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APOLIPOPROTEIN B PLASMA LEVELS, HEPATIC SYNTHESIS AND SECRETION, AND mRNA ABUNDANCE AND EDITING IN COPPER-DEFICIENT RATS

by

Scott Kenneth Reaves

A Dissertation Submitted to the Faculty of the COMMITTEE ON NUTRITIONAL SCIENCES In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA

1995
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Scott Kenneth Reaves entitled Apolipoprotein B Plasma Levels, Hepatic Synthesis and Secretion, and mRNA Abundance and Editing in Copper-Deficient Rats and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: Scott Kenneth Reaves
ACKNOWLEDGEMENTS

Special thanks to Dr. David Lei, Dr. Fumiko Rosenstein, Dr. Yiran Wang, Dr. Yongjian Wu, and Dr. Ron Hoogeveen for their advice, assistance and friendship throughout my graduate career. I would also like to thank Dr. Charles Weber, Dr. Wanda Howell, and Dr. Ann Jerkins for serving on my graduate committee. The time and effort put forth by the Nutritional Sciences Department Staff in support of my graduate work was also very much appreciated.

I would also like to express my thanks to Dr. Mary Pedersen who believed in my abilities and encouraged me to pursue a graduate degree. I am especially grateful to Elizabeth and Gabrielle for their technical assistance in the preparation of this manuscript in addition to their patience and understanding when the hours were long. Finally, I am deeply indebted to my parents, as well as Brently and Cherelle for their moral support and encouragement throughout all of my academic endeavors.
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ABSTRACT

The effects of dietary copper deficiency on plasma apoB levels and hepatic apolipoprotein (apo) B synthesis, secretion and intracellular degradation were examined in rats. In addition, hepatic apoB mRNA abundance as well as editing in the liver and intestine were also determined. Male weanling Sprague-Dawley rats were assigned to copper-deficient (9.0 μmol Cu/kg diet) or copper-adequate (102 μmol Cu/kg diet) dietary treatments, for six weeks. In vitro hepatic apoB synthesis, secretion, and degradation were determined by pulse-chase studies using freshly isolated rat liver parenchymal cells. Two x 10^8 cells were pulsed for 20 minutes in minimum essential medium (MEM) with [3H]phenylalanine and chased for 10, 20, 30 and 45 min in MEM with 0.1 mM PHE. Triton X-100 and Na deoxycholate were added to cell and medium samples and apoB in the detergent soluble fractions was immunoprecipitated with monospecific polyclonal antibody. Proteins were resolved by SDS-PAGE and radioactivities associated with apoB-48 or apoB-100 were counted. In vitro apoB-48 and apoB-100 synthesis was not altered, secretion was increased twofold and intracellular degradation of total apoB was significantly reduced in cells derived from copper-deficient rats. Plasma levels of apoB-48 and apoB-100 were determined by resolving delipidated LDL and VLDL fractions by SDS-PAGE. Apolipoprotein bands were identified and quantitated by loading various amounts of purified apoB-48 and apoB-100 on each gel. Plasma apoB-48 and apoB-100 levels were increased by copper deficiency but only the apoB-48 increase was found to be significant, thereby, indicating a preferential increase in plasma apoB-48. ApoB mRNA editing activity was determined by using the PCR-
cloning-colony hybridization technique. Hepatic apoB mRNA editing, expressed as a ratio of apoB-48 mRNA/apoB-48 plus apoB-100 mRNA, was significantly increased from 60.8% in copper-adequate to 70.2% in copper-deficient rats. Moreover, hepatic apoB mRNA abundance, as determined by dot blot hybridization with a specific [32P]-labelled rat apoB cDNA probe, was not significantly increased by copper-deficiency. Thus, the increased amount of nascent apoB-48 secreted into the medium as well as the enhanced apoB mRNA editing may have contributed to the differential increase in plasma apoB-48 over apoB-100 level in copper-deficient rats.
Apolipoprotein B (apoB) is an essential structural and functional component of the triglyceride-rich very low density lipoproteins (VLDLs) and chylomicrons. It is also an essential component of the cholesterol-rich low density lipoprotein (LDL) which is the main transporter of cholesterol in humans. Therefore, apoB is crucial in the regulation of cholesterol homeostasis as well as energy metabolism since VLDL triglycerides represent a major source of energy for peripheral tissues. In humans, chylomicrons contain apoB-48 while LDL particles and VLDL particles contain apoB-100. Since each particle only contains one molecule of apoB, and apoB-48 and apoB-100 are metabolized differently, the presence of either apoB-48 or apoB-100 determines the metabolic fate of the particle.

ApoB-100, one of the largest proteins known, is a 512 kDa polypeptide that represents a full-length translation product of the apoB gene. ApoB-48 is a truncated translation product that is approximately one-half (48%) the size of apoB-100 but both forms of apoB are coded for by the same gene. ApoB-48 is synthesized from the apoB gene by a novel mechanism in which the apoB mRNA is “edited” by a C to U conversion. The editing reaction results in a CAA glutamine codon to be changed to a UAA translation stop codon, therefore, the amino acid sequence of apoB-48 is colinear with the amino-terminal half of apoB-100. Since the LDL receptor binding domain is located in the carboxy-terminal half of the apoB-100 protein, apoB-48-containing lipoproteins are not taken up by the LDL
receptor mediated endocytosis pathway. ApoB-48-containing particles are cleared from the circulation much faster than apoB-100-containing particles by a process that is mediated by the presence of apolipoprotein E on these particles.

Atherosclerosis develops when macrophages adhere to the arterial endothelium of the coronary arteries, accumulate cholesterol and lipids intracellularly, and initiate plaque formation. Due to the participation of plasma cholesterol in this process, there is a strong positive correlation between LDL cholesterol levels and coronary artery disease (CAD). Recently, it has been reported that plasma apoB levels were even better predictors of CAD than were plasma LDL cholesterol levels. Because atherosclerosis is the leading cause of death in the United States in all persons above age 45, the metabolism of apoB and its role in the regulation of plasma cholesterol levels and development of atherosclerosis is of fundamental interest. The ability to clear cholesterol-rich particles or transport cholesterol in particles that cannot participate in the atherosclerotic process would obviously reduce the risk of CAD.

In the rat, approximately 85% of its plasma cholesterol is associated with high density lipoproteins (HDL), a feature that is probably responsible for the lack of atherosclerosis in this species. The drastic differences between rat and human lipoprotein profiles are most likely due to the ability of the rat to edit apoB mRNA in the liver. This allows the rat to produce apoB-48-containing VLDL particles that are cleared rapidly and not catabolized to LDL particles. The present studies were performed to examine apoB metabolism and mRNA editing in the hypercholesterolemic and hypertriglyceridemic copper-deficient rat model.
Recent cloning of the editing enzyme has provided insights into the mechanisms of the editing reaction which may eventually allow the therapeutic induction of hepatic editing in humans.
CHAPTER 2

LITERATURE REVIEW

Lipoprotein Metabolism

Lipids must be absorbed by the intestine, transported through the body via the circulatory system, distributed to specific tissues and also excreted from the body. These processes are all part of the lipid transport system which is composed of four major classes of plasma lipoproteins. Although each class of lipoproteins has very distinct features, it is the coordination of their metabolism that allows the lipid transport system to perform its required functions.

Chylomicrons are synthesized by intestinal epithelial cells to large triacylglycerol-rich particles that initially transport lipids to other tissues. Chylomicrons are rapidly cleared from the circulation by endothelial cell-bound lipoprotein lipase (LPL). The lipase allows fatty acids to be taken up by adipose tissue and muscle and the remaining particles (chylomicron remnants) are cleared via receptor-mediated endocytosis in the liver. Chylomicron remnants contain apolipoprotein (apo) B-48 as well as E, which facilitates the receptor mediated uptake. Very low density lipoproteins (VLDL) are synthesized in the liver to remove hepatic triacylglycerol (TG). They contain the apolipoproteins B-100 or apoB-48, apoE and apoC. These lipoproteins can also be lipolyzed by adipose and muscle endothelial LPL with the resultant particle being intermediate density lipoproteins (IDL). Most of the IDL particles are rapidly taken up by the liver through LDL receptor (apoB, E receptor)
mediated endocytosis. However, a part of the IDL particles are further lipolyzed, probably by hepatic lipase to form the cholesterol-rich low density lipoproteins (LDL). LDL particles contain B-100 to facilitate receptor uptake in the liver and extrahepatic tissues. These LDL particles are responsible for cholesterol transport to extrahepatic tissues. High density lipoproteins (HDL) are produced in the liver and intestine and are involved in the dynamic exchange of lipids and apolipoproteins between lipoprotein particles as well as the process known as reverse cholesterol transport: This process involves cellular cholesterol transported by HDL directly to the liver for excretion or transfer of cholesterol to VLDL, IDL, and LDL particles via the protein cholesterol ester transfer protein (CETP). The characteristics of plasma apolipoproteins in humans is shown in Table 1.

**Apolipoproteins B-48 and B-100**

Apolipoprotein B (apoB) provides the structural framework for the TG-rich VLDL particle, which is responsible for hepatic triacylglycerol secretion. In 1987 it was first reported that the two major forms of apoB (apoB-100 and apoB-48) are the result of a novel mRNA editing process (Chen et al., 1987; Powell et al., 1987). ApoB mRNA editing is a post-transcriptional intranuclear event that changes a glutamine codon CAA to the UAA translational stop codon. Editing results in apoB-48, a protein that is colinear with the amino terminal of B-100 and contains 48% of the 4536 amino acid residues of B-100.

**Translocation of ApoB and Assembly of VLDL Particles**

Secretory proteins such as B-48 and B-100 are targeted to the endoplasmic reticulum
Table 1. Characteristics of Plasma Apolipoproteins in Humans

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<tr>
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<th>Distribution in lipoproteins</th>
<th>Major tissue source</th>
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<tr>
<td></td>
<td>(mol%)¹</td>
<td></td>
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<tr>
<td>HDL</td>
<td>LDL</td>
<td>IDL</td>
<td>VLDL</td>
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<tr>
<td>ApoA-I</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo-AII</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo-AIV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoB-48</td>
<td></td>
<td>90</td>
<td>8</td>
</tr>
<tr>
<td>ApoB-100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoC-I</td>
<td>97</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Apo-CII</td>
<td>60</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Apo-CIII</td>
<td>60</td>
<td>10</td>
<td>10</td>
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<tr>
<td>ApoD</td>
<td>100</td>
<td></td>
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<tr>
<td>ApoE-II</td>
<td>50</td>
<td>10</td>
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<td>ApoE-III</td>
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<tr>
<td>ApoE-IV</td>
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¹For each apolipoprotein.
(ER) by an mRNA-contained translational signal-sequence. Signal-recognition particle (SRP) binds to the protein’s signal sequence and then interacts with the SRP receptor at the ER membrane, consequently, targeting the protein to this organelle (Wiedmann et al., 1994; Gibbons, 1990). ApoB contains an N-terminal 27-residue signal sequence that is cleaved prior to the simultaneous translation and translocation events. Studies in rat liver, rabbit liver, and HepG2 cells have shown that much of the newly synthesized apoB is bound to ER and Golgi membranes (Cartwright et al., 1993; Dixon and Ginsberg, 1993; Boren et al., 1992). Other studies have indicated that a substantial fraction of the ER-membrane bound apoB is susceptible to protease degradation, suggesting that portions of the protein are located on the outer surface of the ER (Davis et al., 1990; Dixon et al., 1992). The luminal and membrane-bound fractions of apoB appear to represent two pools of apoB that may have distinct fates. The site(s) of apoB degradation is worthy of future studies since it appears to be a major point in regulating the amount of apoB that is secreted by the cell.

The processes involved in apoB synthesis and its association with lipids in the assembly of VLDL particles remain unclear. Numerous studies have indicated that apoB-100 is synthesized in "surplus" and apoB that is not immediately associated with lipid undergoes intracellular posttranslational degradation (Bostrom et al., 1988; Boren et al., 1990; Borchardt and Davis, 1987; Davis et al., 1989; Sato et al., 1990). Boren et al. (1992) have shown apoB-100 can be assembled into lipoproteins cotranslationally in the presence of appropriate lipids as the protein enters the ER lumen. The pool of apoB-100 which remains membrane-bound, in the absence of lipid constituents, is therefore destined for degradation.
In addition, Cartwright et al. (1993) have shown that most of the ER membrane-bound apoB is degraded. They have also shown another degradative pathway in which luminal apoB is degraded or combined with lipid and transferred to the Golgi for eventual secretion. Moreover, a significant amount of apoB in the trans-Golgi and cis-Golgi is membrane-bound but the relevance of this distribution remains unclear. Phospholipid and non-esterified cholesterol are thought to be added to maturing particles in the Golgi (Higgins and Hutson, 1984) while terminal polypeptide glycosylation occurs in the Golgi prior to the "budding off" of secretory vesicles.

