special report

Report on the American Society of Biological Chemists Satellite Conference on Regulation of Intracellular Cholesterol Esterification[1]

Sandra K. Erickson

Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

A one day satellite conference to the annual meeting of the American Society of Biological Chemists on "Regulation of Intracellular Cholesterol Esterification" was held at the University of California School of Medicine in San Francisco on June 5, 1983.

Mammalian cells have an absolute requirement for cholesterol for growth and development. The maintenance of cholesterol homeostasis appears to be vital for proper cellular function. The rates of cellular entrance and exit and synthesis of cholesterol determine cholesterol content.

There are three major cellular compartments for cholesterol: first, a free cholesterol pool, which as a membrane component is "structural"; second, a metabolically active pool of free cholesterol; and third, the cholesteryl ester pool which appears to function primarily as a storage reservoir. The details of regulation of cholesterol entry and exit into these pools and how they relate to various aspects of cholesterol metabolism are areas of active investigation.

In times of high cholesterol uptake or synthesis, an increasing portion of the sterol is converted to cholesteryl esters. Free cholesterol can be esterified with fatty acyl CoA esters via the enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT) and stored in the cell. If the cell at some later time has an increased requirement for free cholesterol, the esters can be hydrolyzed by a neutral cholesteryl ester hydrolase. Maintenance of a free cholesterol:phospholipid ratio within certain limits in membrane has been shown to be necessary for proper membrane function and can play an important role in regulation of membrane-associated enzyme activities. Thus the balance between free and ester cholesterol in the cell may be important for maintenance or alteration of a number of membrane-associated phenomena.

In the whole animal, intracellular cholesterol esterification and metabolism appear to play key roles in steroid hormone, lipoprotein, and biliary lipid metabolism. Most of the dietary cholesterol absorbed by the intestine enters the circulation as cholesteryl esters which are transported in lipoproteins. The liver also secretes cholesteryl esters in lipoproteins. In addition, it secretes biliary lipids. Because only free cholesterol is secreted in the bile and is the precursor for bile acids, regulation of free cholesterol availability may be the key in regulating these processes.

In tissues that actively synthesize steroid hormones, again, because only free cholesterol is a precursor, its availability is important for steroid hormone production.

This conference was designed to bring together individuals from different disciplines to exchange ideas and information on the regulation of cholesterol esterification via ACAT, the cellular metabolism of cholesteryl esters, and the physiological consequences of these processes. In addition, intestinal cholesterol absorption and esterification were discussed.

The first session, chaired by Arthur Spector was on the "Regulation of ACAT at the molecular level." T. Y. Chang discussed the solubilization and reconstitution in phospholipid-cholesterol liposomes of acyl coenzyme A:cholesterol acyltransferase (ACAT), the enzyme responsible for intracellular cholesterol esterification. This method was used to study the regulation of ACAT in Chinese hamster ovary (CHO) cells exposed to low density lipoprotein (LDL). The microsomal enzyme showed 20-fold activation after exposure of the cells to LDL. The increase in activity was independent of protein synthesis. After solubilization and reconstitution of the enzyme, the activities of ACAT derived from control cells, from cells grown in sterol-free medium, and from cells exposed to

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LDL were the same. From these observations, it was suggested that ACAT activity in the intact cell is controlled by the content and composition of cellular lipids associated with the enzyme molecule; especially by the cholesterol content in the vicinity of the enzyme.

Kathleen Gavey discussed the evidence for regulation of ACAT by a phosphorylation/dephosphorylation mechanism. It was observed that microsomal ACAT activity could be inhibited by Mg^{2+} + EDTA and that KF blocked the inhibition. Incubation with a partially purified rat liver phosphoprotein phosphatase also reduced ACAT activity. The activity could be reactivated by incubation with a partially purified protein kinase in the presence of ATP. From these studies, it was suggested that ACAT is inactivated by dephosphorylation and activated by phosphorylation.

