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Original Research Article

Effects of medium chain triglycerides on hepatic fatty acid oxidation in clofibrate-fed newborn piglets

Jinan Zhao [1], Brandon Pike, Jin Huang [2], Zhihua Feng [3], Jack Odle , Xi Lin [*]

Laboratory of Developmental Nutrition, Department of Animal Sciences, North Carolina State University, Raleigh, NC 27695, USA

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ABSTRACT

To investigate whether increasing tricarboxylic acid (TCA) cycle activity and ketogenic capacity would augment fatty acid (FA) oxidation induced by the peroxisome proliferator-activated receptor-alpha (PPARα) agonist clofibrate, suckling newborn piglets ($n = 54$) were assigned to 8 groups following a 2 (± clofibrate) × 4 (glycerol succinate [SUC], triglycerides of 2-methylpentanoic acid [T2M], valeric acid [TC5] and hexanoic acid [TC6]) factorial design. Each group was fed an isocaloric milk formula containing either 0% or 0.35% clofibrate (wt/wt, dry matter basis) with 5% SUC, T2M, TC5 or TC6 for 5 d. Another 6 pigs served as newborn controls. Fatty acid oxidation was examined in fresh homogenates of liver collected on d 6 using [1-^{14}C] palmitic acid (1 mM) as a substrate (0.265 μCi/μmol). Measurements were performed in the absence or presence of L-carnitine (1 mM) or inhibitors of 3-hydroxy-3-methylglutaryl-CoA synthase (L659699, 1.6 μM) or acetoacetate-CoA deacylase (iodoacetamide, 50 μM). Without clofibrate stimulation, ^{14}C accumulation in CO_2 was higher from piglets fed diets containing T2M and TC5 than SUC, but similar to those fed TC6. Under clofibrate stimulation, accumulation also was higher in homogenates from piglets fed TC5 than all other dietary treatments. Interactions between clofibrate and carnitine or the inhibitors were observed ($P = 0.0004$) for acid soluble products (ASP). In vitro addition of carnitine increased ^{14}C-ASP ($P < 0.0001$) above all other treatments, regardless of clofibrate treatment. The percentage of ^{14}C in CO_2 was higher ($P = 0.0023$) in TC5 than in the control group. From these results we suggest that dietary supplementation of anaplerotic and ketogenic FA could impact FA oxidation and modify the metabolism of acetyl-CoA (product of β-oxidation) via alteration of TCA cycle activity, but the modification has no significant impact on the hepatic FA oxidative capacity induced by PPARα. In addition, the availability of carnitine is a critical element to maintain FA oxidation during the neonatal period.

1. Introduction

High neonatal piglet pre-weaning mortality has been a major economic and animal welfare problem in swine industry worldwide for decades. The problem is associated with starvation and diarrhea caused by energy deficiency and an undeveloped immune system. Evidence shows that newborn piglets have a low capacity to oxidize milk fat and generate ketone bodies and a higher fatty acid (FA) esterification rate compared with neonates from other species (Bieber et al., 1973; Pégorier et al., 1983; Adams et al., 1997; Mascaró et al., 1999). Thus, effective hepatic metabolic use of colostrum (milk) is extremely important for both supplying nutrients (energy) and supporting development of the immune system. To improve the efficiency of energy utilization, we have focused on the role of peroxisome proliferator-activated receptor-alpha (PPARα) in

* Corresponding author.
E-mail address: xilin@ncsu.edu (X. Lin).
[1] Present address: Zinpro Corporation, Eden Prairie, MN 55344, USA.
[2] Present address: College of Biological Engineering, Henan University of Technology, Zhengzhou, 450001, China.
[3] Present address: College of Animal Science and Technology, Agricultural University of Hebei, Baoding, 071001, China.

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hepatic FA oxidation and regulation. We found that oral feeding of clofibrate, a potent pharmaceutical PPARα agonist, to newborn pigs significantly increased FA oxidation, and greatly promoted a natural increase in development. Oxidation rates were 1.3- and 2-fold higher in clofibrate fed pigs than control pigs on d 4 and 7 after birth (Bai et al., 2014; Shim et al., 2018). However, it is not known whether the high FA β-oxidation observed in previous studies can be further enhanced. There are 2 lines of evidence indicating that the FA oxidation rate can be affected by the tricarboxylic acid (TCA) cycle activity and by the ketogenic capacity. Firstly, additions of malic acid in mitochondria isolated from newborn pigs within 24 h after birth (Lin et al., 2010) and 2-methylpentanoate in liver homogenate from mice and pig (our unpublished data) increased the hepatic FA oxidation in vitro and modified the accumulative metabolite distribution between CO_2 and acid soluble products (ASP). Secondly, mitochondrial hydroxymethylglutaryl-CoA synthase (mHMGCS) gene expression was significantly increased after feeding clofibrate, but ketone body levels in plasma and tissues remained low (Cheon et al., 2005). Because the excess acetyl-CoA generated from accelerated FA β-oxidation needs to divert into the TCA cycle and the ketogenic pathway for further metabolism, the capacities of the TCA cycle and ketogenic pathways are critical for maintaining the high β-oxidation flux.

The capacity of the TCA cycle can be limited by insufficient intermediates within the cycle. The term "anaplerotic" (Greek, "to fill up") refers to metabolic carbon sources that can contribute net carbon to replenish TCA cycle intermediates. If intermediates are removed from the cycle, they must be replaced via anaplerotic reactions (Owen et al., 2002). Anaplerosis plays an important role in glutamine synthesis and FA oxidation disorders in neonates (Brekke et al., 2016; Amaral and Wajner, 2020), but it has not been evaluated in neonatal pigs. Evidence from our previous study (Lin et al., 2010) indicated that 80% of the acetyl-CoA produced from hepatic mitochondrial β-oxidation was converted to acetate rather than ketone bodies, suggesting that the oxidation rate could be limited by constrained TCA cycle activity (due to insufficient anaplerosis) and/or by a deficit in the pathways of ketogenesis (Adams et al., 1997).

In suckling neonatal pigs, a species with hypoketonemia during the suckling period, the enzyme *mHMGCS* gene is upregulated significantly after administration of clofibrate, but the ketone body level in plasma remains low. That could be due to the low mHMGCS enzyme activity (Adams et al., 1997; He et al., 2017). Because 80% of the acetyl-CoA produced from hepatic mitochondrial β-oxidation was converted to acetate rather than ketone bodies (Lin et al., 2010), the lack of ketogenesis in neonatal pigs has been suggested as an element of a low mHMGCS pathway activity. Thus, the accumulation of incompletely metabolized FA could inhibit the flux of β-oxidation in feedback way. Accordingly, the limited energy production is due to lack of ability to use the acetyl-CoA produced and accumulated by β-oxidation (Duée et al., 1994). However, ketone bodies can be produced via either mHMGCS or the acetoacetyl-CoA deacylase (AACD) pathways in which acetoacetyl-CoA formed from acetyl-CoA is deacylated directly. The acetoacetate and CoA are produced in this reaction, and the activity of the AACD pathway is 20% of the mHMGCS pathway in rat liver (Williamson et al., 1968). The activity of AACD and its role in ketogenesis has never been examined in pigs. In rat liver mitochondria, both pathways of acetoacetate synthesis are stimulated during starvation or in alloxan-induced diabetes, while in cow liver only the mHMGCS pathway was increased during ketosis (Lopes-Cardozo et al., 1975), suggesting that differences in ketogenic pathways exist among species.

