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Frozen storage of ovine and rat tissues adversely affects lipoprotein lipase activity

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The purpose of this study was to determine effects of frozen storage (frozen in liquid N, stored for 6 weeks at −80°C) of ovine adipose, and skeletal and cardiac muscle tissues on dietary induced changes in total (intra- and extracellular) lipoprotein lipase (LPL) activity previously determined in fresh tissue. Ewes were grown on either a low- (LE) or high-energy (HE) diet for 120 days (six per diet). In fresh tissue of HE ewes, LPL activity was higher ($P < 0.05$) for adipose tissue, and lower ($P < 0.05$) for skeletal and cardiac muscles than fresh tissues of LE ewes. Frozen storage altered these dietary effects and decreased ($P < 0.01$) LPL activity in adipose and cardiac tissues; treatment differences were maintained in skeletal muscle, and LPL activity was similar for fresh and frozen skeletal muscle of LE ewes. In subsequent experiments, ovine adipose and cardiac tissues were subjected to frozen storage, and LPL activity assayed with either a protease inhibitor (aprotinin, 2 μg/mL), or with 1 mM/L EGTA and without Ca^{2+} in the medium. Either treatment inhibited loss of LPL activity in frozen tissues. Frozen storage of hearts from five male, Sprague-Dawley rats decreased ($P < 0.01$) LPL activity; no decrease in activity was observed when protease inhibitor was used. However, it is not certain whether inclusion of protease inhibitor and (or) elimination of Ca^{2+} in frozen-thawed tissue prevents loss of effects on LPL activity that would be observed in fresh tissue in response to dietary changes or other physiological perturbations. In conclusion, frozen storage causes loss of LPL activity in ovine and rat tissues. Thus, frozen storage of tissue may be inappropriate when subsequent analysis of LPL activity is of interest. © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7: 577–581, 1996.)

Keywords: lipoprotein lipase; adipose tissue; heart; ovine; rat; frozen

Introduction

Frozen storage of tissue or tissue preparations is a common practice, and for many applications frozen storage does not affect results of subsequent assays. Method of frozen storage, however, can influence the outcome of enzyme assay. For example, glycerolipid biosynthesis activity in porcine adipose tissue homogenates was lost

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when samples were frozen at −20°C, but not when flash-frozen in liquid N.[1] Lipoprotein lipase (LPL, EC 3.1.1.34), which catalyzes hydrolysis of circulating lipoprotein triacylglycerol, is synthesized by parenchymal cells, then secreted and bound to the capillary endothelium.[2] Assay for LPL activity often involves release of the enzyme from the capillary endothelium by preincubation with heparin[3] or by homogenization of tissue in heparin-containing medium[4] to measure total (intra- and extracellular) LPL. Loss of LPL activity in frozen-thawed tissue would not compromise validity of experimental results given that treatment effects observed in fresh tissue preparations were maintained in the frozen-thawed tissue. The purpose of the present study was to determine the influence of frozen storage on known dietary-induced changes in LPL activity in adipose tissue, skeletal muscle, and cardiac muscle of sheep that were fed either a high- (HE) or a low-energy (LE) diet for 120 days.[5] Additional experiments were conducted to investigate methods that alleviate freeze-thaw-induced effects on LPL activity in ovine adipose tissue, as well as in ovine and rat heart.

Methods and materials

Animals

Twelve Columbia ewe lambs, average body weight 31.5 ± 1.0 (SEM) kg, were housed indoors in pairs in 2.4 m^2 pens; a 12-hr light/dark cycle was maintained. Feces and urine were expelled through openings in the floor of each pen and into a ventilated pit that was cleared as needed. Animals were fed daily in the morning and checked again each afternoon or early evening. All lambs had free access to fresh water.

The daily ration of each lamb consisted of 30% corn, 30% barley, 22% dehydrated alfalfa pellets, 10% chopped alfalfa hay, 7% soybean meal, and 1% salt (NaCl). Lambs were randomly assigned by weight to one of two dietary energy intake groups. The HE intake group ($n = 6$) consumed 1.8 kg of diet per day, and the LE intake group ($n = 6$) consumed 1.1 kg per day. Daily energy intakes were calculated[6] as 19.2 and 12.1 Mjoules of metabolizable energy per day for the HE and LE groups, respectively.

After 120 days, lambs were slaughtered at the University of Wyoming Meat Laboratory (Laramie, WY USA); the abattoir is located in the same building as the laboratory where assays were conducted. Lambs were killed by electrical stunning followed by exsanguination according to federal standards for humane lamb slaughter.[7] Abattoir procedures were conducted under the supervision of a Wyoming Department of Agriculture inspector. Some of each carcass was used for other research; remaining meat was available to consumers.

