THE ROLE OF FETUIN-A ON ADIPOSE TISSUE LIPID MOBILIZATION IN DAIRY COWS

By

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ABSTRACT

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Adipose tissue (AT) is a major modulator of metabolic functions by regulating energy storage and acting as an endocrine organ. In periparturient dairy cows, increased AT mobilization of free fatty acids (FFA) is one a major adaptive mechanism to cope with higher energy demand for rapid fetal growth and the onset of lactation. As lactation progresses, lipolysis rates decrease, and lipogenesis replenishes triacylglycerol (TAG) stores in adipocytes. However, dysregulated metabolic responses, characterized by altered AT sensitivity to hormonal and endocrine changes around parturition, lead to a massive release of FFA into circulation and an increased susceptibility of cows to disease. These maladaptive responses are underlined by an altered secretory pattern of adipokines and a marked unbalance in lipolysis and lipogenesis rates, favoring TAG breakdown in adipocytes. Thus, identifying adipokines that modulate AT function in periparturient dairy cows can facilitate the development of novel management, nutritional, or pharmaceutical interventions to reduce disease incidence. Fetuin-A (FetA; alpha-2-Heremans-Schmid glycoprotein, AHSG) is an adipokine that functions as a carrier of FFA in plasma and is associated with insulin-mediated inhibition of lipolysis and stimulation of lipogenesis in humans. FetA increases the incorporation of fatty acids (FA) into intracellular lipids and enhances cellular TAG in human cells. However, the mechanisms by which FetA induces TAG synthesis are not defined. FetA has also anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines and acting as a negative acute-phase protein (APP) in acute inflammation. These findings suggest that FetA may also be involved in lipid mobilization and

inflammation in AT of dairy cows. In our first in vivo study with periparturient dairy cows, we observed that serum and AT FetA expression decreased at the onset of lactation when lipogenesis was downregulated and plasma FFA was increased. FetA expression dynamics in AT were analogous to the patterns of lipogenic markers suggesting its link with lipid mobilization in AT of dairy cows. We also demonstrated that FetA is negative-APP locally in AT of dairy cows. These results suggest that FetA could support physiological adaptations to NEB in AT of periparturient dairy cows. To explore the potential roles of FetA on AT lipid mobilization of dairy cows, we developed an in vitro model for culturing bovine adipocytes that closely mimics the in vivo AT environment. For the first time, we reported an abundant expression and secretion of FetA by primary bovine adipocytes, thus suggesting a potential autocrine effect of FetA in AT of dairy cows. We observed that FetA attenuates lipolytic responses and enhances both, FA uptake and TAG accumulation in bovine adipocytes. Our results reveal that the upregulation of the expression and activity of 1-acylglycerol-3-phosphate acyltransferase (AGAPT2), a rate limiting lipogenic enzyme for TAG synthesis, may be a potential mechanism by which FetA enhances lipogenic function of bovine adipocytes. Overall, our results indicate that FetA is a lipogenic adipokine with anti-inflammatory function in the AT of dairy cows. Our findings provide evidence that FetA could buffer increased plasma FFA during negative energy balance by stimulating AGAPT2 activity and the use of excess FFA for TAG synthesis in AT of dairy cows. The genetic selection of cows by variations of the FetA coding gene associated with its anti-lipolytic and pro-lipogenic functions (already known in humans), the identification of dietary supplements (i.e. FA) that enhance FetA function, as well as the parenteral use of FetA to stimulate AGAPT2 activity, could serve as potential strategies to be tested and implemented in dairy cows.

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KEY TO ABBREVIATIONS

ADIPOQ	Adiponectin
AdipoR1	Adiponectin receptor-1
AdipoR2	Adiponectin receptors-2
AGAPT2	1-acylglycerol-3-phosphate acyltransferase
AHSG	Alpha-2-Heremans-Schmid glycoprotein
AL	ad libitum
AMPK	5'-adenosine monophosphate-activated protein kinase
ANGTLP4	Angiopoietin-like protein-4
aP2	Adipocyte fatty acid-binding protein
APP	Acute-phase protein
ARG1	Arginase-1
AT	Adipose tissue
AUC	Area under the curve
B2M	Beta-2-microglobulin
BCS	Body condition score
BHB	Beta-hydroxybutyrate
BME	Mercaptoethanol
BSA	Bovine serum albumin
Ca	Calcium
CC7	7 d in co-culture
CCL2	Chemoattractant protein-1

CD36	Fatty acid translocase
CD44	Cluster differentiation 44
CD68	Cluster differentiation 68
CEBPA	CCAAT/enhancer-binding protein alpha
CEBPB	CCAAT/enhancer-binding protein beta
COL6A2	Collagen alpha-2
CP	Crude protein
CU	Close-up
DGAT	Diacylglycerol acyltransferases
DGAT1	Diacylglycerol O-Acyltransferase-1
DGAT2	Diacylglycerol O-Acyltransferase-2
DM	Dry matter
DTT	Dithiothreitol
EIF3K	Eukaryotic translation initiation factor 3 subunit K
EL	Early lactation
ELOVL6	Fatty Acid Elongase 6
FA	Fatty acids
FABP4	Fatty acid binding protein 4
FASN	Fatty acid synthase
FASN	Fatty acid synthase
FATP1	Fatty acid transporter-1
FBS	Fetal bovine serum
Fet A	Fetuin-A

FFA	Free fatty acids
FO	Far off
FR	Feed-restricted
GLUT4	Glucose transporter-4
GPAT	Glycerol phosphate acyltransferases
GPAT1	Glycerol-3-Phosphate Acyltransferase-1
GPAT2	Glycerol-3-Phosphate Acyltransferase-2
HBCS	High body condition score
HCAR1	Hydroxycarboxylic acid receptors-1
HDL	High density lipoprotein
HSL	Hormone lipase
IBMX	2 isobutyl-1-methylaxanthine
IFN-γ	Interferon gamma
IL1	Interleukin 1
IL10	Interleukin-10
IL6	Interleukin 6
ISO	Isoproterenol
KRBB	Krebs-Ringer Bicarbonate Buffer
LIPE	Hormone sensitive lipase
LIPIN1	Phosphatidate phosphatase-1
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MBCS	Moderate body condition score

MCP-1	Monocyte chemoattractant protein-1
NDF	Neutral detergent fiber
NE	Net energy of lactation
NEB	Negative energy balance
P	Phosphorus
PA	Phosphatidic acid
PAI-1	Plasminogen activator inhibitor 1
PPARG	Peroxisome proliferator-activated receptor gamma
PPARGC1A	PPARγ co-activator 1α
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor-γ
PRE	Preadipocytes
RBP	Retinol-binding protein
RPLO	50S ribosomal protein L15
RPS9	40S ribosomal protein S9
RPS9	40S ribosomal protein S9
SCD1	Stearoyl-CoA desaturase-1
SD14	14 d in standard differentiation
SD7	7 d in standard differentiation
SIRPA	Signal regulatory protein α
SIRT1	Sirtuin-1
SNP	Single nucleotide polymorphism
SPP1	Osteopontin-1

SVF	Stromal vascular fraction
TAG	Triacylglycerol
TBS	Tris-buffered saline solution
TNF	Tumor necrosis alpha

CHAPTER 1

INTRODUCTION

Periparturient dairy cows are at higher risk of disease due to intense lipid mobilization rates in the adipose tissues (AT) triggered by increased energy demands around parturition (Ospina et al., 2010). Increased lipolysis and decreased lipogenesis are major adaptive mechanisms to cope with negative energy balance (NEB) in periparturient cows. However, dysregulated adaptive responses result in excessive circulating FFA and lead to impaired immune and inflammatory responses (Contreras and Sordillo, 2011). Thus, adequate AT function is essential for a successful transition from gestation to lactation in dairy cattle (McNamara, 2012). Despite the advances on the management of periparturient dairy cows, at least 30% of early lactation cows develop metabolic and inflammatory diseases leading to high economic losses to the dairy industry (Van Saun and Sniffen, 2014). Strategies for effectively modulating AT function and assure an adequate balance between lipolysis and lipogenesis in AT are required. Adipokines are signaling proteins produced by the cellular components of AT including adipocytes and cells of the stromal vascular fraction, such as immune and vascular cells. Adipokines have crucial autocrine and endocrine functions regulating energy homeostasis, insulin sensitivity and inflammatory pathways, thus being suitable targets for the modulation of AT and whole-body metabolic function in dairy cows (Kusminski et al., 2016). In dairy cows, more than 500 adipokines have been identified in subcutaneous AT, but just few of them have been characterized in the context of the periparturient period (Zachut, 2015). Especially during this period, identifying adipokines involved in the autocrine regulation of lipid mobilization and inflammatory responses in AT are particularly relevant for the development of novel strategies to reduce diseases around parturition (Lehr et al., 2012, Zachut, 2015).

In humans, Fetuin-A (FetA; alpha-2-Heremans-Schmid glycoprotein, AHSG) is a recently reported adipokine and an attractive candidate gene for disturbed adipocyte lipolytic function during metabolic diseases, such as obesity and insulin resistance (Dahlman et al., 2004). FetA is involved on insulin-dependent and independent inhibition of lipolysis and stimulation of lipogenesis in human adipocytes (Dahlman et al., 2004). Attenuating lipolysis independently of insulin would be particularly important in early lactation when dairy cows develop hypoinsulinemia concurrently with a state of insulin resistance, thus releasing even higher FFA concentrations into circulation (Contreras et al., 2017). However, whether FetA plays the same roles in AT of dairy cows and the specific mechanisms by which this adipokine modulates lipid mobilization have not yet been described. Because of its adipogenic and lipogenic properties inducing incorporation of extracellular lipids into intracellular TAG (Cayatte et al., 1990), increased circulating concentrations and AT expression of FetA have been associated with obesity and obesity-related metabolic diseases, such as metabolic syndrome (Jialal et al., 2015, Pérez-Sotelo et al., 2016). Fetuin-A is also involved in acute inflammatory responses acting as a negative acute-phase protein (APP) in cases of infection, sepsis and trauma in non-ruminant models (Li et al., 2011, Wang and Sama, 2012, Zhang et al., 2014). Based on these antecedents, FetA could play a crucial role in the AT function of periparturient dairy cows as a modulator of lipid mobilization and inflammatory responses. However, little is known about this adipokine in dairy cows. The first report of FetA abundancy in AT of dairy cows indicates that its expression is downregulated during environmental metabolic stress when cows had increased plasma FFA and were in a pro-inflammatory status (Zachut et al., 2017). If acting similarly to non-ruminants, FetA might help buffering the increased circulating FFA concentrations during NEB in periparturient dairy cows, by potentially stimulating the use of FFA for TAG synthesis. The

better understanding of the roles of FetA in AT function of dairy cows could facilitate its use as, for example, a disease predictor or biomarker, a target for genetic selection, or even as a potential drug for improving AT function of dairy cows.

We hypothesized that FetA is a pro-lipogenic and anti-inflammatory role in the AT of periparturient dairy cows and enhances TAG synthesis in bovine adipocytes by augmenting FA uptake and the expression and activity of lipogenic enzymes. Therefore, our objectives were to (1) determine the roles of FetA on AT lipid mobilization and inflammation in AT of periparturient dairy cows, (2) to evaluate how FetA affects lipogenic and lipolytic functions in bovine adipocytes, and (3) to identify mechanisms and key cellular components underlying the effects of FetA on lipogenic activity of bovine adipocytes.

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CHAPTER 2

LITERATURE REVIEW

ADIPOKINES AS MODULATORS OF METABOLIC FUNCTION IN PERIPARTURIENT DAIRY COWS

Hormonal and endocrine changes and the abrupt increase in energy requirements associated with parturition and the onset of lactation lead to a pronounced negative energy balance (NEB) in dairy cows (McNamara and Hillers, 1986). The major metabolic adaptation to NEB is the increment in the use of lipids as an energy substrate (Bell and Bauman, 1997). Within adipose tissue (AT), enhanced lipolysis and reduced lipogenesis increase FFA flux into circulation. These AT responses are coupled with reduced insulin sensitivity within AT and in other peripheral tissues (i.e. muscle) that prioritize the use of glucose by the mammary gland (Bauman and Currie, 1980b). These metabolic adaptations maintain energy supply for the successful transition from gestation to lactation, however, when dysregulated, trigger an excessive release of FFA that leads to inadequate inflammatory responses and alterations in AT metabolic function (Contreras and Sordillo, 2011a, Contreras et al., 2017b). Currently, the mechanisms that lead to AT dysfunction in periparturient dairy cows are poorly understood and may involve not only alterations in lipid mobilization but also changes in its endocrine function.

Classic literature defined fat depots as connective tissues in which lipids were deposited and FFA were identified as its only secretory products (Wells, 1940). AT is now recognized as a complex organ with sympathetic and sensory innervation that plays a fundamental role in energy homeostasis, and endocrine and immune functions. AT fulfills its function in part through the synthesis and secretion of signaling proteins that are collectively termed adipokines (Blüher, 2012). These signaling proteins are produced by the cellular components of AT including

adipocytes and cells of the stromal vascular fraction (SVF), such as immune, vascular, and adipocyte progenitor cells. Adipokines regulate glucose and lipid metabolism, insulin signaling, and inflammatory pathways through autocrine, paracrine and endocrine actions [Fig. 1]. Although dairy cows express hundreds of adipokines in AT (Zachut, 2015), only a few have been characterized in the context of the periparturient period.

Around parturition, the secretory pattern of adipokines is altered by the overall metabolic status and by within-AT depot factors such as infiltration of immune cells (Contreras et al., 2015, Häussler et al., 2015). The central role of adipokines as modulators of energy homeostasis and AT function opens the possibility of using these signaling proteins as targets in the prevention and treatment of metabolic diseases in periparturient dairy cows (Lehr et al., 2012, Zachut, 2015) [Table 1]. However, only a few studies have analyzed the association of adipokines and diseases in dairy cows (Kasimanickam et al., 2013, Fadden and Bobe, 2016). This review summarizes current knowledge on the dynamics and roles of main adipokines involved on lipid mobilization and inflammatory responses around parturition in dairy cows, and their relationship with disease and metabolic dysfunction.

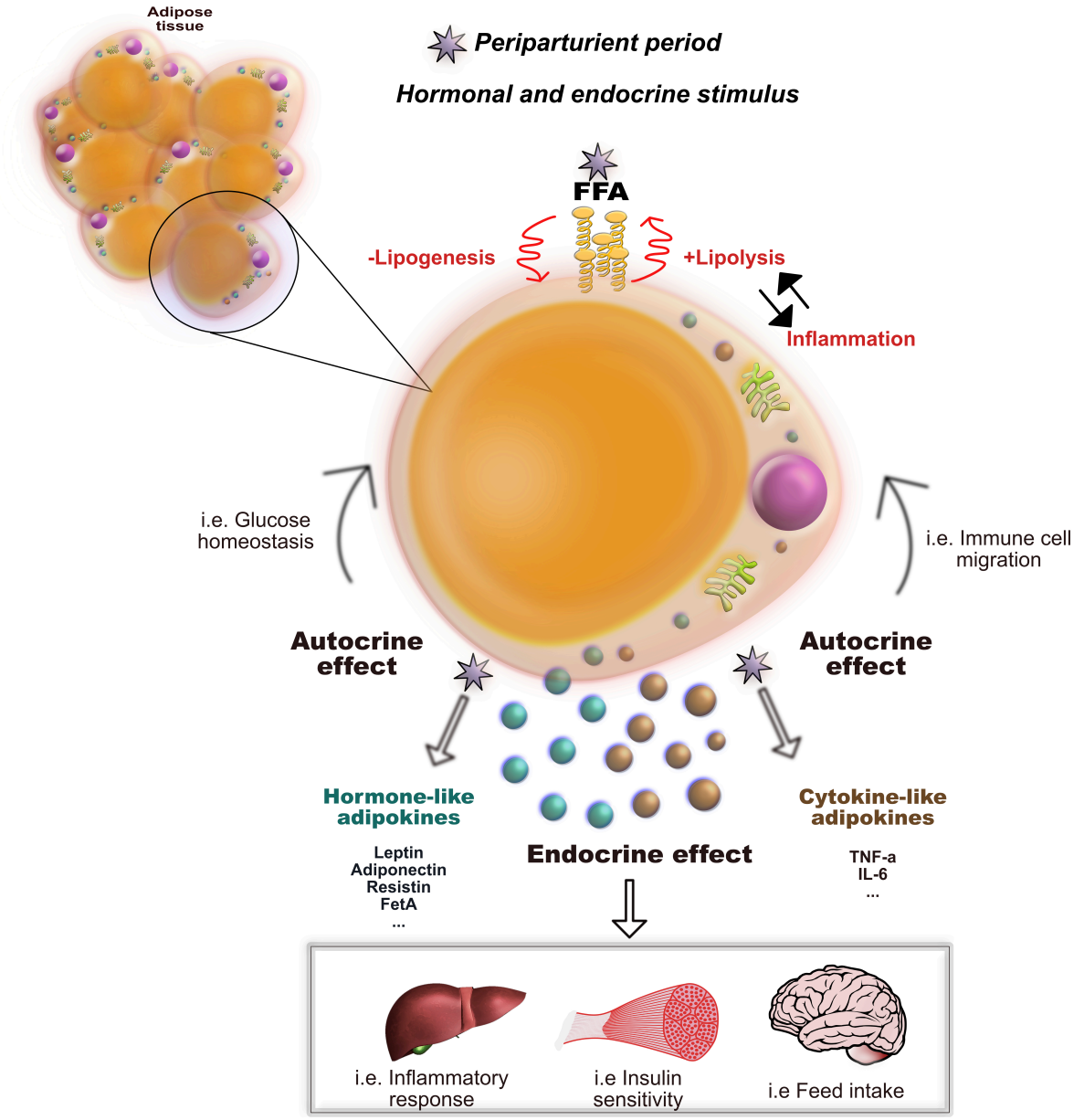

Figure 2. 1. Adipokines and metabolic function in adipose tissue of periparturient dairy cows.

Hormonal and endocrine changes around parturition stimulate changes in lipid mobilization, inflammatory responses, and the endocrine function of adipose tissue through the synthesis of adipokines. Hormone- and cytokine-like adipokines derived from adipose tissue participate in autocrine/paracrine and endocrine functions. Adipokines mediate crosstalk among different adipose tissue depots and cell populations within each depot (i.e. immune cells) and reach other organs/systems such as liver, muscle, and brain to regulate systemic energy metabolism.

Table 2. 1 Metabolic functions, plasma concentrations and known clinical relevance of adipokines in periparturient dairy cows.

