Increased adipocyte differentiation may be mediated by extracellular calcium levels through effects on calreticulin and peroxisome proliferator activated receptor gamma expression in intramuscular stromal vascular cells isolated from Hanwoo beef cattle

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ABSTRACT
Marbling is the intramuscular deposition of adipose tissue and is said to be directly associated with beef quality. Nutritional manipulations are a common practice among beef cattle producers in order to achieve better quality meat. The objective of the current study was to investigate the effects of increasing extracellular calcium on the differentiation of intramuscular stromal vascular cells (IM SVCs) isolated from Hanwoo beef cattle. Primary cell isolates of SVCs were differentiated for 14 days while exposed to increasing concentrations (1.8, 3.6, 7.2, 10.8 mM) of calcium in the media. The correlation between adipogenesis and calcium concentrations was determined through SVC differentiation monitored by RT-PCR, Western blot analysis for PPARγ, C/EBPα, calreticulin, FABP4 and GLUT4 expression. In addition, expression of both phospholipase C gamma (PLC-γ) and protein kinase C (PKC) were investigated since both have possible links to intracellular calcium increase and the expression of several adipogenic genes including FABP4 and GLUT4. Results of the current study provide evidence that stromal vascular cells exposed to the lower concentrations of extracellular calcium have higher rates of adipogenesis possibly due to a decrease in the expression of calreticulin, a known inhibitor of PPARγ expression, and PKC activation followed by PLC-γ activation, leading to the expression of adipogenic genes. Data derived from this study shows that in vitro, decreasing calcium levels present in the microenvironment of fat precursor cells lead to a higher percentage of adipogenesis.

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Introduction
Calcium is the most abundant mineral in an animal’s body and is known to be involved, and is essential, in several physiological processes such as vascular function (contraction and dilation), muscle function, transmission of nerve impulses, intracellular signalling and hormonal secretion. Most of the body’s calcium is stored in the bones and teeth since only less than 1% of the total calcium in the body is needed to drive normal physiological functions. At the cellular level, calcium is an important secondary signalling molecule involved in a wide array of cellular functions including proliferation and differentiation (Hashimoto et al. 2015). The ion is highly regulated and is thus stored in intracellular calcium pools such as the mitochondria and endoplasmic reticulum. Calreticulin is a protein implicated as having the most relevant role to calcium storage in non-muscle cells. It has been known to bind to calcium with high capacity and low affinity, a very important characteristic since the intracellular space only can store large amounts of calcium within a restricted fraction of cellular volume (Mery et al. 1996; Gold et al. 2010; Mendlovic & Conconi 2010).

Marbling, a term used in beef production, refers to all visible intramuscular fat located in between bundles of skeletal muscle fibres on the cross section of meat (Sadowski et al. 2014). The amount of adipose tissue depends primarily on the number and size of the constituent adipocytes. A greater synthesis and storage of lipid droplets in the cytoplasm is attributed to an increase in adipocytes volume (Kawachi 2006). The proliferation and differentiation of intramuscular adipose tissue occurs at 14 months of age (Hood & Allen 1973). It was also reported that during the 13th to 19th month of age there is an increase in the quantity of adipocytes in the longissimus muscle group with this phenomenon correlated with beef marbling.
In addition, it was reported that in Japanese Black steers, fattening starts at 10 months of age, continues by 20 months of age and is completed at 30 months (MAFF 2000). Microscopically, marbling fat appears in specific adipose depots wherein adipocytes are embedded in a connective tissue matrix close to a blood capillary (Albrecht et al. 2006). Intramuscular adipose tissue has direct impact on improving the quality of beef products and is the basis of quality grading (Lengi & Corl 2010; Brooks et al. 2011; Dodson et al. 2010). With the aim to produce livestock with better quality meat for consumers, intramuscular fat content has been at the centre of high quality beef production because it positively affects the sensory quality traits such as taste and flavour. It has already been a generally accepted fact that marbling positively influences flavour, juiciness, tenderness and overall acceptability of meat (Kawachi 2006; Wood et al. 2008; Hocquette et al. 2010).

Nutritional manipulations are a common practice among beef cattle producers in order to achieve better quality meat (Pethick et al. 2004; Kawachi 2006). Some experiments have already shown that micronutrient manipulation of vitamins (vitamin A, vitamin D, vitamin C and biotin) and minerals (zinc, chromium) displays positive effect on fat deposition in cattle. Since manipulations of micronutrients promotes adipocytes differentiation, some producers run on the premise that it can also be beneficial for beef production (Wulf et al. 1996; Kawachi 2006). A number of dietary factors which regulate adipocyte differentiation have already been clarified (Tori et al. 1996; Hida et al. 1998). In fact, Japanese beef cattle raisers are already extensively practicing giving low dietary Vitamin A levels to improve marbling and carcass quality of the Japanese Black cattle (Kawachi 2006). However, it is still unknown if the manipulation of dietary calcium is a useful strategy for the production of beef with excellent marbling characteristics.

