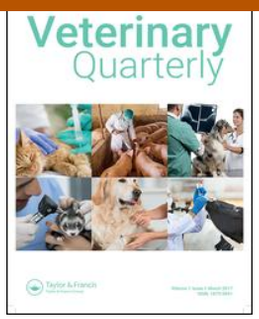

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REVIEW ARTICLE

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Disturbed bovine mitochondrial lipid metabolism: a review

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ABSTRACT

In mammals, excess energy is stored primarily as triglycerides, which are mobilized when energy demands arise and cannot be covered by feed intake. This review mainly focuses on the role of long chain fatty acids in disturbed energy metabolism of the bovine species. Long chain fatty acids regulate energy metabolism as ligands of peroxisome proliferator-activated receptors. Carnitine acts as a carrier of fatty acyl groups as long-chain acyl-CoA derivatives do not penetrate the mitochondrial inner membrane. There are two different types of disorders in lipid metabolism which can occur in cattle, namely the hypoglycaemic-hypoinsulinaemic and the hyperglycaemic-hyperinsulinaemic type with the latter not always associated with ketosis. There is general agreement that fatty acid β-oxidation capability is limited in the liver of (ketotic) cows. In accord, supplemental L-carnitine decreased liver lipid accumulation in periparturient Holstein cows. Of note, around parturition concurrent oxidation of fatty acids in skeletal muscle is highly activated. Also peroxisomal β-oxidation in liver of dairy cows may be part of the hepatic adaptations to a negative energy balance (NEB) to break down fatty acids. An elevated blood concentration of nonesterified fatty acids is one of the indicators of NEB in cattle among others like increased β-hydroxy butyrate concentration, and decreased concentrations of glucose, insulin, and insulin-like growth factor-I. Assuming that liver carnitine concentrations might limit hepatic fatty acid oxidation capacity in dairy cows, further study of the role of acyl-CoA dehydrogenases and/or riboflavin in bovine ketosis is warranted.

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1. Introduction

In mammals, excess energy is stored primarily as triacylglycerols (TAGs) also known as triglycerides, which are mobilized when energy demands arise beyond current nutrient supply via feed (Nakamura et al. 2014). TAG is an ester derived from glycerol and three fatty acids (FAs), mostly found in the various adipose tissue depots of the body which provides high dense energy storage. FAs are the major source of energy for most tissues during periods of negative energy balance (NEB) although FA can, in some circumstances, have pathological effects. In addition, if blood nonesterified fatty acid (NEFA) levels are elevated for prolonged periods, as may occur during lactation or obesity, TAG can accumulate in other tissues including liver, oocytes, regenerating endometrium and muscle cells (myocytes) (Rukkwamsuk, Kruip, Wensing, et al. 1999; Vernon 2005; Wathes et al. 2012). This can have pathological consequences such as the development of fatty liver and ketosis (Herdt et al. 1981; Grummer 1993; Herdt 2000; Drackley et al. 2001; Bobe et al. 2004; Gross et al. 2013), impairment of the immune system (Rukkwamsuk, Kruip, Wensing, et al. 1999; Zerbe et al. 2000), or gynaecological disorders and reduced reproductive efficiency (Rukkwamsuk, Kruip, Meijer, et al. 1999; Zerbe et al. 2000; Jorritsma et al. 2003; Jorritsma et al. 2005; Roche 2006; Raboisson et al. 2014; Rutherford et al. 2016).

Cells package TAG into cytoplasmic lipid droplets for storage. New emerging data, however, shows the lipid droplets as a highly dynamic storage pool of FA that can be used for energy reserve. Lipid excess packaging into lipid droplets can be seen as an adaptive response to fulfilling energy supply without hindering mitochondrial or cellular redox status and keeping low concentration of lipotoxic intermediates (Aon et al. 2014). The ability to store energy in the form of energy-dense TAG and to mobilize these stores rapidly during times of low carbohydrate availability (e.g. fasting) or during increased metabolic demand (e.g. exercise) is a highly conserved process essential for survival (Watt and Steinberg 2008; Lampidonis et al. 2011).

At rest, plasma NEFA are trafficked largely to intramyocellular TAG before they enter long-chain acylcarnitines oxidative pools. Thus, intramyocellular TAGs are an important central pool that regulates the delivery of

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FA to the intracellular environment (Kanaley et al. 2009). Medium-chain TAGs are triacylglycerols with a FA chain length varying between 6 and 10 carbon atoms and they differ from long-chain TAG as they are relatively soluble in water and, hence, rapidly hydrolysed and absorbed. Medium-chain TAGs are transported in the blood through the portal system. As a consequence, they bypass adipose tissue that makes them less susceptible to hormone-sensitive lipase (HSL) and deposition into adipose tissue stores. Due to these properties, medium-chain TAGs have been researched for both benefits to exercise performance and health. However, only two studies to date have shown an improvement in exercise performance (Clegg 2010).

Conceptus energy and nitrogen demands in late pregnancy are mostly met by placental uptake of maternal glucose and amino acids. Within a few days after parturition, mammary demands for glucose, amino acids, and FA are several-fold those of the pregnant uterus before term. Even postparturient feed increases in voluntary intake cannot satisfy this increased nutrient demand. Therefore, rates of hepatic gluconeogenesis and adipose fat mobilization are greatly accelerated (Bell 1995). As a consequence, massive mobilization of NEFA from adipose tissue starting already prior and considerably after parturition in high-yielding dairy cows is the metabolic hallmark of the transition from pregnancy to lactation (Bell 1995; Adewuyi et al. 2005) associated with the phenomenon of NEB when energy requirements (needed for milk production and maintenance) exceed energy intake (Adewuyi et al. 2005). Of note, muscle protein mobilization occurred in advance of fat mobilization periparturient in Holstein cows until approximately four weeks of lactation (van der Drift et al. 2012).

