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Effect of low density lipoproteins, high density lipoproteins, and cholesterol on apolipoprotein A-I mRNA in Hep G2 cells

Juan Carlos Monge, Jeffrey M. Hoeg, Simon W. Law and H. Bryan Brewer, jr

Molecular Disease Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Building 10, Room 7N117, Bethesda, MD 20892, USA

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We have utilized the human hepatocellular carcinoma cell line, Hep G2, to study the effects of low density lipoproteins (LDL), high density lipoproteins (HDL), and free cholesterol on apolipoprotein (apo) A-I mRNA levels. Incubation of the Hep G2 cells with LDL and free cholesterol led to a significant increase in the cellular content of cholesterol without any effect on the yield of total RNA or in the cellular protein content. Our studies established that incubation with LDL or free cholesterol increased the relative levels of apoA-I mRNA in the Hep G2 cells. In contrast with cholesterol loading, HDL had the effect of lowering the levels of apoA-I mRNA. These results indicate the LDL and HDL pathways as well as intracellular cholesterol may be important in apoA-I gene expression and regulation.

HDL; Apolipoprotein A-I

1. INTRODUCTION

The human hepatocarcinoma cell line Hep G2, which retains many characteristics of the normal differentiated hepatocyte, synthesizes and secretes into the culture medium the majority of the human plasma apolipoproteins [1,2]. This cell line has been shown previously to be a good model system for studying the regulation of apolipoprotein biosynthesis, and the effects of estrogen on the secretion and mRNA levels of apoA-I and apoC-II have been well characterized [3–5].

Several different dyslipoproteinemias exhibit abnormally high levels of LDL and low levels of HDL and apoA-I [6–8]. These observations suggest that the synthesis of LDL and HDL could be coordinately linked and the LDL receptor important in the regulation of apoA-I biosynthesis. In the present study we have evaluated the effects of LDL, HDL, and free cholesterol on apoA-I mRNA levels in Hep G2 cells using a cDNA probe to quantitate specific mRNA levels by dot blot hybridization. LDL and free cholesterol led to an increase in the relative levels of apoA-I mRNA, whereas HDL had the opposite effect. These results indicate that intracellular cholesterol, as well as the LDL and HDL receptor pathways, may be linked to apoA-I gene expression and regulation.

Correspondence address: J.M. Hoeg, Molecular Disease Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Building 10, Room 7N117, Bethesda, MD 20892, USA

Abbreviations: apo, apolipoprotein; LDL, low density lipoproteins; HDL, high density lipoproteins; MEM, Eagle's minimum essential medium; LPDS, lipoprotein deficient serum; PBS, phosphate-buffered saline; SSC, standard saline citrate

2. MATERIALS AND METHODS

2.1. *Preparation of lipoproteins and LPDS*

LDL (d = 1.030–1.050 g/ml) and LPDS (d > 1.21 g/ml) were prepared from 500 ml of plasma collected in 0.1% EDTA by plasmapheresis after a 12–14 h fast. Preparative ultracentrifugation was performed at 4°C (24 h for LDL and 48 h for LPDS) utilizing KBr for density adjustment [9,10]. Isolated fractions were dialyzed against PBS. Protein concentration was determined as described by Lowry et al. [11], and cellular content of cholesterol was quantitated by an enzymic fluorometric procedure [12].

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2.2. *Cell culture*

The cell line Hep G2 was grown in T-150 flasks containing 30 ml of MEM supplemented with 10% fetal bovine serum. Cells were grown to near confluence prior to changing the media to serum supplemented with 10% LPDS. After 24 to 48 h in MEM + LPDS the cells were changed to fresh MEM + LPDS (controls) or MEM + LPDS containing one of the following: LDL at a concentration of 200 μg protein/ml medium, HDL at a concentration of 50 μg/ml medium, or cholesterol dissolved in absolute ethanol (stock solution = 5 mg/ml) at a final concentration of 50 μg/ml medium.

2.3. *Isolation of total RNA*

At designated time intervals, culture media were removed and the cells monolayers were washed twice with 10 ml of ice-cold PBS. The cells were then harvested by addition of 4 ml of 4 M guanidinium thiocyanate [13] and removal with a cell scraper. The viscous cell suspension was homogenized using two pulses of 15 s each in a Polytron (Brinkman Instruments).

