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Modulation of apo A-IV Transcript Levels and Synthesis by n-3, n-6, and n-9 Fatty Acids in CACO-2 Cells

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Abstract It has been postulated that apolipoprotein (apo) A-IV plays various significant roles in lipid transport and lipoprotein metabolism. Although it is controlled by fat feeding, so far little else is known about its regulation by specific fatty acids. In this study, we focused on the modulation of apo A-IV mRNA levels, mass, and biogenesis by mono- and polyunsaturated fatty acids (FA) in the human intestinal Caco-2 cell line. In confluent cells incubated with 1 mM oleic (n-9), linoleic (n-6), α-linolenic (n-3), or docosahexaenoic (n-3) acids for a long-term period, both apo A-IV protein levels and de novo synthesis were increased. The induction resulted from the up-regulation of apo A-IV mRNA transcripts. In contrast, an inhibitory effect was evident with short-term incubation. FA chain length and degree of unsaturation had little effect altering apo A-IV transcript and biogenesis. These data offer evidence that isolated fatty acids regulate gene expression and the production of apo A-IV in the enterocyte. J. Cell. Biochem. 75:73–81, 1999.

Key words: oleate; linoleate; linolenate; docosahexaenoate; intestine; apo A-IV mRNA; regulation

Apo A-IV is a 46-kD structural component of intestinal lipoproteins, such as chylomicrons and high-density lipoproteins [Beisiegel et al., 1979; Green et al., 1979, 1980]. Multiple functions have been postulated for apo A-IV. They include the stabilization of lipoprotein structure [Weinberg et al., 1985], the promotion of cellular cholesterol removal (a key component of reverse cholesterol transport) [Stein et al., 1976; Steinmetz et al., 1990], the activation of lipid-metabolising enzymes [Goldberg et al., 1990; Steinmetz et al., 1985], and an antioxidant ability [Qin et al., 1998]. Recent work in transgenic mice has also provided evidence that overexpression of apo A-IV reduces susceptibility to aortic lesion formation and increases plasma HDL levels [Duvenger et al., 1996; Cohen et al., 1997]. Taken together, these observations suggest important anti-atherosclerotic characteristics for apo A-IV. In addition, the reported effects of apo A-IV on food intake and satiety as well as gastric emptying and gastric acid secretion [Fujimoto et al., 1992, 1993; Okumura et al., 1995] point to its physiological gastrointestinal relevance. If, as postulated by the aforementioned features, apo A-IV fulfills so many critical functions, knowledge of the regulation of its production is of major importance.

Apo A-IV seems to be particularly sensitive to dietary control. Fat ingestion results in pronounced apo A-IV output in mesenteric lymph of rats [Kalogeris et al., 1994]. Its mRNA levels and stability are also induced in the presence of triglycerides as shown in piglet intestinal explants [Black et al., 1990]. Increased transcript concentrations were documented with diverse effectors in many studies [Apfelbaum et al., 1987; Black et al., 1990; Gordon et al., 1982], indicating that apo A-IV synthesis is modulated at the transcriptional level. Most of these data were generated from animal experiments. Very often, apo A-IV protein levels were determined in lymph or serum without reference of the tissue of origin. Furthermore, conflicting data have been reported, probably due to different methodologies, the source of dietary lipids and the strains of animal models. For example, some

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investigators have concluded that the intestinal contribution of apo A-IV is unaltered by dietary lipids [Windmueller et al., 1981] and that apo A-IV regulation is more complex, beyond the transcription level and may reside anywhere between translation and clearance from plasma [Srivastava et al., 1994]. Extrapolation to the human species is complicated, since in the latter, apo A-IV is largely restricted to the intestine [Elshourbagy et al., 1987], while in numerous animal species, it is produced by both the intestine and the liver [Go et al., 1988]. Finally, in many studies on apo A-IV, animals were tested by mixtures of lipids, such as triglycerides and phospholipids without reference to fatty acid content. In the present investigation, we have addressed the modulation of apo A-IV gene expression, protein mass, and de novo synthesis by specific fatty acids. Both n-3 and n-6 polyunsaturated as well as n-9 monounsaturated fatty acids were tested employing the Caco-2 cell line, a human enterocyte model that exhibits several morphological and biochemical characteristics of the small intestine.

