The Effect of Individual Amino Acids on ApoB100 and Lp(a) Secretion by HepG2 Cells*

Katherine Cianflone‡, ZuJun Zhang, Hai Vu, Rita Kohen-Avramoglu, David Kalant, and Allan D. Sniderman
From the McGill Unit for the Prevention of Cardiovascular Disease, McGill University, Montreal, Quebec H3A 1A1, Canada

The rate at which HepG2 cells secrete apoB100 lipoproteins is inversely related to the concentration of amino acids in the medium (Zhang, Z., Sniderman, A. D., Kalant, D., Vu, H., Monge, J. C., Tao, Y., and Cianflone, K. (1993) J. Biol. Chem. 268, 26920–26926). The purpose of the present study was to determine the effect of individual amino acids on apoB100 and lipoprotein secretion.

Asparagine was associated with modestly increased secretion. The branched chain amino acids (leucine, isoleucine, and valine) and lysine had minor inhibitory effects. The other amino acids, by contrast, decreased apoB secretion, although the magnitude of the effect varied considerably, the most potent being tyrosine, cysteine, phenylalanine, tryptophan, methionine, and glutamine. Although the effect on Lp(a) generally paralleled that on apoB100, it was usually much less pronounced. No amino acid caused a marked decrease in albumin, apoAI, or total protein secreted from the HepG2 cells. The amino acid effect on apoB was paralleled by similar decreases in secreted cholesterol ester (CE) primarily in the low density lipoprotein density range (d < 1.006–1.063 g/ml), although there was no significant change in intracellular CE. Neither intracellular nor secreted triglycerides (TG) or free cholesterol changed, resulting in a slightly larger TG-enriched particle being secreted. The effect was confirmed in cultured primary hamster hepatocytes, where a mixture of amino acids also caused a decrease in apoB secretion (up to 40%). ApoAI appeared to increase as with the HepG2 cells. Secreted CE paralleled apoB. There was no change in intracellular or secreted TG or free cholesterol, resulting in a substantially larger TG-rich particle being secreted. mRNA for apoB100 increased with asparagine, decreased moderately with branched chain amino acids, and decreased further with glutamine, as shown by dot blot and Northern blotting. Pulse-chase studies indicated that there was no change in apoB secretion efficiency under any condition.

These results extend our previous observations by demonstrating specificity of the amino acid effect on apoB100 secretion. Although an effect on transcription is the likely mechanism, the exact basis for this remains to be determined.

Assembly and secretion of apoB100 lipoprotein particles by hepatocytes is a complex process. Regulation occurs at both transcriptional and post-transcriptional levels (1–3). Only a portion of the apoB100 molecules that are synthesized are eventually secreted from the cell as lipoprotein particles, the rest being hydrolyzed intracellularly shortly after they are synthesized (for review see Refs. 1–3). Rapid association with neutral lipids such as triglycerides (TG) and cholesterol esters (CE) may be essential for a newly synthesized apoB100 molecule to enter the lumen of the endoplasmic reticulum as a nascent lipoprotein particle and thus escape early degradation (4). Although the most important effects of lipids on apoB100 secretion are exerted at this level, it should be noted that smaller influences have also been demonstrated on the transcriptional process (5, 6).

Insulin, at least acutely, has been shown to reduce secretion of these lipoprotein particles possibly at a post-translational level although the mechanism by which it does so has not yet been elucidated (7). Albumin also affects secretion of apoB100 particles, with lower concentrations in the medium being associated with higher rates of apoB100 secretion. This effect may relate to concurrent changes in intracellular CE synthesis (8, 9). By contrast, amino acids alter the rate of apoB100 synthesis but do not alter the secretion efficiency from HepG2 cells (10); i.e. as amino acid concentration in the medium is increased, apoB100 synthetic rates decrease without any change in the proportion of molecules that are degraded intracellularly. In addition, parallel changes between the apoB100 mRNA levels and changes in apoB100 secretion were demonstrated as amino acid delivery to the hepatocyte was altered (10).

In our previous studies of the effects of amino acids on apoB100 synthesis and secretion, only the effects of a standard amino acid mixture were studied (10). The present studies were designed, therefore, to determine whether all amino acids had a similar effect. We also took advantage of the opportunity to examine the effects of these amino acids on the secretion of Lp(a) lipoprotein particles, apoAI, albumin, and total secreted protein. We have previously reported in abstract form that Lp(a) particles are secreted by HepG2 cells and that there is an inverse relation between their secretion and ambient amino acid concentration as there is with apoB100 lipoproteins (11).

* This work was supported by an MRC-Industrial grant sponsored jointly by the Medical Research Council of Canada (UI-11434) and Merck Frosst Pharmaceuticals and a Baxter Extramural Grant (to A. D. S. and K. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a scholarship of the Heart & Stroke Foundation of Canada and Fonds de la Recherche en Santé du Quebec. To whom correspondence should be addressed; McGill Unit for the Prevention of Cardiovascular Disease, Royal Victoria Hospital, 687 Pine Ave. West, Montreal, Quebec H3A 1A1, Canada. Tel.: 514-842-1231 (ext. 5426); Fax: 514-982-0686.

1 The abbreviations used are: TG, triglyceride; CE, cholesterol ester; CHOL, cholesterol; BSA, bovine serum albumin; MEM, minimum essential medium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosassay; LDL, low density lipoprotein; GAPDH, glycerol-dehyade-3-phosphate dehydrogenase; C/EBPs, CCAAT enhancer-binding protein; BCAA, branched chain amino acids.
The present data indicate that the amino acid effect on secretion of these two lipoproteins is not general but varies depending on the specific amino acid being examined. Moreover, although there is considerable similarity, the effects of individual amino acids on apoB100 and Lp(a) secretion are not identical. Finally, this effect was not confined to HepG2 cells. Studies in cultured primary hamster hepatocytes confirmed that amino acids decrease apoB secretion in this model as well.

