtained by clotting blood for 30 min at room temperature. It was aliquoted and stored at −80 °C until use. Animal experiments were permitted by the animal experiment committee of the University of Yamanashi and done in accordance with guidelines and regulations.

2.3. Cells

Rat glomerular mesangial cells (MCs) were obtained from the outgrowth of the isolated glomeruli, according to the method reported previously [13,16]. Cells were cultured in DMEM/F12 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% FBS for passage and expansion. Mouse T cells isolated from the thymus of 3–4-week old male mice and immortalized human T lymphocyte cell line Jurkat cells (ATCC, Rockville, MD) were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic and antimycotic solution in a humidified atmosphere of 5% CO2/95% air at 37 °C. For experiments, cells were cultured in DMEM/F12 or RPMI-1640 containing 1% FBS in the presence or absence of various stimuli.

2.4. Dialysis of NaHS-treated antibodies

Antibodies, pretreated with or without NaHS, were loaded into a 3000 Da cut-off dialysis tube and dialyzed against a large volume of DMEM/F12. After removing the remaining NaHS and its metabolites with this procedure, the antibodies were used for the designated experiments.

2.5. Induction of glomerular MC lysis by differently modified anti-Thy-1 monoclonal antibody together with complements

The anti-thy-1 monoclonal antibody was either pretreated with the indicated concentrations of NaHS or DTT or left untreated for 1 h. Afterward, the remaining NaHS, DTT or their metabolites were removed using the dialysis. The pretreated anti-Thy-1 monoclonal antibody was used for induction of MC lysis at the concentration of 0.5 μg/ml in the presence of 5% rabbit serum as a source of complements. Preliminary experiments showed that this concentration of antibody was localized within the range (0.1–1 μg/ml) for induction of linear MC lysis. Confluently cultured rat glomerular MCs in 96-well plate were exposed to the anti-Thy-1 monoclonal antibody in the presence or absence of rabbit serum. After reaction in 37 °C for 4.5 h, culture supernatants were collected and assayed for LDH release. Cell viability was also determined using calcein-AM/Propidium Iodide (PI) staining and WST assay, as previously reported [13].

2.6. Western blot analysis

Western blot was performed using the enhanced chemiluminescence system following the procedures reported previously [13,16]. Cells were suspended in SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol) together with a freshly added proteinase inhibitor cocktail (Nakalai Tesque, Kyoto, Japan). The cellular proteins were extracted by placing the cellular lysates on ice for 15 min with intermittent mixing, followed by centrifuge at 15,350 ×*g* for 10 min at 4 °C. The collected supernatant was assayed for protein concentration with the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The same amount of protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 3% BSA or 5% milk in PBS for 1 h before reaction with HRP-conjugated anti-rabbit or mouse IgG (Cell Signaling; Beverly, MA, USA) or the primary antibodies, followed by the HRP-labelled second antibodies. After washing, the bands were detected using the enhanced chemiluminescence system (Nacalai Tesque). The chemiluminescent signal is captured with a Fujifilm image LAS-1000 analyzer (Fujifilm, Tokyo, Japan) and quantified with the NIH Image J software (http://rsb.info.nih.gov/ij). β-actin was probed to confirm equal loading of proteins.

2.7. Sulfhydration assay using maleimide

This assay was performed according to the previous reports [17,18]. Purified rabbit IgG (8 μg) in 200 μl PBS were exposed to the indicated concentrations of NaHS at room temperature for 1 h, followed by incubation with a mixture of protein A/G magnet beads for 2 h. Through this process, IgG was precipitated, and the remaining NaHS, as well as its metabolites, were removed. The bead-bound IgG was incubated with Alexa Fluor 680 C2 maleimide (red fluorescence at the final concentration of 2 μM) for 2 h at 4 °C with occasional gentle mixing. After washing out the unlabeled maleimide with PBS, pellet beads were divided equally into two tubes, treated with or without 1 mM DTT at 4 °C for 1 h. After washing, the beads were resuspended in 60 μl electrophoresis sample buffer and heated at 95 °C for 5 min. The supernatants were collected and separated with 10% SDS-PAGE gel or directly loaded onto Nitrocellulose Membrane in the dot blot apparatus under vacuum (Bio-Rad). The fluorescent signal in the membranes was