To maintain or further enhance FA oxidation, the excess production of acetyl-CoA from the elevated FA β-oxidation induced by clofibrate needs to enter the TCA cycle or the ketogenic pathway(s) for further use. Therefore, we hypothesized that maximizing the activities of the TCA cycle and ketogenesis would increase FA oxidation and energy production. To test our hypothesis, the role of the TCA cycle and ketogenesis pathways (during elevated FA oxidation induced by supplemented clofibrate) were evaluated in liver of neonatal piglets receiving diets containing extra anaplerotic carbon sources such as succinate, or/and ketogenic FA such as medium chain fatty acids (MCFA) (Courchesne-Loyer et al., 2013). Succinate, an intermediate of the TCA cycle, was used as a direct anaplerotic carbon source and due to its role in the ketogenesis pathway (potential inaction of mHMGCS via succinyl-CoA; Lowe and Tubbs, 1985 CoA; Lowe and Tubbs, 1985). Medium chain triglyceride (MCT)/MCFA in dietary supplementation have been confirmed to have positive effects on health, production, feed digestibility and lower body and muscle fat in broilers and swine (Baltić et al., 2017), and they are absorbed quickly and oxidized in the mitochondria with no obligate need of carnitine palmitoyltransferase I (CPT I) transport (Odle, 1997). Accordingly, MCFA are more ketogenic than long-chain FA and have been used in ketogenic diets extensively. Furthermore, 2-methylpentanoic acid (a branched-chain MCFA), valeric acid (an odd-chain MCFA), and hexanoic acid (an even-chain MCFA) were used in the diet of this study, because 2-methylpentanoic acid is a novel source of anaplerotic carbon via the propionyl-CoA-succinyl-CoA pathway, and valeric acid is a dual source of both anaplerotic carbon and ketogenic carbon via production of both propionyl-CoA and acetyl-CoA. Furthermore, hexanoic acid is a source of ketogenic carbon for both mHMGCS and AACD pathways via production of both acetyl-CoA and acetoacetyl-CoA. The effects of dietary supplementation of succinate and the various MCFA on hepatic FA oxidation were measured in fresh liver homogenate in vitro after 5 d of feeding.

2. Materials and methods

2.1. Animal ethics statement

The experimental protocol and all animal care and management were approved by the Institutional Animal Care and Use Committee at North Carolina State University, IACUC ID 16-142 (approved on the September 14, 2016).

2.2. Medium chain triglyceride synthesis

To reduce the effects of the irritant odor and pH of the free MCFA, the respective triglycerides were synthesized in our laboratory by direct esterification of free FA (valeric, hexanoic and 2-methylpentanoic acid) and glycerol with p-toluenesulfonic acid as the catalyst. The esterification followed the method described by Wheeler et al. (1940) with slight modifications (Heo et al., 2002). Briefly, a mixture of MCFA, HCl (6 M), glycerol and p-toluenesulfonic acid were heated at 135 °C in a reaction vessel and stirred under the flow of nitrogen for 72 h. The synthesized triglycerides of valeric acid (TC5), hexanoic acid (TC6) and 2-methylpentanoic acid (T2M) were purified via alkaline/ethanoic extraction, and the purity was verified by HPLC (Waters, Milford, MA) with a stationary phase (Lichrospher Si-100) and a mobile phase (500 mL hexane, 20 mL tetrahydrofuran and 0.1 mL acetic acid).

2.3. Animals and experimental design

A total of 54 suckling newborn pigs (with 36 males and 18 females; average body weight 1.31 ± 0.027 kg) from 6 sows (9 pigs/sow) were used in this study. The pigs were moved from the sows and assigned into a newborn control and 8 dietary treatments

following a randomized complete block design with a 2 (±clofibrate) × 4 (glycerol succinate [SUC], T2M, TC5 and TC6) factorial arrangement, in which the pigs were treated as experimental units and the sow was considered as the block. There was a total of 6 blocks, and 9 piglets in each block from the same sow. Piglets in the newborn control group ($n = 6$) were euthanized within 12 h via sedation with isoflurane followed by exsanguination. Blood and liver samples were collected, processed, and stored in a −80 °C freezer for later analyses. All other piglets were housed individually in an intensive care nursery facility at 30 °C, and received a basal milk replacer (Lin et al., 2020) containing 18.5% of soybean oil and 0.5% of either SUC (glycerol + succinate), TC5, TC6 or T2M in triglycerides with or without 0.35% of the clofibrate (wt/wt, dry matter basis). To keep all diets isocaloric, the supplementation of SUC and the T2M, TC5 and TC6 to the basal diets were offset by a reduction in soybean oil. The ingredients and chemical composition of the basal milk replacer and the milk reconstitution are listed in Tables 1 and 2. The piglets were fed 3 times per day for 5 d, and the diets were freshly prepared each day. The milk consumption and pig body weights were recorded in the morning before feeding. On d 5, piglets were euthanized after feeding as per the newborn piglets. Blood was collected and processed for plasma and stored at −80 °C.

2.4. Liver homogenate preparation

Liver samples from the left lobe were homogenized immediately (Yu et al. (1997). The homogenate buffer contained 220 mM mannitol, 0.1 mM ethylenediaminetetraacetic acid, 70 mM sucrose and 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The pH of the buffer was adjusted to 7.2 at 4 °C with potassium hydroxide. The homogenization was completed on ice in the buffer with an initial ratio of the liver sample and buffer 1:7 (wt/vol) using a glass homogenizer (7 mL Dounce Tissue Grinder; Vineland, NJ 08360, USA). Protein concentration of the homogenate was quantified using the Biuret method (Gornal et al., 1949) on a plate reader (BioTek Instruments, Inc.; Winooski, VT 05404, USA).

Table 1
Milk replacer composition and diet formula, g/100 g DM.

Item	Content
Ingredients	
Whey	55.57
Sodium caseinate	14.06
Whey protein concentrate	12.67
Delactosed whey	6.43
Edible lard	5.50
Dicalcium phosphate	2.76
Other[1]	3.01
Total	100.00
Calculated nutrition composition	
Energy, kcal/g	4.23
Crude protein	31.25
Crude fat[2]	7.50
Lactose	43.18
Calcium	1.48
Phosphorus	1.32
Lysine	3.06
Methionine	1.29
Tryptophan	0.47
Isoleucine	1.80
Histidine	0.71
Phenylalanine	1.25
Threonine	1.62
Leucine	3.07
Valine	1.93

[1] Provided the following grams per kilogram diet: mineral premix, 6.3; DL-methionine, 6.2; potassium sorbate, 5.6; L-lysine HCl, 4.5; calcium chloride, 4.1; vitamin premix, 1.4; flavor additive, 0.6; emulsifier, 0.6; flow agent, 0.5; tetrasodium pyrophosphate, 0.3; antioxidant 0.01. Purchased from Milk Specialties (Eden Prairie, MN 55344).
[2] Whey products contributed another 2% of fat to the dry powder.

Table 2
Experimental dietary formulation (1 kg milk).

Components, kg	Diets			
	SUC	T2M	TC5	TC6
Basal dry powder	0.1280	0.1280	0.1280	0.1280
Soybean oil	0.0259	0.0237	0.0237	0.0237
TC6				0.0064
TC5			0.0068	
T2M		0.0064		
Succinate	0.0048			
Glycerol	0.0016			
Water	0.8397	0.8419	0.8415	0.8419
Total	1	1	1	1

SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid.

2.5. Hepatic fatty acid oxidation in vitro

The FA oxidation measurements were completed in whole fresh homogenate using [1-^{14}C] palmitic acid (1 mM; 9.8 kBq/μmol) as a substrate following the procedure described previously (Shim et al., 2018). Briefly, the medium was pre-incubated in a 25-mL Erlenmeyer flask with the liver homogenate (12.26 mg) in a 37 °C water bath. Incubations were conducted in duplicate flasks in the absence or presence of L-carnitine (1 mM), L659699 (1.6 μM), the inhibitor of HMGCS (Skaff et al., 2012), and iodoacetamide (50 μM), the inhibitor of AACD (Suzuki et al., 1987; Mulder et al., 1977). The measurement of FA oxidation was initiated by adding the substrate (4 μmol) after 5 min pre-incubation and terminated by injecting 0.5 mL of 3 M $HClO_4$ into the flask after 30 min. The $^{14}CO_2$ and ^{14}C-labeled-ASP were determined as described previously (Peffer et al., 2005).