**EXPERIMENTAL PROCEDURES**

**Materials and Supplies**—Tissue culture medium, fetal calf serum, and other tissue culture supplies were obtained from Life Technologies, Inc., including a concentrated amino acid solution at 50 times the concentration in minimum essential medium (MEM) (12). Fatty acid-free bovine serum albumin (BSA) and each of the individual amino acids (tissue culture grade) were from Sigma. L-[4,5-3H]leucine (specific activity 40 Ci/mmol) was purchased from DuPont NEN. Hamaster plasma was obtained from BioProducts for Science (Indianapolis, Indiana).

HepG2 Tissue Culture—HepG2 cells obtained from the American Tissue Culture Collection (Rockville, MD) were grown in MEM supplemented with 10% fetal calf serum. Flasks were subcultured every 7 days with a split ratio of 1:3 using 0.25% trypsin in Ca2+- and Mg2+-free saline (PBS). For experiments, cells were plated out at a density of 2 × 105 cells/cm2 in 24-well plates 24 h before dishes (24-well plates). Isolation and Culture of Hamster Hepatocytes—Male Syrian golden hamsters weighing 100–120 g were used for hamster hepatocyte isolation. The liver was perfused with 10 ml HEPES in Ca2+- and Mg2+-free Hanks’ solution at 37°C, 4 ml/min for 20 min. The liver was placed in a sterile Petri dish with 20 ml of digestion solution (0.1% collagenase A, 0.05% hyaluronanase, Boehringer Mannheim) and 0.01% DNase (Sigma) dissolved at 37°C, 4 ml/min for 20 min. The liver was washed in 0.9% saline five or six times between each step. Subsequently, 100 ml warm saline was added, and the liver was placed on ice, and the medium was removed and incubated at 37°C for 24 h. Following digestion, cells were filtered through sterile cotton gauze, washed twice (by centrifugation) with DMEM/F-12 containing 20% fetal calf serum and penicillin G/streptomycin in leucine-free medium. The cells were plated out at a concentration of 2.5 × 105 cells/ml into tissue culture dishes that had previously been coated with a solution of 3 μg/ml collagen type IV (Becton Dickinson Labware). Cells were incubated overnight and then used for experimentation.

**Experimental Conditions**—Before the experiments were performed, cells were allowed to grow for 24 h at 37°C in 5% CO2 in serum-free medium supplemented with 1% BSA and the indicated amino acid concentrations. Cells were then changed to fresh serum-free medium supplemented with 1% BSA and the indicated amino acid concentrations and incubated at 37°C for 24 h. At the end of the incubation period, the cells were placed on ice, and the medium was removed and set aside for analysis. The cells were washed three times with 1 ml of ice-cold PBS, and soluble cell protein was dissolved in 1 ml of 0.1 N NaOH and collected.

**Total Cell Protein and Human ApoA1 Quantification**—Cell protein was measured by the method of Bradford (13) using BSA as a standard. Human apoA1 concentration in the medium was measured by a sandwich ELISA using a standard curve of 0.025–0.400 μg/ml of apoA1.

**Human ApoB100, Human Albumin, Hamster ApoB100, and Hamster ApoA1 Assays**—These medium proteins were measured by competitive ELISA using standard curves of 0.125–4.0 μg/ml for human and hamster apoB100 or human albumin. For hamster apoA1, a standard curve of 0.04–1.25 μg/ml was used. There was no effect of medium BSA concentration or medium amino acid concentration on the assays. Antigen was coated (3 μg/ml hamster or human LDL, 2 μg/ml human albumin or 3 μg/ml hamster apoA1 in PBS, 100 μl/well) overnight at 4°C, and the plate was blocked for 2 h with 250 μl of 1.5% BSA. Plates were washed in 0.9% saline five or six times between each step. Standards, blanks, controls, and samples were diluted appropriately in 0.05%/v/v Tween 20 in PBS, and 150 μl of diluted primary antibody and incubated overnight at 4°C.

For apoB100 antibody, LDL was isolated from normal human plasma or hamster plasma (15) and electrophoresed on a 5% SDS-polyacrylamide gel according to the method of Laemmli (16), and the band corresponding to apoB100 was cut out, emulsified, and injected subcutaneously in a rabbit to generate polyclonal anti-human apoB100 antisera. Anti-hamster apoA1 was produced in the same manner. After several extractions, the antisera was assessed by radial immunodiffusion to determine the appropriate dilution in 0.05% (v/v) Tween 20 in PBS. Rabbit anti-human albumin antibody was obtained commercially and diluted according to the manufacturer’s suggestion at 1:500,000 to 1:1,000,000 (Sigma). This antibody did not cross-react with BSA.

The mixture of sample/antibody was added to the microtiter plate (100 μl/well) and incubated at 37°C for 2 h in a shaking water bath. After washing, 100 μl/well of anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (Sigma) diluted 1:10,000 in 4% polyethylene glycol 6000, 0.05% (v/v) Tween 20 in PBS was added to each well and incubated at 37°C for 30 min. For color development, 100 μl of o-phenylamine diamine (1 mg/ml), 0.018% H2O2, 0.05% (v/v) Tween 20 in 100 mM sodium citrate, pH 5.0, was added to each well. The color was developed over 5–15 min, the reaction was quenched with the addition of 50 μl of 4 N H2SO4, and the optical density was read at 490 nm. Results are calculated from the linear portion of the curve calculated by regression analysis of log(concentration of antigen) versus log(optical density).

