Pergamon

Biochemical Pharmacology, Vol. 49, No. 10, pp. 1381–1386, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00055-0

EFFECTS OF HYPOPHYSECTOMY AND THYROXINE ON THE EXPRESSION OF HEPATIC OESTROGEN, HYDROXYSTEROID AND PHENOL SULPHOTRANSFERASES

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(*Received* 11 *August* 1994; *accepted* 10 *January* 1995)

Abstract—Sulphation in rats, and other mammals, is carried out by a family of sulphotransferase isoenzymes, which can be further subdivided into oestrogen, hydroxysteroid and phenol sulphotransferases. We have examined the effects of hypophysectomy on the activity and expression of representative members of the three major sulphotransferase sub-families in male Wistar rat liver cytosols, and have found that the different sub-families are subject to differential regulation by pituitary hormones. Our data show that in male rat liver hydroxysteroid sulphotransferases activity was increased, oestrogen sulphotransferases activity was not altered and phenol sulphotransferases activity was reduced. Further, we have studied the effect on sulphotransferase expression of administration of thyroxine and dexamethasone to hypophysectomized rats. Treatment of hypophysectomized rats with thyroxine virtually abolished oestrogen sulphotransferase activity in male rat liver but had no effect on hydroxysteroid sulphotransferase or phenol sulphotransferase activity. Treatment of hypophysectomized rats with dexamethasone had no effect on sulphotransferase activities. Quantitative immunoblot analysis of liver cytosols showed that these changes in enzyme activity were related to changes in levels of the respective enzyme proteins.

Key words: sulphotransferase; thyroid hormones; pituitary; oestrogens; rat; immunoblot

Sulphation, catalysed by members of the cytosolic ST§ enzyme family, is an important detoxication mechanism for xenobiotics and endogenous compounds such as steroid hormones, bile acids and neurotransmitters [1–4]. The STs catalyse the transfer of sulphate from the donor molecule PAPS to an appropriate group (usually hydroxyl) on the acceptor molecule. In rats, three different sub-families of ST exist: phenol (or aryl) STs (PST, or AST), which sulphate phenolic xenobiotics (such as 4-nitrophenol and 1-naphthol) as well as monoamines such as dopamine, hydroxysteroid (or alcohol) STs (HST), which sulphate a wide range of steroid hormones (e.g. androsterone and DHEA), bile acids and xenobiotics, and EST, which sulphate endogenous and xenobiotic oestrogens. In rats, these enzymes are subject to sex-specific and age-specific expression: for example, in young adult rats, EST is expressed exclusively in males, where HST is predominantly expressed in females [5, 6]. We have recently purified a PST [7], an EST [5] and an HST [6] from rat liver cytosol, and raised antibodies against these proteins.

Previous studies have shown that hypophysectomy results in a down-regulation of sulphotransferase activity towards cortisol [8] and towards an aromatic hydroxylamine and a hydroxamic acid [9]. The identity of the ST isoenzymes sulphating cortisol remains to be conclusively demonstrated, although the sulphation of aromatic hydroxamic acids such as *N*-hydroxy-2-acetylaminofluorene appears to be carried out principally by the rat ST isoenzyme referred to as ST1C1 [10]. We have studied the effects of hypophysectomy on ST activities associated with each of the three major recognized sub-families of sulphotransferase, EST (oestrone), HST (DHEA) and PST (1-naphthol). Our access to antibodies to these different ST sub-families has also allowed us to investigate the relationship of the effects of hypophysectomy on sulphotransferase enzyme activity and levels of enzyme protein. Our results show that differential regulation of the major rat ST isoenzymes by pituitary hormones occurs.