	Metabolic functions	Plasma concentrations	Clinical relevance
Adiponectin	Through AMPK and PPARα activation (Kadowaki and Yamauchi, 2005): - ↑ glucose uptake and FA oxidation in liver, muscle and AT - ↑ food intake acting through CNS - ↑ insulin sensitivity in AT - ↓ NF-*κ*B activation in AT (Sauerwein and Häußler, 2016) - ↓TNF-α expression in bovine monocytes (Kabara et al., 2014)	-3 to +3 wk from parturition: ~35 ug/mL Calving: ~20 ug/mL (Singh et al., 2014b)	- Plasma concentrations are: o Negatively associated with plasma FFA concentrations (Kabara et al., 2014, Mellouk et al., 2017) o Negatively associated with adiposity (De Koster et al., 2017) o Positively associated with the insulin responsiveness of the glucose and FFA metabolism (De Koster et al., 2017) o Low adipocyte-derived adiponectin increase inflammatory-based disease development during the periparturient period in cattle (Kabara et al., 2014)
Leptin	- ↓ lipogenesis by inhibiting *de novo* synthesis of FA (William et al., 2002) - ↑ lipolysis and FFA oxidation (William et al., 2002) - ↑ lipid oxidation (Wang et al., 1999) - ↑conservation of glucose and energy at the onset of lactation (Ehrhardt et al., 2016)	Prepartum: 7.83 ± 2.84 ng/mL Postpartum: 4.64 ± 2.24 ng/mL (Lemor et al. 2009)	- Experimental parental infusion of leptin (Ehrhardt et al., 2016): o Attenuates accumulation of TAG in the liver o Increases plasma concentrations of T4 and T3 o Increases glucose disposal during an insulin tolerance test - Plasma leptin is directly correlated with fat mass (Leury et al., 2003)

Table 2. 1 (cont'd)

RBP	- Transport system for retinol in circulation (Rezamand et al., 2012a) - ↓ adipogenesis and glucose uptake by adipocytes (Klöting et al., 2007)	1 wk prepartum: 50.7±5.3 mg/ml Calving: 29.8±5.4 mg/ml 2 wks postpartum: 52.1±2.1 mg/ml (Eldaim et al., 2010)	- Negatively associated with hyperketonemia (Gröhn and Lindberg, 1985) - Indicator of the liver activity index (Trevisi et al., 2001) - Visceral AT RBP expression is positively associated with that of TNF-α (Rezamand et al., 2012a)
ANGTLP4	- Inhibits adipocyte uptake of FA for esterification controlled by PPAR-γ (Kersten, 2005) - ↑ lipolysis by increasing cAMP and enhancing the phosphorylation of PKA (Gray et al., 2012) - Inhibits LPL-mediated lipolytic processing of TAG-rich lipoprotein (Preedy and Hunter, 2016)	Lactating cows: 3.22 – 4.00 ng/mL (Li, 2011)	- May signal for lipolysis induction and contribute to the sustained release of FFA and lipid accumulation in the liver (Loor et al., 2007)
Fetuin-A	- ↑ Lipogensesis and adipogenesis factor (Cayatte et al., 1990) - Lipid transporter in plasma (Kumbla et al., 1989) - Negative acute phase protein systemically and in the AT (Strieder-Barboza et al., 2017b, Zachut et al., 2017)	30d prepartum: 0.89 ± 0.13 mg/mL 10d prepartum: 0.96 ± 0.13 mg/mL 10d postpartum: 0.77 ± 0.13 mg/mL (Strieder-Barboza et al., 2017b)	- Expression in adipose tissue: o Decreased during heat stress and lipolysis in late gestation (Zachut et al., 2017) o Negatively associated with pro-inflammatory markers in AT of periparturient cows (Strieder-Barboza et al., 2017b) - Circulating concentrations (Strieder-Barboza et al., 2017b): o Positively associated with serum albumin and calcium o Positively associated with BCS

Table 2. 1 (cont'd)

Resistin	- ↑ lipolysis in subcutaneous AT (Reverchon et al., 2014). - Influence the insulin-dependent glucose uptake in mammary epithelial cells (Komatsu et al., 2003) - May inhibit GLUT4 translocation in AT (Komatsu et al., 2003)	4-2 wk prepartum: 43.25 ng/mL 7-14d postpartum: 75.10 ng/mL - 4-6 wk postpartum: 36.42 ng/mL (Reverchon et al., 2014).	- In adipose tissue: o May contribute to insulin resistance (Komatsu et al., 2003) - Circulating concentrations: o Positively correlated with subcutaneous AT HCAR1 gene expression, an insulin-induced anti-lipolytic factor (Weber et al., 2016a) o Positively associated with plasma FFA (Reverchon et al., 2014). o Positively associated with adiposity (Mellouk et al., 2017). o Negatively associated with plasma leptin (Mellouk et al., 2017).
Visfatin	- Induces insulin secretion and promotes glucose uptake (Fukuhara et al., 2005) - Induces inflammation response through cytokine secretion (Moschen et al., 2007) - ↑ cell survival (Rongvaux et al., 2008)	-3 to +3 weeks to calving: median 6.59 µg/L; interquartile range: 5.48 to 7.86 µg/L (Fadden and Bobe, 2016)	- Plasma concentrations are increased during periparturient disease (Fadden and Bobe, 2016) - Early predictive indicator of retained placenta and other diseases (metritis, mastitis, ketosis, or laminitis) in periparturient dairy cows (Fadden and Bobe, 2016)
TNF-α	- Inhibits adipogenesis by downregulating LPL, FATP and ACS (Arner, 2003) - Regulates the formation of leptin, PAI-1, aP2 and GLUT4 (Hotamisligil and Spiegelman, 1994) - ↑ lipolysis (Arner, 2003) - Induces AT insulin resistance (Sethi and Hotamisligil, 1999) - Activates inflammation systemically and in the AT (Sadri et al., 2010, Contreras et al., 2015)	1d postpartum: 92 ±13 pg/ml 7 weeks portpartum: 85 ± 12 pg/ml (Sadri et al., 2010)	- Serum TNF activity is associated with insulin resistance in cows with fatty liver (Ohtsuka et al., 2001) - Increased expression in the visceral and subcutaneous AT of cows with displaced abomasum (Contreras et al., 2015) - Administration of TNF-α promoted lipolysis and insulin resistance in AT of dairy steers (Kushibiki et al., 2001)

ANGTLP4, Angiopoietin-like protein-4; LPL, lipoprotein lipase; FetA, Fetuin-a; TNF-α, tumor necrosis factor alpha; RBP, retinol-binding-protein.

Adiponectin

Adiponectin is a hormone-like adipokine exclusively produced by adipocytes and secreted directly into the circulation without further tissue accumulation (Sauerwein and Häußler, 2016). Adiponectin is synthesized as a monomer (30 kDa) and then assembled to oligomers detectable as low molecular weight trimers, medium molecular weight hexamers and high molecular weight oligomers (Waki et al., 2003). This adipokine regulates glucose and FA metabolism and has insulin-sensitizing and anti-inflammatory properties in different tissues (Singh et al., 2014a, De Koster et al., 2017). Adiponectin binds to its receptors in the skeletal muscle (AdipoR1) and liver (AdipoR2) to enhance glucose uptake and FA oxidation through the activation of 5'-adenosine monophosphate-activated protein kinase (AMPK) and the peroxisome proliferator-activated receptor alpha (PPARα) (Yamauchi et al., 2003). Adiponectin also acts in the in hypothalamic neurons increasing food intake and decreasing peripheral energy expenditure through the activation of AMPK (Kadowaki and Yamauchi, 2005).

In periparturient dairy cows, adiponectin expression in subcutaneous AT reaches its nadir around parturition and doubles by day 21 postpartum (Singh et al., 2014b), and its expression is greater in cows with high prepartum BCS (Vailati-Riboni et al., 2016). Adiponectin actions are modulated by the expression of its receptors, AdipoR1 and AdipoR2 across different tissues in dairy cows (Sauerwein and Häußler, 2016). AdipoR1 and AdipoR2 expression in AT are downregulated during the first 3 weeks after calving and increases steadily to peak at around 100 DIM in dairy cows (Saremi et al., 2014, Weber et al., 2016b). Decreased AdipoR1 and AdipoR2 mRNA abundance in AT might be the result of a complex regulatory system where expression of Sirtuin-1 (SIRT1) PPARγ co-activator 1α (PPARGC1A) axis modulates the abundance of adiponectin receptors (Weber et al., 2016b). However, the role of this axis on the modulation of

adiponectin signaling is a matter of debate as Giesy et al. (2012) did not observe any changes in the expression of AT adiponectin mRNA and its receptors AdipoR1 and AdipoR2 in mature dairy cows around parturition.

Changes in AT adiponectin expression in periparturient cows are reflected in its plasma concentrations that reach their nadir at calving (Singh et al., 2014a). At 3 weeks prepartum, plasma adiponectin concentrations are ~35 μg/mL and decrease to about 20 μg/mL at the time of parturition. By the third week of lactation plasma adiponectin returns to pre-calving values and is mainly influenced by visceral AT secretion of adiponectin (Singh et al., 2014b). Plasma adiponectin is negatively associated with FFA concentrations and therefore with lipolysis intensity in periparturient cows (Kabara et al., 2014, Mellouk et al., 2017). Adiponectin circulates predominantly in high and medium molecular weight forms (Giesy et al., 2012), and this oligomer distribution is independent of parity and the dietary supplementation with conjugated linoleic acid (Singh et al., 2014b). In dairy cows during late gestation, serum adiponectin was negatively associated with adiposity and positively associated with the insulin responsiveness of the glucose and FFA metabolism (De Koster et al., 2017).

Adiponectin modulates inflammation by suppressing nuclear factor-κß (NFκß) activation (Sauerwein and Häußler, 2016). Accordingly, adiponectin exposure decreased TNF-α expression in bovine monocyte after LPS-stimulation (Kabara et al., 2014). Therefore, decreased adiponectin concentrations could be determinant for increased inflammatory-based disease susceptibility in periparturient cattle (Kabara et al., 2014). In summary, adiponectin acts as an autocrine, paracrine, and endocrine modulator of the AT homeorhetic adaptations and in part of the inflammatory responses that may increase disease susceptibility in periparturient cows. All

together, these data indicate that adiponectin as a biomarker for metabolic function and insulin resistance in dairy cows has great potential for clinical use.

Leptin

Leptin is a 16-kDa adipokine encoded by the *OB* gene and synthesized by white AT (Leury et al., 2003). In ruminants, leptin is also expressed in the rumen, abomasum, duodenum, mammary gland, skeletal muscle and pituitary gland (Chilliard et al., 2001). In adipocytes, leptin reduces lipogenesis by inhibiting *de novo* synthesis of FA and increases lipolysis and FFA oxidation (William et al., 2002). These effects are induced by modulating the expression of enzymes involved in lipid oxidation such as acyl-CoA oxidase, uncoupling protein 2, and PPAR-α (Wang et al., 1999).

Leptinemia dynamics reflect AT leptin gene expression (Block et al., 2001) and are directly correlated with fat mass (Leury et al., 2003). Plasma leptin peaks during the dry period and then decreases from 7.83 ± 2.84 ng/mL two weeks before parturition to 4.64 ± 2.24 ng/mL at 20 days postpartum (Block et al., 2001, Lemor et al., 2009). The reduction in leptinemia during periparturient NEB was implicated as one of the signals promoting conservation of glucose and energy (Ehrhardt et al., 2016) and precedes significant depletion of lipid reserves mediated by the hypoinsulinemia (Block et al., 2003, Leury et al., 2003).

Leptin acts on the hypothalamus to regulate energy metabolism by decreasing food intake and increasing energy expenditure (Ingvartsen and Boisclair, 2001). A fall in plasma leptin concentrations signals to the CNS that a state of energy insufficiency prevails in the periphery (Leury et al., 2003). In early lactation cows, cerebrospinal fluid leptin concentrations decline as NEB and lipolysis increase suggesting that central leptin signals to promote hyperphagia (Laeger et al., 2013). Reduced AT leptin transcription and secretion may also promote a rapid return to

normal feed intake after parturition (Ehrhardt et al., 2016). However, voluntary feed intake becomes unresponsive to variations in plasma leptin during NEB in early lactation indicating that if leptin resistance exists in dairy cows, reduced plasma leptin may not promote higher voluntary feed intake (Leury et al., 2003, Ehrhardt et al., 2016). Therefore, leptin acts in autocrine and endocrine manners to modulate energy expenditure and is especially important during NEB as one of the signals promoting conservation of glucose.

Besides the recent report that circulating concentrations of leptin could be used to assess subclinical uterine diseases (Kasimanickam et al., 2013), a more recent study demonstrated how the manipulation of leptinemia could benefit metabolic function in periparturient cows. By reverting hypoleptinemia in early lactation cows, Ehrhardt et al. (2016) increased the glucose response during an insulin tolerance test, decreased hepatic lipid accumulation in early lactation, and increased (~45%) plasma concentrations of both T4 and T3 (Ehrhardt et al., 2016). These studies revealed that leptin has an important clinical relevance not only as a marker or predictors of diseases, but as a target in the treatment of metabolic diseases in periparturient dairy cows.

Angiopoietin-like protein-4

Angiopoietin-like protein-4 (ANGTLP4), also known as fasting-induced adipose factor, is an adipokine secreted during NEB that under transcriptional control by PPARγ inhibits FA uptake by adipocytes (Kersten, 2005). ANGPTL4 enhances catecholamine-induced lipolysis in adipocytes by increasing cAMP and enhancing the phosphorylation of PKA (Gray et al., 2012). ANGPTL4 inhibits lipoprotein lipase (LPL)-lipolytic activity at the same time that induces intracellular lipolysis and FFA release into circulation (Preedy and Hunter, 2016). In dairy cattle, AT *ANGPTL4* expression increased with the transition from pregnancy to lactation (Sumner-Thomson et al., 2011). *ANGPTL4* transcription was inversely associated with the degree of NEB

during lipolytic states as induced by GH and feed restriction in dairy cows (Koltes and Spurlock, 2012). The relative importance of circulating concentration *vs* local expression of ANGPTL4 in AT depots is currently unknown, and ANGPTL4 protein content in AT from periparturient cows has not been determined (Koltes and Spurlock, 2012). Increased hepatic synthesis of ANGPTL4 during periods of NEB might serve as a signal for lipolysis and contribute to the sustained release of FFA and lipid accumulation in the liver (Loor et al., 2007). Therefore, ANGPTL4 could be a potential sensitive marker of AT lipid mobilization due to its regulation by transcriptional factors of adipogenesis and its potent lipolytic activity in adipocytes.

Retinol-binding protein

Retinol-binding protein (RBP) secreted by adipocytes serves as the main transport system for circulating retinol (vitamin A). RBP inhibits adipogenesis and glucose uptake by adipocytes by suppressing the insulin signaling (Klöting et al., 2007). In visceral AT of cows, the expression of RBP was positively associated with that of TNF-α (Rezamand et al., 2012b), which is a recognized inhibitor of adipocyte differentiation. In plasma, RBP concentrations decrease 3 to 6 days before parturition, peak at calving, and then return to pre-calving levels by the second (Eldaim et al., 2010) or third (Rezamand et al., 2012b) week of lactation when lipogenesis also increase. These data suggest that RBP supports homeorhetic mechanisms of metabolic adaptation during the onset of lactation through the downregulation of adipogenesis and lipogenesis in dairy cows. Reduced plasma RBP have been associated with metabolic disorders such as hyperketonemia (Gröhn and Lindberg, 1985), and has been used as part of an index to evaluate liver activity (Trevisi et al., 2001). The clinical relevance of RPB and its effects on AT metabolism in the dairy cow needs to be elucidated.

Tumor necrosis factor alpha

Tumor necrosis factor-α was the first pro-inflammatory cytokine shown to be constitutively expressed in the adipocytes of insulin-resistant animals (Hotamisligil et al., 1993), and is also secreted by AT macrophages (Gregoire et al., 1998). Besides its pro-inflammatory role, TNF-α modulates AT glucose homeostasis and lipid mobilization in an autocrine manner (Hotamisligil et al., 1993, Vernon and Houseknecht, 2000). These effects are mediated by downregulating the production and activity of LPL, fatty acid transport proteins (FATP) and acetyl CoA synthase (Arner, 2003). TNF-α also regulates the synthesis of leptin and plasminogen activator inhibitor 1 (PAI-1), adipocyte fatty acid-binding protein (aP2) and glucose transporter 4 (GLUT4), and is a major contributor to the development of AT insulin resistance (Sethi and Hotamisligil, 1999). Early studies imply that TNF-α is involved in the control of adipocyte hypertrophy since increasing adipocyte size led to increased production of TNF-α, which in turn inhibits adipogenesis (Hotamisligil and Spiegelman, 1994). The net effect of TNF-α is to decrease lipogenesis (FFA uptake and TAG synthesis) and to increase lipolysis (Arner, 2003). Accordingly, in dairy cows during the first 5 weeks of lactation, higher expression of TNF-α in subcutaneous AT coincides with increased lipolysis activity (Sadri et al., 2010).

TNF-α action in AT appears to be more auto- and paracrine than endocrine as it is not released into circulation (Mohamed-Ali et al., 1998). These results are supported by Sadri et al (2010) who described high abundance of *TNFA* mRNA in AT postpartum despite low plasma TNF-α. Therefore, in dairy cows, adipocyte-derived TNF-α may act locally in a similar way to what has been described for monogastric animals (Ronti et al., 2006, Mukesh et al., 2010). AT TNF-α secretion is affected by proadipogenic PPAR-γ agonist drugs. Administration of such as thiazolidinedione enhanced AT adipogenesis and lipogenesis and increased TNF-α plasma

concentrations in periparturient cows; however, AT TNF-α was not evaluated and the plasma concentrations of TNF-α could have derived from immune cells (Schoenberg et al., 2011). Further investigation is needed to determine the actual importance of adipocyte-derived TNF-α in the endocrine function in dairy cows.

Systemic inflammatory responses can affect TNF-α secretion in AT and modify metabolic function as it inhibits the secretion of leptin and adiponectin (Fasshauer and Paschke, 2003, Vernon, 2005). In visceral and subcutaneous AT of dairy cows, the expression of TNF-α and IL6 increased after a 2h-LPS challenge demonstrating that AT is capable of rapidly synthesizing pro-inflammatory cytokines when animals are exposed to inflammatory conditions arising from a pathogenic insult or because of parturition (Mukesh et al., 2010). The same was observed in subcutaneous AT of heat stressed prepartum dairy cows (Zachut et al., 2017). Besides contributing to AT inflammation, administration of TNF-α promoted lipolysis and insulin resistance in AT of dairy steers (Kushibiki et al., 2001). In dairy cows, TNF-α serum activity was elevated during moderate to severe fatty liver and insulin resistance (Ohtsuka et al., 2001). These results demonstrate that TNF-α not only activates inflammatory pathways in AT but also promotes peripheral insulin resistance and lipolysis to increase glucose flow to the mammary gland in dairy cows (Cawthorn and Sethi, 2008) and has the potential to be used as a biomarker of metabolic homeostasis in periparturient dairy cows.

Resistin

Resistin is a small cysteine-rich secretory protein considered both hormone- and cytokine-like adipokine (Holcomb et al., 2000). Resistin is overexpressed in white AT of murine models of obesity and downregulated by the insulin-sensitizing agents thiazolidinediones (Steppan et al., 2001). Komatsu et al. (2003) were the first to report resistin expression in bovine

AT and that its transcription increased in lactating compared with non-lactating dairy cows, while the inverse was observed in the mammary gland.

Resistin modulates lipid metabolism, glucose homeostasis, and feed intake in monogastrics (Tovar et al., 2005, Vázquez et al., 2008). In dairy cows, recombinant bovine resistin promotes lipolysis in subcutaneous AT explants from animals during the first two months of lactation, and its circulating concentrations are positively associated with plasma FFA (Reverchon et al., 2014). Nevertheless, circulating resistin was positively associated with subcutaneous AT hydroxycarboxylic acid receptors-1 (HCAR1) expression which mediates insulin-induced anti-lipolytic effects in dairy cows; however, this effect was not observed in retroperitoneal AT (Weber et al., 2016a). Plasma resistin is also associated with increased adiposity, and negatively associated with plasma leptin in dairy cows fed a low-energy diet from 1 month before calving through lactation (Mellouk et al., 2017). While some studies report an increase in AT and plasma resistin during the first week of lactation (Komatsu et al., 2003, Reverchon et al., 2014), others report an increase in circulating resistin concentrations toward parturition and a decrease during the first week of lactation (Weber et al., 2016a, Mellouk et al., 2017). During early lactation, AT resistin contributes to the development of insulin resistance via inhibition of GLUT4 translocation to the cytomembrane (Komatsu et al., 2003), in a similar way to what was reported in monogastrics (Steppan et al., 2001).

Resistin stimulates the secretion of pro-inflammatory cytokines by macrophages such as TNF-α, IL-6, and IL-12 (Silswal et al., 2005) and is linked to systemic inflammation in cardiovascular diseases, diabetes, obesity and metabolic syndrome in humans [reviewed by (Abate et al., 2014)]. The inflammatory effect of resistin in dairy cows has not yet been studied. Although conjecture, this adipokine could induce systemic and local AT inflammation through

the stimulation of cytokine production. These findings demonstrate that resistin content in plasma and AT are altered by the metabolic status, diet, and adiposity in periparturient cows. Therefore, the potential of resistin to be a marker of insulin resistance and AT inflammation in dairy cows should be further explored.

