Differential expression of hepatic oestrogen, phenol and dehydroepiandrosterone sulphotransferases in genetically obese (ob/ob) male and female mice

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Abstract

Sulphotransferases (STs) are a family of closely related enzymes playing a key role in regulation of the bioavailability and activity of important endogenous molecules such as steroid hormones. A relationship between the expression of steroid STs and the diabetic state has been demonstrated in various laboratory animal models, and steroid sulphates such as dehydroepiandrosterone sulphate are known to have anti-diabetic properties. In order to further our understanding of the molecular basis for the association of steroid hormone sulphation and diabetes, we have examined the expression of oestrogen, phenol and dehydroepiandrosterone (DHEA) STs in mice carrying the obesity mutation (ob), which in the homozygous state (ob/ob) produces mice which are obese and diabetic. Our data show that, in male mice, ST activities towards oestrone (E1), oestriol (E3), DHEA and the xenobiotic 1-naphthol are elevated in ob/ob mice, whereas in female mice, only the oestrogen ST activities were elevated, with the DHEA and 1-naphthol ST activities reduced. Using antibodies directed against oestrogen ST, it was demonstrated that the induction of E1 and E3 ST activity in ob/ob mice correlated with the expression of an ST isoenzyme not constitutively expressed in control mouse liver.


Introduction

Reversible sulphation of steroid hormones, catalysed by sulphotransferases (STs) and sulphatases, is a sensitive and specific mechanism for regulating their biological activity in target and non-target tissues (Hobkirk 1985, Roy 1992). STs have evolved as a multigene family and, in most species, three subfamilies exist comprising ST isoenzymes with specificity for oestrogens (EST), for other hydroxysteroids, such as androgens, bile acids and pregnenolone (HST) and for non-steroid phenols (PST). A number of ESTs, HSTs and PSTs have recently been cloned and/or purified from human (e.g. Heroux & Roth 1988, Falany et al. 1989, 1993, Ottermans et al. 1992, Dooley et al. 1993, Hondoh et al. 1993, Wilborn et al. 1993, Forbes-Barnforth & Coughtrie 1994, Wood et al. 1994) and non-human species (e.g. Nash et al. 1988, Coughtrie & Sharp 1990, Ogura et al. 1990, Ozawa et al. 1990, Homma et al. 1991, Demyan et al. 1992, Hirshey et al. 1992, Oeda et al. 1992, Borthwick et al. 1993, Kong et al. 1993, Sharp et al. 1993). Within the rat PST enzyme family, the different isoenzymes are subject to independent sex-specific regulation, normally with a higher level of expression in males (e.g. Coughtrie & Sharp 1990).

Much interest has been generated over observations which suggest an association between steroids (and steroid sulphation) and the diabetic state in various laboratory animal species. For example, dehydroepiandrosterone and its sulphate (DHEA, DHEA-S) have been shown to have anti-diabetic effects in mice (Leiter 1981, Coleman et al. 1984a, b, Gordon et al. 1987), and the induction of diabetes by streptozotocin in rats can be inhibited by oestrogens and potentiated by androgens (Paik et al. 1982). A relationship between steroid STs, which inactivate biologically active androgens and oestrogens, to obesity and diabetes in a series of genetically obese and/or diabetic mice has been demonstrated (Leiter et al. 1989, 1991).

We have recently purified an EST (Borthwick et al. 1993), an HST (Sharp et al. 1993) and a PST (Coughtrie & Sharp 1990) from rat liver cytosol, and raised antibodies against these proteins. Access to such molecular tools makes it possible to further our understanding of the molecular basis of the effects of the presence of the obese mutation (ob) in the homozygous form (ob/ob) in male and female mice on the expression of STs. In the current work...
we have used these probes to identify a novel oestrogen-sulphating ST which is induced in mice expressing the ob/ob phenotype, and to demonstrate the down-regulation of HST and PST enzyme synthesis in ob/ob mice. Further, we have conducted kinetic analysis of oestrogen sulphation in mouse liver cytosols from control and ob/ob animals, and have shown that these two enzymes responsible for oestrogen sulphation in control and ob/ob mice have distinct kinetic properties.

