Polyunsaturated Fatty Acids and Acetoacetate Downregulate the Expression of the ATP-Binding Cassette Transporter A1

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Low HDL cholesterol is a frequent cardiovascular risk factor in diabetes. Because of its pivotal role for the regulation of HDL plasma levels, we investigated in vivo and in vitro regulation of the ATP-binding cassette transporter A1 (ABCA1) by insulin and metabolites accumulating in diabetes. Compared with euglycemic control mice, ABCA1 gene expression was severely decreased in the liver and peritoneal macrophages of diabetic mice. Treatment with insulin restored this deficit. Incubation of cultivated HepG2 hepatocytes and RAW264.7 macrophages with unsaturated fatty acids or acetoacetate, but not with insulin, glucose, saturated fatty acids, or hydroxybutyrate, downregulated ABCA1 mRNA and protein. The suppressive effect of unsaturated fatty acids and acetoacetate became most obvious in cells stimulated with oxysterols or retinoic acid but was independent of the expression of the thereby regulated transcription factors liver-X-receptor α (LXRα) and retinoid-X-receptor α (RXRα), respectively. Unsaturated fatty acids and acetoacetate also reduced ABCA1 promotor activity in RAW264.7 macrophages that were transfected with a 968-bp ABCA1 promotor/luciferase gene construct. As the functional consequence, unsaturated fatty acids and acetoacetate inhibited cholesterol efflux from macrophages. Downregulation of ABCA1 by unsaturated fatty acids and acetoacetate may contribute to low HDL cholesterol and increased cardiovascular risk of diabetic patients. *Diabetes* 51: 2922–2928, 2002

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9-*cis*-RA, 9-*cis*-retinoic acid; 22R-HC, 22(R)-hydroxycholesterol; ABCA1, ATP-binding cassette transporter A1; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; LXRα, liver-X-receptor α; IFNγ, interferon-γ; PPAR, peroxisome proliferator–activated receptor; PUFA, polyunsaturated fatty acid; RXRα, retinoid-X-receptor α.

Numerous clinical and epidemiological studies have demonstrated the inverse association between HDL cholesterol and the risk of coronary heart disease events (1). It is controversially discussed whether this relationship is causal or only an epiphenomenon of a more general atherogenic disorder. On the one hand, HDL exerts various potentially antiatherogenic properties. For example, HDL particles transport cholesterol from cells of the arterial wall to the liver and to steroidogenic organs, in which cholesterol is used for the synthesis of bile acids, lipoproteins, vitamin D, and steroid hormones (1). On the other hand, low HDL cholesterol is frequently found as a component of the metabolic syndrome in many populations, i.e., together with overweight or obesity, glucose intolerance or overt diabetes, hypertriglyceridemia, and hypertension, which by themselves contribute to the pathogenesis of atherosclerosis (2). Moreover, many individuals with low HDL cholesterol have elevated fasting plasma levels of insulin, are resistant to exogenous insulin in euglycemic clamp studies, and bear an increased risk for future type 2 diabetes (3–5). The pathogenesis of low HDL cholesterol in insulin resistance is not well understood but may provide an important key to answer the question of whether low HDL cholesterol is a causal factor in the pathogenesis of atherosclerosis in patients with insulin resistance or diabetes.

ATP-binding cassette transporter A1 (ABCA1) has previously been identified as a pivotal gene in the regulation of both HDL cholesterol plasma levels and the cellular cholesterol homeostasis, which is defective in patients with Tangier disease (1,6–9). In these patients and their heterozygous relatives, mutations in the ABCA1 gene cause gene dosage–dependent decreases in plasma levels of HDL cholesterol and in the capacity of skin fibroblasts and monocyte-derived macrophages to release cholesterol in the extracellular presence of apolipoproteins (1,6–9). As the clinical result, homozygous patients with Tangier disease accumulate macrophage-derived foam cells in various tissues and develop tonsil anomalies, hepatosplenomegaly, premature atherosclerosis, and peripheral neuropathy (10).

Cyclic AMP and ligands of the nuclear transcription factors liver-X-receptor α (LXRα) and retinoid-X-receptor α (RXRα), i.e., oxysterols and retinoids, respectively, have

been identified as enhancers of ABCA1 gene expression (11–14). By contrast, interferon-γ (IFNγ) downregulates ABCA1 gene expression (15). Here we investigated the regulation of ABCA1 in a mouse model of diabetes. Since ABCA1 gene expression was substantially decreased in the liver and peritoneal macrophages of diabetic mice, we next investigated the effects of insulin as well as metabolites accumulating in diabetes on ABCA1 gene expression. Thereby, we identified free polyunsaturated fatty acids (PUFAs) and acetoacetate as strong suppressors of ABCA1 gene expression.

RESEARCH DESIGN AND METHODS

Materials. Palmitic acid, oleic acid, eicosapentaenoic acid (EPA), arachidonic acid, stearic acid, linoleic acid, 22(R)-hydroxycholesterol (22R-HC), 9-*cis*-retinoic acid (9-*cis*-RA), acetoacetate, and β-hydroxybutyrate were purchased from Sigma. Insulin was from Hoechst Marion Roussel. ApoA-I was isolated from delipidated HDL by fast-performance liquid chromatography as previously described (16)

Cell culture. The human hepatocyte cell line HepG2 and the murine macrophage cell line RAW264.7 (DSMZ; Braunschweig) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS (GIBCO-BRL). For all experiments, cells were maintained in serum-free DMEM containing 10 g/l BSA and was supplemented with additives (22R-HC, 9-*cis*-RA, insulin, glucose, fatty acids, or ketone bodies) at concentrations and time periods described in the RESULTS section and in the figure legends.

Mice. All animal experiments were approved by the local Animal Care and Usage Committee and the District Governor of Münster. Mice were caged in groups of three to four animals in a 12-h dark rhythm. They were fed a regular chow diet (Furina Altromin 1034; Lage, Germany). At the age of 10 weeks, 10 mice were made diabetic by intraperitoneal injection of 200 mg streptozotocin dissolved in citric buffer (pH 4.0) (17). The manifestation of diabetes was indicated by blood glucose levels >22 mmol/l (>400 mg/dl). After 2 weeks of being diabetic, the animals were killed by inhalation of CO_2. Blood was obtained immediately thereafter by intracardial or intra-aortal puncture. Peritoneal macrophages and livers were obtained by lavage and careful preparation, respectively, and stored in liquid nitrogen. In some experiments the diabetic mice received intraperitoneal injections of PBS or 14 IU insulin per 100 g/body wt as well as subcutaneous injections of PBS or 14 IU insulin per 100 g/body wt every 6 h before being killed.