Visfatin

Visfatin is a cytokine-like adipokine and a multifunctional protein highly conserved across species. Visfatin is expressed ubiquitously (Adeghate, 2008), and was demonstrated to promote cell survival and function by producing nicotinamide mononucleotide, a precursor to NAD+ (Rongvaux et al., 2008). Like insulin, visfatin stimulates glucose uptake and homeostasis (Fukuhara et al., 2005). In dairy cows, AT visfatin mRNA abundance tended to decrease after parturition and was negatively correlated with serum FFA and BHB concentrations (Lemor et al., 2009). Serum visfatin concentrations decreased during the last three weeks before calving and increased back to concentrations observed 3 weeks before parturition by the first week of lactation (Fadden and Bobe, 2016). Visfatin induces pro-inflammatory response through cytokine secretion (Moschen et al., 2007). In cattle, Fadden and Bobe (2016) reported that serum visfatin may serve as chronic disease indicator and could assist in early detection of cows at increased risk for developing retained placenta, ketosis, and metritis (Fadden and Bobe, 2016). These results open the possibility of the study of visfatin as a predictive biomarker for periparturient diseases in dairy cows; however, larger epidemiological studies are needed.

CONCLUSIONS

Adipose tissue functions go beyond energy storage and supply. Through the secretion of adipokines, AT regulates lipid mobilization, insulin sensitivity, and immune and inflammatory functions in an autocrine, paracrine and endocrine manner. In human medicine, adipokines are

used in clinical practice in preventive and diagnostic strategies for metabolic disorders (Blüher, 2012), and are targets for drug development for treatment of metabolic dysfunction (Ouchi et al., 2010), lipodystrophy (Oral et al., 2002), obesity, and diabetes (Blüher, 2014). In dairy cows, adipokines have potential clinical relevance as biomarkers of AT function, lipid mobilization, adiposity, insulin sensitivity, oxidative stress status, and inflammation, however few adipokines have been explored as biomarkers for health status or as disease predictors [Table 1]. In addition to that, providing that their mode of action is fully delineated, and the key molecular targets are identified, adipokines are promising targets for developing novel diagnostic tools, nutritional strategies, and therapies to improve dairy cows' wellbeing and productivity.

FETUIN-A AS A LINK BETWEEN LIPID MOBILIZATION AND INFLAMMATION

Fetuin-A Structure, Biosynthesis and General Properties

Fetuin-A (FetA, *AHSG,* alpha-2-Heremans-Schmid glycoprotein in humans) is a 64-kDa acidic glycoprotein with 3 cystatin-like domains that was first isolated from newborn calf serum in 1944 (Pedersen, 1944). Fetuin-A molecule goes through posttranslational modifications, such as proteolytic processing, glycosylation, phosphorylation (Ser and Thr), and sulfation (Jahnen-Dechent et al., 2011), which may regulate its protein expression levels, stability, and biological activity (Jahnen-Dechent et al., 2011). For example, while bovine FetA is both N- and 0-glycosylated and contains nearly 30% of carbohydrates, that of human contains only 13%. The variation in the degree of glycosylation between species may also be associated with differential activities among humans and bovine FetA (Brown et al., 1992).

Fetuin-A is mainly synthesized and secreted by the liver and to a lesser extent the kidneys, placenta and the tongue (Denecke et al., 2003). In addition to be a hepatokine, FetA has been recently considered an adipokine (Chatterjee et al., 2013, Jialal et al., 2015, Pérez-Sotelo et al., 2016). In these studies, FetA secretion from subcutaneous adipose tissue (AT) was increased in patients with the metabolic syndrome (Jialal et al., 2015), while in rats, FetA was increased in visceral and subcutaneous AT during obesity and high fat diet (Pérez-Sotelo et al., 2016). Besides its involvement in lipid metabolism, as indicated by these studies demonstrating variations in AT abundance depending on AT dysfunction, fat mass, and amounts of fat in the diet, a number of studies suggest that FetA is a multifunctional protein. Among its main functions, FetA is an inhibitor of ectopic calcification (Schäfer et al., 2003) and has a primarily role as a binding and carrier protein like albumin (Jahnen-Dechent et al., 2011). Fetuin-A binds to matrix metalloproteinases (particularly MMP-9) and protects this enzyme from autolytic

degradation (Kübler et al., 2007). Upon its rapid calcium-dependent uptake by cells (Chen et al., 2007), FetA also mediates critical roles for cells' metabolism including the activation of PI3 kinase/Akt (Kundranda et al., 2004) and the mediation of growth signaling in tumor cells (Sakwe et al., 2010). Notably, FetA is an antagonist of TGF-ß in vivo, in that it inhibited intestinal tumor progression (Swallow et al., 2004). FetA also binds and carries lipids in plasma, thus being associated with strong lipogenic activity (Kumbla et al., 1989, Kumbla et al., 1991). Although FetA binding and carrier properties have been consistently described, a specific receptor for FetA on plasma membrane of cells has not yet been identified.

Fetuin-A and lipid metabolism

Early studies have characterized bovine FetA as a lipoprotein-like particle corresponding to the density of a high density lipoprotein (HDL) (Kumbla et al., 1989, Kumbla et al., 1991). Accordingly, FetA binds and transports considerable amounts of lipids including cholesterol, cholesteryl esters, triacylglycerol (TAG), and fatty acids (FA) that nearly account for 33% of the its molecule (Kumbla et al., 1991, Subbiah, 1991, Jialal et al., 2016). Like FA binding proteins (i.e. FABP4, FATP1), FetA reversibly binds hydrophobic ligands, including saturated and unsaturated long-chain FA, and other lipids with high affinity (Furuhashi and Hotamisligil, 2008). Others studies suggest that FetA may also remove cholesterol from cells (Kumbla et al., 1991). In this study, the incubation of FetA with cells increased FetA-binding with cholesterol and cholesteryl ester, but had no changes in cellular cholesterol content, while the cellular TAG content increased markedly (Kumbla et al., 1991).

Potent adipogenic and lipogenic activities have been attributed to FetA. An early in vitro study with 1246 adipogenic cell line and 3T3-L1 cells reported the existence of three adipogenic factors in crude FetA preparation (Zaitsu and Serrero, 1990). In adipogenic models with rabbit

and human's cells, FetA increased incorporation of exogenous FA into cellular TAG by 50 times compared with albumin—the best characterized FA transporter— (Kumbla et al., 1989, Cayatte et al., 1990). However, the mechanisms by which FetA increased TAG accumulation have not yet been identified. Whether the lipogenic activity of FetA is due to the facilitation of FA entry into cells or whether FetA might directly stimulate lipogenic enzymes in TAG synthesis pathway is not known. According to its lipogenic properties, FetA has been associated with increased fat mass, obesity, high plasma FFA concentrations in rodents and humans, and with lipid-induced insulin resistance (Chen et al., 2009, Pal et al., 2012, Pérez-Sotelo et al., 2016). Notably, loss of fat mass induces a reduction in plasma FetA content in obese humans (Brix et al., 2010, Choi et al., 2013). These studies highlight not only the involvement of FetA in lipogenic pathways, but also an effect of TAG breakdown on FetA synthesis, and potentially, on its function in AT.

Fetuin-A has been associated with differential regulation of lipolysis. A common variation, single nucleotide polymorphism (SNP) rs4917 (Thr230Met), in the FetA gene was associated with a marked increase in β2-adrenoceptor sensitivity. This variation affects the sensitivity for lipolysis in adipocytes, and may be of importance in body weight regulation in humans (Lavebratt et al., 2005a). In fact, another common variant of FetA gene (*AHSG*; homozygosity for the rs2593813:G-230:Met238:Ser haplotype) associated with a lower FetA concentrations, was more common among lean than obese and overweight Swedish men (Lavebratt et al., 2005b). In a different study, two FetA SNPs (-469T>G and IVS6+98C>T) were connected to dyslipidemia in human subjects and -469T>G SNP was associated with insulin-mediated inhibition of lipolysis and stimulation of lipogenesis in adipocytes (Dahlman et al., 2004, Andersen et al., 2008). Dahlman et al. (2004) suggested that FetA may control insulin

signaling in AT, thus being an attractive candidate gene for the treatment of disturbed adipocyte lipolytic function in obesity and insulin resistance in humans (Dahlman et al., 2004).

These results consistently demonstrate the roles of FetA on the enhancement of lipogenic activity and modulation of lipolytic responses in adipocytes. However, whether FetA plays similar roles on lipid metabolism of dairy cows and may contribute to the improvement of the lipogenic function during periods of intense lipid mobilization in bovine adipocytes are completely unknown.

Fetuin-A and inflammatory responses

Fetuin-A is traditionally regarded as one of the few negative acute- phase proteins (APP) (Wang et al., 1997, Ombrellino et al., 2001b, Wang and Sama, 2012). While FetA is known to inhibit TNF- α production by immune cells during acute inflammation (Wang and Sama, 2012), this protein is also downregulated by TNF- α, IL-6, IL-1, and interferon gamma (IFN-γ) (Daveau et al., 1988, Li et al., 2011). Low serum FetA concentrations have been strongly associated with inflammatory markers like C-reactive protein (Wang et al., 2005, Metry et al., 2008). The strong anti-inflammatory effects of FetA were verified *in vivo* using several models of inflammation, including lipopolysaccharide-induced miscarriage in rats(Dziegielewska and Andersen, 1998), carrageenan injection(Ombrellino et al., 2001b), cerebral ischemic injury in rodents(Wang and Sama, 2012), and cecal ligation and puncture in mice (Li et al., 2011). In all these reports, FetA was associated with reduced inflammatory response and increased survival and administering additional FetA generally improved outcome. Accordingly, FetA deficiency is linked to features of the malnutrition-inflammation-atherosclerosis syndrome and to cardiovascular events and mortality in dialysis patients (Wang et al., 2005).

Few studies have focused on the roles of FetA on AT inflammation. During chronic obesity-induced inflammation, FetA appears to upregulate pro-inflammatory mediators and impairs the response to insulin in cells of AT, liver and skeletal muscle (Stefan et al., 2008, Dasgupta et al., 2010). Adipose tissue expression of FetA was upregulated during chronic inflammation in obese dyslipidemic humans and rodents (Heinrichsdorff and Olefsky, 2012, Jialal et al., 2015, Pérez-Sotelo et al., 2016). In fact, FetA was identified as an endogenous ligand between FFA and toll-like receptor-4 in adipocytes that triggers lipid-induced inflammation resulting in insulin resistance in AT (Heinrichsdorff and Olefsky, 2012, Pal et al., 2012). In contrast to these results, but in agreement with other reports classifying FetA as a negative APP, in the first report of FetA abundance in AT of dairy cows, FetA expression decreased 1.5 times in the subcutaneous AT of cows in late gestation during environmental heat stress (Zachut et al., 2017). This decrease on AT FetA abundance was associated with a decrease in other negative APP such as albumin, hemopexin, serotransferrin and apolipoprotein A-II (Zachut et al., 2017). One of the potential explanations for this finding is that FetA is downregulated by different pro-inflammatory mediators such as TNF- α and IL6, (Wang and Sama, Zhang et al., 2014), which are known to be upregulated in AT of periparturient dairy cows during intense lipid mobilization (Contreras et al., 2015). While periparturient dairy cows have increased plasma FFA concentrations, which lead to a systemic and local AT inflammation (Saremi et al., 2014, Contreras et al., 2015, Mann et al., 2016), the potential effects of FetA supporting anti-inflammatory responses during exacerbated FFA release around parturition and through early lactation in dairy cows remain unknown.

CONCLUSIONS

Based on the importance of FetA in lipid metabolism and inflammatory responses, FetA

has emerged as a predictor and biomarker of diseases and metabolic dysfunction in several clinical epidemiological studies in human patients. Evidence from in vitro and in vivo studies with non-ruminants, and from the single report describing decreased FetA abundance in AT of periparturient cows during a pro-inflammatory and pro-lipolytic state (Zachut et al., 2017), support the possibility that FetA could also be involved in the modulation of AT functions in dairy cows, especially during the periparturient period when lipid mobilization and inflammatory responses are intensified. Based on previous reports, FetA may have the potential to buffer excessive release of FFA from AT of periparturient dairy cows by (1) stimulating the use of circulating FFA for intracellular TAG synthesis in AT, (2) mediating insulin-inhibition of lipolysis, and (3) preventing dysregulated pro-inflammatory responses, thus also attenuating exacerbated lipolytic activity in AT. However, very little is known about FetA dynamics and roles in dairy cows. A better understanding of the role of FetA on modulating the metabolic and AT function of periparturient dairy cows could lead to the development of novel management, nutritional, or pharmaceutical interventions to reduce around parturition.

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CHAPTER 3

FETUIN-A: A NEGATIVE ACUTE-PHASE PROTEIN LINKED TO ADIPOSE TISSUE FUNCTION IN PERIPARTURIENT DAIRY COWS

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ABSTRACT

Fetuin-A (**FetA**) is a free fatty acid (**FFA**) transporter and an acute-phase protein (**APP**) that enhances cellular lipid uptake and lipogenesis. In non-ruminants, FetA is involved in lipid-induced inflammation. Despite FetA importance in lipid metabolism and inflammation, its expression and dynamics in adipose tissue (**AT**) of dairy cows are unknown. The objectives of this study were to: 1) Determine serum and AT FetA dynamics over the periparturient period and in mid-lactation cows in negative energy balance (**NEB**) after a feed restriction protocol. 2) Characterize how an inflammatory challenge affects adipocyte FetA expression. Blood and subcutaneous AT were collected from 16 cows with high (≥3.75, *n*=8) or moderate BCS (≤3.5, *n*=8) at -26±7 (far off, **FO**) and -8±5 (close-up, **CU**) days before calving, and at 10±2 d after parturition (early lactation, **EL)**, and from 14 non-pregnant mid-lactation cows (>220 DIM) after a feed restriction protocol. Serum FetA concentrations were 0.89±0.13 at FO, 0.96±0.13 at CU, and 0.77±0.13 mg/mL at EL, and 1.09±0.09 and 1.17±0.09 mg/mL in feed-restricted and control cows, respectively. Serum and AT FetA contents decreased at the onset of lactation when lipolysis was higher. No changes in AT and serum FetA were observed after feed restriction induced-NEB in mid-lactation cows. Prepartum BCS had no effect on serum FetA, but AT expression of *AHSG,* the gene encoding FetA, was reduced in periparturient cows with high BCS at dry off throughout all time points. Circulating FetA was positively associated with serum

albumin and calcium, and with BCS variation over the periparturient period. The dynamics of *AHSG* expression were analogous to the patterns of lipogenic markers *ABDH5, ELOVL6, FABP4, FASN*, *PPARy* and *SCD1*. *AHSG* and FetA protein expression in AT were inversely correlated with AT pro-inflammatory markers *CD68, CD44, SPP1*, and *CCL2*. In vitro, bovine adipocytes challenged with lipopolysaccharide (**LPS**) downregulated FetA protein expression. Adipocytes treated with FetA had lower *CCL2* expression compared to those exposed to LPS. Overall, FetA is a systemic and local (AT) negative APP linked to AT function in periparturient cows. Furthermore, FetA may support physiological adaptations to NEB in periparturient cows.
Key words: adipose tissue, biomarker, inflammation, lipolysis, transition cow

CHAPTER 4

IN VITRO ADIPOGENIC DIFFERENTIATION OF BOVINE PREADIPOCYTES: A CO-CULTURE MODEL

ABSTRACT

Reductionists studies of adipose tissue (**AT**) biology require reliable in vitro adipocyte culturing models. Current protocols for adipogenesis induction in stromal vascular fraction (**SVF**)-derived preadipocytes require extended culturing periods and have low adipogenic rates, especially in visceral SVF. We compared the adipogenic efficiency of direct co-culture of adipocytes with preadipocytes and the standard adipocyte differentiation in visceral and subcutaneous bovine adipocytes. SVF-derived preadipocytes and mature adipocytes were obtained by collagenase digestion from subcutaneous and visceral AT of dairy cows ($n = 6$). Primary adipocytes were retained for use in co-culture. Confluent preadipocytes were induced to differentiate with medium containing insulin, acetate, troglitazone, 2-isobutyl-1-methylaxanthine (IBMX) and dexamethasone for 48 h, and then maintained in this medium, excluding the dexamethasone and IBMX, for 7 d in co-culture (**CC7**), and for 7 d (**SD7**) or 14 d (**SD14**) in standard differentiation. For CC7, 900 primary adipocytes/cm^2 were added to 0.4 μm transwell inserts and placed in the wells with preadipocyte cultures for the first 5 d of differentiation. Adipogenic efficiency was evaluated by gene expression of adipogenesis markers, triacylglycerol (**TAG**) accumulation, and glycerol release upon ß-adrenergic stimulation. CC7 and SD14 similarly stimulated higher gene expression of *ADIPOQ, CEBPA, CEBPB*, *AHSG*, *FABP4* and *LIPIN1* in visceral and subcutaneous cells compared with preadipocytes ($P < 0.05$). Mature adipocytes viability in CC7 decreased 50% from the d 1 to d 5 of induction. TAG accumulation in subcutaneous and visceral adipocytes in CC7 incremented 40 and 16-fold compared with 22 and 4-fold increment in SD14, respectively. In the same period as SD7, CC7

incremented TAG accumulation by 8 times in subcutaneous and visceral adipocytes ($P < 0.05$). As measured by glycerol concentrations, responsiveness to ß-adrenergic stimulation was higher in CC7 and SD14 than SD7 in subcutaneous adipocytes ($P < 0.05$); in visceral adipocytes, CC7 had higher glycerol release than SD7 ($P < 0.05$), however SD7 and SD14 were similar ($P > 0.05$). Overall, CC7 is more efficient inducing adipogenesis in visceral and subcutaneous bovine adipocytes than SD14 since it stimulated similar responses in a shorter period of time. This protocol will facilitate the use of reductionist models to study adipocyte physiology in periparturient dairy cows and the assessment of pharmacological or nutritional interventions in conditions closer to those observed *in vivo*.

Key words: Adipocytes, adipogenesis, bovine, co-culture, in vitro

TECHNICAL NOTE

During the periparturient period, dairy cows rely on adipocyte triacylglycerol (**TAG**) reserves to offset the effects of negative energy balance (Contreras and Sordillo, 2011b). Through its secretome, adipose tissue (**AT**) regulates energy balance and storage, nutrient transport, glucose homeostasis, lipid metabolism, and immune function (Trayhurn, 2013). In order to expand our knowledge about the underlying mechanisms of adipocyte functions in periparturient dairy cows, including lipid mobilization and adipokine roles, it is necessary to use reductionist approaches such as *in vitro* adipocyte culturing. Primary adipocyte isolation and culturing is difficult because these cells are buoyant and lack sufficient matrix proteins to facilitate *in vitro* adherence (Sugihara et al., 1987, Picot et al., 2005). An alternate approach is to isolate stromal vascular fraction (**SVF**) and induce the differentiation of those cells into adipocytes. Across species, *in vitro* adipocyte differentiation is induced by preadipocyte exposure to a mixture of hormones that includes glucocorticoids, an agent to increase cAMP activity, and high concentrations of insulin (MacDougald and Mandrup, 2002). In bovines, adipocyte differentiation is complemented by supplementation of individual unbound fatty acids such as acetate and octanoate, partially characterized bovine serum lipids, and pharmacological peroxisomal proliferation activated receptor gamma ligands such as rosiglitazone and troglitazone (Grant et al., 2008, Smith et al., 2009, Lengi and Corl, 2010).

