Diallyl disulfide (DADS) enhances gap-junctional intercellular communication by both direct and indirect mechanisms in rat liver cells

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Diallyl disulfide (DADS), a sulfur compound from garlic, has been shown to exert many biological effects: induction of carcinoen detoxication, inhibition of tumor cell proliferation, etc. These effects are consistent with its anti-carcinogenic properties in animal models and could account for gap-junctional protective effects in humans. Our study demonstrates that DADS can improve gap-junctional intercellular communication (GJIC) in vitro. In rat liver epithelial cells (REL cells), using the dye transfer assay, we observe a temperature-dependent stimulation of GJIC by DADS at non-cytotoxic concentrations. In addition, incubation of cells with DADS for 1 h prevents the inhibition of GJIC induced by 3,5-di-tertio-butyl-4-hydroxytoluene (BHT). We have studied the direct effects of DADS on the regulation of GJIC, and especially on the expression and localization of the connexin expressed in these cells (Cx43): the enhancement of dye transfer (×1.6) by DADS from 1 to 50 μM is associated with an increase (×1.3–1.8) in the amount of Cx43 protein (western blotting) with no alteration of its localization in the cell-cell contact regions of the plasma membrane (immunofluorescence analysis). We have also explored the possibility that DADS might act indirectly on GJIC. On one hand, DADS does not change the amount of E-cadherin, the adhesion molecule expressed in epithelial cells. On the other hand, it induces rapid inhibition of protein glycosylation. The data suggest that DADS could reduce local constraints imposed by glycoproteins, thus facilitating dye transfer. In conclusion, DADS can be included with other plant microconstituents, which have been demonstrated to improve GJIC. Its effect on REL cells can be explained by its ability to enhance the amount of Cx43 and also to diminish the level of glycosylated proteins.

Introduction

Diallyl disulfide (DADS) is one of the sulfur compounds obtained from crushed garlic and represents 40–60% of garlic essential oil (1,2). Increasing interest is being shown for DADS due to its numerous biological activities (3,4). In particular, DADS could account for the observed protective effects of garlic on cancer development. Indeed several epidemiological studies have suggested a protective effect of a high intake of raw or cooked garlic on gastric cancer. Cohort studies show this inverse association for colorectal cancer (5). These results are supported by extensive in vivo data. In rodents, experimental studies have shown that garlic powder and organosulfurs, especially diallyl sulfide (DAS) and DADS, inhibit chemical-induced carcinogenesis in different organs (mammary gland, colon, esophagus, lung and liver) (6–10), when administered during the initiation or the promotion stages.

Various mechanisms of action have been proposed to explain DADS anticarcinogenic effects. As far as the initiation phase is concerned, DADS has been shown to reduce the mutagenicity of N-nitrosopiperidine and benzo[a]pyrene as well as the hepatic DNA breaks induced by aflatoxin B1 or N-nitrosodimethylamine (11–13). These effects could be related to the modulation of drug-metabolizing enzymes, which play a key role in xenobiotic activation as well as detoxication (14). DADS enhances the activities, protein and mRNA levels of microsomal P450 1A2 and P450 2B1/2. It increases the activities of different phase II enzymes such as glutathione S-transferase, UDP-glucuronyl transferase and epoxide hydrolase (15–17).

Up to now, one explanation for DADS anti-promoting effects has been its ability to retard the growth of established tumor cell lines, which has been demonstrated both in vitro and in vivo (18). In particular, DADS has been found to be considerably more efficient than the water-soluble monosulfide S-allyl cysteine in retarding the in vitro growth of human cells from colon, skin or lung tumors (19).

To explore other potential mechanisms of action, we focused our study on gap-junctional intercellular communication (GJIC). Gap junctions (GJ) are transmembrane channels, composed of proteins called connexins (Cx); they permit neighboring cells to communicate directly by sharing small cytoplasmic molecules like ions and second messengers (20). Increasing evidence indicates that GJIC alteration is involved in tumor cell development (21). It is hypothesized that it causes a disruption of the growth control of initiated cells by healthy surrounding cells, allowing the clonal proliferation of initiated cells (22,23). Several articles report that some tumor cells display a reduced communication capacity related to Cx gene alteration (24,25). Transfection of Cx genes in these cells results in GJIC recovery, growth normalization and tumorigenicity suppression (26). It has also been reported that many tumor promoters disrupt GJIC (27,28). While, conversely, a few compounds including dietary chemopreventive

Abbreviations: BHT, 3,5-di-tertio-butyl-4-hydroxytoluene; Cx, connexin; DADS, diallyl disulfide; DAS, diallyl sulfide; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GJ, gap junction; GJIC, gap-junctional intercellular communication; PBS, phosphate-buffered saline; REL cells, rat liver epithelial cells; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TTBS, Tween–Tris-buffered saline.

