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Regulation of apolipoprotein A-I gene expression in Hep G2 cells depleted of copper by cupruretic tetramine

Zhang, Jin, Ph.D.
The University of Arizona, 1994
REGULATION OF APOLIPOPROTEIN A-I GENE EXPRESSION
IN HEP G2 CELLS DEPLETED OF COPPER
BY CUPRURETIC TETRAMINE

by

Jin Zhang

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

1994
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Jin Zhang entitled "Regulation of Apolipoprotein A-I Gene Expression in Hep G2 Cells Depleted of Copper by Cuprulethic Tetramine" and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: Xin Zhang
ACKNOWLEDGEMENTS

It is my greatest pleasure to thank Dr. David K.Y. Lei, my dissertation advisor, for his support and continuous encouragement during my graduate studies in his laboratory.

I would also like to thank Drs. Bobby L. Reid, Charles W. Weber, William F. MaCaughey, and Patsy M. Brannon for serving on my committee. The excellent technical assistance provided by Phyllis Reid in the preparation of this dissertation is also greatly appreciated.

Finally, I would like to acknowledge the encouragement and support of my husband Matt Y. Wang.
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ABSTRACT

Two cupruretic chelators, 2,3,2-tetramine and diamsar were used to deplete copper from Hep G2 cells. Studies using incremental concentrations of chelators, an average maximal depletion of 45% of cellular Cu amounting to 2.4 or 2.3 pmol Cu/μg DNA were attained after 24 h of incubation with 10 μM of tetramine or diamsar in basal medium containing 0.63 μM of Cu. In time-course studies, maximal depletion of Cu was rapidly reached after only 5 h of treatment with 50 μM of these chelators for cells cultured in basal medium. In long-term Cu depletion studies, cellular Cu was reduced more than 40% as compared to controls when cells were cultured in basal medium containing 20 μM tetramine for 4 passages. At the end of the second passage, the synthesis of total protein and albumin was not altered for tetramine-treated cells. After cells were treated with tetramine for 2 passages, pulse-chase studies were performed to determine apolipoprotein synthesis and secretion. Cells were pulsed for 10 min with [3H]leucine and chased for up to 2 h. Trichloroacetic acid precipitation or immunoprecipitation and SDS-PAGE were used to isolate the nascent total protein or specific apolipoproteins, respectively. A two-fold increase in apo A-I synthesis was observed at the end of 10 min pulse in tetramine-treated cells. Although the amount of nascent apo A-I degraded was not affected, that secreted into the medium was increased 56% by tetramine treatment between 30 and 120 min of the chase. A reporter construct -256 A-I.CAT was transiently transfected into cells treated with tetramine for 2 to 3 passages to determine the influence of Cu depletion on apo A-I gene transcriptional
regulation. In these Cu depleted cells, a 40% increase in CAT activity indicated that the apo A-I gene promoter activity was enhanced. In addition, the ability of apo A-I regulatory protein 1 to repress the CAT activity was not affected by tetramine treatment. Furthermore, gel shift assays indicated that the increased apo A-I gene expression was probably mediated by enhanced binding of hepatocyte nuclear factor 4 and other unknown nuclear factors to site A of the apo A-I gene promoter.
CHAPTER I

INTRODUCTION

Hypercholesterolemia induced by copper (Cu) deficiency has been observed in a number of rodent species, nonhuman primates and humans (reviewed by Lei, 1990a). Marked increases in triacylglycerol concentrations and percent composition of lipoprotein fractions, especially that of VLDL, have been reported in rats (Al-Othman et al., 1992) and in hamsters (Al-Othman, 1991). Moreover, increases of apolipoproteins among lipoprotein fractions (Lei et al., 1983; Croswell and Lei, 1985; Lefevre et al., 1986; Lee and Koo, 1988), particularly that of apo A-I (Hoogeveen, 1993) in HDL, have been established in Cu-deficient rats. In vivo and in vitro studies have demonstrated that Cu deficiency enhanced the hepatic synthesis of fatty acids (Lei, 1977; Al-Othman et al., 1993) and secretion of triacylglycerols and phospholipids (Al-Othman et al., 1993). In addition, increased synthesis and secretion of apo A-I have been observed in vivo in hepatic parenchymal cells (Hoogeveen and Lei, 1992) and in vitro by the liver (Hoogeveen and Lei, 1991) of Cu-deficient rats. Thus, at least in rats, the hyperlipoproteinemia associated with depressed Cu status appears to be the result of the enhanced synthesis of these lipoprotein components.

Although the Cu-deficient rat model is a useful experimental animal for the elucidation of mechanisms responsible for the hyperlipoproteinemia, there is a compelling need to substantiate the animal findings with human studies. Since human studies are expensive and limited by ethical concerns, an alternative approach is to perform in vitro
studies using tissues derived from humans. Hep G2, a liver cell line established from a human hepatoblastoma (Javitt, 1990), was selected as the human cell model for the following reason: among the wide variety of liver-specific metabolic functions expressed by confluent Hep G2 cells are the activities related to lipoprotein, cholesterol and triacylglycerol metabolism (reviewed by Javitt, 1990). The ability of Hep G2 cells to synthesize and secret apolipoproteins A-I, A-II, B, C, and E, as well as cholesterol, triacylglycerols and primary bile acids, established them as an attractive human cell model for studying the biochemical and molecular processes involved in lipoprotein metabolism.

In this research project, the first study was designed to examine the effectiveness of 2,3,2-tetramine in the depletion of Cu from a human hepatoblastoma cell line, Hep G2 cells, over a number of passages. Moreover, the influence of 2,3,2-tetramine induced Cu deficiency on the Hep G2 cell’s ability to synthesize total cellular protein (trichloroacetic acid [TCA] precipitable) and albumin was also evaluated in the second study. The first study established for the first time that 2,3,2-tetramine is effective in maintaining a depressed Cu status over 4 passages and does not alter total protein and albumin synthesis at the end of the second passage in Hep G2 cells. In addition, the second study was performed to examine the influence of 2,3,2-tetramine-induced Cu deficiency on the Hep G2 cell’s capacity to synthesize, secret, and intracellulary degrade apo A-I and B. The specific and marked increase in apo A-I synthesis and secretion indicate that this Cu-depleted human liver cell model may be
useful for the elucidation of the responsible molecular mechanism(s) for the regulation of apo A-I gene expression.

The third study was designed to examine the regulation of apo A-I gene expression in copper-depleted Hep G2 cells. The use of a reporter construct -256 A-I. CAT, in a transient transfection study, demonstrated that the apo A-I gene promoter activity was enhanced in these cells. Moreover, gel shift studies indicated that the increase apo A-I gene expression may be mediated by the increased binding of HNF-4 and other unknown factors to site A of the apo A-I promoter.
CHAPTER II

LITERATURE REVIEW

Plasma Lipoproteins

The plasma lipoproteins are large spherical macromolecular structures containing the most hydrophobic lipids, triacylglycerols and cholesteryl esters in the central core of these particles, and hydrophilic lipids, cholesterol, phospholipids and specific proteins (apolipoproteins) on the surface. They are biosynthesized by the intestine and liver. Their major function is to transport dietary or endogenously synthesized cholesterol, triglycerides and phospholipids to those tissues which utilize exogenous lipids for oxidative metabolism, steroid hormone biosynthesis and maintenance of their membrane integrity. According to the density, size, and net surface charge, there are five major classes of lipoproteins (Table 1): chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) (Gotto, Pownall and Havel, 1986).

Lipoprotein Metabolism

Since the major functions of plasma lipoproteins are to transport cholesterol, triglycerides and phospholipids via the bloodstream to various tissues in the animal body, they are metabolized by different lipoprotein metabolic pathways (Goldstein et al., 1985).

The exogenous pathway has been postulated to be involved in the absorption of dietary cholesterol and triglycerides from the intestine by chylomicrons.
**TABLE 1. Major classes of the plasma lipoproteins**

<table>
<thead>
<tr>
<th>Name</th>
<th>Density range (g/mL)</th>
<th>Diameters (nm)</th>
<th>Molecular weight (Dalton $\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>&lt;0.95</td>
<td>&gt;100</td>
<td>400</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.950-1.006</td>
<td>30-80</td>
<td>18-80</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>25-35</td>
<td>5-10</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>18-25</td>
<td>2.5</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.210</td>
<td>6-12</td>
<td>0.2-0.4</td>
</tr>
</tbody>
</table>

\(^1\text{Table from Gotto et al. (1986).}\)
Chylomicrons are the least dense and largest particles which are synthesized by the intestine in response to dietary fat (Gotto et al., 1986). Dietary fat and cholesterol are absorbed through the wall of the intestine and are packaged along with triglycerides into lipoprotein complex called chylomicrons in the bloodstream. The triglyceride ester bond is cleaved by lipoprotein lipase on the surface of endothelial cells of extrahepatic tissues, and the fatty acids are used by adipose tissue for storage and by muscle for oxidation to supply energy (Cryer, 1981). The remnants of the chylomicrons, high in cholesteryl ester, are cleared rapidly by the liver through receptor-mediated endocytosis (Brown and Goldstein, 1974). Their cholesterol is either secreted into the intestine as bile acids or packaged with triglycerides to form VLDL in the liver (Havel, Goldstein and Brown, 1980). VLDL are rich in triglycerides and contain a smaller amount of cholesteryl ester particles. Three predominant proteins, apolipoproteins B-100, E, and C (apo B-100, E and C) are present on their surface. VLDL are secreted from the liver. Endogenous lipids are transported by VLDL, along with IDL and LDL, through circulation in the so-called endogenous pathway (Havel et al., 1980). The triglycerides of VLDL are hydrolyzed to free fatty acids by endothelial lipoprotein lipase of the capillaries of adipose tissue or muscle and resulting in the removal of apo C. Thus a series of IDL particles, which are smaller in size, enriched in cholesteryl esters but retaining their two surface apolipoproteins (apo B-100 and apo E), are formed (Brown, Kovanen and Goldstein, 1981). They are rapidly removed from the circulation by hepatic LDL receptors (apo B and E receptors) to make new VLDL and bile acids. Those IDL particles that escape hepatic clearance will remain in the circulation for much longer
period of time. They will finally lose their apo E and triglycerides and are converted into LDL, with apo B-100 as their only surface protein (Havel et al., 1980). LDL are the major cholesterol transport proteins in human plasma. Elevated levels of plasma LDL are correlated with increased risk of atherosclerosis (Goldstein, Kita and Brown, 1983). LDL receptors (apo B, E receptors) on the liver and other tissues, which recognize apo B-100 of LDL, are responsible for metabolizing about two-thirds of the LDL. The rest are metabolized by the receptor-independent process (Goldstein et al., 1983).

High density lipoproteins are synthesized by both the intestine and the liver. It has been postulated that HDL are involved in a process of reverse cholesterol transport. According to this hypothesis, HDL remove cholesterol from peripheral tissues and transport this cholesterol to the liver for excretion from the body (Mahley, 1981; Mahley and Innerarity, 1983). HDL may be used for the maintenance of cell membrane phospholipids or they may also transfer cholesterol to other lipoproteins which are later degraded by the liver. Therefore, high levels of plasma HDL are correlated with decreased risk of atherosclerosis.

Apolipoproteins are necessary for intracellular assembly and secretion of lipoproteins, and for stability of lipoprotein particles in plasma. They act as cofactors for enzymes involved in lipid metabolism and mediate the clearance of lipoprotein particles from blood by receptor-mediated pathways. Apo A-I is the principal protein component of HDL and is relatively abundant in plasma. The concentration of plasma apo A-I is an indicator of the level of total circulating HDL. In some mammals,
including human, apo A-I has been reported to be synthesized mainly in the liver and intestine, as a preproprotein of 267 amino acids that is processed during secretion and in the plasma, to a mature polypeptide of 243 amino acids (Lin-Su et al., 1981; Nicolosi et al., 1984). Both sequences of mature and preproprotein have been confirmed by isolation of cDNA and genomic clones of the human apo A-I gene (Breslow et al., 1982; Seilhamer et al., 1984).

Apo A-I, and possibly apo C-I and apo A-IV, are activators of lecithin-cholesterol acyltransferase (Fielding, Shore and Fielding, 1972; Soutar et al., 1975; Steinmetz et al., 1985) and therefore participate actively in the process of reverse cholesterol transport. An elevated level of plasma apo A-I level is associated with a decreased incidence of coronary artery disease. Apo C-II is an essential co-factor for the action of lipoprotein lipase (LaRosa et al., 1970). Apo C-III is a major protein constituent of VLDL and chylomicrons, and it appears to play an important role in their metabolism by inhibiting the hydrolysis of triglycerides by lipoprotein lipase (Wang et al., 1985) and inhibiting the removal of chylomicrons and triglyceride-rich lipoproteins by hepatocytes (Shelburne et al., 1980).

Apo B is an obligatory structural component of chylomicrons, VLDL, IDL, and LDL. There are two kinds of apo B according to their molecular weight (Kane, Hardman and Paulus, 1980; Krishnaiah et al., 1980). The lower molecular weight apo B-48 is synthesized by the intestine and is associated primarily with chylomicrons and chylomicron remnants. The higher molecular weight apo B-100 is synthesized by the liver and is associated with VLDL and LDL. A better understanding of the regulation
of apo B synthesis is of considerable interest because of its essential role in the secretion of hepatic VLDL and in the interaction of LDL with its receptors.

Apo E is present in chylomicrons, VLDL and HDL. Apo E-containing HDL can be taken up by the LDL (apo B,E) receptors and apo E receptors. Different types of apolipoproteins, as well as their molecular weights, major biosynthetic sites, plasma distributions, and functions are listed in Table 2.

Lipoproteins and Atherosclerosis

A high percentage of death in Western countries is caused by atherosclerosis. This disease is initiated by the accumulation of cholesterol in arterial walls which progressively leads to the obstruction of arteries and eventually causes a heart attack (Ross, 1979). All animals require cholesterol for their plasma membranes, but the body cannot tolerate excessive amounts of cholesterol in the blood. An excessive amount of plasma cholesterol will promote the deposition of cholesterol in the arterial walls (Mahley, 1985). Since two-thirds of the cholesterol in human plasma are carried by LDL, the cholesterol that accumulates in the arterial wall is derived from LDL. A high level of LDL in blood will accelerate the development of atherosclerosis. High level of LDL receptor and high level of HDL will decrease the risk of this disease.

Since liver and intestine contribute most of the lipoproteins in circulation, they are influenced by some of the risk factors for atherosclerosis. The examination of the regulation of lipoprotein biosynthesis and secretion by nutritional and hormonal factors is important for understanding lipoprotein metabolism and the involvement of lipoproteins
### TABLE 2. Physical properties and functions of the human plasma apolipoproteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Distribution in lipoproteins</th>
<th>Major function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HDL</td>
<td>LDL</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>28,000</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Apo A-II</td>
<td>17,000</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Apo A-IV</td>
<td>44,500</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Apo B-48</td>
<td>264,000</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Apo B-100</td>
<td>550,000</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Apo C-I</td>
<td>6,600</td>
<td>+++</td>
<td>Trace</td>
</tr>
<tr>
<td>Apo C-II</td>
<td>8,900</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>8,800</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Apo E</td>
<td>34,000</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

1 Table from Chan and Dresel (1990).

2 Chylomicron, at various stages of its metabolism, contains small amounts of all the apolipoproteins except apo B-100. Apo B-48 is present exclusively in chylomicron and its remnants.

