special report

1994 Aspen Bile Acid/Cholesterol/Lipoprotein Conference: Fatty acid metabolism in liver and adipose tissue

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The 1994 Aspen Bile Acid/Cholesterol/Lipoprotein Conference "Fatty Acid Metabolism in Liver and Adipose Tissue" was held at the Given Biomedical Institute of the University of Colorado School of Medicine in Aspen, Colorado on August 20–23, 1994. The program was designed to provide a format for critical interdisciplinary review and discussion of recent advances in fatty acid metabolism in liver and adipose tissue, including its relationship to gene regulation, lipid transport, and lipoprotein formation. Four major topic themes were addressed in the oral sessions, each of which consisted of four presentations followed by informal open discussion. The following are brief summaries of those oral presentations.

Regulation of Fatty Acid Metabolism

The first oral session (chaired by R. Ockner) addressed newer concepts in the interaction between hepatocellular and adipocyte fatty acid metabolism. In his presentation entitled **"Probing the Mysteries of Carnitine Palmitoyltransferase", Denis McGarry** reviewed the transport of long chain fatty acids into mitochondria, and its dependence on the sequential actions of carnitine palmitoyltransferase I and II (CPT I and CPT II), together with carnitine-acylcarnitine translocase. CPT I is now recognized as a pivotal control point in fatty acid oxidation by virtue of its unique inhibitability by malonyl-CoA. This applies to liver as well as non-hepatic tissues such as heart and skeletal muscle, and appears to be a key event in glucose-stimulated insulin secretion from the pancreatic β-cell. As a result, the CPT I locus is seen as a potential target for pharmacological intervention in conditions of excessive fatty acid oxidation, such as uncontrolled diabetes mellitus. Inherited mutations at the level of CPT I or CPT II, some with serious consequences, are now being documented with increasing frequency. For all of these reasons, intensive efforts are currently underway to dissect the CPT enzymes in terms of their structure, function, and regulatory relationships.

CPT I catalyzes the conversion of acyl-CoA to acylcarnitine, and is located on the inner aspect of the mitochondrial outer membrane, while CPT II catalyzes the reverse reaction, and resides on the inner side of the inner membrane. CPT I, but not CPT II, is potently inhibited by malonyl-CoA (reversibly) and by compounds of the etomoxir-CoA class (irreversibly). However, there has been uncertainty about whether the two acyltransferase reactions are catalyzed by different proteins, or by the same enzyme located in separate mitochondrial compartments, and whether agents such as malonyl-CoA and etomoxir-CoA interact with the catalytic domain of CPT I or with an associated regulatory subunit.

Recent enzymological and molecular cloning studies have clarified these two critical issues, for both rat and human as follows: *i)* CPT II is loosely attached to the inner aspect of the mitochondrial inner membrane, is detergent stable, malonyl-CoA-insensitive and is expressed as the same protein in all tissues; *ii)* CPT I is distinct from CPT II, is tightly associated with the mitochondrial outer membrane, and its inhibition by malonyl-CoA and compounds of the etomoxir-CoA class reflects interaction with the catalytic entity itself; *iii)* CPT I loses activity when released from its membrane environment by detergents; *iv)* unlike CPT II, CPT I exists as at least two antigenically and kinetically distinct tissue-specific variants, i.e., liver (88 kDa) and skeletal muscle (82 kDa). The kinetic characteristics of rat heart CPT I are intermediate between those of the liver and skeletal muscle enzymes, reflecting cardiac myocyte expression of both of these variants.

Important issues that remain to be addressed include: elucidation of the protein and gene structures of the liver and skeletal muscle CPT I variants; identification of other CPT I isoforms that may exist, and which isoform(s) is (are) expressed in fibroblasts and the pancreatic β-cells; the molecular basis for inherited CPT I and CPT II deficiency syndromes; the functional significance, if any, of the presence of two CPT isoforms in heart and how this may relate to the control of cardiac fatty acid oxidation; and details of the mechanism by which malonyl-CoA interacts with CPT I.

The second speaker, **Carl Grunfeld,** spoke on the topic **"Regulation of Lipoprotein Metabolism by Cytokines: Nutrition or Host Defense?",** and discussed disturbances in lipid metabolism and hypertriglyceridemia that occur during infection or inflammation. Recent studies demonstrate that multiple cytokines, especially TNFα, as well as lymphotoxin, interferons α/β/γ, leukemia inhibitory factor (LIF), interleukin-1 (IL-1) and IL-6, induce catabolic effects in fat cells that are not reversed by insulin. This differs from cachectic states in which hyperalimentation with hyperinsulinemia effectively stores fat without preventing muscle wasting.

In vivo, TNFα and other cytokines induce hypertriglyceridemia within 45 minutes, in contrast to the several hours required for catabolic effects to develop in cultured fat cells. TNF does not delay triglyceride clearance. Instead, TNFα, IL-1 and IL-6 increased hepatic lipogenesis, by increasing the cellular concentration of citrate, an allosteric activator of acetyl CoA carboxylase, and VLDL production rates in rats. IL-4, an anti-inflammatory cytokine that opposes many actions of TNFα, IL-1 and IL-6, was shown to block their enhancement of hepatic fatty acid synthesis by preventing the necessary increase in citrate, whereas IL-4 did not impair the IFNα effect. Thus, TNFα, IL-1 or IL-6 are synergistic with interferon α, which acts by an apparently different and undefined mechanism.

Cholesterol metabolism is also influenced by LPS and cytokines. In general, HMG-CoA reductase activity is increased, LCAT is decreased, and there is increased association of acute phase proteins with lipoproteins, e.g., C reactive protein with LDL, serum amyloid A with HDL, and apoJ with HDL. As a result of these changes, LDL and HDL binding to macrophages is increased.

