Garlic Oil Polysulfides: H$_2$S- and O$_2$-Independent Pro-Oxidants in Buffer and Anti-Oxidants in Cells

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ABSTRACT

The health benefits of garlic and other organosulfur-containing foods are well recognized and have been attributed to both pro-oxidant and antioxidant activities. The effects of garlic are surprisingly similar to those of hydrogen sulfide (H$_2$S) which is also known to be released from garlic under certain conditions. However, recent evidence suggests that polysulfides, not H$_2$S, may be the actual mediator of physiological signaling. In this study we monitored formation of H$_2$S and polysulfides from garlic oil in buffer and in HEK 293 cells with fluorescent dyes, AzMC and SSP4, respectively and redox activity with two redox indicators roGFP and DCF.

Our results show that H$_2$S release from garlic oil in buffer requires other low molecular weight thiols such as cysteine (Cys) or glutathione (GSH), whereas polysulfides are readily detected in garlic oil alone. Administration of garlic oil to cells rapidly increases intracellular polysulfide but has minimal effects on H$_2$S unless Cys or GSH are also present in the extracellular medium. We also observed that garlic oil and diallyltrisulfide (DATS) potently oxidized roGFP in buffer but did not affect DCF. This appears to be a direct polysulfide-mediated oxidation that does not require a reactive oxygen species intermediate. Conversely, when applied to cells, garlic oil became a significant intracellular reductant independent of extracellular Cys or GSH. This suggests that intracellular metabolism and further processing of the sulfur moieties are necessary to confer anti-oxidant properties to garlic oil in vivo.

Key words: allium, dialyltrisulfide, polysulfides,
INTRODUCTION

Garlic and other organo-sulfur bearing plants have been used by humans for medicinal purposes for at least 6,000 years (32). The wisdom of the Ancients has largely been borne-out by current research and in research animals, if not always in humans, there is accumulating evidence that garlic and organo-sulfur compounds have a myriad of beneficial effects. These have been summarized in a number of recent reviews (1, 3, 4, 7,18, 38, 48, 52, 61, 64, 66).

Considerable interest in garlic metabolism has focused on the pathway whereby the endogenous precursor, alliin (S-allyl-L-cysteine sulfoxide), is metabolized to allicin by the carbon-sulfur lyase enzyme, alliinase, the latter being released when garlic cells are damaged. Allicin rapidly undergoes non-enzymatic decomposition into diallyl monosulfide (DAS) and oil-soluble polysulfides, most notably diallyl disulfide (DADS) and diallyl trisulfide (DATS; 54). Much of the biological activity of these polysulfides has been attributed to DATS; the purported biological activity of DADS being questioned by purported DATS contamination common in commercial preparations (30).

The mechanism through which DATS exerts its biological activity has not been completely resolved but recent attention has focused on release of hydrogen sulfide (H₂S). This has been demonstrated in buffer (2) and in the presence of red blood cells (2) or homogenized heart tissue (47). H₂S release from DATS in buffer is not spontaneous but can be accomplished non-enzymatically in the presence of another organosulfur such as glutathione (GSH), cysteine (Cys) or homocysteine (Hcys) with the relative potency, GSH>Cys>Hcys (2). H₂S is not
released from DAS and it is not clear if its release from DADS (2) is also due to DATS contamination.

Interest in H$_2$S as the active component in garlic not only stems from its release from DATS and other polysulfides but also lies in the almost uncanny similarity in their biological actions. Both H$_2$S and garlic oil have been shown to be cardioprotective (6, 13, 18, 25, 26, 31, 35, 67) and contribute to ischemic conditioning (45, 65). Both are anti-inflammatory (7, 56), anti-atherosclerotic (29, 33, 62), induce Ca$^{2+}$ influx in astrocytes (22) and have been reported to act as potent antioxidants (5, 9, 15, 21, 49, 53, 59, 60) and decrease oxidative stress (28, 31, 68).

It is clear from the above that many of the actions of the organosulfur compounds have been linked to their antioxidant properties. Paradoxically, however, many of the effects of these compounds (e.g., opening vascular KATP channels, nuclear translocation of Nrf2 in activation of antioxidant defenses, reduction in TNF-$\alpha$ in Parkinsonism, to name a few) are best explained by a pro-oxidant effect on reactive protein cysteines (see below), or by over-all increases in oxidative stress (27, 46, 51, 57).

Both pro- and anti-oxidant activities have been ascribed to H$_2$S and related polysulfides (PS; H$_2$S$_n$, n=2-8), although, there is increasing evidence that their pro-oxidant effects are considerably more prevalent than originally thought (8, 40). Arguably, the greatest interest in H$_2$S and polysulfide signaling is in their ability to bind to a protein cysteine through a process called sulphydration, or more appropriately persulfidation (11), and thereby change the functional properties of that protein (11, 19, 20, 37, 44). At least 15 structural proteins or enzymes have been shown to be modified through this process (44) and many of these appear to be proteins that
are also activated or inactivated by organosulfur compounds. Polysulfides can directly persulfidate protein cysteines, whereas \( \text{H}_2\text{S} \) cannot as both the cysteine and \( \text{H}_2\text{S} \) sulfur atoms are in their most reduced state. \( \text{H}_2\text{S} \) sulfuration requires a two-electron oxidation of either sulfur or a one-electron oxidation of both; the mechanism through which this occurs is only beginning to be revealed, although the outcome is well documented (23). While \( \text{H}_2\text{S} \) may reduce a very select number of disulfide bridges in proteins (37), there is relatively little evidence to support the common assumption that it is a potent reductant as \( \text{H}_2\text{S} \) does not readily react with peroxide (39).

