Altered glutathione transferase levels in rat skin inflamed due to contact hypersensitivity: induction of the Alpha-class subunit 1

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Since glutathione transferases (GSTs) are suggested to be involved in the prevention of tissue damage by oxidative stress, quantitative and qualitative alterations of GST forms were examined in rat skin after induction of inflammation by 0.6 and 1 % 1-chloro-2,4-dinitrobenzene (CDNB) treatment. With 0.6 % CDNB, the GST activity in supernatant preparations was 1.8-fold higher than that for control skin, with most GSTs in both cases being bound to *S*-hexyl-GSH-Sepharose. Major GST subunits of control skin were identified as subunits 7, 4 and 2 by HPLC and chromatofocusing at pH 11–7. These subunits were increased in inflamed skin by 0.6 % CDNB and, in addition, the subunit 1 of the Alpha class and subunit 6, both hardly detectable in control skin, were expressed. The specific activity value for GST 7-7 from the inflamed skin by 0.6 % CDNB was 2.4-fold

lower than that from control skin. However, in the case of inflamed skin after application of 1 % CDNB, GST activity was decreased to 69 % of the control value and most activity was recovered in fractions binding to a GSH-Sepharose but not a *S*-hexyl-GSH-Sepharose column. GSTs eluted from the former column demonstrated a restored capacity to bind to the latter, suggesting the GSTs in inflamed skin to be partly inactivated and that they regained activity on exposure to GSH. The K_m and V_{max} values for GSH of GST 4-4 from inflamed skin after 1 % CDNB treatment were 6-fold and 2-fold higher, respectively, than those for the enzyme from control skin, suggesting partial enzyme modification. These results suggest that not only quantitative but also qualitative alterations of GST subunits occur with CDNB-induced inflammation *in vivo*.

INTRODUCTION

Cytosolic glutathione transferases (GSTs) are a family of multifunctional dimeric enzymes that catalyse the conjugation of GSH to electrophilic xenobiotics [1,2]. The many molecular forms identified so far have been grouped into five classes, Alpha, Mu, Pi, Theta and Sigma [3,4]. Two additional classes, Kappa and Zeta, have been introduced recently in this enzyme family [5,6]. The major forms in rat liver are GST 1-1, 1-2 and 2-2 in the Alpha class and GST 3-3, 3-4 and 4-4 in the Mu class. The forms in the Alpha class possess high glutathione peroxidase activity towards lipid hydroperoxides [7]. Conjugation reactions of the Mu-class forms are activated by active oxygen species [8], whereas the Pi class is inactivated by hydrogen peroxide [9,10]. Thus, the GST forms have been suggested to play important roles in prevention of tissue damage by oxidative stress [2,4]. Although Pi- and Alpha-class GST forms are known to be up-regulated during rat hepatic carcinogenesis [11], alteration of GST expression in inflammatory conditions resulting in oxidative stress remains to be clarified.

Each organ examined so far has been shown to possess a unique expression profile of GST forms. Extrahepatic organs share some, but not all, forms expressed in the liver [12,13]. Information on GST forms in rat skin is relatively limited [14,15]. 1-Chloro-2,4-dinitrobenzene (CDNB), a common substrate of most GST forms, is known to induce skin inflammation due to a delayed-type hypersensitivity reaction with repeated topical application of the drug [16,17]. A mutant hairless rat strain established in our institute from Sprague-Dawley rats develops contact hypersensitivity to CDNB [18]. In the present

study, we quantified the amounts of GST forms in inflamed rat skin induced by CDNB treatment and also examined kinetic properties of GST 4-4 obtained from such lesions to clarify whether alterations of GST forms occur in inflammation *in vivo*.

MATERIALS AND METHODS

Materials

Epoxy-activated Sepharose 6B, Polybuffer exchanger (PBE 118) and Pharmalyte 10.5-8, were obtained from Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose membranes and anti-rabbit IgG conjugated with horseradish peroxidase were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). GSH was purchased from Kohjin Co. (Tokyo, Japan); CDNB from Wako Pure Chemicals (Osaka, Japan); xanthine and xanthine oxidase from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. *S*-hexyl-GSH- and GSH-Sepharose were prepared according to the methods of Guthenberg and Mannervik [19] and Simons and Vander Jagt [20], respectively.