Although it had been postulated that changes in lipid metabolism caused cachexia, recent studies showed, both in animals treated with cytokines and in patients with AIDS, that hypertriglyceridemia was not necessarily linked to wasting. Moreover, in earlier studies of the effects of endotoxin (lipopolysaccharide, LPS) in which shock-inducing doses were used and metabolic effects examined many hours later, LPS-induced hypertriglyceridemia was associated with decreases in LPL activity and triglyceride clearance. When very low doses of LPS (1/500,000th the lethal dose) were used, however, its effects on lipid metabolism were extremely rapid (within 2 hours). These reflected early increases in hepatic fatty acid synthesis and VLDL production, and not the later decreases in triglyceride clearance. Thus, changes in lipid metabolism are among the most rapid and sensitive host responses to infection.

Because these changes in lipid metabolism were not clearly harmful and appeared to be part of the acute phase response, it was postulated that they might play a role in host defense. Subsequent experiments have shown that VLDL, LDL, and HDL associate with LPS in plasma, thus directing it away from macrophages and to the hepatocyte, with subsequent secretion into bile. As a consequence, macrophage activation, secretion of deleterious cytokines, and toxicity are all decreased. These effects complement observations by others concerning the antiviral and anti-inflammatory properties of lipoproteins.

The third speaker, **Peter Tontonoz,** addressed **"Gene Regulation Relating to Fatty Acid Metabolism and Adipogenesis",** and discussed advances in the current understanding of adipocyte differentiation, a complex process that involves dramatic changes in cell morphology and gene expression. The transcriptional regulation of the adipocyte P2 (aP2) gene was examined in an effort to identify and characterize the regulatory factors that direct this process. Previously, an enhancer from the 5′-flanking region of the aP2 gene that directs high level adipocyte-specific gene expression in both cultured cells and transgenic mice was isolated and characterized. The key regulator of this enhancer is a cell type-restricted nuclear factor termed ARF6, which has now been isolated from cultured adipocytes by sequence-specific DNA affinity chromatography. Chemical sequencing of tryptic peptides derived from the purified material revealed that the ARF6 complex is composed of RXRα and a novel member of the peroxisome proliferator-activated receptor (PPAR) family designated mPPARγ2; the latter has been cloned from an adipocyte cDNA library.

Expression of mPPARγ2 is induced very early during the differentiation of several cultured adipocyte cell lines and is strikingly adipose-specific in vivo. mPPARγ2 and RXRα bind as a heterodimeric complex to ARF6-binding sites in vitro. Forced expression of mPPARγ2 and RXRα activates the adipocyte-specific aP2 enhancer in cultured fibroblasts, and this activation is potentiated by peroxisome proliferators, fatty acids, and 9-*cis*-retinoic acid.

Retroviral vectors were used to create fibroblast cell lines that overexpress mPPARγ2, mPPARα, or RXRα.

Expression of mPPARγ2, but not mPPARα or RXRα, stimulates adipose differentiation of NIH-3T3 and Balbc-3T3 fibroblasts in a PPAR activator-dependent manner. The ability of the PPAR activators eicosatetraynoic acid (ETYA), clofibric acid, and linoleic acid to stimulate differentiation of mPPARγ2-expressing fibroblasts parallels the ability of these compounds to activate transcription through mPPARγ2 in transient transfection assays. When coexpressed in fibroblasts, mPPARγ2 and C/EBPα cooperate to activate the adipogenic program.

These results demonstrate that mPPARγ2, a novel adipocyte-specific nuclear hormone receptor, can activate the adipogenic program in multiple fibroblast cells lines. The results provide direct evidence that the different PPAR family members have distinct biological roles, and define mechanisms whereby peroxisome proliferators, fatty acids and other lipids may regulate adipocyte gene expression and differentiation.

Allan D. Sniderman, the final discussant, spoke on **"The Adipsin-Acylation Stimulating Protein Pathway: Implications for the Regulation and Dysregulation of Adipocyte Triglyceride Synthesis",** and its role in the storage of fatty acids by human adipocytes. Adipocytes synthesize and secrete the three precursor proteins, i.e., factor B, adipsin (factor D), and the third component of complement (C3), that are necessary to generate acylation simulating protein (ASP). This capability, and their response to ASP, develop during differentiation.

Interaction of factor B, adipsin, and C3 results in the production of C3a, after which the terminal arginine of C3a is removed by carboxypeptidases to produce C3adesarg. Several lines of evidence indicate that ASP is identical to C3adesarg. ASP markedly increases triglyceride synthesis in human skin fibroblasts and human adipocytes by increasing specific membrane transport of glucose through enhanced translocation of glucose transporters to the cell surface, in a manner similar to, but independent of, insulin. Although ASP does not directly affect membrane transport of fatty acids, this also increases as a consequence of increased cellular fatty acid utilization resulting from increased diacylglycerol acyltransferase activity, the final enzyme in triglyceride biosynthesis. ASP not only acts on adipocytes, but also stimulates triglyceride synthesis and glucose transport in differentiated L6 rat myocytes. Thus, it could exert a significant influence on overall glucose homeostasis.

Preliminary studies of the mechanism and regulation of human adipocyte synthesis and secretion of the three precursor proteins indicate little effect of fatty acids, insulin, or glucose on ASP generation. By contrast, ASP production was markedly enhanced by addition of chylomicrons, and initial experiments suggest specific roles of the apoproteins in this process. Thus, coordination of chylomicron fatty acid release at the endothelial surface with an adipsin-ASP-mediated increase in adipocyte triglyceride synthetic capacity results in an effective and efficient microenvironmental metabolic regulation.

Normal function of the adipsin-ASP pathway appears to be essential to normal peripheral clearance of triglyceride. Impaired regulation of this pathway may be important in the pathogenesis of hyperapolipoproteinemia B, one of the most common dyslipoproteinemias associated with premature coronary artery disease, and in the pathogenesis of obesity.