Tissue dissection and LPL assay

Immediately after slaughter, skeletal muscle samples were dissected from sternomandibularis muscle. Subcutaneous adipose tissue was sampled perianal and adjacent the caudal vertebrae (tail head). Immediately upon evisceration (approximately 15 min post mortem), cardiac muscle was sampled near the apex. Both muscle samples were placed in ice-cold Krebs-ringer phosphate (KRP) buffer (pH 7.4), whereas adipose tissue was placed in 37°C KRP. As soon as samples were obtained, LPL assays were conducted to determine activity in fresh tissue. Additional samples of each tissue were wrapped in foil, immersed in liquid N, and stored at −80°C for 6 weeks.

Total LPL was released from tissues by homogenizing 100 mg of each muscle or 200 mg of adipose tissue in 2.4 mL of KRP that contained 80 μL of heparin (4.0 mg/mL)[4] for 30 min. Samples were centrifuged at 750 *g* for 15 min, and the clear supernatant fraction used for assay of LPL activity.[4] Triolein was used as substrate and (9,10-^{3}H-oleate)-triolein was used as radiotracer. Ovine serum, used as source of apolipoprotein-CII (LPL activator), was heated to 60°C for 10 min to inactivate endogenous lipases, and aliquots stored at −80°C. Assays were conducted in triplicate at 37°C for 0, 5, and 10 min to quantify zero time values, and to ensure that assays were linear with time. Reactions were terminated by addition of 3.3 mL of chloroform:methanol:heptane, 1.25:1.41:1 (vol:vol:vol) along with 1.0 mL of 0.05 mole/L bicarbonate (pH 10.5) to extract fatty acid hydrolyzed by LPL.[8] Samples were vortex-mixed, centrifuged at 750 *g*, and radioactivity in 1.0 mL of the upper phase quantified by liquid scintillation counting. Correction for sample volume, upper phase volume, and specific activity were performed as previously described.[4] Adipose tissue adipocyte concentration was determined as described by Bailey et al.[9]

Protease inhibitor experiment

To determine if LPL activity of frozen-thawed tissue is stabilized by addition of protease inhibitor to the assay medium, experiments were conducted with adipose and heart tissues

