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INORGANIC COMPOUNDS

Sulfide induces apoptosis and Rho kinase-dependent cell blebbing in Jurkat cells

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Abstract Hydrogen sulfide (H_2S) is a toxic gaseous substance, and accidental exposure to high concentrations of H_2S has been reported to be lethal to humans. Inhaled and absorbed H_2S is partially dissolved within the circulation and causes toxic effects on lymphocytes. However, the mechanisms involved in H_2S toxicity have not been well documented. In this study, we examined the cellular uptake and injury of sulfide-exposed human T lymphocytes (Jurkat). Cells were exposed to a H_2S donor, sodium hydroxysulfide (NaHS), at pH 6.0, 7.0, or 8.0 for 1 h at 37 °C in a sealed conical tube to avoid the loss of dissolved H_2S gas. Cytotoxicity and cellular sulfide concentrations increased dramatically as the pH of the NaHS solution decreased. Sulfide enhanced the cleavage of caspase-3 and poly (ADP-ribose) polymerase and induced early cellular apoptosis. A pan-caspase inhibitor reduced sulfide-induced apoptosis. These results indicate that sulfide induces pH-dependent and caspase-dependent apoptosis. We also found that blebbing of the plasma membrane occurred in sulfide-exposed cells. Both ROCK-1 and ROCK-2 (Rho kinases) were activated by sulfide, and sulfide-induced cell blebbing was suppressed by a ROCK inhibitor, suggesting that a Rho pathway is involved in sulfide-induced blebbing in lymphocytes.

Keywords Sulfide · Apoptosis · Rho kinase (ROCK) · Flow cytometry · Cell blebbing

Introduction

Hydrogen sulfide (H_2S) is a toxic gas with a characteristic rotten egg odor. Exposure to H_2S is widespread owing to the numerous industrial processes and natural sources that emit the gas (Dorman et al. 2002). The immediately dangerous to life or health (IDLH) for H_2S was determined to be 100 ppm based on the maximum concentration that can be endured for 1 h without serious consequences (Centers for Disease Control and Prevention, NIOSH). Accidental acute exposure to high concentrations of H_2S (≥500 ppm) causes respiratory paralysis, unconsciousness, and eventually death within minutes (Beauchamp et al. 1984). H_2S is absorbed in the upper respiratory tract mucosa (Hildebrandt and Grieshaber 2008) and is rapidly metabolized from sub-lethal concentrations of sulfide into thiosulfate by both hemoglobin and hepatic enzymes (Curtis et al. 1972; Hildebrandt and Grieshaber 2008; Li and Moore 2008). An in vivo experiment using sodium ^{35}S-sulfide indicated that orally administered or inhaled sulfide appears in the circulation transiently (Curtis et al. 1972). One of the mechanisms for H_2S toxicity has been shown to be due to an inhibition of cytochrome *c* oxidase in vivo (Dorman et al. 2002) and in vitro (Roberts et al. 2006; Thompson et al. 2003). Conversely, H_2S is synthesized endogenously by two pyridoxal-5′-phosphate-dependent enzymes responsible for the metabolism of L-cysteine in mammalian cells,

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namely, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Hughes et al. 2009; Li et al. 2011). Endogenous H_2S has many physiological effects in mammalian tissues. Recent studies suggest that H_2S is a unique gasotransmitter with regulatory roles in immune system. Endogenous H_2S modulates immune responses, such as neutrophil migration (Dal-Secco et al. 2008) and leukocyte-mediated inflammation (Zanardo et al. 2006). Exogenous H_2S has been reported to reduce local inflammatory responses of peripheral blood lymphocytes (Mirandola et al. 2007). H_2S has also been identified as an autocrine immunomodulatory molecule in T lymphocytes (Miller et al. 2012). However, it is unclear how H_2S changes the function of T lymphocytes.

Apoptosis, a genetically programed cell death, is an essential physiological process required for the normal development and maintenance of tissue homeostasis. It is reported that low levels of H_2S prevent the apoptosis of human polymorphonuclear cells via the inhibition of p38 mitogen-activated protein kinase (MAPK) and caspase-3 (Rinaldi et al. 2006). Yin et al. reported that sodium hydroxysulfide (NaHS) protected 1-methyl-4-phenyl-pyridinium (MPP^+)-induced cytotoxicity and apoptosis in pheochromocytoma cells (Abe and Kimura 1996; Yin et al. 2009). Conversely, it has been reported that H_2S induced apoptosis in various types of cells, including human lung fibroblasts (Baskar et al. 2007), pancreatic acinar cells through the activation of Bax (Cao et al. 2006), and insulin-secreting β cells via p38 MAPK activation (Yang et al. 2007). During apoptosis, cells undergo distinct morphological changes, including cell contraction, membrane blebbing, and nuclear disintegration. Contractile forces generated by actin–myosin cytoskeletal structures lead to cell contractions and membrane blebbing (Coleman et al. 2001).

Rho kinases (ROCK-1 and ROCK-2) are the effectors of Rho GTPase and have a molecular mass of ~160 kDa (Ishizaki et al. 1996; Leung et al. 1995). Activated ROCK-1 induces myosin light chain (MLC) phosphorylation and cellular F-actin and activates the actin–myosin contractile system (Coleman et al. 2001). MLC phosphorylation leads to the formation of membrane blebbing during apoptosis (Orlando et al. 2006). ROCK-2 has been also reported to implicate as a mediator of the morphological changes associated with apoptosis (Song et al. 2002). However, it has not reported whether ROCKs contribute to morphological changes during sulfide-induced apoptosis.

Since H_2S is a weak acid ($pK_{a_1} = 6.76$), it dissociates into [HS^-] and [H^+] with a trace of [S^{2-}] in physiological liquids, including plasma (pH 7.4, 37 °C), according to the Handerson–Hasselbach equation (Dombkowski et al. 2004). The proportion of [H_2S]/[HS^-] is greatly affected by the pH of a solution, and thus, the loss of H_2S from the culture solution in the form of a gas also depends on its pH. It is reported that about 25 % of 1 mM H_2S escapes from the cell-free medium within 2 h (Oh et al. 2006). Thus, exposure of cells to an exact amount of sulfide would not be completed using a conventional in vitro culture system. The membrane transport systems of sulfide are not well understood. It is reported that the membrane transport of sulfide occurs by simple diffusion as H_2S form using planar lipid membrane (Mathai et al. 2009). However, it is not clear until now which species of sulfide are permeable to transport cell membrane and responsible for cell injury. In the present study, we briefly exposed cells to buffered solution in a capped tube to prevent the loss of H_2S and adjust the total amount of sulfur species (H_2S, HS^-, or S^{2-}) in the exposure solution. Herein, we report on the cellular concentration of sulfide, sulfide-induced apoptosis, and involvement of ROCKs during the apoptotic process in Jurkat cells. The term "sulfide" used in this study refers to H_2S, HS^-, and S^{2-}.