Induction of contact hypersensitivity

Hirosaki hairless rats, a mutant strain established from the Sprague-Dawley strain in the Institute for Animal Experiments, Hirosaki University School of Medicine, Hirosaki, Japan [18], were fed a rat diet (MF Oriental Yeast Co., Tokyo) and water *ad libitum*. For induction of skin inflammation by contact hypersensitivity, 12 female rats (250–300 g body weight) were each sensitized by painting 0.4 ml of 1 % (w/v) CDNB dissolved in

Abbreviations used: GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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acetone on their backs. After 14 days, the individual rats were challenged by painting 3 ml of 1% or 0.6% CDNB solution on the whole bodies of the rats except for the heads and limbs. Two days after the challenge, the rats were killed and their skins were removed. Some naive rats were treated with 0.4 ml or 3 ml of 0.6% or 1% CDNB alone. Control rats were treated with equal amounts of the acetone vehicle.

GST assay

GST activity was assayed with 1 mM CDNB and 1 mM GSH as the substrates according to the method of Habig et al. [21]. GST activity (1 Unit) is the amount of enzyme catalysing the conjugation of 1 μ mol of substrate/min at 25 °C. In some experiments to determine the K_m values, CDNB and GSH concentrations were varied from 0.025 to 1 mM. Protein content was measured by the methods of Lowry et al. [22] or Bradford [23], using BSA as a standard.

Purification of GST from rat skin

Rat skins were washed with 0.9% NaCl solution, scraped with a surgical knife to remove dermal fat and cut into small pieces. Separate 100 g batches of control or inflamed skins derived from 3–4 rats were homogenized with a Polytron in 4 vols. of 10 mM Tris/HCl (pH 7.8), containing 0.2 M NaCl. A 0.6% CDNB-treated skin sample from non-sensitized rats was also used as a reference without inflammation. After centrifugation at 105 000 g for 45 min, the supernatant was subjected to *S*-hexyl-GSH–Sepharose affinity chromatography [19] followed by chromatofocusing (pH 11–7), as described previously [24]. Unbound fractions were further subjected to GSH–Sepharose chromatography [20], as reported earlier [25].

Electrophoresis

SDS/PAGE on 12.5% slab gels was carried out as described by Laemmli [26]. Immunoblot analysis was performed according to the method of Towbin et al. [27]. Antibodies against GST 1-2, 3-4 and 7-7 were raised in rabbits as reported earlier [28].

Quantification of GST subunits by HPLC

Individual GST subunits were separated and quantified by HPLC, essentially according to the method of Ostlund-Farrants et al. [29]. HPLC was carried out on a C₁₈ reverse-phase column (3.9 × 150 mm, μ Bondasphere; Waters, Milford, MA, U.S.A.). Elution was performed with the following gradient system of solvent A (water) and solvent B (80% acetonitrile/0.1% trifluoroacetic acid) at a flow rate of 1.4 ml/min; 0–5 min, 100% solvent A; 5–15 min, 0–45% solvent B; 15–90 min, 45–100% solvent B. All runs were performed at 35 °C, and column effluent was monitored at 214 nm. GST 1-1 and 4-4 purified from rat liver [28], GST 7-7 from hepatic hyperplastic nodules [11] and GST 6-6 from brain [30] were also examined to identify the individual peaks.

Treatment of GSTs with xanthine and xanthine oxidase

An appropriate amount (0.2–0.8 Unit/ml) of each purified GST form was incubated at 25 °C for 30 min with 0.4 mM xanthine and xanthine oxidase (15 m-Units/ml) in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA, as reported previously [8].