2.6. Hepatic fatty acid profile

Fatty acids were methylated (Walter et al., 2019) and quantified using GC/MS (Agilent Technologies, Wilmington, DE19808, USA) as described by Lin et al. (2010). Fatty acid methyl esters were separated on a HP-23 capillary column (cis/trans FAME CR), 30 m × 0.25 mm, film thickness 0.3 μm (Agilent Technologies, Wilmington, DE, 19808, USA). Mass spectrometric analysis was conducted using a gas chromatograph (Agilent 6890 N Series GC-Systems, Wilmington, DE, 19808, USA) equipped with a mass spectrometric detector (Agilent 5973N, Wilmington, DE, 19808, USA). The oven temperature was programmed from 50 to 100 °C at 10 °C/min, then to 200 °C at 4 °C/min, held for 2 min, and finally to 220 °C at 4 °C/min, held for 12 min. The average helium velocity was 36 cm/s, and the split ratio was 100:1. The temperatures of the MSD electron ionization source and quadropoles were 230 and 150 °C respectively. One microliter of the methyl ester was manually injected, and the total FA amounts were determined by the areas of the total ions for each FA.

2.7. Enzyme activity of carnitine palmitoyltransferase I (CPT I), acetyl-carboxylase (ACC) and propionyl-CoA carboxylase (PCC)

The activity of CPT I was analyzed using isolated mitochondria from fresh liver homogenate following the method described previously (Lin et al., 2010). The activities of ACC and PCC were analyzed using tissue homogenate following the method reported

by Hugler et al. (2003) with a slight modification (Lin et al., 2020). Briefly, tissue homogenates (0.5 to 0.65 mg protein) were incubated with acetyl-CoA or propionyl-CoA (0.4 mM) and [^{14}C]-Na_2CO_3 (37 kBq/μmol) in a buffer containing 100 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM dithioerythritol, 4 mM ATP, 2 mM NADPH, 10 mM $NaHCO_3$ at 37 °C for 6 min. The reaction was started by adding the substrate (acetyl-CoA or propionyl-CoA) and ended by adding HCl. The unused labeled substrate then was removed from the reactant using an analytical evaporator (MULTVAP, Berlin, MA01503, USA) under N_2.

2.8. Acetate and ketone body concentration

Hepatic acetate and ketone body concentrations were determined using an acetate colorimetric kit from Sigma-BioVision (Milpitas, CA 95035, USA).

2.9. Gene expression

Total RNA was extracted using guanidine isothiocynate. The extracted mRNA was quantified with spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE, 19810, USA) and treated with Turbo DNase (Ambion, Austin, TX, USA). The treated mRNA then was transcribed using an iScripTM Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA 94547, USA). Primers were designed based on the use of GenBank as described previously (Lin et al., 2010). The mRNA abundances were measured with MyiQ Single Color RT-PCR (Bio-Rad Laboratories, Hercules, CA 94547, USA). GAPDH was used as housekeeping gene and data were calculated and analyzed for relative expression using the $2^{-\Delta\Delta CT}$ method as described previously (Shim et al., 2018). Relative expression of each measured gene was normalized to the newborn pigs.

2.10. Chemicals

The clofibrate was purchased from TCI America (Portland, OR 97203, USA). The [1-^{14}C] palmitic acid (specific activity: 50 to 60 mCi/mmol), [^{3}H]carnitine and [^{14}C]Na_2CO_3 (37 kBq/μmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO 63146, USA); L659699 was obtained from Cayman Chemical (Ann Arbor, MI 48108, USA), L-carnitine, iodoacetamide and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO 63103, USA).

2.11. Statistical analysis

The data from hepatic FA oxidation were subjected to Analysis of Variance (ANOVA) according to a split-plot design, using the general linear model (GLM) procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC 27513, USA). The main plot was the 8 dietary treatments (2 × 4 factorial design) on animals. The sub-plot was comprised of the in vitro treatments on liver samples. Multiple comparisons between treatments were performed using Tukey's test. The data from FA profiling, enzymatic analysis and mRNA abundance were analyzed using the GLM procedure following the randomized complete block design with a 2 (±clofibrate) × 4 (SUC, T2M, TC5 and TC6) factorial arrangement. Multiple comparisons between treatments were also performed using Tukey's test. To compare the difference between newborn and the 6-d-old treatment piglets, one-way ANOVA was used following a completely randomized design. Effects were considered significant with a probability level of $P < 0.05$, whereas tendencies were noted when the probability level was $0.05 \leq P < 0.10$, and $P > 0.10$ deemed insignificant.

3. Results

3.1. Growth performance and liver weight

Supplementation of clofibrate and dietary treatment with SUC and the MCT in milk replacer had no effects on pig growth performance (Table 3). The liver weight and its percentage (on average 0.69%) of body weight were not affected by clofibrate ($P > 0.10$). The average liver weight was 75.7 g from pigs with no clofibrate and 71.3 g from pigs with clofibrate. Dietary SUC tended to increase liver weight ($P = 0.06$).

3.2. Hepatic fatty acid oxidation in vitro

Results from hepatic [^{14}C]-palmitic acid oxidation in vitro (Fig. 1) showed that the interaction of clofibrate and dietary treatment tended to be significant for $^{14}CO_2$ production ($P = 0.08$). The ^{14}C accumulation in CO_2 ($^{14}CO_2$) was higher from pigs fed diets containing T2M and TC5 than newborns and the pigs fed diets containing SUC with no supplementation of clofibrate, but was not different from TC6. Supplementation of clofibrate increased the $^{14}CO_2$ production for all dietary treatments, but the increase was significantly higher in pigs fed the diet containing TC5 than all other dietary treatments. No difference was detected for all other dietary treatments (Fig. 1A). No interactions were detected between clofibrate and the dietary treatment for the ^{14}C accumulation in ASP (^{14}C-ASP, Fig. 1B) and the total ($^{14}CO_2$ and ^{14}C-ASP, Fig. 1C). The ^{14}C-ASP was significantly greater in all treatments with clofibrate than without clofibrate ($P < 0.0001$). No difference was detected in pigs fed diets without clofibrate supplementation, but the pigs that received diets T2M, TC5 and TC6 had a greater ^{14}C-ASP

Table 3
Effects of diet and clofibrate on growth performance[1].

Item	Diet							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
IBW, kg	1.260	1.290	1.292	1.267	1.297	0.049	0.97	1.260	1.300	1.273	0.034	0.58	0.77
FBW, kg		2.136	1.860	2.004	2.402	0.090	0.19		2.056	1.965	0.064	0.32	0.47
ADG, kg		0.141	0.119	0.129	0.094	0.015	0.15		0.126	0.115	0.011	0.48	0.78
Total gain, kg		0.846	0.712	0.775	0.563	0.091	0.16		0.756	0.692	0.063	0.49	0.78
ADI, kg		0.825	0.745	0.799	0.753	0.033	0.30		0.808	0.753	0.023	0.10	0.78
Total intake, kg		4.027	3.631	3.924	3.678	0.160	0.26		3.948	3.682	0.114	0.10	0.82
Liver weight, g		70.64	69.00	77.65	67.81	5.983	0.69		75.03	67.52	4.251	0.22	0.84
Liver weight, % BW		3.449	3.724	3.917	3.538	0.227	0.57		3.769	3.545	0.169	0.35	0.84

NB = newborn; SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = no clofibrate administration; Clof+ = clofibrate administration; IBW = initial body weight; FBW = final body weight; ADG = average daily gain; ADI = average daily intake.
[1] Data are least square means ± SEM ($n = 6$).

