Opposing effects of polysulfides and thioredoxin on apoptosis through caspase persulfidation

Received for publication, December 26, 2019, and in revised form, February 6, 2020. Published, Papers in Press, February 10, 2020, DOI 10.1074/jbc.RA119.012357

Ilana Braunstein†1, Rotem Engelman‡1, Ofer Yitzhaki‡, Tamar Ziv§, Erwan Galardon¶, and Moran Benhar†2
From the †Department of Biochemistry, Rappaport Institute for Research in the Medical Sciences, Faculty of Medicine, Technion Israel Institute of Technology, Haifa, 3200003 Israel, ‡Smoler Proteomics Center and Faculty of Biology, Technion Israel Institute of Technology, Haifa, 31096 Israel, and §UMR 8601, CNRS, Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France
Edited by F. Peter Guengerich

Hydrogen sulfide has been implicated in a large number of physiological processes including cell survival and death, encouraging research into its mechanisms of action and therapeutic potential. Results from recent studies suggest that the cellular effects of hydrogen sulfide are mediated in part by sulfane sulfur species, including persulfides and polysulfides. In the present study, we investigated the apoptosis-modulating effects of polysulfides, especially on the caspase cascade, which mediates the intrinsic apoptotic pathway. Biochemical analyses revealed that organic or synthetic polysulfides strongly and rapidly inhibit the enzymatic activity of caspase-3, a major effector protease in apoptosis. We attributed the caspase-3 inhibition to persulfidation of its catalytic cysteine. In apoptotically stimulated HeLa cells, short-term exposure to polysulfides triggered the persulfidation and deactivation of cleaved caspase-3. These effects were antagonized by the thioredoxin/thioredoxin reductase system (Trx/TrxR). Trx/TrxR restored the activity of polysulfide-inactivated caspase-3 in vitro, and TrxR inhibition potentiated polysulfide-mediated suppression of caspase-3 activity in situ. We further found that under conditions of low TrxR activity, early cell exposure to polysulfides leads to enhanced persulfidation of initiator caspase-9 and decreases apoptosis. Notably, we show that the proenzymes procaspase-3 and -9 are basally persulfidated in resting (unstimulated) cells and become depersulfidated during their processing and activation. Inhibition of TrxR attenuated the depersulfidation and activation of caspase-9. Taken together, our results reveal that polysulfides target the caspase-9 cascade and thereby suppress cancer cell apoptosis, and highlight the role of Trx/TrxR-mediated depersulfidation in enabling caspase activation.

Research conducted over the last two decades has established that hydrogen sulfide (H₂S) acts as a signaling molecule that regulates various physiological and pathological responses (1–4). Endogenous H₂S is synthesized by cystathionine β-synthase and cystathionine γ-lyase and cystathionine β-synthase in the transsulfuration pathway and by 3-mercaptopyruvate sulfurtransferase involved in cysteine catabolism. H₂S can be converted via enzymatic or non-enzymatic processes to different reactive sulfur species (RSS), including sulfane sulfur species such as persulfides and polysulfides. These sulfur chemotypes exhibit rich chemistry and biological activities, including antioxidant, anti-inflammatory, and antiangiogenic properties. Additionally, H₂S has been implicated in various physiological and pathological processes, including modulation of cell survival and death, modulation of transcription factors, and metabolic enzymes (15–18).

This work was supported by Israel Science Foundation Grant 15714/14 (to M. B.) and Allen and Jewell Prince Center for Neurodegenerative Diseases of the Brain Grant 86607114 (to M. B.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Fig. S1 and Table S1.

The MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017144.

† These authors contributed equally to this work.
‡ To whom correspondence should be addressed. Tel.: 972-4-8295376; Fax: 972-4-8295412; E-mail: benhar@technion.ac.il.
§ UMR 8601, CNRS, Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France

Opposing effects of polysulfides and thioredoxin on apoptosis through caspase persulfidation

...
However, how persulfidation may regulate the cellular apoptotic machinery remains poorly characterized. In addition, although recent studies have established that the thioredoxin/thioredoxin reductase (Trx/TrxR) system catalyzes persulfide reduction in cells (17, 18), whether and how RSS and the Trx system interact in the context of apoptosis regulation has not been investigated.

At present, it is not fully understood how H2S triggers Cys persulfidation in cells. H2S cannot directly react with Cys thiols because the sulfur atoms in both species have the same oxidation state (–2). Accordingly, de novo protein persulfidation is thought to occur via one of the following mechanisms: (i) the reaction of oxidized Cys derivatives with sulfide or (ii) sulfide oxidation products reacting with Cys thiols (19). Regarding the latter, polysulfides (R-S-Sn-S-R‘; n=1) are oxidation products of H2S and potential intermediates in persulfidation reactions. Polysulfides are abundant in some types of food such as garlic (20), can be detected in mammalian cells (21, 22), and have recently been implicated in cellular regulation (23–26).

In the present study we found that polysulfides inhibited apoptosis signaling in cancer cells through a mechanism that involves inhibitory persulfidation of apoptotic caspases, caspase-3 and -9. In addition, we provide evidence that pro-caspase-3 and -9 are constitutively persulfidated in resting cells and undergo depersulfidation during their activation. Caspase persulfidation is antagonized by the Trx system. Overall, our findings suggest that the balance between persulfidation and Trx-mediated depersulfidation can determine caspase activities and resultant apoptotic cell death.

