

Diallyl Disulfide Increases Rat H-Ferritin, L-Ferritin and Transferrin Receptor Genes In Vitro in Hepatic Cells and In Vivo in Liver

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ABSTRACT Of the oil-soluble organosulfur compounds derived from garlic, diallyl disulfide (DADS) is one of the most abundant. We examined the effect of DADS on gene expression in rat liver. By suppressive subtractive hybridization, we identified the heavy (H)-ferritin gene as a DADS-stimulated gene in the rat liver epithelial (REL) cells. DADS stimulation of H- and L (light)-ferritin mRNA was analyzed in REL cells and in rat liver. Incubation of the REL cells in 10 $\mu\text{mol/L}$ DADS for 4 h increased H-ferritin 1.9 ± 0.2 -fold, $n = 3$) and light(L)-ferritin mRNA 1.5 ± 0.2 -fold, $n = 3$). Stimulation did not occur in the presence of an inhibitor of transcription, actinomycin D. Stimulation of ferritin at the RNA and protein levels was also found in rats administered a DADS-enriched oil solution intragastrically. There was a 3 ± 1.1 -fold increase in H- and 3 ± 0.14 -fold increase for L-ferritin mRNA 24 h after the end of the infusion in the presence of DADS, ($n = 3$). The expression of the transferrin receptor, an iron transporter, was also enhanced by DADS in rat liver. In conclusion, our data suggest that DADS could modify iron homeostasis through the modulation of ferritin and transferrin receptor gene expression. **J. Nutr.** 132: 3638–3641, 2002.

KEY WORDS: • organosulfur compounds • garlic • iron metabolism • subtractive hybridization

Garlic (*Allium sativum*) has been used for its health benefits by different populations over the centuries (1). Diallyl disulfide (DADS)² is one of the most abundant molecules found in processed garlic. When a garlic clove is crushed, the odorless precursor, allin, is rapidly converted into allicin, which decomposes instantly into other oil-soluble compounds, such as diallyl sulfide (DAS) and DADS (Fig. 1A) (2). Among garlic constituents, DADS appears to be the most potent at reducing

the growth of a wide range of cell lines (3). In animals, DADS has been shown to decrease the number and size of chemically induced tumors (4). The chemopreventive aspects of DADS are often correlated with its capacity to modulate activities of drug-metabolizing enzymes at different levels (mRNA, protein or enzymatic activity) (5).

The objective of the present work was to isolate previously unidentified genes whose transcription could be stimulated by DADS. The heavy (H)-ferritin gene has been identified by subtractive screening as a gene with heightened expression in the rat liver epithelial (REL) cells treated with 10 $\mu\text{mol/L}$ DADS. Ferritin composed of both H- and light (L)-subunits is involved in iron sequestering (6). We therefore analyzed H- and L-ferritin mRNA sensitivity toward DADS in REL as well as in the liver of rats receiving 100 mmol/L DADS by gastric infusion. We also tested the effect of DADS on the transferrin receptor (Tfr), which delivers iron into cells (7).

MATERIALS AND METHODS

Chemicals and cell culture. DADS ($\text{CH}_2=\text{CHCH}_2\text{SSCH}_2\text{CH}=\text{CH}_2$; purity 80%), dimethyl sulfoxide (DMSO) and actinomycin D were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesses, France). DADS was used without further purification (8). Two different batches of DADS were used in the study.

REL cells were isolated from newborn rat livers and cultivated as previously described (9,10). DADS was dissolved in 0.1% DMSO and fully homogenized with ultrasonic waves (11). For experiments using actinomycin D, REL cells were incubated with (4 mg/L) actinomycin D for 30 min and 10 $\mu\text{mol/L}$ DADS was then added to the culture medium for an additional 4 h. All treatments were repeated three times.

Subtractive method. All procedures were performed as previously described (12). The driver consisted of cDNA amplicons from REL cells treated for 8 h with DMSO; the tester consisted of the cells treated the same length of time with 10 $\mu\text{mol/L}$ DADS. Progressive subtraction was obtained by mixing the driver and tester pools in a molar ratio increasing from 10 to 1000 and then 10,000. The low quantity of any housekeeping cDNA in the final subtractive population was a criterion for stopping the protocol (data not shown). A total of 26 fragments were sequenced; among them, 19 clones matched H-ferritin cDNA.