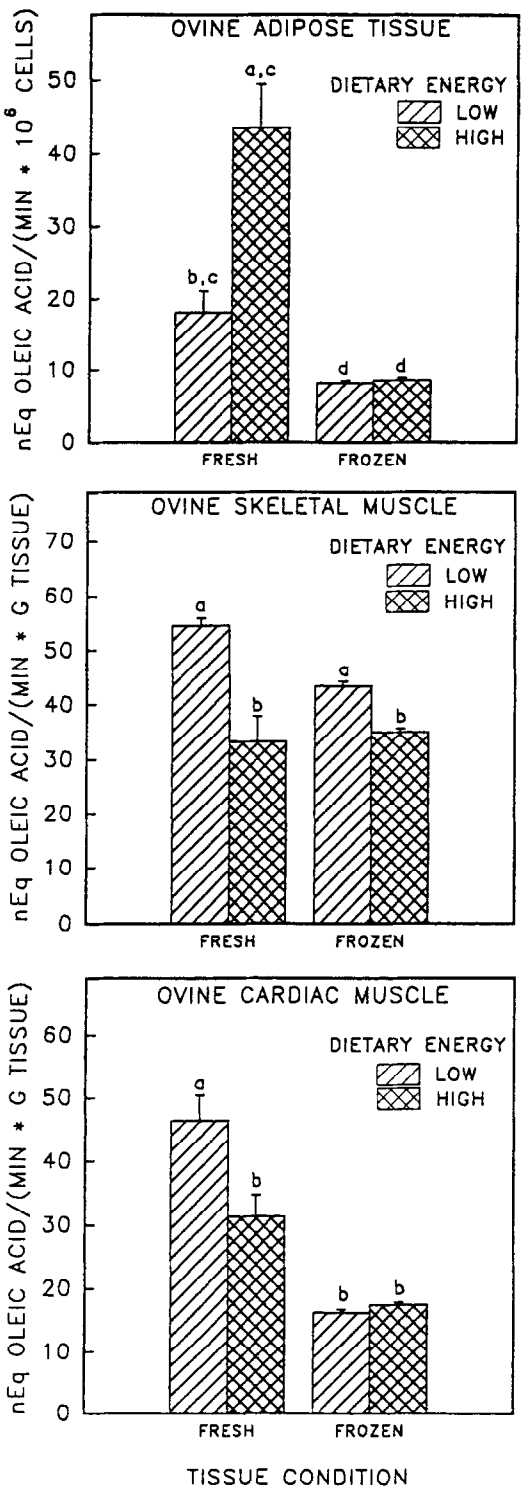

Figure 1 Lipoprotein lipase activity in fresh and frozen-stored adipose tissue, skeletal muscle, and cardiac muscle of sheep fed a low- or high-energy intake regimen for 120 days. For a given tissue and condition, superscripts 'a' and 'b' indicate significant differences between low- and high-energy groups, and superscripts 'c' and 'd' indicate significant differences between fresh and frozen-stored tissue. Low-energy was different from high-energy for each tissue when LPL activity was measured in fresh tissue ($P < 0.01$), but for frozen-stored tissue, differences occurred only for skeletal muscle LPL activity ($P < 0.05$). Fresh tissue LPL activity was greater than that in frozen-stored adipose tissue and cardiac muscle for both dietary energy treatment ($P < 0.01$). Difference in LPL activity between fresh and frozen-stored skeletal muscle approached significance ($P = 0.07$). Bars are means ± SEM of six ewe lambs per treatment. Data for fresh tissue LPL activity were reported elsewhere[5] and are shown here with permission.

from three additional sheep that were of uniform breed, body weight, and rearing. Ovine skeletal muscle LPL activity was minimally affected by freezing and thawing, so this tissue was not used in this experiment. Hearts of five male, Sprague-Dawley rats (approximately 350 g body weight) also were studied. Protocol for LPL assay was the same as that described above except that tissues were kept at −80°C for only 1 week, and a sample of the heparin release homogenate supernatant from rat heart also was flash frozen and stored at −80°C. Ovine adipose tissue LPL activity was expressed on a tissue weight basis in this experiment. Effects of adding aprotinin (a protease inhibitor, 2 μg/mL, Sigma Chemical Co, St. Louis, MO USA) and 1 mM/L ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), and eliminating Ca^{2+} from homogenate and assay media were investigated.

Statistical analysis

Statistical analyses were conducted by using SAS.[10] Dietary energy and tissue condition data were analyzed by two-way analysis of variance (ANOVA). Effects of protease inhibition were analyzed by one-way ANOVA. Duncan's new multiple range test was performed to identify significantly different means when the analysis of variance model indicated an overall significant treatment effect ($P < 0.05$).

Results

Lambs fed HE and LE grew and deposited fat at different rats during the 120 days. HE lambs gained 0.24 kg per day and LE lambs gained at 0.15 kg per day ($P < 0.05$).[5] Depth of subcutaneous adipose tissue measured adjacent the thoracic spinal process between the 12th and 13th rib was 0.56 cm for HE and 0.21 cm for LE lambs, respectively ($P < 0.05$).[5] Increased LPL activity in adipose tissue and decreased LPL activity in skeletal and cardiac muscle was observed in HE lambs, whereas the opposite effect occurred in LE lambs. Discussion of these dietary responses was presented in a companion paper.[5]

Frozen storage and diet effects on ovine LPL

Effects of frozen storage on LPL activity in ovine adipose tissue and muscle are shown in *Figure 1*. In fresh adipose tissue, HE lambs had greater LPL activity than did LE lambs ($P < 0.01$). When frozen tissue was used, however, LPL activity in adipose tissue from both groups was depressed ($P < 0.01$). Moreover, adipose tissue stored frozen from the LE group had similar LPL activity as frozen tissue from the HE group. In contrast, skeletal muscle LPL activity was greater in LE lambs ($P < 0.01$) for both fresh and frozen tissues. Freezing resulted in lower LPL activity ($P < 0.01$) for the LE group, but not for the HE group. Similar to findings in fresh skeletal muscle, fresh cardiac muscle of lambs fed LE had greater LPL activity than those fed HE ($P < 0.01$). Cardiac muscle LPL activity was lower in frozen than in fresh tissue ($P < 0.01$), but with frozen tissue dietary energy effects previously observed in fresh tissue were no longer observed. Thus, several interactions between dietary treatment and tissue condition (fresh and frozen-stored) were observed ($P < 0.01$ for adipose and cardiac tissues; $P < 0.05$ for skeletal muscle), indicating that frozen storage of tissues would not have been appropriate in this study.[5]

