Nutritional milieu of isolated stromal vascular cells determines their proliferative, adipogenic, and lipogenic capacity in vitro

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The objective was to determine the effect of nutritional milieu of isolated stromal vascular (SV) cells on proliferative capacity of preadipocytes, and adipogenic and lipogenic capacity in adipocytes in vitro. Proliferation of the preadipocytes increased over time with 48 and 72 h being greater than 24 h; however, preadipocytes from steers supplemented with corn (LC) had lower proliferation rates compared with those without corn grain supplementation (L) at 72 h. Adipocyte cultures isolated from LC group had higher mean diameter on d 4 and 6, and higher mean volume on d 0, 4, 6, and 12 of culture. Adipocytes from steers supplemented with corn grain (LC) had lower expression of key adipogenic genes during extended days in culture. The results show that prior nutritional treatment of the donor animal used to isolate SV cultures alters their proliferative, adipogenic, and lipogenic capacity in culture. These differences may be related to lower induction/ expression of AP2 gene in the adipose cultures from corn supplemented group. Corn grain supplementation to steers grazing legumes could have stimulated more active adipogenic progenitor cells to differentiate, which would leave fewer behind in the SV pool for subsequent isolation.

Introduction

The use of primary adipocyte cultures for examination of nutritional treatments in vitro is common today. Adipocytes used for these experiments come from stromal vascular cells isolated from adipose tissues of animals typically obtained at slaughter. Research shows that specific differentiation cocktails facilitate the differentiation of these SV cells into adipocytes that are able to incorporate exogenous fatty acids and/or synthesize long-chain fatty acids in vitro. Numerous research articles are available to address the function of these adipocytes in culture and how exogenous compounds alter lipogenesis (for a review see ref. 5). However, little attention has been paid to the nutritional background that the donor animal received prior to the isolation of the SV cell population.

Nutritional content of the animal’s diet prior to slaughter alters fat deposition rates and fatty acid composition of adipose tissues. Comparisons of grain- vs. grass-finishing systems show that adipose tissue deposition is greater and polyunsaturated fatty acid contents are lower for grain-finished steers. The longer time period that cattle are fed high-concentrate diets results in linear increases in subcutaneous fat deposition, intramuscular total lipid content, and monounsaturated fatty acid concentrations due to increased triacylglyceride storage in the adipocyte. Corn grain supplementation to grazing steers can also alter fat deposition, fatty acid composition and lipogenic gene expression of adipose tissue depots. Fat deposition in finishing animals is the result of both hyperplasia and hypertrophy. We hypothesized that the nutritional milieu of the donor animals from which SV cells were isolated from would alter proliferative, adipogenic, and lipogenic capacity of preadipocytes/adipocytes. The specific objective of the experiment was to determine the effect of nutritional milieu of isolated SV cells on proliferative capacity of preadipocytes, and adipogenic and lipogenic capacity in adipocytes in vitro.

Results

The fatty acid composition of the subcutaneous adipose tissues of the donor animals is presented in Table 1. Previous research in our laboratory has shown that the fatty acid composition of subcutaneous and intramuscular adipose tissues is very similar in composition (Duckett, unpublished data). Equinoa et al. compared lipogenic enzyme rates of various fat depots (omentum, perirenal, subcutaneous, and intermuscular) to find...
Table 1. Fatty acid profile (g/100 g adipose tissue) of subcutaneous adipose tissue for donor steers that grazed legume pastures with (LC) or without (L) corn grain supplementation (0.75% of body weight/d).

