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Induction of glutathione-*S*-transferase mRNA levels by chemopreventive selenocysteine *Se*-conjugates

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Abstract

Several selenocysteine *Se*-conjugates (SeCys-conjugates) prevent against chemically induced carcinogenesis. Bioactivation to selenols (RSeH) by β-lyases is thought to be critical, but the mechanism of tumor suppression remains unclear. Induction of phase II biotransformation enzymes is a possible mechanism of chemoprevention. In this study, we evaluated the isoform-selective induction of glutathione-*S*-transferase (GST) at the mRNA level using a quantitative reverse transcriptase polymerase chain reaction assay. In cultured primary rat hepatocytes and H35 Reuber rat hepatoma cells, SeCys-conjugates time-dependently increased mRNA levels of GST Alpha isoforms and GST Pi, but not of GST Mu isoforms. *Se*-allyl-L-selenocysteine, the most potent chemopreventive SeCys-conjugate so far known, was also the most active GST inducer. After exposure for 24 hr, it elevated *GSTA2*, *GSTA3*, *GSTA5*, and *GSTP* mRNA levels in primary hepatocytes 3.2 ± 0.4-, 1.9 ± 0.1-, 4.3 ± 0.3-, and 2.9 ± 0.3-fold, respectively. *Se*-allyl-D-selenocysteine was significantly less active, suggesting that stereoselective conversion of SeCys-conjugates to selenols is involved in GST induction. In H35 Reuber hepatoma cells, where conversion of SeCys-conjugates to selenols was 2–6-fold lower than in primary hepatocytes, GST induction was also much lower than in primary hepatocytes. SeCys-conjugates did not induce cytochrome P450 1A1, 2B1/2, or 3A1. This indicates that SeCys-conjugates are monofunctional inducers of phase II biotransformation enzymes. The present results suggest that induction of GST expression contributes to the chemopreventive activity of SeCys-conjugates.

Keywords: Selenium; Glutathione-*S*-transferase; mRNA; Chemoprevention; Primary rat hepatocyte; β-Lyase

1. Introduction

Epidemiological and animal studies have revealed that several organic and inorganic selenium compounds protect against carcinogen-induced tumor formation [1–6]. A mechanism that could explain the chemopreventive properties of organoselenium compounds, is induction of phase II biotransformation enzymes, such as glutathione-*S*-transferases (GSTs), resulting in enhanced inactivation of carcinogenic intermediates [7]. Indeed, administration of selenium-enriched garlic to rats inhibited the dimethylbenz[a]anthracene-induced formation of mammary gland tumors and increased total GST enzyme activity, in liver, kidney, and mammary tissue up to 2.5-fold [5]. Selenocysteine *Se*-conjugates (SeCys-conjugates; Fig. 1) are important constituents of selenium-enriched garlic and may be responsible for the observed effects. Also exposure of rats to selenomethionine [8] and selenocyanates [9,10] resulted in small increases in GST enzyme activity.

SeCys-conjugates are considered prodrugs that need bioactivation to selenols (RSeH) to become pharmacologically active (Fig. 1) [11,12]. These selenols are formed from SeCys-conjugates by human and rat β-lyase enzymes, such as pyridoxal 5′-phosphate-dependent cysteine-conjugate β-lyases, and amino acid oxidases [13–15]. Furthermore, SeCys-conjugates can be bioactivated by flavin-containing monooxygenases to selenenic acids (RSeOH) [16].

Selenols and selenenic acids are redox-active compounds that possibly induce the expression of genes with

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Abbreviations: GST, glutathione-*S*-transferase; SeCys-conjugates, selenocysteine *Se*-conjugates; ARE, antioxidant responsive element; CYP, cytochrome P450; tBHQ, *tert*-butylhydroquinone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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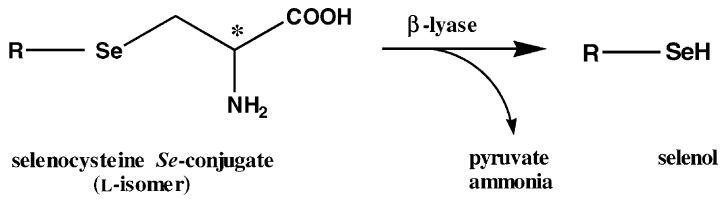


