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LDL versus apolipoprotein B responses to variable proportions of selected amino acids in semipurified diets fed to rabbits and in the media of HepG2 cells

Elzbieta M. Kurowska and Kenneth K. Carroll

Centre for Human Nutrition, Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada

Rabbits fed amino acid diets containing high proportions of several essential amino acids (EAA), mainly Lys and Met, develop hypercholesterolemia associated with elevated low density lipoprotein (LDL) cholesterol and apoprotein B (apo B) levels. This effect is partially prevented by feeding a high proportion of Arg. We previously demonstrated that down-regulation of hepatic LDL receptors contributes to elevation of LDL produced by selected EAA. Additionally, our earlier studies and recent observation in transformed liver cells HepG2 suggested that amino acids can alter apo B production, and that direct interaction between amino acids and hepatic cells is likely to be involved in the mechanism. Presently, these hypotheses were further examined. Our experiment in vivo demonstrated that rates of incorporation of ^{14}C-Lys into LDL apoprotein were elevated in rabbits fed hypercholesterolemic Lys- and Met-enriched diet compared with normocholesterolemic animals fed diets enriched with Lys and Leu. This suggested that increase in hepatic apo B production contributed to hypercholesterolemic response induced by the experimental diet. In vitro, we used HepG2 cells to investigate whether exposure of these cells to amino acid mixtures similar to those tested in rabbits can induce changes in apo B consistent with LDL responses found in vivo. These results showed that apo B responses in cell culture corresponded with LDL changes observed in rabbits in relation to effects of Arg, but not to those of hypercholesterolemic EAA. This suggests that HepG2 cells provide a useful model for studying the mechanism of regulation of apo B metabolism by dietary Arg but not of the regulation by other dietary amino acids. (J. Nutr. Biochem. 7: 418–424, 1996.)

Keywords: dietary amino acids; apoprotein B; HepG2 cells; rabbits

Introduction

Many previous animal and human studies have demonstrated that diets containing casein or other animal proteins produce hypercholesterolemia associated with elevation of low density lipoprotein (LDL), whereas similar diets containing soy protein or other plant proteins maintain normal serum cholesterol levels. Earlier studies from our laboratory provided evidence that these effects are largely due to differences in amino acid composition between animal and plant proteins.[1] In rabbits fed a low-fat, cholesterol-free, semipurified casein diet, replacing casein with its corresponding amino acid mixture led to a comparable hypercholesterolemic response. Replacing soy protein with its amino acid mixture in a similar diet resulted in only a moderate elevation of serum cholesterol.[1]

In our more recent experiments we have shown that selectively increasing proportions of certain dietary amino ac-

Address reprint requests to Elzbieta M. Kurowska at Department of Biochemistry, Centre for Human Nutrition, The University of Western Ontario, London, ON, N6A 5C1 Canada.
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ids can indeed alter cholesterolemic responses in rabbits. High levels of casein amino acids present in semipurified diet caused a progressive increase of serum and LDL cholesterol,[2,3] and this was due to essential amino acids of casein (EAA) rather than due to the non-essential amino acids (NEAA).[3,4] Among the EAA, combinations consisting of all EAA except L-arginine (EAA–Arg), ketogenic EAA (ketoEAA), or L-lysine and L-methionine (Lys + Met) promoted hypercholesterolemia, whereas other combinations, such as (Lys + Leu), (Leu + Met) or (His + Gly + Phe + Thr + Trp) produced a moderate or low response.[5,6] The group of all EAA–Arg was more hypercholesterolemic than the group of all EAA suggesting that L-arginine may counteract the elevation of cholesterol in serum and LDL induced by other EAA.[5] The effects produced by various dietary amino acids were consistent with the fact that animal proteins contain higher levels of nearly all EAA, particularly L-lysine and L-methionine, whereas plant proteins are enriched with L-arginine.[7]

The mechanism(s) by which various dietary amino acids alter metabolism of LDL are still poorly understood. In rabbits fed diets enriched with hypercholesterolemic amino acids, similar as in those fed intact casein, receptor-mediated uptake of LDL in the liver is down-regulated.[5] However, this is not associated with changes in concentrations of cholesterol and its esters in the liver or with alteration of other parameters of cholesterol metabolism suggesting that the effect of amino acids is sterol-independent.[3–5,8] Also, hypercholesterolemic amino acid diets do not produce consistent changes in the postprandial release of hormones known to be involved in the regulation of LDL metabolism[3] and unpublished results. Therefore, the effect of these amino acids is unlikely to be mediated by hormones.

