Adipophilin regulates maturation of cytoplasmic lipid droplets and alveolae in differentiating mammary glands

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Summary

Milk lipids originate by secretion of triglyceride-rich cytoplasmic lipid droplets (CLDs) from mammary epithelial cells. Adipophilin (ADPH)/Plin2, a member of the perilipin family of CLD binding proteins, is hypothesized to regulate CLD production in these cells during differentiation of the mammary gland into a secretory organ. We tested this hypothesis by comparing CLD accumulation in differentiating mammary glands of wild-type and ADPH-deficient mice. ADPH deficiency did not prevent CLD formation; however, it disrupted the increase in CLD size that normally occurs in differentiating mammary epithelial cells. Failure to form large CLDs in ADPH-deficient mice correlated with localization of adipose triglyceride lipase (ATGL) to the CLD surface, suggesting that ADPH promotes CLD growth by inhibiting lipolytic activity. Significantly, mammary alveoli also failed to mature in ADPH-deficient mice, and pups born to these mice failed to survive. The possibility that CLD accumulation and alveolar maturation defects in ADPH-deficient mice are functionally related was tested by in vivo rescue experiments. Transduction of mammary glands of pregnant ADPH-deficient mice with adenovirus encoding ADPH as an N-terminal GFP fusion protein prevented ATGL from localizing to CLDs and rescued CLD size and alveolar maturation defects. Collectively, these data provide direct in vivo evidence that ADPH inhibition of ATGL-dependent lipolysis is required for normal CLD accumulation and alveolar maturation during mammary gland differentiation. We speculate that impairing CLD accumulation interferes with alveolar maturation and lactation by disrupting triglyceride homeostasis in mammary epithelial cells.

Key words: Mammary gland, Adipophilin, Lipid droplet, Adipose triglyceride lipase, Differentiation, Adenovirus

Introduction

Milk lipids, principally triglycerides, provide a large percentage of calories, essential fatty acids and bioactive lipids required for neonatal growth and development (McManaman, 2009). These calorific and nutritional demands are met by an incredible ability of mammary glands of most species to synthesize and secrete large quantities of lipid during lactation (Allen et al., 1991; Rudolph et al., 2007; Schwertfeger et al., 2003). Formation and secretion of milk lipids are not constitutive functions of milk-secreting cells but develop as part of a complex, hormonally driven, program that is initiated during the second half of gestation as the mammary gland differentiates into a secretory organ (Anderson et al., 2007; Russell et al., 2007). Studies in mice have shown that this differentiation process, which we refer to as secretory differentiation, occurs in distinct phases characterized by progressive accumulation and enlargement of phospholipid- and protein-coated cytoplasmic lipid droplets (CLDs) which are the immediate precursors of milk lipids (Hollmann, 1974; Russell et al., 2007).

Perilipin family members have been proposed to be essential regulators of lipid metabolism and trafficking in mammalian cells (Brasaemle, 2007a; Ducharme and Bickel, 2008; Murphy, 2001). Physiological roles of perilipin/Plin1 and ADPH/Plin2 in CLD accumulation and triglyceride metabolism have been established in adipose and hepatic tissues, respectively (Chang et al., 2006; Imai et al., 2007; Martinez-Botas et al., 2000; Tansey et al., 2001). In addition, evidence has been presented indicating that the C-terminal region of ADPH is important for milk lipid secretion (Chong et al., 2011). Observations that *Adph* mRNA transcripts are highly enriched in mammary epithelial cells of mouse mammary glands relative to other cell types, and that their levels increase coordinately with CLD growth and accumulation as mammary epithelial cells undergo secretory differentiation, have led to the hypothesis that ADPH

might also function in the packaging of triglycerides for secretion as milk lipids (Russell et al., 2007).

Efforts to substantiate physiological roles of ADPH in milk lipid formation and secretion were complicated by the discovery that mammary glands of lactating *Adph*-null mice produce an N-terminally truncated form of ADPH that retains at least some biological activity (Russell et al., 2008). However, during gestation, total *Adph* transcript levels in mammary glands of *Adph*-null mice were shown to be less than 1% of wild-type (WT) levels. Furthermore, neither full-length nor truncated ADPH were detected in mammary gland extracts of *Adph*-null mice by immunoblot throughout most of pregnancy, and ADPH immunostaining of CLDs was not detected in mammary epithelial cells of pregnant *Adph*-null mice (Russell et al., 2008). Consequently all forms of ADPH appear to be dramatically reduced, or not present, in mammary glands of *Adph*-null mice during gestation. However, because these mice are not truly *Adph*-null, they will be referred to here as ADPH-deficient. In this study, we take advantage of this gestational ADPH deficiency to investigate the importance of ADPH in regulating CLD accumulation in the differentiating mammary gland. Our data demonstrate that during their first pregnancy ADPH-deficient mice are unable to support their litters and exhibit impaired alveolar maturation and CLD growth during secretory differentiation. In addition we show that ADPH deficiency is associated with relocalization of adipose triglyceride lipase (ATGL) to the CLD surface. Using a novel adenoviral transduction procedure we show that exogenous expression of a green fluorescence protein (GFP)–ADPH fusion protein in differentiating mammary epithelial cells of ADPH-deficient mice prevents ATGL from localizing to CLDs, and rescues defects in CLD accumulation and alveolar maturation. Collectively, these data link ADPH inhibition of ATGL-dependent lipolytic activity to the regulation of both CLD accumulation and alveolar maturation in differentiating mammary epithelial cells.