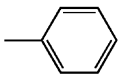
Name	R =	Abbreviation
Se-methyl-L-selenocysteine	— CH_3	MSeC
Se-*n*-propyl-L-selenocysteine	— CH_2 CH_2 CH_3	PSeC
Se-phenyl-L-selenocysteine		PhSeC
Se-allyl-L-selenocysteine	— CH_2 – CH = CH_2	ASeC-L
Se-allyl-D-selenocysteine	— CH_2 – CH = CH_2	ASeC-D

Fig. 1. Chemical structure and bioactivation of SeCys-conjugates.

an antioxidant responsive element (ARE) in the promoter, as was demonstrated for other redox-active agents [7,17–19]. The core sequence of the ARE is 5′-RTGACNNNGCR-3′ [19]. However, additional sequence elements are required to obtain a functional ARE, and therefore a new consensus sequence (5′-TMANNRTGAYNNNGCR-3′) has been defined [20]. Rat GST Alpha 2 (*GSTA2*) and GST Pi (*GSTP*) contain functional consensus ARE sequences [19,20]. In the *GSTA3* and *GSTA5* promoters putative AREs were found that do not fully correspond to the consensus sequence [21,22]. The presence of these ARE-like elements may explain why these isoforms are also induced by redox-active agents [7,23].

In the present study, the effects of various SeCys-conjugates on GST expression were investigated. The expression of GSTs was evaluated at the mRNA level, which enabled us to discriminate between the expression of the different GST isoforms. Evaluation of mRNA levels to measure induction is an established method and GST mRNA levels have been shown to closely correlate with GST protein and enzyme activity levels [24]. To classify SeCys-conjugates as monofunctional or bifunctional inducers, mRNA levels of the major inducible cytochrome P450s (CYPs) were also determined. Furthermore, we examined the rate of conversion of the SeCys-conjugates into the corresponding selenols. The presented data extend the understanding of the mechanisms of chemoprevention by SeCys-conjugates.

2. Materials and methods

2.1. Materials

Se-methyl-L-selenocysteine (MSeC), α-keto-γ-methiolbutyric acid, *tert*-butylhydroquinone (tBHQ), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. *o*-Phenylenediamine was obtained from Janssen Chimica. Se-(*n*-propyl)-L-selenocysteine (PSeC), *Se*-allyl-L-selenocysteine (ASeC-L), and *Se*-phenyl-L-selenocysteine (PhSeC) were synthesized as described before [12–14]. *Se*-allyl-D-selenocysteine (ASeC-D) was prepared as described for ASeC-L [13]. ASeC-D appeared to be contaminated with 12.5% of the L-isomer, as determined by polarimetry. All test compounds were dissolved at a concentration of 200 mM in dimethylsulfoxide (DMSO). TRIZOL and Superscript II^{TM} reverse transcriptase were from Invitrogen. PCR primers were synthesized by Eurogentec. All other chemicals were of the highest grade commercially available.

2.2. Cell cultures and incubations

H35 Reuber rat hepatoma cells (European collection of cell cultures) were grown at 37° under a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1}

streptomycin. Primary rat hepatocytes were isolated and cultured on collagen-S coated plates in serum-free DMEM, containing 0.2 % (w/v) bovine serum albumin (BSA), as was described before [25]. At 24 hr after seeding, cultures (approximately 80% confluent) were incubated with 100 μM of the SeCys-conjugates, or a corresponding volume of DMSO (maximal concentration in medium: 0.05% (v/v)). tBHQ (*GSTA* and *GSTP* induction) and prototypical CYP inducers (phenobarbital and dexamethasone [26]) were included as positive controls. After incubation for 24 hr, cells were lysed in TRIZOL and total RNA was isolated according to the manufacturer's protocol.

2.3. Quantification of mRNA levels

GST and CYP mRNA levels were determined with a radioactive, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay, essentially as described before [25]. To this end, mRNA was reverse-transcribed into cDNA using Superscript II™ and oligo(dT)$_{18}$ primers. The primer sets displayed in Table 1 were used for PCR amplification. The following PCR protocol was applied: 5 min of denaturation at 94°, 20 PCR cycles, consisting of 1 min at 94°, 1 min at 52°, and 1 min at 72°, and a final extension step of 10 min at 72°. At 20 PCR cycles, amplification was in the exponential phase, and 10-fold decreased and increased expression levels would still be within the linear range of the assay. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was co-amplified as an internal housekeeping control. PCR samples were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE). Radioactivity present in the bands of the target gene and *GAPDH* was quantified with ImageQuant™ Software (Molecular Dynamics). mRNA levels in SeCys-conjugate treated cultures were related to levels in vehicle-treated cultures, which were included in each experiment.