Materials and Methods

Chemicals

1-[1-14]C]Naphthol (58 Ci/mmol) was purchased from Amersham International plc, Aylesbury, Bucks, UK. [1,2,6,7-3H]DHEA (100 Ci/mmol), [2,4,6,7-3H]oestrone (E2) (91 Ci/mmol) and [2,4,6,7-3H]oestradiol (E3) (91 Ci/mmol) were from DuPont/NEN, Stevenage, Herts, UK. E1, DHEA, E3, 3'-phosphoadenosine 5'-phosphosulphate (co-substrate for the ST enzyme reaction), nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and alkaline phosphate-conjugated anti- (rabbit IgG) were obtained from Sigma Chemical Co, Poole, Dorset, UK, and 1-naphthol was from Merck/BDH, Glasgow, UK. Scintillation fluid (Emulsifier Safe) was from Canberra Packard, Pangbourne, Berks, UK. All other reagents were of analytical grade and purchased from local suppliers.

Liver samples and preparation of cytosols

Mice (C57BL/GB-Lee) were obtained from the animal facility at the University of Leeds, UK. The colony was maintained by breeding heterozygous male and female mice (ob/+) and selecting those progeny which developed obesity at about 4 weeks of age. To reduce the potential for confusion with interstrain differences, we used as our control group non-obese littersmates of the ob/ob mice, and these therefore were a mixed homozygous normal/
heterozygous (designated +/?) population. Cytosols were prepared from mouse liver by differential centrifugation, with all operations performed at 4 °C. Homogenates (20%, w/v) were prepared in 250 mM sucrose, 5 mM Hapes, pH 7·4 and centrifuged at 10 000 g for 20 min. The resulting supernatants were further centrifuged at 105 000 g for 60 min, and the supernatants (cytosolic fraction) were aspirated, carefully avoiding the lipid layer at the surface. Cytosols were aliquoted (1ml) and stored at −70 °C until used (within 3 months).

Assay for sulphotransferase activity and protein determination

The in vitro sulphation of 1-naphthol (12 µM), DHEA (7·5 µM) E3 (7·5 µM) and E3 (5 µM) was quantified as previously described (Borthwick et al. 1993, Sharp et al. 1993), using radioactively labelled substrates. Following incubation of substrate and PAPS with cytosols (500 µg protein), unreacted substrate was separated from the more polar sulphate using a simple solvent extraction, and after separation of the phases by brief centrifugation, radioactivity in an aliquot of the aqueous layer was determined by liquid scintillation counting. E1, in addition to E2, was used in these studies since little is known of the substrate specificity of mouse liver oestrogen ST(s), and we have previously presented evidence for E2 being a more specific substrate for the rat liver EST enzyme by virtue of its much more marked sexual dimorphism (Borthwick et al. 1993). For determination of apparent kinetic constants for E1 ST, enzyme activities were measured using 100 µg liver cytosolic protein and 1–8 µM (female ob/ob) and 0·05–2 µM (female control) E1. Apparent K_m values were calculated by the linear regression analysis from double reciprocal (Lineweaver–Burke) plots of the data obtained with three separate liver cytosols (FigP for Windows graphics software, BLOSOFT, Cambridge, Cambs, UK). Protein content of cytosols was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrone (7.5 µM)</td>
</tr>
<tr>
<td>Male control (n=9)</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Male ob/ob (n=22)</td>
<td>78 ± 2**</td>
</tr>
<tr>
<td>Female control (n=7)</td>
<td>9.3 ± 2.2</td>
</tr>
<tr>
<td>Female ob/ob (n=25)</td>
<td>82 ± 2**</td>
</tr>
</tbody>
</table>