The current study was undertaken to investigate the effects of increasing levels of extracellular calcium on the differentiation of intramuscular stromal vascular cells isolated from Hanwoo beef cattle.

Materials and methods

Preparation of calcium treatment

The stock solution of calcium (500 mM) was made by dissolving anhydrous calcium chloride (Samchun Chemical) in an appropriate volume of triple distilled water. The solution was then autoclaved and filtered through a 0.22 μm syringe driven filter unit (Millex). Appropriate adjustments were made in all treatments as growth media used in all experiments already contained 1.8 mM of calcium.

Animals

Intramuscular stromal vascular cells were obtained from muscle tissues of beef cattle slaughtered at a commercial abattoir. All cattle used in the experiment were Hanwoo steers aged 15 months old. Samples were collected from the longissimus capitus et atlantis muscle. The collected sample was transported to the laboratory in sterile ice-cold PBS with 2 ng/mL amphotericin B and 200 ng/mL gentamycin. Transport time from the abattoir to the laboratory was 45 min.

Primary cell isolation and culture of intramuscular stromal vascular cells

Collected tissues were aseptically minced then digested with type 1 collagenase (2 mg/mL) and bovine serum albumin (4 mg/mL) in Dulbecco’s Modified Eagle’s Medium (DMEM). The obtained cell suspension was centrifuged then filtered through a 100 μm nylon cell strainer. Primary cell isolates were then cultured in DMEM (Life Technologies) supplemented with 10% heat inactivated bovine calf serum (BCS) (Life Technologies) and Penicillin/Streptomycin (100 U/mL) and 3.7 mg/mL of NaHCO₃ at 37 °C in 5% CO₂.

Cell viability analysis

Cell counting kit-8 (CCK-8, Dojindo, Japan) was used to quantify cell viability of primary culture of IM SVCs according to the manufacturer’s instruction. Briefly, the cells were seeded at a density of 10⁴ per well and incubated in DMEM BCS at 37 °C in 5% CO₂ for 24 and 48 h. The cells were pre-treated with calcium (3.6, 7.2 and 10.8 mM) and incubated for another 24 h. After incubation, the media in the wells were suctioned and 100 μL of fresh media with 10 μL CCK-8 was added per well. The plates were then incubated at 37 °C for 2 h. Absorbance at 450 nm was measured using an ELISA plate reader (Tecan, Switzerland). Viability of the treated cells was expressed as the percentage of control cells.

Intramuscular stromal vascular cells differentiation

Primary cell isolates which were on passage 3–7 were seeded in 6-well plates at a density of 1 × 10⁵ cells/mL. Upon reaching confluence, growth medium...
was changed to DMEM 10% FBS with 1 μM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich) and 10 μg/mL insulin (Sigma Aldrich). After 48 h, the media was replaced with DMEM 10% FBS with 10 μg/mL insulin. The cells were allowed to differentiate for a total of 14 days with media replacement done every 2 days.

**Protein kinase C activity ELISA assay kit**

A non-radioactive PKC kinase activity kit (Enzo) was used to quantify the protein kinase C activity of the calcium treated IM SVCs as recommended by the manufacturer with some modifications. Cells that had undergone calcium treatment for 14 days were lysed with a protein extraction solution (Intron Biotechnology) and the protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Two micrograms of protein was used to obtain the PKC activity of the cells used in the study. The final colour development was stopped with acid stop solution and the intensity of the colour was measured by reading the optical density at 450 nm.

**RNA isolation and reverse transcriptase–polymerase chain reaction**

Total RNA was obtained from calcium-treated IM SVCs using Trizol reagent (Takara) according to the manufacturer’s instructions. One microgram of RNA was used to obtain the complementary DNA (cDNA) using the protocol provided by M-MuLV reverse transcriptase (Fermentas, Lithuania). Specific primers were used to amplify different genes depending on the cell of interest. PCR products were then separated by electrophoresis using 1.5% agarose stained with ethidium bromide and UV transillumination was done afterwards. Relative densities of bands obtained were quantified using ImageJ.