3 Plasma apo A-IV is mainly unassociated with lipoproteins in the 1.21 g/L infranate.
in atherosclerosis. To achieve this goal, the human hepatoblastoma cell line, Hep G2, has been used as an *in vitro* model to examine human hepatic functions in recent years. Hep G2 cells, which are derived from human liver biopsies, have characteristics of hepatoblastomas, and retain many of the functions of normal human liver such as synthesis of albumin, lipoproteins, and other liver-specific proteins (Javitt, 1990). Hep G2 cells also secrete apo B as well as most of the plasma apolipoproteins (Knowles, Howe and Aden, 1980). Since the recent introduction of molecular biology techniques to lipoprotein research, important work is now under way to analyze genetic variations of the apolipoprotein genes and gene regulation which contribute to alterations in plasma triglyceride and cholesterol levels and enhanced risk of coronary heart disease.

**Lipoprotein Metabolism in the Human Hepatoblastoma Cell Line, Hep G2**

One of the important functions of the liver is to synthesize plasma lipoproteins. It has been shown that freshly isolated hepatocytes, liver cells maintained in suspension, and hepatocytes in primary culture can retain the ability to synthesize and secrete plasma lipoproteins (Jeejeebhoy *et al.*, 1975; Gravela *et al.*, 1977; Davis *et al.*, 1979). Efforts to understand lipoprotein metabolism have increased interest in the use of tissues derived from humans in metabolic studies. The Hep G2 human hepatoblastoma cell line has generated enthusiasm as a human hepatocyte model and has been validated as a human hepatocyte model for studying the metabolism of many hepatic lipoproteins (Knowles *et al.*, 1980). Hep G2 cells are capable of synthesizing and secreting a broad spectrum of plasma proteins (Knowles *et al.*, 1980). They have also been used extensively as a
model to study the regulation of the synthesis and secretion of a number of human apolipoproteins including apo A-I and apo B (Knowles et al., 1980; Dashti et al., 1987). Many studies designed to examine the regulation of apo A-I and apo B synthesis and secretion have focused on the effects of various nutrients and hormones on Hep G2 cells (Archer, Tam and Deeley, 1986; Ellsworth, Erickson and Cooper, 1986). The levels of secreted apo A-I and apo C-II were increased by the physiological concentrations of estrogen in Hep G2 cells (Tam, Archer and Deeley, 1985) and the rate of apo A-I synthesis was also elevated by this hormone (Archer et al., 1986). Several groups of scientists have reported that the addition of oleate to the culture medium of Hep G2 cells stimulates the secretion of apo B (Ellsworth et al., 1986; Dixon, Furukawa and Ginsberg, 1991). The mechanism of rapid stimulation of apo B secretion is post-translational in nature, as synthesis of apo B is minimally affected by oleate treatment (Dixon et al., 1991). There was a significant decrease in secretion of apo B by insulin in Hep G2 cells (Dashti et al., 1987).

**Copper**

Copper has been considered as an essential trace element since Cu, in addition to iron, is necessary for hemoglobin formation. The healthy adult human body has been estimated to contain 80 mg of total Cu (Cartwright and Wintrobe, 1964). Newborn and very young animals contain higher Cu per unit of body weight than adults of the same species. The Cu level of a newborn is decreased during growth until the adult value is reached. The distribution of total body Cu among the tissues also varies with the
species, ages, and Cu status of the animal (Underwood, 1977). The liver, brain, kidneys, heart, and hair contain relatively high Cu concentration in the animal body. The pancreas, skin, muscle, spleen, and bones represent tissues of intermediate Cu concentration. The pituitary, thyroid, thymus and prostate glands, and the ovary and testis are examples of organs low in Cu (Carlton and Henderson, 1963).

Liver Cu concentrations vary with the species and age of the animal, the chemical concentration of the diet and in various disease conditions (Underwood, 1977). The liver contains 10 to 50 ppm Cu (dry basis) in most normal adult species (Beck, 1956). Sheep, cattle, ducks, frogs, and certain fish exhibit consistently higher liver Cu levels, with normal range of 100 to 400 ppm. Liver Cu concentrations in rats increase rapidly when dietary Cu levels reach 200 ppm. Sheep and cattle probably have a superior capacity to bind Cu in liver since blood Cu levels do not rise in these species with increased Cu intake (Milne and Weswig, 1968). Evans (1973) observed that the major portion of the total hepatic Cu is located in the cytosol, where Cu is bound to the enzyme superoxide dismutase and metallothionein. Liver Cu levels are sensitive to low-Cu intake and are affected by other dietary factors such as zinc, cadmium, iron, and calcium that influence Cu retention in the body through their effects on Cu absorption and excretion (Underwood, 1977).

Both erythrocytes and plasma contain Cu. About 60% of total red blood cell Cu is bound to a protein called erythrocuprein which functions as a superoxide dismutase. In the plasma, Cu is firmly bound to the blue Cu protein ceruloplasmin which is a true oxidase (ferroxidase) involved in iron utilization and in promoting the rate
of iron saturation of transferrin (Osaki, Johnson and Frieden, 1966). In addition to ceruloplasmin, a small proportion of the plasma Cu also exists in combination with albumin and with amino acids. The Cu concentration is usually higher in the plasma than that in the erythrocytes in mammals. The normal range of Cu concentration in the blood of healthy animal is 0.5 to 1.5 µg/mL. Plasma Cu does not increase after meals or decrease during fasting (Cartwright and Wintrobe, 1964). In most species whole blood and plasma Cu levels are similar in males and females. However, plasma Cu is slightly higher in human females than in males. In humans, the normal range of Cu in the blood has been reported to be 105.5 ± 5.03 µg Cu/100 mL for men and 114 ± 4.67 µg Cu/100 mL for women (Cartwright and Wintrobe, 1964). Serum Cu levels are significantly increased in women taking oral contraceptives. Administration of estrogen or stilbestrol can greatly elevate plasma Cu levels in men (Johnson, Kheim and Kountz, 1959). Elevated plasma Cu levels during pregnancy have been reported in women (Hambidge and Droegemueller, 1974). The levels returned to normal in the first few weeks of postpartum. In newborn human infants, serum Cu and ceruloplasmin levels are about one-third of the normal adult range (Fay, Cartwright and Wintrobe, 1949).

Blood Cu concentration can be affected by the status of diet. Cu deficiency can result from low-Cu intake. Additions of Cu to normal adequate diets exert little effect on blood Cu levels. No increase in plasma Cu was observed in rats when the Cu content of the diet was raised from 10 to 50 ppm. However, the plasma Cu concentration was increased two-fold from 1.13 to 2.34 µg Cu/mL when Cu intake was increased to 100 ppm (Milne and Weswig, 1968). Extremely high Cu intake can cause toxic symptoms
in many species. A severe hypercupremia can be caused by a toxic intake of 750 ppm Cu in pigs. On the other hand, this hypercupremia can largely be prevented by the concurrent administration of 500 ppm zinc (Suttle and Mills, 1966). Elements such as zinc, cadmium, and iron that depress Cu absorption can reduce plasma Cu concentrations by high dietary levels. In addition, plasma Cu levels can be affected by many diseases (Underwood, 1977).

Copper Metabolism

Dietary Cu is absorbed in the stomach and portions of the small intestine, particularly the upper small intestine, although the mechanisms are not yet understood. In most species dietary Cu is poorly absorbed. The extent of Cu absorption is influenced by the amount and chemical form of the Cu intake, by the dietary level of several other metal ions and organic substances and by the age of the animal. Cu absorption from the intestine is regulated according to the body Cu needs. In rats and mice, higher intestinal absorption of Cu is observed when Cu was administered at low dosages. Intestinal Cu uptake and transfer to the serosal solution are elevated in Cu deficiency (Schwarz and Kirchgessner, 1974).

Copper occurs in food in many chemical forms which can affect the absorption. Various compounds labeled with $^{64}$Cu have been used for testing the Cu uptake in many species. Chapman and Bell (1963) reported that the relative rate of appearance of $^{64}$Cu in the blood of beef cattle is in the following order CuCO$_3$ > Cu(NO$_3$)$_2$ > CuSO$_4$ > CuCl$_2$ > Cu$_2$O > CuO (powder) > CuO (needles) > Cu (wire). Many dietary factors
influence the uptake of Cu by the gastrointestinal tract. The acidic environment may promote Cu solubility and enhance its transport across the gastric mucosa (Marceau, Aspin and Sass-Kortsak, 1970). Amino acids and high protein diets (Davis, Norris and Kratzer, 1962) increase copper absorption. Dietary phytate (Davis et al., 1962), ascorbic acid (Van Campen and Gross, 1968), thiomolybdate, and fiber (Kelsay, Jacob and Prathers, 1979) appear to complex with Cu and limit its absorption. Dietary fructose also reduces Cu uptake. Bile reduces the reabsorption of secreted Cu. Several dietary metals also affect Cu absorption. These include particularly calcium, cadmium, zinc, iron, lead, silver, and molybdenum plus sulfur. Van Campen and Scaife (1967) demonstrated that high zinc intakes depressed Cu uptake and provided the first direct evidence that Cu competes with zinc for absorption in the rat duodenal segments. Perfusion of the isolated intestine with solution of high zinc content caused some reduction in Cu uptake in the mucosal cells (Oestreicher and Cousins, 1985). However, it is not clear whether excess zinc competes for a specific Cu receptor on the brush border membrane or at intracellular sites or competes for absorbable binding ligands. A reduction in dietary zinc supply has been found to enhance uptake and absorption of Cu as well as zinc (Schwarz and Kirchgessner, 1974). This suggested that a common site may be involved in the uptake of these metals at the brush border membrane. In mice, a high-Ca diet can reduce Cu absorption, compare to a low-Ca diet, due to an increase in intestinal pH. However, diets high in calcium are capable of enhancing Cu toxicity in pigs, presumably due to a lowering of zinc availability (Suttle and Mills, 1966). Iron also interferes with Cu absorption through competition for absorption
binding sites. Molybdenum and sulfate can either increase or decrease the Cu status of an animal, depending on their intakes relative to that of Cu. High dietary levels of cadmium depress Cu uptake, and even a relatively small increase in cadmium intake can adversely affect Cu metabolism when Cu intakes are marginal. The regulatory mechanism for Cu absorption is not well established. At present it seems clear that metal-binding components are involved and that the inhibition of Cu absorption brought about by various metals results from competition for protein metal-binding sites. Little is known about mechanisms responsible for hepatic uptake of Cu.

After absorption, Cu is immediately attached to albumin or to plasma amino acids and the complex is rapidly transferred to the liver. Henkin (1974) indicated that the equilibrium established between amino acid-bound Cu and albumin-bound Cu, may influence how much copper is accumulated by hepatocyte. The amount of Cu absorbed is far in excess of metabolic needs and most Cu is returned to the intestine in the form of bile. In this manner the liver provides the major pathway of Cu excretion via the bile. Cu that is retained by the liver may be stored, presumably bound to metallothionein or used for the synthesis of various Cu-containing proteins.

Cartwright and Wintrobe (1964) reported that out of the 2 to 5 mg Cu ingested daily by man, 0.6 to 1.6 mg (32%) is absorbed, 0.5 to 1.3 mg is excreted in the bile, 0.1 to 0.3 mg passes directly through the bowel, and 0.01 to 0.06 mg appears in the urine. In many species the biliary route is the major pathway of Cu excretion.
Copper Deficiency

Copper deficiency has been observed in a number of animal species. The symptoms of Cu deficiency are influenced by the species, age, and sex of the animal, as well as the severity and duration of deficiency. However, they include anemia, bone abnormalities, demyelination and degeneration of the nervous system leading to neonatal ataxia, impaired reproductive performance, lesions in cardiovascular system and depigmentation of hair, wool, or skin (Underwood, 1977). Anemia has been reported as a common symptom of Cu deficiency in all species. Furthermore, reduction in ceruloplasmin activity has been reported in Cu deficiency. This enzyme is required for the oxidation of ferrous to ferric iron, a necessary step in the transport of iron from tissue to plasma (Osaki et al., 1966). Thus Cu plays an important role in the metabolism of iron through ceruloplasmin and in mobilizing the absorbed iron for hemoglobin synthesis (Evans and Abraham, 1973).

Copper deficiency can cause skeletal abnormalities in many species. In severely Cu-deficient young dogs, a gross bone disorder develops with fractures and severe deformities. The fragile bones from Cu-deficient chicks fracture with less deformation and torque than bone from controls. This may have resulted from a reduction in bone collagen cross-linking (Rucker et al., 1975), since a marked reduction in amine oxidase activity occurs in the bones of Cu-deficient chicks, and collagen extracted from such bones is more easily solubilized than collagen from control bones.

Copper deficient animals also have a nervous disorder characteristic in many species. The ataxia could be prevented by Cu supplementation of the ewe during...
pregnancy. Neonatal ataxia was also observed in Cu-deficient goats, pigs and rats (Underwood, 1977). Swayback is a nervous disorder observed in lambs and this disease is characterized by uncoordinated movements (Underwood, 1977).

Achromotrichia is one of the manifestations of Cu deficiency in rats, rabbits, guinea pigs, cats, dogs, cattle, and sheep. It describes the reduction in growth and abnormal appearance of hair, fur and wool. The associated depigmentation is caused by a reduction of tyrosinase required to convert tyrosine to melanin (O'Dell, 1976). Impaired keratinization, characterized by the appearance of abnormally straight, "stringy" hair, has been observed in many Cu-deficient species. A reduction in the formation of disulfide groups in keratin synthesis may be responsible for the impaired keratinization (Underwood, 1977). The first evidence of cardiovascular disorders was found in Cu-deficient cattle occurring in Western Australia called "falling disease" (Bennetts and Hall, 1939). An outstanding lesion of this disease is a degeneration of the myocardium with replacement fibrosis. Sudden cardiac failure associated with cardiac hypertrophy has been reported in Cu-deficient rats and pigs. Cardiac hypertrophy, and a marked increase in the mitochondrial area of the heart muscles has also been observed in Cu-deficient rats (Goodman, Warshow and Dallman, 1970). O'Dell et al. (1961) demonstrated a derangement in the elastic tissue of the aortas of Cu-deficient chicks. The mortality in these animals appeared to be caused by a rupture of the major blood vessels. The most significant findings were obtained by studying the role of Cu in elastin (and collagen) biosynthesis. Hill, Starcher and Kim (1968) demonstrated that Cu deficiency causes a reduction of lysyl oxidase activity. Thus less lysine is being
converted to desmosine, which is the cross-linkage group of elastin. This results in fewer cross-linkage to be present in the connective tissue and subsequently yielding less elasticity in the aorta.