Materials and methods

Chemicals

NaHS, Y-27632, cytochalasin D, and nocodazole were purchased from Sigma (St. Louis, MO). Z-VAD-fmk and Z-VDVAD-fmk were purchased from BioVision (Mountain View, CA). Two-amino-5-*N*,*N*-diethylaminotoluene hydrochloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$), zinc acetate, iron chloride ($FeCl_3$), and sodium 1-octansulfonate were purchased from Wako (Osaka, Japan). The solution containing NaHS was prepared immediately before use. Cytochalasin D or nocodazole stock solutions (1 mg/ml) were prepared in dimethyl sulfoxide (DMSO), and these stock solutions were used at a final DMSO concentration of 0.1 %. Y-27632 stock solution (5 mM) was prepared in Hanks' Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA, USA). All chemicals were of analytical grade.

Cell culture

A human T lymphocyte cell line, Jurkat, and a human bronchial epithelial cell line, BEAS-2B, were obtained from the American Type Culture Collection (ATCC). Jurkat and BEAS-2B cells were cultured at 37 °C in 5 % CO_2 atmosphere in RPMI1640 medium (Life Technologies) and Dulbecco's modified minimum essential medium (DMEM, Life Technologies), respectively. The culture media contain 10 % heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of

streptomycin. Unless otherwise specified, the pH of the culture medium was adjusted to 7.4. For experiments using cytochalasin D or nocodazole, Jurkat cells were exposed to 5 mM of NaHS in the presence or absence of 1 μg/ml of cytochalasin D, 1 μg/ml of nocodazole, or 0.1 % DMSO (as control) in HBSS at pH 6.0 for 1 h. After washing, the cells were cultured in fresh medium in the presence or absence of 1 μg/ml of cytochalasin D, 1 μg/ml of nocodazole, or 0.1 % DMSO at the indicated time in each experiment.

For experiments using Y-27632, Jurkat cells were precultured with or without 20 μM of Y-27632 for 2 h. The cells were exposed to 5 mM of NaHS in HBSS at pH 6.0 in the presence or absence of 20 μM of Y-27632 for 1 h. After washing, the cells were cultured in fresh medium in the presence or absence of 20 μM of Y-27632 at the indicated time in each experiment.

Cytotoxicity

Jurkat cells were exposed to 0–5 mM of NaHS in HBSS adjusted to pH 6.0, 7.0, or 8.0 for 1 h in polypropylene tubes (BD Biosciences, Franklin Lakes, NJ, USA) in an incubator set at 37 °C with gentle shaking. In the NaHS exposure of Jurkat cells, the cell suspension was placed in an airtight capped tube, and the air was removed by filling the tube with HBSS to avoid a loss of dissolved H_2S gas from HBSS. After exposure, cells were washed twice with phosphate-buffered saline (PBS, Life Technologies). The cells were plated into a 96-well culture dish and cultured in fresh medium. After 24 h of culture, cell viability was evaluated using AlamarBlue® (Life Technologies), according to the manufacturer's instruction. The fluorescence intensity of the medium was measured with a spectrofluorometer (POLARstar OPTIMA, BMG Labtach, Offenburg, Germany) using the excitation and emission wavelengths of 540 and 588 nm, respectively.

BEAS-2B cells were cultured to early confluence in a 96-well culture plate and exposed to 0–5 mM of NaHS in HBSS adjusted to pH 6.0, 7.0, or 8.0 for 1 h. The plate was sealed using Heat Sealer (Eppendorf, Hamburg, Germany) to prevent the escape of gas. The cell monolayers were washed twice with PBS and further cultured in fresh medium without sealing. After 24 h of culture, cell viability of BEAS-2B cells was evaluated using AlamarBlue®.

Analysis of sulfide

Sulfide was extracted from cells according to a method commonly used for the measurement of blood sulfide concentrations (Nagashima et al. 1995) with minor modifications. The reaction solution was prepared by dissolving 2-amino-5-*N*,*N*-diethylaminotoluene hydrochloride in 0.35 M of sulfuric acid at final concentration of 2 %. Intracellular concentrations of sulfide were measured by an HPLC-fluorescence detector (FLD). The analytical column was an ODS column (Inertsil, ODS-3, 3 μm, 150 mm × 4.6 mm i.d., GL Science, Tokyo, Japan). The HPLC system consists of a pump (LC-10AD) equipped with a UV-FLD (SPD-10A) (Shimadzu, Kyoto, Japan). Fluorescence of the eluate was monitored with the excitation set at 640 nm and emission at 675 nm. A 20-μl sample of the organic layer obtained from the sulfide extraction was injected into the HPLC. Under this condition, the thionine derivative of sulfide was eluted at 4.8 min.

A standard sulfide solution was prepared by dissolving $Na_2S{\cdot}9H_2O$. Sulfide concentrations were determined from the liner regression ($r^2 = 0.998$) of a calibration curve ranging from 0 to 2.5 μM. The recovery rate of 1 μM was 78.2 ± 1.7 %.

Cell morphology

Jurkat cells were exposed to 0, 1, or 5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. After washing twice with PBS, cells were plated into a 24-well culture dish and cultured in fresh medium. After 3–6 h, cell suspensions were prepared using the cytospin apparatus. Cells were cytocentrifuged on a slide glass at 1,200 rpm for 5 min and subsequently air-dried. Lastly, cells were stained with Diff-Quik® (International Reagents, Kobe, Japan).

Caspase-3/7 activity assay

Jurkat cells were exposed to 0, 1, or 5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. After washing twice with PBS, cells were plated into a 96-well culture dish and cultured in fresh medium in the presence or absence of 2 μM of Z-VAD-fmk or 2 μM of Z-VDVAD-fmk for 6 h. Caspase-3/7 activities were measured with a Caspase-Glo™ 3/7 Assay kit (Promega, Madison, MI, USA), according to the manufacturer's instruction. The luminescence was measured with a microplate luminometer (GLOMAX, Promega) at 565 nm.

Western blot analysis

Jurkat cells were exposed to 0, 1, or 5 mM of NaHS in HBSS at pH 6.0. After washing twice with PBS, cells were plated into a 6-well culture dish and cultured in fresh medium in the presence or absence of 2 μM of Z-VAD-fmk for 4 h (for ROCK-1 and ROCK-2) or 6 h [for caspase-3 and poly (ADP-ribose) polymerase (PARP)]. Cells were lysed with RIPA buffer containing the protease inhibitor, PMSF, and sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The lysate was

centrifuged at 10,000*g* for 5 min at 4 °C. Proteins in the supernatant were resolved on SDS-PAGE under reducing conditions and electroblotted onto a PVDF membrane. The membrane was blocked with PVDF blocking reagent (TOYOBO, Osaka, Japan) and probed with anti-PARP, anti-caspase-3, anti-ROCK-1, or anti-ROCK-2 (BD Biosciences), followed by peroxidase (POD)-tagged anti-mouse IgG antibody (Santa Cruz Biotechnology). The immunoreactions on the membrane were visualized by using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK). After probing with anti-ROCK-1 or anti-ROCK-2, the membrane was incubated with stripping buffer (2 % SDS and 100 mM of 2-mercaptoethanol in tween-PBS) at 56 °C for 20 min to remove the antibodies and reprobed with POD-tagged anti-α-tubulin antibody (MBL, Nagoya, Japan). The intensities of the bands were quantified using a chemiluminescence densitometer (Lumino Imaging Analyzer, FAS-1100, TOYOBO, Osaka, Japan).

