Effect Of Exogenous Cholesterol And Dithiothreitol On The Activity Of Human Liver Microsomal Acyl-Coenzyme A:Cholesterol Acyltransferase (ACAT)

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Abstract

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is the intracellular enzyme responsible for the esterification of cholesterol with long-chain fatty acyl-CoA derivatives and has been implicated in atherosclerosis and gallstone disease. The effects of exogenous cholesterol and dithiothreitol (DTT) on the ACAT activity of human liver microsomes have been determined. Pre-incubation of microsomes with exogenous cholesterol gave a marked stimulation of activity. Experiments with [^{3}H]cholesterol and [^{14}C]ololeoyl-CoA indicated the time course of equilibration of exogenous with endogenous cholesterol as ACAT substrates, and showed that ACAT activity could be accurately measured using [^{3}H]cholesterol/Tween 80, provising that the concentration of endogenous microsomal cholesterol was also determined. Pre-incubation of liver microsomes for 90 min in the presence of 2 mmol/1 DTT and exogenous cholesterol/Tween 80 resulted in a 60% reduction in ACAT activity, compared with the corresponding activity when DTT was omitted. If microsomes were pre-incubated with DTT prior to the pre-incubation with exogenous cholesterol/Tween 80, and 85–90% reduction in ACAT activity occurred. In contrast, pre-incubation of microsomes with DTT in the absence of exogenous cholesterol/Tween 80 (only endogenous cholesterol present) resulted, initially in a stimulation of ACAT activity; on further pre-incubation, activity returned to control levels. These results indicate that the supply of cholesterol to the enzyme active site is an important factor in ACAT assays in vitro and that DTT has a major effect on this process, suggesting that these factors may be important in controlling ACAT activity in vivo.

Author Keywords: Acyl-CoA hydrolase; cholesterol availability in ACAT assay; cholesterol transport protein; atherosclerosis; gallstones

1. Introduction

Acyl-coenzyme A:cholesterol acyltransferase (ACAT; E.C. 2.3.1.26) is located in the rough endoplasmic reticulum [1-5] where it catalyses the esterification of cholesterol with long-chain fatty acyl-CoA derivatives. ACAT activity is subject to regulation by diet, substrate supply, circadian rhythm and hormones [6-10] and has been shown to play an important role in diseases such as atherosclerosis [11-14] and cholesterol gallstones [15]. The importance of ACAT in cholesterol absorption from the gut [16-18], its involvement in synthesizing cholesterol ester in the liver for secretion into plasma in very low density lipoproteins [19,20] and its role in converting cholesterol into cholesterol ester in arterial tissues [12-14] have all made ACAT a good target for modulation by therapeutic drugs [12-14,18,21].

It has been previously demonstrated that endogenous cholesterol present in rat, monkey and human liver microsomes can act as a substrate for ACAT and that the addition of exogenous cholesterol to the assay mixture stimulates activity several-fold [2,11,22-28]. In

the present work, we used a dual label method ([^{3}H]cholesterol and [^{14}C]oleoyl-CoA) to compare the utilisation of endogenous and exogenous cholesterol by ACAT in a standard assay [27].

Several groups have reported that inclusion of DTT in the assay decreased ACAT activity in vitro [3,25,27,29], suggesting the possible role of a thiol such as glutathione in the regulation of ACAT activity in vivo. Thiols, including glutathione, have been shown to activate HMG-CoA reductase [30,31] and cholesterol 7α-hydroxylase [32,33], two other important enzymes of cholesterol metabolism. Early work by Goodman et al. [34] indicated that ACAT activity may also be modulated by glutathione. It is therefore possible that the activities of HMG-CoA reductase, cholesterol 7α-hydroxylase and ACAT are co-ordinately controlled by a redox mechanism. In this paper, we describe the effects of pre-incubation of microsomes with DTT, in the presence and absence of exogenous cholesterol/Tween 80, on ACAT activity.

2. Materials and methods

2.1. Liver samples

Human liver tissue was obtained from several organ donors who died either from head injuries, cerebral tumour, meningioma or from hypoxic brain death, with no other known medical condition. The portion of liver not required for transplantation was used. All livers were subject to minimal warm ischaemia (i.e. storage at 37°C in the absence of blood flow) and several hours of cold ischaemia by perfusion in situ with cold flush solution (hyperosmolar Collins C2 solution [35,36]) before storage at –70°C. Microsomal membrane fractions were prepared as previously described [37]. Liver samples obtained and processed by these procedures have been previously found to be suitable for studies on hepatic ACAT activity [15,27,36]. Consent for use of liver tissue for scientific purposes was obtained from the next of kin.