Results

ADPH deficiency impairs alveolar maturation

Although multiparous ADPH-deficient dams lactate and produce sufficient milk to support normal litter growth (Russell et al., 2008), we found that all ADPH-deficient dams tested to date (more than 50 animals) failed to support their first litters beyond day 2 postpartum. Histological analysis of mammary glands of pregnant animals indicated that alveolar maturation in mammary glands of ADPH-deficient dams is impaired relative to that of WT dams. Alveolar morphology of ADPH-deficient mice at day 12 of pregnancy (P12) was indistinguishable from that of WT mice (Fig. 1A). However, their morphology appeared to change over subsequent periods of development. By P14, alveoli in mammary glands of WT mice already exhibited evidence of luminal expansion (Fig. 1A, P14–P18) and milk-filled lumens at lactation day 2 (Fig. 1A, L2). By contrast, even after parturition, many alveoli in mammary glands of ADPH-deficient mice lacked evidence of luminal expansion, and their lumens did not appear to contain secretory product (Fig. 1A). The effects of ADPH deficiency on alveolar maturation were quantified by determining the cross-sectional areas of lumens in WT and ADPH-deficient mice at P18 to avoid the morphological variability normally associated with the initiation of lactation. The average cross sectional area of alveolar lumens in ADPH-deficient mice was significantly smaller than in WT mice (Fig. 1B), suggesting that ADPH has a role in alveolar maturation during mammary gland differentiation.

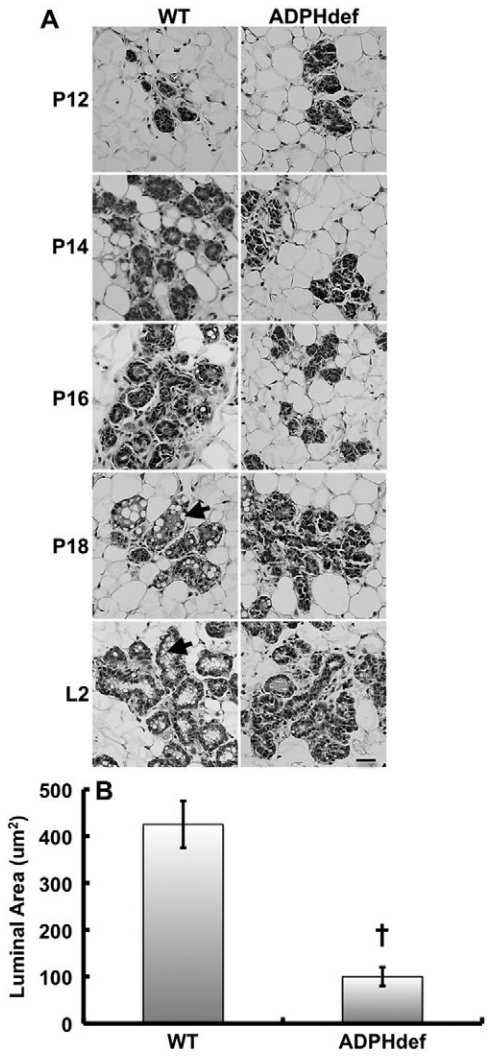

Fig. 1. ADPH deficiency disrupts alveolar maturation in differentiating mouse mammary glands. (**A**) H&E stained mammary gland sections from WT or ADPH-deficient mice at the indicated days of pregnancy (P) or lactation (L) showing reduced alveolar size and impaired CLD (arrows) accumulation in ADPH-deficient mice. Scale bar: 50 μm. (**B**) Alveolar luminal size in mammary glands of WT and ADPH-deficient mice at P18. The values are averages from at least 80 alveoli in five mammary gland sections from three WT and three ADPH-deficient mice. $^{\dagger}P<0.05$.

