Protection Conferred by Selenium Deficiency against Aflatoxin B₁ in the Rat Is Associated with the Hepatic Expression of an Aldo-Keto Reductase and a Glutathione S-Transferase Subunit That Metabolize the Mycotoxin¹

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ABSTRACT

Fischer 344 rats fed on a diet that is deficient in selenium are more resistant to the hepatocarcinogen aflatoxin B₁ (AFB₁) than rats fed on a selenium-sufficient diet. Hepatic cytosol from either selenium-deficient Fischer 344 rats or Hooded Lister rats possesses a marked increase in both reductase activity toward AFB₁-dialdehyde and glutathione S-transferase (GST) activity toward AFB₁-8,9-epoxide than hepatic cytosol from selenium-sufficient rats. The elevation in hepatic AFB₁-aldelyde reductase (AFAR) activity in selenium-deficient animals is accompanied by an increase of 11- and 10-fold in the levels of AFAR protein in liver cytosol from Fischer 344 and Hooded Lister rats, respectively. The amount of AFAR protein in selenium-sufficient and -deficient Fischer rats was modulated by treatment with N-acetylcysteine; this antioxidant reduced basal expression of AFAR but did not modulate the relative overexpression of AFAR during selenium deficiency. The enhanced capacity to conjugate glutathione with AFB₁-8,9-epoxide in selenium-deficient livers from Fischer 344 and Hooded Lister rats is associated with a 5- and 7-fold increase, respectively, in the hepatic levels of the AFB₁-metabolizing α-class GSTA subunit. The elevated levels of AFAR and GSTA protein in the selenium-deficient animals coincided with increases in the steady-state levels of their mRNAs. In selenium-deficient Fischer 344 rats, AFAR and GSTA were both found to be expressed throughout the centrilobular and midzonal areas of the liver lobule but were essentially absent from perportal hepatocytes. The effect of selenium insufficiency is pleiotropic, and it was also noted that the α-class GSTT1 is overexpressed 3- and 10-fold in livers of selenium-deficient Hooded Lister and Fischer 344 rats. Inasmuch as GSTT1 is responsible for the metabolic activation of dihaloalkanes, selenium deficiency may increase the susceptibility of rats to mutagens such as dichloromethane.

INTRODUCTION

Selenium is an essential trace element that exhibits diverse biological properties (1, 2). At normal physiological concentrations, the effects of selenium arise from the fact that it is incorporated covalently into proteins such as GPX⁴, phospholipid hydroperoxide glutathione peroxidase, type I iodothyronine 5′-deiodinase, and thioredoxin reductase (2–5). Selenium deficiency can cause a dramatic reduction in the levels of selenoproteins, and because many of these exhibit antioxidant properties, insufficient intake of selenium would appear to represent a form of oxidative stress (6). Reduced selenium intake has been associated with a number of clinical conditions in both humans and animals (2). Cancer incidence has been linked to selenium deficiency (7–9). For example, dietary selenium intake in some countries has been correlated inversely with cancer incidence in the human population (reviewed in Refs. 7 and 8). Similarly, in experimental animals, selenium deficiency generally increases susceptibility to chemical carcinogens, whereas supranormal intake of both organic and inorganic forms of selenium can be chemopreventive (10–12). A number of mechanisms have been proposed to explain the relationship between the level of selenium in the diet and altered incidence of neoplastic disease, including selenium-dependent modulation in expression of enzymes involved in the metabolism of carcinogens (13–15).

In general, reduction in selenium status is associated with increased risk of carcinogenesis (7, 8). Surprisingly, in apparent contradiction to this generalization, it has been shown that selenium-deficient rats are less susceptible to the toxic effects of AFB₁ (16), a mycotoxin that is arguably one of the most potent naturally occurring hepatocarcinogens (17). In their studies of AFB₁ toxicity in selenium deficiency, Chen et al. (16) showed that male weanling rats that had been fed on selenium-deficient diets for 6 weeks were 60% less susceptible to the formation of AFB₁-DNA adducts and 30% less susceptible to the covalent binding of AFB₁ to protein than were rats fed on selenium-sufficient diets. The basis for protection against AFB₁ in the livers of selenium-deficient rats has not been determined hitherto.

AFB₁ is subject to extensive metabolism in the liver, and the consequence of exposure to the toxin is determined largely by the capacities of the competing toxification and detoxification biotransformation pathways. The ultimate carcinogen formed from AFB₁ is the exo-8,9-epoxide (18), a metabolite produced by the actions of cytochrome P450 (19, 20). This epoxide is a highly reactive electrophile that can form adducts with DNA through the N² atom of guanine (21). Epoxided AFB₁ does not necessarily produce genotoxic damage, because it can be detoxified by the actions of certain α-class GST isoenzymes (22–24). Alternatively, AFB₁-8,9-epoxide can hydrolyze spontaneously at physiological pH to the 8,9-dihydrrodiol, which, in turn, rearranges to form AFB₁-dialdehyde (25). This metabolite can form Schiff bases with primary amino groups in protein and is therefore believed to be cytotoxic. The modification of protein by AFB₁-dialdehyde might be prevented by the actions of an aldo-keto reductase called AFAR, which catalyzes the reduction of the dialdehyde to a dialcohol (26–28).