2.4. *cDNA probe*

A ds-cDNA probe was prepared by digestion with *Msp*I of the apoA-I cDNA clone as described [14]. This probe is 860 base pairs in length and contains the entire sequence coding for mature apoA-I. The cDNA was labeled by nick translation with a [α-^{32}P]CTP to a specific activity of approx. 3×10^8 dpm/μg [15].

2.5. *Northern blot and dot blot analysis of RNA*

Total RNA from Hep G2 cells was fractionated by 1% agarose gel electrophoresis in the presence of 6% formaldehyde. Transfer of RNA to nitrocellulose was performed as described [16,17]. Quantitation of apoA-I mRNA was performed by dot blot hybridization [17]. Total RNA samples were diluted to a volume of 200 μl using sterile doubly distilled water. Quadruplicate samples were spotted onto GeneScreen Plus membranes using a Schleicher and Schuell manifold. After baking at 80°C for 2 h the filters were prehybridized in a solution containing 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 20 mM Na_3PO_4 (pH 7.0), 100 μg/ml salmon sperm DNA, and 50% formamide for 4–6 h at 42°C. Hybridization was performed for 48–72 h in 10 ml of the same solution containing the apoA-I cDNA probe (2×10^7 dpm/filter). Post-hybridization the filters were washed twice in 3 × SSC + 0.1% SDS at room temperature for 30 min, twice in 1 × SSC + 0.1% SDS at 65°C for 30 min, and finally for 30 min in 0.2 × SSC at 65°C. Filters were autoradiographed with Kodak XAR-5 film and double intensifying screens. Dot blots were quantitated with a laser scanning densitometer (LKB).

3. RESULTS

To demonstrate the specificity of our cDNA probe, total RNA samples from human liver, Hep G2 cells, and human fibroblasts were separated by 1% agarose gel electrophoresis and transferred onto nitrocellulose by the Northern blot procedure (fig.1, inset). Hybridization of the probe to the nitrocellulose filter revealed a single complementary mRNA species in the human liver and in the Hep G2 cells, 950 bp in length. Also illustrated is the correlation of the levels of apoA-I mRNA and total RNA from Hep G2 cells quantitated by dot blot hybridization (fig.1). Total RNA was blotted in the range from 1 to 10 μg, and the assay was linear from 1 to 5 μg of total RNA. All subsequent studies to quantitate relative levels of apoA-I mRNA were performed on total RNA within this range.

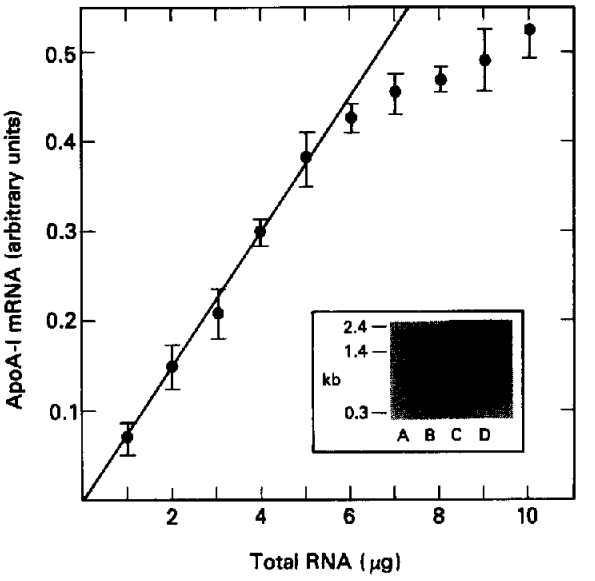

Fig.1. Total RNA from Hep G2 cells was blotted in quadruplicate in the range from 2.5 to 10 μg and hybridized with the apoA-I cDNA probe. The filters were autoradiographed and quantitated in a laser scanning densitometer. Values represent mean ± SD of triplicate samples. Inset illustrates Northern blot analysis of RNA from human fibroblasts, Hep G2 cells and normal human liver utilizing an apoA-I cDNA probe. A, 10 μg total RNA from human fibroblasts; B, 10 μg total RNA from Hep G2 cells; C, 5 μg total RNA from normal human liver; D, 1 μg poly A$^+$ RNA from normal human liver.

Since the cellular cholesterol content may play a role in the regulation of apolipoprotein biosynthesis, we quantitated the effect of cholesterol delivery and incubation with HDL on the total cell protein and cholesterol concentrations in Hep G2 cells. The effects of incubation with LDL, HDL, or free cholesterol did not alter the yields of total RNA and cellular protein content (not shown). In contrast, significant changes were induced in the cellular content of cholesterol by LDL, HDL and cholesterol as illustrated in table 1.