*p<0.01, **p<0.001 ST activities in ob/ob mouse liver compared with same sex control activities (Student's t-test (unpaired, two-tailed and with allowance for unequal variance where appropriate). Excel software package, Microsoft)).
TABLE 2. Sulphotransferase activities in ob/ob mouse liver. Assays were performed in duplicate on liver cytosols prepared from mice aged 10 weeks. Data are expressed as mean specific activity ± S.E.M.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oestrone (7.5 µM)</th>
<th>Oestriol (5 µM)</th>
<th>DHEA (7.5 µM)</th>
<th>1-Naphthol (12 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male ob/ob (n=6)</td>
<td>76 ± 2</td>
<td>303 ± 5</td>
<td>5.5 ± 1.7</td>
<td>1405 ± 102</td>
</tr>
<tr>
<td>Female ob/ob (n=11)</td>
<td>83 ± 2</td>
<td>237 ± 20</td>
<td>19 ± 2.5</td>
<td>1298 ± 84</td>
</tr>
</tbody>
</table>

**SDF/PAGE and immunoblot analysis**

Immunoblotting was performed as described, using anti- (rat liver EST) (Borthwick et al. 1993) and anti- (rat liver paracetamol ST) (Coughtrie & Sharp 1990).

**Results and Discussion**

Steroid ST activities in control mice exhibited marked sex specificity, with the female having E₁, E₃ and DHEA ST activities substantially higher than males (13-fold, 5-fold and 60-fold respectively, Table 1). The sulphation of 1-naphthol was also higher in female mouse liver cytosol, by a factor of fourfold. These observations were in contrast to the sexual dimorphism observed with rat STs, where oestrogen ST in young adults is a male-specific enzyme (Borthwick et al. 1993), and 1-naphthol ST activity is at least twofold higher in males than females (Coughtrie et al. 1990). DHEA ST in rats exhibits female-predominant expression as in the mouse (Sharp et al. 1993). In man, no major sex differences in the expression of any ST isoenzyme have been reported to date (e.g. Falany 1991, Aksoy et al. 1993, Jones et al. 1993).
In male mice, ST activities towards E₁, E₃, 1-naphthol and DHEA were significantly higher in ob/ob compared with control animals (Table 1). However, in female animals only E₁ and E₃ ST activities were higher in ob/ob mice compared with control animals, and 1-naphthol ST activity was reduced (Table 1). Thus, the sex differences in the levels of ST enzyme activities towards E₁, E₃ and 1-naphthol were abolished and towards DHEA greatly reduced (from 60-fold to 3-fold), in ob/ob mice. The data in Table 1 are in contrast to those reported by Leiter et al. (1991), who used DHEA and E₁ as substrates in 10-week-old female C57BL/KsJ normal and ob/ob mice. With 10 µM substrate, these workers reported an approximate tenfold decrease in hepatic DHEA ST activity in ob/ob mice compared with controls, and no significant difference in the E₁ ST activity. Our original experiments (Table 1) were performed on mice in the age range 5–16 weeks; therefore, to eliminate the possibility of a very specific age-related effect, we also measured all our ST activities in additional 10-week-old ob/ob mice. We found that all four ST activities measured in ob/ob male and female 10-week-old mice (Table 2) were not significantly different from the activities in ob/ob mice listed in Table 1. These changes in ST enzyme activity in mouse liver cytosol were different, both in magnitude and direction, to changes previously found in our laboratory following streptozotocin induction of diabetes in the rat (Coughtrie et al. 1990).

For oestrogen ST, our enzyme activity data were confirmed by immunoblot analysis of liver cytosols from male and female ob/ob and control mice with our antibody raised against the 32.5 kDa monomeric rat liver EST (Borthwick et al. 1993), which recognised three immunoreactive polypeptides in the mouse liver cytosols used in these experiments (Fig. 1). These experiments clearly show that liver cytosol from both male and female ob/ob mice express an immunoreactive polypeptide of subunit molecular weight approximately 32 kDa, which does not appear to be present in control mouse liver cytosol from either sex. This observation suggests that ob/ob mice express a unique ST which correlates with the very large induction of oestrogen ST activity. While this manuscript was being evaluated, a report appeared (Leiter & Coleman, 1993).