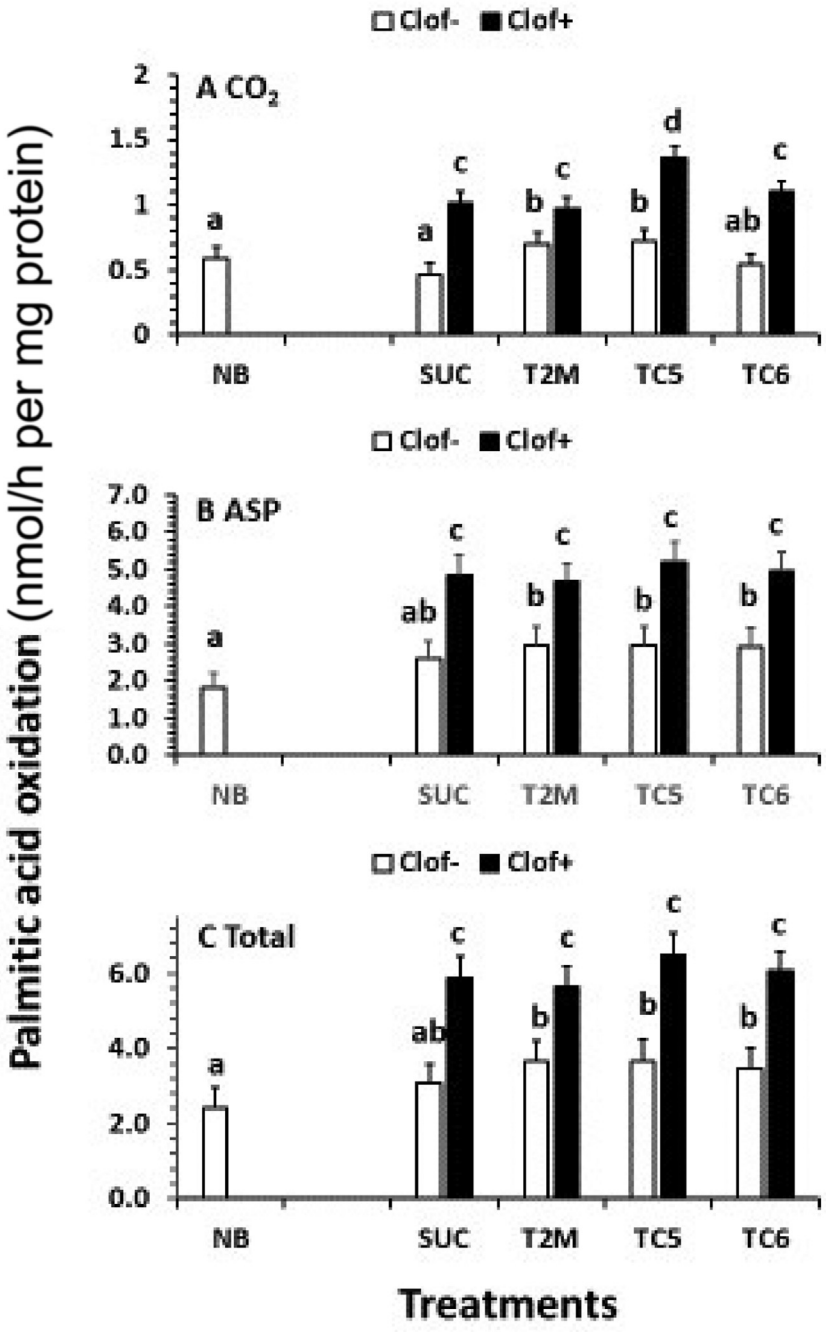

Fig. 1. Effects of dietary medium chain triglycerides (MCT) and clofibrate administration on palmitic acid oxidation in hepatic tissue of suckling pigs. (A) CO_2 = metabolite generated from palmitic acid oxidation in vitro; (B) ASP = metabolites generated from palmitic acid oxidation in vitro; (C) Total = CO_2 + ASP. The unit of CO_2, ASP and CO_2 + ASP is nmol/h per mg protein. NB = newborn; TC5 = triglycerides of valeric acid; TC6 = triglycerides of hexanoic acid; T2M = triglycerides of 2-methylpentanoic acid; SUC = glycerol succinate; Clof- = no clofibrate administration; Clof+ = clofibrate administration; ASP = acid soluble products. Data are least square means ± SEM ($n = 6$). Means lacking a common letter differ ($P < 0.05$).

production than newborn pigs. The total palmitic acid oxidation rate followed the same pattern as ^{14}C-ASP production.

Results from the hepatic palmitic acid oxidation in vitro also showed that there were interactions between clofibrate and incubation factors (carnitine and the inhibitors for ketogenesis) for $^{14}CO_2$, ^{14}C-ASP and the total oxidation (Fig. 2, $P < 0.0001$). The $^{14}CO_2$ production was greater in pigs treated with clofibrate than those without clofibrate and newborn pigs. Addition of carnitine increased $^{14}CO_2$ production, but the increase was much higher in clofibrate treated pigs than non-treated pigs and newborn pigs. Addition of iodoacetamide and L659699, the inhibitors of ketogenic pathways, had no effects on $^{14}CO_2$ production in newborn pigs and pigs without clofibrate, but $^{14}CO_2$ production was reduced by L659699 in pigs that received clofibrate compared to the control (Fig. 2A). The ^{14}C-ASP production was significantly increased by adding carnitine to the incubation in newborn pigs and pigs treated with or without clofibrate compared to the control. However, the increase was much higher in clofibrate treated pigs than pigs without clofibrate and newborn pigs. Addition of the ketogenic inhibitors had no impact on ^{14}C-ASP production in newborn pigs

and pigs without clofibrate compared with the control. The ^{14}C-ASP production was increased in pigs treated with clofibrate, but the increase tended to be attenuated with the ketogenic inhibitors (Fig. 2B). The total ^{14}C accumulation had a similar pattern to ^{14}C-ASP (Fig. 2C).

No interaction between all the dietary treatments (SUC and the MCTs) and the incubation factors was detected ($P > 0.10$).

When the palmitic acid oxidative flux either to CO_2 or ASP was expressed as a percentage of total oxidative flux (CO_2 plus ASP), addition of clofibrate did not change the distributions of ^{14}C accumulation between CO_2 and ASP (Fig. 3A). However, the percentage of $^{14}CO_2$ was significantly higher ($P = 0.0023$) in the TC5 treatment than others, and there was no difference between other dietary treatments and the control group, indicating a greater proportional accumulation of ^{14}C from TC5 in CO_2 (Fig. 3B). No differences were detected between L659699 or iodoacetamide treatment and the control group. However, carnitine significantly decreased ($P < 0.0001$) the percentage of CO_2, indicating a greater relative accumulation in ASP. There was no difference between other incubation factors and the control group ($P > 0.05$, Fig. 3C).

3.3. Hepatic fatty acid composition

Dietary supplementation of T2M decreased hepatic C16:0 concentration (Table 4) and increased C15:0 compared to TC5 and