MATERIALS AND METHODS

Cell Culture

Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown 75 cm^2 flasks (Corning, NY) as described previously [Levy et al., 1996; Mehran et al., 1995]. For individual experiments, cells were subcultured on 24.5-mm polycarbonate Transwell filter inserts with 0.4-μm pores (Costar, Cambridge, MA), in MEM (as above) supplemented with 5% FBS. They were initially plated at a density of 1×10^6 cells/well in a medium refreshed every second day. Experiments were carried out at 20 days, a period at which Caco-2 cells are highly differentiated and appropriate for lipid synthesis [Levy et al., 1996; Mehran et al., 1995; Pinto et al., 1983].

Incubation of Cells With Lipids

Cells were rinsed three times with serum-free MEM before adding 1 mM of one of the fatty acids complexed to bovine serum albumin on the apical side of the cells. The oleate/albumin complex was prepared as specified previously [Levy et al., 1992]. The following fatty acids tested were oleic (18:1n-9), linoleic (18:2n-6), α-linolenic (18:3n-3), and docosahexaenoic (22:6n-3). Table I documents the position of these fatty acids in the elongation and desaturation processes. Cells were incubated with the selected lipid effectors for 30 min or 20 h at 37°C unless stated otherwise. After incubation, apical and basolateral media were collected, pooled and centrifuged to remove cell debris. Apo A-IV was assessed as described below.

Apo A-IV Measurement

Caco-2 homogenates were prepared in phosphate-buffered saline, containing 1% (w/v) Triton X-100 1 mM, phenylmethylsulfonyl fluoride and 1 mM benzamidine, as described previously [Mehran et al., 1997]. Aliquots of cell homogenates and media were centrifuged for 10 min at 18,000*g* and 60 min at 105,000*g*, respectively. Apo A-IV levels were determined on the resulting supernatants by SDS-polyacrylamide and transfer on a nitrocellulose membrane (Amersham), followed by the detection of an enhanced chemiluminescence system of antigen-antibody complexes. Preliminary experiments showed that within the conditions used, there was a direct relationship between the density of the apo A-IV band and the amount of

TABLE I. Major Pathways for the Synthesis of Long Chain Fatty Acids From n-6 and n-3 Precursors*

n-6 series	18:2 n-6 Linoleic	→	18:3 n-6 γ-linolenic	→	20:3 n-6 Dihomo γ-linolenic	→	20:4 n-6 Arachidonic	→	22:4 n-6	→	2:5 n-6	
		(1)		(2)		(3)		(4)		(5)		
n-3 series	18:3 n-3 α-linolenic	→	18:4 n-3	→	20:4 n-3	→	20:5 n-3 Eicosapentaenoic	→	22:5 n-3	→	22:6 n-3 Docosahexaenoc	
		(6)		(7)		(8)		(9)		(10)		
n-9 series	16:0 palmitic	→	18:0 Stearic	→	18:1 n-9 Oleic	→	18:2 n-9	→	20:2 n-9	→	20:3 n-9 Eicosatrienoic	

*(1) Δ6-desaturase; (2) Elongase; (3) Δ5-desaturase; (4) Elongase; (5) Δ4-desaturase; (6) Elongase; (7) Δ9-desaturase; (8) Δ6-desaturase; (9) Elongase; (10) Δ5-desaturase.

either purified apo A-IV or pooled reference plasma. The apo A-IV amount was calculated using arbitrary units in comparison with the latter.