An elevated blood concentration of NEFA is one of the indicators of NEB in cattle among others like increased blood β-hydroxy butyrate (BHB) concentration (Bell 1995; Ferraretto et al. 2014), and decreased concentrations of blood glucose, insulin, and IGF-I (Vazquez-Añon et al. 1994; Grum et al. 1996; Rukkwamsuk, Kruip, Wensing, et al. 1999; Butler et al. 2003; Adewuyi et al. 2005; Gross, van Dorland, Bruckmaier, et al. 2011; Gross, van Dorland, Schwarz, et al. 2011). It has been advised that blood total ketone body concentration should be evaluated in parallel with blood glucose and NEFA with reference to ketosis (Fukao et al. 2014).

This review mainly focuses on the potential role of long chain FAs (over 12 carbon atoms) in disturbed energy metabolism of the bovine species.

2. Lipolysis

Lipolysis is the biochemical pathway responsible for the catabolism of TAG stored in cellular lipid droplets and subsequent release of FA and glycerol into the bloodstream so that they can be used by other tissues. The hydrolytic cleavage of TAG generates NEFA, which are used as energy substrates, essential precursors for lipid and membrane synthesis, or act directly or indirectly as signaling molecules and, when bonded to amino acid side chains of peptides, anchor proteins in biological membranes. Consistent with its central importance in lipid and energy homeostasis, lipolysis occurs in essentially all tissues and cell types; it is most abundant, however, in white and brown adipose tissue (Zechner et al. 2009; Chaves et al. 2011; Lass et al. 2011; Lampidonis et al. 2011). Endocrine and autocrine/paracrine factors cooperate and lead to a fine regulation of lipolysis in adipocytes (Wang 2008; Lafontan and Langin 2009; Zimmermann et al. 2009; Aon et al. 2014), whereas mitochondria and lipid droplets exhibit metabolic interactions (Aon et al. 2014).

In mammals, FAs are predominantly stored as TAG within lipid droplets of white adipose tissue. Lipid droplet-associated TAGs are also present in most non-adipose tissues, including liver, cardiac muscle, and skeletal muscle. The mobilization of FA from all fat depots depends on the activity of TAG hydrolases. Currently, three enzymes are known to hydrolyze TAG, HSL and monoglyceride lipase (MGL), as well as the relatively recently identified adipose triglyceride lipase (ATGL). It has been shown that the consecutive action of ATGL, HSL, and MGL is responsible for the complete hydrolysis of a TAG molecule (Zechner et al. 2009; Lass et al. 2011). ATGL initiates lipolysis followed by the actions of HSL on diacylglycerol, and MGL on monoacylglycerol. HSL is regulated by reversible phosphorylation on five critical residues. Phosphorylation alone, however, is not enough to activate HSL. Probably, conformational alterations and a translocation from the cytoplasm to lipid droplets are also involved. In accordance, perilipin (also known as lipid droplet-associated protein or PLIN) functions as a master regulator of lipolysis, protecting or exposing the triacylglycerol core of a lipid droplet to lipases (Lampidonis et al. 2011; Locher et al. 2011).

The classical pathway of lipolysis activation in adipocytes is cAMP-dependent. The production of cAMP is modulated by G-protein-coupled receptors of the Gs/Gi family and cAMP degradation is regulated by phosphodiesterase. However, other pathways that activate TAG hydrolysis are currently under investigation. Lipolysis can also be started by G-protein-coupled receptors of the Gq family, through molecular mechanisms that involve phospholipase C, calmodulin and protein kinase C. There is also evidence that increased lipolytic activity in adipocytes occurs after stimulation of the mitogen-activated protein kinase pathway or after cGMP accumulation and activation of protein kinase G (Chaves et al. 2011).

Additionally besides perilipin, adipophilin and other proteins of the surface of the lipid droplets like Tip47 (PAT) family of lipid droplet binding proteins

protecting or exposing the TAG core of the droplets to lipases are also potent regulators of lipolysis. Considerable progress has been made in understanding the mechanisms of activation of the various lipases. Lipolysis is under tight hormonal regulation. The best understood hormonal effects on lipolysis in adipose tissue concern the opposing regulation by insulin and catecholamines (Wang 2008; Lafontan and Langin 2009; Zimmermann et al. 2009; Aon et al. 2014). The prototypes for hormonal lipolytic control are beta-adrenergic stimulation and suppression by insulin, both of which affect cyclic AMP levels and hence the protein kinase A-mediated phosphorylation of HSL and perilipin**.** Newly recognized mediators of lipolysis include atrial natriuretic peptide, cyclic GMP, the ketone body 3-hydroxybutyrate, AMP kinase and mitogen-activated kinases (Wang 2008).

Of note, γ-melanocyte stimulating hormone (γ-MSH), a peptide derived from the ACTH precursor, pro-opiomelanocortin (POMC), and belonging to a family of peptides called the melanocortins, is involved in regulating the activity of HSL (Bicknell et al. 2009).

Nicotinic acid (niacin, NA) can suppress lipolysis, but findings on responses to dietary NA in cattle are inconsistent (Minor et al. 1998; Morey et al. 2011). Small intestinal absorption of NA averaged 98.5% in dairy cows (Santschi et al. 2005). In line, in a dose-finding study in nongestating, nonlactating Holstein cows it was not possible to identify a dose of NA that reduced plasma NEFA concentration and prevented the rebound that occurs following termination of NA administration (Pescara et al. 2010). Observations on the NA-stimulated secretion of adiponectin and the mRNA expression of chemerin in bovine adipocytes were suggestive of G-protein coupled receptor signaling-dependent improved insulin sensitivity and/or adipocyte metabolism in dairy cows (Kopp et al. 2014).

It should be realized that lipolysis must be interpreted in its physiological context since similar rates of basal or stimulated lipolysis occur under different conditions and by different mechanisms (Wang 2008).

