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Human serum activates CIDEB-mediated lipid droplet enlargement in hepatoma cells

Ragunath Singaravelu [a,c], Rodney K. Lyn [b,c], Prashanth Srinivasan [c], Julie Delcorde [a,c], Rineke H. Steenbergen [d,e], D. Lorne Tyrrell [d,e], John P. Pezacki [b,c,*]

[a] Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada
[b] Department of Chemistry, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada
[c] National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada
[d] Department of Medical Microbiology and Immunology, University of Alberta, Canada
[e] Li Ka Shing Institute of Virology, Katz Centre for Pharmacy and Health Research, Edmonton, Alberta T6G 2S2, Canada

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ABSTRACT

Human hepatocytes constitutively express the lipid droplet (LD) associated protein cell death-inducing DFFA-like effector B (CIDEB). CIDEB mediates LD fusion, as well as very-low-density lipoprotein (VLDL) maturation. However, there are limited cell culture models readily available to study CIDEB's role in these biological processes, as hepatoma cell lines express negligible levels of CIDEB. Recent work has highlighted the ability of human serum to differentiate hepatoma cells. Herein, we demonstrate that culturing Huh7.5 cells in media supplemented with human serum activates CIDEB expression. This activation occurs through the induced expression of PGC-1α, a positive transcriptional regulator of CIDEB. Coherent anti-Stokes Raman scattering (CARS) microscopy revealed a correlation between CIDEB levels and LD size in human serum treated Huh7.5 cells. Human serum treatment also resulted in a rapid decrease in the levels of adipose differentiation-related protein (ADRP). Furthermore, individual overexpression of CIDEB was sufficient to down-regulate ADRP protein levels. siRNA knockdown of CIDEB revealed that the human serum mediated increase in LD size was CIDEB-dependent. Overall, our work highlights CIDEB's role in LD fusion, and presents a new model system to study the PGC-1α/CIDEB pathway's role in LD dynamics and the VLDL pathway.

1. Introduction

Cytosolic lipid droplets (LDs) were once thought to be static storage centres for excess neutral lipids. However, it is becoming quite apparent that cytosolic LDs are highly dynamic and play crucial roles in cellular processes, such as lipoprotein assembly and secretion in the liver [1]. Their relevance in liver pathologies is also becoming more evident. For example, the hepatitis C virus (HCV) RNA encodes a core protein that localizes to the LD surface [2–4]. This interaction plays a crucial role in proper viral particle assembly [5]. Increased understanding of the host factors which regulate the dynamics of LDs will prove instrumental in creating new therapeutic angles to combat metabolic disorders [6].

The LD surface serves as a platform for several LD binding proteins, including the well-studied PAT (perilipin, ADRP, TIP47, and related proteins) family and CIDE (cell death-inducing DFF45 like effector) family [7]. These host factors determine the diverse functionality of these organelles. While the PAT family represent well-characterized LD-associated proteins [8], the CIDE proteins have only recently emerged as important regulators of lipid homeostasis [9]. The CIDE family consists of CIDEA, CIDEB, and CIDEC (or FSP27). All members of the CIDE family have functional roles in LD clustering and fusion [9–13]. Interestingly, CIDEB is the only member of the family that is constitutively expressed in the liver and implicated in VLDL maturation [11,14].

Aside from primary cell culture and mice models, there are limited models to study CIDEB in the liver. The commonly used hepatoma cell models (e.g. HepG2 and Huh7 derivatives) expressed low to undetectable levels of CIDEB in comparison to primary hepatocytes [14]. To-date, previous reports have demonstrated that CIDEB mRNA expression is controlled by HNF-4α [15,16] and PGC-1α

Abbreviations: CIDE, cell death-inducing DFFA-like effector; ADRP, adipose differentiation-related protein; CARS, coherent anti-Stokes Raman scattering; PPAR, peroxisome proliferator activated receptor; PGC-1α, PPAR-γ co-activator 1α; HNF-4α, hepatocyte nuclear factor 4α; VLDL, very low density lipoprotein; LD, lipid droplet; TG, triglyceride.

* Corresponding author at: National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada. Fax: +1 (613) 941 8447.
E-mail addresses: John.Pezacki@nrc-cnrc.gc.ca, John.Pezacki@nrc.ca (J.P. Pezacki).

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[17]. HNF-4α is a transcription factor associated with the promotion of cellular differentiation [18]. Furthermore, CIDEB expression in the kidney was found to be down-regulated during renal carcinoma [15]. Overall, these studies suggest CIDEB expression may be down-regulated in hepatoma cells due to their de-differentiated state.

In a recent study, human serum (HS) treatment was shown to induce differentiation in Huh7.5 cells. While Huh7.5 cells maintained in the presence of traditional fetal bovine serum (FBS) are unable to secrete nascent VLDL particles, these HS-treated Huh7.5 cells were able to secrete TG-enriched lipoproteins [19]. Since CIDEB plays an important role in VLDL lipidation [11], we hypothesized that HS-induced differentiation of Huh7.5 cells may rescue VLDL secretion through activation of CIDEB expression. In this report, we demonstrate that HS-induced differentiation in Huh7.5 cells promotes CIDEB expression through up-regulation of PGC-1α. Furthermore, we show the increased expression of CIDEB correlates with increased LD size and a drastic drop in ADRP levels. Our work establishes a new model to study CIDEB's role in hepatic LD morphology and dynamics.

