Secretion of apolipoproteins A-I and B by HepG2 cells: regulation by substrates and metabolic inhibitors

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Abstract It was the aim of this study to *i)* compare the effects of glucose and other hexoses with that of oleate on secretion of apolipoproteins (apos) A-I and B by HepG2 cells, and *ii)* document the effect of various metabolic inhibitors on the secretion of these apos in the absence or presence of extra glucose/oleate. *i)* The addition of 10 mM glucose increased secretion of apoA-I and apoB, as measured by enzyme immunoassay, by about 60% when cells were incubated for 48 h in DMEM + 10% fetal calf serum. The addition of extra glucose also increased the mRNA levels for these apos. Increased radioactivity was also found in these apolipoproteins by immunoprecipitation after metabolic labeling with [^{35}S]*methionine* for 48 h. However, in a pulse-chase experiment (15 min labeling, 2 h chase), glucose was found to increase apoA-I synthesis but not apoB synthesis. More labeled apoB appeared in the medium during the chase because glucose inhibited its intracellular degradation. The effect of glucose on secretion of these apos could be mimicked by fructose and mannose but not by 6-deoxyglucose, showing that the hexoses must enter the cells and be phosphorylated. In contrast, the addition of 0.5 mM oleate had a weak inhibitory effect on secretion of apoA-I whereas it increased the secretion of apoB by more than twofold. The combination of 10 mM glucose and 0.5 mM oleate had no greater effect than glucose alone on apoA-I secretion but increased apoB secretion by fourfold. *ii)* Inhibiting glycolysis (by glucosamine) lowered secretion of both apoA-I and apoB, while inhibiting lipogenesis (using 8-Br-cyclic AMP or 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA)) did not affect apoA-I secretion but clearly decreased that of apoB. However, the inhibitory effect of TOFA on apoB secretion was much smaller in the presence of 0.5 mM oleate instead of extra glucose. Actinomycin-D and *cycloheximide strongly suppressed the stimulatory effect of* glucose on secretion of both apolipoproteins. Actinomycin-D also suppressed basal secretion of apoA-I but surprisingly stimulated that of apoB. These observations indicate that in HepG2 cells secretion of apoA-I is strongly dependent on ongoing protein synthesis and can be boosted by glucose, whereas that of apoB is primarily driven by internal (via lipogenesis from glucose) or external supply of fatty acyl-residues.—**Kempen, H. J., A. P. Imbach, T. Giller, W. J. Neumann, U. Hennes, and N. Nakada.** Secretion of apolipoproteins A-I and B by HepG2 cells: regulation by substrates and metabolic inhibitors. *J. Lipid Res.* 1995. **36:** 1796–1806.

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It is well known that feeding a high-carbohydrate diet induces lipogenesis and increases secretion of triglycerides by the liver in the form of VLDL (1, 2). The induction of lipoprotein synthesis, as seen in vivo, could not be modelled by addition of glucose to primary rat hepatocytes cultured in vitro (2), although triglyceride synthesis can clearly be increased in these cells by glucose (3). So far, no information on this point has been reported for human liver cells, but recently some data has become available for the human hepatoma cell line HepG2.

This cell line is derived from a human liver carcinoma (4) and shows similarities to human liver cells in primary culture with respect to regulation of HMG-CoA reductase, LDL-receptor activity, and bile acid synthesis (5, 6). Several studies have reported on the synthesis and secretion of lipoproteins and apolipoproteins by HepG2 cells (for review see refs. 7, 8), and there is unanimity

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; bp, base pairs; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FCS, fetal calf serum; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IgG, immunoglobulin G; PCR, polymerase chain reaction; TOFA, 5-(tetradecyloxy)-2-furancarboxylic acid.

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that apoB secretion by these cells is stimulated by addition of oleate to the incubation medium, whereas apoA-I secretion is not affected. With regard to the regulation by carbohydrate supply, Dashti, Williams, and Alaupovic (9) and Cianflone et al. (10) have reported that addition of extra glucose to the medium stimulated triglyceride synthesis and secretion but did not increase secretion of apoB or that of apoA-I. In contrast, Arrol et al. (11) showed that glucose stimulated secretion of apoB by HepG2 cells (but they did not study apoA-I secretion). Noticeably, the former authors incubated their cells in MEM, whereas the latter used DMEM for their experiments.

We addressed this issue in more detail, and compared the effect of various glucose and other carbohydrates with that of oleate on the secretion of these apolipoproteins. In this paper we confirm the response of apoB to oleate and report a clear stimulation by glucose of both apoA-I and apoB secretion. The glucose effect on apoA-I secretion is evident only when the cells are incubated in DMEM instead of MEM. These findings elicited additional studies, using various metabolic inhibitors, aiming to elucidate the role of glucose metabolism, lipogenesis, and protein synthesis in regulating apolipoprotein secretion.