3. β-oxidation

Beta-oxidation is the process by which FA molecules are broken down in the mitochondria or peroxisomes to generate acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle (also known as citric acid cycle or Krebs cycle), as well as NADH and FADH2, which are used by the electron transport chain. Thiolase enzyme catalyzes the release of the first two carbon units, as acetyl CoA, and a fatty acyl CoA minus two carbons. This process continues until all of the carbon atoms in the FA are broken down into acetyl CoA. In comparison, α-oxidation is the process located in peroxisomes by which certain FA are broken down by removal of a single carbon atom from the carboxyl end (Grevengoed et al. 2014).

Regarding β-oxidation, it is puzzling that hydrogen-rich FAs are used scarcely as fuel in the brain in contrast to glucose. It has been stated that the disadvantages related to using FA as fuel in the brain have created evolutionary pressure on lowering the expression of the β-oxidation enzyme equipment in brain mitochondria to avoid extensive FA oxidation and to favor glucose oxidation in brain. This has been associated with three particular problems: (1) ATP generation linked to β-oxidation of FA demands more oxygen than glucose, thereby enhancing the risk for neurons to become hypoxic; (2) β-oxidation of FA generates superoxide, which, taken together with the poor antioxidative defense in neurons, causes severe oxidative stress; (3) the rate of ATP generation based on adipose tissue-derived FA is slower than that using blood glucose as fuel. Thus, in periods of extended continuous and rapid neuronal firing, FA oxidation cannot guarantee rapid ATP generation in neurons (Schönfeld and Reiser 2013).

Of note, in severely ketotic cows a decrease in the profile density of mitochondria per one mm^2 field and an increase of the volume occupied by mitochondria were shown although not significant (Grohn and Lindberg 1985).

3.1. Mitochondrial β-oxidation

FA oxidation in the mitochondrial matrix is a major source of energy and not only fuels the TCA cycle and oxidative phosphorylation, but also stimulates hepatic synthesis of the ketone bodies (*R*)−3-hydroxybutyrate and acetoacetate, especially when physiological energy demand is increased and exceeds what can be provided via feed, through glycolysis and glycogenolysis (Liang and Nishino 2010; van Houten et al. 2016). A series of enzymes, transporters, and other facilitating proteins are involved in FA oxidation. More specific, approximately 20 different proteins play specific roles in FA oxidation (van Houten et al. 2016). This system requires L-carnitine and is composed of two acyltransferases, carnitine palmitoyltransferases 1 and 2 (CPT1 and CPT2), and carnitine acylcarnitine translocase (CACT), which is a member of the mitochondrial carrier family of proteins. CACT carries out the transport of acylcarnitines across the inner mitochondrial membrane in exchange for a free carnitine molecule (van Houten et al. 2016). Carnitine acts as a carrier of fatty acyl groups from the cytoplasm to the mitochondrion. Long-chain acyl-CoA derivatives do not penetrate the inner mitochondrial membrane. CPT1, located on the external surface of the mitochondrial inner membrane, catalyzes the conversion of cytoplasmic long-chain acyl-CoA and carnitine into acylcarnitine followed by its transport into the mitochondrial matrix in exchange

for free carnitine as mediated by the inner mitochondrial membrane protein CACT. The acylcarnitine is reconverted to intramitochondrial acyl-CoA by the action of CPT2 located in the inner membrane. Now, the acyl-CoA is available for β-oxidation in the matrix. In liver, malonyl-CoA, the first committed intermediate produced during FA synthesis, is proposed to regulate the activity of CPT1 (Hoppel 1982; Drackley et al. 2001; Bartlett and Eaton 2004; van Houten et al. 2016). Malonyl-CoA decreases in response to lowered blood concentrations of insulin and glucagon/adrenalin and increases in line with enhanced blood insulin concentration in ruminants (Bell 1995; Brindle et al. 1985; Knapp and Baldwin 1990). The most pronounced effects of various hormones are on the supply of NEFA to the liver rather than on its intracellular disposal (Drackley et al. 2001).

Three isoforms of CPT1 exist in mammalian tissues, namely the liver isoform (CPT1A or CPT1-L), the muscle isoform (CPT1B or CPT1-M), and the brain isoform (CPT1C) (Lavrentyev et al. 2004). Muscle CPT1α and β mRNA were shown to be upregulated in early lactation in dairy cows (Schäff et al. 2013). With respect to acylcarnitine synthesis in rat mitochondria, the linolenate (C18:2) pool always had the highest fractional turnover rate. Conversely, the stearate (18:0) pool had the lowest fractional turnover rate (Gavino et al. 2003).

Acyl-CoA dehydrogenases are a class of at least 11 enzymes most of which play a role in FA oxidation or amino acid catabolism in the mitochondria of cells (Swigonova et al. 2009; van Houten et al. 2016). Their action results in the introduction of a trans double-bond between C2 (α) and C3 (β) of the acyl-CoA thioester substrate (Thorpe and Kim 1995). Acyl-CoA dehydrogenases have been identified in animals (nine major eukaryotic classes) with five of these nine classes involved in FA β-oxidation (SCAD, MCAD, LCAD, VLCAD, and VLCAD2), and the other four involved in branched chain amino acid metabolism (i3VD, i2VD, GD, and iBD) (Thomas and Sampsom 2013; Wipperman et al. 2013). They can be categorized into three distinct groups based on their specificity for short-, medium-, or long-chain FA acyl-CoA substrates (Kim et al. 1993).

