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Disturbed Regulation of Cholesterol Synthesis in Monocytes of Obese Patients With Hypercholesterolemia

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The aim of the present study was to clarify the influence of obesity on the functions of low-density lipoprotein receptors (LDL-R) and 3-hydroxy-3-methylglutarate-coenyzme A (HMG-CoA) reductase both in healthy control subjects and in patients with hypercholesterolemia (HC). Experiments were performed on monocytes of 15 non-obese (C I) and 11 obese (C II) healthy control subjects and on 22 non-obese (HC I) and 26 obese (HC II) patients with HC. [^{125}I]LDL was used to determine LDL-R activity by measuring binding and intracellular degradation. The rate of endogenous cholesterol synthesis was measured using [^{14}C]acetate incorporation into the cholesterol fraction of monocytes. The binding ability of [^{125}I]LDL was identical across all groups. The [^{14}C]acetate incorporation in resting monocytes was increased only in obese HC group. The 50-μg/mL LDL protein-induced inhibition of [^{14}C]acetate incorporation was significantly diminished ($P < .001$) in the same group. A strong positive correlation was detected between the [^{14}C]acetate incorporation by resting cells and LDL-induced inhibition in all groups except the obese HC group, in which their correlation was negative ($P < .001$). Furthermore, in the obese HC group, a significant positive correlation was detected between body mass index (BMI) and the basal level of [^{14}C]acetate incorporation, whereas a negative correlation was found between BMI and LDL-induced inhibition of [^{14}C]acetate incorporation. The present data suggest that in patients with HC the concomitant obesity results in dysregulation of cholesterol homeostasis, which may contribute to the accelerated atherosclerosis.
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RECEPTORS for low-density lipoprotein (LDL) particles present on the surface of many cell types play a significant role in the regulation of the serum LDL-cholesterol level. The function of the LDL receptors (LDL-R) is binding and incorporation of LDL particles followed by inhibition of endogenous cholesterol synthesis affecting 3-hydroxy-3-methylglutarate-coenyzme A (HMG-CoA) reductase activity.[1,2] The synthesis and function of LDL-R are genetically regulated.[3-5] The different receptor domains, which have different roles in LDL metabolism, are frequently damaged by point-mutation, which results in various types of hypercholesterolemia (HC).[6-8] Most frequently, these DNA mutations trigger a decreased LDL-R binding capacity and consequently lead to an increased LDL level in the circulation. This evidence supports the theory that HMG-CoA reductase, a key enzyme of cholesterol synthesis, is regulated by sterol products via a feedback mechanism. The LDL-R acts at multiple levels, including transcription, translation, and protein degradation.[3,9]

In our earlier study, it was found that in resting monocytes of patients with non–insulin-dependent diabetes mellitus (NIDDM), incorporation of [^{14}C]acetate was elevated in the cholesterol fraction, and its LDL-induced inhibition was decreased. This disturbance of cholesterol homeostasis was independent of the body weight of diabetic patients.[10]

The purpose of this study was to investigate the relationship between obesity and the functions of both LDL-R and HMG-CoA reductase in such a way that both control and HC patients were divided into non-obese and obese groups. A comparison of LDL-R and HMG-CoA reductase functions upon application of [^{125}I]LDL binding and degradation, as well as the [^{14}C]acetate incorporation into the cholesterol fraction of monocytes was performed. Furthermore, we investigated the correlation between different parameters in each group, and the dependence of altered HMG-CoA reductase activity on the body weight of patients.

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Table 1. Demographic Data of Control Subjects and Patients With Hypercholesterolemia

Parameter	C I	C II	HC I	HC II
No. of patients	15	11	22	26
Age (yr)	53.5 ± 7.3	51.8 ± 6.9	54.6 ± 6.3	53.2 ± 7.4
BMI (kg/m^2)	23.8 ± 3.3	34.2 ± 3.6*	24.2 ± 2.7	35.5 ± 4.1*
WHR	0.92 ± 0.11	1.25 ± 0.18*	0.89 ± 0.11	1.29 ± 0.16*
Cholesterol (mmol/L)	4.7 ± 0.42	4.9 ± 0.38	8.2 ± 1.4†	8.5 ± 1.0†
Triglyceride (mmol/L)	1.6 ± 0.21	2.6 ± 0.33*	1.7 ± 0.21	2.9 ± 0.38*
HDL-C (mmol/L)	1.24 ± 0.14	1.18 ± 0.21	1.35 ± 0.21	1.38 ± 0.25
LDL-C (mmol/L)	3.30 ± 0.46	3.42 ± 0.49	5.83 ± 0.68†	5.97 ± 0.70†

NOTE. Each value represents the mean ± SD.

