Selective expression of glutathione S-transferase genes in the murine gastrointestinal tract in response to dietary organosulfur compounds

John H. Andorfer, Tatyan Tchaikovskaya and Irving Listowsky

Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York, NY 10461, USA

To whom correspondence should be addressed. Email: irving@aecom.yu.edu

A short-term feeding regimen was designed to analyze the effects of compounds such as diallyl disulfide (DADS), diallylthiosulfinate (allicin) from garlic and butylated hydroxyanisole (BHA) on glutathione S-transferase (GST) expression in the gastrointestinal tract and liver of male mice. After animals were force-fed these compounds, tissue GSTs were purified and individual subunits resolved by HPLC and identified on the basis of mass spectrometry (ESI MS) and immunoreactivity data. The effects of DADS and allicin on GST expression were especially prominent in stomach and small intestine, where there were major coordinate changes in GST subunit profiles. In particular, the transcripts of the mgSTM1 and mgSTM4 genes, which share large segments of common 5′-flanking sequences, and their corresponding subunits were selectively induced. Levels of α class subunits also increased, whereas mgSTM3 and mgSTP1 were not affected. The inducible mGSTA5 and non-responsive mGSTM3 subunits had not been identified previously. Liver and colon GSTs were also affected to a lesser extent, but this short-term feeding regimen had no effect on GST subunit patterns from other organs, including heart, brain and testis. Real-time PCR (TaqMan) methods were used for quantitative estimations of relative amounts of the mRNAs encoding the GSTs. Effects on the transcripts generally paralleled changes at the protein level, for the most part, however, the greatest relative increases were observed for those mRNAs that were expressed at low abundance constitutively. Mechanisms by which the organosulfur compounds operate to affect GST transcription could involve reversible modification of certain protein sulfhydryl groups, shifts in reduced glutathione/oxidized glutathione ratios and resultant changes in cellular redox status.

Introduction

Some naturally occurring dietary agents, particularly non-nutrient phytochemicals, have been shown to exhibit chemopreventive/chemoprotective effects against development of cancer (1–4). In that regard, several lines of evidence have linked consumption of organosulfur compounds from garlic with chemoprotection against cancer and certain degenerative diseases (5,6). It has been suggested that organosulfur compounds such as allicin and diallyl disulfide (DADS) modify signal transduction pathways, induce apoptosis and suppress tumor cell growth (7–15). Antioxidants such as butylated hydroxyanisole (BHA) have also been shown to have anticarcinogenic effects (16). These types of compounds are known to activate transcription of phase II drug metabolizing enzymes, including glutathione S-transferases (GSTs) (2–4,17).

Mammalian GSTs are products of gene superfamilies that are expressed in a tissue-specific manner (18–21). They have been subdivided into at least seven categories based on sequence homologies, dimeric subunit assembly patterns and other common properties, of which the α, μ, π and θ forms are usually the most abundant (20). GSTs allow cells to adapt to various types of noxious agents by catalyzing nucleophilic addition or substitution reactions between glutathione (GSH) and reactive electrophilic compounds and by reduction of organic hydroperoxides generated from reactive oxygen species (18,20,22–24). GSTs are also intracellular stoichiometric binding proteins for various non-substrate ligands (25). The multiple forms are characterized by discrete differences in substrate specificities and binding properties (20). For instance, stable transfection of the mouse GSTA3 subunit to cells confers protection against aflatoxin B1 mutagenicity (26). The mGSTA4 subunit exhibits selective activities against toxic products of lipid peroxidation, including 4-hydroxy-nonenal (27).

Some classes of GST-inducing compounds activate transcription of GST genes directly and some require further metabolic activation in order to function. The inducing agents are considered to be monofunctional if they increase expression of only phase II drug-metabolizing enzymes and NAD(P)H-quinone oxidoreductase but not cytochrome P450s, and others are bifunctional in that they also induce phase I mixed function oxygenases (cytochrome P450s) (28). Because GSTs are considered to act as cellular chemoprotective proteins by functioning in the direct detoxification of reactive substances, including some carcinogens, some strategies for dietary intervention for the chemoprevention of various diseases include manipulation of GST systems (29–32). In the present study, differential effects of dietary organosulfur compounds on the family of GSTs in mouse tissues are analyzed and molecular mechanisms by which they function are considered.