ADPH deficiency impairs CLD maturation

ADPH expression is hypothesized to mediate increases in CLD size and accumulation in differentiating mammary epithelial cells of WT mice (Russell et al., 2007). As indicated by the images in Fig. 1A, the normal accumulation of CLDs that occurs in mammary epithelial cells of WT mice toward the end of pregnancy appeared to be impaired in ADPH-deficient mice. To better define this defect, we quantified CLD size and number in mammary epithelial cells of WT and ADPH-deficient mice on days 16 and 18 of pregnancy by immunostaining their mammary glands with antibodies against ADPH or the related protein

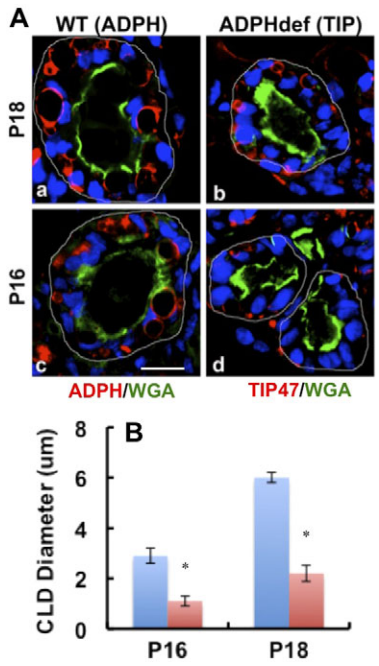

Fig. 2. ADPH deficiency prevents CLD growth. (**A**) Mammary gland sections from WT or ADPH-deficient mice (ADPHdef) mice at pregnancy (P) day16 or 18 were immunostained with antibodies against ADPH or TIP47 to detect CLDs (red). Alexa-Fluor-488-labeled wheatgerm agglutinin (green) was used to identify the luminal surface of alveoli. Nuclei were stained with DAPI (blue). Alveoli are outlined with a dotted white line. Scale bar: 10 µm. (**B**) Quantification of CLDs in mammary epithelial cells from WT (blue bars) and ADPH-deficient (red bars) at P16 and P18. The values are means ± s.e.m. from at least 80 alveoli in five mammary gland sections from three WT and three ADPH-deficient mice. *$P<0.01$.

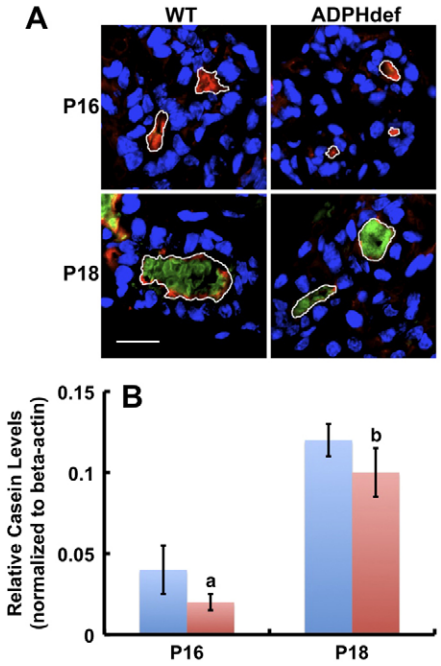

Fig. 3. ADPH deficiency does not prevent expression of milk proteins. (**A**) Representative images of mammary gland sections from WT or ADPH-deficient mice (ADPHdef) mice at P16 and P18 after immunostaining with antibodies against β-casein (green). Alexa-Fluor-594-labeled wheatgerm agglutinin (red) was used to identify the luminal region of alveoli (outlined with a white line). Nuclei were stained with DAPI (blue). Scale bar: 10 µm. (**B**) Quantification of immunoblot analysis of β-casein in mammary gland extracts of WT (blue bars) and ADPH-deficient (red bars) mice at P16 and P18. Values are means ± s.e.m. of actin normalized β-casein staining intensities from mammary gland extracts of three mice. a, $P=0.08$; b, $P=0.3$.

TIP47, which coats CLDs in ADPH-deficient mice (Russell et al., 2008) (Fig. 2A). These times were chosen because previous studies showed that they correspond to a crucial preparturient phase of CLD proliferation and growth in differentiating mammary glands of WT mice (Russell et al., 2007). Similar CLD numbers were detected in mammary epithelial cells of WT and ADPH-deficient mice ($P=0.93$). However, CLD size in ADPH-deficient cells was significantly smaller than that found in cells of WT mice at P16 and P18, and they failed to undergo the increase in size that normally occurs over this period (Fig. 2B). Based on the average CLD diameters shown in Fig. 2B, we estimated that the relative volume of triglycerides stored in mammary epithelial cells of WT mice at P16 and P18, respectively, was 12.5-times and 14.2-times that stored in mammary epithelial cells of ADPH-deficient mice. Thus, although ADPH does not appear to be required for CLD formation, it does appear to be a critical regulator of CLD growth and lipid accumulation in the differentiating mammary epithelial cells.