Rat surgery. Wistar male rats (Iffa Credo, L'Arbresle, France) weighing 300–350 g were housed individually in metabolic cages. They were fed M25 pellets (Dietex; Saint-Gratien, France) composed of wheat, soybean meal, fish meal, dehydrated alfalfa, fine bran, molasses, and a mineral and vitamin mix. This diet contains 23% crude protein, 3% lipids, 3.3% crude fiber and 105 mg/kg iron. The surgery technique was described previously (13). Inside the cage, rats were given free access to food and water. During the 8-d recovery period, all rats gained weight (data not shown). Thereafter, 13 mL of rapeseed oil or rapeseed oil enriched with 0.23 mL DADS (100 mmol/L) was administered through a gastric catheter connected to a peristaltic pump. Gastric administration was started at 1000 h (except for the S12 samples, which were launched at 1700 h) and continued for 4 h. Rat livers were removed immediately (S0), or at 12 (S12), 24 (S24) or 48 (S48) h after the end of the infusion under isoflurane-induced anesthesia (Abbott, Rungis, France). After gastric infusion, we verified as well as possible that the rats had not modified their food intake to any considerable degree (data not shown).

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² Abbreviations used: DADS, diallyl disulfide; DAS, diallyl sulfide; DMSO, dimethyl sulfoxide; REL, rat liver epithelial; Tfr, transferrin receptor.

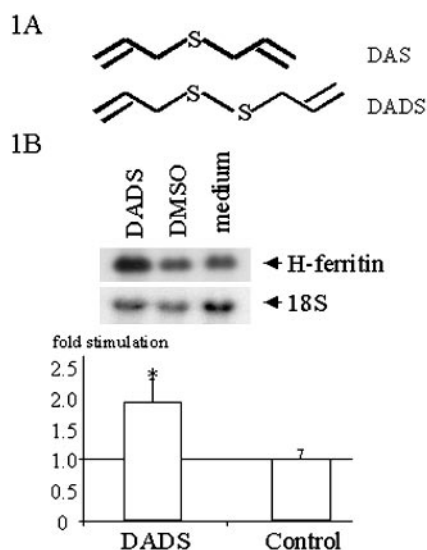


FIGURE 1 Heavy (H)-ferritin is a diallyl disulfide (DADS)-induced gene in rat liver epithelial (REL) cells. (A) Molecular structure of diallyl sulfide DAS and DADS. (B) Northern blot analysis of RNA isolated from REL cells without treatment (medium; $n = 4$), in the presence of 10 $\mu\text{mol/L}$ DADS ($n = 4$) or dimethyl sulfoxide (control) for 8 h ($n = 4$). The same membrane was hybridized with H-ferritin and 18S. Data obtained by quantification of Northern blot membranes were normalized to the 18S signal. *Different from control, $P = 0.017$ (t test).

Rats administered DADS had soft feces, and in a few cases, died ~ 15 h after the end of infusion. In rats, lethal or toxic effects of high DADS doses have been described (14,15). All aspects of the protocol conformed to the International Guiding Principles for Biomedical Research Involving Animals.

Northern blot analysis. RNA (15 μg) from cells or tissues was separated in an agarose-denaturing gel and transferred to a membrane. Blots were hybridized in Quikhyb solution (Stratagene, Amsterdam, Netherlands) with [α - ^{32}P] dCTP probes labeled by the Rediprime II system (Pharmacia Biotech, Orsay, France). The probes were obtained using the RDA procedure (H-ferritin) and by amplification from a rat liver cDNA library (L-ferritin and Tfr). All probes were verified by sequencing. A murine 18S ribosomal RNA probe was used to assess the uniformity of RNA loading.

Western blot analysis. Total proteins were extracted from liver homogenates as previously described (16). Protein (5 and 10 μg) was diluted in Laemmli buffer and loaded onto a 12.5% SDS-polyacrylamide gel. The weak resolution of the gel did not allow separation of the H- and L-ferritin subunits. Ferritin was revealed using peroxidase-conjugated anti-human ferritin (Rockland, Gilbertsville, Pa) (dilution 1:5000) and the enhanced chemiluminescence (ECL+) detection system (Pharmacia Biotech). The protein band was assessed according to its apparent molecular weight compared with standard proteins and to a purified human type IV ferritin (Sigma Aldrich).

Quantification and statistical analysis. The signals on membranes were quantified using a Fla3000 imager (Fujifilm; Paris, France) and data were analyzed using Aida software (Raytes, Paris, France). Data are means \pm SD. Data were analyzed by t test (Fig. 1B) or by ANOVA and the Student-Newman-Keuls test (Fig. 2) with SigmaStat software (L'Isle d'Abeau Chesnes, France). Differences were considered significant at $P < 0.05$.

RESULTS

Identification of H-ferritin as a DADS-responsive gene. The H-ferritin gene was identified by a subtractive method as a gene with heightened expression in the REL cells incubated for 8 h in the presence of 10 $\mu\text{mol/L}$ DADS. The stimulation of H-ferritin mRNA by DADS treatment was confirmed by

Northern blot assay (Fig. 1B; stimulation fold: 1.9 ± 0.4 , $n = 4$).