2.4. Determination of β-lyase activity

β-Lyase activities were determined in cell lysates, which were prepared by scraping of cells in 10 mM sodium phosphate buffer, pH 7.4, containing 2 mM dithiothreitol, 1 mM EDTA, and 50 μM phenylmethanesulfonyl fluoride. The lysates were subjected to short sonication, and subsequently dialyzed overnight against 20 mM sodium phosphate buffer, pH 7.4, as described previously for cytosolic fractions [13]. SeCys-conjugates were incubated for 1 hr at 37° in 50 mM sodium borate buffer (pH 8.6) at a final concentration of 1 mM, and in presence of 500 μM α-keto-γ-methiolbutyric acid (cofactor) and 0.1 mg mL^{-1} of cellular protein (determined with the BioRad protein assay) in a total volume of 100 μL. After 60 min, reactions were terminated by the addition of 500 μL of 0.14% (w/v) *o*-phenylenediamine in 3 N HCl and the amount of pyruvate was determined by HPLC as previously described [13]. As found earlier in experiments with cytosol [13], non-enzymatic β-elimination was not observed.

3. Results and discussion

3.1. Induction of GST expression by SeCys-conjugates in H35 Reuber cells

The effects of different SeCys-conjugates (Fig. 1) on GST expression were investigated by measuring their effects on the mRNA levels of important GST isoforms. The GST mRNA levels were determined with a quantitative, radioactive RT-PCR assay, which has been used before for determination of CYP mRNA levels [25,26], using the primers displayed in Table 1. For each target, it was established that the PCR met the prerequisites for quantitative RT-PCR analyses: PCR amplification was halted in its exponential phase, and the amplification of the target and the co-amplified *GAPDH* internal standard cDNAs

Table 1
PCR primer sequences

Target	GenBank accession	Forward primer	Reverse primer	Amplicon size	Reference
GSTA2	K00136	TTGACATGTATTCAGAGGGT	TTGTTTTGCATCCATGGCTG	362	[a]
GSTA3	X78848	ATGGGAGTTTGATGTTCCAG	GCCAAAGCGCTGGGGTCCAG	386	[a]
GSTA5	X78847	ATGGGAGTTTGATGTTTGAA	TCCACAATGCCTGGGTCCAT	388	[a]
GSTP	X02904	CCTCACCCTTTACCAATCTA	TTCGTCCACTACTGTTTACC	450	[a]
GSTM1	X04229	AGAAGCAGAAGCCAGAGTTC	GGGGTGAGGTTGAGGAGATG	415	[a]
GSTM2	J03914	AGGAGAGGATTCGTGTGGAC	GACTTTGTGGTGCTACTTTG	375	[a]
CYP1A1	NM012540	GGTGAAACAGGGGGATGACT	TAACGGAGGATAGGAATGAA	438	[26]
CYP2B1/2	J00722/M34452	TCAGGAGGAAGCCCAATGTT	CAGCAAAGAAGAGAGAGAGC	458	[26]
CYP3A1	L24207	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC	329	[25]
GAPDH	AF106860	TTCAACGGCACAGTCAAG	CACACCCATCACAAACAT	239	[25]

[a] These primers were newly designed for this study and were directed against non-homologous regions in the different GST genes. Calculated melting temperatures of the primers with non-targeted GST isoforms were at least 10° lower than the annealing temperature used in the PCR reactions, which assures target-specific amplification. Primers were checked for cross-reactivity with other than the intended gene products by performing a BLAST genome search in the rat genome database at NCBI.