Studies into chemoprotection against AFB₁, as well as studies into its selective toxicity, have suggested that tolerance of the mycotoxin is determined primarily by the levels of the Phase I drug-metabolizing enzymes GST and AFAR that catalyze detoxification reactions.
rather than by the levels of the Phase I drug-metabolizing enzyme cytochrome P450, which catalyzes the epoxidation of AFB1 (29). In the rat, which is sensitive to the mycotoxin, many cancer-chemopreventive agents, such as the antioxidants ethoxyquin and oltipraz, induce both the AFB1-metabolizing α-class GSTA5 subunit (also called Yc2 [30]; rat GATA1, GSTA2, GSTA3, GSTA4, GSTA5, GSTM1, GSTM2, GSTP1, GSTT1, and GSTT2 have previously been designated Ya1, Ya2, Yc1, Yk, Yc2, Yb1, Yb2, Yf, subunit 5, and Yrs, respectively) and AFAR (31, 32).

The levels of a number of hepatic proteins, including hemeoxy-hem and Phase II drug-metabolizing enzymes, have been shown to be elevated in selenium-deficient mice and Hooded Lister rats (33, 34). This observation suggests that the reduced ability of AFB1 to bind in vivo to macromolecules in the livers of selenium-deficient Fischer 344 rats may be attributable to increased levels of Phase II enzymes that metabolize the mycotoxin. Specifically, overexpression of GSTA5 may account for the lowered capacity to form AFB1-DNA adducts in selenium-deficient rats. The accompanying reduction in AFB1-protein binding in the selenium-deficient rats might also be attributable to overexpression of AFAR, but no previous evidence exists that might indicate that aldo-keto reductase expression is influenced by selenium status.

During the present study, it has been demonstrated that selenium deficiency results in a marked increase in the level of GSTA5 in rat liver, and this is associated with a corresponding increase in the amount of hepatic AFB1-GSH conjugating activity. Furthermore, selenium deficiency produced a pronounced increase in AFB1-aldehyde reductase activity and AFAR protein in rat liver. To our knowledge, this is the first demonstration that selenium deficiency can influence the expression of an aldo-keto reductase.

MATERIALS AND METHODS

Chemicals. These were of the highest purity available and were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom) unless otherwise stated. The Brownlee C18 reversed-phase HPLC column was from Anachem (Luton, Beds, United Kingdom), and HPLC-grade solvents were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Animals. Both male Hooded Lister and male Fischer 344 rats were used for the study of the effects of selenium status on the expression of AFB1-metabolizing enzymes. Hooded Lister rats were bred on site at the Rowett Research Institute, and the Fischer 344 rats were purchased from Harlan Olac Ltd., Bicester, Oxon, United Kingdom. The preparation of selenium-deficient and selenium-sufficient diets (containing 0.1 mg Na2SeO3/kg of diet) has been described previously (34). All of the animals studied were males obtained from mothers that were placed on synthetic diets during pregnancy and were themselves placed on the same diet from weaning (i.e., 3 weeks) until sacrifice at 10 weeks. In certain experiments, the selenium-deficient and selenium-sufficient diets were supplemented with 0.5% (w/v) NAC for the 4 days immediately prior to sacrifice of Fischer 344 rats.

Livers from Fischer 344 rats fed diets containing the synthetic antioxidant ethoxyquin were also included to allow comparison with results obtained from livers of selenium-deficient animals. For these experiments, 10-week-old Fischer 344 rats that had received normal (selenium-sufficient) animal feed from the time of weaning were fed for 14 days on diets containing 0.5% (w/v) ethoxyquin (22).

Enzyme and Protein Assay. Rat liver (100,000 × g supernatant (cytosol) was routinely prepared in ice-cold 20 mm sodium phosphate buffer (pH 7.0) containing 1 mm DTT (buffer A). GST activity toward CDNBr was determined by measuring the increased absorbance at 340 nm on a Cobas Fara centrifugal analyzer, whereas activity toward ENPP was determined manually by measuring the increased absorbance at 360 nm with a Shimadzu UV 3000 Spectrophotometer (35, 36). GST activity toward AFB1, 8,9-epoxide was measured by HPLC (22). Glutathione peroxidase activity with CuOOH was measured by centrifugal analyzer as described previously (36). Alko-keto reductase activities toward 9,10-PQ (0.05 mm), 2-CBA (0.5 mm), 4-NBA (1 mm), and SSA (1 mm) were measured using NADPH (0.24 mm) or NADH (0.24 mm) by centrifugal analyzer (32). Reductase activity toward AFB1-dialdehyde was measured by HPLC at the same time as the GST assays were carried out (26). Protein concentrations were determined by the dye-binding method of Bradford (37). Statistical analysis of the enzyme data was carried out using the Bonferroni test for pairwise comparisons.