Results

Effects of polysulfides on caspase-3 activity in vitro

We initiated this study by assessing the effects of several polysulfide compounds as well as a persulfide donor on the activity of purified caspase-3. For this, recombinant human caspase-3 was first subjected to DTT reduction and then the reduced protein (1 µM) was incubated for 20 min at 37 °C with SNAP-P* (S-methoxycarbonyl penicillamine disulfide, a persulfide donor) (27); the organic polysulfide diallyl trisulfide (DATS); or the synthetic polysulfides Na2S4, Na2S4, or Na2S4. Thereafter, caspase-3 activity was measured using a fluorogenic assay with the synthetic substrate Ac-DEVD-AMC. As shown (Fig. 1A), caspase-3 activity was effectively inhibited by either SNAP-P* or the polysulfide donors, most potently by Na2S4 and Na2S4 (50% inhibition at 2 µM of either compound). Time course experiments performed at room temperature using 0.1 µM caspase-3 demonstrated rapid inactivation of the enzyme upon incubation with 0.5 or 1 µM Na2S4 (Fig. 1B). To ensure that residual polysulfide did not interfere with the caspase activity assay, we employed another assay in which caspase-3 was immobilized onto nickel-coated microplates, enabling the facile removal of excess reactants (see “Experimental procedures” for more details). This assay also showed that Na2S4 potently and dose-dependently inhibited caspase-3 activity (Fig. 1C).

Mass spectrometric analysis of caspase-3 persulfidation

In view of these results, we next performed mass spectrometric (MS) analysis to identify cysteine residues in caspase-3 that could be persulfidated. For this purpose, untreated, Na2S4-treated, or SNAP-P*-treated caspase-3 were derivatized by iodoacetamide and subjected to tryptic digestion, and the
resulting peptides were analyzed by LC-MS/MS (see “Experimental procedures”). Iodacetamide derivatization was performed to (i) quench thiol/persulfide reactions that may occur during sample processing and to (ii) generate carbamidomethylated persulfide derivatives (R-S-S-CH₂-CO-NH₂) to help differentiate persulfides from sulfinic acid (both causing very similar mass changes). The MS analysis resulted in the identification of persulfidated cysteines in the polysulfide- and persulfide-treated, but not in the untreated, caspase-3 (Table S1, A and B). In both the Na₂S₄ and SNAP-P* samples, the catalytic cysteine (Cys-163) was found to be the predominant site of persulfidation (Fig. 2, A and B; Table S1, A and B). Other, low-abundant modifications, were Cys-170 persulfide, Cys-264 persulfide, and Cys-163–Cys-170 disulfide (Tables S1, A and B).

Effects of polysulfides on cellular caspase-3 via persulfidation

We proceeded to examine possible effects of polysulfides on cellular caspase-3. For this purpose, we exposed HeLa cancer cells to the apoptosis inducer staurosporine (STS) in the presence or absence of Na₂S₄. We used an experimental design in which the cells were exposed to STS for 4 h with the polysulfide added for the final 1 h. Thereafter, the cells were lysed and caspase-3–like activity in cell lysates was measured. These experiments revealed that Na₂S₄ dose-dependently inhibited STS-mediated induction of caspase-3 activity (Fig. 3A). Similar to Na₂S₄, DATS also effectively inhibited STS-dependent caspase activation (Fig. 3A). During its activation, caspase-3 undergoes maturation from its unprocessed, proform, to its processed, cleaved form. It was possible that the polysulfides inhibited the processing of procaspase-3 or disrupted another upstream event. However, immunoblot analysis showed that Na₂S₄ or DATS did not block STS-induced cleavage of procaspase-3 (Fig. 3B), suggesting that the polysulfides directly inhibited the activity of the processed enzyme. Considering these observations, we sought to examine if cellular caspase-3 was persulfidated under these experimental conditions. For this purpose, we employed the biotin thiol assay (BTA) for protein persulfidation (28). BTA results indicated that cellular caspase-3 was indeed persulfidated (Fig. 3B). Notably, procaspase-3 was found to be basally (i.e. constitutively) persulfidated in resting HeLa cells (Fig. 3B). Conversely, cleaved caspase-3 (after STS stimulation) was barely persulfidated; however, its persulfidation was markedly increased in cells exposed to Na₂S₄ or DATS (Fig. 3B). Together, these cellular data suggest that polysulfides trigger the persulfidation of cleaved caspase-3, causing its deactivation. In addition, these results raise the intriguing possibility that endogenous persulfidation of procaspase-3 is involved in the regulation of this protease.

We then examined the effects of Na₂S₄ on other cell types or apoptotic stimuli. Similar to our findings in HeLa cells, we observed that Na₂S₄ inhibited caspase-3 activity in STS-treated THP-1 monocytic cells (Fig. 3C). Whereas STS activates the intrinsic apoptotic pathway, members of the TNF family of cytokines such as TNF and TRAIL trigger the extrinsic apoptotic pathway (7). We found that Na₂S₄ effectively suppressed TRAIL-induced caspase-3 activity in HCT116 colon cancer cells (Fig. 3D). Thus, polysulfides can counteract caspase-3 activation in different cell types, and in response to different apoptotic insults.

Co-regulation caspase-3 by persulfidation and thioredoxin

The extent of protein persulfidation reflects the balance between persulfidation and depersulfidation processes. It is known that the Trx/TrxR system mediates caspase-3 denitrosylation (29) and growing evidence suggests that Trx/TrxR acts as a persulfide reductase (17, 18). Employing our in vitro assay with immobilized caspase-3 (as described above in Fig. 1C and depicted in Fig. 4A) we tested if Trx/TrxR could restore the activity of Na₂S₄-treated caspase-3. The results showed that a 30 min incubation with Trx/TrxR and NADPH resulted in near-complete recovery of caspase-3 activity, an effect almost equal to that achieved by the strong chemical reductant DTT and slightly better compared with the biologically relevant reductant GSH (Fig. 4B).