Materials and methods

Materials

DADS was obtained from LKT Laboratories (St Paul, MN), BHA from ICN (Costa Mesa, CA), 1-chloro 2,4-dinitrobenzene (CDNB) and epoxy-GSH affinity resins from Sigma-Aldrich (St Louis, MO) and other reagents were of high purity. Allicin was a gift from Drs Meir Wilich and Talia Miron (Weizmann Institute of Science, Rehovot, Israel). Peptide sequence-specific antisera prepared in our laboratories were used to distinguish among different subclasses of GST (33–35).

Dietary regimen

Cohorts of 6–7-week-old C57BL/6 male mice (Charles River Laboratories, Wilmington, MA) were fed a laboratory chow diet (505A; Purina Mills, 359...
GSTs were concentrated to 1 ml using 10K MWCO Ultrafree Centrifugal Filter devices (Millipore, Bedford, MA). The GSH concentration was reduced by force-feeding two doses of allicin (2.2 μmol) in citrate buffer (minus GSH) and refiltration in elution buffer (minus GSH).

Homogenates were prepared by disrupting the tissue with a rotor-stator homogenizer in loading buffer (10 mM Tris-HCl pH 7.9, 10 mM NaCl, 1 mM DTT) at 4°C. The homogenates were centrifuged at 35 000 × g for 1 h at 4°C and the cytosolic fractions were used for GST purification. Protein concentrations were determined by a modification of the Bradford assay (Cytoskeleton Inc., Denver, CO). GST enzymatic activities were determined using CDNB as substrate (36). Cytosolic GSTs were purified using epoxy-linked GSH generator in loading buffer (10 mM Tris-HCl pH 9.6, 10 mM GSH, 0.1 mM dithiothreitol). The eluted GSTs were eluted with 30 ml of elution buffer (36). GSTs were eluted with 30 ml of elution buffer (36). GST purification

GSTs were concentrated by dilution and refiltration in elution buffer (minus GSH). GST purification

Homogenates were prepared by disrupting the tissue with a rotor-stator homogenizer in loading buffer (10 mM Tris-HCl pH 7.9, 10 mM NaCl, 1 mM DTT) at 4°C. The homogenates were centrifuged at 35 000 × g for 1 h at 4°C and the cytosolic fractions were used for GST purification. Protein concentrations were determined by a modification of the Bradford assay (Cytoskeleton Inc., Denver, CO). GST enzymatic activities were determined using CDNB as substrate (36). Cytosolic GSTs were purified using epoxy-linked GSH-garoside (Sigma-Aldrich, St Louis, MO) affinity chromatography. The affinity columns (2 ml gel volume) were loaded with cytosolic extracts and washed with ~50 column volumes of loading buffer. The GSTs were eluted with 30 ml of elution buffer (50 mM Tris–HCl pH 9.6, 10 mM GSH, 0.1 mM dithiothreitol). The eluted GSTs were concentrated to 1 ml using 10K MWCO Ultrafree Centrifugal Filter devices (Millipore, Bedford, MA). The GSH concentration was reduced by dilution and refiltration in elution buffer (minus GSH).

Cytosolic GSTs were purified using epoxy-linked GSH-garoside (Sigma-Aldrich, St Louis, MO) affinity chromatography. The affinity columns (2 ml gel volume) were loaded with cytosolic extracts and washed with ~50 column volumes of loading buffer. The GSTs were eluted with 30 ml of elution buffer (50 mM Tris–HCl pH 9.6, 10 mM GSH, 0.1 mM dithiothreitol). The eluted GSTs were concentrated to 1 ml using 10K MWCO Ultrafree Centrifugal Filter devices (Millipore, Bedford, MA). The GSH concentration was reduced by dilution and refiltration in elution buffer (minus GSH).

