Sphingolipid Profiling Reveals Different Extent of Ceramide Accumulation in Bovine Retroperitoneal and Subcutaneous Adipose Tissues

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Research

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Abstract

Background: Sphingolipids are bioactive lipids that can modulate insulin sensitivity, cellular differentiation, and apoptosis in a tissue-specific manner. Previous studies in dairy cattle reported that the retroperitoneal adipose tissue (RPAT) was more active than the subcutaneous adipose tissue (SCAT) in terms of insulin sensitivity, lipolytic activity, and pro-inflammatory signaling. Sphingolipids were discussed to be involved in inflammation, however, their comparative profiles in bovine RPAT and SCAT are currently unknown. We aimed to characterize the sphingolipid profiles using a targeted lipidomics approach and to assess whether potentially related sphingolipid pathways are different between SCAT and RPAT. Holstein bulls (n = 6) were slaughtered, and SCAT and RPAT samples were collected for sphingolipid profiling. A total of 70 sphingolipid species were detected, including 24 species of ceramide (Cer) and dihydroceramide (DHCer), 18 species of sphingomyelin (SM) and dihydrosphingomyelin (DHSM), 11 species of ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P), 9 species of galactosylceramide (GalCer), glucosylceramide (GluCer), lactosylceramide (LacCer), and 8 species of sphinganine (DHSph) and sphingosine (Sph). The concentration of each sphingolipid was quantified by UPLC-MRM/MS.

Results: Our results showed that sphingolipids of the *de novo* synthesis pathway such as DHSph, DHCer, and Cer, were more concentrated in RPAT than in SCAT. Sphingolipids of the salvage pathway and the sphingomyelinase pathway such as Sph, S1P, C1P, glycosphingolipid, and SM were more concentrated in SCAT. Our results indicate that RPAT had a greater extent of ceramide accumulation, and thereby increased the concentration of further sphingolipid intermediates in the *de novo* synthesis pathway.

Conclusion: This distinctive sphingolipid distribution pattern in RPAT and SCAT can potentially explain the tissue-specific activity in insulin sensitivity, pro-inflammation, and oxidative stress in RPAT and SCAT.

Background

Sphingolipid is a class of structural lipids in eukaryotic cells that not only constitutes the cell membrane but also exhibits cell signaling function to modulate insulin sensitivity, differentiation, and apoptosis in a tissue-specific manner [1, 2]. Although associations between bovine metabolic health and sphingolipid function have partially been established [3], most of this research merely focused on ceramides. Even less studies have discussed the physiological role of sphingolipids in bovine adipose tissue. To better understand the sphingolipid function in bovine adipocytes, it is important to consider the dynamics in the metabolic pathways of sphingolipid synthesis, degradation, and modification: the *de novo* synthesis pathway, the salvage pathway, and the sphingomyelinase pathway (Fig. 1). The *de novo* synthesis pathway is essential to the survival and normal metabolic activity of adipocytes. It was demonstrated that an interruption of the *de novo* synthesis pathway by adipocyte-specific serine palmitoyltransferase (SPT) mutation reduces adipose tissue size, decreases the downstream sphingolipid quantity and significantly decreases the circulating level of adipokines leptin and adiponectin in mice [4]. The salvage pathway is important for sphingolipid recycling and turnover. Large sphingolipids such as

glycosphingolipids and ceramide-1-phosphates (C1P) are broken down into sphingosine, and further transformed into ceramide [5]. The sphingomyelinase pathway or sphingomyelin hydrolysis pathway is essential for cellular function and health. It was observed that human adipose tissues affected by inflammation featured greater gene expression levels of sphingomyelinase [6]. This implied that sphingomyelin-driven ceramides could be associated with inflammation, under the transformation by sphingomyelinase [7]. Studying sphingolipid biology, particularly in adipose tissue, is critical in dairy cattle because the physiological function of sphingolipids are suggested to represent a potential link between metabolic stress and physiological adaptation [1].