To increase adipogenic and lipogenic efficiency, addition of phosphatidylcholine, TAG, lipoproteins derived from human plasma, and fatty acids (i.e. oleic and linoleic acids) have been used as alternative approaches to the standard pharmacological induction (Wu et al., 2001, Yanting et al., 2018). However, even lipid-enriched adipocyte differentiation protocols take 12 d to 14 d differentiating preadipocytes into adipocytes. Although sources of lipids and extended

differentiation protocols have been used to develop suitable reductionist models for the study of bovine AT, low efficiency of adipogenesis is still an issue, mainly when using visceral SVF. Visceral adipocytes differentiate poorly, only 30%–40%, compared with the robust differentiation seen in subcutaneous cells (Macotela et al., 2012). As a result, information on visceral-specific regulatory mechanisms and adipokine secretion in bovine are limited. Another limiting characteristic of standard differentiation protocol is the lack on emulating important conditions of *in vivo* AT environment, such as cell by cell communication (Stacey et al., 2009). In adipogenic induction of human adipocytes, the direct co-culture of primary adipocytes with preadipocytes using a transwell insert induces adipogenesis more effectively when compared to standard pharmacological differentiation protocol while maintaining the expression of adipogenic markers (Stacey et al., 2009). It was suggested that mature adipocytes may release factors that promote preadipocyte differentiation and maturation, thus promoting a closer mimic of *in vivo* environment (Considine et al., 1996). Given the need for developing a more efficient and physiological adipogenic model for bovine cells that could also be suitable for the on the differentiation of both, visceral and subcutaneous SVF, the goal of this study was to compare the efficiency of two different culture conditions in stimulating adipogenic differentiation: Direct co-culture of adipocytes with preadipocytes in a transwell insert and the standard adipocyte differentiation medium.

Subcutaneous and omental AT samples were obtained from 6 different dry cows at a local abattoir. Stromal-vascular fraction was isolated as described previously (Contreras et al., 2015, Strieder-Barboza et al., 2018). Briefly, AT samples were collected during carcass processing from the flank (subcutaneous) and omental (visceral) region. Specimens were transported to the laboratory while in Krebs-Ringer Bicarbonate Buffer (KRBB) supplemented

with HEPES 10 mM (pH = 7.4) and gentamicin solution (50µ/mL) at 37°C. Once in the laboratory, 500 mg of AT were washed three times in KRBB and minced in 2 to 3 mm sections in 5 mL collagenase type II solution (2 mg/mL; Worthington Biochemical, Lakewood, NJ) in KRBB with 4% bovine serum albumin (BSA, Millipore-Sigma, USA). Samples were incubated in a 37°C water bath with inversion of the vials every 5 min for 15 min and then transferred to an incubator for further digestion with shaking for 45 min at 37°C and 230 rpm. After incubation, digested material was centrifuged for 10 min at 300 x g to separate primary adipocytes (upper layer) from SVF (lower layer). Primary mature adipocytes were filtered through a 250 µm mesh while washed in 5 mL of KRBB with 4% BSA, then centrifuged for 5 min at 300 x g. Final primary adipocyte population was retained for culture in transwell insert in the co-culture condition. The viability of freshly isolated adipocytes was measured by Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher, Waltham, MA). After digestion, SVF was sequentially filtered through 100 µm and 40 µm cell strainers (Falcon, Corning, NY) and centrifuged for 10 min at 800 x g. Resulting cell pellet was resuspended and incubated in erythrocyte lysis buffer for 5 min at room temperature. After another centrifugation, resultant cells were resuspended in 9 mL of basal medium containing Dulbecco's modified Eagle's medium: F12 (DMEM:F12, Corning, Corning, NY), 10% fetal bovine serum (FBS; Corning), 2 mmol/L of L-glutamine (Corning), 1% (vol/vol) antibiotic-antimycotic (Corning), 44.05 mmol/L of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 100 µmol/L of ascorbic acid (Sigma-Aldrich), 33 µmol/L of biotin (Sigma-Aldrich), 17 µmol/L of pantothenate (Sigma-Aldrich), and 20 mmol/L of HEPES (Corning), and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.. Growth medium replacement was performed every 2 d (Strieder-Barboza et al., 2018). Preadipocytes were obtained by outgrowth of plastic adherent cells from the SVF cells after 2 serial passages in

culture flasks (Corning). Expanded preadipocytes populations were seeded in 24- and 12-well plates (Costar®, Corning) and allowed to proliferate to confluency. Confluent preadipocytes were induced to differentiate with basal medium supplemented with 5 µM (Cayman Chemical, Ann Arbor, MI), 0.5 mmol/L isobutyl-1-methylaxanthine (IBMX; AdipoGen Life Sciences, San Diego, CA) and the following reagents from Sigma-Aldrich: 5 µg/mL insulin, 10 mM acetate, and 1 µmol/L dexamethasone. IBMX and dexamethasone were used only during the first 48 h of induction. For the co-culture condition, a differentiation protocol of 7 d was used (**CC7**). Briefly, 0.4 µm transwell inserts (Greiner Bio-One, Kremsmünster, Austria) were used to generate a co-culture system of adipocytes and preadipocytes. Freshly isolated mature adipocytes (900 cell/cm^2) were added to the inserts and then placed into the wells of 24- or 12-well plates containing preadipocyte cultures for the first 5 d of adipogenic induction. After removing inserts from wells at d 5, attached adipocytes were kept in differentiation media for 2 extra days. For standard differentiation condition, preadipocytes were induced to differentiate using the same medium as for CC7 during 7 d (**SD7**) or 14 d (**SD14**). SD7 served as a control for CC7, which's protocol duration was also 7 d. After treatments, the adipogenic differentiation adipocytes was analyzed via the quantification of gene expression of adipogenic and lipogenic markers, TAG accumulation, and release of glycerol upon ß-adrenergic stimulation. Gene expression of adipocyte activity markers adiponectin (*ADIPOQ),* fatty acid translocase *(CD36),* CCAAT/enhancer-binding protein beta and alpha (*CEBPB, CEBPA),* diacylglycerol O-Acyltransferase -1 and -2 (*DGAT1, DGAT2),* fatty acid binding protein 4 (*FABP4),* fatty acid transporter-1 (*FATP1),* glycerol-3-phosphate acyltransferase-1 and -2 (*GPAT2, GPAT2),* and peroxisome proliferator-activated receptor gamma *(PPARG)* were evaluated (Supplemental Table 1). RNA extraction, purification, conversion to cDNA and quantitative PCR analysis were

performed as described previously by our laboratory (Contreras et al., 2017a). The most stable reference genes eukaryotic translation initiation factor 3 subunit K (*EIF3K),* 50S ribosomal protein L15 (*RPL0),* and 40S ribosomal protein S9 *(RPS9)* were selected using geNorm (Vandesompele et al., 2002). The Cq values were converted to normalized relative gene expression as described by Hellemans et al. (2007b). The calibrator was set as the preadipocytes (PRE) cultured in growth media. AdipoRed™ Adipogenesis Assay (Lonza, Allendale, NJ) and HCS LipidTox™ (Life Technologies, Carlsbad, CA) staining were performed to quantitatively examine TAG accumulation.

Supplemental Table 4. 1 mRNA probes by product and NCBI accession numbers

Gene	Product[1]	RefSeq
AHSG[2]	Bt.23250	NM_173984.2
ADIPOQ	Bt03292341_s1	NM_174742.2
CD36	Bt03234878_m1	NM_001046239.1.
CEBPA	Bt03224529_s1	NM_176784.2
DGAT1	Bt03251718_g1	NM_174693.2
DGAT2	Bt03259837_m1	NM_001253891.1
EIF3K	Bt03226565_m1	NM_001034489.2
FABP4	Bt03213820_m1	NM_174314.2
FATP1	Hs01587911_m1	NM_198580.2
GPAT1	APU63EN	-
PPARG	Bt03217547_m1	NM_181024.2
RPL0	Bt03218086_m1	NM_001012682.1
RPS9	Bt03272016_m1	NM_001101152.2

[1]Thermo Fisher, Waltham, MA, USA. [2] Integrated DNA Technologies, Coralville, IA, USA.
AHSG: Fetuin-A; *ADIPOQ:* Adiponectin; *CD36:* Fatty acid translocase; *CEBPB:* CCAAT/enhancer-binding protein beta; *CEBPA:* CCAAT/enhancer-binding protein alpha; *DGAT1:* Diacylglycerol O-Acyltransferase-1; *DGAT2:* Diacylglycerol O-Acyltransferase-2; *EIF3K:* Eukaryotic translation initiation factor 3 subunit K; *FABP4:* fatty acid binding protein 4; *FATP1:* Fatty acid transporter-1; *GPAT1:* Glycerol-3-Phosphate Acyltransferase-1; *GPAT2:* Glycerol-3-Phosphate Acyltransferase-2, *PPARG:* Peroxisome proliferator-activated receptor gamma; *RPL0:* 50S ribosomal protein L15; *RPS9*: 40S ribosomal protein S9.

Confocal microscopy imaging was performed in HCS LipidTox™ stained cells (red fluorescence) using an Olympus FluoView 1000 Confocal Laser Scanning Microscope (Olympus America, Inc., Center Valley, PA) configured on an IX81 inverted microscope and FV10-ASW software (version 4.2.3.6) using a UPLFLN 20×/0.50 dry objective. Glycerol release was evaluated after a 2 h-β-adrenergic stimulation with 1 μM isoproterenol (ISO, Sigma-Aldrich), a

β-adrenergic receptor agonist. Media alone [KRBB-HEPES containing 3% FA free BSA (Millipore-Sigma)] served as negative control (CON). Glycerol concentrations in the supernatant were analyzed in triplicates (cat. no. MAK117-1KT, Millipore-Sigma) and corrected by the number of cells. Data were analyzed using JMP Statistical Software (SAS Institute Inc., Cary, NC). Normality of the variables was checked using the Kolmogorov-Smirnov test ($P < 0.05$). Non-normally distributed variables were ln transformed. One-way ANOVA Pairwise comparisons were performed using the Tukey's post hoc test. Results are presented as MEAN ± SEM unless otherwise stated. Mean differences were considered significant when $P \leq 0.05$ and tendencies when $P < 0.10$. Adipocyte differentiation or adipogenesis is the result of sequential changes from a fibroblast-like cell to a lipid-filled cell, with the expression of transcription factors, genes, and enzymes indicative of a mature fat cell (Grant et al., 2008). The hallmark of adipogenesis is the end of cellular replication and the beginning of biosynthesis of specialized proteins required for cytoplasmic accumulation of TAG (MacDougald and Mandrup, 2002). Limited information is available on the culture conditions to improve adipogenic efficiency and developing reductionist models that closely emulate the AT conditions in dairy cows. In this study, preadipocytes were differentiated into adipocytes under two different culture conditions and evaluated for adipogenic efficiency: Standard differentiation and co-culture containing mature adipocytes in transwell inserts. This co-culture method has been previously used in the differentiation of human subcutaneous adipocytes (Considine et al., 1996, Janke et al., 2002, Stacey et al., 2009), but has not yet been used in the culture of visceral adipocytes, nor with bovine adipocytes. Therefore, this is the first study to evaluate the adipogenic efficiency of a short 7-d co-culture differentiation protocol between bovine adipocytes and preadipocytes as a model for culturing visceral and subcutaneous adipocytes. In the present study, even though the

viability of adipocytes placed on inserts decreased 50% from d 0 to d5 of differentiation in CC7 ($P < 0.05$; Fig. 4.1), CC7 did demonstrate better adipogenic efficiency than SD7 and SD14.

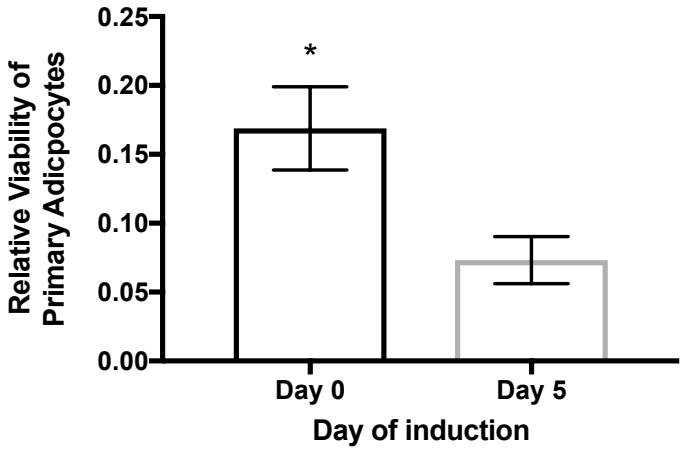

Figure 4. 1 Viability of primary bovine adipocytes placed in inserts during co-culture protocol.
Samples (100 uL) from transwell inserts with primary adipocytes were assayed in triplicates at d 0 and d 5 after adipogenesis induction using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher) following directions of the manufacturer. Values are expressed as mean ± SEM. *Statistically significant ($P < 0.05$).

By the end of the differentiation protocol, CC7 and SD14 similarly stimulated an increase in the gene expression of *ADIPOQ, CEBPA*, *CEBPB* and *AHSG* ($P > 0.05$; Fig. 4.2 and Fig. 4.3) compared with their corresponding preadipocytes ($P < 0.05$). Subcutaneous adipocytes in CC7 and SD14 also increased gene expression of the FA transporter *FABP4,* while in visceral cells, CC7 and SD14 increased LIPIN1, a required lipogenic enzyme for TAG synthesis ($P <0 .05$). While *CEBPB* and *CEBPA* expression directs the differentiation process, expression of *ADIPOQ, FABP4, AHSG* and *LIPIN1* promote FA transport and TAG accumulation and therefore are highly expressed in lipid-filled mature adipocytes (Arimochi et al., 2016). These results demonstrate that in a shorter period of time, CC7 efficiently stimulated the transcription of critical adipogenic and lipogenic regulators in subcutaneous and visceral cells as induced by a 14-d standard differentiation protocol. Adipose tissue function during the periparturient period

supplies the energy necessary to support the rapid fetal growth and the lactation onset, thus depending on TAG reserves in adipocytes to offset negative energy balance. The development of functional and efficient cell culture models of AT is extremely beneficial to *in vitro* assays that would assess adipocyte lipolytic and lipogenic responses during this critical time in dairy cows, thus improving our knowledge of mechanistic adaptations to metabolic challenges.

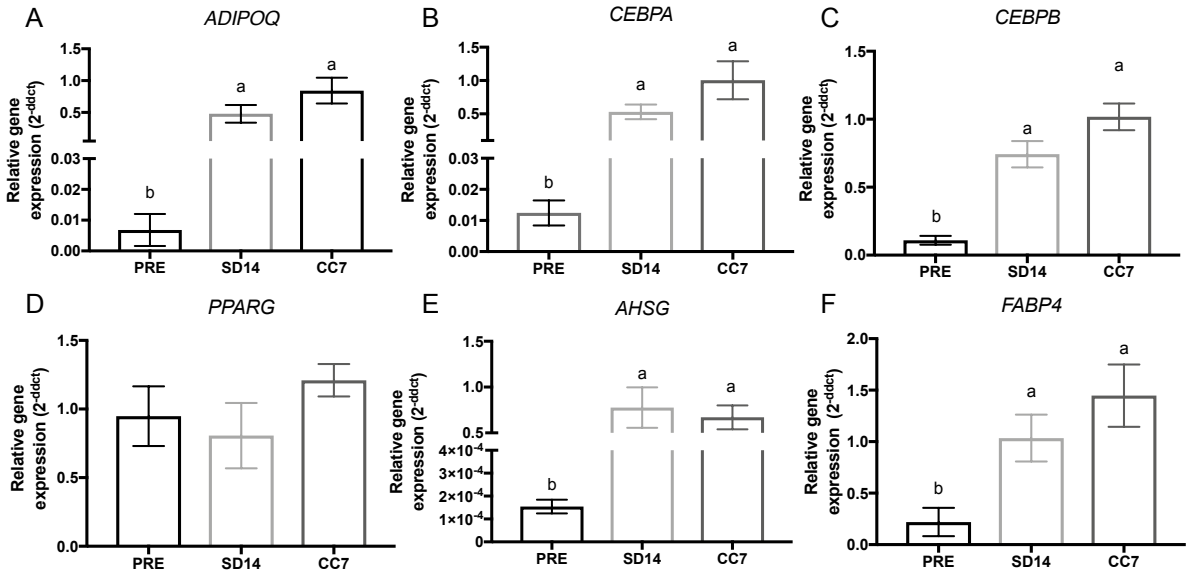

Figure 4. 2 Effect of culture conditions on gene expression of adipogenic markers in bovine adipocytes from subcutaneous adipose tissue depot.
Gene expression of adipogenic and lipogenic markers in subcutaneous bovine adipocytes cultured with a standard differentiation protocol of 14 d (SD14) or using a co-culture model (CC7). Preadipocytes (PRE) from tailhead subcutaneous adipose tissue depot were used as controls. The relative gene expression of (A) Adiponectin (*ADIPOQ*), (B) CCAAT/enhancer-binding protein alpha (CEBPA), (C) CCAAT/enhancer-binding protein beta (CEBPB), (D) Peroxisome proliferator-activated receptor gamma (PPARG), (E) Fetuin-A (AHSG), and (F) Fatty acid binding protein-4 (FABP4) were normalized by control genes 40S ribosomal protein S9 (*RPS9),* Eukaryotic translation initiation factor 3 subunit K (*EIF3K)*, and 50S ribosomal protein L15 (*RPLO)*.Values are shown as $2^{(-\Delta\Delta CT)}$ (where CT = cycle threshold). Results represent means ± SEM.

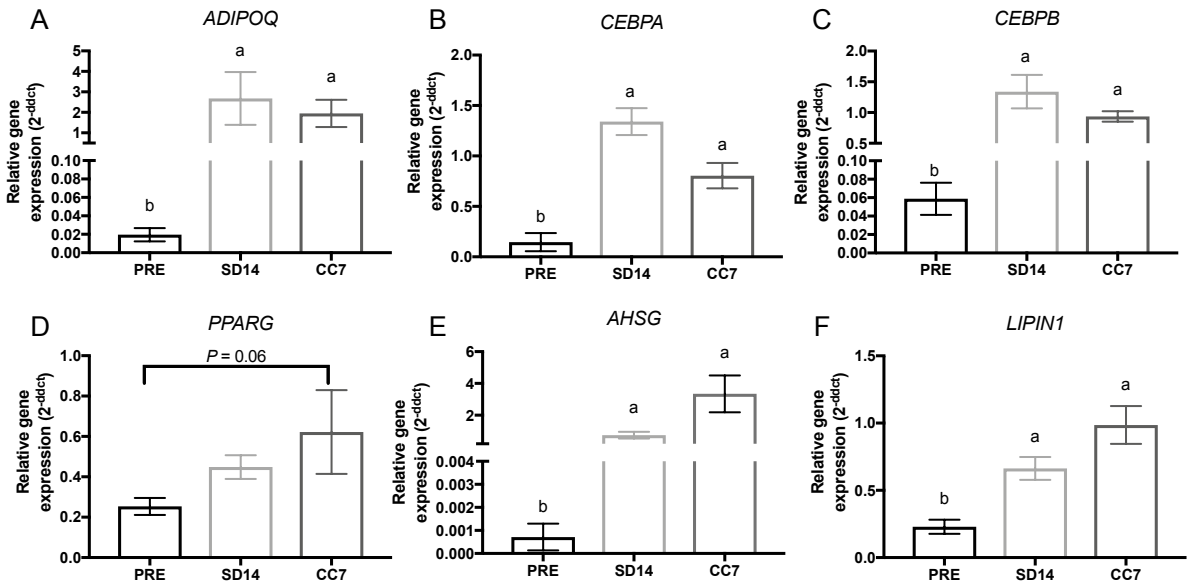

Figure 4. 3 Effect of culture conditions on gene expression of adipogenic markers in bovine adipocytes from visceral adipose tissue depot.
Gene expression of adipogenic and lipogenic markers in visceral bovine adipocytes cultured with a standard differentiation protocol of 14 d (SD14) or using a co-culture model (CC7). Preadipocytes (PRE) from omental visceral adipose tissue depot were used as controls. The relative gene expression of (A) Adiponectin (*ADIPOQ*), (B) CCAAT/enhancer-binding protein alpha (CEBPA), (C) CCAAT/enhancer-binding protein beta (CEBPB), (D) Peroxisome proliferator-activated receptor gamma (PPARG), (E) Fetuin-A (AHSG), and (F) phosphatidate phosphatase-1 (LIPIN1) were normalized by control genes 40S ribosomal protein S9 (*RPS9),* Eukaryotic translation initiation factor 3 subunit K (*EIF3K)*, and 50S ribosomal protein L15 (*RPLO)*.Values are shown as 2(−ΔΔCT) (where CT = cycle threshold). Results represent means ± SEM.

