Effects of milk diets containing beef tallow or coconut oil on the fatty acid metabolism of liver slices from preruminant calves

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(Received 8 February 1999 – Revised 7 January 2000 – Accepted 25 January 2000)

Coconut oil (CO) induces a triacylglycerol infiltration in the hepatocytes of preruminant calves when given as the sole source of fat in the milk diet over a long-term period. Metabolic pathways potentially involved in this hepatic triacylglycerol accumulation were studied by *in vitro* methods on liver slices from preruminant Holstein × Friesian male calves fed a conventional milk diet containing CO (*n* 5) or beef tallow (BT, *n* 5) for 19 d. Liver slices were incubated for 12 h in the presence of 0·8 mM-[^{14}C]oleate or -[^{14}C]laurate added to the medium. Fatty acid oxidation was determined by measuring the production of CO_2 (total oxidation) and acid-soluble products (partial oxidation). Production of CO_2 was 1·7–3·6-fold lower (P 0·0490) and production of acid-soluble products tended to be lower ($P = 0·0625$) in liver slices of CO- than BT-fed calves. Fatty acid esterification as neutral lipids was 2·6– to 3·1–fold higher ($P = 0·0088$) in liver slices prepared from calves fed the CO diet compared with calves fed the BT diet. By contrast with what occurs in the liver of rats fed CO, the increase in neutral lipid production did not stimulate VLDL secretion by the hepatocytes of calves fed with CO, leading to a triacylglycerol accumulation in the cytosol. It could be explained by the reduction of fatty acid oxidation favouring esterification in the form of triacylglycerols, in association with a limited availability of triacylglycerols and/or apolipoprotein B for VLDL packaging and subsequent secretion.

Fatty acid metabolism: Calf hepatocyte: Dietary fat

The preruminant calf was classically given a conventional milk replacer rich in fat (200–230 g/kg DM) in the form of beef tallow (BT). However, during the first weeks of calf life, one-third of the fat source is currently added as coconut oil (CO) which is rich in saturated fatty acids (FA), especially in lauric acid (12 : 0; 40 g/100 g total FA). Indeed, addition of CO in the milk diet improves intestinal FA digestibility of the young calf since the salivary lipase (the lipolytic enzyme mainly active at birth) has a specificity for medium-chain FA (Bauchart & Aurousseau, 1993). Moreover, CO supplementation in the milk diet of preruminant calves increased protein accretion and therefore growth of the animals (Aurousseau *et al*. 1984). However, CO given as the sole source of fat for a long period induced a triacylglycerol (TAG) accumulation in hepatocytes leading to a liver steatosis in the preruminant calf which could impair growth performance (Jenkins & Kramer, 1986; Bauchart *et al*. 1998). The steatosis can lead to liver dysfunction and to health problems (diarrhoea) that impair the well-being of the calves.

Mechanisms involved in this lipid infiltration of the calf liver are still unknown. Among possible explanations, a deficiency in essential FA (linoleic and linolenic acids), poorly represented in CO, has been suggested. However, CO feeding to the rat did not induce a liver steatosis even when fed over a long time period (Ney *et al*. 1991; Yaqoob *et al*. 1995). Moreover, Jenkins & Kramer (1986) reported that healthy calves would have lower essential FA requirements than many monogastric species such as the rat. Another hypothesis could be associated with the hepatic FA metabolism. Indeed, hepatic TAG content resulted from a steady-state between their synthesis by FA esterification and their secretion as part of VLDL particles. The regulation of TAG synthesis occurred at two intracellular major branch-points: (1) the partition of FA between esterification and oxidation pathways; and (2) the conversion of diacylglycerols into TAG or phospholipids. On the other hand, VLDL production requires the availability of TAG at their site of assembly with apolipoprotein B in the endoplasmic reticulum (Gruffat *et al*. 1996; Gordon, 1997). However, microsomal TAG represented only a small part of total cellular TAG compared with the cytosolic TAG storage pool (Pullen *et al*. 1988). Participation of stored TAG to VLDL packaging needs to follow a

Abbreviations: ASP, acid-soluble products; BT, beef tallow; CO, coconut oil; FA, fatty acid; NL, neutral lipid; TAG, triacylglycerol.

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hydrolysis–re-esterification process to reach the endoplasmic reticulum into which VLDL assembly proceeds.

The aim of this present work was to investigate, in preruminant calves, the effects of CO compared with BT added to the milk diet on hepatic FA metabolism (oxidation, esterification and secretion of lipids as part of VLDL), by using the *in vitro* method of liver slices in incubation. For this purpose, we have studied: (1) the oxidative capacities for FA; (2) the subcellular distribution of newly synthesized TAG between cytosol (storage pool) and microsomes (secretory pool); and (3) the secretion rate of VLDL particles in liver slices taken from calves fed a conventional milk diet containing either CO or BT, and incubated in a medium with lauric acid (the most abundant FA in CO) or oleic acid (the most abundant FA in BT).

Materials and methods

Chemicals and materials

CO and BT milk replacers were supplied by Bridel Retiers Society (Bourg Barré, France). The medium used for liver slice incubations (RPMI-1640), bovine serum albumin free of FA, oleic acid, lauric acid and antibiotic–antimycotic cocktail were purchased from Sigma Chemical Corp. (St Louis, MO, USA). [1-^{14}C]oleic acid (3·7 MBq/ml), [1-^{14}C]lauric acid (1·85 MBq/ml), [1-^{14}C]palmitic acid (7·4 MBq/ml), tri[9, 10 (n)-^{3}H]olein (185 MBq/ml) and L-3-phosphatidyl[N-*methyl*-^{3}H]choline 1,2-dipalmitoyl (37 MBq/ml) were purchased from Amersham International (Amersham, Bucks., UK). Hyamine hydroxide was from ICN Biochemicals (Irvine, CA, USA). Perchloric acid and organic solvents (chloroform, methanol, propanol, diethyl ether, acetic acid) were from Prolabo (Paris, France). Ready Safe scintillation cocktail was from Beckman Instruments Inc. (Fullerton, CA, USA). Plastic organ culture Petri dishes were from Beckton Dickinson (Cockeysville, MD, USA). Plastic centre wells were from Kontes Inc. (Vineland, NJ, USA) and amino-propyl-activated silica Sep-Pak® cartridges were from Waters Corp. (Milford, MA, USA).