Adipocyte metabolism is important for sphingolipid biology not only because of its role in lipid storage and release but also because of its active role in regulating homeostasis and inflammatory response [8]. However, adipose metabolism differs to some extent between adipose depots. Retroperitoneal (RPAT) and subcutaneous adipose tissue (SCAT) in dairy cows are suggested to be different in insulin signaling, proinflammatory signaling, and lipolytic activity [9–11]. It was demonstrated that RPAT is more responsive than SCAT regarding the insulin signaling pathway, with greater Akt and AMPK phosphorylation, as well as greater FAS expression, shown by an ex vivo study [9]. Also, RPAT had greater proinflammatory cytokines and chemokines mRNA expression than SCAT during energy overfeeding [10]. LF Locher, N Meyer, EM Weber, J Rehage, U Meyer, S Danicke and K Huber [11] demonstrated that the hormone-sensitive lipase (HSL) activity was higher in RPAT than SCAT as reflected by a greater extent of phosphorylation at Ser 660, an activation marker of HSL. Additionally, RPAT had higher lipolytic activity than SCAT under catecholamine stimulation [12]. In contrast to SCAT, RPAT accumulated and lost adipose mass with greater fluctuation during the peripartum period [13]. Collectively, these studies demonstrated that RPAT responds more dynamically to metabolic stimuli than SCAT, which is in agreement with human adipose biology [14].

To study the systemic relationship of sphingolipids in adipose tissues, particularly in SCAT and RPAT, the comparative distribution pattern of various sphingolipid moieties in these adipose depots has to be determined. However, the sphingolipid profiles of bovine SCAT and RPAT have not yet been reported. We hypothesized that the sphingolipid profiles differ between RPAT and SCAT, particularly ceramides may be more concentrated in RPAT than in SCAT, given that RPAT was shown to be more prone to proinflammatory signaling than SCAT. In this study, we aim to characterize the sphingolipid profiles using a lipidomics approach and to determine whether SCAT and RPAT differ in the concentration of various sphingolipid species. Hence, the objectives of this study were to compare and contrast the sphingolipid species in SCAT and RPAT, and to distinguish the major differences in the sphingolipid biochemical pathways of both tissues.

Materials And Methods

Animals and Sampling

Six German Holstein bulls, intended for beef production, were used for adipose tissue sample collection for this study. Animals were kept at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle (Muenchweiler a.d. Alsenz, Germany). They were a subgroup of a larger cohort used for a nutritional trial, approved by the relevant Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany) in agreement with the German Animal Welfare Act (permit number: G-17-20-070). Bulls were housed on slatted floor with rubber mats in groups of four animals and were fed a total mixed ration based on grass silage and corn silage. Although the current study's objectives did not include the study of nutritional influence on adipose depot dependent sphingolipid abundance, the diet of three of the six bulls included an additional 6 kg per day concentrate for 7 months before slaughter. This is because the larger nutritional trial that these animals derived from had a focus on intensive and moderate fattening regimens (intensive: 11.4 vs. moderate: 10.2 MJ ME /kg of DM). However, the number of replicates per dietary group ($n = 3$) was considered to be insufficient in terms of statistical power to confirm or reject any dietary effects, and so this aspect was not further investigated. Bulls were slaughtered at an age of 20 months (live weight 755 ± 73 kg; means $\pm$ SD), and tissue samples of the subcutaneous adipose depot (at the tail head) and the retroperitoneal adipose depot were collected within 30 minutes. Samples were immediately rinsed in ice-cold physiological saline solution and cut into approx. 100 mg pieces, before snap-freezing them in liquid nitrogen. Samples were then stored at -80°C until analysis.

Sphingolipid Measurement

Samples were submitted to The Metabolomics Innovation Centre (TMIC UVic Node at The University of Victoria, Genome BC Proteomics Centre, Victoria, BC, Canada) for sphingolipid profiling. The detailed experimental procedures were described in previous studies [15, 16]. In brief, lipid extraction was accomplished by methanol-chloroform bilayers separation [17]. After the samples were mechanically homogenized with metal beads, 10 mL per mg of methanol-chloroform (5:2, v/v) was added into the mixture with 0.1 mg/mL of antioxidant butylated hydroxytoluene (BHT). The mixture was then homogenized, sonicated, and centrifuged in an Eppendorf 5420R centrifuge for 15 min at $21,000 \times g$ and 10°C for the first lipid extraction. The precipitated pellet was homogenized with methanol-chloroform (1:1, v/v) at $10 \mu\text{L}/\text{mg}$ tissue weight again using the same setup for the second lipid extraction. After centrifugation, the clear supernatant was pulled and dried by nitrogen gas at 30°C . The residue was dissolved in methanol with two internal standards: C17:0-sphinganine as the positive-ion internal standard, and C17:1-sphingosine-1-phosphate as the negative-ion internal standard.

