special report

International Congress of Biochemistry and Molecular Biology Satellite Conference: Cellular Cholesterol Ester Metabolism[1]

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A 1-day satellite conference to the International Congress of Biochemistry and Molecular Biology entitled "Cellular Cholesterol Ester Metabolism" was held at the University of California, San Francisco on August 23, 1997. It was organized to bring together individuals from different disciplines to exchange ideas and information in this important area. Cellular cholesteryl ester metabolism plays a key role in maintenance of cholesterol homeostasis which is vital for normal cell growth and function.

The Conference began with an overview by **John Dietschy** (UT, Southwestern) on the physiological importance of the cholesterol esterification/hydrolysis system. Although acyl coenzyme A:cholesterol acyltransferase (ACAT) often is said to protect cells from a sudden influx of large amounts of cholesterol, this is an uncommon event physiologically. More commonly, ACAT (along with cholesteryl ester hydrolases, CEH) is responsible for maintaining cellular steady-state levels of cholesteryl esters. In vivo, as in vitro, the level of esterification appears to be driven in part by availability of cholesterol and fatty acids. Feeding cholesterol to a new steady state expands both the unesterified and esterified levels of cholesterol, while feeding different fatty acids significantly shifts the ratio between these two components. In humans, as well as in experimental animals, the levels of both dietary cholesterol and fatty acids profoundly affect the steady-state levels of cholesteryl esters in the liver, the level of hepatic low density lipoprotein (LDL) receptor activity and the outflow of cholesterol in VLDL particles. Further, one of the causes of variable responses of the LDL level to cholesterol challenge is mediated by differences in the amount of cholesterol absorbed across the gastrointestinal tract which in turn appears to be dependent on intestinal ACAT levels. Clearly, it is essential that future work include identification of the genes involved and that appropriate studies be undertaken to identify how these enzymes are regulated and whether they are responsible for genetic differences in responses of the plasma LDL level to dietary challenge.

The first session, "Physiology/Pathophysiology of Cholesterol Esters," chaired by **Richard Havel** (UCSF) and **Jane Glick** (University of Pennsylvania), opened with a discussion by **Larry Rudel** (Bowman Gray) of relationships between hepatic production and secretion of cholesteryl esters and atherosclerosis in African green monkeys. Although plasma apoB and LDL cholesterol concentrations were lower in monkeys fed monounsaturated and polyunsaturated fat than in those fed saturated fat, LDL particles in the monounsaturated fat group were remarkably enriched in cholesteryl oleate and the livers of this group had significantly higher cholesteryl ester concentrations. Liver cholesteryl ester content, plasma cholesterol and apoB levels, liver perfusate cholesteryl ester accumulation rate, hepatic ACAT activity, and coronary artery cholesteryl ester concentration were all highly correlated in each group. These data provide the first direct demonstration of the importance of hepatic cholesteryl ester synthesis and secretion in the development of atherosclerosis in primates.

Next, **Christopher Fielding** (UCSF) presented his and **Phoebe Fielding**'s work on regulation of cell cholesterol content by caveolin. When human skin fibroblast monolayers were loaded with LDL cholesterol in the absence of HDL, about 25% of their free cholesterol was recovered in caveolae (compared to 3–4% in unloaded cells). Balance studies showed that the free cholesterol removed from cells to extracellular HDL was that which had been in caveolae. The transport of free cholesterol to the cell surface paralleled that of caveolin, the major structural protein of caveolae. Oxysterols inhibited both cholesterol efflux and caveolin synthesis. On the basis of these data, caveolin was proposed to act as a "cholesterostat," responding to increased cellular cholesterol by promoting caveolin synthesis, caveolar formation, and the efflux of

[1]Reprints of this report are not available.

cholesterol from caveolae. In further support of this hypothesis, the caveolin gene promoter was found to contain multiple SRE-like G/C-rich boxes, two of which are required for regulation of caveolin expression by sterols. One of these sites is an SREBP-1 binding site.