RNA isolation and RT-PCR. Total RNA was extracted from cells of 9.6-cm^2 flasks using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and treated with DNase to remove residual contaminations with DNA. The RNA was reverse transcribed and the cDNA amplified by PCR using the Superscript II reverse transcriptase, Oligo-dT primers (Invitrogen) and Hotstar *Taq*-DNA polymerase (Qiagen). Sequences of the primers are listed in Table 1. After amplification (activation at 95°C for 15 min, denaturing at 94°C for 0.5 min, primer annealing at 60°C for 0.75 min, and chain elongation at 72°C for 0.75 min) through 16–35 cycles, the whole volume of each PCR product was separated by electrophoresis in 3% agarose gel containing 0.5 μg/ml ethidium bromide. Bands were visualized and photographed under ultraviolet light. Alternatively, ABCA1 gene expression was quantified by real-time PCR using the Applied Biosystems prism model 7700 sequence detection instrument (Weiterstadt, Germany). The amplification was detected using SYBRGreen as the fluorogenic probe specific for double-stranded DNA. Threshold cycle, C_t, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above threshold level using the manufacturer's settings. The mRNA levels for ABCA1 were corrected for GAPDH.

Reporter assay. A firefly luciferase reporter construct was generated by amplifying a 968-bp fragment of human ABCA1 promoter (–803 to +165 bp) (18) with *Kpn*I and *Xho*I restriction sites out of genomic DNA. PCR product was digested with *Kpn*I and *Xho*I, gel purified, and ligated to *Kpn*I-*Xho*I-cut pGL3 Basic vector (Promega, Madison, WI). For transfection, 50–80% confluent RAW264.7 cells were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin G, and 100 mg/ml streptomycin. Cells were transiently transfected with 10 μg of the firefly luciferase reporter plasmids and cotransfected with 1 μg pRL-SV40 vector (Promega) for the control *Renilla* luciferase by electroporation at 950 μF and 220 V using 0.4 ml of 2×10^7 cells/ml per transfection. The cells from one electroporation were seeded into eight wells of a 24-well dish. After transfection, the cells were incubated with 22(R)-HC, 9-*cis*-RA, fatty acids, and/or acetoacetate for 18 h as indicated. The luciferase activities were measured using the dual luciferase assay system (Promega). The firefly luciferase activity was divided by the renilla activity to obtain a normalized value, the relative luciferase activity.

TABLE 1
Sequences of PCR primers

Primer name	Primer sequence
Human ABCA1 forward	5′-TGA GCT ACC CAC CCT ATG AAC A-3′
Human ABCA1 reverse	5′-CCC CTG AAC CCA AGG AAG TG-3′
Mouse ABCA1 forward	5′-CGC TCA ACT TTT ACG AAG GCC-3′
Mouse ABCA1 reverse	5′-GAG CGA ATG TCC TTT CCC CA-3′
Human/mouse 18S forward	5′-GAA GGT GAA GGT CGG AGT C-3′
Human/mouse 18S reverse	5′-GAA GAT GGT GAT GGG ATT TC-3′
Human GAPDH forward	5′-CCC ATG TTC GTC ATG GGT GT-3′
Human GAPDH reverse	5′-TGG TCA TGA GTC CTT CCA CGA TA-3′
Mouse GAPDH forward	5′-AAC GAC CCC TTC ATT GAC-3′
Mouse GAPDH reverse	5′-TCC ACG ACA TAC TCA GCA C-3′
Human LXRα forward	5′-AGC GTC CAC TCA GAG CAA GT-3′
Human LXRα reverse	5′-GGG GAC AGA ACA GTC ATT CG-3′
Human/mouse RXRα forward	5′-TCC TTC TCC CAC CGC TCC ATC-3′
Human/mouse RXRα reverse	5′-CAG CTC CGT CTT GTC CAT CTG-3′
Mouse LXRα forward	5′-GAT CTG GGA TGT CCA CGA GT-3′
Mouse LXRα reverse	5′-CCC TTC TCA GTC TGC TCC AC-3′

Immunoprecipitation and Western blotting of ABCA1. Cells were washed in PBS and lysed in lysis buffer containing 1% (wt/vol) Triton-X 100 (Sigma), 10 mmol/l Tris (pH 7.4), 150 mmol/l sodium chloride, 0.5% (wt/vol) sodium deoxycholic acid, 50 mmol/l 2-mercaptoethanol, 10 μg/ml pepstatin A (Sigma), 10 mmol/l PMSF, and protease inhibitor cocktail (Roche, Mannheim, Germany) for 1 h on a rotating platform at 4°C. Then, 40 μg protein G agarose beads (Pierce, Rockford, IL) were added to 12 ml of the cell lysate. After incubation for 2 h on a rotating platform, protein G beads were separated by centrifugation at 13,000 rpm for 20 s. The cell lysate was then incubated with rabbit anti-ABCA1 antibody for 2 h at 4°C. After addition of 10 μg protein G agarose beads the cell lysates were incubated for additional 2 h. Immune complexes were precipitated by centrifugation at 13,000 rpm for 20 s and washed three times with Triton-Tris–buffered saline (50 mmol/l Tris/HCl pH 7.4, 150 mmol/l NaCl, and 1% Triton-X 100) and PBS. Immune precipitates were denatured by boiling for 5 min in a buffer with 20 g/l SDS and 100 mmol/l mercaptoethanol. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane by electroblotting. Membranes were blocked by incubation for 1 h in a buffer with 50 mmol/l Tris, 150 mmol/l NaCl, 0.05% Tween 20, and 5% milk. ABCA1 was probed with an antibody that was raised in rabbits by immunization with a recombinant ABCA1 fragment encompassing amino acid residues 1,371 through 1,657 (T.E., unpublished observations). The immunoreaction was visualized after incubation of the nitrocellulose sheets with secondary horseradish peroxidase–conjugated anti-rabbit IgG antibodies (Dako) using a chemiluminescent substrate (Lumi-light; Roche Molecular Biochemicals) and a photo imaging system (Raytest, Straubenhardt, Germany).