2.2. Dual label experiments

ACAT activity was determined as previously described [15,27] with the exception that [^{3}H]cholesterol was included in the cholesterol/Tween 80 solution (6 µg cholesterol and 150 µg Tween 80 per assay) and the [^{3}H]cholesterol oleate internal standard was omitted from the assay. The ACAT assay buffer was Tris-HC1, pH 8.4 [27]. Specific activity was calculated by using both the specific activity of the [^{14}C]oleoyl-CoA added to the assay and the specific activity of the [^{3}H]cholesterol, after adjusting for the amount of endogenous cholesterol, assuming that equilibrium existed between the endogenous and exogenous cholesterol.

2.3. Other assays

Microsomal cholesterol concentrations were determined by the method of Salè et al. [38] and protein by the method of Lowry et al. [39] as modified by Peterson [40].

3. Results

3.1. Effect of exogenous [^{3}H]cholesterol/Tween 80 on ACAT activity

The effect of exogenous cholesterol on ACAT activity was investigated by pre-incubating [^{3}H]cholesterol dispersed in Tween 80 with human liver microsomes for various times and subsequently initiating ACAT activity by the addition of [^{14}C]oleoyl-CoA (Fig. 1A). ACAT activity was determined by measuring the rate of incorporation of both ^{3}H from [^{3}H]cholesterol and ^{14}C from [^{14}C]oleoyl-CoA into cholesterol oleate. Results in Fig. 1A demonstrate that the activity determined by both methods was markedly increased by pre-incubation, indicating that activity depends on substrate supply.
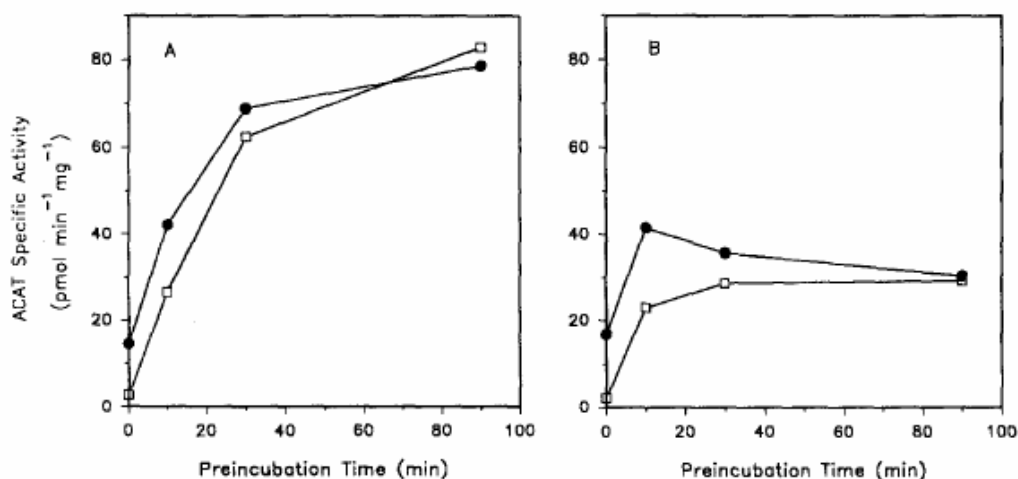
Fig. 1. Effect of pre-incubation of microsomes with exogenous [^{3}H]cholesterol/Tween 80 in the absence (A) and presence (B) of 2 mmol/1 DTT. ACAT specific activity was calculated using both the specific activity of [14C]oleoyl-CoA (●) and [3H]cholesterol (□); both the endogenous (3537 pmol) and exogenous (15 520 pmol) cholesterol were used in the calculation. Duplicates varied by less than 10%.

Furthermore, the specific activities determined by both methods, taking into account the endogenous microsomal cholesterol concentration, were the same following a pre-incubation time of greater than 30 min, indicating that the pools of endogenous and exogenous cholesterol had equilibrated. At shorter pre-incubation times, the specific ACAT activity was lower, if calculated using ^{3}H rather than ^{14}C incorporation, indicating that endogenous cholesterol was being used in preference to exogenous cholesterol.

With zero pre-incubation, only 10-20% of the cholesterol incorporated into cholesterol oleate during the 5 min reaction time was from the exogenous pool (Fig. 1; comparison of ^{14}C and[^{3}H incorporation at zero time).