**Lp(a) Immunoassay**—Lp(a) was measured by a sandwich ELISA assay as described by Fless (17). Monoclonal antibody to apop(a) (Boehringer Mannheim) was coated at a concentration of 3 μg/ml and then blocked with BSA as described above. Then 100 μl of samples, standards (0.0313–1 μg/ml from Boehringer Mannheim), and controls diluted appropriately were added to the well and incubated for 2 h at 37°C. Subsequently, 100 μl of primary polyclonal anti-apoB100 antibody was added and incubated for 2 h at 37°C. Incubation with secondary goat anti-rabbit IgG conjugated to horseradish peroxidase, substrate (o-phenylamine diamine) solution, and quenching H2SO4 solution were as described above. Using this method, only Lp(a) lipoprotein particles are detected, not free apo(a). Western analysis confirmed the supplier’s indication that there was no cross-reaction of the monoclonal antibody with plasminogen. Increasing amounts of LDL did not interfere with the assay.

**Radiolabeled Protein Synthesis and Secretion**—Cells were incubated as described under “Experimental Conditions.” The medium was then removed and replaced with fresh serum-free medium containing 1% BSA and the same concentration of the indicated amino acids (27 mM) supplemented with [3H]leucine. Total radiolabeled protein synthesis and secretion was determined as described previously (10).

**Determination of Intracellular and Secreted Lipids and Lipoprotein Fractionation**—Following experimental incubation and removal of the medium, cells were washed three times with 1 ml of ice-cold PBS, the intracellular lipids were extracted twice with 1 ml of 3:2 (v/v) heptane/isopropanol alcohol at room temperature for 30 min, and the extracts were pooled and concentrated. The extracted mass was quantified as described previously (18). Conditioned medium was fractionated into lipoprotein fractions as described previously (18).

**Pulse-Chase Analysis of ApoB and Albumin**—HepG2 cells were preincubated for 18 h in MEM with 1% BSA with or without supplementation with 5.0 mg/ml amino acid mixture. Cells were then pulsed 10 min with [3H]leucine in leucine-free medium (specific activity, 0.01 mCi/ml) and chased in the same medium for 0–180 min. At the indicated times, the medium was collected, and labeled apoB and albumin were quantified as described previously (10).

**Slot Blot and Northern Analysis of HepG2 mRNA**—HepG2 cells were incubated as described under “Experimental Conditions.” Cells were then rapidly washed, and RNA was extracted as described previously (18). For Northern analysis, RNA was separated on a 1.0% agarose gel and transferred to a nylon membrane for Northern blot. For slot blot, aliquots of RNA were applied directly to the membrane. Biotinylated cDNA probes were prepared, and membranes were probed using an enzymatic chemiluminescent method as described previously (18) according to the manufacturer’s directions (Life Technologies, Inc.).

**Statistical Analysis**—Each point in each experiment is the average of triplicate determinations and is expressed per mg of cell protein ± S.D., and the number of experiments is indicated in the figure legends. Significance was measured by paired Student’s t test or t test of two means and indicated accordingly.

**RESULTS**

Based on our previous results of suppression of apoB100 synthesis and secretion by a mixture of amino acids, we tested each of the amino acids individually. Aspartic acid and glutamic acid were not tested; only asparagine and glutamine were. The general protocol for the experiments was as follows. HepG2 cells were incubated overnight in serum-free medium.
supplemented with 1% BSA and the indicated concentrations of the individual amino acids. The cells were then changed to fresh serum-free medium supplemented with 1% BSA and the individual amino acids and incubated for 24 h. The serum-free incubation medium (MEM) contained 3.46 mM essential amino acids, and this was the lowest concentration of amino acids tested. To this were added the individual amino acids. The cells were then changed to fresh serum-free medium supplemented with 1% BSA and the indicated concentration of individual amino acids (27.7 mM) was tested on albumin secretion, apoAI and Lys), and those amino acids that had a major inhibitory effect on apoB100 secretion (Thr, Ile, Ala, Gly, Arg, Thr, and Lys), and those amino acids that had a major inhibitory effect on apoB100 secretion (right panel; Trp, Met, Gln, Phe, Cys, and Tyr) listed in order of increasing effectiveness. Results are the average of eight experiments.

The data on the effect of the individual amino acids on apoB100 secretion is shown in Fig. 1 (three panels). Basal levels of apoB100 secretion (at 3.46 mM amino acid concentration) are indicated as 100% where the average apoB100 was 8.0 ± 1.5 µg/mg of cell protein for eight experiments. In these panels, the amino acids have been grouped into those that have little inhibitory effect or minor effects (Fig. 1, left panel), those in which a consistent but moderate dose-dependent decrease in apoB100 secretion with increasing amino acid concentration was demonstrated (Fig. 1, center panel), and those that had the largest inhibitory effect on apoB100 secretion by the HepG2 cells (Fig. 1, right panel).

Asn (Fig. 1, left panel) was the only amino acid in which there was a consistent positive relation between its concentration and apoB100 secretion. However, the absolute mass increases in apoB100 were small. Ser, His, and Pro (Fig. 1, left panel) had negative effects only at the lower concentrations tested. The branched chain amino acids, Leu, Ile (Fig. 1, left panel), and Val (Fig. 1, center panel), and Lys (Fig. 1, center panel) had minor but consistent inhibitory effects on apoB100.

In contrast, the other amino acids all caused apoB100 secretion to decrease as their concentration in the medium was increased, but the extent to which they caused apoB100 secretion to be reduced differed. Ala, Gly, Arg, and Thr (Fig. 1, center panel) had moderate inhibitory effects within this group. The most potent inhibitory amino acids appeared to be Tyr, Cys, Phe, Trp, Met, and Gln (Fig. 1, right panel). For comparison, a mixture of amino acids present in the same proportions as in the MEM, added at the same concentrations as shown in Fig. 1, produced an inhibition of 60 ± 7% at the highest concentration tested (27.7 mM), a result that is consistent with our previous study (10). It should be noted that the l-form of amino acids was used in all cases; when the d-form (nonmetabolizable) amino acids were used, no effect was obtained.