The effects on microsomal ACAT activity of modifying the phospholipid fatty acids compositions were discussed by Satya Mathur. Specific phosphatidylcholine (PC) replacements in vitro were accomplished by incubating rat liver microsomes with liposomes of defined fatty acyl composition together with the bovine liver phospholipid exchange protein. This resulted in considerable modification of the fatty acid composition of the microsomes with no change in total phospholipid or cholesterol content. Microsomes enriched with dipalmitoyl PC had 30–45% less ACAT activity than those enriched with dioleoyl PC. Dilinoleoyl or diarachidonyl PC enrichment almost abolished ACAT activity. Lipid peroxidation was shown not to be a factor in this inhibition nor were other microsomal enzymes inactivated. Inclusion of cholesterol in the dilinoleoyl PC liposomes prevented ACAT inactivation. It was concluded that ACAT activity can be modulated either by changes in the lipid environment of the enzyme or by direct interaction of specific PC fatty acyl groups with the enzyme. Furthermore, it appears that the availability of cholesterol has a protective effect on ACAT and can influence such interactions.

Jeffrey Billheimer discussed the effects of sterol structure on expression of ACAT activity. A 3-β-hydroxy group was required for esterification. Additional alkyl groups at C24 or a change in side chain length reduced esterification. Changing the configuration or substituents at C20 showed that only E-17(20)-dehydrocholesterol was esterified. Sterols with additional alkyl groups at C24 or C4 inhibit esterification of exogenous but not endogenous cholesterol. A two-carbon side chain or C20 structural alteration inhibited esterification of both exogenous and endogenous cholesterol. The 25-hydroxy derivative of cholesterol was shown to be converted to the mono-oleate, apparently by an enzyme different from ACAT. In the absence of exogenous cholesterol, the 25-hydroxy derivative increased ACAT activity; this effect was abolished by addition of exogenous cholesterol. It was suggested that the effect of 25-hydroxycholesterol was to apparently increase the amount of microsomal cholesterol available to ACAT for esterification.

In the session "Regulation of cholesterol esterification and hydrolysis in the intact cell" chaired by George Rothblat, Jane Glick discussed factors influencing deposition and clearance of cellular cholesteryl esters in a rat hepatoma cell line, FU5AH. When supplied with free cholesterol-enriched phosphatidylcholine dispersions as a source of exogenous cholesterol, these cells store cholesteryl esters in cytoplasmic inclusions which are anisotropic. However, when oleic acid was also included in the incubations, the cholesteryl esters were stored in inclusions which were isotropic. The mass of ester stored was essentially the same in both cases. The cells with isotropic inclusions did, however, contain significantly more triglyceride.

The kinetics of clearance of cholesteryl esters in these cells were examined during exposure to a free cholesterol acceptor. Both types of cells displayed qualitatively similar kinetics; after a 2-hr lag period, cholesteryl ester clearance was linear. The cholesterol mass cleared per 12 hr was proportional to the initial cellular cholesteryl ester content. However, the fraction cleared was a constant; 34% was cleared from cells with isotropic inclusions and 17% from cells with anisotropic inclusions. This suggests that the clearance rate was related to the physical state of the inclusions.

ACAT activity in cells exposed to cholesterol acceptors was 80% of the control activity after 2 hr and dropped to 10% of control by 10–12 hr. If linolenic acid is added to the medium containing no cholesterol acceptors, the cells with anisotropic inclusions contained 17.5% cholesteryl linolenate, while cells with isotropic inclusions contained 29%. This suggests that the turnover of the esters was also affected by the physical state of the lipids, probably due to differences in ester hydrolysis rate. Inhibition of ACAT with compound 58-035 from Sandoz resulted in a linear decrease in cholesteryl ester content. There was a concomitant increase in cellular free cholesterol showing that cholesteryl ester hydrolase activity is not down-regulated by increased free cholesterol content.

