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Effect of Dietary Conjugated Linoleic Acid and Monounsaturated Fatty Acid Content on Pig Muscle and Adipose Tissue Lipase and Esterase Activity

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Three levels (0%, 1%, and 2%) of conjugated linoleic acid (CLA) were combined with two levels (low and high) of monounsaturated fatty acids (MUFA) for pig feeding. The activity of neutral lipase (NL), acid lipase (AL), phospholipase (PL), neutral esterase (NE), and acid esterase (AE) was measured in extracts from muscle and subcutaneous adipose tissues. The addition of CLA in the diet only affected the lipolytic activity in muscle, whereas differences in MUFA content of pig diets were mainly responsible for the lipolytic enzyme modifications observed in adipose tissue. Nevertheless, a significant effect of the interaction CLA × MUFA on the activity of several lipolytic enzymes was observed in both tissues. The effect of either linoleic acid (LA) or CLA on the activity of muscle and adipose lipolytic enzymes was determined by in vitro assays. Remarkable inhibitory or activation effects were detected depending on the enzyme and kind of tissue.

KEYWORDS: Conjugated linoleic acid (CLA); fatty acids; pig; lipolytic enzymes; muscle; adipose tissue

INTRODUCTION

It has been reported that conjugated linoleic acid (CLA) isomers show a limiting effect on fat accumulation in animal organisms (*1*, *2*). However, it has not been completely elucidated to what extent this effect is due to a decrease in the synthesis of fatty acids, to increased fatty acid oxidation, to a lower growth rate of adipocytes, or to a higher lipolytic activity (*3*, *4*). Regarding the latter hypothesis, different effects of CLA on lipid metabolism have been observed. Park et al. (*5*) found a reduced lipoprotein lipase activity in skeletal muscle and fat pad of mice fed with a CLA-supplemented diet. Pariza et al. (*6*) reported that feeding of CLA enhanced hormone sensitive lipase and carnitine palmitoyltransferase activity in mice. In preadipocytes 3T3-L1, CLA increased the basal lipolysis (*2*). Nevertheless, other authors (*7*) have not observed a clear action of CLA isomers on the lipolytic activity of mice muscle.

In the case of pig production, supplementation of swine feeding with CLA has gained an increasing interest in past decades since it has been suggested as an attractive approach for improving productive traits, carcass, and meat quality (*8*). Lipases play an important role in meat and meat products quality, being responsible for the hydrolysis of lipids, release of free fatty acids, and contribution to flavor development and lipid oxidation (*9*). To date, the effect of CLA feeding of pigs on the different muscle and adipose tissue lipolytic activities and its subsequent effect on meat and meat products quality have not been reported.

On the other hand, the remaining fatty acid profile after pig supplementation with CLA has not been thoroughly examined, despite that interactions with other fatty acids might exist. Thus, multifactorial experiments considering both CLA and other fatty acids contents are needed, because they would better reflect the potential gains under commercial conditions.

Therefore, the aim of this work was to study the effect of CLA and MUFA content of pig diets on the activity of the main lipases and esterases from early postmortem muscle and subcutaneous adipose tissues and the effect of direct addition of CLA on the in vitro activity of the same enzymes in extracts of muscle and subcutaneous adipose tissues.

MATERIALS AND METHODS

Animal Feeding. Three levels (0%, 1%, and 2%) of commercial enriched CLA oil supplementation (CLA-60, BASF, Dortmund), containing approximately 56% of CLA isomers consisting of 28% *cis*-9,*trans*-11 and 28% *trans*-10,*cis*-12, and two levels of MUFA (high and low, see **Table 1**) were combined for pig feedings. 288 female large white ♂ × landrace × large white ♀ pigs were randomly allotted to the six different feeding treatments. Animals weighing 70 kg and at about 140 days of age were blocked in three replicates of 96 pigs. Pigs were housed in an environmentally controlled experimental grower/finisher shed. Pigs were group-housed (12 pigs per pen) and had ad libitum access to feed (single space dry feeders) and water (nipple drinkers). All diets for pigs were formulated to provide similar protein and energy levels (**Table 1**), fulfilling the advised nutritional needs for female pigs at considered ages by the National Research Council.

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Table 1. Ingredients and Chemical Composition of the Experimental Treatments