Table 1

Effect of LDL, HDL and cholesterol on cellular content of cholesterol in Hep G2 cells after HDL (50 μg/ml) preincubation

Medium	Cholesterol content (nmol/mg protein)		
	Total	Free	Esterified
MEM + LPDS	56 ± 13	46 ± 9[a]	12 ± 6
MEM + LPDS + LDL	79 ± 14[bc]	54 ± 13[d]	24 ± 7[e]
MEM + LPDS + HDL	50 ± 14[bf]	46 ± 13	6 ± 7[e]
MEM + LPDS + cholesterol	116 ± 15[cf]	107 ± 20[ad]	11 ± 12

[a-f] Statistically significant differences ($p < 0.05$) between values designated with respective letters

The effects of LDL, HDL and free cholesterol on apoA-I mRNA levels in Hep G2 cells were evaluated by dot blot hybridization. HDL consistently decreased the relative levels of apoA-I mRNA, whereas cholesterol loading by either LDL or free cholesterol dissolved in ethanol increased apoA-I mRNA. The magnitude of the changes in apoA-I mRNA was found to be significantly influenced by the pre-incubation conditions of the pre-confluent Hep G2 cells. Fig.2 illustrates the response of the apoA-I mRNA levels when the cells were pre-incubated for 24 h in MEM supplemented with 10% LPDS and then changed to fresh MEM + LPDS, MEM + LPDS + LDL, MEM + LPDS + HDL or MEM + LPDS + cholesterol (dissolved in ethanol). ApoA-I mRNA levels were increased by LDL and cholesterol compared with the cells grown in MEM + LPDS. In contrast, HDL induced a small decrease in apoA-I mRNA levels but the results did not reach statistical significance. However, the difference between the effects of HDL (decrease) and LDL or cholesterol (increase) was significant.

The magnitude of the changes in apoA-I mRNA levels was greater when the cells were pre-incubated for 24 h in MEM + LPDS containing HDL at a concentration of 50 μg protein/ml medium; these results are illustrated in fig.3. LDL and cholesterol induced an approximately 20% increase in apoA-I mRNA levels. The decrease in the mRNA levels associated with HDL was in this case

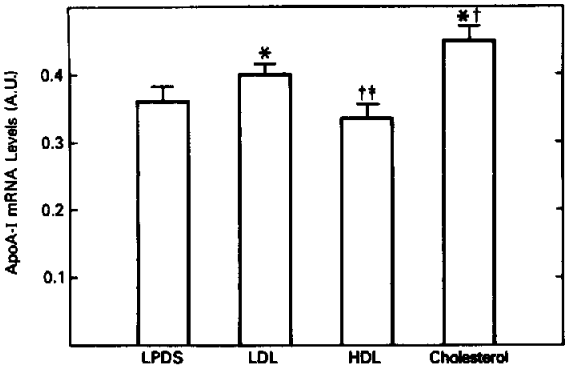

Fig.2. Preconfluent Hep G2 cells were incubated for 24 h in MEM supplemented with 10% LPDS and then changed to fresh MEM + LPDS, MEM + LPDS + LDL, MEM + LPDS + HDL or MEM + LPDS + cholesterol. Bars represent the mean ± SD of quadruplicate samples.

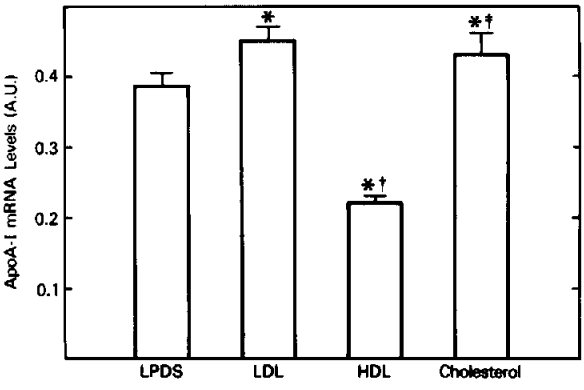

Fig.3. Preconfluent Hep G2 cells were preincubated for 24 h in MEM + LPDS containing HDL at a concentration of 50 μg protein/ml medium before changing the media. Bars represent the mean ± SD of quadruplicate samples.

