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# Esterification of essential and non-essential fatty acids into distinct lipid classes in ruminant and non-ruminant tissues

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ABSTRACT

Extensive microbial biohydrogenation of polyunsaturated fatty acids (PUFA) in the rumen reduces the essential fatty acids (EFA) available for absorption in ruminant animals, but there is no published documentation of ruminants developing EFA deficiency. In ruminants, most circulating PUFA are found in the phospholipid (PL) and cholesteryl ester lipid classes that have slow turn-over compared to other lipid classes. The objective of this experiment was to measure fatty acid esterification patterns of the non-EFA palmitic (16:0) and oleic acid (18:1), and the EFA linoleic (18:2) and linolenic acid (18:3) in small intestine, liver, and muscle tissue of cows and pigs to identify tissues participating in sequestration of these FA in less metabolically active lipid classes in ruminants. Bovine and porcine small intestine, liver, and muscle explants were prepared and incubated in media containing radiolabeled 16:0, 18:1, 18:2, or 18:3 to measure esterification of fatty acids into PL and TG. Neither bovine nor porcine small intestine explants preferentially incorporated non-EFA compared to EFA into PL vs TG. Bovine liver explants esterified a larger proportion of EFA than non-EFA into PL compared to TG, while incorporation was similar among the FA tested in porcine liver explants. Bovine muscle explants showed preferential incorporation of EFA into PL rather than TG. Results show that bovine and porcine liver and muscle esterify EFA and non-EFA differently and that the conservation of EFA in ruminants is a result of preferential incorporation of EFA into PL mediated by bovine liver and muscle, but not the small intestine.



## 1. Introduction

Essential fatty acids (EFA) are components of dietary fat that are vital for normal animal growth and development. The two essential fatty acids are the omega-6 fatty acid (FA) linoleic acid (18:2) and the omega-3 FA α-linolenic acid (18:3). These are 18-carbon polyunsaturated fatty acids (PUFA) with two or three double bonds, respectively. Their classification into omega-6 vs omega-3 FA is based on the position of the first double bond in the carbon chain in relation to the methyl end. Linoleic and α-linolenic acids can be further desaturated and elongated to produce additional long-chain PUFA. These EFA along with their elongation and desaturation products and downstream metabolites are important components of cell membranes, the lipids of the brain and eye, and are involved in the production of eicosanoids that regulate immunity and inflammation (Uauy et al., 2000). Deficiencies in 18:2 and 18:3 in animals can result in dermatitis, growth retardation, hair loss, immune suppression, and abnormalities in brain and retinal development (Gramlich et al., 2015) due to the lack of EFA, as well as the consequent lack of other PUFAs and their downstream metabolites. Essential fatty acids cannot be synthesized by animals because they lack the Δ12- and Δ15-desaturase enzymes capable of inserting double bonds at the 12 and 15 positions of the FA carbon chain. Therefore, animals obtain these FA by consuming plants, which contain abundant EFA, or by consuming herbivorous animals in the case of predators.

Early studies demonstrated that ingestion of EFA-deficient diets resulted in gross dermal lesions in pigs (Sewell and McDowell, 1966; Sewell and Miller, 1966) and increased ratio of trienoic to dienoic and tetraenoic FA in tissues of rats (Rieckehoff et al., 1949), chicks (Bieri et al., 1957), and dogs (Wiese and Hansen, 1951). In ruminants, prior to absorption of dietary fats in the small intestine, the resident bacteria of the foregut biohydrogenate unsaturated FA (Lourenco et al., 2010) leading to almost complete saturation of the carbon chain and loss of function as EFA (Kalscheur et al., 1997a, 1997b; Loor et al., 2004). Despite the small amounts of 18:2 and 18:3 present in the plasma lipids of newborn ruminants and the low concentration of these FA found in their dam's milk, newborn calves and lambs are able to egress borderline EFA deficiency within days of birth (Jenkins et al., 1988; Jensen, 2002; Leat, 1966). In 3-d-old calves, 6 wk of an EFA-deficient diet did

*Abbreviations:* 16:0, Palmitic acid; 18:1, Oleic acid; 18:2, Linoleic acid; 18:3, Linolenic acid; CE, Cholesteryl ester; EFA, Essential fatty acids; FA, Fatty acid; HDL, High-density lipoprotein; IDL, Intermediate-density lipoprotein; LDL, Low-density lipoprotein; LPL, Lipoprotein lipase; PL, Phospholipid; PUFA, Polyunsaturated fatty acid; TG, Triglyceride; VLDL, Very low-density lipoprotein.