Cholesterol efflux. At the state of near confluence, RAW264.7 cells were washed with PBS and incubated for 20 h at 37°C with 2,5 μCi [1,2-^{3}H]-cholesterol (51.7 μCi/mmol, New England Nuclear), which were complexed with BSA. After radiolabeling, cholesterol pools were equilibrated for 15 h by incubation with serum-free DMEM/2 g/l BSA in the presence or absence of additives (oxysterol, fatty acids, or ketone bodies) as described in the RESULTS section. For cholesterol efflux, cells were washed with ice-cold PBS and incubated for 5 h with 300 μl DMEM containing 2 g/l BSA alone or 2 g/l BSA

TABLE 2
Plasma levels of glucose, lipids, and free fatty acids in mice according to diabetes and treatment with insulin or saline

	Control mice treated with PBS	Diabetic mice treated with PBS	Diabetic mice treated with insulin
N	2	4	3
Glucose (mmol/l)	4.0 ± 0.1	35.9 ± 11.1	2.5 ± 0.5
Cholesterol (mmol/l)	2.64 ± 0.21	2.10 ± 0.71	1.95 ± 0.90
HDL cholesterol (mmol/l)	2.14 ± 0.09	1.72 ± 0.74	1.58 ± 0.96
Triglycerides (mmol/l)	0.96 ± 0.06	2.48 ± 0.84	0.70 ± 0.10
Free fatty acids (mmol/l)	1.4 ± 0.5	2.4 ± 2.0	0.8 ± 0.2

Data are means ± SE. Mice were made diabetic by intraperitoneal injection of 200 mg streptozotocin. After at least 2 weeks the mice received intraperitoneal injections of PBS or 14 IU insulin per 100 g body wt as well as subcutaneous injections of PBS or 14 IU insulin per 100 g body wt every 6 h for 24 h. Blood was collected intracardial or intraaortal puncture of mice at once after sacrifice with CO_2.

plus 50 μg apoA-I as well as the additives indicated in the RESULTS section. After incubation for 5 h, cells and media were harvested separately. Radioactivity was measured by scintillation spectrometry of the supernatant and of the cell monolayers after lysis with 500 μl 0.15 mol/l NaCl with 0.2 mol/l NaOH. Fractional cholesterol efflux was calculated as $cpm_{medium}/(cpm_{medium} + cpm_{cells}) \times 100\%$.

Clinical chemistry. Plasma concentrations of glucose, cholesterol, triglycerides, and HDL cholesterol were measured by the use of a Hitachi 917 autoanalyzer (Roche Diagnostics). Plasma levels of free fatty acids were determined by the use of reagents from Roche Diagnostics according to the manufacturer's recommendations.

RESULTS

To test the possible contribution of dysregulated ABCA1 gene expression to low HDL cholesterol, we initially investigated the regulation of ABCA1 in mice that were made diabetic with streptozotocin (Table 2, Fig. 1). Compared with untreated mice, the liver (Fig. 1*A*) and peritoneal macrophages (Fig. 1*B*) of diabetic mice showed a marked decrease in the expression of ABCA1 mRNA. Intraperitoneal injection of insulin abolished the diabetic phenotype (Table 2) and restored the expression of ABCA1 in either liver (Fig. 1*C*) or peritoneal macrophages (Fig. 1*D*).

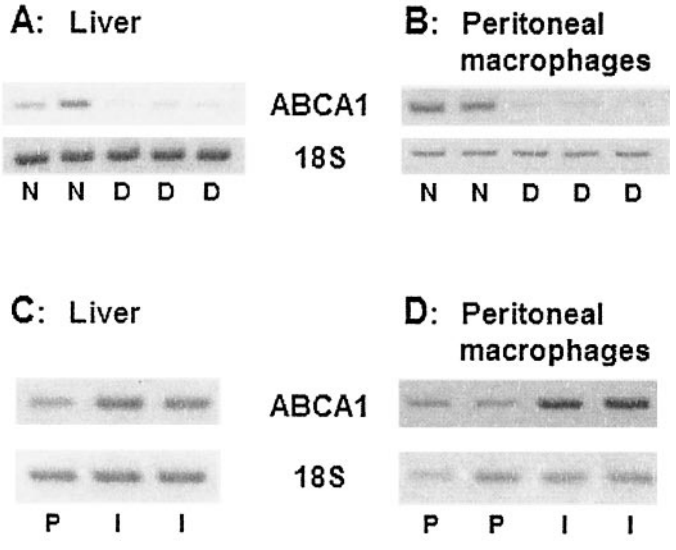

FIG. 1. Effect of diabetes and treatment with insulin on the expression of ABCA1 in liver and peritoneal macrophages. Diabetes was induced by injection of streptozotocin as described in RESEARCH DESIGN AND METHODS section. Control mice received PBS. N, normal mice; D, diabetic mice. Some mice with streptozotocin-induced diabetes received intraperitoneal and subcutaneous injections of insulin for 6 h or saline. P, injection of PBS; I, injection of insulin. The mRNAs of ABCA1 and 18S were demonstrated by RT-PCR in liver (*A* and *C*) and macrophages (*B* and *D*). Note the suppression of ABCA1 in liver and macrophages of diabetic mice (*A* and *B*), which was restored by treatment with insulin (*C* and *D*).

Our next experiments were aimed at the identification of the metabolic basis of the decreased ABCA1 gene expression in diabetes. We incubated HepG2 hepatocytes (Fig. 2*A*) or RAW264.7 macrophages (Fig. 2*B*) for 18 h with increasing dosages of insulin, glucose, fatty acids, or ketone bodies. Addition of insulin or glucose into the cell culture medium had no consistent effects on the expression of ABCA1 in either hepatocytes or macrophages. By contrast, the polyunsaturated EPA as well as the ketone body acetoacetate dose-dependently decreased the expression of ABCA1 in both cell lines (Figs. 2*A* and *B*). The suppressive effects of these fatty acids became most evident in those cells where ABCA1 expression was upregulated with an oxysterol, i.e., an activator of the nuclear transcription factor LXRα, which was previously shown to induce the ABCA1 gene (12,13). In HepG2 cells, the stimulatory effect of 22R-HC (12,13) on ABCA1 was inhibited by ~80% ($P < 0.01$), 60% ($P < 0.01$), and 40% ($P < 0.05$) in the additional presence of physiological dosages of EPA (100 μmol/l), oleic acid (500 μmol/l), or acetotacetate (10 mmol/l), respectively (Fig. 3*A*). In RAW264.7 cells, the respective numbers were 95, 80, and 60% (all $P < 0.01$; Fig. 3*B*). Also, in the presence of 9-*cis*-RA, i.e., an activator of the nuclear transcription factor RXRα that forms heterodimers with LXRα and thereby also induces the ABCA1 gene (14), EPA, oleic acid, and acetoacetate suppressed ABCA1 gene expression in HepG2 and RAW by 50–60% (Figs. 3*C* and *D*, all significant at a level of $P < 0.05$ or $P < 0.01$, except acetoacetate in HepG2 cells). In none of these conditions did palmitic acid or hydroxybutyrate modulate ABCA1 gene expression (Fig. 3*A*– *D*). Likewise, and in agreement with data from Wang and Oram (19), we did not see any suppressive effect of fatty acids or acetoacetate on ABCA1 gene expression in macrophages that were stimulated with Br-cAMP (data not shown).