MATERIALS AND METHODS

Chemicals. [2,4,6,7-^{3}H(*N*)]-Oestrone (90.5 Ci/mmol), [2,4,6,7-^{3}H(*N*)]-oestriol (91 Ci/mmol) and [1,2,6,7-^{3}H(*N*)]-dehydroepiandrosterone (114.7 Ci/mmol) were purchased from Du Pont/NEN (Stevenage, U.K.), 1[1-^{14}C]-naphthol (53 mCi/mmol) was from Amersham U.K. Ltd (Aylesbury, U.K.) and 1-naphthol was obtained from Merck Ltd (Glasgow, U.K.). PAPS, goat anti-rabbit IgG, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt), DL-thyroxine (free

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§ Abbreviations: ST, sulphotransferase; PST, phenol sulphotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; DHEA, dehydroepiandrosterone; EST, oestrogen sulphotransferase; T_4, thyroxine; hypox, hypophysectomized.

acid), dexamethasone and unlabelled steroids were purchased from Sigma Chemical Co. Ltd (Poole, U.K.). Scintillation fluid (Emulsifier Safe) was from Canberra Packard (Pangbourne, U.K.), and all other reagents were of analytical grade and purchased from commonly used local suppliers.

Tissue preparation. Adult male (>12 weeks, 250–300 g) Wistar rats were used throughout—normal rats were from the colony maintained in this institute, and those used for surgical procedures were purchased from Charles River U.K. Ltd (Margate, U.K.). The suppliers performed all hypophysectomy and sham hypophysectomy procedures. For hypophysectomy, the standard parapharyngeal method was used as described [11]. Any animal which gained more than 5 g body weight in the week following the operation was not used, because completely hypox animals lose body weight. After the surgical procedures, 0.5% (w/v) glucose was added to the drinking water of the animals which were allowed to recover for between 2 and 3 weeks prior to sacrifice. Rats (normal and hypox) treated with T_4 received 0.15 mg/100 g body weight (dissolved in water) per day for 4 days before sacrifice and those treated with dexamethasone received 0.4 mg/100 g body weight per day for 4 days. Animals were left for 10–14 days after operation before beginning hormone treatments. All treatments were administered by i.p. injection, and were based on previously published protocols [12, 13]. Tissue cytosols were prepared by differential centrifugation: homogenates (30% w/v) were prepared in 250 mM sucrose, 5 mM HEPES pH 7.4 and centrifuged at 10,000 *g* for 15 min. The resulting supernatants were subjected to further centrifugation at 105,000 *g* for 60 min and the supernatants aspirated, carefully avoiding the lipid layer at the surface, divided into 1 mL aliquots and stored at −70° until used (within 3 months). All handling and processing of tissue was performed at 0–4°.

Enzyme assays and protein determination. ST enzyme activities towards oestrone, oestriol, DHEA and 1-naphthol were measured using radioactive substrates as previously described [5]. These conditions were adopted after extensive optimization of all the assay methods with respect to substrate and PAPS concentrations, pH, cytosolic protein content and incubation time [5]. Protein content of cytosol preparations was estimated by the method of Lowry *et al.* [14] with bovine serum albumin as standard.

SDS–PAGE and immunoblot analysis. Proteins were resolved on denaturing SDS-polyacrylamide gels (11% acrylamide monomer) according to the method first described by Laemmli [15]. For immunoblotting, proteins were electrophoretically transferred to nitrocellulose sheets as described by Towbin *et al.* [16], and immunochemical localization was performed by the alkaline phosphatase method (at room temperature) exactly as described previously [17] with the one exception that the pH of the buffer used for all incubation and washing steps was 9.0. All electrophoresis and blotting apparatus was from Hoefer Scientific Instruments (Newcastle-under-Lyme, U.K.). Blots were probed with antibodies prepared against rat liver EST [5], rat liver paracetamol ST [7] and rat liver HST [6]. These antibodies, raised in rabbits against rat liver STs purified to homogeneity, have been well characterized, and on Western blot analysis recognize the antigens against which they were raised. At higher protein loadings, minor cross reactivity with other ST isoforms can be seen. Densitometric quantitation of immunoblots was performed as follows: a scanned image of a black and white photograph of the blot was made on a Hewlett-Packard ScanJet IIC desktop scanner and imported into the software package Quantiscan (BioSoft, Cambridge, U.K.), and analysis of the areas under the peaks was performed as described by the manufacturer.