The effects of a single high concentration of the individual amino acids (27.7 mM) was tested on albumin secretion, apoAI secretion, total protein secretion, and apoB100 secretion by the HepG2 cells, and these results are shown in Fig. 2. The left are shown the results for the effect of individual amino acids on apoB100 secretion. The results are shown in the same order as in Fig. 1 with the control (basal) shown at the top followed by those amino acids having the least to the most effect on inhibition of apoB100 secretion at the bottom. In the other three panels are shown the effects of individual amino acids on secreted protein (measured as radiolabeled leucine incorporation into trichloroacetic acid-precipitable protein), apoAI, and albumin (both measured by immunosassay). Basal levels for [3H]leucine incorporation into protein were 2.3 ± 0.9 × 10^5 cpm/mg of cell protein, and for apoAI and albumin they were 1.38 ± 0.2 and 16.1 ± 2.6 µg/mg of cell protein, respectively. Basal levels for apoB secretion were 6.6 ± 0.7 µg/mg of cell protein. Inhibitory effects are shown in solid bars, stimulatory effects in hatched bars. In no instance was a major inhibitory effect demonstrated on apoAI, albumin, or total secreted protein. On the contrary, increased concentrations of individual amino acids tended to be associated with an overall increase in total secreted protein (average 25 ± 7% increase over basal amino acid levels in MEM). apoAI secretion changed moderately (average 26 ± 15% increase), whereas there was a larger increase in albumin secretion (average 56 ± 35% increase). All these data stand in contrast to the results observed for apoB100. There was no significant effect of the individual amino acids on overall cell protein synthesis (data not shown).

Lipoprotein Lp(a) mass in a medium of HepG2 cells was also measured. The effect of the individual amino acids on Lp(a) is shown in Fig. 3. Again, the results of the individual amino acids are shown in the same pattern as in Fig. 1. Average basal Lp(a) levels were 1.7 ± 0.25 µg/mg of cell protein at 3.46 mM amino acid concentration in MEM and were taken as 100%. Note that those amino acids that had no effect or little effect on apoB100 also had no or little effect on Lp(a) (Fig. 3, left and center panels). The same pattern did not hold true for the individual amino acids that had the most effect on apoB100. In some cases, those amino acids that had a profound inhibitory effect on apoB100 also increased Lp(a) secretion. This was the case for Tyr, Cys, and Gln. In the presence of tryptophan Trp, which markedly decreased apoB100 levels, Lp(a) was undetectable at concentrations greater than 13 mM. In contrast, Met and Phe, which decreased apoB100 levels 63–88%, had little inhibitory effect on Lp(a) secretion. It has been suggested that a heparin

**Fig. 1.** Effect of individual amino acids on secretion of apoB100 in HepG2 cells. HepG2 cells were incubated overnight in serum-free MEM supplemented with 1% BSA and the indicated concentrations of individual amino acids. ApoB100 accumulation in the medium was measured by competitive ELISA. The average basal level of apoB100 secretion in serum-free MEM (3.46 mM amino acid) was 8.0 ± 1.5 µg/mg of cell protein and is indicated as 100%. The response to amino acids was divided into three groups, those in which the amino acids had little effect or a stimulatory effect (left panel; Asn, Ser, His, Pro, Leu, and Ile), amino acids that had a minor inhibitory effect (center panel; Val, Ala, Gly, Arg, Thr, and Lys), and those amino acids that had a major inhibitory effect on apoB100 secretion (right panel; Trp, Met, Gln, Phe, Cys, and Tyr) listed in order of increasing effectiveness. Results are the average of eight experiments.
or proline wash can release cell membrane bound apo(a) (19). Accordingly, the ELISA was modified to detect free apo(a) by using a polyclonal antibody to apo(a) as detecting antibody; however, even under these conditions, no free apo(a) was detected.

The comparison between the effect of individual amino acids on apoB100 and Lp(a) secretion from the HepG2 cell is shown in Fig. 4. For each concentration curve of amino acids (shown in Figs. 1 and 3), a slope was calculated representing the average percentage change in apoB100/mM amino acid or Lp(a)/mM amino acid. An overall decrease in either apoB100 or Lp(a) results in a negative slope, where the slopes for apoB100 are plotted on the x axis and those for Lp(a) on the y axis. Taken together, there appears to be less effect of amino acids on Lp(a) than there is on apoB100. Note that substantial effects on Lp(a) secretion are only present where there is also a major inhibition of apoB100.

In order to determine the mechanism of the effect of the specific amino acids on apoB secretion we looked at intracellular lipid mass and lipoprotein lipid secretion in the HepG2 cells. Two individual amino acids (Asn and Gln) and a combination of three branched chain amino acids (BCAA; Leu, Ile, and Val at a 1:1:1 ratio) were chosen, which corresponded to amino acids that had a stimulatory effect (Asn), a minor inhibitory effect (BCAA), or a more pronounced inhibitory effect (Gln), as shown in the three panels in Fig. 1. As shown in Fig. 5, top panel, supplementation with Asn (27.7 mM) increased medium apoB slightly as compared with the control. A high concentration of branched chain amino acids or of glutamine (27.7 mM) resulted in substantial and significant decreases in media apoB (252%, *p*, 0.05, and 276%, *p*, 0.025, respectively). This effect was primarily seen in the d 1.006–1.063 g/ml lipoprotein fraction (intermediate density lipoprotein + LDL), which represented the majority of apoB lipoproteins as...
shown in Fig. 5, top panel. By contrast, there was little effect on the $d < 1.006 \text{ g/ml}$ very low density lipoproteins, although this fraction constituted a much smaller percentage of the overall apoB secreted.