We verified the stimulatory effects of 22(R)-HC and 9-*cis*-RA as well as the inhibitory effects of EPA and acetoacetate in a luciferase reporter gene assay, which was performed on a construct that contained 0.968 kb of the human ABCA1 promotor (Fig. 4). As expected from previous reports (12–14), 22R-HC and 9-*cis*-RA increased the ABCA1 promotor activity by a factor up to 30 (Figs. 4*A* and *B*). EPA and acetoacetate suppressed these stimula-

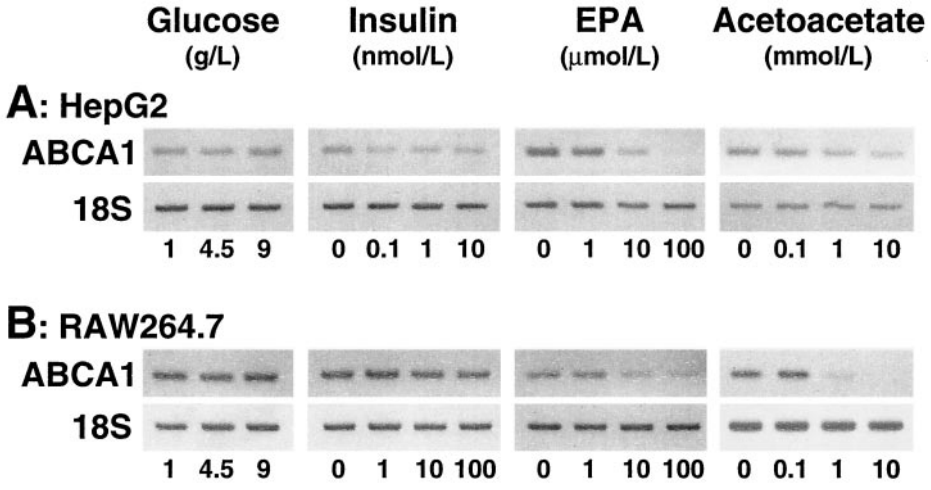

FIG. 2. Dose-dependent effects of insulin, glucose, eicosapentaenoic acid, and acetoacetate on the expression of ABCA1 in HepG2 cells (*A*) and RAW264.7 macrophages (*B*). Confluent cells were incubated for 18 h with increasing dosages of insulin, glucose, EPA, or acetoacetate. The mRNA of ABCA1 was demonstrated by conventional RT-PCR.

tory effects dose dependently by up to 80% (EPA, Figs. 4*C* and *D*) and 50% (acetoacetate, Figs. 4*E* and *F*), respectively. Using this reporter gene assay, we compared the suppressive effects of various fatty acid species at a concentration of 100 μmol/l. Linoleic acid was most effective (−80%), followed by arachidonic acid (−75%), EPA (−70%), and oleic acid (−40%). Palmitic acid had a modest but significant suppressive effect (−10%), whereas stearic acid exerted a moderate but also significant stimulatory effect (+10%, Fig. 5).

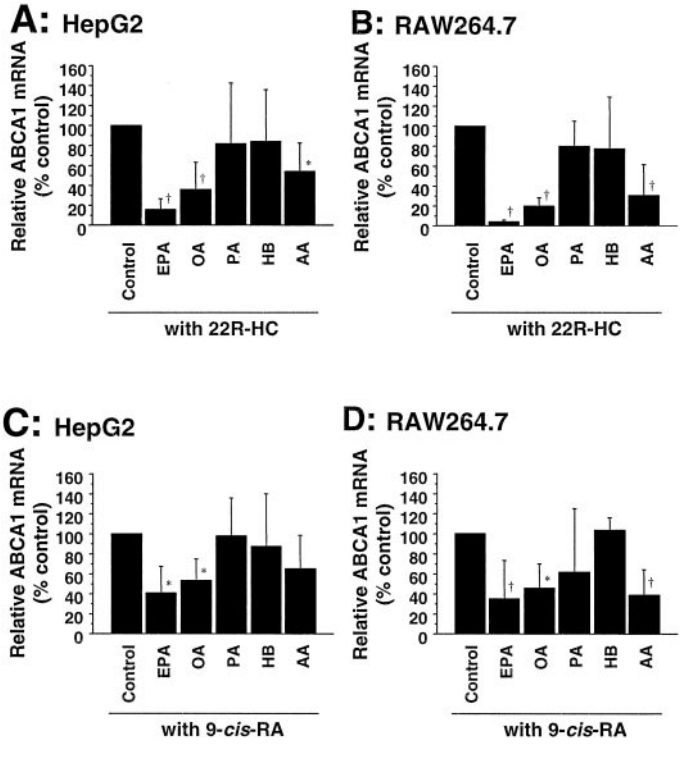

FIG. 3. Interacting effects of 22(*R*)-hydroxycholesterol and 9-*cis*-RA with fatty acids and ketone bodies on the expression of ABCA1 in HepG2 cells and RAW264.7 cells. Confluent hepatocytes (*A* and *C*) and RAW264.7 cells (*B* and *D*) were incubated for 18 h with serum-free medium in the presence of 10 g/l BSA and 10 μmol/l 22R-HC (*A* and *B*) or 10 μmol/l 9-*cis*-RA (*C* and *D*) plus 100 μmol/l EPA, 500 μmol/l palmitic acid (PA), 500 μmol/l oleic acid (OA), 10 mmol/l hydroxybutyrate (HB), or 10 mmol/l acetoacetate (AA). The mRNA of ABCA1 was demonstrated by quantitative real-time PCR. Note that EPA, oleic acid, and acetoacetate (more prominent in macrophages, but not palmitic acid and hydroxybutyrate) (*B*) suppress the stimulatory effect of 22(*R*)-HC on ABCA1 gene expression. EPA and oleic acid have less suppression on ABCA1 expression in the presence of 9-*cis*-RA than in 22(*R*)-HC. Results represent means ± SD from four to six experiments. †$P < 0.01$, *$P < 0.05$ compared with control (one-way ANOVA).

