RESEARCH COMMUNICATION

Microsomal triglyceride transfer protein activity remains unchanged in rat livers under conditions of altered very-low-density lipoprotein secretion

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Microsomal triglyceride transfer protein (MTP) is a heterodimeric protein consisting of a unique 97 kDa subunit and protein disulphide isomerase, that mediates the transfer of lipid between membranes and nascent lipoproteins. Mutations in the gene encoding the 97 kDa subunit of the protein cause the rare autosomal recessive disorder abetalipoproteinaemia. Recent findings in cultured cells indicate that the 5' promoter region contains an insulin-responsive element, suggesting that the gene is responsive to insulin regulation. In this study we examined two

cases where very-low-density lipoprotein (VLDL) secretion is markedly reduced: the streptozotocin-diabetic rat and 10-day-old suckling rats. In both cases MTP activity was unaltered compared with that in control animals. Equal levels of MTP were also apparent in the livers of all groups of animals, as measured by immunoblotting with an anti-MTP antiserum. These findings indicate that factors other than MTP activity are responsible for the reduction in hepatic VLDL triglyceride secretion observed in the suckling and diabetic animals.

INTRODUCTION

Increased entry of hepatically synthesized very-low-density lipoproteins (VLDL) into the plasma, in excess of clearance, leads to hyperlipidaemia. The associated rise in LDL levels is a clear risk factor in the development of premature atherosclerosis. Assembly and secretion of VLDL requires the presence of apolipoprotein B (apoB). Available evidence suggests that the rate of VLDL secretion is not regulated by the rate of apoB production [1]. ApoB is thus constitutively over-produced, and excess apoB that is unable to associate with lipid is degraded in the endoplasmic reticulum (ER) [2–5]. Consequently, it may be the supply of lipid into the ER that governs hepatic VLDL secretion. The discovery of a neutral lipid transfer activity catalysed by the ER microsomal triglyceride transfer protein (MTP) in the ER has given an insight into how triacylglycerol (TAG) is incorporated into the nascent VLDL particle.

MTP is a heterodimeric intracellular lipid transfer protein, present in the ER of liver and small intestine. *In vitro*, it mediates the transfer of TAG, and to a lesser extent cholesterol ester and phospholipid, between unilamellar vesicles [6]. The 97 kDa subunit of MTP has been purified, and the gene has been cloned [7,8] and shown to be defective in abetalipoproteinaemia [9].

Further to this, co-expression of MTP with apoB in heterologous cells confers ability to assemble apoB-containing lipoproteins [10,11]. The function of MTP implies a potential role for this protein in the regulation of lipoprotein secretion. Indeed, both MTP mRNA and protein increased in the intestine of hamsters fed on a high-fat diet. Hepatic mRNA or protein levels were unaffected. Sucrose feeding caused a small increase in hepatic message although with little change in hepatic MTP protein [12].

We chose to determine whether MTP is regulated under conditions in which hepatic VLDL outputs are markedly reduced: in the streptozotocin-diabetic rat [13,14] and the suckling

rat [15]. Both of these states are associated with increased intrahepatic storage of TAG [16,17]. Additionally, we wished to determine whether the rate of TAG output from the liver could be supported by the observed rate of MTP-facilitated lipid transfer measured in the assay system *in vitro*.

MATERIALS AND METHODS

Assay for lipid transfer activity

Lipid transfer activity was determined by a small unilamellar vesicle (SUV) assay previously described [6] with the following modifications. To prepare the donor and acceptor SUV, the appropriate lipids (in chloroform) were dried under a stream of nitrogen, followed by rotary evaporation under vacuum at 40 °C for 4 h. Vesicles were resuspended by vortex mixing in 15:35 buffer [15 mM Tris (pH 7.4), 35 mM NaCl, 1 mM EDTA, 0.02 % Na₂S₂O₈ and 0.5 % fatty-acid-free BSA]. SUV were then prepared by sonication with a Braun microprobe under nitrogen for 30 min. Donor vesicles, per assay, contained 175 nmol egg yolk phosphatidylcholine, 0.175 nmol [¹⁴C]glycerol trioleate and around 4000 c.p.m. [*N*-methyl-³H]dipalmitoylphosphatidylcholine ([³H]DPPC). The acceptor vesicles contained 175 nmol egg yolk phosphatidylcholine, 0.87 nmol TAG and 8.8 nmol cardiolipin. Both sets of vesicles contained 0.1 % butylated hydroxytoluene. Donor vesicles were preabsorbed by the addition of 0.5 ml of DEAE-cellulose/15:35 buffer slurry to 1 ml of vesicles.