Recently we have postulated that dietary amino acids alter metabolism of LDL directly in the liver when high concentrations enter this organ after a meal. In addition, we proposed that changes in de novo LDL synthesis contribute to cholesterolemic responses induced by amino acid diets.[5] In support of our hypotheses, Zhang et al.[9] demonstrated that in human hepatoma cell line HepG2, both apo B synthesis and receptor-mediated LDL catabolism were regulated by varying total level of mixed amino acids present in minimum essential medium (MEM). Regulation of apo B in HepG2 cells was independent of cellular cholesterol metabolism, consistently with the responses found in rabbits fed amino acid diets. However, unlike in rabbits, in HepG2 cells exposure to increasing concentrations of amino acids in medium resulted in lowering total production of apo B.

In the light of these results, present studies were undertaken to investigate (1) whether increased production of LDL can contribute to dietary amino acid-induced hypercholesterolemia in rabbits, (2) whether hypercholesterolemic amino acid mixtures can regulate metabolism of LDL by direct interaction with hepatic cells. In the first experiment, de novo production of LDL was assessed in animals fed hypercholesterolemic Lys + Met versus normocholesterolemic Lys + Leu diet, by measuring rates of incorporation of radiolabeled L-lysine into LDL apoprotein, shortly after injection of the tracer. In the second study, we monitored apo B response in HepG2 cells exposed to amino acid mixtures similar to those tested in rabbits, and compared them with LDL responses found in vivo.

Materials and Methods

Animals and diets

The animal protocol was in accordance with the Canadian Council of Animal Care guidelines and was approved by the Council on Animal Care, University of Western Ontario. Young male New Zealand White rabbits (Reimen's Fur Ranches, Guelph, Ontario, Canada), weighing approximately 1.6 to 1.7 kg, were used in these experiments. The animals were housed individually in galvanized cages with wire bottoms, in a room maintained at 21 to 24°C with a 12-hr light:dark cycle. They were fed ground High Fiber Rabbit pellets (Agway, Syracuse, NY, USA) for 5 days after arrival. At this time, they were randomized, transferred to semipurified amino acid diets over 1 week, as described previously by Hamilton and Carroll,[10] and fed these diets for 3 weeks. The animals were given free access to food and water. Weight changes and food consumption were monitored.

Rabbits were fed low-fat, cholesterol-free, semipurified amino acid diets in which Lys and Met versus Lys and Leu were selectively increased to produce hyper- versus normocholesterolemic responses. The composition of the diets and amino acid mixture formulations were described previously.[4,6] The particular formulations of amino acids used in this study contained Lys and Met or Lys and Leu at levels corresponding to 45% casein amino acid diet. The remaining EAA were as in a 14.7% casein amino acid diet and NEAA were added to increase the nitrogen in both diets to a level corresponding to 29.5% amino acids.

Cholesterol analysis

Blood samples were taken from the marginal ear vein or directly from the heart, after killing the animals by an overdose of Euthanyl (Canada Packers, Cambridge, Ontario, Canada). Cholesterol in serum and in lipoprotein fractions was measured with an enzymatic kit (CHOD-PAP, Boehringer-Mannheim, Montreal, Canada). Very low density lipoprotein (VLDL) ($d < 1.006$ g/mL), LDL ($1.006 < d < 1.063$) and HDL ($1.063 < d < 1.21$) were isolated by discontinuous density gradient ultracentrifugation as described by Redgrave et al.[11] and modified by Terpstra et al.[12]