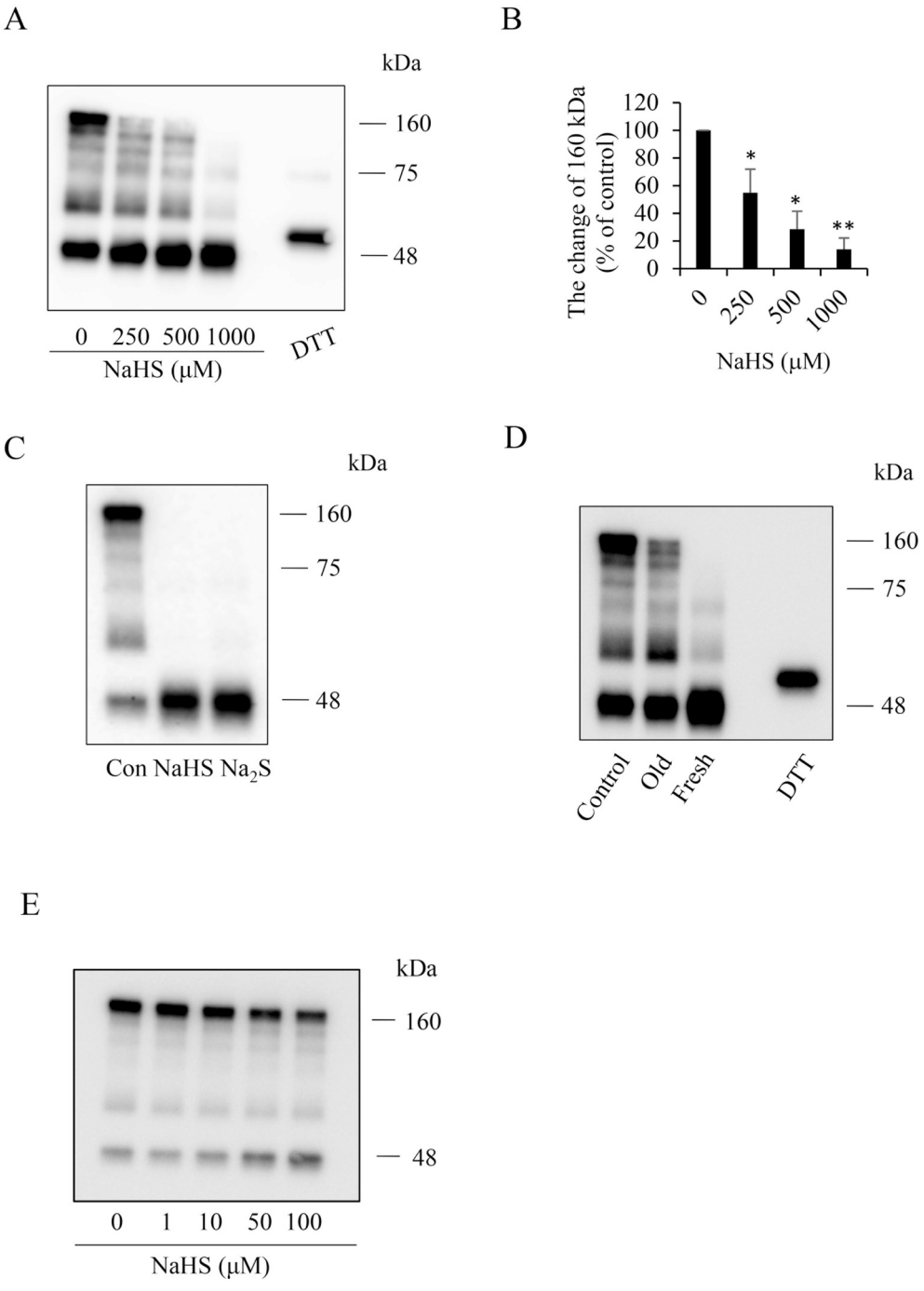


Fig. 1. *H_2S modifies the structure of rabbit IgG.* (A) Time-dependent effect of NaHS on the structure of rabbit IgG. Purified rabbit IgG was incubated with the indicated concentrations of NaHS or 50 mM DTT for 1 h. Afterward, the samples were separated by SDS page and immunoblotted with an HRP-labelled anti-rabbit IgG antibody. Note the shift of the bands from high MW to low MW after NaHS treatment. (B) Densitometric analysis of the intensity of the bands at the MW around 160 kDa. The band intensity was measured using ImageJ software and are expressed as a percentage of the control without NaHS treatment. Data are representative of four independent experiments and values are expressed in mean ± SE. *$P < 0.05$, ** $P < 0.01$ vs. control. (C) Effects of different H_2S donors on the structure of rabbit IgG. Rabbit IgG was incubated with 1 mM NaHS or 1 mM Na2S for 1 h. The samples were subjected to western blot analysis for rabbit IgG. (D) Comparison of the effect of the freshly prepared and pre-placed NaHS solution on the structure of rabbit IgG. Rabbit IgG was incubated either with 1 mM freshly prepared NaHS solution (Fresh) or the same concentration of NaHS that was prepared 24 h before the experiment (Old). The samples were separated by SDS page and immunoblotted for rabbit IgG. Note the weakened effect of old NaHS solution in comparison with the fresh NaHS solution. (E) Effect of the lower concentrations of NaHS on Ig structure. Purified rabbit IgG was incubated with the indicated concentrations of NaHS for 1 h. The samples were separated by SDS page and immunoblotted with an HRP-labelled anti-rabbit IgG antibody. Note the shift of the bands from high MW to low MW at the concentrations starting from 10 to 50 μM.

detected with a Fujifilm image LAS-1000 analyzer (Fujifilm, Tokyo, Japan) and quantified with the NIH Image J software (http://rsb.info.nih.gov/ij). The equal loading of IgG in the membranes was confirmed by immunoblotting the membrane using an HRP-labelled anti-rabbit IgG antibody using the enhanced chemiluminescence system as described above.

2.8. Immunofluorescence staining

Immunofluorescence staining was used to assess the effect of NaHS on the antibody binding to cell surface antigens. Briefly, anti-MC rabbit serum was pretreated with or without the indicated concentrations of NaHS for 1 h and used as the first antibody for immunofluorescence staining of MCs. Primarily cultured MCs were washed with PBS and fixed with 3% paraformaldehyde and allowed to react with the above-mentioned rabbit serum for 2 h at room temperature. After washing with PBS for three times, the cells were incubated with tetramethy1 rhodamine B isothiocyanate-conjugated anti-rabbit IgG antibody for 1 h to detect the membrane-bound IgG, which was followed by 10-min nuclear staining with DAPI. After washing with PBS twice, cells were observed under IF microscopy and photographed using a CCD camera.

2.9. Hoechst staining

Hoechst staining was performed to detect apoptotic cells according to the method reported previously [19]. Briefly, cells were exposed to 10 μg/ml Hoechst 33342 for 10 min before photographed with a CCD camera attached to a fluorescence microscope (Olympus BX50).

