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SREBP-1c regulates glucose-stimulated hepatic clusterin expression

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ABSTRACT

Clusterin is a stress-response protein that is involved in diverse biological processes, including cell proliferation, apoptosis, tissue differentiation, inflammation, and lipid transport. Its expression is upregulated in a broad spectrum of diverse pathological states. Clusterin was recently reported to be associated with diabetes, metabolic syndrome, and their sequelae. However, the regulation of clusterin expression by metabolic signals was not addressed. In this study we evaluated the effects of glucose on hepatic clusterin expression. Interestingly, high glucose concentrations significantly increased clusterin expression in primary hepatocytes and hepatoma cell lines, but the conventional promoter region of the clusterin gene did not respond to glucose stimulation. In contrast, the first intronic region was transcriptionally activated by high glucose concentrations. We then defined a glucose response element (GlRE) of the clusterin gene, showing that it consists of two E-box motifs separated by five nucleotides and resembles carbohydrate response element (ChoRE). Unexpectedly, however, these E-box motifs were not activated by ChoRE binding protein (ChREBP), but were activated by sterol regulatory element binding protein-1c (SREBP-1c). Furthermore, we found that glucose induced recruitment of SREBP-1c to the E-box of the clusterin gene intronic region. Taken together, these results suggest that clusterin expression is increased by glucose stimulation, and SREBP-1c plays a crucial role in the metabolic regulation of clusterin.

1. Introduction

Clusterin, also known as apolipoprotein J (Apo J), aging-associated protein 4 (AAG4), complement lysis inhibitor (CLI), testosterone-repressed prostate message 2 (TRPM-2), Ku70-binding protein 1 (KUB1), X-ray-induced protein 8 (XIP8), and sulfated glycoprotein-2 (SGP-2), was first identified as a glycoprotein that elicited clustering of suspended cells [1]. It is a 75–80 kDa disulfide-linked heterodimeric protein composed of α and β subunits that are generated by a post-translational cleavage of a single-chain precursor protein [2,3]. Clusterin has been proposed to be involved in a variety of important biological processes, including sperm maturation, tissue differentiation, tissue remodeling, membrane recycling, reverse lipid transport, cell–cell or cell–substrate interaction, promotion of erythrocyte aggregation, attenuation of complement activity, cell proliferation, cell survival, and apoptosis [4]. It is expressed ubiquitously in many tissues. Clusterin transcripts are present at relatively high levels in the brain, ovary, testis, stomach, and liver; are less abundant in heart, spleen, lung, kidney, and breast; and are absent in T-lymphocytes [5,6]. Interestingly, clusterin expression is differentially regulated in many pathological conditions, including cancer, atherosclerosis, diabetes, and renal and neurodegenerative diseases [7,8]. A number of studies have shown that clusterin expression levels are associated with prostate cancer [9], gastric cancer [10], and breast cancer [11], as well as colon [12], cervical [13], and ovarian cancers [14].

There is also considerable evidence suggesting a relationship between clusterin and the metabolic diseases such as diabetes and atherosclerosis [15–19]. Type 2 diabetic subjects have higher serum clusterin levels than healthy individuals [17], and these elevated levels of clusterin are positively correlated with blood glucose levels. In addition, an analysis of single nucleotide polymorphisms (SNPs) in Japanese subjects revealed a significant association of clusterin gene polymorphisms with diabetes, serum lipid levels, and the progression of carotid atherosclerosis [19,20]. Given these associations of clusterin with metabolic diseases, it is reasonable to speculate that clusterin expression could be regulated by metabolic signals, such as nutrients. However, to date, no studies

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have addressed the metabolic regulation of clusterin expression. In this study, we sought to investigate whether glucose regulates clusterin expression in hepatocytes and elucidate the underlying regulatory mechanism at the transcriptional level. Our results indicate that glucose stimulation increases clusterin expression, and further show that sterol regulatory element binding protein-1c (SREBP-1c) is involved in glucose-stimulated transcriptional activation of the clusterin gene through tandem intronic E-box motifs.

2. Materials and methods

2.1. Isolation of primary hepatocytes and cell culture

Primary hepatocytes were isolated from 10-week-old mice using the previously described collagenase perfusion method, with minor modifications [21]. Perfusion was performed with Mg^{2+}/Ca^{2+}-free Hanks' balanced salt solution containing 100 U/ml collagenase (Invitrogen, Carlsbad, CA) and 48 μg/ml trypsin inhibitor (Sigma, St. Louis, MO). Cells were plated onto collagen type I-coated culture dishes and maintained in Medium 199. Rat hepatoma FAO and human hepatoma HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator.