Differential Metabolism of ApoB-100 and ApoB-48

Elovson et al. (1988) provided strong evidence that VLDL is comprised of one apoB peptide and that the majority of rat VLDL contain apoB-48 rather than apoB-100. Since each VLDL particle is thought to contain only one molecule of apoB, the presence of apoB-48 or apoB-100 should effect the metabolism of the particle. ApoB-48 lacks the receptor-binding domain present in the carboxyl-terminal region of apoB-100, and it would seem that clearance of B-48 particles would be slower. However, hepatic apoB-48 is cleared from the plasma more rapidly than apoB-100 through the apoE receptor that is responsible for the clearance of chylomicron remnants (Chen et al., 1987; Elovson et al., 1981; Sparks and Marsh, 1981; Stalenhoef et al., 1984). The assumption that apoB-48 containing VLDL from liver is metabolized faster is supported by findings obtained in patients with familial hypobetalipoproteinemia. Point mutations in one allele of the apoB gene result in carboxy terminal truncated isoforms such as apoB-89, apoB-67, apoB-61, apoB-54, apoB-46, apoB-
37, or apoB-31 (Parhofer et al., 1992; Weltry et al., 1991; Pullinger et al., 1992; Wagner et al., 1991; Young et al., 1989; Young et al., 1987; Young et al., 1990). These truncated isoforms are able to form buoyant lipoproteins that clear rapidly from plasma which results in hypobetalipoproteinemia (Parhofer et al., 1992; Parhofer et al., 1990; Vega et al., 1987; Krul et al., 1992). This condition does not cause adverse effects but instead has actually been associated with improved longevity (Maruhama, 1984).

The liver is also the site of origin of the Lp(a) molecule. Lp(a) has received considerable attention because a strong correlation between its plasma concentration and cardiovascular disease has been established (Utermann, 1990). The Lp(a) molecule is similar to LDL in that its lipid composition is similar and Lp(a) also contains B-100. Lp(a) is distinct from LDL because the Lp(a) particle also contains apolipoprotein(a) that is believed to be linked to B-100 molecule via disulfide bonds. As a consequence of its truncation, apoB-48 does not combine with apo(a) to form the atherogenic Lp(a) molecule (Scanu and Fless, 1990).

**ApoB mRNA Editing**

In 1987, two labs simultaneously reported that apoB-48 was produced by the introduction of a stop codon in the apoB mRNA (Chen et al., 1987; Powell et al., 1987). The CAA codon encoding Gln 2153 is changed to an in-frame stop codon (UAA) in a C to U conversion. This process, later termed apoB mRNA editing, was unprecedented at this time and represented a novel means of RNA processing in higher vertebrates.
Species and Tissue Specificity of Editing

ApoB mRNA editing is most active in the intestine in all mammals that have been studied and approximately 90% of apoB mRNA is edited. (Teng et al., 1990a; Patterson et al., 1992; Teng et al., 1990b; Wu et al., 1990) Consequently, apoB-48 is the predominant form of apoB found in the intestine of all animals studied thus far. Greeve et al. (1993) has shown high levels of intestinal apoB RNA editing in 12 different mammalian species suggesting that conservation of intestinal editing is vital. However, in contrast to intestinal editing, there are wide variations in hepatic apoB editing between species. No hepatic editing has been detected in human (Powell et al., 1987; Chen et al., 1987), monkey (Driscoll and Casanova, 1990), pig (Teng et al., 1990b), cow, sheep or cat (Greeve et al., 1993). Low levels (< 1%) of hepatic editing were detected in the rabbit and guinea pig while high levels occurred in the rat (62%), mouse (70%), dog (18%) and horse (43%) (Greeve et al., 1993). ApoB mRNA has also been detected in a number of other tissues (stomach, kidney, colon, and fetal membranes) and a significant part of this mRNA is edited (Teng et al., 1990b). These findings show the necessary editing machinery to be present in tissues where apoB mRNA is very low and could suggest that transcripts other than apoB are also targeted for editing.

Unlike humans and rats, chickens do not exhibit liver or intestinal editing but perhaps the most interesting observation is that chicken enterocyte S100 fractions are capable of enhancing the in vitro editing activity of numerous mammalian extracts (Teng et al., 1992). Thus, chicken enterocytes appear to contain enhancing or complementation factor(s) that are
required in the assembly of a high-order complex but lack one or several key components for editing. This would indicate not only the diverse evolution of editing between species but it would seem that a particular factor of the editing reaction provides the species specificity.

Developmental Regulation of Editing

Developmental modulation of apoB mRNA editing is different in the rat and human and results in substantial hepatic editing in the rat but none in human liver. Human fetal small intestine at eight weeks of gestation contains apoB mRNA but there is no detectable editing. Human intestinal editing increases to 5-10% and approximately 50% at 11 weeks and 15 weeks of gestation, respectively. At 20 weeks of gestation more than 85% of apoB mRNA is edited (Teng et al., 1990a). ApoB-48 and apoB-100 production parallels the changes in editing with apoB-48 being the predominant protein produced in the small intestine by the second trimester (Glickman et al., 1986). Editing also increases during development in other human tissues where editing is detected. However, apoB-48 synthesis and secretion are limited to the small intestine (Teng et al., 1990a). Synthesis of B-100 by numerous fetal tissues may provide a means of meeting the extraordinary cholesterol demands for tissue growth in the fetus. The widespread and efficient editing of apoB transcripts combined with the confinement of apoB-48 synthesis and secretion by the human small intestine represent posttranslational regulation and indicate that editing and protein production are regulated separately. In the rat, intestinal apoB mRNA editing is also fully developed prenatally. However, unlike humans, rat intestinal editing does not reach adult levels until about two days before birth when there is a dramatic increase (from ~1% to >
80%) in editing activity (Wu et al., 1990, Patterson et al., 1992). Complete intestinal editing prenatally in the human and rat would suggest B-48 has a role that is crucial in fetal adaptation to postnatal life. However, editing in the rat liver does not reach the levels seen in adulthood until 2-3 weeks after birth (Patterson et al., 1992; Wu et al., 1990). These findings show editing to be regulated in a temporal, species-specific and tissue-specific manner and while the implications for this regulation are significant the mechanisms responsible for each remain unclear.

Nutritional and Hormonal Modulation of Editing

Rat liver editing activity is responsive to numerous hormonal and nutritional treatments whereas the rat small intestine appears less responsive to the same treatments. The first report of hormonal control of editing in the liver was documented when Davidson et al. (1988) found thyroid hormone treatment in hypothyroid rats increased the amount of apoB mRNA edited. Baum et al. (1990) reported bolus administration of thyroid hormone (T3) to hypothyroid rats caused an increase in hepatic mRNA editing and no change in apoB mRNA abundance while causing apoB-100 synthesis to decrease to undetectable levels. Editing increases were seen by 18 hours following T3 administration (from ~30% to 70%) and peaked after 48 to 72 hours with editing reaching 80%. In contrast to B-100, B-48 synthesis increased two fold 72 hours following T3 administration but the effect was transient as synthesis was reduced by 96 hours (Baum et al., 1990). Effects of thyroid hormone on editing appear to involve a complex interaction between growth hormone and thyroid hormone. Sjoberg et al. (1992) found no effect of thyroid hormone administration in
hypophysectomized rats but growth hormone (GH) treatment increased hepatic editing to near normal levels. These data may suggest that thyroid hormone replacement in hypothyroid rats exerts its effects via GH as previously described (Coiro et al., 1979; Yaffe et al., 1984). However, previous studies found that GH had no effect on editing in hypothyroid rats (Davidson et al., 1988; Davidson et al., 1990) indicating a complex interaction that remains unclear. In support of a complex interaction, hypophysectomized rats treated with T₄, cortisol and GH increased the percentage of edited apoB mRNA in the liver to that of normal rats (Sjoberg et al., 1992).

The effects of insulin on apoB secretion have been controversial with several labs reporting conflicting results. Bartlett and Gibbons (1988) found that chronic exposure to insulin stimulated VLDL secretion in rat hepatocytes. However, numerous studies have found apoB secretion reduced following short-term (<24 hours) insulin exposure in both HepG2 cells and primary rat hepatocyte culture (Pullinger et al., 1989; Dashti et al., 1989; Sparks and Sparks, 1990). Recently, Thomgate et al. (1994) attempted to elucidate the effects of long-term (5 day) exposure of physiologic concentrations of insulin on apoB secretion from primary rat hepatocytes. Insulin caused a specific stimulation of the secretion of apoB which was 4-fold greater than generalized protein synthesis stimulation. Most of the increase in apoB was due to an increase in apoB-48 secretion. ApoB-48 secretion was increased 367% at insulin concentrations of 4ng/ml and 1003% following treatment with insulin at 500ng/ml. ApoB-100 secretion was 240% and 290% at these insulin concentrations. Thus, the predominant effect of insulin appeared to be exerted on B-48
secretion. Insulin increased the absolute amount of apoB-48 secreted as well as the percentage of B-48 to total apoB secreted. Prior to insulin exposure, apoB-48 is 65-70% of the apoB secreted but B-48 increases to approximately 90% of apoB secreted following insulin exposure at 400ng/ml. Insulin did not alter steady-state levels of total apoB mRNA but was found to dramatically increase editing of apoB mRNA. The amount of edited apoB mRNA was ~67% with no insulin and increased to 82% and 88% at concentrations of 4.0 and 400 ng/ml of insulin, respectively (Thorngate et al., 1994). These findings indicate that insulin modulates editing and, in agreement with previous reports (Seishima et al., 1991; and Sjoberg et al., 1992), that the percentage of B-48 to B-100 secreted coincides with the percentage of apoB message that is edited.

Several studies have shown that certain nutritional regimens are capable of modulating editing in the rat liver. Leighton et al. (1990) reported hepatic editing was decreased by 60% in fasted animals while Baum et al. (1990) demonstrated that fasted animals refed a high-carbohydrate diet had decreased editing during fasting but refeeding increased editing significantly above control levels. The fasting-refeeding regimen led to a 30-fold increase in hepatic triacylglycerol content (Baum et al., 1990) whereas thyroid hormone has also been found to increase hepatic triacylglycerol synthesis and secretion rates (Davidson et al., 1988; Baum et al., 1990). Thus, an appealing hypothesis would be that editing is regulated by the lipogenic state of the animal. ApoB-48-containing VLDL have been shown to have a faster plasma clearance rate than those containing apoB-100 (Elovson et al., 1981; Sparks and Marsh, 1981; Stalenhoef et al., 1984) and an increase in editing
could prevent accumulation of the triacylglycerol-rich VLDL particles in the plasma. However, several studies have shown the above hypothesis does not always hold true. For example, dexamethasone administration resulted in hepatic triacylglycerol levels that were 2-fold higher than those seen in the fasting high carbohydrate refeeding study (Baum et al., 1990) but editing was not altered by dexamethasone. In addition, editing activity decreased following supraphysiological doses of estrogen despite enhanced hepatic triacylglycerol accumulation and secretion (Seishima et al., 1991; Srivastava et al., 1992). Thus, although in some cases there seems to be an effect of lipogenesis on the regulation of apoB mRNA editing, there are circumstances in which the two events are uncoupled.

Sequence Requirements and Assembly of the Editing Complex

A single change in a transcript of such immense size leads to intriguing questions of specificity and mechanisms. The apoB gene spans 43 kilobases, containing 28 introns and 29 exons, and is unusual because it is only 3 times larger than the transcript it encodes and it also contains the longest known exon. Exon 26 contains 7572 nucleotides which includes the edited C at residue 6666. Much work has been done to establish how the sequences flanking (encompassing) residue 6666 may effect the editing process. Many advances in the understanding of the editing reaction have been made since it was found that cell extracts possessing editing capabilities could also edit synthetic apoB RNA in vitro (Driscoll et al., 1989). In these early experiments deletion mutants were used with the primer extension assay to show that at least 55 nucleotides of sequence spanning the editing site were required to maintain the editing level (Driscoll et al., 1989; Shah et al., 1991). Transcripts containing
42 nucleotides (residues 6648-6689) and 26 nucleotides (residues 6662-6687) were edited at 62 and 24% efficiency, respectively, of the 55 nucleotide transcript (Shah et al., 1991).