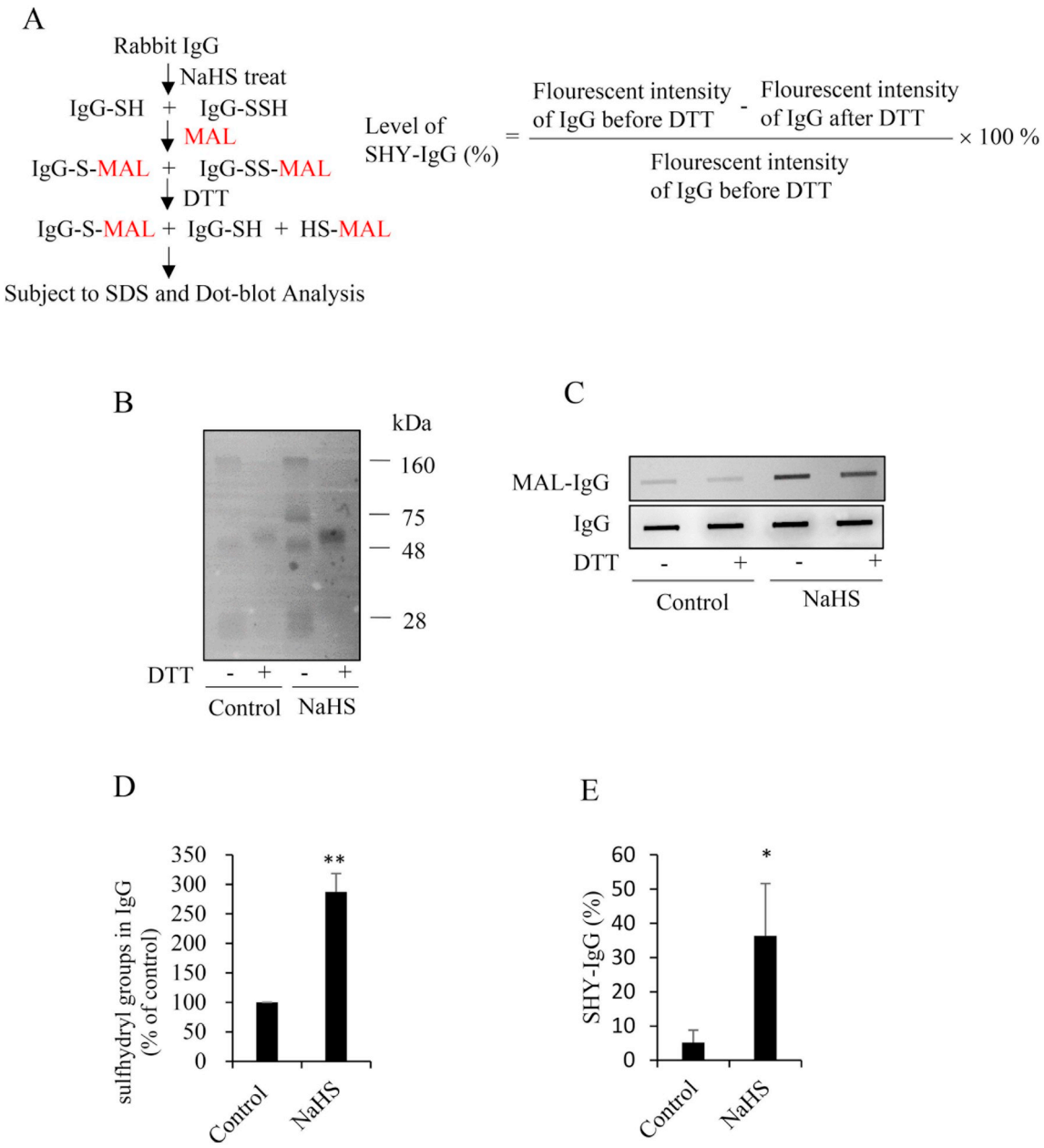


Fig. 2. *H_2S sulfhydrates rabbit IgG.* (A) Schematic diagram of the method used for detection of IgG sulfhydration. In this assay, IgG treated with or without 1000 μM NaHS is immunoprecipitated and allowed to react with Alexa Fluor® 680 C2 maleimide, which labels both sulfhydrated (-SSH) and unsulfhydrated (-SH) Cys in IgG molecules. After removing excess maleimide, DTT is added to split the disulfide bonds, thus resulting in a loss of the fluorescent signal from sulfhydrated (-SSH) but not unsulfhydrated IgG. The level of IgG sulfhydration could be calculated through the fluorescence loss following DTT treatment. (B) Maleimide-labelled samples as analyzed by SDS-PAGE. Purified rabbit IgG treated with or without NaHS was assayed for sulfhydration as described in the section of Materials and Method. The samples were separated by SDS-page and transferred to PVDF membrane, and the fluorescent signal in the membrane was detected. (C) Dot blot analysis of Maleimide-labelled samples. IgG samples were added to dot-blot apparatus and detected for the fluorescent signal as described in Materials and Methods. (D) Densitometric analysis of the intensity of bands between samples treated with or without NaHS. (E) Quantitative calculation of sulfhdrated IgG by H_2S. Data are representative of four independent experiments and values are expressed in mean ± SE. *P < 0.05, ** P < 0.01 vs. control.

2.10. Easy-titer IgG assay

To evaluate the effect of H_2S on the antibody-mediated response, we used an Easy-Titer Mouse IgG Assay Kit from Thermo Scientific. The assay was performed based on the manufacturer's protocol, as we previously reported [13]. Briefly, the standard IgG or diluted samples were treated with or without NaHS for 1 h, followed by the incubation with the same volume of the anti-IgG-sensitized beads. After vigorous mixing, the blocking reagent was added to stop the reaction. The OD at 340 nm was read using a spectrometer. The microagglutination formation was recorded using a CCD camera attached to an Olympus BX50 microscope.

2.11. Red blood cell (RBC) agglutination assay

Mouse whole blood in a plastic anticoagulant collection tube was centrifuged and washed twice with saline. The RBC was adjusted to 1% suspension and added into wells of a 96-well plate together with a serial dilution of anti-mouse RBC antibodies. The formation of RBC agglutination after 2-h incubation was examined under the microscope and photographed.

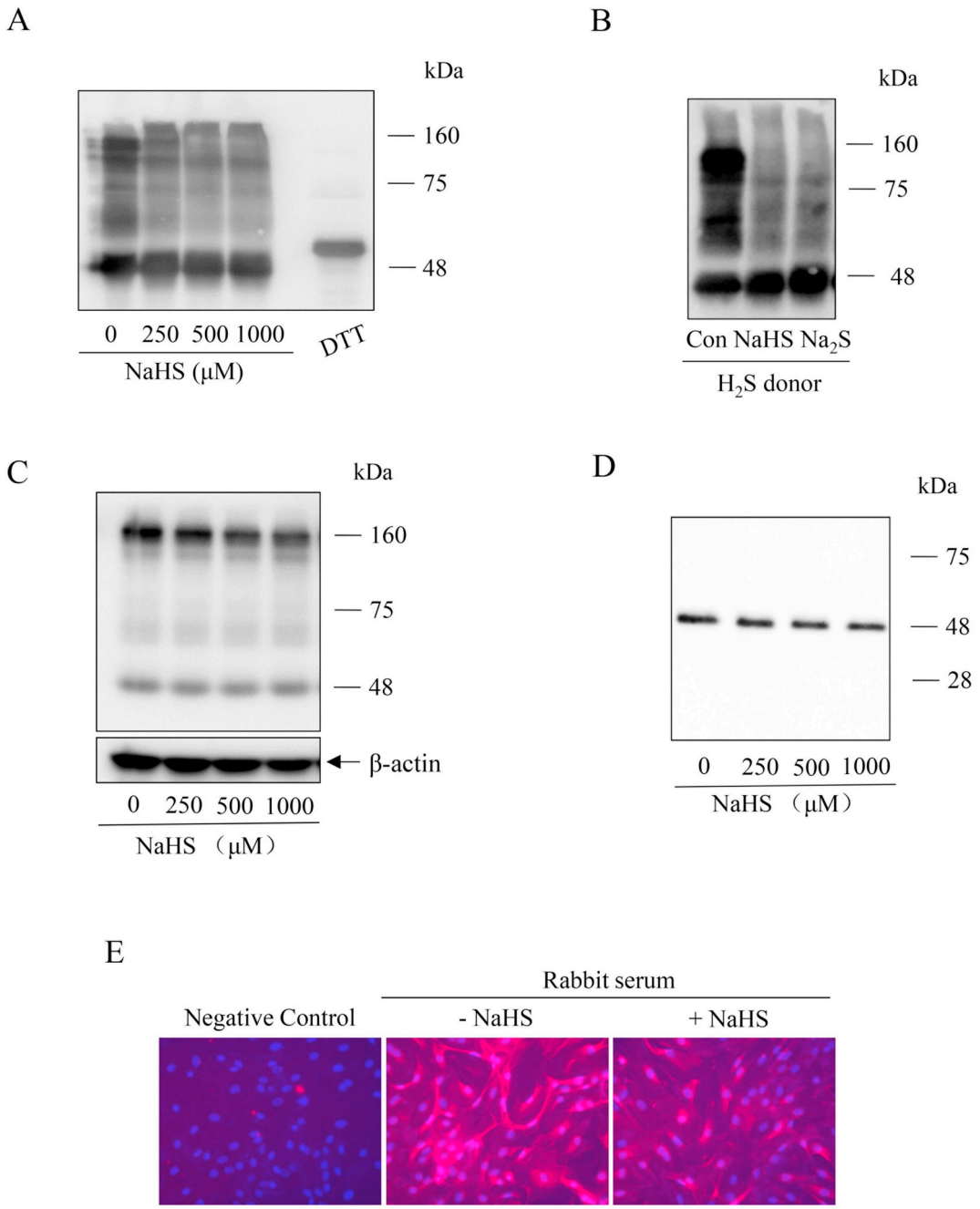


Fig. 3. *H_2S alters the structure of rabbit serum IgG and inhibits its binding to cell surface antigen.* (A and B) Effect of H_2S on the structure of rabbit serum IgG. Rabbit serum was incubated with the indicated concentrations of NaHS (A), 1 mM NaHS or Na2S (B) for 1 h. The treated serum was subjected to Western blot analysis for rabbit IgG. (C) Effect of H_2S on antibody binding to MCs surface. Rabbit serum was treated with or without the indicated concentrations of NaHS for 1 h before addition to MCs culture. After washing out the unbound free Igs, the cells were lysed, and the cell-bound Igs in the cellular lysate were immunoblotted using an HRP-labelled anti-rabbit IgG antibody. β-actin at the bottom was used as a loading control. (D) Precipitation of the cell membrane-bound Igs using protein A/G beads. MCs were treated the same as above and lysed with RIPA. The cell-bound Igs in the lysates were precipitated with proteinA/G beads and subjected to Western blot analysis. (E) Immunofluorescence staining of membrane-bound IgG. Rabbit serum was pretreated with 1 mM NaHS for 1 h and used as the first antibody to react with MC surface antigens. After 1 h incubation, the cells were washed with PBS. The presence of rabbit Igs in cell surface was stained with a rhodamine-conjugated anti-rabbit IgG antibody. Note the reduced red fluorescent signal in NaHS-treated serum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.12. Statistical analysis

All data were presented as mean ± SE. Differences between two groups were tested by Student's *t*-test. When more than two groups were compared, one-way ANOVA with Dunnett test or Student-Newman-Keuls Method was used. *P* values < 0.05 were considered significant.