Our observations raised the question as to whether unsaturated fatty acids and acetoacetate regulate the expression of ABCA1 indirectly via regulation of LXRα or RXRα gene expression. Such a mechanism was previously demonstrated to be responsible for the upregulation of ABCA1 by agonists of peroxisome proliferator–activated receptor (PPAR)-α (i.e., fibrates) and PPARγ (i.e., glitazones) (20). Neither oleic acid nor EPA nor acetoacetate regulated the expression of LXRα or RXRα (data not shown).

Immunoprecipitation and subsequent Western blot analysis clearly detected ABCA1 in RAW264.7 cells (Fig. 6*A*), which were cultivated in the presence of an oxysterol. However, the cellular ABCA1 protein concentration was severely decreased when these cells were incubated in the additional presence of free fatty acids or acetoacetate (Fig. 6*A*). The functional relevance of ABCA1 downregulation by free fatty acids and ketone bodies was investigated in cholesterol efflux experiments (Fig. 6*B*). Lipid-free apoA-I induced significant cholesterol efflux from RAW264.7 cells. As reported previously, cholesterol efflux to apoA-I was stimulated by oxysterols (11–13). The stimulatory effect of oxysterols was completely inhibited by 500 μmol/l oleic acid, 100 μmol/l EPA, and 10 mmol/l acetoacetate (Fig. 6*B*).

Interestingly, the expression of another important gene involved in cholesterol efflux, scavenger receptor BI, was not affected by either fatty acids or acetoacetate (data not shown).

DISCUSSION

Here we have demonstrated that ABCA1 is tremendously downregulated in an animal model of diabetes (Fig. 1). As the most likely explanation, we found suppressive effects of PUFAs, oleic acid, and acetoacetate on ABCA1 gene expression in vitro (Figs. 2–5). Interestingly, the saturated

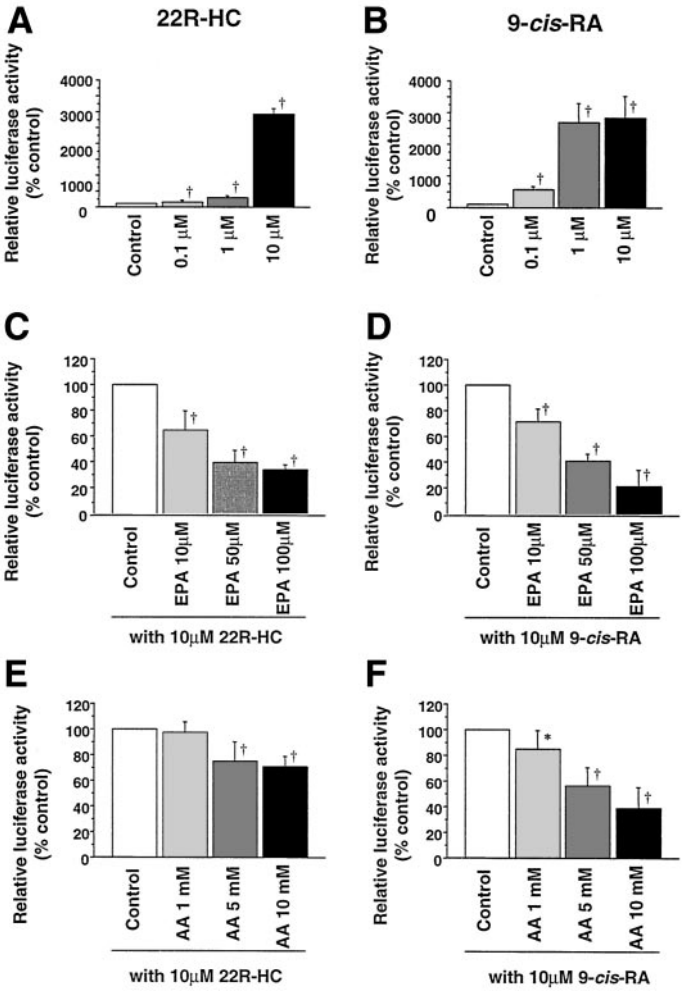

FIG. 4. Dose-dependent effects of 22(*R*)-hydroxycholesterol, 9-*cis*-RA, eicosapentaenoic acid, and acetoacetate on the activity of the human ABCA1 promoter in RAW264.7 cells. The human ABCA1 promoter was inserted into the firefly luciferase reporter gene. RAW 264.7 cells were transfected to ABCA1 gene with pGL3 basic vector together with pRL SV40 as a control reporter plasmid (renilla luciferase). After transfection, the cells were incubated with 22R-HC, 9-*cis*-RA, EPA, and acetoacetate (AA) for 18 h. The firefly luciferase activity was divided by the renilla activity to give a normalized value as the relative luciferase activity, and the values are shown in percent change compared with control. 22R-HC (*A*) and 9-*cis*-RA (*B*) dose-dependently increase the ABCA1 promoter activity. These stimulatory effects of 22R-HC (*C* and *E*) and 9-*cis*-RA (*D* and *F*) are dose-dependently suppressed by EPA (*C* and *D*) and acetoacetate (*E* and *F*). Results represent means ± SD of at least four independent transfections. †$P < 0.01$, *$P < 0.05$ compared with control (one-way ANOVA).