**Western blotting**

The isolation and determination of protein concentration was carried out in the same manner as described in the PKC activity assay. Separation of 30 μg of the extracted protein was carried out in 10 and 12% SDS-PAGE. Afterwards it was transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany) and incubated overnight with 5% skim milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20). The membrane was washed four times with TBST and incubated for 2 h in 2% skim milk containing the primary antibodies (monoclonal anti-PLCγ (abcam), anti-calreticulin (abcam), anti-FABP4 (abcam), anti-GLUT4 (Sta. Cruz Biotechnology) and anti-β-actin(abcam)) at 1:1000 dilution. The blot was washed with TBST buffer four times and incubated with horse radish peroxidase-labelled secondary antibody (Santa Cruz Biotechnology) diluted to 1:2000 for 2 h. The membranes were washed four times and detection was done with the use of the enhanced chemiluminescence system (ECL, Western Blot Analysis System Kit, Amersham Biosciences). Relative densities of bands obtained were quantified using ImageJ.

**Statistical analysis**

Statistical analysis was carried out using IBM SPSS Statistics Version 22. Independent variables were the different concentrations of calcium in the media while the dependent variables were the numerical results obtained from the assays performed. All experiments were done in triplicates and data were expressed as means ± SD. The difference between control and calcium-treated cells were evaluated using one-way analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT). Statistical significant differences were declared at p values less than 0.05.

**Results**

**Cell viability analysis**

Cell viability analysis (Figure 1) using CCK-8 showed that there is no significant change in the quantity of IM SVCs after exposure to increasing levels of extracellular calcium for 24 and 48 h. This data shows that...
calcium exerts neither mitogenic nor cytotoxic effects on the cells used in the study.

**Reverse transcriptase–polymerase chain reaction assay**

RT-PCR assay showed a decrease in two important adipogenic marker genes (PPAR\(_\gamma\) and CEBP/\(\alpha\)) as well as FABP4 and GLUT4 with increasing extracellular calcium concentration (Figure 2). In addition, the mRNA expression of calreticulin increased with increasing calcium concentration suggesting a role of the calcium buffering protein in the adipogenic process. Generally, an inverse relationship can be seen between PPAR\(_\gamma\) and calreticulin.

**Western blotting**

Western blot analysis also showed a decrease in the protein expression of both FABP4 and GLUT4 with increasing extracellular calcium (Figure 3). In addition, the protein expression of PLC gamma also goes down with increasing calcium concentration. The protein expression of calreticulin is seen to increase with increasing extracellular calcium treatment.

**Protein kinase C kinase activity**

Results of the PKC activity determination (Figure 4) using a commercially available kit showed a decrease in the PKC activity of the cells with increasing extracellular calcium concentration. This effect shows that PKC activity decreases with decreasing adipogenesis and increasing extracellular calcium concentration in IM SVCs.

**Discussion**

Adipogenesis is the process wherein fibroblast-like preadipocytes develop into adipocytes. This process is a highly regulated and orchestrated multistep event requiring the sequential activation of many transcription factors before full differentiation is achieved (Ali et al. 2013). It is a course dependent on the expression of several of the so-called adipogenic genes, most important of which is peroxisome proliferator activated receptor gamma (PPAR\(_\gamma\)), also called the “master regulator” of adipocytes biology (Lefterova et al. 2015; Siersbaek et al. 2010; Tontonoz & Spiegelman 2008). The increase in the expression of the said gene is known to be directly correlated with essential aspects of adipocytes biology since it is required for several processes including adipocytes differentiation, regulation of insulin sensitivity, lipogenesis and adipocytes survival and function (Lehrke & Lazar 2005). It has been shown that certain PPAR\(_\gamma\) agonists cause the failure of full expression of the adipocytes phenotype (Cariou et al. 2012). In fact, a study has shown that PPAR\(_\gamma\) is both sufficient and necessary, in itself, for the differentiation of white adipocytes (Farmer 2006). Aside from PPAR\(_\gamma\), others which also increase in expression during adipocyte differentiation are glucose transporter 4 (GLUT4), fatty acid binding protein 4 (FABP4/\(\alpha\)P2) and CCAAT enhancer binding protein alpha (CEBP/\(\alpha\)) (Gregoire et al. 1998). CEBP/\(\alpha\), most especially, is considered alongside PPAR\(_\gamma\) as an important molecule in the adipogenic transcription cascade. The current model for adipocytes differentiation suggests that the entire differentiation process involves both PPAR\(_\gamma\) and CEBP/\(\alpha\). CEBP/\(\alpha\) is known to induce PPAR\(_\gamma\) which in turn heterodimerizes with another nuclear receptor thus regulating the adipogenic program in favour of fat cell differentiation (Brey et al. 2009). What occurs afterwards is a positive feedback loop between PPAR\(_\gamma\) and CEBP/\(\alpha\) that maintains the differentiated state (Wu et al. 1999).