Jerome Strauss discussed cholesterol and cholesteryl ester metabolism in the corpus luteum. Steroidogenesis and cholesteryl ester storage vary with the functional state of the ovary and can be manipulated by chorionic gonadotropic hormone (hCG) and pharmacologic treatments. The role of ACAT in these processes was studied. Treatment of rats with 4-aminopyrazolopyrimidine, which lowers blood cholesterol levels and reduces ovarian sterol ester stores, resulted in lower ACAT activity as measured using endogenous sterol as substrate, but the same activity as control when measured in the presence of exogenous cholesterol. Inhibitors of steroidogenesis (aminogluteth-

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imide or cycloheximide) promoted sterol ester storage and increased ACAT activity as measured with endogenous sterol. However, the activities measured with exogenous cholesterol again were not increased. Treatment with hCG resulted in marked depletion of corpus luteum sterol stores in 2 hr. ACAT activity was also decreased when measured with endogenous cholesterol, but again it was unchanged when compared to control values as measured with exogenous cholesterol. There were no effects of any of the above treatments on neutral cholesteryl ester hydrolase activity. These findings suggested that ACAT activity in the corpus luteum is in excess of that required to fulfill the needs of the cell. It was proposed that the main factor determining sterol esterification and storage is the rate of entry of cholesterol into the ACAT substrate pool. This is determined by the balance between the rate at which lipoprotein-derived cholesterol becomes available to the cells and the rate of conversion of cholesterol to steroid hormones.

Cholesterol metabolism and esterification in human renal cell carcinoma was discussed by Roger Gebhard. Renal clear cell carcinoma cells contain 9–10-fold more cholesterol than normal kidney cells. Seventy percent of this cholesterol was esterified in the carcinoma compared to 21% in normal kidney. Most of the cholesteryl ester fatty acid was oleate. The increase in cholesteryl ester content was shown not to be due to increased cholesterol uptake. Cholesterol synthesis as assessed by HMG CoA reductase activity actually was reduced in the tumor. ACAT activity was increased in the tumor. This increase may be due not only to a change in cholesterol substrate availability but also to an increase in enzyme activity per se. It was concluded that the increased cholesteryl ester content of renal carcinoma cells may be due to either a primary defect resulting in increased ACAT activity, or it may be secondary to a defect in efflux of cholesterol from these tumor cells.

George Vahouny discussed the role of sterol carrier protein$_2$ (SCP_2) in adrenal cholesterol metabolism. SCP_2 stoichiometrically sequesters free cholesterol from adrenal lipid inclusion droplets and transfers it to mitochondria for subsequent synthesis of pregnenolone. When this synthesis was prevented by aminoglutethimide, cholesterol accumulated largely in the mitochondrial inner membrane. This study, together with studies of cytochrome P-450$_{scc}$ reconstituted into liposomes, suggests that SCP_2 does not affect direct interaction of cholesterol with cytochrome P-450.

Adrenal mitochondria from cycloheximide-treated rats showed a marked increase in cholesterol which was not available for pregnenolone synthesis. Addition of SCP_2 enhanced its availability. This enhancement also was observed with cytosol; this was abolished by immunotitration with anti-SCP_2 IgG.

It was suggested that SCP_2 transfers cholesterol from lipid storage droplets to mitochondria and enhances the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane where cytochrome P-450$_{scc}$ is located. SCP_2 does not, however, directly affect interaction of the sterol with the hemoprotein nor does it have a direct effect on P-450$_{scc}$ activity.

Catharine Ross discussed the effects of an experimental drug 58-035 from Sandoz on synthesis of cholesteryl oleate, retinyl esters, and on oleate incorporation into phospholipids and triglycerides. This work used rat hepatoma cell line Fu 5AH. There was no effect on retinol esterification or on phospholipid synthesis. While cholesterol esterification decreased 90%, di- and triglyceride synthesis increased transiently. Similar results were obtained with isolated microsomes. On the basis of these data it was concluded that 58-035 acts selectively on ACAT and that this enzyme is distinct from acyl coenzyme A: retinol acyltransferase (ARAT) and other cellular acyltransferases.

