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Cholesterol Ester Droplets and Steroidogenesis

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Abstract

Intracellular lipid droplets (LDs) are dynamic organelles that contain a number of associated proteins including perilipin (Plin) and vimentin. Cholesteryl ester (CE)-rich LDs normally accumulate in steroidogenic cells and their mobilization is the preferred initial source of cholesterol for steroidogenesis. Plin1a, 1b and 5 were found to preferentially associate with triacylglycerol-rich LDs and Plin1c and 4 to associate with CE-rich LDs, but the biological significance of this remains unanswered. Vimentin null mice were found to have decreased ACTH-stimulated corticosterone levels, and decreased progesterone levels in females, but normal hCG-stimulated testosterone levels in males. Smaller LDs were seen in null cells. Lipoprotein cholesterol delivery to adrenals and ovary was normal, as was the expression of steroidogenic genes; however, the movement of cholesterol to mitochondria was reduced in vimentin null mice. These results suggest that vimentin is important in the maintenance of CE-rich LDs and in the movement of cholesterol for steroidogenesis.

Keywords

lipid droplet; cholesterol; steroidogenesis; perilipin; mice; vimentin

1. Introduction

When increased amounts of cholesterol accumulate within the ER, the integral ER membrane protein fatty acyl coA:cholesterol acyltransferase converts unesterified cholesterol to cholesteryl ester (CE)¹ for storage as lipid droplets (LDs). The prevailing view of LD formation has been based primarily on analogy with triacylglycerol (TAG) LDs and can be interpreted to state that CE are synthesized within the ER and, as CEs accumulate and coalesce within the ER membrane bilayer, the LD buds off from the ER forming a nascent LD possessing a phospholipid monolayer on its surface within the cytosol (Walther and Farese, 2009), though continued physical communication between the ER and the LD is possible. During this process of nascent LD formation, several proteins, primarily of the PERILIPIN family (Kimmel, Brasaemle, McAndrews-Hill et al., 2010), associate with the LD either during budding from the ER or derived from a soluble pool within the cytosol.

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¹Abbreviations: CE, cholesteryl ester; HSL, hormone sensitive lipase; LD, lipid droplet; NSF, N-ethylmaleimide-sensitive factor; PKA, cyclic AMP-dependent protein kinase; Plin, perilipin; SNARE, soluble NSF attachment protein receptor; SR-BI, scavenger receptor type B-I; StAR, steroidogenic acute regulatory protein; TAG, triacylglycerol.

Evidence suggests that some PERILIPIN family members such as Plin3 and Plin4 associate with and coat very small, nascent LDs; subsequently, they are replaced by Plin2 as LDs enlarge and finally by perilipin (Plin1) as LD size enlarges further (Wolins, Quaynor, Skinner et al., 2005). Adrenal LDs containing CE are similarly known to be coated by Plin1 (Servetnick, Brasaemle, Gruia-Gray et al., 1995) and Plin2 (Fong, Yang, Greenberg et al., 2002). In addition to these dynamic changes in surface proteins associated with LDs, there is temporal and spatial movement of TAG-rich LDs within the cell as they enlarge both by accumulation of newly synthesized TAG and via fusion of cytosolic LDs (Nagayama, Uchida and Gohara, 2007) independent of TAG biosynthesis; this fusion appears to require microtubules and the motor protein dynein (Boström, Rutberg, Ericsson et al., 2005), as well as the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complex (Boström, Andersson, Rutberg et al., 2007) and other related SNARE proteins. Whereas TAG-rich LDs tend to coalesce into large LDs, particularly in adipose cells where large LD formation is dependent on fat specific protein of 27 kDa (FSP27) (Nishino, Tamori, Tateya et al., 2008, Puri, Konda, Ranjit et al., 2007), CEs have a propensity to form multiple small LDs in adrenals and gonads (Reaven, Tsai and Azhar, 1995), although LD enlargement also occurs. Proteomic analyses of LDs from yeast, mouse mammary gland, Chinese hamster ovary cells, human hepatoma cells, human squamous epithelial carcinoma cells, mouse 3T3-L1 adipocytes, fly cells and leukocytes have revealed the existence of many LD-associated proteins including specific marker proteins, structural proteins, enzymes involved in various aspects of cholesterol and fatty acid metabolism, and proteins that function as regulators of membrane traffic (Athenstaedt, Zweytick, Jandrositz et al., 1999, Wu, Howell, Neville et al., 2000, Liu, Ying, Zhao et al., 2004, Fujimoto, Itabe, Sakai et al., 2004, Umlauf, Csaszar, Moertelmaier et al., 2004, Brasaemle, Dolios, Shapiro et al., 2004, Miura, Gan, Brzostowski et al., 2002, Paciga, McCudden, Londos et al., 2003, Wolins, Skinner, Schoenfish et al., 2003, Bartz, Zehmer, Zhu et al., 2007, Beller, Riedel, Jansch et al., 2006, Cermelli, Guo, Gross et al., 2006, Sato, Fukasawa, Yamakawa et al., 2006, Wan, Melo, Jin et al., 2007). In recent studies, functional genome-wide screens of *Drosophila* cells using RNA interference revealed that approximately 1.5% of the fly genome is involved in TAG LD formation and regulation (Guo, Walther, Rao et al., 2008, Beller, Sztalryd, Southall et al., 2008). The genes identified as involved in LD homeostasis represent a wide variety of different and diverse cellular processes, including genes involved with TAG and phospholipid synthesis, the proteasome and spliceosome, vesicular transport machinery translational machinery, and several cytoskeletal genes and motor proteins. While it seems reasonable to assume that there are substantial similarities, and perhaps even identity between many of the processes involved in the formation and behavior of predominantly TAG-containing and CE-containing LDs, the fact remains that there is very limited direct evidence proving this. Indeed, there are clearly differences between the synthesis of CE and TAG, as well as differences in the pathways in which TAG and CE enter cells and the cell types where TAG-rich and CE-rich LDs form under normal physiological conditions, with CE-rich LDs forming predominantly in the adrenal and ovary where the stored cholesterol is utilized as a primary source for steroidogenesis (Kraemer, 2007).