MATERIALS AND METHODS

Materials

DMEM, MEM, FCS, concentrated solutions of penicillin/streptomycin, of L-glutamine and L-methionine, Nunclon microwell (96-well) plates were all obtained from Gibco BRL (Life Technologies, Basel, Switzerland). Costar 10-cm diameter dishes were from Tecnomara (Walliselen, Switzerland); Falcon 6-well plates and 75-cm^2 tissue culture flasks were from Becton-Dickinson (Basel, Switzerland). Gelatin for coating culture dishes, BSA, and oleic acid were from Sigma (Buchs, Switzerland). D(+)-glucose was from Fluka. Sheep polyclonal anti-human-apoA-I IgG, anti-human-apoB IgG, and the corresponding peroxidase conjugates, were obtained from The Binding Site (Birmingham, UK). *o*-Phenylenediamine reagent (substrate for peroxidase), as well as standard solutions of human apoA-I and apoB (isolated from human serum) were obtained from Roche Diagnostica (Kaiseraugst, Switzerland). ^{35}S-labeled Tran^{R} (a mixture of labeled methionine and cysteine, specific activity 1173 Ci/mmol) was from ICN, Meckenheim, Germany. Rabbit IgGs against human apoA-I, against human apoB, and against human albumin were from Dako A/S (IG Instrumentengesellschaft, Zürich; catalog numbers Q496, Q497, and A001, respectively). Protein-G-agarose was from Boehringer, Mannheim, Germany. Metabolic inhibitors were from Sigma (Buchs, Switzerland), with exception of TOFA which was synthesized by Dr. U. Fischer, Hoffmann-La Roche, as described by Parker et al. (12).

Cell culture

The established HepG2 cell line was obtained from ATCC (Rockville, Maryland). The cells were cultured at 37°C in 75-cm^2 culture flasks containing 0.24 ml of culture medium per cm^2. Unless mentioned otherwise the standard medium was DMEM (Gibco catalog 041-01885, containing 3700 mg/L NaHCO3, 1000 mg/L D-glucose), supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). The medium was removed and replaced with fresh medium twice a week. Subcultures were made from confluent stock cultures at a split-ratio of 1:6 by trypsinization and 2–3 million cells were seeded per flask. Five days before start of an experiment the cells were split at a ratio of 1:6 and seeded into 96-well plates at a density of about 50,000 cells/well in 0.3 ml standard medium. The medium was renewed after 3 days. At the start of the incubations the cells were grown to confluence.

Experiments with test compounds were carried out in DMEM plus 10% FCS. In some experiments the experimental medium was MEM (Gibco catalog 041-01900, also containing 1000 mg glucose/L) supplemented with penicillin/streptomycin and 10% FCS. Single compounds were added from concentrated stock-solutions in water or DMSO as appropriate. Oleic acid was first complexed with BSA. For this purpose a 10% w/v solution of BSA in DMEM containing 2.5 mM oleate was prepared using a stock solution of 1 M oleic acid in DMSO. The final incubations of HepG2 cells contained 2% BSA and 0.5 mM oleate. The compounds were tested in triplicate, with corresponding triplicate control wells with only the solvent (and in the case of oleate also 2% BSA) added. When not indicated differently, incubation time was 48 h. The conditioned media were harvested, and analyzed for apoA-I and apoB as described below. The cells were washed twice with 200 µl PBS and then dissolved in 25 µl 0.1 M NaOH for protein determination, using the bicinchoninic acid method (Pierce kit, Kontron, Birsfelden Switzerland).

For RNA extraction HepG2 cells were precultured for 5 days in 6-well plates. The medium was changed after 48 h and after 96 h. After a 24-h preincubation period in the growth medium, the cells were then cultured for 48 h in growth medium alone or supplemented with additional glucose as indicated. At the start of the incubations the cells were grown to confluence.

Enzyme-immuno assay of apoA-I and apoB

These assays were performed using a 96-well plate solid-phase immunoassay format, as described in detail previously (13). Intra-assay variation coefficients were 4.4 and 4.5%, and inter-assay variation coefficients were 6.6 and 8.8%, for the assay of apoA-I and apoB, respectively.

Synthesis and secretion rates of apoA-I and apoB as measured by metabolic labeling

In one protocol ("continuous labeling") 3 million HepG2 cells were seeded in 10-cm diameter dishes and cultured for 3 days in 10 ml DMEM + 10% FCS. The medium was then replaced with 10 ml DMEM + 10% FCS containing 500 μCi ^{35}S-labeled Tran^{R} (a mixture of methionine and cysteine, in proportion of about 85/15, according to the supplier), without or with extra 10 mM glucose added. After 48 h incubation in this medium, 9 ml medium was taken off, and mixed with 1 ml 10-fold concentrated PIM (protease-inhibitor mix, containing per 373 mg EDTA, 70 mg PMSF, 2 mg antipain, 2 mg chymstatin, and 2 mg pepstatin-A per 100 ml), and 2.5 ml 5-fold concentrated PBS-TDS (containing 47.25 g NaCl, 7 g Na_2HPO_4, 1.08 g KH_2PO_4, 5 ml Triton-X-100, 2.5 g deoxycholate, and 0.5 g sodium dodecylsulfate per liter). This was centrifuged for 5 min at 4,000 *g*, and the supernatants were kept at −25°C for later analysis of apolipoprotein content and metabolic labeling of apoA-I, apoB and albumin. The cells were washed twice with cold PBS, lysed in PBS-TDS/PIM, and homogenized by drawing through a 8-gauge needle.

In another protocol ("pulse-chase protocol"), two 6-well plates (plate A for pulse-labeling and plate B for pulse-chase labeling) were seeded with HepG2 cells and the cells were grown for 3 days in 3 ml DMEM/FCS per well. Then, in each plate three wells received fresh DMEM/FCS without extra glucose and three wells received DMEM/FCS with 10 mM extra glucose, and incubation was continued for 48 h. The media were then removed and replaced by 3 ml MEM free of cysteine and methionine, in which medium the cells were incubated for 20 min. Thereafter, 3 ml fresh MEM (without cold cysteine and methionine) with 100 μCi ^{35}S-labeled Tran^{R} was added for pulse-labeling during 15 min. After pulse-labeling of plate A, medium was removed and the cells were washed with cold PBS and lysed in PBS-TDS/PIM as above. After pulse-labeling of plate B the hot medium was removed and the cells were washed twice with DMEM/FCS and then incubated for 2 h in DMEM/FCS without or with glucose (chase incubation). Then medium and cells were mixed/lysed with PBS-TDS/PIM as described above.