| Fatty acid       | L  | LC | SEM | P value |
|------------------|----|----|-----|---------|
| n                | 4  | 4  |     |         |
| C14:0            | 2.51 | 3.67 | 0.1 | 0.01    |
| C14:1 cis-9      | 1.02 | 1.36 | 0.07 | 0.01    |
| C16:0            | 20.40 | 24.47 | 0.85 | 0.01    |
| C16:1 cis-9      | 2.99 | 3.69 | 0.17 | 0.01    |
| C18:0            | 9.99 | 10.41 | 0.53 | 0.58    |
| C18:1 cis-9      | 27.54 | 31.7 | 1.34 | 0.04    |
| C18:1 trans-11   | 1.68 | 1.21 | 0.13 | 0.02    |
| C18:2 cis-11     | 0.76 | 0.90 | 0.05 | 0.05    |
| C18:2 n-6        | 0.09 | 0.09 | 0.01 | 0.81    |
| C18:2 cis-9 trans-11 | 0.60 | 0.58 | 0.04 | 0.75    |
| C20:4 n-6        | 0.01 | 0.004 | 0.002 | 0.36    |
| C20:5 n-3        | 0.001 | 0.002 | 0.001 | 0.64    |
| C22:5 n-3        | 0.02 | 0.04 | 0.01 | 0.3     |
| C22:6 n-3        | 0.008 | 0.008 | 0.001 | 0.8     |
| SFA              | 32.90 | 38.55 | 1.38 | 0.01    |
| MUFA             | 33.99 | 38.86 | 1.6  | 0.04    |
| Total fatty acids | 74.34 | 84.99 | 3.27 | 0.02    |

that lipogenic enzyme activities were similar in subcutaneous and intermuscular adipose depots in cattle and that these depots had lower activities than omental and perirenal adipose depots. In a proteomics approach, Gondret et al.\(^\text{17}\) evaluated intramuscular, subcutaneous, intermuscular, and perirenal adipose depots in cattle and that these depots had non-significant differences except for C18:2. Adipocytes from L group had increased \((P < 0.01)\) total fatty acid content after 6 d in culture compared with LC. The L adipocytes had higher \((P < 0.05)\) concentrations of palmitic, palmitoleic, stearic, oleic, arachidonic, docosapentaenoic (DPA), and docosahexaenoic (DHA) acids than LC adipocytes. Addition of linoleic acid to the cultures decreased \((P < 0.05)\) stearic, oleic, arachidonic, DPA, and DHA acid amounts in both L and LC adipocytes. Linoleic acid was increased \((P < 0.05)\) with C18:2 supplementation; however, the amount of the increase was greater \((P < 0.05)\) for L than LC.

Proliferation of the preadipocytes increased \((P < 0.05)\) over time with 48 and 72 h being greater \((P < 0.05)\) than 24 h (Fig. 1). Preadipocytes from LC had lower \((P < 0.05)\) proliferation rates than L preadipocytes at 72 h. These results indicate the preadipocytes from L treatment were able to proliferate longer in culture than those from LC.

The adipocyte mean diameter and volume at different days of culture are presented in Figure 2A and B respectively. Adipocyte diameter (µM) and volume (pL) increased \((P < 0.05)\) over time with the maximum average sizes observed at d 12. The adipocyte cultures isolated from LC group had higher \((P < 0.05)\) mean diameter on d 4 and 6, and higher \((P < 0.05)\) mean volume on d 0, 4, 6, and 12 of culture. The cell size distribution of adipocyte

Table 2. Fatty acid profile (µg/well) of intermuscular adipocyte cultures at 6 d from steers that grazed legume pastures with (LC) or without (L) corn gain supplementation (0.75% of body weight/d). Cultures from each treatment group (L or LC) received 0 or 100 µM addition of linoleic (C18:2)

| TRT   | L  | LC | SEM | P value |
|-------|----|----|-----|---------|
| C18:2 | 0  | 100 |     |         |
| C10:0 | 3.26 | 2.76 | 2.92 | 3.43     |
| C12:0 | 2.9 | 2.41 | 2.07 | 3.04     |
| C14:0 | 0.91 | 0.82 | 0.64 | 1.03     |
| C16:0 | 10.56 | 11.10 | 10.20 | 9.53     |
| C16:1 | 0.54 | 0.55 | 0.39 | 0.25     |
| C18:0 | 13.00 | 12.62 | 12.01 | 10.67     |
| C18:1 cis-9 | 9.62 | 7.79 | 5.46 | 3.23     |
| C18:2 | 2.23\(^c\) | 40.52\(^a\) | 1.37\(^c\) | 19.51\(^a\) |
| C20:4 | 5.62 | 2.89 | 4.97 | 2.48     |
| C20:5 | 1.12 | 0.03 | 0.25 | 0.00     |
| C22:5 | 1.46 | 1.24 | 1.28 | 1.15     |
| C22:6 | 2.22 | 1.64 | 1.90 | 1.36     |
| Total FA | 112.10 | 149.97 | 98.95 | 118.57 |