Inside the mitochondrion, acyl-CoAs are degraded via β-oxidation, a cyclic process consisting of four enzymatic steps. Each cycle shortens the acyl-CoA by releasing the two carboxy-terminal carbon atoms as acetyl-CoA. The cycle is initiated by dehydrogenation of the acyl-CoA to *trans*-2-enoyl-CoA by an acyl-CoA dehydrogenase. This step is followed by ahydration catalyzed by an enoyl-CoA hydratase, generating (*S*)−3-hydroxyacyl-CoA, which is subsequently dehydrogenated to 3-ketoacyl-CoA in a reaction performed by (*S*)−3-hydroxyacyl-CoA dehydrogenase. Finally, a thiolase cleaves the 3-ketoacyl-CoA into a two-carbon chain–shortened acyl-CoA and an acetyl-CoA (van Houten et al. 2016).

On the basis of the different substrate specificities of the individual FA oxidation enzymes, it is assumed that the long-chain acyl-CoAs first undergo two to three β-oxidation cycles by the membrane-bound enzymes VLCAD and mitochondrial trifunctional protein (MTP). The resulting medium-chain acyl-CoAs are then handled by the matrix-localized enzymes MCAD, crotonase, SCHAD, and MCKAT. Finally, the short-chain acyl-CoAs are metabolized by SCAD, crotonase, SCHAD, and MCKAT (van Houten et al. 2016). SCAD is a mitochondrial enzyme that catalyzes the dehydrogenation of short chain FAs (four to six carbons in length) thereby initiating the cycle of β-oxidation. This process generates acetyl-CoA, the key substrate for hepatic ketogenesis or ATP production by the TCA acid cycle (Turpin and Tobias 2005). MTP harbors enoyl-CoA hydratase, (*S*)−3-hydroxyacyl-CoA dehydrogenase, and 3-ketothiolase activities that are specific for long-chain intermediates (van Houten et al. 2016).

In addition, the sirtuins represent a family of NAD (+)-dependent protein deacetylases that regulate cell survival, metabolism, and longevity. Three sirtuins, SIRT3-5, localize to mitochondria. Expression of SIRT3 is selectively activated during fasting and calorie restriction. SIRT3 regulates the acetylation level and enzymatic activity of key metabolic enzymes, such as acetyl-CoA synthetase, long-chain acyl-CoA dehydrogenase, and 3-hydroxy-3-methylglutaryl-CoA synthase 2, and enhances fat metabolism during fasting (Newman et al. 2012). Furthermore, SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative stress (Tseng et al. 2013). The existence of the SIRT1-peroxisome proliferator-activated receptor (PPAR) GC1A-axis has been demonstrated in dairy cows and indicates a functional relationship between SIRT1 and adiponectin type 1 receptor in bovine adipose tissue (Weber et al. 2016).

Facilitation of β-oxidation of NEFA, by 'sparing' glucose for oxaloacetate formation in the major peripheral tissues, increases the diversion of pyruvate to oxaloacetate formation which facilitates the entry of acetyl-CoA derived from FA β-oxidation into the TCA cycle through citrate formation (Eaton et al. 1996; Sugden et al. 2001). However, if the TCA cycle gets overloaded (e.g. by capacious drainage of oxaloacetate for gluconeogenesis), the acetyl-CoA is shunted off to produce ketone bodies (acetoacetic acid, acetone, and β-hydroxy butyrate) in order to prevent cessation of the TCA cycle and accumulation of acetyl-CoA (Sato et al. 1999; Block and Sanchez 2000; Sugden et al. 2001).

3.2. Peroxisomal β-oxidation

Peroxisomes are subcellular organelles which are present in virtually every eukaryotic cell and catalyze a large number of metabolic functions. The importance

of peroxisomes for humans is stressed by the existence of a large group of genetic diseases in which either the biogenesis of peroxisomes is impaired or one of its metabolic functions. Thanks to the work on Zellweger syndrome which is the prototype of the group of peroxisomal disorders, much has been learned about the metabolism and biogenesis of peroxisomes in humans. These metabolic functions include: (1) FA β-oxidation; (2) etherphospholipid biosynthesis; (3) FA α-oxidation, and (4) glyoxylate detoxification. Since peroxisomes lack a TCA cycle and a respiratory chain, peroxisomes are relatively helpless organelles which rely heavily on their cross-talk with other subcellular organelles in order to metabolize the end products of metabolism as generated in peroxisomes (Wanders 2013, 2014; Hunt et al. 2014). Many of the metabolites which require peroxisomes for their homeostasic regulation are involved in signal transduction pathways. These include the primary bile acids, platelet activating factor, plasmalogens, N-acylglycines and N-acyltaurines, docosahexaenoic acid as well as multiple prostanoids (Wanders 2013).

It has been stated that peroxisomal β-oxidation in liver of dairy cows may be a part of the hepatic adaptations to NEB (Grum et al. 2002). Peroxisomal β-oxidation capacity and the ratio of peroxisomal to total β-oxidation in Holstein cows decreased from week 3 to 12 after parturition and then increased at week 42 post-partum (Grum et al. 2002). When hepatic peroxisomal β-oxidation rates were compared in liver homogenates from cows and rats during different nutritional and physiological states, peroxisomal oxidation in liver homogenates from cows represented 50% and 77% of the total capacity for the initial cycle of β-oxidation of palmitate (16:0) and octanoate (8:0), respectively, but only 26% and 65% for rats, whereas lactation or food deprivation did not alter rates of hepatic peroxisomal β-oxidation of palmitate or octanoate in cows (Grum et al. 1994).

Long-chain FAs regulate energy metabolism as ligands of PPARs. PPAR-α expressed primarily in liver is essential for metabolic adaptation to starvation by inducing genes for β-oxidation and ketogenesis and by downregulating energy expenditure through fibroblast growth factor 21. PPAR-δ is highly expressed in skeletal muscle and induces genes for long chain FAs oxidation during fasting and endurance exercise. PPAR-δ also regulates glucose metabolism and mitochondrial biogenesis by inducing FOXO1 and PGC1-α. Genes targeted by PPAR-γ in adipocytes suggest that PPAR-γ senses incoming non-esterified long chain FAs and induces the pathways to store long chain FAs as triglycerides. Adiponectin, another important target of PPAR-γ may act as a spacer between adipocytes to maintain their metabolic activity and insulin sensitivity (Nakamura et al. 2014).