De Novo apo A-IV Synthesis

Pulse labeling of Caco-2 cells and immunoprecipitation were carried out using standard methods [Mehran et al., 1997]. Briefly, Caco-2 cells were incubated in a methionine-free medium, containing (^{35}S)-methionine (300 µCi/ml), (50 mCi/mmol, Amersham Life Sciences, Montreal) with or without specific unlabeled fatty acids. Then, the media from the apical and the basolateral sides were collected and pooled. Cells were scraped off the inserts in a cell lysis buffer (tris 10 mM, NaCl 150 mM, EDTA 5 mM, SDS 0.1%, Triton 1%, Na desoxycholate 0.5%). The medium and cell lysates were supplemented with the anti-protease mixture, as described above. Aliquots were precipitated with 20% trichloroacetic acid, and precipitates were washed three times with 5% trichloroacetic acid before the radioactivity was determined in a liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Media and homogenates were centrifuged as above and supernatants subsequently reacted with excess monoclonal antibodies for 18 h at 4°C. This antibody was purified as described previously [Steinmetz et al., 1988]. Pansorbin (Calbiochem, San Diego, CA) was then added, and the mixture was reincubated at 20°C for 60 min. The immunoprecipitates were washed extensively and analyzed by a linear 4–20% polyacrylamide gradient, preceded by a 3% stacking gel [Mehran et al., 1997]. Gels were sectioned into 4-mm slices and counted after an overnight incubation at 20°C with 1 ml BTS-450 (Beckman Instruments) and 10 ml of liquid scintillation fluid (Ready Solv., Beckman Instruments). Homogenates were used for protein determinations according to Lowry et al. [1951], using bovine serum albumin (BSA) as a standard.

Sample Extraction and RT-PCR Analysis for Gene Expression Assay

After incubating Caco-2 cells in the presence or absence of the fatty acids studied, total RNA was isolated as described by Chomczynski and Sacchi [1987] and treated with RQI Rnase-free Dnase (Promega, Madison, WI). Single-strand cDNA was synthesized after denaturation of total RNA (10µg, 15 min, 65°C) by a reverse transcription reaction, consisting of 0.5 mM of each deoxynucleotide triphosphate (dNATP, Pharmacia Biotech., Gaithersburg, MD), 10 mM DDT (GIBCO BRL, Gaithersburg, MD), 200 U SuperScript II reverse RNAse H transcriptase (Gibco, Grand Island, NY), 30 U RNA guard (Pharmacia, Gaithersburg, MD), RT buffer (50 mM Tris-HCl-pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, and 10 mM DTT; GIBCO BRL). Reverse transcription was performed at 37°C for 2 h followed by inactivation (95°C for 5 min).

PCR amplification was performed with a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Norwalk, CT) in a mixture containing 100 ng cDNA in a labeled amplification buffer containing 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxynucleotide triphosphate (dNTP; Pharmacia), 2µM up and down-stream primers (ACGT, Canada), 2.5 U Taq DNA polymerase (GIBCO BRL), and 2µCi (a-^{32}P(dCTP (3,000 Ci/mmol, Amersham). The amplification of apo A-IV and GAPDH was carried out with 30 cycles of denaturation at 94°C for 30 sec, annealing at the temperature specific to each primer for 1 min and, extension at 72°C for 2 min. The reaction ended with a 10-min incubation at 72°C. GAPDH was used as the housekeeping gene. For electrophoresis, samples (20µl) were applied on 5% polyacrylamide gels. In order to determine A-IV mRNA, gels were exposed and scanned by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The PCR primers used in this study were:

		Annealing T°:
ApoA-IV:	5′:5′-CCCGATCTTCTGGCTCACCTCATT-3′	55°C
	3′:5′-AATGCCAAGGAGGCCGTFFAACAT-3′	
GAPDH:	5′:5′-CCCATCACCATCTTCCGA-3′	56.3°C
	3′:5′-CATCACGCCACAGTTTCC-3′	

Statistical Analysis

Conventional methods were used for calculation of means and standard error. Statistical significance for differences in variables between more than two group was tested by one-way ANOVA.

RESULTS

Cultured Caco-2 Cells

The experimental Caco-2 cell line used in the present study displayed functional and morphological similarities to mature enterocytes, as reported previously [Levy et al., 1996; Mehran et al., 1995]. Confluent cells developed a high

degree of polarization, brush border microvilli, tight junctions and intergitations. This was consistent with recovered high sucrase activity (15–20 IU/g cell protein) and transepithelial cell monolayer resistance (2,000–3,000 ohm × cm^2). These observations indicate the adequacy of differentiated enterocyte-like intestinal Caco-2 for investigating the regulation of apo A-IV.