2. Differential Protein Expression of LDs

Investigators have only very recently begun to examine whether the proteomes of TAG-rich and CE-rich LDs differ. The first published study to address this issue specifically examined whether members of the PERILIPIN family were differentially expressed in TAG-rich and CE-rich LDs (Hsieh, Lee, Londos et al., 2012). The Plin family consists of 5 distinct genes (designated 1-5), with Plin1 having 4 different splice variants (designated a-d) that are essentially C-terminal truncations of the full length Plin1a (Brasaemle, 2007).

2.1 Plin Regulation by TAG or CE

To address this issue, the investigators (Hsieh et al., 2012) incubated Y1 adrenocortical cells with either fatty acids to enhance TAG-rich LDs or with cholesterol to enhance CE-rich LDs. An increased expression of Plin1a and Plin5 was observed with accumulation of TAG-rich LDs, and increased expression of Plin1c and Plin4 was observed with accumulation of CE-rich LDs. Expression of Plin2 and Plin3 did not differ whether cells accumulated TAG-rich or CE-rich LDs, and Plin1b and Plin1d were only weakly detected. This differential expression of Plin family members was observed with lipid loading of other cell types in culture and also when the Plin members were over-expressed in cells using a constitutive promoter.

2.2 Distinct LDs

Using specific fatty acid and cholesterol fluorescent probes to label LDs, the investigators observed relatively limited mixing of lipid species in LDs when loaded simultaneously with fatty acids and cholesterol and observed a distinctive separation of TAG-rich and CE-rich LDs in discrete LDs (Hsieh et al., 2012). Though in most cells studied the different LDs were intermingled, this separation was most prominent in cultured McArdle liver cells where TAG-rich and CE-rich LDs formed in completely separate subcellular locations within the cells. Fluorescent-activated cell sorting was used to separate the differentially fluorescently labeled LDs, and the expression of the different Plin species was examined. Plin1a, Plin1b and Plin5 were preferentially expressed on isolated TAG-rich LDs, whereas Plin1c and Plin4 were preferentially expressed on isolated CE-rich LDs. Plin1b, Plin2 and Plin3 did not display any distinct lipid preference. Ectopic expression of Plin members was found to alter TAG/CE cellular distribution; however, it is unclear whether inhibiting expression of individual Plin members would alter lipid composition in LDs since previous reports have shown that deletion of one Plin member results in the compensatory increase in other Plin members. Nonetheless, these studies document that TAG-rich and CE-rich LDs contain different Plin members and further suggest that each of the Plin members may have unique functions that reflect their differential LD expression; however, the biological significance as it relates to steroidogenesis remains as yet unexplored.

