Dietary and hypothyroid hypercholesterolemia induces hepatic apolipoprotein E expression in the rat: direct role of cholesterol

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Abstract Apolipoprotein E (apo E) exerts a protective effect against atherosclerosis, related to its role in intracellular cholesterol removal and remnants clearance. In this study we investigated the effect of dietary and hypothyroid hypercholesterolemia, induced respectively by a high cholesterol diet and by propylthiouracil, on hepatic apo E expression in Wistar male rats. The Northern and Western blot analysis of hepatic mRNA and protein levels showed a 2–3-fold increase of apo E in hypercholesterolemic rats compared to controls. The incubation of FAO rat hepatoma cells with 25-OH cholesterol and mevalonate led to a three-fold increase of apo E mRNA, demonstrating a direct role of cholesterol on apo E expression. This effect was completely abolished by elevating intracellular cAMP levels with forskolin. Immunoblot and immunofluorescence analysis revealed that 25-OH cholesterol/mevalonate strongly increased also apo E protein synthesis and secretion in FAO cells. Our data demonstrate that hypercholesterolemia, apart of the cause (diet or hypothyroidism) induces liver apo E expression in the rat and that this effect can be directly related, via cAMP, to cholesterol.

Key words: Apolipoprotein E; Hypercholesterolemia; 25-OH Cholesterol; Cyclic AMP; FAO hepatoma cell

1. Introduction

Apolipoprotein E (apo E) plays an important role in lipoprotein metabolism through its interaction with cell surface receptors as low density lipoprotein (LDL) receptor and LDL-related protein receptor [1]. Recently, it has been demonstrated that free apo E secreted by hepatocytes in the sinusoidal space of Disse and associated with very low density lipoprotein (VLDL) and chylomicron remnants [2] is necessary for a correct hepatic clearance of these lipoproteins and therefore for the maintenance of normal fasting and postprandial lipid levels. Moreover, apo E has been suggested to be involved in the reverse cholesterol transport [3]. The importance of apo E in cholesterol homeostasis has been demonstrated in transgenic mice lacking functioning apo E. They develop hypercholesterolemia and early atherosclerosis [4].

Despite the growing evidence of the protective role exerted by this apoprotein against atherosclerosis, the regulation of apo E expression by plasma lipids has not yet been extensively studied. Diet-induced hypercholesterolemia has been previously shown to enhance the hepatic expression of apo E; on the contrary, only few reports exist describing opposite effects of *n*-propylthiouracil (PTU)-induced hypercholesterolemia on hepatic apo E levels [5–7].

The aim of our study was to investigate the effect of hypercholesterolemia on hepatic apo E expression and to clarify the underlying mechanism. On this purpose we used two different models of hypercholesterolemia in the rat: (1) a dietary hypercholesterolemia obtained by feeding the animals with a diet enriched with 1.5% of cholesterol; (2) a hypothyroid hypercholesterolemia induced by PTU treatment. In both models hypercholesterolemia strongly enhanced liver apo E expression. Furthermore, by in vitro studies using FAO cells, a rat differentiated hepatoma cell line, we demonstrated that cholesterol is directly involved in the regulation of hepatic apo E expression through a cAMP-dependent mechanism.

2. Materials and methods

2.1. Materials

25-OH cholesterol, PTU, mevalonate (MVA) and forskolin were purchased from Sigma.

2.2. Animals, diet and treatment procedures

Sixty Wistar male rats weighing 200 ± 50 g were divided in three groups of 20 rats each. The animals of the first (control) and second groups were fed the commercial pellet laboratory chow. After 35 days hypothyroidism was induced in the animals of second group by continuous administration for 25 days of 0.1% (w/v) PTU in the drinking water. The rats of the third group were fed for 2 months a diet enriched with 1.5% of cholesterol, the Nath diet [8]. All animals were given diet and water ad libitum.

At the end of treatments blood was collected (after 16 h fasting) by intracardiac puncture, under a slight diethylether anesthesia. Then the animals were killed by cervical dislocation and livers were immediately collected, frozen in N_2 and stored at −80°C until they were used.

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Abbreviations: apo E, apolipoprotein E; MVA, mevalonate; PTU, *n*-propylthiouracil; FCS, fetal calf serum; PMSF, phenylmethyl-sulfonylfluoride; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline

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2.3. Cell culture

FAO cells, a rat hepatoma cell line, established and characterized by Deschatrette and Weiss [9], were grown in Coon's modified Ham's F12 medium containing 5% fetal calf serum (FCS) under a humidified atmosphere composed of 95% air and 5% CO_2 at 37°C.