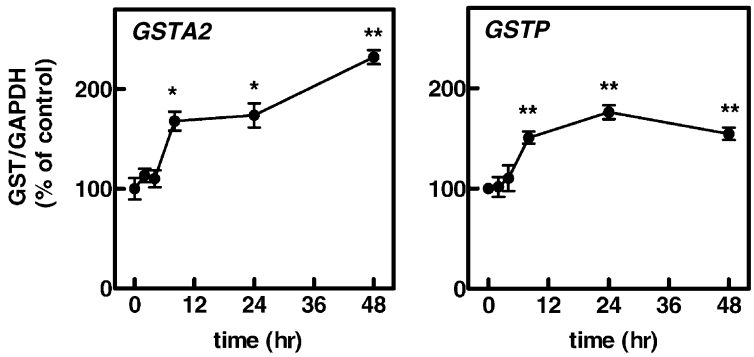


Fig. 2. Time-dependent induction of GSTs by MSeC. H35 Reuber rat hepatoma cells were incubated with 100 μM of MSeC for different time periods. Subsequently, RNA was isolated and levels of *GSTA2* and *GSTP* mRNA were determined with a radioactive, quantitative RT-PCR assay, and related to the co-amplified *GAPDH* housekeeping mRNA. GST mRNA levels are expressed as percentage of mRNA levels in DMSO-treated cultures. Values are means of three separate determinations ± SEM. Differences with respect to controls were tested for significance (unpaired Student's *t*-test). $^{*}P < 0.05$, $^{**}P < 0.0001$.

proceeded with equal efficiencies. *GAPDH* expression levels were not changed by treatment with SeCys-conjugates, as the GAPDH signals from SeCys-conjugate treated cultures were comparable to those from vehicle-treated cultures.

To find the optimal incubation time for analysis of GST induction, we exposed H35 Reuber rat hepatoma cells to 100 μM of MSeC for different time periods. Fig. 2 demonstrates that significant induction of *GSTA2* and *GSTP* was first observed at 8 hr, reaching maximal values at 24–48 hr. Since exposure to 100 μM of MSeC for more than 24 hr decreased cell viability by <30% (as determined by trypan blue exclusion), an incubation time of 24 hr was chosen for all subsequent experiments. The effects of exposure to 100 μM of MSeC or ASeC-L on the expression of *GSTA2*, *GSTA3*, *GSTA5*, *GSTP*, *GSTM1*, and *GSTM2* are shown in Fig. 3. As was found for MSeC, ASeC-L increased *GSTA2* and *GSTP* mRNA levels 1.4- and 1.7-fold, respectively. ASeC-L was the only compound that slightly but significantly affected *GSTA3* mRNA levels (1.2-fold increase). *GSTA5*, GST Mu 1 (*GSTM1*), and *GSTM2* mRNA levels were not affected by any of the tested compounds. In the experiments, tBHQ (100 μM) served as a positive control for ARE-mediated GST induction [18,27,28]. Treatment with tBHQ resulted in 3.1 ± 0.2- and 2.0 ± 0.1-fold increases in the mRNA levels of *GSTA2* and *GSTP*, respectively. The induction of *GSTA2* and *GSTP* by SeCys-conjugates was dose-dependent. A small but significant induction of *GSTA2* and *GSTP* (1.1- and 1.3-fold, respectively) was observed after incubation with 10 μM of MSeC for 24 hr (not shown). DMSO (vehicle control) did not alter GST mRNA levels in H35 Reuber rat hepatoma cells.

3.2. Effect of SeCys-conjugates on GST and CYP expression in primary rat hepatocytes

The effects of SeCys-conjugates on the GST mRNA levels were also evaluated in cultures of primary rat hepatocytes. The test set was extended with PSeC and PhSeC. To assess stereoselectivity in the induction of GSTs, ASeC-D (contaminated with 12.5% of the L-isomer) was also included in the study. The viability of the hepatocytes was not significantly affected by incubation with 100 μM of the SeCys-conjugates for 24 hr, as analyzed in a MTT cytotoxicity assay. We found that the response of primary hepatocytes to SeCys-conjugates and tBHQ was higher than that of H35 Reuber hepatoma cells, which makes them more suitable for the testing of GST induction. All tested compounds enhanced *GSTA2*, *GSTA3*, *GSTA5*, and *GSTP* mRNA levels (Fig. 4). DMSO (vehicle control) increased GST mRNA levels slightly (<15%). ASeC-L was the most potent SeCys-conjugate, elevating *GSTA2*, *GSTA3*, *GSTA5*, and *GSTP* mRNA levels 3.2 ± 0.4-, 1.9 ± 0.1-, 4.3 ± 0.3-, and 2.9 ± 0.3-fold, respectively. ASeC-D was less active than ASeC-L in the induction of *GSTA* isoforms, which indicates that the induction of GST expression is stereoselective. Most studies with chemopreventive SeCys-conjugates have been performed with racemic mixtures of SeCys-conjugates [6,11]. Our results suggest that lower doses can be used when pure L-isomers are applied.