Flow cytometric analysis of apoptotic cells

The detection of apoptotic cells was conducted using an Annexin V-FITC Apoptosis Detection Kit (BioVision). Jurkat cells were exposed to 5 mM of NaHS for 1 h at pH 6.0. After washing twice with PBS, cells were plated into a 24-well culture dish and cultured in fresh medium in the presence or absence of 2 μM of Z-VAD-fmk for 4 h. Cells were then collected by centrifugation at 1,200*g* for 2 min. Then, cells were resuspended with binding buffer and stained with FITC-labeled annexin V and propidium iodide (PI) for 5 min at room temperature in the dark, according to the manufacturer's instruction, and analyzed with a flow cytometer (Guava EasyCyte^{TM} 8HT, MILLIPORE, Hayward, CA, USA).

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni/Dunn's post hoc analysis. The statistical significance level was set at $p < 0.05$.

Results

Sulfide-induced changes in cell viability

Figure 1 and supplemental Fig. 1 demonstrate the cytotoxic effects of NaHS on Jurkat and BEAS-2B cells in HBSS at pH 6.0, 7.0, or 8.0, respectively. Cytotoxicity of Jurkat cells due to NaHS was markedly enhanced with decreases in the pH of the culture solution. When a curve of the secondary degree was fitted to the dose–effect relationship, the effective concentration of 50 % (EC_{50}) for lethality of NaHS at pH 6.0 was calculated to be 2.5 mM, whereas the EC_{50} of NaHS at pH 7.0 and 8.0 was >5 mM. Similar results of cytotoxicity were also observed in BEAS-2B cells. Cytotoxicity was reduced at alkaline pHs and enhanced at acidic pHs when BEAS-2B cells were exposed to 5 mM of NaHS. However, the EC_{50} of NaHS at all pHs was >5 mM. In the absence of NaHS, changes in pH did not affect the viability of both Jurkat and BEAS-2B cells.

Intracellular concentrations of sulfide

Jurkat cells were exposed to 0, 1, or 5 mM of NaHS, and the intracellular concentrations of sulfide were measured immediately after exposure using HPLC-FLD. As shown in Fig. 2, the intracellular concentrations of sulfide increased dramatically as the pH decreased, when the cells were exposed to 5 mM of NaHS. Moreover, the intracellular concentrations of sulfide were still detectable by the current HPLC-FLD method without NaHS treatment.

Effects of sulfide on cell blebbing

Figure 3a–d show photomicrographs of cells stained with Diff-Quik®. Morphological changes were not observed immediately after exposure (data not shown). Cells cultured for 6 h after the cessation of exposure to 5 mM of NaHS at pH 6.0 showed fragmented nuclei and plasma membrane blebbing (Fig. 3b). Conversely, no morphological changes were observed in cells exposed to 5 mM of NaHS at either pH 7.0 (Fig. 3c) or 8.0 (Fig. 3d).

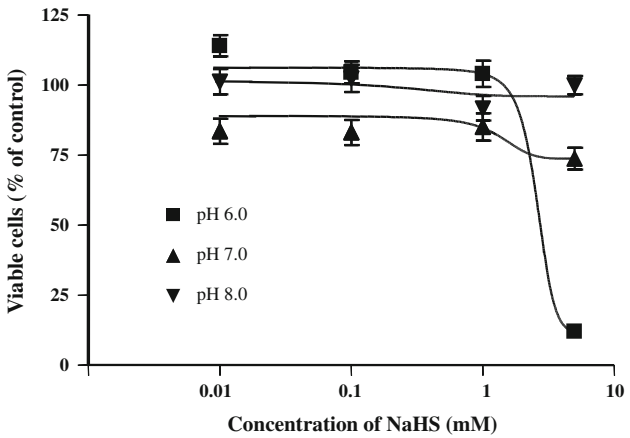

Fig. 1 Effects of pH on cytotoxicity induced by sulfide in Jurkat cells. Jurkat cells were exposed to 0–5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. After washing twice with PBS, cells were plated into a 96-well culture dish and cultured in fresh RPMI1640 complete medium. After 24 h of culture, cell viability was evaluated using AlamarBlue®. Data are presented as mean ± SEM ($N = 5$)

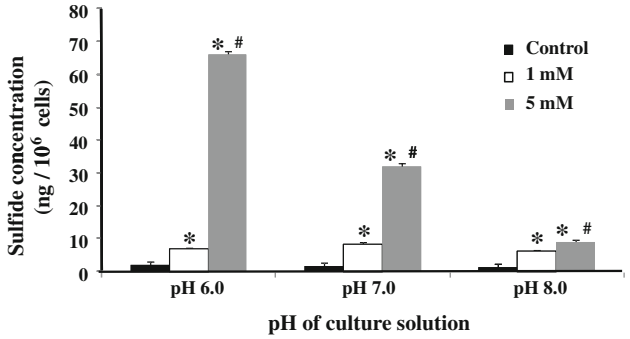

Fig. 2 Effects of pH on cellular uptake of sulfide. Jurkat cells were exposed to 0, 1, or 5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. The cells were washed twice with 10 ml of HBSS, resuspended in 100 µl of fresh HBSS, and transferred into an airtight glass tube. The cell suspension was diluted to a final volume of 4.0 ml with 0.5 % of zinc acetate in H_2O to trap the sulfide, and then, the mixture was sonicated for 5 min to disrupt the cells. A 0.4 ml of the reaction solution and 0.2 ml of 0.1 mM $FeCl_3$ solution in 5 mM of sulfuric acid were added to the sample solution and diluted the solution with H_2O to 5 ml. The mixture was incubated for 60 min at 25 °C in the dark. After the incubation, 0.5 ml of 0.1 M sodium 1-octansulfonate and 0.5 ml of 2-octanol were added to the mixture. The extraction was performed by shaking the tube for 10 min and centrifuging at 3,000 rpm for 10 min to separate the aqueous and organic layers. A 20-µl sample of the organic layer was injected into the HPLC. Analytical column: an ODS column, mobile phase: a mixture of 0.1 M of sodium 1-octansulfonate solution and acetonitrile (1:9, v/v), HPLC analysis condition: isocratically at a flow rate of 0.5 ml/min at 40 °C. $*p < 0.05$, compared to control; $\#p < 0.05$, compared to 1 mM

Furthermore, 1 mM of NaHS did not affect cell morphology even at pH 6.0 (data not shown).