Abbreviations: BMI, body mass index; C I, healthy control non-obese voluteers; C II, healthy obese control volunteers; HC I, non-obese patients with hypercholesterolemia; HC II, obese patients with hypercholesterolemia; WHR, waist-hip-ratio.

*Differences between non-obese and obese groups are significant, $P < .001$.

†Differences between control and HC groups are significant, $P < .001$.

MATERIALS AND METHODS

Patients

We examined 8 male and 7 female (15) non-obese, 6 male and 5 female healthy obese volunteers, 11 male and 11 female (22) non-obese, and 11 male and 15 female (26) obese patients suffering from HC. Body weight (Table 1) is expressed in kilograms per square meter using body mass index (BMI). Both the control volunteers and the HC affected individuals were kept on a National Cholesterol Education Program (NCEP) Step 1 diet. Other demographic data of the subjects are illustrated in Table 1. Exclusion criteria included fever, liver, thyroid and kidney diseases, infective disorders, and antilipidemic or antihypertensive drug use. Serum cholesterol concentration was measured by routine clinical colorimetric, enzymatic method (cholesterol-oxidase, peroxidase), and serum triglyceride levels were determined by the GPO-Trinder method (colorimetric, endpoint) assayed with Boehringer Mannheim GmbH Diagnostic enzyme kit (Mannheim, Germany), while high-density lipoprotein (HDL) cholesterol was measured by the phosphotungstate-magnesium precipitation method. The LDL-cholesterol fraction was calculated indirectly using the Friedewald equation.[11] Apolipoprotein examination was performed with an immunonephelometric assay using the Orion Diagnostic kit (Finland). All patients with HC were previously examined for LDL-R density. We therefore excluded the role of decreased LDL-R density in the pathogenesis of HC in these patients. The venous blood samples (10 to 15 mL) were taken at intervals of 4 to 5 days from 6 to 8 freshly diagnosed patients and 3 to 4 control volunteers for each set of experiments. The interassay coefficient did not exceed 15%.

Monocyte Isolation and Culture

Monocytes were isolated from venous blood according to the method of Boyum.[12] The mononuclear cell suspensions were placed on Nunc petri dishes (100 mm diameter), pretreated with fetal calf serum (FCS), and further separated by the method of Kumagai et al.[13] The monocyte suspensions were 93% to 96% pure for monocytes, and the proportions of viable cells were 90% to 95%. Monocytes (10^6) in 1.0 mL RPMI 1640 (Gibco, UK) containing 5% FCS were placed on a plate (Nunc, Denmark) with 16 wells for incubation. After an overnight incubation in a CO_2 incubator (ASSAB) at 37°C, 5% CO_2, 95% humidity, monolayers were vigorously washed with Hanks' Balanced Salt Solution (HBSS). The cell numbers in wells were counted on 10 randomly selected areas under an inverted microscope having a special appliance. All cell preparations and incubations were performed under sterile conditions.

LDL Isolation and Iodination

LDL was obtained from a pooled sera of healthy male volunteers by KBr-density gradient ultracentrifugation according to the method of Cornwell et al[14] modified by Szondi et al.[15] LDL concentrations were expressed in micrograms LDL protein/milliliters. Labeling of the LDL was performed according to the method of Sheperd et al[16] using Na [^{125}I] with 1.0 GBq/cm^3 activity (Isotope Institute of Hungarian Academy of Sciences, Budapest, Hungary). Specific activity of labeled LDL was 300 to 400 cpm/ng LDL protein.

LDL-R Activity

The measurement of receptor activity (binding and intracellular degradation) was performed by the method of Goldstein and Brown.[17] A total of 50 μg [^{125}I]LDL protein was added to 1.0 mL RPMI 1640 containing 5% FCS and 10^6 monocytes/well both in the presence and absence of 500 μg cold LDL. The cell-bound radioactivities were determined after a 60-minute incubation at 4°C. The amount of intracellularly degraded LDL was also determined using 50 μg [^{125}I]LDL protein/mL in the presence and absence of cold LDL. The monocyte-monolayers were incubated at 37°C for 4 hours. The amount of intracellularly degraded LDL was quantified from trichloroacetic acid soluble fraction of the supernatant. This fraction was used with chloroform to extract the free iodine after hydrogen peroxide treatment.