**Table I.** Primer and probe sequences used for quantitation of mRNA levels by real-time PCR using Taqman® chemistry

<table>
<thead>
<tr>
<th>GST</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-CTGCACTAGTGAGATCTACAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-GGAGGCTACCCATGGTG-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-CGCGGACTGACACACCCGATCC-3'</td>
</tr>
<tr>
<td>M2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-CTGCACTAGTGAGATCTACAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-CAGCCAAACCTAAGCTCTACTACTTTAATG-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-CCGCGGACTGACTCACTCCATCCG-3'</td>
</tr>
<tr>
<td>M3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-TTGAGGACAGAGATGATGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-CCAAGCCTACCCGCTCTGTT-3'</td>
</tr>
<tr>
<td>M4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-TTAGAACACTGGGCTATGGGACAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-GGGCATCCCCCATGACA-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-CGCGGACTGACCTACCTCCATTCCG-3'</td>
</tr>
<tr>
<td>A3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-TGGGGAACACTTCTTCTTCTTCTGAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-GGAACTCTTCTTCTTCTTCTTCTGAA-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-TGAGAAGCAGAGTCAGCAACCTCCCCA-3'</td>
</tr>
<tr>
<td>A4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-TGGGGAACACTTCTTCTTCTTCTGAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-GGAACTCTTCTTCTTCTTCTTCTGAA-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-TGAGAAGCAGAGTCAGCAACCTCCCCA-3'</td>
</tr>
<tr>
<td>P1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-GCAAATATGGCACCATGATCTACAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'</td>
</tr>
<tr>
<td>P2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward primer 5'-GCAAATATGGCACCATGATCTACAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer 5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'</td>
</tr>
<tr>
<td></td>
<td>Labeled probe 5'-AGGGCCTTCACGTAGTCATTCTTACCATTCTCATAGT-3'</td>
</tr>
</tbody>
</table>

Probes were end-labeled with 6-FAM (5'-end) and TAMRA (3'-end). Analysis was carried out using an ABI Prism 7000 Sequence Detection System.

<sup>a</sup> Primer and probe sequences are those published by Chanas et al. (37).

<sup>b</sup> Designated GSTM3 by Chanas et al. (37).
were normalized to that of GAPDH using rodent-specific primers and probes (Applied Biosystems). GST gene-specific probes were fluorescently labeled at the 5'-end with 6-carboxy-fluorescein phosphoramidite (FAM, reporter dye) and at the 3'-end with 6-carboxy-tetramethylrhodamin (TAMRA, quencher dye). The GAPDH probe was 5'-labeled with VIC™ (Applied Biosystems), enabling both the gene-specific and GAPDH probes to be used in the same well with each replicate cDNA sample. Relative changes in gene expression as measured by real-time PCR were calculated by comparing treated samples with untreated controls. Data are presented as the fold change in gene expression normalized to mGAPDH and relative to the control using the 2-ΔΔCT method (38).

**RT-PCR**

To substantiate results obtained by real-time PCR, an independent DADS feeding experiment was carried out according to the procedures outlined above and data for the mGSTA3 transcript analyzed by reverse transcription PCR methods. Thus, total RNA was isolated from five treated and five control mice and first strand cDNA was synthesized using SuperScript II RNase H- Reverse Transcriptase according to the manufacturer's suggestions (Invitrogen). After digestion of RNA–DNA hybrids with RNase H (Invitrogen), the reverse transcriptase reaction was diluted 100× with Ultra-Pure H₂O (Millipore) and 5 μl used directly in a PCR reaction using Platinum Pfx DNA Polymerase (Invitrogen). Forward and reverse primers (GeneLink) were designed that contained 3'-ends specific for mGSTA3 (5'-GGTTTGGGAAACTCTTCCTC-3' and 5'-CTGACTCACAACATTTTGCGCATC-3', unique mGSTA3 nucleotides shown in bold). Temperature cycling was carried out for 31 cycles and the reaction products loaded onto a 1.5% agarose gel containing ethidium bromide. The gel was digitally imaged using an Image Station 2000R (Eastman Kodak, Rochester, NY) and bands were analyzed using Kodak 1D Image Analysis software.

