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Diabetes alters LDL receptor and PCSK9 expression in rat liver

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Abstract

Since the hepatic LDL receptor is regarded as a major determinant of plasma LDL levels, the effect of diabetes on the expression of this receptor was examined in rat liver. Inducing diabetes with streptozotocin caused a significant reduction in hepatic LDL receptor mRNA levels in concert with an increase in serum cholesterol levels. However, LDL receptor protein levels were unaffected by the diabetic state. Further investigation revealed that protein levels of PCSK9, which has been shown to enhance the degradation of the LDL receptor protein, were significantly decreased in the diabetic rats explaining the lack of reduction in LDL receptor protein levels. These observations indicate that the rate of LDL receptor cycling (function) in diabetic rats is decreased resulting in higher serum LDL levels.

Keywords: Diabetes mellitus; Liver; LDL receptor; PCSK9; Gene regulation

In general, diabetic patients have several risk factors for an increased occurrence of cardiovascular disease [1–3]. These include endothelial dysfunction, dyslipidemia [decreased high density lipoprotein (HDL)[1]-cholesterol levels, hypertriglyceridemia, and increased levels and oxidation of small dense low density lipoprotein (LDL) particles], and platelet activation leading to a prothrombotic state [1–3]. Of these risk factors, the high levels of LDL particles in the serum appear to be of great concern [1–4]. In fact, it has been recently suggested that LDL particle number or size should be included as a marker for assessing risk of developing type 2 diabetes or cardiovascular disease in patients with metabolic syndrome [4].

Several studies have shown that the major determinant of plasma LDL levels is activity of the hepatic LDL receptor [5–11]. The primary function of this receptor is the removal of highly atherogenic LDL particles from the circulation [5,6]. The LDL receptor pathway mediates at least 60–75% of LDL turnover in rats [7,8], 67% in rabbits [9], and 56–80% in man [7,10]. Since the liver contains about 70% of total LDL receptor present in the body [11], changes in plasma LDL levels are generally due to changes in hepatic LDL receptor activity. The importance of the LDL receptor to the cardiovascular physiology of humans has been demonstrated by the identification of mutations either in the gene encoding the LDL receptor, in the gene encoding the liver specific LDL receptor adaptor protein ARH (autosomal recessive hypercholesterolemia), in the gene encoding the protease PCSK9 (proprotein convertase subtilisin/kexin type 9), or in genes encoding the ligands of the LDL receptor [12–22]. Thus, a greater understanding of the regulatory mechanisms that control expression of the hepatic LDL receptor is essential.

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[1] *Abbreviations used:* HDL, high density lipoprotein; LDL, low density lipoprotein; ARH, autosomal recessive hypercholesterolemia; PCSK9, proprotein convertase subtilisin/kexin type 9; STZ, streptozotocin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gels; ANOVA, analysis of variance; Tris–HCL, tris–hydrochloride; PPRE, peroxisomal proliferator response element; SRE, sterol regulatory element;WT, wild-type; EGF-A, epidermal growth factor-like repeat.

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The aim of this study was to examine the effects of diabetes on the expression of the hepatic LDL receptor.

Materials and methods

Animals

Normal male Sprague–Dawley rats weighing 125 to 150 g were purchased from Harlan Industries (Madison, WI). All experiments involving animals were carried out according to the regulations of the University of South Florida Institutional Animal Care and Use Committee, protocol #2511. Rats were fed Tekland 22/5 rodent chow *ad libitum* and housed in a light-controlled reversed cycle room with 12 h of light followed by 12 h of darkness and had free access to water. Animals were made diabetic by injecting subcutaneously 65 mg/kg of streptozotocin (STZ) in 20 mM sodium citrate, pH 4.5/0.15 M NaCl. Diabetes was confirmed by the presence of glucose in the urine which was determined using Clinistix (Bayer, Elkhard, IN). Ten days after STZ injection, rats were euthanatized at the fourth hour of the dark period by an isoflurane overdose. Liver portions were quickly removed for preparation of RNA and protein samples. Serum samples were collected from all rats. Serum cholesterol levels were determined using the Cholesterol Reagent Set from Pointe Scientific, Inc (Canton, MI). Serum glucose levels were measured by the glucose oxidase method using a kit from Sigma–Aldrich Co. (St. Louis, MO). Serum insulin levels were determined using the Ultra Sensitive Rat Insulin ELISA Kit from Crystal Chem Inc. (Downers Grove, IL). Serum glucagon levels were measured with the Rat Glucagon ELISA Kit from Wako Chemicals USA, Inc. (Richmond, VA).