After pretreatment of the extracts with protein-G-agarose beads (to remove proteins that nonspecifically bind to these beads) the media and cell extracts were mixed with predetermined amounts of rabbit anti-human apoA-I or rabbit anti-human apoB, and incubated overnight at 4°C. The media of the continuous labeling experiment were also incubated with anti-human albumin. The immune complexes were then harvested by incubation with Protein-G agarose for 45 min at 4°C and 30 min at room temperature followed by a brief centrifugation. The pellets were washed twice with PBS-TDS, resuspended in 100 μl 1% SDS/100 mM DTT, and heated for 5 min at 95°C. After centrifugation, 50 μl of the supernatant was loaded on a 1.5-mm-thick SDS-containing polyacrylamide gel (3–17% gradient), which was electrophoresed for 1 h at 120 V and 3 h at 180 V. The gel was fixed and dried using standard techniques and then exposed (Molecular Dynamics Phosphorimager) to detect and quantify the amount of label incorporated. For quantitation and correction of recovery losses during electrophoresis, a known amount of ^{14}C-labeled carbonic anhydrase (Amersham) was applied and run on separate lanes in each gel.

Quantitation of mRNA of apoA-I and apoB in HepG2 cells

Total cellular RNA was isolated from cells by the method of Chomczynski and Sacchi (14), by adding 0.5 ml of the guanidinium thiocyanate lysis buffer per well directly to the cells in each well. Agarose electrophoresis and Northern blotting was performed essentially as described previously (13). DNA fragments used as probes for assay of apoA-I, apoB mRNA or ribosomal 18s RNA by Northern blot analysis were obtained by PCR amplification using the GeneAmp PCR Kit from Perkin-Elmer. A 651 bp apoA-I exon 3 DNA fragment was amplified from human genomic DNA purified from Caco-2 cells, using the oligonucleotides 5′-ctgggacagcgtgacctc-3′ and 5′-tctgagcaccgggaaggg-3′ as primers. A 627 bp DNA fragment of the apoB exon 26 region was created by PCR from human placental DNA (Oncogene Science Inc.) using the oligonucleotides 5′-tatcagatctcttctgctgccttatc and 5′-atctgcagggcttctcaacggcatctc as primers. The apoA-I and the apoB DNA fragments were ligated into the multiple cloning site of the vector pSPT19 (SmaI for apoA-I or BglII and PstI for apoB) and sequenced.

The probe for the 18s ribosomal RNA was generated by PCR from human placental DNA using the oligonucleotides 5′-ccggacacggacagg and 5′-gacgggcggtgtgta. The 437 bp fragment was purified on low melt agarose and used directly for labeling as northern probe.

Probes were created by labeling DNA fragments with [α^{32}P]dCTP (Amersham), using a random priming labeling kit (Prime-It, Stratagene). The probes were boiled for 5 min and added to the prehybridization buffer

which was supplemented with 1/4 volume of 50% dextransulfate. The filters were hybridized at 42°C for 48 h, and finally washed with a buffer containing 0.1 × SSC, 0.1% SDS at 60°C for 90 min. Signal quantitation was done using a Phosphorimager (Molecular Dynamics).

Other analytical techniques

Glucose concentrations in the incubation media were determined using the *o*-toluidine reagent (Sigma diagnostic kit catalog number 635), lactate concentrations using lactic dehydrogenase and NAD (Sigma diagnostic kit catalog number 826). Triglyceride contents in HepG2 cells were determined by densitometry after extraction of total lipids (15) and thin-layer silica gel separation, essentially as described by Schmitz and Assmann (16).

RESULTS

Secretion rate of apoA-I and apoB: effect of glucose and other energy substrates

In preliminary experiments we observed that secretion of apoA-I and apoB by HepG2 cells, as measured by enzyme immuno-assay, was higher when the cells were cultured in DMEM formulated with high glucose (4500 mg/L = 24.8 mM) than in DMEM formulated with normal glucose (1000 mg/L = 5.5 mM).

This led us to study in more detail the effect of addition of extra glucose to the DMEM/normal glucose. HepG2 cells were incubated for 24, 48, or 72 h. As shown in **Fig. 1,** upper panels, the secretion rates of both apoA-I and apoB were higher when 15 mM glucose was added to the incubation medium. Direct measurement