We (8) recently observed that \( \text{H}_2\text{S} \) and related Polysulfides readily oxidize a number of indicators commonly used to detect redox state and reactive oxygen species including redox sensitive green fluorescent protein (roGFP), \( 2',7' \)-dichlorofluorescein (DCF), MitoSox Red and Amplex Red. In the present studies we used two of these indicators, roGFP and DCF, along with specific fluorescent indicators of \( \text{H}_2\text{S} \) (7-azido-4-methylcoumarin, AzMC) and polysulfides (SSP4) to examine \( \text{H}_2\text{S} \) and polysulfides release from garlic oil and DATS and to determine their potential to act as an oxidant or reductant. We also examined \( \text{H}_2\text{S} \) and Polysulfides production in cells exposed to garlic oil in normoxia and hypoxia. To our knowledge, this study is the first to directly measure the effects of garlic oil on intracellular \( \text{H}_2\text{S} \) and polysulfides and to evaluate the redox properties of garlic oil in buffer and cells.

**MATERIALS AND METHODS**

*Chemicals*
SSP4 was generously provided by Dr. Ming Xian, Washington State University. DCF and LB Broth, Miller was purchased from ThermoFisher Scientific (Grand Island, NY) and roGFP from the University of Oregon, Dept. of Health Sciences (Eugene OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**General protocol**

Buffer and the reporting fluorescent indicator were aliquoted into black 96 well plates in a darkened room. A black cover with a parafilm liner was placed over the plates to further minimize photobleaching and reduce H₂S volatization. The edge of the plate was also wrapped with parafilm. Baseline fluorescence was measured on a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA; see *note below) every 10 min over 30 min (t = -30, -20, -10 min). The plates were then uncovered, the experimental compounds were added, the plate resealed and fluorescence recorded an additional 100 min (t 0-90 min) with sampling at 10 min intervals. In order to reduce well-to-well variation the samples were normalized to the baseline fluorescence at t = -10 min (10 min prior to addition of drugs). All experiments were done at room temperature in a darkened room. A minimum of three replicates were obtained. Hypoxia experiments were performed with the plate reader in a model 856-HYPO hypoxia chamber (Plas Labs, Inc. Lansing, MI) under 100% N₂ which lowered the ambient O₂ to less than 0.35%.

Under normal barometric conditions (~747±2 mmHg) this produced an O₂ concentration less than 3.8 μM in the buffer. *Note: due to occasional malfunctions in our plate reader we used a colleague’s for several of the normoxia experiments. Because this instrument was set up
differently the absolute fluorescence was not identical to ours, however this did not affect the relative changes in fluorescence and suitable controls were run with each experiment.

\[H_2S \text{ and polysulfide production from garlic oil and diallyl} \text{trisulfide (DATS)}\]

Varying concentrations of garlic oil blend consisting of 30-50 % by wt. diallyl disulfide (DADS), 10-13 % diallyl trisulfide (DATS) and 5-13 % allyl sulfide (AS) were used. Pure DATS was also examined for comparison. AzMC (10 μM) and SSP4 (50 μM) were used to detect H₂S and polysulfides, respectively. 2 mM cysteine (Cys) and 2 mM glutathione (GSH) were added to release H₂S. In pilot experiments we confirmed that AzMC was insensitive to polysulfides and that SSP4 was insensitive to H₂S.

\[roGFP\]

Plasmid containing a roGFP2 sequence, an ampicillin resistance sequence, and lac operon promoter sequence were transfected into BL21D3 \textit{E. coli} cells. Transfection success was verified via purification and spectroscopy.

In order to purify the roGFP protein, three colonies were chosen at random and grown in LB Broth, Miller in 100 μM ampicillin to an optimum optical density of 0.800 absorbance units at 600nm. The cells were then pelleted using an Avanti J-30I centrifuge at 6,000 xg for 6 min. The pellet was weighed, resuspended in a 5x volume of lysis buffer for 20 min, then sonicated to complete cell lysis. The lysate was then centrifuged at 50,000 xg for 30 min and the supernatant successively filtered through 0.8 μm and 0.45 μm syringe filters. Ni-NTA Agarose solution was
added to the filtered supernatant and after 2 hours the supernatant was passed through a Thermo Scientific 5mL polypropylene column, washed, and eluted.

From initial studies, the protein appeared to be nearly completely oxidized (fraction oxidized = 1.0) once purified. To obtain a partially reduced protein, the roGFP was incubated with 1 mM dithiothreitol (DTT) for 20 min and then dialyzed with two rinses overnight in 100% N₂ sparged Sorensen’s (phosphate) buffer (pH of 7) using Spectra/Por membrane tubing with 3.5 kD molecular weight cutoff. This yielded protein that was 25-50% oxidized (fraction oxidized 0.2-0.5). The purified protein was diluted 1:50 and a volume of 200 μL of diluted protein was then added to each well of a 96 well plate; final protein concentration was ~20 μM. Samples were read on a plate reader (General protocol) at excitation wavelengths of 405 and 488 nm and emission wavelength of 510 nm. The fraction oxidation was determined by ratiometric analysis and comparisons to roGFP completely oxidized with 10 mM H₂O₂ and completely reduced with 30 mM DTT as described previously (34).

DCF

H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) is a non-fluorescent ester that when oxidized fluoresces upon exposure to blue light (17). DCF reportedly responds indirectly to H₂O₂ or O₂•− after their oxidation to HO• (17). DCF (1 or 10 μM) was used with 500/525 nm excitation/emission (e/m) wavelength. Samples were processed as described in general protocol.

HEK293 cells
HEK293 cells were plated in 96-well plates and examined when near confluence.

AzMC, SSP4 and DCF (all 10 μM) were added 2 h prior to experimentation. The medium was replaced 2x prior to the start of the experiment.

Transfection of HEK-293 cells was done using a roGFP primer targeted to the cytoplasm (also purchased from the University of Oregon). The cells were transfected following the procedure from Lipofectamine (Fisher Scientific), washed and plated in 96-well plates. They were used when near confluence.