RESULTS

To ensure the validity of data obtained from hypox animals, sham-operated controls were included in the study as there were some effects on the ST activities measured following sham treatment,

Table 1. Hepatic cytosolic ST activities in normal and hypox adult male rats

Treatment	ST activity (pmol/min/mg)			
	Oestrone	Oestriol	DHEA	1-Naphthol
Normal (N = 6)	61 ± 1	161 ± 19	12 ± 1	1954 ± 101
Sham hypox (N = 3)	123 ± 12§	319 ± 5§	27 ± 9§	2110 ± 86
Hypox (N = 4)	129 ± 8	97 ± 20*	78 ± 2†	1030 ± 179*
Hypox + Dex (N = 3)	115 ± 9	84 ± 26	74 ± 3	949 ± 69
Hypox + T_4 (N = 3)	3 ± 2‡	ND	57 ± 19	830 ± 118
Normal + T_4 (N = 4)	34 ± 23‖	80 ± 17‖	13 ± 2	2238 ± 198

Data are expressed as mean ± SEM for duplicate determinations on the number of liver samples indicated. Statistical analysis was performed using Student's *t*-test with the 'Instat' software package for Apple Macintosh computers.

* $P < 0.001$, † $P < 0.0001$ hypox versus sham hypox.

‡ $P < 0.001$, hypox + T_4 versus hypox.

§ $P < 0.05$, sham hypox versus normal.

‖ $P < 0.05$, normal T_4 versus normal.