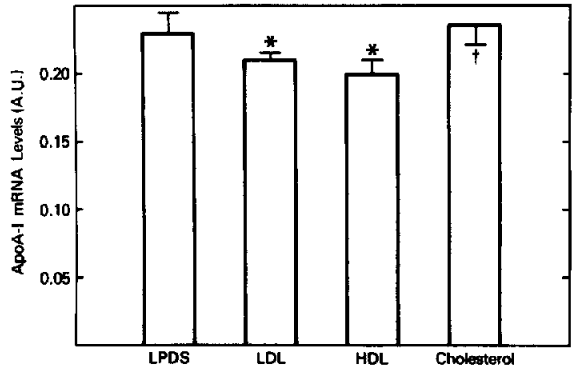

Fig.4. Hep G2 cells were incubated in MEM + LPDS containing 200 μg protein/ml medium of LDL followed by the addition of the different types of media as indicated. Bars represent the mean ± SD of quadruplicate samples.

greater than 40%. A comparison between the apoA-I mRNA levels following incubation with HDL with the incubations with LDL or free cholesterol demonstrated an even greater difference, with cholesterol loading by either method being associated with levels that were at least 2-fold higher than with HDL. In contrast with these results, minimal changes were observed when the pre-incubation conditions were 24 h in MEM + LPDS containing 200 μg/ml of LDL protein (fig.4). Interestingly, further incubation in the presence of media containing LDL did not increase the apoA-I mRNA levels. Time course experiments were also carried out to further characterize the response of apoA-I mRNA levels to HDL. There was a decrease in apoA-I in RNA levels within 2 h after the addition of HDL (not shown). Therefore, the modulation of apoA-I mRNA by HDL was rapid and sustained up to 24 h.

4. DISCUSSION

The liver plays a major role in virtually every aspect of lipoprotein synthesis and catabolism [18]. VLDL, HDL and the majority of the known apolipoproteins are synthesized by the liver. Kinetic studies have led to great advances in our understanding of lipoprotein and apolipoprotein biosynthesis [19]. However, because of the limitations with the availability of human cells, the basic molecular mechanisms responsible for those processes have not been fully defined.

In the studies reported here we have utilized the Hep G2 cells as a model system to evaluate the effects of LDL, free cholesterol and HDL on the levels of apoA-I mRNA. We have established that cholesterol loading, either by LDL uptake or addition of free cholesterol to the media, led to a significant accumulation of cholesterol within the cells, without significantly affecting cell growth or number when compared with control cells incubated in MEM supplemented with LPDS. Both LDL uptake and free cholesterol loading were associated with an increase in the levels of apoA-I mRNA in the Hep G2 cells. In contrast, HDL led to a 37–57% decline in intracellular total and a 45–75% reduction in free cholesterol compared to LDL treated cells. These changes in cellular sterol content were associated with a decrease in the levels of apoA-I mRNA.

The mechanisms of the increased apoA-I levels with cholesterol loading and their decrease by HDL remain to be elucidated. It is very likely that there are distinct intracellular pools of cholesterol with different functions. In these studies only the total content of cholesterol per cell was quantitated. In addition the pathways (e.g. LDL receptor, HDL receptor, or alternate) that cholesterol utilizes to enter or exit the cell may be of great importance and ultimately determine the effect(s) of cholesterol in the regulation of apolipoprotein biosynthesis.

In the Hep G2 cells, as in any other system, the levels of a particular mRNA species may be determined by several factors. The changes in apoA-I mRNA levels induced by cholesterol loading or HDL could be due to modifications at the level of the transcriptional rate resulting from the interaction of regulatory elements in the apoA-I gene with a *trans*-acting regulatory ligand. Other potential sites of regulation are at the level of processing of the primary transcript and at the mechanisms that control the stability of the mRNA [20].

The results of the present study provide additional insights in our understanding of HDL and, in particular, apoA-I metabolism. Another potential implication of our results relates to the mechanism whereby certain drugs increase the plasma levels of HDL and apoA-I [21]. Drugs like lovastatin and niacin, by up-regulating the LDL receptor lead to increased uptake of LDL which could then be followed by an increase in apoA-I mRNA levels and, consequently, by an enhanced synthetic rate of apoA-I and HDL. Further studies will be necessary to test these and other hypotheses, and to further understand the mechanisms regulating the cellular levels of apoA-I mRNA.

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