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Hot topic: Ceramide inhibits insulin sensitivity in primary bovine adipocytes

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ABSTRACT

In nonruminants, the sphingolipid ceramide inhibits insulin sensitivity by inactivating protein kinase B (AKT) within the insulin-signaling pathway. We have established that ceramide accrual develops with impaired systemic insulin action in ruminants during the transition from gestation to lactation, dietary palmitic acid supplementation, or controlled nutrient restriction. We hypothesized that ceramide promotes AKT inactivation and antagonizes insulin sensitivity in primary bovine adipocytes. Stromal-vascular cells were grown from bovine adipose tissue explants and cultured in differentiation media. To modify ceramide supply, we treated differentiated adipocytes with (1) myriocin, an inhibitor of de novo ceramide synthesis, or (2) cell-permeable C2:0-ceramide. Insulin-stimulated AKT activation (i.e., phosphorylation) and 2-deoxy-D-[^{3}H]-glucose (2DOG) uptake were measured. Treatment of adipocytes with myriocin consistently decreased concentrations of ceramide, monohexosylceramide, and lactosylceramide. The insulin-stimulated ratio of phosphorylated AKT to total AKT was increased with myriocin but decreased with C2:0-ceramide. Moreover, adipocyte insulin-stimulated 2DOG uptake was decreased with C2:0-ceramide and increased with myriocin. We conclude that ceramide inhibits insulin-stimulated glucose uptake by downregulating AKT activation in primary bovine adipocytes.
Key words: bovine adipocyte, ceramide, insulin sensitivity

Hot Topic

The sphingolipid ceramide has received considerable attention for its ability to antagonize insulin action in obese nonruminants experiencing type 2 diabetes or nonalcoholic fatty liver disease (Holland and Summers, 2008; Pagadala et al., 2012). Specifically, nutrient excess increases de novo ceramide synthesis in adipose and liver tissues (Summers, 2006; Pagadala et al., 2012). In turn, ceramide promotes the dephosphorylation of serine-473 and inactivation of protein kinase B (**AKT**) to suppress glucose transporter translocation to the plasma membrane (Schubert et al., 2000). In adipocytes, impaired insulin action by ceramide promotes lipolysis (Mei et al., 2002), which is important because adipose tissue ceramide accrual and the uncontrolled lipolytic release of fatty acids are key metabolic features of heightened adiposity (Shah et al., 2008; Karpe et al., 2011). Interestingly, decreasing the activation of serine palmitoyltransferase (**SPT**) within the de novo ceramide synthesis pathway is a means to lower ceramide and improve localized and systemic insulin sensitivity (Holland et al., 2007).

Ceramide has emerged as an associative biomarker for impaired systemic insulin tolerance in ruminants. First, we established that the accumulation of ceramide in liver, skeletal muscle, or plasma develops with hyperlipidemia and decreased insulin sensitivity in cattle transitioning from gestation to lactation (Rico et al., 2015, 2017b). These relationships were more pronounced in cows with increased subcutaneous adiposity. Second, palmitic acid feeding increases circulating ceramide while inhibiting glucose-stimulated fatty acid disappearance (Rico et al., 2016). Third, a controlled nutrient restriction protocol to induce lipolysis increases hepatic and plasma ceramide while impairing systemic glucose tolerance (Davis et al., 2017a). Collectively, these studies repeatedly demonstrate that ceramide supply is inversely related to direct measurements of systemic insulin action; however, the ability of ceramide to mediate insulin antagonism in ruminants is uncertain. Therefore, our objective was to modulate ceramide concentrations in primary bovine adipocytes and measure insulin response.

To test our hypothesis, we utilized subcutaneous adipose tissue collected from 14 Angus or Angus-cross steers to obtain primary bovine adipocytes, using methodology developed in part by Lengi and Corl (2010). Subcutaneous adipose tissue was harvested from the flank. Stromal-vascular cells were grown from adipose

