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Effect of garlic-derived organosulfur compounds on mitochondrial function and integrity in isolated mouse liver mitochondria

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Abstract

The objectives of this work were to evaluate the direct effects of diallylsulfide (DAS) and diallyldisulfide (DADS), two major organosulfur compounds of garlic oil, on mitochondrial function and integrity, by using isolated mouse liver mitochondria in a cell-free system. DADS produced concentration-dependent mitochondrial swelling over the range 125–1000 μ M, while DAS was ineffective. Swelling experiments performed with de-energized or energized mitochondria showed similar maximal swelling amplitudes. Cyclosporin A (1 μ M), or ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, 1 mM) were ineffective in inhibiting DADS-induced mitochondrial swelling. DADS produced a minor (12%) decrease in mitochondrial membrane protein thiols, but did not induce clustering of mitochondrial membrane proteins. Incubation of mitochondria with DADS (but not DAS) produced an increase in the oxidation rate of 2',7' dichlorofluorescein diacetate (DCFH-DA), together with depletion of reduced glutathione (GSH) and increased lipid peroxidation. DADS (but not DAS) produced a concentration-dependent dissipation of the mitochondrial membrane potential, but did not induce cytochrome c release. DADS-dependent effects, including mitochondrial swelling, DCFH-DA oxidation, lipid peroxidation and loss of mitochondrial membrane potential, were inhibited by antioxidants and iron chelators. These results suggest that DADS causes direct impairment of mitochondrial function as the result of oxidation of the membrane lipid phase initiated by the GSH- and iron-dependent generation of oxidants.

Keywords

Diallyl disulfide; Diallyl sulfide; Garlic; Mitochondria; Oxidative stress

1. Introduction

Organosulfur compounds (OSCs) are phytochemicals of the *Allium* genus, which are especially abundant in garlic and onion bulbs (Arranz et al., 2007). Garlic-derived OSCs are

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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primarily classified as lipid-soluble, such as diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), or water-soluble, such as S-allylcysteine and S-allylmercaptocysteine (Wang et al., 2010a). Lipid-soluble OSCs derived from garlic induce dose-dependent impairment of mitochondrial function in mammalian cells in tissue culture. For example, DAS and DADS produced a decrease in mitochondrial membrane potential (widely considered an indicator of mitochondrial functionality, Rhein et al., 2009) in primary rat hepatocytes (Truong et al., 2009) and human glioblastoma cells (Das et al., 2007); DAS and DADS also induced cytochrome c release associated with mitochondrial damage in human neuroblastoma cells (Karmakar et al., 2007). In addition, DADS decreased mitochondrial membrane potential in human lung adenocarcinoma cells (Wu et al., 2009), human cervical cancer cells (Lin et al., 2008), human colon cancer cells (Yang et al., 2009), and mouse-rat hybrid retina ganglion-lymphoma cells (Lin et al., 2006).

Activation of cellular oxidative stress seems to be a central pathway by which lipid-soluble garlic-derived OSCs induce mitochondrial damage. OSCs including DAS, DADS and DATS have been reported to increase levels of reactive oxygen species (ROS) in cultured cells, and induce mitochondrial impairment indirectly by: i) releasing calcium from intracellular stores (Das et al., 2007; Lin et al., 2006; Lin et al., 2008; Karmakar et al., 2007); ii) activating redox-sensitive kinases such as JNK (Lee et al., 2011; Lin et al., 2006); and iii) activating p53 through DNA damage (Wang et al., 2010a; Lin et al., 2008). In addition, DADS induced mitochondrial toxicity partly by disrupting microtubule structure (Xiao et al., 2005).

While it is clear that garlic-derived OSCs can induce mitochondrial impairment indirectly, the contribution of direct effects of OSCs on mitochondrial damage is less clear. The direct interaction between a test chemical and mitochondria can be determined by the capacity of the chemical to affect mitochondrial function and/or integrity in a cell-free system (Fulda et al., 2010). Therefore, the objectives of our work were to evaluate the direct effects of DAS and DADS, two major OSCs of garlic oil (Sheen et al., 1999) on mitochondrial function and integrity in isolated mouse liver mitochondria. The ability of DAS and DADS to induce by themselves large amplitude mitochondrial swelling, dissipation of the mitochondrial membrane potential and cytochrome c release was evaluated, along with possible mechanisms for these effects.

2. Materials and Methods

2.1. Isolation of mitochondria

Adult female CD-1 mice (4–8 weeks, 20–25 g, Charles River Laboratories, Wilmington, MA) were fed *ad libitum* a commercial diet and maintained on a 12:12-h dark/light cycle. Mice were euthanized with ketamine/xylazine and the livers were removed and washed with ice-cold saline (Zhao et al., 2002). The livers were homogenized in a buffer composed of 0.22 M mannitol, 70 mM sucrose, 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 2 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid) (Hepes), 0.1% essentially fatty acid-free bovine serum albumin and pH 7.4 at a 10:1 buffer to liver v/w ratio (Sarkela et al., 2001). Mitochondria were isolated by differential centrifugation of the liver homogenate: first, unbroken cells and nuclei were pelleted at 600 x g for 10 min at 4°C; second, the supernatant was centrifuged at 10,000 x g for 10 min at 4°C to pellet the mitochondria (Sarkela et al., 2001). Finally, the mitochondrial pellet was resuspended into the same buffer as above but without bovine serum albumin, at a final concentration of 20 mg protein/mL, determined by the Bradford reagent (Sigma-Aldrich, St. Louis, Mo). The average respiratory control ratio (defined as the ratio of state 3 to state 4 respiration using succinate as respiratory substrate) of the mitochondrial suspension was 2.7±0.3, reflecting a high level of functional integrity. All studies with mitochondria were

performed within 3 h of isolation. All procedures involving the mice were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Hendrix College, Conway, AR.