3.2. Effect of DTT on equilibration of exogenous and endogenous cholesterol pools on ACAT activity

Previous studies from our laboratory demonstrated that the inclusion of 2 mmol/1 DTT in ACAT assays produced maximal inhibition of ACAT activity [27]; higher concentrations (5 and 10 mmol/1) had no additional effect on activity. Therefore, 2 mmol/1 DTT was chosen for the current experiments. When DTT (2 mmol/l) was included in the pre-incubation mixture together with [^{3}H]cholesterol/Tween 80, the nett effect of pre-incubation was also to increase ACAT activity but to a lesser extent than when DTT was absent from the assay (Fig. 1B compared with Fig. 1A). DTT did not prevent the equilibration of endogenous and exogenous cholesterol, since the ^{3}H and ^{14}C specific activities after 90 min pre-incubation were the same. The initial rise in activity presumably reflects this equilibration. The final specific activity (30 pmol ⋅ min^{-1} ⋅ mg^{-1} at 90 min pre-incubation) shows that the effect of the 2 mmol/l DTT was to decrease ACAT activity by approximately 60% (Fig. 1B compared with Fig. 1A).

To examine whether DTT had a direct effect on ACAT activity, human liver microsomes were incubated for 20 min in the presence and absence of DTT (2 retool/l) before incubating with [^{3}H] cholesterol/Tween 80 for various times prior to addition of [^{14}C]oleoyl-CoA (Fig. 2). Incubation of microsomes in the absence of DTT had no effect on the subsequent stimulation by exogenous cholesterol, as expected (compare Figs. 2 and 1A). However, if microsomes were incubated with DTT prior to incubation with exogenous cholesterol, the activity was decreased by 85-90% following 30 min incubation with exogenous cholesterol (Fig. 2).
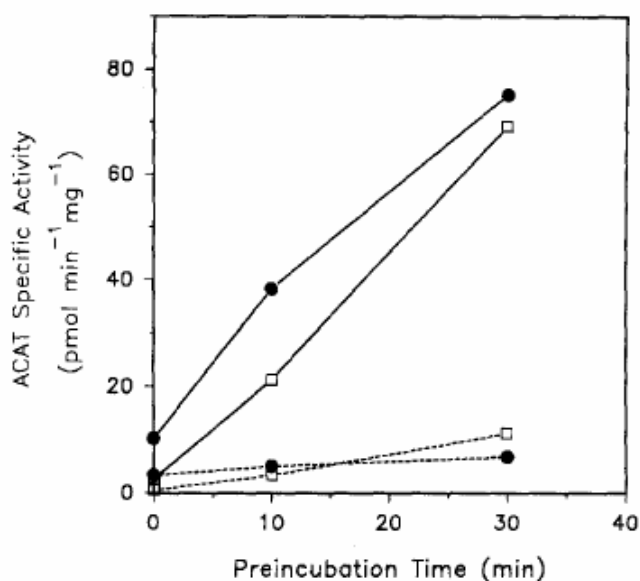
Fig. 2. Effect of pre-incubation of microsomes with exogenous [³H]cholesterol/Tween 80 following incubation of microsomes for 20 rain in the absence (solid lines) and presence (dashed lines) of 2 retool/1 DTT. ACAT specific activity was calculated as described in Fig. l. (●) [¹⁴C]oleoyl-CoA; (□) [³H]cholesterol. Duplicate assays varied by less than 10%, except for the zero and 10 rain time points of dashed lines in which the variation was considerably higher owing to the low specific activities at these time points.

Thus, pre-treatment of microsomes with DTT prior to the addition of exogenous cholesterol/Tween 80 resulted in a greater reduction in activity than when DTT was added simultaneously with exogenous cholesterol/Tween 80 (compare Figs. 2 and 1B). The final ACAT activities in the presence of DTT of Fig. 2 (11.3 and 6.9 pmol $\cdot$ min$^{-1}\cdot$ mg^{-1}) were similar to those obtained when activity was determined in the absence of exogenous cholesterol and DTT after 90 min pre-incubation of the microsomes, i.e.12 pmol $\cdot$ min$^{-1}\cdot$ mg^{-1}.

3.3. Effect of DTT on ACAT activity in the absence of exogenous cholesterol
To investigate the mechanism of DTT inhibition of ACAT activity, the effect of pre-incubation with 2 mmol/1 DTT on the activity in the absence of exogenous cholesterol was determined (Fig. 3).
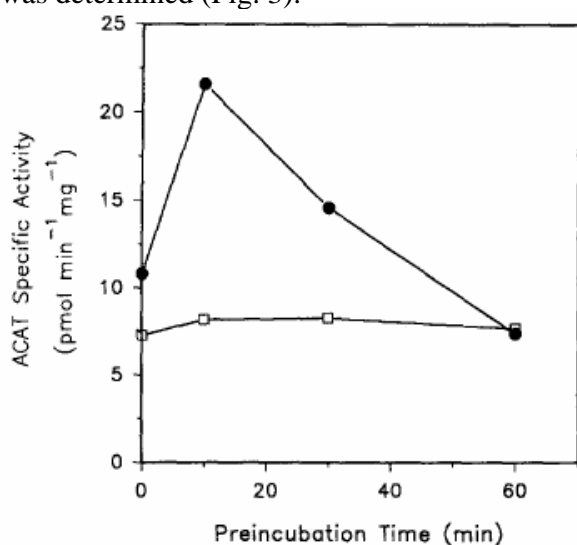