RESULTS

Alteration of GST activity in inflamed skin

Sensitized rats were challenged by CDNB to induce skin inflammation due to contact hypersensitivity. Rats treated with 1 or 0.6% (w/v) CDNB exhibited strong and weak redness of the skin, respectively, 2 days after the challenge. The total GST activity of supernatant of inflamed skin in the 0.6% CDNB case was 73.3 Units/100 g of skin, 1.8-fold higher than the control value (40.9 Units/100 g of skin, Table 1). Most supernatant GST activity in both cases was due to forms binding to *S*-hexyl-GSH–Sepharose. However, in the case of inflamed skin after 1% CDNB, GST activity of supernatant was decreased to 69% of the control value, and most activity was recovered in the *S*-hexyl-GSH–Sepharose unbound fractions. However, binding to GSH–Sepharose was apparent and the value for enzyme bound to columns of the latter (16.5 Units/100 g of skin) was 2.3-fold

Table 1 Changes in GST activity in rat inflamed skin after challenge with 0.6 or 1% CDNB

Supernatant fractions prepared from homogenates of 100 g samples of rat control or inflamed skin after challenge with 0.6 or 1% CDNB were applied individually to *S*-hexylglutathione–Sepharose columns. A CDNB (0.6%)-treated skin sample from naive rats was also used as a reference without inflammation. GST forms bound to the column were eluted with 5 mM *S*-hexylglutathione. Unbound GST forms were further applied to a column of glutathione–Sepharose and eluted with 25 mM GSH. Values in parentheses indicate activities recovered in the bound fractions of the latter column. Details of the procedures are described in the Materials and methods section.

Fraction	GST activity (units/100 g of skin)			
	Control	Naive rats treated with 0.6% CDNB	Rats challenged with 0.6% CDNB	Rats challenged with 1% CDNB
105 000 g Supernatant	40.9	36.7	73.3	28.4
<i>S</i> -Hexyl-GSH–Sepharose bound	28.3	25.2	62.6	8.2
<i>S</i> -Hexyl-GSH–Sepharose unbound	11.6 (7.3)	10.2 (6.9)	10.4 (8.2)	19.3 (16.5)

Figure 1 SDS/PAGE of rat skin GSTs bound to *S*-hexyl-GSH– (A) and GSH–Sepharose (B) and eluates from a chromatofocusing column (C)

S-Hexyl-GSH– and GSH–Sepharose affinity chromatography was performed as described in the text. SDS/PAGE was carried out with 12.5% acrylamide gels. (A, B) each lane contained 3–30 μ g of protein, the amounts equivalent to 0.4 g of the individual tissues. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, control skin; lane 2, 0.6% CDNB-inflamed skin; lane 3, 1% CDNB-inflamed skin. The numbers on the left indicate the GST subunits. (C) *S*-Hexyl-GSH–Sepharose-bound GSTs derived from 0.6% CDNB-inflamed skin were resolved by chromatofocusing as described for Figure 3. Eluates from the column were subjected to SDS/PAGE. Lane 1, fraction 26 of Figure 3B; lane 2, fraction 32; lane 3, fraction 60; lane 4, fraction 78; lane 5, fraction 119.

Figure 2 Separation of rat skin GST subunits by HPLC

Aliquots (20–200 µg of protein) of GSTs partially purified by *S*-hexyl-GSH affinity chromatography from 100 g samples of control rat skin (A), and inflamed skin after challenge with 0.6% CDNB (B), or 1% CDNB (C) were subjected to HPLC. The amounts applied were equivalent to 3.0 g of the individual tissues. Elution was performed as described in the text. Numbers by peaks denote the respective GST subunits, identified using the individual homodimers.

higher than that for the control skin (Table 1). When GSTs eluted from GSH-Sepharose were again applied to *S*-hexyl-GSH-Sepharose after dialysis, most activity was retained by the column (results not shown). When non-sensitized naive rats were treated with 0.4 or 3 ml of 0.6% CDNB, no skin redness was exhibited within 14 days and the GST activity of skin preparations obtained at 2 days after the treatment was not different from the control value (Table 1). When 1% CDNB was applied to non-sensitized rats, GST activity was also similar to the control value (results not shown).