Next, to examine the role of the Trx system in regulating persulfidation in cells we employed auranofin (AUR), a highly potent and selective TrxR inhibitor (30). We found that AUR markedly potentiated the inhibitory effects elicited by Na₂S₄. Indeed, whereas treatment with 25 μM Na₂S₄ alone failed to inhibit caspase-3, in the presence of AUR ~75% inhibition was observed (Fig. 4C). Immunoblot analysis showed that cleavage of procaspase-3 was not inhibited by Na₂S₄ in the presence of AUR (Fig. 4C), further arguing for a direct effect on the cleaved enzyme. We extended these findings by using Tri-1, a recently described potent and specific inhibitor of TrxR1 (31). We found that Tri-1, which has a different chemical structure and a different mechanism of action compared with AUR (31), also augmented Na₂S₄-mediated inhibition of caspase-3 (Fig. 4S), thus corroborating the role of TrxR1 in these effects. We then monitored the cleavage of a well-established caspase-3 substrate, inhibitor of caspase-activated DNase (ICAD). After its cleavage by caspase-3 the cleaved ICAD loses its affinity for CAD, and CAD is thereby released from ICAD and digests the chromosomal DNA (7). Despite the above-noted effects on caspase-3 activity, STS-dependent cleavage of ICAD was not altered in cells exposed to AUR and Na₂S₄. This lack of effect on ICAD processing is very likely because the polysulfide was added 3 h after STS stimulation, a time point where the key apoptotic processes (including ICAD cleavage) are well under way.

Regulation of apoptosis signaling via reversible persulfidation of caspase-9

Based on the above observations, we next sought to investigate cellular effects induced by polysulfide exposure that precedes or coincides with the initiation of apoptosis. Accordingly, cells were exposed simultaneously to STS and different combinations of Na₂S₄ and AUR. As shown in Fig. 4D, in the absence of AUR, an early exposure to Na₂S₄ did not affect STS-induced caspase-3 activation. In contrast, in the presence of AUR, Na₂S₄ treatment effectively and dose-dependently inhibited caspase activation. Importantly, in this setting, the cleavage of procaspase-3 was also attenuated (Fig. 4D). These observations suggest that under conditions of suppressed Trx/TrxR activity, early administration of Na₂S₄ disrupts the apoptotic signaling upstream of caspase-3. Of note, analysis of ICAD processing
Figure 2. LC-MS/MS analysis of untreated and Na$_2$S$_4$-treated caspase-3. Reduced caspase-3 (1 μM) was incubated with or without Na$_2$S$_4$ (1 μM) for 15 min at 37 °C and then alkylated with 10 mM iodoacetamide for 2 h at room temperature. Thereafter, the enzyme was digested with trypsin and the resulting peptides were analyzed by LC-MS/MS (see “Experimental procedures” for more details). A, extracted ion chromatogram showing specific peaks (m/z = 526.77, retention time (RT) 35.72 min) detected in Na$_2$S$_4$-treated but not in the untreated caspase-3. B, MS/MS spectra of the specific peak shown in A, which was found to be doubly charged LFIIQAC(sulfide)R peptide. NL, normalized intensity level (counts per second). The figure displays the main fragmentation series, y (C-terminal series) and b (N-terminal series) and related species obtained by collision-induced fragmentation of the peptide backbones. CAM indicates carbamidomethylation by iodoacetamide.
showed that AUR/Na$_2$S$_4$ co-treatment led to decreased ICAD cleavage, indicating a weaker apoptotic response (Fig. 4D).

In light of these observations, we postulated that AUR/Na$_2$S$_4$ treatment may disrupt the activity of caspase-9, the initiator caspase that cleaves and activates procaspase-3 during intrinsic apoptosis (7). Supporting this notion, we observed that Na$_2$S$_4$ dose-dependently inhibited caspase-9 activity in apoptotic cell lysates, which was quantitatively similar to the effect toward proenzyme cleavage (Fig. 5A). Next, we found that although cell treatment with Na$_2$S$_4$ alone had very little effect on STS-induced caspase-9 activity, in the presence of AUR, Na$_2$S$_4$ effectively inhibited the enzyme (Fig. 5B). Immunoblot analysis showed that treatment with AUR/Na$_2$S$_4$ (but not Na$_2$S$_4$ alone) partially inhibited the cleavage of procaspase-9 (Fig. 5C); however, the inhibitory effect on caspase-9 activity was far greater than the effect on proenzyme cleavage (Fig. 5, B and C). For example, treatment with 250 μM Na$_2$S$_4$ (in the presence of AUR) led to >90% inhibition of caspase-9 activity as compared with ~50% decrease in procaspase processing. These data suggested the possibility that caspase-9 persulfidation could be involved in these effects. We therefore performed BTA analysis, the results of which provided support and further insight about this hypothesis. Specifically, the data showed that procaspase-9 was constitutively persulfidated in resting cells whereas after STS stimulation the cleaved caspase-9 was mostly nonpersulfidated (Fig. 5C, bottom blot, compare lanes 1 and 2). Notably, treatment with AUR alone attenuated STS-induced depersulfidation of cleaved caspase-9 (Fig. 5C, compare lanes 2 and 5). This result combined with the activity data (Fig. 5B) suggests that Trx/TrxR mediated stimulus-coupled depersulfidation of caspase-9, promoting its activation. In addition, AUR enhanced Na$_2$S$_4$-dependent persulfidation of cleaved caspase-9 (Fig. 5C, compare lanes 3 and 6).