Fig. 3. Effect of pre-incubation of microsomes (in the absence of exogenous cholesterol and Tween 80) in the absence (□) or presence (●) of 2 mmol/1 DTT. ACAT specific activity was calculated by using the specific activity of [¹⁴C]oleoyl-CoA. [³H]Cholesterol oleate was included as an internal standard as previously described [27]. Duplicate assays varied by less than 10%.

Pre-incubation of microsomes alone had very little effect on ACAT activity using endogenous cholesterol as substrate, confirming our previous studies [27]. If DTT was included in the reaction mixture, ACAT activity was initially stimulated (2.6 fold upon 10 min pre-incubation) and then gradually decreased to control (DTT omitted) levels by 60 min pre-incubation. Thus, under these assay conditions, DTT appeared to activate ACAT activity at some pre-incubation times and not others, arguing against a direct inhibitory effect of DTT on ACAT. However, this experiment differed from the earlier experiments in that the detergent, Tween 80 (as well as exogenous cholesterol), was not present in the reaction mixture.

The effect of Tween 80 on DTT inhibition of ACAT activity was therefore examined. Microsomes were pre-incubated for 90 min in the presence of Tween 80, with or without DTT. Tween 80 alone increased ACAT activity by 2.2 fold compared with a control containing no DTT (Table 1).

Table 1
Effect of Tween 80 and DTT on ACAT Activity[a]

Additions	ACAT specific activity (pmol $\cdot$ min$^{-1}\cdot$ mg^{-1})
None	6.3
Tween 80[b]	14.1
Tween 80 + DTT[c]	3.6
DTT + Tween 80[c]	2.9

a Microsomes were pre-incubated in assay buffer in the absence of exogenous cholesterol but with the additions specified for 90 min prior to initiation of the assay by addition of [14C]oleoyl-CoA. Duplicates varied by less than 10%.
b The amount of Tween 80 in all experiments was 150μg/assay.
c The first named reagent was added at zero time and the second at 5 min. All additions were at 37°C.

This presumably reflects an effect of the detergent on the microsomal membrane, perhaps leading to greater accessibility of the active site to the substrates. In the presence of Tween 80, pre-treatment with 2 mmol/1 DTT caused a 74-79% decrease in ACAT activity compared with the control. This decrease was similar to that obtained when DTT was added to the assay mixture before exogenous cholesterol/Tween 80 (compare results in Table 1 and Fig. 2). Thus, the inhibitory effect of DTT on ACAT activity in the presence of Tween 80 is the same, whether or not exogenous cholesterol is added.

4. Discussion

A reliable assay for the determination of ACAT activity is of fundamental importance, particularly when the activity of this enzyme has been implicated in diseases such as atherosclerosis and gallstones. The supply of cholesterol to the active site of ACAT is an important factor in assays of ACAT activity of liver microsomes and presumably also in the endoplasmic reticulum of intact liver. Using exogenous [3H]cholesterol and [14C]oleoyl CoA, it is possible to determine the fraction of product cholesterol oleate which is derived from exogenous cholesterol. Results in Fig. 1A show the time course for equilibration between exogenous and endogenous cholesterol pools under standard assay conditions (in the presence of the detergent Tween 80). Equilibration was complete after 30 min of pre-incubation. This experiment has delineated conditions in which factors affecting equilibration of exogenous and endogenous cholesterol may be investigated. It also shows that the much less expensive [3H]cholesterol could be used instead of [14C]oleoyl-CoA in routine ACAT assays, if sufficient time is allowed for pre-equilibration prior to addition of oleoyl-CoA (provided the concentration of endogenous cholesterol in the enzyme sample is measured to allow correction of the specific radioactivity of the [3H]cholesterol added). Failure to allow

sufficient time for pre-incubation would result in a serious underestimation of ACAT activity (as shown by the zero time points of Fig. 1A).