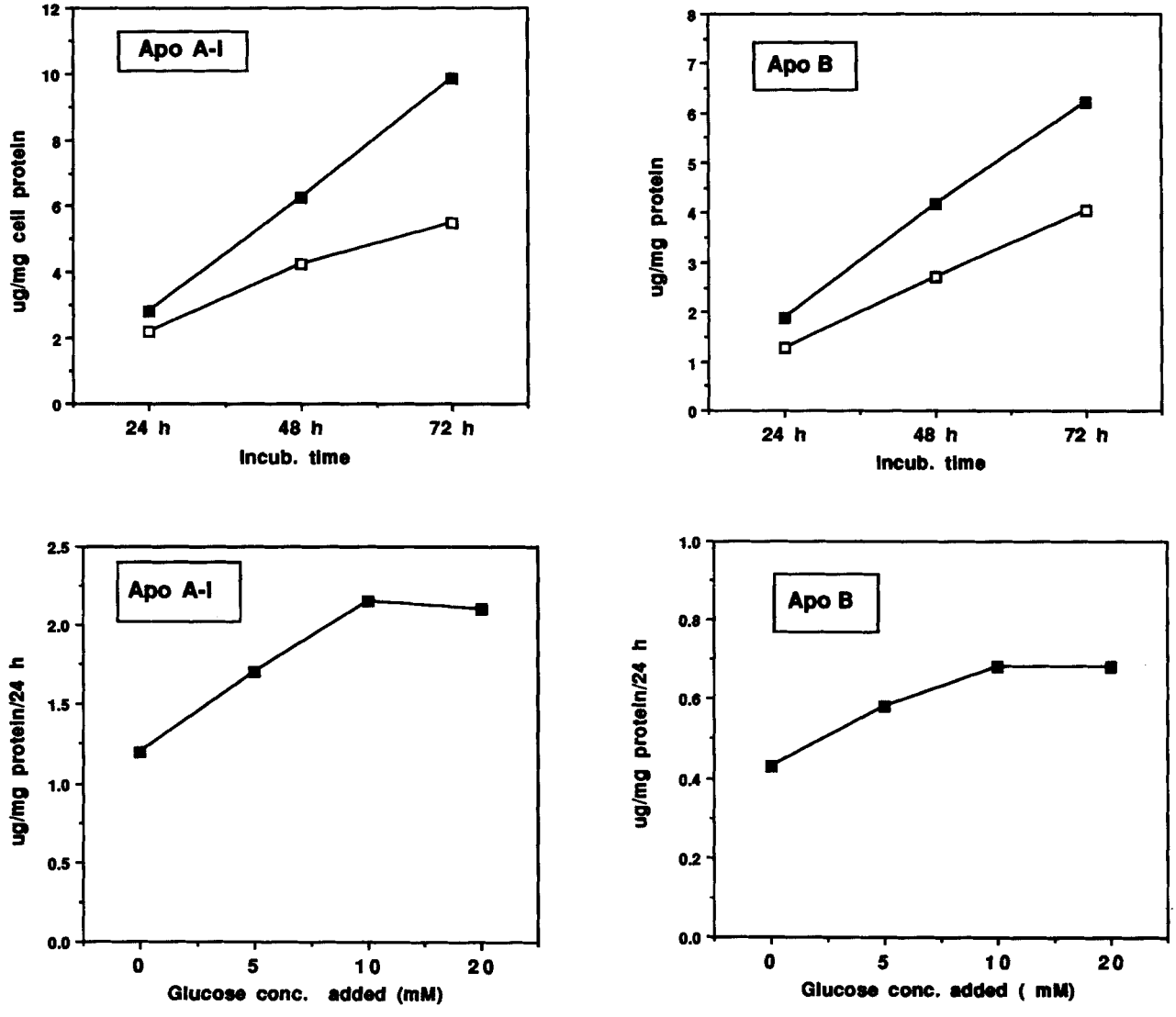

Fig. 1. Effect of addition of glucose to the incubation medium on secretion of apoA-I (left panels) and apoB (right panels) by HepG2 cells cultured in 96-well plates. Upper panels: time course; open symbols: no extra glucose added; closed symbols: 15 mM glucose extra added to the medium. Lower panels: dependence on glucose concentration added. Data are means of triplicate wells.

of glucose levels showed that the glucose concentration dropped to below 0.5 mM after 24 h incubation in DMEM/normal glucose. When 15 mM extra glucose was added at the beginning of the incubation (making the initial glucose concentration 20.5 mM) the glucose concentration had dropped to 8.7 mM after 24 h. As an indicator of glucose metabolism via glycolysis, we measured production of lactate. After 24 h incubation in DMEM/low glucose, there was 9.0 mM lactate in the medium, while 18.7 mM lactate was found after incubation with 15 mM extra glucose added.

In an experiment testing increasing concentrations of glucose, the effect was maximal when 10 mM extra glucose was added (Fig. 1. lower panels). In the experiments described below either no or 10 mM extra glucose was added to DMEM/normal glucose and the incubation time was 48 h.

It can be seen from **Table 1** (top row) that although absolute secretion rates varied considerably between different experiments, the addition of 10 mM glucose consistently increased both apoA-I secretion and apoB secretion. The stimulation amounted to 64% (range 28–110%) above control for apoA-I and to 61% (range 6–98%) above control for apoB. Total cellular protein at the end of the 48-h incubation period also was higher when the medium was supplemented with 10 mM extra glucose (27 ± 3% above low-glucose control; range 14–41%). It should be stressed, however, that the data in Table 1 (columns A and B) are expressed per mg cell protein, so that the stimulation of apoA-I and apoB secretion by glucose is already corrected for the increase of total cell protein.

As shown in Table 1, second row, when HepG2 cells were incubated in MEM instead of DMEM, the basal rate secretion rates are higher, and addition of 10 mM extra glucose increased apoA-I secretion by only 12 ± 6% above control (significantly less than the stimulation seen in DMEM) and apoB secretion by 35 ± 9% (not significantly different from the effect in DMEM) (Table 1). The effect of extra glucose on total cellular protein also was smaller (8 ± 7%; significantly less than the effect in DMEM) in this condition.

Addition of other metabolizable monosaccharides such as fructose and mannose also stimulated secretion of apoA-I and apoB (Table 1), although to a lesser extent for apoB than glucose. However, addition of the non-metabolizable monosaccharide 6-deoxyglucose had no effect (Table 1). As glucose was to a major extent metabolized to lactate (see above), this substance was tested as well. Addition of lactate (20 mM) had no significant effect on secretion of apoA-I of apoB (Table 1). Addition of 0.5 mM oleate (complexed with BSA) to DMEM/low glucose decreased apoA-I secretion by 13% while it stimulated apoB secretion by more than twofold (as compared to controls also containing the extra BSA) (Table 1). Combined addition of 10 mM glucose and 0.5 mM oleate had no greater effect on apoA-I secretion than glucose alone, but increased apoB secretion more than fourfold as compared to the control without extra glucose and oleate (Table 1). In other words, both in the absence and in the presence of extra glucose the addition of oleate had no or little effect on apoA-I secretion but stimulated apoB secretion more than twofold.