Effect of Fatty Acids on apo A-IV Protein Level

Following the incubation of Caco-2 cells with 1 mM oleic, linoleic, linolenic, or docosahexaenoic acid, the amount of apo A-IV was determined by the laser densitometric scanning of immunoblots prepared from native gels. As shown in Figure 1, there was a marked decrease of apo A-IV in Caco-2 monolayers exposed to fatty acids for 30 min. Cellular apo A-IV content was affected by all types of fatty acids during this short period, in contrast with the medium levels. As expected from these data, the total amount (equivalent to the apo A-IV accumulation in cell and medium) was substantially lowered by the presence of each n-3, n-6, and n-9 fatty acid tested.

On the other hand, a long-term incubation period (20 h) with the same fatty acids resulted in an increase of apo A-IV, recorded mainly in the cells. In sum, maximal effect was generally noted on the total amount following supplementation with oleic and docosahexaenoic acids.

Effect of Fatty Acids on De Novo Synthesis and Secretion

The next series of experiments were conducted to assess the modulation of apo A-IV biogenesis and delivery by fatty acids. The estimation of these processes was evaluated by the incorporation of [^{35}S]-methionine into apo A-IV, immunoprecipitation and electrophoresis. Figure 2 shows a representative gel scan, which reveals that apo A-IV de novo synthesis exceeded that of apo A-I in Caco-2 cells.

Caco-2 cells treated with free fatty acids attached to BSA demonstrated a propensity to synthesize and export more apo A-IV only when incubation was performed for 20 h (Fig. 3). Docosahexaenoic acid tended to potentiate more induction of total apo A-IV production. The administration of fatty acids for 30 min strongly affected the accumulation of newly synthesized apo A-IV in cell and medium, corroborating our findings relative to apo A-IV protein levels.

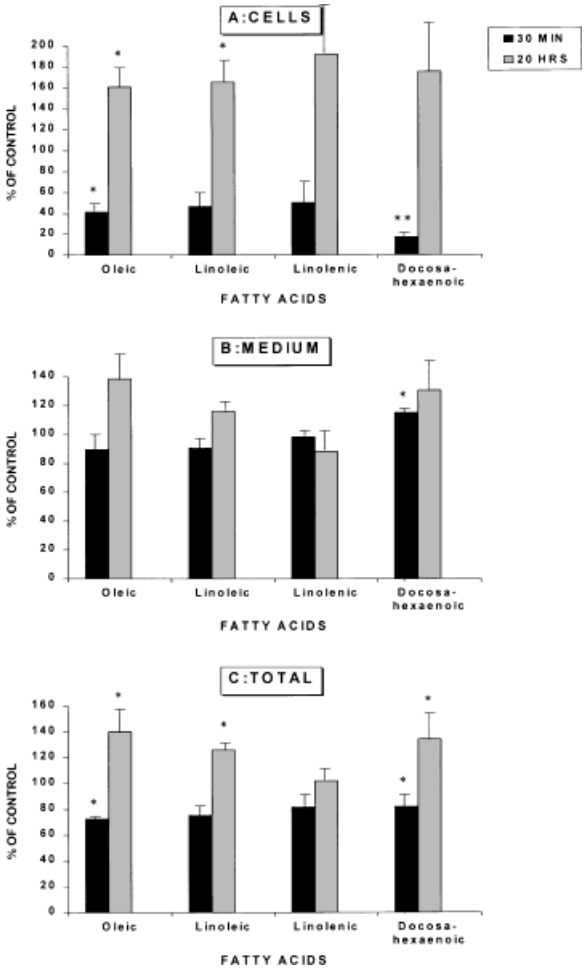

Fig. 1. Effect of fatty acids on apo A-IV levels. Differentiated Caco-2 cells were incubated with 1 mM oleic, linoleic, α-linolenic, or docosahexaenoic acid for 30 min or 20 h. The medium was then collected from the apical and basolateral chambers and pooled. Apo A-IV from cell homogenates (**A**) and media (**B**) was determined as described in Methods. **C**: Cell + medium values. Results are expressed as percentage relative to control values for n = 3–5. *$P < 0.05$, **$P < 0.005$.

