Growth hormone- and testosterone-dependent regulation of glutathione transferase subunit A5 in rat liver

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The class Alpha glutathione S-transferase (GST) subunit A5 is expressed in the livers of young male and female rats. After sexual maturation, this protein is no longer detectable in the livers of male rats, but is still expressed in female rats. We have previously demonstrated that the sexually dimorphic secretion of growth hormone regulates the levels of certain class Mu GSTs in rat liver, and this study was designed to investigate the hormonal regulation of GSTA5. Control and hypophysectomized rats of both sexes were used to study the role of growth hormone in the

regulation of hepatic GSTA5; and the influence of testosterone on the expression of this same subunit was investigated in intact females and castrated males. Liver cytosols were subjected to SDS/PAGE and immunoblotting using antibodies directed towards rat (r)GSTA5, and to affinity purification on glutathione-Sepharose followed by reverse-phase HPLC in order to quantify the relative levels of rGSTA1, A2, A3, A4, M1 and M2 subunits. These analyses revealed that the expression of rGSTA5 is, indeed, regulated by both growth hormone and testosterone.

INTRODUCTION

The glutathione S-transferases (GSTs) are a family of isoenzymes expressed in all eukaryotic organisms investigated to date. The cytosolic isoenzymes are divided into seven different classes, i.e. Alpha, Mu, Pi [1], Theta [2], Sigma [3], Kappa [4] and Zeta [5], and are active as homo- or hetero-dimers containing subunits belonging to the same class. The isoenzymes have different, but overlapping, substrate specificities and catalyse the conjugation of glutathione to a broad range of electrophilic compounds, which might otherwise react with nucleophilic sites in proteins and nucleic acids. This conjugation with glutathione results in a less reactive and more water-soluble and readily excreted compound.

In addition to detoxifying xenobiotics, the GSTs also catalyse the conjugation of endogenous substrates, including cholesterol α -oxide [6], prostaglandins [7] and leukotriene A4 [8,9]. Although poorly understood, GSTs may also act as binding proteins for bile acids [10,11], steroid hormones [12–14] and neurotransmitters [14]. (For a review, see Hayes and Pulford [15].)

GSTs can be up-regulated by various enzyme-inducing agents, including *trans*-stilbene oxide [16] and butylated hydroxyanisole [17]. In addition to induction by exogenous compounds, the pattern of GST expression varies during development [18,19] and aging [20,21] and is, furthermore, organ-, sex- and species-specific [22,23]. Such observations indicate that the expression of GSTs is also regulated by endogenous factors. Lamartinière [24] demonstrated pituitary regulation of rat hepatic GSTs. In addition, specific hormones, including adrenocorticotrophic [25], growth (GH) [26], thyroid [27] and steroid hormones [28] have been demonstrated to regulate the levels of cytosolic GSTs in certain target organs.

Regulation of GST expression can have a profound effect on sensitivity to chemical insult. For example, aflatoxin B₁ (AFB₁), which is produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*, has been implicated as a causal factor in several

human diseases, including Kwashiorkor and cancers of the colon, liver and lung [29,30]. The toxic effects of AFB₁ are caused directly by the *endo*-AFB₁-8,9-epoxide and *exo*-AFB₁-8,9-epoxide, generated via metabolism by the cytochrome P-450 system [31]. GST-catalysed conjugation of AFB₁-8,9-epoxide to glutathione is a major pathway for detoxication of these reactive metabolites [32]. The *endo*-epoxide is less harmful and is metabolized by the class Mu GSTs, whereas the more toxic *exo*-epoxide is metabolized by class Alpha GSTs [33]. Protection against AFB₁ toxicity in the rat can be conferred by dietary administration with ethoxyquin, butylated hydroxyanisole and oltipraz [34–36]. This protection has been found to be correlated with up-regulation of GSTA5, a subunit with high activity towards *exo*-AFB₁-8,9-epoxide [37].