TABLE 1. Effect of various substrates on secretion of apolipoproteins A-I and B by HepG2 cells

			ApoA-I			ApoB		
Substrates/Medium	n	Conc. Substrate	Basal (A)	+ Added Substrate (B)	B as % of A	Basal (A)	+ Added Substrate (B)	B as % of A
		mM	*μg/48 h/mg cell protein*			*μg/48 h/mg cell protein*		
Glucose/DMEM	30	10	3.42 ± 0.23	5.58 ± 0.42	164 ± 4[a]	1.72 ± 0.16	2.77 ± 0.24	161 ± 5[a]
Glucose/MEM	3	10	7.60 ± 1.39	8.42 ± 1.29	112 ± 6[b]	3.23 ± 0.12	4.42 ± 0.17	135 ± 9[a]
Fructose/DMEM	3	10	1.95 ± 0.07	2.90 ± 0.34	149 ± 14[a]	0.67 ± 0.03	0.85 ± 0.08	127 ± 7[a,b]
Mannose/DMEM	3	10	2.08 ± 0.12	3.36 ± 0.13	161 ± 5[a]	0.61 ± 0.05	0.78 ± 0.04	128 ± 8[a,b]
6-Deoxy/DMEM	3	10	7.40 ± 3.05	7.60 ± 3.25	103 ± 5[b]	2.39 ± 1.12	2.42 ± 1.20	101 ± 11[b]
Lactate/DMEM	3	20	3.34 ± 0.93	3.76 ± 1.33	105 ± 11[b]	1.36 ± 0.16	1.10 ± 0.23	79 ± 10[b]
Oleate[d]/DMEM	6	0.5	3.80 ± 0.38	3.29 ± 0.37	87 ± 3[a,b]	1.83 ± 0.12	4.03 ± 0.51	222 ± 8[a,b]
Oleate[d] + glucose/DMEM	4	0.5 ± 10	3.50 ± 0.29	5.83 ± 1.08	162 ± 17[a]	1.86 ± 0.14	7.86 ± 0.83	420 ± 17[a,b,c]

Values are given as means ± SEM for the indicated number (n) of separate experiments. Incubations were carried out in DMEM or MEM (containing 5.5 mM glucose of its own).

[a]Different from 100% ($P < 0.05$; *t*-test for paired obs.).

[b]Different from value for glucose/DMEM ($P < 0.05$; *t*-test for unpaired obs.).

[c]Different from value for oleate/DMEM ($P < 0.05$; *t*-test for unpaired obs.).

[d]Incubations with oleate, and with the corresponding controls, contained 2% BSA.

Effect of glucose on apoA-I and B mRNA levels, and on synthesis and turn-over of apoA-I and apoB

To further investigate the mechanism by which glucose increased secretion of apoA-I and apoB, the influence of additional glucose on the levels of apoA-I and apoB mRNA was investigated. After 48 h incubation with 15 mM extra glucose the mRNA levels for apoA-I and apoB were elevated by 70% and 100%, respectively, above the low glucose control (**Table 2,** top row). In contrast, glucose did not affect the amount of mRNA for GAPDH (not shown).

As a next step in these mechanistic studies, immunoprecipitation experiments were performed to assess the incorporation of ^{35}S-labeled Tran^{R} (mainly labeled methionine) in these apolipoproteins in cells and medium. Two protocols were used for these experiments. In the first protocol the cells were incubated for 48 h in DMEM with normal or extra glucose in the continuous presence of ^{35}S-Tran^{R}. As shown in Table 2 (bottom row) the amounts of ^{35}S-labeled apoA-I and apoB in the medium were about 80% higher when the incubation medium was supplemented with 10 mM extra glucose. (Using this protocol, radioactivity in apoA-I and apoB in cellular extracts was less than 1% of that in these proteins recovered from the medium.) To monitor the specificity of the glucose effect the incorporation in secreted albumin was also determined. Addition of extra glucose stimulated the amount of label in this protein about twofold (Table 2).

TABLE 2. Content of mRNA coding for apoA-I and apoB (mRNA), and synthesis of apoA-I and apoB assessed by immunoprecipitation after metabolic labeling (I.P/M.L.), in HepG2 cells incubated 48 h in DMEM without or with extra glucose

	ApoA-I	ApoB	Albumin
mRNA[a]	168 ± 16	198 ± 48	N.D.
I.P./M.L.[b]	186 ± 24	180 ± 41	213 ± 52

Results represent the value for the incubation with extra glucose expressed as % of the value for the incubation without extra glucose, and are calculated from the means ± SEM of three dishes/wells for the low or high glucose situation. All values are significantly different from 100% ($P < 0.05$; *t*-test for paired obs.).

[a]Cells were incubated in 6-well plates with 3 ml DMEM without or with 20 mM extra glucose. After 48 h, cellular total RNA was extracted and mRNAs for apoA-I and apoB were determined by Northern blotting as described in Materials and Methods. For each well, the mRNA amounts of the apolipoproteins were first normalized to the amount of 18S rRNA.

[b]Cells were incubated in 10-cm diameter plates with 10 ml DMEM without or with 10 mM extra glucose and supplemented with 0.5 mCi ^{35}S-labeled Tran^{R}. After 48 h the medium was harvested and radioactivities in immunoprecipitated apoA-I, apoB, or albumin were determined as described in Materials and Methods.