The partial inhibition of procaspase-9 processing noted above (Fig. 5C) prompted us to assess the possible effect of Na$_2$S$_4$/AUR on release of Cyt c from mitochondria, a process required for apoptosome formation and activation of procaspase-9 (7). The results showed that STS-induced release of Cyt c to the cytosol was largely intact (albeit slightly reduced) when comparing AUR- or AUR/Na$_2$S$_4$-treated cells to their
respective (minus AUR) controls (Fig. 5D). Taken together, the data thus suggest that AUR/Na₂S₄ treatment targets the apoptotic cascade mainly at the level of caspase-9, which in turn results in diminished activation of caspase-3 and cleavage of ICAD. To further assess the functional relevance of these findings, we measured DNA fragmentation, a hallmark of apoptotic cell death. Consistent with our findings regarding the activities of caspase-9 and -3 and ICAD cleavage, we found that, AUR/Na₂S₄ co-treatment resulted in decreased DNA fragmentation, indicative of attenuated apoptosis (Fig. 5E).

Mammalian TrxR enzymes are susceptible to inactivation by various oxidants (32), yet it is unknown if their activity is modulated by polysulfides. To address this issue, we measured the enzymatic activity of cellular TrxR under different treatment combinations. As expected, we found that AUR treatment substantially inhibited TrxR activity. Of note, cell treatment with Na₂S₄ did not affect TrxR activity; however, the polysulfide markedly potentiated the inhibitory effect of AUR (Fig. 5F). Specifically, whereas AUR treatment resulted in ~50% inhibitory effect on TrxR activity, ~90% inhibition was seen in cells exposed to AUR plus Na₂S₄ (Fig. 5F). In conjunction with the previous results, these new data highlight a correlation between the extent of apoptosis and the level of TrxR activity, supporting the notion that a functional Trx/TrxR system is required for efficient transmission of the apoptotic signal.

The last set of experiments pointed to caspase-9 as an important target of polysulfide action. To further examine how polysulfides affect caspase-9 we employed a well-established cell-free assay for Cyt c–dependent activation of the caspase-9 and -3 cascade (33). The results showed that Na₂S₄ effectively...
Regulation of apoptotic caspases by thiol persulfidation

Figure 5. Inhibition of TrxR augments polysulfide-mediated suppression of caspase-9 activity and apoptosis. A, apoptotic cell lysates were prepared from HeLa cells treated with STS for 3 h. Then, equal amount of lysates (200 μg) were incubated for 30 min at 37 °C with the indicated concentrations of Na2S4 followed by determination of caspase-3 and -9 activities. *, p < 0.05; **, p < 0.01 versus vehicle control. B, HeLa cells were simultaneously treated for 4 h with AUR, STS, and Na2S4 as indicated. Thereafter, caspase-9 activity in cell lysates was determined. *, p < 0.05; **, p < 0.01 versus STS alone. C, cells were simultaneously treated with (+) or without (-) AUR (2 μM), STS (1 μM), and Na2S4 (250 μM), followed by determination of pro- and cleaved-caspase-9 persulfidation using the BTA. D, cells were treated with (+) or without (-) AUR/STS/Na2S4 as in C. Thereafter, the presence of cytochrome c (Cyt c) in cytosolic fractions was determined by immunoblotting and TrxR1 served as loading control. E, cells were treated as in C followed by analysis of DNA fragmentation. F, cells were treated as in C followed by determination of TrxR activity. **, p < 0.01 versus vehicle control unless noted. G, cytosolic extracts from THP-1 cells were treated with Cyt c and dATP in the presence of the indicated concentrations of Na2S4 followed by assessment of caspase-9 activity and cleavage. *, p < 0.05; **, p < 0.01 versus Cyt c/dATP alone. Data in the graphs represent mean ± S.D. (n = 3).

Discussion

A large body of evidence has shown that H2S serves as a signaling molecule in various cellular processes and cell types (1–4). Recently, there has been growing interest in sulfane sulfur species, including polysulfides, as possible mediators of H2S signaling (3, 4, 36). There is accumulating evidence for the presence and effects of polysulfides in various cell types, including cancer cells, provoking a range of mechanistic and analytical investigations (21, 23–26). Nonetheless, the role of endogenous polysulfides in cellular signaling remains a subject of considerable debate and ongoing investigation (15). Alongside this, there is also increased interest in the application of exogenous polysulfides for modulating cellular processes and pathophysiological responses. For example, a recent study demonstrated that administration of exogenous polysulfides inhibits Toll-like receptor signaling in macrophages and reduces endotoxic shock in mice (37). These advances highlight the need to further characterize the effects of polysulfides on cellular physiology and elucidate the underlying mechanisms. In the present study, we revealed that polysulfides suppress apoptosis signaling in cancer cells via inhibitory persulfidation of apoptotic caspases.

Redox regulation of mammalian caspases has been the subject of previous investigations. Among caspases, the redox control of caspase-3, an enzyme that bears a highly nucleophilic thiol at its active site, has been mostly studied. It was discovered that either S-nitrosylation or S-glutathionylation negatively regulates caspase-3 activation (38, 39). Studies in lymphocytes have shown that cytosolic procaspase-3 is non-nitrosylated under basal steady-state conditions, whereas mitochondria-associated procaspase-3 is constitutively nitrosylated (40). It was further established that denitrosylation and activation of mitochondrial procaspase-3 is triggered by certain apoptotic stimuli such as Fas, and that Trx proteins mediate both basal and stimulus-induced denitrosylation (29, 40). Notably, in the present study, we found that procaspases-3 and -9 (which are mostly localized in the cytoplasm) are basally persulfidated in unstimulated HeLa cells, and that upon apoptotic stimulation they undergo depersulfidation. Furthermore, during...
Regulation of apoptotic caspases by thiol persulfidation

apoptosis, TrxR inhibition attenuated caspase depersulfidation and activation. These results support the notion that reversible persulfidation regulates the activation of cytosolic caspase-9 and -3. Moreover, to our knowledge, these findings provide the first demonstration of stimulus-coupled protein depersulfidation. In addition, we showed that cell exposure to exogenous polysulfide promotes the persulfidation of cleaved caspase-3 and -9, blocking protease activities.