2.4. Western blot

Livers from each group of animals were pooled, homogenized in 10 volumes of PBS in the presence of 1 mM of PMSF and centrifuged for 5 min at 4°C at 900×*g*. Cell extracts were prepared from subconfluent cells grown in 100 mm Petri dishes. Cells were washed twice in PBS, scraped in PBS and pelleted by centrifugation. Cell pellets were resuspended in lysis buffer (10 mM Tris–HCl pH 7.2, 1 mM PMSF, 2 μM aprotinin, 10 μM leupeptin) disrupted by sonication and centrifuged at 3000 rpm for 10 min at 4°C. Fifty micrograms protein of liver homogenates and cell supernatants were electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose membrane and blocked with 7.5% milk in TBS–Tween 20 for 1 h at room temperature. The filters were then probed with polyclonal rabbit antibodies directed against anti rat apo E (kindly provided by Dr. E. Bond, Istituto dei Tumori 'Pascale', Naples) at a 1:100 dilution. Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated donkey anti rabbit IgG (Amersham), using the enhanced chemiluminescence system (ECL, Amersham). The coefficient of analytical variations of Western blot experiments was 4%.

2.5. Northern blot

Total RNA was isolated from a pool of liver samples for each group of animals or from FAO cells by the guanidium thiocyanate-acid phenol procedure [10]. Samples of total RNA were fractioned by electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane and crosslinked by ultraviolet (UV) irradiation. The membrane was then hybridized with a ^{32}P-labelled 330 bp fragment of rat apo E cDNA (kindly provided by Dr. J. Taylor, Gladstone Foundation, San Francisco, CA, USA) [11] in 7% SDS and 0.5 M Na-phosphate pH 7.2 at 60°C. Labelling of the fragment was done as described [12]. After 16 h the filters were washed at 55°C and autoradiographed using Kodak XAR5 film for 16 h at −80°C [10]. The coefficient of analytical variations of Northern blot experiments was 5%.

2.6. Densitometry

Protein and mRNA bands were quantified by densitometric scanning of autoradiographs using NIH-image software.

2.7. Immunofluorescence microscopy

FAO cells grown on coverslip glasses, were incubated for 24 h in culture medium containing 0.2% FCS in the presence or in the absence of 1 μg/ml 25-OH cholesterol and 0.76 mM MVA; then were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100. The primary antibodies anti rat apo E were the same utilized for Western blot experiments. The secondary antibodies were FITC-conjugated anti rabbit IgG (sc-521, Santa Cruz, Ca, USA). Non-specific labeling was determined similarly, except that non-immune rabbit antiserum substituted for anti apo E antibodies. Coverslips were mounted on 50% glycerol in PBS and examined by fluorescence microscopy [13].

2.8. Analytical determinations

Cholesterol and triglycerides were determined by the enzymatic-colorimetric method of Siedel et al. [14] and Wahlefeld [15], respectively. The protein concentrations were determined according to the procedure of Lowry et al. [16], using bovine serum albumin as standard.

Table 1
Plasma cholesterol and triglyceride levels in normal, Nath and PTU-treated rats

	Control	Nath	PTU
Cholesterol(mg/dl)	63 ± 8	205 ± 22[a]	85 ± 10[b]
Triglycerides (mg/dl)	140 ± 16	203 ± 20[a]	73 ± 9[a]

Values are means ± S.E.M.
[a] $P < 0.001$.
[b] $P < 0.05$, compared to control.

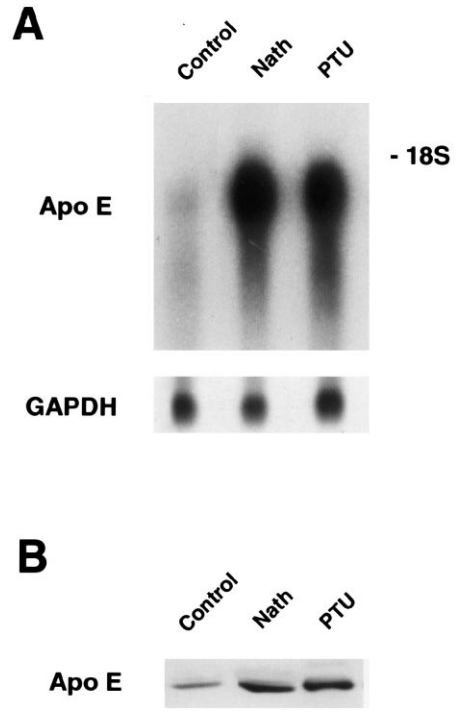

Fig. 1. Expression of hepatic apo E in control and hypercholesterolemic (Nath and PTU) rats. A: Northern blot analysis of apo E mRNA. In each lane 20 μg of total RNA was loaded. In the top panel the blot was probed with rat apo E cDNA. In the bottom panel the same blot was stripped and reprobed with a rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe, as control. B: Western blot analysis of apo E. In each lane 50 μg of tissue protein extracts was loaded.