	low MUFA feed			high MUFA feed		
ingredients (%)	0% CLA	1% CLA	2% CLA	0% CLA	1% CLA	2% CLA
barley	53.26	53.26	53.26	53.26	53.26	53.26
wheat	15.00	15.00	15.00	15.00	15.00	15.00
bran	8.00	8.00	8.00	8.00	8.00	8.00
soybean meal 44%	16.00	16.00	16.00	16.00	16.00	16.00
palm oil	1.60	1.10	0.60	1.00	0.50	0.00
soy olein	0.40	0.40	0.40	0.00	0.00	0.00
olive olein	0.00	0.00	0.00	3.00	3.00	3.00
hydrogenated stearin palm	3.00	2.50	2.00	1.00	0.50	0.00
CLA	0.00	1.00	2.00	0.00	1.00	2.00
carbonate	1.20	1.20	1.20	1.20	1.20	1.20
phosphate	0.40	0.40	0.40	0.40	0.40	0.40
salt	0.40	0.40	0.40	0.40	0.40	0.40
L-lysine 50	0.17	0.17	0.17	0.17	0.17	0.17
L -threonine	0.03	0.03	0.03	0.03	0.03	0.03
coline 75	0.04	0.04	0.04	0.04	0.04	0.04
vitamin and mineral premix	0.50	0.50	0.50	0.50	0.50	0.50
	Chemical Composition (%)					
dry matter	89.23	89.59	89.42	89.34	89.48	89.60
ash	4.93	5.09	4.95	5.10	5.63	5.32
crude fiber	4.23	4.26	4.07	4.68	4.26	4.63
crude fat	7.65	6.88	7.30	7.20	7.06	6.67
crude protein	16.41	15.96	15.84	16.66	16.45	15.81
nitrogen free extractives	62.77	64.07	64.03	62.35	62.67	63.81
calculated ME (kcal/kg)	3238.75	3240.75	3242.75	3257.75	3259.75	3261.75
	Fatty Acid Composition (%)					
C14:0	0.75	0.63	0.54	0.45	0.31	0.25
C16:0	35.33	30.41	25.61	25.42	19.66	15.04
C16:1 *n*-7	0.13	0.13	0.08	0.54	0.40	0.38
C18:0	22.83	20.13	16.58	11.36	7.60	4.62
C18:1 *n*-9	18.10	17.95	18.66	37.79	37.87	37.83
C18:2 *n*-6	19.87	20.19	19.84	20.60	22.24	22.54
C18:3 *n*-3	1.67	1.68	1.62	1.79	2.12	2.13
cis-9,*trans*-11 CLA	0.00	3.85	7.97	0.00	4.33	7.88
trans-10,*cis*-12 CLA	0.00	3.73	7.85	0.00	4.18	8.12
SFA	59.71	51.99	43.53	38.83	28.38	20.63
MUFA	18.75	18.56	19.19	38.78	38.75	38.71
PUFA	21.54	21.87	21.46	22.39	24.36	24.67

Representative samples of mixed diets were taken after elaboration and before the beginning of the trial. Animals were slaughtered at a local slaughterhouse by electrical stunning and ex-sanguination.

Samples. Eight animals from each treatment were randomly selected for sampling. Samples from slaughtered animals were taken within 10 min after bleeding. The whole *Longissimus dorsi* muscle and a 100 g subcutaneous adipose tissue sample were vacuum packaged and frozen at −80 °C until required.

Proximate composition of diets was analyzed following AOAC procedures (*10*): nitrogen content by the Kjeldahl method (976.05), crude protein (954.01), crude fat (920.39), crude fiber (962.09), and ash (942.05). Feed analysis is shown in **Table 1**. Total lipids from feeds and muscle samples were extracted with chloroform:methanol (*11*). Lipids from subcutaneous fat samples were extracted with diethyl ether and subsequent filtration. Fatty acid methyl esters (FAMEs) from total extracted lipids were obtained by acidic transesterification (*12*) and analyzed by gas chromatography (*13*). Briefly, 20 mg of extracted lipids placed in a glass vial was thoroughly mixed with 1 mL of 5% sulfuric acid in methanol and kept 30 min at 80 °C in an oven. Afterward, FAMEs were extracted with 1 mL of hexane. Hexane was evaporated to dryness under a nitrogen stream, and FAMEs were dissolved in 1 mL of hexane. FAMEs were analyzed by gas chromatography, using a Hewlett-Packard HP-5890-II gas chromatograph, equipped with a flame ionization detector. Separation was carried out on a polyethyleneglycol capillary column (60 m long, 0.32 mm i.d., 0.25 μm film thickness) (Supelcowax-10, Supelco, Bellafonte, PA). Oven temperature programming started at 180 °C. Immediately, it was raised 4 °C/min to 200 °C, held for 35 min at 200 °C, increased again at 5 °C/min to 250 °C, and held for 11 min at 250 °C. Injector and detector temperatures were 250 °C. Carrier gas was nitrogen at a flow rate of 0.8 mL/min. Individual compounds were identified by comparing their retention times with those of standards (Sigma, St. Louis, MO).

Preparation of Enzymatic Extracts for Lipase and Esterase Assays. Muscle and subcutaneous adipose extracts were prepared as previously described by Motilva et al. (*14*). In the case of muscle tissue, 5 g of ground sample was homogenized in 25 mL of 50 mM phosphate buffer, pH 7.5, containing 5 mM of EGTA by using an ultraturrax homogenizer (IKA, Germany) (3 × 10 s each at 24 000 rpm, while cooling in ice). For adipose tissue, 3 g of ground sample was homogenized under the same conditions but in 30 mL of 0.2 M Tris-HCl buffer, pH 8.2, containing 2 mg/mL of sodium deoxycholate, 0.08 mg/mL of Nonidet P-40, 0.05 mg/mL of heparin, 10 mg/mL of bovine serum albumin (BSA), and 25 mM of sucrose. The homogenates were centrifuged at 10 000*g* for 20 min, and the resulting supernatant of the muscle sample, or the aqueous phase in the case of adipose tissue, was filtered through glass wool and collected for further enzyme assays. All operations were carried out at 4 °C.