We determined that polysulfides very potently inhibited caspases under cell-free conditions, with effects observed in the low micromolar range. Higher concentrations of polysulfides were needed to effectively inhibit caspases in intact cells, likely reflecting both the presence of competing targets and the robust activities of thiol/persulfide reducing systems. Indeed, we demonstrated that inhibition of the cellular Trx system greatly sensitized caspases to inactivation by polysulfides. Collectively, our results indicate that the effects of polysulfides on caspases depend upon both the timing of cell exposure to the polysulfide and the activity of the Trx system. Effective blockade of the apoptotic process occurred upon early administration of polysulfides (i.e., concurrently with application of the apoptotic stimulus) and under conditions of low TrxR activity. In this setting, caspase-9 was markedly persulfidated and repressed, resulting in diminished activation of caspase-3 and downstream molecules. It is noteworthy that, even without addition of exogenous polysulfide, TrxR inhibition had functional consequences, evident by the attenuation of caspase-9 depersulfidation and activation after apoptotic stimulation (Fig. 5, B and C). These observations lend support to the idea that Trx/TrxR-mediated depersulfidation plays a regulatory role in caspase-9 activation. More generally, the current results expand the emerging concept that the Trx system plays an important role in sulfide homeostasis and signaling (15, 17, 18).

The cellular mechanism that mediates endogenous persulfidation of procaspases is currently unknown. More generally, fundamental mechanistic questions remain unresolved regarding the formation and targeting of persulfides in cells (15, 23, 24). In this regard, a recent study suggested a model whereby H₂S oxidation by Cyt c may facilitate protein persulfidation, with procaspase-9 being studied as a potential target protein (41). It was reported that cell treatment with the H₂S donor GYY4137 caused elevated persulfidation and decreased activation of procaspase-9 (41). However, the functional downstream consequences of procaspase-9 persulfidation were not analyzed, nor was the mechanism of depersulfidation addressed. The present study, by revealing dynamic caspase persulfidation and its regulation by Trx, significantly extends upon previous observations. Of note, our cell-based and cell-free assays indicate that endogenous and exogenous RSS have distinct effects, targeting the inactive procaspase precursors and the active (processed) enzymes, respectively. Additional research is necessary to elucidate the cellular mechanism mediating persulfidation of procaspases-9 and -3 and address the question of persulfide targeting and turnover. Our findings nonetheless reveal rapid turnover of persulfides which allows dynamic regulation of caspase signaling. They further indicate decreased stability and faster turnover of persulfide on cleaved caspases as compared with the proenzymes. On the basis of the present data, we can speculate that structural changes that occur upon the proteolytic maturation of caspase-3 and -9 render the active site thiol more accessible to react with polysulfides on the one hand and Trx on the other hand. This notion is consistent with structural analyses, which have shown that caspase processing involves significant rearrangements in the active site and its surroundings, rendering the catalytic cysteine more exposed to the solvent (42). Altogether, our observations may help guide future experimental approaches to manipulate caspases using polysulfides or related compounds.

In conclusion, our findings show that polysulfides exert an antiapoptotic effect through persulfidation of components of the core apoptotic machinery, namely, caspase-3 and -9. The results further reveal that persulfidation and Trx antagonistically regulate apoptotic signaling in cancer cells. Altogether, the new findings advance a mechanistic understanding of sulfide-based regulation of cellular apoptosis. We should emphasize that the present studies do not exclude the possibility that the effects of H₂S/RSS on apoptosis involve other targets and mechanisms. Indeed, recent proteomic studies indicate that a large number of cellular proteins are susceptible to persulfidation (28, 43, 44). Hence, additional roles of persulfidation in apoptotic signaling are likely to be discovered in the future. Further elucidation of the mechanisms by which H₂S/RSS regulate apoptosis and other cell death pathways may ultimately enable the development of new therapeutic approaches for cancer treatment.

Experimental procedures

Antibodies and reagents

The following antibodies were used throughout this study. Anti-caspase-3 (catalogue no. 9662) was from Cell Signaling Technology. Anti-caspase-9 (MAB8301) was from R&D Systems. Anti-ICAD (sc-17816) was from Santa Cruz Biotechnology. Anti Cyt c (556432) was from BD Biosciences. Anti TrxR (ab16840) was from Abcam. Auranofin (BML-EI206-0100) was from Enzo Life Sciences. Synthetic polysulfides (Na₂S₂, Na₂S₃, and Na₂S₄) were from Dojindo Laboratories. SNAP-P⁺ was synthesized as described previously (27). Stauroporine and fluorescent caspase substrates Ac-DEVD-AMC and Ac-LEHD-AFC were from Cayman Chemical Company. Recombinant human TRAIL (310-04) was from PeproTech. EZ-link-PEG-maleimide-biotin (21901BID) and streptavidin-agarose resin (20361) were from Thermo Scientific. cOmplete, EDTA-free protease inhibitor was from Roche. Tissue culture media and reagents were from Biological Industries (Beit Haemek, Israel). Recombinant rat TrxR1 (specific activity of 28 units/mg) and the TrxR1 inhibitor Tri-1 were kindly provided by Elias Arnér (Karolinska Institute, Stockholm, Sweden). Other materials were obtained from Sigma unless otherwise indicated.