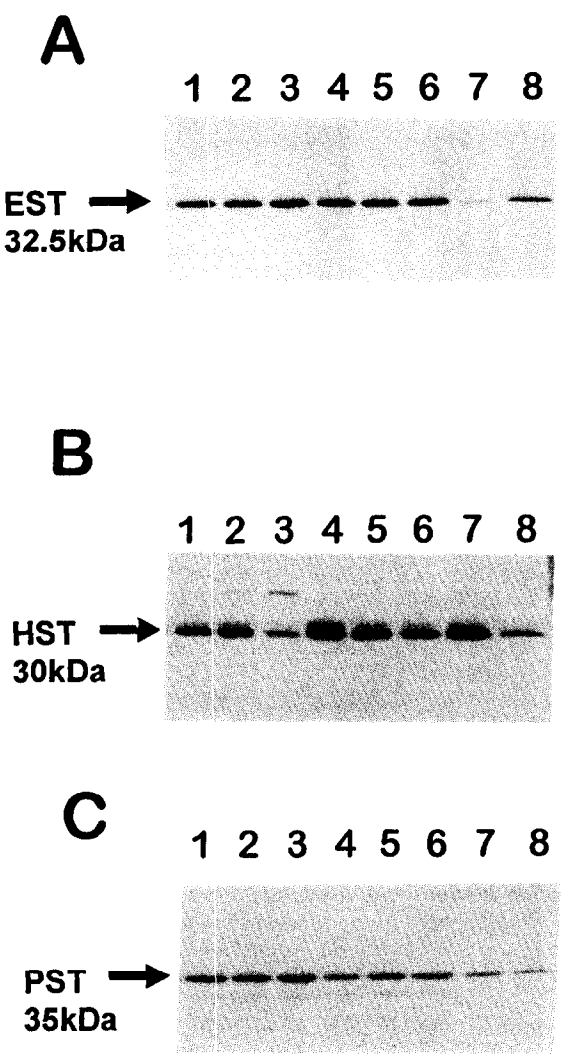

Fig. 1. (A) Immunoblot analysis of EST expression in male rat liver cytosols. Cytosol prepared from rat liver (5 μg protein) was resolved on polyacrylamide gels (11% acrylamide monomer) in the presence of 0.1% SDS and electroblotted onto nitrocellulose. Blots were exposed to anti-(rat liver EST) at a concentration of 2.4 μg/mL for 60 min at room temperature and immunoreactive proteins visualized by alkaline phosphatase-conjugated secondary antibody. Lanes 1–3, sham hypox; lanes 4–6, hypox; lanes 7 and 8, hypox + T_4. (B) Immunoblot analysis of DHEA ST expression in male rat liver cytosols. Cytosol prepared from rat liver (5 μg protein) was resolved on polyacrylamide gels (11% acrylamide monomer) in the presence of 0.1% SDS and electroblotted onto nitrocellulose. Blots were exposed to anti-(rat liver DHEA ST) at a concentration of 8 μg/mL and immunoreactive proteins visualized by alkaline phosphatase-conjugated secondary antibody. Lanes 1–3, sham hypox; lanes 4–6, hypox; lanes 7 and 8, hypox + T_4. (C) Immunoblot analysis of PST expression in male rat liver cytosols. Cytosol prepared from rat liver (25 μg protein) was resolved on polyacrylamide gels (11% acrylamide monomer) in the presence of 0.1% SDS and electroblotted onto nitrocellulose. Blots were exposed to anti-(rat liver paracetamol ST) at a concentration of 6.7 μg/mL and immunoreactive proteins visualized by alkaline phosphatase-conjugated secondary antibody. Lanes 1–3, sham hypox; lanes 4–6, hypox; lanes 7 and 8, hypox + T_4.

indicating that the operation, the subsequent dietary supplements and/or minor strain differences had some effect on sulphation (Table 1), and all comparisons are made with sham-operated animals. A potential problem with all hypophysectomy experiments is incomplete removal of the pituitary, especially in the hands of the inexperienced. For this reason, the operations were performed by experts at Charles River U.K. Ltd. As a control, we assayed glucose-6-phosphatase activity (which is not sexually dimorphic, and which is known to be reduced following hypophysectomy [12]). In all untreated hypox rats, the expected decrease in glucose-6-phosphatase activity was found [18].

Hypophysectomy had no significant effect on the sulphation of oestrone (Table 1). Hypophysectomy resulted in a significant increase in DHEA ST activity (approx. 3-fold), in a significant decrease in EST activity (approx. 3-fold) and in a significant decrease (approx. 2-fold) in the sulphation of 1-naphthol (Table 1). Administration of dexamethasone (Table 1) to hypox male animals did not affect ST activities compared to untreated hypox rats; however, a dramatic effect on EST activity (as assessed with both oestrone and oestriol) was observed when hypox male rats were treated with T_4, almost completely abolishing the enzyme activity (Table 1). T_4 treatment had no significant effect on the sulphation of DHEA and 1-naphthol, but EST activity (oestrone and oestriol) was decreased by a factor of 2 (Table 1). This decrease in EST activity in normal rats was not nearly as dramatic as that which occurred when hypox rats were administered T_4 (Table 1).

We performed immunoblot analysis on liver cytosols prepared from rats subjected to various treatments described, using antibodies previously prepared against EST, HST and PST isoenzymes [5–7] to investigate whether the effects of these treatments on ST enzyme activity were the result of changes in levels of individual ST isoenzyme proteins. In an attempt to correlate enzyme activity data (Table 1) with levels of immunodetectable protein (Figs 1–3), we also performed densitometric analysis of these immunoblots (Fig. 2). Figure 1(A) shows that EST enzyme protein levels were dramatically reduced in liver cytosol prepared from hypox rats which had received T_4, compared with both the sham operated and the hypox animals, and quantitation of the levels of immunoreactive protein (Fig. 2) confirmed that the dramatically decreased enzyme activity in hypox T_4 treated animals was accompanied by a large decrease in the amount of immunodetectable protein. Figure 1(B) demonstrates that there was a substantial increase in the level of protein immunodetectable with our anti-(rat liver DHEA ST) antibody preparation in both hypox and hypox T_4-treated animals, and again the results of this immunochemical analysis were shown to relate to the enzyme activity data (Fig. 2). The appearance of an additional immunoreactive polypeptide of slightly higher subunit molecular weight probably represents minor cross-reactivity with another (unidentified) ST isoenzyme. The results obtained on immunoblot analysis with our antibody against a member of the rat PST enzyme family (Fig. 1(C))