**Peptide mass fingerprinting**

Unidentified mGST HPLC fractions from DADS-treated intestine were subjected to peptide mass fingerprinting analysis. Briefly, the HPLC fraction was collected and the organic solvent (acetonitrile or TFA) removed by vacuum evaporation. Ammonium bicarbonate was added to a final concentration of 100 mM and digestion was conducted with 8 μg trypsin (Promega, Madison, WI) at 25°C for 16 h. The resulting peptides were resolved by loading the digest onto a Vydac reversed phase C18 column (1 mm × 15 cm) connected to an HP-1100 (Hewlett Packard) HPLC apparatus. Peptides were eluted with increasing concentrations (0.2%/min) of buffer B (0.1% TFA in acetonitrile) at a flow rate of 50 μl/min. Peptides were subjected to analysis by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) using a Voyager-DE mass analyzer (PerSeptive Biosystems, Framingham, MA). A portion of each fraction was mixed 1:1 with MALDI matrix (α-cyano-4-hydroxy-cinnamic acid; Sigma) in 50% acetonitrile and 0.1% TFA and spotted on a MALDI target before analysis. The peptide masses obtained were compared to theoretical tryptic digests of known mouse GSTs. Additionally, sequences were determined for a few abundant peptides using Edman chemistry on a Procise-494 sequencer (Applied Biosystems).

**Results**

Dietary protocols using short-term feeding regimens of naturally occurring organosulfur compounds were established for studies on induction of GSTs in the gastrointestinal tract and liver of mice. Cytosolic extracts from those tissues were initially screened for overall GST enzymatic activities and probed by immunoblots with specific antisera to distinguish among the major α, μ, and π class GSTs. Two courses of 20 μmol DADS resulted in almost 3-fold increases in GST enzymatic activities of stomach and small intestine (Table II). There were smaller changes in GST activities of liver and colon (Table II).

**Basis for GST nomenclature**

It has been pointed out that many studies on regulation of expression of mouse GSTs failed to distinguish among individual subunits which were either incompletely resolved or not identified (37). Accordingly, retention times of each subunit, molecular masses determined by ESI MS with molecular masses based on deduced sequences from the database.

![Fig. 1. Immunoblots of cytosolic proteins from intestine (columns 1 and 2) and liver (columns 3 and 4). Homogenates were prepared according to the methods described in Materials and methods. Columns 1 and 3 show data obtained for untreated control mice and columns 2 and 4 show the effect of DADS treatment on the indicated classes of GSTs.](image)

**Table III. Mouse GST subunits, resolved by HPLC and identified by ESI MS**

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Mouse GST subunit</th>
<th>Molecular mass (a.m.u.</th>
<th>Database (accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.4</td>
<td>μ1</td>
<td>25 839</td>
<td>25 839 (P10649)</td>
</tr>
<tr>
<td>22.0</td>
<td>μ4</td>
<td>25 571</td>
<td>25 570 (P19639)</td>
</tr>
<tr>
<td>23.0</td>
<td>μ2</td>
<td>25 586</td>
<td>25 585 (P15626)</td>
</tr>
<tr>
<td>23.5</td>
<td>μ6</td>
<td>25 494</td>
<td>25 497 (O35660)</td>
</tr>
<tr>
<td>24.5</td>
<td>α3</td>
<td>25 571b</td>
<td>25 229 (P30115)</td>
</tr>
<tr>
<td>24.8</td>
<td>n2</td>
<td>23 403</td>
<td>23 406 (P46425)</td>
</tr>
<tr>
<td>25.5</td>
<td>n1</td>
<td>23 477</td>
<td>23 478 (P19157)</td>
</tr>
<tr>
<td>25.6</td>
<td>μ3</td>
<td>25 579</td>
<td>25 579 (AK002213)</td>
</tr>
<tr>
<td>29.5</td>
<td>α5</td>
<td>25 491</td>
<td>No entry</td>
</tr>
<tr>
<td>30.0</td>
<td>α1</td>
<td>25 517b</td>
<td>25 477 (P13745)</td>
</tr>
<tr>
<td>31.0</td>
<td>α2</td>
<td>25 450b</td>
<td>25 402 (P10648)</td>
</tr>
<tr>
<td>32.0</td>
<td>μ5</td>
<td>26 546b</td>
<td>26 504 (P48774)</td>
</tr>
<tr>
<td>34.7</td>
<td>μ7</td>
<td>25 384</td>
<td>25 388 (AAM67419)</td>
</tr>
<tr>
<td>35.2</td>
<td>α4</td>
<td>25 478</td>
<td>25 477 (Q9CQ81)</td>
</tr>
</tbody>
</table>

aObserved mass ± 3 a.m.u. Results were obtained for at least three different preparations of the GST subunit.