Although the latter results indicate that glucose enhances net production of apoA-I and apoB (and albumin) under these conditions, they, in fact, just confirm the results of the enzyme-immunoassay (mass) measurements but are not informative with respect to the underlying reason for this enhancement. In order to assess whether glucose directly affects rate of protein synthesis and/or intracellular protein turn-over, a pulse-chase experiment was conducted. Under this protocol the cells were first incubated for 48 h without or with extra glucose, then washed and pulse-labeled for a short period (15 min) with ^{35}S-Tran^{R}, whereafter the label was removed and the cells were either directly extracted, or further incubated for 2 h in fresh medium without label (chase). As shown in **Table 3,** label incorporation in apoA-I during the pulse was nearly threefold higher in cells pre-incubated in DMEM with extra glucose as compared to cells incubated in normal DMEM. In contrast, label incorporation in apoB during this pulse was not affected by pretreatment with extra glucose. After the chase incubation, the amount of label in cellular apolipoproteins (both apoA-I and apoB) was much lower than after the pulse. For apoA-I this loss was totally accounted for by secretion of the labeled protein into the medium, the sum of label in cells and medium after the chase being closely similar to the amount of label after the pulse for both the low and the high glucose condition. In contrast, for apoB the loss of label from cellular protein during the chase was not made up by appearance of labeled protein in the medium. This indicates that a major part of the apoB newly synthesized during the pulse has been intracellularly degraded during the chase. In the cells pre-incubated in DMEM/low glucose this loss accounted for about 94% of the newly made protein; in the cells pre-incubated in DMEM/high

TABLE 3. Synthesis, intracellular turnover, and secretion of apoA-I and apoB as assessed by pulse-chase experiment

	ApoA-I		ApoB	
	Low Gluc	High Gluc	Low Gluc	High Gluc
After pulse, cells	63 ± 7	170 ± 7[a]	2,343 ± 277	2,248 ± 71
After chase; cells	18 ± 4	15 ± 2	127 ± 12	171 ± 10[a]
After chase; medium	52 ± 17	146 ± 16[a]	28 ± 3	55 ± 6[a]
After chase, total	70 ± 15	159 ± 14[a]	155 ± 13	226 ± 12[a]
Chase, as % of pulse	111	94	6.5	10.0

The experiment was performed in two 6-well plates (one for pulse, one for pulse-chase) as described in Materials and Methods. Counts are thousands dpm and represent means ± SEM of three wells for low or high glucose each.

[a]Different from low glucose ($P < 0.05$; *t*-test for unpaired obs.)

glucose there was twofold more labeled apoB secreted in the medium, which apparently had been salvaged from intracellular degradation.

Effect of inhibiting glycolysis and lipogenesis

To further study the need of glucose to be metabolized for it to support/stimulate apolipoprotein secretion, we tested the effect of inhibitors of glycolysis and lipogenesis in experiments in which 10 mM extra glucose was provided. Care was taken that the inhibitors were used at concentrations that had no general cytotoxic effect. As a criterion for this we used the total cell protein recovered at the end of the incubation, which should remain above 95% of control (in the absence of inhibitor). For the experiments described in this section this requirement was fulfilled.

First, we checked glucosamine, assumed to be taken up and phosphorylated, but then unable to be further metabolized. This compound has been described to inhibit glucose metabolism by blocking 6-phosphohexose isomerase and possibly other steps (17), and also is known as inhibitor of liver glucokinase (18). Its antiglycolytic effect in the HepG2 cells was confirmed by measuring changes in lactate production. As shown in **Table 4,** glucosamine decreased the lactate concentration in the medium by 23%, and suppressed the secretion of apoA-I by 33% and that of apoB by 58%.

8-Bromo cyclic AMP was tested as a cell-permeable analog of cyclic AMP, with the expectation of inhibiting both glycolysis and lipogenesis because in hepatocytes it is known to decrease pyruvate kinase at the transcriptional and mRNA stability level (19) while in rat liver glucagon decreases acetyl CoA carboxylase activity (20). Surprisingly, 8-Br-cyclic AMP hardly affected lactate formation by HepG2 cells, but clearly decreased the intracellular triglyceride content (Table 4), evidencing its effect on lipogenesis. The compound caused a small and nonsignificant reduction of apoA-I secretion whereas it clearly suppressed apoB secretion (Table 4).

Next, the effect of TOFA was investigated, a known inhibitor of lipogenesis at the level of acetyl-CoA carboxylase (21), and recently reported to suppress triglyceride and apoB secretion in hamster hepatocytes (22). This compound, like 8-Br-cyclic AMP, did not affect lactate production but decreased intracellular triglyceride content. It decreased apoA-I secretion only to a small extent but inhibited apoB secretion by 63% (Table 4). Interestingly, when the medium was supplemented with 0.5 mM oleate instead of with extra glucose, the inhibitory effect of TOFA on apoB secretion was virtually abolished (Table 4), while apoA-I secretion tended to be increased.

Effects of inhibitors of mRNA synthesis and protein synthesis

Finally, in order to judge whether the stimulation by glucose required ongoing gene expression or de novo protein synthesis, the effect of inhibitors of gene expression/protein synthesis were studied in incubations without or with 10 mM extra glucose. As shown in **Table 5,** in this case these compounds clearly inhibited cell growth/proliferation (as judged by the low or zero increase of cell protein) in DMEM/low glucose, and virtually abolished the additional increase in cell protein caused by extra glucose. In incubations in DMEM/low glucose, actinomycin-D (10 μM) suppressed secretion of apoA-I but stimulated that of apoB. Cycloheximide at 1 μM clearly inhibited apoA-I secretion but did not affect that of apoB. At 10 μM, cycloheximide inhibited secretion of both apolipoproteins but the effect on apoA-I secretion was stronger than that on apoB secretion. Both inhibitors suppressed or even abolished the stimulatory effect of glucose on secretion of both apolipoproteins (Table 5).