Binding and Transport Proteins in Lipid Metabolism

The second session (chaired by M. Schotz) addressed advances in selected membrane-associated and soluble cellular proteins. The first speaker, **Peter Meier,** spoke on the topic, **"ATP-Binding Cassette Proteins",** and reported on the characteristics and possible role of a large multigene family of ATP-dependent membrane transport proteins, the so called ATP-binding casette proteins, ABC-transporters, or traffic ATPases. Their common characteristics include *a)* mediation of ATP-driven uniport of a wide range of substrates, *b)* conservation of the ATP-binding motif in two closely related protein complexes, and *c)* widespread distribution among prokaryotes (e.g., bacterial periplasmic permeases) and eukaryotes. Members of the eukaryotic ABC-transporter gene family include the a-type mating factor export pump of *Saccharomyces cerevisae* (STE6), the antimalaria drug efflux pump of *Plasmodium falciparum* (pfmdr1), the peptide transporter associated with MHC class I antigen processing in the endoplasmic reticulum (TAP1, 2), the cystic fibrosis transmembrane conductance regulator (CFTR), and the multidrug resistance (mdr) gene products. The latter represent drug efflux pumps and are also called P-glycoproteins.

Mammalian P-glycoproteins are encoded by small gene families, containing two members in humans (MDR1 and MDR2) and three members in mice (mdr1a or mdr3, mdr1b or mdr1, and mdr2) and in hamster (pgp1, pgp2, and pgp3). MDR1, mdr1a, and mdr1b cDNAs can confer multidrug resistance, while the closely related MDR3 and mdr2 cDNAs apparently cannot. Recently, an mdr1a knock-out mouse has been generated and shown to exhibit a greatly increased sensitivity towards the xenobiotics ivermectin and vinblastine. Tissue concentrations of these drugs were greatly increased in the mdr1a (-/-) mouse and the mdr1a gene product was not detectable in brain capillaries as it was in the wild type mouse. Otherwise the physiology, anatomy, and histology of the mdr1a deficient mouse was normal.

An mdr2 knock-out mouse has also been generated. Mdr2 is predominantly localized at the canalicular membrane of rodent liver, and mdr2 (-/-) mice demonstrated significant liver pathology including decreased hepatic clearance of bilirubin and increased alkaline phosphatase and liver transaminases in serum. However, mdr2 (-/-) mice did not secrete any phosphatidylcholine into bile. These findings support the hypothesis of Higgins and Gottesman that P-glycoproteins may function as flippases rather than transmembrane efflux pumps. For mdr2 this flippase concept has recently been further substantiated in vesicles isolated from transfected sec 6-4 yeast mutants. These studies indicated ATP-dependent flipping of a fluorescent phosphatidylcholine derivative from the outer to the inner leaflet of the membrane lipid bilayer in mdr2 overexpressing vesicles, but not in mdr3 overexpressing vesicles or wild type sec 6-4 yeast vesicles. Collectively these studies strongly suggest that the mdr2 P-glycoprotein is directly involved in the canalicular secretion of phosphatidylcholine into bile in mammalian liver. ATP-dependent in-to-out flipping could cause accumulation of phosphatidylcholine in the outer layer of the canalicular membrane, thereby promoting release of phosphatidylcholine vesicles from the outer membrane leaflet into bile.

The next speaker, **David Bernlohr,** in his lecture entitled **"Structure and Function of the Adipocyte Lipid Binding Protein",** reviewed the properties of the intracellular lipid binding proteins (iLBP), an 18-member multigene family that encodes monomeric 15 kDa polypeptides that bind cytosolic hydrophobic ligands, thus facilitating their solubilization and forming a metabolically accessible ligand pool. In all reported cases, the genes consist of three introns of varying size and invariant position relative to the coding sequence. In general, regulation is at the transcriptional level and family members are named by the dominant cell type in which they are expressed: thus, liver fatty acid binding protein (L-FABP), intestinal FABP, testis lipid binding protein, adipocyte lipid binding protein. Some members form high-affinity complexes with retinol (cellular retinol binding proteins I and II) or retinoic acid (cellular retinoic acid binding proteins I and II).

The polypeptides exhibit 20 to 70% sequence identity among members of the family from the same species, whereas sequence identity between the same protein of different species is often 95% or greater. Dissociation constants for fatty acids binding to FABPs are generally between 100 nM and 1 μM, while those for retinoids binding to the CRBPs or CRABPs are often near 1 nM.

Dr. Bernlohr's laboratory has focused on the characterization of the major fatty acid binding protein expressed in both white and brown fat of mice as well as in a variety of adipogenic cell lines, termed adipocyte lipid binding protein (ALBP or aP2). ALBP is most closely related to the FABP from peripheral nerve myelin and striated muscle. The binding site of ALBP is a large interior, water-filled cavity wherein the ligand resides, the interior surface of which is comprised of large numbers of polar and charged residues. The carboxyl group of the bound fatty acid typically forms direct hydrogen bonds with R126, Y128 and, through an intervening water molecule, R106. Despite amino acid sequence variations, the structures of the binding sites of several LBPs are similar. Enthalpic factors dominate the binding process, consistent with the crystallographic analysis of the fatty acid carboxylate-arginine interaction.

Kinetic studies suggest that ALBP donates its bound ligand to acceptor membranes or other proteins via a collisional rather than a diffusional mechanism. Surface lysine residues are critical for this interaction. While not directly tested, it is speculated that ALBP acquisition of ligand also requires physical interaction with a donating protein molecule or membrane. Several candidate membrane proteins with which the adipocyte lipid binding protein may interact are under investigation.