bThese subunits are N-acetylated at their N-termini.
Differential induction of GSTs by DADS

Typical HPLC profiles of GST subunits isolated from small intestines of control and DADS-treated animals are shown in Figure 2A. mGSTP1 is the predominant subunit in the small intestine and usually comprises >60% of the total GST content of that organ. After acute feeding of DADS, however, sharp increases in the mGSTA1 and mGSTM4 subunits were observed. Consequently, in response to DADS administration mGSTM1 subunit levels exceeded those of mGSTP1 and mGSTM4 was elevated to levels comparable with those of mGSTP1. The α class subunits, mGSTA1, mGSTA4 and mGSTA5 also increased after feeding of DADS. α class GSTs were shown to be restricted mainly to the epithelial cell layer of the villi and crypts of the small intestine (43). In addition, the low abundance mGSTM6, mGSTM7 and mGSTA2 subunits, which were not detected in control small intestines, were clearly discernible in small intestines of DADS-treated animals. On the other hand, no substantial changes were observed in mGSTP1, mGSTM2 and mGSTM3 levels. A consistent pattern of induction of GSTs in small intestine (as represented in Figure 2A) was observed for three different animals.

In mouse stomach, the major GST subunits are mGSTM1 (~25% of total GST), mGSTP1 (~30%) and mGSTA5 (~10% each). In contrast to the small intestine, the stomach has extremely low levels of mGSTM4 in untreated animals. Effects of direct force-feeding of DADS into the stomach yielded a typical pattern of GST induction as shown in Figure 2B, with little variation among animals. Similar to findings in the small intestine, the greatest increases caused by DADS in the stomach were in the mGSTM1 and mGSTM4 subunits. The levels of mGSTA4 and mGSTA5 also increased, as did the minor subunits mGSTA1 and mGSTA2. Subunits mGSTM2, mGSTM3 and mGSTM5 did not undergo substantial changes.

In colon of chow-fed animals, subunits mGSTM1 and mGSTP1 together comprise >85% of the GST composition (Figure 3A). Although the effects of DADS were less pronounced than in stomach and small intestine, increases in the mGSTM1 and mGSTM4 subunits were also observed in the colon and the relative proportions of minor subunits mGSTA5 and mGSTA1 increased substantially (Figure 3A).

Mouse liver GSTs consist primarily of three subunits, mGSTM1, mGSTA3 and, mostly, mGSTP1 (~65% of the total GST in livers of male mice) (Figure 3B). mGSTM4 subunits were not detected in control livers. The greatest changes in livers of animals after administration of DADS were in the α class GSTs. In particular, mGSTA5 and mGSTA1, which were present at very low levels in livers of control mice (Figure 3B, inset), showed the greatest percentage increase in DADS-treated animals.

GSTs from other organs, such as heart, brain and testis, were not substantially influenced by the short-term DADS feeding regimen (Figure 3C–E). The data in Figures 2 and 3 illustrate the discrete tissue-specific GST expression patterns in mice and Figure 3C–E underscores the small degree of experimental variability in the tissue GST subunit patterns among different mice. Testicular and brain GSTs are characterized by a relatively high level of mGSTM5 subunits, which are present at low or below detectable levels in most other tissues. Testicular GSTs
are also notable for the absence of mGSTM4 subunits and for greater expression of the mGSTM3 and mGSTM6 subunits as compared with their expression in most other tissues.