The good correlation between physiological expression of GSTA5 and relative resistance to aflatoxin-induced carcinogenesis provides further evidence for the protective role of this subunit with respect to AFB₁ toxicity. Specifically, rGSTA5 is expressed in the livers of young rats of both sexes [30], but is no longer detectable in male rat liver at 10 weeks of age. As would be predicted, mature male rats are more sensitive than are females to the carcinogenic effect of AFB₁ [38].

We have previously demonstrated that the sexual dimorphism in the expression of certain GST subunits (i.e. rGSTA1, A2, M1 and M2) in rat liver is due to the sexually dimorphic secretion of GH [26]. The secretion of this hormone is pulsatile in both sexes, but the amplitudes of the pulses are lower in females than in males, resulting in more continuous secretion in females [39]. By continuous administration of GH to intact males or hypophysectomized animals of either sex, it is possible to feminize the pattern of expression of GST isoenzymes in the liver.

The onset of the sexually differentiated GH secretion in rats occurs at puberty, which is also the period when rGSTA5 expression in the livers of male rats is down-regulated. The present study was designed to elucidate the endocrine regulation of rat hepatic GSTA5. Initially, the role of growth hormone in

Abbreviations used: GST, glutathione S-transferase; rGST, rat GST; GH, growth hormone; AFB₁, aflatoxin B₁; CDNB, 1-chloro-2,4-dinitrobenzene.

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this regulation was examined. In addition, the effects of castration and testosterone treatment on GSTs in general and rGSTA5 in particular were characterized.

MATERIALS AND METHODS

Materials

Human GH was kindly given by Pharmacia and Upjohn (Stockholm, Sweden). Thyroxine, cortisone acetate, testosterone propionate and 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma, St. Louis, MO, U.S.A.), Alzet osmotic minipumps (Alza Corp., Palo Alto, CA, U.S.A.), glutathione–Sepharose affinity gel (Pharmacia Biotech, Sollentuna, Sweden) and Sprague–Dawley rats, either sham-operated, hypophysectomized or castrated (Møllegaards Avlslaboratorium, Skensved, Denmark) were obtained from the sources indicated. All other chemicals and reagents were of the highest grade obtainable from common commercial sources.

Animals, treatment and preparation of cytosolic fractions

The first experiment will be referred to as the GH experiment and the second as the testosterone experiment. Table 1 lists the experimental groups and sexes used in these two experiments, and Figure 1 illustrates the hormone treatments employed.

Table 1 A summary of the approaches employed in the GH and testosterone experiments

Human GH was administered continuously through osmotic minipumps, and testosterone propionate was administered as daily subcutaneous injections as described in the Materials and methods section. Abbreviations: M, males; F, females.

Experiment	Treatment	Sex
Control GH	Sham-hypophysectomized	M, F
	Hypophysectomized	M, F
	Hypophysectomized + GH	M, F
Control Testosterone	Sham-castrated	M
	Testosterone	F
	Castrated	M
	Castrated + testosterone	M

Figure 1 Schematic illustration of the treatments employed

In the GH experiment, animals of either sex were hypophysectomized (hx) or sham-hypophysectomized (sham) at 6 weeks of age; whereas in the testosterone (test) experiment, males were either castrated (cx) or sham-castrated (sham) at 4 weeks of age. The hormone treatments are indicated with bold lines. One group of female rats was treated with testosterone in the same manner as the males. The ends of the lines represent the time of killing.

Sham-operated rats of both sexes were used as controls in both experiments. The first experiment involved animals of both sexes and three experimental groups: controls, hypophysectomized rats and hypophysectomized rats administered GH continuously. Hypophysectomy was performed at 6 weeks of age (i.e. immediately before puberty), and hormone treatment was initiated 4 weeks later, at a time-point that was shown previously to yield maximal or near maximal increases in hepatic GST activity [26].