Effect of Fatty Acids on apo A-IV mRNA Levels

Fatty acids were delivered to the apical surface of confluent monolayers in order to examine their effect of apo A-IV mRNA levels. Under our experimental conditions, PCR revealed abundant apo A-IV mRNA in Caco-2 cells. A preliminary experiment, consisting in the incubation of the cells with 1 mM of oleic acid, for 30, 60, 120 min and 20 h allowed us to observe that the apo A-IV transcript levels were decreased in short term experiments (30 and 60 min). However, at 20 h, this fatty acid provoked an increase in apo A-IV mRNA levels as documented by a representative experiment de-

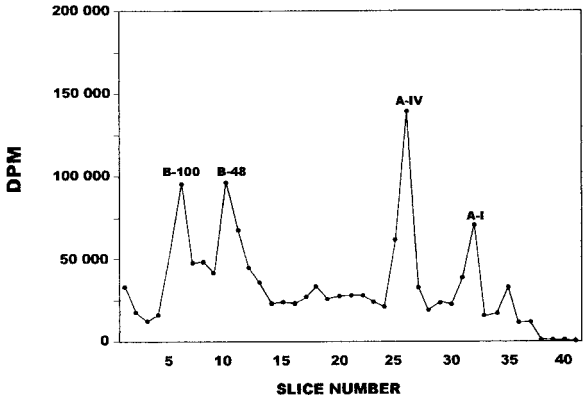

Fig. 2. Profile of ^{35}S-labeled apo A-IV vs. other apolipoproteins synthesized by Caco-2 cells. Following 20 h incubation with unlabeled oleic acid and [^{35}S]-methionine, apolipoproteins were immunoprecipitated and analyzed by SDS-PAGE. Data from a representative experiment are illustrated for cell homogenate.

picted in Figure 4. Due to these findings, all further experiments were conducted at 20 h. Thus, when the apical culture medium was supplemented with the different fatty acids for 20 h, there was a significant increase in apo A-IV mRNA levels in the case of oleic, linoleic, and docosahexaenoic acids (Fig. 5), ranging from 125 to 160% when compared to controls. Although α-linolenic acid had some stimulatory effect, it did not reach statistical significance.

DISCUSSION

Human colonic adenocarcinoma Caco-2 cells have largely been used as an intestinal model to determine the impact of different lipids on apolipoprotein synthesis and transport [Levy et al., 1995]. We took advantage of this convenient and accessible model for studying human apo A-IV regulation by isolated fatty acids. The data reported in this paper provide evidence that monounsaturated and polyunsaturated fatty acids of n-3, n-6, and n-9 families are able to promote protein accumulation and apo A-IV biogenesis. As shown by our findings, such effects involved the modulation of apo A-IV mRNA levels. Conditions for the stimulatory effects of fatty acids included a relatively long incubation time (20 h), in contrast to short periods (30 min) during which fatty acids exhibited inhibitory actions. Only a small influence on apo A-IV protein mass, de novo synthesis and gene expression was noticeable by the degree of unsaturation without regard of chain length.