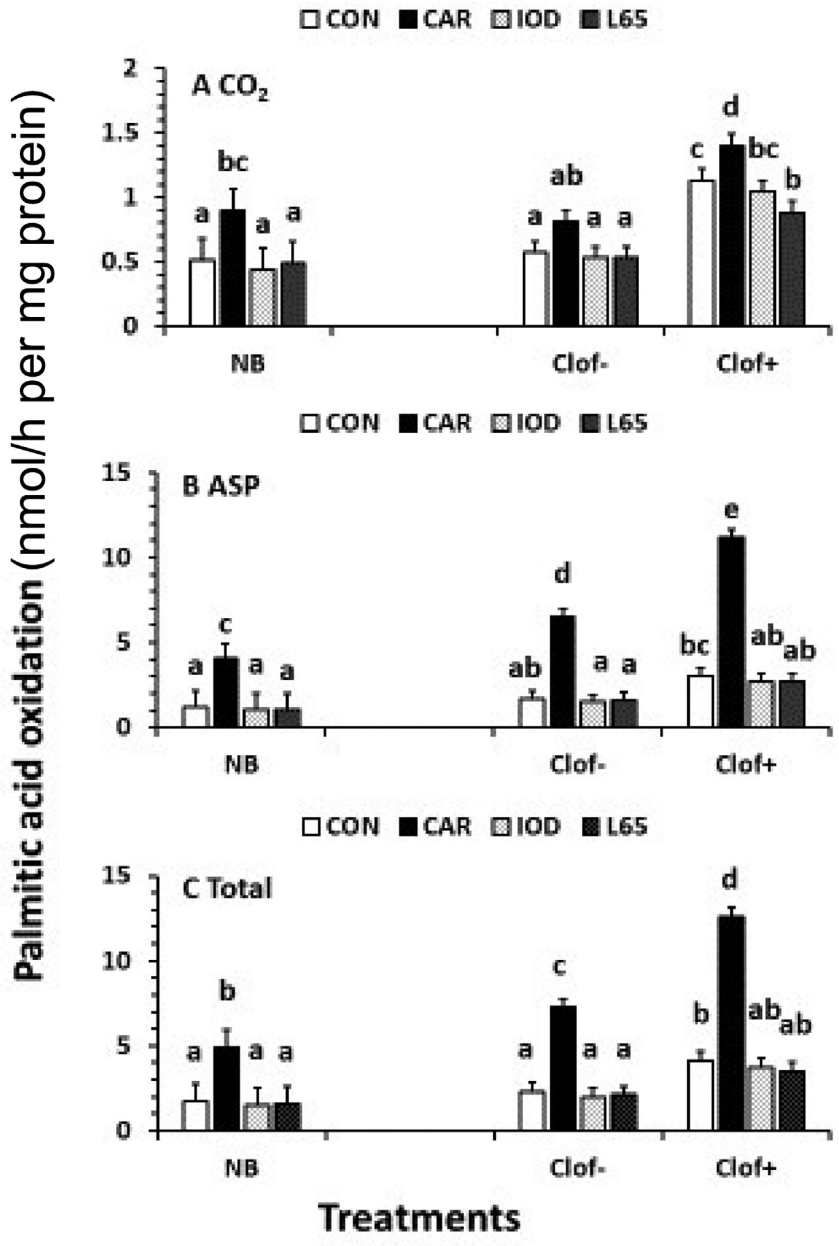

Fig. 2. Effects of in vitro supplementation of carnitine and ketogenic inhibitors on palmitic acid oxidation in hepatic tissue of suckling pigs. (A) CO_2 = metabolite generated from palmitic acid oxidation in vitro; (B) ASP = acid soluble metabolites generated from palmitic acid oxidation in vitro; (C) Total = CO_2 + ASP. The unit of CO_2, ASP and CO_2 + ASP is nmol/h•mg protein. NB = newborn; Clof- = no clofibrate administration; Clof+ = clofibrate administration; CON = control (no carnitine supplementation and inhibitor addition); CAR = carnitine (1 mM); L65 = L659699 (1.6 μM), inhibitor of hydroxymethylglutaryl-CoA synthase; IOD = iodoacetamide (50 μM), inhibitor of acetoacetyl-CoA deacylase; ASP = acid soluble products. Data are least square means ± SEM ($n = 6$). Means lacking a common character differ ($P < 0.05$).

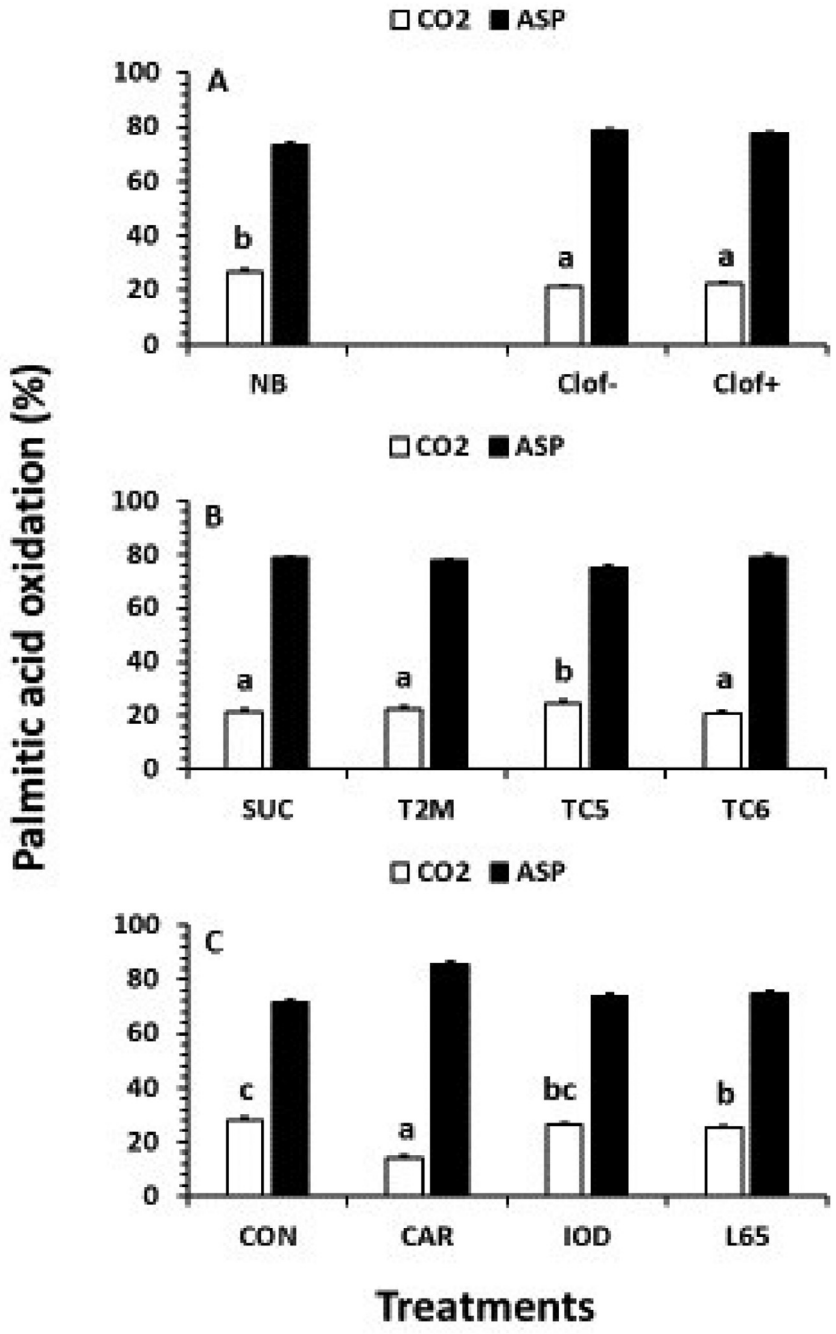

Fig. 3. Effects of the medium chain triglycerides (MCT), clofibrate, carnitine and ketogenic inhibitors on palmitic acid oxidative distribution between CO_2 and ASP in hepatic tissue of suckling pigs. (A) Effects of clofibrate on % of CO_2 and ASP, the metabolites generated from palmitic acid oxidation in vitro; (B) effects of MCT on % of CO_2 and ASP (acid soluble products), the metabolites generated from palmitic acid oxidation in vitro; (C) effects of carnitine and ketogenic inhibitors on % of CO_2 and ASP, the metabolites generated from palmitic acid oxidation in vitro. NB = newborn; TC5 = triglycerides of valeric acid; TC6 = triglycerides of hexanoic acid; T2M = triglycerides of 2-methylpentanoic acid; SUC = glycerol succinate; Clof- = no clofibrate administration; Clof+ = clofibrate administration. CON = control (no carnitine supplementation and inhibitor addition); CAR = carnitine (1 mM); L65 = L659699 (1.6 μM), the inhibitor of hydroxymethylglutaryl-CoA synthase; IOD = iodoacetamide (50 μM), the inhibitor of acetoacetyl-CoA deacylase; ASP = acid soluble products. Data are least square means ± SEM ($n = 6$). Means lacking a common character differ ($P < 0.05$).