Protocol for injection of radiolabelled L-lysine

L-[^{14}C(U)] lysine, 100 μCi/mL, 0.31 μM/mL was purchased from Du Pont Canada Inc., Markham, Ontario, Canada. At the end of the third week on diet, rabbits were deprived of food for 16 to 18 h and subsequently allowed to eat similar, small amounts (1.4 to 1.8 g) of the respective diets. Radioactive tracer (10 μCi/kg) was injected intravenously in 1 mL saline 50 min after food withdrawal. At this time, postprandial plasma concentrations of amino acids reached peak levels (unpublished results) and that could increase the efficiency of incorporation of ^{14}C-Lys into newly synthesized proteins. Blood was collected on ice into tubes containing heparin (14 U/mL) and sodium azide (1 mg/mL). Samples from each rabbit were taken at 2.0 hr, and terminally at 3.5 hr after tracer injection, and analyzed for incorporation of the label into LDL. The time intervals between the isotope injection and blood collection were short relative to the half-life of LDL in rabbits.[13] Therefore, calculated rates of incorporation of the tracer into LDL apoproteins provided an estimate of rates of LDL apoprotein synthesis.[14]

Incorporation of ^{14}C tracer into plasma LDL components and into liver lipids

Plasma samples collected from rabbits after injection of the radioactive tracer were dialysed against two exchanges of 0.01 M L-

lysine in 0.1 M KBr followed by one exchange against 0.1 M KBr, to remove free [^{14}C]L-lysine.[14] The LDL fraction (1.019 < d < 1.063) was then isolated by sequential ultracentrifugation[15] and recentrifuged under the same conditions. Purified, concentrated LDL was dialyzed against 0.05 M NH_4HCO_3, delipidated with diethyl ether:ethanol 3:1 (v/v) and dissolved in 3% SDS as described earlier.[14] Aliquots of delipidated LDL were taken for determination of protein by the method of Lowry et al.[16] as modified by Markwell et al.[17] and for measurement of radioactivity with a 1214 Rackbeta liquid scintillation counter. Part of the remaining LDL lipid fraction was analyzed for incorporation of label and another part was used to measure cholesterol with the enzymatic kit. Total counts were also measured in VLDL.

Total lipids were extracted from liver tissue by the method of Folch et al.[18] and assayed for radioactivity after separation into unsaponifiable (nonpolar) lipids, free fatty acids, and polar components as described by Edmond.[19]

Cell culture experiment

Tissue culture medium, fetal calf serum and other tissue culture supplies, including 50× concentrated MEM amino acid solution without L-glutamine and 100× concentrated MEM non-essential amino acids, were obtained from Life Technologies Inc. (Burlington, ON, Canada). Fatty acid-free bovine serum albumin (BSA) fraction V and single amino acids were from Sigma, St. Louis, MO. Disposable Falcon culture flasks and plates were purchased from Becton Dickinson (Meyland Cedex, France) and from Corning Laboratories (Richmond Hill, Ontario, Canada). Human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD, USA).

Cells were grown and maintained in 75 cm^2 flasks at 37°C in a humidified atmosphere of 95% air-5% CO_2 in MEM containing 10% fetal calf serum (FCS) and 100 IU penicillin/streptomycin. Flasks were subcultured at a 1:5 ratio every 7 days, using 0.25% trypsin in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline for 10 min at 37°C. For experiments, cells were seeded in 24-well plates (6×10^5 cells/plate) and used at confluence, usually 7 days after plating. The medium was changed every 2 to 3 days.

Before the experiments, cells were preincubated for 24 hr with MEM containing 1% BSA instead of FCS. During the next 24 hr, control cells were exposed to the same medium (containing 0.5 mg/mL of standard cell culture EAA), whereas tested cells were incubated with medium supplemented with 5.0 mg/mL of various amino acid mixtures. The concentrations of amino acids did not have any toxic effect on HepG2 cells in the previous studies.[9]