We were unable to achieve satisfactory reduction of the HEK293 cells with 10 mM DDT and therefore could not accurately calibrate the fraction oxidation of roGFP in the cytoplasm. However, the ratiometric analysis still permitted an evaluation of the relative change in redox status of the cells and these changes were evaluated.

Data Analysis

Data was analyzed and graphed using QuatroPro (Corel Corporation, Ottawa Ont, Canada) and SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). Statistical significance was determined using one-way ANOVA and the Holm-Sidak test SigmaPlot 13.0. Results are given as mean ± SE; significance was assumed when p≤0.05.

RESULTS

Selectivity and sensitivity of AzMC and SSP4
We first verified that AzMC and SSP4 were selective for H$_2$S and polysulfides and relatively insensitive to potential interferences from cysteine (Cys), cystine (CSSC) and reduced and oxidized glutathione (GSH and GSSG, respectively). As shown in Fig. 1, AzMC is only slightly sensitive to 1 and 3 mM H$_2$S$_n$, the response to H$_2$S$_n$ is likely due to H$_2$S which exists as an impurity in K$_2$S$_n$ salts (12). AzMC does not respond to up to 3 mM CSSC or GSSG or to Cys or GSH at the 2 mM concentration used to release H$_2$S from garlic oil. As shown in Fig. 2, SSP4 is insensitive to H$_2$S, but responds well to H$_2$S$_n$; high H$_2$S$_n$ concentrations may decrease fluorescence. SSP4 does not respond to Cys or GSH at the 2 mM concentrations used to release H$_2$S from garlic oil nor does it respond to GSSG between 1 μM and 3 mM. However, 1 and 3 mM CSSC slightly increase SSP4 fluorescence.

H$_2$S formation from garlic oil and DATS

H$_2$S production from garlic oil in normoxia and hypoxia (<5 μM oxygen) is shown in Fig. 3. Garlic oil alone did not produce appreciable amounts of H$_2$S in either normoxia or hypoxia, whereas a concentration-dependent steady rate of H$_2$S formation was observed between 1 and 300 μM garlic oil when in the presence of either 2 mM cysteine (Cys) or 2 mM reduced glutathione (GSH). H$_2$S production from garlic oil in the presence of either Cys or GSH decreased when the garlic oil concentration was 1 and 3 mM. Hypoxia did not appreciably affect H$_2$S production from garlic oil in the presence of either Cys or GSH. H$_2$S was not generated from garlic oil in the presence of either cystine (CSSC) or oxidized glutathione (GSSG) in normoxia or hypoxia.
Because garlic oil contains a mixture of DAS, DADS and DATS, we used a 10-fold lower concentration of DATS than garlic oil to measure H$_2$S production in normoxia (bottom left panel Fig. 3). As with garlic oil, H$_2$S production from DATS was highly dependent on the presence of Cys and GSH and these two were essentially equally efficacious. Over the 90 min experimental period H$_2$S production from DATS in the presence of Cys or GSH was linear.

Polysulfides in garlic oil and DATS

More SSP4-reactive polysulfides were observed in garlic oil without either Cys or GSH and the least were observed in the presence of GSH (Fig. 4). The amount of polysulfide in garlic oil concentration-dependently increased from 1 to 10 μM, was maximal at 10 and 30 μM, and then decreased thereafter. With 2 mM Cys, polysulfides were not apparent until garlic oil reached 30 μM, they were maximal at 100 μM, and decreased thereafter. Garlic oil polysulfide concentrations were lowest in 2 mM GSH. Estimated maximum polysulfide concentrations in the presence of Cys and GSH were ≥100 μM. SSP4 fluorescence from 1 μM garlic oil alone was similar to that from 30 μM garlic oil with either Cys or GSH. Oxidized low molecular weight thiols, cystine (CSSC) and glutathione (GSSG) increased SSP4 fluorescence at the lowest garlic oil concentrations. (Note: variations in the y-axis were due to different instruments which altered the absolute fluorescence but did not affect the relative changes.)

Polysulfides in DATS were greatest with 100 μM DATS in 2 mM Cys followed by 100 μM DATS in 2 mM GSH and 10 μM DATS alone (Fig. 4). However, unlike garlic oil, there was considerably more polysulfide with DATS in the presence of Cys and slightly more in the
presence of GSH than with DATS alone. Furthermore, 2 mM Cys produced a rapid increase in polysulfide concentration with both 10 and 100 μM DATS, whereas polysulfide concentration increased slowly, nearly linearly, with GSH or DATS alone.

Effects of garlic oil on H₂S and polysulfide in HEK293 cells

The effects of garlic oil alone and in combinations with Cys or GSH on intracellular H₂S (AzMC fluorescence) and polysulfides (SSP4 fluorescence) in HEK293 cells exposed to normoxia (21% O₂) or hypoxia (<0.4% O₂) are shown in Figs. 5, 6. Intracellular H₂S gradually increased in all cells over time. 100 μM garlic oil modestly increased H₂S in cells in normoxia and essentially doubled it in hypoxia; 1 mM garlic oil was without effect in normoxia but also increased H₂S in hypoxia. Conversely, 1 mM garlic oil was more efficacious than 100 μM garlic oil in increasing intracellular polysulfides and there was no significant effect of hypoxia.

Addition of either Cys (2 mM) or GSH (2 mM) to garlic oil greatly increased intracellular H₂S under all conditions, although hypoxia blunted the effect of 1 mM garlic oil in the presence of GSH. Intracellular polysulfides were increased ~5- and 25-fold by 100 μM and 1 mM garlic oil, respectively and these responses were unaffected by either Cys or GSH. GSH alone did not affect either H₂S or polysulfide in these cells.