Assays for lipid transfer activity were carried out by incubating donor and acceptor SUV with 50 µl of a microsomal fraction in 1 ml of 15:35 buffer at 37 °C for 1 h, after which donor and acceptor vesicles were separated by incubation (5 min) with 0.5 ml of DEAE-cellulose/15:35 buffer. After low-speed centrifugation, the supernatant (containing the donor vesicles) was analysed by scintillation counting to determine the ratio of [¹⁴C]glycerol trioleate to [³H]DPPC. The percentage lipid transfer

Abbreviations used: VLDL, very-low-density lipoprotein; apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; TAG, triacylglycerol; ER, endoplasmic reticulum; SUV, small unilamellar vesicle; [³H]DPPC, [*N*-methyl-³H]dipalmitoylphosphatidylcholine.

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was calculated from the decrease in this ratio. Enzyme activities were expressed as, mol of [^{14}C]TAG transferred/h per mg of microsomal protein.

Preparation of rat liver microsomes

Adult male Wistar rats (200–250 g) were fed *ad libitum* a pelleted chow diet consisting of 46.7% carbohydrate, 3.1% fat and 15.4% protein [16]. Five days before the experiment, one group of rats was made insulin-deficient by injection of a solution of streptozotocin (50 mg/kg body weight in 0.01 M citrate buffer/ml; pH 4.5) via a tail vein [18]. After 5 days, the urinary glucose concentration was greater than 25 mM. The animals were not ketotic. Diabetic and non-diabetic animals were killed by cervical dislocation and the livers were removed, chilled, minced and homogenized in a Potter–Elvehjem homogenizer in 20 ml of homogenization buffer [50 mM Tris (pH 7.4)/250 mM sucrose/1 mM EDTA/1 mM PMSF/1 mM benzamidine/5 $\mu\text{g}/\text{ml}$ leupeptin]. The homogenate was then centrifuged at 10000 *g* for 30 min and the supernatant centrifuged at 18000 *g* for 30 min. A final centrifugation of the above supernatant was carried out for 1 h at 100000 *g*. The microsomal pellet obtained from each whole liver was suspended overnight in 1 mM Tris (pH 7.4) containing 0.02% saponin. Aliquots were taken for protein estimation and immunoblotting. The microsomal membranes were pelleted by centrifugation and the supernatant was retained. The supernatant was concentrated by membrane filtration and tested for MTP activity. Protein estimation was by a modified method of Lowry (Bio-Rad kit) [19]. Microsomes were prepared in a similar manner from 12-day-old suckling animals, except, in these cases, the livers from ten rats were pooled before homogenization.

Detection of the large subunit of MTP by immunoblotting

Samples containing 20 μg of microsomal protein were resolved by 8% SDS/PAGE. An immunoblot of the resolved gel was carried out using a 10% solution of methanol in 3-cyclohexylamino-1-propanesulphonic acid buffer (pH 11.3) onto pretreated polyvinylidene difluoride membrane. The membrane

was blocked in 5% non-fat milk powder dissolved in a solution of 10 mM Tris, 1 M NaCl and 0.5% Tween-20 (high-salt Tween) for 1 h. The membrane was incubated for 1 h in high-salt Tween with the addition of rabbit anti-MTP serum at a 1:2000 dilution. The membrane was washed in high-salt Tween and incubated for 30 min in blocking solution with the addition of goat anti-(rabbit IgG) conjugated to horseradish peroxidase and again washed in high-salt Tween. Immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical methods

Values shown for control and diabetic rats are the means $\pm$ S.E.M. of six animals in each group. Results shown for the suckling animals are an average of three separate experiments in which each experiment consisted of the livers from ten animals. Mean values $\pm$ S.E.M. for the rate of VLDL TAG secretion were taken from [20]. Values were checked for statistical significance using an unpaired Student's *t*-test.