\(^a\)Means in the same row with uncommon superscripts differ \((P < 0.05)\).
cultures are presented in Figure 3A–C. Adipocytes grew larger ($P < 0.05$) with increasing days in culture. Approximately 78–82% of the adipocytes were $<18 \mu M$ in size at d $-2$. By d 12 of culture, the percentage of small ($<18 \mu M$) adipocytes was reduced to 40–50% of the total cellular population. Adipocyte populations with larger sizes ($>24 \mu M$) were seen in d 6 and 12 of culture. The cellular size distribution during culture differed ($P < 0.05$) between nutritional treatments, L and LC. Adipocytes from LC had greater percentages ($P < 0.05$) of larger adipocytes than L adipocytes.

Relative gene expression of key adipogenic genes in L and LC adipocytes at various days in culture is shown in Figure 4. The expression of $AP2$ was downregulated ($P < 0.05$) in LC adipocytes at 2, 4, 6, and 12 d in culture compared with L. Expression of C/EBPa was downregulated in LC compared with L adipocytes at d 6 and 12 of culture. PPAR-$\gamma$ mRNA expression was upregulated in LC at d $-2$ of culture but downregulated in d 2 and 4 compared with L. SCD-1 mRNA expression was downregulated ($P < 0.05$) in LC vs. L adipocytes at d 4 and 6 in culture.
The expression of GPDH mRNA was downregulated in LC adipocytes at d 4, 6, and 12 of culture compared with L. Overall, adipocytes from steers supplemented with corn grain (LC) had lower expression of key adipogenic genes during extended days in culture.

The enrichment of precursor 1,2-13C2 estimated by MIDA did not change within the time points suggesting isotopic steady-state in the adipocyte cultures (Fig. 5A). However, the enrichment of the precursor was lower (P < 0.05) in LC adipocyte cultures than L. The enrichment of C16:0 (M+2), expressed as TTR of C16:0, increased with time from 6 to 48 h, with no difference due to nutritional treatment (Fig. 5B). The TTR of M+4 did not differ between the treatments at 6, 12, and 24 h; however at 48 h, M+4 was lower (P < 0.05) in LC adipocytes (Fig. 5C). The TTR of C18:0 (M+2) increased from 6 to 48 h but TTR was lower (P < 0.05) for LC at 12, 24, and 48 h (Fig. 5D). C18:0 (M+4) increased from 24 to 48 h in both the adipocyte cultures but TTR was lower (P < 0.05) for LC than L adipocytes (Fig. 5E). Lipogenesis, as indicated by FSR16:0, increased from 12 to 48 h; however, LC adipocytes had lower (P < 0.05) lipogenic rates than L adipocytes (Fig. 5F). Stearic acid is formed in culture via elongation of palmitic acid and therefore is also part of the lipogenesis rate. The FSR18:0 was greater (P < 0.05) in the LC adipocyte cultures than L from 24 to 48h (Fig. 5G). GPDH activity (nmol/mg protein/ min), another index of lipogenesis, was reduced (P < 0.05) in LC compared with L on both d 6 and 12 of culture (Fig. 6). Activity of GPDH was higher (P < 0.05) in L cells at d 12 compared with d 6.