TABLE 4. Effect of inhibitors of glycolysis or lipogenesis on apolipoprotein secretion, lactate production, and intracellular triglyceride content in HepG2 cells incubated for 48 h in DMEM with extra glucose (10 mM) or oleate (0.5 mM)

Substance (Concentration)	Substrate Added	n	ApoA-I	ApoB	Lactate	TG
Glucosamine, 5 mM	glucose	3	67 ± 1[a]	42 ± 11[a]	77 ± 4[a]	ND
8-Br-cAMP, 1 mM	glucose	7	89 ± 5	62 ± 3[a]	91 ± 5	47 ± 4[a]
TOFA, 10 μM	glucose	7	91 ± 3[a]	37 ± 4[a]	103 ± 3	33 ± 4[a]
TOFA, 10 μM	oleate	4	113 ± 6	91 ± 2[a,b]	ND	ND

Data represent % of corresponding no-substance controls and are means ± SEM for the indicated number (n) of separate experiments; ND, not done.

[a]Different from 100% ($P < 0.05$; t-test for paired obs.).

[b]Different from value for glucose/TOFA ($P < 0.05$; t-test for unpaired obs.).

TABLE 5. Effect of actinomycin-D or cycloheximide on total cell protein and on apolipoprotein secretion by HepG2 cells, incubated for 48 h in DMEM without (Basal) or with 10 mM extra glucose (+ Extra)

	n	mg Cell Protein[a]		ApoA-I		ApoB	
		Basal	+ Extra	Basal	+ Extra	Basal	+ Extra
Control	3	46 ± 1	60 ± 2[b]	2.7 ± 0.2	4.4 ± 0.4[b]	1.3 ± 0.3	1.8 ± 0.3[b]
Act-D, 10 μM		39 ± 2[c]	39 ± 4[c]	1.9 ± 0.3[c]	2.1 ± 0.3[c]	2.2 ± 0.3[c]	2.3 ± 0.3
Control	3	41 ± 2	50 ± 3[b]	4.1 ± 0.7	6.3 ± 0.7[b]	1.9 ± 0.4	2.7 ± 0.4[b]
Cycloh., 1 μM		39 ± 2	42 ± 3[c]	1.9 ± 0.3[c]	2.3 ± 0.5[c]	2.0 ± 0.3	2.5 ± 0.2[b]
Cycloh., 10 μM		32 ± 1[c]	29 ± 2[c]	0.6 ± 0.1[c]	0.7 ± 0.1[c]	1.0 ± 0.2[c]	0.8 ± 0.2[c]

Data represent μg/well (for total cell protein) or μg/48 h per mg cell protein (for apolipoprotein mass secreted), and are means ± SEM for the indicated number (n) of separate experiments.

[a]Total cell protein was 36 ± 2 and 32 ± 2 μg/well at t = 0 for the experiments with actinomycin-D (Act-D) and cycloheximide (Cycloh.), respectively.

[b]Different from basal ($P < 0.05$; *t*-test for unpaired obs.).

[c]Different from control ($P < 0.05$; *t*-test for unpaired obs.).

DISCUSSION

The data show that glucose is able to stimulate secretion rates of apolipoproteins B and A-I by HepG2 cells, as measured by a solid-phase enzyme-immuno assay (Table 1) and by immunoprecipitation after metabolic labeling (Table 2 bottom row). The effect was smaller when the cells were cultured in MEM than in DMEM, which partly explains the conflicting evidence about the effect of glucose reported previously: two groups incubating these cells in MEM reported no effect on secretion of apoA-I or apoB (9, 10), whereas a third group incubating the cells in DMEM observed a stimulatory effect of glucose on apoB secretion (11). The two media have equal contents of glucose (1000 g/L = 5.5 mM) but differ considerably in their contents of amino acids, with DMEM having approximately double the concentration. It has been reported recently (23) that addition of amino acids led to a reduced secretion rate of apoB but not of apoA-I by HepG2 cells. We indeed observed a lower absolute secretion of apoB and apoA-I in DMEM than in MEM (see Table 1). Therefore, the stimulatory effect of glucose occurring in DMEM may consist partly in relieving the inhibitory effect of the extra amino acids present in DMEM.

Addition of extra glucose was also found to increase cellular apoA-I and apoB mRNA contents (Table 2, top row), and actinomycin-D and cycloheximide abolished the stimulation by glucose (Table 5). However, these findings do not necessarily mean that the glucose effect on apolipoprotein secretion is due to an increased transcription of these apolipoprotein genes or enhanced translation of their mRNAs. As elaborated below, there are good reasons to assume that enhanced expression/biosynthesis of other proteins (e.g., lipogenic enzymes) is key for the glucose effect on apoB secretion, mediated by a decrease of its intracellular breakdown.