[^{14}C]Acetate Incorporation Into the Cholesterol Fraction of Monocytes

The assay was performed as described by McNamara et al.[18] Monocytes (10^6 cells) in 1.0 mL HBSS containing 2.5 nmol/L [2-^{14}C]acetate with 1.8 GBq/mmol specific activity (Isotope Institute of the Hungarian Academy of Sciences) were incubated with and without 50 μg/mL LDL protein for 4 hours at 37°C. The reaction was terminated with 0.5 mL of 1.0 mol/L KOH at the end of the incubation, and the samples were saponified for 90 minutes at 70°C. As an internal reference standard, [1,2-^{3}H]cholesterol (1480 GBq/mmol) was used. The unsaponified lipids were extracted with hexane, the extracts were placed on aluminium oxide columns, and the steroid fraction was eluted with a 1:1 mixture of acetone/diethylether. Radioactivities were counted and the values expressed as picomoles synthesized cholesterol/hour/10^6 monocytes after drying.

Statistical Evaluation

Results are expressed as means ± SD. using the Windows-compatible version 9.0 program SPSS (SPSS, Chicago, IL). Statistical analysis

Table 2. Binding and Intracellular Degradation of [^{125}I]LDL, Basal Level of [^{14}C]Acetate Incorporation, and LDL-Induced Decrease in [^{14}C]Acetate Incorporation by Monocytes of Controls and Patients With HC

		[^{125}I]LDL*		[^{14}C]Acetate†		Δ‡ [^{14}C]Acetate†
Groups	No.	Binding	Intracellular Degradation	Incorporation by Resting Cells	Incorporation After LDL Treatment	Basal-LDL-Induced [^{14}C]Acetate Incorporation
C I	15	111.73 ± 15.7	146.4 ± 13.4	11.4 ± 1.6	6.2 ± 0.8	5.2 ± 1.9
C II	11	114.53 ± 12.2	145.2 ± 12.7	11.4 ± 0.8	6.4 ± 0.7	5.1 ± 1.4
HC I	22	113.6 ± 14.6	149.1 ± 17.6	12.2 ± 2.4	6.2 ± 0.6	5.9 ± 2.2
HC II	26	113.3 ± 10.2	134.7 ± 15.7§	18.5 ± 2.5‖	15.4 ± 2.9‖	3.1 ± 0.4‖

NOTE. Each value represents the mean ± SD.

*Expressed as nanograms LDL protein/10^6 monocytes; †expressed as pmol/h/10^6 monocytes; ‡Δ = basal minus LDL-induced [^{14}C]acetate incorporation; §differences between HC I and HC II groups are significant, $P < .05$; ‖differences between C I, C II, HC I, and HC II groups are significant, $P < .001$.

was performed using the same program. Data were analyzed by analysis of variance (ANOVA) followed by multiple comparisons between the means using the least-significant-difference test. A probability of $P < .05$ was considered statistically significant.

RESULTS

The binding and intracellular degradation of [^{125}I]LDL were determined in the monocytes from non-obese and obese healthy subjects on one hand and non-obese and obese patients with HC on the other. The basal level of [^{14}C]acetate incorporation into the cholesterol fraction of resting monocytes and the incorporation after treatment with 50 μg/mL LDL are shown in Table 2. Data suggest that the binding capacity of monocyte-monolayers was identical in each group of subjects. In contrast, in monocytes obtained from obese HC patients, the intracellular degradation of LDL was slightly decreased as compared with the non-obese HC group ($P < .05$). The [^{14}C]acetate incorporation into the cholesterol fraction of monocytes either by resting or by 50 μg LDL protein-treated monocytes was measured. Table 2 shows that we found a significant increase in [^{14}C]acetate incorporation by resting cells in the obese HC group ($P < .001$) and a diminished LDL-induced inhibition after LDL treatment ($P< .001$). Consequently, Δ [^{14}C]acetate incorporation (incorporation by resting monocytes minus incorporation after LDL treatment) in the obese HC group was significantly decreased ($P< .001$). In Table 3, the correlation coefficients are demonstrated for all patient groups. A significant positive correlation was found in all groups between the LDL binding and degradation. The correlation between the LDL binding and [^{14}C]acetate incorporation after LDL treatment was significantly negative ($P < .001$) in both control and non-obese HC groups. It should be noted that there was no significant correlation in the obese HC group between LDL binding and LDL-induced decrease of [^{14}C]acetate incorporation ($P = .3827$). Similarly, there was no correlation between LDL degradation and [^{14}C]acetate incorporation after LDL treatment ($P = .9344$). The correlation coefficient in the obese HC group between [^{14}C]acetate incorporation of resting cells and Δ [^{14}C]acetate incorporation was strongly negative ($P < .001$). In control groups and in the non-obese HC group, this correlation was significantly positive ($P < .001$).