Materials

[^3H]DPPC (81 mCi/mmol), [^{14}C]glycerol trioleate (61 mCi/mmol) and chemiluminescence detection reagents were purchased from Amersham International (Aylesbury, Bucks., U.K.). Egg yolk phosphatidylcholine, bovine heart cardiolipin, triolein, butylated hydroxytoluene, saponin, fatty-acid-free BSA and protease inhibitors were purchased from Sigma (Poole, Dorset, U.K.). Goat anti-rabbit serum, conjugated to horseradish peroxidase, was purchased from Bio-Rad UK (Hemel Hempstead, Herts., U.K.).

RESULTS

Triacylglycerol transfer rates

Liver microsomes were prepared from diabetic, suckling and control rats, and MTP activity was released with saponin. Comparable TAG transfer activities were found in diabetic, suckling and control animals (Figure 1). TAG transfer rates were

Figure 1 MTP activities in the livers of normal, diabetic and suckling rats

Liver microsomal extracts were prepared as described in the Materials and methods section. Comparisons were made of MTP activity in the livers of normal (a), diabetic (b) and suckling (c) rats. Each value represents the mean $\pm$ S.E.M. of either three (suckling animals) or six (normal and diabetic animals) independent microsomal preparations.

Table 1 Specific activities of MTP in the livers of normal, diabetic and suckling rats

Calculations were based on the protein concentrations corresponding to the linear range shown in Figure 1. Each value represents the mean $\pm$ S.E.M. of three (suckling animals) or six (normal and diabetic animals) independent microsomal preparations.

State of rats	MTP specific activity (nmol TAG transferred/h per mg of total microsomal protein)
Normal	10.6 $\pm$ 0.6
Diabetic	10.9 $\pm$ 0.8
Suckling	11.8 $\pm$ 0.8

Figure 2 Immunoblot analysis of hepatic microsomal luminal proteins

Lane 1, control; lane 2, diabetic; lane 3, suckling. All samples contain the same quantity of total microsomal protein (20 μ g). Partially purified bovine liver MTP was used as a standard (lane 4). Blots were incubated with anti-MTP antiserum (1:2000 dilution; see the Materials and methods section).

measured in the linear range of the assay in which the transfer of TAG did not exceed 25% (up to 50 μ g of protein). The specific activities were expressed as nmol of TAG per mg of microsomal protein and were similar in value in all three animal models examined (Table 1). Similar transfer rates were also observed at pH 6 (results not shown).

Detection of MTP protein by immunoblotting

An anti-(MTP peptide) serum was raised in rabbits against residues 841–861 of the human MTP sequence. The anti-MTP serum recognizes purified bovine MTP (Figure 2) and also the large subunit of human MTP expressed either as a bacterial fusion protein or following transfection into Cos cells (results for the latter two experiments not shown). Samples containing equal amounts of microsomal protein were resolved by 8% SDS/PAGE. An immunoblot of these samples showed an immunoreactive band corresponding to the large subunit of MTP (previously shown to have an apparent molecular mass of 88 kDa on SDS/PAGE [6]. In agreement with measurements of activity (Figure 1; Table 1) equal amounts of immunoreactive protein were seen in samples from each type of rat (Figure 2).

Comparison of MTP activity and VLDL TAG secretion rate

Rates of TAG transfer in the liver microsomes were compared with the known rates of VLDL TAG secretion from primary rat hepatocytes derived from diabetic and control animals. Rates of TAG transfer per total hepatocyte protein were re-calculated from the original data on the basis that microsomal protein constitutes 20% of the total. The rates of VLDL TAG secretion in hepatocytes from diabetic and control animals have been determined previously [20]. Table 2 shows that TAG transfer rates measured by assay *in vitro* are well in excess of the amount

Table 2 Comparison of MTP specific activities with rates of VLDL TAG secretion

Rates of MTP-catalysed TAG transfer activity were calculated from the data shown in Figure 1, assuming that microsomal protein constitutes 20% of total liver protein [27]. Values for VLDL TAG secretion from cultured hepatocytes were taken from the references cited.