Protease inhibitor experiment

Additional studies were performed to determine if LPL activity can be stabilized in frozen-thawed tissue. Results of assays conducted in the presence of protease inhibitor and in the absence of Ca^{2+} are illustrated in *Figure 2*. No losses of ovine LPL activity were observed for both adipose and cardiac tissues when either 2 μg/mL of aprotinin, or 1 mM/L EGTA without Ca^{2+} in the KRP were used in the heparin-release and LPL assay media. An additional experiment was conducted to verify loss of LPL activity from frozen storage of ovine adipose and cardiac muscle tissues when Ca^{2+}-containing medium was used. In the presence of Ca^{2+}, LPL activities in adipose tissue and cardiac muscle were 61% and 18%, respectively, of values obtained by eliminating Ca^{2+} and using 1 mM/L EGTA, or by including aprotinin (data not shown).

Lipoprotein lipase activity in fresh rat heart was compared with activity in both

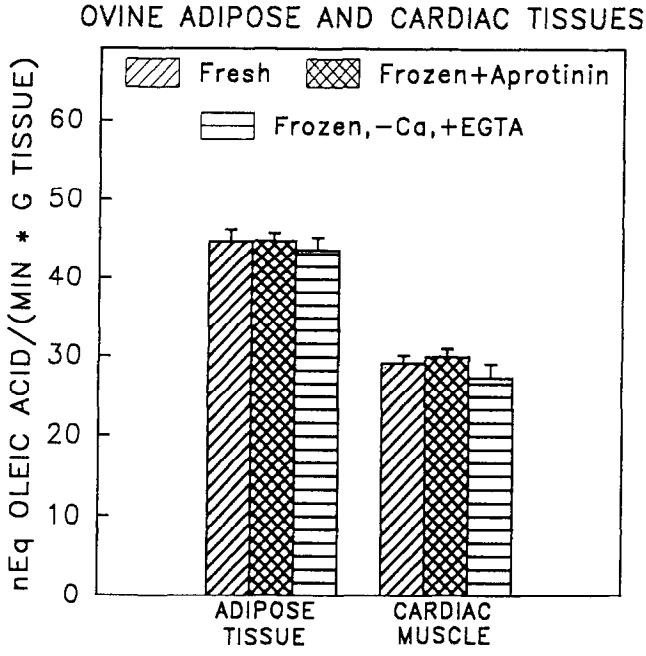

Figure 2 Lipoprotein lipase activity in fresh and frozen-stored ovine adipose tissue and cardiac muscle. Frozen-stored tissue was homogenized in KRP that contained 2 μg/mL of the protease inhibitor, aprotinin, or homogenized in KRP that contained 1 mM/L EGTA and devoid of Ca^{2+}. Bars are means ± SEM of three sheep. No differences in LPL activity were observed within a tissue ($P > 0.05$).

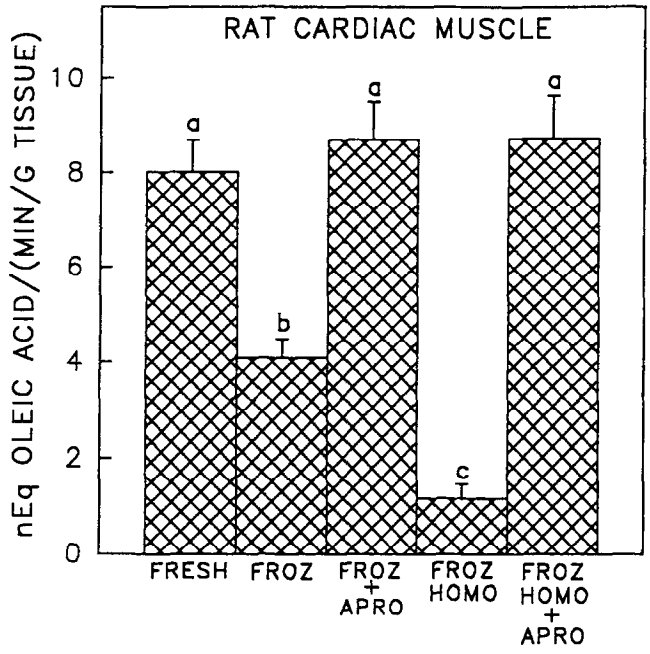

Figure 3 Lipoprotein lipase activity in fresh and frozen-stored rat heart. Heart LPL activity was determined in fresh, frozen-stored (FROZ), and frozen-stored tissue with aprotinin (2 μg/mL; FROZ + APRO). Aliquots of homogenates were stored frozen (FROZ HOMO) with and without aprotinin (2 μg/mL; FROZ HOMO + APRO). Bars are means ± SEM of five male, Sprague-Dawley rats. Different superscripts indicate significant differences ($P < 0.01$).