Table 4 demonstrates the correlations between BMI and LDL degradation, BMI and [^{14}C]acetate incorporation by resting monocytes, BMI and [^{14}C]acetate incorporation by LDL-treated-monocytes, and lastly between BMI and the Δ [^{14}C]acetate incorporation. The data suggest in the obese HC group there is a significantly positive correlation between BMI and basal or LDL-induced [^{14}C]acetate incorporation ($P < .001$). Consequently there is a negative correlation between BMI and Δ [^{14}C]acetate incorporation ($P < .001$), which occurs only in the obese HC group. There is a slight negative correlation ($P < .05$) between BMI and LDL degradation in the non-obese HC group despite a nonsignificant correlation between the same parameters in the obese HC group.

DISCUSSION

Based on the present results, we concluded that in patients with HC, depending on the body weight, the LDL-induced inhibition of endogenous cholesterol synthesis through HMG-CoA reductase activity was disturbed.

The strong and significant negative correlation between the binding or intracellular degradation of LDL and acetate incorporation after LDL treatment in both control and non-obese groups and the failure of this correlation in the obese HC groups (Table 3) suggest the existence of an abnormal intracellular cholesterol metabolism in the monocytes of these patients. In addition, the increased basal [^{14}C]acetate incorpora-

Table 3. Correlation Coefficients Between Parameters of Control Non-obese, Control Obese, HC Non-obese, and HC Obese Groups

Correlation Between	Control Non-obese	Control Obese	HC Non-obese	HC Obese
LDL binding *v* degradation*	.9462‡	.8186‡	.8186‡	.8913‡
LDL binding *v* [^{14}C]acetate incorporation† + LDL	−.9061‡	−.8623‡	−.8623‡	−.1236
LDL degradation *v* [^{14}C]acetate incorporation† + LDL	−.8803‡	−.8620‡	−.8620‡	−.0117
Basal *v* Δ§ [^{14}C]acetate incorporation†	.9277‡	.9274‡	.9274‡	−.8997‡

*Expressed as nanograms LDL protein/10^6 cells; †expressed as pmol/h/10^6 cells; ‡the correlations are significant, $P < .001$; §Δ = difference of [^{14}C]acetate incorporation into resting minus LDL-treated monocytes.

Table 4. Correlation Coefficients Between BMI and Other Parameters in Control Non-obese, Control Obese, HC Non-obese, and HC Obese Patients

Correlation	C I	C II	HC I	HC II
BMI *v* LDL degradation*	.0187	−.0909	−.4911§	.1030
BMI *v* basal [^{14}C]acetate incorporation†	.3237	−.0458	−.0731	.7883‖
BMI *v* [^{14}C]acetate incorporation + LDL†	−.0194	.3120	.1895	.5573‖
BMI *v* Δ [^{14}C]acetate incorporation‡	.3643	.0992	−.1259	−.6657‖

*Expressed as nanograms LDL protein/10^6 cells; †expressed as pmol/h/10^6 cells; ‡Δ = difference of acetate incorporation into resting cells, decreased incorporation into LDL treated cells. The correlations were significant; §$P < .05$; ‖$P < .001$.

tion was associated with a diminished LDL-induced acetate incorporation. Consequently, the correlation between basal and Δ [^{14}C]acetate incorporation was strongly negative, whereas in the controls and non-obese HC groups, the correlation coefficients were significantly ($P < .001$) positive. Our results suggest that obesity is associated with an increased basal cholesterol synthesis and with a decrease in LDL-induced inhibition of synthesis. If that is true, a closer relationship between being overweight and cholesterol metabolism is more probable than was expected.

The dependence of different parameters in the obese HC group on the extent of obesity suggests that concomitant obesity and HC are required for disturbed intracellular cholesterol homeostasis (Table 4). It should be noted that the LDL-R and [^{14}C]acetate incorporation were independent from body weight except in the obese HC group. A significant positive correlation was detected between BMI and [^{14}C]acetate incorporation by resting and LDL-treated monocytes in the obese HC group. Thus, the correlation between BMI and Δ [^{14}C]acetate incorporation was negative. As yet, the exact location of point(s), which disrupt the cascade of genetically regulated processes of intracellular cholesterol metabolism remains unclear.