C23:0 concentrations compared to TC5 and TC6. Dietary supplementation of TC6 and SUC increased C18:0 concentration compared to TC5 ($P < 0.001$). Supplementation of clofibrate reduced C14:0, C20:0 and C22:0 concentrations ($P < 0.05$). Concentrations of C18:0, C22:0, C23:0 and C24:0 were higher in 6-d-old piglets than newborn pigs, while C14:0, C15:0 and C16:0 concentrations were higher in newborn pigs than 6-d-old piglets ($P < 0.05$). No interactions were detected between dietary treatment and clofibrate ($P > 0.10$).

Dietary supplementation of T2M increased C17:1 (Table 5) compared with all other dietary treatments and C19:1 and C22:5 compared with TC6 and C22:6 compared with SUC. Dietary supplementation of SUC increased C18:2 compared to other dietary treatments and C18:3n3 compared to TC5. Supplementation of clofibrate increased C20:3n6, C22:5n3 and C22:6n3 but reduced C20:5n3 compared to the control. The monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) C22:2 and C22:5n3 were higher in newborns than 6-d-old piglets, but C18:2, C18:3n3, C20:5n3 and

Table 4
Effects of diet and clofibrate on hepatic saturated fatty acid of suckling pigs (%). [1]

Item	Diets							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
SFA													
C12:0	0.015	0.005	0.005	0.008	0.004	0.003	0.208	0.015	0.006	0.004	0.003	0.077	0.774
C14:0	0.835^{b}	0.340^{a}	0.270^{a}	0.384^{a}	0.309^{a}	0.047	0.0001	0.835^{c}	0.392^{b}	0.229^{a}	0.044	0.0001	0.18
C15:0	0.214^{b}	0.067^{a}	0.238^{b}	0.255^{b}	0.075^{a}	0.029	0.0001	0.214	0.184	0.131	0.034	0.217	0.20
C16:0	21.02^{c}	17.74^{b}	13.78^{a}	17.08^{b}	16.05^{b}	0.757	0.0001	21.10^{b}	16.54^{a}	15.34^{a}	0.767	0.0002	0.43
C18:0	13.94^{a}	21.85^{c}	21.30^{bc}	19.05^{b}	21.99^{c}	1.019	0.0001	13.94^{a}	21.24^{b}	21.31^{b}	1.130	0.0005	0.49
C20:0	0.344	0.420	0.448	0.394	0.434	0.030	0.225	0.344^{a}	0.461^{b}	0.393^{a}	0.026	0.0087	0.97
C22:0	0.239^{a}	0.432^{b}	0.451^{b}	0.401^{b}	0.425^{b}	0.031	0.0009	0.239^{a}	0.476^{c}	0.381^{b}	0.026	0.0001	0.48
C23:0	0.092^{a}	0.260^{bc}	0.318^{c}	0.248^{b}	0.262^{bc}	0.023	0.0001	0.092^{a}	0.302^{c}	0.248^{b}	0.020	0.0001	0.16
C24:0	0.738^{a}	1.392^{b}	1.457^{b}	1.310^{b}	1.389^{b}	0.076	0.0001	0.738	1.485	1.325	0.067	0.0001	0.29

FA = fatty acid; SFA = saturated fatty acids; NB = newborn; SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = No clofibrate administration; Clof+ = clofibrate administration.
[1] Data are least square means ± SEM ($n = 6$). Means within a row with lacking a common superscript differ ($P < 0.05$).

C22:6n3 were lower in the newborns than 6-d-old piglets. No interactions were detected between dietary treatment and clofibrate ($P > 0.10$).

The total saturated fatty acids (SFA) was increased (Table 6), and the total USFA was decreased by dietary SUC compared to TC5, T2M and the newborn groups. The ratio of SFA to USFA was lower in SUC than T2M. Clofibrate had no impact on total SFA, USFA and the ratio of SFA to USFA.

Dietary supplementation of T2M decreased the total MUFA but increased the total PUFA with no impact on the ratio of MUFA to PUFA. However, the ratio was lower in 6-d-old pigs than newborns. Clofibrate had no impact on the total of MUFA and PUFA. In general, the total MUFA decreased and the total PUFA increased in the 6-d-old pigs compared to newborns.

Dietary supplementation of T2M also increased both total n3 and n6 PUFA compared to other dietary treatments, and no impact was detected on the ratio of n6 to n3. Clofibrate administration increased n3 PUFA, which correspondingly decreased the n6 to n3 ratio. The total MUFA and PUFA increased/tended to increase in the 6-d-old pigs compared to newborns.

3.4. Hepatic enzyme activity

Supplementation of clofibrate increased CPT I and ACC activity ($P < 0.001$) and decreased PCC activity ($P < 0.01$, Table 7). Dietary MCT treatments had no impact on the enzymatic activities ($P > 0.10$). No interactions were detected between clofibrate supplementation and the dietary treatments ($P > 0.10$).

3.5. Acetate and ketone body concentrations

Dietary treatments and clofibrate administration had no impacts on the hepatic acetate concentration ($P > 0.10$). The concentrations of acetoacetate (AcAc), 3-β-hydroxybutyrate (BHO) and total ketone bodies were also not affected by dietary treatment and clofibrate supplementation ($P > 0.10$, Table 8).

3.6. Hepatic mRNA abundance

Clofibrate administration increased *CPT II* mRNA ($P < 0.05$) and tended to increase acyl CoA oxidase (*ACO*) mRNA enrichment

Table 5
Effect of diets and clofibrate on hepatic unsaturated fatty acid (USFA) of sucking pigs (%).[1]

Item	Diets							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
MUFA													
C15:1	0.020	0.024	0.031	0.026	0.031	0.009	0.879	0.020	0.030	0.024	0.008	0.662	0.27
C16:1	5.019^{b}	1.573^{a}	1.632^{a}	2.021^{a}	1.595^{a}	0.244	0.0001	5.019^{b}	1.693^{a}	1.615^{a}	0.227	0.0001	0.60
C17:1	0.345^{b}	0.122^{a}	0.716^{c}	0.383^{b}	0.135^{a}	0.067	0.0001	0.345	0.379	0.319	0.080	0.793	0.83
C18:1	27.13^{c}	19.87^{ab}	18.06^{a}	21.32^{b}	19.60^{ab}	1.073	0.0001	27.13^{b}	19.93^{a}	18.98^{a}	1.139	0.0001	0.32
C19:1	0.082^{c}	0.006^{a}	0.071^{c}	0.076^{c}	0.012^{b}	0.010	0.0001	0.082	0.043	0.041	0.013	0.145	0.97
C20:1	0.495^{b}	0.157^{a}	0.175^{a}	0.195^{a}	0.161^{a}	0.019	0.0001	0.495^{b}	0.189^{a}	0.155^{a}	0.018	0.0001	0.71
C24:1	0.588^{b}	0.338^{a}	0.397^{a}	0.366^{a}	0.324^{a}	0.032	0.0001	0.588^{b}	0.374^{a}	0.336^{a}	0.033	0.0001	0.62
PUFA													
C18:2	11.36^{a}	18.15^{c}	17.63^{bc}	15.45^{b}	17.28^{b}	0.896	0.0001	11.36^{b}	17.23^{a}	17.28^{a}	1.059	0.0031	0.94
C18:3n6	0.471	0.434	0.553	0.524	0.516	0.077	0.804	0.471	0.476	0.542	0.071	0.656	0.27
C18:3n3	0.114^{a}	0.547^{c}	0.471^{bc}	0.392^{b}	0.438^{bc}	0.055	0.0002	0.114^{a}	0.435^{b}	0.496^{b}	0.063	0.0024	0.71
C20:2	1.025	0.755	1.119	1.022	0.843	0.128	0.203	1.025	0.099	1.070	0.117	0.099	0.62
C20:3n6	0.706	0.686	0.800	0.776	0.741	0.070	0.742	0.706^{ab}	0.669^{a}	0.847^{b}	0.059	0.023	0.68
C20:4n6	11.5^{a}	11.47^{a}	14.87^{b}	13.77^{ab}	13.29^{ab}	0.935	0.049	11.50	12.92	13.99	0.927	0.243	0.15
C20:3n3	0.065	0.087	0.107	0.080	0.097	0.013	0.254	0.065	0.097	0.090	0.015	0.416	0.51
C20:5n3	0.043^{a}	0.117^{b}	0.114^{b}	0.110^{b}	0.130^{b}	0.013	0.002	0.043^{c}	0.130^{b}	0.103^{a}	0.012	0.0001	0.36
C22:2	0.064^{c}	0.002^{a}	0.011^{ab}	0.026^{b}	0.015^{ab}	0.006	0.0001	0.064^{b}	0.011^{a}	0.014^{a}	0.005	0.0002	0.52
C22:4	0.497	0.423	0.609	0.560	0.473	0.054	0.086	0.497	0.490	0.547	0.048	0.526	0.46
C22:5n3	1.324^{c}	0.384^{a}	1.027^{c}	0.748^{bc}	0.523^{ab}	0.108	0.0001	1.324^{c}	0.500^{a}	0.883^{b}	0.101	0.0001	0.43
C22:6n3	1.624^{a}	2.191^{ab}	3.131^{c}	2.861^{bc}	2.673^{bc}	0.278	0.0072	1.624^{a}	2.483^{b}	3.000^{c}	0.265	0.0082	0.18