Table 1. Composition of the experimental milk diets (% of DM)

Ingredients	Beef-tallow diet	Coconut-oil diet
Spray-dried skimmed milk powder	71·6	71·6
Beef tallow	22·4	–
Coconut oil	–	22·4
Corn starch	5·0	5·0
Vitamin and mineral mixture*	1·0	1·0
Fatty acid composition (g/100 g total fatty acids)		
8 : 0	0·1	0·6
10 : 0	0·1	4·0
12 : 0	2·9	42·4
14 : 0	4·2	17·9
16 : 0	22·3	12·8
18 : 0	19·2	5·0
Saturated fatty acids	48·8	82·7
16 : 1*n*-7	2·4	0·5
18 : 1*n*-9	37·8	12·1
Monounsaturated fatty acids	40·2	12·6
18 : 2*n*-6	2·4	3·0
18 : 3*n*-3	0·4	0·3
Polyunsaturated fatty acids	2·8	3·3
Others	8·2	1·4
Saturated : unsaturated ratio	1·13	5·20

* The vitamin and mineral mixture contained (per kg mixture): $MgSO_4$ 0·15 g, MgO 0·30 g, $FeSO_4$ 8 mg, $CuSO_4$ 10 mg, $ZnSO_4$ 80 mg, $MnSO_4$ 48 mg, $CoSO_4$ 0·6 mg, CaI_2 0·18 mg, Na_2SeO_3 0·15 mg, retinol 8·6 mg, cholecalciferol 125 μg, α-tocopherol 50 mg, thiamine 5 mg, riboflavin 10 mg, panthothenic acid 25 mg, niacin 40 mg, cyanocobalamin 0·08 mg, pyridoxine 4 mg, menadione 2·5 mg, ascorbic acid 100 mg, biotin 0·1 mg, folic acid 1·2 mg, methionine 0·6 g, lysine chloride 0·8 g, choline 0·8 g, virginiamycin 0·05 g, sorbitol 3 g (Celtic Langlois, St. Jacques de la Lande, France).

Calves and diets

Ten preruminant Holstein × Friesian male calves (15 d old; 50·7 (SE 2·2) kg) were divided into two groups matched in age and body weight. Each group received, for 19 d, a conventional milk diet containing 224 g lipids/kg DM, given either in the form of BT or CO. The chemical composition of the milk diets and their FA content were given in Table 1. Calves were adapted to the new living conditions and to the milk replacers for 1 week according to recommendations given by Toullec (1978) and then were given food corresponding to an average daily weight gain of 0·65 kg/d for the two milk diets. The experimental period began just after the adaptive period and lasted 19 d during which time calves were given the BT or the CO diets in amounts corresponding to an average daily weight gain of 1·1 kg/d (Toullec, 1978). Calves were fed four times (at 22.00, 01.00, 04.00 and 07.00 hours) on the night before the experiment to ensure a constant post-absorptive state (Durand & Bauchart, 1986).

Liver slice preparation and incubations

Liver samples were metabolically labelled using a method developed from previously described intestinal organoculture (Bouma *et al.* 1990) adapted to the calf liver by Graulet *et al.* (1998). For each of the ten calves of the experiment, the metabolic labelling was carried out in kinetics on liver slices incubated for either 3, 6, 9 or 12 h. At the end of the dietary experimental period, liver tissue samples were taken up by surgical biopsies under general anaesthesia (2 % isoflurane at 0·5 litre/min). Biopsies were quickly rinsed in an ice-cold saline solution (0·4 g KCl/l, 6 g NaCl/l, 0·8 g $NaHPO_4$/l pH 7·4) containing D-glucose (2 g/l), trimmed of blood and connective tissue, and cut into slices 0·5 mm thick. Approximately 200 mg fresh liver (i.e. 4–5 slices) were placed on stainless-steel grids positioned either on a plastic organ culture Petri dish (Beckton Dickinson) or in a 25 ml flask equipped with suspended plastic centre wells (Kontes Inc.; for CO_2 measurements) in the presence of RPMI-1640 medium free of FA (Sigma Chemical Corp.; 0·9 ml in dishes and 1·4 ml in flasks). The samples were placed in an incubator for 2 h at 37°C under a water-saturated O_2–CO_2 (95 : 5, v/v) atmosphere in order to deplete hepatocytes of intracellular FA and to create a requirement of substrate in the cells. Medium was then replaced by fresh RPMI-1640 media (Sigma Chemical Corp.; 0·9 ml in dishes and 1·4 ml in flasks) supplemented with an antibiotic–antimycotic cocktail (Sigma Chemical Corp.; 100 U penicillin/ml, 0·1 mg streptomycin/ml and

0·25 μg amphotericin B/ml) and either 0·8 mM-[^{14}C]oleate or 0·8 mM-[^{14}C]laurate (Amersham International; both 148 MBq/mmol) complexed to bovine serum albumin (FA : albumin molar ratio 4 : 1). The FA concentration (0·8 mM) used in this experiment was in the range of the physiological plasma non-esterified FA concentrations in the preruminant calf (Leplaix-Charlat *et al*. 1996*a*). Moreover, previous experiments have demonstrated that the response of the hepatocytes was globally proportional to the increase in the concentration of FA in the incubation medium between 0·1 and 0·8 mM and led to higher statistical significance at 0·8 mM than at the other concentrations tested (Graulet *et al*. 1998).