Cleavage of caspase-3 and PARP in sulfide-exposed cells

The activation of caspase-3 is a typical apoptotic event. We investigated the activation of caspase-3 and caspase-3/7 in NaHS-exposed cells, and the effect of Z-VAD-fmk, a pan-caspase inhibitor, by Western blotting and chemiluminescence method. Figure 4 demonstrates that the inactive form of pro-caspase-3 at 32 kDa was decreased and the cleaved active form of caspase-3 at 17 kDa was increased with NaHS exposure. Additionally, Z-VAD-fmk suppressed the conversion of pro-caspase-3 to the active form of caspase-3. Apoptotic responses in NaHS-exposed cells were further confirmed by detecting the degradation of PARP, which is known to be cleaved by activated caspase-3. PARP at 116 kDa and cleaved PARP fragment at 89 kDa were detected in 5-mM NaHS-exposed cells, and this was also inhibited by Z-VAD-fmk.

As shown in Fig. 5, caspase-3/7 activity was increased with 5 mM of NaHS at both pH 6.0 and 7.0, but not pH 8.0. The caspase activities induced by 5 mM of NaHS at both pH 6.0 and 7.0 were completely inhibited by Z-VAD-fmk. In the absence of NaHS, the changes in pH did not affect caspase-3/7 activity. Furthermore, Z-VDVAD-fmk, a caspase-2-specific inhibitor, inhibited caspase-3/7 activity

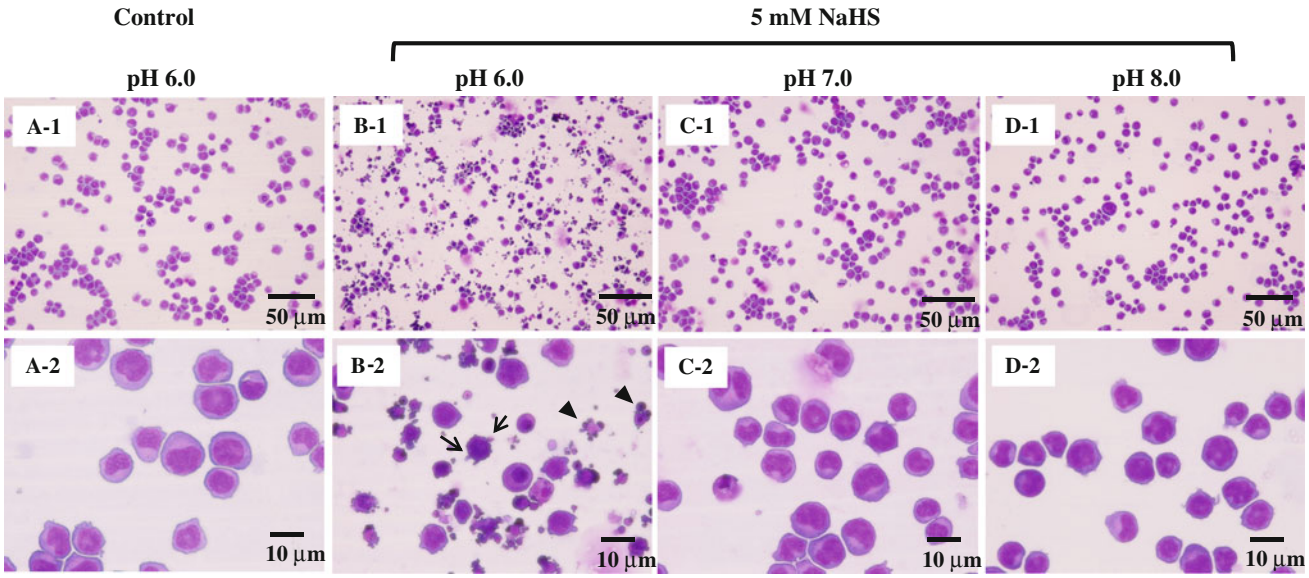

Fig. 3 Photomicrographs of sulfide-exposed Jurkat cells stained with Diff-Quik®. Jurkat cells were exposed to 0 or 5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. After washing twice with PBS, cells were plated into a 24-well culture dish and cultured in fresh RPMI1640 complete medium. After 6 h of culture, the cell suspension was cytocentrifuged and stained with Diff-Quik®. The *lower panel* is a higher magnification of each *upper panel*. *Arrows* and *arrow heads* indicate plasma membrane blebbing and fragmented nuclei, respectively

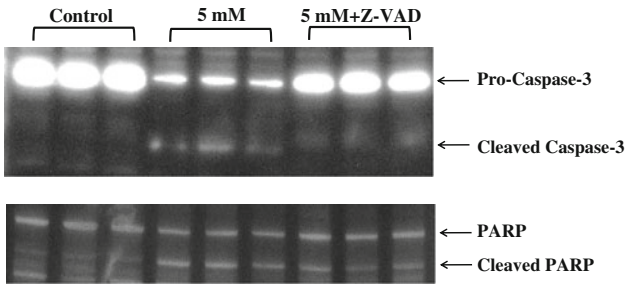

Fig. 4 Western blot analyses for the detection of caspase-3 and PARP cleavage in sulfide-exposed Jurkat cells. Jurkat cells were exposed to 5 mM of NaHS in HBSS at pH 6.0 for 1 h. After washing twice with PBS, cells were plated into a 6-well culture dish and cultured in fresh RPMI1640 complete medium in the presence or absence of 2 μM of Z-VAD-fmk for 6 h. The lysate was centrifuged, and the proteins in the supernatant were resolved by SDS-PAGE and electroblotted onto a PVDF membrane. The blot was probed with anti-PARP or anti-caspase-3 followed by a corresponding POD-tagged secondary antibody

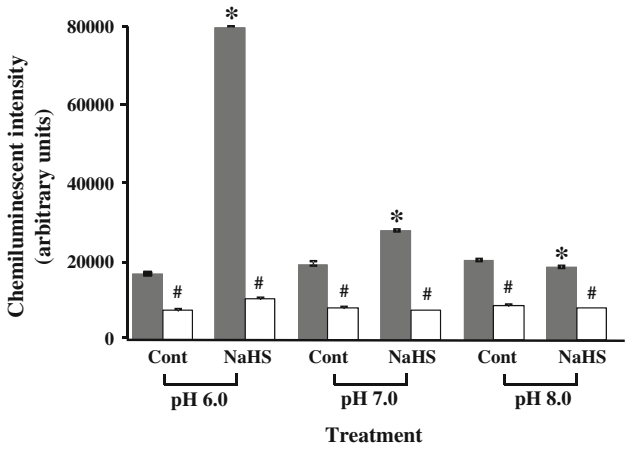

Fig. 5 Changes in caspase-3/7 activity in sulfide-exposed Jurkat cells. Jurkat cells were exposed to 0 (control) or 5 mM of NaHS in HBSS at pH 6.0, 7.0, or 8.0 for 1 h. After washing twice with PBS, cells were plated into a 96-well culture dish and cultured in fresh RPMI1640 complete medium in the presence (*open column*) or absence (*closed column*) of 2 μM of Z-VAD-fmk. After 6 h, caspase-3/7 activities were measured with a Caspase-Glo^{TM} 3/7 Assay Kit. Data are presented as means ± SEM ($N = 3$). *$p < 0.05$, compared to control at each pH; #$p < 0.05$, compared to the corresponding Z-VAD-fmk group

induced by 5 mM of NaHS at pH 6.0 (see supplemental Fig. 2).