Copper deficiency in human adults is rare in the United States. Severe Cu deficiency has been reported in the human infant and children with protein-energy malnutrition and in some individuals maintained on total parenteral alimentation (Mason, 1979). The common manifestations of Cu deficiency are severe anemia, leukopenia, neutropenia, occasionally demineralization of bone, decreased pigmentation of skin, and neurological abnormalities (Mason, 1979). Cartwright and Wintrobe (1964) estimated the dietary Cu requirements of infants and children to be 50 to 100 \( \mu g/kg \) body weight/day. The Food and Nutrition Board of the U.S. National Academy of Sciences recommends 2 to 3 mg Cu/day for adults. However, a number of studies have reported that various United States diets provided only slightly more than 1 mg Cu/day (Wolf, Holden and Greene, 1977; Klevay, Reck and Barcome 1979; Lei et al., 1980).

Copper Chelators

\( D \)-penicillamine (DPA) and triethylene tetramine (2,2,2-tetramine) have been used to treat Wilson's disease, a genetic disorder in which excessive copper is accumulated in human body (Walshe, 1982). DPA and 2,2,2-tetramine are capable of binding Cu to their \( \alpha \)-amino and sulfhydryl groups and electronegative nitrogens. Therefore, these two drugs are able to enhance excretion and reduce absorption of excessive Cu stored in the patient. Because of the relatively low inefficiency of both
drugs and the side effects of DPA, scientists have attempted to develop better Cu
chelators. In mouse hepatocyte culture studies, the "cage" chelators such as sar and
diamsar were found to deplete Cu more efficiently than DPA (McArdle et al., 1989).
Cu appears to be bound and encapsulated by the 6 secondary nitrogen atoms of these
caged chelators. They explained that the formation of these stable ring structure complex
may contribute to the enhanced effectiveness in depleting Cu. In addition, various
tetramines and DPA have been used to test their cupruretic responses in experimental
animals (Borthwick, Benson and Schugar, 1980). It has been found that 2,3,2-tetramine
(N,N'-bis [2-aminoethyl]-1,3-propanediamine) is more effective than either DPA or 2,2,2-
tetramine in Cu depletion. In 1987, Allen, Twedt and Hunsker demonstrated that 2,3,2-
tetramine induced a four-fold to nine-fold greater Cu cupruresis than 2,2,2-tetramine in
dogs without any toxic side effects after 23 days of treatment. In addition, Cu deficiency
and hyperlipoproteinemia were induced in rabbits by only 10 days of 2,3,2-tetramine
treatment (Hing and Lei, 1991).

Copper Deficiency and Lipoprotein Metabolism

An important relationship between dietary zinc and Cu on lipid metabolism has
been demonstrated in rats. Hypercholesterolemia was observed by Klevay (1973) in rats
by increasing the ratio of zinc to Cu ingested. He postulated that the high ratio of
dietary zinc to Cu is associated with hypercholesterolemia and further hypothesized that
coronary heart disease is predominantly a disease of imbalance in regard to zinc and Cu
metabolism. By using large scale factorial experiments, with various levels of dietary
zinc and Cu, Petering, Murthy and O’Flaherty (1977) indicated that there is an inverse relationship between dietary Cu, serum Cu and levels of serum cholesterol, triglycerides and phospholipids. They suggested that dietary Cu governs the metabolic fate of cholesterol more than the dietary zinc to Cu ratio. Lei (1977) suggested that the hypercholesterolemia could be partially caused by a shift of cholesterol from the liver pool to the plasma pool in Cu-deficient rats. Allen and Klevay (1978) also suggested that a more rapid clearance of cholesterol from the liver to the plasma pool, with this cholesterol being unavailable for excretion as biliary steroids, may be responsible for the hypercholesterolemia in Cu-deficient rats. Subsequent studies demonstrated that cholesteryl ester, newly synthesized from [2-14C]mevalonate, cleared the liver faster in Cu-deficient rats than controls (Shao and Lei, 1980). Lin and Lei (1981) observed marked increases in the size and half-life of the rapidly exchangeable cholesterol pool, which consisted of cholesterol from serum and tissues, such as the red blood cell and liver, which equilibrated rapidly with serum cholesterol in Cu-deficient rats. Furthermore, a prolonged half-life of the free cholesterol and total cholesterol carried by the high density lipoprotein (HDL) was observed in Cu-deficient rats (Lin and Lei, 1981). The influence of Cu deficiency on cholesterol clearance from plasma and on half-life of HDL cholesterol suggest copper may play an important role in lipoprotein metabolism.

Alterations in the distribution of cholesterol and lipids among the various lipoprotein fractions have been observed in Cu-deficient rats. In rats, plasma HDL were found to carry about 85% of total plasma cholesterol in normal rats and the percentage
was decreased in Cu-deficient rats, but the absolute amount of cholesterol carried by HDL was markedly increased by Cu deficiency (Allen and Klevay, 1980; Lei and Lin, 1981). Lei, Hassel and Allen (1983) observed similar results when the lipoprotein fractions were partitioned by ultracentrifugation and column chromatography into high, low, and very low density lipoproteins (HDL, LDL, VLDL). Elevations in protein and cholesterol contents of HDL and LDL and in triglyceride content of LDL were observed in Cu-deficient rats (Lei et al., 1983).

The protein and cholesterol contents of plasma HDL were elevated as a result of Cu deficiency in rats (Croswell and Lei, 1985; Al-Othman, Rosenstein and Lei, 1992) and in hamster (Al-Othman et al., 1991). An increase in Apo A-I of HDL was observed in Cu-deficient rats (Hoogeveen, 1993). In vivo apo A-I synthesis was increased two-fold in the liver of Cu-deficient rats (Hoogeveen and Lei, 1991). In addition, the synthesis and the secretion of apo A-I were found to be stimulated in freshly isolated hepatocytes derived from Cu-deficient rats (Hoogeveen and Lei, 1992). Furthermore, Cu deficiency also enhanced the hepatic synthesis of fatty acids and secretion of triacylglycerols and phospholipids in both in vivo and in vitro studies (Lei, 1977; Al-Othman et al., 1993).

Apolipoprotein Genes and Gene Regulations

In recent years the methods of molecular and cell biology have provided important insights into the structure and regulation of the genes that code for proteins involved in lipid metabolism. Almost all apolipoprotein gene structures and sequences have been determined by now (Scott, 1987). Intensive work is focused on analyzing the
regulation of apolipoprotein genes and the genetic variations of these genes which may contribute to an accelerated development of atherosclerosis. The tissue sites of expression of the apolipoprotein genes have been determined in humans and experimental animals. Human apo A-I mRNA is found mainly in liver and small intestine (Elshourbagy et al., 1985). In liver cells, the expression of apo A-I gene depends on a powerful liver-specific transcriptional enhancer located in the 5' -flanking region of the human apo A-I gene (Widom et al., 1991). This enhancer contains three sites, site A, B, and C, each of which has been shown to bind nuclear proteins and regulate apo A-I gene expression in liver cells (Widom et al., 1991). Several steroid-thyroid hormone receptor superfamily of transcriptional factors have been shown to play a role in apolipoprotein gene regulation. Apo A-I regulatory protein 1 (ARP-1) and Ear3/COUP-TF, which are found to bind to site A in the apo A-I gene promoter, have been shown to decrease apo A-I gene transcription in Hep G2 cells (Ladias and Karathanasis, 1991). Retinoid X receptor alpha (RXR-α), which also binds to site A, activates transcription of apo A-I gene (Rottman et al., 1991). Recently, hepatocyte nuclear factor 4 (HNF-4) has been shown to bind to site C within apo A-I promoter and to stimulate activity of apo A-I gene promoter (Chan, Nakabayashi and Wong, 1993). The discovery of the mechanisms involved in gene regulation will contribute to the understanding of how gene expression affects plasma lipoprotein levels as well as coronary heart disease.
CHAPTER III

TETRAMINE DEPLETES CELLULAR COPPER BUT DOES NOT ALTER TOTAL PROTEIN OR ALBUMIN SYNTHESIS IN HEP G2 CELLS

Abstract

Two amine chelators, namely 2,3,2-tetramine and diamsar (a "cage" hexaamine), were used to deplete copper (Cu) from cultured Hep G2 cells. Cells were seeded and cultured in 2 levels of Cu: 1) 0.63 μM of Cu (0.04 ppm), the basal level routinely used to culture Hep G2 cells; and 2) 1.57 μM of Cu (0.1 ppm), a higher level used for primary culture of hepatocytes. After 5 days, 90% confluency was reached, the media were replaced by basal medium containing 0, 10, 20, 50 and 100 μM of 2,3,2-tetramine or diamsar and incubated for 24 h. For cells cultured in 0.63 μM of Cu, maximal depletion of cellular Cu amounting to 2.45 ± 0.20 or 2.29 ± 0.18 pmol Cu/μg DNA were attained with 10 μM of 2,3,2-tetramine or diamsar, respectively, which resulted in an average reduction of 45% of cellular Cu as compared to controls. In contrast, maximal depletion of cellular Cu amounting to 13.00 ± 0.12 or 15.01 ± 1.76 pmol Cu/μg DNA were attained with 20 μM of 2,3,2-tetramine or diamsar, respectively, for cells cultured in 1.57 μM of Cu. An average reduction of 33% of cellular Cu was accomplished with 20 μM of these chelators. When cells were incubated with 50 μM of 2,3,2-tetramine or diamsar, as a function of time, near maximal Cu depletion was reached after 5 h for cells cultured at 0.63 μM of Cu. In addition, when cells were cultured for 4 passages continuously at 0.63 μM of Cu with 20 μM 2,3,2-tetramine,
cellular Cu was reduced more than 40% as compared to controls and no abnormal morphologic changes were observed. Furthermore, no alterations in the Hep G2 cell’s capacity to synthesize total cellular protein or albumin were detected at the end of the second passage for cells cultured continuously at 0.63 μM of Cu with 20 μM 2,3,2-tetramine. In view of the large and reproducible reduction in cellular Cu, this approach may serve as a useful human hepatocyte system to examine the influence of Cu status on apolipoprotein synthesis and secretion.

Introduction

D-penicillamine (DPA) and triethylene tetramine (2,2,2-tetramine) are metal chelating drugs used in the treatment of Wilson's disease, a genetic disorder in which excessive copper (Cu) is deposited in the body. Although DPA is most often prescribed for this disease (Walshe, 1956; 1982), 10% of patients treated with DPA develop intolerance to this drug (Walshe, 1982; Brewer et al., 1983). When patients develop adverse side effects to DPA, 2,2,2-tetramine is used as an alternate therapy (Walshe, 1982). Cu appears to be chelated by the α-amino and sulphydryl groups of DPA and by electronegative nitrogens of 2,2,2-tetramine. The use of these orally active chelators, enhanced excretion and reduced absorption of Cu resulted in the depletion of excessive Cu stores and clinical improvements. In general, the enhanced excretion has been perceived to be derived from the mobilization of hepatic Cu (Scheinberg and Sternlieb, 1986). Wilson's disease patients have been successfully maintained for years with chelator therapy.
Both DPA and 2,2,2-tetramine are capable of inducing Cu deficiency in experimental animals (Keen et al., 1983a,b). However, DPA was found to be ineffective in depleting Cu from cultured mouse hepatocytes as compared to "cage" chelators, such as sar and diamsar (McArdle et al., 1989). The formation of a stable complex, when Cu is bound and encapsulated by the 6 secondary nitrogen atoms of these caged chelators, may contribute to their effectiveness in depleting Cu from hepatocytes. In contrast, Cu is not firmly bound to DPA and may be released to other cellular ligands.

A comparison of cupruretic responses to various tetramines and DPA has been performed by Borthwick et al. (1980) in laboratory animals. A greater cupruresis was induced by 2,3,2-tetramine (N,N'-bis [2-aminoethyl]-1,3-propanediamine) than either penicillamine or 2,2,2-tetramine (the common alternative drug for Wilson's disease). In dogs, 2,3,2-tetramine induced a four-fold to nine-fold greater cupruresis than 2,2,2-tetramine (Allen et al., 1987). This was attributed to the 370-fold higher formation constant of the Cu-2,3,2-tetramine complex than that of Cu-2,2,2-tetramine (Weatherburn, Billo and Jones, 1970). No laboratory or clinical evidence of toxic side effects to both tetramines were detected during the 23-day treatment period. In rabbits, 2,3,2-tetramine administration was also found to be effective in the rapid induction of Cu deficiency and hyperlipoproteinemia after only 10 days of treatment (Hing and Lei, 1991).

The present study was designed to examine the effectiveness of 2,3,2-tetramine in the depletion of Cu from a human hepatoblastoma cell line, Hep G2 cells, over a number of passages. Moreover, the influence of 2,3,2-tetramine induced Cu deficiency
on the Hep G2 cell's ability to synthesize total cellular protein (TCA precipitable) and albumin was also evaluated in the present study. The present study established for the first time that 2,3,2-tetramine is effective in maintaining a depressed Cu status over 4 passages and does not alter total protein and albumin synthesis in Hep G2 cells. In view of the enlarged plasma pool size (Al-Othman et al., 1992) and enhanced hepatic synthesis of apo A-I (Hoogeveen, 1993) in Cu-deficient rats, this human hepatic cell line with a depressed Cu status may aid in the elucidation of mechanisms responsible for the regulation of apo A-I gene expression.

Materials and Methods

Cell Culture

Hep G2 cells (purchased from American Type Culture, Rockville, MD) were cultured in GIBCO tissue culture flasks coated with collagen. A sterile-filtered collagen solution (50 μg/mL of 1% acetic acid) was added to the flasks (5 mL/25 cm²) and incubated at 37°C overnight. Flasks were rinsed twice with phosphate-buffered saline (PBS). In general, cells were seeded into flasks (8 × 10⁵ cells/25 cm² flask or 3 × 10⁶ cells/75 cm² flask) and cultured in a basal medium containing: minimum essential medium (GIBCO BRL, cat. no. 320-1095, Gaithersberg, MD) with 0.1 mM nonessential amino acids (GIBCO BRL, cat. no. 320-1140), 1 mM Na pyruvate, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. This basal medium contained the basal Cu content of 0.63 μM (0.04 ppm) as measured by atomic absorption spectrophotometry (Hitachi, 1980). Cells spread out as a monolayer and reached 90%
confluency \((6 \times 10^6 \text{ cells}/25 \text{ cm}^2 \text{ flask})\) after 5 days of incubation, at 37°C with 95% air and 5% CO₂, with replenishment of medium after 3 days.