2.2. RNA preparation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from primary hepatocytes using the TRIzol reagent (Invitrogen). Purified total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI), according to the manufacturer's protocol. Quantitative gene expression analyses were performed on a Roche LightCycler 480 System (Roche, Basel, Switzerland) using SYBR Green PCR Master Mix. PCR primers were designed using Primer Express 3.0 software with the manufacturer's default settings, and were validated for identical efficiencies. 18S rRNA was used as the internal control. Ratios of target genes to 18S rRNA expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method [22]. The oligonucleotide primers used for qRT-PCR were as follows: mClusterin, 5′-ATA AGG AGA TTC AGA ACG CC-3′ and 5′-GCT CTG CGT TGG TTT TTT CTA TG-3′; mSREBP-1c, 5′-AGC CAT GGA TTG CAC ATT TGA-3′ and 5′-CAA ATA GGC CAG GGA AGT CA-3′; 18s rRNA, 5′-CCG CGG TTC TAT TTT GTT GGT-3′ and 5′-CTC TAG CGG CGC AAT ACG A-3′. The oligonucleotide primers used for conventional RT-PCR were as follows: mClusterin, 5′-AGG AGC TAA ACG ACT CGC T-3′ and 5′-CTT TTC CTG CGG TAT TCC T-3′; mSREBP-1c, 5′-GGC GCA TGG ATT GCA CAT TT-3′ and 5′-GCA GGC TGT AGG ATG GTG A-3′; 18s rRNA, 5′-CGT CCC CCA ACT TCT TAG AG-3′ and 5′-CAC CTA CGG AAA CCT TGT TAC-3′.

2.3. Preparation of cell lysates and Western blotting

Protein extracts of hepatocytes were prepared in lysis buffer containing 20 mM Tris–Cl (pH 7.5), 100 mM KCl, 5 mM EDTA (pH 8.0), 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_2VO_4, and 1% NP-40. Cells were incubated in lysis buffer for 1 h on ice. Cell debris was removed by centrifuging the lysates at 15,000*g* for 10 min. Protein concentrations were determined using the Bradford assay. For Western blot analysis, 50–100 μg of lysate protein was separated by electrophoresis on 7.5–10% polyacrylamide gels under denaturing conditions and then transferred to PVDF membranes (Millipore, Billerica, MA). Blots were incubated with antibodies to clusterin (1:1000 dilution; Santa Cruz), SREBP-1c (1:500 dilution; Santa Cruz), and β-actin (1:10,000 dilution; Sigma).

2.4. Construction of luciferase reporter genes and luciferase assay

The −2285/+430 and +2158/+4426 clusterin promoter regions used to construct promoter–luciferase reporter were amplified from human genomic DNA by PCR and were used as templates for the construction of other reporters. Deletion and site-specific mutations within the promoter were created using PCR cloning strategies. The −2285/+430 clusterin promoter and corresponding deletion constructs +230/+430 and +331/+430 were amplified using the unique forward primers 5′-CGG GGT ACC AAA CCC AGC TGT GTA AGT CCA TAA-3′ (-2285), 5′-CGG GGT ACC GGC ATT CTT TGG GCG TGA GTC-3′ (+230), and 5′-CGG GGT ACC CGC GGC GTC GCC AG-3′ (+331), and the common reverse primer 5′-CCG CTC GAG CAT CCG TCC TGG TGT GGC TCT-3′ (+430). The +2158/+4426 clusterin promoter was amplified using the primers 5′-CGG GGT ACC AAG TGG TTT AAG CCT TCT TAG G-3′ (forward) and 5′-CCC CCC GGG AGA GAA CAG GAG ACC AT-3′ (reverse). Two mutant promoters were prepared using the following primer pairs: M1, 5′-AGT GCT CAT CAG AGA CCC GTG AGA CCA CA-3′ (forward) and 5′-GGT CTC TGA TGA GCA CTG CCC ACT GAG C-3′ (reverse); and M2, 5′-GGT CAG AGA TGG TCA AGA CCA CAG CCT TC-3′ (forward) and 5′-CTT GAC CAT CTC TGA CCC CAG CTG CCC A-3′ (reverse). The authenticity of plasmid constructs was confirmed by DNA sequencing. For luciferase reporter assay, HepG2 cells were cultured in DMEM supplemented with 10% FBS. Transfections were performed in 24-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. pActin-βgal plasmid (50 ng) was included in each transfection experiment to control for the efficiency of transfection. Luciferase activity was measured using a luminometer (Centro LB 960, Berthold Technologies), and values were normalized to β-galactosidase activity.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously, with minor modifications [23]. Briefly, HepG2 cells were transfected with SREBP-1c expression plasmids and incubated overnight. After stimulation with 25 mM glucose for 6 h, cells were fixed with 1% formaldehyde for 15 min at room temperature. The cells were washed with cold phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 1% SDS, and 10 mM EDTA. Soluble chromatin was prepared by sonication and immunoprecipitated with antibodies against SREBP-1c (sc-13551; Santa Cruz Biotechnology) or preimmune IgG. The final DNA extractions were analyzed by qRT-PCR using two primer pairs: +3093/+3113 (5′-TTC TGG CTG GCT TTG TCT CTC T-3′) and +3149/+3166 (5′-TGC CCA CTG AGC CCT GAA-3′), encompassing the first intronic E-box-containing region; and +915/+936 (5′-TTC TGC CTC CTA ATG CAT CTG A-3′) and +956/+975 (5′-AGG CCT GGT GGA TCT TGT GT-3′), encompassing a control region containing no E-boxes.

2.6. Statistical analysis

Results are expressed as means ± standard errors (SEs). Statistical significance was assessed using unpaired Student's two-tailed *t*-tests. Differences with a *P*-value <0.05 were considered statistically significant.

3. Results

3.1. High glucose concentration increases clusterin mRNA and protein levels

The expressions of many metabolic enzymes and related factors are regulated by metabolic signals, such as nutrients. Clusterin is