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constituents have been found to stimulate GJIC or to counteract its inhibition by tumor promoters (29–35).

We therefore studied the ability of DADS to modulate GJIC. Rat liver epithelial cells (REL cells) are particularly useful to study the modulatory effect of compounds whose target is GJIC and their mechanisms of action (36), so such a line was used in our study. With this model, we have already observed that retinoic acid and two flavonoids, apigenin and tangeretin, can increase GJIC (29).

The effect of DADS on the amount and localization of Cx43, the main Cx expressed in REL cells, was investigated. In addition, we hypothesized that the modulation of GJIC by DADS could result from a modification of cellular adhesion or glycosylation patterns and we therefore examined E-cadherin expression and protein glycosylation.

Materials and methods

Chemicals

DADS (purity 80%) was obtained from Aldrich Chemical Co. (France) and used without further purification, the remaining 20% being diallyl trisulfide and DAS (17). Lucifer yellow CH, dimethyl sulfoxide (DMSO) and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St Quentin Fallavier, France). α-[6-3H]Glucosamine hydrochloride (15 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Cell culture products, phosphate-buffered saline (PBS), Ham’s F10 Glutamax and antibiotics (penicillin, streptomycin, and gentamycin) were obtained from Invitrogen (Cergy Pontoise, France), and fetal calf serum (FCS) was purchased from Dutscher (Brumath, France). Monoclonal mouse anti-Cx43 and monoclonal mouse anti-E-cadherin were purchased from Transduction Laboratories (Le Pont de Claix, France). Monoclonal mouse anti-β-tubulin was purchased from Sigma-Aldrich. Peroxidase-conjugated anti-mouse IgGs and fluorescein isothiocyanate (FITC)-anti-mouse IgGs were from Jackson ImmunoResearch Laboratories (West Grove, PA), Materials, chemicals and molecular weight markers for western blot came from Bio-Rad (Marnes la Coquette, France).

Cell culture

REL cells were cloned from a REL cell line, established in our laboratory (37) and cryopreserved in liquid nitrogen. Cells were grown in Ham’s F10 medium with Glutamax supplemented with 10% FCS, 0.1% gentamycin and 1% penicillin (50 U/ml)–streptomycin (50 μg/ml). Cells were cultivated in a humidified incubator (5% CO2) at 37°C. For all experiments except cell proliferation curve, cells were seeded at the same density (44 000 cells/cm²): 4 x 10⁴ in 35-mm Petri dishes, 8 x 10³ in 24-well microplates, 2 x 10³ in 96-well microplates or 3 x 10⁴ on epoxy-treated slides (Merck Eurolab, Strasbourg, France). This density made it possible to obtain a confluent monolayer 48 h later. DADS was dissolved in DMSO and added to pure FCS, then sonicated later. DADS was dissolved in DMSO and added to pure FCS, then sonicated for 2 min. Ham’s-F10 was then added to give a 0.1% DMSO final concentration (this concentration did not affect GJIC, data not shown).

Cytotoxicity assay

Cell density determination. Cells were seeded into each of the 35-mm Petri dishes (three dishes per treatment). Twenty-four hours after seeding, cells were incubated with DMSO (0.1%) or DADS (1, 10, 25 or 100 μM) for 24 h. After trypsinization with 0.05% trypsin and 0.02% EDTA, cells from each dish were counted with a Coulter counter-channelizer (Coultronics, Roissy, France).

Neutral red uptake incorporation (38). For each treatment, incorporation of neutral red was measured in five wells from a 96-well microplate. Cells were incubated for 24 h with DMSO (0.1%) or DADS (1, 10, 25 or 50 μM), and then they were treated 3 h at 37°C with the neutral red solution (50 μg/ml of culture medium) (Sigma-Aldrich). Cells were washed three times with PBS and fixed with destain solution (1% glacial acetic acid, 50% ethanol, 49% distilled water). The neutral red uptake was then determined by spectrophotometry (Labsystems Multiskan MCC340 type 347, Les Ulis, France) at 540 nm.