Transcriptional activation profiles

Gene-specific primers were designed to distinguish among closely related genes encoding for the GST subclasses (Table I) and for quantitative estimation of their RNA levels by real-time PCR (TaqMan) methods. In general, DADS treatment enhanced transcription of individual GST genes (Figure 4), which paralleled the patterns observed for the corresponding protein subunits (Figures 2 and 3). Thus, increased transcripts of mGSTM1 were observed in stomach, small intestine, liver and colon of DADS-fed animals. Expression of mGSTM4 and some α class GSTs also increased in stomach and small intestine after DADS feeding. Neither mGSTP1 (a major π class GST transcript) nor mGSTP2 (expressed at lower levels and which encodes for a subunit that is catalytically deficient) (44,45), was substantially affected by DADS. The mGSTA4 transcript increased in small intestine, with smaller changes in that transcript in stomach. This feeding regimen caused little change in mGSTM2 transcripts and those of mGSTM3 were not affected.

From a quantitative perspective, however, the apparent increases in mRNA and protein levels were sometimes divergent; thus, the greatest increases were often observed for the low abundance mRNAs. For instance, the constitutive levels of mGSTM4 mRNA expression are much lower in stomach (normalized average Ct = 34) and liver (Ct = 32) as compared with those of small intestine (Ct = 25). Accordingly, greater proportional increases in its mRNA were observed in stomach and liver after DADS feeding (Figure 4). Large changes in mGSTM4 mRNA levels (>80-fold) were also observed in stomach after feeding allicin (data not shown). In small intestine, the more...
abundant mGSTM1 subunit exhibited the greatest increase in absolute amount at the protein level (Figure 2A), but increases in terms of fold increase of its mRNA was not proportionally so great as that of mGSTM4 and those that occurred for the less abundant GST mRNAs (Figure 4). The selective increase in transcription of mGSTA3 in small intestine was unexpected (Figure 4) in view of the limited increase in protein level (Figure 2). Real-time PCR data were therefore reproduced for eight different sets of treated animals. In addition, semi-quantitative RT–PCR followed by densitometric analysis of agarose gel electrophoretic bands was carried out on five separate control and five DADS-treated animals from independent feeding experiments (Figure 4, inset). The 9-fold increase in mGSTA3 transcripts for animals treated with DADS thereby confirmed the results obtained by real-time PCR (Figure 4).

**Effects of allicin and BHA**

HPLC patterns of GSTs from small intestine and stomach of mice force-fed with allicin or BHA are shown in Figure 5. Although some quantitative differences were evident between the effects of these compounds and the effects of DADS, certain general features of induction were consistent for the different substances and for animals of different ages. For instance, the three compounds selectively induced expression of mGSTM1 and mGSTM4 subunits to approximately the same extent in small intestine and stomach. The α class subunits, in addition to mGSTM6 and mGSTM7 that are induced by DADS, were also increased by administration of allicin or BHA. In liver, BHA, which is a prototypic inducer of GSTs (43,46–49), appeared to be more effective than the organosulfur compounds for induction of mGSTM1 and the low abundance mGSTM4 subunits.

**Discussion**

The short-term feeding regimen of organosulfur compounds or BHA to mice resulted in substantial and coordinate increases in some GST subunits in the stomach and small intestine. The rapid induction of GSTs by DADS and allicin documented in this study could in fact be an immediate response required to counteract noxious dietary substances encountered by organs that are directly exposed to them. Moreover, a reduced risk of gastric cancer due to garlic consumption has been reported (50,51). Liver and colon GSTs were also induced, but to a lesser extent. Each tissue is marked by characteristic cell type-specific patterns of expression of the multiple GSTs, with mGSTM1 and mGSTP1 usually being the most abundant and widely distributed mouse subunits (52). The mGSTM1, mGSTM4 and some α class GSTs are considerably induced by the treatments (Figures 2 and 3).