Preliminary kinetic measurements after 3, 6, 9, 12 and 24 h of incubation demonstrated significant and reproducible VLDL production by liver slices from both calf groups at 12 h incubation. Moreover, appearance of CO_2 and acid-soluble products (ASP), FA esterification into lipids, and VLDL production increased linearly with the time of incubation between 3 and 12 h for both FA tested and calf group. Therefore, liver-slice incubations for each of the five calves of each dietary group, corresponding to a dish and a flask for each FA tested were performed for kinetic assays for 12 h. The incubations were stopped after 3, 6, 9 or 12 h by harvesting the medium. Then, the liver slices were washed in 2 ml saline solution which was pooled with the incubation medium. Finally, the liver slices were homogenized in 2 ml 25 mM-Tris-HCl (pH 8·0), 50 mM-NaCl buffer with a Dounce homogenizer (Polylabo, Strasbourg, France).

Determination of fatty acid oxidation

CO_2 excreted by the liver slices in the atmosphere was complexed with hyamine hydroxide (150 μl) introduced into the suspended plastic centre wells (Kontes Inc.) inside flasks at the beginning of the incubation and further 150 μl aliquots were successively added at 3, 6 and 9 h of incubation. At the end of the incubation, the centre wells were placed into scintillation vials containing 4 ml Ready Safe scintillation cocktail (Beckman Instruments Inc.) and the radioactivity was counted (Graulet *et al*. 1998). Ketone bodies were purified as ASP from aliquots of cell homogenates (250 μl) and media (500 μl) by an ice-cold perchloric acid treatment (0·2 mM final concentration) as previously described (Graulet *et al*. 1998).

Determination of ^{14}C-labelled fatty acid incorporation into microsomal and cytosolic lipids

Microsomal and cytosolic fractions of hepatocytes were purified from whole homogenates according to a method based on multiple steps of centrifugation to successively eliminate large debris, nuclei, mitochondria and other large components of cells as described by Graulet *et al*. (1998). Then, the residual supernatant was ultracentrifuged for 1 h at 100 000 ***g*** and 4°C (Kontron Centrikon T-2060 ultracentrifuge with a TFT 65.13 rotor; Kontron Instruments, Champagne au Mont d'Or, France). The supernatant containing the floating fat (corresponding to the cytosolic fraction) was carefully collected and the microsomal pellet was suspended in 200 μl 1 mM-Tris-HCl (pH 7·4), 50 mM-KCl and 5 mM-$MgCl_2$. The cytosolic and microsomal fractions of hepatocytes were kept at −20°C until subsequent analysis.

Microsomal and cytosolic lipids were extracted according to the method of Folch *et al*. (1957) after the addition of standard non-radioactive liver homogenate (850 μl containing approximately 10 mg lipids) as a lipid carrier and of glycerol [^{3}H]trioleylglycerol (67 Bq) and [^{3}H]phosphatidylcholine (100 Bq) as internal TAG and phospholipid standards (Graulet *et al*. 1998). The lipid classes were separated by solid phase chromatography on aminopropyl-activated silica Sep-Pak® cartridge (Waters Corp.) according to the method of Kaluzny *et al*. (1985). The lipid fractions were collected in scintillation vials, evaporated to dryness under an air stream, and counted for radioactivity. Preliminary experiments were carried out to determine the recovery of FA when [^{14}C]palmitic acid (118 Bq) was added to the homogenates as a lipid carrier. The average yields were 85·4, 84·5 and 81·4 % for TAG, FA, and PL, respectively.

Determination of secreted ^{14}C-labelled VLDL

Medium (3 ml) supplemented with purified calf VLDL (0·3 mg VLDL-TAG per tube) as a carrier, was brought to a density of 1·063 g/l with KBr and overlaid with 9 ml KBr solution (density 1·006 g/l). VLDL were purified by ultracentrifugal flotation at 100 000 ***g*** for 16 h at 15°C in a Kontron Centrikon T-2060 ultracentrifuge with a TST 41–14 rotor (Kontron Instruments). A portion (2 ml) of the supernatant from the top of each tube were recentrifuged in the same conditions except that pure albumin (50 mg/tube) was added to remove traces of [^{14}C]-labelled fatty acids adsorbed onto VLDL particles. Finally, the purified VLDL were collected at the top of the tube (five fractions of 500 μl) and counted for radioactivity in scintillation vials.

Biochemical analysis

The total protein content in liver homogenates was determined according to the colorimetric method of Lowry *et al*. (1951). Determination of the hepatic DNA content was performed according to the method described by Labarca & Paigen (1980).

Statistical analysis

ANOVA of the data was done using the GLM procedure of SAS (Version 6, 1987; Statistical Analysis Systems Inc., Cary, NC, USA). For the liver-slice experiments, the effects tested in the model included dietary FA treatment (BT or CO) presented as diet, calf tested within dietary FA treatment, FA tested (oleate or laurate) in the incubation medium of the liver slices (presented as FA) and the interaction between dietary FA treatment and the FA tested (presented as diet × FA). The diet factor was tested against calves within groups. The residual mean square was used as the error term for other effects. Results are expressed as the mean values of five independent experiments (calves) per group (BT or CO). Comparisons among FA (oleate *v.* laurate) within an animal of a dietary group (BT or CO)

Table 2. Zootechnical variables of calves fed on coconut-oil or beef-tallow diets for 19 d*
(Mean values and standard errors for five calves per group)

	Beef-tallow diet		Coconut-oil diet		
	Mean	SE	Mean	SE	*P* value†
Live-body weight (kg) at:					
15 d old	53·00	3·39	50·60	3·14	0·711
34 d old (slaughter)	68·20	5·46	66·90	1·87	0·831
Liver weight at slaughter (g)	1710	145	1899	81	0·259
Liver weight/body weight (g/kg)	25·2	0·9	28·4	0·7	0·027
Average daily weight gain (g/d)	855	47	905	103	0·756
Average daily ME intake (MJ/d)	25·1	0·5	25·6	0·2	0·340

ME, metabolizable energy.
* For details of diets see Table 1.
† Mean values were tested for a significant effect of the lipid source in the diet (beef tallow or coconut oil) by Student's *t* test.

were analysed using the Student's *t* test for paired data. Comparisons of the mean values between calves of different dietary groups (BT or CO) were made using the Student's *t* test for unpaired data. The effect of dietary lipids on values of calf performances was tested by a Student's *t* test. $P < 0{\cdot}05$ was considered statistically significant for Student's *t* tests.