Cell proliferation curve. 1.5 x 10⁴ cells were seeded into each of the 60-mm Petri dishes. One day after seeding, DMSO (0.1%) or DADS (10, 25, 50 or 100 μM) was added to the culture medium for an additional 1, 3, 6 or 8 days. We counted the cells of three separate 60-mm Petri dishes per treatment, with the Coulter counter.

Dye transfer

Intercellular coupling was determined using the microinjection dye transfer method described by Enomoto et al. (39). Cells were plated in each of the 35-mm Petri dishes. From day 1 (for 24 h incubation) or 2 (for short incubations) cells were incubated with DADS or 0.1% DMSO. In kinetic studies, DADS 10 or 25 μM was added for 1, 4, 8 or 24 h. In dose-response studies, two Petri dishes with DMSO or DADS at 1, 5, 10, 25 or 50 μM and all microinjections were performed at day 2. Single cells were microinjected for 1 s with 5% (w/v) lucifer yellow CH (diluted in 0.33 M lithium chloride) using capillaries driven by a micromanipulator (IMT2-SYF, Tokyo, Japan) coupled to a pressure control unit (Eppendorf model 5242, Hamburg, Germany). All manipulations were performed under a microscope equipped with epifluorescence (Olympus, Rungis, France). The glass capillaries used were prepared in a microinjection setup (Clark Electromedical Instruments) and were prepared by an automatic horizontal puller (Harishige, Tokyo, Japan). Ten minutes after the last microinjection, cells were fixed with 4% formaldehyde in PBS and the dye transfer was quantified by counting the number of fluorescent cells surrounding the microinjected cell as described previously (40). As only about half of the microinjections were successful, 30–40 microinjections/dish were necessary to allow precise quantification of GJIC competency.

For each treatment, data are the mean ± SD of 10–20 fluorescent areas. Data from independent or pooled experiments were examined by an analysis of variance (ANOVA). Then, differences between control (untreated cells) and treated groups were assessed using the Student–Newman–Keuls test (parametric) if used when possible) or Dunn’s method (non-parametric test). To compare results from independent experiments, the modulating factor was calculated, as the ratio of the number of dye-coupled cells in treated dishes on the number of dye-coupled cells in control dishes.

Western blot analysis

Cells were plated in 35-mm Petri dishes. Twenty-four hours after seeding, one Petri dish was incubated with DADS 1, 5, 10, 25 or 50 μM or 0.1% DMSO. Total proteins were recovered the following day. Cells were washed twice with PBS and scraped in 2 x sample buffer (2 x sample buffer: 250 mM Tris–HCl, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, 200 mM DTT). After sonication, 2 x sample buffer was diluted 1 x with water. Proteins (corresponding to 25 x 10³ cells) were loaded on an 8 or 15% SDS-polyacrylamide gel for electrophoresis, and then electro-transferred (60 mA, 4°C for 2 h) to a PVDF-membrane (Hybond ECL, Amersham, Orsay, France). Brain protein extracts (Transduction laboratories) were used as Cx43 positive control and liver protein extracts as negative control. The membrane was blocked for 2 h with 5% skimmed milk powder in Tween–Tris-buffered saline (TTBS) containing 0.1% Tween 20 (Merck Eurolab), 2.5 mM NaCl and 20 mM Tris–Cl. Membranes were hybridized overnight at 4°C with anti-Cx43 (1:250) or anti-E-cadherin (1:2500) antibodies, diluted in TTBS + 5% skimmed milk powder. Membranes were then washed four times with TTBS and incubated with peroxidase-conjugated anti-mouse IgGs (1:12 000). The immunopositive reaction was detected using an ECL Plus kit (Enhanced Chemiluminescence plus) (Amersham) and revealed using an autoradiography film (Hyperfilm ECL, Amersham). Proteins were quantified by densitometry of the autoradiogram using a Las 1000 camera (Fujifilm, Paris, France) and Aida software (Raytest, Strasbourg, France). To verify the homogeneity of sample deposition, membranes were stripped and re-probed with a monoclonal anti-β-tubulin (1:20 000) and then with peroxidase-conjugated anti-mouse IgGs (1:12 000). In this case, the immunopositive reaction was detected using an ECL kit (Enhanced Chemiluminescence) (Amersham).