3. Vimentin

As opposed to Plin, vimentin, a protein identified to be associated with LDs, has been proposed for a number of years to play a role in steroidogenesis (Almahbobi and Hall, 1990). Vimentin is an intermediate filament that constitutes part of the network of the cytoskeleton (Fuchs and Weber, 1994). It is expressed in mesenchymal cells, including adrenal cells where it is attached to and forms a capsule around LDs (Almahbobi and Hall, 1990, Almahbobi, Williams and Hall, 1992, Hall, 1997), as opposed to the cage or scaffold it has been described to form around LDs in adipose cells (Franke, Hergt and Grund, 1987), although it does not appear to surround all LDs. Vimentin has been reported to interact with a number of different proteins, including some with motor-like properties (Chou, Flitney, Chang et al., 2007) and sterol binding properties (Wang, JeBailey and Ridgway, 2002, Wyles, Perry and Ridgway, 2007), as well as with agonist-stimulated β_3 -adrenergic receptors, where this interaction appears to be important for activation of ERK and stimulation of lipolysis (Kumar, Robidoux, Daniel et al., 2007). Moreover, we recently reported that hormone sensitive lipase (HSL) can interact with vimentin and that the interaction affects lipolysis, as well as the translocation of HSL to the LD (Shen, Patel, Eriksson et al., 2010). Thus, recent studies examined the importance of vimentin in CE-rich LDs and the utilization of CE for steroidogenesis (Shen, Zaidi, Patel et al., 2012).

3.1 Steroid Production *in vivo*

These studies were carried out in vimentin null mice, which are known to develop and reproduce normally (Colucci-Guyon, Portier, Dunia et al., 1994). Using both male and female wild type and vimentin null animals, steroid hormone levels, as well as steroid responses to trophic hormonal stimulation, were surveyed. Due to issues related to stress, corticosterone levels were not measured at baseline, but only 1h after cosyntropin injection. Compared with wild-type mice, serum corticosterone concentrations were reduced 35% in male (17 ± 3 vs 11 ± 3 $\mu\text{g/dl}$, $p < 0.05$, mean $\pm$ SD) and $>50\%$ in female (27 ± 7 vs 13 ± 6 $\mu\text{g/dl}$, $p < 0.01$) vimentin null mice, suggesting a defect in adrenal steroidogenesis. Next progesterone concentrations during the course of the estrus cycle were examined. While the absence of vimentin did not alter the timing of the estrus cycle, peak concentrations of progesterone were significantly lower in vimentin null mice than in wild-type control (4.4 ± 1.5 vs 2.9 ± 0.1 ng/ml, $p < 0.05$, mean $\pm$ SD), suggesting a defect in ovarian steroidogenesis. In order to evaluate ovarian steroidogenesis further, serum progesterone concentrations were measured 4h following the administration of chorionic gonadotropin in animals that had been primed with pregnant mares serum gonadotropins 56h previously. Stimulated serum progesterone concentrations were reduced 60% in vimentin null mice (0.13 ± 0.05 vs 0.05 ± 0.01 nM, $p < 0.001$, mean $\pm$ SD), again suggesting a defect in ovarian steroidogenesis. In contrast to the adrenal and ovary, serum testosterone concentrations measured 4h following chorionic gonadotropin were similar in vimentin null and wild type mice, revealing no defect in testicular steroidogenesis.

3.2 Steroid Production *in vitro*

In order to explore the apparent abnormalities in steroid production further, primary adrenocortical, granulosa and Leydig cells were isolated from both wild type and vimentin null mice and basal and cyclic AMP-stimulated steroid production examined *in vitro*. In general, steroid production is very low in primary cells in the absence of cyclic AMP stimulation, but basal corticosterone production was 70% lower in adrenocortical cells isolated from vimentin null mice compared with wild type. In the presence of dibutyryl cyclic AMP, corticosterone production was 80% lower in adrenocortical cells isolated from vimentin null mice compared with wild type (2.2 ± 0.5 vs 0.3 ± 0.1 $\mu\text{g/ml}$, $p < 0.01$), consistent with the reduction seen *in vivo*. Likewise, consistent with the reduction observed *in vivo*, progesterone production was 45% lower in luteinized granulosa cells isolated from vimentin null mice compared with wild type (40.5 ± 1.8 vs 22.1 ± 5.5 ng/ml, $p < 0.001$) when treated with dibutyryl cyclic AMP and HDL as an exogenous source of cholesterol, whereas basal progesterone production was very low and similar between luteinized granulosa cells isolated from vimentin null and wild type mice. Not surprisingly, testosterone production was similar in Leydig cells isolated from vimentin null and wild type mice under basal and stimulated conditions. Thus, the absence of vimentin appears to result in defective steroidogenesis in the adrenal and ovary, but not the testis. This tissue specific difference may be due to the preferential utilization of endogenously synthesized cholesterol for testosterone production in the testis as opposed to the preferred utilization of lipoprotein-derived cholesterol in the adrenal and ovary.