To determine whether defects in alveolar maturation and CLD accumulation are associated with a general impairment of secretory differentiation, we quantified β-casein levels in mammary glands of WT and ADPH-deficient mice. Fig. 3A shows representative confocal immunofluorescence images of β-casein staining in mammary alveoli of these mice at P16 and P18. Consistent with evidence that β-casein expression increases significantly during the later stages of secretory differentiation in the mouse mammary gland (McManaman et al., 2002; Robinson et al., 1995), we found that β-casein immunofluorescence in the lumens of mammary alveoli at P18 was significantly greater than that in P16 lumens of both WT and ADPH-deficient mice. Furthermore, immunoblot analysis of mammary gland extracts from WT and ADPH-deficient mice at P16 and P18 (Fig. 3B) showed that ADPH deficiency did not affect β-casein levels at either of these time points. Thus, despite defects in alveolar maturation and CLD growth in ADPH-deficient mice, these data suggest that ADPH-deficiency does not appear to impair β-casein expression or its initial secretion during mammary gland differentiation.

ADPH promotes CLD maturation by inhibiting binding of adipose triglyceride lipase (ATGL) to the CLD surface

ATGL catalyzes the initial step in the hydrolysis of triglycerides in adipose tissue and many non-adipose tissues (Zechner et al., 2009). Evidence from cell culture studies suggests that ATGL-dependent triglyceride turnover reduces CLD size, and that ADPH inhibits this activity by preventing access of ATGL to the CLD surface (Listenberger et al., 2007; Smirnova et al., 2006). Our initial immunoblot analyses indicated that ATGL is present in extracts of mammary glands of WT and ADPH-deficient mice at P18 (Fig. 4A). As an initial test of whether the association of ATGL with CLDs potentially contributes to the formation of smaller CLDs in mammary epithelial cells of ADPH-deficient mice, we determined its localization in mammary gland sections