FIGURE 2. Immunoblot of liver cytosol from control and ob/ob mice probed with anti-(rat liver PST). Cytosols (50 µg protein) were subjected to immunoblot analysis as described in the legend to Fig. 1, except that blots were exposed to anti-(rat liver paracetamol ST) at a concentration of 6.7 µg/ml. Lanes 1 and 2, male control (10–11 weeks); lanes 3 and 4 male ob/ob (12–13 weeks); lanes 5 and 6, female ob/ob (10–11 weeks).
Steroid sulphotransferase expression in ob/ob mice  · E B BORTHWICK and others

![Diagram](image)

**Figure 3.** Kinetic analysis of E$_1$ ST activity in liver cytosols from ob/ob and control female mice. Cytosols (100 µg protein) from (a,c) ob/ob and (b,d) controls were incubated with various concentrations of E$_1$, and the enzyme activities determined. Plots of specific activity against substrate concentration (a and b) display the mean ± S.E.M. for determinations on three separate samples of mouse liver cytosol, and the Lineweaver–Burke plots of these data (c and d) were used to estimate the apparent kinetic constants for E$_1$ ST activity by linear regression analysis (FigP for Windows software, BIOSOFT, Cambridge, UK).

We have also compared the enzyme activity data for 1-naphthol ST with the immunoreactive protein present in mouse liver cytosol as determined with an antibody against a rat liver phenol ST (Coughtrie et al. 1990). Figure 2 shows the results of these experiments, which demonstrated that only one major immunoreactive polypeptide was recognised in both control and ob/ob mouse liver cytosol.

Male and female ob/ob mouse liver cytosol had a higher level of 1-naphthol ST enzyme activity than control male (+/?) mouse liver (Table 1), and this was confirmed, qualitatively at least, upon immunoblot analysis, which showed a stronger immunostaining with male and female ob/ob liver cytosols (Fig. 2). Additionally, we performed immunoblot analysis using our anti-(rat liver DHEA ST) antibody preparation (Sharp et al. 1993) on these mouse liver samples. Unfortunately, this antibody, although specific for DHEA ST in rat and human liver cytosol

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1994) describing the induction of the expression of an EST mRNA in mice homozygous for the db mutation, which also gives rise to obesity and diabetes, and which results in altered expression of steriod ST activity. These data are in agreement with those presented here, in that the induction of a novel EST was observed in the diabetic/obese mice (db/db). Control female (Fig. 1, lanes 7 and 8) and male (Figure 1, lanes 3 and 4) mouse liver cytosols contain a polypeptide of approximately 31 kDa (middle of the three major immunoreactive polypeptides) immunoreactive with our anti-(rat liver EST) antibody preparation, and which may possibly be responsible for the low level of oestrogen ST activity observed in these samples. This idea is supported by the observation that this 31 kDa protein is expressed at higher levels in control female liver than in control male liver, as is the oestrogen ST enzyme activity (Table 1).
Table 3. Apparent kinetic constants for \( E_1 \) ST activity in \( ob/ob \) and control female mouse liver cytosol. Values were calculated by linear regression analysis from double reciprocal plots of the data in Fig 3a and b, and therefore represent the means of kinetic analysis performed on separate samples of liver cytosol from three separate mice.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( V_{\text{max}} ) (pmol/min/mg)</th>
<th>( K_m ) (( \mu \text{m} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( ob/ob )</td>
<td>2500</td>
<td>40</td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>0.9</td>
</tr>
</tbody>
</table>

(Sharp et al. 1993), recognised a number of different polypeptides in mouse liver cytosol, and it was not possible to discern which one(s) represented the principal mouse DHEA ST(s) (not shown).

Leiter et al. (1991) also measured DHEA ST activity using 0-2 \( \mu \text{m} \) substrate and reported, based on only these two substrate concentrations (0-2 \( \mu \text{m} \), 10 \( \mu \text{m} \)) that the tenfold decrease in DHEA ST activity was due to a \( V_{\text{max}} \) effect. These authors reported this to be the result of non-competitive inhibition, but also demonstrated the loss of DHEA ST mRNA in female \( ob/ob \) mice, an observation not compatible with an inhibitory effect. In contrast, using 0-2 \( \mu \text{m} \) \( E_1 \) (non-saturating) as substrate, a tenfold increase in ST activity was observed (Leiter et al. 1991), and assumed to reflect a reduction in \( E_1 \) ST \( K_m \) in \( ob/ob \) mice.