Results

Animal characteristics at slaughter

Calves were fed the BT and CO milk replacers to ensure a similar average daily weight gain which amounted to 855 (SE 47) and 905 (SE 103) g/day for BT and CO respectively (Table 2). Live-weight gains were 25·1 (SE 0·5) and 25·6 (SE 0·2) MJ metabolizable energy/d for BT and CO calves respectively. Mean live weights at slaughter were not different between the two groups (68·20 (SE 5·46) *v.* 66·90 (SE 1·87) kg for BT and CO calves respectively). However, the liver weight (g/kg body weight) at slaughter was significantly higher with the calves of the CO group (28·4 (SE 0.07)] than of the BT group (25·2 (SE 0.09), $P = 0{\cdot}027$).

In vitro *assay of fatty acid metabolism in liver slices*

Liver-slice incubations were performed for kinetic assays for 12 h. For a better understanding of the physiological significance of the results, only the values obtained after 12 h incubation are presented. The hepatic contents of protein and DNA were not different between dietary groups of calves (data not shown). The statistical analysis of the data was performed both with values expressed as nmol FA/mg protein or as nmol FA/mg DNA and gave the same level of significance. Thus, the data presented were expressed as nmol FA/mg protein rather than nmol FA/g fresh liver to take into account the possible variations in active cell mass.

The difference in the amounts of FA in the medium at the end of the incubation period was estimated after perchloric acid treatment. The results indicated that lauric acid would be more easily removed from the medium than oleic acid (1·3–1·7-fold, $P = 0{\cdot}0124$, data not shown). Then, after 12 h incubation, the difference in the amounts of FA taken up by the liver slices would be close to 10 nmol/mg protein for BT-fed calves and 6 nmol/mg of protein in the liver slices of CO-fed calves (data not shown). The remaining FA in the medium would represent 62·9–76·2 % of the initial amounts added in the RPMI-1640 medium (Sigma Chemical Corp.).

Fatty acid oxidation

The complete oxidation of laurate into CO_2, determined after 12 h of incubation (Fig. 1), was far higher than for oleate ($P = 0{\cdot}0001$), both in liver slices from BT (9·57-fold) and CO calves (20·03-fold). Moreover, liver slices from the CO group had the lowest capacity to oxidize FA completely into CO_2 as indicated by the statistical analysis ($P = 0{\cdot}0490$). Similarly, the partial oxidation of laurate into ASP after 12 h incubation (Fig. 2) was higher than of oleate ($P = 0{\cdot}0001$) both in liver slices from BT (4·30-fold) and CO calves (7·07-fold). Liver slices from the CO group tended to have the lowest capacity of partial oxidation of FA compared

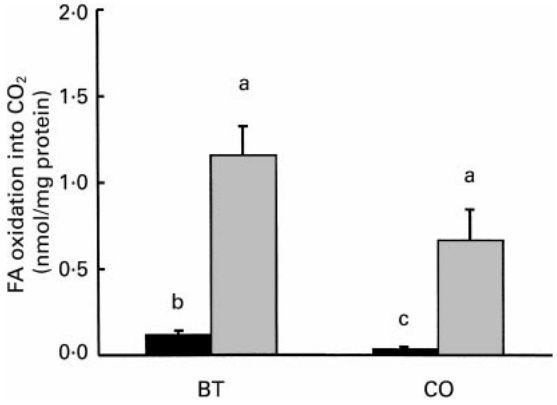

Fig. 1. Carbon dioxide production by liver slices from calves fed beef-tallow (BT) or coconut-oil (CO) diets for 19 d, after 12 h of incubation in the medium containing oleate or laurate. For details of diets see Table 1. Liver slices from calves fed either BT or CO were incubated in medium containing 0·8 mM-[^{14}C]oleate (■) or -[^{14}C]laurate (■) for 12 h. Secreted carbon dioxide was complexed to hyamine hydroxide and the radioactivity was measured in a scintillation counter. Values are corrected for specific activity and expressed per mg of protein. Values are means with standard errors represented by vertical bars for five animals per dietary group. Significant effects of dietary fatty acid (FA) composition and of FA in the incubation medium were observed ($P = 0{\cdot}0490$ and $P = 0{\cdot}0001$ respectively). ^{a,b,c}Mean values with unlike superscript letters were significantly different: $P < 0{\cdot}05$.

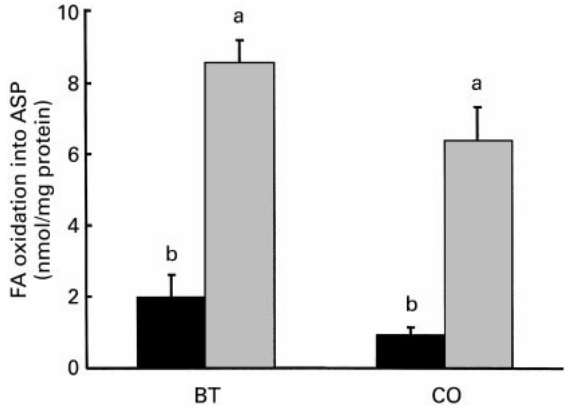

Fig. 2. Acid-soluble products (ASP) production by liver slices of calves fed beef-tallow (BT) or coconut-oil (CO) diets for 19 d after 12 h of incubation in the presence of oleate or laurate in the medium. For details of diets see Table 1. Liver slices from calves fed either BT or CO were incubated in medium containing 0·8 mM-[^{14}C]oleate (■) or -[^{14}C]laurate (■) for 12 h. ASP produced by liver slices for 12 h were purified from cell homogenate and medium by perchloric acid treatment and the radioactivity was measured in a scintillation counter. Values are corrected for specific activity and expressed per mg of protein. Values are means with standard errors represented by vertical bars for five animals per group. Significant effects of dietary fatty acid (FA) composition and of FA in the incubation medium were observed ($P=0·0625$ and $P=0·0001$ respectively). [a,b] Mean values with unlike superscript letters were significantly different: $P<0·05$.