Interaction of reduced/oxidized cysteine and glutathione and garlic oil with the reactive oxygen species (ROS) indicator, roGFP
In order to examine the redox properties of garlic compounds on roGFP we first determined the effects of reduced and oxidized cysteine (Cys and CSSC, respectively) and reduced and oxidized glutathione (GSH and GSSG, respectively) on roGFP (Fig. 7 top panels). Cys reduced roGFP at 1 and 3 mM, the same concentrations of GSH also initially reduced roGFP, although to a lesser extent. roGFP was concentration-dependently oxidized by >3 μM CSSC with 1 and 3 mM producing nearly complete oxidation at 90 min. All GSSG concentrations oxidized roGFP, albeit at a slower rate and lower extent oxidation at 90 min compared to CSSC.

roGFP was concentration-dependently oxidized by both garlic oil alone (Fig. 7) and DATS (not shown). Significant roGFP oxidation was produced by 1 μM, the lowest concentration of garlic oil and DATS employed. The maximum responses were attained at 30-100 μM garlic oil and 300 μM DATS. Low concentrations of garlic oil or DATS were initially unable to overcome Cys reduction of roGFP, although the protein became progressively more oxidized thereafter. roGFP was nearly completely oxidized in the presence of 2 mM CSSC masking nearly all of the effects of garlic oil or DATS. GSH and GSSG had similar effects on garlic oil and DATS as Cys and CSSC, respectively, although the effects were less pronounced.

If the roGFP was not reduced prior to experimentation and remained ~100% oxidized (fraction oxidized ~1.0; Fig. 8) it was slightly, but concentration-dependently reduced by high concentrations (≥ 300 μM) of garlic oil. Cys (2 mM) and GSH slightly enhanced garlic oil’s efficacy, although the latter to a lesser extent. Neither CSSC nor GSSG affected the garlic oil response.
Effects of garlic oil on roGFP oxidation in HEK293 cells

In spite of our inability to accurately calibrate intracellular redox conditions, we were able to monitor relative changes in cytoplasmic redox after application of garlic oil (Fig. 9). In normoxia, 10 μM garlic oil slightly reduced the cytoplasm after 2 h, whereas 100 μM garlic oil immediately and substantially reduced the cytoplasm and it remained so for the entire 24 h experimental period. One mM garlic oil produced the greatest cytoplasmic reduction immediately after application but the cytoplasm became progressively more oxidized thereafter. These responses were unaffected by the simultaneous application of 1 mM Cys or GSH.

Cytoplasmic reduction by 100 μM garlic oil was even more pronounced when cells were exposed to hypoxia, whereas 1mM garlic oil was considerably less efficacious of a reductant. In hypoxia both Cys and GSH augmented the reductive effects of 100 μM and 1 mM garlic oil.

Effects of garlic on the reactive oxygen species (ROS) indicator, 2',7'-dichlorofluorescein (DCF)

Garlic oil alone (1 μM-3 mM) did not produce DCF fluorescence, whereas fluorescence was concentration-dependently increased by Cys, GSH, CSSC and GSSG (Figs. 10, 11); the relative potency was CSSC > Cys > GSH >> GSSG. When garlic oil was added in conjunction with Cys or GSH it concentration dependently augmented DCF fluorescence between 1 and 30 μM and progressively decreased it thereafter. Low concentrations of garlic oil did not affect maximum CSSC or GSSG fluorescence, while higher garlic oil concentrations progressively
inhibited fluorescence. Hypoxia did not affect the inability of garlic oil alone to produce DCF fluorescence nor did it inhibit DCF fluorescence produced by Cys or GSH (not shown).
DISCUSSION

We examined the production of H\textsubscript{2}S and polysulfides from garlic oil and DATS and determined their redox properties using two commonly employed redox indicators, roGFP and DCF. These experiments were performed in buffer to evaluate direct chemical interactions and in cells to ascertain their ultimate fate or effects in a living and dynamic system. A number of conclusions can be drawn from the results. First, H\textsubscript{2}S is released from garlic oil by reduced, but not oxidized, low molecular weight thiols, as shown previously, but garlic oil alone has modest effects on intracellular H\textsubscript{2}S concentration, whereas it greatly increases the concentration of intracellular polysulfides. Second, the large increases in intracellular H\textsubscript{2}S following garlic oil application are largely due to extracellular H\textsubscript{2}S formation concomitant with application of other low-molecular weight thiols. Third, the physiological effects of garlic oil that are mediated through reactive protein cysteines may be accomplished without H\textsubscript{2}S formation. Fourth, garlic oils are effective oxidants but poor reductants in buffer solutions and this appears to occur independent of reactive oxygen intermediates. Fifth, when garlic is taken up by cells the cytoplasm becomes more reduced and this is likely due to secondary metabolism of the garlic oil polysulfides.

I-3.) \textit{H\textsubscript{2}S and polysulfide formation from garlic oil in buffer and cells}

Generation of H\textsubscript{2}S from garlic oil in buffer or homogenized tissue has been shown to require the presence of low-molecular weight thiols and subsequent H\textsubscript{2}S signaling is believed to play a major role in the physiological effects of garlic (2, 47; see also Introduction). Our results
confirm that H$_2$S is generated from garlic oils in buffer (Fig. 3) but they also indicate that there is a threshold concentration above which the garlic oil inhibits Cys- and GSH-mediated H$_2$S release. There was less evidence, however, that increases in H$_2$S in buffer are directly translated into substantial increases in intracellular H$_2$S unless low molecular weight reduced thiols (Cys or GSH) were also present in the extracellular medium (Fig. 5). We believe that the large (3-7 fold) increase in intracellular H$_2$S in the presence of extracellular Cys or GSH was due to rapid H$_2$S production in the extracellular milieu and subsequent diffusion of H$_2$S into the cells. One would have expected that intracellular GSH, which is also present in millimolar concentrations, would foster similar H$_2$S production with extracellular garlic oil alone, but that did not occur. Perhaps this is because garlic oil diffusion into the cells is slower than that of H$_2$S and with the slower delivery of substrate, H$_2$S metabolism would better be able to keep pace with H$_2$S formation. This hypothesis is supported by the observation that intracellular H$_2$S concentration after garlic oil application was greater when the cells were hypoxic (Fig. 6) and by previous observations that cellular H$_2$S metabolism is to a large extent O$_2$-dependent (41). Alternatively, the garlic oil sulfides could be metabolized to other persulfides or thiols without H$_2$S formation (see below).