Discussion

Subcutaneous adipose tissue samples from the donor steers that were on two different nutritional treatments, grazing legume
pastures with (LC) or without (L) corn grain supplement, differed in total fatty acid content and composition. The increase in total fatty acid content of the adipocytes would indicate the increased hypertrophy or filling with lipid occurred with the supplementation of corn grain. Robelin\textsuperscript{15} reported that postnatal adipose tissue growth is a combination of both hyperplasia of preadipocytes and hypertrophy of adipocytes; however as animals reach mature size, hypertrophy or lipid filling of adipocytes predominates (70%). Cianzio et al.\textsuperscript{14} also reported that hypertrophy of the adipose tissue is the major contributor of adipose tissue
mass expansion of steers in the growing phase from 11 to 17 mo of age. Corn grain supplementation also increased the content of saturated (myristic and palmitic acids) and monounsaturated (myristoleic, palmitoleic, and oleic acids) fatty acids. Supplementation increased cis-11 vaccenic acid, which is an elongation product of myristoleic, palmitoleic, and oleic acids. Pratt et al. showed that AP2 is upregulated by 100-fold by d 2 after addition of primary differentiation cocktail. Similarly, AP2 gene expression was upregulated by over 100-fold in L cells in this study; however, LC cells only showed about 20-fold increase in AP2 expression. Expression of SCD-1 increases after differentiation of SV cells and precedes lipid filling in 3T3-L1 and bovine preadipocytes. In this study, SCD-1 expression increased (P < 0.05) above d −2 and 2 levels at d 4 in L and d12 in LC. The SV cells from both nutritional treatments (L and LC) differentiated to mature adipocytes with our differentiating protocol as evidenced both by measuring the expression of key genes involved in adipogenesis, activity of GPDH, and incorporation of 13C2 into long-chain fatty acids. In addition, further confirmation was also evaluated by histological examination of the cell culture to show change in cell shape and evidence of lipid droplets (data not shown). All these parameters unequivocally suggested that the SV cells in both the groups were differentiated to fully mature adipocytes. However, clear differences in the markers of adipose differentiation suggested that the source of SV cells could influence the differentiating capabilities of adipocytes. Adipocytes from steers supplemented with corn grain (LC) had lower relative expression of AP2, C/EBPα, PPARγ, SCD-1, and GPDH during extended days in culture. Thus, adipocytes isolated from steers supplemented with corn grain had lower expression of key adipogenic genes that likely influenced their capacity for de novo lipogenesis, desaturation, and elongation in culture. Indeed, adipocytes from steers supplemented with corn grain had lower total fatty acid content after 6 d in culture than L adipocytes. Fatty acid composition was also altered with lower amounts of saturated (palmitic and stearic acids), monounsaturated (palmitoleic and oleic acids), and polyunsaturated (arachidonic, DPA, DHA) fatty acids. Addition of linoleic acid to the cultures also altered uptake of C18:2 into the adipocytes dependent on nutritional milieu of donor cells. Adipocytes from L were able to incorporate more C18:2 into the cells than LC (40.5 vs. 19.5 μg/well). Therefore, the lower expression of key fatty acid synthesis enzymes by LC adipocytes may have negatively influenced their ability to incorporate C18:2 into the cells.
adipogenic genes in LC altered their capacity for de novo lipogenesis, desaturation, and uptake of long-chain fatty acids in culture.

Lipogenic rate as measured by $^{13}$C2 incorporation into fatty acids and GPDH activity at d 6 and 12 showed differences among SV cells dependent on nutritional milieu of donor samples. Activity of GPDH, another index of lipogenesis, was lower for LC than L adipocytes at d 6 and 12 of culture. Lipogenesis, as determined by FSB16:0 and FSB18:0, was lower in LC adipocytes than L. The lipogenic capacity of adipocytes isolated from steers supplemented with corn grain was reduced in culture.

Together these results show that prior nutritional treatment of the donor animal used to isolate SV cultures alters their proliferative, adipogenic and lipogenic capacity in culture. These differences may be related to lower induction/expression of AP2 gene in the adipose cultures from corn supplemented group. Corn grain supplementation to steers grazing legumes could have stimulated more active adipogenic progenitor cells to differentiate, which would leave fewer behind in the SV pool for subsequent isolation. Additional research in this area is needed to further determine how corn grain supplementation altered the SV pool isolated for primary adipocyte culture experiments. This study highlights the importance of knowing that details regarding the nutritional treatments of the donor animal and source of SV cells used in subsequent primary adipocyte culture experiments in order to attain appropriate in vitro results.