2.2. Determination of mitochondrial swelling

Isolated mitochondria were resuspended at a final concentration of 0.5 mg protein/ml in a buffer composed of 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, 20 μ M Ca^{2+} , pH 7.2 (buffer A), with the additions indicated in the text. Mitochondrial swelling was evaluated by the decrease in light scattering at 540 nm or by the increase in the forward scatter parameter determined by flow cytometry.

a) Decrease in light scattering—The absorbance of the mitochondrial suspension was monitored at 540 nm for up to 10 min at 25 °C using a Biotek Synergy 2 microplate reader with constant shaking and temperature control (Kessova and Cederbaum, 2007).

b) Increase in forward scatter parameter—The mitochondrial suspension was incubated at room temperature for 5 min under constant mixing in a nutator and samples were immediately analyzed using an Accuri C6 flow cytometer (Kaufmann et al., 2005; Kluza et al., 2006). A minimum of 10,000 events was evaluated per sample, and the forward scatter parameter, side scatter parameter, and fluorescence in the F11 channel (530 $\pm$ 30 nm emission, with excitation at 488 nm) were evaluated for each event.

2.3. Determination of mitochondrial membrane potential

Isolated mitochondria were resuspended at a final concentration of 0.25 mg protein/ml in buffer A with 0.2 μ M rhodamine 123 and the additions indicated in the text. The change in fluorescence at an excitation of 485 $\pm$ 20 nm and emission of 528 $\pm$ 20 nm was monitored continuously at 25 °C using a Biotek Synergy microplate reader with constant shaking and temperature control (Kessova and Cederbaum, 2007).

2.4. Evaluation of the generation of reactive oxygen species

Isolated mitochondria were resuspended at a final concentration of 0.25 mg protein/ml in buffer A with 10 μ M 2',7' dichlorofluorescein diacetate (DCFH-DA) and the additions indicated in the text. The change in fluorescence at an excitation of 485 $\pm$ 20 nm and emission of 528 $\pm$ 20 nm was monitored continuously at 25 °C using a Biotek Synergy microplate reader with constant shaking and temperature control (Young et al., 2002).

2.5. Quantitation of mitochondrial reduced glutathione (GSH)

Isolated mitochondria were resuspended at a final concentration of 0.5 mg protein/ml in buffer A with the additions indicated in the text. Mitochondria were then incubated at room temperature for 15 min under constant mixing in a nutator. The mitochondrial suspension was treated with trichloroacetic acid to a final concentration of 10% (w/v) to extract mitochondrial glutathione. The mixture was centrifuged at 13,000 $\times g$ for 1 min to remove denatured proteins. Total glutathione, including reduced glutathione (GSH) and glutathione disulfide (GSSG), was determined in the resulting supernatant according to Mari and Cederbaum (2000) using an enzymatic recycling assay. For the determination of GSSG, GSH was first conjugated with 2-vinylpyridine, followed by the same enzymatic recycling assay. The concentration of GSH was calculated as the difference between the concentration of total glutathione and the concentration of GSSG (Mari and Cederbaum, 2000).

2.6. Determination of mitochondrial lipid peroxidation

Lipid peroxidation was determined by the thiobarbituric acid-reactive substances (TBARS) assay. Because sucrose interferes with the TBARS assay (Plumb et al., 1996), isolated mitochondria were washed once (to remove sucrose) in ice-cold 3-(N-norpholino)propanesulfonic acid (MOPS)-KCl buffer (50 mM MOPS, 100 mM KCl, pH 7.4), and resuspended to 2.0 mg protein/mL in MOPS-KCl buffer (Bacon et al., 1986) with the additions indicated in the text. Mitochondria were then incubated at room temperature for 15 min under constant mixing in a nutator. At the end of the incubation, the mitochondrial suspension was mixed with twice its volume of 15% trichloroacetic acid, 0.375% thiobarbituric acid (TBA), 0.24 N HCl plus 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and heated for 15 min at 100°C (Caro and Cederbaum, 2001). After centrifugation, the absorbance of the supernatant was measured at 535 nm, and the concentration of the malondialdehyde (MDA)-TBA adduct calculated using the extinction coefficient $\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Woods and Ellis, 1995).

2.7. Quantitation of mitochondrial membrane protein thiol groups

Isolated mitochondria were resuspended at a final concentration of 0.5 mg protein/ml in buffer A with the additions indicated in the text. Mitochondria were then incubated at room temperature for 15 min under constant mixing in a nutator. The mitochondrial suspension was submitted to three subsequent freeze-thawing steps to release matrix proteins, and then centrifuged for 10 min at 10,000 $\times$ g and 4 °C. The pellet was resuspended in 0.5 mL of a medium containing 80 mM sodium phosphate pH 7.4, 2% sodium dodecyl sulfate and 200 μM 5,5'-dithiobis(2-nitrobenzoid acid) (DTNB). After a 10 min incubation at room temperature, the absorbance at 412 nm was measured ($\epsilon^{412} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) (Velho et al., 2006).