After the lipid extraction, the mixed standard stock solution (S1) was serially diluted with methanol in a ratio of 1:4 (v/v) into stock solution S2 to S10. $10 \mu\text{L}$ of sample was injected into UPLC-MS/MS for lipid detection. The Waters UPLC system coupled with 4000 QTPAP mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The mobile phase in UPLC was made of 0.01% formic acid in water and acetonitrile-isopropanol (2:1, v/v) for binary-solvent gradient elution. The UPLC-MRM/MS data were documented with Sciex Analyst software and processed with Sciex MultiQuant software. The concentrations of sphingolipids were quantified from the calibration standard curves with the measured

peak areas. 77 sphingolipid species were targeted and 70 species were detected, including 24 species of ceramide (Cer) and dihydroceramide (DHCer), 16 species of sphingomyelins (SM), 2 species of dihydrosphingomyelin (DHSM), 11 species of ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P), 9 species of galactosylceramide (GalCer), glucosylceramide (GluCer), lactosylceramide (LacCer), and 8 species of 3-ketosphinganine, sphinganine (DHSph) and sphingosine (Sph).

Statistical Analyses

To visualize the fold-change and P-value of a t-test analyzed between SCAT and RPAT, a volcano plot was drawn in *R version 4.0.0* with the package *ggplot2* [18]. No criteria were set for fold-change, and the threshold for P-value was set at 0.1. The data frames were reshaped with the packages *dplyr* and *reshape* [19, 20]. To show the distribution of sphingolipids in SCAT and RPAT, bar plots of the three pathways of interest were drawn in GraphPad Prism version 8.3.0 for Mac (GraphPad Software, La Jolla, CA). P-values between SCAT and RPAT were calculated by paired Student's t-test with false discovery rate (FDR) correction. The standard error of mean (SEM) was shown by the error bar. Levels of statistical trends and statistical significance were indicated as: † $P < 0.1$, * $P < 0.05$; ** $P < 0.01$.

Results

Comparative Sphingolipid Distribution in SCAT and RPAT

The fold-change of RPAT to SCAT over respective P-values of sphingolipids are shown in the volcano plot in Fig. 2. In the *de novo* synthesis pathway (red), 11 out of 28 sphingolipid species were more concentrated in RPAT ($P < 0.1$, $\log_2FC > 0$), while 3 out of 28 sphingolipid species were more concentrated in SCAT ($P < 0.1$, $\log_2FC < 0$). In the salvage pathway (green), 16 out of 24 sphingolipid species were more concentrated in SCAT ($P < 0.1$, $\log_2FC < 0$), while none of them were more concentrated in RPAT with a significant difference. In the sphingomyelinase pathway (blue), 15 out of 18 sphingolipid species were more concentrated in SCAT ($P < 0.1$, $\log_2FC < 0$), and C22:0-SM was the only sphingolipid in this category which was more concentrated in RPAT ($P = 0.014$, $\log_2FC > 0$). Collectively, RPAT was more concentrated with the sphingolipids of the *de novo* synthesis pathway, and SCAT was more concentrated with the sphingolipids of the salvage and sphingomyelinase pathways.

Distribution of Sphingolipids in the De Novo Synthesis Pathway

The distribution of sphingolipids of the *de novo* synthesis pathway in SCAT and RPAT is shown in Fig. 3. 3-ketosphinganine concentration was not significantly different between SCAT and RPAT. Among the sphinganines, C16:0- and C18:0-DHSph did not show significant differences, while C20:0-DHSph was more concentrated in RPAT than in SCAT ($P = 0.082$). Among the dihydroceramide, C17:0-DHCer did not show significant differences, while C12:0-DHCer was more concentrated in SCAT than RPAT ($P = 0.091$),

and the rest of DHCer were more concentrated in RPAT than in SCAT: C16:0-, C18:0-DHCer ($P < 0.1$), C14:0-, C20:0-, C22:0-, C24:0-DHCer ($P < 0.05$). Among the dihydroceramide-1-phosphates, none of the detected species, C16:0-, C18:0-, C18:1-, C20:0-DHCer1P, showed significant differences between SCAT and RPAT. Among the ceramides, C12:0-, C17:0-, C18:1-, C20:4-, C24:1- and C26:0-Cer did not show significant differences, while C14:0- and C16:0-Cer were more concentrated in SCAT than in RPAT ($P < 0.05$), and the rest of Cer were more concentrated in RPAT than in SCAT: C18:0-, C20:0-, C24:0-Cer ($P < 0.05$), and C22:0-Cer ($P = 0.0092$). In summary, the majority of the sphingolipid species in the *de novo* synthesis pathway were more concentrated in RPAT, compared with SCAT, except for C12:0-, C14:0- and C16:0-Cer, which were more concentrated in SCAT.