Chelator Treatment

In short-term chelator treatments, cells were grown in 2 levels of Cu: 1) basal 0.63 μM of Cu (0.04 ppm) and 2) 1.57 μM of Cu (0.1 ppm). Cu carbonate was added to basal medium (0.63 μM Cu) to attain the higher Cu levels. After 5 days of culture, cells were washed and subjected to several short-term chelator treatments as described in figure legends. To evaluate the effectiveness of chelators on Cu status, cells were treated with graded levels of 2,3,2-tetramine \((N,N'\text{-bis \[2\text{-aminoethyl}\]-1,3-propanediamine})\) or diamsar (a "cage" hexa-amine; Sargeson, 1986) for 24 h. Cells were also treated with 50 μM of 2,3,2-tetramine or diamsar for various intervals up to 24 h. The 2,3,2-tetramine was a kind gift from Dr. K.G.D. Allen, Department of Food Science and Human Nutrition, Colorado State University, Colorado 80523-0001. This chelator was also purchased from Eastman Kodak Co. (cat. no. 9995, Rochester, NY 14650). Diamsar was a kind gift from Professors A. Sargeson and D.M. Danks, Murdoch Institute for Research into Birth Defects Ltd., Royal Children’s Hospital, Flemington Rd., Parkville 3052, Melbourne, Australia.

In long-term 2,3,2-tetramine studies, cells were evaluated for 4 passages in basal medium under the following conditions: 1) basal 0.63 μM Cu and 2) basal 0.63 μM Cu plus 20 μM 2,3,2-tetramine. At the end of each passage, cells were detached by rinsing with 1 mL of a trypsin (0.05%)-EDTA (0.02%) solution and then split and
subcultured under the same respective conditions. At the end of each of the four passages, confluent cells were washed twice in PBS, sonicated and assayed for cellular DNA, protein and Cu contents. Cellular DNA was determined by the method of Cuttiolico and Gibbs (1975) using a model 650-15 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Protein determinations were performed with the Bio-Rad Protein Assay Reagents (Bio-Rad Lab., cat. no. 500-0006, Hercules, CA) using bovine serum albumin as standard. Cellular Cu was determined by flame atomic absorption spectrophotometry (Hitachi, 1980) using a model 180-70 atomic absorption spectrophotometer (Hitachi Ltd., Mountain View, CA).

For cellular total protein (TCA precipitable) and albumin synthesis studies, cells were grown in basal medium (0.63 μM Cu) with or without 20 μM 2,3,2-tetramine for 2 passages. At the end of the second passage, cells (90% confluency) were used for synthesis studies.

Labeling of Cells for Synthesis Studies

At the end of the second treatment passage, confluent cells (in 25 cm² flask) were preincubated with 4 mL of serum-free/leucine-free medium with or without 20 μM 2,3,2-tetramine. This preincubation medium was prepared from leucine/glutamate-free medium (GIBCO cat. no. 320-1890) and supplemented with the same amount of nonessential amino acids, Na pyruvate, penicillin/streptomycin and glutamate as the basal medium but with no fetal bovine serum. After 70 min of preincubation, cells were labeled with 400 μCi of L-[4,5-3H]leucine (135 Ci/mmol, Amersham Corp. cat. no.
TRK, 683, Arlington Heights, IL) in 2 mL of fresh serum-free leucine-free medium for various times, as indicated in table footnotes or figure legends.

Immediately after the pulse, cells were rapidly rinsed twice in 5 mL of ice-cold PBS and collected in 5 mL of PBS by using a cell scraper. Cells were pelleted by centrifugation at 3,000 rpm for 5 min at 4°C and lysed in 1 mL of ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 62 mM sucrose, 0.5% Na deoxycholate, 1.0% Triton X-100, and 50 mM Tris, pH 7.4) containing 50 μg/mL leupeptin, 50 μg/mL pepstatin A, 0.1% Na azide and the mixture of protease inhibitors used for the media. The cell lysate will be repeatedly frozen in dry ice and thawed in a 37°C water bath for 3 times to ensure complete cell lysis, vortex-mixed, and centrifuged (16,000 g for 30 min at 4°C) to provide a supernatant (detergent soluble cell extract) for immunoprecipitation or TCA precipitation.

**Immunoprecipitation**

Equal volumes of medium or cell extract and NET buffer (150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 5 mM Tris, pH 7.4) plus monospecific polyclonal rabbit antihuman albumin antibodies (Boehringer Mannheim, BH cat. no. 605001, Indianapolis, IN) were gently mixed on a rocking platform for 12 h at 4°C. Thirty μL of a 10% suspension of protein A (Ig G sorb from Enzyme Center, Malden, MA) were added and the mixture was incubated at 4°C for an additional hour with gentle rocking. Samples were centrifuged in a microfuge (16,000 g, 30 min, 4°C). Supernatants were removed and subjected to immunoprecipitation one more time to
ensure complete recovery of labeled albumin from cell extract. Complete recovery was accomplished usually with one immunoprecipitation. Pellets from the two immunoprecipitations were combined, washed twice with 0.3 mL of dilution buffer (0.1% Triton X-100 in PBS with 0.1% Na azide), twice with 0.3 mL of PBS and once with 0.3 mL of 0.05 M Tris HCl, pH 6.8. Fifty to 70 μL of PAGE buffer (2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.002% bromophenol blue, and 0.125 M Tris HCl, pH 6.8) were added and heated for 4 min in a boiling water bath to solubilize the apolipoproteins. The supernatant obtained after centrifugation was subjected to SDS-PAGE (15% acrylamide for albumin) at 20 mA for 5 h. The gel was stained with Coomassie blue and then destained. In preliminary studies, the gel was autoradiographed to assure that radioactivity was associated mainly with the respective apolipoproteins. The respective apolipoprotein band was excised and dissolved in 1 mL of 30% H2O2 for 6 h at 60°C. The solubilized gel was mixed with 5 mL of Ecolite (ICN, cat. no. 882475, Cleveland, OH) and counted by a liquid scintillation counter.

Trichloroacetic Acid (TCA) Precipitation

Eight μL of cell extract or medium were added to 100 μL of 20% TCA (wt./vol.), mixed, and allowed to stand at room temperature for 30 min. The mixture was microcentrifuged for 2 min and the supernatant was discarded. The pellet was washed once with 100 μL of 20% TCA, transferred to a scintillation vial containing 5 mL of Ecolite, and the radioactivity was counted.
Statistical Analysis

All data were analyzed by analysis of variance (Winer, 1962). Data in Figure 1 for the chelator concentration effect of tetramine or diamsar were analyzed by one-way ANOVA. Data for Figures 2 to 5 were analyzed by two-way ANOVA. Individual degree of freedom was partitioned for independent or interactive effects of chelator and duration of treatment.

Results and Discussion

Maximal depletion of cellular Cu was attained after 24 h of treatment with 10 μM of either 2,3,2-tetramine or diamsar (Figure 1) from cells cultured for 5 days in 0.63 μM (0.04 ppm) of Cu. A 48% reduction from 5 to 2.6 pmol Cu/μg DNA or a total of 2.4 ± 0.20 pmol Cu/μg DNA were depleted by 10 μM of 2,3,2-tetramine (Figure 1). Similarly, a 44% reduction from 5.3 to 3.0 pmol/μg DNA or a total of 2.29 ± 0.18 pmol Cu/μg DNA were depleted by 10 μM of diamsar (Figure 1). After cells were cultured for 5 days in 1.57 μM (0.1 ppm) of Cu, similar response curves demonstrating that near maximal depletion was attained with 20 μM of either chelator were observed (data not shown). A similar amount of Cu was depleted by 20 μM of 2,3,2-tetramine or diamsar, 13.00 ± 0.12 or 15.01 ± 1.76 pmol Cu/μg DNA, respectively, from cells cultured in 1.57 μM (0.1 ppm) Cu. This resulted in an average of 33% of cellular Cu being depleted by 20 μM of these chelators. In addition, near maximal depletion of cellular Cu was reached after 5 h, when cells cultured with 0.63 μM (0.04 ppm) of Cu were treated with 50 μM of 2,3,2-tetramine (Figure 2) or diamsar (Figure 3) for intervals
FIGURE 1. Effect of chelator concentration on Cu status of Hep G2 cells cultured with 0.63 μM (0.04 ppm) of Cu. Cells were seeded at $8 \times 10^5$ cells/25 cm$^2$ flask and cultured for 5 days and then treated with graded levels of 2,3,2-tetramine (TETA) or diamsar for 24 h. Cellular Cu concentrations were determined as described in Materials and Methods. Results are means ± SE of 4 experiments. Data were analyzed by one-way ANOVA, individual degrees of freedom (DF=1) was partitioned for chelator concentration (C) effects: $C_1$ = none vs 10, 20, 50 and 100 μM; $C_2$ = 10 vs 20, 50 and 100 μM; $C_3$ = 20 vs 50 and 500 μM; $C_4$ = 50 vs 100 μM. $C_1$ with $P<0.0001$ for TETA or diamsar; $C_2$ to $C_4$, NS for both chelators.
FIGURE 2. Depletion of copper (Cu) from Hep G2 cells treated with 50 μM of 2,3,2-tetramine (TETA) for increasing periods of time. Cells were seeded at 8 × 10^5 cells/25 cm^2 flask, cultured for 5 days at 0.63 μM (0.04 ppm) of Cu and then treated with 50 μM of TETA for various time periods. Cellular Cu levels were measured as described in Materials and Methods. Results are means ± SE of 4 experiments. Data were analyzed by two-way ANOVA, individual degrees of freedom (DF = 1) was partitioned for main effects — TETA and time (T_1 = 0 vs 1, 3, 5, 8 and 24 h; T_2 = 1 vs 3, 5, 8 and 24 h; T_3 = 3 vs 5, 8 and 24 h; T_4 = 5 vs 8 and 24 h; T_5 = 8 vs 24 h) and their interactions — TETA × T_1, TETA × T_2, TETA × T_3, TETA × T_4, and TETA × T_5. TETA effect, P<0.0001; TETA × T_1, P<0.05.
FIGURE 3. Depletion of copper (Cu) from Hep G2 cells treated with 50 μM of diamsar for increasing periods of time. Cells were seeded at $8 \times 10^5$ cells/25 cm$^2$ flask, cultured for 5 days at 0.63 μM (0.04 ppm) of Cu and then treated with 50 μM of diamsar for various time periods. Cellular Cu levels were measured as described in Materials and Methods. Results are means ± SE of 4 experiments. Data were analyzed by two-way ANOVA, individual degrees of freedom (DF = 1) was partitioned for main effects — diamsar (D) and time ($T_1 = 0$ vs $1, 3, 5, 8$ and 24 h; $T_2 = 1$ vs $3, 5, 8$ and 24 h; $T_3 = 3$ vs $5, 8$ and 24 h; $T_4 = 5$ vs 8 and 24 h; $T_5 = 8$ vs 24 h) and their interactions — $D \times T_1, D \times T_2, D \times T_3, D \times T_4,$ and $D \times T_5$. Diamsar effect, $P<0.0001$; $D \times T_1, P<0.005$. 
up to 24 h. In these time-course studies, the amounts of Cu depleted were comparable to those observed in the concentration studies (Figure 1). Thus, both chelators appeared to be equally effective and rapid in depleting Cu from Hep G2 cells at relatively low concentrations.

The long-term influence of 2,3,2-tetramine on the Cu status of Hep G2 cells was evaluated over 4 passages. Diamars was not studied further due to the limited supply of this chelator. The relatively low dosage level of 20 μM 2,3,2-tetramine was selected since the maximal amount of cellular Cu depleted was attained with only 10 μM of 2,3,2-tetramine for cells cultured in 0.63 μM (0.04 ppm) of Cu (Figure 1). Reductions in cellular Cu appeared to be fairly reproducible over the 4 passages, with the exception of a slightly lower Cu status at the end of the first passage (Figure 4). The amount of Cu depleted was similar to that observed in the dosage and time-course studies (Figure 1) for cell cultured in 0.63 μM of Cu (the basal level routinely used to culture Hep G2 cells). No abnormal changes in cell morphology were observed during the 4 passages. The substantial quantity of cellular Cu depleted, which provided a reduction of more than 40% of cellular Cu, indicates that the depressed Cu status can be effectively maintained for at least 4 passages using the same culture and dosage conditions.

After culturing Hep G2 cells for 2 passages, in the basal 0.62 μM Cu level with or without 20 μM 2,3,2-tetramine, the ability of cells to synthesize total cellular protein (TCA precipitable) and albumin appeared not to be compromised by the chelator treatment. Since no significant differences were observed between treated and control cells, the values were pooled to provide one response curve to depict the rate of synthesis
FIGURE 4. Effect of 20 μM of 2,3,2-tetramine (TETA) treatment on cellular copper (Cu) levels of Hep G2 cells over 4 passages. Cells were seeded at $3 \times 10^6$ cells/75 cm$^2$ flask, cultured at 0.63 μM of Cu with or without 20 μM of TETA for 4 passages. Cellular Cu levels were determined as described in Materials and Methods. Results are means ± SE from at least 2 experiments. Data were analyzed by two-way ANOVA, individual degrees of freedom (DF = 1) was partitioned for main effects — TETA and passage time ($T_c = \text{time linear}; T_q = \text{time quadratic}; T_c = \text{time cubic}; T_T = \text{time quartic}$) and their interactions — TETA $\times$ T$L$, TETA $\times$ T$q$, TETA $\times$ T$c$, TETA $\times$ T$_T$. TETA effect, $P<0.0001$; TETA $\times$ T$q$, $P<0.005$; TETA $\times$ T$c$, $P<0.05$. 
for total protein or albumin. The ability of the cells to incorporate $[^3]$H]leucine into nascent total protein and albumin was fairly linear during the 20 min labeling period (Figure 5). However, the incorporation of $[^3]$H]leucine may be leveling off after 20 min of labeling. Thus, in subsequent studies, a 10 min labeling period was used to examine apolipoproteins A-I and B synthesis in Hep G2 cells (Zhang, Wang and Lei, 1994). Since the total protein and albumin synthesis was not altered, the protein synthetic machinery appeared to be intact and not compromised by the depressed Cu status induced by 2,3,2-tetramine treatment. Similarly, in vivo hepatic total protein and albumin synthesis was found not to be affected by dietary Cu deficiency in rats (Hoogeveen and Lei, 1991).

The elegant cultured mouse hepatocyte studies performed by McArdle et al. (1989) demonstrated that 50 $\mu$M diamsar was able to deplete appreciable amounts of accumulated cellular $^{64}$Cu as well as capable of preventing the uptake of $^{64}$Cu from a diamsar-Cu complex. In contrast, penicillamine was found to be ineffective. When cells were labeled with $^{64}$Cu (1 $\mu$Ci/mL at 1.2 - 2.3 $\mu$M Cu) for 18 h and then treated with graded levels of chelators, maximal depletion of $^{64}$Cu accumulated ($\sim$20% reduction) was attained with 20 $\mu$M of diamsar. Again penicillamine was ineffective up to 100 $\mu$M. The percent reduction of $^{64}$Cu accumulated in mouse hepatocytes appeared to be only slightly lower than the 33% reduction observed for the cellular nonradiolabeled Cu depleted by 20 $\mu$M diamsar from Hep G2 cells cultured in 1.57 $\mu$M of Cu. Moreover, in the present study, the rate of nonradioactive Cu depleted and the dosage of chelator
[^3H]LEU–LABELED PROTEIN
DPM X 10^5/MG PROTEIN

TOTAL PROTEIN

ALBUMIN

TIME (MINUTES)

FIGURE 5. Total protein and albumin synthesis in Hep G2 cells. Cells were cultured for two passages at 0.63 μM (0.04 ppm) of Cu with or without 20 μM 2,3,2-tetramine. After a 70 min preincubation with serum-free/leucine-free medium, cells were labeled with 400 μCi of L[4,5-^3H]leucine in 2 mL of fresh serum-free/leucine-free medium for 5, 10, 15 and 20 min. Cellular total protein and albumin were isolated and their associated radioactivities counted as described in Materials and Methods. Since no significant differences were observed between treated and control cells, the data were pooled to provide one response curve to depict the rate of total protein or albumin synthesis. Results are means ± SE from at least 4 experiments. Data were analyzed by two-way ANOVA, individual degrees of freedom (DF = 1) was partitioned for main effects — TETA and time (T_L = time linear; T_Q = time quadratic; T_C = time cubic) and their interactions — TETA × T_L, TETA × T_Q, TETA × T_C. T_L effect, P<0.0001. All other comparisons were NS.
required for maximal depletion are comparable to those reported by McArdle et al. (1989) using $^{64}\text{Cu}$ labeling technique.