State of rats	Rate of TAG transfer activity (nmol/24 h per mg of total liver protein)	Rate of VLDL TAG secretion (nmol/24 h per mg of total liver protein)
Control	72.0 $\pm$ 5.1	148 $\pm$ 24 [20]
Diabetic	81.6 $\pm$ 6.2	11.0 $\pm$ 5.6 [20]
Suckling	76.8 $\pm$ 7.2	1.0 [21]

of TAG secreted in cultured primary hepatocytes from both suckling and diabetic rats. In the normal rats these values were comparable. A comparison of TAG transfer rate with that of VLDL secretion in the suckling animals was calculated from secretion rates measured as described previously [21]. Hepatic rates of TAG secretion are substantially reduced in suckling animals in which virtually all the plasma TAG is derived from intestinal secretion [21]. This large decrease in secretion was not, however, accompanied by any change in MTP activity (Figure 1; Table 1).

DISCUSSION

Recent studies in the hamster have sought to determine whether MTP is regulated by dietary manipulation [12]. In these studies, the mRNA of the large subunit of MTP was acutely regulated by fat feeding in the intestine, but in the liver, marginal changes were observed only after chronic feeding of the high-fat diet. Chronic sucrose feeding also resulted in increased hepatic MTP mRNA levels, but in this case, levels in the intestine were unchanged. Other studies showed that the 5' promoter region of MTP contains a modified positive sterol-responsive element and a negative insulin-responsive element. Together these findings would indicate that cholesterol and insulin could regulate the activity of the promoter in opposite directions [22]. While acute dietary manipulation may have marginal effects on the liver, hepatic VLDL output responds strongly to pathophysiological and developmental changes. For instance, insulin-deficient diabetes [13,14] and the suckling stage of development [15,21] both substantially reduce hepatic VLDL output.

The purpose of our studies was to evaluate whether a fall in the lipid transfer activity of MTP could explain the inhibition of VLDL output in hepatocytes from diabetic and suckling animals. However, in both situations where VLDL output was suppressed, the level of lipid transfer activity remained comparable to that of control animals. Immunoblotting with an anti-MTP antiserum of the hepatic microsomes of these animals confirmed that MTP protein was also unchanged. We cannot of course, at present, completely rule out the possibility that there were changes in MTP mRNA which, due to slow MTP protein turnover, were not reflected by measurable changes in MTP activity. We consider this unlikely, however, because if this were the case, MTP turnover must have been exceptionally slow, since we were unable to detect any change even after 5 days of insulin deficiency and 12 days of suckling.

It has previously been reported that insulin directly suppresses the secretion of VLDL *in vitro* (for a review, see [23]), and the human MTP gene promoter is also negatively regulated by insulin [22]. Whether these insulin effects in cultured cells are

causally linked is not known. However, if this situation applied in intact animals, then in insulin-deficient states such as diabetes, VLDL secretion and the expression and activity of MTP should be de-repressed. This was not the case. Instead, the secretion of VLDL was substantially decreased under these conditions. These decreases were accompanied neither by any significant change in the capacity of MTP to transfer TAG (Figure 1; Table 1) nor by a change in the quantity of the large subunit present in the microsomal fraction of the liver (Figure 2). Together, the above observations suggest that *in vivo* there are other insulin-dependent factors which regulate hepatic VLDL assembly. It may be of relevance in this context that the rate of *de novo* lipogenesis in diabetic rats is very low [16] and this rate appears to bear a strong positive correlation with VLDL secretion [24,25]. It might, of course, be argued that streptozotocin may affect certain metabolic parameters via a mechanism which does not directly involve lack of insulin. The resistance of MTP to streptozotocin treatment suggested, however, that neither the lack of insulin nor, for that matter, any other concomitant metabolic disturbance had any effect on MTP activity and abundance. The cause(s) of the low rates of VLDL secretion at the molecular level in the diabetic and suckling rat models is not known, but may possibly be related to an impaired mobilization of the intrahepatic pool of TAG [26]. Another question arising from the present study was whether the rate of TAG transfer activity measured in the assay *in vitro* was sufficient to account for the level of VLDL TAG secretion measured from rat livers. Rates of MTP-facilitated TAG transfer measured from the microsomal fraction of diabetic, suckling and control animals was shown to be within the range or in excess of that secreted as VLDL. However, a number of issues have to be considered in the comparison of TAG transfer activity with rates of secretion. First the rate of TAG secretion was measured in cultured hepatocytes and may be altered *in vivo*. Secondly, microsomal extracts were prepared from whole liver, which contains cells other than hepatocytes. Thus MTP activity per mg of microsomal hepatocyte protein may have been higher. Finally, limitations in the measurement of net transfer of TAG due to design of the assay *in vitro* may underestimate the level of MTP-mediated lipid transfer *in vivo*. We consider it likely, therefore, that the activity of MTP, as measured, is sufficiently high to maintain the observed rates of VLDL TAG secretion. In conclusion, our results indicate that factors other than the rate of MTP-facilitated TAG transfer act to suppress VLDL output in both suckling and streptozotocin-induced diabetic rats.