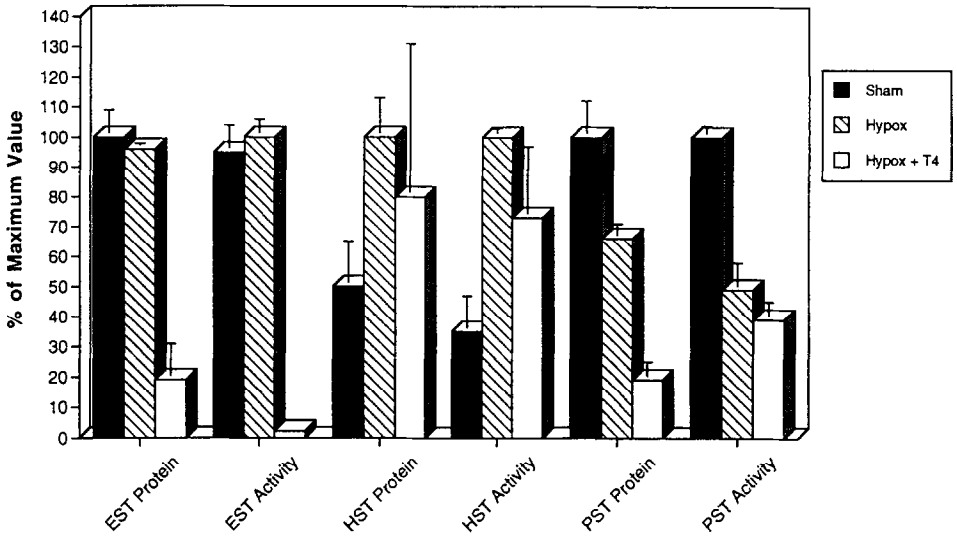

Fig. 2. Comparison of ST enzyme activity and immunoquantitated enzyme protein levels. Immunoreactive protein was quantified by densitometric analysis, and all data are expressed as a percentage of the maximum level of activity or protein for each enzyme family. Enzyme activity values are derived from the data in Table 1, and enzyme protein values are derived from densitometric analysis of the immunoblots shown in Figs 1(A)–(C). For immunoblots (Figs 1(A)–(C)), data shown are derived from the means ± SEM of the replicates analysed.

show a reduction in the immunoreactive protein detectable in samples of liver cytosol from hypox rats and from hypox rats treated with T_4. There was, however, a somewhat larger reduction in the level of immunodetectable protein in liver cytosol from hypox T_4 treated animals than expected from the enzyme activity levels (Fig. 2)—the overlapping substrate specificities of the different PST isoenzymes in rat liver are well known (e.g. [1] and [7]), and may account at least in part for this observation.

It is important to note that quantitation of immunoblots based on only one protein concentration may not always give a numerically accurate indication of the differences between samples since, like most enzyme reactions, the immunochemical reaction employed to visualize the proteins is saturable. Such a consideration can explain small quantitative differences observed between data obtained with enzyme activity measurements and immunoquantitation, such as those in Fig. 2; however, it does not detract from the value of such comparisons.

DISCUSSION

The data presented here indicate that the effects of hypophysectomy on ST expression are complex, and isoenzyme-specific. Previously, only down-regulation of ST expression following hypophysectomy had been shown [8, 9, 13], whereas we have for the first time demonstrated that the sulphation of DHEA in male rat liver, and the level of DHEA ST enzyme protein, is increased after hypophysectomy (Table 1, Fig. 1(B)). In contrast, sulphation of 1-naphthol, a substrate for at least two forms of PST in rat liver, was decreased following hypophysectomy (Table 1) and the enzyme protein recognized by our anti-(rat liver paracetamol ST) was reduced (Fig. 1(C)). Similar results have been shown with the aryl hydroxamic acid *N*-hydroxy-2-acetylaminofluorene [9], which is now known to be a substrate for the ST isoenzyme termed 1C1 [10]. The identity of the purified paracetamol ST isolated in our laboratory and against which the anti-PST antibody used in these experiments was raised [7] is still not entirely clear, but it is unlikely to be 1C1 because the 1C1 isoenzyme exhibits much more extensive sexual dimorphism (as determined by immunoblot analysis [7, 10]). Delineation of the substrate specificities of the different rat PST isoenzymes requires comparison (by enzyme kinetic analysis) of the substrate specificities of the cloned and heterologously expressed cDNAs.