Our data, however, show that the large increase which we also observed in \( E_1 \) ST activity in \( ob/ob \) mice was in fact due to a concomitant increase in the level of expression of a protein (approx. 32 kDa) immunoreactive with an antibody raised against purified rat liver \( E_1 \) ST (Fig. 1). This protein is essentially absent in control male mouse liver, therefore a logical explanation for the data of Leiter et al. (1991) is that \( E_1 \) ST activity in control mouse liver is in fact the result of the action of different ST isoenzymes with different kinetic properties using \( E_1 \) as substrate. There is a precedent in the rat for such an observation, where in young adult female rat liver, when EST is not expressed, the residual ST activity towards oestrogens is primarily the result of the action of DHEA ST (Borthwick et al. 1993).

To confirm this hypothesis further, we attempted to determine kinetic constants for \( E_1 \) ST activity in control and \( ob/ob \) female mouse liver cytosol (Fig. 3 and Table 3). For the control female mouse liver, a \( K_m \) value of 0-9 \( \mu \text{m} \) (substrate concentration range of 0-05–2 \( \mu \text{m} \)) was obtained (Fig. 3a and b), but the female \( ob/ob \) mouse liver cytosol displayed marked non-Michaelis–Menten kinetics, and it was only possible to estimate an apparent \( K_m \) value of 40 \( \mu \text{m} \) (Fig. 3c and d) over a substrate concentration range of 1–8 \( \mu \text{m} \), since concentrations of \( E_1 \) above this range caused a dramatic inhibition of the enzyme activity. These data are consistent with the idea that different ST isoenzymes, with different affinities for \( E_1 \), are responsible for the sulphation of \( E_1 \) in control and \( ob/ob \) female mouse liver. Thus, the difference between our data for \( E_1 \) ST and those of Leiter et al. (1991) can be explained by the induction in our female \( ob/ob \) mice of an ST isoenzyme with greatly different kinetic properties (45-fold higher apparent \( K_m \), 52-fold higher \( V_{\text{max}} \)) towards \( E_1 \) than the enzyme responsible for this activity in control female mice. The possibility that the effect of this differential expression of STs metabolising oestrogens may be compounded by potential differences in the genetic background of the \( ob/ob \) mice used in the two laboratories cannot be ruled out.

A relationship between sulphation of steroid hormones and experimental diabetes in laboratory animal models has been established (e.g. Leiter 1981, Coleman et al. 1984b, Leiter et al. 1989, 1991, Coughtrie et al. 1990). The results presented here confirm that in laboratory animals (a) there are differences in the response of ST activities towards oestrogens, androgens and phenols to the induction of diabetes, (b) ST activities in mice and rats respond differently to the presence of the diabetic state, and (c) ST activities in male and female animals respond differently to the presence of the diabetic state. The data also indicate that these effects on ST activity and enzyme protein expression are secondary to the mutation(s) resulting in the \( ob/ob \) phenotype. In addition, a full understanding of which ST isoenzymes are involved in the sulphation of these steroid hormones in diabetes and of the factors governing their expression is essential if we are to fully appreciate the relationship between steroid ST gene expression and the pathogenesis and progression of diabetes and obesity. These observations also have important implications for the extrapolation of the data obtained with laboratory animals to the human situation, and confirm that the ideal animal model system(s) for studying the role of sulphation and of the STs in diabetes remains to be identified. They further emphasise the need for a more detailed understanding of the molecular biology of STs, both in laboratory animals used as models for disease and in humans.

**Acknowledgements**

We are grateful to the Scottish Hospitals Endowment Research Trust for their generous financial support. A B is a Lister Institute Research Fellow, and M W H C was a Caledonian Research Foundation/Royal Society of Edinburgh Research Fellow.

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Received 4 May 1994
Revised manuscript received 1 August 1994
Accepted 15 August 1994