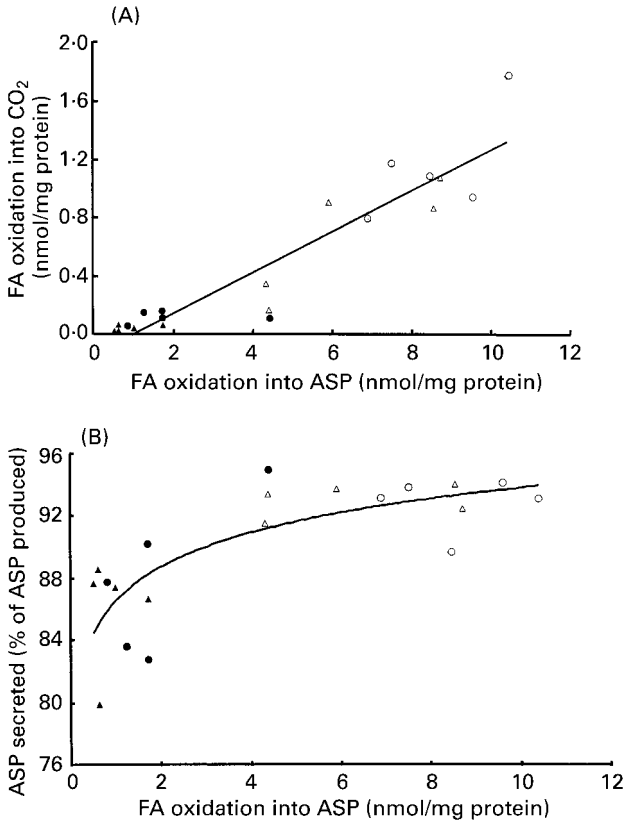

Fig. 3. Relationships (A) between total (carbon dioxide) and partial (acid-soluble products; ASP) fatty acid (FA) oxidation and (B) between ASP secretion rate and ASP production by liver slices. Liver slices from calves fed either beef tallow (BT) (●, ●) or coconut oil (CO) (▲, ▲) for 19 d, were incubated in the presence of 0·8 mM-[^{14}C]oleate (●, ▲) or -[^{14}C]laurate (●, ▲) for 12 h. For details of diets see Table 1. Carbon dioxide secreted into the atmosphere was complexed to hyamine hydroxide and ASP produced by liver slices were purified from cell homogenates and media by perchloric acid treatment. After counting for scintillation, values were corrected for specific activity and expressed per mg of protein. The ASP secretion rate corresponded to the amount of ASP secreted into the medium : the amount of total ASP produced. Analyses of correlation were made between the values of carbon dioxide and ASP production (n 20, r 0·964, $P<0·01$) and between the values of ASP production and ASP secretion (n 20, r 0·773, $P<0·01$).

with the BT group as indicated by the statistical analysis ($P=0·0625$). ASP and CO_2 production rates were highly correlated (r 0·964, $P=0·01$) whatever the nature of the FA tested and the group of calves (Fig. 3(A)).

Secretion rate of ASP, calculated as the amount of ASP secreted into the medium : the amount of total ASP produced by hepatocytes, was not affected by the nature of dietary lipids (Fig. 4). However, ASP secretion rates were slightly but significantly higher (1·1-fold, $P=$ 0·0021) when the liver slices were incubated with laurate (92·7 and 93·0 % for BT and CO respectively) than with oleate (87·9 and 86·0 % for BT and CO respectively). Moreover, the relationship between the amounts of ASP secreted in the medium and the amounts of total ASP produced by the liver slices gave a logarithmic shape with a significant correlation between these two variables (r 0·773, $P=0·01$) (Fig. 3(B)).

Incorporation of fatty acids into lipids

FA incorporation into the lipids of the two subcellular precursor pools of VLDL particles (cytosol and microsomes which represented a fraction of the total cellular lipids) was studied in liver slices incubated with [^{14}C]laurate or [^{14}C]oleate. There was no significant difference in lipid synthesis according to the nature of the FA tested in both groups of calves (Table 3). By contrast, amounts of total lipids (corresponding to neutral lipids (NL) and phospholipids) produced after 12 h incubation were 2·6-fold higher in liver slices of CO fed calves than in BT calves whatever the FA marker ($P=0·0106$).

The FA incorporation into phospholipids corresponded to a minor pathway for FA esterification in our experimental conditions since it accounted for less than 5 % of total FA incorporation into lipids (Table 3). Esterification of FA into phospholipids was not modified by the nature of the FA tested and the dietary lipids. Conversely, FA were mainly esterified into NL (mainly TAG) in the slices since they represented 82·9 to 97·2 % of the total newly-synthesized lipids (Table 3). Mean rates of incorporation of FA into NL were similar in each group of calves when slices were incubated either in a medium containing oleate or laurate. However, esterification of FA into NL was much lower in the liver of BT calves (0·255–0·287 nmol FA incorporated into NL/mg protein), than that of CO calves (0·661–0·888 nmol FA incorporated into NL/mg protein) indicating a possible stimulating effect of saturated FA (abundant in the CO diet) in this process ($P=0·0088$).

The analysis of the subcellular distribution of the newly-synthetized NL between the cytosolic droplets and the