The remaining experiments investigated the effect of DTT treatment on ACAT activity. The results of Fig. 1A and Fig. 1B show that the presence of 2 mmol/l DTT during the pre-incubation period in the standard ACAT assay had no effect on equilibration of endogenous and exogenous cholesterol. However, the final rate after 90 min of pre-incubation with DTT was approximately 60% lower than when DTT was omitted. This inhibitory effect of DTT on ACAT activity may be a general one, having also been reported in rat [25,29] and observed in hamsters (J.L. Smith and C. Lutton, unpublished observations). The results of Fig. 2 show that pre-treatment of microsomes in the assay buffer with 2 mmol/1 DTT prior to addition of cholesterol/Tween 80 had an even greater effect, resulting in loss of 85-90% of the ACAT activity compared with a control in the absence of DTT. DTT may have a direct effect on ACAT protein or an effect on the supply of substrates to the enzyme active site. A further possible explanation for the apparent inhibitory effect of DTT on ACAT activity was that DTT activates other enzymes which use oleoyl-CoA. This could, in principle, lead to a significant depletion of oleoyl-CoA during the assay and an apparent decrease in ACAT activity. We have previously found [J.L. Smith, unpublished observations] that when DTT is included in the ACAT assay, the activity of acyl-CoA hydrolase (also present in the microsomes) is increased several-fold.

In the experiments of Fig. 1B, the maximal amount of oleoyl-CoA converted to oleic acid (hydrolase activity) was 11%. Work by Fiorica [29] showed that if 2 mmol/l DTT was included in the assay of rat liver ACAT activity, a significant amount (about equal to the amount of oleic acid) of unidentified polar material was produced. This material may be the thioester formed from oleoyl-CoA and DTT. These results suggest that depletion of oleoyl-CoA sufficient to account for the loss of activity had not occurred during the assay period. In this context, we have shown that fatty acid ethyl esters are formed when ethanol is included in the ACAT assay [41].

The results of Fig. 3 and Table 1 show the importance of the detergent Tween 80 in understanding the effect of DTT. When microsomes were pre-incubated with DTT in assay buffer and then assayed for ACAT activity in the absence of detergent and exogenous cholesterol, the activity at short pre-incubation times was significantly increased ∼2.6 fold after 10 min; Fig. 3). After 60 min, the activity had returned to the baseline level (i.e., the level in the absence of DTT). Thus, the ACAT activity of intact microsomes using endogenous cholesterol and added oleoyl-CoA as substrates (in the absence of Tween 80), was stimulated by DTT, in marked contrast to the results of Figs. 1 and 2. The ACAT activity of human liver microsomes using endogenous cholesterol was also stimulated (2.2-fold) by pre-treatment with Tween 80 for 90 min (Table 1). If 2 mmol/l DTT was added with the Tween 80, the ACAT activity was decreased by 74-79%, consistent with the results of Figs. 1 and 2. Therefore, pre-incubation of human liver microsomes separately with DTT and Tween 80 both led to stimulation of ACAT activity with endogenous cholesterol, whereas pre-incubation with DTT/Tween 80 together led to an ∼ 80% decrease in ACAT activity.

Our hypotheses to explain these results are as follows (see also Fig. 4):

(1) Treatment of microsomes with Tween 80 alone caused an increase in activity by making the active site more accessible to either or both of the substrates, cholesterol and oleoyl-CoA. Many microsomal enzymes exhibit marked stimulation by detergents *(latency)* which has been attributed to increased accessibility of the enzyme to the substrates. Notably, acyl-CoA hydrolase activity (which can be measured concurrently with ACAT activity) is stimulated by Tween 80 in the standard ACAT assay [J.L. Smith, unpublished observations].

(2) ACAT activity of intact microsomes is stimulated by DTT (Fig. 3) because the enzyme itself, or an auxiliary protein, requires a sulfhydryl group (cysteine side chain) for activity [42]. Alternatively, DTT may stabilize microsomes, maintaining the interaction of ACAT with the membrane components required for activity. The transient nature of the stimulation could be explained by depletion of the DTT during the pre-incubation period. An effect of

DTT on ACAT itself may be masked by other factors in the presence of exogenous cholesterol/Tween 80 (see Fig. 4).

(3) ACAT activity is inhibited by treatment of microsomes with DTT and Tween 80 because Tween 80 exposes an essential component of the ACAT system to DTT. The differential effects of DTT in the presence and absence of Tween 80 seem difficult to explain without invoking a second component required for activity (i.e. in addition to ACAT). One possibility is a transport protein, required for the transfer of cholesterol to the active site of ACAT.
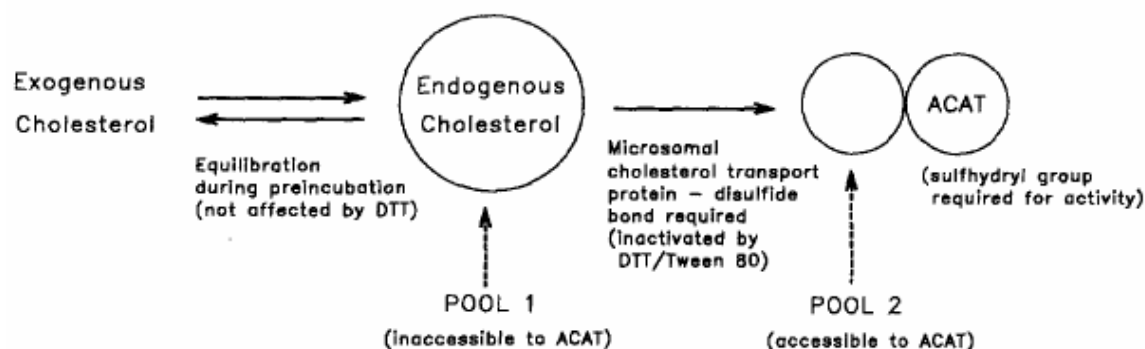