2.8. SDS-polyacrylamide gel electrophoresis of mitochondrial membrane proteins

The pellets obtained in the previous assay (mitochondrial membrane protein thiol groups) were resuspended in 0.1 M Hepes buffer pH 7.4, and protein concentration was evaluated with the Bradford assay (Sigma-Aldrich, St. Louis, Mo). An aliquot containing 50 μg of mitochondrial protein was diluted into non-reducing Laemmli buffer, and electrophoresis of the membrane proteins was performed in a discontinuous SDS-polyacrylamide gel consisting of a 3.5% stacking gel and a 12% resolving gel. Proteins in the gel were stained with Coomassie Brilliant Blue (Fagian et al., 1990).

2.9. Evaluation of the release of cytochrome c

Mitochondria (1 mg of protein/ml) were incubated at room temperature for 15 min in buffer A with the additions indicated in the text. The reaction mixture was then centrifuged at 12,000 $\times$ g for 10 min at 4 °C. The supernatant was concentrated by ultrafiltration through Amicon Ultra 10K centrifugal filters (Millipore, Billerica, MA). The concentrated supernatants were subjected to 12% SDS-PAGE, transferred to nitrocellulose membranes and analyzed by Western blotting using an anti-cytochrome c antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (Kessova and Cederbaum, 2007).

2.10. Statistics

Data are expressed as mean $\pm$ standard error of the mean from three to five independent experiments. One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffé's test was performed. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mitochondrial swelling induced by garlic-derived organosulfur compounds

Figure 1A shows representative swelling traces of mouse liver mitochondria exposed to 1 mM DAS, 1 mM DADS, or 1% ethanol (solvent control) in swelling buffer. Only DADS produced significant swelling with respect to the solvent control. In the following experiments, maximal swelling amplitude was used to quantify mitochondrial swelling data (Brookes and Darley-Usmar, 2004). DADS produced mitochondrial swelling in a concentration-dependent manner over the range 125–1000 μ M, while DAS was ineffective (Fig. 1B). Swelling experiments performed in de-energized mitochondria (i.e. in the absence of succinate and glutamate) showed similar maximal swelling amplitudes with respect to energized mitochondria (i.e. in the presence of succinate and glutamate) (Fig. 1C).

DADS-induced mitochondrial swelling was confirmed by flow cytometry. Initially, mitochondria were selected by gating the largest particle population in a SSC (side scatter) versus FSC (forward scatter) dot plot (P1, Fig. 2A). Mitochondria were then stained for 1 min with 1 nM 10-N-nonyl acridine orange (NAO), a fluorescent dye that specifically binds to cardiolipin, a glycerophospholipid present primarily in the inner mitochondrial membrane (Petit et al., 1992). Of the events gated in P1, 99% were positive for NAO staining, suggesting that the gated events represent mitochondrial particles (Fig. 2B). Mitochondrial swelling is indicated by an increase in FSC evaluated by flow cytometry (Kluza et al., 2006). DADS at 1 mM caused a 2.4-fold increase in the mean FSC parameter (FSC-H), while DAS at 1 mM did not produce a significant change in FSC-H with respect to the solvent control (Fig. 2C).

3.2. Mitochondrial swelling by DADS is not inhibited by cyclosporin A and is not dependent on Ca^{2+}

If DADS-dependent mitochondrial swelling is caused by the opening of the regulated permeability transition pore, then swelling should be inhibited by cyclosporin A or Ca^{2+} chelators such as EGTA (He and Lemasters, 2002). Cyclosporin A at 1 μ M, or EGTA at 1 mM were ineffective in inhibiting mitochondrial swelling caused by DADS at 1 mM (Fig. 3A and Fig. 3B, respectively) or 0.25 mM (data not shown). In order to confirm that cyclosporin A and EGTA were effective in inhibiting the opening of the regulated permeability transition pore under our incubation conditions, a positive control experiment was done using 1 mM inorganic phosphate (Pi), an agent that induces the opening of the regulated form of the mitochondrial permeability transition pore (Broekemeier et al., 1989). Cyclosporin A (Fig. 3C) or EGTA (Fig. 3D) were both effective in inhibiting swelling when swelling was induced by 1 mM Pi.

3.3. Effect of garlic-derived organosulfur compounds on mitochondrial membrane protein thiols

In several *in vitro* mitochondrial swelling models, Ca^{2+} - and cyclosporin A-insensitive swelling depended on oxidation of membrane protein thiols with subsequent membrane protein cross-linking (Brookes et al., 2007; Puntel et al., 2010; Gadelha et al., 1997; Santana et al., 2009). It is important to evaluate if mitochondrial swelling caused by DADS in isolated mitochondria is associated with this mechanism. In isolated mouse mitochondria, DADS (but not DAS) produced a significant, minor decrease (–12%) in mitochondrial membrane protein thiols (Fig. 4A). In contrast, a typical protein thiol-oxidizing agent such as ebselen (Puntel et al., 2010) produced a significant major decrease in mitochondrial membrane protein thiols (–83%) (Fig. 4A). Ebselen also induced mitochondrial swelling under our conditions (data not shown). Reagents that induce the oxidation of mitochondrial membrane protein thiols also induce the formation of protein aggregates due to cross-

linkage of thiol groups resulting from the formation of disulfide bonds (Santana et al., 2009). These protein aggregates are usually evidenced as high molecular weight protein bands located at the top of non-reducing SDS-polyacrylamide gels after electrophoresis (Cruz et al., 2010; Puntel et al., 2010; Fagian et al., 1990). In DADS- or DAS-treated mitochondria, there was no significant change in the intensity of protein bands located at a high molecular weight (higher than 250 kDa), with respect to the solvent control (Fig. 4B). In contrast, ebselen-treated mitochondria accumulated high molecular weight protein aggregates (Fig. 4B), suggesting that DADS does not behave as an effective membrane protein thiol-oxidizing agent.