In the long-term culture study, the average Cu content of treated Hep G2 cells during the 4 passages was reduced more than 40% as compared to controls. This reduction may appear to be smaller when compared to the large reduction in hepatic Cu content observed in frank Cu-deficient rats. A recent review (Lei, 1990) provided a range of 37 to 87% reduction in hepatic Cu content for weanling rats fed Cu-deficient diets for 6 to 9 weeks. Nevertheless, the Hep G2 cells with a 40% reduction in cellular Cu can at least be considered to be marginally deficient in Cu. Furthermore, the sizable and reproducible reduction in cellular Cu and the intact protein synthetic capacity of the treated cells demonstrated that the present system may be used as an alternative approach to examine the influence of Cu status on cellular processes such as apolipoprotein synthesis and secretion in a human hepatic cell line.
CHAPTER IV

APOLIPOPROTEIN A-I SYNTHESIS AND SECRETION ARE INCREASED IN HEP G2 CELLS DEPLETED OF COPPER BY CUPRURETIC TETRAMINE

Abstract

The cupruretic chelator, 2,3,2-tetramine, was used to maintain a 50% reduction in cellular copper (Cu) in Hep G2 cells for 2 passages. Cells were then pulsed for 10 min with [3H]leucine and chased for various times up to 2 h. Radioactivity precipitated by trichloroacetic acid (TCA) was measured to determine the amount of nascent total protein synthesized in the cell and that secreted into the medium. Cellular and media apo A-I and apo B radioactivities were isolated by immunoprecipitation and SDS-PAGE to determine the synthesis, intracellular degradation and secretion of these lipoproteins. At the end of the 10 min pulse, the amount of [3H]leucine incorporated into apo A-I was increased more than two-fold in tetramine-treated cells. No difference was detected for the synthesis of apo B and total protein. During the 2 h chase, the cellular depletion curves for apo A-I, apo B and total protein were not altered by tetramine treatment. From 30 min to 120 min of the chase period, the amount of nascent apo A-I degraded was small and not influenced by tetramine treatment; but that secreted into the medium was increased 56% in the tetramine-treated cells. Thus, the depressed Cu status induced by tetramine may have promoted an increased apo A-I secretion mainly by an enhanced synthesis in Hep G2 cells.
Introduction

Hypercholesterolemia induced by copper (Cu) deficiency has been observed in a number of rodent species, nonhuman primates and humans (reviewed by Lei, 1990a). Marked increases in triacylglycerol concentrations and percent composition of lipoprotein fractions, especially that of very low density lipoproteins (VLDL), have been reported in rats (Al-Othman et al., 1992) and in hamsters (Al-Othman et al., 1991). Moreover, increases of apolipoproteins among lipoprotein fractions (Lei et al., 1983; Croswell and Lei, 1985; Lefevre et al., 1986; Lee and Koo, 1988), particularly that of apo A-I (Hoogeveen, 1993) in high-density lipoproteins (HDL), have been established in Cu-deficient rats. In vivo and in vitro studies have demonstrated that Cu deficiency enhanced the hepatic synthesis of fatty acids (Lei, 1977; Al-Othman et al., 1993) and secretion of triacylglycerols and phospholipids (Al-Othman et al., 1993). In addition, increased synthesis and secretion of apo A-I have been observed in vitro in hepatic parenchymal cells (Hoogeveen and Lei, 1992) and in vivo by the liver (Hoogeveen and Lei, 1991) of Cu-deficient rats. Thus, at least in rats, the hyperlipoproteinemia associated with depressed Cu status appears to be the result of the enhanced synthesis of these lipoprotein components.

Although the Cu-deficient rat model is a useful experimental animal for the elucidation of mechanisms responsible for the hyperlipoproteinemia, there is a compelling need to substantiate the animal findings with human studies. Since human studies are expansive and limited by ethical concerns, an alternative approach is to perform in vitro studies using tissues derived from humans. Hep G2, a liver cell line established from
a human hepatoblastoma (Javitt, 1990), is selected as the human cell model for the following reasons: 1) Tetramine, a cupruretic chelator, has been used successfully to maintain a consistently depressed Cu status but allow optimum growth in Hep G2 cells for 4 continuous passages (Zhang and Lei, 1991); 2) Among the wide variety of liver-specific metabolic functions expressed by confluent Hep G2 cells are the activities related to lipoprotein, cholesterol and triacylglycerol metabolism (reviewed by Javitt, 1990). The ability of Hep G2 cells to synthesize and secret apolipoproteins A-I, A-II, B, C, and E, as well as cholesterol, triacylglycerols and primary bile acids, established them as an attractive human cell model for studying the biochemical and molecular processes involved in lipoprotein metabolism.

The present study was performed to examine the influence of 2,3,2-tetramine-induced Cu deficiency on the Hep G2 cell’s capacity to synthesize, secrete, and intracellularly degrade apo A-I and B. The specific and marked increase in apo A-I synthesis and secretion indicate that this Cu-depleted human liver cell model may be useful for the elucidation of the responsible molecular mechanism(s).

**Materials and Methods**

**Cell Culture**

Hep G2 cells (purchased from American Type Culture, Rockville, MD) were cultured in GIBCO tissue culture flasks coated with collagen. A sterile-filtered collagen solution (50 μg/mL of 1% acetic acid) was added to the flasks (5 mL/25 cm²) and incubated at 37°C overnight. Flasks were rinsed twice with phosphate-buffered saline
In general, cells were seeded into flasks \((8 \times 10^5 \text{ cells}/25 \text{ cm}^2 \text{ flask or } 3 \times 10^6 \text{ cells}/75 \text{ cm}^2 \text{ flask})\) and cultured in a basal medium containing: minimum essential medium (GIBCO BRL, cat. no. 320-1095, Gaithersberg, MD) with 0.1 mM nonessential amino acids (GIBCO BRL, cat. no. 320-1140), 1 mM Na pyruvate, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. This basal medium contained the basal Cu content of 0.63 μM of Cu (0.04 ppm) as measured by atomic absorption spectrophotometry (Hitachi, 1980). Cells spread out as a monolayer and reached 90% confluency \((6 \times 10^6 \text{ cells}/25 \text{ cm}^2 \text{ flask})\) after 5 days of incubation, at 37°C with 95% air and 5% CO₂, with replenishment of medium after 3 days.

**Chelator Treatment**

Cells were cultured for two passages in basal medium (0.63 μM of Cu) with or without 20 μM of 2,3,2-tetramine \((N,N'\text{-bis [2-aminoethyl]-1,3-propanediamine})\). The 2,3,2-tetramine was a kind gift from Dr. K.G.D. Allen, Department of Food Science and Human Nutrition, Colorado State University, Colorado 80523-0001. This chelator was also purchased from Eastman Kodak Co. (cat. no. 9995, Rochester, NY 14650). At the end of each passage, cells were detached by rinsing with 1 mL of a trypsin (0.05%)-EDTA (0.02%) solution and then split and subcultured under the same respective conditions. In addition, confluent cells were washed twice in PBS, sonicated and assayed for cellular DNA, protein and Cu contents, at the end of each passage. Cellular DNA was determined by the method of Cuttiolico and Gibbs (1975) using a model 650-15 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT).
Protein determinations were performed with the Bio-Rad Protein Assay Reagents (Bio-Rad Lab., cat. no. 500-0006, Hercules, CA) using bovine serum albumin as standard. Cellular Cu was determined by flame atomic absorption spectrophotometry (Hitachi, 1980) using a model 180-70 Hitachi atomic absorption spectrophotometer (Hitachi Ltd., Mountain View, CA).

For apolipoprotein synthesis and secretion studies, cells were grown in basal medium (0.63 μM of Cu) with or without 20 μM 2,3,2-tetramine for 2 passages. At the end of the second passage, cells (90% confluency) were used for synthesis and secretion studies.

Labeling of Cells for Synthesis and Secretion Studies

At the end of the second treatment passage, confluent cells (in 25 cm² flask) were preincubated with 4 mL of serum-free/leucine-free medium with or without 20 μM 2,3,2-tetramine. This preincubation medium was prepared from leucine/glutamate-free medium (GIBCO BRL cat. no. 320-1890) and supplemented with the same amount of nonessential amino acids, Na pyruvate, penicillin/streptomycin and glutamate as the basal medium but with no fetal bovine serum. After 70 min of preincubation, cells were labeled with 400 μCi of L-[4,5-3H]leucine (135 Ci/mmol, Amersham Corp. cat. no. TRK, 683, Arlington Heights, IL) in 2 mL of fresh serum-free/leucine-free medium for various times, as indicated in table footnotes or figure legends. Cells were rapidly washed twice in 5 mL of ice-cold PBS and immediately processed for immunoprecipitation to establish apolipoprotein synthesis rates. Alternatively, cells were
immediately chased to establish intracellular depletion and degradation as well as secretion.

Pulse-Chase Experiments

Cells were labeled for 10 min, rapidly rinsed twice in 5 mL of PBS, and then chased in 2 mL of chase medium containing 10 mM unlabeled leucine (preincubation medium supplemented with cold leucine) with or without 20 μM 2,3,2-tetramine for various times up to 2 h. After each chase period, the medium was rapidly transferred to a tube containing a protease inhibitor mixture (which provided a final concentration of 1 mM benzamidine, 5 mM EDTA, 100 kallikrein-inactivating units of aprotinin/mL, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Heps, pH 8.0) and processed for immunoprecipitations or TCA precipitation. Immediately after the chase, cells were rinsed twice in 5 mL of ice-cold PBS and collected in 5 mL of PBS by using a cell scraper. Cells were pelleted by centrifugation at 3,000 rpm for 5 min at 4°C and lysed in 1 mL of ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 62 mM sucrose, 0.5% Na deoxycholate, 1.0% Triton X-100, and 50 mM Tris, pH 7.4) containing 50 μg/mL leupeptin, 50 μg/mL pepstatin A, 0.1% Na azide and the mixture of protease inhibitors used for the media. The cell lysate will be repeatedly frozen in dry ice and thawed in a 37°C water bath for 3 times to ensure complete cell lysis, vortex-mixed, and centrifuged (16,000 g for 30 min at 4°C) to provide a supernatant (detergent soluble cell extract) for immunoprecipitation or TCA precipitation.
Immunoprecipitation

Equal volumes of medium or cell extract and NET buffer (150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 5 mM Tris, pH 7.4) plus monospecific polyclonal sheep antihuman apo B antibodies (Boehringer Mannheim, BH cat. no. 1001-400, Indianapolis, IN) or apo A-I (BH cat. no. 1001-388) were gently mixed on a rocking platform for 12 h at 4°C. Thirty μL of a 10% suspension of protein A (Ig G sorb from Enzyme Center, Malden, MA) were added and the mixture was incubated at 4°C for an additional h with gentle rocking. Samples were centrifuged in a microfuge (16,000 g, 30 min, 4°C). Supernatants were removed and subjected to immunoprecipitation one more time to ensure complete recovery of labeled apo A-I or apo B from medium or cell extract. Complete recovery was accomplished usually with one immunoprecipitation. Pellets from the two immunoprecipitations were combined, washed twice with 0.3 mL of dilution buffer (0.1% Triton X-100 in PBS with 0.1% NaN₃), twice with 0.3 mL of PBS and once with 0.3 mL of 0.05 M Tris HCl, pH 6.8. Fifty to 70 μL of PAGE buffer (2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.002% bromophenol blue, and 0.125 M Tris HCl, pH 6.8) were added and heated for 4 min in a boiling water bath to solubilize the apolipoproteins. The supernatant obtained after centrifugation was subjected to SDS-PAGE (7% acrylamide for apo B and 15% acrylamide for apo A-I) at 20 mA for 5 h. The gel was stained with Coomassie blue and then destained. In preliminary studies, the gel was autoradiographed to assure that radioactivity was associated mainly with the respective apolipoproteins. The respective apolipoprotein band was excised and dissolved in 1 mL of 30% H₂O₂ for 6 h at 60°C.
The solubilized gel was mixed with 5 mL of Ecolite (ICN, cat. no. 882475, Cleveland, OH) and counted by a liquid scintillation counter.

Trichloroacetic Acid (TCA) Precipitation

Eight µL of cell extract or medium were added to 100 µL of 20% TCA (wt./vol.), mixed, and allowed to stand at room temperature for 30 min. The mixture was microcentrifuged for 2 min and the supernatant was discarded. The pellet was washed once with 100 µL of 20% TCA, transferred to a scintillation vial containing 5 mL of Ecolite, and the radioactivity was counted.

Statistical Analysis

All data were analyzed by analysis of variance (Winer, 1962). Data for Tables 3 and 4 were tested by one-way ANOVA. Intracellular and media data for Figures 6 to 8 were analyzed by two-way ANOVA. Individual degree of freedom (DF = 1) was partitioned for main effects - tetramine, time 1 (30 vs 60 min), time 2 (30 and 60 vs 120 min) and for interactions - tetramine × time 1 and tetramine × time 2.

Results and Discussion

Cellular Cu was reduced 50 ± 4% in cells treated with tetramine for two passages prior to the pulse-chase studies. This magnitude of depletion in cellular Cu is similar to that reported in a previous study when tetramine-treated cells were monitored for 4 passages (Zhang and Lei, 1991). In view of the marked reduction in cellular Cu, the tetramine-treated cells could at least be considered as marginally deficient in Cu.
In the present synthesis study, a relatively short 10 min pulse period was selected since preliminary studies have established that incorporation of $[^3H]$leucine into the various proteins was linear for at least 15 min for both control and tetramine-treated cells (data not shown). In tetramine-treated cells, the incorporation of $[^3H]$leucine into the various proteins (total protein and apo B) was not altered after the 10 min pulse, with the exception of the increase for apo A-I, when the data were expressed as dpm $\times 10^3$/mg protein or as a % of TCA precipitable radioactivity (Table 3). Thus the two-fold increase in apo A-I synthesis appeared to be specific.