Fig. 4. Diagram showing possible pools of cholesterol in ACAT assay mixture and the effects of DTT.

It is plausible to suggest that a transport protein is involved in the transport of cholesterol to the active site of ACAT (see Fig. 4) as such a transfer protein, microsomal triglyceride and cholesterol ester transfer protein (MTP), has been previously described [43] and its structure and physiological role elucidated [44]. While MTP has highest specificity for cholesterol ester and triglyceride it can bind and transport significant amounts of cholesterol [45]. Purified MTP from bovine liver contains two subunits with molecular weights of 88 000 and 55 000. The 55 000 subunit has been recently identified as a multifunctional protein disulfide isomerase (PDI) which appears necessary to maintain the active structure of MTP [46]. It is conceivable that MTP or a similar cholesterol transport protein may be involved in the transport of cholesterol to the ACAT active site and that this process is inactivated by DTT/Tween 80 (as suggested in Fig. 4). Several cytosolic lipid transport proteins have been reported to transfer cholesterol from unilamellar vesicles/liposomes to membranes [47-49].

In the present work we have investigated the effects of exogenous cholesterol/Tween 80, pre-incubation time and DTT on the ACAT activity of human liver microsomes. The results suggest that the supply of cholesterol to the active site of ACAT may be inhibited by DTT in the presence of Tween 80 and we have outlined a possible mechanism by which this may occur. The present work also delineates a framework in which factors affecting ACAT activity may be further investigated. The possible in vivo importance of these findings is the subject of further investigation.

References

1. S. Balasubramaniam, S. Venkatesan, K.A. Mitropoulos and T.J. Peters, The submicrosomal localization of acyl-coenzyme A-cholesterol acyltransferase and its substrate, and of cholesteryl esters in rat liver. *Biochem J* **174** (1978), pp. 863–872.

2. A.H. Lichtenstein and P. Brecher, Properties of acyl-CoA:cholesterol acyltransferase in rat liver microsomes. Topological localization and effects of detergents, albumin and polar steroids. *J Biol Chem* **255** (1980), pp. 9098–9104.

3. S.K. Erickson, M.A. Shrewsbury, C. Brooks and D.J. Meyer, Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation in vivo and some of its properties in vitro. *J Lipid Res* **21** (1980), pp. 930–941.

4. S. Hashimoto and A.M. Fogelman, Smooth microsomes. A trap for cholesteryl ester formed in hepatic microsomes. *J Biol Chem* **255** (1980), pp. 8678–8684.

5. S. Balasubramaniam, K.A. Mitropoulos, S. Venkatesan *et al.*, Analytical fractionation of human liver microsomal fractions: localization of cholesterol and of the enzymes relevant to its metabolism. *Clin Sci* **60** (1981), pp. 435–439.

6. A.A. Spector, S.N. Mathur and T.L. Kaduce, Role of acylcoenzyme A:cholesterol *O*-acyltransferase in cholesterol metabolism. *Prog Lipid Res* **18** (1979), pp. 31–53.

7. S.K. Erickson, Report on the American Society of Biological Chemists Satellite Conference on Regulation of Intracellular Cholesterol Esterification. *J Lipid Res* **25** (1984), pp. 411–415.

8. K.E. Suckling and E.F. Stange, Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* **26** (1985), pp. 647–671.

9. B. Jackson, A.N. Gee, M. Martinez Cayuela and K.E. Suckling, The effects of feeding a saturated fat-rich diet on enzymes of cholesterol metabolism in the liver, intestine and aorta of the hamster. *Biochim Biophys Acta* **1045** (1990), pp. 21–28.

10. A. Szanto, J. Ruys and S. Balasubramaniam, Coordinate diurnal regulation of hepatic acyl-coenzyme A:cholesterol acyltransferase and cellular levels of esterified cholesterol. *Biochim Biophys Acta* **1214** (1994), pp. 39–42.