Western Blotting. SDS-PAGE and protein blotting were carried out using methods described elsewhere (32). Most of the antibodies used in the immunoblotting experiments were polyclonal IgGs, raised previously in rabbits against purified native rat AFAR (27) and α-, μ-, and π-class GSTs (38). However, during the present study, monoclonal antibodies against heterologously expressed rat GSTA5 were generated in the mouse myeloma NSO cell line using standard techniques (39). In addition, polyclonal antibodies against θ-class GST were raised in rabbits using heterologously expressed recombinant protein; antibodies against GSTT1 were obtained against the COOH-terminally his-tagged human transerase synthesized in Escherichia coli from pET20b (40), whereas antibodies against GSTT2 were obtained against the NH2-terminally his-tagged rat transerase synthesized in E. coli from pET15b. Proteins resolved by SDS-PAGE were transferred to Immobilon P membranes, which were in turn blocked using a 10% solution of semi-skimmed dried milk in 50 mm Tris and 140 mm NaCl (pH 7.4) containing 0.025% Tween before being probed with antibodies at the following dilutions: anti-AFAR, 1:3,000, anti-GSTA1-2, 1:30,000; anti-GSTA3-3, 1:1,000; anti-GSTA4-4, 1:4,000; anti-GSTA5-5, 1:500; anti-GSTM1-1, 1:2,000; anti-GSTP1-1, 1:1,000; anti-GSTT1-1, 1:10,000; and anti-GSTT2-2, 1:1,000. Blots were also probed with anti-LDH as a loading control (32). All of the Western blots were repeated on three separate occasions using fresh liver samples. ECL was used to visualize immunoreactive protein.

Quantitation of GST Subunits. Cytosol was prepared at 4°C in buffer A from 5-g portions of rat liver. Cytosolic GSTs were isolated by affinity chromatography on 1.6 × 8.0 cm columns of glutathione-agarose. During the study, routine examination of the flow-through fractions from the glutathione-agarose column showed that the affinity matrix retained between 85 and 92% of the hepatic GST activity toward CDNB. After extensive washing of the column with buffer A until the A280 of the eluate was less than 0.05, GST was recovered by elution (20 ml, 4°C) with a freshly prepared solution of 200 mm Tris/HCl buffer (pH 9.0) containing 10 mm GSH. The GST-containing eluate was mixed immediately with an equal volume of 200 mm sodium phosphate buffer (pH 6.0, to reduce the pH) before the subunits were resolved by reversed-phase HPLC, on a 4.6 × 250 mm Brownlee C18 column (7-μm particle size and 300-Å pore size). The subunits were resolved using a 35–56% acetonitrile gradient that was formed in 0.1% trifluoroacetic acid. The eluate was monitored at 214 nm, and the relative amounts of the different subunits were estimated using published extinction coefficients (41).

Northern Blotting. Total RNA was prepared from rat liver by the method of Chomczynski and Sacchi (42) and was separated by denaturing electrophoresis in formaldehyde-agarose gels. The RNA was transferred to nylon membranes and was probed with radioactively labeled cDNA. For AFAR, the probe was generated using the 1.2-kb EcoRI fragment from pEE60 that comprises the entire cDNA (28). For GSTA5, the 0.7-kb BamHI fragment containing the entire coding sequence of the gene was prepared from pEE80, which contains the GSTA5 cDNA (i.e., from pET-GSTYc2-K; Ref. 24) cloned into the vector pTZ19r. As a loading control, a cDNA probe generated from the rat GAPDH gene was used. Blots were washed in 2× SSC and 0.1% SDS at 65°C before being subjected to autoradiography.

Purification of AFAR. Cytosol, prepared from livers (6-g portions) of selenium-deficient and -sufficient rats, was dialyzed at 4°C against three changes, each of 5 liters, of 10 mM Tris-HCl buffer (pH 8.2; prepared at 20°C) containing 1 mm EDTA, 1 mm Na2SeO3, and 1 mm NaCl. The buffer was dialyzed to remove precipitated protein and was applied to 1.6 × 45.0-cm columns of Q-Sepharose equilibrated and eluted (32 ml/h) with buffer B. The columns were each developed with a linear 0–120 mm NaCl gradient formed in buffer B (32). To ensure comparability, heparin cytosols from selenium-deficient and selenium-sufficient rats were processed simultaneously and subjected to Q-Sepharose chromatography in parallel using the same gradient-forming reservoirs. Once the salt gradient had been applied to the column, the remaining acidic carbonyl-reducing enzymes were eluted from Q-Sepharose in a stepwise fashion with 300 mm NaCl in buffer B. The eluate from the anion exchanger with 2-CBA reductase activity was applied to a 1.6
Table 1 AFB1-metabolizing activities in selenium-deficient and selenium-sufficient rat liver cytosol