Cell culture

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, and 1% sodium pyruvate. THP-1 and HCT116 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, and 1% sodium pyruvate. Cells were seeded in 6-well plates or
Regulation of apoptotic caspases by thiol persulfidation

10-cm dishes and grown in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Experiments were initiated 24 h after plating.

Preparation of recombinant proteins

Human caspase-3 was expressed in Escherichia coli strain BL21(DE3). The His₆-tagged protein was purified by affinity chromatography on nickel-nitrilotriacetic acid-agarose as described elsewhere (29). Briefly, bacterial lysates were prepared in buffer (50 mM Tris, 100 mM NaCl, pH 8.0) and incubated with nickel-nitrilotriacetic acid-agarose for 1 h. Caspase-3 was eluted with an imidazole linear gradient from 0–200 mM. Fractions containing caspase-3 were pooled and concentrated by ultrafiltration. For the experiments, the protein was reduced by incubation with 50 mM DTT for 30 min at room temperature followed by removal of excess reductant by gel filtration using Sephadex G-25 columns (GE Healthcare).

Human Trx was prepared as described previously (45). Briefly, E. coli cells expressing His-tagged Trx were cultured in 500 ml LB and induced by the addition of 0.5 mM isopropyl-β-d-1-thiogalactopyranoside for 6 h. Cells were harvested by centrifugation at 3000 rpm for 10 min at 4 °C, resuspended in 10 ml of extraction buffer (300 mM KCl, 50 mM KH₂PO₄, 5 mM imidazole, with protease inhibitors, pH 7.4) and disrupted by ultrasonication. After centrifugation at 12,000 × g for 30 min at 4 °C, the supernatant was applied to nickel-nitrilotriacetic acid beads overnight at 4 °C. The beads were washed with extraction buffer containing imidazole (10 mM) and finally the protein was eluted with 250 mM imidazole. Fractions were analyzed by SDS-PAGE, pooled and stored at −80 °C. Protein purity was at least 95% as judged by SDS-PAGE.

Assessment of the effects of polysulfides on caspase activities

The effects of polysulfides on recombinant caspase-3 were analyzed as follows. DTT-reduced caspase-3 (1 mM) was incubated at 37 °C for 20 min with persulfide/polysulfide donors as indicated in the legend to Fig. 1. The activity of caspase-3 (diluted to 5 mM) was determined using fluorogenic substrate Ac-DEVDA-AMC (100 μM) and quantified by fluorescent detection of free AMC with excitation at 360 nm and emission at 465 nm.

Assays using immobilized caspase-3 were performed as follows. Reduced caspase-3 (0.5 mM) was incubated in 96-well His-Grab plates (Pierce) in 5 μl buffer H (50 mM Hepes, 100 mM NaCl, and 0.1% CHAPS, pH 7.5) for 1 h at room temperature. Unbound material was removed by three washes with buffer H and the immobilized enzyme was incubated with Na₂S₄ (concentrations and durations as indicated in the legends). In some experiments, after sulfide treatment, samples were subjected to additional incubation with reductants. Each incubation step was followed by three washes with buffer H. Finally, caspase-3 activity was determined using fluorogenic substrate Ac-DEVDA-AMC (100 μM).

Measurements of caspase-3 and -9 activity in cell lysates were performed as follows. After treatments, cells were lysed in lysis buffer (20 mM Hepes, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 20 mM β-glycerophosphate, Complete EDTA-free protease inhibitors, pH 7.5). Lysates were centrifuged at 13,000 rpm for 20 min at 4 °C to remove insoluble material. Thereafter, activity was measured in buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 3 mM MgCl₂, pH 7.5) using 125 μg of lysate and 100 μM substrate (Ac-DEVDA-AMC for caspase-3 or Ac-LEHD-AFC for caspase-9).

Cell-free assay of Cyt c–induced caspase activation

The assay was performed using THP-1 cells according to Ref 46. Briefly, cells were harvested in PBS by centrifugation at 1500 rpm for 5 min at 4 °C. Cells were then incubated for 15 min in three volumes of ice-cold cell extract buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin), followed by homogenization using a B-pestle. Lysates were then centrifuged at 15,000 × g for 20 min to remove nuclei, mitochondria, and cellular debris. To analyze the effects of polysulfides on caspase activation, DTT was first removed by ultrafiltration using Amicon Ultra Filters (10 kDa, Millipore). Then, extracts (50 μg, 2 mg/ml) were incubated with bovine heart Cyt c (50 μg/ml) and dATP (1 mM) and with different concentration of Na₂S₄ for 60 min at 37 °C. Caspase activation was analyzed by immunoblotting or enzyme activity assay.

Analysis of modifications of caspase-3 by MS

Reduced caspase-3 (1 μM) was incubated with Na₂S₄ (1 or 2 μM) or with SNAP-P⁺ (50 or 100 μM) for 15 min at 37 °C. Thereafter, samples were subjected to alkylation with 10 mM iodoacetamide for 2 h at room temperature. The proteins in the samples were separated on a nonreducing SDS-PAGE gel. A gel slice corresponding to molecular weights from 10 to 50 kDa was excised and the proteins in the gel were digested in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:10 enzyme-to-substrate ratio, overnight at 37 °C. The resulting peptides were desalted using C18 tips (homemade stage tips) and then subjected to LC coupled with tandem MS (LC-MS/MS). The peptides were resolved by reverse-phase chromatography on 0.075 × 180-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr. Maisch GmbH, Germany). The peptides were then eluted over 30 min with a linear gradient of 5 to 28% acetonitrile with 0.1% formic acid in water, 15 min gradient of 28 to 95% acetonitrile with 0.1% formic acid in water, and 15 min at 95% acetonitrile with 0.1% formic acid in water, at a flow rate of 0.15 μl/min. The MS/MS analyses were performed with a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) in a positive mode using repetitively full MS scan followed by collision inducing dissociation of the 10 most dominant ions selected from the first MS scan. Raw data were subjected to the SEQUEST search algorithm using Proteome Discoverer Software version 1.4 (Thermo Fisher Scientific). Detected peptides were searched against human and E. coli UniProt databases as fully tryptic peptides and in addition against the human caspase-3 sequence as semi-tryptic peptides. All the analysis was performed with mass tolerance of 15 ppm for the precursor masses and 0.05 atomic mass units for the fragment ions. Oxidation of Met and several modifications of Cys (oxidation, dehydro, carbamidomethyl, carbamidomethylated persulfide, and propionamidation) were accepted as variable modifica-
Analysis of cytochrome c release from mitochondria