The session closed with **Jill Morris** (NIH) describing her group's recent work on cloning of the first Niemann-Pick C (NP-C) related gene. The primary cellular lesion of NP-C was elucidated from studies of a spontaneous mutant mouse colony in which a high cholesterol diet induced massive hepatosplenomegaly and abnormal tissue accumulation of free cholesterol. In vitro studies with cultured mutant murine fibroblasts revealed a block in intracellular metabolism of LDL-derived cholesterol. Similarities in the pathological and clinical presentation of the murine disorder and the progression of human NP-C disease led her group to confirm a similar lesion in all forms of NP-C, viz, an anomalous accumulation of free cholesterol in lysosomes. Her group then initiated and directed an international effort to identify the *NPC* gene by positional and functional cloning strategies. The mutant gene was identified in the chromosomal region 18Q11. It encoded a protein with a calculated molecular mass of 143 kDa which appears to contain multiple membrane spanning regions. It has sequence homology to the morphogen receptor, PATCHED, and to the putative sterol sensing regions of proteins involved in cellular cholesterol homeostasis. The mutations documented in 30 NP-C families represented a wide array spanning the length of the protein.

The second session, "Neutral Cholesterol Ester Hydrolases," was co-chaired by **Fredric Kraemer** (Stanford) and **Cecelia Holm** (University of Lund, in absentia). **Henry Choy** (UCLA) and colleagues reported on the enhancement of cholesteryl ester hydrolysis by overexpression of hormone-sensitive lipase (HSL) in macrophage cell lines. They showed, by antibody titration, that HSL accounts for essentially all neutral cholesteryl ester hydrolase (nCEH) activity in extracts of both RAW cells, a murine macrophage cell line, and freshly isolated peritoneal macrophages. Stable overexpression of HSL in transfected RAW cells effectively depleted accumulated stores of esterified cholesterol. These cells degraded cholesteryl esters two to three times faster than control cells, leaving little ester after 24 h in the presence of an ACAT inhibitor. cAMP addition to cultures, known to stimulate HSL activity, enhanced hydrolysis 6-fold resulting in a dramatic depletion of cholesteryl ester in 9 h. Thus, cholesteryl ester hydrolysis in macrophage foam cells was manipulated by HSL overexpression to deplete accumulated stores of intracellular cholesterol, in effect, reversing the result of cholesterol loading.

Next, **Takako Tomita** (University of Shizuoka) discussed her group's work on mechanisms of cholesterol-mediated changes in nCEH activity in macrophages and adipocytes. They previously showed that incubation of J774 A1. macrophages with LDL inhibited nCEH activity while incubation with HDL enhanced it, and that the change was mediated by cellular cholesterol. To study this further, J774 A1. cells, mouse peritoneal macrophages, and adipocytes differentiated from 3T3-L1 cells were cultured with or without cholesterol or 25-hydroxycholesterol (25OH). Cholesterol inhibited nCEH activity in J774 A1. cells and peritoneal macrophages time- and concentration-dependently, but only 25OH did in adipocytes. Although expression of nCEH mRNA was unaltered, its mass decreased markedly both in J774 A1. and 3T3-L1 cells. Pulse–chase experiments indicated that the turnover rate of the enzyme was accelerated in the presence of sterol, suggesting post-transcriptional regulation.

Ramesh Natarajan (Medical College of Virginia) presented a molecular analysis of the rat hepatic cytosolic nCEH promoter. There were no typical TATA-box sequences 1.3 kb upstream of the ATG initiation site, but there was a consensus GC-box. Functional glucocorticoid response elements, phorbol ester and sterol responsive sequences were mapped by transient transfection assays in HepG2 cells of deletion constructs linked to the luciferase reporter gene.

Susana Cristobal (University of the Basque Country) described the first purification and characterization of membrane-associated nCEH. On PAGE analysis, the purified protein was negative to esterase staining and exhibited only one silver-staining band with CEH activity. On the basis of differential purification, lack of affinity for heparin and concanavalin A, kinetic properties, sensitivity to metal ions and to serine and sulfhydryl specific reagents, this nCEH was clearly distinguished from the cytosolic CEH, lysosomal CEH, hepatic lipase, triacylglycerol lipase, nonspecific microsomal carboxylesterases, and bile salt-dependent esterase.

Zafural Beg (Aligarh Medical University) presented his group's data on regulation of rat liver nCEHs. In vitro studies demonstrated that nCEHs were activated by a cAMP-dependent kinase and inactivated by a novel ATP-Mg-dependent phosphatase. In vivo, a 36-h fast or cAMP injection led to increased activities of both cytosolic and microsomal nCEHs, suggesting that modulation by phosphorylation/dephosphorylation is an important regulatory mechanism.