In order to substitute for the deficiency in the thyroid and adrenal glands in the hypophysectomized animals, thyroxine and cortisone acetate at doses of 10 µg and 500 µg/kg body weight, respectively, were administered daily as subcutaneous injections, starting 2 days before initiation of GH treatment [40]. (Administration of thyroxine and cortisone acetate alone, without GH, does not affect the expression of hepatic GST in hypophysectomized animals [26].) GH was administered at a rate of 50 m-units/h and kg body weight for 7 days through osmotic minipumps implanted subcutaneously under the neck.

In the testosterone experiment we examined intact female rats treated with testosterone, castrated male rats and castrated males treated with testosterone. Castration was carried out at 4 weeks of age, when the males still express rGSTA5, and testosterone treatment was initiated at 8 weeks of age in both female and castrated male rats. Testosterone propionate, a biological equivalent to testosterone, was dissolved in corn oil and administered by daily injections at a dose of 5 mg/kg body weight for 10 days [41,42].

After completion of these hormone treatments, the animals were killed by decapitation, and the livers were excised and placed in ice-cold 0.25 M sucrose, homogenized and centrifuged at 13000 g for 10 min. Cytosolic fractions were obtained by further centrifugation of the first supernatant at 105000 g for 1 h.

Biochemical analyses

Determination of protein concentration was performed according to Lowry and co-workers [43], and GST activity towards CDNB was assayed spectrophotometrically using the method described by Habig and co-workers [44]. Western blotting was carried out using monoclonal antibodies raised against heterologously expressed rGSTA5-5, as described elsewhere [45]. Samples from each of the treated animals were analysed on several different occasions, and the results shown are representative of each treatment group.

GST subunits were purified by affinity chromatography. Liver cytosols were applied to a glutathione–Sepharose gel pre-equilibrated with 20 mM Tris/HCl, pH 7.8 (buffer A). The gel was first washed with buffer A and then with buffer A + 0.2 M NaCl. Subsequently, the GSTs were eluted using 10 mM hexyl glutathione in buffer A + 0.2 M NaCl. The transferase activity towards CDNB in the flow-through fractions from the affinity gel was measured and was typically found to be less than 10 % of that of the cytosol, indicating that about 90 % of hepatic class Alpha and Mu GSTs are retained by the matrix.

The affinity pool was injected on to a reverse-phase HPLC column [46] (YMC-Pack Protein-RP, 5 µm, 250 mm × 4.6 mm; YMC GmbH, Schermbach/Weselerwald, Germany), and the GST subunits were separated using the following gradient of water/acetonitrile, both supplemented with 0.1 % trifluoroacetic acid: from 0–9 min, 42 % acetonitrile; from 9–52 min, a linear increase to 58 % acetonitrile; from 53–63 min, washing with 100 % acetonitrile; and from 63–80 min, re-equilibration with 42 % acetonitrile. Absorbance of peptide bonds was measured at 214 nm.

RESULTS

Confirmation of the effectiveness of hypophysectomy and GH treatment

The effectiveness of hypophysectomy and treatment with GH were verified by measurement of GST activity towards CDNB (Table 2). This was up-regulated in hypophysectomized animals compared with sham-operated animals, and administration of GH to hypophysectomized rats resulted in GST activities similar to those observed in the sham-operated rats [26].

Regulation of rGSTA5

Using Western blotting, we confirmed earlier findings [30] that mature female, but not male, rats express GSTA5 in the liver (Figure 2). Hypophysectomy resulted in an increased hepatic level of GSTA5 in females and up-regulated the expression of this protein in males as well. Furthermore, when hypophysectomized animals of either sex were treated with GH, the hepatic level of GSTA5 was decreased. This down-regulation is more apparent in hypophysectomized females than in hypophysectomized males, but clearly indicates that GH is a pituitary factor decreasing the expression of this enzyme. Testosterone alone was not able to down-regulate the rGSTA5 level in hypophysectomized male rats, but GH and testosterone administered together resulted in rGSTA5 levels below the limit of detection.