Flow cytometric analyses of sulfide-exposed cells

Figure 6 shows that the proportion of cells in the upper left quadrant (annexin V positive and PI negative), which denotes early apoptotic cells, was increased about 17 % after exposure to NaHS. The proportion of NaHS-induced early apoptotic cells was decreased by treatment with Z-VAD-fmk. These results indicate that NaHS-induced cell death was caused by caspase-mediated apoptosis.

Effects of cytochalasin D or nocodazole on cell blebbing

As described above, cell blebbing occurred in NaHS-exposed cells at 6 h after exposure, as examined by microscopic examination (Fig. 3). It is known that membrane blebbing is dependent on intracellular force generated by the actin–myosin cytoskeleton (Croft et al. 2005). The depolymerization of microtubules is also reported to contribute to apoptotic events (Ndozangue-Touriguine et al. 2008). Thus, we further investigated the effects of actin filament-destabilizing or microtubule-destabilizing agents to examine the mechanisms of cell blebbing in NaHS-exposed cells. Microscopic examination revealed that cytochalasin D, but not nocodazole, suppressed cell blebbing induced by 5 mM of NaHS (Fig. 7). Neither cytochalasin D nor nocodazole alone without NaHS treatment changed cell morphology as observed by photomicroscopy (data not shown). Conversely, caspase-3/7 activity was not suppressed by these agents significantly (see supplemental Fig. 3). These results suggest that the disruption of actin filaments, but not microtubule structures, prevents cell blebbing without affecting caspase activity.

Cleavage of ROCK-1 and ROCK-2 in sulfide-exposed cells

To elucidate whether ROCK-1 and -2 are involved in the NaHS-induced apoptotic process, we examined the cleavage of ROCKs and the effects of Z-VAD-fmk by Western blotting. As shown in Fig. 8a, b, both ROCK-1 and ROCK-2 were cleaved by 5 mM of NaHS, respectively. The amount of cleaved ROCK-1 following exposure to 5 mM of NaHS decreased with Z-VAD-fmk treatment, however, not completely (Fig. 8a). Conversely, the amount of cleaved ROCK-2 was suppressed completely following Z-VAD-fmk treatment (Fig. 8b). The relative amount of cleaved ROCK-2 was lower than that of ROCK-1.

Effects of a ROCK inhibitor on cell blebbing

We examined the effects of Y-27632, a ROCK inhibitor, on cell blebbing. Microscopic examination revealed that Y-27632 suppressed cell blebbing induced by 5 mM of NaHS (Fig. 9). Conversely, caspase-3/7 activity was not affected by Y-27632 (see supplemental Fig. 5). These results indicate that this particular ROCK inhibitor prevents cell blebbing without affecting caspase activity.

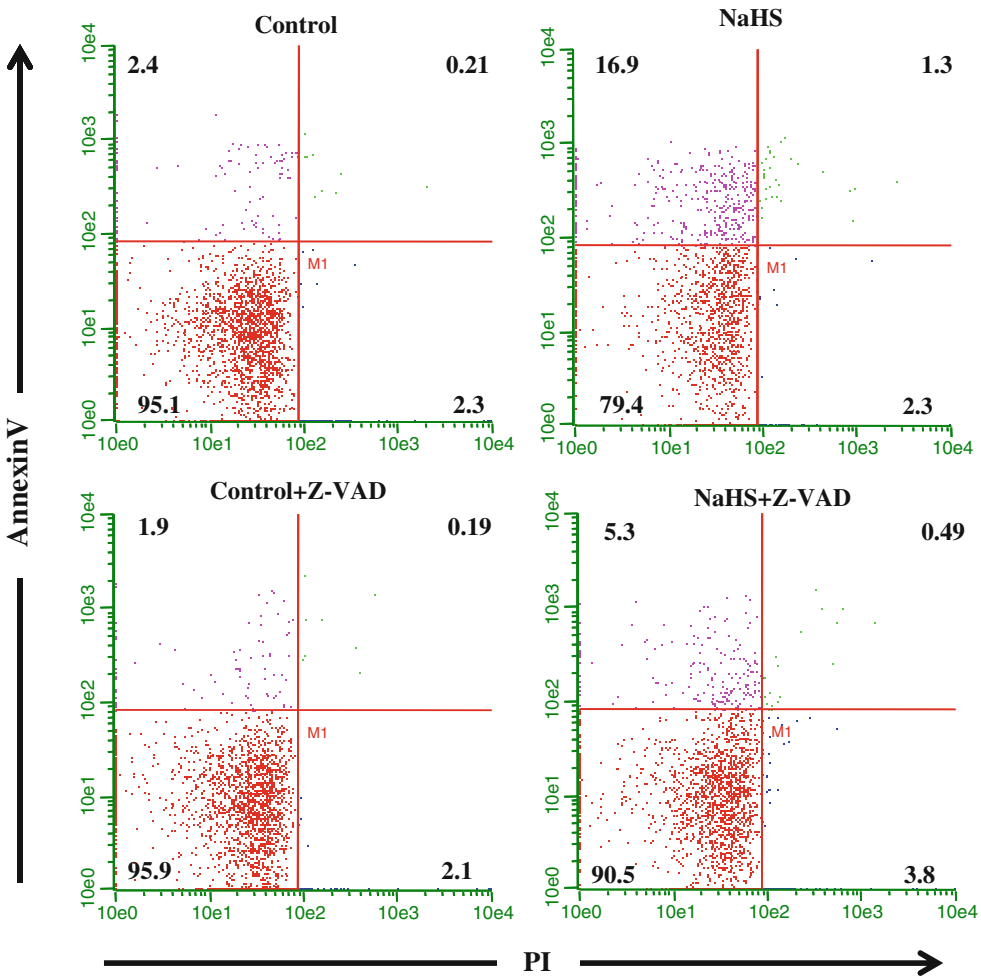

Fig. 6 Flow cytometric analyses of sulfide-exposed Jurkat cells. Jurkat cells were exposed to 0 (control) or 5 mM of NaHS in HBSS at pH 6.0 for 1 h. After washing twice with PBS, cells were plated into a culture dish and cultured in fresh RPMI1640 complete medium in the presence or absence of 2 μM of Z-VAD-fmk for 4 h. Cells were centrifuged, resuspended with binding buffer, stained with annexin V and PI for 5 min at room temperature in dark, and analyzed with a flow cytometer. The *number* inside each quadrant represents the percentage of intact (*lower left*, LL), apoptotic (*upper left*, UL), and necrotic (*lower* and *upper right*, LR and UR) cells