The amino acid composition of control and experimental media is presented in *Table 1*. With the exception of mixture 3 (selectively enriched with all NEAA), mixture 6 and mixture 11, all other formulations contained a total of 5.5 mg amino acids/mL and were designed to determine whether exposing HepG2 cells to selectively high levels of certain EAA can alter the content of apo B in the cell culture medium. Combinations of EAA selectively increased in these mixtures resembled the combinations previously tested in rabbits, but some groups of EAA were raised to levels 10 times higher than in MEM, to emphasize apo B responses. The mixtures were designed as follows: all EAA were raised in mixture 2, all EAA except Arg in mixture 4, Arg only in mixture 5, Lys and Met in mixture 7, Lys and Leu in mixture 8, Met and Leu in mixture 9, His, Phe, Thr and Trp in mixture 10. To those mixtures that contained fewer than the 12 EAA, selected NEAA (Asp only or Ser Pro Asp in the proportions of MEM non-essential amino acids) were added to bring the total level of amino acids to 5.5 mg/mL. These particular combinations of NEAA, when added to HepG2 cells at a concentration of 5.0 mg/mL (mixtures 6 and 11), had no effect on the apo B content of the media, and are referred to as "neutral."

Media apo B concentration and cell protein were subsequently determined as reported previously.[9] Briefly, media were collected and frozen for apo B determination and cells were dissolved in 1

Table 1 Composition of amino acid mixtures used in cell culture experiments

	Mixtures										
	1	2	3	4	5	6	7	8	9	10	11
	mg amino acid/L medium										
Essential amino acids (EAA)											
L-Arg	104	1129	104	104	1129	104	104	104	104	104	104
L-Lys	58	629	58	629	58	58	629	629	58	58	58
L-Met	15	163	15	163	15	15	163	15	163	15	15
L-Leu	52	564	52	564	52	52	52	564	564	52	52
L-His	31	337	31	337	31	31	31	31	31	337	31
L-Phe	32	347	32	347	32	32	32	32	32	347	32
L-Thr	48	520	48	520	48	48	48	48	48	520	48
L-Trp	10	108	10	108	10	10	10	10	10	108	10
L-Cys	24	258	24	258	24	24	24	24	24	24	24
L-Ile	52	564	52	564	52	52	52	52	52	52	52
L-Tyr	36	390	36	390	36	36	36	36	36	36	36
L-Val	46	499	46	499	46	46	46	46	46	46	46
Sum EAA	508	5508	508	4483	1533	508	1227	1591	1168	1699	508
Nonessential amino acids (NEAA)											
L-Ser	—	—	645	302	1178	1485	—	—	—	—	—
L-Pro	—	—	706	332	1293	1630	—	—	—	—	—
L-ASP	—	—	817	384	1496	1885	4273	3909	4332	3801	5000
L-Asp-NH_2	—	—	921	—	—	—	—	—	—	—	—
L-Ala	—	—	547	—	—	—	—	—	—	—	—
Gly	—	—	461	—	—	—	—	—	—	—	—
L-Glu	—	—	903	—	—	—	—	—	—	—	—
Sum NEAA	—	—	5000	1018	3967	5000	4274	3909	4332	3905	5000
Total AA	508	5508	5508	5500	5500	5508	5500	5500	5500	5500	5508

mL of 0.1 N NaOH following three 1 mL washes with ice-cold phosphate-buffered saline. Cell protein was measured by the method of Lowry et al.,[16] using BSA as a standard. Apo B was measured by enzyme-linked immunosorbent assay according to Young et al.,[20] as modified by Ortho Diagnostics (La Jolla, CA, USA). Human LDL for coating microtiter plates and for preparing a standard curve (range 0.025 to 0.400 μg/mL apo B) was isolated from fresh human EDTA-plasma, by sequential density gradient centrifugation.[15] Antibody against human LDL was supplied by Hoechst (Montreal, Canada) and other reagents for the assay were obtained from Sigma.

Results

Growth performance, serum and LDL cholesterol and LDL protein in rabbits fed the Lys Met and Lys Leu diets are presented in *Table 2*. The results show that concentrations of cholesterol in serum and LDL, as well as concentrations of LDL protein, were significantly higher in the animals fed the Lys Met-enriched diet than in those fed diet enriched with Lys and Leu ($P < 0.05$). There was no significant difference between the body weight changes and between VLDL and HDL cholesterol of the two groups.