Distribution of Sphingolipids in the Salvage Pathway

The distribution of sphingolipids of the salvage pathway in SCAT and RPAT is shown in Fig. 4. Among the sphingosines, C20:1-Sph did not show significant differences, while the rest of Sph were more concentrated in SCAT than in RPAT: C17:1-Sph ($P = 0.051$), and C16:1-, C18:1-Sph ($P < 0.05$). Concerning sphingosine-1-phosphate, C18:1-S1P was more concentrated in SCAT than in RPAT ($P = 0.028$). Among the ceramide-1-phosphates, C18:0-, C20:0- and C22:0-C1P did not show significant differences, while the rest of C1P was more concentrated in SCAT than in RPAT: C12:0-, C14:0-, C16:0-, C18:1-, C24:0-, C26:0-C1P ($P < 0.05$). Among the glucosylceramides, C18:1-GluCer did not show significant differences, while C18:0-GluCer was more concentrated in SCAT than in RPAT ($P = 0.04$). Among the lactosylceramides, C18:0- and C18:1-LacCer were not significantly different between adipose tissues, while C16:0-LacCer was more concentrated in SCAT than in RPAT ($P = 0.052$). Among the galactosylceramides, C24:1-GalCer were not significantly different, while the rest of the GalCer were more concentrated in SCAT than in RPAT: C16:0-, C22:0-, C24:0-GalCer ($P < 0.05$). Collectively, all sphingolipid species in the salvage pathway were more concentrated in the SCAT, compared with RPAT.

Distribution of Sphingolipids in the Sphingomyelinase Pathway

The distributions of sphingolipids of the sphingomyelinase pathway in SCAT and RPAT are shown in Fig. 5. Concerning dihydrosphingomyelin, C12:0-DHSM was more concentrated in SCAT than in RPAT ($P = 0.027$). Among the sphingomyelins, C18:0-, C20:0-SM did not show significant differences, C22:0-SM was more concentrated in RPAT than in SCAT ($P = 0.014$), and the rest of the SM were more concentrated in SCAT than in RPAT: C20:1-, C28:0-SM ($P < 0.1$), and C12:0-, C14:0-, C16:0-, C16:1-, C17:0-, C18:1-, C18:2-, C20:4-, C22:6-, C24:0-, C24:1-, C26:0-SM ($P < 0.05$). Collectively, sphingolipids in the sphingomyelinase pathway were generally more concentrated in SCAT, compared with RPAT, except for C22:0-SM, which was more concentrated in RPAT.

Discussion

This study analyzed 70 sphingolipid species, and the concentrations of 46 species were found to be statistically different between RPAT and SCAT ($P < 0.1$), indicating that the sphingolipid profiles of these

two adipose tissue depots were remarkably different. The differential concentration of sphingolipid species can reflect dissimilar sphingolipid pathway activities, affecting downstream signaling function [2]. For instance, ceramide synthesis via the *de novo* pathway was more related to insulin resistance [21], whereas ceramide formation from the sphingomyelinase pathway was more associated with inflammation, oxidative stress, and apoptosis through the one-step rapid transformation from SM to ceramide [7]. Little was known about the salvage pathway in sphingolipid signaling, but it was reported that ceramides from the salvage pathway could be responsible for the dephosphorylation of the p38 cascade and the PKC signaling pathway [5]. Combining the potential signaling function of various sphingolipids to the sphingolipid profile of RPAT and SCAT, we could better understand the physiologic role of these two adipose tissues. Limitations of this study include the relatively small sampling size, restricting the assessment of any possible dietary influence. Sphingolipid metabolism was shown to be altered by dietary factors in dairy cows [22], however, whether the diet has an effect on adipose dependent distribution of sphingolipids should be tested in further studies. The novelty of this study is confirming that bovine SCAT and RPAT are distinct in their metabolism, by providing the sphingolipid profiles of these two depots in a lipidomics approach.