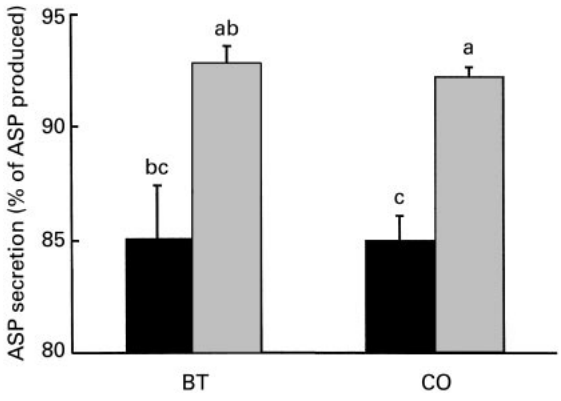

Fig. 4. Acid-soluble products (ASP) secretion rate by liver slices of calves fed beef-tallow (BT) or coconut-oil (CO) diets after 12 h of incubation in the presence of oleate or laurate in the medium. For details of diets see Table 1. Liver slices from calves fed either BT or CO for 19 d were incubated in the presence of 0·8 mM-[^{14}C]oleate (■) or -[^{14}C]laurate (■) for 12 h. ASP produced by liver slices were purified from cell homogenates and media by perchloric acid treatment and counted for scintillation. Values were corrected for specific activity and expressed per mg of protein. The ASP secretion rate corresponded to the amount of ASP secreted into the medium : amount of total ASP produced. Values are means with standard errors represented by vertical bars for five animals per group. Significant effects of dietary fatty acid composition and of fatty acid in the incubation medium were observed ($P = 0·6067$ and $P = 0·0021$, respectively). [a,b,c] Mean values with unlike superscript letters were significantly different: $P < 0·05$.

Table 4. Incorporation of fatty acids into neutral lipids (nmol/mg protein) of the cytosolic and the microsomal fractions in liver slices from calves fed beef-tallow or coconut-oil diets for 19 d, after 12 h incubation in medium containing oleate or laurate*

(Mean values for five calves per diet group)

		Neutral lipids	
Dietary group†	FA tested	Cytosol	Microsomes
BT-fed calves	Oleate	0·236[b]	0·051[ab]
	Laurate	0·218[b]	0·037[ab]
CO-fed calves	Oleate	0·822[a]	0·066[a]
	Laurate	0·628[a]	0·033[b]
SED‡			
Between animals		0·095	0·019
Within animals		0·071	0·011
Statistical effect of (P=):			
Diet		0·0060	0·8425
FA		0·3259	0·1600
Diet × FA		0·4104	0·5576

FA, fatty acid; BT, beef tallow; CO, coconut oil.
[a,b] Mean values within a column with unlike superscript letters were significantly different: $P < 0·05$ (Student's t test).
* Liver slices from calves fed either BT or CO were incubated in medium containing either 0·8 mM-[^{14}C]oleate or -[^{14}C]laurate for 12 h. Lipids of the precursor pools of VLDL were purified after subcellular fractionation by an extraction with chloroform–methanol (2 : 1, v/v) and separation of the different classes of lipids on cartridge of silica activated with aminopropyl. [^{14}C]-labelled NL were measured in a scintillation counter. Values are corrected for specific activity and expressed per mg of protein.
† For details of diets see Table 1.
‡ SED = $\sqrt{\textit{mean square/total number of observations}}$.

microsomal compartment indicated that cytosol was the major site for NL in calf hepatocytes since it contained close to 90 % of the newly-synthetized NL (Table 4), whatever the nature of the FA tested (oleate or laurate) or the group of calves. However, dietary FA influenced significantly the rate of FA incorporation into the cytosolic NL ($P = 0·006$) since it was 3·5-fold and 2·9-fold higher for oleate and laurate respectively, in the liver slices of CO than those of BT calves. The rate of incorporation of FA into microsomal NL was almost constant in all the conditions studied (diet or FA tested) and corresponded to only 5–17 % of the total NL.

FA secretion as part of VLDL lipids was 2·2–4·1-fold higher ($P = 0·0472$) in liver slices incubated with oleate than with laurate for BT and CO calves respectively (Table 5).

Table 3. Incorporation of fatty acids into total lipids (microsomes plus cytosol) and their main constituents (neutral lipids and polar lipids) (nmol/mg protein) in liver slices from calves fed beef-tallow or coconut-oil diets for 19 d, after 12 h incubation in medium containing oleate or laurate*

(Mean values for five calves per diet group)

Dietary group†	FA tested	Total lipids	Phospholipids	Neutral lipids
BT-fed calves	Oleate	0·346[b]	0·059	0·287[b]
	Laurate	0·267[b]	0·012	0·255[b]
CO-fed calves	Oleate	0·914[a]	0·027	0·888[a]
	Laurate	0·692[a]	0·031	0·661[a]
SED‡				
Between animals		0·105	0·011	0·103
Within animals		0·074	0·009	0·070
Statistical effect of (P=):				
Diet		0·0106	0·6762	0·0088
FA		0·1909	0·1256	0·2287
Diet × FA		0·5125	0·0704	0·3538

FA, fatty acid; BT, beef tallow; CO, coconut oil.
[a,b] Mean values within a column with unlike superscript letters were significantly different: $P < 0·05$ (Student's t test).
* Liver slices from calves fed either BT or CO were incubated in medium containing either 0·8 mM-[^{14}C]oleate or -[^{14}C]laurate for 12 h. Lipids of the precursor pools of VLDL were purified after subcellular fractionation by an extraction with chloroform–methanol (2 : 1, v/v) and separation of the different classes of lipids on cartridge of silica activated with aminopropyl. ^{14}C-labelled lipids were measured in a scintillation counter. Values are corrected for specific activity and expressed per mg of protein.
† For details of diets see Table 1.
‡ SED = $\sqrt{\textit{mean square/total number of observations}}$.

Table 5. Fatty acid incorporation into secreted VLDL particles and rates of secretion of newly synthesized lipids by liver slices from calves fed beef-tallow or coconut-oil diets for 19 d, after 12 h incubation in medium containing oleate or laurate*

(Mean values for five calves per dietary group)

Dietary group†	FA tested	VLDL production (nmol fatty acid /mg of protein)	Lipid secretion rate (% lipids in cytosol and microsomes)
BT-fed calves	Oleate	0·00487[a]	2·83
	Laurate	0·00224[ab]	1·19
CO-fed calves	Oleate	0·00423[ab]	0·66
	Laurate	0·00102[b]	0·18
SED‡			
Between animals		0·00084	0·699
Within animals		0·00088	0·665
Statistical effect of (P=):			
Diet		0·4572	0·1456
FA		0·0472	0·2946
Diet × FA		0·8206	0·5564

FA, fatty acid; BT, beef tallow; CO, coconut oil.