Effects of feeding the experimental diets on rates of ^{14}C-Lys incorporation into LDL apoprotein are presented in *Figure 1*. Increases in the amount of label incorporated into LDL apoproteins expressed as dpm/mL plasma LDL/hr were significantly higher in animals fed Lys + Met than in those fed Lys + Leu diet. Differences in rates of plasma enrichment with radiolabeled LDL were maintained for the samples taken 2.0 hr and 3.5 hr after injection of the tracer. Increases in apparent specific activity of LDL apoproteins (dpm/mg total LDL protein/h) were similar for both groups, and also did not differ for LDL isolated from plasma samples collected 2.0 hr and 3.5 hr after injection of the tracer. No measurable radioactivity was present in the LDL lipid fraction and in VLDL fraction.

Table 3 shows the ^{14}C-Lys incorporation into liver lipids. Most of the label was found in the polar lipids (60 to 70%) and only little (7 to 8%) was present in neutral sterols. Incorporation of label into total fat, neutral sterols and fatty acids was comparable between the groups and this was associated with similar content of liver cholesterol (3.3 ± 0.5 and 3.1 ± 0.2 mg/g liver for animals fed Lys Met and Lys Leu diet, respectively). Incorporation of label into polar lipids was significantly higher in the animals fed Lys Met than in those fed Lys Leu diet.

Changes in apo B production by HepG2 cells induced by addition of 5.0 mg/mL of various amino acid mixtures to MEM medium are presented in *Figures 2–4*. Addition of 10 times higher than normal levels of either all MEM EAA or all MEM non-essential amino acids to standard MEM media resulted in a significant, two fold reduction of apo B in media of HepG2 cells after 24-hr incubation ($P < 0.05$) (*Figure 2*). A supplementation of control MEM medium with 10 fold excess of all EAA except Arg, and with (Ser + Pro + Asp) added as neutral NEAA, induced a moderate increase of apo B. In contrast, supplementation of MEM with Arg only (also in presence of Ser + Pro + Asp) caused a significant reduction of apo B in the media ($P < 0.05$). As a result, apo B production was two times lower in cells exposed to high proportion of L-arginine than in those exposed to excess of other EAA (*Figure 3*). The effect of high versus low levels of L-arginine was similar when Asp instead of (Ser + Pro + Asp) was added to the medium as a neutral amino acid (data not shown). Supplementation of MEM with four other mixtures of EAA corresponding to those studied in vivo, (Lys + Met), (Lys + Leu), (Leu + Met) and (His + Phe + Thr + Trp) (and with Asp added to equalize total level of amino acids to 5.5 mg/mL) caused moderate reduction of apo B in cell culture medium, without apparent differences in responses between tested combinations (*Figure 4*).

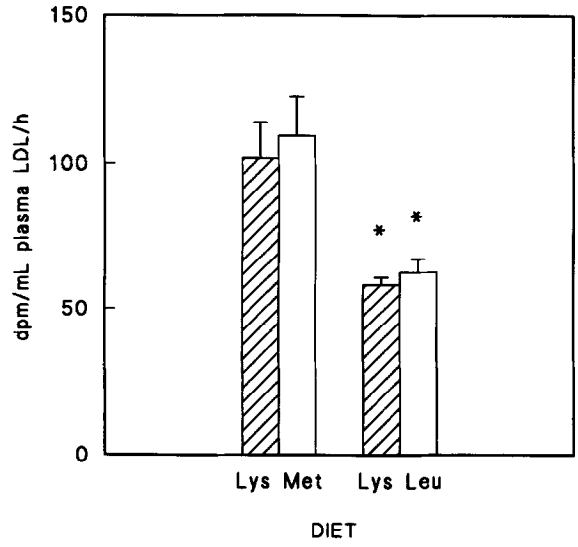

Figure 1 Effects of Lys Met-enriched versus Lys Leu-enriched amino acid diets on rates of incorporation of ^{14}C-Lys into LDL protein. Results for blood samples collected 2.0 hr and 3.5 hr after label injection are represented by hatched and open bars, respectively. Error bars represent SEM. Three rabbits per group. * –P < 0.05.

Table 2 Growth performance, plasma total and LDL cholesterol, and LDL protein in rabbits fed experimental diets

Diet	Initial weight (g)	Change in body weight g/day	Cholesterol mmol/L plasma		LDL protein g/L plasma
			Total	LDL	
Lys Met	1626 ± 17	–5.8 ± 1.7	9.67 ± 1.53*	7.99 ± 1.16*	1.64 ± 0.18*
Lys Leu	1580 ± 21	1.8 ± 3.5	4.01 ± 1.03	3.18 ± 0.96	0.71 ± 0.16

Values are given as means ± SEM. Three animals per group.
*– significantly different from Lys Leu, $P < 0.05$.