Assay of Lipase and Esterase Activities from Muscle and Adipose Tissue Extracts. Five enzymatic activities were measured in muscle samples: neutral lipase (NL), acid lipase (AL), acid phospholipase (PL), neutral esterase (NE), and acid esterase (AE). The assays were carried out as previously described by Motilva et al. (*14*) with minor modifications.

The reaction mixture consisted of 50 μL of enzyme extract and 250 μL of reaction medium containing the specific substrate. Lipase and esterase activities were determined using 4-methylumbelliferyl oleate and 4-methylumbelliferyl propionate (Sigma Chemical Co., St. Louis, MO) as substrates, respectively.

Acid activities were assayed in 0.1 M citric acid/0.2 M disodium phosphate buffer, pH 5.0, containing 0.8 mg/mL of BSA, 0.05% of Triton X-100, and 0.2 mM of the respective substrate, with the addition of 150 mM of sodium fluoride when measuring the PL activity. NL was assayed in Tris-HCl 0.22 M, pH 7.5, containing 5 mg/mL of BSA, 0.01 mg/mL of heparin, and 1.2 mM of the specific substrate. Composition of reaction medium for NE was the same as that used for acid activities but at pH 7.5.

In the case of subcutaneous adipose tissue, only the activities of NL, AE, and NE were assayed, because PL and AL are not relevant in lard (*15*, *16*). The composition of reaction medium for NL was the same as that used for muscle samples but at pH 7.0 and 0.8 mM of the specific substrate. AE and NE were assayed in the same buffers used for these enzymes in the case of muscle samples but with a substrate concentration of 0.8 mM for both activities.

All reaction mixtures were incubated at 37 °C and measured at 20 min (lipases) and 10 min (esterases) by a Fluoroskan Ascent fluorimeter (Thermo Electron Co., Finland) with excitation and emission wavelengths of 355 and 460 nm, respectively. In all cases, three measurements were made for each sample. One unit of enzyme activity (U) is defined as the amount of enzyme capable of hydrolyzing 1 μmol of substrate in 1 h at 37 °C.

In Vitro Study of the Effect of CLA on Lipase and Esterase Activities from Muscle and Adipose Tissue Extracts. The effect of CLA on in vitro lipolytic activities from muscle and subcutaneous adipose tissues was evaluated by adding CLA at different concentrations to the reaction mixtures. CLA was previously bound to BSA forming a CLA−BSA complex that allows the solubilization of the fatty acid in the aqueous reaction medium (*17*).

A BSA control free of fatty acid was prepared to distinguish between the effect on enzymatic activities due to the BSA or CLA. In addition to the study of the effect of CLA on in vitro lipolytic activities, the effect of γ-linoleic acid (C18:2 c9, c12) (LA) was assayed.

Preparation of fatty acid−BSA complexes was based on the procedure previously described by Lin et al. (*18*) with some modifications. BSA at 1.5 mM was dissolved in a buffer containing 40 mM of sodium hydrogen carbonate, 100 mM of sodium chloride, 0.9 mM of sodium hydrogen phosphate anhydrous, and 5 mM of potassium chloride to form BSA-medium. This BSA-medium was adjusted to pH 10 with 1 M NaOH. CLA−BSA complex was made by adding a standard mixture of CLA isomers consisting of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 octadecadienoic acid (Sigma Chemical Co., St. Louis, MO) to BSA-medium up to a final CLA concentration of 6 mM. LA−BSA

Table 2. Lipase and Esterase Activity (U/g of Sample) in Muscle and Adipose Tissue from Pigs Fed with Different Levels of MUFA and CLA[a]

	low MUFA feed			high MUFA feed				*p*		
	0% CLA	1% CLA	2% CLA	0% CLA	1% CLA	2% CLA	SEM	CLA	MUFA	CLA × MUFA
					Muscle Tissue					
NL	0.95abc	1.07a	0.82bc	0.77c	1.02ab	0.87abc	0.03	0.002	0.193	0.137
AL	0.20	0.23	0.21	0.23	0.20	0.21	0.00	0.773	0.910	0.042
PL	0.049	0.050	0.050	0.050	0.055	0.053	0.00	0.777	0.350	0.906
NE	13.48ab	13.73ab	13.70ab	14.34a	13.03b	13.34b	0.10	0.060	0.723	0.003
AE	3.54ab	3.72a	3.51ab	3.26b	3.69a	3.85a	0.04	0.001	0.914	0.002
					Adipose Tissue					
NL	3.37a	2.75bc	3.13ab	2.43c	3.05ab	2.41c	0.07	0.490	0.000	0.000
NE	21.28a	20.06ab	20.48a	16.25b	17.83ab	17.56ab	0.46	0.966	0.000	0.346
AE	1.10a	0.88a	1.02a	0.52b	0.78ab	0.90a	0.04	0.143	0.000	0.006

[a] Data are expressed as mean values, $n = 8$ samples. Within a row, numbers lacking common letters (a–c) denote significant differences ($p \leq 0.05$).

complex was prepared at the same final concentration for the fatty acid (6 mM) in BSA-medium by using a standard of linoleic acid 99% (Sigma Chemical Co., St. Louis, MO). Each fatty acid−BSA mixture was sonicated in an ice bath until optically clear solutions were obtained. The media was later adjusted to pH 7.5 with 1 M HCl.