Our results show that SSP4 clearly measures polysulfides from garlic oils in buffer (Fig. 4) and in cells (Fig. 5) and that this does not require the presence of either Cys or GSH (Fig. 4). High concentrations of the garlic oils inhibit SSP4 fluorescence but this is not specific for garlic oil as similar effects were observed using a mixed polysulfide (H$_2$S$_n$, n=1-8). More importantly, our results show that garlic oil polysulfides enter HEK293 cells and that this is independent of
other low-molecular weight thiols (Figs. 5, 6). We believe these polysulfides are the most relevant signaling moieties of the garlic oils.

3.) The physiological effects of garlic oil mediated through reactive protein cysteines are accomplished without H$_2$S formation.

While addition of 2 mM Cys or 2 mM GSH to garlic oil was necessary to substantially increase intracellular H$_2$S in HEK293 cells (Fig. 5), neither Cys nor GSH were necessary for the effect of garlic oil on intracellular redox status of roGFP (Fig. 9). This indicates that the effects of garlic oil on cellular redox status is due to garlic oil polysulfides and independent of H$_2$S.

A number of studies have shown that sulfur signaling via regulatory protein cysteines is effectively accomplished by polysulfides through the process of persulfidation (11, 20, 37, 42-44). In this process one (or more?) sulfur atoms becomes covalently bound to a protein cysteine thereby altering protein function. Over 15 proteins have been shown to be regulated via sulfuration and this number is rapidly increasing (44). However, H$_2$S cannot persulfidate protein cysteines because sulfur is in its most reduced form (-2) as are the reactive protein cysteines and their reaction is not favored (11, 55). In order for H$_2$S to persulfidate, either it, or the protein cystine sulfur, must first undergo a two electron oxidation to sulfane sulfur (0 formal oxidation state), or both must undergo a one-electron oxidation, which is less likely. The sulfane (thiosulfoxide) sulfur in DADS (RSSR) and DATS (RSSSR), where R denotes the allyl groups, appears to be especially important in this regard as it can tautomerize to RS(S)R and RS(S)SR and this sulfur is highly reactive (55).
The sulfane sulfur in garlic oil has the potential to be both non-enzymatically and enzymatically transferred to other thiols or polysulfides. Non-enzymatic transfer is suggested in Fig. 4 whereby SSP4 fluorescence is greatly increased by even the lowest garlic oil concentration when CSSC or GSSG are present. The inability of oxidized thiols, CSSC and GSSG to release H$_2$S in either normoxia or hypoxia suggests that this process is a typical thiol-disulfide exchange reaction of the type described by Liang et al. (30) which is O$_2$-independent and generates a variety of polysulfide intermediates but does not generate H$_2$S.

Enzymatic processes can also catalyze sulfur transfer and formation of a variety of polysulfide moieties. Dihydropersulfides (H$_2$S$_2$), dihydropolysulfides (H$_2$S$_n$), hydopersulfides (RS$_2$H) and hydropolysulfides (RS$_n$H), where n = 3-5, have been shown to occur in cells and they can be endogenously generated. Kimura et al. (23) identified H$_2$S$_2$, H$_2$S$_3$ and H$_2$S$_5$ in mouse brain and showed that the enzymes 3-mercaptopyruvate sulfur transferase (3MST) and rhodanase catalyzed the formation of H$_2$S and H$_2$S$_3$ from 3-mercaptopyruvate, an endogenous substrate. Ida et al. (14) showed that the enzymes originally linked to H$_2$S biosynthesis, cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), catalyze the formation of cysteine and glutathione hydopersulfides and/or hydropolysulfides (RSSH, RSSSH and RSSSSH, where R = Cys or GSH) from cystine. They also suggest that the intracellular concentration of these GSH hydopersulfides and hydropolysulfides exceeds 100 μM. Recently, Yadav et al. (63) showed that under more physiological conditions CSE and CBS are more likely to generate H$_2$S than either hydopersulfides or hydropolysulfides from cystine which questions the intracellular concentrations of the latter two reported by Ida et al. (14) and suggests they are far lower. Yadav
et al. (63) also suggested that SSP4 is unlikely to detect low molecular weight persulfides in solution but instead detects thiosulfoxides (RS(=S)SR) and that SSP4 itself could bind to cysteine persulfides. The issue is further complicated by the possibility of tautomerization between thiosulfoxides and polysulfides (55). We have shown previously (8) and again in Fig. 2 that SSP4 readily responds to dihydropersulfides and dihydropolysulfides, however the decrease in fluorescence at high H$_2$S$_n$ or garlic oil concentrations (see below) may reflect this persulfide-SSP4 interaction. Clearly, additional studies are necessary to fully sort out the reactivity of SSP4.

Although never measured, it is quite possible that similar mono and dihydropersulfides and polysulfides are generated from DADS and DATS by 3MST, CSE and CBS and these products would be expected to be quite reactive. The rapid cellular uptake of the garlic polysulfides and their comparatively higher intracellular titers (Figs. 5, 6) and reactivities suggest that they are far more relevant than H$_2$S to the biological actions of garlic. In addition, these enzymatically generated persulfides and polysulfides may act as either electrophiles or nucleophiles (11, 37, 42, 43).