2.9. Statistical analysis

Statistical differences in cholesterol and triglyceride levels between groups were evaluated by Student's *t*-test.

3. Results

On the purpose to study the effect of increased plasma cholesterol levels on hepatic apo E expression, we induced hypercholesterolemia in the rat using two different strategies: by feeding the animals with a cholesterol enriched diet (Nath) and by PTU treatment which leads to an hypothyroid status and a consequent increase of plasma cholesterol levels [17].

Plasma cholesterol and triglyceride levels in control, Nath diet and PTU-treated rats are reported in Table 1. Nath diet increases plasma levels of cholesterol and triglycerides. Plasma cholesterol levels increased also in PTU-treated rats, even if at a lower extent, whereas the triglyceride levels decreased compared to control.

In both the experimental conditions of hypercholesterolemia a large increase in liver apo E mRNA levels was observed (three and two-fold in Nath and PTU treated animals, respectively) (Fig. 1A). Increased levels (about two-fold) of apo E proteins, as revealed by immunoblotting experiments, were also evidenced in both groups of hypercholesterolemic animals (Fig. 1B).

Moreover, as also previously described [18–21], elevated plasma apo E levels were observed in hypercholesterolemic rats compared to controls (50% and 40% of increase in Nath and PTU treatment, respectively).

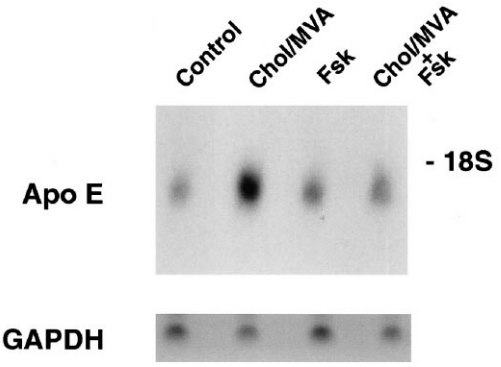

Fig. 2. Northern blot analysis of the apo E mRNA expression in FAO cells. In each lane 20 μg of total RNA was loaded. Cells were preincubated in the presence or in the absence of 25-OH cholesterol/MVA, forskolin (Fsk) or both for 24 h in complete medium before harvesting. In the top panel the blot was probed with rat apo E cDNA. In the bottom panel the same blot was stripped and reprobed with a rat glyceraldheyde phosphate dehydrogenase (GAPDH) cDNA probe, as control. The figure is representative of three independent experiments.

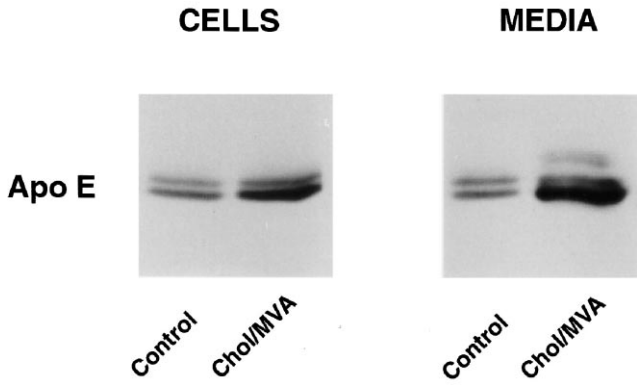

Fig. 3. Western blot analysis of apo E in cells and media of FAO cells. Cells were preincubated for 24 h in the presence or in the absence of 25-OH cholesterol/MVA in 0.2% FCS. Twenty-five microliters of media and 50 μg of cellular protein extracts were loaded. The figure is representative of three independent experiments.

In order to establish a direct role of cholesterol on apo E expression, we performed in vitro experiments in FAO cells. The incubation of cells with 1 μg/ml of 25-OH cholesterol and 0.76 mM of MVA in complete medium for 24 h, led to a three-fold increase of apo E mRNA (Fig. 2). The addition of 25-OH cholesterol alone resulted in a slightly lower increase of apo E mRNA, whereas MVA alone did not cause any major variations (data not shown). A time-course experiment showed that the effect was already evident after 9 h of incubation with 25-OH cholesterol and MVA and reached a plateau after 24 h (data not shown). When 25-OH cholesterol and MVA were incubated in the presence of forskolin, an agent that elevates intracellular cAMP levels, the effect was completely abolished (Fig. 2).