For their use on the enzymatic assays, CLA−BSA and LA−BSA complexes (6 mM) were diluted with BSA-medium and added to the reaction mixtures at the different concentrations assayed (from 0.01 to 1 mM depending on the enzyme).

The effect of CLA and LA on lipase and esterase activities was determined following the same procedure previously described. The muscle and adipose enzymatic extracts obtained from pigs fed with a 0% CLA feed were used for the analysis and assayed per triplicate. Results are expressed as a percentage of activity relative to the BSA control. In the case of those enzymes that resulted inhibited at any treatment, the fatty acid concentration that inhibited 50% of the enzyme activity (IC_{50}) was reported.

Statistical Analysis. The study of lipase and esterase activities from muscle and adipose tissue extracts of different pig feeding was a 3 factor (0%, 1%, and 2% CLA) × 2 factor (high MUFA and low MUFA) model. Mean values ($n = 8$) and pooled standard errors of means (SEM) are reported. The main effects (concentration of CLA and level of MUFA) and their interaction were analyzed using SPSS software (V.12.0) with a two-way analysis of variance (ANOVA). Differences were considered statistically significant at $p \leq 0.05$. When the effect of any of the factors was significant, differences between groups were analyzed by Tukey's post-hoc test.

In the case of fatty acid−BSA complexes, data were analyzed with a two-way (concentration and fatty acid) ANOVA. Differences of each value from that of the BSA control and between the two fatty acids (CLA or LA) for each concentration were compared by Tukey's post-hoc test. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The complex lipolytic enzyme system of mammalian muscles is mainly composed by lysosomal acid lipases (pH optimum 4.5−5.5), responsible for the hydrolysis of neutral lipids (*19*), lipoprotein lipases (pH optimum 8.5), which remove triglycerides form plasma lipoproteins (*20*) and neutral lipases (pH optimum 7.0−7.5) (*16*). Phospholipases A2 and A1 also play an important role in the biochemical degradation of lipids, because they catalyze the hydrolysis of 2-acyl and 1-acyl esters, respectively, from the 3-*sn*-phosphoglycerides (*16*). In the case of mammalian adipose tissue, a lipoprotein lipase activity (pH optimum 8−8.5) also stands out together with other important enzymes such as the hormone-sensitive lipase (pH optimum 7.0−7.5), which hydrolyzes triglycerides and diglycerides, and the monoacylglycerol lipase, responsible for the complete hydrolysis of monoglycerides (*15*). Neutral and acid esterase activities, which release short chain fatty acids from tri-, di-, and monoglycerides, have also been detected both in muscle and in adipose tissues (*21*).

The direct relationship existing between the fatty acid content of pig diets and the fatty acid profile of different tissues is well known (*22*). This fact was also observed in our case. Thus, an increasing proportion of CLA in muscle and adipose tissue was obtained with the increasing level of CLA on the feeds. For example, at the highest level of CLA on the feeds (2%), total CLA content (sum of both considered isomers) in muscle was 6 times higher than that observed for pigs fed with a 0% CLA feed (0.85% vs 0.2%, respectively). In adipose tissue, this relation was of 10 times higher proportion of total CLA in pigs with a 2% CLA in the diet with respect to those with no CLA added in the feeding (3.2% vs 0.35%, respectively). In the same way, higher or lower proportions of MUFA were observed in the subcutaneous tissue of pigs fed with a high or low MUFA level, respectively (40% vs 37%, respectively).

Basic, neutral, and acid lipolytic activities were assayed in early postmortem muscle and adipose tissues of pigs fed with different levels of CLA and MUFA. Values for neutral and acid lipolytic activities are shown in **Table 2**. There was a spontaneous hydrolysis of the 4-methylumbelliferyl oleate substrate at the pH used for assaying basic lipolytic activities, and thus results for this activity were not considered.

Lipase and Esterase Activities from Muscle Tissue Extracts. The different MUFA content of pig diets did not exert any significant effect on the activity of any of the lipolytic enzymes assayed in muscle tissue extracts ($p > 0.05$) (**Table 2**). Thus, it seems that the extent of the lipolytic metabolism carried out by the assayed enzymes is not affected by differences in the MUFA content (18−39%) of the tested pig diets. These results do not agree with those previously reported by Cava et al. (*23*), who measured the lipolytic enzyme residual activities from *Longissimus dorsi* of pigs fed diets with different MUFA content. These authors found differences in AL and AE activities between groups but not in the case of NL and PL activities. Nevertheless, animals in that study were from different pig breeds, and, therefore, genetics might also have played a decisive role in their results.