Although, Davies et al. (1989) found that a 26 nucleotide transcript also had comparable editing compared to larger transcripts when these were transfected into the McArdle 7777 rat hepatoma cell line. This study also provided tissue-specificity evidence for the reaction since transfection of cell lines that do not synthesize B-48 did not result in the editing of transfected transcript. Chen et al. (1990) found the sequence requirements to be lax when changes involved the 3 bases on either side of the edited C in the *in vitro* reaction. Numerous experiments have shown other more distal regions on the transcript are involved in the editing efficiency and specificity. Shah et al. (1991) reported that mutations in the region spanning nucleotides 6671-6681 resulted in drastic reductions in editing activity (< 20% of wild type transcripts) and the authors speculated that this region may represent a sequence-specific binding site for the editing enzyme.

Another group simultaneously reported the requirement of the 6671-6681 region but suggested that two additional sequence elements are also necessary for efficient editing. They presented evidence for a 'spacer' sequence located at nucleotides 6667-6670 which has lax sequence specificity but critical length requirements for editing. A 'regulator' region between nucleotides 6661-6665 was reported to modulate editing efficiency (Backus and Smith, 1991; Backus and Smith; 1992). Backus and Smith proposed the 11-nt sequence at 6671-6681 is a 'mooring sequence' reported to be necessary for the apparent scanning model that positions cytidine deaminase over the site to be edited without actually docking it to a
specific nucleotide (Smith et al., 1991).

To prove the importance of the 3 sites described above, Smith et al. (1991) were able to induce editing at the usually non-edited cytidine 6434 by correcting flanking sequences to match those of the mooring sequence 3' to the edited C-6666. Support of the mooring sequence model was also provided in a study that showed induction of editing in a heterologous mRNA, the product of a luciferase construct (Driscoll et al., 1993). Based on these data, Backus and Smith (1991) proposed that the 'mooring sequence' directs assembly of an editisome complex which is a high order complex resembling that of a spliceosome or polyadenylation complex. Native gels and glycerol gradient sedimentation of rat liver cytosolic S100 extracts were used to show that a 27S complex with editing activity assembled after 3 hours corresponding to the accumulation of edited apoB mRNA (Smith et al., 1991; Harris et al., 1993). However, whether or not the assembly of a complex is required remains controversial as several labs have disputed this phenomenon with their data generated in an effort to characterize editing. The protein or proteins responsible for editing have an apparent molecular mass of 125 kDa (Driscoll and Casanova; 1990) and sediment through glycerol gradients at 11S (240 kDa) (Greeve et al., 1991). Smith et al. (1991) demonstrated that editing factors assemble first into an 11S complex and then a 27S (1400 kDa) complex over a 3 hour period but other researchers found no lag time was required for catalysis as would be expected for complex assembly (Greeve et al., 1991). Splicesomes and polyadenylation complexes require small RNA molecules to function properly but RNase and DNase treatment was found to have no effect on editing (Garcia et al., 1992; Greeve et al.,
Several strategies have been used to identify protein-RNA interactions at or near the editing site. Lau et al. (1990) used an RNA gel mobility shift assay to show sequence-specific binding of a protein(s) from rat liver nuclear extract to a synthetic 65-nucleotide apoB transcript spanning the editing site. UV-crosslinking and SDS-PAGE demonstrated that a 40 kDa protein-RNA complex formed at or near the editing site (Lau et al., 1990). Recent work has confirmed the UV-crosslinking of a protein of approximately 40-43 kDa and, in addition, report binding of a 60-66 kDa protein (Harris et al., 1993; Navaratnam et al., 1993a). Harris et al. (1993) demonstrated that the two proteins co-sedimented with editing activity and were present in liver and enterocyte extracts although yield and kinetics differed between extracts. Work by another lab indicated that p60 (60 kDa) binding is centered on nucleotides 6671-6674 and the authors hypothesized that p60 contains the RNA sequence recognition component of the apoB editing enzyme (Navaratnam et al., 1993).

Classification of the Editing Reaction

The simplest and most likely mechanism for the conversion of C to U in the editing
reaction is site-specific cytidine deamination. Driscoll et al. (1989) and Bostrom et al. (1989) first proposed the reaction to be a hydrolytic deamination of the specific cytosine at position 4 and numerous experiments have supported this hypothesis. In vitro editing of RNA labelled with $[\alpha-^{32}\text{P}]\text{CTP}$ was shown to result in $[^{32}\text{P}]\text{UMP}$ at the editing site, thus ruling out the possibility of nucleotide excision, replacement and religation since the $^{32}\text{P}$-labelled phosphate of the base remains at the site (Hodges et al., 1991). However, these findings do not rule out the possibility of base exchange through transglycosylation. A dual-labeled RNA substrate in which $[5-^{3}\text{H}]\text{CTP}$ and $[\alpha-^{32}\text{P}]\text{CTP}$ were incorporated was used in in vitro editing reactions. Analysis of the edited RNA showed that $[^{3}\text{H}]$-cytidine also was converted to $[^{3}\text{H}]$-uridine, eliminating base exchange as a possible mechanism (Johnson et al., 1993). Although, these data were unable to formally exclude transamination, the inability to identify a reversible reaction or required cofactors strongly argue for deamination. In view of these findings, it appears that editing is performed by a cytidine deaminase or that if a higher order complex is formed then one of the components would have cytidine deaminase activity. Based on these likely conclusions, the dependency of the editing reaction on zinc was investigated because all known deaminases contain a zinc atom within their active sites (Frick et al., 1989; Yang et al., 1992). Recent work showed that preincubation of rat liver editing-competent extracts with the zinc-specific chelator 1,10-o-phenanthroline virtually inhibited editing activity (98% inhibited relative to control) in in vitro reactions (Barnes and Smith, 1993).
Cloning and Characterization of the Editing Enzyme

An elaborate functional complementation assay was developed by Teng et al. (1993) that resulted in the cloning of a rat intestinal protein, designated apoB mRNA editing protein (REPR), of 27 kDa that appears to be the catalytic subunit of the editing enzyme. In support of the multicomponent editing complex is the finding that p27 is not the same size as any of the putative editing site binding proteins identified by UV-crosslinking. In addition, oocyte extracts expressing REPR only edited apoB mRNA in the presence of chicken or rat intestinal S100 extracts, suggesting an interaction of REPR with another factor or factors (Teng et al., 1993). In order to determine if p27 could confer editing activity in mammalian cells, McArdle 7777 cells were transfected with p27. These cells have inherent low editing efficiency. But following transfection with editing enzyme, the enzyme level was increased 10-fold and editing of endogenous apoB mRNA was also increased 2-fold (Driscoll and Zhang, 1994). Transfection of REPR into HepG2 cells, which normally produce only apoB-100, resulted in editing of endogenous apoB mRNA and secretion of apoB-48 protein. In the same study, S100 fractions from human liver when combined with Xenopus oocytes expressing REPR were found to edit a synthetic apoB template. This finding indicated that human liver contains the necessary complementation factors but cannot edit only because it lacks the deaminase enzyme (Giannoni et al., 1994).

Recent cloning of the human editing protein (HEPR) has shown that the enzyme in humans, unlike the rat, is present in substantial amounts only in the small intestine with none detected in the liver (Hadjiagapiou et al., 1994). REPR shares sequence homology with
cytidine deaminase and deoxycytidylate (dCMP) deaminase from bacteria, viruses, yeasts and humans with the principal homology existing around the predicted active site residues that coordinate a zinc atom (Moore et al., 1987). Sequence alignment of human and rat editing protein show high overall homology as each contain His-61, Cys-93 and Cys-96, residues thought to be essential for zinc coordination possibly resulting in a zinc-finger structural motif. COS cells, which normally do not synthesize apoB and lack editing activity, were used as an expression system for REPR in an effort to elucidate the significance of the putative zinc-finger. Site-directed mutagenesis was used to introduce conservative mutations at His-61 and Cys-96 which resulted in abolishing the editing activity (Driscoll et al., 1994). The importance of zinc was once again illustrated when the zinc-chelator 1,10 o-phenanthroline abolished editing activity of rat enterocyte extracts (Navaratnam et al., 1993). The precise role of zinc remains unclear at this time but it could have a structural role in that it is required to form the appropriate conformation that enables the editing protein to interact with RNA or other proteins in essential protein-protein interactions.

A breakthrough in the structural characteristics of the editing protein occurred when Lau et al. (1994) recently reported that the HEPR exists as a homodimer. A complex with an apparent Mr of ~56,000 in its native form was obtained by HPLC, suggesting that the editing protein exists as a homodimer. Further evidence was provided in an experiment that used an antibody to specifically immunoprecipitate an epitope-tagged HEPR and stoichiometric data were consistent with dimer formation (Lau et al., 1994). An intriguing feature of HEPR and REPR is the presence of leucine-zipper-like sequences that could form
a structural motif that reportedly stabilizes the interaction of homo- and heterodimeric protein complexes (Landschulz et al., 1988). Editing activity was abolished by the elimination of the leucine zipper domain of REPR. This suggested a crucial role for the leucine zipper motif although deletions of this magnitude could result in a single structural aberration (Teng et al., 1993). Lau et al. (1994) eliminated disulfide bridge formation, as a possible dimerization mechanism, with the reducing agent DTT but observed no effect on homodimerization.

**Copper Metabolism**

Copper is a transition metal that has an atomic mass of 63.54 daltons. $^{67}$Cu and $^{64}$Cu are the two radioisotopes with the longest half-lives of 61.9 and 12.9 hours, respectively. $^{63}$Cu and $^{65}$Cu are the two stable isotopes of copper and $^{65}$Cu is often used in tracer studies. Copper has three oxidation states although it rarely exists in the Cu$^{3+}$ form. It is usually present as Cu$^{+}$ or Cu$^{2+}$ and shifts between these states depending upon cellular conditions or enzyme action. Copper is most often found in a bound form as free copper ions are highly insoluble. Although it can exist in several oxidative states, in biological systems it is most often present as Cu$^{2+}$ and is a potent oxidizer.

**Absorption and Excretion**

The major site of absorption of copper is in the small intestine but it has also been shown that the human stomach has absorptive capabilities (Bush et al., 1955). Although the precise mechanism of absorption is not known, it appears to involve both a saturable and an
unsaturable process. Low concentrations of copper appear to be absorbed by the active (saturable) transporter while high concentrations rely on the passive (unstaurable) transporter mechanism. Absorption efficiency is influenced by the amount of copper present in the diet. Absorption has been reported to be approximately 56% when 0.8mg Cu/day was ingested and was lowered to 12% when 7.5mg Cu/day was consumed (Tumlund et al., 1989). Metallothionein also appears to have a regulating role in copper absorption. Metallothionein is itself regulated by zinc with the presence of zinc increasing thionein synthesis. Although metallothionein binds both copper and zinc, it has a much higher affinity for copper. High levels of metallothionein could bind copper present in the epithelial cells and sequester the copper in these cells until they are sloughed off and excreted. Therefore, high dietary zinc would reduce the amount of copper that is absorbed and utilized. Precise levels of zinc and copper required for such physiologic interactions are not known.

Like absorption, excretion of copper also appears to be regulatable and therefore provides another mechanism to provide copper homeostasis. The majority of copper excreted in the feces is unabsorbed copper. However, active excretion also occurs via bile into the gastrointestinal tract. When dietary copper levels are high, excretion is elevated in animals and humans but excretion of endogenous copper is reduced with lowered levels of copper intake (Tumlund et al., 1989; Linder, 1990). Regulation of both absorption and excretion provide the necessary homeostatic mechanisms to protect from copper toxicity as well as deficiency.
Transport and Storage

Copper is transported to the liver bound mostly to albumin and transcuprein in a short period following absorption (Linder, 1990; Frieden, 1986). Although the majority of newly absorbed copper is transported to the liver a small amount is also transported to the kidney. Copper transported to the liver is immediately incorporated into ceruloplasmin (ferroxidase I), a protein of approximately 150 kDa that contains six (possibly seven) copper atoms. Unlike its loose association with albumin, copper is bound tightly to ceruloplasmin. Ceruloplasmin leaves the liver and enters the blood to deliver the copper to extrahepatic cells via ceruloplasmin-specific receptors (Harris and Percival, 1990). It has been suggested that after part of the copper is removed from ceruloplasmin at the cells, these ceruloplasmin molecules return to the liver where copper and protein are transferred to the bile for excretion. The liver represents the main organ of copper metabolism as illustrated by its high levels of storage, relative to other tissues. Copper is taken up by the liver rapidly by a saturable transporter and once inside the cell is dispersed between the cytosol and nucleus with about one-fourth of cellular copper accumulating in the nucleus (Smeyers-Verbeke et al., 1977). The majority of cytosolic copper is bound to metallothionein although copper enzymes also bind a significant amount (Owen, 1982).