Immunolocalization

For immunohistochemistry, cells were seeded onto epoxy-treated slides. Twenty-four hours after seeding, cells were treated with 0.1% DMSO or DADS (1, 5, 10, 25 or 50 μM) for 24 h. They were then briefly washed three times with PBS, permeabilized with 0.25% Triton X-100–4% paraformaldehyde for 2 min and fixed with 4% paraformaldehyde in PBS for 30 min. Cells were pre-hybridized for 1 h in PBS–2% bovine serum albumin then incubated with anti-Cx43 (1:50) or anti-E-cadherin (1:250) monoclonal antibodies, followed by FITC anti-mouse IgGs (1:200). After mounting in a Vecta-Shield (Vector Laboratories, Biovalley, Marne-la Vallée, France), samples were observed (×400) using an Olympus fluorescent microscope equipped with a camera (DPS5) (Olympus, Rungis, France).

Measurement of glycosylation

To study protein glycosylation, we used the [1H]glucosamine-incorporation method (41). To avoid competition between the glucose from the medium and [1H]glucosamine, 5% FCS in MEM medium without glucose (Invitrogen) was used.
Results

Up to 50 μM DADS has no cytotoxic effect

As the cell density of a monolayer influences the ability of cells to come into contact and to communicate, we first determined the effect of increasing concentrations of DADS on cell density and cell viability. We observed that after a 24 h exposure, DADS up to 50 μM had no effect on cell density (Table I), whereas 100 μM DADS caused a 40% decrease. However, even at 100 μM the neutral red uptake was not significantly decreased, suggesting that DADS had a cytostatic effect rather than a cytotoxic one. To confirm this cytostatic effect we studied the effect of DADS in conditions of exponential cell proliferation (Figure 1). We observed an inverse relationship between the concentration of DADS (from 10 to 100 μM) and cell proliferation. Exposure to 10 μM DADS for 8 days did not cause any alteration of the growth rate. DADS reduced the cell number by half (at 50 μM and totally at 100 μM), without any detachment of cells from the dishes, which confirms the absence of cytotoxicity. These observations prompted us to use DADS in the following experiments at concentrations comprised between 1 and 50 μM for 24 h incubations.

DADS enhances GJIC and prevents the inhibition of dye transfer by BHT

To assess GJIC, cell–cell transfer of the fluorescent tracer lucifer yellow was analysed as a function of DADS concentration and time. Subconfluent REL monolayers were incubated with DADS at 10 or 25 μM for 1, 4, 8 or 24 h. A stimulation of GJIC by DADS was observed. As reported in Table II, in the presence of DADS 10 or 25 μM, the enhancement was

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Treatment</th>
<th>Average number of dye-coupled cells/ microinjectiona</th>
<th>Modulation factorb</th>
<th>Total number of fluorescent areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>Control</td>
<td>314 ± 17</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>DADS 10 μM</td>
<td>334 ± 17</td>
<td>1.1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>DADS 25 μM</td>
<td>337 ± 34</td>
<td>1.1</td>
<td>14</td>
</tr>
<tr>
<td>4 h</td>
<td>Control</td>
<td>244 ± 13</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>DADS 10 μM</td>
<td>240 ± 21</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>DADS 25 μM</td>
<td>242 ± 11</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>8 h</td>
<td>Control</td>
<td>260 ± 12</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>DADS 10 μM</td>
<td>325 ± 24</td>
<td>1.2</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>DADS 25 μM</td>
<td>345 ± 21</td>
<td>1.3</td>
<td>18</td>
</tr>
<tr>
<td>24 h</td>
<td>Control</td>
<td>262 ± 14</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>DADS 10 μM</td>
<td>378 ± 39c</td>
<td>1.4</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>DADS 25 μM</td>
<td>400 ± 40c</td>
<td>1.5</td>
<td>19</td>
</tr>
</tbody>
</table>

GJIC was detected by lucifer yellow dye transfer in confluent monolayered REL cells. Cells were untreated (control) or treated with 10 or 25 μM DADS during 1, 4, 8 or 24 h before microinjection.

Table II. Kinetic analysis of the stimulation of junctional transfer by DADS

aValues are means ± SD of fluorescent cells in one representative experiment.

bThe modulation factor represents the ratio: number of dye coupled cells in treated dishes/number of dye-coupled cells in control dishes.

cFor these treatments, the average number of dye coupled cells is significantly (by Dunn’s method) higher than the corresponding control value (P < 0.05).
significant after 8 h of treatment (×1.2 at 10 μM) and 24 h (×1.5 at 25 μM). Figure 2 illustrates the stimulation of GJIC in REL cells treated with 25 μM DADS for 24 h in comparison with control cells.