Next, **Shobha Ghosh** (Medical College of Virginia) reported her studies on identification of a nCEH in J774 cells that was similar to hepatic cytosolic nCEH as determined by Western blot, immunocytochemistry, and Northern blot. CEH activity decreased 36% after transfection with a specific hepatic nCEH antisense oligonucleotide, suggesting that a CEH similar to the hepatic enzyme is responsible for a portion of CE hydrolysis in J774 cells.

The first afternoon session entitled "Cellular Cholesterol/Cholesterol Ester Transport" was chaired by **Ira Tabas** (Columbia) and **Phoebe Fielding** (UCSF). **Salman Azhar** (VAMC, Palo Alto) described his and **Eve Reaven's** findings on the selective cholesteryl ester (CE) uptake pathway in a steroidogenic cell model, rat ovarian granulosa cells. These are physiologically normal cells that show no selective uptake of HDL-CE and no progestin production until stimulated by trophic hormones or adenylate cyclase stimulators. At every cell stage studied, SR-BI protein content and mRNA level were linked to changes in HDL-

CE uptake; SR-BI was not present in basal cells, but it increased after hormone treatment in parallel with selective uptake and the progestin response. SR-BI appears to be associated with cell surface sites showing high levels of CE uptake, in particular, with microvilli and microvillar channel regions on these surfaces. Thus, development of the SR-BI receptor system and the HDL-CE selective uptake pathway appears to be linked morphologically, biochemically, and functionally.

Fredrick Maxfield (Cornell University Medical College) presented his group's data on intracellular localization of ACAT and free cholesterol. Using CHO fibroblasts, they found that a cholesterol analog, dehydroergosterol (DHE) colocalizes with endocytosed transferrin, an endocytic recycling compartment (ERC) marker and with TGN38, a marker for the trans-Golgi network (TGN) indicating that the ERC and TGN contain major intracellular cholesterol pools. Sterol levels in the ERC may play an important role in regulating membrane traffic through this organelle because reduction of cellular cholesterol by 40% caused GPI-anchored proteins to exit the ERC at the same rate as fluorescent sphingomyelins or transferrin receptors. By immunofluorescence and confocal microscopy, they found that much of the cellular ACAT colocalized with endoplasmic reticulum markers in mouse peritoneal macrophages; however, some ACAT also was found in a perinuclear site slightly separated from the ERC that colocalized with TGN38, suggesting that trafficking of cholesterol-rich vesicles derived from the plasma membrane or lysosomes to the TGN or the ERC is involved in the regulation of cholesterol esterification.

Arthur Tinklenberg (Columbia) discussed his work in **Steve Sturley's** group on NCRI, which they cloned recently and which is a yeast homologue of the NP-C-related gene. Preliminary evidence for the role of this protein in intracellular sterol homeostasis was presented.

Ira Tabas (Columbia) discussed phospholipid metabolism in free cholesterol (FC)-loaded macrophages. FC appears to signal a series of protein dephosphorylation reactions that lead to post-translational activation of the phosphatidyl choline (PC) biosynthetic enzyme, CTP:phosphocholine cytididyltransferase (CT). The hypothesis that induction of PC biosynthesis represents an adaptive response to help prevent FC-mediated cellular toxicity was supported by the observation that a blunted PC biosynthetic response in macrophages due to cellular choline depletion led to accelerated FC-mediated toxicity. This suggested that enhancement of PC biosynthesis in macrophages might be protective. Dr. Tabas' laboratory created macrophage-targeted CT transgenic mice in the apoE knockout (i.e., atherogenic) background to test the hypothesis that the advanced lesions of these mice will have less macrophage necrosis because they will be able to overcome FC-mediated cytotoxicity.

The final session, "Synthesis of Cholesterol Esters," was chaired by **Robert Farese** (Gladstone, UCSF) and **Catherine Chang** (Dartmouth). The first presentation was by **Sylvaine Cases** (Gladstone and CVRI, UCSF) who presented data indicating that additional ACAT genes exist in mice. Studies of mouse *Acact* gene tissue expression suggested that it is not responsible for cholesterol esterification in the liver or small intestine. She described the cloning of two other ACAT gene family members (A-2 and A-3), which are candidates for sterol esterification enzymes. The A-2 gene product is expressed at highest levels in mouse intestine and liver but at very low levels in most other tissues in both humans and mice. The A-3 gene product is expressed in most tissues with the highest levels in small intestine and testes. Mouse cDNAs for these genes were expressed in insect cells which are being assayed for ACAT activity.