3.4. Effect of garlic-derived organosulfur compounds on mitochondrial oxidative stress

The mitochondrial generation of oxidants in the presence of garlic-derived OSCs was evaluated using DCFH-DA. DCFH-DA is a redox-indicator probe which is oxidized by several one-electron oxidizing species including thiyl radicals, hydroxyl radical, alkoxyl and peroxy radicals, and redox-active metals in the presence of O₂ or H₂O₂ (Kalyanaraman et al., 2012; Eruslanov and Kusmartsev, 2010). DADS by itself, in the presence or absence of GSH, did not induce significant oxidation of DCFH (data not shown). Isolated mitochondria produced a linear time-dependent oxidation of DCFH-DA (Fig. 5A). Incubation of mitochondria with DADS at 1 mM produced a 2.7-fold increase in the oxidation rate of DCFH-DA, with respect to untreated mitochondria (Fig. 5A and Table 1). In contrast, mitochondria incubated with DAS at 1 mM did not show a significant change in DCFH-DA oxidation rate with respect to untreated organelles (Fig. 5A and Table 1). The oxidation rate of DCFH-DA in mitochondria increased with the concentration of DADS in the mixture (Fig. 5B). In order to evaluate the nature of the oxidizing species, mitochondria were incubated in the presence of DADS and inhibitors including: i) amphipathic free radical scavengers such as Trolox and 2,2,5,7,8-pentamethyl-6-chromanol (PMC); ii) iron chelators that inhibit the iron-driven Fenton reaction at high chelator/metal ratio such as EDTA and DTPA (Engelmann et al., 2003); iii) an enzymatic scavenger of hydrogen peroxide such as catalase; and iv) a scavenger of hydrogen sulfide such as hydroxocobalamin (Truong et al., 2009). Iron chelators and amphipathic free radical scavengers significantly inhibited the DADS-induced oxidation of DCFH-DA, while catalase and hydroxocobalamin were ineffective (Fig. 5C).

Garlic polysulfides can induce oxidative stress through redox-cycling reactions initiated by glutathione oxidation (Munday, 2012; Munday et al., 2003; Filomeni et al., 2008). If mitochondrial oxidative stress is promoted by reactions of DADS with reduced glutathione (GSH), then GSH should be depleted in mitochondria incubated with DADS. DADS at 1 mM produced a 76% decrease in the concentration of mitochondrial GSH with respect to un-treated organelles. In contrast, DAS at 1 mM did not change the concentration of mitochondrial GSH (Table 1).

Because reactive oxidant species can initiate lipid peroxidation, mitochondrial lipid peroxidation was evaluated in the presence or absence of garlic OSCs. Lipid peroxidation levels, evaluated as the concentration of TBARS and expressed as nano equivalents (neq) of MDA per milligram of mitochondrial protein, were not affected by incubation with DAS at 1 mM; on the contrary, incubation of mitochondria with DADS at 1 mM produced a 2.3-fold increase in TBARS with respect to untreated mitochondria (Table 1).

3.5. Effect of antioxidants on mitochondrial swelling and oxidative stress initiated by DADS

If DADS causes mitochondrial swelling via oxidative stress and lipid peroxidation, then mitochondrial swelling should be inhibited by antioxidants. DADS-induced mitochondrial

swelling was inhibited by lipid soluble antioxidants BHT and α -tocopherol; these agents also decreased DADS-induced lipid peroxidation (Table 2). Iron chelators that are effective inhibitors of lipid peroxidation reactions, such as EDTA and DTPA (Puntarulo and Cederbaum, 1988), significantly inhibited DADS-induced mitochondrial swelling, and acted as antioxidants decreasing DADS-induced mitochondrial lipid peroxidation (Table 2). In addition, catalase and hydroxocobalamin did not inhibit DADS-induced mitochondrial swelling (data not shown).

3.6. Effect of garlic-derived organosulfur compounds on mitochondrial membrane potential and cytochrome c release

Alterations in mitochondrial permeability can induce osmotic swelling of the mitochondrial matrix, together with dissipation of the mitochondrial membrane potential and mitochondrial outer membrane permeabilization. This may lead to the release into the cytosol of cytotoxic proteins, such as cytochrome c, which are normally confined within the mitochondrial intermembrane space (Fulda et al., 2010). Therefore, membrane potential and cytochrome c release was evaluated in mitochondria exposed to garlic-derived OSCs. Figure 6A shows that the respiratory substrates succinate and glutamate energized mitochondria (i.e. induce mitochondrial accumulation of rhodamine 123 followed by quenching of its fluorescence), an effect abolished by carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a protonophore. This confirms that isolated mitochondria were intact and fully functional. DADS (but not DAS) induced a concentration-dependent depolarization of mitochondria (Fig. 6B). Depolarization by DADS started after a lag period of around 150 s, and the depolarization rate increased with the concentration of DADS, in the range between 125 and 500 μ M (Fig. 6B). DADS-induced depolarization was completely prevented by amphiphilic antioxidants such as Trolox (Fig. 6C) or 50 μ M PMC (data not shown), and by iron chelators such as DTPA (Fig. 6D) or 1 mM EDTA (data not shown).