**4a.) Garlic oils are effective oxidants but poor reductants**

Our experiments with roGFP, arguably the “gold standard” indicator for ROS (50) show that garlic oil alone acted as a potent oxidant and was effective at concentrations as low as 1 μM (Fig. 7). This is similar to our previous report (8) where 1 μM of the mixed polysulfide, H$_2$S$_n$ also effectively oxidized roGFP. Furthermore, while cysteine and glutathione reduced roGFP
these effects could be overcome by garlic oil oxidation at substantially lower concentrations of garlic oil. Conversely, high concentrations (~1-3 mM) of garlic oil were able to reduce the oxidized roGFP (Fig. 8) which was also similar to the effects of H₂Sn at these same two high concentrations (8). Our previous experiments (8) and those in the present study also suggest that this “oxidative” process is independent of oxygen or its reduced species, superoxide anion, hydrogen peroxide and hydroxyl radical.

4b. Garlic oil oxidation of roGFP does not require reactive oxygen species

Most scenarios of sulfur compounds, especially thiols and polysulfides acting as oxidants indicate that this is ultimately achieved by generation of reactive oxygen species, not by the thiols themselves (10, 16, 36, 58). Munday (36) describes a catalytic cycle whereby an oxidized transition metal reacts with a thiol to form a thyl radical (eq. 1) which reacts with another thiol to form the disulfide radical anion (eq. 2) that then auto-oxidizes forming a disulfide and superoxide anion (eq. 3). Superoxide can further react with another thiol to produce peroxide and regenerate the thyl radical (eq. 4). The reduced metal can also reduce oxygen to superoxide or peroxide to hydroxyl radical.

\[
\begin{align*}
\text{RS}^- + M^{n+} & \rightarrow \text{RS}^+ + M^{(n-1)+} \\
\text{RS}^+ + \text{RS}^- & \rightarrow \text{RSSR}^{2-} \\
\text{RSSR}^{2-} + O_2 & \rightarrow \text{RSSR} + O_2^{2-} \\
\text{RS}^- + O_2^{2-} & \rightarrow \text{RS}^+ + H_2O_2
\end{align*}
\]

(eq. 1) (eq. 2) (eq. 3) (eq. 4)

Oxyhemoglobin can also react with thiols to form peroxide (eq. 5; ref 36) and H₂S binding to cytochrome c also leads to superoxide production (58).
\[ \text{RS}^- + \text{Fe}^{II}\text{HbO}_2 + 2\text{H}^+ \rightarrow \text{RS}^+ + \text{Fe}^{II}\text{Hb} + \text{H}_2\text{O}_2 \]  

(eq. 5)

We recently showed that H$_2$S and dihydropolysulfides (H$_2$S$_n$) mimic ROS activation of a variety of sensors designed to measure ROS including roGFP, DCF, MitoSox Red and Amplex Red (8). Furthermore, we showed that sulfide “oxidation” of roGFP was actually increased in hypoxia (O$_2$ <4 μM). Our present experiments show that garlic oil are also efficacious roGFP oxidants (Fig. 7), and although we did not examine this in hypoxia, parallel experiments with DCF support our conclusion of an oxygen-independent process.

DCF has been shown to be relatively non-specific for reactive oxygen species (ROS) but because it appears insensitive to direct oxidation by either O$_2$•$^-$ or H$_2$O$_2$ it has been suggested that DCF oxidation requires hydroxyl radicals generated from H$_2$O$_2$ and transition metal ions via Fenton-type reactions (17). However, in the present experiments we found no evidence for garlic oil oxidation of DCF in normoxia and presumably in the presence of trace metals (Fig. 10). This suggests that, a) garlic oil does not produce ROS, and, therefore, b) garlic oil oxidation of roGFP is accomplished through some ROS-independent mechanism, most likely sulfur-sulfur interactions. Furthermore, the apparent paradoxical ability of garlic oil to greatly enhance DCF fluorescence, which only occurs in the presence of Cys or GSH (Fig. 10), can be readily explained by Cys and GSH liberation of H$_2$S from garlic oil (Fig. 3) and subsequent H$_2$S-mediated increase in DCF fluorescence, as we have previously shown (8). The ability of Cys and GSH to reduce roGFP (Fig. 7), yet oxidize DCF (Fig. 10) is further evidence that these reactions do not have ROS generation as the common denominator.
5. Garlic oil as an intracellular reductant

To our knowledge, this study is the first to measure the effects of garlic oil on intracellular H$_2$S, polysulfides and redox conditions. As we found little evidence that garlic oil or DATS is a reductant in buffer (except at high concentrations; Fig. 8), the conundrum is how can it become one in cells (Fig. 9)? Furthermore, why is this reversed at higher garlic oil concentrations and why does hypoxia amplify both the reductive effect of 100 μM garlic oil and the reversal of this effect by 1 mM garlic oil. While there are a number of possibilities, these must include short- and long-term effects as we observed both over the 24 h experimental period.

The short-term responses which were seen immediately upon application of the garlic oil must be explained by reaction of garlic oil with an intracellular molecule or molecules that subsequently reduce roGFP. The most likely are direct sulfide or disulfide exchange processes and/or enzymatic reactions as described above. The long-term effects could be explained by persulfidation of Keap1. This dissociates the Keap1-Nrf2 complex allowing the latter to translocate to the nucleus and activate the antioxidant response elements (24).

PERSPECTIVES

Humans have known for eons that some foods bring more to the table than just calories. Clearly this is the case with garlic. Sulfur springs also have a long history in human culture and at the crossroads of these seemingly disparate and malodorous medicinals is sulfur. Since the original description that hydrogen sulfide is a physiologically relevant signaling molecule considerable emphasis has been placed on its metabolism and mechanisms of action. Not
surprisingly, it has also been demonstrated that hydrogen sulfide is released from garlic under certain conditions. Very recently, however, the ability of hydrogen sulfide to directly interact with regulatory proteins has been questioned and focus has now turned towards polysulfides as the biologically relevant mediators. In our studies we show that polysulfides derived from garlic readily enter cells and may in fact be directly responsible for garlic’s attributes. But this is not the final answer. Considerable questions remain regarding intracellular trafficking of the sulfur compounds as well as their metabolism and signaling attributes. With the considerable emphasis now being placed on the therapeutic benefits of synthetic hydrogen sulfide “donating” compounds perhaps we should take an even more rigorous look at these natural sulfides as they can be cultivated under a wide range of conditions with minimal expenditure of energy or labor.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.R.O. designed the study, E.R.D. and Y.G. performed the experiments. K.R.O. and E.R.D analyzed the data interpreted the results. K.R.O. wrote the manuscript and all authors discussed the results and commented on the manuscript.