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tissue explants in Dulbecco's modified Eagle medium (**DMEM**)/F-12 growth medium containing 17.5 m*M* glucose and 10% fetal bovine serum (**FBS**). Cells were harvested by trypsinization and replated, undergoing 3 or 4 passages. Seeding densities were 1×10^4 cells/cm^2 during the proliferation phase, and 2×10^4 cells/cm^2 for differentiation. Once confluent, cells were differentiated using DMEM/F-12 medium containing 5 m*M* sodium acetate, 17.5 m*M* glucose, 1 m*M* octanoate, 5% FBS, 1.7 m*M* bovine insulin, 0.25 m*M* dexamethasone, 0.5 m*M* isobutylmethylxanthine, and 2 m*M* rosiglitazone. Following 8 to 10 d of differentiation induction, adipocytes were used in experiments and incubated in DMEM/F-12 treatment medium containing 1% FBS and 0.6 n*M* insulin. Depending on the experiment, the treatment medium contained 1 or 5 m*M* sodium acetate with 5 or 17.5 m*M* glucose, respectively. Cell differentiation was confirmed by visually observing lipid droplet formation and measuring intracellular triacylglycerol accumulation using colorimetry (kit no. K622-100; BioVision, Mountain View, CA). Cells were incubated in treatment medium in the absence or presence of myriocin (10 μ*M*; Cayman Chemical, Ann Arbor, MI) or C2:0-ceramide (100 μ*M*; Cayman Chemical) for 18 or 2 h, respectively. For future ceramide quantification, cells were washed and pelleted in ice-cold PBS. For immunoblotting purposes, cells were rinsed with 50 m*M* NaF in PBS and harvested in lysis buffer [20 m*M* Tris base, 50 m*M* NaCl, 50 m*M* NaF, 5 m*M* $Na_4O_7P_2$, 250 m*M* sucrose, 1% (vol/vol) Triton X-100, 500 m*M* dithiothreitol, and protease inhibitor]. Pellets and cell lysates were immediately stored at −80°C. Insulin stimulation was performed using 200 n*M* insulin for 15 min. Assessment of 2-deoxy-D-[^{3}H]-glucose (**2DOG**) uptake was measured at treatment conclusion. Specifically, cells were incubated with 2DOG (0.75 mCi/mL; a nonmetabolizable glucose derivative; American Radiolabeled Chemicals Inc., St. Louis, MO) and 0.1 m*M* 2-deoxy-D-glucose for 5 min. Following washing with PBS, cells were harvested in 1% SDS-PBS and radioactivity was measured using a liquid scintillation counter (Beckman Coulter Inc., Brea, CA).

Ceramide, monohexosylceramide (**GlcCer**), and lactosylceramide (**LacCer**) were extracted from adipocytes using a modified Bligh and Dyer procedure and quantified using liquid chromatography (ExionLC AD; Sciex, Framingham, MA) and electrospray ionization coupled with tandem mass spectrometry (QTRAP 5500; Sciex) as previously described (Davis et al., 2017a; Phipps et al., 2017). Cell lysate protein quantification was determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA). For analysis of AKT phosphorylation, 50 μg of protein was loaded onto a gradient gel and separated using electrophoresis. Proteins were transferred to a nitrocellulose membrane and incubated overnight with phosphorylated AKT (**pAKT**, Ser-473, 1:1,000; Cell Signaling Technology, Danvers, MA) or total AKT (**tAKT**, 1:1,000; Cell Signaling Technology) antibodies. Following washing, membranes were incubated with secondary antibody (1:2,000; anti-rabbit IgG, horseradish peroxidase-linked; Cell Signaling Technology) followed by chemiluminescence detection using an Azure c600 imager (Azure Biosystems, Dublin, CA). Densitometry was performed using ImageJ (Schneider et al., 2012). Data were normalized to a loading control (β-actin; Cell Signaling Technology; Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13983). Utilizing a previous approach (Amstalden et al., 2005; McFadden and Corl, 2009), 3 independent experiments (animals) with 3 replicates per experiment were performed for triacylglycerol and ceramide quantification, whereas 2 independent experiments with 3 replicates per experiment were completed for all other measurements. Of importance, our conclusion is supported by observations utilizing cultures obtained from 4 independent experiments. Data were analyzed under a mixed model including the fixed effect of treatment and the random effect of experiment and replicate within treatment. All results are expressed as least squares means and their standard errors. Significance was declared at $P < 0.05$.

Relative to undifferentiated adipocytes, triacylglycerol accumulation was 736% greater in differentiated adipocytes ($P < 0.01$) with lipid droplet formation. Differentiated adipocytes were treated with myriocin, which is a common approach to inhibit SPT and de novo ceramide synthesis (Chen et al., 1999; Holland et al., 2007). Treating primary bovine adipocytes with myriocin for 18 h significantly decreased ceramide, GlcCer, and LacCer concentrations (Table 1; Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13983). Although myriocin lowered all ceramides, very long chain ceramides were most responsive to SPT inhibition. For instance, myriocin decreased C24:0-ceramide concentration by 74% ($P < 0.01$). In nonruminants, the ceramide-lowering ability of myriocin improves AKT activation, in turn improving adipocyte and whole-body insulin sensitivity (Chavez et al., 2003; Holland et al., 2007). Within our in vitro culture system, the inhibition of adipocyte de novo ceramide synthesis by myriocin developed with enhanced AKT activation, as demonstrated by an increased ratio of pAKT to tAKT (Figure 1A). To support our findings, we treated primary bovine adipocytes with C2:0-ceramide, which is a cell membrane–permeable analog that inhibits insulin-stimulated AKT activation in rodent models (Obanda et al., 2012; Hsieh et al., 2014). We demonstrated that treating bovine adipocytes with C2:0-ceramide for 2 h