Apo E protein levels, evaluated by immunoblotting experiments, were downregulated when FAO cells where preincubated in medium containing low FCS concentrations (0.2%) or 5% lipoprotein deficient serum (LPDS); this effect was rescued by the addition of purified lipoproteins (data not shown). The incubation of cells with 25-OH cholesterol and MVA in 0.2% FCS containing medium led to a three-fold increase of intracellular apo E levels; moreover, the apo E present in the medium, representing the major secreted apoprotein, was also strongly increased (about three-fold) (Fig. 3). The increase in the apo E in the media can not be ascribed to an increased cellular lysis since the levels of the cytosolic enzyme lactic dehydrogenase (LDH) in the incubation media of control and 25-OH-cholesterol/MVA treated cells were almost identical accounting for only the 0.7% of total. Intracellular apo E was also evidenced by immunofluorescence microscopy (Fig. 4). The main fluorescent signal was observed in control cells intracellularly in a perinuclear region probably associated with compartments of the secretory pathway. In cells preincubated for 24 h with 25-OH cholesterol/MVA, the intracellular fluorescent signal strongly enhanced and in several cells a cell surface fluorescence was evidenced.

4. Discussion

In this study we showed that dietary and hypothyroid hypercholesterolemia induced hepatic apo E expression in the rat. These two experimental conditions were characterized by increased levels of plasma cholesterol but differ in the ability to affect plasma triglyceride levels, that are elevated in Nath and decreased in the PTU-treated rats. This suggests that the hypercholesterolemia, and not the other variations in

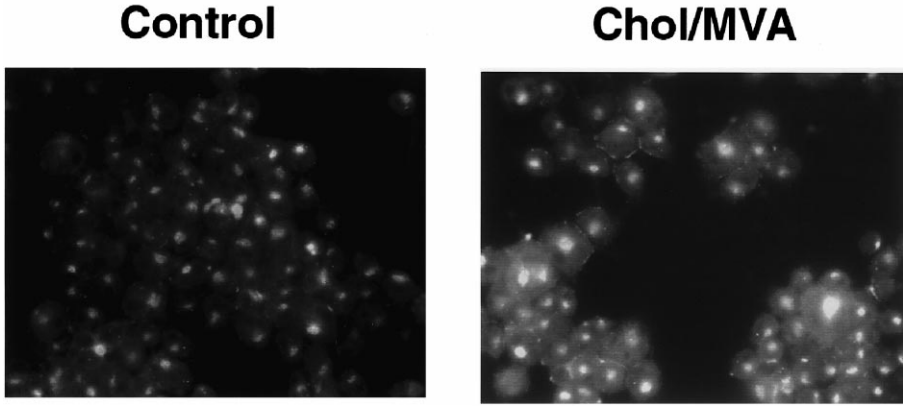

Fig. 4. Immunofluorescence of apo E in FAO cells. Cells were preincubated for 24 h in the absence (control) or in the presence of 25-OH cholesterol and MVA before processing for immunostaining of apo E. The figure is representative of three independent experiments.

plasma lipid levels, were responsible for the induced hepatic apo E expression.

Experiments performed in FAO cells, a rat differentiated hepatoma cell line previously characterized to express apoproteins and, in particular, apo E [22–24], demonstrated a direct role of cholesterol on apo E expression. The addition of 25-OH cholesterol and MVA to the FAO cells caused a strong increase in Apo E mRNA and protein levels.

A down-regulation of apo E expression was observed when cells were grown in medium containing low lipoprotein levels. Moreover, it has been previously demonstrated [22] that lipoproteins or a lipoprotein-derived lipid extract induces apo E synthesis in FAO cells. These effects could be ascribed to the cholesterol carried by plasma lipoproteins, as suggested by the direct effect exerted by cholesterol.

A transcriptional down-regulation of apo E expression by cyclic AMP has been described [25]. We have shown that forskolin mostly does not modify apo E mRNA levels in FAO cells, but completely inhibits the cholesterol-induced apo E expression, leading to the hypothesis that cAMP is involved in this regulation. The low cAMP levels required to observe the effect of cholesterol suggest that it could induce apo E expression by a factor that is, at the same time, negatively regulated by cAMP.