Response curves for the various proteins depleted from the cell and secreted into the medium were constructed with data from 30, 60, and 120 min of the chase period (Figures 6-8). For both treatments, the depletion of newly synthesized total protein and apo A-I appeared to be more rapid during the 30-60 min than the 60-120 min period (Figures 6 and 7). In contrast, during the 30-60 min, the depletion of nascent cellular apo B tended to be less rapid than for the 60-120 min period (Figure 8). Moreover, the secretion of nascent apo A-I into the medium by cells from both treatments appeared to increase linearly with time during the chase period (Figure 7). However, the secretion of nascent total protein and apo B into the medium, for both treatments, seemed to be more rapid during the 30-60 min period and may have reached a plateau by 120 min (Figures 6 and 8). Analysis of variance detected no significant difference among the response curves, with the exception of the elevated response curves for the amount of apo A-I and total protein secreted into the medium by tetramine-treated
### TABLE 3. Effect of tetramine on the synthesis of total protein, apo A-I and apo B by Hep G2 cells

<table>
<thead>
<tr>
<th>Measurements after 10 min pulse</th>
<th>Control cells</th>
<th>Tetramine-treated cells</th>
<th>ANOVA P value</th>
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<tbody>
<tr>
<td><strong>dpm × 10^6/mg protein</strong></td>
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<td></td>
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<tr>
<td>Total protein (TCA precipitable)</td>
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<tr>
<td></td>
<td>112.9 ± 5.9</td>
<td>127.8 ± 6.3</td>
<td>NS</td>
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<tr>
<td><strong>dpm × 10^3/mg protein</strong></td>
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<td></td>
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</tr>
<tr>
<td>Apo B</td>
<td>2590 ± 191</td>
<td>3332 ± 743</td>
<td>NS</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>215 ± 7</td>
<td>483 ± 104</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>% of control TCA radioactivity</strong></td>
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<tr>
<td>Total protein (TCA precipitable)</td>
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<tr>
<td></td>
<td>100.0 ± 5.3</td>
<td>113.2 ± 5.6</td>
<td>NS</td>
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<tr>
<td><strong>% of own TCA radioactivity</strong></td>
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<tr>
<td>Apo B</td>
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<td>NS</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>0.192 ± 0.009</td>
<td>0.381 ± 0.080</td>
<td>&lt;0.05</td>
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</table>

Cells in T25 flasks (90% confluent) were incubated in 4 mL of labeling medium (0.2 mCi of [3H]leucine/mL). After 10 min of pulse, cells were washed twice in ice cold PBS and processed for the immunoprecipitation and SDS-PAGE isolation of apo A-I and apo B. Data represent the mean ± SEM from 5 experiments.
FIGURE 6. Depletion and secretion of Hep G2 cellular total protein during pulse-chase experiments. Nascent total protein (trichloroacetic acid precipitable) from control (◇ □) and 2,3,2-tetramine-treated (◆ ■) cells were labeled with [3H]leucine and chased with cold leucine for 30, 60, 90 and 120 min. Cellular (◇ ◆) and media (□ ■) total protein radioactivities were determined as described in Materials and Methods. Ordinate — nascent total protein expressed as dpm × 10⁷ of [3H]leucine-labeled total protein per mg cell protein. Abscissa — time after cold leucine chase. Data represent means ± SEM from 3 experiments. The tetramine effect was significant for total protein secreted into the media (P < 0.001). Both time 1 (30 vs 60 min) and time 2 (30 plus 60 vs 120 min) effects were significant for cellular (P < 0.05 and P < 0.005, respectively) and media (P < 0.001 and P < 0.001, respectively) total protein. All other comparisons were nonsignificant.
FIGURE 7. Depletion and secretion of Hep G2 cellular apo A-I during pulse-chase experiments. Nascent apo A-I from control (◇□) and 2,3,2-tetramine-treated (◆■) cells were labeled with [3H]leucine and chased with cold leucine for 30, 60, 90 and 120 min. Cellular (◇◆) and media (□■) apo A-I were isolated by immunoprecipitation and SDS-PAGE, and their radioactivities were determined as described in Materials and Methods. Ordinate — nascent apo A-I expressed as dpm × 10^5 of [3H]leucine-labeled apo A-I per mg cell protein. Abscissa — time after cold leucine chase. Data represent means ± SEM from 3 experiments. The tetramine effect was significant for apo A-I secreted into the media (P < 0.001). Both time 1 (30 vs 60 min) and time 2 (30 plus 60 vs 120 min) effects were significant for cellular (P < 0.005 and P < 0.01, respectively) and media (P < 0.001 and P < 0.001, respectively) apo A-I. All other comparisons were nonsignificant.
FIGURE 8. Depletion and secretion of Hep G2 cellular apo B during pulse-chase experiments. Nascent apo B from control (◇ □) and 2,3,2-tetramine-treated (◆ ■) cells were labeled with $[^{3}H]$leucine and chased with cold leucine for 30, 60, 90 and 120 min. Cellular (◇ ◆) and media (□ ■) apo B were isolated by immunoprecipitation and SDS-PAGE, and their radioactivities were determined as described in Materials and Methods. Ordinate — nascent apo B expressed as dpm $\times 10^{5}$ of $[^{3}H]$leucine-labeled apo B per mg cell protein. Abscissa — time after cold leucine chase. Data represent means ± SEM from 3 experiments. Both time 1 (30 vs 60 min) and time 2 (30 plus 60 vs 120 min) effects for media apo B were significant ($P<0.005$ and $P<0.01$, respectively). In addition, the time 2 effect for cellular apo B was significant ($P<0.001$). All other comparisons were nonsignificant.
cells (Figures 6 and 7). However, when the absolute amount of protein secreted during 30-120 min was derived from the difference between the value at 30 min and that at 120 min of the chase, only an elevated amount of apo A-I (56% increase) was found to be secreted into the medium by tetramine-treated cells (Table 4). The absolute amount of nascent total protein secreted into the medium was not influenced by tetramine treatment. In addition, the amount of apo A-I and total protein depleted from the cells or degraded intracellularly (calculated as a difference between the amount depleted and that secreted into the medium) were not different among the two treatments (Table 4). Furthermore, no differences in the amount of apo B depleted, degraded or secreted were detected among the two treatments. Nevertheless, a substantial amount of the newly synthesized apo B was estimated to be degraded among the two treatments. An average of 60.2% of the nascent cellular apo B depleted from the two treatments, during 30-120 min of the chase period, was unaccounted for and was assumed to be degraded intracellularly. Similarly, Dixon et al. (1991) reported that 58% of nascent apo B was degraded in Hep G2 cells during the early period of the chase.

In view of the relatively small numerical values and the exceptionally large standard errors associated with the estimated amount of nascent apo A-I degraded intracellularly, the amount of apo A-I actually degraded may be considered to be negligible. This contention is also supported by the lack of a significant difference between the amount of apo A-I depleted and secreted. Thus, the enhanced secretion of apo A-I was resulted mainly from an increased synthesis in the tetramine-treated, Cu-depleted, Hep G2 cells.
TABLE 4. Influence of tetramine on the depletion, secretion, and intracellular degradation of total protein, apo A-I and apo B by Hep G2 cells.

<table>
<thead>
<tr>
<th>Time Interval (30 to 120 min)</th>
<th>Control Cells</th>
<th>Tetramine-treated cells</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm x 10^6/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(TCA precipitable)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depleted</td>
<td>18.10 ± 3.30</td>
<td>28.80 ± 12.40</td>
<td>NS</td>
</tr>
<tr>
<td>Secreted</td>
<td>4.59 ± 0.44</td>
<td>7.41 ± 1.11</td>
<td>NS</td>
</tr>
<tr>
<td>Degraded</td>
<td>13.48 ± 3.39</td>
<td>21.42 ± 13.15</td>
<td>NS</td>
</tr>
<tr>
<td>dpm x 10^3/mg protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apo A-I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depleted</td>
<td>140 ± 36</td>
<td>180 ± 57</td>
<td>NS</td>
</tr>
<tr>
<td>Secreted</td>
<td>89 ± 11</td>
<td>139 ± 8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Degraded</td>
<td>51 ± 45</td>
<td>41 ± 63</td>
<td>NS</td>
</tr>
<tr>
<td>Apo B-100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depleted</td>
<td>729 ± 161</td>
<td>563 ± 21</td>
<td>NS</td>
</tr>
<tr>
<td>Secreted</td>
<td>230 ± 36</td>
<td>270 ± 58</td>
<td>NS</td>
</tr>
<tr>
<td>Degraded</td>
<td>498 ± 169</td>
<td>293 ± 54</td>
<td>NS</td>
</tr>
</tbody>
</table>

Depleted protein (total protein, apo A-I or apo B) was the difference between the intracellular level at 30 min and that at 120 min for each experiment. Secreted protein was the difference between the level in the medium at 30 min and that at 120 min for each experiment. Secreted protein was subtracted from depleted protein to provide the intracellular degraded protein for each experiment. Data represent the mean ± SEM from 3 experiments.
Available data provide an overview of the new steady state in lipid and lipoprotein metabolism induced by dietary Cu deficiency in rats. First, an enhanced hepatic cholesterol synthesis (Yount et al., 1991; 1992) provided the nascent cholesteryl esters to sustain the accelerated clearance from the liver to the plasma (Shao and Lei, 1980). An increased hepatic uptake of plasma HDL cholesteryl esters (Carr and Lei, 1990) demonstrates that an elevated amount of plasma cholesterol may be recycled back to the liver. The increased supply of cholesterol from de novo synthesis and that taken up from the plasma is not diverted for excretion, since bile acid production and biliary cholesterol excretion are not affected by Cu deficiency (Lei, 1978). In order to sustain the accelerated secretion into the plasma, cholesterol is derived from the increased supply and liver store, resulting in a small reduction in hepatic cholesterol content (Lei, 1990b).

Second, an enhanced hepatic lipogenesis (Lei, 1977; Al-Othman et al., 1993) supplied fatty acids for lipoprotein assembly, resulting in a six-fold elevation in plasma VLDL triacylglycerol pool and a two-fold increase in HDL triacylglycerol pool size. Lastly, a marked increase in hepatic apo A-I synthesis (Hoogeveen and Lei, 1991; 1992) supplied the apolipoproteins to sustain the increase in lipoprotein synthesis and secretion.

An enhanced synthesis of apo A-I has been reported in vivo by the liver of diet-induced, Cu-deficient rats (Hoogeveen and Lei, 1991). After an injection of a flooding dose of [3H]phenylalanine, a two-fold increase in the incorporation of [3H]phenylalanine into hepatic immunoprecipitable apo A-I was observed when the data were expressed as dpm/liver/100 body weight. In addition, a two-fold increase in the amount of nascent apo A-I was detected in the plasma pool of deficient rats. Similarly, an enhanced
synthesis and secretion of apo A-I has been reported in vitro in liver parenchymal cells derived from Cu-deficient rats (Hoogeveen and Lei, 1992). Two-fold increases in apo A-I synthesis and secretion were observed after a 20 min pulse with $[^{3}H]$phenylalanine and during the 2 h chase period, respectively. However, in Cu-deficient rats, the relative hepatic apo A-I mRNA abundance was increased only 28% over that of adequate rats when the data were expressed on the basis of per liver per 100 g body weight (Hoogeveen, 1993). Since this small increase in apo A-I mRNA abundance could not account for the marked increase in apo A-I synthesis, an enhanced translational process may also play an important role in the hyperapolipoproteinemia A-I in copper deficiency.

At present, the possibility that tetramine may promote the increase in apo A-I synthesis via a mechanism other than its cupruretic action cannot be discounted. Nevertheless, in view of the two-fold increase in hepatic apo A-I synthesis observed in the diet induced Cu-deficient rats (Hoogeveen and Lei, 1991; 1992), there is a high probability that the enhanced apo A-I synthesis may have resulted from the depressed Cu status induced by tetramine treatment. Regardless of the exact mechanism(s) involved, this valuable human hepatic cell line offers an alternative Cu-depleted cell model which may aid in the elucidation of the molecular mechanisms involved in the induction of apo A-I synthesis. Information generated from this model will provide valuable insights into the regulation of apo A-I gene expression.
CHAPTER V

REGULATION OF APOLIPOPROTEIN A-I GENE EXPRESSION IN HEP G2 CELLS DEPLETED OF COPPER BY CUPRURETIC TETRAMINE

Abstract

The cupruretic chelator, 2,3,2-tetramine, was used to maintain a 40% reduction in cellular copper (Cu) in Hep G2 cells for up to 3 passages. The influence of the tetramine depleted Cu status on the transcription regulation of apolipoprotein A-I (apo A-I) gene in Hep G2 cells was examined by using a transient transfection assay. The promoter sequence for Hep G2-specific expression located between -256 to -41 upstream of apo A-I gene transcription start site (+1) was used to prepare the reporter construct -256 A-I.CAT for transfection into tetramine-treated and untreated cells. A 40% increase in CAT activity for this reporter construct was observed in treated Hep G2 cells. However, the ability of apo A-I regulatory protein 1 (ARP-1) to repress the CAT activity of -256 A-I.CAT was not affected by the depressed Cu status in Hep G2 cells. Additional gel shift experiments demonstrated that the increased apo A-I gene expression may be mediated by enhanced binding of hepatocyte nuclear factor 4 (HNF-4) and other unknown nuclear factors to site A, one of three regulatory sites of the apo A-I gene promoter. Thus the transcription of apo A-I gene may be dependent on the intracellular balance of enhancing and repressing regulatory factors.
Introduction

High density lipoprotein (HDL) is involved in the process of "reverse cholesterol transport" (Glomset, 1968; Miller, Laville and Crook, 1985), which returns excess cholesterol from peripheral tissues back to the liver for excretion. Apolipoprotein A-I (apo A-I), the major protein component of HDL, is inversely correlated with the risk of cardiovascular disease (Castelli et al., 1986; Rubin et al., 1991). Thus, one possible approach to reduce the risk of cardiovascular disease is to increase plasma level of apo A-I, especially in subjects with low apo A-I levels. In order to accomplish this goal, the molecular mechanism(s) that enhance apo A-I expression must be established.