In this study, we quantitatively evaluated adipogenic efficiency through the measurement of cytoplasmic TAG accumulation in adipocytes. Even though CC7 and SD14 had similar TAG accumulation ($P > 0.05$; Fig. 4.4 and Fig. 4.5), respectively, CC7 was more efficient than SD14 in both, visceral and subcutaneous adipocytes, because CC7 synthetized similar amounts of cytoplasmic TAG in the half of time took by SD14. Relative to preadipocytes TAG accumulation, CC7 incremented in 40- and 16-fold the total lipid accumulation in subcutaneous and visceral adipocytes compared with an increment of 22 and 4-fold in SD14, respectively. In the same differentiation time-point (d 7), CC7 incremented by 8 times the TAG accumulation in

subcutaneous and visceral adipocytes compared with SD7 ($P < 0.05$; Fig. 4.4N, 4.5N). The augmentation of TAG accumulation in co-culture conditions compared with standard differentiation was also previously observed in subcutaneous human adipocytes (Stacey et al., 2009). Based on previous reports and on our results, the secretome of the mature adipocytes present in the inserts during co-culture may aid in the differentiation of adjacent cells enhancing lipogenic activity of adipocytes (Considine et al., 1996).

As important as the capacity of synthetizing and accumulating TAG, is the ability of responding to lipolytic stimulus to release glycerol and free fatty acids during periods of negative energy balance as the peripartum in dairy cows. As exacerbated lipolysis in closely related with metabolic disorders and high economic losses in the dairy industry, the development of reliable reductionist models for the study of lipolytic pathways and strategies to modulate them are crucial. In our study, we evaluated the functionality of co-culture and standard protocols through the quantification of glycerol release upon the adipocytes stimulation with a ß-adrenergic receptor agonist. In subcutaneous adipocytes, responsiveness to ß-adrenergic stimulation was higher in CC7 and SD14 compared with SD7, as demonstrated by higher glycerol release in ISO over CON ($P < 0.05$; Fig. 4.6A). Total glycerol released by subcutaneous adipocytes upon stimulation of lipolysis were 272 ± 131 nM, 987 ± 303 nM, and 1362 ± 208 nM for SD7, CC7 and SD14, respectively, and were similar between CC7 and SD14 ($P > 0.05$), and higher in SD14 compared with SD7 ($P < 0.05$). Visceral adipocytes released approximately half of glycerol concentrations compared with subcutaneous cells (Fig. 4.6C, 4.6D) and did not differ between SD7, SD14 and CC7 ($P > 0.05$). Upon ISO-stimulation, visceral adipocytes released 300 ± 79 nM, 621 ± 80 nM, and 480 ± 135 nM glycerol for SD7, SD14 and CC7, respectively. Two possible reasons for these findings are (1) the lower TAG accumulation in visceral

adipocytes compared with subcutaneous, and (2) lower ß-agonist effectivity of ISO in the stimulation of lipolysis in mesenteric adipocytes, as previously reported (Umekawa et al., 1997). Notably, while SD7 and SD14 responses to lipolysis stimulation were similar in visceral adipocytes ($P > 0.05$), CC7 had greater responsiveness than SD7 ($P < 0.05$, Fig. 4.6B). These results suggest that co-culture may be a suitable model for evaluating lipolytic responses and pathways in visceral adipocytes. This is particularly important because many *in vitro* studies have been performed with only subcutaneous adipocytes due to the low adipogenic efficiency of visceral SFV, potentially missing visceral-specific regulatory mechanisms and differential physiological functions compared with other AT depots (Macotela et al., 2012).

In this study, we demonstrated that a 7-d co-culture protocol with visceral or subcutaneous bovine adipocytes improves adipogenic efficiency by promoting similar responses than a 14-d standard differentiation protocol in a shorter period of time. Co-culture model may better mimic the complex differentiation stimuli provided *in vivo* and may be a more suitable model for studying visceral adipocyte functions compared with standard model. Therefore, co-culture is physiologically relevant for reductionist models of adipocyte biology studies in periparturient dairy cows and the assessment of pharmacological or nutritional interventions in conditions closer to those observed *in vivo*.

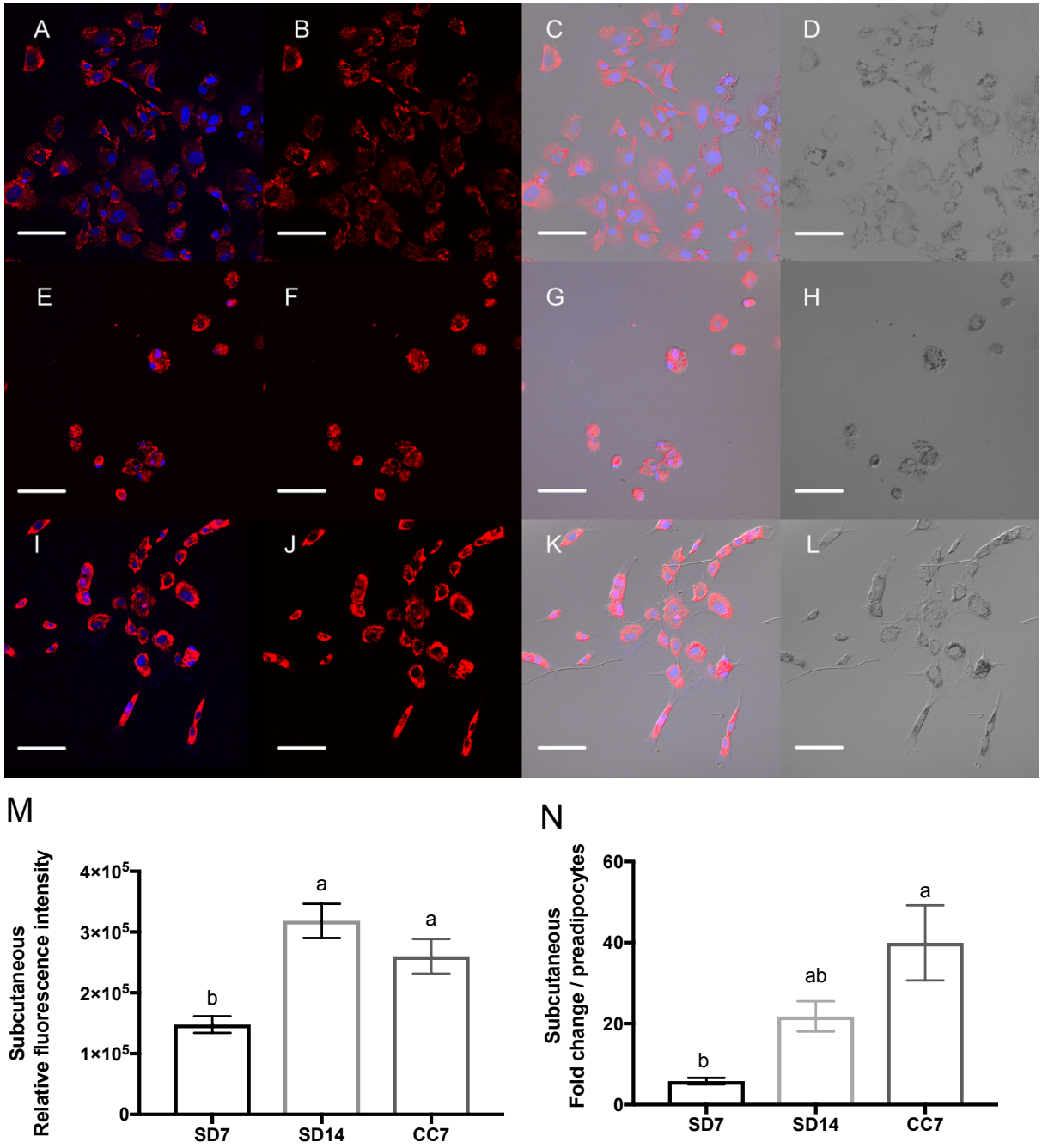

Figure 4. 4 Lipid accumulation in cultured bovine adipocytes derived from subcutaneous adipose tissue depot in dairy cows.

Images A-L: Laser scanning confocal microscopy imaging of lipid droplets in bovine adipocytes derived from subcutaneous AT using (A-D) standard induction for 7 d (SD7); (E-H) standard induction for 14 d (SD14); and (I-L) a co-culture model for 7 d (CC7). (M) Relative fluorescence intensity measured through confocal microscopy images using ImageJ software (means ± SEM). (N) Lipid accumulation fold change of visceral adipocytes over subcutaneous preadipocytes (AdipoRed™ assay). For confocal microscopy images, from left to right: 1st column: Bovine adipocytes co-stained with the lipid droplet stain HCS LipidTox™ and nuclei stain NucBlue™.

Figure 4. 4 (cont'd)
2nd: HCS LipidTox™ red fluorescence. 3rd: HCS LipidTox™ and NucBlue™ fluorescence overlaid on differential interference contrast image. 4th: Differential interference contrast image of bovine adipocytes. Scale bars: 100 µm. Bars with different letters (a–c) are significantly different ($P \leq 0.05$).

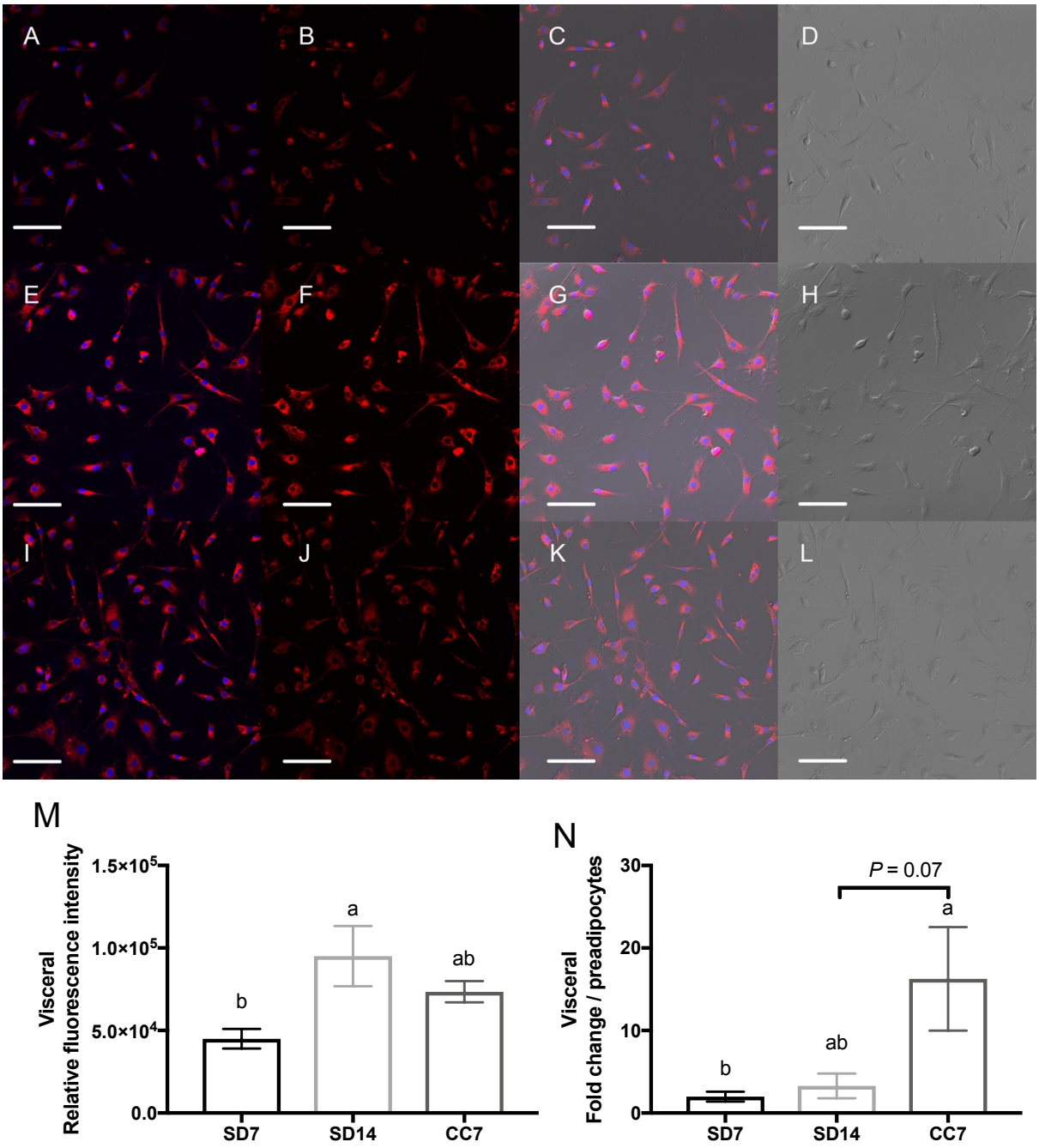

Figure 4. 5 Lipid accumulation in cultured bovine adipocytes derived from omental visceral adipose tissue in dairy cows.
Images A-L: Laser scanning confocal microscopy imaging of lipid droplets in bovine adipocytes derived from omental visceral adipose depot using (A-D) standard induction for 7 d (SD7);

Figure 4. 5 (cont'd)
(E-H) standard induction for 14 d (SD14); and (I-L) a co-culture model for 7 d (CC7). (M) Relative fluorescence intensity measured through confocal microscopy images using ImageJ software (means ± SEM). (N) Lipid accumulation fold change of visceral adipocytes over omental preadipocytes (AdipoRed™ assay). For confocal microscopy images, from left to right: 1st column: Bovine adipocytes co-stained with the lipid droplet stain HCS LipidTox™ and nuclei stain NucBlue™. 2nd: HCS LipidTox™ red fluorescence. 3rd: HCS LipidTox™ and NucBlue™ fluorescence overlaid on differential interference contrast image. 4th: Differential interference contrast image of bovine adipocytes. Scale bars: 100 µm. Bars with different letters (a–c) are significantly different ($P \leq 0.05$).

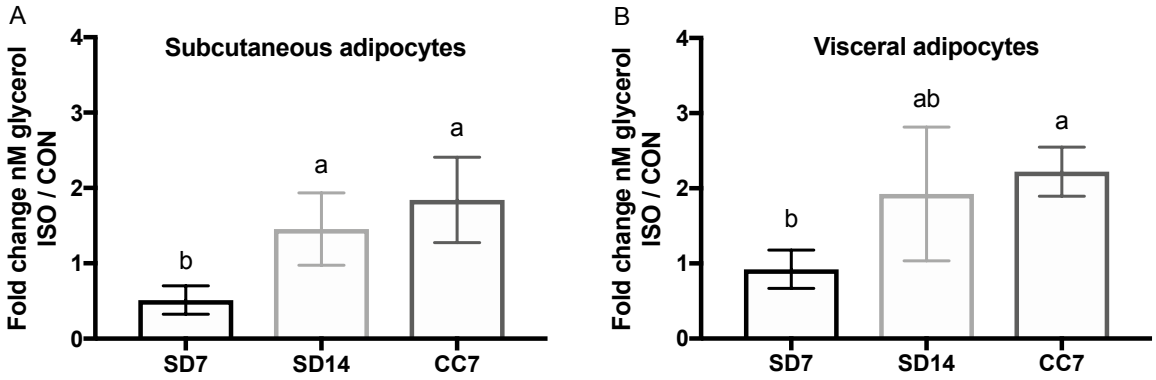

Figure 4. 6. Responsiveness to β-adrenergic stimulation in subcutaneous and visceral adipocytes differentiated in different culture conditions.
Concentrations of glycerol (nM) released in the medium during 2 h-stimulation with (ISO) or without (CON) isoproterenol (1 uM). Glycerol concentration fold change increase over CON in (A) subcutaneous and (B) visceral adipocytes induced to differentiate using a standard protocol for 7 (SD7, control) or 14 d (SD), and a co-culture (CC) in vitro model. Glycerol concentrations were calibrated by the number of cells per well and are expressed as mean fold change over CON ± SEM. Bars with different letters (a–c) are significantly different ($P \leq 0.05$).

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CHAPTER 5

FETUIN-A MODULATES LIPID MOBILIZATION IN BOVINE ADIPOSE TISSUE BY ENHANCING LIPOGENIC ACTIVITY OF ADIPOCYTES.

ABSTRACT

Fetuin-A (**FetA**) is an adipokine and free fatty acids (**FFA**) transporter in plasma linked to adipose tissue (**AT**) function in transition dairy cows. Plasma and AT FetA decrease after parturition coinciding with reduced lipogenesis and increased lipolysis. In monogastrics, FetA enhances lipogenesis, but its role on lipid mobilization of ruminants is unclear. We hypothesized that FetA modulates lipid mobilization by enhancing lipogenic activity in bovine adipocytes. Our objective was to determine the effects of FetA on lipogenesis and lipolysis in cultured primary adipocytes from dairy cows. Preadipocytes from tailhead subcutaneous AT depot of dairy cows were induced to differentiate in a 7-day co-culture in vitro model. Lipolytic responses of adipocytes were evaluated after 2h-β-adrenergic stimulation with 1 μM isoproterenol (**ISO**) alone or combined with 0.1 mg/mL of FetA (**FETA+ISO**), and in cells treated with media alone (**CON**) or with 0.1 mg/mL of FetA (**FETA**). Lipogenic responses of adipocytes treated with CON or FETA from day 5 to 7 of differentiation were assessed by FFA uptake quantification and triacylglycerol (**TAG**) accumulation, and the gene and protein expression of lipogenic markers. Cultured bovine adipocytes abundantly expressed *AHSG* and FetA protein, and secreted 48 ± 3.5 ng FetA. Adrenergic stimulation with ISO increased lipolysis compared with CON, as reflected in the release of glycerol (12±0.04 vs 0.04±0.02 nM) and FFA (15±13 vs 6.2±2.4 nM). Lipolysis induced by ISO was attenuated by the addition of FetA (FETA+ISO) as reflected by a lower glycerol (0.06±0.04 nM) and FFA (5.7±2.7 nM) release. The treatment with FetA enhanced lipogenic responses compared with CON as demonstrated by an increment in FFA uptake and 1.7 times in the accumulation of TAG. Exposure to FetA upregulated the AGAPT2 gene

expression and protein content, as well as its activity as demonstrated by increased concentrations of phosphatidic acid, the final product of its activity in adipocytes' synthesis of TAG. In conclusion, FetA attenuates lipolytic responses and enhances lipogenesis in bovine adipocytes. The upregulation of the rate-limiting lipogenic enzyme AGAPT2 by FetA suggests a potential pathway by which this adipokine promotes TAG synthesis in adipocytes. FetA is a potential target for lipid mobilization modulation in AT of periparturient dairy cows.
Key words: adipokine, adipocytes, dairy cow, lipogenesis, lipolysis

INTRODUCTION

Increased lipid mobilization in adipose tissues (**AT**) is the major metabolic adaptation to negative energy balance in periparturient dairy cows. Lipid reserves are used to meet the energy needs of milk synthesis thus favoring lipolysis over lipogenesis in AT (Contreras and Sordillo, 2011). During lipolysis, one molecule of triacylglycerol (**TAG**) is broken down into 3 free fatty acids (**FFA**) molecules and 1 molecule of glycerol through the activity of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Released FFA are either re-esterified into TAG within the adipocytes or used as energy source elsewhere in the body (Vernon and Pond, 1997). Lipogenesis involves the biosynthesis of TAG from fatty acids (**FA**) either synthesized from FFA released from blood TAG or *de novo* within the adipocytes. Glycerol phosphate pathway is the major pathway for *de novo* synthesis of TAG by adipocytes, which consists in stepwise addition of fatty acyl groups catalyzed by distinct enzymes: glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), lipins (phosphatidate phosphatases), and diacylglycerol acyltransferase (DGAT) to finally form TAG (Takeuchi and Reue, 2009). Adipocyte TAG reserves serve as the main source of energy to offset the effects of negative energy balance in periparturient cows (McNamara, 1995).