3. Results

3.1. H_2S donor NaHS alters rabbit antibody structure and inhibits antibody binding to cell surface antigens

First, we examined whether H_2S modified the structure of Ig. For this purpose, purified rabbit IgG was incubated with NaHS, a well-used

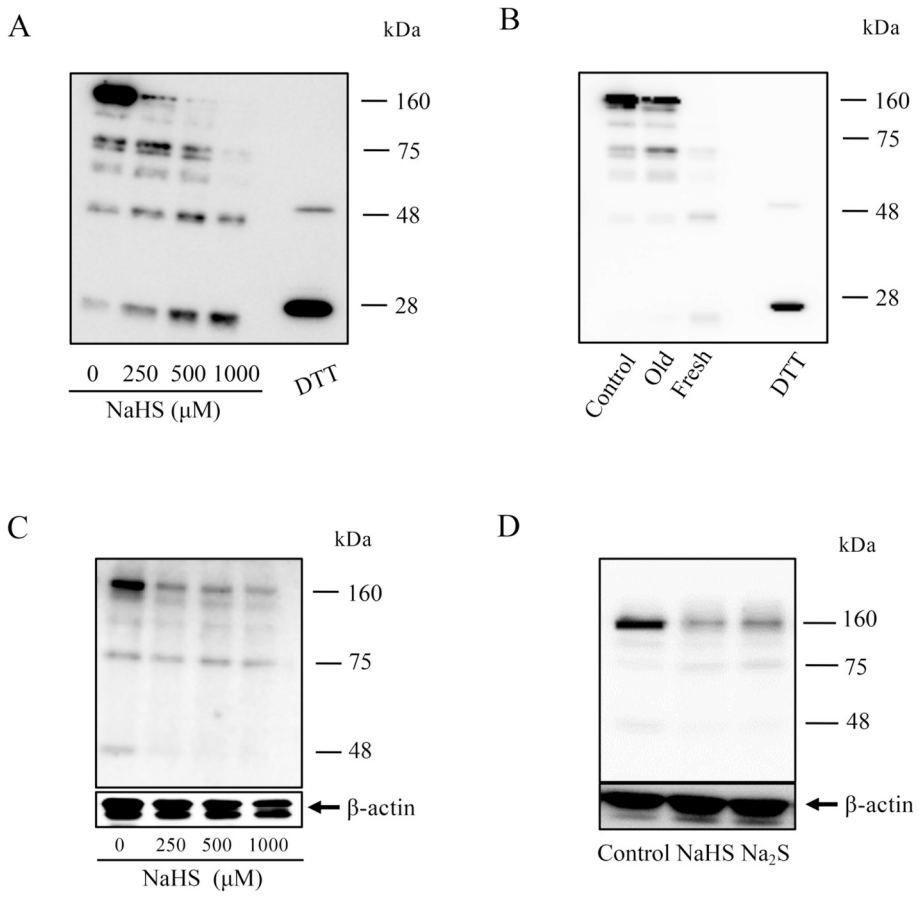


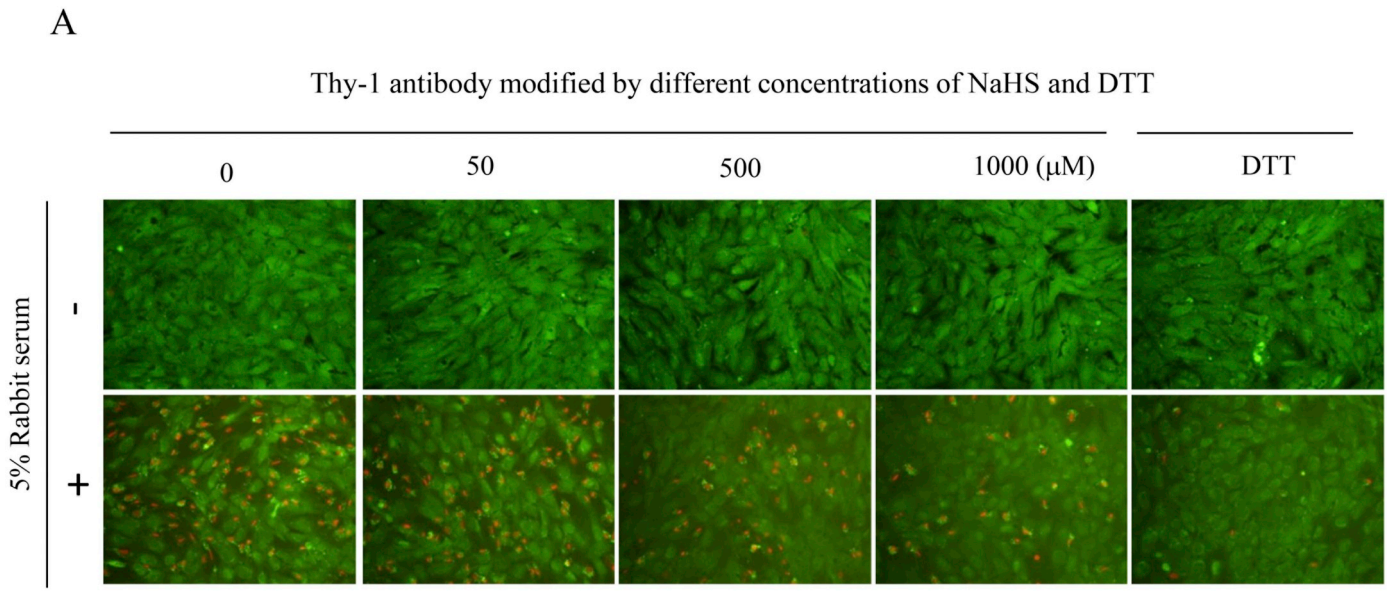
Fig. 4. *H_2S alters the structure of an anti-Thy-1 monoclonal antibody and inhibits its binding to cell surface Thy-1 antigen.* (A) Time-dependent effect of NaHS on the structure of the monoclonal anti-Thy-1 antibody. The monoclonal anti-Thy-1 antibody was incubated with the indicated concentrations of NaHS or 50 mM DTT for 1 h. Afterward, the samples were separated by SDS page and immunoblotted with an HRP-labelled anti-mouse IgG antibody. Note the shift of the bands from high MW to low MW after NaHS treatment. (B) Comparison of the effect of the freshly prepared and pre-placed NaHS solution on the structure of rabbit IgG. The anti-Thy1 monoclonal antibody was incubated either with 1 mM freshly prepared NaHS solution or the same concentration of NaHS that was prepared 24 h before addition to the assay system (old NaHS) for 1 h. The samples were separated by SDS page and immunoblotted for mouse IgG. Note the weakened effect of pre-placed NaHS solution in the modification of IgG structure in comparison with the freshly prepared NaHS solution. (C, D) Effect of H_2S on anti-Thy-1 antibody binding to Thy-1 molecules in thymus T cells. The anti-Thy-1 monoclonal antibody was pretreated with the indicated concentrations of NaHS (C), 1 mM NaHS or Na_2S (D) for 1 h, and dialyzed against culture medium. Afterward, it was added into the freshly isolated thymus T cells. After 30 min, the unbound free Igs were washed out, and the cells were lysed and assayed for the cell-bound Igs with Western blot analysis of the lysate using an HRP-labelled anti-mouse IgG antibody. β-actin at the bottom of the blot was used as loading control to indicate the equal loading of the cellular lysates.