Table 2 Confirmation of the effectiveness of hypophysectomy (hx) and GH administration

GST activities in control animals were compared with those in hypophysectomized and hormone-treated animals. Values given in the Table are the means of three animals, and statistical significance was assessed using Student's *t* test. *, ** and *** indicate $P < 0.05$, 0.01 and 0.005 respectively, compared with the female control value, and † indicates $P < 0.01$ compared with the value obtained for control males.

Experimental group	Mean GST activity (μ mol of CDNB/min per mg)
Female, control	0.69 ± 0.07
Female, hx	$1.64 \pm 0.21^{***}$
Female, hx + GH	1.03 ± 0.24
Male, control	$1.18 \pm 0.17^{***}$
Male, hx	$1.69 \pm 0.11^{***\dagger}$
Male, hx + GH	$1.03 \pm 0.14^*$

Figure 2 Western blotting of rGSTA5 in rats from the GH experiment

A 10 μ g portion of cytosolic protein from each sample and 0.1 μ g of rGSTA5 standard were loaded on to a polyacrylamide gel, and electrophoresis was performed. Proteins were then blotted on to nylon membranes, and rGSTA5 was detected using anti-rGSTA5 antibodies, as described in the Materials and methods section. Abbreviations: f, female; m, male; Std, rGSTA5 standard.

Figure 3 Western blotting of rGSTA5 in rats from the testosterone propionate experiment

A 10 μ g portion of cytosolic protein from each sample and 0.1 μ g of rGSTA5 standard were loaded on to a polyacrylamide gel, and electrophoresis was performed. Proteins were then blotted on to nylon membranes, and rGSTA5 was detected using anti-rGSTA5 antibodies, as described in the Materials and methods section. Abbreviations: f, female; m, male; Std, rGSTA5 standard.

The effects on the rGSTA5 levels caused by castration and/or administration of testosterone are depicted in Figure 3. Interestingly, castration of males resulted in detectable levels of rGSTA5, and administration of testosterone to castrated males was found to down-regulate the levels of this isoenzyme to below the limit of detection. Furthermore, testosterone was also able to decrease the level of rGSTA5 in intact female rats. Signals in Figure 2 can, however, not be compared with those in Figure 3, as their respective samples have been analysed on different SDS/polyacrylamide gels and incubated with antibodies against rGSTA5 at different occasions.

Characterization of the possible effects of testosterone on other GST subunits

In order to screen for possible effects of castration and/or testosterone administration on hepatic class Alpha and Mu GSTs, the GSTs from the cytosols in the testosterone experiment were purified by affinity chromatography, and the relative amounts of different subunits present were analysed by reverse-phase HPLC. Figures 4(a)–4(e) show representative chromatograms from each experimental group in the testosterone experiment. Under the conditions employed here, the level of rGSTA1 in castrated male rats was decreased to 70 % of the control value, and this level was restored to 90 % of the control value by treatment with testosterone (based on comparison of the areas under the peaks containing this subunit). The rGSTA2, A3, A4, M1 and M2 subunits were not affected by these treatments. rGSTA5 co-eluted with rGSTA3 and was expressed in very low amounts compared with rGSTA3. Thus, rGSTA5 did not contribute significantly to the area of this common peak and could not be quantified by this approach.

DISCUSSION

The level of GST expression is an important factor in determining resistance to chemical toxicity. The regulation of these enzymes by cancer chemopreventive inducing agents has attracted much interest. However, relatively little is known about the endogenous control of GST. In broad terms, it is known that class Alpha GSTs are expressed in female rat liver in higher amounts than in male rat liver. Conversely, class Mu GSTs are expressed in male rat liver at higher amounts than in female liver. However, little is known about the hormonal regulation of specific GST genes. In the present study, we have investigated the regulation of GSTA5, since in rat liver it is a sex-specific transferase that provides a major detoxification mechanism for AFB₁.