Saturated long-chain FAs (mainly 16:0 and 18:0 fed at 250 g/day) were effective in upregulating the bovine adipose tissue PPARγ-gene network. In contrast, only saturated long-chain FAs led to sustaining that response. Overall, the observed expression patterns are suggestive of an adipogenic regulatory mechanism particularly responsive to saturated long-chain FAs (Schmitt et al. 2011). Overfeeding energy upregulates peroxisome PPARγ-controlled adipogenic and lipolytic gene networks but does not affect proinflammatory markers in visceral and subcutaneous adipose depots of Holstein cows. Overfeeding energy also may predispose cows to greater lipolytic potential by stimulating expression of TAG hydrolysis genes while inhibiting signaling via hydroxycarboxylic acid receptor 1, which is a novel antilipolytic regulator (Ji et al. 2014). In line, liver from overfed cows (1.62 Mcal/kg BW) responded to postpartal NEB by up-regulating expression of PPARα-targets in the FA oxidation and ketogenesis pathways, along with gluconeogenic genes. Hepatokines (fibroblast growth factor 21 and angiopoietin-like 4) and apolipoprotein A-V were up-regulated postpartum to a greater extent in overfed than controls (1.34 Mcal/kg BW)(Khan et al. 2014). Although overfeeding compared with restricted feeding did not significantly alter the *in vitro* lipolytic response to 3-hydroxybutyrate or glucose, adipose tissue from overfed cows tended to be less inhibited by these substances, which may contribute to higher lipolytic rates *in vivo* and a greater triacylglycerol accumulation in the liver after parturition (Rukkwamsuk et al. 1998).

The mRNA abundance of muscle PPARγ increased in early lactation and was higher in cows with high mean liver fat content compared to low mean liver fat content, whereas the abundance of PPARα continuously decreased after parturition. The mRNA abundance of muscle PPARδ, uncoupling protein 3, and the β-oxidative enzymes 3-hydroxyacyl-coenzyme A dehydrogenase, very long-chain acyl-CoA dehydrogenase, and 3-ketoacyl-CoA was greatest at day three after parturition, whereas the abundance of PPARγ coactivator 1α decreased after parturition (Schäff et al. 2013).

Of note, peroxisomal β-oxidation in liver of dairy cows was not affected by feeding supplemental fat or NA during weeks 4–42 of lactation (Grum et al. 2002).

4. Carnitine

Carnitine is an amino acid derivative of lysine and methionine found in high energy demanding tissues (skeletal muscles, myocardium, the liver and the adrenal glands). It is essential for the intermediary metabolism of FAs and is indispensable for β-oxidation of long-chain FAs in the mitochondria but also regulates CoA concentration and removal of the produced acyl groups. Acyl-CoA acts as restraining factor for several

enzymes participating in intermediary metabolism. Transformation of acyl-CoA into acylcarnitine is an important system also for removing the toxic acyl groups (Hoppel 1982; Evangeliou and Vlassopoulos 2003). In rodents and pigs, it has been shown that carnitine synthesis and uptake of carnitine into cells are also regulated by PPARα, a transcription factor which is physiologically activated during fasting or energy deprivation (Schlegel et al. 2012). Feed restriction downregulated BBOX, a key enzyme involved in L-carnitine biosynthesis in cows (Akbar et al. 2013). Schlegel et al. (2012) showed that the expression of hepatic genes of carnitine synthesis and cellular uptake of carnitine is enhanced in dairy cows during early lactation. These changes might provide an explanation for increased hepatic carnitine concentrations observed in the first postpartum period and might be regarded as a physiologic means to provide liver cells with sufficient carnitine required for transport of excessive amounts of NEFA during a NEB (Schlegel et al. 2012).

As L-carnitine is required for mitochondrial FA oxidation, abomasal infusion of L-carnitine indeed increased *in vitro* hepatic FA oxidation, decreased liver lipid accumulation, and supported higher fat-corrected milk yield in feed-restricted lactating Holstein cows (Carlson et al. 2006; Akbar et al. 2013), whereas infusion of up to 12 g/day of carnitine into the abomasum did not improve milk yield or nutrient digestibilities (LaCount et al. 1996). More specific, abomasal infusion of L-carnitine (from day 25 relative to expected calving date until 56 days in milk at 6, 50, or 100 g/day) decreased liver total lipid and triacylglycerol accumulation on day 10 after calving. In addition, carnitine-supplemented cows had higher liver glycogen during early lactation. In general, carnitine supplementation increased *in vitro* palmitate (C16:0) β-oxidation by liver slices, with medium and high treatments affecting *in vitro* palmitate metabolism more potently than did lower treatment, whereas the concentration of NEFA in serum was not affected by carnitine. As a result of greater hepatic FA β-oxidation, plasma BHB was higher for the medium and high treatments. By decreasing liver lipid accumulation and stimulating hepatic glucose output, carnitine supplementation might improve glucose status and diminish the risk of developing metabolic disorders during early lactation (Carlson, McFaden, et al. 2007). Similarly, in liver slices from cows during early lactation, carnitine increased oxidation and total utilization of palmitate and decreased palmitate esterification (Drackley et al. 1991). Abomasal L-carnitine infusion (at 20 g/day during nine days in midlactation) increased total carnitine in plasma, liver, muscle, and milk during feed restriction, whereas feed restriction alone increased carnitine concentrations in muscle and milk but not in the liver. Carnitine infusion increased the concentration of each milk carnitine fraction as well as milk carnitine output on days five to six after start of the infusion. Remarkably, on the last two days of the nine-day infusion period, all carnitine fractions were increased in carnitine-infused, feed-restricted cows, whereas all except short-chain acylcarnitine were increased in milk from water-infused, feed-restricted cows. As a consequence, it has been concluded that liver carnitine concentrations might limit hepatic FA oxidation capacity in dairy cows during the periparturient period. Therefore, supplemental L-carnitine has been advocated in order to decrease liver lipid accumulation in periparturient cows (Carlson, Woodworth, et al. 2007).