Nada Abumrad, the third speaker, described her presentation, **"Cloning and Expression of an Adipocyte Membrane Protein Implicated in the Transport of Long-Chain Fatty Acids".** This 88-kDa membrane protein was recently identified by labeling with sulfo-N-succinimidyl derivatives of long-chain fatty acids, most notably oleate, and with DIDS. Both labeling conditions were associated with strong inhibition of fatty acid transport. The 88 kDa protein was purified, its amino-terminal sequence was determined, and a cDNA for the protein was isolated from a rat adipocyte cDNA library, and designated fatty acid transport protein (FAT). The cDNA encodes a protein with 472 amino acids and a predicted molecular mass of 53 kDa, having two potential transmembrane segments and ten potential glycosylation sites. In vitro transcription and translation of the cDNA in the presence of microsomal membranes yielded an 88 kDa protein. The deduced protein sequence had 85% similarity to that of glycoprotein IV (CD36), which had been identified in human platelets and in lactating mammary epithelium. Further, a polyclonal antibody against CD36 detected a single 88 kDa protein band in rat adipocyte membranes. FAT mRNA was abundant in intestine, adipose, heart, and muscle tissues. While FAT mRNA was not detected in cultured adipose cell lines (3T3 F442A, Ob-1771 and BFC-1) at the fibroblastic stage, it was strongly induced on differentiation into adipocytes and by dexamethasone treatment, i.e., conditions that are also associated with increased cellular oleate uptake. Fibroblastic cell lines that

do not differentiate into adipocytes in culture do not express FAT mRNA, as late as two weeks after confluence.

Transfection of cells with an expression vector containing FAT cDNA in the sense or antisense orientation was associated with alterations in fatty acid uptake and esterification. Overexpression of the sense mRNA enhanced oleate uptake in fibroblastic cells; in contrast, antisense mRNA decreased fatty acid uptake and lipid deposition by 3T3 F442A following confluence. Regulation of FAT gene expression was demonstrated in response to long-chain fatty acids in preadipocytes and to cyclic AMP in adipocytes. Also, changes in FAT mRNA abundance were observed in adipose tissue from insulin-deficient and insulin-resistant animals. These effects, and their relevance to cell differentiation, are currently being characterized in greater detail.

Jerome Strauss, the final speaker, addressed the topic **"Sterol Carrier Protein 2: a Lipid Transfer Protein?",** and the role of this 13 kDa basic protein (SCP2) in intracellular lipid transport and regulation of membrane-bound enzymes involved in cholesterol biosynthesis and metabolism. SCP2 is encoded by an 80 kb gene consisting of 16 exons, mapped to human chromosome 1p32. The gene gives rise to two different transcripts under the influence of two independent promoters: a 2.7 kb transcript encoding a 58 kDa protein (SCPx) and a 1.5 kb transcript encoding SCP2. Homology searches suggest that SCPx has structural features of a thiolase. SCP2 is synthesized as a pro-protein with a 20 amino acid N-terminal sequence that is cleaved to yield the mature protein. The site of processing and the potential function of the 20 amino acid sequence remain unknown, although it may serve to target the protein to mitochondria. SCP2 is a component of the carboxyl terminus of SCPx. The C-terminal three amino acid sequence of the two proteins, A-K-L, represents a peroxisome-targeting sequence, and the SCPx promoter has characteristics of a peroxisomal protein gene. Subcellular fractionation and immunocytochemical analyses indicate that SCPx is localized to peroxisomes; SCP2 is also present in peroxisomes, as well as in association with other organelles, including mitochondria. Levels of SCP2 are markedly reduced in cells from subjects with known peroxisomal defects, including Zellweger syndrome and neonatal adrenoleukodystrophy. These observations suggest that both SCPx and SCP2 play roles in peroxisomal metabolism. The SCPx/SCP2 amino acid sequence is highly conserved across species lines.

Participation of SCP2 in sterol metabolism is suggested by studies of steroid hormone synthesis. Thus, SCP2 stimulates conversion of cholesterol to pregnenolone by isolated adrenal and ovarian mitochondria, anti-SCP2 antibodies introduced into adrenal cells diminish adrenal steroid biosynthesis, and expression of SCP2 in COS cells engineered to synthesize steroid hormones increases steroid synthesis. In the ovary, the SCPx/SCP2 gene is highly expressed, especially in compartments most actively involved in steroidogenesis, and expression is stimulated by tropic hormones that increase steroid secretion.

SCP2-mediated sterol transfer between vesicles and mitochondria in vitro may reflect its ability to bind to acidic membrane lipids and thus to promote sterol desorption. Structural studies based on NMR indicate that SCP2 is comprised of three α helical domains and a five-stranded β sheet. Site-directed mutagenesis suggests that residues in the first helix and fifth β sheet strand function in in vitro lipid transfer.

Peroxisomal Metabolism of Long-Chain Fatty Acids

The third session (chaired by R. Rachubinski) dealt with recent advances in the understanding of the regulation of peroxisomal function and the first talk, entitled **"Structural Comparisons between Peroxisomal and Mitochondrial Fatty Acid β-Oxidation Enzymes",** was given by **Takashi Hashimoto.** Dr. Hashimoto described the two different fatty acid oxidation systems that are present in almost all mammalian cells, one located in mitochondria and the other in peroxisomes. Specialization of these two systems in the same cell is clearly revealed by the study of hereditary diseases of the two systems. The mitochondrial system is responsible for the production of ATP, whereas the peroxisomal system oxidizes potentially toxic, very-long-chain fatty acids.

Long-chain fatty acyl-CoA synthetases in mitochondria and peroxisomes are indistinguishable at the protein level. Importantly, however, very-long-chain fatty acyl-CoA synthetase apparently is present in peroxisomes but not in mitochondria. In addition, mitochondria have a carnitine-dependent transport system for long-chain acyl-CoA transport into the mitochondrial matrix while the peroxisomal system is carnitine-independent.