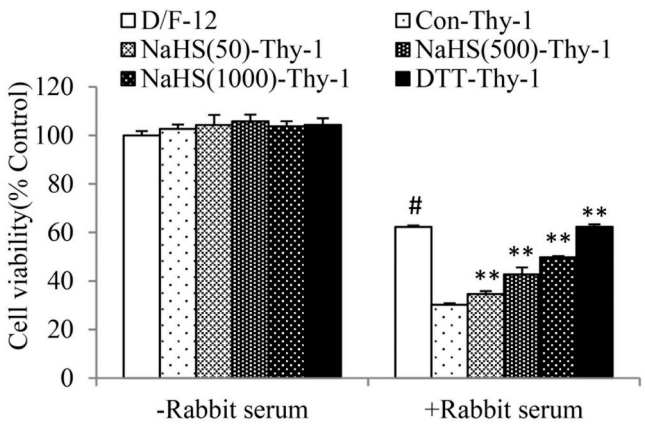
H_2S donor [1], for 1 h and assessed for the alterations in structure with Western blot analysis. Fig. 1A shows that the rabbit IgG was mainly localized at 160 kDa, corresponding to the predicted molecular mass of a homodimeric IgG consisting of two heavy and two light chains, linked by disulfide bonds. NaHS treatment caused a shift of IgG from 160 kDa to 50 kDa, corresponding to the molecular weight (MW) of a single heavy chain. Furthermore, this effect was also reproduced by another structurally different H_2S donor Na_2S [1] (Fig. 1C), indicating a possible involvement of their common product H_2S in this reaction. Consistent with this notion, NaHS prepared 24 h before the experiments (to release gaseous H_2S in the solution) exhibited a reduced ability in causing the shift, as compared with freshly prepared NaHS. This effect of NaHS was reproduced by DTT (Fig. 1, A and D), a reducing chemical that splits disulfide bonds in proteins, indicating that H_2S disrupts the disulfide bonds in IgG (Fig. 1D). Of note, the antibody used for detection of rabbit IgG poorly reacted with IgG light chain at 25 kDa.

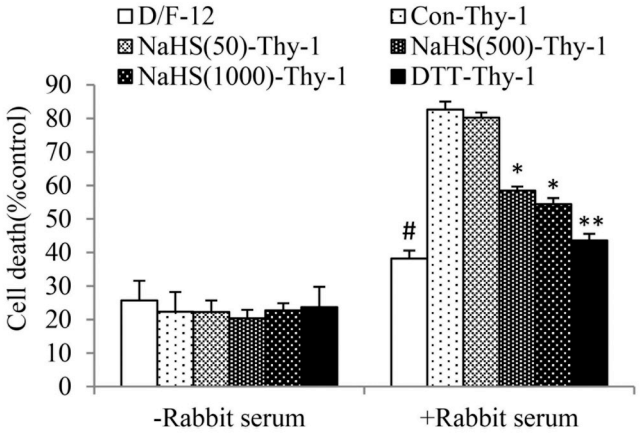
We also observed the effects of NaHS at the relatively lower concentrations (10–100 μM) and found that NaHS at the concentration of 10 μM was enough to induce a structural change in IgG, as demonstrated by the shift of the IgG band from 160 kDa to 50 kDa in Western blot (Fig. 1E).

Protein sulfhydration, i.e., the formation of a persulfide (–SSH) bond by adding additional sulfur to sulfhydryl (-SH) groups of Cys in proteins, is one of the major mechanisms by which H_2S exerts its antioxidative, anti-inflammatory and vasodilative actions [4,20]. To examine the effect of NaHS on Ig sulfhydration, we have employed an Alexa Fluor 680-conjugated C2 maleimide assay, reported by Sen and Liu, etc. [17,18] with minor modification [15]. This assay is based on the principle that the sulfhydryl (-SH) groups of Cys in Igs, including sulfhydrated (-SSH) and unsulfhydrated (-SH) Cys, are labelled by fluorescent maleimide. Addition of the reducing chemical DTT leads to the split of disulfide bonds in sulfhydrated (-SSH) IgG, causing a loss of the labelled maleimide, i.e., the fluorescence. Thus, the change in fluorescence intensity reflects the extent of sulfhydration (Fig. 2A). Fig. 2B shows that NaHS treatment increased fluorescence intensity in IgG molecules at several different MWs in an undenatured condition (Fig. 2B). In the presence of DTT, the fluorescent signal was only detected at the location of the heavy and light chains after separation with SDS-PAGE (Fig. 2B). The intensity of fluorescent signal in NaHS-treated IgG was stronger than that in untreated IgG, suggesting that NaHS treatment increased the number of maleimide-reactive sulhydryl (-SH) groups. Dot blot analysis confirmed that the total amount of maleimide-labelled IgG was indeed increased in NaHS-treated IgG (Fig. 2, C and D). DTT treatment caused a significant loss of the fluorescent signal in NaHS-treated IgG, but not in control, suggesting the presence of sulfhydration (-SSH; Fig. 2, C and E). These observations suggest that H_2S increases both sulfhydrated (-SSH) and unsulfhydrated (-SH) Cys residues in Igs.