Table 3 Effects of amino acid diets on distribution of label in liver lipids after injection of ^{14}C-lysine

Diet	Total fat	Neutral sterols	Fatty acids	Polar lipids
	dpm/g liver			
Lys Met	646 ± 35	43 ± 15	151 ± 34	456 ± 16*
Lys Leu	522 ± 116	50 ± 32	190 ± 60	386 ± 28

Values are means ± SEM. Three animals per group.
*– significantly different from Lys Leu, $P < 0.05$.

Discussion

In our in vivo experiment, the rate of appearance of radiolabeled LDL apoprotein in plasma was higher in rabbits fed the hypercholesterolemic Lys Met diet than in those fed a similar but normocholesterolemic diet enriched with Lys and Leu. The difference in rates of tracer incorporation between the two groups was consistent at two early time points following intravenous injection of ^{14}C-Lys. This suggested that hypercholesterolemia induced by amino acid diets is associated with increased rates of synthesis of LDL apoprotein, which in rabbits is 85 to 90% apo B. Consistently, increased rates of production of LDL apo B were previously found in rabbits fed casein versus soy protein diet.[13]

Our results also confirmed that in rabbits, hypercholesterolemic amino acid diets do not alter metabolism of cholesterol in the liver.[3–5] As expected, the rates of incorporation of ^{14}C-Lys into liver neutral lipids and the concentration of cholesterol in the liver were similar in the animals fed Lys + Met and Lys + Leu. The significantly increased incorporation of the label into hepatic polar lipids observed on Lys + Met versus Lys + Leu diet may indicate that compounds from this group are involved in the mechanism by which amino acids regulate metabolism of LDL. Changes in concentrations of some polar lipids were previously reported in mice and rats fed casein versus soy protein diet.[21,22]

The results of the experiment in vitro demonstrated that in HepG2 cells, total apo B production can be influenced directly by selectively increasing proportions of some amino acids in isonitrogenous media. However, among various combinations of amino acids tested, only some produced apo B responses corresponding to those found in rabbits. Combination containing relatively low versus high levels of L-arginine (EAA–Arg) (*Figure 3*) increased apo B content in cell culture medium whereas combinations containing excess of L-arginine alone (*Figure 3*) or with other MEM EAA (*Figure 2*) reduced it. In agreement, LDL levels were higher in animals fed a diet enriched with all EAA except Arg than in those fed excess of all EAA of casein.[5] Furthermore, our recent results have demonstrated that in rabbits, addition of L-arginine to Lys + Met diet partially reverses hypercholesterolemia unpublished results.

Changes produced by other combinations of amino acids in cell culture were inconsistent with those obtained in vivo. In HepG2 cells, amino acid mixtures enriched with either all NEAA or all EAA caused a significant reduction of apo B in the medium. In contrast, diets enriched with EAA increased hypercholesterolemia in rabbits, and similar diets containing high levels of all NEAA had little effect on LDL.[3–4] Additionally, enrichment of the medium with groups of EAA known to cause various degrees of hypercholesterolemia in rabbits, such as Lys + Met, Lys + Leu, Leu + Met and His + Phe + Thr + Trp, failed to produce corresponding apo B responses in the medium of HepG2 cells.

These discrepancies between in vitro and in vivo results

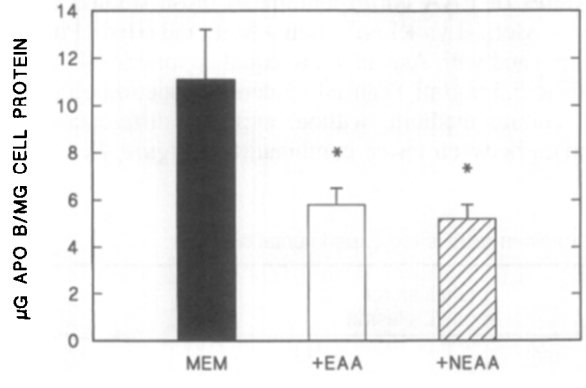

Figure 2 Effect of incubation of HepG2 cells with high concentrations of EAA or NEAA (mixtures 2 and 3 from *Table 1*) on apo B accumulation in medium. Results are the average of six experiments. Left bar represents apo B responses in control (cells incubated with mixture 1, Table 1). Error bars represent SEM. * $<P <$ 0.05.