Thyroid hormones and their metabolites are sulphated and the sulphation is believed to promote deiodination, the principal metabolic pathway for these compounds [19, 20]; however, the sulphation of thyroid hormones is believed to be carried out by members of the PST enzyme sub-family [21], which did not appear to be significantly affected by T_4 in our experiments, as assessed with 1-naphthol as a substrate (Table 1). Our finding that DHEA ST enzyme activity and protein levels were increased upon hypophysectomy (Table 1, Figs 1 and 2), in contrast to levels of cortisol ST which decrease [8], provides strong evidence to support the suggestion that these two ST enzyme activities reside with different isoenzymes [22].

EST activity and enzyme protein level were not affected by hypophysectomy (Table 1, Fig. 1(A)); however, the sulphation of oestriol was reduced

approximately 3-fold (Table 1). It is possible that oestriol, in addition to being a substrate for the rat EST isoenzyme [5], may be sulphated by another isoenzyme, for instance a PST. Indeed, on gel filtration chromatography of male rat liver cytosol, a distinct peak of oestriol ST activity was observed which co-eluted with ST activity towards 1-naphthol [5]. Sulphation of 1-naphthol (a substrate for more than one PST in rats) was reduced approximately 2-fold in hypox rats compared with normal animals (Table 1). However, the most interesting effect we observed was that of T_4 treatment on the level of EST following hypophysectomy (Table 1, Fig. 1(A)), treatment which abolished EST activity and enzyme protein almost completely. Regulation of rat liver EST expression is believed to be related to hepatic androgen sensitivity, with high EST levels occurring with high androgen sensitivity—i.e. in young adult male but not female animals [23]. Our results demonstrate that in adult male rats EST expression *per se* may not be directly dependent on pituitary hormones, since there was no change in oestrone ST activity or enzyme protein recognized by our anti-(rat liver EST) antibody preparation following hypophysectomy (Table 1, Figs 1 and 4). However, rat liver EST is obviously responsive to administered T_4 in the absence of pituitary hormones, since the enzyme activity (with both oestrone and oestriol) and enzyme protein were dramatically reduced in hypox animals which received T_4 (Table 1, Fig. 1(A)). In normal animals, treatment with T_4 reduced EST activity (both oestrone and oestriol) by 50% (Table 1). Removal of the pituitary dramatically reduces circulating levels of thyroid hormones [24], and a logical explanation for our data would be that some other factor(s), either produced by the pituitary or regulated by pituitary hormone(s), prevents exogenous T_4 from affecting EST expression in the normal animal and that hypophysectomy on its own produces no effect on EST expression since the procedure not only removes this factor(s) but also reduces endogenous thyroid hormone levels. Therefore, only in the absence of this factor(s) and endogenous thyroid hormones is it possible to virtually abolish EST activity by exogenous T_4.

In conclusion we have shown that the three major sub-families of rat STs are differentially regulated by pituitary hormones and T_4. We observed that changes in enzyme activity were principally due to changes in levels of the individual enzyme proteins, rather than to activation or inhibition of the activity of existing proteins. These data further our appreciation of the hormonal regulation of ST expression.

Acknowledgements—This work was supported by a grant from the Scottish Hospitals Endowment Research Trust. AB is a Lister Institute Research Fellow, and MWHC was a Caledonian Research Foundation/Royal Society of Edinburgh Research Fellow.

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