The enzymes involved in the peroxisomal β-oxidation spiral are acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacylCoA dehydrogenase bifunctional protein, and 3-ketoacyl-CoA thiolase. Dr. Hashimoto described two newly discovered mitochondrial enzymes designated very-long-chain acyl-CoA dehydrogenase and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein. The trifunctional protein is an enzyme complex consisting of four α-subunits and four β-subunits. Both of these recently recognized enzymes are associated with the inner mitochondrial membrane, in contrast to the location of the previously described β-oxidation enzymes which are

located in the matrix. Severe symptoms associated with deficiencies of these new enzymes suggest they have important physiological roles.

Structural comparisons were made, based on deduced amino acid sequences from the cDNAs. Short-chain, medium-chain and long-chain acyl-CoA dehydrogenases are homotetramers of approximately 40 kDa subunits. The primary structures are quite similar to each other. Very-long-chain acyl-CoA dehydrogenase is a homodimer, with the subunits being approximately 75 kDa in size, with an N-terminal 40 kDa that is very similar to the classical dehydrogenases. The sequence of peroxisomal acyl-CoA oxidase has no significant homologies with other gene products. Peroxisomal bifunctional protein and the α-subunit of the mitochondrial trifunctional protein have similar sizes. The sequence of the N-terminal portions of these proteins are similar to enoyl-CoA hydratase (crotonase), and the C-terminal sequences are similar to 3-hydroxyacyl-CoA dehydrogenase sequences. Peroxisomal and mitochondrial 3-ketoacyl-CoA thiolase and the β-subunit of the mitochondrial trifunctional protein are very similar in both size and primary sequence.

Barry Foreman presented the second talk of the session, entitled **"Peroxisome Proliferator-Activated Receptors: a Family of Transcription Factors Activated by Fatty Acids".** Peroxisome proliferators and a number of fatty acids comprise a class of structurally unrelated compounds that cause liver enlargement as a consequence of increases in the size and number of hepatic peroxisomes and an associated hepatocellular hypertrophy and hyperplasia. Proliferation of peroxisomes is accompanied by an increase in peroxisomal fatty acid β-oxidation due to an increase in the synthesis of enzymes required for peroxisomal β-oxidation including acyl-CoA oxidase and bifunctional enzyme. Induction of these and other proteins by peroxisome proliferators is the result of transcriptional regulation.

Peroxisome proliferator-activated receptors (PPAR) are a group of structurally related transcription factors whose activity is regulated by peroxisome proliferators and/or fatty acids. PPARs are members of the nuclear hormone receptor superfamily and possess sequence similarity to receptors for steroids, retinoids, thyroid hormone, and vitamin D_3. PPAR form heterodimers with the retinoid X receptor (RXR) that are capable of binding with high affinity to response elements consisting of imperfect copies of the sequence AGGTCA n AGGTCA. Such response elements have been identified in the gene promoters of acyl CoA oxidase, bifunctional enzyme and fatty acid binding protein.

Despite these findings, it is still unclear how a wide variety of compounds can activate PPAR. One hypothesis is that all of these compounds are metabolized to or induce the synthesis of a natural ligand for PPAR. In an attempt to identify such a compound, Dr. Foreman and his colleagues examined the ability of fatty acids to activate PPARα. They found that a number of saturated and unsaturated fatty acids of chain length 12–20 were capable of activating PPARα, suggesting that a common metabolite of all fatty acids may act as a natural ligand for PPARα. Using metabolic inhibitors they concluded that ω-hydroxylation, cyclooxygenase, and lipoxygenase pathways are not required for the generation of a PPARα activator.

Interestingly, the majority of peroxisome proliferators contain a free COOH group or a functional group capable of being metabolized to a free COOH. Furthermore, although a number of fatty acids activate PPARα, their corresponding fatty alcohols are inactive. This suggested that a free carboxyl group may be required for further metabolism to a PPARα activator. Taken together, these findings raised the possibility that potential PPARα activators require activation by fatty acyl-CoA synthetase or subsequent steps in β-oxidation. On the basis of studies in which inhibitors of fatty acyl-CoA synthetase, carnitine palmitoyltransferase and medium chain acyl-CoA dehydrogenase/acyl CoA oxidase were used, it was concluded that long-chain fatty acyl CoA synthase is involved in the formation of a PPARα agonist. Dr. Foreman presented a model suggesting how some compounds may act as true ligands for PPARα, whereas others act to prevent metabolism of these true ligands. The related PPARγ and δ subtypes are somewhat divergent in their ligand binding domains, compared to PPARα, and exhibit distinct profiles of responsiveness to a number of fatty acids/peroxisome proliferators. Foreman concluded by suggesting that PPAR α, γ, and δ encode transcription factors that respond to a distinct set of fatty acid ligands.

Nathan Bass was the third speaker of the session, on the topic **"Peroxisome Proliferation and the Regulation and Function of Liver Fatty Acid Binding Protein".** Dr. Bass has investigated the mechanism of peroxisome proliferation and how it relates to the regulation and function of the liver fatty acid binding protein (L-FABP), the abundant cytoplasmic fatty acid binding protein present in hepatocytes. Although many functions have been proposed for L-FABP, including intracellular transport and utilization of long chain fatty acids, limitation of the cytoplasmic concentration of unbound fatty acids, and modulation of cell growth, definitive evidence for these functions is lacking. Studies in primary cultured hepatocytes confirmed the in vivo observation that L-FABP is markedly induced by fibrate peroxisome proliferators at a pre-translational level. L-FABP contains a candidate peroxisome proliferator responsive element in its 5′ promoter region that ap-

pears to be involved in the transcriptional regulation of L-FABP by peroxisome proliferator-activated receptors (PPAR). PPAR are activated by fatty acids, and animals fed high fat diets exhibit a modest increase in peroxisome proliferator-responsive genes, suggesting that the physiological significance of the induction of these genes is that it comprises an adaptive process to increased hepatocellular fatty acid flux.