Discussion

H_2S is a harmful gas and is rapidly metabolized in mitochondria and converted to thiosulfate (Li and Moore 2008). When human body is accidentally inhaled H_2S, H_2S is absorbed by the upper respiratory tract mucosa (Hildebrandt and Grieshaber 2008). Therefore, bronchial epithelium is one of the targets affected by H_2S as a gaseous material directly. Next, inhaled and absorbed H_2S is partially dissolved H_2S within the circulation. On the other hand, sulfide is known to be present in normal human blood at concentrations of 10–100 μM (Richardson et al. 2000). H_2S dissociates to HS^- and S^{2-} depending on pH, and, therefore, we examined cytotoxicity of sulfide in Jurkat cells (Fig. 1) and also in BEAS-2B cells (supplemental Fig. 1) at different pHs. The cytotoxicity of sulfide was markedly enhanced with decrease in pH of the solution in both Jurkat and BEAS-2B cells. The pH-dependent remarkable change in cytotoxicity was not limited to sulfide. It was reported that the toxicity of sodium fluoride in macrophages was enhanced when the pH of the culture medium was decreased and the generation of hydrogen fluoride (HF) in acidic condition was responsible for the entry of fluoride into the cells (Hirano and Ando 1997). Thus, it is plausible that cytotoxicity of sulfide depends on entry of H_2S, which is generated in the culture medium at acidic condition, into the cells.

We, then, measured the intracellular concentrations of sulfide immediately after exposure using the HPLC-FLD method, as we speculated that cytotoxicity may be due to an intracellular accumulation of sulfide. The morphological changes in NaHS-exposed cells were not observed at this point (data not shown). Interestingly, the sulfide concentration in cells exposed to 5 mM of NaHS increased dramatically as the pH of the culture solution decreased (Fig. 2). The entry systems through cell membrane of H_2S are not well understood. It has been reported that H_2S is a highly lipophilic molecule and readily crosses the cell membrane by diffusion (Hughes et al. 2009). It is suggested that HS^- is protonated, and only the uncharged H_2S permeates the planar lipid membrane (Mathai et al. 2009). In physiological solution (pH 7.4), 18.5 % of sulfide is in the form of H_2S and 81.5 % is in the form of HS^- and more than 90 % of the sulfide is present as H_2S at pH 6.0,

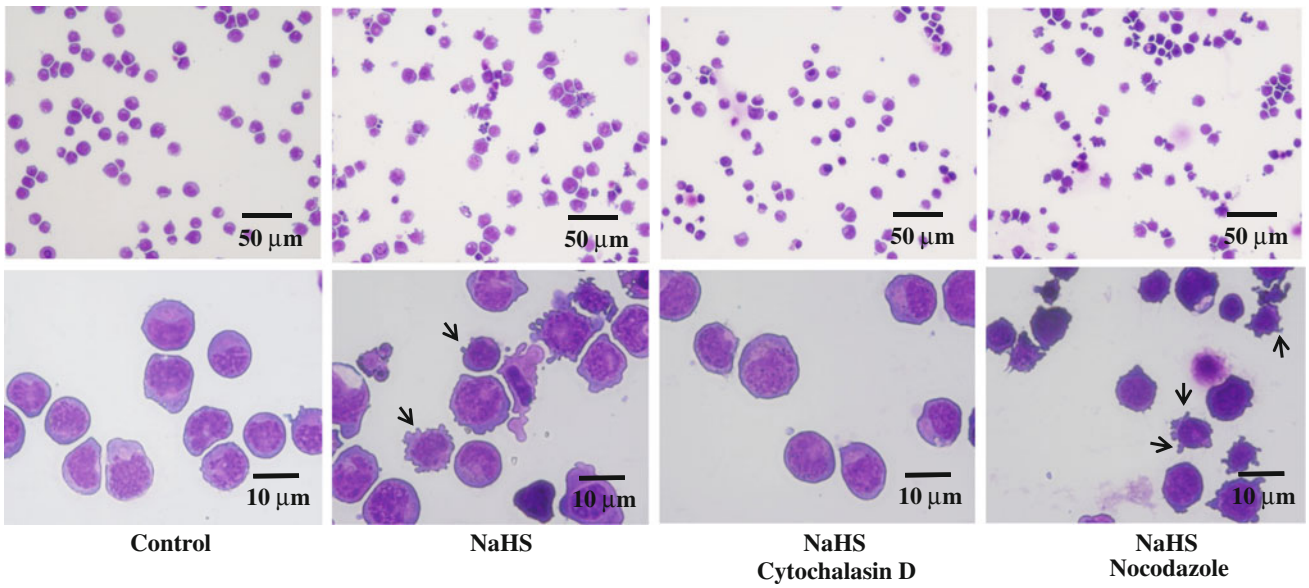

Fig. 7 Effects of cytochalasin D or nocodazole on morphological changes in sulfide-exposed Jurkat cells. Jurkat cells were exposed to 0 (control) or 5 mM of NaHS in the presence or absence of 1 μg/ml of cytochalasin D or 1 μg/ml of nocodazole in HBSS at pH 6.0 for 1 h. After washing twice with PBS, cells were plated into a culture dish and cultured in fresh RPMI1640 complete medium in the presence or absence of cytochalasin D or nocodazole. After 3 h of culture, the cell suspension was cytocentrifuged and stained with Diff-Quik®. The *lower panel* is a higher magnification of each *upper panel*. *Arrows* indicate plasma membrane blebbing

as predicted by the Handerson–Hasselbach equation (Dombkowski et al. 2004). At pH 6.0, the proportion of H_2S, which is permeable through the cell membrane, to less permeable HS^- and S^{2-} forms is higher compared to that at pHs 7.0–8.0. Therefore, the pH-dependent cytotoxicity in NaHS-exposed cells was probably caused by an increased uptake of sulfide at lower pH. Flow cytometric analyses revealed that pH-dependent cell death caused by NaHS was apoptotic rather than necrotic, if any (Fig. 6).

It has been reported that blood sulfide concentration obtained from victims who had died of acute exposure to H_2S was up to 0.995 ppm (Maebashi et al. 2011). It is plausible that the function of T lymphocytes is deteriorated after exposure to lethal level of H_2S.

In this study, we observed cell blebbing in cells treated with NaHS (Figs. 3, 7 and 9) and staurosporine (data not shown) by microscopic examination and flow cytometry. Jurkat cells have been used to investigate the release of microvesicles (MVs) and cell blebbing from during apoptosis induced by a variety of chemotherapeutic agents or protein kinase C inhibitors, such as staurosporine and 7-hydroxy staurosporine (Azuma et al. 2011; Ullal and Pisetsky 2010). The cell blebbing in NaHS-exposed cells suppressed following treatment with cytochalasin D, an actin filament-destabilizing agent, but not nocodazole, a microtubule-destabilizing agent (Fig. 7). Similar inhibitory effects on cell blebbing by these agents were observed in staurosporine-treated Jurkat cells (data not shown). On the other hand, caspase-3/7 activity and ROCK cleavage were not affected by either agent (supplemental Figs. 3 and 4). It has been reported that cytochalasin D blocks bleb formation, but not apoptosis, as the two processes do not occur in the same linear cascade (Huot et al. 1998), and disruption of only actin filament, but not microtubule structure, prevents membrane blebbing or apoptotic nuclear breakdown (Croft et al. 2005). Our results are consistent with those of previous reports. Only actin filaments, but not microtubule structure, were involved in blebbing without affecting caspase activity and ROCK cleavage.