The effects of selected amino acids on secreted lipids are shown in the remaining three panels of Fig. 5 for CE (second panel), TG (third panel), and cholesterol (CHOL) (bottom panel). Incubation with asparagine had no effect on secreted CE mass, either the total $d < 1.006 \text{ g/ml}$ lipoprotein fraction or the $d = 1.006–1.063 \text{ g/ml}$ lipoprotein fraction. However, incubation with BCAA and Gln does result in a significant decrease in both total medium CE mass and in $d = 1.006–1.063 \text{ g/ml}$ lipoprotein CE mass. Although the decrease in the $1.006–1.063$ fraction is small, it is significant ($p < 0.05$ for branched chain amino acids; $p < 0.025$ for glutamine). By contrast, although there is a proportion of the total medium CE that is secreted in the $d < 1.006 \text{ g/ml}$ fraction, there is no significant change with any of the amino acid incubations. With respect to both TG and CHOL, there is no significant change in the amount of lipid mass secreted with any of the amino acids in either total medium or lipoprotein fractions. As a result of the decrease in the apoB secretion into the media, the lipid/apoB ratio increased. Thus, the TG/apoB ratio increased in both the medium (4.3-fold, $p < 0.01$) and in the $d = 1.006–1.063 \text{ g/ml}$ lipoproteins (6.5-fold, $p < 0.01$) following glutamine supplementation. CE mass appeared to follow that of apoB, however, and overall there was no significant change in the CE/apoB ratio.

The intracellular lipid mass under the same conditions is shown in Table I. There was no change in intracellular CE mass, CHOL mass, or TG mass following incubation with any of the specific amino acids: asparagine, branched chain amino acids, and glutamine. Although the secretion of apoB is clearly decreased, consequently affecting secretion of lipid, the specific amino acids do not appear to affect intracellular lipid mass.

In order to verify these observations in HepG2 cells, we also investigated the effect of a mixture of amino acids on lipid and lipoprotein secretion in a primary hepatocyte model. Primary hamster hepatocytes were isolated as described under "Experimental Procedures." The cells were then exposed to basal control media with and without supplementation of amino acids (27.7 mM), as shown in Fig. 6. The cells were cultured for 24 h immediately after plating and also for an additional 24 h (48 h total). Primary hepatocytes are routinely cultured in an enriched medium (DMEM) containing 3 mg/ml glucose to increase viability and durability of the cells. However, we also conducted these experiments in a standard glucose medium (MEM containing 1 g/liter of glucose) in order to mimic the same conditions as those that were used in the HepG2 cells.
Amino Acids and ApoB Secretion in HepG2 Cells

Table I

|            | CE  | TG  | CHOL |
|------------|-----|-----|------|
| Control    | 5.9 | 82  | 18.0 |
| Asn        | 5.5 | 64  | 19.0 |
| BCAA       | 6.3 | 84  | 19.0 |
| Gln        | 5.6 | 69  | 18.6 |

(MEM, 48 h total in amino acid-supplemented media). As shown in Fig. 6 (top panel), apoB secretion was greater in the DMEM as compared with the MEM. In addition, there was more apoB secreted after a 48-h incubation time as compared with a 24-h incubation time. However, in all cases, there was a significant and substantial decrease in apoB under conditions where amino acid supplementation was used ranging from 20–40% inhibition. As shown in the bottom panel in Fig. 6, there was greater apoAI secretion in the presence of the DMEM as compared with the MEM and more apoAI accumulation in the medium following a 48-h incubation as compared to a 24-h incubation, similar to the results with apoB. By contrast, there was no inhibitory effect of amino acids. If anything, there was a trend toward a positive effect as was seen in the HepG2 cells.

The effect of the amino acid mixture on hamster hepatocyte intracellular lipid mass and lipid secretion was also examined as shown in Fig. 7. Results are given for CE (top panel), TG (middle panel), and CHOL (bottom panel) secretion from the media under the same conditions as shown in Fig. 6. In all cases, there is greater secretion of lipids in the presence of DMEM as compared to MEM. Similarly, there is also greater lipid accumulation in the media following 48 h as compared to 24 h. In the case of CE, as with the HepG2 cells (see Fig. 5), there is a decrease in CE secreted following incubation with the amino acid-supplemented media. This is true for all conditions: MEM versus DMEM and 24 h versus 48 h. Overall, the decrease in CE secretion ranges from −28% to −45%, p < 0.01 to p < 0.005. By contrast, there is no change in either TG mass secreted into the media or CHOL mass secreted into the media following incubation with amino acids (Fig. 7, center and bottom panels). Thus, the CE/apoB100 ratio did not significantly change in the hamster hepatocytes, similar to what was seen in the HepG2 cells. However, the TG/apoB100 ratio did change substantially, increasing by 42% (p < 0.025), after a 24-h incubation and by 44% (p < 0.05) after a 48-h incubation. Similarly, the free CHOL/apoB ratio increased slightly (±22%) after 24 h, and by 54% (p < 0.025) at 48 h.

The results for the intracellular lipid mass at 48 h are given in Table II. Although in general there appears to be greater lipid mass following incubation in enriched DMEM as compared with MEM, there appears to be little effect of supplemented amino acids on intracellular lipid levels. This is similar to what was seen with the HepG2 cells (Table I). It should be noted that the primary hamster hepatocytes secrete a much greater proportion of their lipid than do the HepG2 cells. Only 7% of total CE was secreted in the HepG2 cells, whereas 60–70% of total CE was secreted under basal conditions in the hamster hepatocytes. Amino acid supplementation, however, reduced the percentage of CE secreted so that only 39–44% of the total CE was secreted (a 30–48% decrease, p < 0.005). By contrast, since there was no change in intracellular or secreted TG or CHOL mass, there was no change in the proportion secreted. Nonetheless, the proportion of TG and CHOL secreted was much greater than in HepG2 cells. Only 21% of TG was secreted in HepG2 cells, whereas 44–49% was secreted in the hamster hepatocytes. Only 6% of CHOL was secreted in HepG2 cells, and 54–61% was secreted in the hamster hepatocytes.