The cholesterol-mediated increase of apo E expression in FAO cells was accompanied by an increase in its secretion. These data suggest that the elevated plasma apo E levels found in hypercholesterolemic rats can be the consequence of an increased hepatic secretion, considering that liver is a major site of apo E synthesis, and as also suggested by our previous finding on increased levels of VLDL-IDL apo E content [20].

Finally, by immunofluorescence studies we evidenced in 25-OH cholesterol/MVA treated cells that apo E is associated with the cell surface. This signal could be ascribed to the secreted free apo E bound extracellularly to the plasma membrane [26]. Cell surface-associated apo E has been demonstrated to play an important role in the binding of lipoproteins to the hepatic receptors and in the reverse cholesterol transport [2]. Therefore, the increased expression and secretion of apo E in hypercholesterolemic conditions could represent a protective mechanism aimed to lower the increased plasma cholesterol levels.

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References

[1] Mahley, R.W. and Innerarity, T.L. (1983) Biochim. Biophys. Acta 737, 197–222.
[2] Mahley, R.W. and Ji, Z.-S. (1999) J. Lipid Res. 40, 1–16.
[3] Johnson, W.J., Mahlberg, F.H., Rothblat, G.H. and Phillips, M.C. (1991) Biochim. Biophys. Acta 1085, 273–298.
[4] Zhang, S.H., Reddick, R.L., Piedrahita, J.A. and Maeda, N. (1992) Science 258, 468–471.
[5] Apostolopoulos, J.J., Howlett, G.J. and Fidge, N. (1987) J. Lipid Res. 28, 642–648.
[6] Lin-Lee, Y.-C., Tanaka, Y., Lin, C.-T. and Chan, L. (1981) Biochemistry 20, 6474–6480.
[7] Go, M.F., Schonfeld, G., Pfleger, B., Cole, T.G., Sussman, N.L. and Alpers, D.H. (1988) J. Clin. Invest. 81, 165–1620.
[8] Nath, N., Wiener, R., Harper, A.E. and Elvehjem, C.A. (1959) J. Nutr. 67, 289–307.
[9] Deschatrette, J. and Weiss, M.C. (1974) Biochimie 56, 1603–1606.
[10] Laezza, C., Di Marzo, V. and Bifulco, M. (1998) Proc. Natl. Acad. Sci. USA 95, 13646–13651.
[11] Taylor, J.M., Mahley, R.W. and Fukazawa, C. (1986) Methods Enzymol. 128, 801–811.
[12] Feinberg, A.B. and Vogelstein, B. (1983) Annal. Biochem. 132, 6–13.
[13] Bifulco, M., Laezza, C., Aloj, S.M. and Garbi, C. (1993) J. Cell Physiol. 155, 340–348.
[14] Siedel, J., Schlumberger, H., Klose, S., Ziegenhorn, J. and Wahlefeld, A.W. (1981) J. Clin. Chem. 19, 838–839.
[15] Wahlefeld, A.W. (1974) in: Methods of enzymatic analysis (Bergmeyer, H.V., Ed.), 2 Engl. Edn., vol. 4, pp. 1831–1835, Academic Press, New York
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Synkes, M., Cnoop-Koopmans, W.M., Julien, P. and Angel, A. (1981) Metabolism 30, 733–738.
[18] Mondola, P., Santangelo, F., Falconi, C. and Belfiore, A. (1987) Horm. Metabol. Res. 19, 407–410.
[19] Dory, L. and Roheim, P.S. (1981) J. Lipid Res. 22, 287–296.
[20] Santillo, M., Santangelo, F., Belfiore, A., Mastursi, M. and Mondola, P. (1993) Horm. Metabol. Res. 25, 156–159.
[21] Santillo, M., Mondola, P., Santangelo, F., Gioielli, A., Iossa, S., Basilisco, A. and De Mercato, R. (1995) Int. J. Biochem. Cell Biol. 27, 257–262.
[22] Pietrangelo, A., Tiozzo, R., Ghisellini, M., Cingi, M.R., Albertazzi, L., Ventura, E. and Calandra, S. (1988) J. Hepatol. 7, 258–268.
[23] Scarino, M.L. and Howell, K.E. (1987) Exp. Cell Res. 170, 1–14.
[24] Scarino, M.L. and Howell, K.E. (1987) Exp. Cell Res. 170, 15–30.
[25] Andreani-Mangeney, M., Vandenbrough, Y., Janvier, B., Girlich, D. and Bereziat, G. (1996) FEBS Lett. 397, 155–158.
[26] Lilly-Stauderman, M., Brown, T.L., Balasubramanian, A. and Harmony, J.A. (1993) J. Lipid Res. 34, 190–200.