On the other hand, the lipolytic activity from muscle extracts was significantly affected by the CLA content of diets (**Table 2**). Thus, a significant effect of CLA on NL activity was observed ($p = 0.002$). The presence of CLA at 1% in pig diets implied a higher activity of lipases with a neutral optimum pH. Nevertheless, the extent of such an effect was very limited (around 10% increased from control). However, CLA at 2% in the feed did not significantly affect the activity of NL. No study

dealing with the effect of CLA on the activity of neutral lipases in pig muscle has been found in the scientific literature available.

The activities of AL and PL from muscle extracts were not affected by CLA in the feed ($p > 0.05$). Lysosomal acid lipase is the most important lipase of muscle with an acid optimum pH. Obtained results suggest that the assayed CLA levels have no effect on triglycerides metabolism regulated by acid lipases in muscle tissues. With respect to PL enzymes, very little is known about the effect of CLA on such activities. Bonorden et al. (*24*) found an inhibitory effect of CLA on phospholipase C in tumor-induced Swiss-mice 3T3 cells. However, Park et al. (*25*) did not ascribe any effect of *trans*-10,*cis*-12 CLA on the same enzyme in cancer cells. Al-Madaney et al. (*26*) pointed out evidence for stimulation of platelet phospholipase A_2 activity by *cis*,*cis* CLA isomers. However, our results suggest that the addition of the studied mixture of CLA isomers in the feed has no effect on the muscle phospholipase activity measured in the enzymatic extract.

Esterase activity measured at neutral pH was not affected by CLA content on pig feeding ($p > 0.05$). However, a significant interaction of CLA × MUFA ($p = 0.003$) was observed on the activity of this enzyme. Levels of 1% and 2% of CLA combined with a high MUFA feed caused a significant decrease of NE activity, but this result was not observed in the case of low MUFA feeds, showing the same values for the three levels of CLA evaluated (**Table 2**). At any rate, such activities were always within a range of 10% variation.

There was a significant effect on AE activity due to both CLA ($p = 0.001$) and the interaction of CLA × MUFA ($p = 0.002$). Contrary to NE, a higher enzymatic activity was observed in the presence of CLA in the diet in the case of high MUFA feeds. However, as it was observed for NE, the same values of enzymatic activities were obtained for the three levels of CLA in the case of a low MUFA feed. Thus, it seems that the presence of CLA in the pig diets has an effect on both neutral and acid esterase activities when this fatty acid is combined with high levels of MUFA in feeding, but this effect is opposite depending on the acid or neutral esterase activity. Nevertheless, it must be taken into account that the role of esterase enzymes on the lipolytic phenomena in muscle tissues is of limited importance due to the lack of adequate substrates, because they mainly act on short chain fatty acid hydrolysis (*21*). Moreover, differences in esterase activities between dietary treatments found in this study were very limited.

Lipase and Esterase Activities from Subcutaneous Adipose Tissue Extracts. As can be observed in **Table 2** and contrary to the results obtained for muscle extracts, CLA feeding did not show any significant effect on the activity of the evaluated enzymes ($p > 0.05$), but there was a significant effect of MUFA content of diet on the three measured enzymatic activities ($p = 0.000$). Thus, in the case of samples from pigs fed CLA-free diets, a higher activity of NL, NE, and AE was observed for low MUFA feedings than for high MUFA ones. These findings could suggest that a higher lipolytic metabolism takes place in the adipose tissue from animals with a higher level of SFA in the feeding due to the action of NL, NE, and AE.

One of the most important enzymes in adipose tissue with a neutral optimum pH is the hormone-sensitive lipase. Feeding CLA has been reported to enhance the activity of such lipase in adipocytes from CLA-fed mice (*6*). In our case, despite that CLA showed no significant effect on the NL activity from adipose tissue, a significant effect of the interaction between CLA and MUFA content of the diet ($p = 0.000$) was obtained on such activity. A decrease of NL was observed when CLA at 1% was combined with low levels of MUFA, but an increase of activity was caused by the same percentage of CLA combined with high levels of MUFA in diets. Thus, at the assayed CLA contents of the pig diets, NL seems to show opposite activity depending on the fatty acid profile of the adipose tissue.

In the case of esterase activities measured at acid pH, a significant interaction between CLA and the level of MUFA was also observed ($p = 0.006$). A trend toward higher enzymatic activity was found with the presence of CLA on the adipose tissue in the case of high MUFA feeds. However, similar enzymatic activities were obtained for the three levels of CLA in the case of low MUFA feeds. It must be pointed out that this behavior was the same as that observed for AE from muscle extracts.

