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Characterization of lipid droplets in steroidogenic MLTC-1 Leydig cells: Protein profiles and the morphological change induced by hormone stimulation

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ABSTRACT

Lipid droplets (LDs) are functional subcellular organelles involved in multiple intracellular processes. LDs are found in nearly all types of eukaryotic cells, but their properties are highly variable in different types of tissues. Steroidogenic cells synthesize steroid hormones *de novo* from the cholesterol deposited in cytosolic LDs. However, the roles of LD proteins in steroidogenesis under pituitary hormone stimulation have not been well elucidated. The protein profile of isolated LDs from the mouse Leydig tumor cell line MLTC-1 was distinct from that of hepatic cells or macrophages. By proteomic analysis of the components using mass spectrometry, two enzymes for steroidogenesis, 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) and 17 β-hydroxysteroid dehydrogenase type 11 (17βHSD11), were identified in two strong bands in the LD fractions. The LD fraction of MLTC-1 cells also included CYP11A1 and CYP17, suggesting that the LDs contain all the enzymes needed for testosterone synthesis. The steroidogenesis in Leydig cells is activated by luteinizing hormone through a PKA-dependent pathway. Stimulation of MLTC-1 cells with luteinizing hormone or 8-bromo-cAMP caused drastic changes in the morphology of the LDs in the MLTC-1 cells. Upon stimulation, large perinuclear LDs are turned into much smaller LDs and dispersed throughout the cytosol. These results raise the possibility that LDs are involved in a regulatory pathway of steroidogenesis, not just by serving as a storage depot for cholesterol esters, but also by providing enzymes and generating sites for enzymatic activity.

1. Introduction

Lipid droplets (LDs), neutral lipid storage sites surrounded by a phospholipid monolayer, are now recognized to be a functional subcellular organelle rather than metabolically inactive lipid depots [1,2]. They are involved in multiple intracellular processes, such as membrane trafficking, lipid metabolism and cell signaling [1]. LDs are found in nearly all types of eukaryotic cells, but their properties are highly variable in different tissues. Mature adipocytes contain massive LDs that occupy the entire cell volume and function as a long-term energy storage depot [3]. The LDs in other cell types are usually much smaller than those in adipocytes and they are thought to be involved in the temporal storage and effective utilization of lipids.

It is believed that the protein components on LDs contribute to LD function. A number of proteomic studies have made it clear that there are many different proteins in the LD fractions, and the protein profiles of LDs are different among different cell types [4–9]. In mammals, the major LD-associated proteins belong to the perilipin (PLIN) family which consists of five members. PLIN family proteins are characterized by a PAT domain and 11mer-repeats, but each has a unique tissue distribution, subcellular localization and characteristic features [5]. All of the mammalian LDs have one or more PLIN proteins as the major LD component, reported to date. PLIN1 (the classical perilipin) is the most abundant phosphoprotein in adipocytes and has major roles in regulating both LD size and access of lipases to LDs [2]. PLIN2 (ADRP) is expressed ubiquitously and has an important role in the recruitment, interaction and *de novo* formation of LDs from the ER [10]. PLIN3 (TIP47), another ubiquitously-expressed LD protein, was shown to be a major LD protein in HL60-derived neutrophils and involved in PGE_2 production [11]. PLIN4 is mainly expressed in adipocytes and is supposed to be a regulator of LD formation [12]. PLIN5 forms very small LDs in heart and skeletal muscle [13], while in a study using PLIN5-knockout mice, PLIN5 was revealed to have a crucial role in quickly supplying lipid fuel to mitochondria [14].

The morphology of LDs dynamically converts when the energy demand in cells or the surrounding environment changes. Upon activation of lipolysis by adrenergic agents in adipocytes, protein kinase A (PKA)

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phosphorylates a number of downstream targets, notably PLIN1 and hormone sensitive lipase (HSL). During this activation, large unilocular LDs in mature adipocytes change into numerous micro-sized LDs that appear all over the cytoplasm [15,16]. Some of the LD-associated proteins are actively involved in this remodeling system. Upon stimulation, CGI-58 dissociates from phosphorylated PLIN1 and forms a complex with adipose triglyceride lipase (ATGL) on the micro-sized LDs, playing an important role in its robust lipolytic activity [17]. The mechanism of micro-sized LDs formation in adipocytes has yet to be clarified, but fatty acid availability and intracellular TG synthesis could be involved [18].

Steroid hormones are synthesized *de novo* from cholesterol in mitochondria and the ER, and are also secreted from specialized endocrine cells in the adrenal cortex, testes and ovaries. LDs are also prominent constituents of steroidogenic cells, while cholesterol ester (CE) is deposited in numerous small cytosolic LDs that are 0.5–1.5 μm diameter [3]. Although triacylglycerol (TAG) and CE are accumulated in LDs, they form distinct LD particles even in a single cell. The PLIN family consists of five distinct genes, with PLIN1 having four different splice variants. Steroidogenic cells are able to synthesize all of the *PLIN1* variants as well as the other *PLIN* genes [19,20]. It has been shown that distinct PLINs differentially locate to either TAG- or CE-LDs, namely PLIN1a, PLIN1c and PLIN5 associate with TAG-containing LDs while PLIN1d and PLIN4 preferentially bind to CE-LDs in several different types of cells, including Y1 adrenocortical cells [21]. Transfection of these PLIN proteins altered the relative intracellular TAG/CE ratio toward the preferentially targeted lipid. Therefore, CE-LDs may be regulated differently from TAG-LDs. However, the profiles of the associated proteins and the physiological significance of the CE-LDs, especially in steroidogenic cells, remain unclear.