The effect of selected amino acids on apoB secretion, therefore, does not appear to be mediated through intracellular lipids. We therefore examined the synthesis and secretion of the apoB molecule itself in the HepG2 cells. Pulse-chase analysis of apoB secretion was conducted in basal cells, cells treated with a high concentration of asparagine (27.7 mM) (which had a positive effect on apoB secretion), and cells treated with a high concentration of glutamine (27.7 mM) (which had a negative effect on apoB secretion). As shown in Table III, following incubation with amino acids, HepG2 cells were pulse-labeled with tracer [3H]leucine, and apoB secretion was measured at time points up to 3 h. The amount of radiolabeled apoB present at 10 min was taken as 100%. The secretion efficiency of apoB in the HepG2 cells was similar under all incubation conditions. In basal control cells, the amount of apoB secreted plateaued at...
Amino Acids and ApoB Secretion in HepG2 Cells

Fig. 7. Effect of mixed amino acid supplementation on secreted lipids in primary hamster hepatocytes. Primary hamster hepatocytes were isolated and incubated as described under “Experimental Procedures.” Aliquots of the media were extracted, and lipids were separated by thin layer chromatography. The mass of CE (upper panel), CHOL (bottom panel), and TG (middle panel) are expressed as μg/mg of cell protein for an average of six experiments ± S.D., where * represents p < 0.05, ** represents p < 0.01, and *** represents p < 0.005.

55 ± 3.7%. This was no different from that obtained in cells that had been incubated with asparagine (48 ± 5%) or with glutamine (51 ± 3.9%). The secretion of radiolabeled albumin was also measured under the same conditions. As shown in Table IV, under basal, asparagine-supplemented, or glutamine-supplemented conditions, 100% of radiolabeled albumin was secreted. The plateau for albumin secretion was obtained earlier than apoB (30–60 min), and in all cases, as would be expected, secretion efficiency was very high (close to 100%). These results with individual amino acids are comparable with results obtained previously with a mixture of amino acids (10).

The effect of the same amino acids on apoB100 mRNA was examined by both slot blot and Northern analysis to determine the mechanism of the decrease in apoB100, and these data are displayed in Fig. 8. HepG2 cells in T75 flasks were incubated with 27.7 mM of the indicated amino acids in MEM for 24 h as described under “Experimental Procedures.” As shown in Fig. 8 (top panel), values for mRNA are expressed as the apoB100/glycereraldehyde-3-phosphate dehydrogenase (GAPDH) ratio (slot blot analysis). It has previously been demonstrated that GAPDH is unaffected by changes in ambient acids (22, 38). Asparagine, which was associated with increased apoB100 secretion (Fig. 1, left panel), results in significantly increased apoB100 mRNA. In contrast, with high concentrations of a mixture of the branched chain amino acids (leucine, isoleucine, and valine), there is a moderate decrease in the apoB100/GAPDH ratio. Finally, glutamine results in significant decreases in apoB100 mRNA levels compared with the control amino acid medium, again consistent with its profound inhibitor effects on apoB100 secretion. These results were confirmed by Northern blot analysis as shown in Fig. 8 (bottom panel).

Last, in rat hepatocytes, changes in amino acid supplementation have resulted in modulation of certain transcription factors including CCAAT enhancer-binding protein (C/EBPα) (20). C/EBPα as well as a number of other transcription factors has been shown to bind to the promoter region of apoB and modulate transcription (21–25). Accordingly, we attempted to examine the levels of C/EBPα mRNA in HepG2 cells. Using primers based on the published rat sequence (26), we amplified (by polymerase chain reaction) human liver and rat liver cDNA. The probes generated were identical in length based on gel electrophoresis (142 base pairs predicted). However, although both of these probes hybridized with rat liver RNA and human liver RNA, the signal was extremely low in HepG2 cells (results not shown). Polymerase chain reaction amplification of HepG2-derived cDNA yielded a product of the expected length; however, no consistent difference in levels could be detected in cells that had been treated with amino acid supplementation (results not shown).

DISCUSSION

In our previous study, an inverse relation between the overall supply of amino acids to HepG2 cells and secretion of apoB100 lipoproteins was documented (10). No changes in either intracellular lipid synthesis or secretion efficiency of apoB100 molecules occurred, and there was no evidence, therefore, of an effect of amino acids on any post-transcriptional event. On the contrary, the change in apoB100 secretion correlated with changes in apoB100 mRNA levels, thus indicating that a change in transcription was responsible for the amino acid effects on apoB100 secretion.

The present studies were undertaken to extend these observations. Our first objective was to determine whether the effect of amino acids was a general one or whether it varied among the individual amino acids. Our second objective was to determine what the effects were on other hepatic secreted proteins (albumin, apoAI, and Lp(a)). No inhibitory effect was found on either apoAI or albumin. However, there was an inhibitory effect of some amino acids on Lp(a). This effect was less than that seen for apoB100, and in general, only those amino acids that had marked effects on apoB100 had a major effect on Lp(a).