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diet</th>
<th>Drug treatment</th>
<th>AFB1-(CHO)2 reductase (pmol/min/mg)</th>
<th>AFB1-epoxide conjugation (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hooded Lister</td>
<td>Se-</td>
<td>None</td>
<td>5.0 ± 1.0 (1)</td>
<td>85.0 ± 0.0 (1)</td>
</tr>
<tr>
<td>Fischer 344</td>
<td>Se-</td>
<td>None</td>
<td>50.0 ± 0.0 (10)</td>
<td>596.0 ± 5.2 (7)</td>
</tr>
<tr>
<td>Se+</td>
<td>None</td>
<td>0.8 ± 1.1 (1)</td>
<td>28.7 ± 1.3 (1)</td>
<td></td>
</tr>
<tr>
<td>Se+</td>
<td>NAC</td>
<td>0.2 ± 0.2 (0.25)</td>
<td>11.3 ± 4.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Se-</td>
<td>None</td>
<td>10.7 ± 0.0 (13)</td>
<td>174.0 ± 0.2a (6)</td>
<td></td>
</tr>
<tr>
<td>Se+</td>
<td>NAC</td>
<td>7.1 ± 1.3 (9)</td>
<td>114.7 ± 4.7 (5.5)</td>
<td></td>
</tr>
</tbody>
</table>

* Se-, selenium sufficient; Se+, selenium deficient.

Table 2 Aldehyde reductase, GST and glutathione peroxidase activities in selenium-deficient (Se-) and -sufficient (Se+) rat liver cytosol

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diet</th>
<th>Drug treatment</th>
<th>2-CBA (NADH)</th>
<th>2-CBA (NADPH)</th>
<th>SSA</th>
<th>4-NBA</th>
<th>9,10-PQ</th>
<th>CDNB</th>
<th>ENPP</th>
<th>CuOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hooded Lister</td>
<td>Se+</td>
<td>None</td>
<td>6.8 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>8.2 ± 0.2</td>
<td>32.7 ± 0.2</td>
<td>76.4 ± 12.0</td>
<td>711 ± 11</td>
<td>NDa</td>
<td>1490 ± 10</td>
</tr>
<tr>
<td>Fischer 344</td>
<td>Se+</td>
<td>None</td>
<td>21.7 ± 0.3b</td>
<td>4.2 ± 0.2b</td>
<td>9.5 ± 0.2</td>
<td>30.7 ± 0.2b</td>
<td>69.5 ± 12.0</td>
<td>1200 ± 1b</td>
<td>ND</td>
<td>910 ± 10</td>
</tr>
<tr>
<td>NAC</td>
<td>None</td>
<td>6.5 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>11.0 ± 0.2</td>
<td>36.8 ± 0.2</td>
<td>69.5 ± 12.0</td>
<td>1460 ± 45</td>
<td>0.29 ± 0.01</td>
<td>1175 ± 10</td>
<td></td>
</tr>
<tr>
<td>Se-</td>
<td>NAC</td>
<td>5.4 ± 0.3</td>
<td>3.3 ± 0.1</td>
<td>8.9 ± 0.4</td>
<td>29.4 ± 0.2</td>
<td>71.8 ± 10.0</td>
<td>1170 ± 15</td>
<td>0.20 ± 0.02</td>
<td>1000 ± 10</td>
<td></td>
</tr>
<tr>
<td>Se+</td>
<td>None</td>
<td>21.5 ± 0.4</td>
<td>4.8 ± 0.1</td>
<td>9.8 ± 0.1</td>
<td>28.3 ± 0.3</td>
<td>64.5 ± 10.0</td>
<td>1884 ± 10b</td>
<td>0.51 ± 0.01</td>
<td>235 ± 10</td>
<td></td>
</tr>
<tr>
<td>Se-</td>
<td>NAC</td>
<td>16.5 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>9.4 ± 0.1</td>
<td>26.0 ± 0.1b</td>
<td>71.1 ± 11.0</td>
<td>1730 ± 25</td>
<td>0.53 ± 0.01</td>
<td>220 ± 10</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.

** Values that are significantly greater than selenium sufficient control (P < 0.01).

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OVEREXPRESSION OF AFAR AND GSTA5 IN SELENIUM DEFICIENCY

Fig. 1. Increase of AFAR protein in the livers of selenium-deficient rats. Portions of purified AFAR (0.1 μg of protein) and hepatic cytosol (4 μg of protein) from both selenium-sufficient (+) and -deficient (−) rats were subjected to SDS-PAGE before transfer to immobilon P; the identity of samples applied to each of the lanes is shown. The blots were probed with antibodies raised against AFAR and developed by ECL (32). Immunoreactive AFAR protein was visualized by exposing the blot to X-ray film (A), and the light emission was quantitated directly using a Bio-Rad Phosphorimager (B). As a loading control, the blot was stripped of antibody and reprobed with antisera raised against LDH. The samples were analyzed on three separate occasions, and the data shown represent typical results. Std, standard.

Effect of Selenium Status on the Expression of AFAR and GST Subunits. Immunoblotting experiments demonstrated that the elevated AFB1-aldehyde reductase activity that occurs in selenium deficiency is associated with an approximate 11- to 15-fold increase in the amount of AFAR protein in rat liver cytosol (Fig. 1). The increase in AFAR protein produced by selenium deficiency was greater in Hooded Lister rats than in Fischer rats; this is in agreement with the treated with NAC, the AFAR activity was increased only 9-fold. It was also noted that the increase in 2-CBA reductase activity that accompanied selenium deficiency was, like AFB1-aldehyde reductase activity, offset by treatment with NAC.