Backward Accumulation in the De Novo Synthesis Pathway in RPAT

The distribution patterns of the salvage pathway and the sphingomyelinase pathway were more similar than that of the *de novo* synthesis pathway (Fig. 2). Connecting all sphingolipids with the sphingolipid metabolic map, a unique distribution pattern was shown in Fig. 6. Our results showed that the sphingolipid species that act downstream of 3-ketosphinganine, such as sphinganine, DHCer, and Cer were more concentrated in RPAT (red in Fig. 6), while sphingolipids that act downstream of DHCer and Cer, such as DHSM, Sph, S1P, SM, and glycosphingolipids, were more concentrated in SCAT (blue in Fig. 6). This pattern suggested that there was a ceramide accrual, and even a possible backward accumulation in the *de novo* synthesis pathway in RPAT either due to a higher influx rate of substrates at the origin of the *de novo* synthesis, or due to a lower transformation rate of ceramide at the end of the synthesis, or due to a combination of both [2].

The higher substrate influx rate in RPAT could be supported by the greater dynamics of adipose mass, and higher HSL activity in bovine RPAT [23]. It was shown that the adipose mass of RPAT in periparturient German Holstein cows had a greater fluctuation than that of SCAT [13, 24]. Additionally, RPAT had a higher HSL phosphorylation at residues 563 and 660 detected by Western blot analysis, indicating greater enzyme activation [11]. A similar experiment performed in rodents also showed a higher HSL phosphorylation at residues 563 and 660 in the visceral adipose tissue under forskolin stimulation, compared with the subcutaneous adipose tissue [25]. Together with the greater adipose mass in abdominal adipose depot (66.7% of total body fat) than the subcutaneous adipose tissue (17.9% of total body fat) [26], it is suggested that triglycerides stored in RPAT undergo greater facilitated hydrolysis into NEFA than SCAT. Meanwhile, palmitic acid is one of the most prevalent fatty acids among circulating

NEFA [27, 28]. The influx of palmitic acid could drain into the *de novo* synthesis pathway, and result in the accumulation of ceramide [29, 30].

Besides the high influx rate at the origin of the *de novo* synthesis pathway, the inhibition in the transformation, or a backward synthesis of ceramide should also be considered as a possible explanation for the accrual of ceramides [2]. The balance between ceramides, sphingomyelins, sphingosines, and other sphingolipid metabolites are controlled by the enzymes involved in their biotransformation. For instance, acid sphingomyelinase (ASMase) is the enzyme converting sphingomyelin into ceramide in the sphingomyelinase pathway, under the activation of oxidative stress, pathogens, and the proinflammatory cytokine interleukin-1 β (IL-1 β) [31, 32]. In dairy cows, Ji et al. demonstrated that the IL-1 β mRNA signal was higher in the mesenteric adipose tissue than the subcutaneous depot [10]. Hence, the ceramide accumulation in the RPAT profile could be the consequence of the increased enzyme activity of sphingomyelinase, driven by pro-inflammatory signals. The enzyme that acts in the opposite direction, sphingomyelin synthase (SMS), converts ceramide to sphingolipid in the endoplasmic reticulum [33]. Although the activation mechanism and the physiological role of SMS in adipose tissue in dairy cattle has not yet been identified, it is evident that SMS could downregulate the reactive oxidative species (ROS) level by breaking down sphingomyelin into ceramide, triggering the release of ROS [34, 35], and opposing the action of ASMase. Further mechanistic studies are warranted to elucidate the role of these metabolic pathways in driving adipose depot specific sphingolipid distribution.

Comparing two sources of ceramide accrual, Rico et al. demonstrated that the *de novo* synthesis pathway might play a more crucial role than the sphingomyelinase pathway in dairy cattle physiology [36]. It was shown that cows with an intravenous triglyceride (TAG) infusion had a higher ceramide synthase 2 (CerS2) mRNA expression, compared with the control. High level of CerS2 indicated an upregulation of the *de novo* synthesis pathway as CerS2 is the enzyme promoting the synthesis of C22:0- and C24:0 ceramide [37]. Additionally, it was shown that the SM concentration was not altered with the TAG level, indicating that the sphingomyelinase pathway was not involved in the surge of ceramide. Thus, this provided compelling evidence that the *de novo* synthesis pathway was more important than the sphingomyelinase pathway in contributing to the ceramide accrual [1]. The salvage pathway could be, but to a lesser extent, contributing to the accumulation of ceramide.