We also tested the effect of H_2S on IgG in a rabbit serum, which we have previously reported to contain antibodies against several MC surface antigens [13,16]. H_2S donors, NaHS and Na_2S, also disrupted the disulfide bonds of the rabbit IgG in serum (Fig. 3, A and B). Given that the disulfide bonds in Igs are indispensable for antibody function [21,22], we, therefore, assessed the influence of H_2S on the antigen-antibody binding. For this purpose, rabbit serum was pretreated with or without NaHS and allowed to react with MCs for 1 h. After washing out the unbound IgG, the membrane-bound IgG was detected using immunoblotting or IF staining. Fig. 3 shows that NaHS treatment caused a reduction in IgG binding to the cell surface antigens. The amount of IgG in the cellular lysates was reduced, as revealed by Western blot detection of the cellular lysates (Fig. 3C) or the precipitated protein using an anti-rabbit IgG antibody (Fig. 3D), or IF staining of the cells with a fluorescently labelled antibody (Fig. 3E). These observations thus indicate that H_2S cleaves the disulfide bonds of serum rabbit IgG and







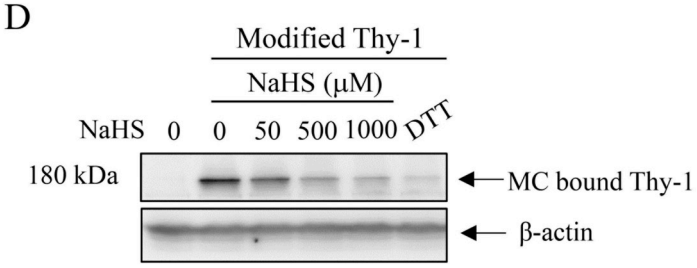


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Fig. 5. P*revention of anti-Thy-1 antibody and complement-induced MC lysis by H_2S.* (A–C) Effect of the pretreatment of anti-Thy-1 antibody with NaHS on antibody and complement-initiated MC lysis. MCs cultured in 96-well were exposed to 0.5 μg/ml differently modified anti-Thy-1 antibodies in the presence or absence of 5% native rabbit serum for 4.5 h. Cell viability was determined with calcein-AM (green, living cells)/PI (red, dead cells) staining (panel A), WST assay (panel B) and LDH release (panel C). Note the obviously reduced number of PI-positive dead cells and a significantly higher level of viability in the cells exposed to H_2S- or DTT-pretreated anti-Thy-1 antibody in comparison with anti-Thy-1 antibody control. Data shown in panels B and C are mean ± SE ($n = 4$). * $P < 0.05$, ** $P < 0.01$ vs. anti-Thy-1 control, #$P < 0.01$ vs control. (D) Western blot analysis of MC bound IgG. MCs at 12-well plate were treated the same as above for 2 h. The cellular lysates were harvested and cell-bound IgG was determined using Western blot analysis. The band shown was IgG localized at the MW about 160 kDa. β-actin was used as an internal control to demonstrate the equal loading of the samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduces its binding to cell surface antigens.

3.2. H_2S modifies the structure and function of a monoclonal anti-Thy-1 antibody

We also assessed the effects of H_2S on the structure and function of a monoclonal anti-Thy-1 antibody 1-22-3 [23]. Fig. 4A shows that NaHS also split the disulfide bonds of the monoclonal IgG in a concentration-dependent manner. This effect of NaHS was weakened after releasing the gaseous H_2S through the exposure of the prepared solution to air for 24 h before experiments (Fig. 4B). The NaHS-treated anti-Thy-1 monoclonal antibody exhibited a reduced binding ability to Thy-1 molecules in mouse thymus T cells (Fig. 4, C and D), as revealed by Western blot analysis of mouse IgG in the cellular lysates (Fig. 4, C and D). Of note, the H_2S donor-modified Thy-1 antibodies used in these experiments were dialyzed against culture medium to exclude the potential influence of the remaining H_2S donor and its metabolites on cell surface antigens.

Anti-Thy-1 monoclonal antibody-initiated and complement-mediated glomerular MC lysis is a well-used model for investigation of cell lysis in vitro [13]. Given that H_2S inhibited anti-Thy-1 antibody reaction with Thy-1 molecules, the cell lysis resulted from activation of classic complement pathway following antibody and antigen binding in the presence of complements should also be suppressed. This was, in fact, exactly the case. As shown in Fig. 5A to C, incubation of rat MCs with anti-Thy-1 antibody plus 5% rabbit serum as a source of complements resulted in MC lysis, as evidenced by the increased number of PI-positive dead cells (Fig. 5A), the decreased formazan formation (Fig. 5B), as well as the increased LDH release (Fig. 5C). This effect was completely abolished by the pre-modification of anti-Thy-1 antibody with a reducing chemical DTT. Similarly, it was also significantly reduced by NaHS in a concentration-dependent manner. Consistently, the amount of anti-Thy-1 antibody bound to MCs was reduced after pre-modification with NaHS or DTT (Fig. 5D). Of note, chemicals (NaHS or DTT) used for the modification of anti-Thy-1 antibody and their metabolites have also been removed through dialysis in this experiment. Therefore, it is unlikely that the observed effects were caused by the effect of these chemicals on cells. Collectively, these results indicate that H_2S potently prevents antibody/antigen binding via disruption of disulfide bonds in immunoglobulin.

3.3. H_2S inhibits Jurkat cell apoptosis initiated by an anti-human CD95 IgM antibody

To further establish the role of H_2S in inhibition of antibody-mediated biological actions, we examined its effect on anti-CD95 antibody-induced Jurkat cell apoptosis [24,25]. Fig. 6A shows that pretreatment of anti-CD95 IgM antibody with NaHS caused a marked reduction in its ability in the induction of Jurkat T cell apoptosis as compared with the control antibody. Hoechst staining revealed that the number of cells with nuclear fragmentation and condensation was significantly reduced (Fig. 6, A and B). Consistenly, there was a markedly reduction in caspase-3 activation (Fig. 6, C and D). To confirm that H_2S also altered the structure of anti-CD95 IgM antibody, we detected the change of antibody with Western blot analysis. Fig. 6E shows that NaHS treatment caused a concentration-dependent shift of IgM bands from high MWs to lower MWs, an effect that was similarly induced by DTT, suggesting a disruption of disulfide bonds in IgM. The intact structure of IgM was indispensable for its induction of Jurkat cell apoptosis because DTT treatment completely abolished its apoptosis-inducing activity. These results together suggest that H_2S also alters IgM structure and function.

3.4. H_2S inhibits the antibody-mediated aggregation of microbeads and agglutination of RBCs

To further confirm that H_2S modified the structure and function of Igs, we determined the effect of H_2S on antibody-mediated agglutination of microbeads. To this end, we have taken advantage of an Easy-Titer Mouse IgG Assay Kit. In this kit, polystyrene beads are coated with anti-mouse IgG antibody. The reaction between the coated antibodies and IgG in the samples bring two or more beads together, causing bead agglutination and a reduction in light absorption at 405 nm in a way proportional to the IgG concentration. One would expect that the cleavage of disulfide bonds in the IgG sample would lead to a reduced aggregation. That was the exact case. In the presence of NaHS, the size of the aggregated microbeads was greatly reduced (Fig. 7A), and the estimated concentrations of IgG was significantly lower than the actual concentration (Fig. 7B). These results suggest that H_2S modifies IgG structure and interferes with Easy-Titer Mouse IgG Assay.

To further confirm that H_2S regulated the antibody-mediated immune responses, we also examined the effect of NaHS on antibody-initiated red blood cell agglutination. Fig. 7C shows that NaHS also inhibited the agglutination of RBCs initiated by anti-mouse RBC antibody, as indicated by the reduced number of RBCs in the aggregates.

4. Discussion

In this study, we demonstrated, for the first time, that H_2S donor NaHS directly modified the structure and function of Igs through modification of Cys residues. Our study thus provides novel mechanistic insight into the immune suppressive action of H_2S and suggests that it could be used to treat certain antibody-mediated immune diseases.