Organosulfur compounds including DAS and DADS did not induce cytochrome c release from isolated mitochondria; in contrast, cytochrome c release was evident in mitochondria treated with Triton X-100 as a positive control (Guicciardi et al., 2000) (Fig. 7).

4. Discussion

Osmotic mitochondrial swelling can occur according to two basic mechanisms: a) energy-dependent electrophoretic uptake of monovalent cations, and b) passive diffusion of chemical species following a decrease of the inner mitochondrial membrane permeability barrier (Bernardi et al., 1999). This last type of swelling can arise from the opening of the mitochondrial permeability transition pore, or from direct actions upon the membrane lipid phase (Pfeiffer et al., 1995). In turn, the permeability transition pore can exist in two functional modes: a regulated mode activated by Ca^{2+} and inhibited by cyclosporin A, or an unregulated mode that is Ca^{2+} -independent and insensitive to cyclosporin A (He and Lemasters, 2002). A hypothetical model proposed by He and Lemasters (2002) and supported by many experimental reports (Brookes et al., 2007; Puntel et al., 2010; Gadelha et al., 1997; Santana et al., 2009) maintains that the unregulated mitochondrial permeability transition pore forms by clustering of misfolded inner membrane proteins; protein misfolding is triggered by oxidation and cross-linking of membrane protein thiol groups. In our work, DADS at micromolar concentrations (but not DAS) induced mitochondrial swelling in isolated mouse liver mitochondria. Our results suggest that swelling caused by DADS is a passive phenomenon caused by direct oxidation of the membrane lipid phase, and not by opening of the permeability transition pore. This conclusion is supported by the following observations: a) DADS-dependent swelling occurred to the same extent in energized and de-energized mitochondria; b) DADS-dependent swelling was not inhibited by EGTA or cyclosporin A; c) DADS-dependent swelling was not associated with

significant membrane protein thiol oxidation or protein cross-linking; d) DADS-dependent swelling occurred together with lipid peroxidation, and both lipid peroxidation and swelling were inhibited by antioxidants.

What is the mechanism by which DADS induces mitochondrial lipid peroxidation? There is literature evidence that redox-cycling of disulfides produces reactive species that might induce oxidative stress (Fig. 8) (Munday, 2012; Munday et al., 2003; Filomemni et al., 2008). The initial step in the redox cycle is the reduction of disulfides (RSSR) to thiols (RSH) by GSH (Fig. 8, reaction 1). After ionization (Fig. 8, reaction 2), the thiolate anion (RS^-) undergoes one-electron oxidation in the presence of a high oxidation state transition metal (mainly Fe^{3+}), forming the thiyl radical $RS^\bullet$ (Fig. 8, reaction 3). In the presence of excess thiol, the thiyl radical forms the disulfide radical anion $RSSR^{\bullet-}$ (Fig. 8, reaction 4) which rapidly autoxidizes, yielding superoxide anion (O_2^-) and regenerating the disulfide, thereby closing the redox cycle (Fig. 8, reaction 5). The transition metal also cycles, because the high oxidation state of the transition metal is regenerated through autoxidation (Fig. 8, reaction 6). In the presence of excess thiol, superoxide anion might produce thiyl radical and hydrogen peroxide (H_2O_2) (Fig. 8, reaction 7). This set of reactions (1–7) generates reactive intermediates that can ultimately initiate lipid peroxidation. Lipid peroxidation could be initiated by:

- i. lipid alkoxyl radicals ($LO^\bullet$) resulting from the Fe^{2+} -mediated decomposition of preformed lipid hydroperoxides (LOOH, reaction 8):

- ii. an Fe(III):Fe(II): O_2 complex, of unknown structure, represented as perferryl ion (reaction 9):

- iii. hydroxyl radical ($^\bullet OH$) resulting from the Fe^{2+} -mediated decomposition of H_2O_2 (reaction 10):

- iv. thiyl radicals ($RS^\bullet$) generated in reactions 3 and 7.

All these reactive species have the oxidation potential to abstract a hydrogen atom from polyunsaturated fatty acids in mitochondrial membranes (LH) and initiate lipid peroxidation (reaction 11) (Caro and Cederbaum, 2004; Tweeddale et al., 2007).

Specific experimental evidence supports this mechanism: a) DADS (but not DAS) induced depletion of mitochondrial glutathione, in accordance with reaction (1); b) a key role for redox-active iron in the mechanism is suggested by the fact that DADS induces the oxidation of DCFH-DA, a redox probe that responds to changes in intracellular iron signaling (Kalyanaraman et al., 2012), and DCFH-DA and lipid oxidation are significantly blocked by iron chelators that form redox-inactive complexes; and c) the inhibition of DADS-induced oxidation of DCFH-DA by amphipathic free radical scavengers suggests the active generation of one-electron oxidants such as Fe^{2+}/O_2 , thiyl radicals and/or alkoxyl radicals (Kalyanaraman et al., 2012; Eruslanov and Kusmartsev, 2010) as anticipated in the

mechanism. The fact that catalase did not inhibit DADS-induced mitochondrial swelling and DCFH-DA oxidation suggests that hydrogen peroxide does not significantly contribute to the generation of oxidative stress in this model. Oxidative stress was evaluated in non-energized mitochondria (i.e. in the absence of respiratory substrates), under conditions where the generation of ROS (mainly H_2O_2 , because superoxide is rapidly converted to H_2O_2 by Mn-superoxide dismutase) by the mitochondrial electron transport chain is minimal (Liu et al., 2002). Therefore, the fact that catalase did not inhibit DCFH-DA oxidation in mitochondria incubated with DADS under non-energized conditions also suggests that the electron transport chain is not a significant source of oxidants under these conditions. An alternative mechanism for the generation of ROS by isolated mitochondria exposed to DADS involves the generation of hydrogen sulfide (H_2S) by the reaction of DADS with GSH, and the direct inhibition of cytochrome c oxidase by H_2S , followed by increased reduction of mitochondrial electron carriers (Truong et al., 2009). The observation that hydroxocobalamin, a scavenger of H_2S , does not inhibit DADS-induced DCFH-DA or swelling suggests that H_2S is not a significant source of oxidants in our model.