Insulin Resistance, Inflammation, and Oxidative Stress in RPAT and SCAT

Our data showed that ceramides were more concentrated in RPAT, and less concentrated in SCAT. As ceramide is the upstream regulator of Akt, confirmed in an ex vivo study in Holstein steers [38], this ceramide distribution suggested that RPAT would be more associated with insulin resistance because of its ceramide profile. Ceramide has been suggested to be a mediator of obesity and insulin resistance. Previously, it was shown that ceramide could bind with SET, releasing its inhibitory function to PP2A for the inactivation of Akt [39]. Recently, it was shown that ceramide could inhibit Akt by dephosphorylating

Ser 473 [40], which was in line with bovine adipocytes research using C2:0-ceramide [38]. Besides, it was demonstrated that dairy cows with ceramide accrual in plasma, liver, and skeletal muscle had higher lipolytic activity and lower insulin sensitivity [3]. This provides evidence that adipose tissue with more ceramides would more suppress insulin sensitivity and have an enhanced sphingolipid dynamic. In contrast to SCAT, Kenéz et al. demonstrated that Akt and HSL phosphorylation were greater in the insulin signaling pathway in dairy cow RPAT during the peripartum period [9]. This indicated that RPAT may be more sensitive and responsive in insulin signaling than SCAT. As RPAT had a greater concentration of ceramides, RPAT may likely have a sphingolipid profile more associated with insulin resistance. Hence, RPAT may contribute to the total insulin sensitivity greater in dairy cattle.

Not only insulin sensitivity but also the inflammatory response was found to be different between RPAT and SCAT in cows [10]. Here, we observed that ceramides were more concentrated in RPAT, whereas sphingosines and S1P were more concentrated in SCAT. The distribution of Cer, Sph, and S1P in RPAT and SCAT may provide an explanation in the different expression of pro-inflammatory cytokines in the two adipose depots. Ceramide, derived from the transformation of sphingomyelin, was shown to affect the proinflammatory cytokine IL-1 β and tumor necrosis factor (TNF) signaling pathway [41]. Down to the ceramide species level, Brodlie et al. showed that C16:0, C18:0, C20:0-ceramide levels were increased in the lower airway epithelium in human patients with lung inflammation [42]. Furthermore, sphingomyelinase, the enzyme transforming SM to Cer, was shown to be expressed to a greater extent in inflamed adipose tissues than the non-inflamed adipose tissues in humans [6]. Collectively, these studies showed a strong correlation between ceramides and inflammation via the sphingomyelinase pathway. Besides ceramides, Samad et al. demonstrated that Sph and S1P could also increase the mRNA expression of pro-inflammatory proteins (TNF- α , MCP-1, IL-6, and KC) [23]. In particular, Sph induced more TNF- α expression, and S1P induced more IL-6 mRNA expression in 3T3-L1 adipocytes cell culture, compared with C2:0- and C6:0-ceramide. These studies showed that not only ceramide but also its downstream sphingolipid species such as Sph and S1P could be associated with inflammation. In dairy research, Ji et al. demonstrated that the pro-inflammatory cytokines (IL-1 β , IL-6R, CCL2, CCL5) mRNA expression of omental and mesenteric adipose tissue was greater than the subcutaneous adipose tissue in overfed dairy cows [10]. As RPAT had a greater pro-inflammatory cytokines signal and a greater concentration of ceramides, it is suggested that RPAT is associated with a stronger inflammatory response, compared with SCAT.