Table 2 Contents of GST subunits in rat inflamed skin after challenge with 0.6 or 1% CDNB

GST subunits bound to an *S*-hexyl-GSH-Sepharose column were resolved by HPLC and their protein contents quantified. Details of the procedures are described in the text. Abbreviation: n.d., not detectable.

Rat skin	GST subunit ...	Content (mg/100 g of tissue)			
		1	2	4	7
Control		n.d.	0.6	0.9	1.6
Naive rats treated with 0.6% CDNB		n.d.	0.5	0.7	1.4
Rats challenged with 0.6% CDNB		0.6	2.1	1.5	2.4
Rats challenged with 1% CDNB		n.d.	0.2	0.2	0.3

Alterations of GST subunits in inflammation

To identify altered GST subunits in inflamed skin, fractions bound to *S*-hexyl-GSH- and GSH-Sepharose were analysed by SDS/PAGE (Figure 1) and HPLC (Figure 2). These analyses revealed the major GST subunits of control skin to be subunits 7, 4 and 2. These subunits were increased in inflamed skin induced by 0.6% CDNB and an additional subunit was detected by SDS/PAGE (Figure 1A, lane 2). This was identified as subunit 1 by immunoblot analysis with anti-GST 1-2 antibody (results not shown) and the elution position on HPLC (Figure 2), and was also detected in the GSH-Sepharose-bound fraction. All three subunits binding to *S*-hexyl-GSH-Sepharose for control skin were decreased markedly in inflamed skin after 1% CDNB. However, significant amounts of subunits 4 and 7 were recovered in the GSH-Sepharose-bound fractions (Figure 1B, lane 3), consistent with their GST activities being higher than those for control skin. Amounts of GST subunits in control and inflamed skins determined by HPLC employing *S*-hexyl-GSH-Sepharose-bound fractions are summarized in Table 2. Subunit content of 0.6% CDNB-treated skin from naive rats was similar to the control value. Subunits 1 and 2, both belonging to the Alpha class, were increased markedly in inflammation induced by 0.6% CDNB, as compared with those in control skin. Subunits 4 and 7 were also increased in the inflamed skin but not as markedly as the Alpha-class subunits. In the case of inflammation due to 1% CDNB, the individual subunits bound to *S*-hexyl-GSH-Sepharose were decreased to between one third and one fifth of their levels in control skin. The total amount of GST subunits, including those bound to GSH-Sepharose, was about half that for control skin (results not shown). GST forms bound to *S*-hexyl-GSH-Sepharose were also resolved by chromatofocusing and the constituent subunits identified by SDS/PAGE. Control skin GSTs were separated into three active peaks corresponding to GST 2-2, 7-7 and 4-4 (Figure 3A), consistent with the HPLC results. Furthermore, an acidic GST form(s) possessing isoelectric point value(s) less than 7.0 was eluted with 1 M NaCl. In the case of inflamed skin after 0.6% CDNB treatment, an additional peak was detected before that for GST 2-2 (Figure 3B). Although the separation of the two peaks was incomplete, the lower part of the preceding peak (fractions 24–28) gave the two bands of subunits 1 and 2 with equal amounts on SDS/PAGE (Figure 1C), suggesting that a heterodimer GST 1-2 was eluted in the fractions. The activity of GST 7-7 derived from the inflamed skin

Figure 3 Separation of rat skin GST forms by chromatofocusing

Purified GSTs as described for Figure 2 were subjected to chromatofocusing at pH 11–7. Elution was performed as described in the text. Flow rate was 15 ml/h and fractions of 2.4 ml were collected. Numbers by peaks denote the respective GST forms. **A**, **B** and **C** are the same as those in Figure 2. ●, GST activity with CDNB; ---, pH. Arrows indicate the elution position with 1 M NaCl.