**FIGURE LEGENDS**

**Figure 1.** Sensitivity and selectivity of AzMC to H$_2$S, polysulfides (H$_2$S$_n$), cysteine (Cys), glutathione (GSH), cystine (CSSC) and oxidized GSH (GSSG). AzMC is selective for H$_2$S over H$_2$S$_n$ but it is insensitive to CSSC, GSSG and to Cys and GSH at the concentrations used in this study (2 mM). Note: scales are expanded to those of Fig. 3 for comparison. Mean ±SE, n=3 experiments.

**Figure 2.** Sensitivity and selectivity of SSP4 to H$_2$S, polysulfides (H$_2$S$_n$), cysteine (Cys), glutathione (GSH), cystine (CSSC) and oxidized GSH (GSSG). SSP4 is sensitive to H$_2$S$_n$, somewhat sensitive to CSSC, but insensitive to H$_2$S or to 2 mM Cys or GSH (concentrations used in this study). Note: the ordinate is expanded for CSSC, GSSG and 2 mM Cys or GSH to correspond to comparable experiments in Fig. 4. Mean ±SE, n=3 experiments.

**Figure 3.** H$_2$S production from garlic oil and DATS. In normoxia (top panels) garlic oil alone did not produce appreciable amounts of H$_2$S, whereas a concentration-dependent steady rate of H$_2$S formation was observed between 1 and 300 μM garlic oil in the presence of either 2 mM cysteine (Cys) or 2 mM reduced glutathione (GSH). H$_2$S production from garlic oil in the presence of either Cys or GSH decreased when the oil concentration exceeded 300 μM. Essentially similar results were observed in hypoxia (<5 μM O$_2$, middle panels). H$_2$S was not generated from garlic oil by either cystine (CSSC) or oxidized glutathione (GSSG) in normoxia or hypoxia (bottom right panels). H$_2$S is also produced in normoxia from DATS in the presence
of Cys or GSH (bottom left panel). Dashed lines indicate 100 μM H₂S from simultaneous Na₂S
calibration curves. Garlic oil or DATS was added at -10 min; values are mean ±SE, n=3
experiments.

Figure 4. Polysulfides in garlic oil and DATS. In garlic alone polysulfide concentration
increased between 1 and 10 μM, was maximal at 10 and 30 μM and decreased thereafter. With 2
mM Cys, polysulfides were not apparent until garlic oil reached 30 μM, were maximal at 100
μM and decreased thereafter. Garlic oil polysulfide concentrations were lowest in 2 mM GSH.
Oxidized thiols, cystine (CSSC, 2mM) and to a lesser extent, glutathione (GSSG, 2 mM),
increased SSP4 fluorescence by low (1-30 μM) garlic oil concentrations. Polysulfides in DATS
were greatest with 100 μM DATS in 2 mM Cys folowed by 100 μM DATS in 2 mM GSH and
10 μM DATS alone. Garlic oil and DATS were added at -10 min; values are mean ±SE, n=3
experiments. Use of different instruments produced the variations in the y axis scale.

Figure 5. Effects of garlic oil (GO; 100 μM and 1 mM) alone and in combinations with cysteine
(Cys; 2 mM) or glutathione (GSH; 2 mM) on intracellular H₂S (AzMC fluorescence; A, B) and
polysulfides (SSP4 fluorescence; C, D) in HEK293 cells exposed to normoxia (21% O₂; A, C)
and hypoxia (<0.4% O₂, B, D). A, B, both Cys and GSH increased intracellular H₂S, although
hypoxia blunted the effect of 1 mM CO and GSH. C, D, GO alone concentration-dependently
increased intracellular polysulfides and this was not affected by Cys but reduced when GSH was
added to 100 μM GO. These responses were unaffected by hypoxia. bkg, autofluorescence of untreated cells; AzMC/SSP4, cells treated with dye only; values are mean ±SE, n=3 experiments. **Figure 6.** Expanded scale of data in Fig. 5 showing the effects of 100 μM and 1 mM garlic oil on H₂S and polysulfides in HEK293 cells in normoxia and hypoxia. H₂S appeared to steadily increase over time in untreated cells although only t= 0 and t= 24 h were significantly different (p = 0.046 and 0.035 for normoxia and hypoxia, respectively). In all other cells, t = 2-24 h was significantly (p < 0.001) greater than t = 0 h. Addition of 100 μM garlic oil increased intracellular H₂S more than 1 mM garlic oil and hypoxia augmented the effect of 100 μM garlic oil. Conversely, 1 mM garlic oil was ~3-fold more efficacious than 100 μM garlic oil in increasing intracellular polysulfides and this was unaffected by hypoxia. Autofluorescent cells and t = -1h have been omitted for clarity.

**Figure 7.** Reduction/oxidation of roGFP by cysteine (Cys), reduced glutathione (GSH), cystine (CSSC) and oxidized glutathione (GSSG; top panels) and by garlic oil alone or in combination with 2 mM of the low-molecular weight thiols (middle and bottom panels). Cys and CSSC were more efficacious reductants or oxidants, respectively of roGFP than GSH and GSSG. Garlic oil alone concentration-dependently oxidized roGFP. Simultaneous addition garlic oil and reduced glutathione (GSH) or cysteine (Cys) decreased the rate and extent of roGFP oxidation. Low concentrations (1-10 μM) of garlic oil were initially unable to overcome the reductive effects of Cys, although roGFP was progressively oxidized thereafter. The effects of GSH were similar to those of Cys, although less pronounced. roGFP was nearly completely oxidized at all garlic
concentrations in the presence of cystine (CSSC). The effects of oxidized glutathione (GSSG) were similar to those of CSSC although less pronounced. Mean ±SE, n=3 experiments.