Bass' initial studies in primary hepatocytes pointed to a role for long-chain dicarboxylic fatty acids as mediators of the peroxisome proliferative response. Evidence for this included the fact that long-chain monocarboxylic fatty acids had only limited ability to induce proliferator-responsive genes in cultured hepatocytes although they did so dramatically when there was inhibition of carnitine palmitoyltransferase I, and hence entry into the mitochondrial β-oxidation pathway. Furthermore, inhibition of fatty acid ω-hydroxylase (cytochrome P450 4A) activity in cultured hepatocytes markedly inhibited the induction of peroxisome proliferator-responsive genes, indicating a role for the products of the ω-oxidation pathway, namely dicarboxylic fatty acids, in mediating this induction. Finally, long-chain dicarboxylic fatty acids were shown to directly induce mRNA levels of peroxisomal fatty acyl-CoA oxidase and L-FABP, while medium-chain dicarboxylic fatty acids and long-chain monocarboxylic fatty acids had little or no effect under experimental conditions.

Dr. Bass described recent studies in which the ability of dicarboxylic fatty acids to activate PPAR and the role of L-FABP in modulating the activation of this receptor were investigated. Using transient transfection assays, he and his colleagues showed that the long-chain dicarboxylic fatty acid, hexadecanedioic acid (DC-16), is a potent activator of PPAR in HTC rat hepatoma cells. Contrary to their lack of ability to induce peroxisome proliferator-responsive genes in primary hepatocytes, long-chain monocarboxylic fatty acids were also found to activate PPAR in transient transfection assays, suggesting the presence of factors in hepatocytes that differentially modulate the potency of PPAR-activating molecules. A potential role for L-FABP as a negative modulator of proliferator response genes has been suggested from its lobular distribution in the liver, which runs in the opposite direction to the gradient of peroxisomal enzymes. In transient transfection assays, co-transfection of expression vectors containing L-FABP in anti-sense orientation had no effect upon the activation of PPAR by either long-chain monocarboxylic or dicarboxylic fatty acids. However, co-transfection with an L-FABP cDNA in sense orientation led to a profound inhibition of PPAR activation by both classes of fatty acids. Co-transfection with the L-FABP sense construct also produced a profound inhibitory effect upon the activation of PPAR by both bezafibrate and clofibrate. Although the former binds to L-FABP with a K_d of ~9 μM, the latter is a poor ligand for this protein. This raises the interesting question of whether the fibrates act directly as PPAR activators, or rather activate this receptor via a primary disruption in fatty acid metabolism, with fatty acids being the ultimate inducers.

The results of these experiments imply that there is an important role for L-FABP in modulating the activation of PPAR by fatty acids and xenobiotics. Dr. Bass concluded by suggesting that L-FABP might serve to increase the "threshold" for fatty acid and xenobiotic activation of PPAR, and thus prevent chronic induction of the peroxisomal β-oxidation pathway and its generation of reactive oxygen species that can produce toxic and neoplastic changes in cells.

The last talk of the session, **"The Mechanisms of Transcriptional Induction by Peroxisome Proliferators",** was presented by **Richard Rachubinski.** He discussed how the pleiotropic cellular responses to peroxisome proliferators are mediated in part by the transcriptional induction of a number of genes whose products regulate lipid metabolism. Human, rat, mouse, and *Xenopus* have multiple PPAR-related genes, perhaps reflecting a requirement for different PPARs in regulating specific target genes or in mediating responsiveness to a variety of stimuli. PPARs can be activated by structurally diverse peroxisome proliferators and by synthetic and natural fatty acids. However, none of these agents bind directly to PPARs, and the true ligands for these receptors remain unknown.

Peroxisome proliferator-responsive elements (PPREs) consist of direct repeats of the core half-site motif TGACCT. This motif is also found in the cognate response elements of other nuclear hormone receptors. Target specificity is determined in part by the sequence of the halfsites, the number and relative spacing of their direct repeats, and the ability of some receptors to bind to cognate response elements as homodimers and/or heterodimers. The potential for promiscuous binding, combinatorial interactions, and crosstalk among receptors serves to modulate transcription of hormone-responsive genes in multiple ways, thereby contributing to the complexity and the diversity in signaling pathways. PPAR function is dependent upon interactions with, and can be subject to modulation by, other members of the nuclear hormone receptor superfamily.

To date, much of the information on PPAR function has come from transient transfection assays done in mammalian cell cultures. These studies are complicated by the presence of endogenous nuclear hormone receptors and putative activators of the peroxisome proliferator-response pathway. The potential of various mammalian cellular proteins for heterodimerization with RXR,

and possibly with PPAR, and the finding that other orphan receptors can also bind to PPREs and modulate PPAR function have made it difficult to investigate directly the autonomous or cooperative functioning of individual PPARs and RXRs in transcriptional activation of specific target genes. Therefore, it has not yet been established whether PPAR functions exclusively, or necessarily, through cooperativity with RXR in vivo.

The yeast *Saccharomyces cerevisiae* does not have endogenous nuclear receptors or retinoids. Mammalian hormone receptors have been shown to function in *S. cerevisiae* in both ligand-dependent and ligand-independent manners. Dr. Rachubinski's group has now shown that mouse PPAR and human RXRα cooperate in yeast to synergistically activate transcription via cognate PPREs, and that this activation is potentiated by at least one exogenously added fatty acid, petroselinic acid, known to activate PPARs in mammalian cells. Using yeast peroxisomal assembly mutants, they have also shown that peroxisomes are essential for the stimulation of PPAR by fatty acid. This finding should open up new opportunities for the investigation of the complex biology of transcriptional regulation by peroxisome proliferators, PPARs, and PPREs.

Control of Hepatic Secretion of Triacylglycerol-Rich Lipoproteins

The fourth session (chaired by R. Gregg) was a forum for discussing the regulation of the synthesis of lipids and the mechanism by which lipid and protein macromolecular complexes are assembled in the lumen of the endoplasmic reticulum (ER). **Grahame Hardie,** in his opening lecture entitled **"The AMP-Activated Protein Kinase: Stress-Induced Regulation of Fatty Acid and Cholesterol Metabolism",** started the session by presenting a novel hypothesis concerning the regulation of cellular energy metabolism through protein phosphorylation in response to stress to the cell.