the frozen-stored tissue and the heparin-release homogenate supernatant that was flash-frozen in liquid N and stored at −80°C (*Figure 3*). Enzyme activity was decreased by half when frozen tissue was used ($P < 0.01$), and decreased nearly 8 fold when the frozen supernatant was used ($P < 0.01$). When aprotinin was used to prepare the frozen tissue homogenate or added to the homogenate supernatant before freezing, no differences in LPL activity were observed when compared to fresh tissue.

Discussion

Results of these studies clearly indicate that differences in dietary energy intake influence LPL activity in ovine adipose tissue, cardiac muscle, and skeletal muscle. Decreases in LPL activity in response to frozen storage of tissues were substantial for adipose tissue and cardiac muscle. More importantly, dietary effects observed with fresh tissue were no longer observed or were different (*Figure 1*). Skeletal muscle LPL activity was not influenced enough to change treatment effects; however, the magnitude of difference was decreased when frozen tissue was assayed.

Homogenizing frozen tissue in KRP devoid of Ca^{2+} and including EGTA to chelate endogenous Ca^{2+} mimicked the effects of aprotinin on LPL activity. Also, observations in the present study were not species-specific because similar responses to protease inhibitor and Ca^{2+} removal were observed with rat heart. Others, however, have not observed consistent protection of LPL activity in swine adipose tissue with either EGTA or with a protease inhibitor (H.J. Mersmann, personal communication).

Ovine skeletal muscle LPL activity was not significantly affected by freeze-thaw. In contrast, adipose tissue and cardiac muscle subjected to freeze-thaw had reduced LPL activity. The only difference between handling of ovine skeletal and cardiac muscle, as well as adipose tissue, was the time from exsanguination to dissection. Sternomandibularis muscle was sampled within seconds after exsanguination; these muscle samples were placed in an ice-cold KRP, whereas cardiac muscle remained in the carcass at body temperature for 15 min before it was sampled, and adipose tissue was maintained at 37°C from dissection through assay. These results suggest that the process(es) responsible for LPL activity loss in frozen-stored tissue may become active very soon after death (or exsanguination), and that chilling of the tissue slows this process.

The mechanism of LPL activity loss in frozen-thawed tissues is not clear. Ca^{2+}-activated proteases[12,13] may be involved because amino acids of LPL were shown to include significant proportions of sequences containing proline, glutamate, serine, and threonine,[14] which were suggested to promote degradation of LPL by Ca-dependent proteases.[15] However, freeze-thaw-induced loss of LPL activity by enzyme protein degradation has not been determined.

Storage of post-heparin plasma for later LPL activity measurement would also be of concern. Whether or not frozen-storage of post-heparin plasma adversely affects LPL activity is difficult to suggest because the mechanism of LPL activity loss is unclear. If the loss of tissue LPL activity in frozen-thawed tissue occurs because of tissue-borne substances, then post-heparin plasma LPL may not be affected because samples would be stored without contact with the tissue of origin. However, stability of LPL activity in post-heparin plasma should be evaluated in a subsample of the treated population to determine if loss of activity occurs, and to determine if activity changes that occur from treatment are maintained in frozen-thawed samples.

Overall, the present study determined that LPL activity in ovine cardiac muscle and subcutaneous adipose tissue responded differently to dietary energy, and that selected tissues had reduced LPL activity following freeze-thaw. Moreover, differences observed in LPL activity in fresh adipose and cardiac tissues in response to differences in energy intake by sheep were not sustained when frozen-thawed tissues were assayed. Compared with other tissues studied, skeletal muscle LPL activity was not adversely affected by freeze-thaw; reasons for the tissue difference in response to freezing were not clear. Addition of aprotinin to the medium

or eliminating Ca^{2+} from the medium abolished the loss of LPL activity observed in frozen-thawed adipose tissue (sheep) and heart (sheep, rat).

Although LPL activity from homogenates of various tissues appear to respond differently to freezing-thawing, it is concluded that frozen storage of tissue may be inappropriate when subsequent analysis of LPL activity is of interest. Additionally, efficacy of LPL activity stabilization by protease inhibitor and (or) elimination of Ca^{2+} to sustain treatment effects should be ascertained in pilot experiments because species variation was evident and the effect of frozen storage of different tissues in response to all physiological perturbations is uncertain.

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