These results turn out very interesting because they report the first information about the activity of the main lipolytic enzymes in muscle and adipose tissues from pigs fed with diets showing different CLA content and fatty acids profiles. CLA seems to have an effect on the lipolytic metabolism of muscle but not on the adipose tissue one, whereas MUFA content of diet appears to be mainly responsible for modifications on lipolytic activities from adipose tissue but not on those from muscle tissue. Furthermore, this work reveals the existence of interactions between CLA and the proportion of MUFA in the feed on the effect of several lipase enzymatic activities, both in adipose and in muscle extracts.

Effect of CLA on in Vitro Lipase and Esterase Activities from Muscle and Adipose Tissues Extracts. The enzymatic extracts used for the analysis (both from muscle and from lard) were free of fat. Therefore, the assessment of the activity in such extracts did not allow one to detect the direct effect of CLA on the assayed enzymes. The potential role of dietary CLA and its resulting increase in tissues on lipolytic enzymes activity could only be evidenced if such an effect was related to the expression of such enzymes. However, several in vitro studies have demonstrated the inhibitory role of CLA on the activity of different enzymes implied in metabolic paths and other important processes, such as oxidative or anticarcinogenic reactions. Thus, evidence for affected enzyme activities has been reported for Δ^9 desaturase (*27*), lipoprotein lipase (*28*), heparin-releasable-lipoprotein lipase (*18*, *29*), cicloxigenase, lipoxigenase, ornithine decarboxylase (*30*), protein kinase C (*31*), and catalase or superoxide dismutase (*32*). Taking this into account, we carried out an in vitro experiment to study the role of CLA as a possible inhibitor of lipase and esterase enzymes from pig muscle and adipose tissue extracts. Such an assay was performed by adding CLA as a CLA−BSA complex on the reaction mixtures. To distinguish between the effect on enzymatic activities due to the presence of γ-linoleic acid (C18:2 *cis*-9,*cis*-12) or due to its conjugated isomers (CLA), the effect of a LA−BSA on the studied enzymatic activities complex was also measured.

The considered concentrations of CLA were within the found range of CLA content in muscle tissue (0.85% CLA in neutral lipids, which was the percentage found in muscle for animals fed a 2% CLA diet, would approximately represent a 0.3 mM concentration) and were lower than those found in subcutaneous tissue (3% CLA in subcutaneous fat, which was the percentage of CLA found in animals fed a 2% CLA diet, would approximately represent a 100 mM concentration). Nevertheless, the calculated concentrations would be those in the whole tissue (either fat or muscle). However, the distribution of CLA is not homogeneous within the whole volume of any of these tissues (muscle and adipose). Therefore, depending on the enzyme