AMP-activated protein kinase was originally described as an activity from rat liver that phosphorylated and inactivated HMG-CoA reductase, and was named HMG-CoA reductase kinase. Hardie's laboratory subsequently discovered that it has additional targets in vivo (e.g., acetyl-CoA carboxylase, hormone-sensitive lipase/cholesterol esterase) and renamed it the AMP-activated protein kinase (AMPK). 5′-AMP allosterically activates AMPK, and also promotes phosphorylation and activation of it by an upstream kinase (AMPK kinase). The two effects multiply together such that elevation of the AMP:ATP ratio could produce >100-fold activation of AMPK. The AMP:ATP ratio appears to be primarily controlled in vivo by the enzyme adenylate kinase, which maintains its reaction (2ADP<–>ATP + AMP) close to equilibrium. Most, if not all, treatments that produce the cellular stress response (e.g., heat shock, arsenite, hypoxia) cause depletion of ATP, and consequent elevation of AMP due to displacement of the adenylate kinase reaction. They have now shown that in isolated rat hepatocytes, stress treatments (heat shock, arsenite) cause elevation of the AMP:ATP ratio, phosphorylation and activation of AMPK, and severe inhibition of both fatty acid and sterol synthesis, the latter accompanied by phosphorylation and inactivation of HMG-CoA reductase. AMPK also phosphorylates key proteins involved in other pathways (particularly biosynthetic pathways) in cell-free assays, but further work is necessary to test whether these are physiological substrates.

Purified rat liver AMPK consists of a 63 kDa protein (p63) tightly associated with two additional subunits (p38 and p35). Cloning of the rat liver p63 by Carling's group has revealed that it is closely related to the snf1 gene product from yeast. Yeast strains carrying a disrupted snf1 gene do not respond appropriately to starvation for glucose. In particular, they cannot derepress genes required for aerobic metabolism or metabolism of alternative carbon sources, and fail to arrest in G1 phase. Recent work also shows that yeast acetyl-CoA carboxylase is phosphorylated and inactivated by snf1 protein kinase both in vitro and in vivo.

Thus, a system that appears to have evolved to protect unicellular eukaryotes against starvation for a key nutrient may have been adapted in multicellular eukaryotes to provide protection against environmental stress. Inhibition of fatty acid and sterol synthesis under these conditions represents one facet of its physiological function.

The second speaker, **David Gordon,** spoke on **"Reconstitution of Lipoprotein Assembly in a Non-Lipoprotein Producing Cell Line".** He described the recent investigations of his group into the role of microsomal triglyceride transfer protein (MTP) in the assembly of apoB-containing lipoprotein particles in the ER lumen of enterocytes and hepatocytes. While it is clear that assembly and secretion of VLDL particles requires synthesis of apoB, phosphatidylcholine (PC), and neutral lipids, additional factor(s) appear to be involved. ApoB-53 (the amino terminal 53% of apoB-100) expressed in hepatocyte-derived cell lines is secreted in a lipoprotein particle, while apoB-53 expressed in non-lipoprotein producing cells is not secreted, but is degraded intracellularly.

A candidate for this missing factor is the MTP. MTP is a soluble ER luminal protein that catalyzes the transfer of triacylglycerol (TG), cholesterol ester (CE), and PC between membranes. It is a heterodimer composed of protein disulfide isomerase (PDI, 58 kDa) and a unique 97 kDa subunit. While PDI is a multifunctional, ubiquitous protein, the large subunit of MTP has only been

found in the liver and intestine. In addition, recent studies have demonstrated that mutations in MTP are the proximate cause of abetalipoproteinemia. Thus, it is clear that MTP is required for the efficient assembly and secretion of apoB-containing lipoprotein particles.

Although studies of abetalipoproteinemic patients indicate that MTP is required for the production of plasma lipoproteins containing apoB, it is not known if MTP is the only tissue-specific factor needed by cells to synthesize and secrete these particles. To address this, apoB-53 was expressed either in a non-lipoprotein producing cell line (HeLa cells) or a derivative of this cell line stably expressing the large subunit of MTP (HLM-40 cells). The results of this study demonstrated that MTP activity expressed in HeLa cells is sufficient to reconstitute the assembly of apoB-53 and lipid into a macromolecular lipoprotein particle that is secreted from the cell. Thus, lipoprotein secretion from HeLa cells only required the expression of apoB and MTP.

Similar to apoB-53 expressed in McArdle RH-7777 rat hepatoma cells, apoB-53 lipoprotein particles produced by HLM-40 cells were predominantly of the HDL density class, although their buoyant density varied somewhat depending upon the availability of lipid. The mass of apoB-53 secreted was also increased after addition of lipid to the cell culture media. Such up-regulation of apoB secretion is characteristic of other lipoprotein producing cell lines. This suggests that MTP is not only sufficient to direct secretion of apoB-containing lipoproteins in a non-lipoprotein producing cell, but is also sufficient to confer a phenotype similar to that of the McArdle RH-7777 cell in regard to the buoyant density distribution of the secreted lipoprotein particles.

The third speaker of the session was **Nicholas O. Davidson,** addressing the **"Regulation of Apolipoprotein B Gene Expression through Post-Transcriptional RNA Editing".** ApoB is a large, hydrophobic protein that is an integral component of triacylglycerol-rich lipoproteins in both the mammalian liver and small intestine. Mammalian apoB circulates in two distinct forms, apoB-100 and apoB-48. In humans, apoB-100 is synthesized in the liver while apoB-48 is synthesized in the small intestine. The molecular basis for this tissue-specific production of distinct protein isomorphs is a posttranscriptional modification referred to as apoB mRNA editing. In this process, a genomically templated CAA codon is modified in the primary RNA transcript to a UAA codon, and this alters the reading frame from a glutamine amino acid to a stop codon. The intestinal transcript is thus translated as an abbreviated protein (apoB-48) which is colinear with the amino-terminus of apoB-100.