After treatments, cells were harvested, washed in PBS, resuspended in hypotonic lysis buffer (10 mM Heps, 3 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, pH 7.5), and disrupted by passage (five times) through a 25-gauge needle. Lysates were centrifuged at 600 × g for 10 min at 4 °C to remove nuclei and unbroken cells. Supernatants were then centrifuged at 15,000 × g for 30 min at 4 °C. Supernatants from this step were designated as cytosolic fractions and subjected to immunoblot analysis using anti-caspase-3 or anti-caspase-9 antibodies.

Analysis of DNA fragmentation

After treatments, cells were collected by centrifugation and washed with PBS and then lysed with lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). The lysates were incubated with proteinase K (0.1 μg/μl) for 2 h at 50 °C. Thereafter, the lysates were incubated with RNase (0.1 μg/μl) for an additional 2 h at 37 °C. After isopropanol precipitation, DNA was extracted and subjected to electrophoresis in a 1% (w/v) agarose gel in 40 mM Tris acetate buffer (pH 7.4) at 100 V. After electrophoresis, DNA was visualized by staining with ethidium bromide.

Determination of activity of cellular TrxR

TrxR activity in cell lysates was measured using the insulin reduction end point assay as detailed before (47). In brief, 25 μg of protein cell lysate was incubated in a final volume of 50 μl containing 0.3 mM insulin, 660 μM NADPH, 2.5 mM EDTA, and 5 μM human Trx1 in 85 mM Heps (pH 7.5) for 20 min at room temperature. Control reactions excluding Trx1 were used for background subtraction. Then, 250 μl of 1 mM DTNB, 240 μM NADPH, and 200 mM Tris-HCl, pH 8, in 6 M guanidine hydrochloride was added and the absorbance was determined at 412 nm.

Statistical analysis

All data are presented as the mean ± S.D. Statistical differences were analyzed by one-way analysis of variance followed by Tukey post-test using Prism software (GraphPad). A p value < 0.05 was considered statistically significant.
Regulation of apoptotic caspases by thiol persulfidation

15. Filipovic, M. R., Zivanovic, J., Alvarez, B., and Banerjee, R. (2018) Chemical biology of H2S signaling through persulfidation. Chem Rev 118, 1253–1337 CrossRef Medline

16. Sen, N., Paul, B. D., Gadalla, M. M., Mustafa, A. K., Sen, T., Xu, R., Kim, S., and Snyder, S. H. (2012) Hydrogen sulfide-linked sulphhydration of NF-κB mediates its antiapoptotic actions. Mol Cell 45, 13–24 CrossRef Medline

17. Dóka, É., Pader, I., Bér, A., Johannson, K., Cheng, Q., Ballagó, K., Prigge, J. R., Pastor-Flores, D., Dick, T. P., Schmidt, E. E., Arnér, E. S., and Nagy, P. (2016) A novel persulfide detection method reveals protein persulfide- and polysulfide-reducing functions of thioredoxin and glutathione systems. Sci Adv 2, e1500968 CrossRef Medline

18. Wedemann, R., Onderka, C., Wei, S., Szijártó, I. A., Miljkovic, J. L., Mitrovic, A., Lange, M., Savitsky, S., Yadav, P. K., Torregrossa, R., Harrer, E. G., Harrer, T., Ishii, I., Gollasch, M., Wood, M. E., et al. (2016) Improved tag-switch method reveals that thioredoxin acts as depersulfidase and controls the intracellular levels of protein persulfidation. Chem Sci 7, 3414–3426 CrossRef Medline

19. Nagy, P. (2015) Mechanistic chemical perspective of hydrogen sulfide signaling. Methods Enzymol 554, 3–29 CrossRef Medline

20. Münchberg, U., Anwar, A., Mecklenburg, S., and Jacob, C. (2007) Polysulfides as biochemically active ingredients of garlic. Org Biomol Chem 5, 1505–1518 CrossRef Medline

21. Ida, T., Tawa, T., Ihara, H., Chisai, Y., Watanabe, Y., Kumagai, Y., Sue-matsu, M., Motohashi, H., Fuji, S., Mutsanaga, T., Yamamoto, M., Ono, K., Devarie-Baez, N. O., Xian, M., Fukuto, J. M., and Akaie, T. (2014) Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proc Natl Acad Sci U S A 111, 7606–7611 CrossRef Medline

22. Kimura, H. (2019) Signaling by hydrogen sulfide and polysulfides via protein S-sulfuration. Br J Pharmacol CrossRef Medline

23. Fukuto, J. M., Ignarro, L. J., Nagy, P., Wink, D. A., Kevil, C. G., Feilisch, M., Cortese-Krott, M. M., Bianco, C., Lum, K., Yohbs, A. J., Lin, J., Ida, T., and Akaie, T. (2018) Biological hydropersulfides and related polysulfides—a new concept and perspective in redox biology. FEBS Lett 592, 2140–2152 CrossRef Medline