We assessed to what extent glycolytic metabolism of glucose and de novo synthesis of fatty acids are involved in the effects of glucose on apolipoprotein secretion. We confirmed an earlier report that HepG2 cells have a very high capacity for glycolysis and lactate formation (24). Furthermore, no stimulation of apolipoprotein secretion was obtained with 6-deoxyglucose (Table 1), which cannot be phosphorylated, and inhibition of glycolysis by glucosamine clearly reduced apolipoprotein secretion (Table 4). However, the stimulatory effect of glucose is not solely brought about by generating more glycolytic products as added lactate had no effect on apolipoprotein secretion (Table 1). How does glucose then work? One possibility is by directly (or after conversion into glucose-6-phosphate) inducing the expression or mRNA stability of the lipogenic enzymes acetyl CoA carboxylase or fatty acid synthetase as shown in HepG2 cells (25) and other cell types (26, 27), and so generating more intracellular fatty acids and triglycerides. Glucose indeed increased the intracellular triglyceride content after 48 h incubation, testifying to an increased rate of lipogenesis. For the stimulation of apoB secretion by glucose this may well be the main mechanism involved, as secretion in the presence of extra glucose was effectively suppressed with TOFA, an inhibitor of acetyl CoA carboxylase (Table 4). Glucose, by inducing de novo synthesis of endogenous fatty acids, seems to act similarly as exogenous oleate, which is known to stimulate apoB secretion in HepG2 cells (28–30) by sequestering newly synthesized apoB in the endoplasmatic reticulum or Golgi compartment and so protecting it against early proteolytic degradation, without increasing apoB biosynthesis (31, 32). Direct evidence that glucose enhances apoB secretion via this mechanism is given in Table 3.

The observation that the inhibitory effect of TOFA on apoB secretion was much smaller when oleate was

added instead of extra glucose also fits with this viewpoint, because in that situation triglyceride synthesis and sequestration of apoB are driven by the supply of exogenous fatty acid and the rate of lipogenesis is much lower. The finding that lactate addition does not stimulate apoB secretion seemed to be discordant with this hypothesis because lactate or pyruvate are excellent stimulators of lipogenesis in rat hepatocytes (33). However, this paradox was resolved by a recent publication showing that in HepG2 cells, in contrast to rat hepatocytes, lactate addition does not lead to increased lipogenesis (34).

8-Bromo cyclic AMP was tested because we expected it to decrease glycolytic flux through inhibition/suppression of pyruvate kinase as shown in cultured rat hepatocytes (19) as well as to decrease de novo lipogenesis through inhibition of acetyl-CoA carboxylase as shown for glucagon in rat liver (35). To our initial surprise, this compound only slightly affected lactate production. However, it was recently published that the expression of the pyruvate kinase gene was not affected by cyclic AMP in a mouse hepatoma cell line (36) and this may apply for HepG2 cells as well. The inhibitory effect of the compound on apoB secretion therefore is probably solely due to suppression of the amount of acetyl CoA carboxylase, and/or inhibition of its activity by protein kinase-mediated phosphorylation (20, 35, 37), resulting in less fatty acid generation as testified by a lower triglyceride content (Table 4). The suppression of apoB secretion by 8-bromo-cAMP is in agreement with earlier findings showing inhibition of VLDL secretion by dibutyryl cyclic AMP or glucagon in isolated rat hepatocytes (38) or in hepatocyte cultures (39). Previously, a similar albeit smaller inhibitory effect of this compound was observed in HepG2 cells (28).

The stimulation of apoA-I synthesis and apoA-I mRNA content by glucose remains to be explained in molecular terms. Increased lipogenesis seems to be much less important for this effect as apoA-I secretion was hardly suppressed by 8-Br-cyclic AMP or TOFA. Also arguing against a role of fatty acid/triglyceride synthesis is the fact that exogenous oleate, in contrast to glucose, is unable to stimulate apoA-I secretion (9, 30, 32; Table 1 of this paper). A direct effect of glucose-6-phosphate on apoA-I gene transcription for the moment is the simplest hypothesis, but remains to be directly demonstrated.

Interestingly, in DMEM/low glucose actinomycin-D inhibited secretion of apoA-I but clearly increased that of apoB (Table 5). Also, cycloheximide at 1 μM strongly inhibited apoA-I secretion but had no effect on that of apoB. At 10 μM, cycloheximide still had less effect on apoB secretion than on apoA-I (Table 5). Such findings have, to our knowledge, not been described before. First, it suggests that secretion of apoB during the 48-h incubation period does not require ongoing expression of the apoB gene. This may seem in contrast to the reported half-life of 16 h for the apoB mRNA in HepG2 cells (28). However, there may be an excess of this mRNA in the cells, and the translation and/or secretion rates may be unaltered or increased even if the amount of mRNA would go down. Second, it raises the question of how the stimulatory effect of actinomycin-D on apoB secretion is achieved. The answer we submit is that the compound inhibits the intracellular breakdown of the apoB protein. As shown in Table 3, a large fraction of newly synthesized apoB is rapidly degraded before it is sequestered in the Golgi compartment and becomes destined for secretion (40, 41). Recently, the protease involved in this intracellular breakdown was characterized as a short-lived non-lysosomal calpain-I-like thiol-protease (31). A similar short-lived protease or protein regulator was inferred to play a role in the regulation of HMG-CoA reductase (42, 43), because cycloheximide increased the amount of this enzyme by preventing its degradation. Actinomycin may, therefore, increase net apoB secretion by preventing expression of this protease. A similar explanation may hold for the lack of inhibitory effect of 1 μM cycloheximide on apoB secretion in the face of a clear inhibitory effect on apoA-I. (Cycloheximide is likely to inhibit apoB synthesis equally strong, but the effect thereof on apoB secretion is counteracted by inhibiting the synthesis of this protease at the same time).

Finally, the question arises whether these findings are relevant for human liver cells exposed to high glucose concentrations. Although this is currently uncertain, the occurrence of elevated plasma levels of HDL-cholesterol and apoA-I in patients with insulin-dependent diabetes mellitus (44, 45) is at least congruent with the possibility that hyperglycemia by itself leads to increased synthesis and secretion of apoA-I in humans.

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