Lp(a) is a lipoprotein found in human plasma that is made up of lipid, principally CE, and a molecule of apoB100 to which is joined a molecule of apo(a), an apoprotein with structural similarity to plasminogen (for review see Refs. 27 and 28). As with the level of apoB100 lipoproteins, the risk of premature coronary disease relates to the level of Lp(a) lipoprotein in human plasma, and it is therefore of importance to search out the factors that might regulate its levels in plasma. We have shown
Amino Acids and ApoB Secretion in HepG2 Cells

Table II

| Lipid | MEM | Change ± AA | DMEM | Change ± AA |
|-------|-----|-------------|------|-------------|
| CE    | -AA | +AA         | -AA  | +AA         |
| Mass  | 1.81 ± 0.25 | 2.84 ± 0.8 | 3.4 ± 0.5 | 4.5 ± 0.5 |
| % secreted | 75 ± 3% | 39 ± 8% | -48% (p < 0.005) | 63 ± 5% | 44 ± 4% | -30% (p < 0.025) |
| TG    | Mass | 81 ± 11 | 97 ± 7 | 94 ± 5.5 | 101 ± 13.3 |
| % secreted | 49 ± 2% | 47 ± 2% | -4% (p.n.s.) | 44 ± 4% | 46 ± 1% | +3% (p.n.s.) |
| CHOL  | Mass | 3.1 ± .4 | 3.9 ± 0.7 | 4.6 ± 0.4 |
| % secreted | 54 ± 5% | 56 ± 5% | +8% (p.n.s.) | 61 ± 2% | 63 ± 3% | -5% (p.n.s.) |

Table III

Pulse-chase analysis of radiolabelled apoB secretion in HepG2 cells

HepG2 cells were incubated overnight in serum-free MEM supplemented with 1% BSA with or without supplementation with additional amino acids: Asn or Gln at a concentration of 27.7 mM. Following overnight incubation, cells were changed to leucine-free media, pulsed with [3H]leucine, and chased for up to 3 h. Results are expressed as the percentage of radiolabeled apoB immunoprecipitated at the indicated time periods as compared to the total amount of radiolabeled protein immunoprecipitated at 10 min (taken as 100%) for each of the experimental conditions. Results are the average of three experiments and are expressed as mean ± S.D.

| Time min | Control | Asn | Gln |
|----------|---------|-----|-----|
| 10       | 15 ± 1  | 10 ± 1 | 23 ± 2 |
| 30       | 10 ± 1  | 24 ± 6 | 51 ± 5 |
| 60       | 45 ± 6  | 17 ± 1 | 50 ± 5 |
| 120      | 56 ± 5  | 54 ± 6 | 41 ± 1 |
| 180      | 68 ± 2  | 50 ± 2 | 51 ± 7 |

that the same general relation holds between the secretion of Lp(a) by HepG2 cells and the concentration of amino acids as was demonstrated for secretion of apoB100 particles; i.e. lower concentrations of amino acid in the medium were associated with higher concentrations of Lp(a) in the medium at the end of the incubation period, and higher concentrations of amino acids decreased Lp(a) accumulation in medium. It was of interest, therefore, to determine if individual amino acids affected Lp(a) in the same manner as they might affect apoB100. We found, in fact, that very few amino acids had a marked effect on Lp(a) and that the effect on Lp(a) was always less than that on apoB100. It is important to note that most of the amino acids that decreased apoB100 markedly, such as tyrosine, cysteine, glutamine, and tryptophan also had an effect on Lp(a). It has been suggested that apo(a) is secreted directly from liver cells and that the Lp(a) complex of apo(a) and LDL apoB100 occurs extracellularly with the formation of a cysteine bond between the proteins apo(a) and apoB100 to generate plasma Lp(a) (29, 30). In such circumstances, the formation of Lp(a) may be dependent not only on translational efficiency and post-translational processing and secretion of apo(a), but also on the concentration of the precursors apo(a) and apoB100. The reduction in Lp(a) seen experimentally in the presence of specific amino acids may be the consequence of a limiting amount of apoB100 available for complexing with apo(a).

The changes in apoB demonstrated in both the HepG2 and primary hamster hepatocytes were not accompanied by decreases in secretion of either TG or CHOL, resulting in a TG- or CHOL-rich particle being secreted. Although the mass of CE secreted did decrease, it was less than that seen with apoB, and intracellular CE mass did not change, suggesting that the decrease in CE secretion is secondary to decreased apoB secretion. Thus, in this particular instance, modulation of apoB secretion does not appear to be driven by post-translational lipid substrate availability but occurs at an earlier stage.

That amino acids have an effect on lipid metabolism should not be surprising. There is, in fact, a body of evidence demonstrating effects of dietary protein on plasma lipid levels (for review see Refs. 31–33). Studies in humans (34, 35) and normal or obese Zucker rats (36, 37) have shown that increasing the proportion of protein in the diet at the expense of carbohydrate will result in decreases in plasma CHOL and TG. On this basis, a high protein diet has been recommended in the treatment of obesity (34) and of hypercholesterolemia (35) in human subjects. However, dietary protein has effects on lipid and lipoprotein levels that are independent of its substitution for carbohydrate. Although a substantial number of animal and plant proteins have been examined, by far the largest number of studies have compared casein (animal) to soy (plant) protein in both animal and human studies with the results demonstrating a hypocholesterolemic effect of soy protein. Although the mode of action is not clear, the hyper-/hypocholesterolemic effects of the specific dietary proteins have been ascribed to the amino acid composition of the diets. An indirect effect mediated through changes in insulin/glucagon ratios may be responsible (31–33, 38). However, direct effects such as modulation of LDL receptor-mediated catabolism (39–46) and changes in bile acid secretion are also potential mechanisms, although they are not supported by all studies (47–49). In HepG2 cells, Lovati (50) demonstrated a dose-dependent increase in uptake and degradation of 125I-LDL by 7 S storage globulin from soy protein. On the other hand, our previous studies on human hepatoma HepG2 cells demonstrated that an amino acid mixture decreased LDL receptor-mediated catabolism and could not, therefore, explain the hypocholesterolemic effect (10). However, apoB100 synthesis and secretion was markedly decreased in the presence of a high amino acid medium concentration as was the mRNA for apoB100, suggesting regulation at the transcriptional level (10). This is consistent with other studies reporting a decrease in very low density lipoprotein secretion in normal rats fed a soy diet (51, 52), or in obese Zucker rats fed a high protein diet (37); such a direct negative effect of amino acids on hepatic apoB100 secretion would result in a hypocholesterolemic effect.