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not result in development of external deficiency signs (Jenkins and Kramer, 1986), whereas feeding 3-wk-old pigs an EFA-deficient diet for the same period of time resulted in the development of dermal lesions (Sewell and McDowell, 1966). Because biohydrogenation reduces EFA ingested by ruminants to almost negligible levels before absorption, it is interesting that there is no documentation of ruminants developing EFA deficiency despite the small amounts of absorbed EFA. Conservation of EFA in ruminant animals and not non-ruminants indicates the development of physiological and biochemical mechanisms to cope with rumen bacterial modification of EFA.

Most PUFA, including 18:2 and 18:3, circulate in the phospholipid (PL) and cholesteryl ester (CE) lipid classes in ruminants. These are the two most abundant lipid classes in ruminant plasma (Christie, 1978), making EFA the most abundant FA in ruminant plasma. Additionally, plasma PL and CE have a slow turn-over (Mattos and Palmquist, 1977; Palmquist and Mattos, 1978) compared to other lipid classes such as triglycerides (TG) and non-esterified FA. Conservation of EFA in ruminants might be the result of sequestration of these FA in less metabolically active plasma lipid classes.

We hypothesized that bovine tissues esterify greater proportions of EFA into PL than into TG compared to the pig, a non-ruminant ungulate. The objective of this experiment was to measure lipid esterification patterns of the non-EFA palmitic (16:0) and oleic acid (18:1), and the EFA 18:2 and 18:3 acids in small intestine, liver, and muscle tissue of cows and pigs to identify tissues participating in sequestration of these FA in less metabolically active lipid classes in ruminants.

## 2. Materials and methods

### 2.1. Reagents

Medium 199, fatty-acid-free bovine serum albumin, bicarbonate, and insulin (bovine pancreas) were purchased from Sigma–Aldrich (St. Louis, MO). Fatty acids were purchased from Nu-Chek Prep, Inc. (Elysian, MN). [1-$^{14}$C]-labeled fatty acids were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Hexane, isopropanol, sodium sulfate, ethyl ether, acetic acid, and liquid scintillation cocktail (ScintiSafe 30%) were purchased from Fisher Scientific (Pittsburg, PA).

### 2.2. Tissue collection

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Virginia Tech. A portion of the left lobe of the liver, the jejunum of the small intestine, and diaphragm muscle were collected from four cattle (>16 months of age) and four pigs (>8 months of age) and immediately transported to the lab in 1X DPBS (pH 7.3) on ice.

### 2.3. In vitro cultures

Liver tissue samples were trimmed to remove capsule and nonparenchymal tissue. Small intestine samples were opened longitudinally and pinned epithelial side up on a dissection board and cored using an MD5000 Tissue Coring Press (Alabama Research and Development, Munford, AL) to obtain disks of tissue. Afterwards, the serosa layer of the disk of tissue was removed. Liver and muscle cores were also prepared using the Coring Press, and sliced at 600-μm thickness with a MD4000 Krumdieck automated tissue slicer (Alabama Research and Development). Tissue slices and small intestine disks were blotted, immediately weighed, and ~150 mg of tissue were placed in 25 mL Erlenmeyer flasks with 3 mL bicarbonate-supplemented Medium 199 with Earle's salts and L-glutamine (pH 7.3), containing 0.15 IU/mL insulin, 0.25 mM 16:0, 0.25 mM 18:1, 0.25 mM 18:2, 0.25 mM 18:3, and 1 μCi of [1-$^{14}$C]16:0, [1-$^{14}$C]18:1, [1-$^{14}$C]18:2, or [1-$^{14}$C]18:3. Incubations were prepared in triplicate. Fatty acids were complexed to bovine serum albumin (4:1 molar ratio) to facilitate solubility in the aqueous culture media. Flasks were gassed with $O_2$:$CO_2$ (95:5), sealed with rubber stoppers and placed in a 37 °C shaking water bath for 2 h. Incubation was terminated by the addition of 0.5 mL 1 M $H_2SO_4$ to the media.