FA = fatty acid; NB = newborn; SUC, glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5, triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = no clofibrate administration; Clof+ = clofibrate administration. MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[1] Data are least square means ± SEM ($n = 6$). Means within a row with lacking a common superscript differ ($P < 0.05$).

Table 6
Effects of diet and clofibrate on ratio of hepatic fatty acids.[1]

Item	Diets							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
SFA	37.43^{a}	42.51^{c}	38.26^{a}	39.13^{ab}	40.95^{bc}	1.068	0.011	37.43	41.07	39.37	1.246	0.166	0.83
USFA	62.52^{c}	57.34^{a}	61.56^{c}	60.73^{bc}	58.90^{ab}	1.050	0.009	62.52	58.90	60.34	1.246	0.180	0.82
USFA:SFA ratio	1.677^{b}	1.418^{a}	1.651^{b}	1.572^{ab}	1.458^{a}	0.061	0.011	1.677	1.479	1.567	0.072	0.209	0.80
MUFA	33.70^{c}	22.06^{ab}	21.04^{a}	24.33^{b}	21.83^{ab}	1.250	0.0001	33.70^{b}	22.60^{a}	21.43^{a}	1.250	0.0001	0.33
PUFA	28.82^{a}	35.26^{b}	40.47^{c}	36.33^{b}	37.04^{bc}	1.450	0.0001	28.82^{a}	36.26^{b}	38.88^{b}	1.477	0.0006	0.42
MUFA:PUFA ratio	1.253^{b}	0.678^{a}	0.542^{a}	0.703^{a}	0.618^{a}	0.070	0.0001	1.253^{b}	0.660^{a}	0.578^{a}	0.066	0.0001	0.27
N3FA	3.170^{a}	3.326^{a}	4.850^{b}	4.190^{ab}	3.861^{a}	0.352	0.0085	3.171^{a}	3.646^{a}	4.572^{b}	0.338	0.013	0.16
N6FA	23.72^{a}	30.63^{b}	33.65^{c}	30.35^{b}	31.66^{bc}	1.178	0.0001	23.72^{a}	31.13^{b}	32.49^{b}	1.255	0.0003	0.62
N6:N3 ratio	8.306	10.70	7.451	7.953	9.631	0.959	0.085	8.306^{ab}	9.949^{b}	7.768^{a}	0.845	0.060	0.57

FA = fatty acid; NB = newborn; SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = no clofibrate administration; Clof+ = clofibrate administration; SFA = saturated fatty acids; USFA, unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[1] Data are least square means ± SEM ($n = 6$). Means within a row with lacking a common superscript differ ($P < 0.05$).

($P < 0.1$). Dietary supplementation of TC5 also tended to increase *CPT II* mRNA enrichment ($P < 0.1$). The interaction between dietary treatment and clofibrate administration tended to be significant for long-chain acyl-CoA dehydrogenase (*LCAD*) and very long-chain acyl-CoA dehydrogenase (*VLCAD*) expressions ($P < 0.1$). No effects of dietary treatments or administration of clofibrate were detected on relative mRNA abundance of all other measured genes (Table S2).

4. Discussion

Clofibrate is a potent pharmaceutical PPARα agonist and its effects on hepatic FA oxidation in neonatal pigs were investigated in our previous in vitro (Shim et al., 2018) and in vivo studies (Bai et al., 2014). As the main source of energy for cells and a critical part of aerobic respiration, the TCA cycle plays an important role in supporting FA oxidation. To evaluate the role of TCA cycle capacity in neonatal FA oxidation with and without activation of PPARα, we fed newborn pigs diets containing different levels of anaplerotic carbon derived from SUC, T2M, TC5 and TC6. The results showed that there was no interaction between clofibrate and the dietary anaplerotic carbon levels. As observed in our previous studies (Bai et al., 2014; Shim et al., 2018), administration of clofibrate significantly increased ^{14}C accumulation in CO_2, ASP and total FA oxidative metabolites. The average rate of total FA oxidation induced by clofibrate was 1.8-fold higher than unstimulated controls and aligned with increased CPT I activity (Bai et al., 2014; Shim et al., 2018). Clofibrate also increased hepatic n3 FA enrichment, and docosapentaenoic acid (C22:5) and docosahexeneoic acid (C22:6) were the primary contributors. An increased enrichment of C22:6 in the phosphatidylserine of liver was reported previously in clofibrate-fed Swiss-Webster mice (Wheelock et al., 2007), and the increase was due to an activation of phosphatidylserine synthetase 2. Thus, the high enrichments of C22:5 and C22:6 in liver, like observed in the mice, might be associated with the phosphatidylserine synthetase 2. However, a similar result was not detected in kidney tissues (Lin et al., 2020), suggesting that the effect on activation of phosphatidylserine synthetase 2 might be tissue specific. Because phosphatidylserine is an essential constituent of eukaryotic membranes and is the most abundant anionic phospholipid in the eukaryotic cells (Kay and Fairn, 2019), this finding may imply that clofibrate could promote the development of hepatic cell membrane function during the suckling period.