Figure 4 Representative HPLC chromatograms depicting hepatic GST isoenzyme patterns in (a) control female rats, (b) females treated with testosterone propionate, (c) control male rats, (d) castrated males, and (e) castrated males treated with testosterone propionate

A 5 mg portion of cytosolic protein from each sample in the testosterone experiment was applied to the glutathione–Sepharose affinity gel. The GSTs were eluted by 10 mM hexyl glutathione in 20 mM Tris/HCl (pH 7.8)/0.2 M NaCl/1 mM EDTA and analysed by reverse-phase HPLC. The GST subunits contained in the different peaks are indicated in the chromatograms.

Regulation of rGSTA5 by GH

The results presented here show that GSTA5 is regulated in a unique fashion: male GH secretion (with no detectable GH in the blood between peaks) is associated with failure to express GSTA5 in rat liver, whereas with the more continuous female secretion of GH expression of GSTA5 occurs. These observations suggest that the female pattern of GH secretion up-regulates hepatic rGSTA5. However, two other observations indicate that GH is a down-regulator of rGSTA5: firstly, hypophysectomy leads to increased levels of expression in both sexes; and secondly, continuous administration of GH to hypophysectomized rats of either sex down-regulates the hepatic rGSTA5 level to a value which is similar to that seen in the normal mature female.

A remaining possibility is that GH serves to down-regulate

this protein, with the male pattern of secretion down-regulating the A5 subunit more potently than the female pattern of secretion does. In this respect it would be interesting to see whether the hepatic expression of rGSTA5 in hypophysectomized animals of both sexes can be ‘masculinized’ (i.e. eliminated) by administration of GH intermittently as two daily injections. Such treatment has proven to be effective in masculinizing the expression of rat hepatic cytochrome *P*-450 isoenzymes, which are also known to be regulated by the sexually dimorphic secretion of GH. *P*-450 2C7, 2C11, 2C12 and 2C13 demonstrate sex-specific expression [40,47,48]. Among these, 2C7 is expressed at higher levels in female than in male rat liver, whereas 2C12 is found only in females. Hypophysectomy suppresses the expression of these isoenzymes in both sexes [49,50], indicating an up-regulation of these isoenzymes by GH.

Comparison of the expression of cytochromes *P*-450 2C7 and 2C12 with that of rGSTA5 is of interest: all three enzymes are female specific, but the two former are down-regulated by hypophysectomy, whereas the latter is up-regulated by this same treatment. There is, to our knowledge, no other GH-regulated, female-specific enzyme other than rGSTA5, which is up-regulated by hypophysectomy in both sexes. Thus, there might be another down-regulating factor that is specific for males and the effect of which is eliminated by hypophysectomy.

Our results concerning the regulation of rGSTA5 by GH should be compared with our earlier findings for the hepatic class Mu transferases: The higher basal level of GH in female than in male rats results in lower levels of these isoenzymes in females. Hypophysectomy, which results in elimination of circulating GH (the half-life of rat plasma GH is 5–10 min [51,52]), up-regulates the class Mu GSTs in both sexes as much as 6-fold. Furthermore, this up-regulation can be partially reversed by administration of GH. It is thus clear that the regulation by GH of GSTA5 differs from that of the class Mu GSTs.

Regulation of rGSTA5 by testosterone

Testosterone represents a factor that may inhibit GSTA5 expression, since it fulfills the two criteria described above, since the level of testosterone receptors in rat liver has been shown to be dramatically decreased by hypophysectomy and castration [53]. Indeed, hypophysectomized male rats and males castrated at the age of 6 weeks (when they still express the rGSTA5) maintain detectable levels of this enzyme 5 weeks later, which is not the case for normal male rats. Furthermore, testosterone enhances the down-regulation of rGSTA5 in hypophysectomized male rats treated with GH, and treatment of castrated males with testosterone eliminates their expression of rGSTA5, indicating that testosterone is indeed involved in the down-regulation of this enzyme.

HPLC chromatograms from rats treated with testosterone demonstrated that the effect of this hormone on the expression of GSTA5 is highly specific, since most of the other GST isoenzymes are not affected by this same treatment. GSTA1 is, in fact, the only other GST subunit affected by castration and subsequent administration of testosterone. GSTA1 appears to be up-regulated by testosterone, although only to a minor extent.