5. Riboflavin

Vitamin B_2 (or riboflavin) is an essential dietary compound used for the enzymatic biosynthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The human genome contains 90 genes encoding for flavin-dependent proteins, six for riboflavin uptake and transformation into the active coenzymes FMN and FAD as well as two for the reduction to the dihydroflavin form. Flavoproteins utilize either FMN (16%) or FAD (84%) while five human flavoenzymes have a requirement for both FMN and FAD. The majority of flavin-dependent enzymes catalyze oxidation-reduction processes in primary metabolic pathways such as the TCA cycle, β-oxidation and degradation of amino acids. Flavin-dependent proteins also play an important role in the biosynthesis of other essential cofactors and hormones such as coenzyme A, coenzyme Q, heme, pyridoxal 5'-phosphate (the active form of vitamin B_6), steroids and thyroxine (Henriques et al. 2010; Lienhart et al. 2013).

Little is known about riboflavin in relation to fat metabolism in the bovine species. Small intestinal absorption in calves averaged 25% (Zinn et al. 1987) and 99% in dairy cows (Santschi et al. 2005). Given the fact that riboflavin is an essential dietary compound used for the enzymatic biosynthesis of FMN and FAD, and that flavoproteins utilize either FMN or FAD in order to catalyze processes in primary metabolic pathways such as the β-oxidation (Henriques et al. 2010; Lienhart et al. 2013), its role in bovine ketosis needs further study.

6. Ketogenesis

Ketogenesis is the branch of mammalian metabolism concerned with the synthesis of ketone bodies. In this process, the small, water-soluble compounds acetoacetate, D-3-β-hydroxybutyrate and propanone are produced by the liver in response to reduced glucose availability. Although ketone bodies are always present at a low level in healthy individuals, dietary manipulation and certain pathological conditions can increase the levels of these compounds *in vivo* (McPherson and McEneny 2012). Rather than metabolization via the TCA cycle, acetyl-CoA might also be used instead in

biosynthesis of ketone bodies via acetoacetyl-CoA and β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). Ketone bodies are important vectors of energy transport from the liver to extrahepatic tissues, especially during fasting, when glucose supply is low. Blood total ketone body levels should be evaluated in parallel with blood glucose and free FAs (Fukao et al. 2014).

It has been shown that ketone bodies inhibit protein degradation and thereby gluconeogenesis and also are able to spare glucose by inhibiting glucose utilization (Zarrin et al. 2013; Zarrin et al. 2014a, 2014b; Zarrin et al. 2017). They also can inhibit lipolysis and function as a regulatory safety system, replacing insulin, in situations when the activity of this hormone is low, as in type I bovine ketosis. Ketone bodies thus have important functions as substrates replacing glucose in many tissues including the immune system and also as signal substances in the regulation of energy metabolism (Holtenius and Holtenius 1996; Zarrin et al. 2014a, 2014b).

Under fasting conditions, hepatic FA binding protein (L-FABP) contributes to hepatic long chain FAs oxidation and ketogenesis by a nontranscriptional mechanism, whereas L-FABP can activate ketogenic gene expression in fed mice. Thus, the mechanisms whereby L-FABP affects FA oxidation may vary with physiological status (Erol et al. 2004).

Messenger RNA and protein levels of long-chain acyl-CoA synthetase were significantly higher in livers of ketotic cows than those in nonketotic cows. In contrast, mRNA levels of CPT1 and mRNA and protein levels of CPT2, long chain acyl-CoA dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA synthase, and acetyl-CoA carboxylase were decreased in the liver of ketotic cows. Serum NEFA concentrations positively correlated with long chain acyl-CoA synthetase protein levels and negatively correlated with protein levels of CPT2, long chain acyl-CoA dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA synthase, and acetyl-CoA carboxylase. In addition, serum BHB concentration negatively correlated with protein levels of CPT2, acyl-CoA dehydrogenase long chain, and 3-hydroxy-3-methylglutaryl-CoA synthase. Overall, FA β-oxidation capability was reduced in the liver of ketotic compared with nonketotic cows (Murondoti et al. 2004; Carlson, Woodworth, et al. 2007; Li et al. 2012; Gross et al. 2013). It should also be realized that deficiencies of various acyl-CoA dehydrogenases lead to FA oxidation disorders involving episodes of metabolic derangement in man (Wanders et al. 1989, Wanders et al. 1992, van Houten et al. 2016). Of note, around parturition oxidation of FA in skeletal muscle is highly activated in dairy cows, which may contribute to diminish the FA load on the liver. The decline in muscle FA oxidation within the first four weeks of lactation accompanied with increased feed intake refer to greater supply of ruminally derived acetate, which as the preferred fuel of the muscle, saves long-chain FAs for milk fat production (Schäff et al. 2013).

Of note, it cannot be excluded that reduced phenolics from the rumen inhibit metabolism in liver cells including β-oxidation. Benzoic acid, 3-phenylpropionic acid, trans-cinnamic acid, and 3-(4-hydroxyphenyl)propionic acid in ruminal fluid are presumed to be the products of chemical reduction of dietary phenolic monomers by ruminal microorganisms. These reduced phenolics, which are representative of those in ruminal fluid, inhibited metabolism of bovine liver tissue *in vitro* at supraphysiological concentrations (Cremin et al. 1994).