3.3 Enzyme Expression and Lipoprotein Uptake

To ensure that vimentin deficiency did not alter the expression of genes important in steroidogenesis, RT-PCR was used to document that there were no alterations in the expression of CYP11A1, CYP11B1, CYP21A2, CYP17A1, 3 β -HSD1, the LDL receptor, the scavenger receptor type B-I (SR-BI) or LRP-1 in adrenals or ovaries of vimentin null mice. In addition, normal protein expression of HSL, Plin1 and SR-BI was documented in adrenals of vimentin null mice. Although neither trophic hormone receptors nor cyclic AMP-dependent protein kinase (PKA) expression or activity were measured in these studies,

it is unlikely that any observed changes in steroidogenesis in the setting of vimentin deficiency were due to alterations in the cAMP pathway since ovarian CYP11A1 mRNA levels, which are transcriptionally regulated by cyclic AMP (LaVoie and King, 2009), were similar in vimentin null and wild type mice 24h after gonadotropin treatment. Moreover, using double-labeled lipoprotein particles, it was documented that there were no changes in either the endocytic or selective uptake of lipoprotein cholesterol into adrenals or ovaries of vimentin null mice, further confirming that cellular cholesterol delivery and steroidogenic pathways were not disrupted by vimentin deficiency.

3.4 Mitochondrial Cholesterol

In view of the normal delivery of lipoprotein cholesterol to adrenal and ovaries, it was not surprising that total cholesterol content in the adrenals was not altered in vimentin null mice. However, the size of adrenal LDs was significantly reduced ($p < 0.001$) in vimentin null mice, with a shift in the entire population towards smaller adrenal LDs. Finally, to further explore the defect in steroidogenesis in vimentin null mice, animals were pretreated with aminoglutethimide (to inhibit CYP11A1) prior to stimulation with ACTH or saline and then isolated mitochondria from the adrenals and measured cholesterol content. Interestingly, adrenal mitochondrial cholesterol content was 65% lower in vimentin null compared to wild type mice, suggesting that there is a defect in the movement of cholesterol into mitochondria in the absence of vimentin. In combination with the observations that vimentin is important in supporting mitochondrial morphology (Tang, Lung, Wu et al., 2008) and modulating mitochondrial motility through its specific binding to mitochondria (Nekrasova, Mendez, Chernov Ivanenko et al., 2011), it appears that vimentin plays a functional role in cholesterol trafficking between LDs and mitochondria. However, it is possible that vimentin deficiency leads to reductions in steroidogenesis through additional mechanisms, such as increased production of reactive oxygen species (Tolstonog, Shoeman, Traub et al., 2001), with the ensuing excessive oxidative stress inhibiting steroidogenesis (Abidi, Zhang, Zaidi et al., 2008).

4. Conclusion

The cartoon in Figure 1 depicts adrenal steroidogenesis and a summary of these observations. ACTH induces steroidogenesis by binding to its cognate G protein-coupled receptor, leading to activation of adenylate cyclase, which generates cyclic AMP and activates cyclic AMP-dependent protein kinase (PKA). Stimulation of the cyclic AMP cascade exerts both acute and chronic effects on the regulation of steroid hormone production. The acute response is characterized by a rapid increase in the rate of steroid hormone biosynthesis as a result of the induction of steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996) and delivery of cholesterol to mitochondrial CYP11A1, the first enzymatic step in the conversion of cholesterol to steroid hormones that mediates the conversion of cholesterol to pregnenolone (Stocco, 2001). Additionally, there is an increase in the expression of enzymes of the steroidogenic pathway (Waterman and Keeney, 1996), along with an increase in the selective uptake of HDL-CE via scavenger receptor type B-I (SR-BI) (Kraemer, 2007). PKA also activates Plin1 and HSL, leading to the hydrolysis of CEs stored within LDs, releasing unesterified cholesterol that can be supplied to the mitochondria as precursor substrate for steroid production. LD movement and cholesterol delivery to mitochondria appear to rely on SNAREs and vimentin, but the exact mechanisms involved remain to be identified. Alternatively, the ER and mitochondria have been shown to interact through a tethering complex (Kornmann, Currie, Collins et al., 2009) that could possibly facilitate LD movement to mitochondria.

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Highlights

Cholesteryl ester-rich and triacylglycerol-rich lipid droplets have unique expression of perilipin family members

Vimentin is associated with lipid droplets

Vimentin deficiency results in defective ACTH-stimulated corticosterone production

Vimentin deficiency impairs cholesterol movement to mitochondria

Figure 1.
Cartoon of adrenal steroidogenesis and movement of cholesterol to mitochondria.