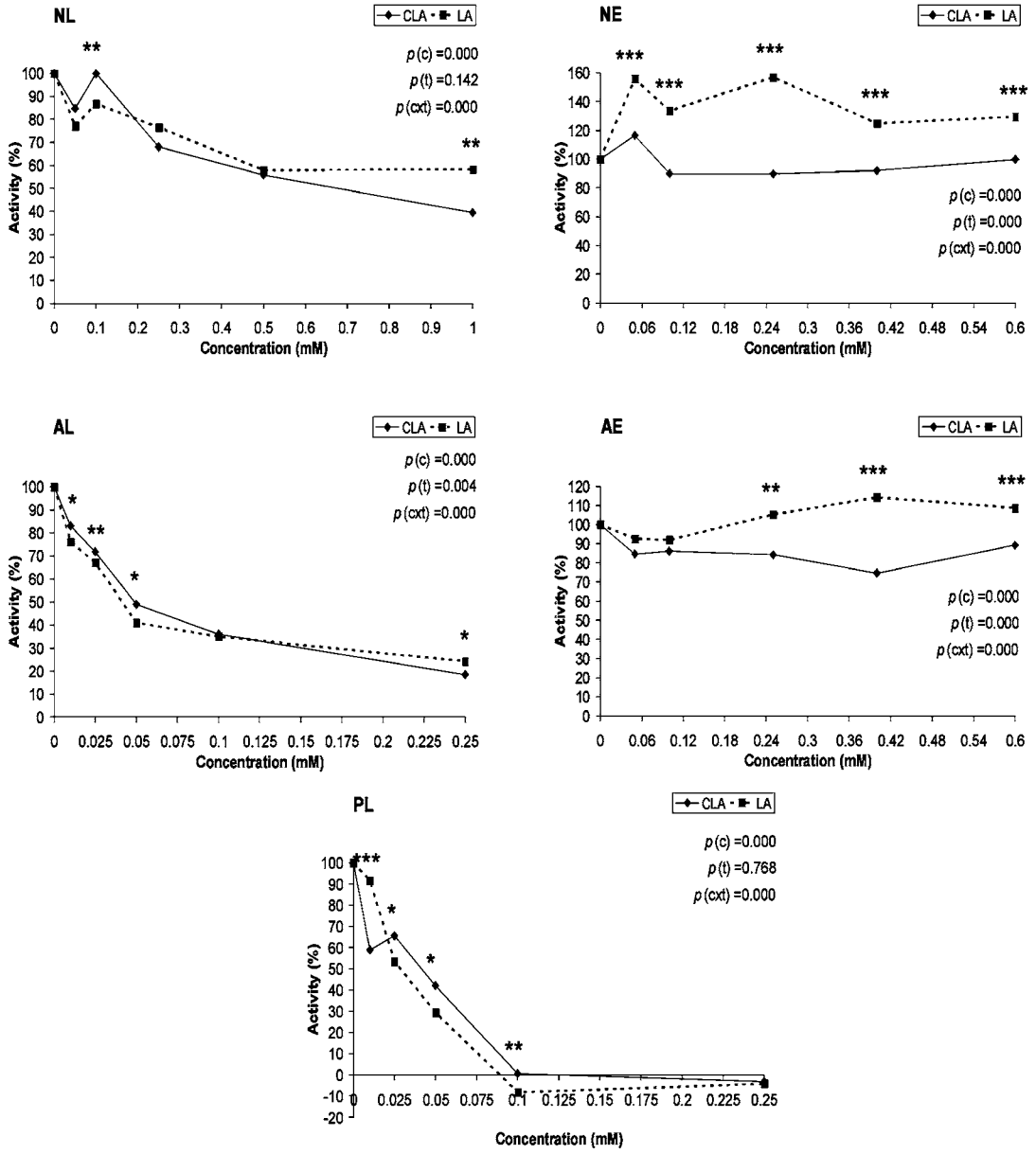

Figure 1. Effects of different concentrations of either CLA or LA on enzymatic activities from muscle tissue extracts. Data are presented as mean values of percentage of the respective mean value of BSA control. The values of the two treatments at the corresponding concentration are significantly different if $p \leq 0.05$ (*), $p \leq 0.01$ (**), or $p < 0.001$ (***). c = concentration, t = treatment, c × t = concentration × treatment.

location within the cell, each lipolytic enzyme would be in a medium with a different CLA concentration. Thus, it is very difficult to know the proximate real CLA concentration to which enzymes are exposed.

Figures 1 and **2** show the percentage of enzymatic activity with respect to the BSA control (fatty acid free BSA) for each studied concentration of CLA or LA in the reaction mixture. In the case of muscle extracts (**Figure 1**), there was a significant decrease of the NL, AL, and PL enzyme activities caused by higher concentration of both fatty acids added ($p = 0.000$). These results suggest that the CLA shows an inhibitory effect on the activity of these enzymes at the evaluated concentrations similar to that showed by the LA in its *cis*-9,*cis*-12 configuration. The concentration of each fatty acid that inhibited 50% of the enzymatic activity (IC_{50}) was 0.7 mM CLA and LA in the case of NL and 0.04 mM CLA and 0.03 mM LA for PL. The IC_{50} for both fatty acids was also highly similar for AL activity, 0.06 mM with CLA and 0.05 mM with LA.

With respect to PL enzymes, as it has been previously explained, different effects of CLA have been observed by other authors in this activity (*24*−*26*), from inhibitory effects in cancer cells to stimulating activity in platelets. Our results suggest that, in muscle samples and at the concentrations evaluated, CLA acts as an inhibitor of PL activity, but this effect is not different from that caused by the γ-linoleic acid isomer.

Esterase activities from muscle extracts showed different behaviors for both fatty acids assayed. At all concentrations evaluated for the NE activity, a significant increase ($p = 0.000$) of the enzymatic activity with respect to the BSA control was observed when this enzyme was exposed to LA. However, the presence of CLA only increased NE activity at 0.05 mM of this fatty acid in the medium (116.5% of activity), causing a significant but slight decrease of activity ($p = 0.000$) at higher concentrations. This enzymatic behavior due to the presence of CLA is similar to that found by Lin et al. (*18*) for the activity of heparin-releasable-lipoprotein lipase measured in cultured