7. Pathophysiology of ketogenesis

In dairy cows, overfeeding during the dry period leads to overcondition at calving and to depression of appetite after calving. As a consequence, at calving overconditioned high-producing dairy cows inevitably go into a more severe NEB postpartum than cows that have a normal appetite. During the period of NEB, the energy requirements of the cow are satisfied by lipolysis and proteolysis. Lipolysis results in an increased concentration of NEFA in the blood. In the liver, these NEFA are predominantly esterified to TAGs that are secreted in very low density lipoproteins (VLDL). In early lactation in cows with a severe NEB, the capacity of the liver to maintain the export of the TAG in the form of VLDL in balance with the hepatic TAG production is not always adequate. As a result, the excess amount of TAG accumulates in the liver, leading to fatty infiltration of the liver (hepatic lipidosis or fatty liver) (Rukkwamsuk, Kruip, Wensing, et al. 1999) and ketosis (Herdt et al. 1981; Herdt 2000).

Reported defects in human ketogenesis include mitochondrial HMG-CoA synthase deficiency and HMG-CoA lyase deficiency. Mitochondrial HMG-CoA synthase deficiency should be considered in nonketotic hypoglycemia if a FA β-oxidation defect is suspected, but cannot be confirmed. Human patients with HMG-CoA lyase deficiency can develop hypoglycemic crises and neurological symptoms even in adolescents and adults. Succinyl-CoA-3-oxoacid CoA transferase (SCOT) deficiency and β-ketothiolase (T2) deficiency are two defects in ketolysis. Permanent ketosis is pathognomonic for SCOT deficiency. However, patients with 'mild' SCOT mutations may have nonketotic periods. T2-deficient patients with 'mild' mutations may have normal blood acylcarnitine profiles even in ketoacidotic crises. T2 deficient patients cannot be detected in a reliable manner by newborn screening using acylcarnitines (Fukao et al. 2014). Furthermore, acetylcarnitine accounts for a major fraction of the acylcarnitines excreted in the ketotic conditions. The contribution of acetylcarnitine to the change in acylcarnitines as ketosis appears or disappears is

significantly less in obese human subjects than in normal-weight subjects or in diabetic patients. This difference may reflect an alteration in the production or disposition of acetyl-CoA and acetylcarnitine in obesity (Hoppel and Genuth 1982).

Although primary carnitine deficiency is unusual, depletion due to secondary causes, such as a disease or a medication side effect, can occur. Primary carnitine deficiency is caused by a defect in the plasma membrane carnitine transporter (OCTN2) in muscle and kidneys. Secondary carnitine deficiency is associated with several inborn errors of metabolism and acquired medical or iatrogenic conditions (Hoppel 1982; Evangeliou and Vlassopoulos 2003).

The most likely cause of bovine ketosis is the limited capacity of the bovine liver for complete oxidation of NEFA, leading to an increased formation of ketone bodies, reesterification, and accumulation of triglycerides in the liver (Murondoti et al. 2004; Carlson, Woodworth, et al. 2007; Li et al. 2012; Gross et al. 2013; Schäff et al. 2013). Of note, no inborn errors in ketolysis and primary carnitine deficiency have been reported in the bovine species yet.

8. (Subclinical) Ketosis

The beginning of lactation requires huge metabolic adaptations to meet increased energy demands for milk production of dairy cows (Bell 1995; Rukkwamsuk, Kruip TA, Wensing, et al. 1999; Vernon 2005; Wathes et al. 2012; Gross et al. 2013; Schäff et al. 2013). Dairy cows pass through a period of NEB as they transition from late gestation to early lactation (Herdt et al. 1981; Bell 1995; Rukkwamsuk, Kruip, Wensing, et al. 1999; Gross, van Dorland, Bruckmaier, et al. 2011; McArt et al. 2013) during which they are highly susceptible to developing ketosis and liver lipidosis, which are costly diseases to farmers (Loor et al. 2007). Subclinical ketosis is usually defined as a blood BHB concentration beyond 1.2 mmol/L (McArt et al. 2012).

Of interest, a ketosis model can be induced in dairy cows by restricting feed intake plus feeding 1,3-butanediol. Increases in BHB in blood result from metabolism of the 1,3-butanediol. Production of BHB from butyrate by the tissues was greatest for liver and rumen and much less for kidney in nonlactating, nonpregnant Holstein cows (Drackley et al. 1990). By using this model, ketosis begun at day 15 postpartum, caused ketonemia and gradual development of clinical ketosis by day 40–45 in cows. Concentrations of NEFA in plasma of cows that became ketotic increased 3.0-, 2.6-, and 1.9-fold at three weeks before, two weeks before, and at ketosis, respectively, but increased nonsignificantly for glucose-treated cows. Concurrently, BHB increased 3.5-, 5.8- and 8.4-fold for cows that became ketotic but 1.6-fold or less for glucose-treated cows. Plasma acetate increased dramatically two weeks before ketosis. Liver glycogen content decreased to nearly 0 by two weeks before ketosis occurred, but it increased to prepartal values in glucose-treated cows. Liver triglycerides averaged 2.0% of wet weight at day five for all cows but increased to 8%–10% for about two weeks before ketosis occurred. Hepatic *in vitro* gluconeogenic capacity decreased significantly for ketosis induction protocol cows when clinical ketosis was detected. Results indicate that experimental ketosis was preceded by metabolic abnormalities up to two weeks before clinical ketosis occurred (Veenhuizen et al. 1991). In addition, the increased lipolysis after parturition led to a vast increase in the hepatic triacylglycerol concentration and to a shift in hepatic FA composition. In cows with fatty liver, the percentages of two of the four major bovine FAs palmitic (C16:0) and oleic (C18:1) acids were higher at 0.5 week after parturition than at one week before parturition, whereas percentages of stearic (C18:0) and linoleic (C18:2) acids decreased (Rukkwamsuk, Kruip, Meijer, et al. 1999). However, different feeding regimens during the dry period do not influence the composition of FAs in adipose tissue (Rukkwamsuk et al. 2000).