Next, **Steve Sturley** (Columbia) presented his group's recent data on sterol esterification multi-gene families in yeast and in humans. They had previously found two genes for sterol esterification in *S. cerevisiae*. **Dr. Peter Oelkers**, in Sturley's laboratory, identified two human ACAT-like genes by sequence conservation, which are similar to the genes described by Dr. Cases. They were expressed in human hepatic cell lines to determine their role(s) in sterol esterification. In addition, site-directed mutagenesis of the first cloned ACAT gene is being used to identify key residues in the C-terminus of the ACAT protein that are necessary for activity.

Akira Miyasaki (Dartmouth) presented results of studies on regulation of ACAT expression in human atherosclerotic lesions and in cultured human monocyte-macrophages. Immunohistochemical studies showed that ACAT is abundantly expressed in atherosclerotic lesions of human aorta, predominantly in monocyte-macrophages but not in smooth muscle cells. When human monocytes were cultured in vitro, ACAT protein content and activity increased 5- to 10-fold within the first 2 days of culture, suggesting that increase in ACAT is an early event in differentiation of monocytes to macrophages and may explain its abundant expression in human atherosclerotic lesions.

Robert Farese (Gladstone, USCF) presented his group's recent work on AKR mice that exhibit adrenocortical lipid depletion that is conditional and manifested predominantly at the time of sexual maturation. These mice have mutations in the *Acact* cDNA that lead to a truncated protein. Nevertheless cholesterol esterification appears to be normal in in vitro assays suggesting that the *Acact* mutation in AKR mice leads to cholesteryl ester depletion only when combined with modifying factors that occur at the time of sexual maturation.

Finally, **Catherine Chang** (Dartmouth) presented data on purification and biochemical characterization of recombinant human ACAT protein, the product of the first gene cloned originally by her group. Using recombinant DNA technology, they isolated a CHO cell line that stably expresses the ACAT protein bearing a hexa-histidine tag at its N-terminus. ACAT was purified by subcellular fractionation, detergent solubilization, and two affinity column chromatography steps. The purified protein responds to cholesterol concentration in a sigmoidal manner supporting the hypothesis that ACAT is an allosteric enzyme.

A series of posters also were presented. **Hideki Hakamata's** group (Kumamoto University School of Medicine) described the molecular cloning and tissue distribution of rat ACAT that showed high mRNA and protein expression in adrenals but low expression in liver. **Jeffrey Smith** (University of Queensland) presented his studies with **Claude Lutton** (Paris) suggesting a role for ACAT in regulating secretion of cholesterol into bile in the LPN hamster cholesterol gallstone model. **Julian Chang** (USCF) presented his group's work on the crystal structure of bovine pancreatic cholesterol esterase, which has an unusual active site environment around the catalytic triad including the presence of a hydrophobic sequence at its C-terminus thereby restricting access of substrate to the active site region, and a truncated amphipathic helical lid suggesting a new mode of lipase activation. **A. Koudinov** (Russian Academy of Medical Science) and colleagues presented their work on the effects of Alzheimer's Aβ peptide on plasma cholesterol esterification and lipid biosynthesis in HepG2 cells. **H. Shige** and colleagues demonstrated that α-tocopherol suppressed cholesteryl ester accumulation in macrophages induced by atherogenic lipoproteins.

The Organizing Committee included Michael Schotz (UCLA), David Williams (SUNY, Stony Brook), John Watson (UCSF), Marguerite Engler (UCSF), and Sandra Erickson (UCSF and VAMCSF), Chair. The Conference was supported by the American Heart Association–California Affiliate, Wyeth-Ayerst Research, The Council for Tobacco Research USA, Inc., Pfizer Inc., and Merck Research Laboratories. Special thanks are due to Maggie Chow and Diane Heininger for secretarial support, Steven Lear for projectionist duties, and the Ludvik family for their able assistance in helping to organize the Conference. We thank Nicole Herranz for typing the manuscript.