The apo A-I gene is expressed mainly in the liver and small intestine (Elshourbagy et al., 1985; Zannis et al., 1985) and is highly conserved in the region of -240 to -15 in both humans and rats (Sastry, Seedorf and Karathanasis, 1988). Three sites within this region, namely site A (-214 to -192), site B (-169 to -146) and site C (-136 to -119), have been shown to regulate the human apo A-I gene (Higuchi et al., 1988; Widom et al., 1991). Site A has been established as a positive and negative response element for the transcription factors, retinoid X receptor alpha (RXR-α) and apo A-I regulatory protein 1 (ARP-1), respectively (Ladias and Karathanasis, 1991; Rottman et al., 1991), which are members of the steroid-thyroid receptor superfamily. In addition, a number of factors has been shown to bind to site B (Papazafir et al., 1991). Furthermore, hepatocyte nuclear factor 4 (HNF-4), another member of the steroid-thyroid hormone receptor family, has been found to interact with site A (Widom et al., 1991) and with site C (Chan et al., 1993) to enhance activity of the apo A-I promoter.
Hypercholesterolemia is well established, during the diet induced copper deficiency, in rats and other rodent species, as well as in non-human primates and humans (reviewed by Lei, 1989). In addition, the protein component of plasma HDL is markedly increased in copper-deficient rats (Croswell and Lei, 1985; Al-Othman et al., 1992) and hamsters (Al-Othman et al., 1991). In rats, this elevation in plasma HDL protein is associated with a more than two-fold increase in plasma apo A-I pool size (Hoogeveen, 1993). Moreover, a two-fold increase in apo A-I synthesis by the liver of copper-deficient rats has been established in vivo (Hoogeveen and Lei, 1991). Similarly, an enhanced synthesis and secretion of apo A-I has been observed in vitro in freshly isolated hepatocytes derived from rats deficient in copper (Hoogeveen and Lei, 1992). Furthermore, a two-fold increase in apo A-I synthesis has been reported in Hep G2 cells depleted of copper by a cupruretic chelator, 2,3,2-tetramine (Zhang et al., 1994). Thus, a depressed copper status, either by the use of diet or chelator, appears to enhance hepatic apo A-I synthesis.

The present study was designed to examine the regulation of apo A-I gene expression in copper-depleted Hep G2 cells. The use of a reporter construct -256 A-I.CAT, in a transient transfection study, demonstrated that the apo A-I gene promoter activity was enhanced in these cells. Moreover, gel shift studies indicated that the increase apo A-I gene expression may be mediated by the increased binding of HNF-4 and other unknown factors to site A of the apo A-I promoter.
Materials and Methods

Preparation of Competent *E. coli* Cells (Strain HB101) for Plasmid DNA Transformation

Competent *E. coli* cells (strain HB101) were prepared according to the protocol in Sambrook, Fritsch and Maniatis (1989) and is briefly described as follows. Frozen cells were streaked and cultured on a 100 mm Luria-Bertani (LB) agar petri dish. A single colony was inoculated into 100 mL of 2X YT medium (Sambrook *et al.*, 1989) and grown overnight at 37°C in a series 25 shaker incubator (New Brunswick Scientific Co. Inc., Edison, NJ) with constant shaking at a rate of 250 rpm. Fifty mL of the overnight culture were added to 450 mL of LB medium (Sambrook *et al.*, 1989) (i.e. 1:10 dilution) in a 2-liter flask and incubated at 37°C with shaking (250 rpm) for 2-3 h until the OD$_{660}$ reached 0.8. Cultured cells were pelleted by centrifugation at 4°C for 10 min at 4000 rpm in a Beckman centrifuge (Model J6-HC, Fullerton, CA). Cell pellets were resuspended in 250 mL of ice-cold 50 mM CaCl$_2$ and incubated on ice for 30 min. The resuspended cells were centrifuged at 4000 rpm for 10 min at 4°C. Cell pellets were kept on ice and gently resuspended in 50 mL (1/10 of the original cell culture volume) of CaCl$_2$-glycerol buffer (2:1 ratio of 50 mM CaCl$_2$ : glycerol). The cell suspension was then aliquoted into 100 μL in Eppendorf tubes, frozen in dried ice-ethanol, and kept at -80°C. Competent cells thus prepared can be stored at -80°C for at least one year.
Transformation of \textit{E. coli} Cells with Plasmid DNA

A vial of 100 $\mu$L of competent HB101 cells were quickly thawed at room temperature. One $\mu$L of plasmid DNA (10 ng/$\mu$L) was added to the cells and incubated on ice for 30 to 60 min. Cells were then heat-shocked at 42°C (water bath) for 90 sec and placed on ice. One mL of 2X YT medium were then added and the cells were incubated in a 37°C incubator for 60 min. Since all of the plasmid DNA used in this study encode an ampicillin-resistant gene, ampicillin-resistance was used as a selection marker. Twenty-five to one hundred $\mu$L of the culture were plated onto a LB plate containing 100 $\mu$g/mL of ampicillin and incubated at 37°C overnight for colonies to develop. The plates with colonies were used within two weeks.

Large Scale Preparation of Plasmid DNA for Hep G2 Cell Transfection

Large quantities of plasmid DNA were prepared by using Qiagen-tip 500 columns (Qiagen, Chatsworth, CA) according to the manufacture’s instructions. Typically, more than 1 mg of plasmid DNA were obtained from 500 mL of overnight \textit{E. coli} cell culture.

Verification of Plasmid DNA by Restriction Enzyme Digestion and DNA Sequencing

Identities of plasmid DNA prepared from Qiagen columns were verified before being used for any purpose. Most of the plasmid DNA were verified by restriction endonuclease digestion followed by agarose gel electrophoresis. Restriction enzyme (New England Biolab, Beverly, MA) digestion of plasmid DNA was performed according to the supplier’s instruction. Two plasmid DNA (plasmids -41 A-I.CAT and -256
A-I.CAT) were verified by DNA sequencing analysis with the T7 sequence kit (US Biochemicals, Cleveland, OH) using M13 reverse primer.

**Hep G2 Cell Maintenance and Transfection**

Reagents for Hep G2 cell culture were all purchased from GIBCO BRL (Gaithersburg, MD). Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal calf serum, 1% penicillin-streptomycin (GIBCO BRL cat. no. 15140-015) was used for cell maintenance. Cells were grown and maintained in a fully humidified incubator at 37°C in a 5% CO₂ and 95% atmosphere. A vial of frozen Hep G2 cells (ATCC, Rockville, MD) was quickly thawed at 37°C and transferred to a 100 mm culture dish containing 10 mL medium. The cells were replenished with fresh medium the next day. After confluency was reached, the cells were split at 2 million cells per plate. At the end of the second passage, cells were split and divided into two groups. Group 1 was used as the control in which cells were grown in the normal medium condition. Group 2 was used as an experimental group in which the culture medium contained 20 μM 2,3,2-tetramine (Kodak, Rochester, NY). When cells became confluent at the end of the second passage of tetramine treatment, cells were split again at 2 millions cells per plate for transfection. The experimental plates were cultured in medium containing tetramine throughout the experiment.

Twenty-four h after seeding, cells were replenished with fresh medium. Three h later, transfected DNA (see preparation of transfected DNA) was added to the cells and incubated overnight at 37°C. On the next day, transfected cells were treated as follows.
Medium was removed and cells were washed with 10 mL of serum-free DMEM. Two mL of 15% glycerol in 1X HEBS (25 mM Hepes, 140 mM NaCl, 0.75 mM Na₂HPO₄, pH 7.2) were added to each plate and left at room temperature for 90 sec. Thereafter, 10 mL of serum-free DMEM were added to wash off the glycerol. Cells were incubated with 10 mL of medium with or without 20 µM tetramine at 37°C for 48 h.

Transfected cells were harvested 48 h after glycerol shock as follows. Medium was removed and cells were washed twice with 10 mL D-PBS (GIBCO BRL) and then scraped into 10 mL D-PBS. Cell suspensions were transferred into a 15 mL falcon tube and centrifuged at 3000 rpm at room temperature in a table top centrifuge (Beckman GS-6 centrifuge). The supernatant was decanted. Cells were resuspended in 1 mL of buffer (250 mM Tris, pH 7.8, 5 mM EDTA, and 150 mM NaCl), transferred to a 1.5 mL Eppendorf tube, and microcentrifuged for 5 min at room temperature. The supernatant was again removed and cell pellet was resuspended in 300 µL of reaction buffer (250 mM Tris-HCl, pH 7.8, 5 mM EDTA), and frozen in dried ice-ethanol. Cell suspensions were frozen and thawed (dried ice-ethanol vs 37°C water) three times. Cell lysates were collected by spinning the tube at 4°C for 15 min in a microcentrifuge. After the measurement of β-galactosidase activity, the remaining supernatant was frozen in dried ice-ethanol and kept at -80°C for analyzing chloramphenicol acetyltransferase (CAT) activity in the future.
Preparation of Transfected DNA

Transfected DNA was prepared according to the calcium-phosphate coprecipitation method as follows. The various types of DNA used for transfection were: 3 \( \mu \)g of pRSV-\( \beta \)-gal (Edlund \textit{et al}., 1985), 5 \( \mu \)g of reporter construct (for -41 A-I.CAT and -256 A-I.CAT), 2 \( \mu \)g of pMT2-ARP-1 (Ladias and Karathanasis, 1990) where indicated, and pMT2-UT3’ (Mietus-Snyder \textit{et al}., 1992) to make up a total of 15 \( \mu \)g DNA. Inclusion of plasmid DNA encoding \( \beta \)-galactosidase (pRSV-\( \beta \)-gal; Edlund \textit{et al}., 1985) was used to correct for DNA uptake efficiency by cells. Various amounts of DNA and 31 \( \mu \)L of Ca buffer (161.3 mM Tris-\( \text{HCl} \) pH 7.6, 2 M CaCl\(_2\)) were added to Eppendorf tubes. Water was added to make up to 500 \( \mu \)L. Five hundred \( \mu \)L of 2X HEBS (50 mM Hepes, 280 mM NaCl, 1.5 mM Na\(_2\)HPO\(_4\), pH 7.2) were placed in a 15 mL Falcon tube. While gently vortexing the falcon tube, the DNA mixture was added to the Falcon tube drop by drop in a 1 min time period. DNA precipitate was kept at room temperature for a minimal of 30 min before adding to the cells.

\( \beta \)-Galactosidase Assay

The activity of \( \beta \)-galactosidase (\( \beta \)-gal) was used as an internal control for transfection efficiency and was assayed as follows. The reaction mixture (300 \( \mu \)L) contained 4 \( \mu \)L of cell extracts, 66 \( \mu \)L of 53.1 mM ONPG (\( \sigma \)-nitrophenyl \( \beta \)-D-galactopyranoside), 3 \( \mu \)L of 0.1 M MgCl\(_2\), 5 M \( \beta \)-mecaptoethanol solution, and 227 \( \mu \)L 0.1 M sodium phosphate, pH 7.3. After 30 min of incubation at 37°C, the reaction was stopped by adding 500 \( \mu \)L of 1 M Na\(_2\)CO\(_3\). Absorptions at 420 nm was used to
determine the β-gal enzymatic activity. One β-gal unit is defined as the amount of extracts which produced 1 absorption unit at 420 nm at the above reaction conditions.

Chloramphenicol Acetyltransferase (CAT) Assays

Apo A-I promoter activity in reporter constructs containing various portions of apo A-I promoter was analyzed by CAT assays using cell extracts from transient cotransfection experiments as described below. The reaction mixture (160 μL) was consisted of cell extracts with 6 β-gal units, 1 μL 14C-chloramphenicol (1 μCi/μL, Amersham, Arlington Heights, IL), and reaction buffer (250 mM Tris-HCl, pH 7.8, 5 mM EDTA). Five min after incubation at 37°C, 20 μL of acetyl-CoA (Pharmacia LKB, Piscataway, NJ) prepared in the above reaction buffer were added to the reaction. After mixing, the reactions were incubated for another h and then stopped by adding 500 μL of ice-cold ethyl acetate. After vortexing for 15 sec, the mixture was microcentrifuged for 10 min at room temperature. Four hundred and twenty μL of supernatant (the ethyl acetate layer) were transferred to a new tube and dried for 30 min in a speed vac. Twenty μL of ethyl acetate were then added to each dried tube and the tube was vortexed for 15 sec. All of the 20 μL of ethyl acetate suspension were then spotted onto a thin layer chromatography (TLC) plate (20 × 20 cm, Baker) and acetylated chloramphenicol was then separated from substrate by TLC in 200 mL of 5% methanol:95% chloroform. The TLC plate was removed from the tank when the solvent front was about 1 inch from the top edge, air-dried, and counted by beta-scope (Betagen, Waltham, MA) for 30 min. The CAT activity was calculated as the percent of 14C-chloramphenicol converted to
acetylated chloramphenicol. The expression of apo A-I CAT constructs was normalized to β-gal activity and expressed as CAT/β-gal activity (Gorman, Moffat and Howard, 1982).

Preparation of Whole Cell Extracts for Gel Shift Assays

Plates of confluent Hep G2 or tetramine-treated Hep G2 cells were washed twice with D-PBS and cells were scraped into 10 mL cold D-PBS. Cells were transferred to a 15 mL Falcon tube and centrifuged at 4°C at 3,000 rpm for 10 min. Supernatant was decanted and cell pellets were suspended in 1 mL cold D-PBS containing 0.5 mM PMSF. Cells were then transferred to an Eppendorf tube and pelleted in a microcentrifuge for 5 min at 4°C. Supernatant was removed and cell pellets were resuspended in 100 µL of buffer (20 mM Hepes pH 7.8, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, and 5 µg/mL of aprotinin, leupeptin, and pepstatin). All of the protease inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN). Cells were lysed by 3 cycles of quick freeze/thaw and cell extracts were collected by spinning in a microcentrifuge for 15 min at 4°C. The whole cell extracts in the supernatant were transferred to a new tube, aliquoted into 25 µL each, quickly frozen in dried ice-ethanol and stored at -80°C. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad).

Preparation of Probe for Gel Shift Assay

Single-stranded site A oligos (the sense strand 5’-gatc-ACTGAACCCTTGACCCCTGC-CCT-3’ and the antisense strand 5’-gatc-
AGGGCAGGGGTCAAGGGTTCAAGT-3'), purified by anion-exchange high pressure liquid chromatography, were purchased from the Midland Certified Reagent Company (Midland, Texas). The small letters gatc in the oligonucleotides represent BamHI linker and were designed for cloning purpose, the capital letters correspond the apo A-I promoter sequence from -214 to -192 (Widom et al., 1991). Double-stranded oligos were formed by annealing the two single-stranded oligos in TES buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 100 mM NaCl). Annealing was accomplished in a heat block at 94, 67, 37, 25 and 4°C for 4 min at each temperature and then stored at -20°C.

Double-stranded (ds) site A oligos (50 ng) in TES buffer were labeled in a mixture consisting 2 μL of 10X kinase buffer (provided with the purchase of T4 polynucleotide kinase from New England Biolabs), 12 μL of fresh gamma-32P-ATP (10 μCi/μL, Amersham), 1 μL of T4 polynucleotide kinase (10 units/μL, New England Biolabs). After 30 min of incubation at 37°C, 1 μL of kinase was again added and the reaction was continued for another 30 min. The unincorporated 32P-ATP was then removed by G-50 Sephadex Quick Spin column (Boehringer Mannheim). The labeled ds site A oligos were adjusted with TES buffer to a final concentration of 0.1 ng/μL and used within a week.

Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE)

The nondenaturing PAGE was carried out using a Protein II xi vertical electrophoresis apparatus (Bio-Rad, Richmond, CA). A mixture of 88 mL of water, 20 mL of 30% acrylamide stock [(30:1) acrylamide:bis-acrylamide], 12 mL of 5X TBE
buffer (Sambrook et al., 1989), 750 μL of 10% ammonium persulfate, and 75 μL of TEMED was prepared to cast two 5% polyacrylamide gels. The gels were allowed to polymerize for about 1 hour at room temperature and then pre-electrophoresed at 200 volts for 30 minutes before the loading of samples.