The focus of interest regarding the regulation of the cholesterol metabolism centers on LDL-R and their genetic control.[19] Mutations in the LDL-R genes produce familial hypercholesterolemia (FH), which exists clinically in 2 forms.[20] FH heterozygotes, who inherit 1 mutant LDL-R allele develop a premature coronary heart disease with elevated plasma LDL-cholesterol after age 35. In FH homozygotes, the elevation in plasma LDL-cholesterol is more significant, and patients are likely to experience myocardial infarction in the first 2 decades. Functionally, the LDL-R mutations have been classified by Hobbs et al[5] into 5 groups based on biosynthetic and functional studies of fibroblast cell strains derived from skin biopsy specimens. In 4 of these 5 groups, the genetic alteration results in a variable decrease in LDL-R number, including the complete absence of LDL-R (class 1 mutations: null alleles). The alleles resulting in transport defective, binding defective, and recycling defective variants cause a diminished binding of LDL. In our experiments, the 4 variants may be uninvolved in the alteration of cholesterol homeostasis in monocytes of the obese HC group, as the binding ability of cells remained unchanged in all groups, including the obese HC patients (Table 2). The class 4 mutation, which is characterized by internalization-defective alleles, resulted in a slightly decreased binding ability followed by the absence of internalization and degradation. The subclass 4 A mutation alters the cytoplasmic domain alone, whereas in subclass 4 B, the internalization-defective alleles result in the absence of both the membrane-spanning domain and the cytoplasmic tail of LDL-R.

Balanced cholesterol metabolism in mammalian cells occurs through the feedback regulation of key proteins involved in cellular uptake by LDL-Rs and endogenous cholesterol synthesis. A major control point is at the level of the gene transcription that encodes important proteins of both processes.[21,22] The identified DNA sequences coding for important regulatory enzymes of cholesterol synthesis are similar to the target site for cholesterol regulation in the promoter region of the LDL-R and HMG-CoA synthase.[23-25] The sterol regulatory element 1 (SRE-1) of LDL-R binds a specific subfamily of basic-helix-loop-helix-zipper DNA binding protein called sterol regulatory element binding proteins (SREBP) as described by Goldstein and Brown et al.[26-28] Decreased intracellular cholesterol is a signal for both SREBP-1 and SREBP-2. SREBPs are cleaved from the membrane of endoplasmic reticulum, and the soluble amino-terminal fragment containing DNA binding and transcriptional activation functions is translocated to the nucleus, where it activates the expression of appropriate target genes. The SREBPs, as transcription factors, activate more than 20 genes that produce enzymes required for the synthesis of cholesterol affecting among others, HMG-CoA synthase and reductase. The biologically active fragment, which contains about 500 amino acids, enters the nucleus and stimulates the transcription of enzymes for both HMG-CoA reductase synthesis and genes encoding the LDL-R biosynthesis. The cholesterol analogue, 25-hydroxysterol, kills mammalian cells by blocking the proteolytic activation of both SREBP-1 and SREBP-2, resulting in a dramatic decrease in cholesterol. The opposite is true in an oxisterol-resistant cell line, in which investigators determined an overproduction of LDL-R, HMG-CoA reductase and synthase.[29] SREBP-1 also regulates key lipogenic enzymes, ie, fatty-acid synthase (FAS), which is present in all steps of biosynthesis of long chain fatty acids derived from acetyl CoA precursors.[30,31] In adipose tissue of obese Zucker rats, Boizard et al[32] found a region on the FAS promoter, which was responsible for obesity due to overexpression of the FAS gene, serving as a target for SREBP-1. Furthermore, subsequent studies have supported the observation that FAS gene expression is dysregulated in some pathologic conditions including obesity, and that SREBPs are implicated in the increase of adipocyte energy stores and obesity.

The previously cited literature[30-32] describes the possibility of a close association between obesity and lipid metabolism. These studies suggest that SREBPs play some role in the body

weight dependent disorders of LDL-R and HMG-CoA reductase activity in obese HC patients. However, the assumption that the increase in cholesterol synthesis depends on overweight appears to be controversial in humans. According to Dietz[33] and other investigators,[34-36] there is an elevated cholesterol synthesis the liver cells of both obese and lean rats and humans, whereas another group of investigators did not find any such differences.[37-39] Based on our results, another question arose, namely that in patients with obesity and a manifest hypercholesterolemia, elevated cholesterol, and the failure of its inhibition may be a possible mechanism for the hypercholesterolemia.

In summary, we have concluded from the present study that in obese-HC patients there exists a disturbed intracellular cholesterol metabolism, which disrupts or alters regulation of cholesterol synthesis by negative feedback of intracellularly elevated LDL-cholesterol. This damage does not occur in individuals with simple obesity or HC, without obesity.

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