induced by 0.6% CDNB was less than that for control skin, despite the higher amount of the subunit 7 protein in the former case (Table 2). The specific activity value of this form purified from inflamed skin was calculated to be 5.2 Units/mg of protein, about 2.4-fold lower than the value for GST 7-7 from control skin (12.5 Units/mg of protein). Such a difference in specific activity was not evident for GST 2-2 or 4-4. GST activity in fractions eluted with 1 M NaCl was about 10-fold higher in the inflamed skin than in control skin. These fractions showed two bands on SDS/PAGE: one was identical with the subunit 4 in mobility and another band migrated between subunit 4 and subunit 1 (Figure 1C). The latter band stained by immunoblotting with anti-GST 3-4 antibody (results not shown). HPLC analysis revealed that these fractions contained the subunits 4 and 6 (Figure 4). Thus, the band between subunits 4 and 1 on SDS/PAGE could have been subunit 6. Unlike the increase after 0.6% CDNB, the individual GST forms were decreased in the inflamed skin in the 1% CDNB case (Figure 3C).

Comparison of kinetic parameters between GST 4-4 from control skin and GST 4-4 from 1% CDNB-inflamed skin

The K_m and V_{max} values for GSH of GST 4-4 from inflamed skin were 0.5 mM and 20 $\mu\text{mol}/\text{mg}$ of protein per min, respectively, 6-fold and 2-fold higher than the values for the enzyme in control

Figure 4 HPLC analysis of acidic GST forms derived from 0.6% CDNB-inflamed skin

An aliquot (20 μg of protein) of inflamed-skin GST forms eluted from a chromatofocusing column by 1 M NaCl was subjected to HPLC to examine the subunit composition. Elution was performed under the same conditions as for Figure 2. Numbers by peaks denote the respective GST subunits.

skin (Figure 5). Treatment of control-skin GST 4-4 with xanthine oxidase and xanthine resulted in a 3-fold increase in the V_{max} value, 30 $\mu\text{mol}/\text{mg}$ of protein per min, confirming our previous result [8]. Thus, the V_{max} value of inflamed-skin GST 4-4 was intermediate between those of control-skin GST 4-4 and the enzyme treated with xanthine oxidase, whereas the K_m value of inflamed-skin enzyme was the highest among these three preparations. At 0.025–0.10 mM GSH concentrations, GST 4-4 from inflamed skin showed lower activities than that from control skin and that treated with xanthine oxidase. On the other

Figure 5 Lineweaver-Burk plots of GST 4-4s purified from control and inflamed skin and the same enzyme treated with xanthine oxidase and xanthine

The enzyme assay was performed with various concentrations of GSH at 1 mM CDNB in 0.1 M potassium phosphate (pH 6.5). ●, GST 4-4 from control skin; ○, GST 4-4 from inflamed skin after challenge with 1% CDNB; △, GST 4-4 treated with xanthine oxidase and xanthine. Conditions for xanthine oxidase treatment are described in the text.

hand, the K_m and V_{max} values for CDNB were not different between the inflamed and control skin cases (results not shown).

Although the V_{max} value (6 $\mu\text{mol}/\text{mg}$ of protein per min) of GST 7-7 from inflamed skin induced by 0.6% CDNB was a half of that of GST 7-7 from control skin (12 $\mu\text{mol}/\text{mg}$ of protein per min), the K_m value (0.1 mM) for GSH was not different between the two preparations.