DADS-induced mitochondrial swelling correlated with lipid oxidative stress and oxidation of thiols groups in glutathione but not in mitochondrial membrane proteins. In contrast, experimental reports show that other thiol-reactive drugs acting on mitochondria induced mitochondrial swelling, in the absence of oxidative stress and associated with the oxidation of membrane protein thiols but not glutathione. For example, thioridazine (Cruz et al., 2010), organotelluranes (Pessoto et al., 2007) and palladacycles (Santana et al., 2009) induced swelling in isolated rat liver mitochondria, associated with oxidation of membrane protein thiols, in the absence of glutathione oxidation or oxidative stress. Therefore, although lipid peroxidation is a central mechanism by which DADS induce mitochondrial membrane permeabilization, lipid peroxidation is not absolutely required to produce mitochondrial impairment by thiol-oxidizing agents. Our results suggest that the mechanism by which thiol-reactive drugs induce mitochondrial membrane permeabilization and swelling depends critically on their selective reactivity towards glutathione or membrane protein thiols. Our work has identified DADS as a selective glutathione-oxidizing agent. This selective reactivity might depend on selective access to the oxidizable thiol groups, and/or relative redox potentials of the reacting partners.

In experimental models where swelling is induced by a decrease of the inner mitochondrial membrane permeability barrier, swelling occurs together with: i) decrease of the mitochondrial membrane potential caused by dissipation of the transmembrane proton gradient, and ii) mitochondrial outer membrane permeabilization because the surface area of the inner membrane considerably exceeds that of the outer membrane, followed by release of intermembrane space proteins such as cytochrome c (Fulda et al., 2010). In isolated mitochondria, DADS-induced swelling occurred together with mitochondrial depolarization, but in the absence of cytochrome c release. In contrast, DADS induced cytochrome c release in cultured cells (Lin et al., 2006; Yang et al., 2009; Lin et al., 2008). It has been reported that cytochrome c release from isolated mitochondria occurs by a two-step process: the first step involves the detachment of cytochrome c from the inner membrane which could occur by oxidation of mitochondrial lipids, particularly cardiolipin, and the second step involves the permeabilization of the outer membrane and the release of cytochrome c into the extramitochondrial medium, mediated by pore-forming Bcl-2 family members such as Bax or Bak (Ott et al., 2002). This suggests that the direct effects of DADS on mitochondria produce lipid peroxidation, but that for cytochrome c release to occur, extramitochondrial factors such as pro-apoptotic Bcl-2 family members may need to be present.

Alterations in mitochondrial membrane permeability and membrane potential are now thought to be a central regulatory mechanism for cell death induction (Susin et al., 1998;

Grimm and Brdiczka, 2007; Kroemer et al., 2007). DADS in the concentration range of 0.05 to 1 mmol/L induced cytotoxicity in human cells from neuroblastoma (Karmakar et al., 2007), colon adenocarcinoma (Xiao et al., 2005), melanoma (Wang et al., 2010b) and glioblastoma (Das et al., 2007), and rat hepatocytes (Truong et al., 2009). Our work suggests that direct effects of DADS on mitochondria, including mitochondrial permeabilization and depolarization, at concentrations in the same range as those reported to produce cytotoxic effects in intact cells, could be the first step in the induction of cell death by OSCs. Because mitochondrial depolarization by DADS was inhibited by antioxidants and iron chelators which also prevented lipid peroxidation and DCFH-DA oxidation, our work suggests that oxidative stress has a central role in the direct impairing effects of DADS on mitochondrial functionality.

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Abbreviations

OSCs	organosulfur compounds
DAS	diallyl sulfide
DADS	diallyl disulfide
DATS	diallyl trisulfide
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
DCFH-DA	2',7' dichlorofluorescein diacetate
GSH	reduced glutathione
GSSG	glutathione disulfide
Hepes	N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid)
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
buffer A	125 mM sucrose, 65 mM KCl, 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid)-KOH, 20 μ M Ca^{2+} , and pH 7.2
CsA	cyclosporin A
MOPS	3-(N-Morpholino)propanesulfonic acid
ROS	reactive oxygen species
TBARS	thiobarbituric acid-reactive substances
TBA	thiobarbituric acid
MDA	malondialdehyde
DTNB	5'-dithiobis(2-nitrobenzoid acid)
FSC	forward scatter
SSC	side scatter
NAO	10-N-nonyl acridine orange
Pi	inorganic phosphate

PMC	2,2,5,7,8-pentamethyl-6-chromanol
FCCP	4-(trifluoromethoxy)phenylhydrazine

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Highlights

- The direct effect of DAS and DADS on mitochondria was evaluated.
- DADS (not DAS) induced mitochondrial oxidative stress, swelling and depolarization.
- DADS-induced mitochondrial swelling did not involve the permeability transition pore.
- DADS-induced effects were inhibited by lipid antioxidants and iron chelators.
- Direct oxidative effects of DADS on mitochondrial lipids impair membrane permeability.