An additional important feature of NAC treatment is that besides modulation of Phase II metabolism of AFB1, it also decreased reductase activity toward 2-CBA, SSA, and 4-NBA as well as GST activity toward CDNB in the livers of selenium-sufficient rats (Table 2). This result suggests that the basal levels of these enzyme activities may be influenced by redox status.

Fig. 2. Selective increase of GSTA5 and GSTT1 subunits in livers of selenium-deficient rats. Portions of purified GST (0.1 μg of protein) and hepatic cytosol (4 μg of protein) from control and treated rats were resolved by SDS-PAGE and transferred to immobilon P membranes. The blots were probed with antibodies raised against individual α, μ, θ, and ι-class GSTs and developed using ECL. Immunoreactive protein was visualized by autoradiography (A). As a loading control, the blot was stripped and reprobed using antisera raised against LDH. The differences in the levels of immunoreactive GSTA5 and GSTT1 observed in the samples were quantitated using a Bio-Rad Phosphorimager (B). +, selenium-sufficient rats; −, selenium-deficient rats. Std, standard.
OVEREXPRESSION OF AFAR AND GSTA5 IN SELENIUM DEFICIENCY

Fig. 3. Overexpression of GSTA2 and GSTM1 subunits in selenium-deficient rat liver. Hepatic GSTs were isolated by affinity chromatography on glutathione-agarose, and individual transferase subunits were resolved by reversed-phase HPLC, as described in the text. Approximately 30 μg of a mixture of purified GST subunits were applied to a Brownlee 4.6 × 250-mm (7-μm) column that was developed over 60 min with a 35-56% acetonitrile gradient, formed in 0.1% aqueous trifluoroacetic acid; the gradient was initiated at 10 min. A and B show the subunits resolved (between 28 min and 56 min) from the livers of selenium-deficient and selenium-sufficient rats, respectively.

Table 3 Quantitation of GST subunits in selenium-sufficient (Se⁺) and -deficient (Se⁻) Fischer rat liver cytosol

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>GSTM1 (mg/g protein)</th>
<th>GSTM2 (mg/g protein)</th>
<th>GSTP1 (mg/g protein)</th>
<th>GSTP2 (mg/g protein)</th>
<th>GSTA1 (mg/g protein)</th>
<th>GSTA2 (mg/g protein)</th>
<th>GSTA3 (mg/g protein)</th>
<th>GSTA4 (mg/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se⁺</td>
<td>None</td>
<td>3.37 ± 0.25</td>
<td>5.50 ± 0.33</td>
<td>0.57 ± 0.10</td>
<td>4.88 ± 0.36</td>
<td>&lt;0.01 ± &lt;0.01</td>
<td>2.62 ± 0.18</td>
<td>0.93 ± 0.08</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>Se⁺</td>
<td>NAC</td>
<td>2.08 ± 0.01</td>
<td>3.45 ± 0.01</td>
<td>0.61 ± 0.01</td>
<td>3.49 ± 0.26</td>
<td>&lt;0.01 ± &lt;0.01</td>
<td>2.73 ± 0.19</td>
<td>1.01 ± 0.08</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>Se⁻</td>
<td>None</td>
<td>3.80 ± 0.13</td>
<td>5.40 ± 0.11</td>
<td>0.47 ± 0.01</td>
<td>6.38 ± 0.02</td>
<td>1.35 ± 0.11</td>
<td>4.27 ± 0.01</td>
<td>4.85 ± 0.11</td>
<td>0.51 ± 0.33</td>
</tr>
<tr>
<td>Se⁻</td>
<td>NAC</td>
<td>3.03 ± 0.08</td>
<td>4.31 ± 0.06</td>
<td>0.61 ± 0.11</td>
<td>5.34 ± 0.05</td>
<td>1.27 ± 0.04</td>
<td>3.18 ± 0.18</td>
<td>2.84 ± 0.02</td>
<td>0.97 ± 0.05</td>
</tr>
</tbody>
</table>

a Values that are significantly less than untreated (−NAC) control (P < 0.01).
b Values that are significantly greater than selenium sufficient control (P < 0.01).

The enzyme data in Table 1. The data in Fig. 1 show that the ratio of the hepatic AFAR levels in NAC-treated selenium-deficient rats and NAC-treated selenium-sufficient rats was similar to the ratio of AFAR in selenium-deficient rats and selenium-sufficient rats that had not been treated with NAC. This suggests that NAC serves to reduce the basal expression of AFAR rather than modulate the mechanism responsible for its overexpression.