Biological and Physiologic Functions

In the body, copper functions as part of copper-enzymes, as well as binds to storage and transport proteins. The copper-containing enzymes are involved in many different areas of metabolism which consequently can effect a large number of physiologic processes. A
list of the copper-containing proteins in humans is shown in Table 2 (Tummlund, 1994). Some of the enzymes are briefly discussed in the following section in order to provide information as to how and why certain copper deficiency conditions develop.

**Amine Oxidases.** There are several important amine oxidases that require copper to function. Monoamine oxidase inactivates catecholamines and reacts with serotonin, norepinephrine, tyramine and dopamine. Diamine oxidases are involved in histamine inactivation and are therefore involved in vital processes such as acid secretion in the small intestine and the allergic response. Diamine oxidase also inactivates polyamines which are involved in cell proliferation. The cupro-enzyme lysyl oxidase deaminates the lysine and hydroxylysine side chains of collagen and elastin. Deamination is required for the essential cross-linking in the connective tissue which includes bone, blood vessels, skin, lungs and teeth.

**Ceruloplasmin.** This enzyme catalyzes the oxidation of ferrous iron and is appropriately also called ferroxidase I. It is involved in the conversion of stored iron to the form of iron that is involved in hemoglobin synthesis. Ferroxidase II also contains copper and oxidizes ferrous iron but in humans it is believed to only be responsible for about 5% of the ferroxidase activity. About 60% of the plasma copper has been estimated to be associated with ceruloplasmin (Wirth and Linder, 1985).

**Cytochrome C Oxidase.** This enzyme is part of the last reaction in the mitochondrial electron transport chain. It is required to reduce \( \text{O}_2 \) to \( \text{H}_2\text{O} \) which allows production of the high energy compound ATP. The molecule contains two or three copper atoms and
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represents probably the most important enzyme in mammalian cells because it is the rate-limiting step in the electron transport chain (Schoenemann et al., 1990).

**Dopamine β-Hydroxylase.** Dopamine in the brain is converted to the neurotransmitter norepinephrine by this enzyme. The enzyme is also present in the adrenal gland where it is required for the production of epinephrine. The most recent evidence estimates there to be about eight atoms of copper per molecule of enzyme (McCracken et al., 1988).

**Superoxide Dismutase.** Copper/zinc superoxide dismutase (SOD) contains two atoms of copper and is present in the cytosol within most cells of the body. The enzyme converts the superoxide ion to hydrogen peroxide thereby protecting against oxidative damage. Extracellular superoxide dismutase (EC-SOD) also reduces superoxide levels but does not contain zinc.

**Tyrosinase.** This enzyme is essential for melanin synthesis by converting tyrosine to dopamine and dopamine to dopaquinone. Tyrosinase is present in the melanocytes of the eye and skin and is required for the color in these organs.

Copper Deficiency in Animals

The information in the previous section helps to explain many of the symptoms that have been observed in copper-deficient animals. Many conditions manifest in all species while others only manifest in a particular species which complicates the deduction that it is due solely to copper-deficiency. Anemia, neutropenia and osteoporosis have been seen in all copper-deficient species studied. There are numerous other manifestations in animals that
include skeletal abnormalities; neonatal ataxia; impaired keratinization of hair, fur, and wool; reproductive failure, including low fertility, and fetal death; cardiovascular disorders including degeneration of myocardium, cardiac hypertrophy and failure, rupture of blood vessels, and electrocardiographic changes; and impaired immune function.

Copper Deficiency in Rats

Many studies have been conducted to examine and explain the manifestations of copper-deficiency in the rat model. Due to the overwhelming amount of work performed on this topic, only a few of the more recent and pertinent findings will be discussed in this section.

One of the most dramatic findings in this area is the effect of copper deficiency on cholesterol metabolism. Numerous investigators have reported the development of hypercholesterolemia in copper-deficient rats (reviewed by Lei, 1990). In rats, about 85% of plasma cholesterol is associated with HDL and most of the increase in copper deficiency is seen in the HDL fraction, however, there is also an increase in LDL cholesterol (Lei et al., 1983; Al-Othman et al., 1989). Numerous studies have been performed to determine the mechanism(s) responsible for the increases in plasma cholesterol. Increases in plasma cholesterol concentrations occur even though there is a drastic increase in the plasma pool size of copper-deficient rats (Lei et al., 1983). If the plasma volume increase is considered, there is a twofold increase in the plasma cholesterol pool size (Carr and Lei, 1990). Increases in plasma cholesterol occur for both free and esterified cholesterol and neither form appears to be preferentially increased (Lei, 1978).
Despite the increase in plasma cholesterol there is a small decrease in hepatic free and esterified cholesterol (Lei, 1978; Lefevre et al., 1985). Even though hepatic cholesterol decreased, there was an increase in the enzymatic activity of hepatic HMG-CoA reductase (Yount et al., 1990), the rate-limiting step in cholesterol biosynthesis. In support of this finding, in vivo studies using $[^{14}\text{C}]$mevalonate and $[^{3}H]$water as precursors were used to examine cholesterol synthesis (Shao and Lei, 1980; Yount et al., 1991). Data from both studies confirmed there to be increased cholesterol synthesis in the liver of copper-deficient rats.

The liver can also accumulate cholesterol by binding and removing cholesterol from plasma lipoproteins. In a recent study, Koo et al. (1992) showed that hepatic uptake of LDL by the apoB, E receptor is increased by copper deficiency. HDL also transports cholesterol and it is through a process called ‘reverse cholesterol transport’ that cholesterol from peripheral tissues is transported back to the liver in HDL particles. Carr and Lei (1990) provided data supporting that copper deficiency increases hepatic uptake of HDL cholesterol esters in conjunction with its increased plasma clearance. In contrast, no significant change in the elimination of hepatic cholesterol either by bile acid production or biliary sterol excretion was observed (Lei, 1978; Allen and Klevay, 1978). Therefore, cholesterol efflux from the liver must be increased in copper deficiency to account for the increase in synthesis, no change in excretion and a slight decrease in cholesterol concentration. In support of this apparent phenomenon, Shao and Lei (1980) observed an increase in the efflux of cholesterol from the liver to plasma in copper-deficient rats.
Other changes in lipid metabolism also result from copper deficiency in the rat. An in vitro study showed there to be a twofold increase in the capacity of rat liver slices from copper-deficient rats to synthesize fatty acids (Lei, 1977). Another study confirmed the increase in fatty acid synthesis also occurs in vivo. In the in vivo study, the radioactive substrate [1-\textsuperscript{14}C] acetate was injected into anesthetized rats and incorporation into various lipids determined. Data were expressed as dpm/liver/100g body weight to correct for the relative increase in liver weight and decreased body weight observed in copper deficiency. A twofold increase in hepatic fatty acid synthesis was observed in copper-deficient rats. Another objective of this study was to determine the fate of the newly synthesized fatty acids. The incorporation of \textsuperscript{14}C-acetate into fatty acids of triacylglycerols and phospholipids (dpm/liver/100g body weight) was elevated 2.0 and 2.3-fold, respectively, in the copper-deficient rats. In spite of the increased hepatic lipogenesis, no difference in liver triacylglycerol concentrations was observed (Al-Othman et al., 1993). This might suggest that the increased lipogenesis is used to support lipoprotein production and the hyperlipidemia observed in copper deficiency. Another study was undertaken to examine changes in the lipoprotein profiles and specific lipoprotein composition in each lipoprotein fraction (Al-Othman et al., 1992). Data revealed the percent composition of protein was reduced by one-half and triacylglycerols were increased by 1.6 and 2.7-fold in LDL and VLDL fractions, respectively. VLDL particles actually exhibited a decreased percentage of cholesterol and phospholipids in the copper-deficient rats. When data were corrected for the increased plasma pool per body weight in copper deficiency, drastic composition changes
were evident. The plasma pool size of protein, phospholipids and cholesterol of LDL and HDL were increased twofold or more. A 36% reduction in cholesterol and no change in protein or phospholipid pool size of VLDL were observed in copper deficiency. Triacylglycerols were most affected by copper status. VLDL triglyceride pool size was increased sixfold while LDL and HDL triacylglycerols were increased approximately 5 and 2-fold, respectively (Al-Othman et al., 1992). Therefore, copper deficiency appears to enlarge VLDL particle size but not number, increase LDL particle size and number, and increase HDL particle number but not size.

Since apoA-I is the major apolipoprotein component of HDL particles, the effects of copper deficiency on apoA-I metabolism were recently examined. Hoogeveen et al. (1995) used an in vivo study to show apoA-I synthesis was significantly increased by copper deficiency. A pulse-chase study using freshly isolated hepatocytes confirmed that apoA-I synthesis was increased along with secretion while intracellular degradation was not altered by copper deficiency. Increased apolipoprotein production coincides with the apparent increased lipoprotein production in the liver of copper-deficient rats.

Although numerous studies have reported hyperlipidemia in copper deficiency, the purpose or requirement of this condition as well as lipoprotein profile changes have not been elucidated. One of the major roles of triacylglycerols in the body is to function as substrate to provide metabolizable energy. Triacylglycerols would be more heavily relied upon in times of glucose deprivation or an impaired glucose utilization. Several studies have documented an impairment of glucose metabolism in copper-deficient rats (Hassel et al.,
Therefore, increased plasma triacylglycerol transport could be in response to a shift in energy substrate utilization from carbohydrate to fat. In support of this possibility, indirect calorimetry was used to reveal a reduction from 0.85 to 0.80 in the respiratory quotients of copper-deficient rats versus controls. Fat utilization was higher (3.6 vs. 5.1 g/day) while carbohydrate utilization was lower (7.9 vs. 5.2 g/day) in the copper-deficient rats (Hoogeveen et al., 1994).

As with most other trace minerals, many of the manifestations of copper deficiency cannot be explained by the known biochemical functions or properties of copper. In addition, many of the conditions associated with copper deficiency cannot be directly attributed to copper because of its interrelationships with other trace minerals and compounds. However, as research continues and experimental technology improves, the molecular mechanisms of many of the vital functions of copper will be explained.
CHAPTER 3

APOLIPOPROTEIN B PLASMA LEVELS, HEPATIC SYNTHESIS AND SECRETION, mRNA ABUNDANCE AND EDITING

Specific Aims

The project was designed to determine the influence of dietary copper on:

1. In vivo plasma apoB-48 and apoB-100 levels and the plasma apoB-48/apoB-100 ratio.
2. In vitro apoB-48 and apoB-100 synthesis, secretion and intracellular degradation in freshly isolated rat hepatocytes.
3. The abundance of apoB mRNA in the liver.
4. The level of apoB mRNA editing in the liver and intestine.

Study Design

Four experiments were performed to examine the effects of copper deficiency on apoB metabolism. The first experiment examined the influence of dietary copper on in vivo apoB metabolism by determining plasma apoB levels in five copper-deficient and seven copper-adequate rats. Plasma VLDL and LDL fractions were obtained by ultracentrifugation. Following delipidation of the apoB-containing lipoprotein fractions, apolipoproteins were resolved using SDS-PAGE and apoB-48 and apoB-100 were quantitated with a laser densitometer. The second experiment was an in vitro study that utilized a pulse-chase design and freshly isolated hepatocytes derived from four copper-adequate and four copper-deficient rats. This design allowed hepatic apoB-48 and apoB-100 synthesis, secretion and
intracellular degradation to be determined in both dietary treatment groups. The third and fourth studies were performed to determine the influence of copper on pretranslational events in apoB synthesis. The third study examined the effects of copper on hepatic apoB mRNA abundance as determined by dot blot analysis in six rats from each treatment group. Hepatic and intestinal apoB mRNA editing were measured by the PCR-cloning-colony hybridization method in five copper-adequate and five copper-deficient rats for the fourth and final experiment of this study.