Figure 8. Completely oxidized roGFP was slightly but concentration dependently reduced by garlic oil alone. Cys (2 mM) increased garlic oil efficacy as did 2 mM GSH, although to a lesser extent. Garlic oil oxidization was not affected by either CSSC (2 mM) or GSSG (2 mM). Note: expanded scale relative to Fig. 7. Mean ±SE, n=3 experiments.

Figure 9. Effects of garlic oil (GO) and GO in combination with 1 mM cysteine (Cys) or 1 mM glutathione (GSH) on oxidation/reduction of roGFP in the cytosol of HEK293 cells in normoxia or hypoxia. In normoxia, 10 μM garlic oil produced a slight time-dependent reduction of roGFP, 100 μM produced a sustained reduction, whereas after 1 mM roGFP was initially reduced and then became progressively oxidized. These responses were unaffected by either Cys or GSH. Compared to normoxia, in hypoxia, 10 μM and 1 mM garlic oil were less effective, whereas 100 μM was more potent. Both Cys and GSH enhanced the reductive effect of 100 μM and 1 mM garlic oil. Green bars (t = -1h) are normalized fluorescence 1 h prior to addition of drugs, bkg is background fluorescence of cells without roGFP. Mean ±SE, n=3 experiments.

Figure 10. Cysteine (Cys), glutathione (GSH) and garlic oil-induced fluorescence from the ROS indicator, 2',7'-dichlorofluorescein (DCF). Both low molecular thiols dose-dependently increased DCF fluorescence, whereas garlic oil alone did not affect DCF fluorescence. Addition of low
concentrations of garlic oil augmented 2 mM Cys and 2 mM GSH induced fluorescence, whereas higher concentrations of garlic oil were inhibitory. Reducing the concentration of either Cys or GSH decreased total fluorescence (right panels; note change in y-axis scale) without affecting the general influence of garlic oil. Mean ±SE, n=3 experiments.

**Figure 11.** Cystine (CSSC) and oxidized glutathione (GSSG) induced fluorescence from the ROS indicator, 2',7'-dichlorofluorescein (DCF) in the presence or absence of garlic oil. Both low molecular weight thiols dose-dependently increased DCF fluorescence, although GSSG was far less effective (note scale of y-axis). Addition of low concentrations of garlic oil to 2 mM CSSC or 2 mM GSSG did not affect fluorescence compared to 3 mM CSSC or GSSG alone, whereas higher concentrations of garlic oil concentration-dependently fluorescence. Reducing the concentration of either CSSC or GSSG decreased total fluorescence (right panels; note change in y-axis scale) without affecting the general influence of garlic oil. Mean ±SE, n=3 experiments.
Fig. 1

Time (min) vs. Relative Fluorescence for different concentrations of AzMC and 2 mM CyS or GSH with H$_2$S, CSSC, GSSG.

- AzMC concentrations: 1 uM, 3 uM, 10 uM, 30 uM, 100 uM, 300 uM, 1 mM, 3 mM
- CyS or GSH: 2 mM

Fluorescence levels are shown for each condition over a time course of 100 minutes.
Fig. 2

H₂S

CSSC

GSSG

2 mM CyS or GSH
Fig. 3

**normoxia**

- **Garlic oil**
- **Garlic + Cys**
- **Garlic + GSH**

**hypoxia**

- **Garlic oil**
- **Garlic + Cys**
- **Garlic + GSH**

**DATS** (normoxia)

- **Garlic + CSSC/GSSG** (normoxia)

**Garlic + CSSC/GSSG** (hypoxia)
Fig. 4

**Garlic oil**

- Relative Fluorescence vs. Time (min)

**Garlic + Cys**

- Relative Fluorescence vs. Time (min)

**Garlic + GSH**

- Relative Fluorescence vs. Time (min)

**DATS**

- Relative Fluorescence vs. Time (min)

**Garlic+CSSC**

- Relative Fluorescence vs. Time (min)

**GSSG+GO**

- Relative Fluorescence vs. Time (min)

Legend:
- buffer
- SSP4
- 10 uM DATS
- 100 uM DATS
- 10 uM DATS+GSH
- 100 uM DATS+GSH
- 10 uM DATS+Cys
- 100 uM DATS+Cys

Different concentrations of SSP4, DATS, and GSH are indicated by different colors and symbols.
Fig. 5

A. H$_2$S Relative Fluorescence in normoxia and hypoxia conditions.

B. Time (h) for H$_2$S Relative Fluorescence in hypoxia.

C. Polysulfide Relative Fluorescence under different conditions.

D. Polysulfide Relative Fluorescence with time in hypoxia conditions.
Fig. 6

H$_2$S

Polysulfide

Relative Fluorescence

Time (h)

0 2 4 8 24

normoxia hypoxia

Time (h)

0 2 4 8 24

normoxia hypoxia
Fig. 9
Fig. 10

Cys

Cys(1 mM)+Garlic

Cys(300 uM)+Garlic

GSH

GSH(1 mM)+Garlic

GSH(100 uM)+Garlic

Garlic oil
Fig. 11

CSSC

CSSC(1M)+Garlic

CSSC(300 uM)+Garlic

GSSG

GSSG(1mM)+Garlic

GSSG(300uM)+Garlic

Relative Fluorescence vs. Time (min)

DCF 1 uM 3 uM 10 uM 30 uM 100 uM 300 uM 1 mM 3 mM