The question arises as to whether the effects on GST subunits caused by castration and subsequent testosterone administration are in fact due to altered pulsatile secretion of GH. This does not seem to be the case, since the rGSTA1 and A5 subunits are affected in opposite ways by such treatment, whereas both are down-regulated by GH. Prepubertal gonadectomy of male rats does not suppress the adult pulsatile secretion of GH [54], although the troughs between GH bursts are not as deep (i.e. the plasma level of GH never decreases below the level of detection) – an effect reversed by testosterone administration.

Eagon and co-workers [53] have demonstrated that androgen binding in female rats is low, but can be induced by administration of testosterone. Indeed, in our study, testosterone was able to down-regulate the levels of hepatic rGSTA5 in intact females.

In mice, testosterone has been demonstrated to up-regulate the hepatic GST Pi isoenzyme [28]. Castration of male mice results in a decrease in the hepatic level of GST Pi to female levels, and administration of testosterone to intact females increases this level to that present in normal males.

Model of rGSTA5 regulation by GH and testosterone

The following model would explain all of our present observations regarding rGSTA5: It is proposed that the promoter

region of the gene for rGSTA5 contains two separate *cis*-acting elements that are responsive to GH and testosterone. Binding of factors to these elements in response to treatment with GH or testosterone alone results only in partial suppression of the transcription of the gene. On the other hand, upon exposure to both of these hormones at the same time this transcription is totally suppressed. It is apparent that this hypothesis assumes that down-regulation of rGSTA5 by both GH and testosterone occurs at the transcriptional level, which, although highly probable, remains to be demonstrated. Of interest in this context is the observation by Srivastava and Waxman [55] that the regulation of rat hepatic class Alpha and class Mu GSTs by growth hormone is pretranslational.

The existence of elements in the rGSTA5 gene that are responsive to GH and testosterone would predict the following: in systems where both GH (irrespective of the pattern of secretion) and testosterone are present and can exert their hormonal effects—i.e. normal mature male rats; female and castrated male rats treated with testosterone; hypophysectomized male rats treated with both GH and testosterone—no hepatic expression of rGSTA5 should be seen. Under conditions where GH alone is present and can exert its hormonal effects—i.e. immature rats of both sexes; normal mature female rats; hypophysectomized rats of both sexes treated with GH; castrated male rats—rGSTA5 should be expressed at an intermediate level. Finally, when neither GH nor testosterone are present—i.e. hypophysectomized female rats—or GH is absent and testosterone cannot exert its physiological effects—i.e. hypophysectomized male rats, then the expression of rGSTA5 should be maximal. With the exception of females treated with testosterone, this is exactly what was observed in our present study. The fact that females treated with testosterone do express rGSTA5 might be explained by sub-optimal dosage of testosterone or by the fact that the androgen binding cannot be sufficiently up-regulated to suppress completely rGSTA5 expression in these animals. It should also be remarked that class Mu GSTs are down-regulated by the pattern of GH secretion, with no apparent contribution by testosterone.

Concluding remarks

The functional significance of hormonal regulation of GSTs is intriguing. None of the known functions of GSTs—i.e. conjugation of xenobiotics; binding of fatty acids, bile acids, haem, etc.; reduction of lipid hydroperoxides; and isomerase activity—provides an obvious explanation as to why endocrine regulation of this family of enzymes occurs. Many of these functions have been demonstrated both *in vivo* and *in vitro*, but their physiological significance remains unclear.

The patterns of GST isoenzymes expressed in different tissues [56], in different cell types in the same tissue [57], and even during different phases of the cell cycle of one and the same cell [58] can differ considerably. Furthermore, the expressions of different GSTs in many different tissues have been shown to vary during development and aging [18,19,59–61]. Such observations, together with our growing information concerning hormonal regulation of GSTs, may offer clues concerning the endogenous function(s) of these enzymes.

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