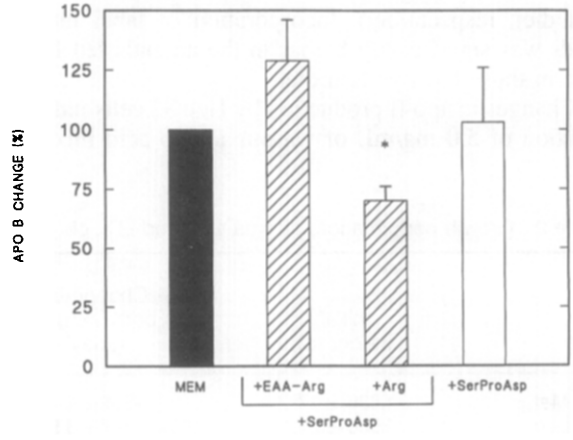

Figure 3 Effect of incubation of HepG2 cells with high concentrations of various amino acids (mixture 4–6 from *Table 1*) on changes in apo B accumulation in medium. Description as in *Figure 2.*

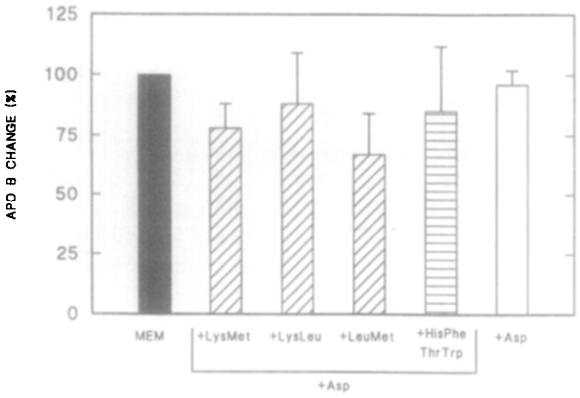

Figure 4 Effect of incubation of HepG2 cells with high concentrations of various amino acids (mixtures 7–11 from *Table 1*) on changes in apo B accumulation in medium. Description as in *Figure 2*.

may be related to the fact that the comparisons were made between the whole animal and cells in culture, and also between two different species. In our experiment, HepG2 cells were exposed to relatively high concentrations of amino acids and to substantial imbalances in their proportions while the total levels and proportions of amino acids fed to the animals were much closer to physiological requirements. Moreover, some of the metabolic responses in HepG2 cells, such as poor ability to secrete VLDL,[23] are unique for this cell line. It is also well known that in humans, which are the source of HepG2 cells, cholesterolemic responses to dietary proteins are quite different from those in rabbits. Thus, most of previous studies showed that substitution of dietary soy protein for animal proteins led to only a minor reduction of serum total and LDL cholesterol in normocholesterolemic subjects.[24] In contrast, cholesterol metabolism in rabbits was found to be extremely sensitive both to dietary proteins and to various mixtures of amino acids.[25]

Overall, our results in vitro suggest that dietary amino acids are capable of playing a role in direct regulation of LDL metabolism in the liver. However, HepG2 cells apparently did not provide the best model for studying the mechanism of amino acid-induced hypercholesterolemia in rabbits, and for future investigation, use of cultured rabbit hepatocytes should be considered.

The apo B responses induced in HepG2 cells by Arg-rich versus Arg-poor mixtures of amino acids may help to explain the mechanisms by which dietary proteins and some of their constituent amino acids regulate LDL metabolism in humans. Although these mechanisms do not seem to play a major role in normocholesterolemic subjects, they may become important when hypercholesterolemia is present. Previous studies suggested that substituting dietary soy protein for animal proteins usually causes a moderate reduction of LDL cholesterol levels in individuals with pre-existing hypercholesterolemia.[24]

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