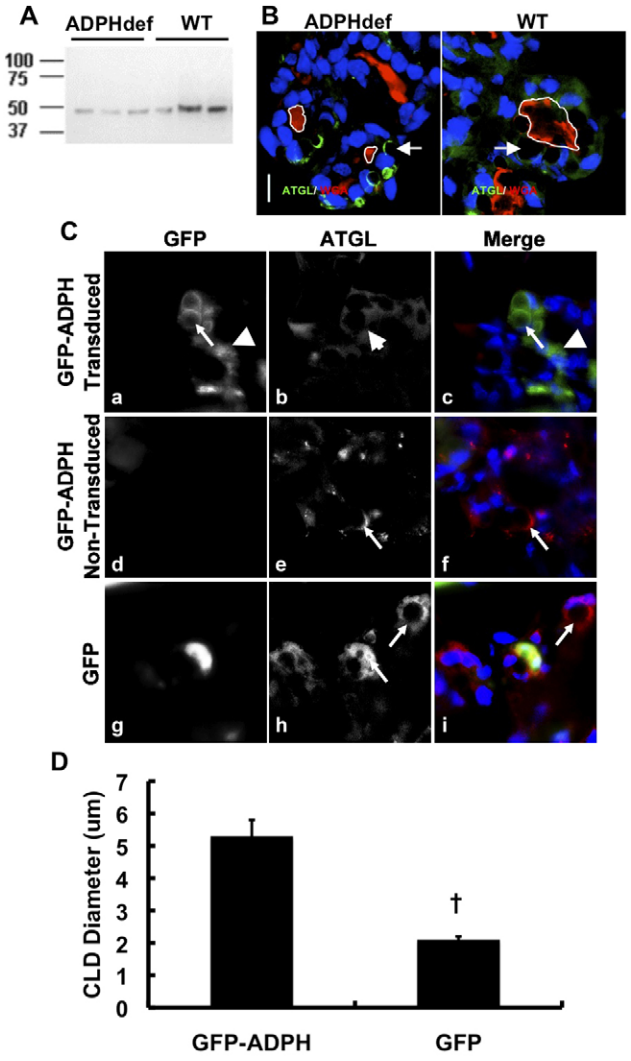

Fig. 4. ADPH promotes CLD growth by inhibiting access of ATGL to their surface. (**A**) ATGL immunoblot showing the presence of ATGL in mammary gland extracts of WT and ADPH-deficient mice. (**B**) Representative images of mammary gland sections from WT or ADPH-deficient mice at P18 immunostained with antibodies against ATGL (green), showing that ATGL localizes to CLDs in ADPH-deficient mice (arrow in left panel) but not to CLDs in WT mice (arrow in right panel). Differential interference contrast (DIC) optics were used to aid in CLD identification. Alexa-Fluor-594-labeled wheatgerm agglutinin was used to identify luminal region of alveoli (outlined with a white line). DAPI-stained nuclei are shown in blue. Scale bar: 10 μm. (**C**) Representative images of ATGL (red) immunolocalization in mammary epithelial cells of ADPH-deficient mice at P17 after transduction with GFP–ADPH adenovirus (a–c) or GFP adenovirus (g–i) on P16. Images in d–f show ATGL localization in representative non-transduced cells. Panels a,d and g show GFP-specific fluorescence; panels b,e and h show ATGL-specific immunofluoresence; panels c,f and i show merged GFP and ATGL fluorescence. Arrows in panels a,c,e,f,h and i indicate CLD. The arrowheads in a and c show small GFP-ADPH positive CLD clusters that fail to resolve well when larger CLD are in focus. The arrowhead in panel b indicates ATGL localization in cytoplasm of MEC of GFP-ADPH-transduced cells. (**D**) CLD size quantification in mammary epithelial cells of ADPH-deficient mice at P18 following transduction with GFP–ADPH or GFP adenovirus on P14. Nuclei in all images were stained with DAPI (blue). The values are means ± s.e.m. from at least 80 GFP-positive alveoli in five mammary gland sections from three ADPH-deficient mice. $^{\dagger}P<0.01$.

from WT and ADPH-deficient mice on P18. Fig. 4B (left panel) shows that ATGL is present in mammary epithelial cells of ADPH-deficient mice, and that it selectively localizes to CLDs. ATGL was also present in mammary epithelial cells of WT mice; however in these cells it had a diffuse appearance, and did not appear to exhibit significant association with CLDs (Fig. 4B, right panel). These results are consistent with the hypothesis that the increase in CLD size in differentiating mammary epithelial cells is dependent on the ability of ADPH to prevent ATGL from binding to the CLD surface.

To obtain additional validation of this hypothesis, we next determined whether exogenously expressed ADPH in mammary epithelial cells of ADPH-deficient mice prevents ATGL from localizing to CLDs and rescues CLD size defects. We previously demonstrated that milk-secreting cells of intact virgin or pregnant mice can be selectively and efficiently transduced with adenovirus by injecting viral particles into the ductal network of the mammary gland (Russell et al., 2003). Using this approach, we transduced one of the inguinal (fourth) mammary glands of ADPH-deficient mice with adenovirus encoding ADPH as an N-terminal GFP fusion protein (GFP–ADPH). The contralateral gland was transduced with adenovirus encoding GFP only (GFP) as a transduction control. We have previously shown that GFP–ADPH localizes to CLDs and promotes their accumulation in cultured cells (Orlicky et al., 2008), and that adenovirus-mediated expression of GFP in mammary epithelial cells does not alter normal mammary gland development (Russell et al., 2003). Fig. 4C shows the localizations of ATGL and GFP–ADPH or GFP in mammary epithelial cells of ADPH-deficient mice at P17 following transduction at P16. In GFP–ADPH-transduced cells (Fig. 4C,a–c), GFP–ADPH was selectively detected on CLDs, whereas ATGL staining was diffusely localized within the cytoplasm. By contrast, in non-transduced cells (Fig. 4C,d–f) or in cells transduced with GFP (Fig. 4C,g–i), ATGL was enriched around CLDs. These results verify that ADPH expression is necessary and sufficient to prevent ATGL from localizing to CLDs in differentiating mammary epithelial cells.

If the association of ATGL with CLDs is responsible for deficits in CLD maturation in mammary epithelial cells of ADPH-deficient mice, then the exogenous expression of ADPH is predicted to rescue this defect. To test this prediction, we transduced inguinal mammary glands of ADPH-deficient mice with GFP–ADPH or GFP on P14 and quantified CLD size on P18. CLD diameter in GFP–ADPH-transduced glands was significantly larger than that in GFP-transduced glands (Fig. 4D). Significantly, the average CLD diameter in GFP–ADPH-transduced glands (5.4 μm) was comparable to that found in WT glands at the same gestational age (6 μm), which indicates that exogenous expression of GFP–ADPH from P14 to P18 rescues the defects in CLD size observed in ADPH-deficient mice.