In addition to pro-inflammatory signaling, it was shown that ceramide and sphingosine could also be the downstream mediators of oxidative stress, and regulate the apoptosis signaling pathway in both human and rat cell line [34]. Here, we observed that ceramides were more concentrated in RPAT, whereas sphingosines were more concentrated in SCAT. These are two important mediators in regulating apoptosis under oxidative stress. Goldkorn et al. demonstrated that ROS such as hydrogen peroxide (H₂O₂) could activate sphingomyelinases and promote the transformation of SM to Cer in the tracheobronchial epithelial cells [43]. The surge of ceramide from the transformation could, therefore, activate cathepsin D and pro-apoptotic protein BID to induce apoptosis [2, 44]. Besides, the elevated

ceramide could be converted into sphingosine, and act as a second messenger to induce apoptosis by inhibiting MAP kinase activity [45, 46]. In specific, Osawa et al. demonstrated that C16:0-ceramide induced apoptosis in rat primary hepatocytes [47], and Seumois et al. showed that C16:0 and C24:0-ceramide are pro-apoptotic signals in human blood neutrophil cells [48]. These findings showed that ceramide and sphingosine are important mediators in response to oxidative stress, and therefore induce apoptosis. Although the oxidative stress level of two adipose tissues in dairy cattle was not measured, it is shown that the visceral depots in mice are more sensitive to oxidative stress than the subcutaneous depots by comparing the stress signaling pathway JNK and MAPK [49]. Thus, the visceral adipocytes might be more susceptible to apoptosis than the subcutaneous adipocytes [50]. As ceramide is the precursor of sphingosine, ceramide might take a more important role in apoptosis signaling. Therefore, it is suggested that the sphingolipid profile of RPAT would be more associated with oxidative stress and apoptosis, compared with SCAT.

The Third Ceramide Regulating Pathway: Phosphorylation Pathway

In addition to the ceramide profile, we observed that DHCer1P and C1P were highly concentrated in both adipose tissues, particularly in SCAT. The concentration of DHCer1P was roughly 10 folds higher than that of DHSph, DHCer, and Cer; and the concentration of C1P was roughly 4 folds higher than that of Sph, S1P, and glycosphingolipid. To explain the high concentration of DHCer1P, it seems likely that the DHCer kinase was more active, or the dihydroceramide desaturase (Des1) was less active in the adipocytes [2]. Des1 is the enzyme promoting the transformation from DHCer to Cer [51]. When the kinase activity is higher than Des1, DHCer may shift the synthesis from Cer to DHCer1P. However, the DHCer kinase activity in bovine cells was not reported yet. The physiological role and the downstream signaling pathway of DHCer1P are still elusive. More research has to be done to understand the high concentration of DHCer1P in both RPAT and SCAT. Similarly, the high concentration of C1P might because of a high level of ceramide kinase (CerK), or lower expression of phosphatase [52]. On the whole, as the concentration of DHCer1P, C1P, and SM were similarly concentrated in the sphingolipid profile, the phosphorylated sphingolipids could regulate the synthesis of ceramides through its one-step transformation with phosphatase, like the one-step SM-Cer pathway, to give a rapid response. Thus, besides two major regulating pathways: *de novo* synthesis pathway and sphingomyelinase pathway, the phosphorylation pathway could be the third pathway in the sphingolipid metabolic network regulating the synthesis of ceramide, in agreement with JW McFadden and JE Rico [1].

Conclusion

To conclude, this study revealed that the sphingolipid profiles differed between bovine RPAT and SCAT, in particular the concentration of ceramides were higher in RPAT than in SCAT. This suggested that the activity of the pathways of sphingolipid metabolism, such as the *de novo* synthesis of ceramide, were also different in RPAT and SCAT. Consistently with previous findings, this indicated that the physiological role of RPAT could be more responsive than SCAT in insulin signaling, pro-inflammatory signaling, and

oxidative stress response. More research has to be done to understand the metabolic stimuli and signaling pathways of other sphingolipids, such as DHCer, Sph, S1P and C1P in adipose tissue, to provide a comprehensive comparison of the physiological role of RPAT and SCAT.

Abbreviations

C1P: Ceramide-1-phosphate

Cer: Ceramide

CerK: Ceramide kinase

CerS: Ceramide synthase

Des1: Dihydroceramide desaturase

DHCer: Dihydroceramide

DHCer1P: Dihydroceramide-1-phosphate

DHSM: Dihydrosphingomyelin

DHSph: Sphinganine

FDR: False discovery rate

GalCer: Galactosylceramide

GluCer: Glucosylceramide

HSL: Hormone-sensitive lipase

LacCer: Lactosylceramide

RPAT: Retroperitoneal adipose tissue

S1P: Sphingosine-1-phosphate

SCAT: Subcutaneous adipose tissue

SM: Sphingomyelin

Sph: Sphingosine

SPT: Palmitoyltransferase

UPLC-MRM/MS: Ultra-performance liquid chromatography electrospray ionization-tandem mass spectrometry in multiple reactions monitoring mode.