One of the most interesting findings in the present study is that both ROCK-1 and -2 were cleaved to generate a truncated active form during the apoptotic process in 5-mM NaHS-exposed cells (Fig. 8a, b). To our knowledge, this is the first report to describe both ROCK-1 and -2 cleavage are involved in apoptosis via caspases, with the exception of ouabain-induced apoptosis (Ark et al. 2010). ROCK-1 is cleaved to generate active form by activated caspase-3 during apoptosis (Sebbagh et al. 2001). It has been shown that caspase-3-cleaved ROCK-1 induces membrane blebbing during apoptosis (Coleman et al. 2001; Sebbagh et al. 2005). In this study, ROCK-1 cleavage induced by NaHS was suppressed by Z-VAD-fmk, a pan-caspase inhibitor (Fig. 8a), indicating that ROCK-1 caused cell blebbing following activation by caspases. On the other hand, the cleavage of ROCK-2 induced by NaHS was completely suppressed by Z-VAD-fmk (Fig. 8b). ROCK-2 has been reported to be cleaved primarily by either granzyme B (Sebbagh et al. 2005), which is a serine protease derived

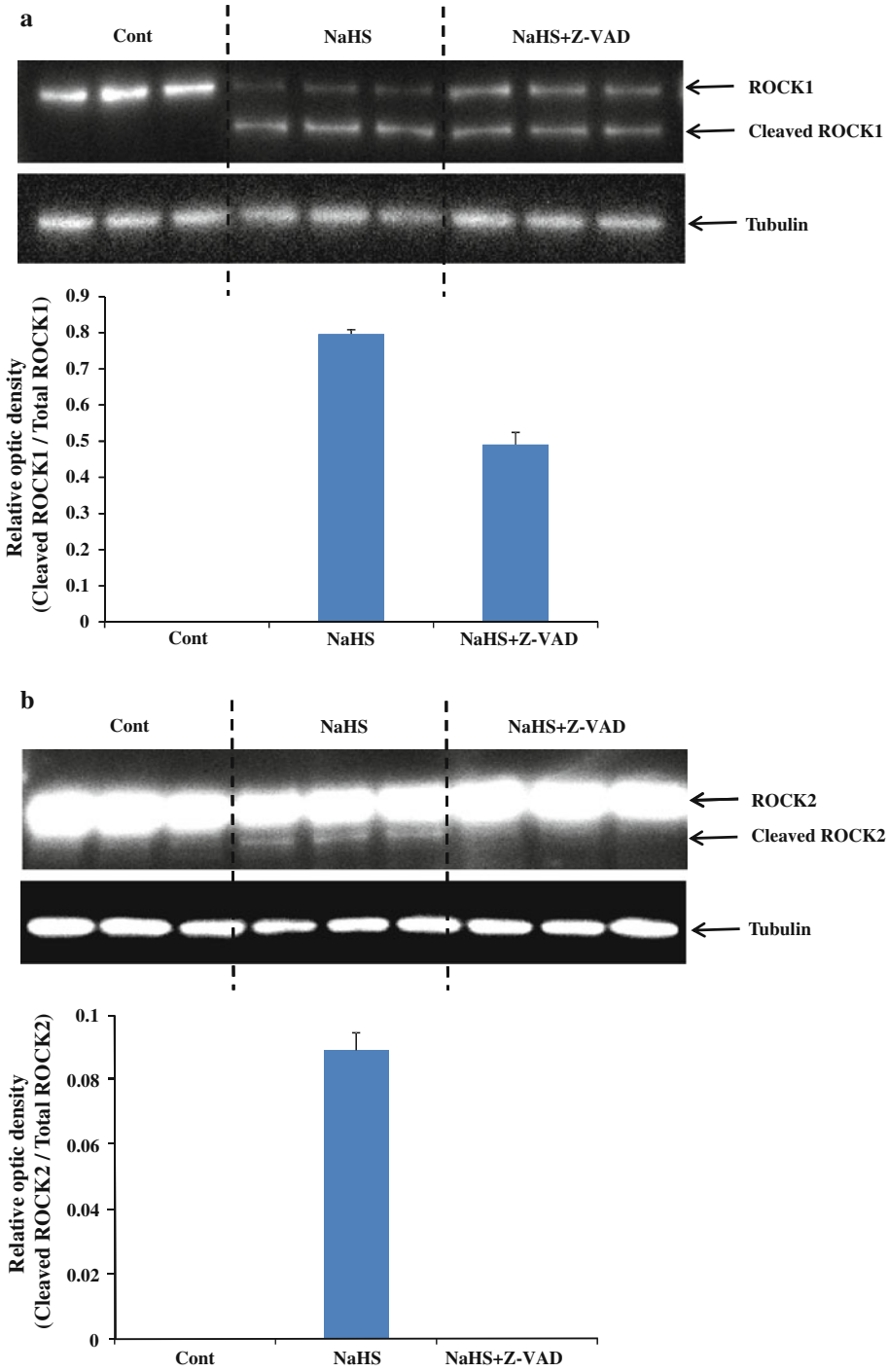

Fig. 8 Western blot analyses for the detection of cleavage of ROCK-1 (**a**) and ROCK-2 (**b**) in sulfide-exposed Jurkat cells. Jurkat cells were exposed to 5 mM of NaHS in HBSS at pH 6.0 for 1 h in the presence or absence of 2 μM of Z-VAD-fmk. After washing twice with PBS, cells were plated into a 6-well culture dish and cultured in fresh RPMI1640 complete medium in the presence or absence of Z-VAD-fmk for 4 h. The lysate was centrifuged, and proteins in the supernatant were resolved by SDS-PAGE and electroblotted onto a PVDF membrane. The blot was probed with anti-ROCK-1 or anti-ROCK-2 followed by a corresponding POD-tagged secondary antibody. The membranes were stripped and reprobed with POD-tagged anti-tubulin antibody. The intensity of the bands was quantified with chemiluminescence densitometry

from cytotoxic T lymphocytes, or caspase-2 (Ark et al. 2010). Our current results indicate that ROCK-2 may be cleaved by caspases in NaHS-exposed cells. ROCK-1 and ROCK-2 have been proposed to perform the same functions that induce membrane blebbing and chromatin condensation (Sebbagh et al. 2005). However, ezrin–radixin–moesin phosphorylation mediated by ROCK-1, but not ROCK-2, is involved in anti-Fas-induced apoptosis in Jurkat cells (Hebert et al. 2008). The activation of ROCK-2 by cleavage is a prerequisite for the generation of MVs, which are small cell fragments released from membrane blebs during apoptosis, in response to thrombin (Sapet et al. 2006). Though further studies are needed to understand the functions of each ROCK in sulfide-induced apoptosis, ROCKs may have sulfide-specific physiological roles involving cell blebbing. In this study, Z-VAD-fmk suppressed caspase-3/7 activity (Fig. 5) and the cleavage of ROCK-1 and ROCK-2 (Fig. 8a, b), and accordingly,