GFP–ADPH rescues alveolar maturation defects in ADPH-deficient mice

Collectively, the results thus far suggest that defects in CLD accumulation in mammary epithelial cells of ADPH-deficient mice are functionally linked to defects in alveolar maturation. We have previously documented that hepatic triglyceride accumulation in response to a high-fat diet is altered in these mice (Chang et al., 2006). Thus it is possible that the observed effects of ADPH deficiency on mammary gland maturation

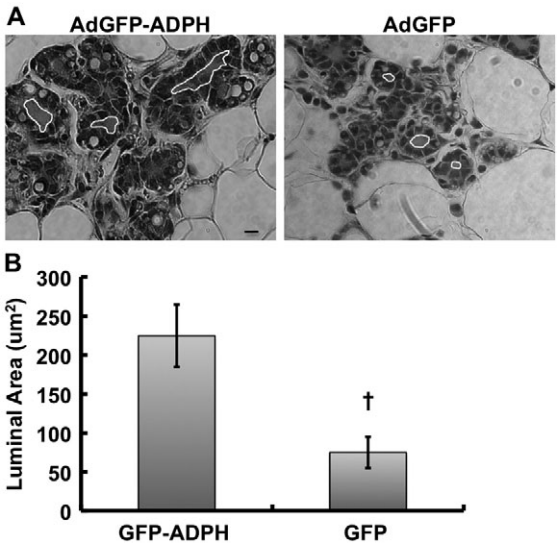

Fig. 5. GFP–ADPH expression rescues alveolar maturation defects in ADPH-deficient mice. (**A**) Representative images of H&E-stained mammary gland sections from ADPH-deficient mice at P18 following transduction with GFP–ADPH or GFP adenovirus on P14. White lines outline luminal regions of alveoli. Scale bar: 10 μm. (**B**) Quantification of luminal areas of mammary alveoli at P18 following transduction with GFP–ADPH or GFP adenovirus at P14. The values are means ± s.e.m. from at least 80 GFP-positive alveoli in five mammary gland sections from three ADPH-deficient mice. $^{\dagger}P<0.001$.

reflect general metabolic alterations rather than, or in addition to, mammary-gland-specific effects. To distinguish between these alternatives, and formally establish the importance of ADPH in alveolar maturation, we quantified the effects of adenoviral expression of GFP–ADPH, or GFP, on the luminal area of differentiating alveoli in ADPH-deficient mice. Histological staining showed that at P18, alveoli in mammary glands transduced with GFP–ADPH on P14 exhibited expanded lumens, and their epithelial cells possessed large CLDs (Fig. 5A, left panel). By contrast, alveoli in contralateral glands transduced with GFP had unexpanded lumens and their cells lacked large CLDs (Fig. 5A, right panel). Measurement of luminal areas in transduced mammary glands (Fig. 5B) showed that the average luminal area of those transduced with GFP-ADPH were more than 4-times that of those transduced with GFP, which demonstrates that expression of ADPH in differentiating mammary epithelial cells is able to rescue defects in alveolar maturation. However, the average luminal area in P18 ADPH-deficient mammary glands transduced with GFP–ADPH (220 μm^2) was smaller than that of P18 WT glands (428 μm^2), suggesting either that exogenous expression of ADPH between P14 and P18 is not sufficient to fully rescue the alveolar phenotype of ADPH-deficient mice, or that GFP–ADPH does not fully substitute for endogenous ADPH in this process.

Discussion

The ability of mammary epithelial cells to produce the triglycerides that are the source of milk lipids is developmentally regulated in conjunction with the differentiation of the mammary gland into a secretory organ during pregnancy (Russell et al., 2007). Data presented here implicate ADPH in both the regulation of triglyceride accumulation and the formation of mature alveoli in the differentiating mouse mammary gland. The ability of exogenous ADPH to rescue CLD accumulation and alveolar maturation defects in ADPH-deficient mice further suggests that efficient triglyceride storage has a role in the functional development of the mammary gland. This concept is supported by studies showing that deletion of diacylglycerolacyltransferase-1 (DGAT1), the enzyme catalyzing the last step in triglyceride synthesis in mammary epithelial cells (Cases et al., 2004), interferes with CLD accumulation and secretory differentiation in the mouse mammary gland (Cases et al., 2004). The mechanism(s) by which triglyceride storage influences mammary gland differentiation remains to be established. However, cellular triglycerides are recognized to contribute to the regulation of cellular functions by limiting the availability of free fatty acids (FFAs) and the associated production of bioactive lipid metabolites (Browning and Horton, 2004; Listenberger et al., 2003). Mammary glands of lactating mice are highly lipogenic (Schwertfeger et al., 2003), and pathways controlling de novo fatty acid synthesis and dietary fatty acid uptake are upregulated during differentiation to meet milk lipid production demands once lactation ensues (Jensen et al., 1994; McManaman, 2009; Rudolph et al., 2003). Thus, impairing triglyceride synthesis or storage during mammary gland differentiation might increase the availability of fatty acids and/or bioactive lipid metabolites that interfere with the processes regulating the transition of the mammary gland to a secretory organ. Previous observations that ADPH-deficient mice express a truncated variant of ADPH during lactation (Russell et al., 2008), have led to speculation that the variant might be able to substitute for some of the functions of intact ADPH. However, ADPH-deficient mice lack detectable amounts of any form of ADPH throughout most of pregnancy (Russell et al., 2008), thus it is unlikely that the variant form of ADPH contributes to defects in CLD accumulation and alveolar maturation observed in differentiating mammary glands of this strain.