In the current study, we isolated LDs from the mouse Leydig tumor cell line MLTC-1, a model cell line of studying steroidogenesis, and conducted proteomic analysis of the components by mass spectrometry. We found that the LD fraction from MLTC-1 has a unique protein profile in which the enzymes for steroidogenesis, especially 3β-hydroxysteroid dehydrogenase (HSD) type 1 (3βHSD1) and 17β-HSD type 11 (17βHSD11). It was also shown that drastic morphological changes of the LDs in MLTC-1 are induced upon the activation of steroidogenesis by luteinizing hormone (LH).

2. Experimental procedures

2.1. Cell culture

The mouse Leydig tumor cell line MLTC-1 and mouse macrophage J774 cells were cultured in RPMI-1640 medium, and the mouse hepatoma cell line Hepa1 was maintained in DMEM. Both media were supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 atmosphere. J774 cells were activated by 10 μg/mL lipopolysaccharide for 24 h and then cultured in the medium supplemented with 37.5 μM cholesterol-containing liposomes (dioleoylphosphatidylcholine:dioleoylphosphatidylserine:cholesterol= 2:2:3) for 24 h before harvesting the cells.

2.2. Antibodies and reagents

Guinea pig polyclonal antibodies (pAb) against PLIN1 (GP29) and PLIN2 (GP40 mN1) were purchased from Progen Biotechnik (Heidelberg, Germany). An anti-PLIN3 rabbit pAb (3883) was from ProSci Inc. (Poway, CA, USA). Rabbit pAb against 17βHSD11 (ab55804) was from Abcam (Cambridge, UK). Mouse monoclonal antibody (mAb) against GM130 was from Gibco Transduction Laboratories (Carlsbad, CA, USA). Goat anti-3βHSD pAb (sc-30820), rabbit anti-oxidoreductase protein disulfide isomerase (PDI) pAb, mouse anti-β-actin mAb and mouse anti-HSL mAb were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-calnexin pAb was from Sigma (St. Louis, MO, USA). FITC donkey anti-guinea pig IgG was from Chemicon International (Billerica, MA, USA). Alexa Fluor 594 donkey anti-guinea pig IgG, Alexa Fluor 488 donkey anti-rabbit IgG were from Invitrogen (San Diego, CA, USA). HCS LipidTOX™ Red neutral lipid stain was from Life Technologies (Carlsbad, CA, USA). The amount of progesterone in the culture medium was measured with a progesterone EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA). Silver staining of the SDS-PAGE gel was performed using a Silver staining kit (Wako Pure Chemical, Tokyo, Japan). The amounts of total cholesterol and TAG were measured using enzymatic assay kits (Wako Pure Chemical, Tokyo, Japan).

2.3. Isolation of LDs and proteomic analysis of LD proteins

Preparation of LDs was basically carried out according to a previous report [22]. Cells were grown in ten 100 mm dishes, washed with PBS, collected by centrifugation, resuspended in a hypotonic medium (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, protease inhibitor cocktail) and incubated for 10 min on ice. Cells were homogenized by nitrogen cavitation. The homogenate was centrifuged at 1000 ×*g* for 5 min at 4 °C to obtain post-nuclear supernatant (PNS). For gradient centrifugation, PNS was adjusted to 20% sucrose and layered on top were hypotonic media with 5% and 0% sucrose. Centrifugation was carried out at 28,000 ×*g* for 1 h at 4 °C using an RPS40T rotor (Hitachi, Tokyo, Japan). LD fractions were collected from the top and delipidated with cold acetone overnight at −20 °C. Proteins were solubilized with Laemmli sample buffer, analyzed by 10% SDS-PAGE, and visualized by Coomassie Brilliant Blue (CBB) or silver staining. Each lane was sliced into 19 bands that were separately digested with trypsin, followed by LC–MS/MS analysis using a Q-TOF micro mass spectrometer (Waters), as described previously [23]. The MS data were analyzed using Mascot software to identify proteins. The cut-off value of 100 was used for protein identification.

2.4. Subcellular fractionation by sucrose density gradient

For the detection of endogenous HSDs, the PNS fraction, prepared as above, was adjusted to 1.1 M sucrose. Four mL of the post-nuclear supernatants were placed into the bottom of a 13-mL centrifuge tube and overlaid with ice-cold disruption buffer containing 0.25 M sucrose. The two-step gradient was centrifuged at 110,000 ×*g* at 4 °C for 2 h. Fractions were taken from the top as follows: the top 2 mL, the LD fraction; the next 3.5 mL, the intermediate fraction; the next 4 mL including the phase boundary between 0.25 and 1.1 M sucrose, the floating membranes; and the last 3.5 mL, the bottom fraction [24].

For the study of intracellular distribution, the sucrose concentration of the PNS fraction was adjusted to 35% and on top of this a 2–35% stepwise sucrose gradient was formed. The ultracentrifugation was carried out at 110,000 ×*g* for 2 h at 4 °C. Following centrifugation, the samples were collected from the top (1.0 mL each) and 12 fractions were recovered [25].

2.5. Plasmids and DNA transfection

Clones containing full-length 3βHSD1 and 17βHSD11 cDNA were obtained from Image clone (Open Biosystems). The entire open reading frames were amplified by PCR, and subcloned into a GFP expression vector, phGFP [26], using XhoI-BamHI sites for 3βHSD1 and EcoRI-BglII sites for 17βHSD11. MLTC-1 cells grown on coverslips were transfected with expression vectors encoding GFP–3βHSD1 and GFP–17βHSD11. Transfection was carried out using Lipofectamine Plus (Invitrogen) according to the manufacturer's directions.

2.6. Fluorescent microscopy

MLTC-1 cells were fixed with PBS containing 4% paraformaldehyde, permeabilized in 0.1% digitonin in PBS and blocked with 2% BSA in