Experimental Procedures

Experimental Animals and Diets

Male weanling Sprague-Dawley rats were randomly assigned to either a copper-deficient (9.0 μmol Cu/kg diet) or copper-adequate (102 μmol Cu/kg diet) dietary group. Rats were housed individually in stainless steel cages with a room temperature of 22±2°C and 12 hour light (0700 to 1900 H):dark cycle. Animals were provided free access to their respective diets and distilled demineralized water for 6 weeks. Rats were anesthetized by subcutaneous injection of 10 mg sodium pentobarbital/100 g body weight and exsanguination performed by ether inhalation. Animals were deprived of food overnight prior to sacrifice and all procedures were approved by the Animal Care and Use Committee of the University of Arizona. Basal diet was prepared according to the AIN recommendations (AIN, 1980). Copper carbonate was added to the basal diet to provide 102 μmol Cu/kg diet. Copper levels in the diets were determined by atomic absorption spectrophotometry (model 180-70, Hitachi). The composition of the copper-adequate and
Table 3. Diet Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Copper Adequate</th>
<th>Copper Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Monohydrate</td>
<td>64.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Cellulose (fiber)</td>
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<td>5.0</td>
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<tr>
<td>Corn Oil</td>
<td>5.0</td>
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<tr>
<td>AIN Mineral Mix</td>
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<tr>
<td>AIN Vitamin Mix</td>
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<td>1.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
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<td>0.3</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
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<td>0.2</td>
</tr>
<tr>
<td>Cupric Carbonate - Mix</td>
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<td>0.0</td>
</tr>
<tr>
<td>Dietary Copper Content</td>
<td>102.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

\( \mu \text{mol Cu/kg diet} \)

1Contains all minerals except copper.

2As determined by atomic absorption spectrophotometry.
copper-deficient diet are both shown in Table 3.

Plasma Lipoprotein Isolation and Apolipoprotein Quantitation

Animals were anesthesized and blood withdrawn by cardiac puncture and collected into tubes containing 1mg/ml EDTA. Plasma was isolated by centrifugation at 1000 x g for 20 min at 4°C. VLDL and LDL particles were floated into overlayer buffer during ultracentrifugation by a method described by Radding and Steinberg (1960). Solid KBr was added to achieve a density of $\rho_{1.063}$ and then 5 ml of overlayer buffer of the same density were placed on top. Samples were centrifuged at 171,000 x g for 18 hrs at 4°C and then the top 3 ml were removed by aspiration. Samples were concentrated and KBr removed by washing with PBS in ultrafiltration membrane cones (CF25, Amicon Corp.) and centrifuged at 1000 x g for 15 min at 4°C. This method provides >95% protein retention and is used routinely in our lab because we have found it to be superior to dialysis. Lipoprotein fractions from plasma were delipidated according to the method of Herbert et al. (1978) with slight modifications developed in our lab. Lipoprotein solution (250 µl) was added slowly to 3 ml of vortexing methanol, then 7 ml of diethyl ether was added and the mixture placed on ice for 10 min. These tubes were centrifuged at 200 x g for 15 min at 4°C and supernatant removed by aspiration. Ten ml of diethyl ether were added to the remaining slurry before tubes were placed on ice for 10 min and then centrifuged as before. Supernatant was removed by aspiration and the remaining ether evaporated under a stream of nitrogen. Protein was then solubilized in 6M urea and quantitated by the method of Bradford (1976), using BSA as the working standard, prior to adding sample buffer (6M urea, 1% SDS, 0.05M
Tris-HCl, 2% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8). Purified apoB-48 and apoB-100, previously used by our lab for antibody production, was loaded in several concentrations to provide calibration curves for band quantitation. Samples were heated at 90°C for 5 min and applied to a discontinuous 3.5-15% SDS polyacrylamide vertical slab gel (Laemmli, 1970). Gels were stained with 0.25% Coomassie Brilliant Blue R-250, destained and scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Bands were identified by comparison to molecular weight standards (Amersham Corp., Arlington Heights, IL) and quantitated using standard curves generated from purified apoB-48 and apoB-100. A linear relationship existed between the amounts of purified apoB applied to the gel and the absorbances determined by laser scanning.

ApoB Purification and Antibody Production

Rats were injected with 250 mg/kg body weight of Tyloxapol (Sigma, St. Louis, MO) via the femoral vein and blood collected 24 hours post-injection into a syringe containing 10 mg EDTA. Plasma was obtained and the VLDL and LDL fractions collected, washed and delipidated as described above. Dried protein was reconstituted in SDS-PAGE sample buffer (6M Urea, 1% SDS, 0.05M Tris-HCl pH 6.8, 2% B-mercaptoethanol, 0.01% bromophenol blue) and electrophoresed in 4% polyacrylamide. ApoB-48 and apoB-100 bands were each cut from the gel and transferred to dialysis tubing, followed by electroelution at 30V for 24 hours. Dialysis tubing volumes were reduced with Aquaside III (Calbiochem, La Jolla, CA) and ApoB-48 and apoB-100 were quantitated colorimetrically (Bradford, 1976) prior to aliquotting and lyophilization. Five-hundred µg of combined apoB-48 and apoB-100 were
emulsified in a mixture containing Freund's complete adjuvent (Sigma) and subcutaneously injected into the sheep. Three-hundred μg of antigen were used for secondary and tertiary injections with 30 days between each injection. Antisera was screened for anti-rat apoB activity and monospecificity by Western blot analysis against rat plasma proteins using anti-sheep IgG alkaline phosphatase-labeled antibody (Sigma). Titer of antisera continued to improve following each injection and final antisera collection was performed 2 weeks following the third immunization and prepared as described (Harlow and Lane, 1988). Preimmune sera did not contain anti-rat apoB antibodies as determined by Western blot analysis and immunoprecipitations.

Cell Isolations and Pulse-Chase Analysis

Rat hepatocytes were isolated from Cu-deficient and Cu-adequate rats via in situ collagenase perfusion. The cell isolation method was based on that of Berry and Friend (1969) including modifications recently described in detail by Zhang and Lei (1990). Isolated cells were washed and then kept on ice in M199 culture medium with Earle's salt solution (Sigma) while cell number and viability was determined by trypan blue exclusion. Cell average viability was ≥90% with no differences between dietary groups. 210 x 10^6 viable cells were then immediately suspended in 7 ml of phenylalanine-free MEM containing 0.1mM nonessential amino acids [MEM Select-amine kit, Life Technologies Inc., Grand Island, NY], 20mM HEPES (pH 7.4), 1mM sodium pyruvate, penicillin 100 units/ml, and streptomycin 100μg/ml. The medium contained L-[2,3,4,5,6-3H] phenylalanine (130 Ci/mmol, Amersham Corp.) at 142 μCi/ml for labeling of proteins. Following 20 minutes
of pulse, the cell suspension was centrifuged at 300 x g for 1 min and the medium removed. Cells were washed one time with PBS (37°C) and then centrifuged at 300 x g for 1 min. Pelleted cells were then resuspended in MEM containing 0.1mM phenylalanine at 30 x 10^6 cells/ml. Cell suspension was chased at 37°C and at each time point 1 ml (30 x 10^6 cells) was removed and immediately placed on ice for 2 min. Cells were pelleted at 300 x g for 30 seconds at 4°C and the clear supernatant was removed and adjusted to 150mM Tris-HCl (pH 7.4), 150mM NaCl, 5mM EDTA, 0.1875M sucrose, 0.5% Triton X-100, 0.5% sodium deoxycholate, and protease inhibitors (2mM PMSF, 100μM TPCK, 100 KIU aprotinin/ml, 10μg leupeptin/ml and 100μg benzamidine/ml) prior to centrifuging at 15,000 x g for 15 min at 4°C. This 15,000 x g supernatant was frozen immediately and represents the medium samples. The 300 x g pellet was resuspended in 150mM Tris-HCl (pH 7.4), 150mM NaCl, 5mM EDTA, 0.1875M sucrose, 0.5% Triton X-100, 0.5% sodium deoxycholate, together with protease inhibitors in a volume of 1.5 ml and then centrifuged at 15,000 x g for 15 min at 4°C. Cell samples (300 x g pellet) were sonicated for 3 cycles of 20 seconds and then snap frozen. The 15,000 x g supernatant from both medium and cell samples represent the detergent soluble fraction (DSF) used for analysis.

Immunoprecipitations and SDS-PAGE

One-hundred μl of the detergent soluble fraction from each sample were combined with an equal volume of NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris-HCl, pH 7.4) and anti-rat apoB antisera (adjusted to 100μg/ml PMSF) and incubated overnight at 4°C. Protein G (Pharmacia LKB, Piscataway, NJ) was then added to each sample followed by a
1 hour incubation at 4°C. The amount of antisera and protein G required were determined empirically. Supernatant from the first precipitation was again precipitated to assure that all labeled apoB was quantitatively immunoprecipitated. Following protein G incubation, samples were centrifuged at 16,000 x g for 30 seconds at 4°C and precipitates washed 3 times with 1 ml of buffer (150mM NaCl, 5mM EDTA, 50mM Tris-HCl, pH 7.4, 0.5 % Triton X-100, 0.1% SDS). Final precipitates were resuspended in sample buffer and electrophoresed on 3.5-15% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue and apoB-100 and apoB-48 bands in the samples were identified according to purified apoB on the same gel. ApoB-100 and apoB-48 bands were excised from the gel and radioactivity associated with each band was determined by liquid scintillation counting. ApoB-100 and apoB-95 bands were excised from the gel as one slice so that apoB-100 counts also include apoB-95 counts. ApoB was not precipitated when preimmune serum was used for immunoprecipitations.

Isolation of Total Cellular RNA from Rat Liver and Intestine

Sprague-Dawley rats were killed and livers perfused with 35 ml ice-cold phosphate saline (10mM Na phosphate, pH 7.4, 154mM NaCl 1mM EDTA) and then quickly excised. Upper intestinal segments were also excised and flushed with the same solution. RNA was isolated using TRIzol Reagent (Life Technologies Inc.), a mono-phasic solution of phenol and guanidine isothiocyanate, developed to simplify the method of Chomzynski and Sacchi (1987). Two ml of TRIzol were added to 0.2 g of tissue and samples were homogenized using a Polytron at 10,000 rpm for 30 seconds. The remainder of the procedure was
performed according to the manufacturer's protocol.

Northern Blot and Dot Blot Analyses

For Northern blot analysis, rat liver total RNA was fractionated on 0.8% agarose, 6% formaldehyde gels prior to capillary transfer to Zeta-Probe (Bio-Rad, Richmond, CA) nylon membranes. For dot blot analysis, serial dilutions of RNA from 0.5 to 8μg were blotted using a vacuum manifold (Bio-Rad, Richmond, CA). Linearity of the calibration curve allowed the quantitation of RNA in the samples. Filters were hybridized with the following 32P-labeled probes: a 2.9Kb rat apoB cDNA which spans the editing site, a mouse β-actin cDNA fragment of 270bp and a rat 18S rRNA antisense oligomer (Omiecinski et al., 1990). Membranes were stripped of each probe by washing twice for 20 min in 0.1X SSC/0.5% SDS at 95°C and then hybridized to the next probe. Autoradiography was performed at -80°C and films were analyzed by laser densitometry (Molecular Dynamics).