Results of the study have shown that exposure of the IM SVCs to increasing extracellular calcium concentration after initiation of differentiation causes a decrease in the expression of the adipogenic genes. This suggests a negative correlation between high extracellular calcium levels and the adipogenic process. This inverse relationship is further demonstrated by the increase in the mRNA and protein expression of calreticulin, a high capacity calcium buffering protein present in the lumen of the endoplasmic reticulum. Calreticulin has been shown to inhibit the commitment of 3T3-L1 cells to full adipocytes (Szabo et al. 2008). In addition, they have also stated that increasing the ER luminal calcium content causes a decrease in the full differentiation of the preadipocyte cell line (Szabo et al. 2008). It can therefore be assumed that an inverse relationship exists between calreticulin and PPAR\(_\gamma\), along with other adipogenic genes due to an increase in the extracellular calcium levels.

Phospholipase C is an important protein present on the intracellular domain of the plasma membrane. PLC\(_\gamma\) catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to diacylglycerol and inositol 1,4,5-triphosphate. The two latter molecules are known as secondary messengers involved in both the activation of protein kinase as well as the release of calcium from intracellular stores (Rhee & Bae 1997; Noh et al. 1995). It has been shown that PLC has an increased expression in fully differentiated adipocytes as compared to
Figure 2. Effect of increasing extracellular calcium on the mRNA expression of genes related to intramuscular stromal vascular cells differentiation. The PCR products visualized after electrophoresis (A) with their corresponding densitometric graphs (B) are shown. Data are means ± SE (n = 3). Bars with different superscripts are significantly different (p < 0.05).
Results of Western blot analysis have shown that at low extracellular calcium, the expression of \( \text{PLC}_c \) is higher in correlation with the increased expression of adipogenic genes. \( \text{PLC}_c \) has already been shown to be of abundance in adipocytes due to its involvement in the insulin-stimulated glucose transport within these cells. Fully differentiated adipocytes, having more insulin receptors, and thus a greater rate of glucose uptake for fat storage, have a higher \( \text{PLC}_c \) content compared to preadipocytes (Kayali et al. 1998).

Protein kinase C is a member of the serine/threonine protein kinase family known to play essential roles in the control of many cellular functions (Zhou et al. 2006). It is already known that PKC has several isoforms (-beta, -mu, -epsilon, -alpha, etc.) and the function and location of these isoforms vary depending on the type of cell or what stage of growth the cell is. It has already been

| Protein    | Extracellular calcium concentration (mM) |
|------------|------------------------------------------|
|            | 1.8 | 3.6 | 7.2 | 10.8 |
| Beta-actin | ![Image](image1)                      |
| PLC-γ      | ![Image](image2)                      |
| Calreticulin | ![Image](image3)                  |
| GLUT4      | ![Image](image4)                      |
| FABP4      | ![Image](image5)                      |

**Figure 3.** Effect of increasing extracellular calcium concentration on the expression of adipogenesis-related proteins in IM SVCs. The relative protein expression (A) and the quantitative protein expression obtained through densitometry analysis (B) are shown. Data are means ± SE (\( n = 3 \)). Bars with different superscripts are significantly different (\( p < 0.05 \)).
determined previously that PKC-epsilon promotes adipogenic commitment and is essential for the termination of the preadipocytes cell line 3T3-F442A (Webb et al. 2003). Also, it has been shown that the treatment of 3T3-L1 cells with a broad-spectrum PKC inhibitor lead to the suppression of adipogenesis (Zhou et al. 2006). Results of the PKC kinase activity kit has shown that there is a decrease in the PKC activity with increasing calcium treatment. As previously described, the occurrence of the increase in extracellular calcium concentration coincides with a decrease in adipogenesis as shown by the decreased expression of the adipogenesis-related genes used in this study.

Conclusions

Our current study has shown that exposure of primary isolates of IM SVCs to high extracellular calcium levels cause a decrease in the adipogenesis of the cells in a dose-dependent manner as seen in the results of the RT-PCR and Western blot analyses. In addition, the PLC-γ and PKC data shows that the treatments with increasing calcium also have an increase in the protein expression and activity of the aforementioned molecules, respectively. Data derived from this study shows that, in vitro, increasing the calcium levels present in the microenvironment of fat precursor cells leads to a lower percentage of adipogenesis in between muscle fibres.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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