Western blotting also showed that the increased AFB1-GSH conjugating activity in selenium-deficient rat liver is associated with an increase of between 5- and 7-fold in the levels of GSTA5. As was noted above for AFAR, selenium deficiency caused a greater increase in the hepatic levels of GSTA5 in Hooded Lister rats than in Fischer rats. It was found that treatment of Fischer 344 rats with NAC did not influence the levels of GSTA5. The hepatic content of a number of other transferase subunits was examined to establish whether the marked overexpression of GSTA5 in selenium deficiency is unique within this superfamily. Fig. 2 shows that among α-class GSTs, the A5 subunit was the most dramatically overexpressed, although modest increases in A1 and/or A2 and A3 were also observed. The combined level of the μ-class GSTM1/2 subunits was not altered substantially by selenium status, but it was evident that livers from Hooded Lister rats contained substantially more μ-class GST than those from Fischer rats.

The data in Fig. 2 suggest that selenium deficiency results in a preferential overexpression of α-class rather than μ-class GSTs. However, because immunoblotting cannot discriminate between the GSTA1 and GSTA2 subunits nor between the GSTM1 and GSTM2 subunits, this apparent selectivity was investigated further using reversed-phase HPLC (Fig. 3). Using samples from the Fischer rat, reversed-phase HPLC showed that in addition to GSTA5, the GSTA2 subunit is overexpressed about 5-fold during selenium deficiency (Table 3). By contrast with GSTA2 and GSTA5, the levels of the GSTA1, GSTA3, and GSTA4 subunits showed smaller changes in selenium deficiency (Table 3). It was also found that the expression of neither GSTM1 nor GSTM2 is influenced by selenium deficiency. No significant changes were observed in the levels of GSTP1 in these animals. Furthermore, these experiments indicated that NAC treatment reduced marginally the level of the GSTA2 subunit.

During this study, we also probed the above samples with antibodies obtained recently against θ-class GSTs, and it was found that the amount of the GSTT1 subunit in hepatic cytosol was increased about 3-fold by selenium deficiency in Hooded Lister rats and about 10-fold in Fischer rats. Immunoblotting demonstrated that this polypeptide is expressed at higher basal levels in the Hooded Lister rats than in Fischer 344 rats. It is possible that higher constitutive levels of GSTT1 in Hooded Lister rats detracts from its relative overexpression during selenium deficiency. By contrast, the levels of the θ-class GSTT2
denaturing electrophoresis before being transferred to a nylon membrane. The blot was probed with a radioactively labeled cDNA encoding AFAR, GSTA5, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Regulation of AFAR and GSTA5 mRNA by Selenium Status.**

Northern blotting experiments revealed a substantial increase in the steady-state AFAR and GSTA5 mRNA levels in livers of selenium-deficient rats (Fig. 4). This shows that the control of these two genes is at the mRNA level and may involve either transcriptional activation or stabilization of message.

**Demonstration That AFAR Is the Major 2-CBA Reductase in Selenium-deficient Rat Liver.**

The fact that reductase activity toward both 2-CBA and AFB1-dialdehyde was increased in selenium-deficient rat liver, whereas reductase activities toward 9,10-PQ, 4-NBA, and SSA were relatively unaltered by selenium status suggested that AFAR may represent the principal 2-CBA reductase in the rat. To test this hypothesis and examine whether the levels of reductases other than AFAR are influenced by selenium status, aldo-keto reductases from the livers of selenium-deficient and selenium-sufficient rats were resolved by anion-exchange chromatography on Q-Sepharose (Fig. 5). In hepatic cytosol from selenium-deficient rats, reductase activity toward 2-CBA was found to be associated exclusively with acidic protein that was only eluted from the anion exchanger [equilibrated with 20 mM Tris-HCl buffer (pH 8.2)] in fractions 130—135 by the final 0.5 M NaCl wash step. Although this acidic peak of 2-CBA reductase activity was also observed in hepatic cytosol from selenium-deficient rats, the major peak of reductase activity toward 2-CBA in the deficient rat was eluted in fractions 80—85, by 50 mM NaCl. The position of this earlier eluting peak and the fact that it possessed reductase activity with SSA, suggested that it represented AFAR. Following purification of the 2-CBA reductase activity from fractions 80—85 of Q-Sepharose by affinity chromatography on Matrix Orange, this hypothesis was supported by the finding that the homogenous 2-CBA reductase comigrated during SDS-PAGE with heterologously expressed recombinant rat AFAR and cross-reacted with antibodies raised against purified rat AFAR (Fig. 6). Additional separate experiments (data not shown) revealed that this 2-CBA reductase activity in selenium-deficient rat liver coeluted with the major ethoxyquin-inducible aldo-keto reductase peak III from Q-Sepharose (See Ref. 32), suggesting that it is identical to the protein we have characterized previously from the livers of ethoxyquin-treated rats.

Besides the additional peak of 2-CBA activity, comparisons between the two elution profiles shown in Fig. 5 reveal a small increase in 4-NBA reductase activity and a small decrease in SSA reductase activity in the Q-Sepharose flow-through fractions (fractions 11—22) obtained from selenium-deficient rat livers. It therefore appears that among carbonyl-reducing enzymes in rat liver, only AFAR expression is increased markedly by selenium deficiency.