In periparturient cows, the main endocrine factors enhancing lipolysis and diminishing lipid synthesis in adipocytes include decreased plasma insulin and glucose concentrations, impaired insulin sensitivity in AT and other peripheral tissues, and increased concentrations of catecholamines, growth hormone and glucocorticoids (Bauman and Currie, 1980). In addition, a decrease in the transcription and activity of key lipogenic enzymes lead to a marked decrease in the ability of adipocytes to synthesize FA and TAG thus enhancing FFA release from AT (McNamara, 1995).

The relative balance between lipolysis and lipogenesis rates in AT controls the release of FFA from adipocytes and is critical to assure a successful transition from gestation to lactation in dairy cows. Dysregulated lipid mobilization leads to a massive release of FFA and a pro-inflammatory response that are major underlying factors of health disorders in periparturient dairy cows (Vernon and Houseknecht, 2000, Contreras et al., 2018). Currently, a major challenge in the field of periparturient dairy cows' management is the adequate regulation of AT lipolysis and lipogenesis rates to ensure that released FFA will be fully metabolized for energy needs, thus avoiding the detrimental FFA accumulation in blood and tissues.

We have recently determined the dynamics of a novel adipokine, Fetuin-A (**FetA**; alpha-2-Heremans-Schmid glycoprotein), in serum and AT of dairy cows during the periparturient period (Strieder-Barboza et al., 2018). Our results indicated that FetA is a negative acute-phase adipokine in the subcutaneous AT and is potentially involved in AT lipid mobilization in periparturient dairy cows (Strieder-Barboza et al., 2018). We also reported the gene and protein expression of FetA in bovine adipocytes suggesting a potential autocrine role for this adipokine; however, its secretion has not yet been reported (Strieder-Barboza et al., 2018). In humans and mice, FetA secretion by visceral and subcutaneous adipocytes is augmented during obesity and in animals fed a high fat diet (Jialal et al., 2015, Pérez-Sotelo et al., 2016). This effect is probably related with lipogenic properties of FetA. Fetuin-A carries lipids in plasma and facilitates the incorporation of exogenous FA into intracellular TAG in non-ruminants' adipogenic models (Kumbla et al., 1989, Cayatte et al., 1990). Variation in *AHSG*, the gene encoding FetA, was strongly associated with stimulation of lipogenesis and insulin-mediated inhibition of lipolysis in women (Dahlman et al., 2004). Because we previously observed that decreased FetA coincided with low lipogenesis and high lipolysis rates, and given the fact that FetA was associated with

pro-lipogenic states in non-ruminant species, we hypothesized that FetA modulates lipid mobilization by enhancing lipogenic activity in bovine adipocytes. Our objective was to determine the effect of FetA on lipogenesis and lipolysis and identify potential mechanisms by which this adipokine modulates lipid mobilization of bovine adipocytes.

MATERIALS AND METHODS

All animal procedures were approved by the Michigan State University Animal Care and Use Committee.

Tissue Collection and Processing

Subcutaneous AT from the tailhead depot from seven dairy cows were collected in KRBB supplemented with HEPES 10 mM (pH = 7.4) at a local slaughterhouse as previously described (Strieder-Barboza et al., 2018). In brief, AT (500 mg) was digested with 5 mL collagenase type II solution (2 mg/mL; Worthington Biochemical, Lakewood, NJ) and then centrifuged to separate the primary adipocytes from the stromal vascular fraction (SVF). Primary mature adipocytes were washed in 5 mL of KRBB with 4% bovine serum albumin (BSA, Millipore-Sigma, USA) and centrifuged. Final adipocyte population was retained for use in transwell inserts for inductions using a co-culture protocol. The SVF was then sequentially filtered through 100 μm and 40 μm cell strainers (Falcon, Corning, NY) and centrifuged. Resulting cell pellet was resuspended and incubated in erythrocyte lysis buffer. After another centrifugation, resultant cells were resuspended in basal preadipocyte media containing Dulbecco's modified Eagle's medium: F12 (Corning, Corning, NY), 10% fetal bovine serum (FBS; Corning), 2 mmol/L of L-glutamine (Corning), 1% (vol/vol) antibiotic-antimycotic (Corning), 44.05 mmol/L of sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 100 μmol/L of ascorbic acid (Sigma-Aldrich), 33 μmol/L of biotin (Sigma-Aldrich), 17 μmol/L of pantothenate

(Sigma-Aldrich), and 20 mmol/L of HEPES (Corning) with replacement every 2 d as described previously (Strieder-Barboza et al., 2018). Preadipocytes were obtained by outgrowth of plastic adherent cells from the SVF cells after 2 serial passages in culture flasks (Corning).

Cell Induction and Differentiation

Expanded preadipocytes populations were seeded in 6-well plates (Corning) and allowed to proliferate to confluency. Preadipocytes were induced to differentiate after 48 h at 100% confluency (d 0) using a co-culture model. Nine hundred mature adipocytes/cm2 were placed in 0.4 μm transwell inserts (Greiner Bio-One, Kremsmünster, Austria) over the attached preadipocytes for the first 5 days of differentiation. Induction basal media was supplemented with 10% FBS, 5 μmol/L troglitazone (Cayman Chemical, Ann Arbor, MI), 0.5 mmol/L 2 isobutyl-1-methylaxanthine (IBMX; AdipoGen Life Sciences, San Diego, CA) and the following reagents from Sigma Aldrich: 5 μg/mL insulin, 10 mM acetate, and 1 μmol/L dexamethasone. IBMX and dexamethasone were used only during the first 48 h of induction and media changes were performed every 48 h for 7 d. Adipocyte lipid accumulation was assessed quantitatively in triplicates per experimental unit using a 96-well plate for the AdipoRed™ assay (Lonza, Allendale, NJ) using a Synergy H1 Microplate Reader (Biotek, Vermont, MA).

Lipolysis-induction Assay

β-adrenergic induction of lipolysis was performed using isoproterenol (ISO, Sigma-Aldrich), an agonist of β-adrenergic receptors in adipocytes. Briefly, cultured adipocytes (n = 6) were removed from 6-well plates (Corning) using Trypsin (ThermoFisher, Waltham, MA), seeded in triplicate at $1x10^5$ cells/well in black wall 96-well plates (Nunc, Roskilde, Denmark) and allowed to attach for 4 h at 37 °C in a humidified atmosphere with 5% CO_2. Adipocytes were then starved for 4 h with serum free-basal preadipocyte media supplemented with 0.1% FA

free BSA (Millipore-Sigma, USA). Lipolytic responses of adipocytes were evaluated after 2 h incubation at 37 °C with Krebs Ringer Bicarbonate HEPES buffer (KRBH, pH 7.4) containing 3% FA free BSA (Millipore-Sigma) supplemented with 1 μM isoproterenol alone (ISO), 0.1 mg/mL of FetA alone (FETA; cat. no. 341506; Millipore, Darmstadt, Germany) or the combination of 0.1 mg/mL of FetA and 1 μM isoproterenol (ISO+FETA). Basal lipolysis was determined without addition of any reagent (CON). FetA dose was stablished based on previous studies with adipocytes (Heinrichsdorff and Olefsky, 2012, Pal et al., 2012). All reagents were prepared fresh on the day of the experiment. Lipolytic responses were assessed by the concentrations of glycerol (cat. no. MAK117-1KT, Millipore-Sigma) and FFA (HR Series NEFA-HR (2), FUJIFILM Wako Diagnostics U.S.A) released in the culture medium during 2 h-assay. To evaluate whether FetA is secreted by bovine adipocytes, we analyzed FetA concentrations in CON cell culture media. FetA concentrations were determined by ELISA following the manufacturer's guidelines (cow AHSG/fetuin A, cat. no. LS-F6106; LSBio, Seattle, WA) as reported previously (Strieder-Barboza et al., 2018). Concentrations of glycerol, FFA and FetA were adjusted by the number of cells/well as determined by CyQUANT Assay (Life Technologies, Carlsbad, CA) post functional analysis. All conditions were performed in triplicate. Statistical analysis was performed using the average glycerol and FFA concentrations of the triplicates corrected by the number of cells.

Fatty Acid Uptake Assay

Fatty acid uptake analysis was performed using the kinetic QBT Fatty Acid Uptake Assay (cat. no. R8132; Molecular Devices, Sunnyvale, CA). Cultured adipocytes (n = 6) were detached and seeded as described for lipolysis assay. Adipocytes were then serum-starved overnight and pre-incubated with 0.1mg/mL of FetA, 10 nM insulin (INSULIN, positive control;

Sigma-Aldrich, St. Louis, MO) or serum-free basal preadipocyte media (CON; basal FFA uptake) during 30 min at 37°C in a humidified atmosphere with 5% CO_2. Reagent from the QBT Fatty Acid Uptake assay containing a fluorescent labelled FA analog was added to the cells (100 µL/well) and kinetic uptake was measured every 20 sec for 1 h using a Synergy H1 Microplate Reader (Biotek). After the assay, to calculate area under the curve for each treatment, triplicate values per sample were averaged, subtracted from blank and calibrated by basal values (CON). Values are expressed as fold change over CON.

Triacylglycerol Accumulation

Intracellular TAG accumulation in adipocytes was assessed by Adipogenesis Assay Reagent (AdipoRed™, Lonza) and the lipid droplet staining HCS LipidTox™ (Life Technologies, Carlsbad, CA) analyzed through confocal microscopy imaging. On d 5 of differentiation, cultured adipocytes were incubated with basal adipocyte differentiation media supplemented with 0.1 mg/mL of FetA (FETA) for 48 h (d 5 to d 7). Basal TAG accumulation was determined in adipocytes incubated with basal differentiation media without addition of FetA (CON).

Lipid Droplets Staining and Confocal Microscopy Imaging. On d 5 of differentiation, cultured adipocytes (n = 8) were seeded in a glass bottom 24 well plate (Corning) at a concentration of 20,000 cells/cm^2 and incubated with or without FetA for 48 h. DAPI (NucBlue™, Life Technologies) and Alexa Fluor® 594 (HCS LipidTox™, Life Technologies) were used to visualize adipocytes' nuclei and intracellular TAG, respectively, through confocal laser scanning microscopy. These dyes were utilized according to the manufacturer's protocols. Briefly, adipocytes were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (1X PBS). 200 µL of HCS LipidTOX™ neutral lipid

stain 1:200 in 1X PBS was added to each well (Corning) and incubated at room temperature for 30 min. Buffer was removed and 200 µL DAPI (300 nM in 1X PBS) was added to each well and incubated at room temperature for 5 min. Images were acquired using an Olympus FluoView 1000 Confocal Laser Scanning Microscope (Olympus America, Inc., Center Valley, PA) configured on an IX81 inverted microscope and FV10-ASW software (version 4.2.3.6) using a PLAPON 60×/1.42 oil objective. Alex Fluor® 594 fluorescence (577 nm excitation/609 nm emission) was excited with the 543 nm HeNe gas laser, detected using a BP 560IF emission filter, and displayed in red color. DAPI fluorescence (358nm excitation/461nm emission) was excited using the 405nm diode laser, detected using a 430-470nm band pass emission filter, and displayed in blue color. Images were obtained using sequential single confocal XY scan mode. Control images for TAG accumulation included preadipocytes and non-stained adipocytes. Relative fluorescence intensity of intracellular TAG per cell was determined by ImageJ software.

Adipogenesis Assay Reagent. AdipoRed™ is a solution of Nile Red, a hydrophilic stain, that specifically partitions into the lipid droplets of differentiated adipocytes. On d 7 of differentiation, the assay was performed in triplicates in 24 well plates (Corning). Briefly, adipocytes (n = 5) were washed once with 1X PBS, incubated with AdipoRed™ assay reagent for 20 min at room temperature, and then analyzed for fluorescence intensity at 572 nm using a Synergy H1 Microplate Reader (Biotek). Preadipocytes served as negative controls. Relative fluorescence units (RFU) data of adipocytes was calibrated by their correspondent preadipocytes RFU and are presented as fold change over preadipocytes lipid accumulation.

Gene Expression Analysis of Adipogenesis and Lipogenesis Markers

After treating adipocytes (n = 5) for 48 h with or without FetA, culture media was removed, and cells were rinsed twice with ice-cold 1X PBS. Adipocytes' RNA was extracted

using Promega simplyRNA Cells Kits (Cat# AS1390, Promega, Madison, WI) in the Maxwell® RSC Instrument (Promega, Madison, WI) as described previously (Strieder-Barboza et al., 2018). 200 μL of 1-thioglycerol/homogenization solution were added to each well of 6-well plates with adipocytes and then transferred to a microfuge tube. Next, 200 μL of lysis buffer were added and homogenate was vortexed and then placed in Maxwell® RSC Cartridges, which were previously loaded with 10 μL of DNase I. Purity, concentration, and integrity of AT and cells' RNA were evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). All samples had a RNA integrity number ≥6. Conversion to cDNA was performed using the Applied Biosystems High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Transcriptional studies were performed on the cDNA samples using qPCR reactions on QuantStudio 7 Flex (Applied Biosystems). All qPCR reactions were performed in duplicates and no template controls (NTC) were included on each plate for each TaqMan gene expression assay.

Gene expression data of 5 endogenous control genes (*B2M, EIF3K, GAPDH, RPLP0* and *RPS9*) were analyzed using qBase+ analysis software, which calculates the stability of endogenous control genes (M-value). Following qBase+ analysis of gene expression data, endogenous control genes *EIF3K* and *RPS9*, were ranked best. The Cq values of the target genes (*AHSG, FABP4, CD36, FATP1, DGAT1, DGAT2, GPAT1, GPAT2, AGAPT2, PPARG, ADIPOQ* and *CEBPB*) were converted to normalized relative gene expression as described previously (Hellemans et al., 2007)(30). The quantitative PCR assays were conducted with TaqMan gene expression assays from Applied Biosystems, with the exception of FetA (*AHSG)* which was provided by Integrated DNA Technologies (IDT; Coralville, IA) as described previously (Strieder-Barboza et al., 2018) (Supplemental Table 1).

Supplemental Table 5. 1 mRNA probes by product and NCBI accession numbers

Gene	Product[1]	RefSeq
AHSG[2]	Bt.23250	NM_173984.2
ADIPOQ	Bt03292341_s1	NM_174742.2
AGAPT2	Bt03244182_m1	NM_001012727.1
B2M	Bt03251628_m1	NM_173893.3
CD36	Bt03234878_m1	NM_001046239.1.
DGAT1	Bt03251718_g1	NM_174693.2
DGAT2	Bt03259837_m1	NM_001253891.1
EIF3K	Bt03226565_m1	NM_001034489.2
FABP4	Bt03213820_m1	NM_174314.2
FATP1	Hs01587911_m1	NM_198580.2
GAPDH	Bt03210913_g1	NM_001034034.2
GPAT1	APU63EN	-
GPAT2	APGZFWZ	-
PPARG	Bt03217547_m1	NM_181024.2
RPL0	Bt03218086_m1	NM_001012682.1
RPS9	Bt03272016_m1	NM_001101152.2

[1]Thermo Fisher, Waltham, MA, USA. [2] Integrated DNA Technologies, Coralville, IA, USA.
AHSG: Fetuin-A; *ADIPOQ:* Adiponectin; *AGAPT:* 1-acylglycerol-3-phosphate acyltransferase-2; *B2M*: beta-2-microglobulin; *CD36:* Fatty acid translocase; *CEBPB:* CCAAT/enhancer-binding protein beta (*CEBPB*); *DGAT1:* Diacylglycerol O-Acyltransferase-1; *DGAT2:* Diacylglycerol O-Acyltransferase-2; *EIF3K:* Eukaryotic translation initiation factor 3 subunit K; *FABP4:* fatty acid binding protein 4; *FATP1:* Fatty acid transporter-1; *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase; *GPAT1:* Glycerol-3-Phosphate Acyltransferase-1; *GPAT2:* Glycerol-3-Phosphate Acyltransferase-2*, PPARG:* Peroxisome proliferator-activated receptor gamma; *RPL0:* 50S ribosomal protein L15; *RPS9*: 40S ribosomal protein S9.

Western Blotting

Western blots were performed as previously described (Strieder-Barboza et al., 2018). Protein extraction from cultured adipocytes (n = 8) were extracted using ice-cold RIPA buffer (Teknova, Hollister, CA) containing protease (Roche, San Francisco, CA) and phosphatase (Thermo Scientific, Waltham, MA) inhibitors. Estimation of protein content was carried out using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). FetA content was analyzed using reducing conditions. Briefly, samples were added to a reducing buffer containing 10 mmol/L dithiothreitol (DTT) and 5% -mercaptoethanol (BME) and denatured at 95°C for 4 min. Equal amounts of total protein (20 µg) were electrophoresed on a 4-20% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in tris-

buffered saline solution (TBS) with 0.01% tween-20 (TBST) and 5% fat free milk. Membranes were incubated with a conjugated polyclonal rabbit anti-bovine AGAPT2 antibody (C-terminal region cat. no. ARP44636_P050; Aviva Systems Biology Corporation, San Diego, CA) in 1ug/mL concentration in TBST-5% milk for 16 h at 8 °C. αβ-tubulin (1:1,000; Cat# 2148; Cell Signaling Technology, Danvers, MA) served as loading controls for adipocytes protein. Membranes were then exposed to horseradish peroxidase substrate (cat. no. WBLUR0100; Millipore, Darmstadt, Germany), and visualized by chemiluminescence using the ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA). Band densitometry was determined using the Image Lab software (Bio-Rad, Hercules, CA). Values of AGAPT2 protein content are expressed as means of relative band density using αβ-tubulin band intensity values as calibrator.

Phosphatidic Acid Analysis

Phosphatidic acid (**PA**) concentrations in adipocytes were measured using the enzymatic fluorometric PicoProbe™ Phosphatidic Acid Assay Kit (cat. no. K748, BioVision Incorporated, Milpitas, CA) following the manufacturer instructions. Briefly, after treating adipocytes (n = 5) with or without 0.1 mg/mL of FetA from d 5 to d 7 of differentiation, culture media was removed, and cells were rinsed twice with ice-cold 1X PBS, scraped from cell culture plates, and collected in 1X PBS. For lipid extraction, $1x10^6$ adipocytes were mixed with 1 mL of assay buffer (BioVision) and sonicated using an ultrasonic liquid processor (Misonix; Farmingdale, NY) for 2 min. Protein content was estimated at this point using a BCA Protein Assay Kit (Thermo Scientific). Following, 3.75 ml of chloroform/methanol/12N HCl (2 : 4 : 0.1 v/v) were added to the cell homogenate and mixed thoroughly. Then, 1.25 ml of chloroform were added to the solution, vortexed for 30 sec and added to 1.25 ml of 1 M NaCl. Followed by centrifugation, the lower organic layer containing solubilized lipids was collected and transferred to a glass tube.

Chloroform was evaporated in a vacuum concentrator (Savant SPD121P, Thermo Fisher) for 2 h at 40°C. Before performing the analysis, samples were solubilized in 50 µl 5% Triton X-100 solution. For PA measurement, 10 µl of sample were added to a 96-well plate and incubated for 1 h at 45°C with a compound that hydrolyzes PA to form an intermediate. Following, samples were incubated for 30 min at 37°C in the presence of a developer and enzyme mix that converts a non-fluorescent probe to a fluorescent product (Ex/Em= 535/587 nm) that can be quantified (Synergy H1 Microplate Reader; Biotek).