A major advance was the discovery made in Dr. Davidson's laboratory that chicken apoB RNA was not editable in in vitro incubations using rat S-100 extracts. When chicken small intestinal S-100 extracts were assayed using chicken apoB RNA as a substrate, again no editing was found. However, when chicken and rat S-100 extracts were mixed and added to rat synthetic apoB RNA, there was a striking enhancement of editing activity compared to that seen with rat S-100 extracts alone. This enhancement was found to be mediated by a protein factor(s), most abundant in small intestinal enterocytes.

This editing enhancement allowed these workers to establish a genetic complementation system to clone a novel gene involved in apoB mRNA editing. Rat intestinal RNA was injected into Xenopus oocytes. Oocyte extracts were added to an in vitro apoB mRNA editing assay along with chicken intestinal S-100 extracts. From an initial pool of 10 million clones, a single clone was isolated and sequenced to reveal a novel gene originally referred to as REPR (rat intestinal apoB mRNA editing protein). The current nomenclature for this gene and its protein product is APOBEC-1 (apolipoprotein B mRNA editing protein, catalytic polypeptide #1). Injection of Xenopus oocytes with RNA transcribed from this cDNA produced 50% editing of a rat apoB RNA template, but only in the presence of chicken intestinal extracts. These findings implied the existence of other components in the editing complex. Extending this suggestion, these workers established that human liver S-100 extracts acquired apoB mRNA editing ability when mixed with oocyte extracts expressing APOBEC-1. In further studies, HepG2 cells, a human liver-derived hepatoma cell line, when transfected with APOBEC-1, was found to synthesize and secrete both apoB-100 and apoB-48. These findings suggest that human liver fails to edit endogenous apoB mRNA as a result of the absence of APOBEC-1. Furthermore, the findings suggest the feasibility of targeted gene therapy with APOBEC-1 for syndromes characterized by excess concentrations of apoB-100.

Davidson's laboratory has also cloned a human small intestinal cDNA corresponding to the catalytic subunit of the apoB mRNA editing enzyme. This cDNA encodes a protein that is predicted to be seven residues longer than the rat protein (236 versus 229). Overall homology was less than 70% at both the nucleotide and amino acid levels. Interestingly, the predicted amino acid sequence of the human homolog includes a conserved motif (His-[X]-Cys-X-X-Cys) corresponding to the zinc binding domain of other, previously identified, cytidine deaminases. Additionally, the tissue distribution of APOBEC-1 mRNA in humans was found to be quite distinct from that demonstrated in the rat. APOBEC-1 mRNA was restricted essentially to the small intestine in humans, with lower levels detectable by RT-PCR in other gastro-

intestinal tissues such as colon and stomach. By contrast, APOBEC-1 mRNA was detectable in all rat tissues examined. The functional significance of APOBEC-1 in rat tissues other than the small intestine is a question that will require further investigation.

In the final lecture, **"How the Endoplasmic Reticulum Deals with the Protein Folding Problem", Ari Helenius** described his research group's studies in cells and in isolated ER vesicles on the conformational maturation process that glycoproteins undergo, focussing on the interactions between newly synthesized glycopolypeptides and the unique folding machinery present in the ER. The folding and oligomeric assembly of glycoproteins in the ER lumen determines how efficiently, how fast, and in which form they are secreted, expressed on the plasma membrane, or delivered to the membranes or lumen of vacuolar organelles.

Helenius and his associates have found that newly synthesized glycoproteins associate transiently during their folding with several chaperones. One is BiP/GRP78, an HSP70 homologue with peptide binding and ATPase activity. By following the folding of carboxypeptidase Y in temperature sensitive BiP mutants of *S. cerevisae,* they were able to show that BiP/GRP78 plays a crucial role not only in the translocation of polypeptides into the ER, but also in the folding process within the ER lumen.

Calnexin, a membrane bound ER protein, is another protein that binds transiently to glycoproteins, with attachment already starting on the nascent chain as the polypeptide emerges from the ER membrane. Using viral and cellular proteins as model glycoproteins, Helenius and colleagues demonstrated that this association is prevented not only by inhibitors of N-linked glycosylation (tunicamycin), but also by inhibitors of the glucose trimming enzymes (castanospermine and 1-deoxynojirimycin) responsible for the rapid removal of three glucoses from newly added N-glycans. Further results indicated that calnexin binds cellular and viral glycoproteins only when they have partially trimmed N-linked oligosaccharides. The monoglucosylated ER forms ($Glc_1Man_{9-7}GlcNac_2$) are optimal.

For vesicular stomatitis virus G protein, they have further demonstrated that association with calnexin is necessary for normal folding in the ER. They found that G protein undergoes sequential chaperone interactions during its folding. It first binds to BiP/GRP78, and, after partial folding, it transfers to calnexin for completion of its disulfide bond formation program.

On the basis of their results, Dr. Helenius and colleagues propose that the ER contains, in addition to the standard heat shock protein family members, unique folding and quality control machinery specific for glycoproteins. This includes calnexin as a lectin-like retention molecule, glucosidases I and II as signal modifiers, and UDP-glucose:glycoprotein glucosyltransferase (a lumenal ER enzyme) as a folding sensor. Glycoproteins undergo de- and reglucosylation until they reach a folded or assembled conformation that leads to permanent release from calnexin. The folding itself involves formation of intramolecular disulfide bonds in a strict hierarchial sequence. These findings help to account for the retention of glycoproteins in the ER until they are fully folded, and provide a rationale for the glucose-trimming events.

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