[a,b] Mean values within a column with unlike superscript letters were significantly different: $P < 0·05$ (Student's t test).

* Liver slices from calves fed either BT or CO were incubated in medium containing either 0·8 mM-[^{14}C]oleate or -[^{14}C]laurate for 12 h. VLDL were purified by ultracentrifugal flotation and the radioactivity was measured in a scintillation counter.

† For details of diets see Table 1.

‡ SED = $\sqrt{\textit{mean square/total number of observations}}$.

However, no significant effect was observed inside each group of calves (BT or CO). Furthermore, the production of VLDL by liver slices would not be affected by the FA composition of the diet since the secretion rate of lipids, calculated as VLDL production : lipid amounts in the precursor pools, was not significantly affected by the diet and the FA tested (Table 5). Mean rates of VLDL secretion (as a percentage of the newly-synthesized lipids secreted as VLDL/mg protein) ranged from 2·83 to 0·18 after 12 h incubation.

Discussion

Zootechnic performances of calves

Although the apparent coefficient of digestibility of the lipids was higher with the CO (96·4 %) than the BT diet (92·8 %) (Jenkins *et al*. 1985), the average daily weight gains obtained in our experiment over 19 d of dietary treatment, did not differ between calves of the BT and CO groups. This was in agreement with previous results described by Jenkins *et al*. (1985) in newborn calves (3 d old) with a similar feeding trial for 28 d. In our experimental conditions, the weight of the liver (expressed per kg body weight) which was significantly higher for the calves fed the CO than for those receiving the BT diet, probably reflected the accumulation of TAG in the liver of CO calves as described previously by Jenkins & Kramer (1986). Moreover, the TAG content in the liver of the calves used in the present experiment has been previously published by Bauchart *et al*. (1998) who reported mean values at the level of a moderate steatosis for the calf of the CO group (40·1 *v*. 3·3 mg/g fresh tissue for the calves of the BT group). Such a TAG infiltration in hepatocytes had been already observed in our laboratory in calves fed a milk-based diet into which lipids were provided as soyabean oil, rich in polyunsaturated FA (Leplaix-Charlat *et al*. 1996*b*). In this latter case, the steatosis was associated with a reduction in the food intake and led to health problems and to a reduction of growth performances (Leplaix-Charlat, personal communication). We could expect that the steatosis induced by CO-feeding over a long period of time would have the same effect on calves. The metabolic origin of the steatosis in the CO-fed calves is still undetermined and several hypotheses can be proposed. Among them, a differential repartition of FA between oxidation and esterification pathways in the hepatocytes, and the availability of the NL (mainly TAG) in the secretory compartment (endoplasmic reticulum) for VLDL assembly and secretion were studied.

Fatty acid metabolism in calf-liver slices

Previous experiments have shown that ASP were mainly composed of ketone bodies and acetate, and in a smaller proportion, of intermediate products of the Krebs cycle (Jesse *et al*. 1986). This was also probably the case in our experimental conditions since 85 to 95 % of ASP produced by the liver slices were secreted in the medium. In the present experiment, products of FA oxidation by liver slices from preruminant calves were composed mainly of ASP which represented 88 to 96 % of the total oxidation products, the other part being composed of CO_2, in agreement with a previous report in the rat and in calf liver (Graulet *et al*. 1998). Additionally, it could be noted that FA oxidation to CO_2 increased linearly with the oxidation to ASP.

The FA incorporation into the lipids purified in cytosol and microsomes (which are the main sources of TAG secreted into VLDL but represented only a fraction of the total lipids of the cell) purified from calf-liver slices showed that NL are the major product of FA esterification (83 to 97 % of total lipids). They were principally composed of

TAG since previous experiments have shown that TAG represented 72 to 90 % of the NL in the liver of BT calves (Leplaix-Charlat *et al.* 1996*b*) and 87 % in the liver of CO calves (Jenkins & Kramer, 1986). In the former study, the authors had shown that high amounts of cholesteryl esters could compete with TAG for VLDL secretion in the liver of preruminant calves fed soyabean oil. However, cholesteryl esters were probably not limiting for VLDL synthesis and secretion in the hepatocytes of CO calves in our experimental conditions since: (1) cholesterol (free and esterified) content in the liver of CO-fed calves was not different from that in BT-fed calves (Bauchart *et al.* 1998); and (2) CO-feeding did not modify the cholesterol secretion as part of VLDL-lipids by the liver of CO-fed calves (Durand *et al.* 1998).

Effects of coconut-oil diet

Several studies have demonstrated that the dietary lipids, when provided as fish oil rich in polyunsaturated FA, were able to orientate the FA metabolism towards the oxidation pathway through a coordinated action on key enzymes and proteins of hepatic FA metabolism (Moir *et al.* 1995; Ikeda *et al.* 1998). In the present experiment, intensive CO feeding (average weight gain 905 g/d) to calves over a 19 d period reduced FA oxidation in the liver slices. We might hypothesize that CO feeding decreased FA oxidation in the liver of the preruminant calf as it did in the rat (Mohan *et al.* 1991). Indeed, in the rat, CO feeding inhibited the activity of some key enzymes of the FA oxidation pathways such as carnitine palmitoyltransferase I which is the main limiting step of FA oxidation (Guzman & Geelen, 1993), acyl-CoA dehydrogenase and acyl-CoA oxidase (Power *et al.* 1994; Kabir & Ide, 1995).