The data of several independent experiments focused on a 24 h incubation with DADS are summarized in Table III. They indicate that the increase of the dye transfer by DADS was observed from 1 μM, and reached a maximum level at 25 μM with a corresponding modulation factor of 1.6.

We also examined the effects of DADS on the inhibition of GJIC induced by a tumor promoter, BHT. As shown previously (31), when cells were treated with BHT at 10 μM, dye transfer was reduced in as little as 10 min (Table IV). In co-incubation experiments, 10 μM DADS caused a half reduction of the GJIC inhibition induced by BHT (20% instead of 40% of the corresponding control).

**DADS increases the amount of Cx43 but does not modify its phosphorylation state nor its localization**

One possible mechanism by which GJIC might be increased is the up-regulation of Cx expression. We studied the amount and/or localization of Cx43 after a 24 h treatment with or without DADS (1, 5, 10, 25 or 50 μM). In order to measure the effect of DADS on the total amount of Cx43, we used a 15% polyacrylamide, which concentrates the different Cx43 species in a single band (Figure 3A). A marked increase in immunolabeling of Cx43 was observed, with 5, 10, 25 and 50 μM DADS, and the corresponding factors were 1.3, 1.2, 1.4 and 1.8 (Figure 3B).

Using a more resolvent gel (8% polyacrylamide gel), which separates non-phosphorylated Cx43 (43 kDa) and phosphorylated Cx43 (45–47 kDa) (42), we observed that DADS did not change the number of detected bands (Figure 4), but rather increased all bands uniformly. The stimulation factors for different concentrations of DADS were approximately the same as in the experiments above (data not shown). Thus, DADS increased the amount of Cx43 without affecting its phosphorylation state.

To assess cellular distribution of Cx43, immunocytochemical staining of Cx43 was performed under the same conditions of seeding and incubation. Under control conditions (without DADS treatment), Cx43 staining appeared as brightly fluorescent spots on plasma membranes, at cell-cell contacts (Figure 5). No modification in Cx43 distribution was detected after cell exposure to 10 and 25 μM DADS.

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**Table III.** Level of communication in confluent cells exposed to DADS for 24 h

<table>
<thead>
<tr>
<th>Treatment (24 h)</th>
<th>Average number of dye-coupled cells/microinjection(^a)</th>
<th>Modulation factor(^b)</th>
<th>Total number of fluorescent areas(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>228 ± 17</td>
<td>1</td>
<td>99 (7)</td>
</tr>
<tr>
<td>DADS 1 μM</td>
<td>275 ± 28(^d)</td>
<td>1.2</td>
<td>109 (7)</td>
</tr>
<tr>
<td>DADS 5 μM</td>
<td>324 ± 55(^d)</td>
<td>1.4</td>
<td>90 (7)</td>
</tr>
<tr>
<td>DADS 10 μM</td>
<td>340 ± 30(^d)</td>
<td>1.5</td>
<td>96 (7)</td>
</tr>
<tr>
<td>DADS 25 μM</td>
<td>358 ± 30(^d)</td>
<td>1.6</td>
<td>56 (5)</td>
</tr>
<tr>
<td>DADS 50 μM</td>
<td>366 ± 44(^d)</td>
<td>1.6</td>
<td>55 (5)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means ± SD of microinjections from six independent experiments.

\(^b\)Modulation factor represents ratio: number of dye-coupled cells (treated dishes)/number of dye-coupled cells (control dishes).

\(^c\)Values in parentheses are numbers of independent experiments.

\(^d\)For these treatments, the average number of dye-coupled cells is significantly higher (by Student–Newman–Keuls test) than the corresponding control value (\(P < 0.05\)).

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**Table IV.** Level of communication in confluent cells exposed to DADS alone or in combination with a tumor promoter BHT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average number of dye-coupled cells/microinjection(^a)</th>
<th>Modulation factor(^b)</th>
<th>Total number of fluorescent areas(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134 ± 14</td>
<td>1</td>
<td>49 (6)</td>
</tr>
<tr>
<td>DADS 10 μM, 1 h</td>
<td>137 ± 10</td>
<td>1</td>
<td>45 (6)</td>
</tr>
<tr>
<td>BHT 10 μM, 10 min</td>
<td>76 ± 7(^d)</td>
<td>0.6</td>
<td>67 (6)</td>
</tr>
<tr>
<td>DADS 10 μM 1 h + BHT 10 μM 10 min</td>
<td>108 ± 10(^d)</td>
<td>0.8</td>
<td>55 (6)</td>
</tr>
</tbody>
</table>

\(^a\)Values are means ± SD of microinjections from six independent experiments.