Statistical Analysis

Data were analyzed using JMP Statistical Software (SAS Institute Inc., Cary, NC). Normality of the variables was checked using the Kolmogorov-Smirnov test ($P < 0.05$). Non-normally distributed variables (FFA concentrations in lipolysis assay, FA uptake assay, adipocytes AGAPT2 protein content) were ln transformed. One-way ANOVA Pairwise comparisons were performed using the Tukey's post hoc test. Mean differences were considered significant when $P \leq 0.05$ and tendencies when $P < 0.10$.

RESULTS

Bovine Cultured Adipocytes Abundantly Express and Secrete Fetuin-A

Based in a potential autocrine effect of FetA in AT lipid mobilization, we first evaluated the capacity of bovine adipocytes to express and secrete FetA. We observed that cultured bovine adipocytes abundantly express *AHSG* and FetA protein at similar level as AT of periparturient cows ($P > 0.05$; Fig. 5.1) and secrete 48 ± 3.5 ng of FetA (mean ±SEM).

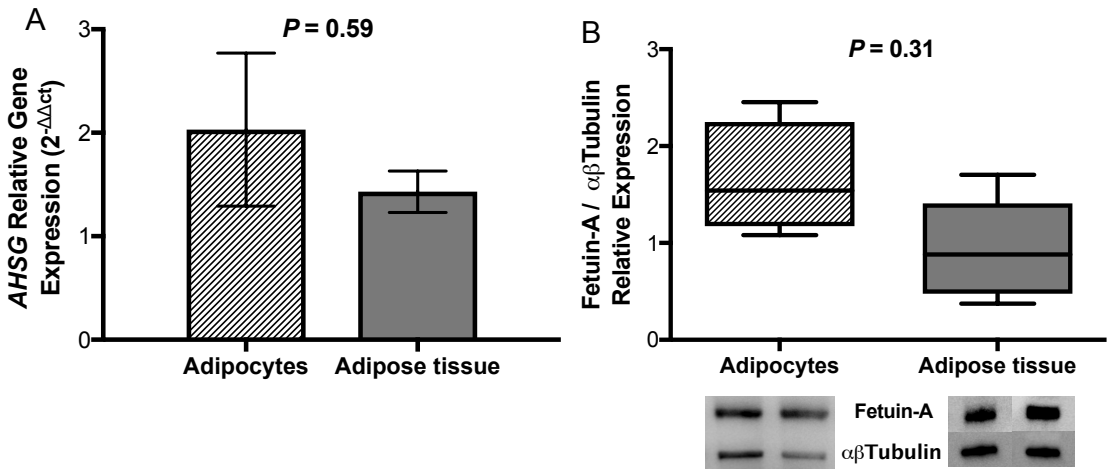

Figure 5. 1 Fetuin-A expression in cultured bovine adipocytes and subcutaneous adipose tissue of dairy cows.
(A) Fetuin-A gene (*AHSG*) and (B) protein expression in cultured bovine adipocytes (n=5) at 7th day of differentiation *in vitro* and subcutaneous adipose tissue of dairy cows (n=5) at 8 ± 3 d after parturition. Cultured adipocytes were derived from tailhead subcutaneous adipose depot of dairy cows. For qPCR results, values are shown as relative gene expression 2(−ΔΔCT) (where CT = cycle threshold), normalized by control genes RPS9 and EIF3K, and represent mean ± SEM. For Western blot analysis, αβ-tubulin was used as a loading control, and values are expressed as a ratio between Fetuin-A and αβ-tubulin adjusted band intensity (mm2). Results represent means ± SEM.

Fetuin-A Attenuates Lipolytic Responses in Bovine Adipocytes

After assessing the secretory ability of bovine adipocytes to secrete FetA, we were interested in evaluating the effect of FetA on the modulation of lipid mobilization. Therefore, we first assessed the effect of FetA on lipolytic responses of bovine adipocytes. β-adrenergic stimulation with ISO increased lipolysis compared with CON, as reflected in the release of glycerol (12±0.04 vs 0.04±0.02 nM, $P = 0.003$; Fig. 5.2A) and FFA (15±13 vs 6.2±2.4 nM, $P = 0.04$; Fig. 5.2B). Lipolysis induced by ISO was attenuated by FetA (FETA+ISO) as reflected by a lower glycerol (0.06±0.04 nM, $P = 0.02$) and FFA (5.7±2.7 nM, $P = 0.01$) release compared with ISO alone.

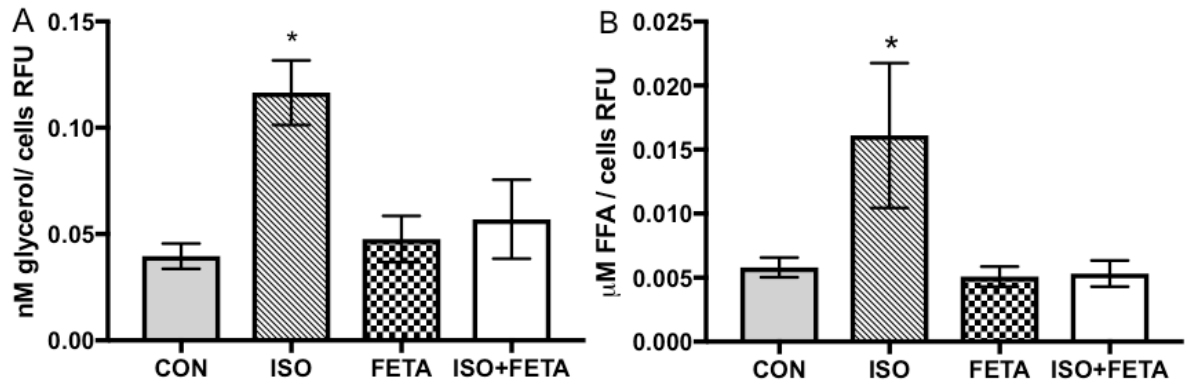

Figure 5. 2 Effect of Fetuin-A on β-adrenergic induced-lipolysis in bovine adipocytes. (A) Concentrations of glycerol and (B) FFA released in the supernatant during 2 h-lipolysis induction. Values were calibrated by the number of cells per well and are expressed as mean ± SEM. *Statistically different ($P < 0.05$).

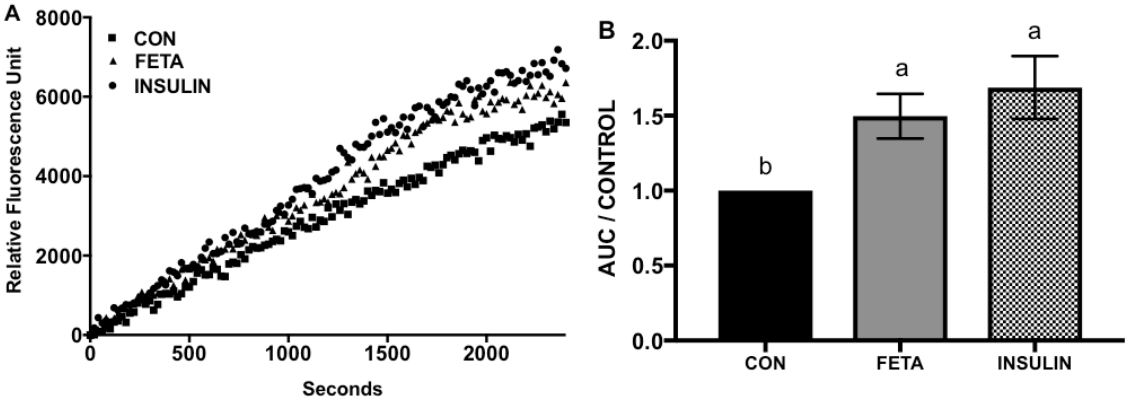

Figure 5. 3 Effect of Fetuin-A on fatty acid (FA) uptake by bovine adipocytes *in vitro*. (A) Example of Kinetic QBT FA uptake output from bovine adipocytes over 1 h-assay. (B) Area under the curve (AUC) from FA uptake assays of bovine adipocytes treated with 0 (CON) or 0.1 mg/mL FetA (FETA) or 10 nM insulin (INSULIN). Bovine adipocytes were treated and pre-incubated with treatments for 30 minutes at 37C before the assay. Values are expressed as AUC using CON FA uptake as the calibrator (means ± SEM). Bars with different letters (a–c) are significantly different ($P \leq 0.05$).

Fetuin-A enhances lipogenesis in bovine adipocytes

Since lipolysis and lipogenesis are continuous processes occurring simultaneously within adipocytes, after evaluating lipolysis, we were interested on evaluating the effect of FetA on lipogenic activity. We assessed lipogenesis by evaluating FFA uptake and accumulation of

intracellular TAG. Exposure of adipocytes to FetA during 1.5 h (pre-incubation and 1 h-kinetic assay; Fig. 5.3A) incremented FFA uptake by 1.5 times ($P = 0.02$; Fig. 5.3B) compared with CON and stimulated similar response as induced by INSULIN ($P = 0.65$; Fig. 5.3B). Insulin increased FFA uptake by 1.69 times compared with CON ($P = 0.004$; Fig. 5.3B).

In agreement with the higher incorporation of FFA, we observed that cells treated with 0.1 mg/mL of FetA during 48 h (d 5 to d 7 of differentiation) increased their adipogenic capacity as demonstrated by higher accumulation of TAG compared with non-treated adipocytes (CON), analyzed by both, lipid droplets staining with HCS LipidTOX™ ($P = 0.003$; Fig. 5.4A-C) and AdipoRed™ assay ($P = 0.04$; Fig. 5.4D-E). There was 1.7 times increment in the accumulation of TAG in FETA compared with CON ($P = 0.04$; Fig. 5.4D-E) and 37 times compared with preadipocytes ($P < 0.0001$). CON increased TAG accumulation by 22 times compared with preadipocytes ($P = 0.006$).

Fetuin-A Upregulates AGAPT2 Expression and Activity in Bovine Adipocytes

To explore potential drivers of FetA lipogenic function, we evaluated the effect of FetA treatment on key regulators of adipogenesis, such as *CEBPB, ADIPOQ* and *PPARG*, on components of FA uptake cascade including *FABP4, FATP1* and *FAT/CD36,* and on lipogenic enzymes controlling *de novo* TAG synthesis in adipocytes, such as *AGAPT2, DGAT1, DGAT2, GPAT1, GPAT2* and *LIPIN1.* We observed that the treatment with 0.1 mg/mL of FetA increased the gene expression of *AGAPT2* ($P = 0.02$; Fig. 5.5A) and *AHSG* ($P = 0.05$; Fig. 5.5B) and tended to increase the expression of *CEBPB* ($P = 0.07$; Fig. 5.5C) and *FATP1* ($P = 0.07$; Fig. 5.5D). There was no difference on the gene expression of *PPARG, ADIPOQ, DGAT1, DGAT2, GPAT1, GPAT2, LIPIN1, FAT/CD36* and *FABP4* between FETA and CON ($P > 0.05$). Given the specific upregulation of AGAPT2 gene expression by FetA, we also analyzed protein content of

AGAPT2 treated with FetA. In agreement with the increase on gene expression, protein content of AGAPT2 was also increased in FETA compared with CON ($P = 0.04$; Fig. 5.5B).

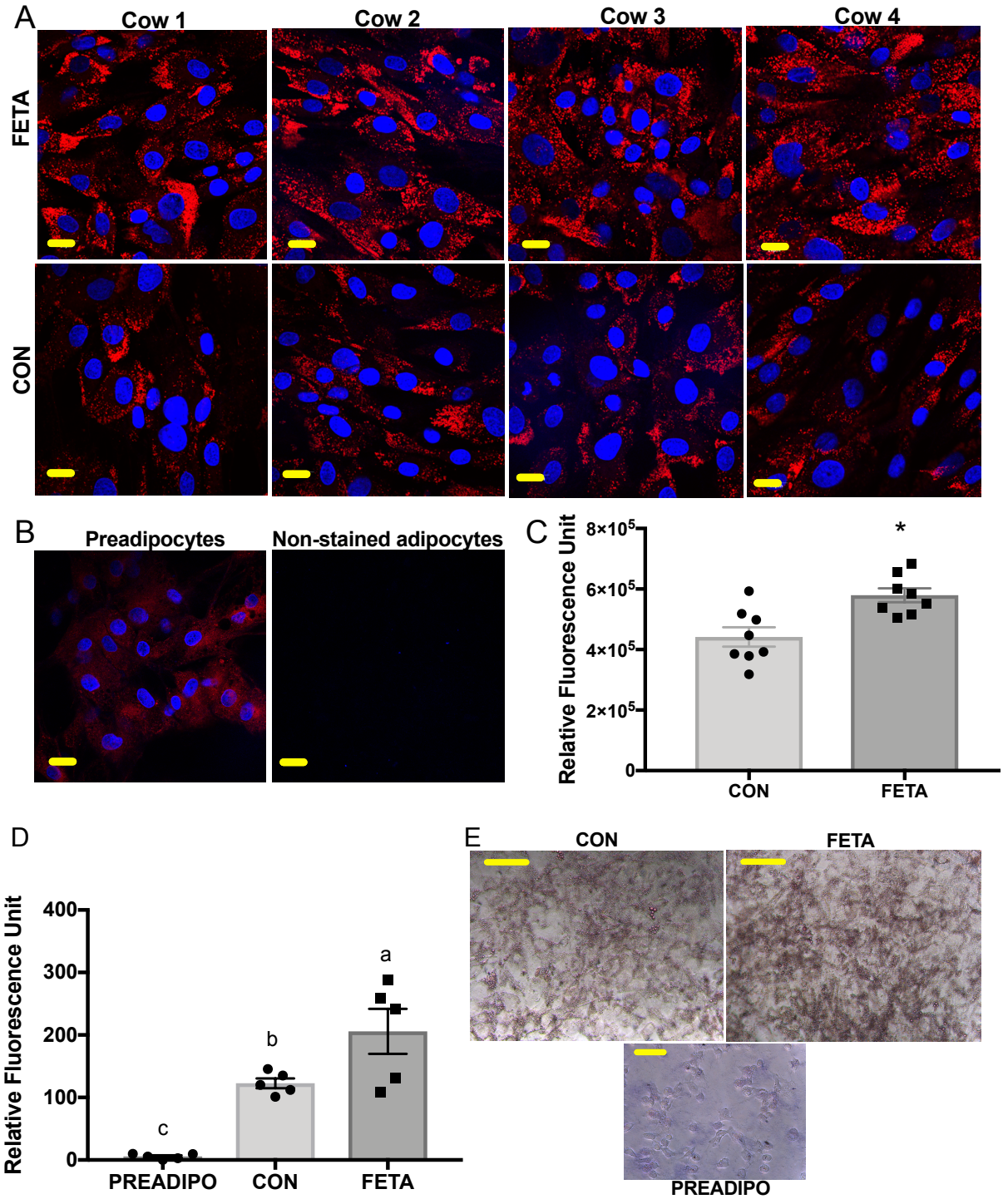

Figure 5. 4 Effect of Fetuin-A on triacylglycerol accumulation in cultured bovine adipocytes.

Figure 5. 4 (cont'd)
(A) Laser scanning confocal microscopy imaging (60 X) of lipid droplets in cultured bovine adipocytes (*n*= 8) treated with treated with 0 (CON; bottom panel) or 0.1 mg/mL FetA (FETA; upper panel). (B) Preadipocytes and non-stained cells served as controls. Lipid droplets of bovine adipocytes were stained with Alexa Fluor 594 (HCS LipidTox™; red fluorescence) and nuclei were stained with DAPI (NucBlue™; blue fluorescence). (C) Plotted relative fluorescence intensity measured through ImageJ software (means ± SEM). Scale bars: 20 µm. (D) Relative fluorescent unit of AdipoRed™ assay analysis in cultured bovine adipocytes (*n*= 5) treated with with 0 (CON) or 0.1 mg/mL FetA (FETA). (E) Microscopy imaging (20 X) of lipid droplets (dark red dots) in bovine adipocytes and preadipocytes (PREADIPO) incubated with AdipoRed™ for 20 minutes. Scale bars: 100 µm. Bars with different letters (a–c) are significantly different ($P \leq 0.05$).

The enzymatic activity of AGAPT2 during TAG synthesis corresponds to transferring an additional FA to lyso-phosphatidic acid (LPA) to produce phosphatidic acid (PA). We assessed the effect of FetA on AGAPT2 activity by quantifying the concentrations PA in bovine adipocytes treated or not with 0.1 mg/mL of FetA from d5 to d7 of differentiation. Adipocytes treated with FetA synthetized more PA (0.27 ± 0.002 nmol) compared with non-treated CON adipocytes (0.20 ± 0.01 nmol; $P = 0.060$; Fig. 5.5F).

DISCUSSION

The balance between lipogenesis and lipolysis rates in AT of periparturient dairy cows is critical to assure adequate adaptive responses and a healthy and successful transition to lactation. In our previous study (Strieder-Barboza et al., 2018), we identified a negative association between FetA and markers of lipolysis, and similar dynamics between AT FetA and other lipogenic markers. In addition to that, previous in vivo and in vitro studies in non-ruminants have consistently reported strong lipogenic properties of FetA (Cayatte et al., 1990, Pérez-Sotelo et al., 2016), however, the mechanisms by which FetA increases TAG synthesis in adipocytes have not yet been studies. In this study, for the first time, we report that FetA modulates lipid mobilization in bovine adipocytes and identify AGAPT2 activity as a novel potential driver of lipogenic function of FetA in bovine adipocytes.

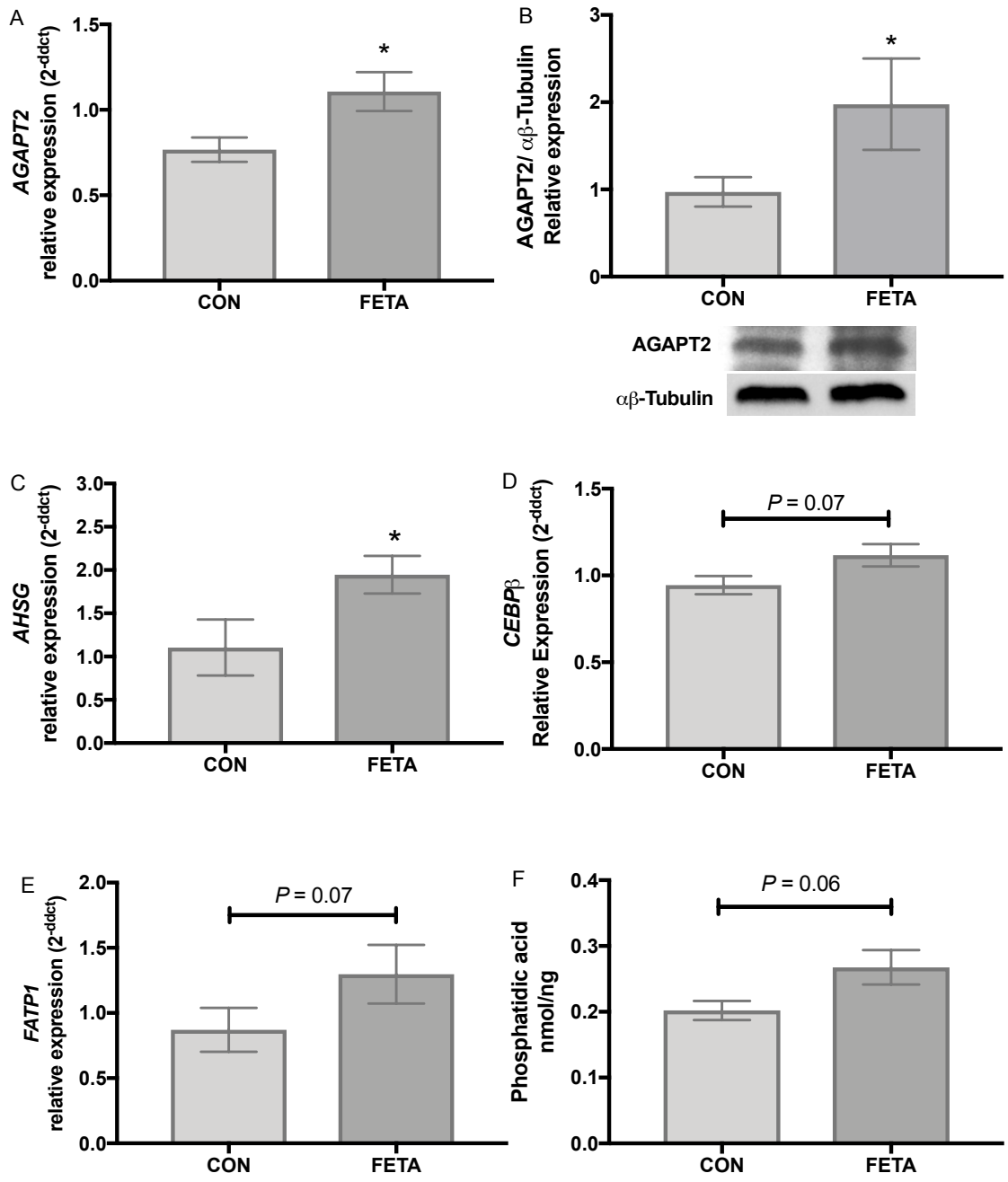

Figure 5. 5 Effect of Fetuin-A on gene expression of lipogenic markers and phosphatidic acid concentrations in bovine adipocytes.