Apo-48 to ApoB-100 mRNA Ratio

The amount of edited apoB mRNA in liver and intestine total cellular RNA was determined by the PCR-cloning-colony hybridization technique as previously described (Wu et al., 1990). PCR was used to amplify a 541-base pair fragment that spans the editing site. Primers used for cDNA synthesis and subsequent PCR were 5'-AATGATG CCTTTGACGAGCCC-3' at the 5' end and 5'-CGGATCTGATTTACACGG ATATGA-3' at the 3' end. A 20μl reaction mixture (2μl of 10X RT Reaction buffer, 2μl of 10mM dNTP mixture, 2μl of RNAsin 40KU/ml, 5μl DEPC H2O, 1μg each of RNA and primers, and 50
U reverse transcriptase) was incubated at 42°C for 45 min for cDNA synthesis. A 100μl PCR reaction mixture was set up by adding 80μl of reagent mixture (10μl Gene Amp 25mM MgCl₂, 10μl PCR buffer II 10X, 16μl 1.25mM dNTP, 10μl DMSO, 34μl DEPC H₂O) to the 20μl cDNA reaction sample. A Perkin-Elmer Cetus DNA amplification kit was used to establish the reaction conditions above. The reaction was incubated at 92°C for 1 min before adding 5 units of Taq polymerase. Thirty cycles of 58°C/6- min extension and annealing and 92°C/1 minute denaturation with a 12 min last cycle for equalization of ends were performed using a thermocycler (Perkin Elmer Cetus, Norwalk, CT). Fragments were partitioned on a 4.5% agarose gel, extruded through a 20g needle, extracted with phenol and ethanol precipitated followed by EcoRI/BamHI digestion. pGEM-3Z was also cut with EcoRI/BamHI and purified, linearized plasmid was ligated with the 541-bp PCR fragment. The ligated product was used to transform competent JM109 bacterial cells by the method of Hanahan (1983). Cells were seeded on X-gal, IPTG, ampicillin plates and incubated overnight at 37°C. Colonies were lifted onto nitrocellulose membranes and amplified on chloramphenicol plates (170μg/ml) at 37°C overnight. Membranes were then placed in 10% SDS; denatured with 0.5M NaOH, 1.5M NaCl; neutralized with 0.5 M Tris-HCl (pH8.0), 1.5M NaCl; rinsed 3 times with 6X SSC; and baked in a vacuum dryer at 80°C for 2 hours. Membranes were then washed in 6X SSC, 0.5% SDS at 58°C for 2 hours and then prehybridized overnight in 6X SSC, 5% Denhardt’s solution, 0.5% SDS and 0.2μg/ml denatured E. coli DNA, at 58°C. The sequence-specific hybridization probes used were B-STOP(5'-TACTGATCAAAATTATATCG-3') and B-GLN(5'-TACTGATCAAAATTGTGA
TCG-3') each of which was end-labeled with $[\gamma^{32}\text{P}]-\text{ATP}$ and T4 polynucleotide kinase. Hybridization of B-STOP was performed at 48°C while 50°C was used for the B-GLN probe. Hybridization was performed for 16 hours in prehybridization solution, followed by washing in 6X SSC, 0.1% SDS at room temperature for 20 minutes and then 2X SSC, 0.1% SDS at 50°C for 1.5 minutes for B-STOP or 52°C for 2 minutes for B-GLN. Membranes were hybridized to B-STOP probe and then exposed to Kodak X-Omat AR film at -80°C. B-STOP probe was then stripped by heating to 90°C, and membranes were then hybridized to B-GLN probe followed by film exposure. The ratio of the colonies expressing B-48 or B-100 mRNA allows the percentage of the apoB mRNA that is edited in the tissue RNA sample to be determined.

Data Analysis

Intracellular and media response curves for the in vitro study were analyzed by Repeated Measures Analysis of Variance. Plasma data from the in vivo study as well as all other tests for variances between two means were analyzed by F-Test. Statistical analyses were performed using SAS Software Release 6.09 (SAS Institute, Inc., Cary, NC).

Results

Plasma ApoB-48 and ApoB-100 Levels

Increases in relative liver and heart weights and plasma volume, as well as reductions in body weight, and hematocrit were observed in rats fed the copper-deficient diet for 6 weeks (Table 3). All of these responses are well-established features of copper-deficiency
in rats (Lei, 1991). Thus, the experimental rats were indeed deficient in copper. Apolipoproteins derived from the VLDL and LDL fractions were resolved on 3.5-15% polyacrylamide-SDS gels and scanned with a laser densitometer. Purified apoB-48 and apoB-100 were also used for identification and quantitation of apoB in the samples. Figure 1 shows a tracing in O.D. units generated by the densitometer when the two plasma apoB samples shown in the insert were scanned. Values for each band were calculated by integrating the area under each peak using Imagequant analysis software (Molecular Dynamics).

An almost twofold increase (p<0.05) in plasma apoB-48 level was observed in copper-deficient (63.1 ± 5.0 μg/ml) as compared to copper-adequate (33.0 ± 1.9 μg/ml) rats. Although the plasma apoB-100 level appeared to be higher in copper-deficient (122.3 ± 17.3 μg/ml) than in copper-adequate (88.6 ± 11.9 μg/ml) rats, the difference was not significant (Table 5; Fig. 2). Nevertheless, the average apoB-100 level was significantly higher than the apoB-48 level among the two treatments. In the copper-adequate rats, the plasma apoB-100 level was more than 2.5-fold higher (p<0.001) than the apoB-48 level. However, in the copper-deficient rats, plasma apoB-100 was less than twofold higher (p<0.05) than the apoB-48 level. A differential increase in the amount of plasma apoB-48 became evident when the plasma apoB values were expressed as a ratio of apoB-48/apoB-100. The plasma apoB-48/apoB-100 ratio was significantly higher in copper-deficient (0.55 ± 0.6) than in copper-adequate (0.39 ± 0.03) rats (Table 5). Similarly, when the data were expressed as a molar ratio to reflect changes in the number of apoB molecules, a significantly higher molar ratio
of apoB-48/B-100 was observed in copper-deficient (1.09 ± 0.12) than in copper-adequate (0.79 ± 0.06) rats (Table 5). Thus, the plasma data demonstrated a marked differential increase in apoB-48.

ApoB Synthesis, Secretion and Intracellular Degradation

A pulse-chase study was performed to examine the influence of copper deficiency on apoB metabolism in freshly isolated hepatocytes. The amount of nascent apoB in hepatocytes after a 20 min pulse, which is equivalent to 0 min of chase, was used as a measure of apoB synthesis (Fig.3). Copper-deficiency appeared to have no effect on hepatic apoB synthesis since the amount of newly synthesized intracellular apoB-100 and apoB-48 were not significantly different between the two treatments. Response curves of intracellular and secreted apoB-100 and apoB-48, derived from the chase study, were depicted in Figures 4 and 5. The kinetics of cellular nascent apoB were similar for both treatments in that the amount of labeled apoB-100 and apoB-48 peaked at 10 min chase in both treatment groups. In addition, labeled apoB-100 or apoB-48 were not detected in the medium up to 10 minutes of the chase, but substantial amounts were detected after 20 minutes of chase following the rapid decline in intracellular levels between 10 and 20 minutes. Intracellular apoB-100 and apoB-48 response curves appeared to be higher for cells derived from copper-deficient rats during the chase period although differences between curves did not reach significance. However, a significant cubic time effect was observed for the average apoB-100 intracellular response curve and a significant cubic and quartic time effect was observed for the average intracellular apoB-48 curve among the two treatments. Moreover, the secretion response
curves for both apoB-100 and apoB-48 were significantly elevated for cells derived from copper-deficient rats. Markedly higher apoB-100 and apoB-48 secretion by cells derived from copper-deficient (11.6 ± 4.6 % and 13.9 ± 3.6 %, respectively) than copper-adequate (4.8 ± 1.0 % and 8.3 ± 1.4 %, respectively) rats, were evident by 20 minutes of chase (Figures 4 and 5; Table 6). At this time, the average amount of apoB-48 secreted was higher (p<0.05) than the average amount of apoB-100 secreted by cells from the two treatments. Furthermore, the molar ratio of apoB-48/apoB-100 present intracellularly at the end of the chase period was higher (p<0.06) in the copper-deficient (2.7 ± 0.2) than in the copper-adequate (2.0 ± 0.2) rats.

Marked reductions in intracellular apoB-100 and apoB-48 observed between 10 and 20 minutes of the chase indicated that large amounts of nascent apoB were depleted from the cells (Figures 4 and 5). In addition, the large difference between the amounts of apoB-100 and apoB-48 depleted intracellularly and that secreted into the medium suggest that the majority of nascent cellular apoB-100 and apoB-48 were degraded prior to secretion. When the amount of apoB degraded was expressed as a percentage of apoB depleted intracellularly, within 10 to 20 minutes of the chase, the percentage of intracellular apoB-100 degraded amounted to 95.2 ± 1.0 % and 88.4 ± 4.6 % in cells derived from copper-adequate and copper-deficient rats, respectively (Table 6). Similarly, the percentage of apoB-48 degraded amounted to 91.7 ± 1.4 % and 86.1 ± 3.6 % in cells derived from copper-adequate and deficient rats, respectively. Although, the differences between the treatments did not reach significance for apoB-48 or apoB-100 alone, the percentage of total intracellular apoB (apoB-
100 plus apoB-48) degraded was significantly lower (p<0.05) in the copper-deficient (89.1 ± 1.8 %) than in the copper-adequate (93.4 ± 0.7 %) cells. Moreover, the average percentage of intracellular apoB-48 degraded (88.9 ± 2.1 %) was lower (p>0.05) than the mean percentage of apoB-100 degraded (91.8 ± 2.5 %) for the two treatment groups. Thus, these data suggest that a reduction in intracellular degradation may contribute to the increase in apoB-100 and apoB-48 secretion by the copper-deficient cells.

In view of the large range of variations associated with the intracellular apoB-48 and apoB-100 observed at the end of the pulse, an additional pulse study was performed with more observations and two time-points, 10 min and 20 min, to evaluate the influence of copper deficiency on apoB synthesis (Fig. 3). The intracellular nascent apoB-48 and apoB-100 were expressed as a percentage of nascent apoB-100 observed at 20 min of pulse for the copper-adequate cells (which was set at 100%) to reflect the non-linear response over time and the distinctly elevated amount of newly synthesized apoB-48 over apoB-100 for both treatments. No significant difference in the amount of nascent apoB-48 or apoB-100 were detected among the treatments over time. The accumulation of nascent apoB-48 and apoB-100 between 10 to 20 min of pulse appeared to be half that of the first 10 min of pulse for both treatments. This decline as well as the divergence of the means at 20 min may suggest that in addition to synthesis, other processes such as intracellular degradation, may already be exerting a substantial influence on the amount of nascent apoB-48 and apoB-100 present in the cells. Data from this additional pulse study confirmed that copper deficiency does not alter apoB-48 and apoB-100 synthesis. In addition, relatively short pulse studies of no more
than 10 min should be used in the future to determine intracellular nascent apoB as a measure of apoB synthesis.

ApoB mRNA Abundance and Editing Activity

To examine RNA integrity and specificity of probes, Northern blot analyses were performed on hepatic total RNA. The apoB cDNA probe was found to hybridize to a single mRNA band, with a size of approximately 14.1kb, within the hepatic total cellular RNA samples derived from copper-deficient and adequate rats (Fig. 6). In addition, the 18S synthetic oligo probe detected a single hybridized RNA band of approximately 2,300 nucleotides for both treatments. Thus, no evidence of RNA degradation or non-specific hybridization of radio-labeled probes were detected. Dot blot analyses demonstrated a linear correlation existed between the amount of RNA loaded and the optical density detected for each of the probes (Fig. 7). No significant difference in hepatic total apoB mRNA abundance was detected, when the data were expressed as apoB mRNA per 18S RNA or per μg total cellular RNA, between the two treatments (Fig. 8). Thus, copper deficiency appeared not to alter hepatic apoB mRNA abundance. However, dot blot analysis does not determine the proportion of apoB mRNA that encodes apoB-48 or apoB-100. To establish the influence of copper on apoB mRNA editing, the ratio of apoB-48 mRNA to total apoB mRNA (apoB-48 plus apoB-100 mRNA) was measured by the PCR-cloning-colony hybridization method. This method was developed by Wu et al. (1990) and has proven to be extremely accurate and reliable for detecting small changes in apoB mRNA editing. When editing was expressed as apoB-48 mRNA/total apoB mRNA, it was found to be
significantly increased from 60.8 ± 3.1 % in control animals to 70.2 ± 1.0 % in the copper-adequate rats (Fig. 9). Thus, hepatic editing was elevated 20% in copper-deficient as compared to copper-adequate rats. In contrast, intestinal apoB mRNA editing (Fig. 9) was not altered by dietary copper status (91.4 ± 0.8 % and 87.1 ± 4.4 % editing for copper-adequate and copper-deficient rats, respectively).
Table 4. Effect of Dietary Copper on Body Weight, Organ Weight, and Plasma Hematocrit and Volume

<table>
<thead>
<tr>
<th></th>
<th>Cu-Adequate</th>
<th>Cu-Deficient</th>
<th>ANOVA$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)$^1$</td>
<td>306 ± 7</td>
<td>232 ± 6</td>
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<tr>
<td>Liver weight (g)$^2$</td>
<td>10.86 ± 0.33</td>
<td>10.10 ± 0.47</td>
<td>NS</td>
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<tr>
<td>Liver weight (g/100 g body wt)$^2$</td>
<td>3.48 ± 0.06</td>
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<td>Heart weight (g)$^3$</td>
<td>1.09 ± 0.03</td>
<td>1.65 ± 0.09</td>
<td>&lt;0.001</td>
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<tr>
<td>Heart weight (g/100 g body wt)$^3$</td>
<td>0.35 ± 0.01</td>
<td>0.72 ± 0.06</td>
<td>&lt;0.001</td>
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<tr>
<td>Hematocrit (% PCV)$^2$</td>
<td>50.1 ± 0.40</td>
<td>26.6 ± 2.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma volume (ml/100 g body weight)$^2$</td>
<td>3.87 ± 0.04</td>
<td>6.00 ± 0.25</td>
<td>&lt;0.001</td>
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</tbody>
</table>

$^1$Values represent mean ± SEM from 18 rats.