\(^b\)Modulation factor represents ratio: number of dye-coupled cells (treated dishes)/number of dye-coupled cells (control dishes).

\(^c\)Values in parentheses are numbers of independent experiments.

\(^d\)For each independent experiment, the average number of dye-coupled cells is significantly higher (by Student–Newman–Keuls test) than the corresponding control value (\(P < 0.05\)).
It is admitted that before the formation of GJs a strong adhesive interaction between neighboring cells is required. E-cadherin, a transmembranous Ca$^{2+}$-dependent cell–cell adhesion molecule specifically expressed in epithelial cells and involved in epithelial formation and integrity (43), is necessary for Cx assembly in GJ (44).

Thus, we investigated the possible relationship between DADS stimulation of GJIC and E-cadherin expression. Cells were treated or not with 1–50 μM DADS for 24 h. Under control conditions, E-cadherin protein of REL cells was detected by western blot (8% polyacrylamide gel) as a band corresponding to a molecular mass of 120 kDa (Figure 6A). No difference was observed between control and DADS-treated cells (Figure 6B), indicating that DADS GJIC stimulation did not result from an induction of E-cadherin expression.

**DADS inhibits the glycosylation state of cell proteins**

Wang and Mehta (41,45) show, in various normal and transformed cells, that the treatment by tunicamycin, a glycosylation inhibitor, induces an enhancement of GJIC. They suggest that oligosaccharide moieties of glycoproteins of the plasma membrane impose local constraints that are unfavorable to GJ assembly.

To explore whether the DADS stimulation of GJIC is the consequence of an inhibition of protein glycosylation, we measured the incorporation of the glycosylation precursor [$^{3}$H]glucosamine into proteins (TCA-insoluble material) (Figure 7) under two different experimental conditions. When cells were incubated with DADS and [$^{3}$H]glucosamine for 1 h simultaneously, we observed that DADS inhibited the precursor incorporation in a dose-related manner, without any alteration of the total cellular protein content (data not shown). The inhibition of glucosamine incorporation reached 85% at 50 μM. When cells were treated with DADS for 24 h and with [$^{3}$H]glucosamine for the last hour, a weak inhibition was observed (40% at 50 μM). The data suggest that the inhibition of glycosylation by DADS is dose-dependent and rapid.

**Discussion**

Until now, only one study concerning the effects of garlic oil on GJIC has been available (46): using the photobleaching method, it was shown that garlic oil increased GJIC in gastric cells; however, the active compounds and their mechanisms of action were not studied. Our study is the first to demonstrate the ability of DADS to enhance GJIC and to prevent its inhibition by a tumor promoter. This sulfur compound is thus a new type of food-borne GJIC stimulator in addition to retinoids (30,34), carotenoids (32,47) and flavonoids (29,33).

In REL cells incubated with DADS, we observed a significant enhancement of dye transfer from 8 h of treatment. The maximum stimulating factor (1.6 at 24 h) is similar to those obtained with retinoic acid and flavonoids on the same cells (29,30). DADS is active on GJIC at a rather low dose. After a 24 h treatment, its effect is significant from 1 μM and up to a maximum at 25 μM. At these concentrations, DADS has no effect on cell proliferation, but it has a cytostatic effect at higher concentrations (50 and 100 μM). This result obtained from liver cells is consistent with studies focused on proliferation of different tumor cell lines which showed a cytostatic effect of DADS at high concentrations (25 μM to 2 mM) (18). Moreover, we found that DADS, is a more potent stimulator of GJIC than DAS (data not shown), which contains a single sulfur atom. Our results suggest the importance of the number of sulfur atoms, already emphasized by Bose et al. for the induction of detoxication enzymes (48).

We explored the possible mechanisms involved in the enhancement of GJIC by DADS and found that DADS can modify GJIC regulation by both direct and indirect mechanisms. Interestingly, western blotting showed DADS increased the amount of Cx43 without changing the phosphorylation state of the protein. Such effects had also been observed for retinoic acid and flavonoids in previous studies (29,30). The increase of Cx43 detected in DADS-treated cells was not
associated with any modification of protein localization. We hypothesize that either DADS does not enhance the density of GJ in the plasma membrane or this enhancement is too diffuse to be detected by immunochemistry. As for retinoic acid, in REL cells, DADS did not increase the amount of Cx43 mRNA (not shown), suggesting that the modification of the amount of Cx43 protein could result from an increase in its half-life.