DISCUSSION

The present study revealed that the major GST subunits in female rat skin are 2, 4 and 7, in agreement with the recent result of Hiratsuka et al. [15]. In inflamed skin after 0.6% CDNB treatment, these subunits were increased and the subunit 2, in particular, was expressed strongly (Table 2). Furthermore, another subunit of the Alpha class, subunit 1, not detected in normal skin, was found (Figure 1). When non-sensitized rats were treated with 0.6% CDNB, neither inflammation nor alterations in GST activity were observed, suggesting that such induction of GST subunits depends on inflammation rather than the direct effects of the compound itself. Although GSTs are known to be inactivated by CDNB *in vitro* [31], the present study has revealed that topical application of the drug hardly affects GST activity and subunit content of the skin *in vivo*. Induction of Alpha- and Pi-class GST forms has been reported in cultured cells exposed to oxidative-stress conditions [32–34], and the expression of Alpha-class forms is regulated at the transcriptional level via the electrophile-responsive elements of their genes [35,36]. The present findings thus partly support the conclusion that induction of GST subunits occurs in inflammation *in vivo*. Since Alpha-class GST forms possess glutathione peroxidase activity towards lipid hydroperoxides [7], induced GST forms may play some role in removal of such oxidants. In addition, since GST 1-1 and GST 1-2 also possess prostaglandin $\text{H} \rightarrow \text{E}$ isomerase activity [37], this might be involved in inflammation.

Chromatofocusing analysis of *S*-hexyl-GSH–Sepharose-bound fractions revealed the activity of acidic GST forms eluted with 1 M NaCl to be 10-fold increased in the 0.6% CDNB-inflamed skin (Figures 3A and 3B), apparently with subunit 6 as one of the components. Although this subunit was not evident on SDS/PAGE of *S*-hexyl-GSH–Sepharose-bound fractions (Figure 1A, lane 2), HPLC analysis exhibited a small shoulder adjacent to the peak of subunit 2 (Figure 2B) at an elution position corresponding to that of subunit 6 purified from rat brain (Figure 4). GST 6-6 and 4-6 are known to possess 5–10-fold higher specific-activity values than GST 4-4 [30]. An increase of subunit 6 in inflamed skin would be interesting since GST 6-6 has been reported to produce leukotriene C_4 [30], a mediator of inflammation [38], from leukotriene A_4 , in the same way as the membrane-bound leukotriene C_4 synthase [39].

In the case of severe inflammation induced by 1% CDNB, however, GST activity of supernatant was slightly decreased and most of the enzyme did not bind to *S*-hexyl-GSH–Sepharose but only to GSH–Sepharose (Table 1). Several GST forms including rat GST 8-8 are reported to show a preferential affinity for GSH–Sepharose [25,40]. The fact that GSTs eluted from GSH–Sepharose seemed identical with GST 4-4 and 7-7 and restored binding capacity to *S*-hexyl-GSH–Sepharose suggests that GSTs in severely inflamed skin are partly inactivated. Our previous study revealed that GST 7-7 is reversibly inactivated after hydrogen peroxide treatment due to intra- or intersubunit disulphide-bond formation between the 47th cysteine and other cysteine residues [10]. In addition, inactive GST 7-7 was partly reactivated by GSH and restored binding capacity to *S*-hexyl-GSH–Sepharose [9]. Thus, after elution from GSH–Sepharose

by GSH, GSTs are likely to display restored activities. Moreover, GST 7-7 purified from *S*-hexyl-GSH–Sepharose-bound fractions of 0.6% CDNB inflamed skin exhibited a 2.4-fold lower specific-activity value than that for GST 7-7 from control skin, suggesting that partial inactivation had also occurred in the former case. GST 4-4 purified from *S*-hexyl-GSH–Sepharose-bound fractions of inflamed skin by 1% CDNB showed different K_m and V_{max} values from GST 4-4 from control skin (Figure 5), providing further supportive evidence of qualitative alteration in subunits. Although both GST 4-4 from inflamed skin and GST 4-4 treated with xanthine oxidase shared increased V_{max} and K_m values as compared with the control-skin enzyme, the two enzyme preparations showed quite different activities at low GSH concentrations. Since xanthine oxidase treatment results in the production of superoxide anions [41], these results suggest that the enzyme from inflamed skin may be partly modified by unidentified factors different from the superoxide anion.

In conclusion, GST subunits may be increased in inflammation *in vivo*, with the extent of induction varying with the individual subunits, but severe inflammation might cause a decrease in expression and/or activity. The GST subunits 7 and 4 appear sensitive to oxidative stress, with a resultant alteration in their catalytic properties.

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