The third session, chaired by John Dietschy dealt with "Intestinal cholesterol absorption and cholesterol esterification." Linda Gallo discussed the roles of cholesteryl ester hydrolase (CEase) and ACAT in cholesterol absorption by rat intestine. The relative role of each enzyme was assessed by studying a series of in vivo models prepared with cannulas in the mesenteric lymphatic ducts and in the common bile ducts. The rats then received a variety of infusions containing fluid, salts, taurocholate, and pancreatic juice which was either *a*) untreated, *b*) anti-CEase–IgG-treated, or *c*) nonimmune IgG-treated. A fourth group (*d*) was given no pancreatic juice. In all groups, 77–89% of the absorbed [^{14}C]cholesterol was esterified. In groups *b* and *d* in which CEase activity should have been low, CEase activity and the mass of cholesterol absorbed were both reduced 81–73%. ACAT levels were the same in all groups. In addition to this evidence for a physiological role for CEase in cholesterol absorption, further evidence indicated that *1*) CEase of pancreatic origin is protected from proteolysis in the intestine by bile salts; *2*) CEase is taken up into the absorptive cells as demonstrated by immunocytochemical studies; *3*) preliminary studies suggest CEase uptake is mediated by receptor specific endocytosis; and *4*) the concentration of bile salts in the intestinal mucosa is greater than that required to support CEase activity. On the basis of these findings, it was concluded that CEase plays a key role in cholesterol absorption.

John Heider discussed the role of ACAT in cholesterol absorption studied using the Sandoz compound 57-118 (N-(-1-oxo-9-octa-decenyl)-ol-tryptophan ethyl ester). This compound prevents the accumulation of cholesteryl esters in cells in tissue culture by inhibiting ACAT. Administration of this compound to the cholesterol-fed rabbit

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prevented the development of atherosclerosis and lowered blood cholesterol levels. Using the Zilversmit dual isotope method, it was shown that cholesterol absorption was inhibited. There was no effect on CEase but ACAT activity was inhibited. Kinetic studies in vitro suggested that this compound was acting as a competitive inhibitor of ACAT. After administration in vivo the compound was present in intestinal microsomes at levels sufficient to inhibit ACAT activity in vitro. In the animals fed a normal diet, compound 57-118 had no effect on cholesterol absorption. From these results it was suggested that ACAT is involved in cholesterol absorption in cholesterol-fed animals, but that CEase possibly may play a predominant role in animals on a normal diet.

Further evidence for a role for ACAT in cholesterol absorption was presented by Jeffrey Field. The effects of 25-hydroxycholesterol on rabbit intestinal ACAT were studied in isolated microsomes and in isolated intestinal cells in culture. As in other cell types, ACAT activity was increased by 25-hydroxycholesterol. After cholesterol enrichment of the microsomes by incubation with cholesterol-dipalmitoylphosphatidylcholine liposomes, there was an increase in ACAT activity which correlated with the degree of cholesterol enrichment. The effect of 25-hydroxycholesterol on ACAT activity decreased with cholesterol enrichment of the microsomes. No effect on activation was observed at the highest levels of activity and cholesterol enrichment. In cells isolated from cholesterol-fed rabbits, 25-hydroxycholesterol was not as effective in stimulating oleate incorporation into cholesterol oleate as it was in cells isolated from untreated animals. The esterification rate of cholesterol newly synthesized from mevalonate also was increased by 25-hydroxycholesterol. However, again as the amounts of cholesterol increased, the stimulatory effect of 25-hydroxycholesterol decreased. It was suggested that the regulation of ACAT activity by 25-hydroxycholesterol is dependent on the availability of cholesterol to the enzyme, and that either newly synthesized cholesterol or cholesterol of dietary or other origin can serve as substrates for this reaction.