**Immunohistochemical Localization of AFAR and GSTA5 in Livers of Selenium-deficient Rats.**

Both AFAR and GSTA5 showed widespread induction in the livers of animals fed on the selenium-deficient diet. In control animals, there was some staining for AFAR localized in hepatocytes adjacent to the central veins (Fig. 7A), whereas selenium deficiency caused widespread induction of this enzyme throughout the lobule with the exception of perportal hepatocytes which generally remained negative (Fig. 7B). A similar pattern was observed with antibody against the GSTA5 subunit, where there was virtually no staining in control livers, but there was a pronounced induction extending from the central vein to midzonal regions of the lobule in animals with selenium deficiency. Staining for γ-glutamyl transferase activity, which was also carried out as a control, showed that in both selenium-sufficient and -deficient animals this enzyme was confined to biliary epithelial cells.

**DISCUSSION**

The fact that the intake of dietary selenium is below the recommended level in many human populations is of concern, because epidemiological data suggest that this predisposes individuals to several degenerative diseases, including cancer. Much remains to be learned about the pathophysiological responses to selenium deprivation and how low selenium status increases the risk of carcinogenesis. Whereas selenium deficiency causes loss of antioxidant capacity through the absence of GPX, it also produces complex metabolic and biochemical changes through the influence of selenoproteins on the activity of the transcription factors AP-1 and NFκB (47), on prosta glandin biosynthesis (48), and on thyroid hormone status (2).

Contrary to expectations based on epidemiological data (7, 8), selenium-deficient rats are less susceptible to the toxic effects of AFB1 than their selenium-sufficient counterparts (16). Furthermore, those regions of southern Asia where individuals are exposed to the mycotoxin (17, 49) are also regions where the dietary intake of selenium is low (50). We therefore chose to examine AFB1 metabolism in selenium-deficient rats, because this experimental model appeared both relevant to the human situation and likely to provide new insights into the alterations in gene expression that accompany low selenium status.

In this study, we examined both the Fischer 344 rat, because this was the strain in which resistance to AFB1 was noted originally during selenium deficiency (16), and the Hooded Lister rat, because this was the strain in which alterations in hepatic GST subunits were noted originally during selenium deficiency (34). Our data show that selenium deficiency produces a marked overexpression of AFAR and GSTA5 proteins in the livers of both Fischer and Hooded Lister rats, and we propose that the elevation of these two detoxification enzymes is responsible for the resistance to AFB1 noted earlier by others in the Fischer 344 rat. This is a particularly interesting observation, because the fact that hepatic levels of AFAR, or indeed the levels of any aldo-keto reductase, can be modulated by selenium status has not been recognized previously. The magnitude of the increase in AFAR and GSTA5 is considerably greater than has been noted before for detox-

**Fig. 4. Modulation of hepatic AFAR and GSTA5 mRNA by selenium status.** Total mRNA from the livers of selenium-sufficient (+) and -deficient (−) rats was separated by denaturing electrophoresis before being transferred to a nylon membrane. The blot was probed with a radioactively labeled cDNA encoding AFAR, GSTA5, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
Fig. 5. Resolution of a major 2-CBA reductase activity that is elevated in selenium-deficient rat liver. Hepatic cytosol was prepared from selenium-deficient and -sufficient Fischer rats. The cytosolic material from both sets of animals was dialyzed against 10 mM Tris-HCl (pH 8.2; buffer B) before being applied in parallel to two separate columns (1.6 × 45.0 cm) of Q-Sepharose. These columns were equilibrated with buffer B and were developed simultaneously with a linear 0–120 mM NaCl gradient formed in 600 ml, between fractions 30 and 125, in the same buffer; the strongly anionic protein retained by Q-Sepharose was finally eluted by a stepped elution with 500 mM NaCl. The flow rate was 32 ml/h, and fractions of 6 ml were collected. Reductase activity toward 2-CBA with NADH (●) or NADPH (○) as cofactor was determined. Reductase activity with NADPH as cofactor was also determined toward SSA (□) and 4-NBA (△). A and B, elution profiles of hepatic aldo-keto reductase activities from selenium-sufficient and -deficient rats, respectively.
Aldo-keto reductases are not widely regarded as inducible enzymes. With the exception of AFAR in rat liver, only 3α-hydroxysteroid dehydrogenase in human colon HT-29 cells has been shown to date to be regulated by drugs (60, 61). In addition, aldose reductase has been shown that it is transcriptionally activated to a greater extent by exposure to 1,2-dithiole-3-thione than any other drug-metabolizing enzymes are primarily localized (51, 52). By contrast with AFAR and GSTA5, GPX is located primarily in the perportal region of the liver (53, 54). This disparity between the localization of AFAR and GSTA5 on the one hand and GPX on the other suggests that if the absence of GPX leads to AFAR and GSTA5 overexpression, then the effect is indirect. In this situation, it would be necessary for the signal involved to be transmitted from the perportal to centrilobular region of the liver.