Of interest, a total of 2415 genes were altered by ketosis in dairy cows with downregulation of genes associated with oxidative phosphorylation, protein ubiquitination, and ubiquinone biosynthesis. Other molecular adaptations included upregulation of genes and nuclear receptors associated with cytokine signaling, FA uptake/transport, and FA oxidation. Genes downregulated during ketosis included several associated with cholesterol metabolism, growth hormone signaling, proton transport, and FA desaturation (Loor et al. 2007).

Field studies have shown that subclinical ketosis often affects 40% of cows in a herd although the incidence can be as high as 80% (McArt et al. 2013). Both peak incidence and prevalence of subclinical ketosis occurred at five days in milk with values of 22% and 29%, respectively (McArt et al. 2012). Herds with more than a 15%–20% prevalence of excessively elevated concentrations of NEFA and BHB in early lactation have higher rates of negative subsequent events (McArt et al. 2013; Raboisson et al. 2014). Cows first testing subclinical ketosis positive from three to five days in milk were 6.1 times more likely to develop a displaced abomasum than cows first testing subclinical ketosis positive at six days in milk or later, whereas cows first testing subclinical ketosis positive from three to seven days in milk were 4.5 times more likely to be removed from the herd, were 0.7 times as likely to conceive to first service, and produced 2.2 kg less milk per day for the first 30 days in milk than cows first testing positive at eight days in milk or later. Each 0.1 mmol/L increase in BHB at first subclinical ketosis-positive test increased the risk of developing a displaced abomasum by a factor of 1.1, increased the risk of removal from herd by a

factor of 1.4, and was associated with a decrease in milk production by 0.5 kg/day for the first 30 days in milk. These results show that time of onset and BHB concentration of first subclinical ketosis-positive test are important indicators of individual cow performance (McArt et al. 2012).

Besides increased lipolysis, low insulin/glucagon ratios and malonyl-CoA concentrations are prerequisites for ketogenesis. From an etiological viewpoint, there are two quite different types of metabolic disorders in which ketosis can occur, the hypoglycaemic-hypoinsulinaemic and the hyperglycaemic-hyperinsulinaemic type. The former, type I, generally occurs 3–6 weeks after calving in cows whose milk secretion is so extensive that the demand for glucose exceeds the capacity for glucose production. To protect the body from hazardous protein degradation by a high rate of gluconeogenesis, this process is inhibited and the increased energy requirements are met by the elevated utilization of ketone bodies. The hyperglycaemic-hyperinsulinaemic form, type II, generally occurs earlier in lactation. An important etiologic factor is overfeeding in the dry period, which can lead to disturbances in the hormonal adaptation of metabolism at calving with increased plasma levels of insulin and glucose and often out not always also with hyperketonaemia (Holtenius and Holtenius 1996).

By defining subclinical ketosis as (1) BHB concentration $>$1.4 mM; (2) NEFA concentration $>$ 0.4 mM prepartum; or (3) NEFA concentration $>$ 1.0 mM, postpartum corrected the underestimated risk of developing various diseases, reproductive disorders, and changes in milk production (Raboisson et al. 2014). Subclinically ketotic cows also showed an elevated proportion of C18:1 cis-9 in milk fat (van Haelst et al. 2008), thereby potentially indicating a acyl-CoA dehydrogenase deficiency.

The pathway for oxidation of energy involves a balanced oxidation of C2 and C3 compounds. During early lactation in dairy cattle, this C2/C3 ratio is out of balance, due to a high availability of lipogenic (C2) products and a low availability of glycogenic (C3) products relative of the C2 and C3 products required for milk production. It is clear that dietary energy source can affect the balance of the C2/C3 ratio, as indicated by plasma NEFA, BHB, and glucose levels. It has been shown that glycogenic nutrients increase glucose and insulin concentrations and decrease NEFA and BHB plasma levels. Extra lipogenic nutrients elevate NEFA and β-hydroxybutyrate and decrease plasma glucose concentrations. Lipogenic nutrients generally increase milk fat percentage and decrease milk protein percentage, suggesting a surplus of C2 compounds. The inverse is the case for feeding extra glycogenic nutrients, implying reduced deamination and oxidation of glycogenic amino acids. Feeding extra glycogenic nutrients improved the energy balance, in contrast to ambiguous results of lipogenic nutrients on energy balance. Moreover, glycogenic feed may reduce the severity of ketosis and fatty liver, but increased the incidence of (sub)clinical acidosis (van Knegsel et al. 2005).

9. Conclusions

The capacity of the bovine liver for complete oxidation of NEFA is limited, leading to an increased formation of ketone bodies, reesterification, and accumulation of triglycerides in the liver when dairy cows pass through a period of NEB as they transition from late gestation to early lactation. As no inborn errors in ketolysis have been reported in the bovine species yet, secondary deficiencies are regarded more likely to occur. For instance, mRNA levels of CPT1 and long chain acyl-CoA dehydrogenase were decreased in the liver of ketotic cows pointing towards the role of long chain acylcarnitines in bovine ketosis. As a consequence, further study of the role of acyl-CoA dehydrogenases in bovine ketosis is warranted as deficiencies of these enzymes lead to FA oxidation disorders in other species. Furthermore, it has been shown that carnitine administration to dairy cows decreased liver total lipid and triacylglycerol accumulation *in vitro* and as a consequence the role of potential carnitine deficiency in bovine ketosis needs further study as well. Carnitine synthesis and uptake of carnitine into cells might be regulated by PPARα. In line, the abundance of PPARα continuously decreased after parturition. Given the fact that riboflavin is an essential dietary compound used for the enzymatic biosynthesis of FMN and FAD, and that flavoproteins utilize either FMN or FAD in order to catalyze processes in primary metabolic pathways such as β-oxidation its role in bovine ketosis needs further study too.

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