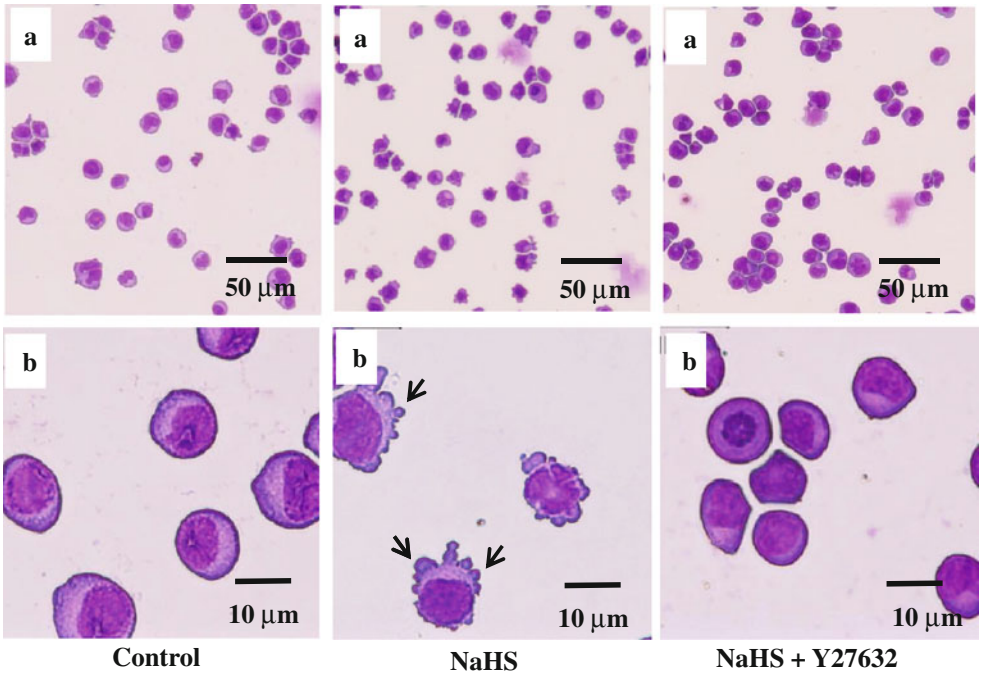

Fig. 9 Effects of ROCK inhibitor on cell morphological changes in sulfide-exposed Jurkat cells. Jurkat cells were pre-cultured with or without 20 μM of Y-27632 for 2 h. Cells were exposed to 5 mM of NaHS in HBSS at pH 6.0 in the presence or absence of Y-27632 for 1 h. After washing twice with PBS, cells were plated into a 24-well culture dish and cultured in fresh RPMI1640 complete medium in the presence or absence of Y-27632. After 3 h of culture, the cell suspension was cytocentrifuged and stained with Diff-Quik®. The *lower panel* (**b**) is a higher magnification of each *upper panel* (**a**). *Arrows* indicate plasma membrane blebbing

suppressed cell blebbing (data not shown). Conversely, Y-27632, a ROCK inhibitor, suppressed cell blebbing (Fig. 9) without affecting caspase-3/7 activity (supplemental fig. 5). These results indicate that sulfide-induced cell blebbing requires ROCK activity (Fig. 10).

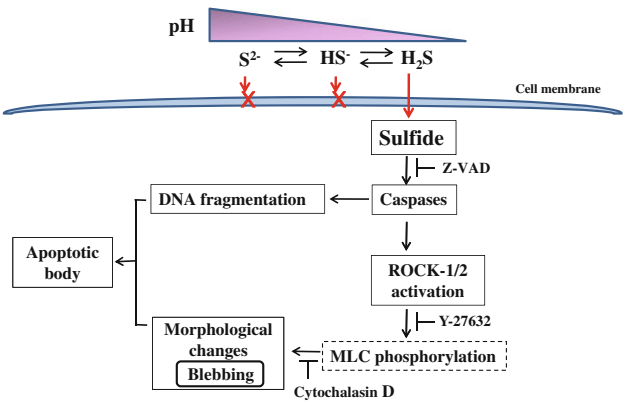

Fig. 10 Schematic representation of the sulfide-induced apoptotic pathway in Jurkat cells. A larger amount of sulfide is accumulated at lower pH. Intracellular sulfide triggers caspase-dependent apoptosis. Activated caspases induce cell blebbing through both ROCK-1 and ROCK-2 cleavages and DNA fragmentation. The sulfide-induced cell blebbing was suppressed by a ROCK inhibitor, indicating that sulfide-induced cell blebbing requires ROCK activity

Sulfide-induced caspase-3/7 activity was completely blocked by Z-VDVAD-fmk, a caspase-2 inhibitor (supplemental Fig. 2), suggesting that caspase-2 acts upstream of caspase-3/7 in the apoptotic pathway. In a recent study, caspase-2 was implicated in the release of cytochrome *c* from mitochondria (Guo et al. 2002). It is reported that NaHS induces the release of cytochrome *c* from mitochondria via the apoptotic pathway in human lung fibroblast cells (Baskar et al. 2007). These previous reports and our current results indicate that the activation of caspase-2 might result in a similar apoptotic pathway in NaHS-exposed Jurkat cells.

This study was designed to examine which species of sulfide are permeable to transport cell membrane and responsible for cell injury. The pH adjustments of NaHS-containing buffered solution to pH 6.0, 7.0 or 8.0 resulted in changing only proportion of sulfur species (H_2S, HS^-, or S^{2-}) without changing total amount of sulfide. However, exposure of cells to an exact amount of sulfide would not be completed using a conventional in vitro culture system because H_2S is easy to escape from culture solution. Thus, we briefly exposed cells to NaHS-containing buffered solution in a capped tube to prevent the loss of H_2S and adjust the total amount of sulfur species (H_2S, HS^-, or S^{2-}) in the exposure solution. The measurement of intracellular sulfide concentrations revealed pH-dependent cytotoxicity

correlated with the proportion of H_2S in culture solution. High concentration of H_2S uptaken into the cells at acidic condition caused apoptosis in Jurkat cells, whereas low concentration of H_2S did not affect cytotoxicity.

In summary, we demonstrate that the intracellular accumulation of sulfide was caused by a penetration of H_2S through the cell membrane in a pH-dependent manner, and sulfide-induced cytotoxicity is due to caspase-dependent apoptosis. Furthermore, we revealed that sulfide induces both ROCK-1 and ROCK-2 cleavage and causes ROCK-dependent cell blebbing during apoptosis.

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Conflict of interest The authors declare no conflict of interest.

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