SeCys-conjugates and tBHQ consistently differed in their GST isoform induction profile (Fig. 4). Whereas SeCys-conjugates induced *GSTA5* to the highest extent, tBHQ increased *GSTA2* expression much more than other GST isoforms. The promoter of *GSTA2* contains a consensus ARE [19,20], while the promoters of *GSTA3*, *GSTA5* contain imperfect ARE motifs [21,22]. Apparently, tBHQ is most efficient in the induction of transcription *via* a consensus ARE sequence. SeCys-conjugates, on the other hand, appear to be less potent inducers of transcription mediated by a consensus ARE, but are potent inducers of *GSTA* isoforms, that contain AREs in their promoters that do not fully correspond with the consensus sequence. The existence of differentially responsive AREs was suggested before [20]. It has been reported that *GSTM* isoforms are not subject to regulation by antioxidants [20,23]. We found a small but significant up-regulation of *GSTM1* mRNA by MSeC, PSeC, and PhSeC. *GSTM2* mRNA levels were not increased by the SeCys-conjugates. In contrast, ASeC-L and tBHQ decreased *GSTM2* mRNA levels by 24 ± 5 and 51 ± 5%, respectively.

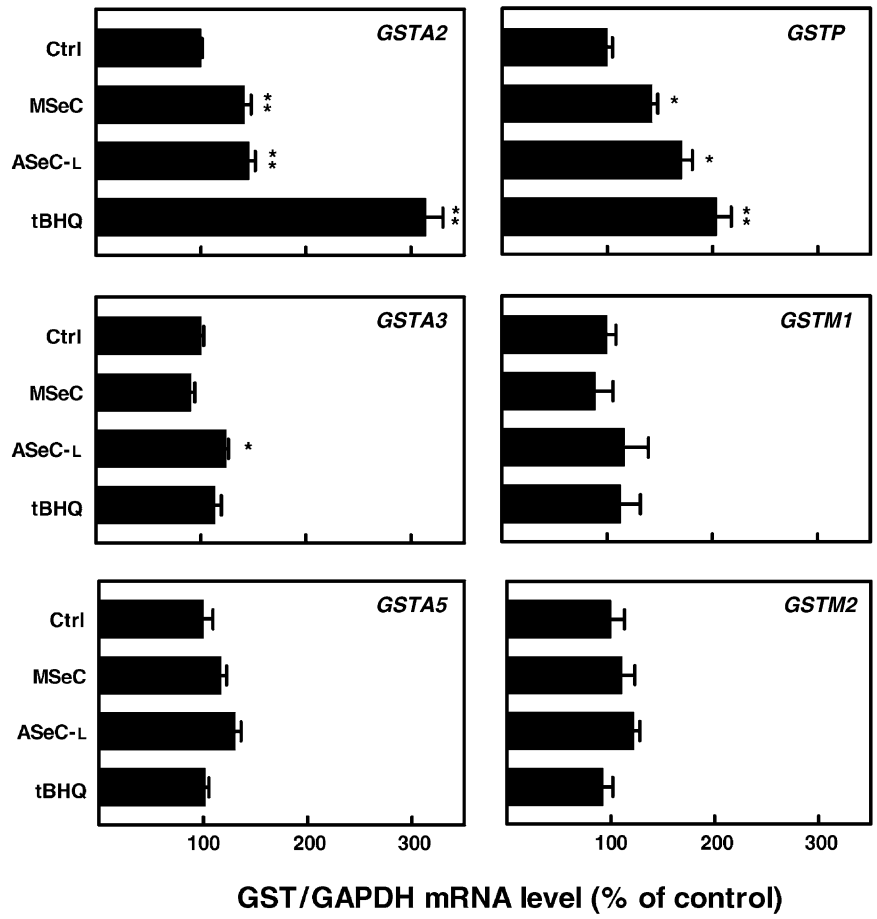


Fig. 3. Induction of GSTs by SeCys-conjugates in H35 Reuber rat hepatoma cells. H35 Reuber rat hepatoma cells were incubated with 100 μM of MSeC, ASeC-L, or tBHQ. After 24 hr, RNA was isolated and levels of *GSTA2*, *GSTA3*, *GSTA5*, *GSTP*, *GSTM1*, and *GSTM2* mRNA were determined with a radioactive, quantitative RT-PCR assay, and related to the co-amplified *GAPDH* housekeeping mRNA. GST mRNA levels are expressed as percentage of mRNA levels in controls (DMSO-treated cell cultures: Ctrl). Values are means of three to six separate determinations from two separate cultures ± SEM. Differences with respect to controls were tested for significance (unpaired Student's *t*-test). $^{*}P < 0.05$, $^{**}P < 0.0001$.