It was found that the increases in AFAR enzyme activities in selenium-deficient rat liver could be decreased by the administration of NAC. For example, AFB1-aldehyde reductase and 2-CBA reductase activities in selenium-deficient Fischer rats were significantly greater in animals that had not received NAC than in those that did receive NAC (Tables 1 and 2). The fact that NAC is known to be an antioxidant and free radical scavenger (45, 46), suggests that oxidative stress may be a significant factor in the mechanism leading to overexpression of AFAR, and that other mechanisms such as altered thyroid hormone status are not involved in this process. It is likely that the increased intracellular levels of H2O2 caused by absence of GPX in the selenium-deficient rat liver may be responsible for overexpression of AFAR. If this hypothesis is correct, the mechanism whereby NAC inhibits expression of these two proteins would appear to be indirect, given that it is unlikely that NAC interacts directly with H2O2. The most likely explanation for the effect of NAC is that it serves to help restore the normal redox status in the hepatocyte or that it reacts directly with some pro-oxidant species generated from H2O2.

It is recognized that reactive oxygen species can influence the activity of several transcription factors including AP-1 and NFκB (47, 55). In the situation of selenium deficiency, genes that are regulated by these factors may be sensitized to oxidative stress because of failure of normal redox control of AP-1 and NFκB brought about by absence of thioredoxin reductase, a selenoprotein that is required to generate the reductant thioredoxin (55, 56). It is therefore possible that the thioredoxin reductase/thioredoxin redox system is involved in the functional control of transcription factors that regulate the expression of AFAR and GSTA5. It is not known whether the promoter of the AFAR gene contains AP-1 or NFκB binding sites. However, examination of 2.0 kb of the 5′-flanking region of the GSTA5 gene has identified putative SP-1 and ARE enhancers rather than AP-1 and NFκB binding sites (57). Understanding the molecular basis for the regulation of AFAR is of considerable interest, because recent work has shown that it is transcriptionally activated to a greater extent by the cancer chemopreventive agent 1,2-dithiole-3-thione than any other gene in rat liver (58, 59).
shown to be inducible by H$_2$O$_2$ in rat vascular smooth muscle cells (62). Examination of these reductases by Q-Sepharose anion-exchange chromatography revealed that AFAR alone is overexpressed during selenium deficiency in rat liver (Fig. 5). The column profile provided no evidence that either 3α-hydroxysteroid dehydrogenase or aldose reductase is significantly overexpressed in the livers of selenium-deficient rats. Possible overexpression of aldo-keto reductases in extrahepatic tissues of selenium-deficient rats has not been examined, but aldose reductase may be overexpressed in smooth muscle. Interestingly, the Q-Sepharose chromatography data also indicated that 2-CBA is a relatively specific substrate for AFAR. The relationship between AFAR and the acidic reductase-containing peak (eluted by 0.5 M NaCl) which also exhibited activity for 2-CBA awaits clarification, but preliminary data have shown the two peaks to contain immunochemically related protein.

During this study, we have found that the α-class GSTT1 subunit is overexpressed in selenium-deficient rats. This result provides the first evidence that members of this class of GST can be regulated by environmental factors. The fact that the level of GSTT1 is increased in selenium deficiency is important, because this transferase is uniquely involved in the activation of a number of chemical carcinogens (30). Through catalyzing GSH conjugation reactions, the GSTT1 subunit activates small bifunctional electrophiles, including dichloromethane, dibromomethane, dibromochloromethane, and 1,2,3,4-diepoxybutane (40, 63). In the rat, it would be predicted that animals that are selenium deficient would be more sensitive to the toxic effects of these compounds than those fed on a selenium-sufficient diet. It should be noted that compounds such as dichloromethane are distributed widely in the environment and represent a serious health risk. In humans, GSTT1 is subject to population polymorphisms, with about 15% of the population being homozygous null (64). Nonetheless, it would be interesting to discover whether in humans who express GSTT1 selenium deficiency leads to overexpression of this transferase. In this eventuality, it would be important to determine whether the increase in GSTT1 is associated with increased risk of toxicity from dihaloalkanes and butadiene diepoxide.

In conclusion, we have demonstrated that AFB$_1$ resistance in the selenium-deficient rat is associated with a marked elevation in the hepatic levels of AFAR and GSTA5. The overexpression of these two AFB$_1$-metabolizing enzymes is accompanied by an increase in GSTT1, which is likely to result in increased sensitivity to dihalomethanes. Thus, although selenium deficiency confers resistance to the mycotoxin AFB$_1$, it may also sensitize animals to other carcinogens such as dichloromethane. Whether AFAR, GSTA5, and GSTT1 are themselves regulated directly by redox status is unproven, but the fact that they all possess activity toward organic hydroperoxides and reactive aldehydes suggests that their induction in selenium deficiency is an appropriate adaptive response to oxidative stress.

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Protection Conferred by Selenium Deficiency against Aflatoxin B$_1$ in the Rat Is Associated with the Hepatic Expression of an Aldo-Keto Reductase and a Glutathione S-Transferase Subunit That Metabolize the Mycotoxin

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