Declarations

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Authors' contributions

CK, SB, TS performed the animal experiment at the Educational and Research Centre for Animal Husbandry (Muenchweiler a.d. Alsenz, Germany); AK, KH, SD conceived and designed the adipose tissue study; SB, CK, TS, UM performed the laboratory experiments; YHL, AK analyzed the data; YHL, SB, CK, TS, UM, SD, AK, KH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The experiment was approved by the relevant Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany) in agreement with the German Animal Welfare Act (permit number: G-17-20-070).

Consent for publication

Not applicable

Competing interests

The author declare that they have no competing interests.

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Figures

Figure 1

Sphingolipid metabolic pathways according to M Maceyka and S Spiegel [53], AH Merrill, Jr. [54]. The de novo synthesis pathway (red) starts with the condensation of serine and palmitoyl CoA to form ceramide through a series of reactions including the conversion of 3-ketosphinganine, sphinganine, and dihydroceramide. Ceramide can be modified into sphingomyelin via the sphingomyelinase pathway (blue), or into glycosphingolipid, sphingosine, and ceramide-1-phosphate via the salvage pathway (green). Double arrows indicate reversible reactions.

Figure 2

Volcano plot visualizing the fold change (RPAT to SCAT) over the P-value of sphingolipids in two adipose tissues. The P-value of each sphingolipid species is calculated by paired Student's t-test with FDR correction. The dotted horizontal line indicated a statistical threshold of $P < 0.1$. The figure shows that sphingolipid species in the de novo synthesis pathway were more concentrated in the retroperitoneal adipose tissue (RPAT). In contrast, sphingolipid species in the salvage pathway and the sphingomyelinase pathway were more concentrated in the subcutaneous adipose tissue (SCAT). Cer: ceramide; C1P: ceramide-1-phosphate; DHCer: dihydroceramide; GalCer: galactosylceramide; Sph: sphingosine; SM: sphingomyelin.

Figure 3

The distribution of sphingolipids in the de novo synthesis pathway in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) in German Holstein bulls (n = 6). Data are presented as mean $\pm$ standard error of mean (SEM). 3-Keto: 3-ketosphinganine concentration; DHSph: sphinganine concentration; DHCer: dihydroceramide concentration; DHCer1P: dihydroceramide-1-phosphate concentration; Cer: ceramide concentration. Asterisks indicate significant differences († P < 0.1; * P < 0.05; ** P < 0.01) between two adipose tissues by paired Student's t-test with FDR correction.

Figure 4

The distribution of sphingolipids in the salvage pathway in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) in German Holstein bulls (n = 6). Data are presented as mean ± standard error of mean (SEM). Sph: sphingosine concentration; S1P: sphingosine-1-phosphate concentration; C1P: ceramide-1-phosphate concentration; GluCer: glucosylceramide concentration; LacCer: lactosylceramide concentration; GalCer: galactosylceramide concentration. Asterisks indicate significant differences († P < 0.1; * P < 0.05) between two adipose tissues by paired Student's t-test with FDR correction.

Figure 5

The distribution of dihydroceramide and sphingolipids of the sphingomyelinase pathway in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) in German Holstein bulls (n = 6). Data are presented as mean $\pm$ standard error of mean (SEM). DHSM: dihydrosphingomyelin concentration; SM: sphingomyelin concentration. Asterisks indicate significant differences ($\dagger$ $P < 0.1$; * $P < 0.05$) between two adipose tissues by paired Student's t-test with FDR correction.

Figure 6

The concentration of sphingolipids across the sphingolipid pathways in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT). Sphinganine, dihydroceramide, and ceramide were more concentrated in RPAT (red) than in SCAT. Dihydrosphingomyelin, sphingomyelin, ceramide-1-phosphate, sphingosine, sphingosine-1-phosphate, glucosylceramide, galactosylceramide, and lactosylceramide were more concentrated in SCAT (blue) than RPAT. There was no statistical difference in 3-ketosphinganine and dihydroceramide-1-phosphate between RPAT and SCAT. The sphingolipid pathway map was drawn according to Maceyka and Spiegel [53], Merrill [54].