Both subunits of ferritin were stimulated by DADS. Because ferritin is a multimeric protein composed of 24 subunits of H- and L-chains, we analyzed the stimulation of H- and L-ferritin mRNA after 10 $\mu\text{mol/L}$ DADS treatment for 4 h (Fig. 2). Stimulations were 1.9 ± 0.2 -fold ($n = 3$) and 1.5 ± 0.2 -fold ($n = 3$) induction above the basal value for H- and L-ferritin, respectively (Fig. 2A). No further enhancement of either mRNA was found at 50 $\mu\text{mol/L}$ DADS (Fig. 2A). When REL cells were preincubated for 30 min in the presence of actinomycin D, DADS did not stimulate H- and L-ferritin mRNA (Fig. 2B).

Stimulation of ferritin by DADS was observed in vivo. We tested the ability of DADS to stimulate H- and L-ferritin in rats that were infused by a gastric catheter with rapeseed oil containing 100 mmol/L DADS for 4 h. Infusion of DADS increased H- and L-ferritin mRNA levels at S12, with a maximum at S24 (Fig. 3A). At S24, H- and L-ferritin levels increased 3 ± 1.1 -fold for H and 3 ± 0.14 -fold for L ($n = 3$). Nevertheless, a slight increase in both mRNA levels occurred in rats administered rapeseed oil alone at S24 and S48, which may have been due to the oil itself. Consistent with the Northern blot analysis, the ferritin protein content was also enhanced in rats infused by DADS at S24 (Fig. 3B). A human purified protein was loaded to control the migration profile even though it migrated slightly faster than the rat version.

Transferrin receptor was sensitive to DADS. The effect of DADS on the Tfr, a protein involved in iron transport (Fig. 4), was explored in REL cells and in rats. REL cells synthesized a high basal level of Tfr mRNA (Fig. 4A), whereas it was virtually undetectable in rat livers at S0 (Fig. 4B). In REL

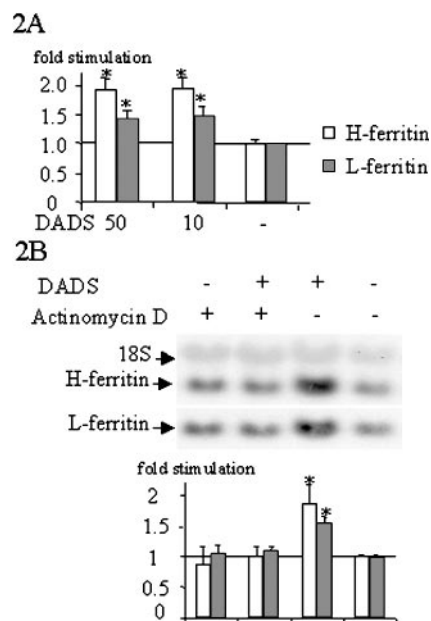


FIGURE 2 Descriptive responsiveness of heavy (H)- and light (L)-ferritin mRNA to diallyl disulfide (DADS) in rat liver epithelial (REL) cells. (A) Data obtained by quantification of Northern blot obtained from REL cells without treatment ($n = 4$) incubated for 4 h with 10 ($n = 4$) or 50 $\mu\text{mol/L}$ DADS ($n = 3$). (B) Typical autoradiography film obtained from REL cells preincubated or not with actinomycin D for 30 min and incubated with DADS (10 $\mu\text{mol/L}$) for an additional 4 h. For quantification, data represent mean, $n = 3$. From each lane, values were standardized to the 18S signal. *Different from the untreated control, $P < 0.05$ (Student-Newman-Keuls test).

cells, the addition of 10 $\mu\text{mol/L}$ DADS for 4 h did not further increase Tfr mRNA. In contrast, a 3.6 ± 0.5 -fold ($n = 3$) Tfr mRNA stimulation occurred in DADS-treated rats at S24 (compare control and DADS-treated rats at S24) and the level remained high for 48 h. To a lesser degree Tfr mRNA increased slightly at S24 and S48 in control rats given rapeseed oil alone.

DISCUSSION

We sought to examine previously unidentified DADS-activated genes. We have shown that ferritin was increased in rat livers at the mRNA and protein levels by DADS. Moreover, DADS increased Tfr mRNA. Ferritin plays a key role in iron storage and detoxification by sequestering intracellular iron, whereas Tfr is involved in iron transport *via* endocytosis of transferrin (7). These results suggest that DADS administration could modify iron homeostasis. Interestingly, DAS, despite its structural similarity to DADS (Fig. 1A), does not appear to stimulate ferritin in REL cells or *in vivo* (data not shown, preliminary results). The stimulation of H- and L-ferritin expression was observed in REL cells at a low DADS concentration (10 $\mu\text{mol/L}$). In the gastric infusion protocol, rats were given such high doses of DADS (667 $\mu\text{mol/kg}$ body weight) that the nutritional relevance of our results to humans is difficult to assess. We are currently testing lower doses of DADS in rats.