Eduard Stange reported on the independent regulation of ACAT, sterol synthesis, and LDL uptake in rat intestine. Basal ACAT activity could be increased by preincubation of cell homogenates or isolated microsomes with cholesterol-rich liposomes and, in an additive fashion, by an apparent ATP-dependent in vitro phosphorylation. ACAT activity was highest in villus cells with 90% of the total activity recovered in the absorptive cells. Jejunal but not ileal ACAT activity increased after cholesterol feeding. Feeding cholestyramine, surfomer, or corn oil had no effect. The increase in ACAT activity induced by the cholesterol diet was not observed in cell homogenates which were pre-incubated with cholesterol-rich liposomes suggesting that much of the effect was due to an increase in substrate supply.

In contrast to cholesterol esterification, sterol synthesis and LDL uptake were preferentially localized in the lower villus-crypt region. Sterol synthesis was enhanced by feeding cholestyramine and surfomer to inhibit luminal sterol uptake or by feeding corn oil to increase chylomicron production. LDL uptake was not affected by these dietary manipulations and was quantitatively of minor importance as a source of cholesterol aquisition by gut epithelial cells.

It was concluded that ACAT-dependent esterification, cholesterol synthesis, and LDL uptake are regulated independently in the gut and that the major substrate for ACAT is luminal sterol rather than newly synthesized or LDL cholesterol.

The role of cholesterol esterases as possible mediators of cholesterol movement across the intestine was discussed by Howard Brockman. The everted gut sac model was used to study the effects on cholesterol absorption of the removal of endogenous cholesterol esterase, of inhibition of the esterase, or of addition of exogenous esterase. Washing gut sacs in bile salt solutions resulted in loss of enzyme activity from the tissue accompanied by decreased cholesterol uptake. This suggested involvement of a readily soluble surface factor. The inhibitors of CEase, diisopropylfluorophosphate and p-bromophenylboronic acid, decreased cholesterol accumulation. Addition of purified pig CEase to the medium stimulated cholesterol uptake. The stimulation was dependent on enzyme concentration, was saturable, and occurred without a measurable time lag. The results were interpreted as suggesting that pancreatic CEase stimulates cholesterol uptake as a result of its catalytic activity at the luminal surface of the villus membrane. It was hypothesized that pancreatic CEase catalyzes the formation of cholesteryl esters on the villus membrane. This apolar lipid then traverses the membrane where it is acted upon by a cytoplasmic CEase which catalyzes ester hydrolysis. Because the reaction equilibrium favors hydrolysis, the net effect is to enhance free cholesterol movement across the membrane. In the presence of a suitable sink for cholesterol removal; i.e., chylomicron formation in the gut, CEase would thus enhance the net rate of cholesterol absorption.

The last session, chaired by Christopher Fielding dealt with "Physiological consequences of cholesterol esterification and hydrolysis."

Pleiotropic effects of ACAT on hepatic lipid metabolism were discussed by Roger Davis. It was hypothesized that ACAT, by determining the availability of intracellular free cholesterol pool(s), affects several important hepatic processes, for example, membrane function, biliary cholesterol content, bile acid synthesis and secretion, VLDL

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assembly and secretion. Using cultured hepatocytes, cholesterol availability was altered by changing its rate of synthesis or by changing its cellular content. This was accomplished by feeding cholesterol to animals before hepatocyte preparation or by adding cholesterol-rich lipoproteins to the hepatocyte culture medium. In all cases ACAT activity and hepatocyte cholesterol availability were altered in parallel. Bile acid synthesis also varied in parallel. This suggested that both cholesteryl ester synthesis and bile acid synthesis were regulated by cholesterol availability. A pleiotropic effect of increased ACAT activity was replacement of triacylglycerol in the VLDL core by cholesteryl esters with accumulation of triacylglycerol within the hepatocyte. This suggested that these two hydrophobic components compete with one another for secretion as core components of VLDL.