### Materials and Methods

**Cell isolation**

Bovine primary stromal vascular (SV) cells were isolated from intermuscular adipose tissue immediately underneath the subcutaneous adipose tissue layer at the lateral edge of the longissimus muscle from the 10–12th rib of Angus × Hereford steers ($n = 8$; 21 mo of age; 503 ± 10 kg) that grazed legume pastures (alfalfa, *Medicago sativa*, Alfagraze 600R, Pennington Seed, and forage soybeans, *Glycine max*, Large Lad RR, Eagle Seed) with or without corn grain supplementation (0.75% of body weight). Subcutaneous adipose tissue samples from each steer were collected for subsequent long-chain fatty acid analyses by GLC. In our laboratories, we found that SV cells could be isolated from the intermuscular fat from the 12th rib area from adipose tissue immediately underneath the subcutaneous fat layer and adjacent to the lateral edge of the longissimus muscle. Isolating fat from this location provided us with a much cleaner sample and less contamination than removing the subcutaneous fat from the similar region because it was exposed to numerous contaminates during the slaughter process. It is important to note that these samples were from forage-finished beef, which have a lower amount of subcutaneous adipose tissue for sampling making more difficult to isolate subcutaneous adipose tissues that have not been exposed to contaminates during the slaughter process. Upon development of this method, we compared these SV cells from intermuscular adipose tissue to previous research with SV cells isolated from subcutaneous fat and found that they behaved similarly to subcutaneous SV cells in culture (i.e., characteristic increase in AP2 expression after differentiation, increase in lipid filling and specific fatty acid composition as demonstrated by GC analyses, uptake of supplemental fatty acids, lipogenic gene expression and miRNA expression, etc.).

Together these results show that prior nutritional treatment of the donor animal used to isolate SV cultures alters their proliferative, adipogenic and lipogenic capacity in culture. These differences may be related to lower induction/expression of AP2 gene in the adipose cultures from corn supplemented group. Corn grain supplementation to steers grazing legumes could have stimulated more active adipogenic progenitor cells to differentiate, which would leave fewer behind in the SV pool for subsequent isolation. Additional research in this area is needed to further determine how corn grain supplementation altered the SV pool isolated for primary adipocyte culture experiments. This study highlights the importance of knowing that details regarding the nutritional treatments of the donor animal and source of SV cells used in subsequent primary adipocyte culture experiments in order to attain appropriate in vitro results.

**Cell experiments**

Individual cultures from 3 steers per nutritional treatment (L and LC) were used in duplicate for this study. Cells were thawed, cultured in growth media for 3 passages, and seeded in 6-well plates (1.6 cm$^2$/well) at 1 × 10$^5$ cells/cm$^2$. Three experiments were conducted to assess proliferation capacity of preadipocytes (Expt. 1), fatty acid composition and uptake of linoleic acid in differentiated adipocytes (Expt. 2), and cell size/volume, relative expression of key adipogenic genes and lipogenesis in differentiated adipocytes (Expt. 3). In experiment 1, preadipocytes were seeded in 6-well plates as described above and then proliferation capacity assessed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). In experiment 2 and 3, cells were seeded in 6-well plates as described above and then allowed to reach confluence. Cells were held for an additional 2 d and then differentiated on d 0 with primary differentiation media (DMEM containing 5% FCS, 2.5 μg/mL insulin, 0.25 μM dexamethasone [DEX], 1 μM rosiglitazone [ROSI], 0.5 mM 2-isobutyl-1-methylxanthine [IBMX], and 10 mM acetate). Secondary differentiation media (DMEM containing 5% FCS, 2× AB/AM, 2.5 μg/mL insulin, 1 μM ROSI, and 10 mM acetate) was applied for 10 d from d 2 to 12. The differentiation hormones and media used in our experiment have been tested for their efficacy in inducing differentiation in bovine SV cultures (see refs. 1–2, and 24). In our laboratory, our SV cultures show the characteristic changes from fibroblastic to round following differentiation and become lipid-filled (see refs. 3 and 18). Cells were washed 3 times with PBS and released from the
cultureware with trypsin to harvest the cells. In experiment 2, L and LC cells were plated in quadruplicate. Bovine serum albumin bound linoleic acid (C18:2 n-6; 0 or 100 μM; Sigma-Aldrich Corp) was added to the culture media of two replicates from d 2 to 6 of differentiation to assess the fatty acid composition and linoleic acid uptake in adipocytes. Cell cultures without FA supplementation received equal amounts of fatty acid free-bovine serum albumin as their counterparts. In experiment 3, cell lines (n = 3 per treatment) from both treatments (L and LC) were plated in duplicate and then examined for cell size distribution, adipogenic gene expression, and lipogenic rate in culture. Pictures of the preadipocytes before differentiation and adipocytes after differentiation are shown in Figure 7. Three fluorophores were used to identify cell nucleus (POPOP™-1 iodide, Life Technologies; blue color), F-actin cytoskeleton (Alexa Fluor 488® phalloidin, Life Technologies; green color), and neutral lipids (HCS LipidTOX™ red neutral lipid stain, Life Technologies; red color). As would be expected, no lipid staining occurred in the preadipocyte cultures; however after differentiation, lipid staining is evident in both nutritional treatment groups.