With some notable exceptions, induction of GSTs generally enhances the detoxification potential, whereas induction of CYPs might result in an increased risk at bioactivation of pro-carcinogens. Thus, chemopreventive compounds ideally should not induce CYPs [7]. Therefore, we determined in cultured rat hepatocytes the effects of SeCys-conjugates on the expression of the major inducible CYP isoforms, *CYP1A1*, *CYP2B1/2*, and *CYP3A1*. The prototypic CYP inducers phenobarbital and dexamethasone were included as positive controls for CYP inducibility [25,26]. *CYP2B1* mRNA levels were induced 10-fold after exposure to 2 mM of phenobarbital, and *CYP3A1* mRNA levels were induced 44-fold after exposure to 10 μM of dexamethasone. None of the SeCys-conjugates elevated, at a concentration of 100 μM, the mRNA levels of the mentioned CYP isoforms (data not shown).

3.3. Determination of β-lyase-dependent conversion of SeCys-conjugates into selenols

SeCys-conjugates are known to be bioactivated by β-elimination, catalyzed by different types of β-lyases, e.g. cysteine conjugate β-lyases, amino acid oxidases, and flavin-containing monooxygenases [13,16]. The observed differences in the induction of GSTs by ASeC-L and ASeC-D may indicate that enzymatic conversion is required for induction of GST expression. Therefore, the enzyme-catalyzed conversion of the different SeCys-conjugates to selenols, determined by stoichiometric pyruvate formation, was measured in dialyzed lysates of rat hepatoma cell cultures and rat hepatocytes. In both cell homogenates significant β-elimination activity was observed (Fig. 5). In rat hepatocyte lysates, high β-elimination rates were observed for MSeC, ASeC-L, and PhSeC, while PSeC was less efficiently converted to the corresponding selenol. MSeC and ASeC-L are also potent inducers of GST expression, whereas PSeC is less active in this regard. However, PhSeC was efficiently converted into the corresponding selenol, while it was a poor GST inducer. The lower β-elimination rate of ASeC-D compared with ASeC-L correlates with the lower induction of *GSTA* by SeCys-conjugates. The combined observations may point to a role for selenols in GST induction and to a relationship between the chemical structure of the selenol and the potency of the compound.