Materials

Primers were synthesized by Integrated DNA Technologies (Coralville, IA). TRI Reagent was obtained from Molecular Research Center (Cincinnati, OH). The Turbo DNase Kit was purchased from Ambion (Austin, TX). The Reverse Transcriptase System was from Promega Corp (Madison, WI). Pre-stained protein molecular weight markers and SYBR Green Real-time PCR supermix were obtained from Bio-Rad Labs (Hercules, CA). The BCA protein assay kit, pre-cast 4–20% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE), and the SuperSignal West Pico Chemiluminescent Substrate were purchased from Pierce (Rockford, IL). Nitrocellulose membrane and BioMax-MR films were purchased from Fisher Scientific (Norcross, GA). Rat LDL receptor specific antibody was kindly provided by Dr. Gene C. Ness (Department of Molecular Medicine, University of South Florida, Tampa, FL). The PCSK9 specific antibody was generously provided by Dr. Kara N. Maxwell (Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, NY). The horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Fisher Scientific or Sigma–Aldrich Co.

Quantitative real-time RT-PCR

Total RNA from rat liver was isolated using TRI Reagent. Two micrograms of DNA-free RNA was reverse transcribed using Promega's Reverse transcriptase system and random primers as per the manufacturer's instructions. Real-time PCR reactions were performed using 200 ng of ssDNA, the Bio-Rad SYBR Green real-time RT-PCR kit, and the iCycler real-time PCR system. The rat LDL receptor specific primers used were: 5′-GGGCTGGCGGTAGACTGGATC-3′ (sense) and 5′-CAATC TGTCCAGTACATGAAGC-3′ (antisense). The rat PCSK9 specific primers used were: 5′-GCACTGGAGAACCACACAGG-3′ (sense) and 5′-TGGCTGCATGACATTGCTTCTC-3′ (antisense). Primers specific for 18S (5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAAT CGGTAGTAGCG-3′) were used as the internal control for these studies. The sizes of the LDL receptor, PCSK9, and 18S fragments that were amplified using these primers were 200, 102, and 152 bp, respectively. The parameters for the PCR reactions were: denaturation at 95 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s. Quantitation of the results was performed using the Comparative CT method. LDL receptor and PCSK9 mRNA levels were expressed relative to 18S rRNA levels. Data from the individual parameters were compared by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test when applicable. A $p < 0.05$ was considered significant for all tests.

Western blotting analysis

Liver samples (0.2 g) were homogenized in 3 ml of lysing buffer (25 mM MES, pH 6.5, 0.15 M NaCl, 1% v/v Triton X-100, 60 mM octylglucoside, and 1× protease inhibitors) using a Dounce homogenizer. After incubating on ice for 20 min, homogenates were centrifuged at 12,000*g* for 15 min at 4 °C. Supernatants were collected and protein concentrations determined using the BCA protein assay. Protein samples (10–50 μg) were denatured at 100 °C in loading buffer and subjected to electrophoresis on pre-cast 4–20% SDS–PAGE gels. After electrophoresis, samples were electroblotted onto nitrocellulose membranes (0.2 μm pore) in buffer containing 25 mM Tris–hydrochloride (Tris–HCl), pH 8.3, 0.192 M glycine, and 20% methanol for 16 h at 4 °C. To verify equal protein loading, nitrocellulose membranes were stained with 0.1% Ponceau S (in 5% acetic acid) and destained in water. Western blot analysis for LDL receptor and PCSK9 protein was carried out with 1:2000 dilutions of rabbit polyclonal antisera to the LDL receptor and PCSK9, respectively, in 2% milk. Immunoreactive proteins were visualized using a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antisera in 2% non-fat dried milk and the SuperSignal West Pico Chemiluminescent Substrate method. The hepatic LDL receptor migrated as a 160 kDa band, while precursor and mature PCSK9 were detected at approximately at 74 and 60 kDa, respectively. Multiple exposures ranging 5 s to 20 min were made. Densitometric quantitations of the autoradiograms were performed using the UVP imaging system and the Labworks software (UVP Laboratory Products, Upland, CA).

Results

We wished to determine whether diabetes affects expression of the hepatic LDL receptor. To carry out these experiments, rats were made diabetic by injecting with streptozotocin (STZ) as described under Materials and methods. Serum samples from diabetic and control rats were examined for insulin, glucagon, glucose, and cholesterol levels. Serum insulin and glucagon levels for controls were 1.179 and 0.503 ng/ml, respectively. For diabetic animals, serum insulin and glucagon levels were 0.8 and 0.943 ng/ml, respectively. Insulin/glucagon ratio and serum glucose levels for these animals are shown in Table 1. Diabetic animals had serum glucose levels 4.5-fold higher than control animals ($p < 0.05$; Table 1). Serum cholesterol levels were also significantly ($p < 0.05$) increased from

Table 1
Measurement of insulin, glucagon and glucose levels in control and streptozotocin (STZ)-induced diabetic rats

Condition	Insulin/glucagon ratio	Glucose levels (mg/dL)
Control	2.29 ± 0.23	142.8 ± 0.82
Diabetic	0.85 ± 0.05[a]	646.9 ± 7.83[b]

Values are presented as means ± SEM for serum samples prepared from five rats per each condition.
[a] Differs from control at $p < 0.05$.
[b] Differs from control at $p < 0.01$.