H_2S has anti-inflammatory actions. It attenuated a variety of inflammatory diseases [2,5,6]. Currently, our understanding of H_2S on immunity is limited to cellular immunity, relating to its regulation on intracellular redox status and modification of Cys residues of the important intracellular signaling molecules [1–4]. In this study, we demonstrated that H_2S donor NaHS also directly modified effector molecules in humoral immunity, as exemplified by its regulation on Ig structure and function. The antibody is a tetrameric polypeptide structure, composed of two identical heavy and light chains, covalently linked by disulfide bridges [21]. In the presence of H_2S, we found that the structure of Igs was altered in a way similar to the reducing chemical DTT [26], suggesting a cleavage of disulfide bonds between the light and heavy chains. This effect of H_2S appeared to be Ig-isotype- and species-independent. It similarly affected the structure of IgG and IgM from mouse and rabbit. Of note, apart from Igs, we have also documented that H_2S also cleaves the disulfide bonds in TGFβ and thioredoxin [14,15].

H_2S has been shown to exert many biological functions through modification of Cys residues and disulfide bonds of the target proteins

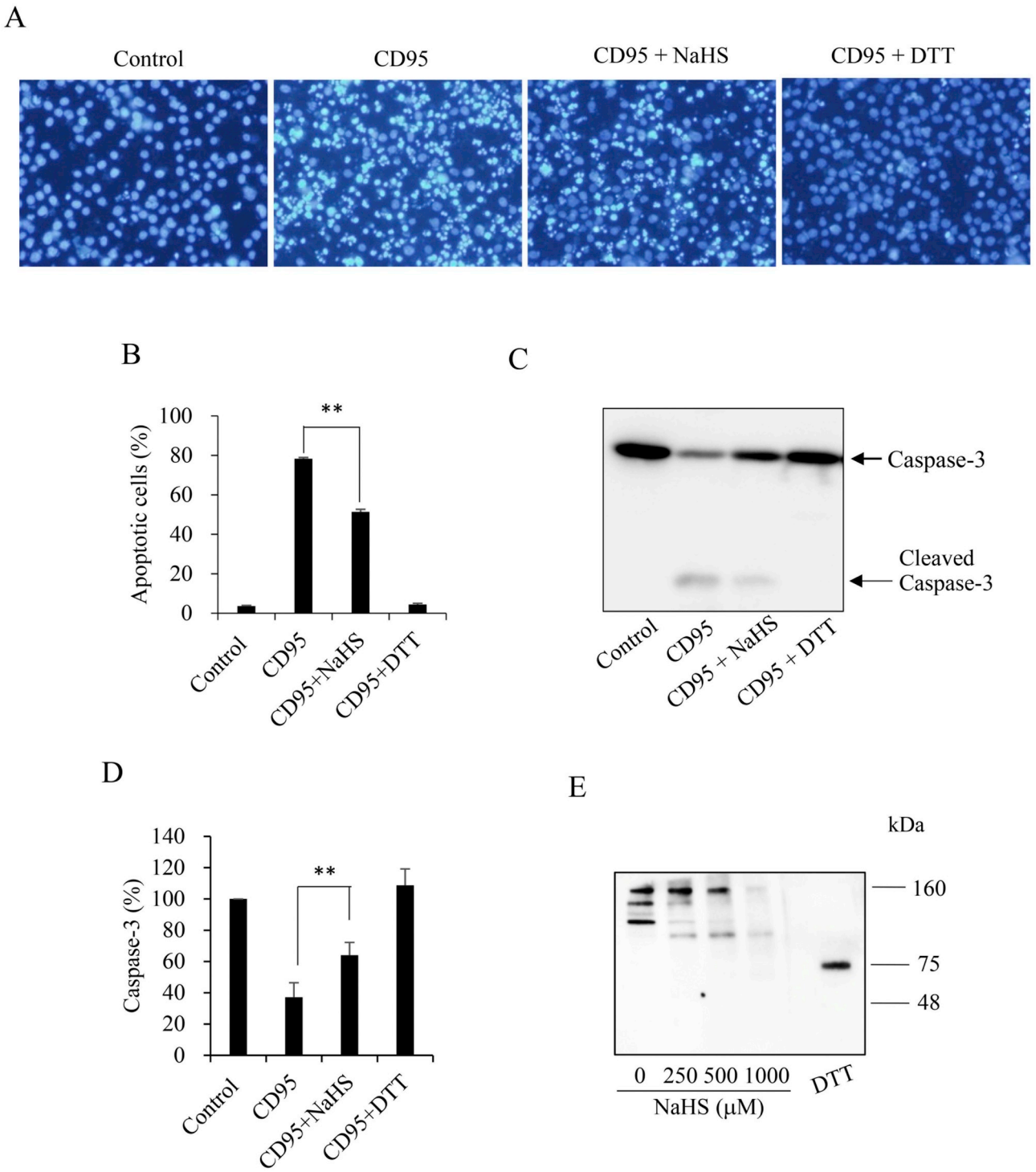

Fig. 6. *Pretreatment of anti-CD95 IgM with H_2S causes a loss of its ability in the induction of Jurkat cell apoptosis.* (A, B) Hoechst staining of apoptotic cells triggered by the anti-CD95 antibody. Anti-CD95 antibodies were treated with or without 1 mM NaHS or 10 mM DTT for 1 h and dialyzed against culture medium to remove the remaining NaHS, DTT or their metabolites. The same concentration of the anti-CD95 antibody was incubated with Jukart cell culture for 3 h. The cells were stained with 10 μg/ml Hoechst 33342 for 10 min. Note the obvious nuclear condensation and fragmentation in the presence of anti-CD95 antibody and its prevention by NaHS and DTT. (B) Percentage of apoptotic cells in (A). (C) Induction of caspase 3 activation by the anti-CD95 antibody. Jukart cells treated the same as above. The cellular lysates were subjected to Western blot analysis for caspase-3. Note the appearance of a cleavage band of caspase-3. (D) Densitometric analysis of the intensity of the latent caspase-3 in (C). The data shown are mean ± SE (n = 4). $^{**}P < 0.01$. (E) Concentration-dependent effect of NaHS on the structure of rabbit IgM. Anti-human CD95 mouse IgM antibody was incubated with the indicated concentrations of NaHS or 50 mM DTT for 1 h. Afterward, the samples were separated by SDS page and immunoblotted with an HRP-labelled anti-mouse IgM antibody. Note the obvious shift of the bands from high MW to low MW after NaHS treatment.

[14,15,20,27,28]. In this study, we observed that H_2S increased the number of maleimide-reactive groups. It split disulfide bonds of Igs through reactions involving both sulfhydryl (-SH) formation and sulfhydration (-SSH). Of note, the number of maleimide-reactive groups was detected not only in the heavy and light chains but also the intact homodimeric IgG at the location of 160 kDa in SDS-PAGE, suggesting

that H_2S also modified other Cys residues, other than the disulfide bonds linking heavy and light chains. In consistence with this notion, Ig is known to have many Cys and disulfide bonds in structure [21].

Reduction of the disulfide bonds in antibody by H_2S led to an altered antibody function, as evidenced by the reduced antibody binding to cell surface antigens and the disruption of antibody-mediated aggregation of microbeads and RBCs. Previous studies showed that reduction of IgM with DTT led to a loss of IgM function [22]. In this study, we found that DTT treatment caused a complete abolishment of CD95-induced apoptosis, indicating a requirement of intact IgM structure in the induction of apoptosis. There also a report demonstrating that incubation of IgG with DTT preferentially reduced the hinge region disulfide bonds. The reduced IgG (rIgG, or half IgG) was monovalent and lost its ability to agglutinate its target bacterial [29]. These data indicate that H_2S-mediated the reduction of the disulfide bond in Igs could be the mechanism causing the reduced antibody activity.