11. S.N. Mathur, M.L. Armstrong, C.A. Alber and A.A. Spector, Hepatic acylcoenzyme A:cholesterol acyltransferase activity during diet-induced hypercholesterolemia in cynomolgus monkeys. *J Lipid Res* **22** (1981), pp. 659–667.

12. J.L. Kelley, C.A. Suenram, M.M. Rozek, C.J. Schaffer and C.J. Schwartz, Influence of the acyl-CoA:cholesterol *O*-acytransferase inhibitor, CL 277 082, on cholesteryl ester accumulation in rabbit macrophage-rich granulomas and hepatic tissue. *Biochim Biophys Acta* **960** (1988), pp. 83–90.

13. D.R. Sliskovic and A.D. White, Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends Pharmacol Sci* **12** (1991), pp. 194–199.

14. T.M.A. Bocan, S.B. Mueller, P.D. Uhlendorf, R.S. Newton and B.R. Krause, Comparison of CI-976, an ACAT inhibitor, and selected lipid-lowering agents for antiatherosclerotic activity in iliac-femoral and thoracic aortic lesions. A biochemical, morphological and morphometric evaluation. *Arterioscler Thromb* **11** (1991), pp. 1830–1843.

15. J.L. Smith, I.R. Hardie, S.P. Pillay and J. de Jersey, Hepatic acyl-coenzyme A:cholesterol acyltransferase activity is decreased in patients with cholesterol gallstones. *J Lipid Res* **31** (1990), pp. 1993–2000.

16. F.J. Field, A.D. Cooper and S.K. Erickson, Regulation of rabbit intestinal acylcoenzyme A-cholesterol acyltransferase in vivo and in vitro. *Gastroenterology* **83** (1982), pp. 873–880.

17. E.F. Stange, K.E. Suckling and J.M. Dietschy, Synthesis and coenzyme A-dependent esterification of cholesterol in rat intestinal epithelium. Differences in cellular localization and mechanisms of regulation. *J Biol Chem* **10** (1983), pp. 12868–12875.

18. F.J. Field, E. Albright and S. Mathur, Inhibition of acylcoenzyme A:cholesterol acyltransferase activity by PD128042: effect on cholesterol metabolism and secretion in CaCo-2 cells. *Lipids* **26** (1991), pp. 1–8.

19. M.W. Huff, D.E. Telford, P.H.R. Barrett, J.T. Billheimer and P.J. Gillies, Inhibition of hepatic ACAT decreases apo B secretion in miniature pigs fed a cholesterol-free diet. *Arterioscler Thromb* **14** (1994), pp. 1498–1508.

20. R. Musanti, L. Giorgini, P. Lovisolo, A. Pirillo, A. Chiari and G. Chiselli, Inhibition of acyl-CoA:cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. *J Lipid Res* **37** (1996), pp. 1–14.

21. B. Jackson, A. Gee, S. Black and K.E. Suckling, Effects of octimibate, an inhibitor of acyl coenzyme A:cholesterol acyltransferase, on cholesterol metabolism in the hamster and rat. *Biochem Pharmacol* **39** (1990), pp. 1487–1489.

22. S. Balasubramaniam, K.A. Mitropoulos and S. Venkatesan, Rat-liver acyl-CoA:cholesterol acyltransferase. *Eur J Biochem* **90** (1978), pp. 377–383.

23. S. Balasubramaniam, K.A. Mitropoulos, N.B. Myant, M. Mancini and A. Postiglione, Acylcoenzyme A-cholesterol acyltransferase activity in human liver. *Clin Sci* **56** (1979), pp. 373–375.

24. J.T. Billheimer, D. Tavani and W.R. Nes, Effect of a dispersion of cholesterol in Triton WR-1339 on acyl CoA:cholesterol acyltransferase in rat liver microsomes. *Anal Biochem* **111** (1981), pp. 331–335.

25. S. Synouri-Vrettakou and K.A. Mitropoulos, Acyl-coenzyme A:cholesterol acyltransferase. Transfer of cholesterol to its substrate pool and modulation of activity. *Eur J Biochem* **133** (1983), pp. 299–307.

26. K.E. Suckling, E.F. Stange and J.M. Dietschy, In vivo modulation of rat liver acyl-coenzyme A:cholesterol acyltransferase by phosphorylation and substrate supply. *FEBS Lett* **159** (1983), pp. 29–32.

27. J.L. Smith, J. de Jersey, S.P. Pillay and I.R. Hardie, Hepatic acyl-CoA:cholesterol acyltransferase. Development of a standard assay and determination in patients with cholesterol gallstones. *Clin Chim Acta* **158** (1986), pp. 271–282.