24. Yuan, S., Shen, X., and Kevil, C. G. (2017) Beyond a gasotransmitter: Hydrogen sulfide signaling in cardiovascular health and immune response. Antioxid Redox Signal 27, 634–653 CrossRef Medline

25. Greiner, R., Pálníkás, Z., Bäsell, K., Becher, D., Antelmann, H., Nagy, P., and Dick, T. P. (2013) Polysulfides link H2S to protein thiol oxidation. Antioxid Redox Signal 19, 1749–1765 CrossRef Medline

26. Artaud, I., and Galardon, E. (2014) A persulfide analogue of the nitrosoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. J Biol Chem 287, 6023–6034 CrossRef Medline

27. Gromer, S., Arscott, L. D., Williams, C. H., Jr., Schirmer, R. H., and Becker, K. (1998) Human placenta thioredoxin reductase. Isolation of the sel-enzyme, steady state kinetics, and inhibition by therapeutic gold compounds. J Biol Chem 273, 20096–20101 CrossRef Medline

28. Stafford, W. C., Peng, X., Olofsson, M. H., Zhang, X., Luci, D. K., Lu, L., Cheng, Q., Trésaugues, L., Dexheimer, T. S., Cousens, N. P., Augsten, M., Ahtzén, H.-S. M., Orwar, O., Östman, A., Stone-Elander, S., et al. (2018) Irreversible inhibition of cysteolic thioredoxin reductase 1 as a mechanistic basis for anticancer therapy. Sci Transl Med 10, eaaf7444 CrossRef Medline

29. Arnér, E. S. J. (2009) Focus on mammalian thioredoxin reductases—important selenoproteins with versatile functions. Biochim Biophys Acta 1790, 495–526 CrossRef Medline

30. Liu, P., Nijhawan, D., Buddharijo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cytochrome c and DATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489 CrossRef Medline

31. Fujita, E., Gashira, J., Urase, K., Kuida, K., and Momoi, T. (2001) Caspase-9 processing by caspase-3 via a feedback amplification loop in vivo. Cell Death Differ 8, 335–344 CrossRef Medline

32. Johnson, C. R., and Jarvis, W. D. (2004) Caspase-9 regulation: An update. Apoptosis 9, 423–427 CrossRef Medline

33. Toohey, J. I., and Cooper, A. J. L. (2014) Thiosulfoxide (sulfane) sulfur: New chemistry and new regulatory roles in biology. Molecules 19, 12789–12813 CrossRef Medline

34. Zhang, T., Ono, K., Tatsuki, H., Ihara, H., Islam, W., Akaie, T., and Sawa, T. (2019) Enhanced cellular polysulfides negatively regulate TLR4 signaling and mitigate lethal endotoxin shock. Cell Chem Biol 26, 686–698.e4 CrossRef Medline

35. Rössig, L., Fichtlscherer, B., Breitschopf, K., Haendeler, J., Zeiher, A. M., Mülsch, A., and Dimmelmer, S. (1999) Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. J Biol Chem 274, 6823–6826 CrossRef Medline

36. Pan, S., and Berk, B. C. (2007) Glutathiolation regulates tumor necrosis factor-α-induced caspase-3 cleavage and apoptosis: Key role for glutaredoxin in the death pathway. Circ Res 100, 213–219 CrossRef Medline

37. Mannick, J. B., Schonhoff, C., Papeta, N., Ghaforifar, P., Szibor, M., Fang, K., and Gaston, B. (2001) S-nitrosylation of mitochondrial caspases. J Cell Biol 154, 1111–1116 CrossRef Medline

38. Wittvitsky, V., Miljkovic, J. L., Bostelma, T., Adhikari, B., Yadav, P. K., Steiger, A. K., Torregrossa, R., Pluth, M. D., Whiteman, M., Banerjee, R., and Filipovic, M. R. (2018) Cytochrome c reduction by H2S potentiates sulfide signaling. ACS Chem Biol 13, 2308–2307 CrossRef Medline

39. Shi, Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9, 459–470 CrossRef Medline

40. Longen, S., Richter, F., Köhler, Y., Wittig, L., Beck, K.-F., and Pfleischer, J. (2016) Quantitative persulfide site identification (qPerS-SD) reveals protein targets of H2S releasing donors in mammalian cells. Sci Rep 6, 29808 CrossRef Medline

41. Fu, L., Liu, K., He, J., Tian, C., Yu, X., and Yang, J. (2019) Direct proteomic mapping of cysteine persulfidation. Antioxid Redox Signal CrossRef Medline

42. Engelmann, R., Weismann-Shomer, P., Ziv, T., Xu, J., Arnér, E. S. J., and Benhar, M. (2013) Multilevel regulation of 2-Cys peroxiredoxin reaction cycle by S-nitrosylation. J Biol Chem 288, 11312–11324 CrossRef Medline

43. Walsh, J. G., Logue, S. E., Lüthi, A. U., and Martin, S. J. (2011) Caspase-1 promiscuity is counterbalanced by rapid inactivation of processed enzyme. J Biol Chem 286, 32513–32524 CrossRef Medline

44. Engelmann, R., Ziv, T., Arnér, E. S. J., and Benhar, M. (2016) Inhibitory nitrosylation of mammalian thioredoxin reductase 1: Molecular characterization and evidence for its functional role in cellular nitroxido-redox imbalance. Free Radic Biol Med 97, 375–385 CrossRef Medline

45. Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, K., et al. (2018) Improved eaaf7444 CrossRef Medline