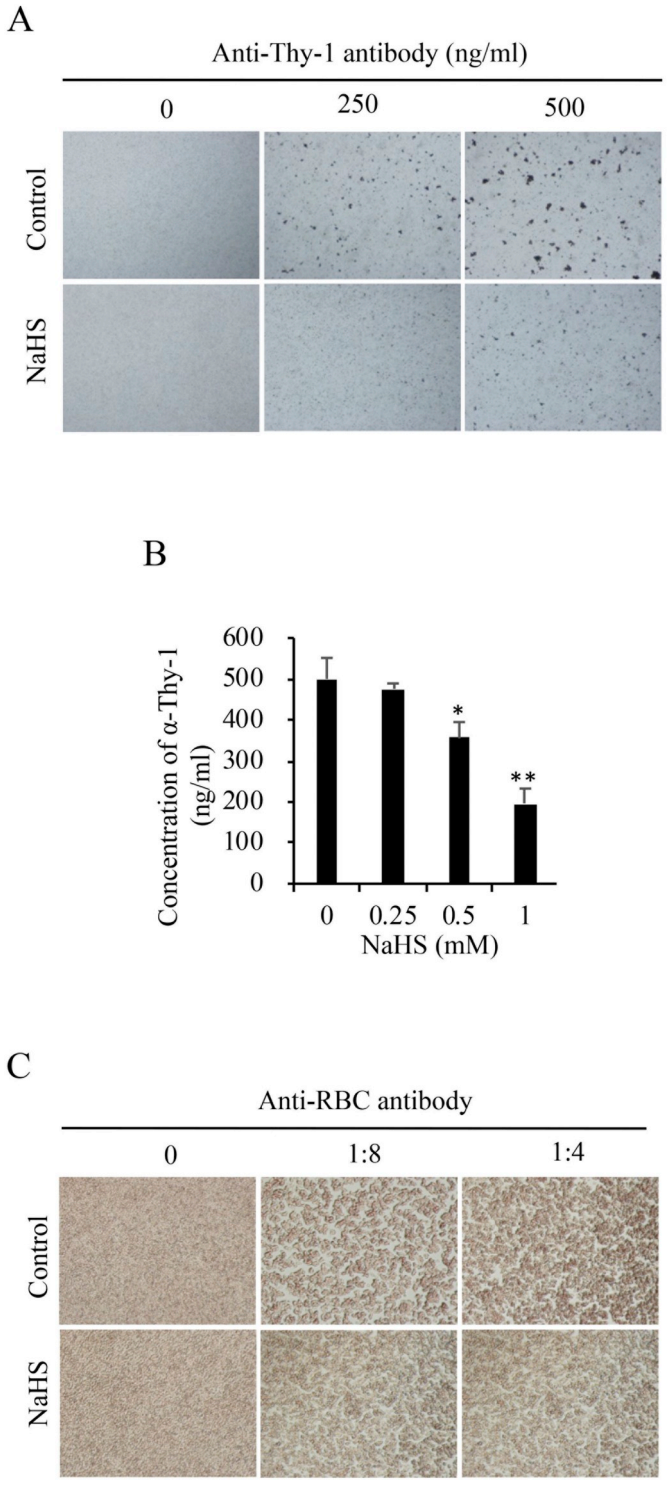

Fig. 7. *H_2S prevents the antibody-mediated agglutination of microbeads and RBCs.* (A) Effect of H_2S on antibody-elicited agglutination of the microbeads. The assay beads were incubated with the indicated concentrations of mouse anti-Thy-1 antibody that was pretreated with the indicated concentrations of NaHS for 1 h. After the reaction for 5 min, the agglutination of the microbeads was photographed. Note the obvious difference in the size of the aggregates between control and NaHS-treated wells. (B) Influence of different concentrations of NaHS on Easy-Titer IgG assay. The same amount of IgG was treated with the indicated concentrations of NaHS for 1 and assayed for IgG concentration using Easy-Titer IgG assay. The concentration of IgG was calculated based on the standard curve generated from a dilution series of a standard. Data shown are mean ± SE (n = 4), * $P < 0.05$; ** $P < 0.01$. (C) Effect of NaHS on antibody-mediated RBC agglutination. Mouse RBCs were exposed to the indicated dilutions of anti-mouse RBC antibody in the presence or absence of 1 mM NaHS for 1 h. The formation of agglutinated RBCs was photographed.

It is worth mentioning that, apart from its action on antibodies, our preliminary study showed that H_2S also significantly inhibited complement activation. It inhibited the formation of C5b-9 elicited by an activator of the alternative pathway (Supplementary Fig. 1). This effect could also be due to the reduction of disulfide bonds in complement components.

Of note, we have used NaHS as an H_2S donor in this investigation. As a widely used donor, it has been extensively documented in the literature relating to the pathophysiological role of H_2S in both in vivo and in vitro models. The concentrations (up to 1 mM) used in this study is also compatible with others. Intriguingly, NaHS at the concentration of 50 μM was enough to cause Ig structural changes and sulfhydration (data not shown). This concentration may also be achievable in vivo. In brain tissue, the physiological concentration of H_2S was reported to be 50–160 μM [6,30]. The highest concentrations of H_2S in the body occur in the lumen of the colon, where the concentrations of H_2S could reach the millimoles [6,31]. Recently, there is a growing interest in the therapeutic application of H_2S in the clinic. In fact, more and more H_2S donors are available for this purpose. It is conceivable that the pharmacological administration of H_2S donor could also lead to a rapid and marked elevation in local H_2S concentration [6]. It remains to be tested whether the pharmacologic H_2S donors could cause the similar changes in antibody structure and function under in vivo situation and whether this property of H_2S could be applied for the treatment of certain immune diseases. This will be the topic of our future investigation.

Our findings could have a great clinic and basic significance. Our study provided novel mechanistic insights into the effect of H_2S on inflammatory diseases. Besides the well-documented actions of H_2S on ROS and cellular signaling pathway, H_2S also directly modified effector molecules in humoral immunity through the mechanisms involving sulfhydration. Because many important functional proteins in the blood are rich in disulfide bonds [32,33], the direct modification of the effector molecules in blood or other body fluid by H_2S could be an important mechanism by which H_2S regulates immune and other pathophysiological responses. This property of H_2S might be exploited to treat certain humoral immune diseases. H_2S has also been reported to be critically implicated in the pathogenesis of colitis and periodontal diseases [34,35]. The toxic levels of sulfide produced by the microflora in the periodontal pockets or the colon may annihilate antibody and complement-mediated opsonization of the bacteria, thus providing a way for the bacteria to escape the host immune system. This possibility warrants to be tested in future studies.

5. Conclusion

In summary, we demonstrated, for the first time, pharmacologic levels of H_2S inhibited antibody-mediated immune responses via direct modification of the disulfide bonds in its structure. Our study thus provided novel mechanistic insights into the pharmacologic actions of H_2S in humoral immunity and suggested that this property of H_2S may be exploited to treat certain humoral immune diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.052.

Conflict of interest statement

The authors confirm that they have no competing interests.

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