Figure 1.

Mitochondrial swelling induced by organosulfur compounds. A) Time curve of the effects of OSCs on mitochondrial swelling. Mouse liver mitochondria (0.5 mg protein/mL) were added to buffer A containing 1 mM DAS (■), 1 mM DADS (●) or ethanol at a final concentration of 1% v/v (○, solvent control). The absorbance at 540 nm was monitored for up to 10 min. B) Concentration curve of the effect of OSCs on mitochondrial swelling. Mouse liver mitochondria (0.5 mg protein/mL) were added to buffer A containing increasing concentrations (in the range 0.125–1 mM) of DAS (■) or DADS (●). Mitochondrial swelling was quantified as the maximal swelling amplitude after 10 min of incubation. C) Mouse liver mitochondria (0.5 mg protein/mL) were added to buffer A in the absence (succinate/glutamate –) or presence (succinate/glutamate +) of 5 mM succinate and 5 mM glutamate together with increasing concentrations of DADS in the range 0.25–1 mM. Mitochondrial swelling was quantified as the maximal swelling amplitude after 10 min of incubation.

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with the solvent control.

Figure 2.

Flow cytometry analysis of mitochondrial swelling. A) Selection of mitochondria based on forward and side scattering of light of individual mitochondria by flow cytometry. A mitochondrial suspension (0.5 mg protein/mL) was analyzed by flow cytometry, and forward scattering (FSC-A) and side scattering (SSC-A) parameters for each event were represented in a dot plot. 10,000 events were collected on gate P1, which represents the major event population. B) Selection of mitochondria based on NAO staining. An unstained (–NAO) or stained (+NAO) mitochondrial suspension at 0.5 mg protein/mL was analyzed by flow cytometry. After gating for the major event population in a FSC vs SSC dot plot, a histogram representing number of events as a function of fluorescence intensity in the FL1 channel (488 nm excitation, 530 ± 30 nm emission) was constructed. Numbers represent the percentage of events in the high fluorescence fraction of the event population. C) Effect of garlic OSCs on light scattering properties of isolated liver mitochondria. The mitochondrial suspension (0.5 mg prot/mL) was incubated in buffer A in the presence of 1 mM DAS, 1 mM DADS, or solvent (ethanol) control at 1% v/v for 5 minutes. Then, the suspension was immediately analyzed by flow cytometry, and after selecting for the P1 gate, a histogram representing number of events as a function of the intensity of forward scatter (FSC-H) was constructed for each condition. Numbers represent the percentage of events in the high forward scatter parameter fraction of the event population.

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with the solvent control.

Figure 3.

Effect of cyclosporin A or EGTA on mitochondrial swelling induced by DADS (A–B) or inorganic phosphate (Pi) (C–D). A) Mitochondria were incubated in buffer A in the presence of 1% v/v ethanol (solvent control), 1 mM DADS, 1 μM cyclosporin A (CsA) or the combination of 1 mM DADS and 1 μM CsA, and the change in absorbance at 540 nm was determined for 10 min. Mitochondrial swelling was quantified as the maximal swelling amplitude after 10 min of incubation. B) Mitochondria were incubated buffer A in the presence of 1% ethanol (solvent control), 1 mM DADS, 1 mM EGTA or the combination of 1 mM DADS and 1 mM EGTA, and the absorbance at 540 nm was determined for 10 min. Mitochondrial swelling was quantified as the maximal swelling amplitude after 10 min of incubation. C) Mitochondria were incubated in buffer A in the absence of any addition, or in the presence of 1 mM Pi, 1 μM CsA or the combination of 1 mM Pi and 1 μM CsA, and the change in absorbance at 540 nm was determined continuously for 10 min. D) Mitochondria were incubated in buffer A in the absence of any addition, or in the presence of 1 mM Pi, 1 mM EGTA, or the combination of 1 mM Pi and 1 mM EGTA, and the absorbance at 540 nm was determined continuously for 10 min.

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with the solvent control.

Figure 4.

Effect of garlic organosulfur compounds (OSCs) on mitochondrial membrane protein thiol groups. Mitochondria were incubated under the conditions described in Materials and Methods, in the presence of solvent control (1% v/v ethanol or 5% v/v DMSO for OSCs or ebselen, respectively), 1 mM DAS, 1 mM DADS, or 40 μ M ebselen. Mitochondrial membranes were prepared by the freeze-thawing technique described in Materials and Methods. A) The reduced thiol content of the mitochondrial membrane proteins was measured using DTNB. B) Representative SDS-polyacrylamide gel after electrophoresis of membrane proteins under non-denaturing conditions. The box represents the area of the gel with proteins with a molecular weight higher than 250 kDa, as determined by a protein molecular weight standard.

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with solvent control.

Figure 5.