Dietary supplementation of SUC and TC6 had no effect on ^{14}C accumulation in CO_2 production as compared to newborn pigs, suggesting that increasing the TCA cycle intermediates directly (SUC) or promoting generation of acetyl-CoA alone (TC6) could not accentuate TCA cycle activity sufficiently to enhance FA oxidation. In contrast with newborns and pigs fed SUC, the pigs receiving dietary T2M and TC5 increased ^{14}C accumulation in CO_2, suggesting that the propionate, as an anaplerotic carbon source, indeed, augmented TCA cycle activity via the propionyl-CoA-succinyl-CoA pathway. However, the stimulation was only observed for TC5 in clofibrate-fed pigs. The anaplerotic influences of odd-chain FA on the TCA cycle were also reported in isolated hepatocytes from piglets, in which the oxidation of heptanoic (C7) or nonanoic (C9) acids to CO_2 and ASP was greater than that of even-chain octanoic (C8) or decanoic (C10) acids (Odle et al., 1991). Thus, TC5 might be the same as other odd-chain medium FA, promoting the FA oxidation rate via the production of both acetyl-CoA and propionyl-CoA. Vockley et al. (2015) proposed that diets containing even-chain MCT only provide acetyl-CoA for the TCA cycle without propionyl-CoA, which could result in a substrate imbalance for the TCA cycle due to the depletion of the latter. Similarly, TC7 can generate 2 molecules of acetyl-CoA and 1 molecule of propionyl-CoA, which can sustain the appropriate substrate balance for the TCA cycle. In our study, dietary T2M, a methyl-branched MCT, generated 2 molecules of propionyl-CoA by β-oxidation and TC6 generated 3 molecules of acetyl-CoA by β-oxidation. In contrast with T2M and TC6, TC5 produces 1 molecule of acetyl-CoA and 1 molecule of propionyl-CoA (Vockley et al., 2015). Although T2M may act as a novel anaplerotic carbon resource when TCA intermediates are diminished, appropriate substrate balance may be more important. This was also supported by supplementation of succinate, a direct substrate of the TCA cycle, which was unable (alone) to improve FA oxidation. Moreover, the increase in $^{14}CO_2$ production from T2M and TC5 did not modify total FA oxidation, suggesting that the ^{14}C-palmitate oxidation rate primarily is controlled by β-oxidation that is associated with CPT I activity. Modifying TCA cycle activity only changed the ratio of CO_2 and ASP. As observed in CO_2 production, the ASP and total metabolites were not different between the diets, although it was significantly increased in pigs receiving dietary T2M, TC5 and TC6 as compared to newborn pigs. Due to the physiological roles of succinate in many metabolic pathways, the effect of SUC on hepatic FA oxidation could be related to its feedback suppression of mitochondrial β-oxidation induced by an increased mitochondrial NADH/NAD + ratio (Zhang et al., 2021).

Dietary treatments had great impacts on the FA composition in the liver. The dietary SUC and TC6 significantly increased saturated FA, while T2M and TC5 increased the concentrations of odd-chain FA C15:0, C17:1 and C19:1. T2M also increased unsaturated FA, especially C20:4 and C22:6. Since succinate inhibits saturated FA de novo synthesis by intact liver mitochondria (Whereat et al., 1967), it is likely that the increase was not associated with FA synthesis.

Table 7
Effects of diet and clofibrate on hepatic enzyme activities (nmol/μg protein)[1].

Enzyme	Diet							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
CPT I	26.37	28.87	31.46	32.93	31.04	4.31	0.78	26.37[a]	24.50[a]	37.65[b]	1.95	0.0001	0.82
PCC	6.59	3.36	3.67	3.41	3.24	0.37	0.82	6.59[c]	3.78[b]	2.83[a]	0.46	0.001	0.28
ACC	1.50	2.79	2.73	2.58	3.41	0.58	0.74	1.50[a]	2.07[a]	3.70[b]	0.55	0.011	0.14

CPT I = carnitine palmitoyl transferase I; PCC = propionyl-CoA carboxylase; ACC = acetyl-CoA carboxylase; NB = newborn; SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = no clofibrate administration; Clof+ = clofibrate administration.
[1] Data are least square means ± SEM ($n = 6$). Means within a row with lacking a common superscript differ ($P < 0.05$).

Table 8
Effects of diet and clofibrate on liver acetate, acetoacetate and β-hydroxybutyrate concentrations (μmol/g) [1].

Item	Diet							Clofibrate					Interaction
	NB	SUC	T2M	TC5	TC6	SEM	*P*-value	NB	Clof-	Clof+	SEM	*P*-value	*P*-value
Acetate	169.88	180.59	189.11	197.65	191.62	9.99	0.70	169.88	191.32	188.35	7.15	0.77	0.30
AcAc	–	7.76	11.46	15.41	16.36	5.39	0.66	–	15.21	10.28	3.75	0.37	0.47
BOH	–	65.70	81.22	26.52	110.28	0.58	0.37	–	47.36	94.49	23.55	0.16	0.33
Total		73.46	92.68	41.93	126.64	35.19	0.40	–	62.59	104.77	24.94	0.24	0.31

AcAc = acetoacetate; BOH = 3-β-hydroxybutyrate; NB = newborn; SUC = glycerol succinate; T2M = triglyceride of 2-methylpentanoic acid; TC5 = triglyceride of valeric acid; TC6 = triglyceride of hexanoic acid; Clof- = no clofibrate administration; Clof+ = clofibrate administration.
[1] Data are least square means ± SEM ($n = 6$). Least square means on each row with different characters differ ($P < 0.05$).

Because excessive succinate oxidation may increase hepatic lipid deposition while suppressing mitochondrial β-oxidation (Zhang et al., 2021), the increase in saturated FA might be related to an increased lipid deposition. The increase of saturated FA in liver of pigs fed TC6 might be related to the elongation of the medium-chain FA. Similarly, because odd- and branched-chain FA in the body are primarily from the diet and can also be metabolized into their elongated or chain-shortened products (Yan et al., 2017), the increase in odd-long-chain FA could be due to elongation of valeric acid and propionic acid from T2M.

To assess the effect of ketogenic capacity on β-oxidation, we found that inhibition of ketogenesis pathways in vitro by addition of L659699 and iodoacetamide in liver homogenate from newborn pigs or dietary treated pigs had no effects on the total FA oxidation, although L659699 decreased CO_2 production in clofibrate-fed pigs compared to the control. This demonstrated that the influence of the ketogenic pathway on FA β-oxidation is minimal in neonatal pigs during the suckling period. This was also supported by the results from the assay of hepatic ketone bodies. Interestingly, in vitro supplementation of carnitine greatly increased total FA oxidation in liver from both newborn pigs and pigs with and without clofibrate administration, especially increasing ASP 6.9-fold of CO_2 in control and clofibrate pigs. This implies that a great proportion of the produced acetyl-CoA may have been converted into acetyl-carnitine. We previously observed a similar response in kidney tissue (Lin et al., 2020). This result suggests that TCA cycle capacity could limit FA oxidation when CPT I is fully activated, and does not constrain the transfer of FA into mitochondria.

When the oxidative flux to CO_2 and ASP were expressed as a percentage of total oxidative flux to CO_2 plus ASP, the percentage of ASP increased with age (Newborn vs. 6-d-old), suggesting that the capacity of use of acetyl-CoA generated from β-oxidation was improved with age, which might relate to an increase in carnitine availability in the liver. Addition of clofibrate increased FA oxidation but had no effect on the percentage ratio of CO_2 to ASP, suggesting that the impact of activation of PPARα on FA oxidation is primarily related to CPT I and/or ACO. It is interesting that TC5 changed the percentage ratio between CO_2 and ASP, but not other dietary treatments. Only supplementation of TCA cycle intermediate SUC or propionyl-CoA precursor and acetyl-CoA could not modify the distribution between CO_2 and ASP. Both carnitine and L659699 decreased the percentage of CO_2, but the decrease in carnitine was much higher than observed for L659699, demonstrating the importance of carnitine availability to FA oxidation due to the low capability of generating ketone bodies in neonatal pigs.

5. Conclusion

In conclusion, results from this experiment indicated that the capacity of the TCA cycle and ketogenic pathways indeed affect hepatic FA oxidation, but among the various MCT used in the study, only TC5 influences the oxidative flux induced by clofibrate. A fitting substrate balance may be more important for maximizing energy generation. Carnitine availability is a critical factor for FA β-oxidation flux rate, especial under the status of PPARα activation. However, further metabolism of the acetyl carnitine generated from a high FA β-oxidation rate needs to be investigated in vivo in neonatal pigs.

Author contributions

Jinan Zhao: conducted the experiment, and, collected and analyzed the samples and experimental data, Formal analysis, and summary as well as wrote the manuscript, (original draft preparation). **Brandon Pike:** conducted the experiment and helped with sample collection. **Jin Huang:** Formal analysis. **Zhihua Feng:** Formal analysis, All authors have read and agreed to the published version of the manuscript. **Jack Odle:** Investigation, Writing – review & editing, and, Funding acquisition. **Xi Lin:** Project administration, and, Supervision, Investigation, Writing – review & editing, and, Funding acquisition.

Declaration of conflicting of interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

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