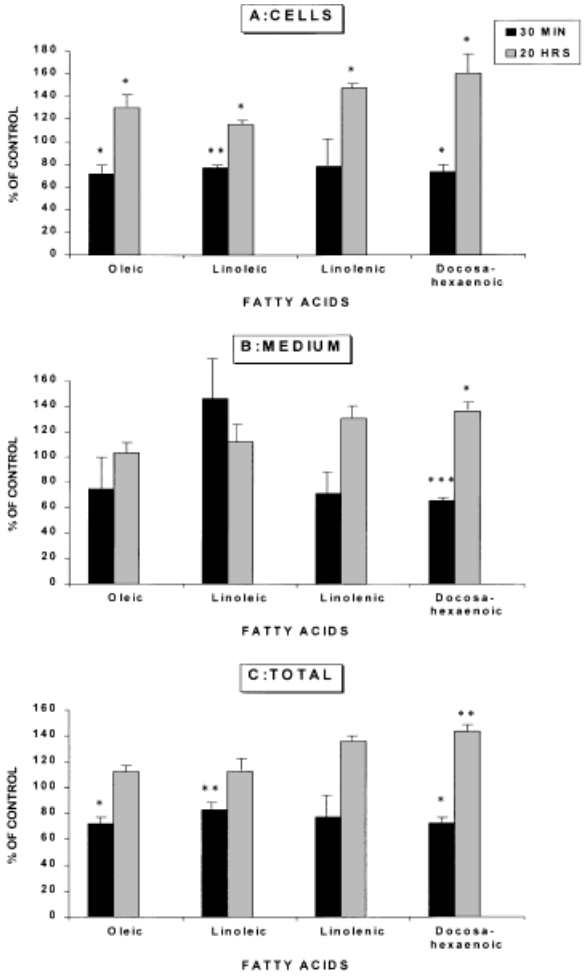

Fig. 3. Effect of individual fatty acids on de novo apo A-IV synthesis. Caco-2 cells were incubated with [^{35}S]-methionine in the presence or absence of different fatty acids. Apo A-IV was immunoprecipitated and analyzed by SDS-PAGE. Data (calculated first as % of TCA-precipitable/mg tissue protein) represent a percentage relative to control values. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Many studies reported that the synthesis and secretion of apo A-IV are regulated by dietary triglycerides [Kalogeris et al., 1994; Apfelbaum et al., 1987; Black et al., 1990; Gordon et al., 1982]. The output of apo A-IV into rat intestinal lymph as well as in human and animal blood circulation was stimulated by the ingestion of a fatty meal or alimentary triglycerides. Furthermore, in a variety of human pathologies involving impaired fat absorption, such as abetalipoproteinemia [Bisgaier et al., 1985], chronic pancreatitis, and malabsorptive syndromes [Koga et al., 1985], and in subjects receiving total parenteral nutrition [Sherman et al., 1988], plasma apo A-IV levels were shown to be substantially decreased. Thus, Hayashi et al.

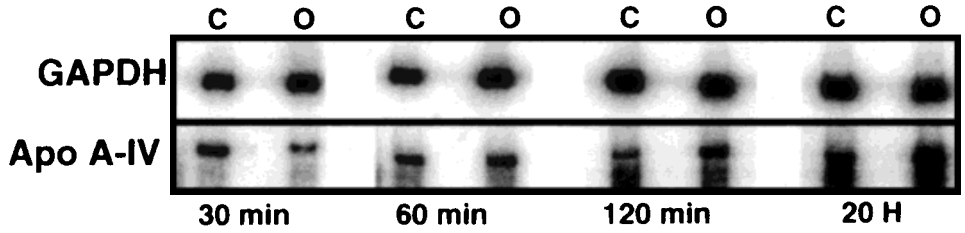

Fig. 4. Representative effect of oleic acid on apo A-IV mRNA levels. Caco-2 cells were incubated with oleic acid during different periods of time and apo A-IV mRNA levels were determined by RT-PCR as described in Materials and Methods.