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REFERENCES

- 1 Pullinger, C. R., North, J. D., Teng, B.-B., Rifici, V. A., Ronhild de Brito, A. E. and Scott, J. (1989) *J. Lipid Res.* **30**, 1065–1077
- 2 Dixon, J. L. and Ginsberg, H. N. (1993) *J. Lipid Res.* **34**, 167–179
- 3 Borchardt, R. A. and Davis, R. A. (1987) *J. Biol. Chem.* **262**, 16394–16402
- 4 Borén, J., Wettesten, M., Rustaeus, S., Andersson, M. and Olofsson, S.-O. (1993) *Biochem. Soc. Trans.* **21**, 487–493
- 5 Wang, H., Chen, X. and Fisher, E. A. (1993) *J. Clin. Invest.* **91**, 1380–1389
- 6 Wetterau, J. R., Aggerbeck, L. P., Lapland, P. M. and McClean, L. R. (1991) *Biochemistry* **30**, 4406–4412
- 7 Sharp, D., Blinderman, L., Combs, K. A. et al. (1993) *Nature (London)* **365**, 65–69
- 8 Shoulders, C. C., Brett, D. J., Bayliss, J. D. et al. (1993) *Human Mol. Genet.* **2**, 2109–2116
- 9 Kane, J. P. and Havel, R. J. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 6th edn., pp. 1139–1164, McGraw-Hill, New York
- 10 Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J. and Shoulders, C. C. (1994) *J. Biol. Chem.* **269**, 21951–21954
- 11 Gordon, D. A., Jamil, H., Sharp, D. et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7628–7632
- 12 Lin, M. C. M., Arbeeny, C., Bergquist, K., Kienzie, B., Gordon, D. A. and Wetterau, J. R. (1994) *J. Biol. Chem.* **269**, 29138–29145
- 13 Berry, E. M., Ziv, E. and Bar-On, H. (1981) *Diabetologia* **21**, 402–408
- 14 Reaven, G. M. and Greenfield, M. S. (1981) *Diabetes* **30**, 66–75
- 15 Frost, S. C., Clark, W. A. and Wells, M. A. (1983) *J. Lipid Res.* **24**, 899–903
- 16 Duerden, J. M. and Gibbons, G. F. (1988) *Biochem. J.* **255**, 929–935
- 17 Jamdar, S. C., Moon, M., Bow, S. and Fallon, H. J. (1978) *J. Lipid Res.* **19**, 763–770
- 18 Schein, P. S., Alberti, K. G. M. M. and Williamson, D. H. (1971) *Endocrinology* **89**, 827–834
- 19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, P. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 20 Duerden, J. M. and Gibbons, G. F. (1993) *Biochem. J.* **294**, 167–171
- 21 Coleman, R. A., Haynes, E. B., Sand, T. M. and Davis, R. A. (1988) *J. Lipid Res.* **29**, 33–42
- 22 Hagan, D. L., Kienzie, B., Jamil, H. and Hariharan, N. (1994) *J. Biol. Chem.* **269**, 28737–28744
- 23 Sparks, J. D. and Sparks, C. E. (1994) *Biochim. Biophys. Acta* **1215**, 9–32
- 24 Boogaerts, J. R., Malone-McNeal, M., Archambault-Schexnayder, J. and Davis, R. A. (1984) *Am. J. Physiol.* **246**, E77–E83
- 25 Yamamoto, M., Yamamoto, Y., Tanaka, Y. and Ontko, J. A. (1987) *J. Lipid Res.* **28**, 1156–1165
- 26 Gibbons, G. F. and Wiggins, D. (1995) *Adv. Enzyme Regul.* **35**, 179–198
- 27 *Biochemist's Handbook* (1961) (Long, C., ed.), pp. 816–817, Spon and Spon, London