Oxidative stress induced by garlic organosulfur compounds. A) Time curve of the effects of OSCs on oxidative stress. Mouse liver mitochondria (0.5 mg protein/mL) were suspended in buffer A containing 10 μ M DCFH-DA with the addition of 1 mM DAS (■), 1 mM DADS (●) or ethanol at a final concentration of 1% v/v (○, solvent control). The fluorescence at 485/528 nm excitation/emission was monitored for up to 6 min. B) Concentration curve of the effect of OSCs on oxidative stress. Mouse liver mitochondria (0.5 mg protein/mL) were suspended in buffer A containing 10 μ M DCFH-DA with the addition of increasing concentrations of DADS up to 1 mM. Oxidative stress in each condition was quantified as the linear rate of DCFH-DA oxidation. C) Effect of inhibitors on DADS-induced oxidative stress. Mouse liver mitochondria (0.5 mg protein/mL) were suspended in a buffer containing 10 μ M DCFH-DA and 0.25 mM DADS, in the absence or presence of 1 mM trolox, 50 μ M PMC, 1 mM EDTA, 1 mM DTPA, 100 U/mL catalase, or 100 μ M hydroxocobalamin. Oxidative stress in each condition was quantified as the linear rate of DCFH-DA oxidation. * significantly different ($p < 0.05$, ANOVA) with respect to untreated mitochondria. # significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with DADS in the absence of inhibitors

Figure 6.

Effect of garlic organosulfur compounds on mitochondrial membrane potential.

Mitochondrial membrane potential was monitored by fluorescence quenching of 0.2 μM rhodamine 123 in a mitochondrial suspension (0.5 mg protein/mL) made in buffer A. A) Mitochondria (mito) were energized with respiratory substrates succinate and glutamate both at 5 mM (succ/glu), and subsequently de-energized with a protonophore (100 nM FCCP). Fluorescence at 485/528 nm excitation/emission was continuously recorded. B) Mitochondria were incubated in the presence of respiratory substrates, and solvent (ethanol) control at 1% v/v (0 μM DADS), 500 μM DAS, or increasing concentrations of DADS (in the range 125–500 μM). Fluorescence at 485/528 nm excitation/emission was continuously recorded. C) Mitochondria were incubated in the presence of respiratory substrates and 250 μM DADS in the absence or presence of 1 mM Trolox. Fluorescence at 485/528 nm excitation/emission was continuously recorded. D) Mitochondria were incubated in the presence of respiratory substrates and 250 μM DADS in the absence or presence of 1 mM DTPA. Fluorescence at 485/528 nm excitation/emission was continuously recorded.

Figure 7.

Cytochrome c release induced by organosulfur compounds. Mitochondria (1 mg/mL) were incubated in buffer A with ethanol 1% v/v (solvent control), 1 mM DAS, 1 mM DADS or 0.1% Triton X-100 v/v for 15 min at 25 °C. The reaction mixture was then centrifuged at 12,000 x g for 10 min at 4 °C, and the supernatant was concentrated by ultrafiltration through Amicon 10K membranes. Proteins from the concentrated supernatant were size-fractionated by SDS-PAGE, transferred onto nitrocellulose, and probed with a polyclonal antibody specific to cytochrome c.

Figure 8. Proposed mechanism of redox cycling of diallyl disulfide. Please see the Discussion section for details. Numbers in parentheses represent the reaction described in the text.

Table 1

Oxidative stress indices. Mitochondria were exposed to 1 mM DAS, 1 mM DADS, or to solvent control (1% ethanol v/v), as described under Materials and Methods. After incubation, oxidative stress indices (including oxidation of DCFH-DA, content of reduced glutathione, and lipid peroxidation products assessed as thiobarbituric acid-reactive substances or TBARS) were quantified.

Addition	DCFH-DA oxidation rate (AU/min)	Reduced Glutathione (nmol/mg prot)	TBARS (neq MDA/mg prot)
Solvent control	27.4 ± 2.2	3.3 ± 0.9	20.5 ± 1.2
DAS 1 mM	28.8 ± 2.0	3.0 ± 0.9	21.8 ± 2.4
DADS 1 mM	73.1 ± 2.1 *	0.8 ± 0.5 *	48.4 ± 4.4 *

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with the solvent control.

Table 2

Effect of inhibitors on DADS-induced mitochondrial swelling and lipid peroxidation. Mitochondria were suspended in buffer containing lipid soluble antioxidants such as butylated hydroxytoluene (BHT) or alpha-tocopherol (α T), or iron chelators such as ethylenediamine tetracetic acid (EDTA) or diethylenetriamine pentaacetic acid (DTPA), or left untreated (addition, none). The mitochondrial sample was then incubated in the absence or presence of 0.25 mM DADS for up to 15 min at room temperature. Mitochondrial swelling (quantified as maximal swelling amplitude) and lipid peroxidation (evaluated as thiobarbituric acid-reactive substances) were assessed as described under Materials and Methods.

DADS (mM)	Addition	Maximal swelling amplitude (% ΔA^{540})	TBARS (neq MDA/mg prot)
0	None	1.2 $\pm$ 0.2	21.6 $\pm$ 2.1
0.25	None	11.3 $\pm$ 0.4 [#]	37.1 $\pm$ 2.8 [#]
0.25	BHT 1 mM	1.3 $\pm$ 0.1 [*]	20.2 $\pm$ 1.9 [*]
0.25	α T 0.1 mM	3.9 $\pm$ 0.3 ^{*#}	19.3 $\pm$ 1.5 [*]
0.25	EDTA 1 mM	5.1 $\pm$ 0.2 ^{*#}	22.2 $\pm$ 1.7 [*]
0.25	DTPA 1 mM	4.9 $\pm$ 0.2 ^{*#}	23.1 $\pm$ 1.3 [*]

* significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated with DADS and without any addition.

significantly different ($p < 0.05$, ANOVA) with respect to mitochondria incubated without DADS and without any addition.