Induction of a GST subunit by these compounds (Figures 2 and 3) is likely to occur by transcriptional activation of its corresponding gene (Figure 4). However, some apparent inconsistencies between changes in amount of a particular subunit (Figures 2 and 3) relative to the change in its corresponding mRNA (Figure 4) are noteworthy. For instance, although mGSTM4 became a major GST subunit in small intestine after induction by DADS (Figure 2), the increase in level of its mRNA (5-fold, Figure 4) was much less than corresponding DADS-induced increases that occurred in stomach (>100-fold). Yet, the stomach is an organ in which the mGSTM4 protein subunit remained a minor component even after induction. Thus, in stomach and liver, mRNAs encoding for mGSTM4, which are of relatively low abundance, exhibited greater proportional increases after administration of DADS. Moreover, the redox-active mGSTM4 subunit, which

![Fig. 5. Effects of allicin and BHA on HPLC profiles of affinity-purified mouse GST subunits. The pattern of GST subunits for small intestine (A and C) and stomach (B and D) of treated (indicated by black lines) and untreated (indicated by grey lines) animals are shown. Treatments consisted of two oral doses of allicin (A and B) or BHA (C and D) administered by gavage. The allicin-treated mice and their untreated paired controls were 6 months old when gavaged (which is different from the mean age of ~7 weeks for both BHA- and DADS-treated animals used in all other experiments). Refer to Figure 2 for subunit identification.](image-url)
has an active site Cys115 residue (40), although expressed at very low levels in most tissues, increased considerably in response to these inducers (Figures 2-4). Likewise, the low abundance small intestinal mRNA for mGSTA3 increased >300-fold after feeding of DADS (Figure 4), while significant changes of this transcript did not occur in liver, an organ in which its constitutive expression is much greater. It is noteworthy that the mGSTA3 subunit has been shown to exhibit selective activity against certain substrates, such as aflatoxin B1 (26). Conversely, very large increases were observed for the low abundance mRNA encoding mGSTM4 in liver (>30-fold), whereas the relative amount of this protein subunit remained relatively low even after induction (Figure 3B). Although there are few studies on GST protein turnover in tissues, the results obtained here may be related, in part, to differences in translational efficiencies of the mRNAs and the relatively long half-lives observed for some GST proteins (53).

Some components involved in the transcriptional activation process of various redox-sensitive gene products, including GSTs, have recently been linked to antioxidant-responsive element (ARE) motifs in the 5'-flanking regions of their genes (54-58). However, with the possible exception of the mGSTA1 gene (which contains tandem repeat ARE elements, also designated an electrophile response element, EpRE) (54), no other mouse GST genes are known to contain functional AREs, even though ARE-like sequences are present in some of their 5'-flanking regions. In that connection it is noteworthy that the DADS-responsive mGSTM1 and mGSTM4 genes have extensive sequence homologies in their 5'-flanking regions that include a consensus ARE element (Figure 6), whereas the non-responsive mGSTM2 and mGSTM3 genes have 5'-flanking sequences that are distinct from those of mGSTM1 and mGSTM4. However, there is no direct evidence that these ARE elements are functional.

**Fig. 6.** Sequence alignment of the 5'-flanking region of the mGSTM1 and mGSTM4 genes. Portions of the 5'-flanking region of mGSTM1 (5000 bp) and mGSTM4 (9000 bp) obtained from the mouse genome database were aligned using CLUSTAL W (v.1.8). A region of high homology is shown and the upstream nucleotides are numbered relative to the start of exon 1 of both genes. There are additional homologous regions in the 5'-flanking region of these genes that are not shown. The 5'-flanking segments of mGSTM1 and mGSTM4 were scanned for the presence of antioxidant-responsive elements (AREs) using the TEIRESIAS algorithm (IBM Bioinformatics Group). The perfect AREs are indicated by bold type and the consensus core regions are underlined. The AREs are located at 1653 bp and 111 bp for mGSTM1 and mGSTM4, respectively.
A common mechanism by which many of the structurally diverse chemoprotective compounds could affect transcription and function of GSTs is by modification of cellular redox status and chemical stress. Many of the known inducers of GSTs are electrophilic, including alkylating agents, free radical-generating compounds (59, 60) or, in the case of DADS and diverse chemoprotective compounds could affect transcription and function of GSTs is by modification of cellular redox status from the National Institutes of Health.

Acknowledgements

The authors thank Dr. John D. Hayes, Sam Seifert and John A. Milner for their valuable comments about this manuscript. This work was supported by grant CA42448 from the National Cancer Institute of the National Institutes of Health. J.A. was supported by Hepatology Training Grant T32DK07218 from the National Institutes of Health.

References

Transcriptional activation of GST by organosulfur