Stimulation of hepatic ACAT by ethinyl estradiol treatment causes accumulation of cholesteryl esters and expression of high affinity LDL receptors. Under normal circumstances there is an inverse relationship between these processes. From these observations it appears that pharmacologic stimulation of ACAT activity results in a decrease in availability of cholesterol for regulation of LDL receptors. It was concluded that ACAT is regulated by cholesterol availability, but that the enzyme itself can regulate availability of free cholesterol within the cell.

The role of ACAT in regulating biliary cholesterol secretion was discussed by Bradford Stone. The amount of free cholesterol in the liver cell is determined by a variety of metabolic processes. One of these is entrance into the cell via lipoprotein uptake. De novo synthesis also increases cellular cholesterol content. Cholesterol is lost from the liver via lipoprotein secretion, bile salt synthesis, and biliary cholesterol output. Cholesterol can also be esterified and stored for future use. It is known from previous work with the rat that the rate of cholesterol secretion into bile is not determined per se by lipoprotein uptake, the rate of cholesterol synthesis, or total hepatic cholesterol levels. Thus, it was hypothesized that there is a metabolically active pool of free cholesterol within the hepatocyte which is available for biliary secretion. Changes in input to and output from this pool are balanced such that the size of the precursor pool and biliary output remain relatively constant. It was further postulated that ACAT plays a key role in this process. 25-Hydroxycholesterol, which inhibits cholesterol synthesis and enhances cholesterol esterification specifically, decreased biliary cholesterol output in parallel. Mevalonate, which increases both cholesterol synthesis and esterification, had no effect on biliary cholesterol output. Mevinolin, which decreases synthesis and has no effect on esterification, produced a modest (10%) decrease in biliary cholesterol output. It was concluded that there is indeed a precursor pool of cholesterol destined for biliary secretion and that the size of this pool is regulated in part by changes in synthesis and esterification rates. Compounds altering this pool size may be useful for regulating biliary cholesterol output.

Robert Mahley discussed the role of HDL in cholesterol transport in and out of cells. It was postulated that HDL that does not contain apoE acquires cholesterol and apoprotein E from peripheral tissues and in this process is converted to larger particles. Cholesterol was delivered to plasma from cholesterol-celite particles or from cholesterol-loaded mouse peritoneal macrophages. The HDL was then isolated and analyzed. There were substantial increases in HDL subclasses enriched in apoE and cholesterol. The macrophages synthesized and secreted apoE which was associated 65–90% with lipoproteins in the d = 1.02–1.09 g/ml range. The predominant lipoproteins were HDL and HDL_c. After incubation of ^{125}I-HDL with macrophages, there was a marked increased of label in the HDL and HDL_c classes. There was a corresponding marked elevation in ^{125}I-HDL_3. Incubation with human serum or HDL_3 gave similar results.

Three distinct subclasses of apoE-rich HDL were formed, differing in size and number of layers of cholesteryl esters. It was suggested that HDL_3 is converted to cholesterol-enriched larger HDL containing apoE and progressively more cholesteryl esters. These esters may then be transferred to other lipoproteins or delivered to the liver for excretion from the body.

Miriam Kaplan discussed intracellular transport of cholesterol from the endoplasmic reticulum to the plasma membrane. Fibroblasts in culture were grown in lipid-deficient media and pulsed with [^{3}H]acetate, followed by an unlabeled substrate chase. Transport was stopped by cooling and the cells were homogenized and membranes were isolated. Cholesterol which is synthesized in the endoplasmic reticulum moves to the plasma membrane with a half-life of 15 min. The process is dependent on metabolic energy. Colchicine, cytochalsin B, and monensin have no effect on transport. The results argue against passive diffusion as a mechanism and suggest an active process is involved for intracellular transport of cholesterol.

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