28. K. Einarsson, L. Benthin, S. Ewerth, G. Hellers, D. Ståhlberg and B. Angelin, Studies on acyl-coenzyme A:cholesterol acyltransferase activity in human liver microsomes. *J Lipid Res* **30** (1989), pp. 739–746.

29. E. Fiorica, Effects of thiol concentration in the assay of liver microsomal acylCoA:cholesterol acyltransferase. *Ital J Biochem* **35** (1986), pp. 272–281.

30. I. Dotan and I. Shechter, Properties of latent and thiol-activated rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase and regulation of enzyme activity. *Arch Biochem Biophys* **226** (1983), pp. 401–410.

31. R.E. Cappel and H.F. Gilbert, Thiol/disulfide exchange between 3-hydroxy-3-methylglutaryl-CoA reductase and glutathione. A thermodynamically facile dithiol oxidation. *J Biol Chem* **263** (1988), pp. 12204–12212.

32. A.S. Hassan, J.J. Hackley and E.H. Jeffery, Role of glutathione in the regulation of hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme of bile acid biosynthesis. *Steroids* **44** (1984), pp. 373–380.

33. A.S. Hassan, Role of hepatic glutathione and glucocorticoids in the regulation of hepatic cholesterol 7α-hydroxylase. *Biochem Pharmacol* **35** (1986), pp. 4592–4594.

34. D.S. Goodman, D. Deykin and T. Shiratori, The formation of cholesterol esters with rat liver enzymes. *J Biol Chem* **239** (1964), pp. 1335–1345.

35. I.R. Hardie, G.A. Balderson, L. Hamlyn, D. McKay and G.J.A. Clunie, Extended ice storage of canine kidneys using hyperosmolar Collins solution. *Transplantation* **23** (1977), pp. 282–283.

36. J.L. Smith, S.P. Pillay, J. de Jersey and I.R. Hardie, Effect of ischaemia on the activities of human hepatic acyl-CoA:cholesterol acyltransferase and other microsomal enzymes. *Clin Chim Acta* **184** (1989), pp. 259–268.

37. C.T. Kwok, W. Burnett and I.R. Hardie, Regulation of rat liver microsomal cholesterol 7α-hydroxylasee: presence of a cytosolic activator. *J Lipid Res* **22** (1981), pp. 570–579.

38. F.O. Salè, S. Marchesini, P.H. Fishman and B. Berra, A sensitive enzymatic assay for determination of cholesterol in lipid extracts. *Anal Biochem* **142** (1984), pp. 347–350.

39. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, Protein measurement with the Folin phenol reagant. *J Biol Chem* **193** (1951), pp. 265–275.

40. G.L. Peterson, Determination of total protein. *Methods Enzymol* **91** (1983), pp. 95–119.

41. J.L. Smith, L.J. Madden and J. de Jersey, Effects of ethanol on the assay of acylCoA:cholesterol acyltansferase (ACAT). *Clin Chim Acta* **215** (1993), pp. 233–236.

42. P.M. Kinnunen, C.A. Spilburg and L.G. Lange, Chemical modification of acyl-CoA:cholesterol *O*-acyltransferase. 2. Identification of a coenzyme A regulatory site by *p*-mercuribenzoate modification. *Biochemistry* **27** (1988), pp. 7351–7356.

43. J.R. Wetterau and D.B. Zilversmit, A triglyceride and cholesteryl ester transfer protein associated with liver microsomes. *J Biol Chem* **259** (1984), pp. 10863–10866.

44. D. Sharp, L. Blinderman, K.A. Combs *et al.*, Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. *Nature* **365** (1993), pp. 65–69.

45. H. Jamil, J.K.J. Dickson, C.H. Chu *et al.*, Microsomal triglyceride transfer protein. Specificity of lipid binding and transfort. *J Biol Chem* **270** (1995), pp. 6549–6554.

46. J.R. Wetterau, K.A. Combs, L.R. McLean, S.N. Spinner and L.P. Aggerbeck, Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. *Biochemistry* **30** (1991), pp. 9728–9735.

47. R.C. Crain and D.B. Zilversmit, Two non-specific phospholipid exchange proteins from beef liver. 1. Purification and characterization. *Biochemistry* **19** (1980), pp. 1433–1439.

48. G.P.H. Van Heusden, T.P. van der Krift, K.Y. Hostetler and K.W.A. Wirtz, Effect of non-specific phospholipid transfer protein on cholesterol esterification in microsomes from Morris hepatomas. *Cancer Res* **43** (1983), pp. 4207–4210.

49. K.L. Gavey, B.J. Noland and T.J. Scallen, The participation of sterol carrier protein$_2$ in the conversion of cholesterol to cholesterol ester by rat liver microsomes. *J Biol Chem* **256** (1981), pp. 2993–2999.