**Gel Shift Assays**

Gel shift assay reactions were set up individually in Eppendorf tubes. The final reaction volume was 20 μL which contained 2 μL of 10 × binding buffer (100 mM Hepes pH 7.9, 500 mM KCl, 10 mM EDTA, 70% glycerol), 1 μL of 20 mM DTT, 1 μL of 20 ng/μL of single-stranded oligo unrelated to the probe, 2.5 μL of 0.5 μg/μL polydI:polydC (Boehringer Mannheim) and indicated amount of extracts. The reaction was incubated at room temperature for 10 minutes. Two μL of 0.1 ng/μL of 32P-labeled probe (site A probe) were then added and the reaction was continued for 20 minutes at room temperature. When antibodies were used, they were added 10 minutes after the addition of the probe (The COUP-TF antisera was a kind gift from Dr. M.-J. Tsai, Baylor College of Medicine. The HNF4 antisera was a kind gift from Dr. F.M. Sladek, U.C. Riverside). One μL of tracking dye (0.4% bromophenol blue in 1X reaction buffer) was added. The entire reaction mixture was loaded into a 5% polyacrylamide gel (20 × 20 × 0.15 cm). The electrophoresis was carried out with 0.5X TBE buffer at 200 volts, cooled with tap water, and stopped when the tracking dye bromophenol blue was about 4 inches away from the bottom of the gel. After gel electrophoresis, the gel was placed on top of two pieces of Whatman paper, covered with Saran wrap, dried at 80°C for
about 80 min. The dried gel was exposed to a film with two intensifying screens at -70°C for about 3 h to overnight. The film was then developed. The relative radioactivity of various bands was then quantitated using a beta-scope (Betagen).

**Results**

A two-fold increase in hepatic apolipoprotein A-I (apo A-I) synthesis (Hoogeveen and Lei, 1991; 1992) and a 28% increase in total hepatic apo A-I mRNA (Hoogeveen, 1993) have been observed in Cu-deficient rats. The following experiments were designed to examine the influence of Cu deficiency on the transcriptional regulation of apo A-I gene in tissue culture, human liver hepatoma cell line Hep G2, using the transient co-transfection assay.

**Treatment of Hep G2 Cells with Tetramine Enhances Apo A-I Gene Expression in Transient Transfection Assay**

The promoter sequence for Hep G2-specific expression has been defined to be located between -256 to -41 upstream of apo A-I gene transcription initiation site (Sastry et al., 1988). Reporter constructs -256 A-I.CAT and -41 A-I.CAT (Sastry et al., 1988) were transfected into normal and tetramine-treated Hep G2 cells. CAT activity from these cell extracts were analyzed after correcting DNA uptake with β-galactosidase assay and presented as bar graph in Figure 9. A 27.4% and a 39.2% increase in CAT activity for reporter construct -256 A-I.CAT were observed in Hep G2 cells treated with tetramine as compared to the untreated controls, in the absence or presence of ARP-1 co-transfection, respectively.
FIGURE 9. Tetramine treatment of Hep G2 cells enhances apo A-I gene expression. Reporter construct -256 A-I.CAT (5 μg) was transfected into Hep G2 cells or tetramine (TETA)-treated Hep G2 cells (treated for 2 passages) in the presence or absence of 3 μg ARP-1 expression vector. Reporter construct -41 A-I.CAT containing only the basal promoter of apo A-I gene was used as a control. The normalized CAT activity was expressed as the ratio of CAT/β-galactosidase activity in extracts from these cells. The transfection study was performed in triplicate. Near identical results were obtained from another independent study.
The transcriptional factor ARP-1 (apo A-I regulatory protein 1) has been reported to repress apo A-I gene expression in transient transfection assays in Hep G2 cells (Ladias and Karathanasis, 1991). In order to examine whether tetramine treatment may exert a direct effect on the ability of ARP-1 to repress apo A-I gene expression, transfection assays were performed in the presence and absence of ARP-1 co-transfection. The results (Figure 9) indicated that the ability of transcription factor ARP-1 to repress CAT activity of -256 A-I.CAT is not affected by Cu-deficiency.

Tetramine-Treatment Enhances Binding of Transcriptional Factors to Site A of the Apo A-I Promoter

So far only potential factors targeting site A of the apo A-I promoter have been identified (Widom, Rhee and Karathanasis, 1992). In order to establish that the enhanced CAT activity of -256 A-I.CAT associated with tetramine treatment may have resulted from an altered ability of various transcriptional factors to bind to site A, DNA binding assays were conducted. In this study, a gel shift assay was employed to measure the binding of Hep G2 cell extracts to site A of the apo A-I promoter. Cell extracts were prepared from normal Hep G2 cells and from the second and the third passage of tetramine-treated Hep G2 cells since transfection were carried out between the second and the third passage of tetramine treatment. Figure 10A depicts the autoradiograph of a typical gel shift assay. The intensities of the shifted band or protein/site A complex in tetramine-treated cell extracts (from both passage 2 and 3) are much stronger than that from the untreated cell extracts. The actual difference was better illustrated when the
FIGURE 10A. Comparison of the binding of whole cell extracts from Hep G2 cells and tetramine (TETA)-treated Hep G2 cells to site A probe using gel shift assays. Preparation of whole cell extracts and gel shift assays were described in Materials and Methods. Cell extracts from untreated, TETA-treated (for 2 and 3 passages) Hep G2 cells were compared for their ability to bind to $^{32}$P-labeled site A probe. Lanes 1 to 5 represented increasing concentration of Hep G2 cell extract (3, 6, 12, 24, 48 µg proteins respectively). Lanes 6 to 10 depicted increasing concentration of extracts from cells treated with TETA for 2 passages (3, 6, 12, 24 and 48 µg protein respectively). Lane 11 was without any extracts. Lanes 12 to 16 represented increasing concentration of extracts from cells treated with TETA for 3 passages. $P=$ Protein-site A probe complex. $NS=$ Non-specific binding. $FP=$ Free probe.
relative radioactivities of each band were quantitated in a beta-scope. As can be seen in Figure 10B, when equal amounts of cell extracts were used, those from tetramine-treated cells bind more efficiently to the site A probe than those from untreated cells. For example, at 24 μg/mL of extracts, there were more than twice as much binding in tetramine-treated than untreated. Data from this experiment suggest that tetramine treatment enhanced the ability of site A factors in Hep G2 cells to bind to site A.

The Enhanced Binding Activity of Tetramine-Treated Cell Extracts Is Due at Least in Part to Enhanced HNF-4 Activity

Several members of steroid/thyroid hormone receptor superfamily, which include ARP-1, HNF-4 (hepatocyte nuclear factor 4), and RXR-α (retinoid X receptor alpha), have been implied to be involved in regulation of apo A-I gene expression (Karathanasis, 1992). To investigate if one or several of these factors are influenced by tetramine treatment of Hep G2 cells, gel shift assays were conducted using antibodies made against HNF-4 and COUP-TF. Anti-HNF-4 antibody interacts specifically with HNF-4, whereas anti-COUP-TF antibody will interact with all of the members of the COUP-TF family including ARP-1. In this experiment, cell extracts from normal Hep G2 cell and from Hep G2 cells treated for two passages with tetramine were used. The gel shift results were depicted in Figure 11. As can be seen from the figure, the binding of cell extracts to site A was specific and could be competed by 100-fold molar excess of unlabeled site A (compare lanes 1 and 2 and lanes 6 and 7) but not by unrelated oligos (data not shown). Both normal Hep G2 cells and tetramine-treated cells appeared to contain HNF-4 and members of COUP-TF/ARP-1 family because in the presence of their
FIGURE 10B. Quantitative comparison of binding activity of cell extracts from tetramine (TETA) treated and untreated Hep G2 Cells. The relative intensity in the bands of protein-site A probe complex was quantitated in a beta-scope and expressed as relative radioactivity. TETA-2 and TETA-3 represent Hep G2 cells treated with tetramine for 2 and 3 passages, respectively.
FIGURE 11. Comparison of HNF-4 and ARP-1/COUP-TF binding activities in Hep G2 cells treated or untreated with tetramine (TETA). Whole cell extracts from untreated (lanes 1 to 5) and TETA-treated (for 2 passages, lanes 6 to 10) Hep G2 cells were used in gel shift assays in various conditions. Lanes 1 and 6: cell extracts only. Lanes 2 and 7: 100-fold excess of site A oligo. Lanes 3 and 8: in the presence of 1 µL of 1 to 10 dilution of antiserum against HNF-4 (Ab-H). Lanes 4 and 9: in the presence of 1 µL of 1 to 10 dilution of antiserum against COUP-TF (Ab-C). Lanes 5 and 10: in the presence of both antisera. P = Protein/site A complex. NS = Non-specific binding. FP = Free site A probe.
corresponding antibodies, supershifted complexes of antibody—specific nuclear protein—site A were detected. The double supershift assays (lanes 5 and 10) when both antibodies were present revealed that there were still significant amount of unshifted protein/site A complex, which implied the presence of other unidentified factors capable of binding to site A. In order to compare the intensity of the bands supershifted by HNF-4 and COUP-TF antibodies, the relative radioactivity of these bands were determined using beta-scope. The results indicated (Table 5) that the relative radioactivity of the bands shifted by COUP-TF antibody in tetramine-treated cell extracts was slightly higher than untreated cells. Whereas the relative radioactivity of the bands super-shifted by HNF-4 antibody in tetramine-treated cells were more than twice as much as that of untreated cells, indicating that treatment of Hep G2 cells with tetramine enhanced the binding of HNF-4 to site A. In addition, there were more HNF-4 activity, with respect to site A binding, than ARP-1/COUP-TF activity even in normal Hep G2 cells.

Discussion

Gene expression in general is controlled by a balance of positive and negative regulators. In the case of apo A-I gene expression, only a limited number of transcriptional factors have been implicated in its regulation. All of them belong to the members of the steroid/thyroid hormone receptor superfamily and they all bind to site A, one of the three regulatory sites in the apo A-I gene promoter (Karathanasis, 1992). ARP-1, a member of COUP-TF family, has been found to repress apo A-I gene
TABLE 5. Comparison of the relative radioactivities of the bands supershifted by anti-HNF-4 and anti-COUP-TF antibodies

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Hep G2</th>
<th>TETA-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Ab-H</td>
<td>Ab-C</td>
<td>Ab-C</td>
</tr>
<tr>
<td>6995</td>
<td>3173</td>
<td>11965</td>
</tr>
</tbody>
</table>

The relative radioactivities of the bands supershifted by anti-HNF-4 (Ab-H) and anti-COUP-TF (Ab-C) antibodies or both in Figure 3 were quantitated by beta-scope. Hep G2 represents cells not treated. TETA-2 represents cells treated with tetramine for two passages.
expression, and this repression can be alleviated by the presence of retinoid receptor RXR-α. Therefore changes in the concentration of different regulators could affect the level of apo A-I gene expression.

The present study demonstrated that treatment of Hep G2 cells with tetramine, a copper chelator, stimulated apo A-I gene expression in transient transfection assays. Additional experiments using gel shift assays indicated that the increased apo A-I gene expression was probably mediated by enhanced binding of HNF-4 and some other unknown factors to site A, one of three regulatory sites in apo A-I gene promoter. HNF-4 has been shown to be a positive transcriptional factor for apo A-I gene expression. Therefore, treatment of Hep G2 cells with tetramine may have further enhanced the binding of HNF-4 to site A and induced an increased apo A-I gene expression. At present, the mechanism responsible for the stimulatory effect of tetramine on HNF-4 activity is not clear. One possibility is that tetramine treatment may affect the stability of transcriptional factors. For example, transcriptional factors HNF-4 and RXR-α are highly susceptible to intracellular protease degradation whereas ARP-1 is very stable under similar conditions.

HNF-4 has been shown to be a positive transcriptional factor which can overcome the repression of ARP-1, a negative factor, on apo C-III gene expression in transient transfection assays (Mietus-Snyder et al., 1992). However, in our study, though tetramine treatment of Hep G2 cells enhanced the ability of HNF-4 to bind to site A, the enhanced HNF-4 activity could not overcome the ARP-1 repression on apo A-I gene expression (Figure 9). The dominant effect of ARP-1 on apo A-I gene expression
suggests that HNF-4 may act differently in different promoters. Probably other factors, as implied by our data presented in Figure 11, are required in order for HNF-4 to overcome the ARP-1 repression.
CHAPTER VI

SUMMARY

Two amine chelators, namely 2,3,2-tetramine and diamsar, at various levels were used to deplete copper (Cu) from cultured Hep G2 cells. For cells cultured at 0.63 \( \mu \text{mol of Cu/L} \), maximal depletions were attained after 24 h of incubation with 10 \( \mu \text{mol} \) of either chelator/L, which resulted in an average significant depletion of 45% of cellular Cu. In addition, when cells were cultured for 4 passages continuously at 0.63 \( \mu \text{mol of Cu/L} \) with 20 \( \mu \text{mol of 2,3,2-tetramine/L} \), cellular Cu was significantly depleted more than 40% compared to controls. Furthermore, after 2 passages of 2,3,2-tetramine treatment, cells were pulsed for 10 min with \(^{3}\text{H}\)leucine and chased up to 2 h. At the end of the pulse, the amount of \(^{3}\text{H}\)leucine incorporated into apo A-I was twofold higher in treated than control cells. No difference was detected for the synthesis of apo B and total protein. During the chase, the cellular depletion curves for apo A-I, apo B and total protein were not altered by 2,3,2-tetramine treatment. From 30 min to 120 min of the chase, the amount of nascent apo A-I degraded was small and not altered, but that secreted into the medium was 56% higher in the Cu-depleted than control cells.

A 40% reduction in cellular copper (Cu) in Hep G2 cells was maintained for up to 3 passages by the use of the 2,3,2-tetramine treatment. The influence of the tetramine depleted Cu status on the transcription regulation of apolipoprotein A-I (apo A-I) gene in Hep G2 cells was examined by using a transient transfection assay. The promoter sequence for Hep G2-specific expression located between -256 to -41 upstream
of apo A-I gene transcription start site (+1) was used to prepare the reporter construct -256 AI. CAT for transfection into tetramine-treated and untreated cells. A 40% increase in CAT activity for this reporter construct was observed in treated Hep G2 cells. However, the ability of apo A-I regulatory protein 1 (ARP-1) to repress the CAT activity of -256 AI. CAT was not affected by the depressed Cu status in Hep G2 cells. Additional gel shift experiments demonstrated that the increased apo A-I gene expression may be mediated by enhanced binding of hepatocyte nuclear factor 4 (HNF-4) and other unknown nuclear factors to site A, one of three regulatory sites of the apo A-I gene promoter. Thus the transcription of apo A-I gene may be dependent on the intracellular balance of enhancing and repressing regulatory factors.
REFERENCES