The present studies have focused on the effects of specific amino acids on apoB100 production in HepG2 cells in order to determine which amino acids are hypocholesterolemic in an in vitro system. Although there are no studies to our knowledge that examine the effect of specific amino acids on apoB100 production in hepatocytes, there is an abundance of studies that do examine the effects of specific amino acids on gene regulation and protein synthesis and secretion of liver-specific
proteins in hepatocytes (20, 53–60). Proteins such as albumin, transferrin, transthyretin, carbamyl phosphate synthetase-I, urate oxidase, class I alcohol dehydrogenase (all liver-specific proteins), α-tubulin, and insulin-like growth factor-1 all increase with amino acid supplementation or decrease with amino acid deprivation (20, 56, 58, 59). This is consistent with our results where we found that the majority of individual amino acids tested resulted in an increase in total protein secreted in the medium, as well as moderate to major increases in apoA1 and albumin. This is not a generalized phenomenon, however, since a number of genes are unaffected by ambient amino acid concentration such as β-2 microglobulin, hypoxanthine-guanine phosphoribosyl transferase, H-ferritin, ubiquitin B, γ-actin, RNA polymerase II, and glyceraldehyde-3-phosphate dehydrogenase (20, 60).

In contrast, the expression of a number of genes are inversely related to the amino acid concentration in the same way as apoB100, including a number of growth-associated genes, c-jun, c-myec, c-fos and junB (60) in addition to other proteins such as C/EBPα, Gadd153, ornithine decarboxylase, phosphoglycerate kinase-1, insulin-like growth factor-binding protein-1, amino acid starvation-induced mRNA, and ubiquitin C (20, 53, 54, 60). A number of these reports have demonstrated that particular amino acids (phenylalanine, tryptophan, methionine, and leucine) had potent effects on modulating gene expression, protein synthesis, and hormone levels (20, 54, 57, 60–64). In our results, while leucine had only a minor effect on apoB100 secretion, the remaining amino acids were in that group of amino acids that had the most potent effects on apoB100 expression and secretion. Thus, amino acids may mediate apoB gene transcription through modulation of a transcription factor or through effects on mRNA stability.

How do the effects we observed of specific amino acids on apoB100 secretion compare with studies that have examined the hyper-/hypcholesterolemic effects of individual amino acids? Although Kritchevsky (65) suggested that the lysine/arginine ratio was important, experimental studies by others failed to show a correlation (66). Lysine and branched chain amino acids (leucine, isoleucine, and valine) were associated with hypercholesterolemia and hypertriglyceridemia (38, 67, 68) in humans and rats. In our studies, these amino acids were not “hypercholesterolemic,” but they had only minor inhibitory effects on apoB100 secretion from HepG2 cells. Histidine and leucine, which were found to have no effect on plasma CHOL in feeding studies in humans (69, 70), also had little effect on apoB100 secretion in HepG2 cells in our studies. Arginine, alanine, and glycine were associated with a hypercholesterolemic effect in humans, rats, and rabbits (38, 71, 72), and in our studies, these amino acids had moderate but consistent inhibitory effects on apoB100 secretion. Strikingly, a number of amino acids that we found to be the most potent in our in vitro cell studies have also been shown to have hypcholesterolemic effects in in vivo human and animal studies, including tryptophan (73, 74), glutamic acid (75–77), cysteine, and methionine (78–83).

Overall, therefore, there is a parallel in the changes in apoB100 secretion in HepG2 cells and the hypercholesterolemia effects of specific dietary amino acids in humans. Although much remains to be understood about the physiological influences of amino acids, the effect of amino acids on the transcription of apoB100 may be a potential mechanism by which certain dietary amino acids can influence plasma levels of the apoB100 lipoproteins.

## REFERENCES

1. Snieder, A. D., and Cianflone, K. (1993) Arterioscler. Thromb. 13, 629–636
2. Yao, Z., and McLeod, R. (1994) Biochem. Biophys. Acta. 1212, 152–166
3. Dixon, J., and Ginsberg, H. (1993) J. Lipid Res. 34, 167–179
4. Dixon, J. L., Furukawa, S., and Ginsberg, H. N. (1991) J. Biol. Chem. 266, 5080–5086
5. Murphy, S. Albright, E., Mathur, S., Davidson, N., and Field, F. (1992) Arterioscler. Thromb. 12, 691–700
6. Dashti, N. (1992) J. Biol. Chem. 267, 7160–7169
7. Sparks, J., and Sparks, C. (1993) Curr. Opin. Lipidol. 4, 177–186
8. Cianflone, K., Vu, H., Zhang, Z., and Snieder, A.D. (1994) Atherosclerosis 107, 125–135
9. Davis, R. A., Engelhorn, S. C., Weinstein, D. B., and Steinberg, D. (1980) J. Biol. Chem. 255, 2039–2045
10. Zhang, Z., Snieder, A. D., Kalant, D., Vu, H., Monge, J. C., Tao, Y., and Cianflone, K. (1993) J. Biol. Chem. 268, 26920–26926
11. Snieder, A. D., Zhang, Z., Vu, H., Kalant, D., and Cianflone, K. (1993) Circulation 88, 1-176 (abstr.)
12. Eagle, H. (1959) Science 130, 432
13. Bradford, M. M. (1976) J. Biol. Chem. 252, 685–688
14. Young, G. S., Smith, R. S., Hogle, D. M., Curtiss, L. K., and Witztum, J. L. (1986) J. Clin. Chem. 32, 1484–1490
15. Havel, R., Eder, H., and Bragdon, J. (1955) J. Clin. Invest. 34, 1245–1353
16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Fless, G., Snyder, M., and Scanzu, A. (1989) J. Lipid Res. 30, 651–662
18. Kohen-Avramoglou, R., Cianflone, K., and Snieder, A. D. (1995) J. Lipid Res. 36, 2513–2526