2. Materials and methods

2.1. Cell culture and reagents

Adherent cells were cultured as previously described [19]. Huh7 and Huh7.5 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON) supplemented with either HS (Invitrogen, 34005-100, pooled human AB serum, lot number 1215151) or 10% fetal bovine serum (FBS) and 100 nM non-essential amino acids (NEAA), 50 U/mL penicillin, and 50 mg/mL streptomycin. The plasmid expressing CFP was previously described [20]. The human CIDEB gene (GenBank Accession Number AAH35970.1) was cloned from cDNA obtained from the HepG2 hepatoma cell line using primers that are listed in Table S1 for cloning into pCMVβ and pIRES2-EGFP (bicistronic construct expressing EGFP).

2.2. Transfection

siRNA transfections were performed using Lipofectamine RNAiMax (Invitrogen), following manufacturer's protocols. CIDEB (SMARTpool, Thermo Fisher Scientific, Waltham, MA, USA) and negative control (Ambion, Austin, TX) siRNAs were transfected in HS cultured Huh7.5 cells at a concentration of 50 nM for 96 h, followed by a second transfection at 50 nM for 72 h. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen), following manufacturer's protocols.

2.3. Immunoblot analyses

After treatment with appropriate media, cells were washed twice with PBS and lysed with an SDS lysis buffer consisting of 50 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol. A protease inhibitor cocktail mix (Roche Diagnostics, Penzberg, Germany) was added to each extract. The protein concentration of each sample was quantified using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Prior to loading, 10% v/v of DTT and bromophenol blue (1:1) were added to each sample, and 30–60 μg/well was loaded onto a SDS–PAGE gel (10% resolving, 4% stacking gel). The resolved proteins were transferred to a Hybond-P (Amersham Biosciences, Piscataway, NJ) polyvinylidene difluoride membrane. The membrane was probed using a mouse anti-CIDEB (sc-101244; 1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-PGC-1α, (sc-13067; 1:200 dilution; Santa Cruz Biotech. Inc.), goat anti-HNF-4α (sc-6556; 1:1000 dilution; Santa Cruz Biotech. Inc.), mouse anti-ADRP (1:100; Progen Biotechnik, Heidelberg, Germany) or mouse anti-PTP1D (1:10,000 dilution; Sigma, Saint Louis, MO) primary antibodies followed by a secondary (HRP)-conjugated goat anti-mouse, donkey anti-goat, or donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA). Protein bands were visualized by Western Lightning Western Blot Chemiluminescence reagents (GE Healthcare, Baie d'Urfé, QC) according to the manufacturer's protocol.

2.4. RNA isolation and qRT-PCR

RNA isolation from hepatocytes was performed using TRIzol (Invitrogen) as per the manufacturer's protocol. RNA integrity was confirmed by electrophoresis on a 0.8% agarose gel in 1× TBE (Ambion, Austin, TX). For mRNA levels, 500 ng of total RNA was used for cDNA synthesis using the Superscript II kit (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Quantitative PCR (qPCR) was subsequently performed on an iCycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad), as per manufacturer's protocol. Primer sequences are listed in Table S1. A 20 μL reaction was assembled according to the manufacturer's protocol. For data analysis, the $2^{-\Delta\Delta Ct}$ method was used, and mean fold changes in expression are shown relative to cells maintained in FBS supplemented media [20].

2.5. CARS microscopy/two-photon fluorescence (TPF) and image analysis

Imaged cells were washed twice with PBS, followed by a 15 min incubation at room temperature with fixing solution (4% formaldehyde, 4% sucrose, 1 mL). The fixed cells were washed twice with PBS for 3 min and then stored at 4 °C in PBS prior to imaging. The CARS microscopy system was setup as previously described [20]. The imaging of TG content was performed as previously described [20]. Lipid droplet counting and sizing was performed using the ImageJ plugin for Particle Counting and Analysis (National Institutes of Health).

2.6. Statistics

Student's *t*-test was used to analyze the data, and *p*-values less than 0.05 were deemed significant.

3. Results

3.1. Human serum activates the expression of CIDEB and PGC-1α

Previous work demonstrated that human serum (HS) treatment over a period of 30 days gradually rescued ApoB lipidation and VLDL secretion in hepatoma cells [19]. We sought to determine how HS treatment and hepatoma cell differentiation affects CIDEB expression. We treated Huh7.5 cells with HS supplemented media during a similar time course and isolated protein or RNA samples. qRT-PCR analysis revealed that, after 14 and 21 days of HS treatment, mRNA levels of CIDEB increased over fivefold compared to FBS treated Huh7.5 cells. This increase was less prominent, but still significantly higher than FBS treated cells, at later time points (after 30 days). Western blot analysis revealed a maximal induction of CIDEB expression after 21 days of HS treatment.

We hypothesized that HNF-4α, a transcriptional regulator of CIDEB, may be responsible for its up-regulation as the transcription factor is a known promoter of hepatic differentiation [18]. Surprisingly, there were only modest changes in HNF-4α levels (Fig. 1A,