ATGL is a crucial triglyceride lipolytic enzyme in adipose tissue and some non-adipose tissues (Zimmermann et al., 2009). Our study provides the first evidence that ATGL is present in mammary epithelial cells, and that inhibiting its association with CLDs contributes to their maturation during mammary gland differentiation. In contrast to adipocytes, in which ATGL appears to be constitutively associated with CLDs (Brasaemle et al., 2009; Granneman et al., 2007), our data suggest that it is either directly or indirectly excluded from CLDs in mammary epithelial cells by ADPH. These in vivo data are consistent with cell culture evidence indicating that ADPH promotes triglyceride accumulation by excluding ATGL from CLDs (Listenberger et al., 2007). This function of ADPH appears to be distinct from that of other perilipin family members. For example, significant colocalization of ATGL and perilipin has been demonstrated in 3T3-L1 adipocytes under both basal and lipase-stimulated conditions (Granneman et al., 2007), and there is increasing evidence that interactions between perilipin and ATGL are mediated by CGI58/Abhd5, a protein activator of ATGL (Brasaemle, 2007b). ATGL was similarly shown to bind to CLDs coated with TIP47 in HeLa (Smirnova et al., 2006) and HEK293 cells (Listenberger et al., 2007). Indeed, evidence in HeLa cells showing colocalization of ATGL and TIP47 on the CLD surface (Smirnova et al., 2006) suggests that ATGL also forms functionally important interactions with TIP47. The apparent ability of ADPH to exclude ATGL from CLDs thus

indicates that its mechanism of regulating cellular lipase activity might be different from that of other perilipin family members.

A functional consequence of excluding ATGL from CLDs in differentiating mammary epithelial cells appears to be an increase in CLD size. Accumulation of large CLDs in these cells has been proposed to involve cooperative effects of increased ADPH expression and stimulation of lipogenic activities (McManaman, 2009; Russell et al., 2007). Our study validates the importance of ADPH in regulating accumulation of large CLDs, and suggests that the mechanism by which ADPH mediates this effect is due in part to preventing ATGL-dependent lipolysis. Cell culture evidence showing that CLD size inversely correlates with cellular ATGL activity in HeLa cells supports this conclusion (Smirnova et al., 2006). However, additional studies are required to determine how limiting ATGL activity enhances accumulation of large CLDs in mammary epithelial cells. For example, elevated ATGL activity has been proposed to reduce CLD size by increasing their turnover (Smirnova et al., 2006). However, our observation that similar numbers of CLDs are present in mammary epithelial cells of WT and ADPH-deficient mice suggests that this mechanism does not contribute to the reduction in CLD size in ADPH-deficient cells. Indeed it is possible that lipolytically driven increases in cellular fatty acids could indirectly inhibit increases in CLD size by reducing lipogenic activity (Natali et al., 2007).

Multiparous ADPH-deficient dams were previously shown to be capable of supporting normal litter growth (Russell et al., 2008). However, in this study we found that these dams did not support their first litters. This finding suggests that parity might influence the sensitivity of the mammary gland to ADPH deficiency. Mammary gland differentiation is known to be affected by parity, with evidence from humans, cattle and rodents demonstrating that both glandular growth and the development of secretory function are accelerated in multiparous animals relative to animals that were pregnant for the first time (Miller et al., 2006; Wagner et al., 2002). In addition, in mammary glands of cattle, the development of key lipid-synthesis genes, *ACC* (acetyl-CoA carboxylase) and *FAS* (fatty acid synthase), during differentiation is accelerated in multiparous animals relative to animals in their first pregnancy, suggesting that parity specifically affects lipid metabolism (Miller et al., 2006). Thus the apparent greater sensitivity of mice in their first pregnancy to ADPH deficiency could reflect slower rates of glandular development and activation of lipid synthetic pathways in nulliparous relative to multiparous animals, which when combined with decreased ADPH might result in the inability of ADPH-deficient first-time dams to support their litters.

In summary, we present direct evidence substantiating the hypothesis that ADPH is a primary physiological regulator of triglyceride accumulation in the developing mammary gland (Russell et al., 2007). By demonstrating that ADPH is specifically required for CLD enlargement but not formation, our data indicate that the processes governing increases in triglyceride storage within CLDs are distinct from those governing its initial packaging in these structures. Although our data support the concept that ADPH increases CLD size by inhibiting ATGL-dependent triglyceride lipolysis (Listenberger et al., 2007; Smirnova et al., 2006), it is unlikely that this is the only mechanism regulating CLD growth, because CLD size in differentiating mammary epithelial cells can exceed 20 μm (Russell et al., 2007), whereas the size of ADPH-coated CLDs in cultured cells rarely exceeds a few micrometers, even when triglyceride synthesis is stimulated by incubation in high concentrations of fatty acids (Orlicky et al., 2008). Nevertheless, the finding that ADPH is necessary for formation of large CLDs in differentiating milk-secreting cells indicates that its levels might be a limiting factor in their production.