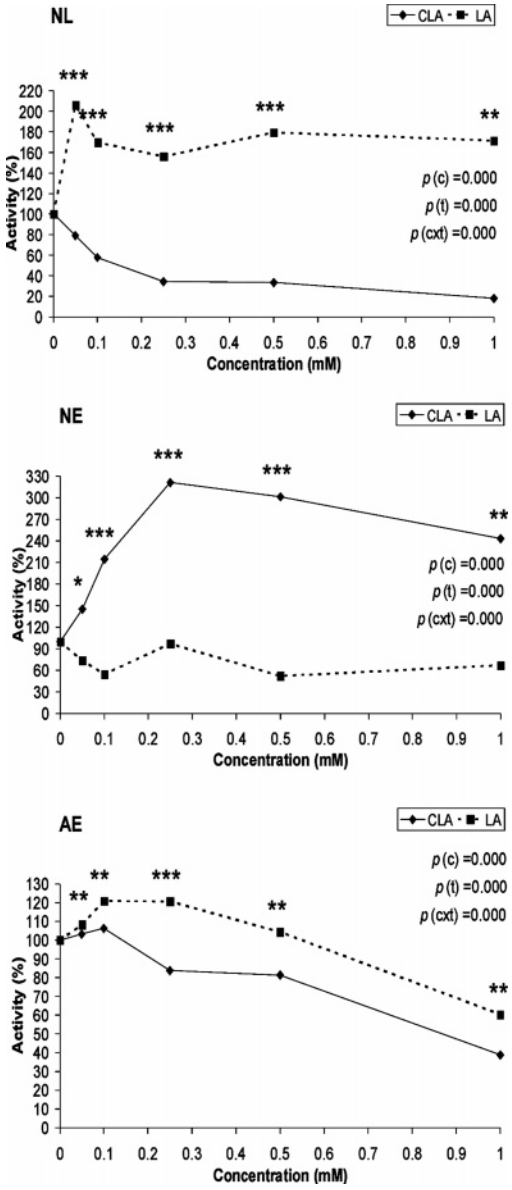

Figure 2. Effects of different concentrations of either CLA or LA on enzymatic activities from subcutaneous adipose tissue extracts. Data are presented as mean values of percentage of the respective mean value of BSA control. The values of the two treatments at the corresponding concentration are significantly different if $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p < 0.001$ (***). c = concentration, t = treatment, c × t = concentration × treatment.

adipocytes with CLA isomers in the medium. At low concentrations of any of the two studied isomers of CLA (*cis*-9,*trans*-11 and *trans*-10,*cis*-12) (<0.03 mM), these authors found a slight increase in the activity of this enzyme, but at higher concentrations (0.1 mM), CLA isomers suppressed the activity of this lipase.

Behavior similar to that of NE was observed for the esterase activity measured at acid pH in muscle extracts. A trend to increasing activity is produced in the presence of LA in the reaction mixture ($p = 0.000$). However, the opposite trend is observed in the case of CLA ($p = 0.000$). The highest reduction of activity was observed for CLA at 0.4 mM. Thus, these findings indicate that the assayed conjugated isomers of LA have a slight inhibitory effect on the activity of esterase enzymes from muscle tissue, while the *cis*-9,*cis*-12 isomer of LA tends to stimulate the activity of such enzymes.

The effects of added CLA− or LA−BSA complexes on lipolytic activities from adipose tissue extracts are shown in **Figure 2**. Contrary to the results obtained in the case of muscle extracts, a significant increase ($p = 0.000$) of NE activity was caused by CLA, showing a totally opposite behavior to that caused by the c9c12 isomer of the LA, which even tended to decrease the NE activity, with an IC_{50} value of 0.12 mM.

In the case of AE, both CLA and LA caused a decrease of esterase activity with increasing concentration of the fatty acid ($p = 0.000$). Nevertheless, an initial trend to a higher activity was observed in the presence of LA. The inhibition produced by CLA was higher that the caused by LA at the same concentrations. Thus, to produce a 50% enzyme inhibition, a lower concentration of CLA was required ($IC_{50} = 0.83$ mM) than that necessary for LA ($IC_{50} = 1.22$ mM).

Lipase activity measured at neutral pH (NL) in the presence of CLA was inhibited with increasing concentrations of the fatty acid in the reaction mixture ($p = 0.000$). At the highest concentration evaluated (1 mM), only 18% of the initial activity remained. The IC_{50} value corresponded to a concentration of 0.15 mM of CLA. This contrasted with the activating effect of the c9c12 LA isomer on the NL activity at all of the concentrations assayed. Moreover, this result is not in agreement with that found by Pariza et al. (*8*) about the enhancing effect of CLA on the main neutral lipolytic activity (hormone-sensitive lipase) from adipose tissue.

This in vitro assay shows that the c9t10 and t10c12 CLA isomers at the studied concentrations act as inhibitors or stimulants depending on the enzyme and the kind of tissue. The effect of CLA on studied enzymatic activities is clearly different from that due to LA in the case of NL activity from adipose tissue and esterase activities from both muscle and adipose tissue. However, CLA acts in a way on the activity of NL, AL, and PL from porcine muscle tissue similar to that evidenced for the c9c12 isomer of LA.

In conclusion, modification of CLA content of lipid tissues in pigs through feeding CLA-enriched diets seems to slightly affect the enzymatic activity of several lipolytic enzymes in fat-free muscle extracts, whereas its effect on subcutaneous adipose tissue extracts is very limited. However, when studying the direct effect of the presence of CLA on the lipolytic enzyme activity of the same extracts as compared to that caused by the major linoleic isomer in animal tissues (C18:2 c9c12), the observed results reveal a different effect depending on the considered enzymatic activity and tissue, ranging from a stimulating effect on the esterase activities in muscle or the neutral lipase activities in subcutaneous adipose tissue to an inhibitory effect on the neutral esterase activity in the latter tissue.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; MUFA, monounsaturated fatty acids; NL, neutral lipase; AL, acid lipase; PL, phospholipase; NE, neutral esterase; AE, acid esterase; LA, γ-linoleic acid; FAMEs, fatty acid methyl esters; EGTA, ethylene glycol bis-2-aminoethyl ether-*N*,*N*′*N*″,*n*′-tetraacetic acid; BSA, bovine serum albumin; SEM, standard error of the mean; ANOVA, analysis of variance; ME, metabolizable energy.

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