$^2$Values represent mean ± SEM from 8 rats.

$^3$Values represent mean ± SEM from 14 rats.

$^4$p-values from one-way analysis of variance; NS=not significant (p>0.05).
Figure 1. Plasma apoB-100 and apoB-48 isolated from copper-adequate and copper-deficient rats.

Apolipoproteins were isolated from rat plasma as described in “Experimental Procedures”. Equal amounts of total protein were applied to SDS-PAGE and quantitated by laser densitometry using purified rat apoB as standards. The curves shown above correspond to the respective apoB bands (insert) and are representative samples of each treatment group.
Table 5. Plasma ApoB-48 and ApoB-100 Concentrations and Ratios

<table>
<thead>
<tr>
<th>Plasma ApoB</th>
<th>Cu-Adequate¹</th>
<th>Cu-Deficient²</th>
<th>ANOVA³</th>
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</thead>
<tbody>
<tr>
<td>ApoB-48 (μg/ml)</td>
<td>33.0 ± 1.9⁴</td>
<td>63.1 ± 5.0⁵</td>
<td>p&lt;0.001</td>
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<tr>
<td>ApoB-100 (μg/ml)</td>
<td>88.6 ± 11.9⁴</td>
<td>122.3 ± 17.3⁵</td>
<td>NS</td>
</tr>
</tbody>
</table>

ApoB-48 / ApoB-100 Ratio | 0.39 ± 0.03 | 0.55 ± 0.06 | p<0.05 |

moles B-48 / moles B-100 | 0.79 ± 0.06 | 1.09 ± 0.12 | p<0.05 |

¹Values represent means ± SEM from seven rats.

²Values represent means ± SEM from five rats.

³p-values from one-way analysis of variance; NS=not significant.

⁴The concentration of apoB-100 is higher than apoB-48 (p<0.001) in copper-adequate rats.

⁵The concentration of apoB-100 is higher than apoB-48 (p<0.05) in copper-deficient rats.
Table 6. Hepatic ApoB Secretion and Intracellular Degradation

<table>
<thead>
<tr>
<th></th>
<th>Cu-Adequate(^1)</th>
<th>Cu-Deficient(^1)</th>
<th>ANOVA(^2)</th>
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<tr>
<td><strong>ApoB-100</strong></td>
<td></td>
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<tr>
<td>Secreted (%)</td>
<td>4.81 ± 0.96</td>
<td>11.61 ± 4.61</td>
<td>NS</td>
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<tr>
<td>Degraded (%)</td>
<td>95.19 ± 0.96</td>
<td>88.39 ± 4.61</td>
<td>NS</td>
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<tr>
<td><strong>ApoB-48</strong></td>
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<tr>
<td>Secreted (%)</td>
<td>8.29 ± 1.38</td>
<td>13.94 ± 3.64</td>
<td>NS</td>
</tr>
<tr>
<td>Degraded (%)</td>
<td>91.71 ± 1.38</td>
<td>86.06 ± 3.64</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Total ApoB</strong></td>
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</tr>
<tr>
<td>Secreted (%)</td>
<td>6.14 ± 0.73</td>
<td>10.91 ± 1.77</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Degraded (%)</td>
<td>93.86 ± 0.73</td>
<td>89.09 ± 1.77</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\(^1\)Values represent the means ± SEM from four rats, expressed as a percentage of the decline in intracellular nascent apoB from 10 to 20 min of the chase study.

\(^2\)p-values from one-way analysis of variance; NS=not significant.
Figure 2. Influence of dietary copper on plasma apoB-48 and apoB-100 levels.

Values of each dietary treatment group represent means ± SEM from 7 rats in the copper-adequate group and 5 rats in the copper-deficient group. Differences in apoB-100 between Cu-adequate and Cu-deficient rats and differences in apoB-48 between Cu-adequate and Cu-deficient rats were analyzed by one-way ANOVA. * denotes significant difference from Cu-adequate apoB-48; p<0.05.
Figure 3. Influence of dietary copper on $^3$H-phenylalanine incorporation into newly synthesized immunoprecipitable hepatic apoB.

Values were expressed as a percentage of apoB-100 in Cu-adequate cells after 20 min pulse. Values of each dietary treatment group represent the mean ±SEM from 8 experiments at each time point. Synthesis curves were analyzed by Repeated Measures ANOVA. For the apoB-100 data: Linear time effect (df=1) p<0.001; all other comparisons were nonsignificant. For the apoB-48 data: Linear time effect (df=1) p<0.001; and all other comparisons were nonsignificant.
Figure 4. Influence of dietary copper on $^3$H-phenylalanine incorporation into immuno-precipitable cellular and secreted apoB-100.

Values represent means ±SEM from 4 experiments. Each experiment involved the use of isolated cells, derived from a pair of copper-deficient and adequate rats, for the construction of a complete cellular and secretion response curve for each treatment. Response curves were analyzed by Repeated Measures ANOVA. For the cellular data: Cubic time effect (df=1) $p<0.01$; and all other comparisons were nonsignificant. For the media data: Cu effect (df=1) $p<0.05$; linear time effect (df=1) $p<0.005$; quadratic time effect (df=1) $p<0.05$; and all other comparisons were nonsignificant.
Figure 5. Influence of dietary copper on $^3$H-phenylalanine incorporation into immuno-precipitable cellular and secreted apoB-48.

Values represent means ±SEM from 4 experiments. Each experiment involved the use of isolated cells, derived from a pair of copper-deficient and adequate rats, for the construction of a complete cellular and secretion response curve for each treatment. Response curves were analyzed by Repeated Measures ANOVA. For the cellular data: Cubic time effect ($df=1$) \( p<0.05 \); quartic time effect ($df=1$) \( p<0.005 \); and all other comparisons were nonsignificant. For the media data: Cu effect ($df=1$) \( p<0.05 \); linear time effect ($df=1$) \( p<0.001 \); quadratic time effect ($df=1$) \( p<0.001 \); Cu x time linear ($df=1$) \( p<0.05 \); and all other comparisons were nonsignificant.
Figure 6. Northern blot analysis of hepatic apoB mRNA and 18S rRNA.

RNA was fractionated on 0.8% agarose-formaldehyde gels (50 μg RNA/lane), transferred to nylon membranes, hybridized to 32P-labeled probes and exposed to film. Positions of the 28S and 18S rRNA bands as indicated by ethidium bromide staining are shown.
Figure 7. ApoB mRNA standard curve for the dot blot analysis.
Figure 8. Influence of dietary copper on hepatic apoB mRNA abundance.

Values of each group represent means ±SEM from 6 rats as obtained by dot blot hybridization and laser densitometry. Data were analyzed by one-way ANOVA and no significant difference was found between groups.
Figure 9. Influence of dietary copper on apoB mRNA editing in rats.

Ratio of apoB-48 to total apoB mRNA was determined by the PCR-cloning-colony hybridization method as outlined in "Experimental Procedures." Values of each group represent means ±SEM from 5 rats. * denotes a significant difference between Cu-deficient and Cu-adequate liver when analyzed by one-way ANOVA; p<0.05.
Discussion

Adequate energy substrates must be delivered to tissues by a reliable and yet adaptable process. During times of optimal intake or supply of energy, the system must be able to store substrates in a location and in a form which can be readily utilized later in times of deprivation. Obviously, during these times of deprivation, the stored energy sources must be accessed and utilized in a regulatable fashion. VLDL particles have a vital role in maintaining this whole-body energy equilibrium by participating in both the storage and mobilization of energy substrates. When excessive triacylglycerols are present they are transported by VLDL to the adipose tissue for storage and in times of energy need, VLDL triacylglycerols are utilized by muscle tissue in substantial amounts. In the rat, approximately 50% of the total amount of lipids used for energy in muscle is derived from VLDL triacylglycerols (Wolfe and Durkot, 1985). In short, the major physiological role of VLDL is to transport hepatic triacylglycerols to peripheral tissues. There are four potential sources of VLDL triacylglycerols: 1) hepatic storage pools; 2) non-esterified fatty acids from the plasma; 3) incoming lipoprotein triacylglycerols; and 4) fatty acids synthesized de novo. An increased fatty acid synthesis has been shown to increase triacylglycerol secretion but in rats the newly synthesized fatty acids appear to provide only a minor contribution to the total amount of triacylglycerols secreted in VLDL. The most significant contribution to VLDL triacylglycerols appears to be derived from recycled lipoprotein triacylglycerols (Weiland et al., 1980). HDL, LDL and VLDL particles can all participate in the recycling of lipoprotein triacylglycerols back to the liver. The composition, size, and number of VLDL particles can
vary in accordance with the secretion requirements of the liver. An increase in the size of VLDL particles enhances the amount of triacylglycerols that can be secreted by the liver and the larger particles also have an increased affinity for lipoprotein lipase (LPL) of peripheral tissues. Following hydrolysis by LPL and removal of core triacylglycerols, the size of the particles is reduced and these particles are now more likely to bind hepatic lipase which has a higher affinity for smaller particles (Gibbons, 1990).

Alterations in hepatic lipid and lipoprotein metabolism in copper-deficient rats have been reported by numerous investigators. Hepatic fatty acid synthesis and assembly into triacylglycerols and phospholipids were significantly elevated in the copper-deficient rats. When these data were expressed as per liver per 100g body weight the elevations were found to be more than twofold higher (Al-Othman et al., 1993). A previous study also demonstrated a 2.7-fold increase in the percent composition of VLDL triacylglycerols and when the enlarged plasma pool was considered, this would result in a sixfold increase in the plasma pool size of VLDL triacylglycerols in copper-deficient rats (Al-Othman, et al., 1992).

In order for VLDL production to be increased, the amount of apoB available for VLDL assembly must also be increased. The current study indicates that copper deficiency may increase the amount of nascent apoB-48 and apoB-100 in hepatocytes available for secretion. Because apoB mRNA editing occurs in the rat liver, this animal can secrete both apoB-48 and apoB-100 containing VLDL particles. Other researchers have reported that a high rate of VLDL output results in an elevated apoB-48/apoB-100 ratio, (Windmueller and Spaeth, 1985; Azain et al., 1985). VLDL particles contain only one molecule of apoB per
particle, and due to their distinct functional properties the presence of either apoB-48 or apoB-100 will determine the metabolic fate of the particles. ApoB-48 containing particles have been shown to be catabolized and cleared from the circulation much faster than those containing apoB-100 (Elovson et al., 1981; Wu and Windmueller, 1981; Sparks and Marsh, 1981). Our data also demonstrated an increased apoB-48/apoB-100 molar ratio in hepatocytes from copper-deficient rats at the end of the chase study. Moreover, marked increases in hepatic triacylglycerol synthesis have been reported previously in copper-deficient rats. These findings suggest that copper deficiency may differentially regulate the metabolism of apoB-48 and apoB-100 intracellularly as well as within the circulation.

The major cellular process which regulates the apoB production pathway has been determined, by numerous investigators, to be the intracellular degradation of newly synthesized apoB prior to secretion. Borchardt and Davis (1987) first provided evidence that a large proportion of newly synthesized apoB was degraded prior to secretion and that the intracellular degradation of apoB-48 was lower than that of apoB-100. The current study also found that the majority of newly synthesized apoB was degraded intracellularly. Interestingly, copper-deficiency significantly decreased the amount of total apoB degraded. The data also suggest that differential amounts of degradation may exist between apoB-48 and apoB-100, although these values were not found to be significant. Nevertheless, other researchers, reported higher amounts of apoB-100 to be degraded compared to apoB-48, regardless of treatment (Windmueller and Spaeth, 1985; White et al., 1992). In addition, the amount of secretion was approximately twofold higher in the copper-deficient rats for both
apoB-48 and apoB-100. A number of studies have suggested that both forms of apoB are synthesized in surplus and apoB that does not associate with lipids during translation is destined for degradation (Bostrom et al., 1988; Boren et al., 1990; Borchardt and Davis, 1987; Davis et al., 1989; Sato et al., 1990). Therefore, the presence of appropriate amounts of lipids during apoB translation/translocation would dictate the amount of apoB degraded and, hence, the amount of apoB secreted.