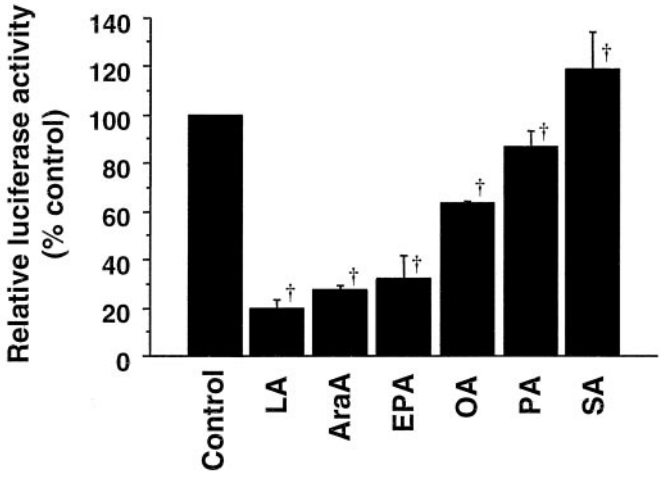

FIG. 5. The effects of various fatty acids on the activity of the human ABCA1 promoter in RAW264.7 cells. For experimental details see legend of Fig. 4. After transfection, the cells were incubated with 100 μmol/l EPA, oleic acid (OA), palmitic acid (PA), linoleic acid (LA), arachidonic acid (AraA), or stearic acid (SA) in the presence of 10 μmol/l 22R-HC and 10 g/l BSA for 18 h. Results represent means ± SD of four independent transfection expriments. †$P < 0.01$ compared with control (one-way ANOVA).

palmitic and stearic acids as well as the ketone body 3-hydroxybutyrate did not alter ABCA1 gene expression so that the inhibitory effects appear to be specific for unsaturated fatty acids and acetoacetate.

Suppression of ABCA1 gene expression by unsaturated fatty acids became most obvious in the additional presence of an oxysterol (Fig. 3) but did not occur in the presence of Br-cAMP (data not shown, 19). This raised the possibility that unsaturated fatty acids downregulate ABCA1 via suppression or inhibition of the nuclear transcription factor LXRα, which is activated by oxysterols and which upregulates ABCA1 (12,13,21). Free fatty acids were previously shown to interact with LXRα on both the transcriptional and the posttranslational level (22,23). Free fatty acids induce the transcription of the LXRα gene via activation of PPARs (22,24). However, unsaturated fatty acids do not suppress ABCA1 via transcriptional regulation of LXRα, since in our experiments oleic acid and EPA did not modulate the expression of LXRα (data not shown) and since PPARα, PPARγ, and PPARδ rather upregulate ABCA1 via stimulation of LXRα transcription (14,25). On the posttranslational level, unsaturated fatty acids antagonize the activation of LXRα by oxysterols and thereby inhibit the transcription of the sterol regulatory element-binding protein-1c (23,26). This posttranslational inhibition of LXRα well explains the transcriptional suppression of ABCA1 by unsaturated fatty acids. Interestingly, PUFAs turned out as more suppressive than the monounsaturated oleic acid, whereas saturated fatty acids exerted only moderate or even no suppressive effects at all. This order is in agreement with the effects of dietary fatty acids on HDL cholesterol. Compared with habitual Western diets being rich in saturated fatty acids, diets rich in PUFAs substantially lower HDL cholesterol, whereas diets rich in oleic acid have only a little HDL cholesterol–decreasing effect (27).

The suppressive effect of acetoacetate on ABCA1 gene expression was more obvious in macrophages than in hepatocytes (Figs. 3–5). Like PUFAs and oleic acid, acetoacetate did not suppress the expression of LXRα or RXRα. Downregulation of ABCA1 by acetoacetate is, to the best of our knowledge, the first example of a gene regulatory effect exerted by this ketone body. Previous in vivo and in vitro studies demonstrated that ketoacidosis decreases hepatic apoA-I gene expression in rats (28). However, this effect was linked to low pH rather than to ketone bodies (28). In vitro we also observed that acidosis suppresses ABCA1 gene expression (data not shown); however, in our experiments the addition of acetoacetate did not acidify the cell culture medium. Moreover, in those experiments, suppression of apoA-I was accomplished by butyrate (28), which did not suppress ABCA1 in our experiments. Thus, the suppressive effect appears to be

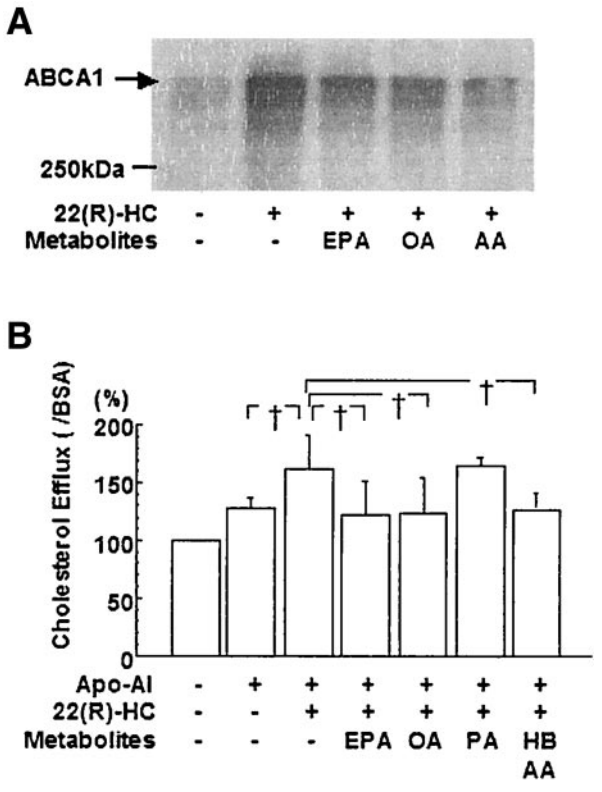

FIG. 6. Effects of 22(*R*)-hydroxycholesterol, fatty acids, and ketone bodies in RAW264.7 cells on the abundance of ABCA1 protein (*A*) and the apoA-I–induced cholesterol efflux (*B*). ABCA1 protein was analyzed by SDS-PAGE and Western blotting of proteins from RAW264.7 cells that were incubated in the absence or presence of 500 μmol/l oleic acid (OA), 100 μmol/l EPA, or 10 mmol/l acetoacetate (AA) (*A*). For cholesterol efflux, RAW264.7 cells were radiolabed with ^{3}H cholesterol. The cells were then equilibrated with 10 μmol/l 22R-HC in the absence or presence of the unsaturated fatty acids or 500 μmol/l palmitic acid (PA) ketone bodies in the forementioned dosages. Cholesterol efflux was induced by a 5-h incubation of 50 μg/ml apoA-I in the respective equilibration medium (*B*).