Iron's regulation of ferritin and transferrin genes is mainly at a post-transcriptional level in opposite directions. When the intracellular iron pool is low, the translation of ferritin mRNA is impaired through an RNA/protein partnership (17,18), whereas the stability of Tfr mRNA is increased (19,20). This balance is reversed by an intracellular iron

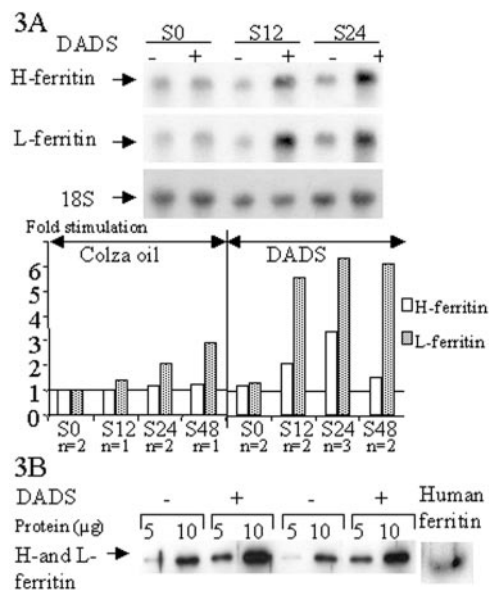


FIGURE 3 Up-regulation of ferritin in liver of rats infused by diallyl disulfide (DADS). (A) Typical autoradiography film of Northern blot. Heavy (H)-ferritin, light (L)-ferritin and 18S were used as probes. S0, S12, S24, S48: time (h) of killing after the end of infusion. The histogram shows data obtained by quantification of Northern blot membranes for the number of rats indicated. Values were normalized to the 18S signal. (B) Immunoblot assay with an antibody against ferritin (without distinction between H- and L-subunits). Protein (5 and 10 μg) from livers of two control rats (S24) and two DADS-treated rats (S24) and a human ferritin were loaded.

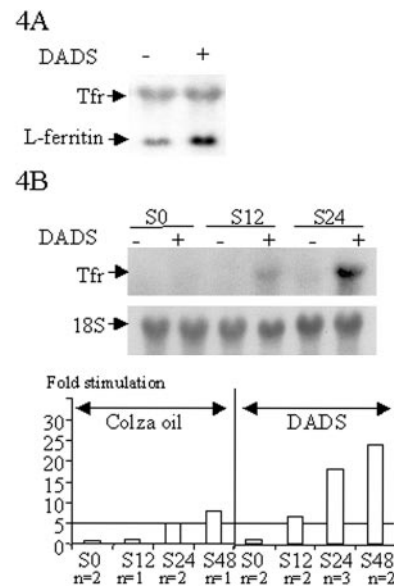


FIGURE 4 Response of transferrin receptor mRNA to diallyl disulfide (DADS) in rat liver epithelial (REL) cells (A) and in rats (B). Representative autoradiography films of Northern blot analysis are shown. Transferrin receptor (Tfr), L-ferritin and 18 S fragments were used as probes. The histogram shows data obtained by quantification of Northern blot membranes for the number of rats indicated. Values, which were normalized to the 18S signal, are means expressed as fold stimulation of the Tfr signal obtained in control rats at S0.

overload. The mechanism involving DADS-enhanced H- and L-ferritin mRNA seems to operate at the transcriptional level in REL cells (Fig. 2B). Such a transcriptional regulation has previously been described for the H- (21,22) as well as for the L-subunits (23). Although DADS seems to act at the level of transcription in REL cells, it remains to be determined what the actual mechanism underlying H- and L-ferritin stimulation in the rat liver is and whether it is compatible with concomitant enhancement of Tfr mRNA. In that receptor, both transcriptional and post-transcriptional mechanisms could account for the DADS-induced stimulation, as previously described for the responsiveness of Tfr to stimulation by erythropoietin (24). The apparent discrepancy between an enhanced Tfr mRNA and an enhanced ferritin content has already been described in regenerating rat liver triggered by carbon tetrachloride administration (25).

To our knowledge, the effect of DADS on H- and L-ferritin and Tfr gene expression has never been described. Associations between organosulfur compounds and iron metabolism (26,27) are seldom suggested. Our data suggest that a high consumption of garlic could modify iron metabolism through changes in ferritin and Tfr. However, the nutritional relevance of such data to humans remains to be determined.

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