Fatty acid analysis

Fatty acid composition of the intermuscular adipose tissue from the donor animals as well as cultured adipocytes was determined. Cellular FA were extracted according to Folch et al.25 and transmethylated to fatty acid methyl esters (FAME) using sodium methoxide followed by boron trifluoride.26 The FAME were analyzed using an Agilent 6850 GLC equipped with Agilent 7673A automatic sampler (Agilent Technologies, Inc.). Separations were accomplished using a 100 min SP-2560 (Supelco) fused silica capillary column (0.25 mm i.d. and 0.20 μM film thickness) according to Duckett et al.5 Individual fatty acids were identified by comparison of retention times with standards (Sigma-Aldrich; Matreya). Fatty acids were quantified by incorporating an internal standard, methyl tricosanoic (C23:0) acid into each sample during methylation and expressed as amount (ug) per well (cells) or weight percentages.

Lipogenic rate

To determine de novo lipogenesis in the adipocytes from the two nutritional treatments, 1,2-13C2 (10 mM) was supplemented in place of unlabelled acetate on d 4 and the samples harvested directly into 2:1 chloroform:methanol (v/v) to terminate the enzyme activity at 0, 6, 12, 24, and 48 h for GC-MS analysis. The cellular FAs were prepared for GC-MS analysis as described above. The enrichment of 13C16:0 were analyzed using Agilent 6890N gas chromatograph (GLC; Agilent Technologies, Inc.) equipped with 30 min Agilent 19091 S-433 (Agilent Technologies, Inc.) capillary column (0.25 mm i.d. and 0.25 μM film thickness) and Agilent 5975C mass spectrometer (MS). Peaks of mass-to-charge ratio (m/z) 270 (m), 272 (m + 2), and 274 (m + 4) were measured using selected ion monitoring to calculate the isotopic enrichment of C16:0, the primary product of de novo lipogenesis. The calculations were done using Mass Isotopomer Distribution Analysis (MIDA) according to Wolfe and Chinkes27 as detailed in our previous publications (see refs. 3 and 4). Briefly, the FA enrichment was calculated as tracer-to-
trace Ratio (TTR) by accounting for background noise and natural abundance of $^{13}$C-isotope i.e., $TTR_{16:0} = (R_{sample} – R_{blank}) \times (1 – A)^N$, where $A$ was the natural abundance of $^{13}$C, $N$ was the number of C in the fatty acid molecule, $n = 16$; RA was relative abundance. The blank samples used for background correction did not have labeled FA isotope in the media. Precursor enrichment in molar percent excess (MPE) was estimated by: MPE$\text{precursor} = \{2 \times [TTR(m + 4) / TTR(m + 2)] + [p - 1] / [2 \times [TTR(m + 4) / TTR(m + 2)]\}$ where, $p$ is the number of precursor monomers present in the polymer, $P = 8$. The estimate of lipogenesis was derived by calculating fractional synthesis rate (FSR). FSR$\text{lipogenesis} = \{TTRc_{16:0} \times \text{time1} - TTRc_{16:0} \times \text{time0}\} / \{\text{p} \times \text{MPE}_{\text{precursor}}\} - 1$. The Lipogenesis was calculated using a commercial kit (MPE). Statistical analyses

Data are presented as mean ± SEM. The mixed procedure of SAS (SAS Enterprise Guide, version 4.3, SAS Institute Inc.) was used with treatments as fixed effects and replicates as random effects. Differences in the treatments were separated using the Fisher least square significant difference pair-wise comparisons. The results were considered significant at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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