[1990] proposed that the intestinal absorption and transport of lipids were stimuli for intestinal apo A-IV. Our findings in Caco-2 cells, in response to the administration of fatty acids that differed in chain length and double bonds, are consistent with the general observations documenting the control of apo A-IV synthesis by lipid nutrients. As the amounts of specific mRNA in Caco-2 increase in the presence of individual fatty acids, one may suggest that apo A-IV synthesis is regulated at the transcriptional level. However, it appears from our experiments that the threshold time required to induce apo A-IV is above 30 to 60 min, whereas periods equivalent to or shorter than 30 min favor the inhibition of apo A-IV synthesis by fatty acids. These data suggest that a minimal period is necessary for the assembly and secretion of chylomicrons, supposedly responsible for stimulating the production of apo A-IV by the small intestine [Hayashi et al., 1990]. Alternative mechanisms may include mRNA instability, insufficient intracellular fatty acid pool or decreased availability of fatty acids. The 5′ nontranscribed region of the apo A-IV gene, as proposed by Gordon et al. [1982], contains a short nucleotide sequence that may constitute a promoter region sensitive to increased intracellular fatty acid concentration. A minimal period may be required to replete the critical cellular pool of fatty acids/triglycerides resulting in apo A-IV stimulation. The association between the long-term incubation of fatty acids and the highly detectable levels of apo A-IV also corroborates the elevated apo A-IV levels found after chronically fed high fat diets [Green et al. 1980; Bisgaier et al., 1985; Appelbaum et al., 1987].

It is generally accepted that the lowering of TG levels by fish oil occurs via the impairment of the assembly of hepatic VLDL, and/or by inhibiting the secretion of newly synthesized apo B. The intestine would also contribute to the reduced plasma concentration of triacylglycerol after the ingestion of long-chain n-3 fatty acids. Recently, many studies have reported a decrease in the secretion of both triglyceride and apo B mass in cultured cells incubated with n-3 fatty acids [Murthy et al., 1992]. In our experiments, docosahexaenoic and oleic acids were more potent than linoleic and α-linolenic acids in increasing the gene expression and synthesis/secretion of apo A-IV. This indicates that the assembly of chylomicrons rather than the secretion process is actually responsible for the efficient stimulation of apo A-IV. Field et al. [1988] explored the regulatory effect of saturated and unsaturated fatty acids on the synthesis and secretion of triglyceride-rich lipoproteins by Caco-2 cells. They reported that oleic acid potently stimulated triglyceride production by Caco-2 cells, followed in descending order by linoleic, α-linolenic, palmitic, and myristic acids. However, eicosahexaenoic acid has been described as stimulating TG production in the way oleic acid does [Ranheim et al., 1992]. With respect to apo A-IV, a similar pattern was seen in our studies with unsaturated fatty acids, pointing out the importance of triglyceride synthesis. However, additional efforts are required for the elucidation of the mechanisms involved in lipid-regulated intestinal apo A-IV expression, in view of the unexpected, but controversial, observations related to medium-chain triglyceride effects. These lipids, which are secreted directly into the portal circulation rather than being re-esterified and incorporated into chylomicrons in the enterocyte, are as effective as long-chain triglycerides in up-regulating apo A-IV expression [Black et al., 1996].

Docosahexaenoic acid elicited an evident stronger effect on cellular apo A-IV levels at 30 min compared with the other fatty acids. This