Materials and Methods

Materials

Rabbit antibodies against amino acids 409–425 of murine ADPH or against amino acids 1–16 of murine TIP47 were generated and characterized as described previously (Russell et al., 2008; Russell et al., 2007). Guinea pig antibodies against ADPH were obtained from Research Diagnostics (Flanders, NJ). Rabbit antibodies against murine β-casein were obtained from Margaret Neville (University of Colorado, Denver, CO). Rabbit antibodies against ATGL were obtained from Cell Signaling Technology (Beverly, MA). Alexa-Fluor-488- and Alexa-Fluor-594-labeled wheat germ agglutinin (WGA) were obtained from Invitrogen/Molecular Probes (Carlsbad, CA).

Animals and tissue preparation

B6.129-Adfp^{tm1Chan} mice deficient in ADPH were generated as previously described (Chang et al., 2006). WT C57B6 mice were obtained from Jackson Laboratories. Both strains were maintained as breeding colonies in the Animal Resource Center of the University of Colorado Anschutz Medical Campus (UCAMC) and housed individually. Pregnancy was timed by the observation of vaginal plugs after mating. The first day of pregnancy was taken as the day of vaginal plug detection. Parturition occurs on approximately day 19 of pregnancy; the day of birth is also designated as day one of lactation. Mammary tissue was removed from animals sacrificed by carbon dioxide inhalation at times indicated in the text and flash frozen in liquid nitrogen or processed for immunofluorescence microscopy (Russell et al., 2008; Russell et al., 2007). For rescue experiments proximal regions of ADPH–GFP- or GFP-transduced mammary glands were removed by dissection under fluorescence illumination to identify transduced portions of the gland. All animal procedures were approved by the Institutional Animal Care and Use Committee of the UCAMC.

Adenovirus and intraductal injections

Adenovirus encoding GFP was constructed as previously described (Fagan et al., 2000). The adenovirus encoding GFP–ADPH was constructed by cloning the fused full-length ADPH coding sequence in frame with GFP-coding sequence into the plasmid pShuttleCMV (He et al., 1998). Virus was grown and purified as previously described (Orlicky and Schaack, 2001) except that, after CsCl gradient purification, the viruses were dialyzed into an optimized storage buffer (Evans et al., 2004) containing 50% v/v glycerol as cryoprotectant.

Imaging

Freshly dissected mouse mammary tissue was immediately processed for histology and immunofluorescence imaging. For histology, mammary tissue was fixed in 10% neutral buffered formalin and processed for paraffin imbedding. Paraffin sections (5 μm) were cut, mounted on slides and stained with hematoxylin and eosin (H&E) for histological examination by IHCtech (Aurora, CO). For immunofluorescence analysis, mammary tissue was processed as previously described (Russell et al., 2003). Cryostat sections (8 μm) were cut and stained with Alexa Fluor 488 or Alexa Fluor 594 WGA to identify luminal boarders of alveoli (Russell et al., 2008). Nuclei were identified by DAPI (4′,6-diamidino-2-phenylindol) staining as described (Russell et al., 2003). Differential interference contrast (DIC) optics were used to aid in CLD identification (Russell et al., 2008). Immunofluorescence images were captured at room temperature on a Nikon Diaphot fluorescence microscope equipped with a Cooke SensiCam charge-coupled device camera (Tonawand, NY) using Slidebook software (Intelligent Imaging Innovations, Denver, CO) as described previously (Russell et al., 2007). All fluorescent images were digitally deconvolved using the No Neighbors algorithm (Slidebook), converted to TIFF files and processed using Photoshop (Adobe Systems, Mountain View, CA).

CLD quantification and statistical methods

Average CLD diameters were determined by analysis of individual ADPH- or TIP47-coated CLDs in 60–80 randomly chosen alveoli at 600× magnification from five sections per animal. Three animals were analyzed per experimental point. Diameters of individual CLDs were enumerated using the masking functions of Slidebook. A lower limit of 200 nm was used in quantifying CLDs, because structures below this size were not adequately resolved. Alveolar luminal area was quantified in 60–80 randomly chosen alveoli at 400× magnification from five sections per animal using masking functions of Slidebook. Three-four animals

were analyzed per experimental point. All values are means ± s.e.m. Statistical significance was determined using Student's *t*-test.

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