Bovine adipocytes were treated with 0 (CON) or 0.1 mg/mL of Fetuin-A (FETA) for 48 h during late differentiation *in vitro*. (A) Gene expression and (B) protein content (n=8) of 1-acylglycerol-3-phosphate acyltransferase-2 (AGAPT2). Gene expression of (C) Fetuin-A (*AHSG*), (D) adipogenesis promoter CCAAT/enhancer-binding protein beta (*CEBPB*), and (E) fatty acid transporter-1 (*FATP1*). (F) Phosphatidic acid concentrations calibrated by total protein

Figure 5. 5 (cont'd)
concentrations (mg) in sampled bovine adipocytes. For qPCR results (n=6), values are shown as relative gene expression 2(−ΔΔCT) (where CT = cycle threshold), normalized by control genes RPS9 and EIF3K, and represent mean ± SEM. For Western blot analysis, αβ-tubulin was used as a loading control, and values are expressed as a ratio between Fetuin-A and αβ-tubulin adjusted band intensity (mm2). Results represent means ± SEM.

In our study, cultured bovine adipocytes derived from subcutaneous AT of dairy cows abundantly express and secrete FetA. Previous secretome studies using visceral and gonadal AT of rats detected significant amounts of FetA (Roca-Rivada et al., 2011). More recently, the same authors reported an abundant secretion of FetA by C3H10T1/2 cells as a murine model of pre-adipocyte differentiation, but only the secretome from mature differentiated adipocytes contained FetA (Pérez-Sotelo et al., 2016). We have recently reported an abundant mRNA and protein expression of FetA in subcutaneous and omental AT of periparturient and mid-lactation dairy cows, as well as in cultured bovine adipocytes (Strieder-Barboza et al., 2018).In a proteomics study, Zachut et al. (2017) also reported an abundant FetA in subcutaneous AT of dairy cows in late gestation. However, this is the first study reporting FetA secretion by adipocytes. Secretion of FetA was demonstrated to decrease during fasting, weight loss and anorexia and to be increased during weight gain and obesity in rodents and humans (Jialal et al., 2015, Pérez-Sotelo et al., 2016). Similarly, serum concentrations and AT gene and protein expression of FetA decreased during a marked negative energy balance in early lactation dairy cows (Strieder-Barboza et al., 2018). The changes in the FetA secretion pattern by the AT of animals and humans going through lipid mobilization suggest the participation of FetA in adipocytes' lipogenesis and lipolysis.

In the present study, FetA attenuated lipolytic responses stimulated by β-adrenergic activation with ISO. In humans, lipolysis in adipocytes appears to be a phenotype that is

particularly sensitive to variation in *AHSG*, the gene encoding FetA (Dahlman et al., 2004, Lavebratt et al., 2005). A common variation (Thr230Met) in the *AHSG* is associated with a marked increase in β2-adrenoceptor sensitivity in subcutaneous adipocytes, which may be of importance in body weight regulation (Lavebratt et al., 2005). In a large study screening adipocyte phenotype in obese and non-obese healthy women, single-nucleotide polymorphisms (−469T>G) in *AHSG* was strongly associated with insulin inhibition of lipolysis (Dahlman et al., 2004). Our results suggest that the effect of FetA during induced-lipolysis in bovine adipocytes was insulin-independent since no insulin was added to the medium during stimulated lipolysis. Previous studies suggest that FetA may control insulin signaling in AT and is an attractive candidate gene for disturbed adipocyte lipolytic function in obesity and insulin resistance in humans (Dahlman et al., 2004). Adipose tissue lipolytic responsiveness and sensitivity to adrenergic agents, including the natural catecholamines, are increased in dairy cows around parturition (Bell, 1995), and are one of the mechanisms leading to the very high plasma concentrations of FFA and disease development during the periparturient period (Grummer, 1993). Therefore, attenuating lipolysis and/or regulating insulin function in early lactation would be particularly important in dairy cows that develop hypoinsulinemia concurrently with a state of insulin resistance (Contreras et al., 2017). Even though FetA seems to be involved in the pathways that control β-adrenergic lipolytic responses in bovine adipocytes, the mechanisms by which this adipokine may play an anti-lipolytic roles, how to modulate them, and the identification of potential *AHSG* alleles that could be particularly involved in AT lipolytic responses in dairy cows remain to be stablished.

In the present study, we observed that incubation of bovine adipocytes with FetA increased FA uptake compared with non-treated cells, and induced similar responses as cells

treated with insulin, a strong lipogenic factor (Stahl et al., 2002). Although speculative at this time, FetA could have stimulated the translocation of plasma membrane FA transporters (i.e. FATP1 and CD36), similar to known effects of insulin (Stahl et al., 2002). Variants of FetA gene had a local impact in AT on insulin regulation of lipogenesis and lipolysis in humans (Dahlman et al., 2004). Other possibility is that FetA could have bound FA and translocated them into the intracellular compartment. FA transporters, such as albumin and FetA, are known by carrying lipids in plasma and facilitating FA uptake by cells (Glatz et al., 2010). Previously, FetA was reported to translocate into 3T3-L1 and human adipocytes (Dasgupta et al., 2010), and to accumulate in vesicles in the cytosol (Reynolds et al., 2005); nevertheless, its relationship with FA transport was not reported by these studies. Fetuin-A carries high amounts of cholesterol, cholesteryl esters, TAG, and FA in plasma, which correspond to nearly 33% of its molecule (Kumbla et al., 1989, Kumbla et al., 1991). In rabbit and human's cells, FetA increased the incorporation of exogenous FA into intracellular TAG by nearly 50-fold compared with albumin (Cayatte et al., 1990). Similarly, we observed that FetA increases TAG accumulation in cultured bovine adipocytes compared with non-treated cells. This suggests that increased FetA-stimulated FA uptake by adipocytes might have enhanced the intracellular synthesis of TAG. Due to its lipogenic properties, increased serum concentrations and AT expression and secretion of have been associated with obesity and obesity-related disorders in humans and animals, such as metabolic syndrome (Chen et al., 2009, Jialal et al., 2015, Pérez-Sotelo et al., 2016). In dairy cows, we previously reported a decrease in FetA serum concentrations and in AT expression from 2 wks prepartum to 10 DIM, and was more pronounced in cows that lost more fat mass from dry off to early lactation (Strieder-Barboza et al., 2018). This period of decreased FetA coincided with the downregulation in the transcription of lipogenic enzymes in the subcutaneous

AT and with a marked increase of plasma FFA concentrations in periparturient cows (Strieder-Barboza et al., 2018). Similarly, FetA protein abundancy in subcutaneous AT of dairy cows during late gestation also decreased during environmental heat stress and increased circulating FFA (Zachut et al., 2017). Together, these results provide evidence that FetA may promote lipogenesis in vitro and in vivo in the AT of dairy cows.

Although it is clear that FetA stimulates the synthesis of TAG, the mechanisms involved are yet to be studied. Whether the stimulation of TAG accumulation in bovine adipocytes is mainly due to the entry of FA into cells or whether FetA might directly stimulate lipogenic enzymes is not known. To start addressing these questions, we evaluated the gene expression of key enzymes involved in the TAG synthesis through the glycerol-3-phosphate pathway in adipocytes: (1) Glycerol phosphate acyltransferases (GPAT), which is involved in glycero-P synthesis; (2) 1-acylglycerol-3-phosphate acyltransferase (AGPAT), catalyst for the first step in the formation of phosphotidic acid (PA); (3) phosphatidate phosphatase (Lipin), which forms diacylglycerol; and diacylglycerol acyltransferases (DGAT) (Coleman and Lee, 2004, Takeuchi and Reue, 2009). In our study, we observed that FetA upregulated the gene expression of AGAPT2 in bovine adipocytes. In agreement with that, we also observed an increased AGAPT2 protein content in FETA. AGPAT2 is the predominant AGAPT isoform in AT and catalyzes acylation of its strict substrate lysophosphatidic acid to PA (Takeuchi and Reue, 2009). In cows, AGAPTs play an important role in the TAG synthesis in the mammary gland (Mistry and Medrano, 2002), but have not yet been reported in AT. AGAPT2 is strongly induced by PPARγ agonists (Blanchard et al., 2016) and is required for TAG mass accumulation in mature adipocytes (Gale et al., 2006). Knocking down AGPAT2 decreased gene expression of adipogenesis regulators such as *PPARγ* and *C/EBPB*, delayed induction of mature adipocyte

markers such as *FABP4* and *GLUT4* , and reduced TAG accumulation in adipocytes (Blanchard et al., 2016). The crucial function of AGAPT2 for adipogenesis is highlighted by the near complete absence of AT and a range of metabolic changes, such as extreme insulin resistance, in humans with congenital generalized lipodystrophy as a consequence of AGAPT2 deficiency (Agarwal and Garg, 2006). These results demonstrate that other AGAPT family members (AGAPT1, and AGAPT3-10) cannot compensate AGAPT2's activity for the synthesis of TAG, thus being a specific and rate limiting enzyme during TAG synthesis (Agarwal et al., 2002, Takeuchi and Reue, 2009).

AGAPT2 controls adipogenesis through modulation of the synthesis of phospholipids and TAG precursors, especially PA (Gale et al., 2006). Impaired AGPAT2 activity affects availability of PA for TAG synthesis but not overall PA synthesis nor utilization of PA for phospholipid synthesis (Gale et al., 2006). In our study, we indirectly evaluated the effect of FetA on AGAPT2 activity by measuring adipocyte concentrations of PA. We observed that FetA increased not only gene expression and protein content of AGAPT2 in bovine adipocytes, but also PA concentrations. Phosphaditic acid was highlighted as one of the main lipid regulators of the size of lipid droplets, an important lipid-storage organelle (Fei et al., 2011). Our results suggest that FetA increases TAG accumulation by increasing AGAPT2 activity, and therefore PA concentrations for TAG synthesis in bovine adipocytes. Knowing that FetA may modulate TAG synthesis precursors in adipocytes provide valuable insights into potential targets for modulating flux of lipids in dairy cows' AT.

Our study haa limitations. First, we analyzed the effect of FetA on a limited pool of genes involved on adipogenesis, FA uptake and lipogenesis. Although we identified an effect of FetA on a few of these selected genes, it is possible that other key lipogenic markers could be driving

the lipogenic function of FetA. Second, we cannot conclusively affirm that AGPAT2 signaling is required for FetA lipogenic effect. To do so, further studies using AGAPT2 and FetA knockout models are necessary to evaluate the potential interdependent function of these components on TAG synthesis in bovine adipocytes.

CONCLUSIONS

Results from this study demonstrate that FetA modulates lipid mobilization by attenuating lipolytic responses and enhancing lipogenesis in bovine adipocytes. FetA upregulates the expression and activity of AGAPT2, a rate-limiting lipogenic enzyme, and suggests a potential mechanism by which this FetA promotes TAG synthesis in adipocytes. Our study provides novel knowledge on how FetA promotes TAG synthesis in adipocytes and opens the possibility of using FetA as a potential therapeutic target for the modulation of lipid mobilization in AT of periparturient dairy cows and humans with similar AT disorders.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The main objective of the present dissertation was to evaluate the dynamics of FetA systemically and in the AT of dairy cows during the periparturient period, and to elucidate the roles of FetA on adipocyte lipid mobilization. We demonstrated that serum and AT FetA decreases after parturition and has similar dynamics as other lipogenic markers in AT of periparturient dairy cows (Strieder-Barboza et al., 2018). Although FetA is increased during obesity in non-ruminants (Chen et al., 2009, Jialal et al., 2015), we were unable to detect an effect of adiposity on serum concentrations of FetA in periparturient cows. In contrast, we observed decreased gene expression of FetA in subcutaneous AT of overconditioned cows. These results may indicate a local effect of excess adiposity on the expression and secretion of FetA by AT and therefore in the mechanisms of lipid mobilization in adipocytes. Previous studies demonstrated that FetA knockout mice have decreased body fat content, altered lipid metabolism and higher energy expenditure (Mathews et al., 2002). These findings suggest that FetA may play a significant role in regulating glucose disposal, insulin sensitivity, weight gain and fat accumulation. In dairy cows, whether lower FetA expression in AT of overconditioned animals is a cause or an effect of decreased lipogenesis or excessive lipolysis is still unknown and requires further investigation.

FetA has been reported as an anti-inflammatory factor during acute inflammation such as during infections, cerebral ischemic injury, and lethal endotoxemia in monogastrics (Wang and Sama, 2012). Accordingly, we observed that serum FetA follows the dynamics of a negative acute-phase protein in periparturient dairy cows and its protein content in AT decreases upon an acute inflammatory stimulus (Strieder-Barboza et al., 2018). A potential anti-inflammatory effect

of FetA on bovine adipocytes was also supported by the decreased CCL2 expression in cells treated with FetA compared with LPS (Strieder-Barboza et al., 2018). Following up on these observations, we generated initial epidemiological data that may suggest a beneficial role of FetA on inflammatory responses in periparturient dairy cows: we observed that at time of dry off and 2 weeks before parturition, serum FetA concentrations were lower in cows that were later diagnosed with retained placenta and metritis, respectively (data not shown). Even though these results suggest that FetA plays an anti-inflammatory role systemically and in the AT of dairy cows, further studies are needed. Future studies will evaluate the expression and secretion of inflammation markers (e.g., TNF, IFN-γ, and IL-6) by bovine adipocytes in response to inflammatory stimulus followed by the treatment with different doses of FetA. Based on our results and previous studies, we also expect to find an anti-inflammatory effect of FetA in future studies with bovine cells.

One of the main goals of this dissertation was to elucidate the effects of FetA on AT lipid mobilization in dairy cows. For this purpose, we developed a novel in vitro model for efficiently inducing adipogenesis in bovine adipocytes and reported, for the first time, that primary adipocytes not only express FetA gene and protein, but also secrete FetA. Our findings support that FetA is a pro-lipogenic and anti-lipolytic adipokine in AT of dairy cows. We demonstrated potential modes of action for FetA during lipid mobilization and identified a potential molecular target for its pro-lipogenic effect. For the first time, we revealed that the upregulation of the expression and activity of AGAPT2, a rate limiting lipogenic enzyme, is a potential mechanism by which FetA enhances triacylglycerol synthesis in bovine adipocytes. When performing these in vitro assays, we chose to use a single dose of 0.1 mg/mL of FetA. This was based on previous studies with human and mice adipocytes (Dasgupta et al., 2010, Pal et al., 2012), on the

biological range of FetA concentrations in serum of dairy cows (Strieder-Barboza et al., 2018), and on the results of a dose-response to FetA using bovine adipocytes in our laboratory. When performing a dose-response analysis on the effects of FetA (0, 0.01, 0.1, 0.25, 0.5 and 1 mg/mL) on the gene expression of adipogenesis and lipogenesis regulators, we observed a nonlinear effect with the expression of ADIPOQ, CD36 and FABP4 tending to decrease when treated with 0.25 mg/mL or higher doses of FetA. The effects of a wider dose range of FetA on the expression and secretion of lipogenesis regulators and other proteins involved on lipolysis regulation and inflammatory responses in the AT of dairy cows require further investigation. Besides the treatment of adipocytes with different doses of FetA, the analysis of the potential autocrine effects of FetA on adipocytes through silencing or inhibiting the transcription of FetA gene, could also reveal intrinsic effects of FetA on AT function in dairy cows.

Based on our in vivo and in vitro results, we hypothesize that the lipogenic and negative acute phase protein role of FetA is beneficial for periparturient cows' health. First, we observed a positive association of FetA with health biomarkers, such as serum albumin and calcium, and an inverse association with pro-inflammatory markers from 2 weeks prepartum to 10 DIM in dairy cows. Second, we observed that FetA dynamics are similar to a negative acute-phase protein with potential anti-inflammatory effects in AT. Third, we observed an anti-lipolytic and pro-lipogenic effect of FetA in adipocytes of dairy cows, suggesting that this adipokine could play a beneficial role during the intense lipid mobilization in periparturient dairy cows. Therefore, strategies to enhance FetA synthesis could potentially improve metabolic function and prevent diseases, mainly during periods of negative energy balance such as the peripartum. Previous studies demonstrated that high-fat diet induced an increase in circulating concentrations and adipose tissue secretion of FetA in rats (Pérez-Sotelo et al., 2016). In vitro, Dasgupta et al.

(2010) demonstrated a dose-dependent lipid-induced secretion of FetA by rat hepatocytes, being higher in cells treated with palmitic, myristic and stearic fatty acids compared with oleic, linolenic and arachidonic acids (Dasgupta et al., 2010). These studies suggest that the secretion of FetA could be potentially augmented by the dietary supplementation with fatty acids. Determining whether feeding supplemental fats with diverse fatty acid profiles affects FetA concentrations and function, as well as dairy cow's health will be valuable for defining potential novel nutritional strategies for the periparturient period.

Genetic studies identified that variations on FetA gene (AHSG) are associated with anti-lipolytic and pro-lipogenic properties in adipocytes, and highlighted AHSG as an attractive candidate gene for disturbed adipocyte lipolytic function in obesity and insulin resistance in humans (Dahlman et al., 2004). In ruminants, evidence from global gene expression profiling studies revealed that AHSG may be involved in regulating energy metabolism in dairy cattle (Chen et al., 2011), and lipid accumulation in several adipose tissue depots in beef cattle (Robinson and Oddy, 2004). Additionally, we found an anti-lipolytic and pro-lipogenic effect of FetA in adipocytes and observed that FetA is a negative acute phase protein in adipose tissue. We envision a genetic study identifying potential variations on AHSG associated with these valuable functions in adipose tissue of periparturient dairy cows. This will be important because AHSG could be used through genetic selection by dairy producers to help reduce disease incidence and improve profitability.

Even though we were able to demonstrate the up-regulatory effect of FetA on AGAPT2 activity, we could not conclusively affirm that AGPAT2 signaling is required for FetA lipogenic effects. Further studying the potential interdependent relationship between FetA and AGAPT2 through the use of knockout models will be conclusive to unravel additional mechanisms and

potential key targets of FetA in the lipogenic pathways in adipocytes. Additionally, AGAPT2 deficiency causes a human disease characterized by impaired triacylglycerol synthesis in white adipose tissue and leads to the development of serious insulin resistance and other metabolic dysfunctions (Agarwal et al., 2002). Therefore, the better understanding of the molecular signaling pathways connecting AGAPT2 and FetA could be comparatively important in developing therapies for human metabolic diseases.

In this study, we were able to take advantage of in vivo studies with dairy cows to develop an in vitro model with bovine adipocytes to better understand the role of FetA on lipid mobilization and inflammatory responses in adipose tissue of dairy cows. Our research provided data that can be further integrated into nutritional, genetic, and even clinical studies to prevent metabolic diseases in dairy cows. Future understandings on pathways involved on FetA modulation of lipid mobilization can potentially be translated to humans with similar adipose tissue disorders.

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