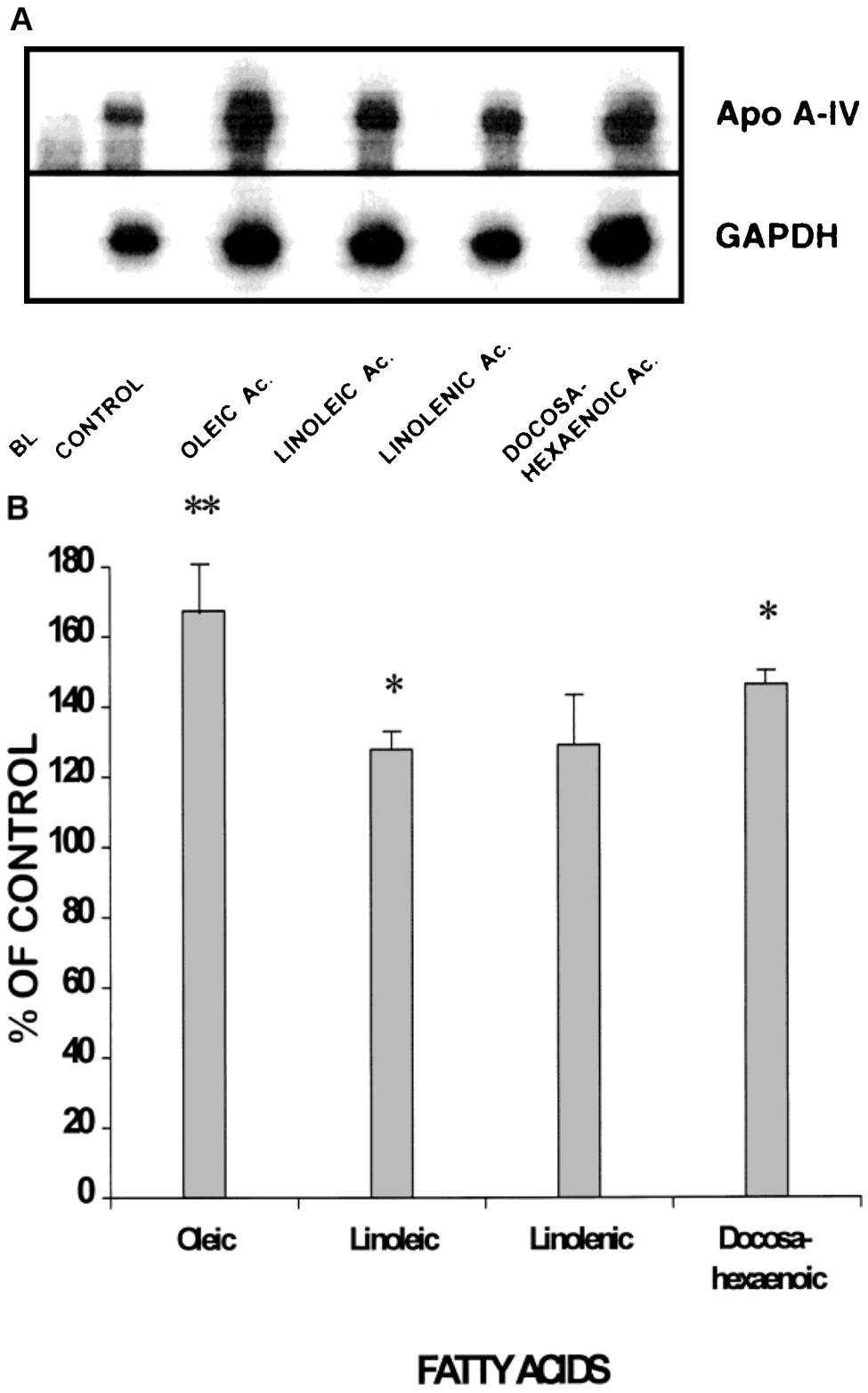

Fig. 5. Representative effect of fatty acids on apo A-IV transcript levels (**A**) and influence of fatty acids on apo A-IV mRNA levels (**B**). Following the incubation of Caco-2 cells with 1 mM oleic, linoleic, α-linolenic, or docosahexaenoic acid, the abundance of apo A-IV transcripts was assessed by RT-PCR as described in Methods. Data are expressed as a percentage of control apo A-IV mRNA/control GAPDH mRNA ratio (n = 3–5), GAPDH being the housekeeping gene. *$P < 0.01$, **$P < 0.005$.

phenomenon could be due to the compensatory increase in apo A-IV secretion into the medium (Fig. 1). Another reason might be the preferential incorporation of docosahexaenoic acid into cellular phospholipids, limiting its esterification into triglycerides and the assembly of chylomicrons [Chen et al., 1985], thereby delaying apo A-IV induction.

In conclusion, the data reported here show the ability of Caco-2 to elaborate substantial amounts of apo A-IV that can respond to dietary monounsaturates and polyunsaturates. The regulation of apo A-IV synthesis by most of these fatty acids involves switching on its gene. Predominant effects are triggered by oleic and docosahexaenoic acids. Since a short-term incubation period with fatty acids leads to the inhibition of apo A-IV gene expression and biogenesis, one can suggest that a minimal period required for mRNA stability and/or chylomicron assembly is necessary to enhance apo A-IV production.

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