Triacylglycerols and cholesterol have both been implicated in the regulation of apoB degradation. In the copper-deficient rats, an increased recycling of both triacylglycerols and cholesterol back to the liver has been observed by our lab (Carr and Lei, 1990). However, as mentioned previously hepatic fatty acid synthesis and assembly of triacylglycerols are both significantly elevated by copper-deficiency. This observed increase in fatty acid synthesis may at least be partly responsible for decreasing apoB degradation in the copper-deficient rats, based on the work of other researchers. Dixon et al. (1991) reported that the addition of oleic acid to HepG2 cells increased apoB secretion by reducing intracellular degradation. Addition of oleate to the perfusate was also found to stimulate apoB secretion in perfused livers from rats fasted for 24 hours (Salam et al., 1988). Although the exact mechanism is not known, it should be noted that triacylglycerol synthesis occurs in both the rough and smooth ER of rat liver cells (Glauman et al., 1975). Recent work by Furukawa et al. (1992) provided convincing evidence that the ER is the site of early apoB degradation and the availability and abundance of triacylglycerols in the ER may protect nascent apoB from degradation. Davis and his colleagues (1984) have shown that an increase in newly
synthesized fatty acids is associated with an increase in the secretion of newly synthesized apoB. In copper-deficient rats the secretion of triacylglycerols associated with VLDL is increased substantially but newly assembled triacylglycerols appeared not to be accumulated in the cytosolic storage pools since hepatic triacylglycerol levels are not increased (Al-Othman et al., 1993). This would suggest that the triacylglycerols in the liver cells of copper-deficient rats may be located mainly in the ER triacylglycerol pool which is rapidly secreted and would also protect apoB from degradation.

As mentioned earlier, cholesterol has also been proposed to influence the degradation of apoB. The influence of cholesterol on apoB and triacylglycerol secretion appears to be complicated and may differ depending on the cell type and experimental design used. For example, the direct addition of cholesterol to the medium of human primary hepatocytes (Kosykh et al., 1985) or HepG2 cells (Fuki et al., 1989) increased the secretion of apoB. In contrast, Dashti (1992) reported that the addition of free cholesterol to HepG2 cells had no effect on apoB secretion. In the same study, the addition of 25-hydroxycholesterol, a form that is preferentially esterified by the cell was found to increase triacylglycerol, cholesteryl ester, and apoB secretion by 1.4-, 3.2-, 2.5-fold, respectively. The form of cholesterol and method of presentation to the cell may be the vital factors involved in the regulation of apoB secretion. For example, in the study by Dashti (1992), the addition of LDL to the medium of HepG2 cells was found to increase apoB secretion by 50%. This would suggest that recycling of lipoprotein cholesterol could also regulate apoB secretion in vivo. Koo et al. (1992) recently provided evidence that hepatic uptake of LDL by the apoB,E receptor is
upregulated by copper deficiency. "Reverse cholesterol transport" was also shown to be increased since nearly all of the HDL [3H]cholesteryl esters cleared from the plasma of copper-deficient rats were taken up by the liver (Carr and Lei, 1990). In addition, the hepatic activity of HMG-CoA reductase and synthesis of cholesteryl esters were also found to be significantly increased in copper-deficient rats (Yount et al., 1991; Al-Othman et al., 1993). The increased synthesis of triacylglycerols and cholesterol in addition to their increased uptake as lipoprotein components by the liver would provide the liver with a substantial supply of these lipids. Interestingly, hepatic triacylglycerol content is not altered but hepatic cholesterol level is significantly lower in the copper-deficient rats (Lei, 1978; Lefevre et al., 1985; Al-Othman et al., 1993). Moreover, the depressed hepatic cholesterol level is not resulted from increased degradation or biliary excretion (Lei, 1978; Allen and Klevay, 1978). Therefore, hepatic lipid levels appear to be regulated by their increased secretion in the form of apoB-containing lipoproteins, as our data would support. Recycled lipoprotein lipids would be "redirected" into another lipoprotein class (VLDL) that could be more readily catabolized by the extrahepatic tissues. The rat liver is able to enhance peripheral uptake of lipids from lipoproteins by secreting particles containing apoB-48. The present study supports the previous findings that high rates of hepatic triacylglycerol synthesis, as in copper deficiency, are associated with a high rate of hepatic VLDL output and a high apoB-48/apoB-100 ratio (Windmueller and Spaeth, 1985; Azain et al., 1985). In copper deficiency there is a preferential increase in VLDL triacylglycerols (2.7-fold increase in percent composition) instead of cholesterol (75% decrease in percent composition) suggesting that the enhanced
availability of triacylglycerols may be responsible for the reduction in degradation and the increase in secretion of apoB. The preferential increase in VLDL triacylglycerols, as well as an increased cellular apoB-48/apoB-100 molar ratio in copper deficiency may suggest that triacylglycerol delivery to peripheral tissues is the vital process to be maintained. Increased cholesterol synthesis and reuptake by the liver appear to be essential in the maintenance of triacylglycerol-rich VLDL secretion for the optimal delivery of energy to tissues.

That cholesterol may not be the principle regulator of apoB metabolism appear to be supported by the present apoB synthesis and apoB mRNA data. In addition, the supplementation of 25-hydroxycholesterol to HepG2 cells was found to increase both apoB synthesis and the apoB mRNA abundance (Dashti, 1992). In contrast, our data revealed that copper-deficiency did not significantly increase apoB synthesis (apoB-48 or apoB-100) or apoB mRNA abundance. However, copper deficiency did increase the ratio of hepatic apoB-48 to apoB-100 mRNA. Hepatic apoB mRNA editing was increased by 20% in the copper-deficient rats while there was no change in intestinal apoB mRNA editing. Other studies have shown that an increase in hepatic editing is associated with an increase in hepatic lipogenesis (Baum et al., 1990; Harris and Smith, 1992) This could be the mechanism responsible for the increase in hepatic apoB mRNA editing observed in copper deficiency. An increased ratio of apoB-48 to apoB-100 mRNA may contribute to the elevation in apoB-48/apoB-100 cellular molar ratio and would provide another possible mechanism, in addition to the decrease in intracellular degradation, for the elevation in plasma apoB-48 levels. The elevated intracellular apoB-48/apoB-100 molar ratio at the end of the chase period also
suggests that apoB-48 may accumulate in an intracellular pool or compartment that is not susceptible to degradation, which would result in the retention of apoB-48 over a period of time. In times of enhanced apoB secretion, as in the case of copper deficiency, the secretory pathway may be saturated and nascent apoB may be diverted to a protected pool which is not subjected to intracellular degradation. Furthermore, a more rapid association of apoB-48 than apoB-100 with available lipids would preferentially protect apoB-48 from degradation and allow more apoB-48 to accumulate intracellularly than apoB-100. In contrast, copper deficiency appears to regulate the metabolism of apoA-I by increasing the hepatic rate of synthesis and secretion of apoA-I while not altering intracellular degradation (Hoogeveen et al., 1995).

Data from the current study indicated that plasma levels of apoB-48 were significantly higher in the copper-deficient rats when compared to apoB-48 level in controls. In addition, plasma apoB-100 levels appeared to be increased by copper deficiency, although increases were not found to be significant. Normally, apoB-48 containing lipoproteins are catabolized and cleared from the circulation faster than those particles containing apoB-100 (Windmueller and Spaeth, 1985; Elovson et al., 1981; Wu and Windmueller, 1981). The differential increase in plasma apoB-48 could be due to a decreased endothelial lipoprotein lipase (LPL) activity as has been reported in copper deficiency (Lau and Klevay, 1982; Koo et al., 1988). LPL normally preferentially catabolizes apoB-48 particles faster than apoB-100 particles (Elovson et al., 1981) and its activity is increased by insulin levels (Raynolds et al., 1990; Carneheim and Alexson, 1989; Semenkovich et al., 1989) In copper-deficient rats
several studies have documented impaired glucose metabolism that appears to be due to an impaired insulin sensitivity (Hassel et al., 1983; Cohen et al., 1992). Recently, the use of the newly developed mRNA differential display technology (Wang and Lei, 1995) in our lab has detected an increase in hepatic fetuin mRNA abundance. Since fetuin is a potent inhibitor of the insulin receptor tyrosine kinase activity, the observed glucose intolerance and hyperinsulinemia may have resulted from the enhanced expression of the hepatic fetuin gene in copper deficiency. In addition, LPL also requires the presence of apoC-II on the lipoprotein surface for enzyme activation. Our plasma data also showed that in copper-deficient rats, apoC-II levels were reduced to approximately one-half the level of controls (unpublished data). A decreased or compromised activity of LPL, which normally prefers apoB-48 containing particles as substrates, could at least partly explain the sustained increase in plasma apoB-48 to apoB-100 ratios in the copper-deficient rats.

A new whole-body energy equilibrium, resulting from a shift in substrate utilization from carbohydrate to fat induced by copper deficiency, may be the main driving force behind the changes in apoB and lipoprotein metabolism. Indeed, recent findings have established a reduction in respiratory quotients as well as increases in fat utilization and lean body mass in copper-deficient rats (Hoogeveen et al., 1994). Therefore, it would appear that impaired glucose metabolism may force the copper-deficient rats to utilize fat as the major energy source. This shift is accomplished by adjusting apoB metabolism in a manner which would maximize the output of VLDL particles containing optimal amounts of triacylglycerols to support the energy needs of peripheral tissues.
CHAPTER 4

SUMMARY

The present studies were performed to examine the effects of copper deficiency on apoB metabolism in the rat. An in vivo study was used to determine the influence of copper on plasma apoB-100 and apoB-48 levels. Plasma apoB associated with the VLDL and LDL fractions was significantly elevated in copper-deficient as compared to control rats. The overall increase in apoB was mostly due to the significant and preferential increase of apoB-48 in the copper-deficient rats since apoB-100 increases were not found to be significant. Plasma apoB-48 concentrations were increased almost twofold in the copper-deficient rats (63 vs. 33 μg/ml) and when the enlarged plasma pool of copper deficiency was considered, the apoB-48 plasma pool size was increased by more than 2.5-fold.

An increased production or a decreased clearance of apoB-containing lipoproteins could each contribute to the observed increases in plasma apoB. Therefore, an in vitro study was performed to determine the effect of copper status on hepatic apoB metabolism. Hepatic apoB-48 and apoB-100 synthesis, secretion, and intracellular degradation were determined with a pulse-chase design using freshly isolated liver parenchymal cells from copper-adequate and copper-deficient rats. This study revealed that copper-deficiency did not significantly alter the hepatic synthesis of apoB-48 or apoB-100. However, secretion of both apoB-48 and apoB-100 was significantly higher and intracellular degradation of total apoB was significantly lower in cells derived from copper-deficient rats. Moreover, intracellular
levels of newly synthesized apoB-48 and apoB-100, as indicated by the response curves throughout chase, appeared to be higher in the deficient cells but the difference did not reach significance. Lowered rates of degradation could provide the mechanism responsible for the higher levels of apoB secretion by the copper-deficient cells. When the hepatocyte apoB values were expressed as moles apoB-48 per mole apoB-100, a higher ratio of cellular apoB-48 to apoB-100 in the copper-deficient cells was observed at the end of the chase period. This result also suggested the possibility of a preferential retention of apoB-48 over apoB-100 with time. Lowered rates of intracellular degradation and the concomitant increase in secretion of apoB could provide the mechanisms by which plasma apoB levels were significantly elevated in the copper-deficient rats.

Other studies were performed to determine if pre-translational mechanisms may also contribute to the alteration in the metabolism of apoB observed in copper deficiency. Northern blot analysis was performed to verify that the integrity of the isolated RNA and specificity of probes was acceptable for accurate quantitation. Dot blot analysis was then used to determine hepatic apoB mRNA abundance in both dietary groups. No significant difference in hepatic apoB mRNA abundance was detected between groups. However, dot blot analysis does not determine the proportion of apoB mRNA that encodes apoB-48 or apoB-100. Thus, the influence of dietary copper on apoB mRNA editing activity was determined by the PCR-cloning-colony hybridization technique. When editing was expressed as apoB-48 mRNA/total apoB mRNA, it was found that copper deficiency
significantly elevated hepatic editing from 60.8% to 70.2%. In contrast, intestinal apoB mRNA editing was not altered by dietary copper status.

In conclusion, the findings of these studies are in agreement with numerous other reports stating that a major regulatory point in apoB metabolism is located at the co- or post-translational intracellular degradation of nascent apoB. We also found that synthesis of apoB as well as mRNA abundance were not altered, while hepatic editing was indeed modulated by dietary treatment. We have proposed that the main driving force that is responsible for the drastic changes in lipid and lipoprotein metabolism observed in copper-deficient rats is a shift from carbohydrate to fat as the main energy source. Findings from the current studies support this hypothesis and provide new mechanistic information as to how these changes are accomplished.
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