In the present study, CO feeding to calves induced an increase in TAG synthesis concomitant to the decrease in FA oxidation, in agreement with data obtained in the rat (Gaiva Gomez da Silva *et al.* 1996). However, in the preruminant calves, the increase of TAG synthesis in the hepatocytes of CO calves led to their storage in cytosolic droplets since it was not associated with an increase of VLDL-TAG production. By contrast, no alteration of the TAG content was observed in the hepatocytes of rats fed a CO diet for 5–6 weeks compared with rats fed a BT diet (Lai *et al.* 1991; Ney *et al.* 1991). Moreover, plasma VLDL-TAG concentrations were higher in the BT than in the CO fed rats, mainly due to a higher secretion rate of VLDL particles by the liver (Nicolosi *et al.* 1976; Lai *et al.* 1991; Gaiva Gomez da Silva *et al.* 1996). Thus, the lack of difference in the VLDL-TAG secretion rate between the two calf groups could partially explain the lipid infiltration that we observed specifically in the liver of the preruminant calves fed the CO diet (Jenkins & Kramer, 1986; Bauchart *et al.* 1998). It could be explained by: (1) the chronic low secretion of VLDL by the bovine liver which has been previously described (Pullen *et al.* 1990; Graulet *et al.* 1998; Gruffat-Mouty *et al.* 1999); (2) the lack of an efficient regulation of VLDL-TAG secretion according to the hepatic TAG content by the bovine liver. This has already been described in the liver of the high-producing dairy cow where the increase in the uptake of the circulating FA resulting from the mobilization of the body stores favours FA esterification and TAG storage since VLDL-TAG secretion rates are unchanged (Grummer, 1993).

The numerous factors acting on the TAG secretion as part of VLDL can be pooled into two main groups. First, the TAG transfer from the cytosolic droplets to the lumen of the endoplasmic reticulum could be poorly efficient due to the low activity of the intracellular TAG hydrolase (Lehner *et al.* 1999), the microsomal carnitine palmitoyltransferase or the intramicrosomal diacylglycerol acyltransferase (Owen *et al.* 1997). Other factors implied in the VLDL assembly process such as the microsomal TAG transfer protein and apolipoprotein B_{100} could then be limiting (Gruffat *et al.* 1996). All of these factors could be involved in the metabolic disorders which are the cause of the steatosis developed by the liver of the preruminant calves fed a CO diet. The mechanism of the regulation by the dietary FA of TAG partitioning between the cytosolic and the microsomal pools is still unknown, especially in the bovine species. By contrast, it has been shown, in the liver of the hamster that microsomal TAG transfer protein expression was increased by diets enriched in myristic and palmitic acids compared with oleic acid (Bennett *et al.* 1995). Such a regulation by dietary FA would not occur in the liver of calves fed the CO diet or microsomal TAG transfer protein activity would not be limiting for TAG export since the VLDL-TAG secretion was similar in CO and in BT-fed calves leading to a lipid infiltration in the hepatocytes.

Finally, apolipoprotein B availability in the endoplasmic reticulum could also be limiting for VLDL secretion in the bovine liver. However, we have recently shown that the rate of apolipoprotein B synthesis was similar in liver slices of rats and calves although the rate of VLDL secretion was higher in rats than in calves (Gruffat-Mouty *et al.* 1999). Nevertheless, we can speculate that hepatic apolipoprotein B synthesis and/or its intracellular degradation might be modified by the composition of the FA in the CO diet similarly to that with fish-oil based diet (Brown *et al.* 1997), thus limiting TAG secretion as part of VLDL particles.

Compared metabolism of lauric and oleic acids

To compare the FA metabolism in the liver of preruminant calves, we decided to use the *in vitro* approach of metabolic labelling on liver slices. To take into account the possible metabolic particularities of a given FA, we decided to follow the metabolic fate of the main representative FA (oleic and lauric acids) of each of the experimental diets (BT and CO respectively). The results seemed to indicate that lauric acid would be more easily taken up from the medium and greater amounts would be metabolized by the liver slices. Indeed, the amount of lauric acid oxidized into CO_2 and ASP by the liver slices from calves was far higher than the amount of oleic acid, as has been observed previously in cultured hepatocytes of the rat (Christensen *et al.* 1989). The better oxidation of lauric acid in comparison with oleic acid could favour and then essentially explain the higher values in FA uptake. Thus, the amounts of lauric acid esterified by the liver slices would not be different from those of oleic acid. The lower capacity of VLDL secretion in the liver

slices incubated with lauric rather than with oleic acid also confirmed previous data obtained on the perfused liver of rats indicating that the VLDL-TAG secretion was twice as high with unsaturated FA (such as oleate) than with saturated FA (such as palmitate and myristate) (Dave & Mayes, 1979).

As has also been observed in the rat (Christensen *et al*. 1989; Mohan *et al*. 1991), we have shown that, by contrast with the CO diet which reduced the oxidative capacity both in the calf and in the rat hepatocytes, lauric acid was highly oxidized compared with oleic acid. This is surprising since lauric acid is the main FA (40 g/100 g total FA) in CO. Although smaller differences exist for the other main saturated (myristic, palmitic and stearic acid) and unsaturated FA (oleic acid) in the composition of BT and CO diets, it is likely that the high content of lauric acid in the CO diet and its metabolic fate in hepatocytes are the main cause of variations in the metabolism of the TAG, although myristic and palmitic acids could have some synergic effects with lauric acid, the stearic acid being considered as metabolically neutral. Indeed, based on our results of liver slice incubation in a medium containing lauric acid, the CO diet would correspond to high uptake and oxidation of lauric acid by the hepatocytes. However, during the experimental period, some metabolic adaptations would occur in the hepatocytes leading to the decrease in the oxidative capacities of FA and to the stimulation of the esterification pathway that we observed after 19 d CO feeding. It would be interesting to know what factor was responsible for the partial inhibition of the oxidation pathway and whether it preceded or followed the fatty-liver development in the CO calves.

In conclusion, we demonstrated that CO feeding to calves induced a modification of the FA partition between oxidation and esterification in favour of TAG synthesis. The secretion of newly-synthesized lipids as part of VLDL was not modified by the chemical composition of the dietary FA in spite of increased hepatic TAG content. A chronic low availability of TAG and/or apolipoprotein B at the site of VLDL packaging probably partly explains the TAG infiltration of the liver in calves fed milk diet containing CO.

Acknowledgements

The authors wish to gratefully acknowledge Dr M. E. Samson-Bouma (INSERM U.327, Faculté de Médecine Xavier Bichat, Paris) for teaching us the method of metabolic labelling developed for liver organ culture, F. Duboisset for technical assistance, and R. Souchet and C. Leoty for maintenance and care of the calves.

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