specific for acetoacetate. Our data do not allow any conclusion of whether acetoacetate exerts the inhibitory effect directly by itself or indirectly by a metabolite or a signal elicited thereof. It is unlikely that the regulatory effect of acetoacetate is exerted by a metabolite because ketolysis is a hallmark of heart and skeletal muscle as well as kidney and brain, which in contrast to liver and macrophages have a high activity of the rate-limiting enzyme in ketolysis, i.e., succinyl-CoA-oxoacid transferase (29,30). Because acetoacetate but not β-hydroxybutyrate downregulates the expression of ABCA1 and because the suppressive effect of acetoacetate was more obvious in macrophages than in hepatocytes, it is interesting to note that acetoacetate by contrast to β-hydroxybutyrate generates oxygen radicals in cells (31). Because oxygen radicals are known to regulate gene expression via various signaling pathways (32), it will be important to investigate the effects of oxygen radicals on the expression of ABCA1.

Our findings contribute to the understanding of the pathogenesis of the low HDL cholesterol syndrome in patients with latent or manifest type 2 diabetes. Insulin was previously shown to stimulate hepatic apoA-I gene expression (33,34), to inhibit hepatic VLDL production (35,36), and to stimulate the release of lipoprotein lipase from adipose tissue (37). Insulin resistance hence reduces the hepatic production of nascent HDL and induces hypertriglyceridemia, which secondarily causes low HDL cholesterol levels via disturbed release of surface remnants (1,2,38) and by enhancing the exchange of triglycerides from VLDL against cholesteryl esters from HDL (1,2,39). Free fatty acids were previously shown to downregulate the expression of apoA-I (40) and to upregulate the expression of cholesteryl ester transfer protein (41). In addition, we have demonstrated here that unsaturated fatty acids and acetoacetate suppress the expression of ABCA1 in liver and in macrophages. By the latter effect, hyperacylemia and ketosis in diabetes and insulin resistance will probably not only interfere with the formation of HDL but also facilitate lipid accumulation in vascular macrophages.

Patients with type 1 diabetes have increased cardiovascular risk but normal or even slightly elevated levels of HDL cholesterol (42). This is, at first sight, in partial contrast to our finding of suppressed ABCA1 expression in liver and macrophages of streptozotocin-induced diabetic mice (i.e., a model for type 1 diabetes) as well as in cultivated cells treated with unsaturated fatty acids and acetoacetate (which are also elevated in patients with type 1 diabetes). However, patients with type 1 diabetes have abnormal HDLs, which are enriched with triglycerides so that in vivo the HDL-decreasing effect of low ABCA1 expression may be counterbalanced by other deregulations of HDL metabolism (42). In addition, low HDL cholesterol is the most frequent dyslipidemia in patients with type 1 diabetes, which becomes manifest especially in patients with insufficient euglycemic control (43). Thus, in patients with type 1 diabetes, HDL cholesterol decreases with increasing blood levels of glycated hemoglobin and increasing albuminuria (44,45), which both are associated with acylemia and ketosis.

In conclusion, here we have provided strong evidence that ABCA1 is suppressed in diabetes by unsaturated long chain fatty acids and acetoacetate. This may be an important etiological factor for the increased cardiovascular risk of patients with insulin resistance or overt diabetes by lowering HDL cholesterol and by impairing cholesterol efflux from vascular cells.

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REFERENCES

1. von Eckardstein A, Nofer JR, Assmann G: HDL and coronary heart disease: role of cholesterol efflux and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 20:13–27, 2001
2. Després JP, Marette A: Relation of components of insulin resistance to coronary disease risk. *Curr Opin Lipidol* 5:274–289, 1994
3. Hergenc G, Schulte H, Assmann G, von Eckardstein A: Associations of obesity markers, insulin, and sex hormones with HDL-cholesterol levels in Turkish and German individuals. *Atherosclerosis* 145:147–156, 1999
4. Karhapää P, Malkki M. Laakso M: Isolated low HDL cholesterol: an insulin-resistant state. *Diabetes* 43:411–417, 1994
5. von Eckardstein A, Schulte H, Assmann G: Risk for diabetes mellitus in middle-aged Caucasian male participants of the PROCAM study: implications for the definition of impaired fasting glucose by the American Diabetes Association. *J Clin Endocrinol Metab* 85:3101–3108, 2000

6. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HOF, Loubser O, Ouelette BFF, Fichter K, Ashbourne-Excoffon KJD, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJP, Genest J, Hayden MR: Mutations in ABCA1 in Tangier disease and familial high density lipoprotein deficiency. *Nat Genet* 22:336–345, 1999
7. Bodzioch M, Orso E, Klucken J, Langmann T, Böttcher A, Diedrich W, Drobnik W, Barlage S, Büchler C, Porsch-Özcürümez M, Kaminski W, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G: The gene encoding ATP binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet* 22:347–351, 1999
8. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G: Tangier disease is caused by mutations in the gene encoding ATP binding cassette transporter 1. *Nat Genet* 22:352–255, 1999
9. Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM, Oram JF: The Tangier disease gene product control subjects: the cellular apolipoprotein-mediated lipid removal pathway. *J Clin Invest* 104: R25–R31, 1999
10. Assmann G, von Eckardstein A, Brewer HB Jr: Familial analphalipoproteinemia: Tangier disease. In *The Metabolic and Molecular Bases of Inherited Disease*. 8th Ed. Scriver, Beaudet, Sly, Valle, Eds. New York, McGraw-Hill, 2000, p. 2937–2960
11. Oram JF, Lawn RM, Garver MR, Wade DP: ABCA1 is the 8-Br-cAMP-inducible receptor that mediates cholesterol secretion from macrophages. *J Biol Chem* 275:34508–34511, 2000
12. Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, Tontonoz P: Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. *Proc Natl Acad Sci U S A* 97:12097–12102, 2000
13. Costet P, Luo Y, Wang N, Tall AR: Sterol-dependent transactivation of the ABC1 pomoter by LXR/RXR: LXRs transactivate ABC1 promotor. *J Biol Chem* 275:28240–28245, 2000
14. Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ: Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 289: 1524–1529, 2000
15. Panousis CG, Zuckerman SH: Interferon-γ induces downregulation of Tangier disease gene (ATP binding cassette transporter 1) in macrophage-derived foam cells. *Arterioscler Thromb Vasc Biol* 20:1565–1571, 2000
16. von Eckardstein A, Funke H, Walter M, Altland K, Benninghoven K, Assmann G: Structural analysis of apolipoprotein A-I variants: amino acid substitutions are nonrandomly distributed throughout the apolipoprotein A-I primary structure. *J Biol Chem* 265:8610–8617, 1990
17. Lakshmanan MR, Nepokroeff CM, Porter JW: Control of the synthesis of fatty-acid synthetase in rat liver by insulin, glucagon, and adenosine 3′:5′ cyclic monophosphate. *Proc Natl Acad Sci U S A* 69:3516–3519, 1972
18. Santamarina-Fojo S, Peterson K, Knapper C, Qiu Y, Freeman L, Cheng JF, Osorio J, Remaley A, Yang XP, Haudenschild C, Prades C, Chimini G, Blackmon E, Francois T, Duverger N, Rubin EM, Rosier M, Denefle P, Fredrickson DS, Brewer HB Jr: Complete genomic sequence of the human ABCA1 gene: analysis of the human and mouse ATP-binding cassette A promoter. *Proc Natl Acad Sci U S A* 97:7987–7992, 2000
19. Wang Y, Oram JF: Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1. *J Biol Chem* 277:5692–5697, 2002
20. Chinetti, G. Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart JC, Clavey V, Staels B: PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nature Medicine* 7:53–58, 2001
21. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ: An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383:728–731, 1996
22. Tobin KA, Steineger HH, Alberti S, Spydevold O, Auwerx J, Gustafsson JA, Nebb HI: Cross-talk between fatty acid and cholesterol metabolism mediated by liver X receptor-alpha. *Mol Endocrinol* 14:741–752, 2000
23. Ou J, Tu H, Shan B, Luk A, DeBose-Boyd RA, Bashmakov Y, Goldstein JL, Brown MS: Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc Natl Acad Sci U S A* 98:6027–6032, 2001
24. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P: A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell* 7:161–171, 2001
25. Oliver WR Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM: A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc Natl Acad Sci U S A* 98:5306–5311, 2001
26. Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T, Nakakuki M, Tomita S, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Takahashi A, Sone H, Osuga Ji J, Gotoda T, Ishibashi S, Yamada N: Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J Biol Chem* 277:1705–1711, 2002
27. Grundy SM, Denke MA: Dietary influences on serum lipids and lipoproteins. *J Lipid Res* 31:1149–1172, 1990
28. Haas MJ, Pun K, Reinacher D, Wong NC, Mooradian AD: Effects of ketoacidosis on rat apolipoprotein A1 gene expression: a link with acidosis but not with ketones. *J Mol Endocrinol* 25:129–139, 2000
29. Laffel L: Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabete Metab Res Rev* 15:412–426, 1999
30. Newsholme P, Gordon S, Newsholme EA: Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages. *Biochem J* 242:631–636, 1987
31. Jain SK, Kannan K, Lim G: Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. *Free Radic Biol Med* 25:1083–1088, 1998
32. Kamata H, Hirata H: Redox regulation of cellular signalling. *Cell Signal* 11:1–14, 1999
33. Murao K, Wada Y, Nakamura T, Taylor AH, Mooradian AD, Wong NC: Effects of glucose and insulin on rat apolipoprotein A-I gene expression. *J Biol Chem* 273:18959–18965, 1998
34. Zheng XL, Matsubara S, Diao C, Hollenberg MD, Wong NC: Epidermal growth factor induction of apolipoprotein A-I is mediated by the Ras-MAP kinase cascade and Sp1. *J Biol Chem* 276:13822–13829, 2001
35. Foretz M, Guichard C, Ferre P, Foufelle F: Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc Natl Acad Sci U S A* 96:12737–12742, 1999
36. Shimomura I, Bashmakov. Y., Ikemoto S, Horton JD, Brown MS, Goldstein JL: Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96:13656–13661, 1999
37. Fried SK, Russell CD, Grauso NL, Brolin RE: Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *J Clin Invest* 92:2191–2198, 1993
38. Strauss JG, Frank S, Kratky D, Hammerle G, Hrzenjak A, Knipping G, von Eckardstein A, Kostner GM, Zechner R: Adenovirus mediated rescue of lipoprotein lipase-deficient mice: lipolysis of triglyceride-rich lipoproteins is essential for HDL maturation in mice. *J Biol Chem* 276:36083–36090, 2001
39. Rye KA, Clay MA, Barter PJ: Remodelling of high density lipoproteins by plasma factors. *Atherosclerosis* 145:227–238, 1999
40. Berthou L, Saladin R, Yaqoob P, Branellec D, Calder P, Fruchart JC, Denefle P, Auwerx J, Staels B: Regulation of rat liver apolipoprotein A-I, apolipoprotein A-II and acyl-coenzyme A oxidase gene expression by fibrates and dietary fatty acids. *Eur J Biochem* 232:179–187, 1995
41. Hirano R, Igarashi O, Kondo K, Itakura H, Matsumoto A: Regulation by long-chain fatty acids of the expression of cholesteryl ester transfer protein in HepG2 cells. *Lipids* 36:401–406, 2001
42. Valabhji J, McColl AJ, Schachter M, Dhanjil S, Richmond W, Elkeles RS: High-density lipoprotein composition and paraoxonase activity in type I diabetes. *Clin Sci (Lond)* 101:659–670, 2001
43. Perez A, Wagner AM, Carreras G, Gimenez G, Sanchez-Quesada JL, Rigla M, Gomez-Gerique JA, Pou JM, de Leiva A: Prevalence and phenotypic distribution of dyslipidemia in type 1 diabetes mellitus: effect of glycemic control. *Arch Intern Med* 160:2756–2762, 2000
44. Idzior-Walus B, Mattock MB, Solnica B, Stevens L, Fuller JH: Factors associated with plasma lipids and lipoproteins in type 1 diabetes: the EURODIAB IDDM Complications Study. *Diabet Med* 18:786–796, 2000
45. Mattock MB, Cronin N, Cavallo-Perin P, Idzior-Walus B, Penno G, Bandinelli S, Standl E, Kofinis A, Fuller JH: Plasma lipids and urinary albumin excretion rate in type 1 diabetes mellitus: the EURODIAB IDDM Complications Study. *Diabet Med* 18:59–67, 2001