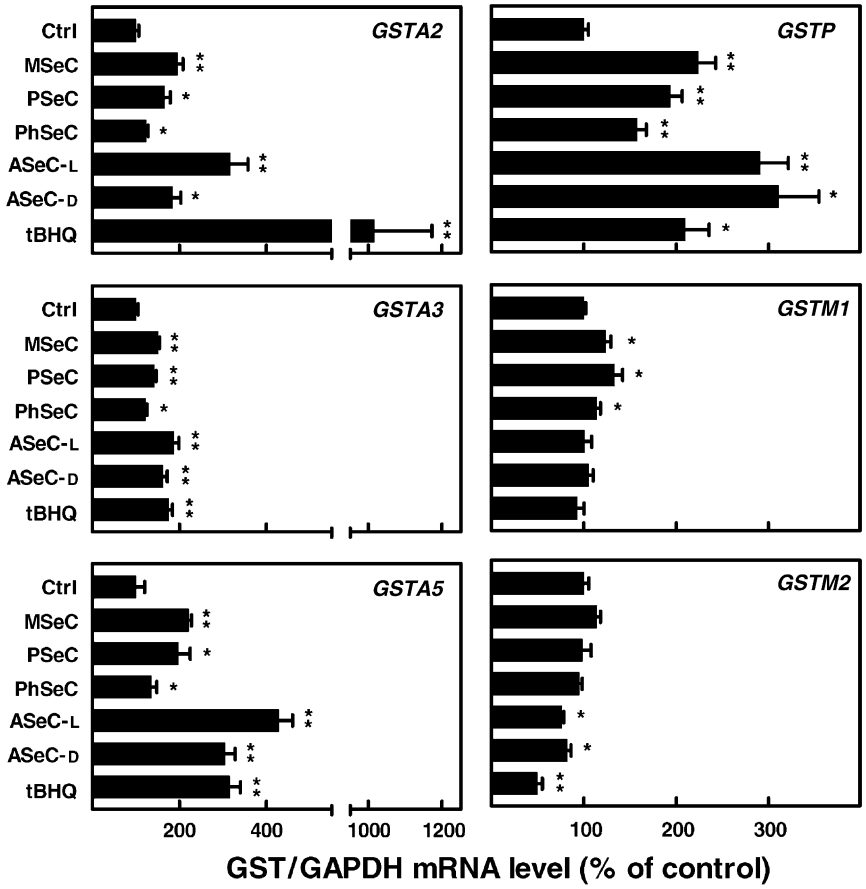


Fig. 4. Induction of GSTs by SeCys-conjugates in cultured primary rat hepatocytes. Cultured primary rat hepatocytes were incubated with 100 μM of MSeC, PSeC, PhSeC, ASeC-L, ASeC-D, or tBHQ. After 24 hr, RNA was isolated and levels of *GSTA2*, *GSTA3*, *GSTA5*, *GSTP*, *GSTM1*, and *GSTM2* mRNA were determined with a radioactive, quantitative RT-PCR assay, and related to the co-amplified *GAPDH* housekeeping mRNA. GST mRNA levels are expressed as percentage of mRNA levels in controls (DMSO-treated cell cultures: Ctrl). Values are means of nine separate determinations from three separate cultures ± SEM. Differences with respect to controls were tested for significance (unpaired Student's *t*-test). $^{*}P < 0.05$, $^{**}P < 0.0001$.

In H35 Reuber rat hepatoma cells, the β-elimination rates of the SeCys-conjugates were 2–6-fold lower than in cultured primary rat hepatocytes (Fig. 5). This may explain why induction of GSTs by SeCys-conjugates was lower in H35 Reuber rat hepatoma cells than in cultured primary rat hepatocytes. However, differences in the GST induction capacity between the two cell types may play a role as well, because the response to tBHQ is also lower in H35 Reuber rat hepatoma cells.

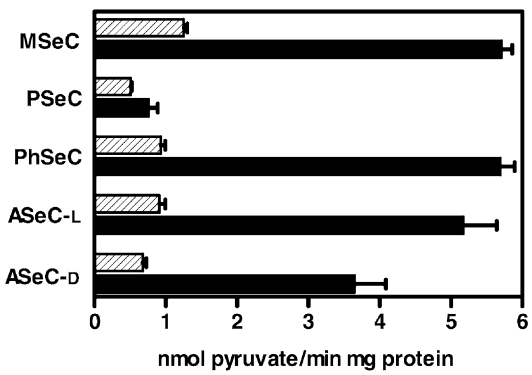


Fig. 5. Rates of β-elimination of SeCys-conjugates in H35 Reuber rat hepatoma cells and primary rat hepatocytes. SeCys-conjugates were incubated for 1 hr at 37° with dialyzed lysates from H35 Reuber rat hepatoma cells (hatched bars) or cultured primary rat hepatocytes (closed bars) at a concentration of 1 mM, in the presence of 500 μM α-keto-γ-methiolbutyric acid. Stoichiometric pyruvate formation was determined as a measure for β-lyase-dependent conversion of the SeCys-conjugates to the corresponding selenols. Values are means of three experiments ± SEM.

4. Concluding remarks

In summary, we demonstrate for the first time isoform-selective induction of GSTs by selenium compounds at the mRNA level. SeCys-conjugates increase *GSTA2*, *GSTA3*, *GSTA5* and *GSTP* mRNA levels in cultured primary rat hepatocytes and, to a lesser extent, in H35 Reuber rat hepatoma cells. ASeC-L was the most potent compound in the test series. This compound has also been demonstrated to be the most potent SeCys-conjugate in the prevention of chemically induced carcinogenesis [6]. The tested SeCys-conjugates did not induce CYP expression, which is an important beneficial property of chemopreventive compounds. Selenols, formed from SeCys-conjugates by β-lyases, may be involved in the transcriptional induction of

GSTs. We conclude that induction of GSTs and concomitant increased phase II metabolism of carcinogens may, at least partially, explain the tumor suppression properties of SeCys-conjugates. Several selenium-containing compounds are now in clinical trials to evaluate their chemopreventive activity. It would be highly interesting to see whether exposure to selenium-containing compounds also leads to elevated GST expression levels in humans.

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