Indirect mechanisms of GJIC regulation also deserved study. Cell–cell recognition and adhesion mediated by E-cadherin seems to be a prerequisite for GJ assembly in the plasma membrane (43). Some modulators of GJIC are known to have indirect effects mediated by E-cadherin expression (49). In our study, whereas 5–50 μM DADS treatment for 24 h increased the amount of Cx43, it did not modify E-cadherin protein expression in REL cells. Another possible indirect mechanism of GJIC regulation was analyzed: the modulation of protein glycosylation, which affects a lot of membrane proteins (but not Cx43, as they are not glycosylated) (45). We observed that DADS, in the concentration range of 5–50 μM, was capable of decreasing [3H]glucosamine incorporation in proteins under two experimental conditions: when cells were incubated with DADS for 23 h prior to tracer incorporation, and when they were co-incubated for 1 h. DADS was more efficient in the latter case, indicating an early effect that is partly lost after a 23 h treatment.

As proposed previously by Wang and Mehta (41) for the glycosylation inhibitor, tunicamycin, DADS inhibition of protein glycosylation could diminish the ratio of bulky glycoproteins in the membranes of adjacent cells. In turn, it could help cells to come into close contact and hemichannels to interlock and form diffuse GJ. This hypothesis is consistent with the increase in Cx43 amount induced by DADS. The latter could reflect an increase of Cx43 half-life due to better stability of GJ in the membranes.

To complete this new insight into the mechanisms of DADS action and since Cx43 is present in many tissues and organs such as the stomach, the colon, the bladder, the breast, the brain, it would be interesting to further analyze its role in GJIC modulation by studying whether DADS modulates GJIC in other cell types expressing Cx43. In particular, one could verify whether DADS is responsible for the effect of garlic oil on GJIC stimulation in gastric cells (46). Recently, Qin et al. (50) have demonstrated that Cx proteins can mediate tumor growth independently of GJIC. Further studies should therefore examine the relationship between the increase in Cx43 amounts and cell functions other than GJIC.

Fig. 5. Immunofluorescent staining of Cx43 protein in control cells and cells treated with DADS for 24 h. Cells were plated on epoxy-treated slides and Cx43 proteins were detected with Cx43 antibodies followed by an FITC-conjugated second antibody.
DADS is formed from its natural precursor allicin when a garlic clove is crushed, but the fate of DADS in the body after consumption of garlic has not been clearly established. DADS is rapidly and extensively metabolized. It would therefore be interesting to test if metabolites have the same effects as DADS on GJIC, in order to identify the active compounds. For pharmacological applications, their ability to restore GJIC in non-communicating cancer cells could be investigated, as other plant microconstituents exhibit such a property (54).

In other respects, we have shown that, whereas DADS treatment alone for 1 h did not have any rapid effect on dye transfer, DADS pre-treatment for 1 h partly antagonized the BHT-induced inhibition of GJIC. Such an effect has been observed previously with two flavonoids, apigenin and tangeretin, in REL cells (31). BHT at rather low concentration (10 μM) reduced the dye transfer by half in 10 min, probably, as suggested by Guan et al. (55), through a modification of GJ permeability. The mechanisms by which DADS can counteract this inhibition remain to be elucidated. Taken together, the ability of DADS to enhance GJIC alone and its capacity to counteract the inhibition induced by a tumor promoter confers to this molecule interesting properties that complete the range of its potential anticarcinogenic activities.

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References


![Fig. 6. Effect of DADS on the amount of E-cadherin protein. (A) Western blot analysis (8% polyacrylamide gel) was performed with total proteins extracted from rat liver, rat brain, untreated cells and cells treated with DADS 1–50 μM. (B) Three independent experiments were performed. Each spot was standardized with α-tubulin protein. The amount of E-cadherin was expressed as the percentage of control. Results are mean ± SEM of triplicates.](image)

![Fig. 7. Inhibition of glycosylation by DADS in REL cells. The incorporation of 3H-glucosamine into TCA-insoluble material from 50 μl cell lysate was measured as described in Materials and Methods. The data represent the mean ± SEM from six separate experiments. CPM = counts per min.](image)