### 2.4. Measurement of fatty acid incorporation into total lipids

Lipids were extracted according to the method of Hara and Radin (1978) with some modifications. Following the 2 h incubation, tissue explants were removed from the Erlenmeyer flasks using forceps, washed with water from a squirt bottle to remove residual media, blotted on tissue paper, and placed in a 17 × 100 polypropylene test tube containing 3 mL 3:2 hexane-isopropanol. A PRO200 laboratory homogenizer (PRO Scientific, Oxford, CT) was used to homogenize the tissue samples. The homogenate was washed with 2 mL sodium sulfate solution (1 g/15 mL), centrifuged at 2000 × *g* for 5 min at 4 °C to separate the aqueous and organic phases, and the organic phase was transferred to a new 17 × 100 test tube. The solvent was evaporated in a water bath at 40 °C under a stream of $N_2$. Extracted lipids were dissolved in 200 μL of hexane. Half of the extracted lipids were transferred to a liquid scintillation vial with liquid scintillation cocktail to quantify $^{14}$C incorporated in total lipids/2 h/150 mg tissue. The remainder of the extracted lipids was separated into lipid classes.

### 2.5. Measurement of fatty acid incorporation into lipid classes

Triglycerides and PL in extracted lipids were separated from non-esterified FA (substrate) using 250 μm Silica Gel G thin-layer chromatography plates (Analtech Inc., Newark, DE). Extracted lipids were spotted onto thin-layer chromatography plates and developed with hexane-ethyl ether-acetic acid (85:20:2 v/v). After plate development, plates were allowed to dry for 10 min and were then exposed to a PhosphorImager screen (Bio-Rad, Hercules, CA) for 3 d. Radioactivity in bands corresponding to lipid classes was quantified using a Molecular Imager® FX Pro Plus® MultiImager System (Bio-Rad, Hercules, CA). The densitometry of all bands (complete lane) was measured and the percentage of radioactivity in each lipid class was calculated by dividing the densitometry value of a lipid class by the total of all the bands. Total nmol of FA incorporated into a lipid class/2 h/150 mg tissue was calculated by multiplying the percentage of total radioactivity in a lipid class by total nmol of FA incorporated/2 h/150 mg tissue.

### 2.6. Statistical analyses

The data were tested for normality and equality of variances and then analyzed using the mixed models procedure of SAS (v. 9.2, SAS Institute Inc., Cary, NC). For total FA incorporation, the statistical model included the fixed effects of species, FA, species by FA interaction, and the random effect of animal nested within species. The effect of species was tested using animal within species. Treatment effects were further analyzed using preplanned orthogonal contrasts. Preplanned orthogonal contrasts were used to compare EFA with non-EFA, 16:0 with 18:1, and 18:2 with 18:3 overall and within species.

## 3. Results

### 3.1. Total fatty acid incorporation and PL to TG ratio in bovine and porcine small intestine, liver, and muscle explants

In small intestine, liver, and muscle, explants of bovids and porcids esterified similar amounts of radiolabeled FA during the 2 h incubation period ($P > 0.12$; Fig. 1a). Furthermore, there was no significant difference in the PL:TG ratio of bovids versus porcids ($P > 0.13$; Fig. 1b) in either small intestine, liver, or muscle explants. The PL:TG ratio represents a measure of incorporation of FA into PL vs TG fractions. A ratio ≈1 indicates that FA were similarly esterified in the PL and TG fractions; a