Effects of Zinc on Lipogenesis of Bovine Intramuscular Adipocytes

Young Sook Oh and Chang Bon Choi[1,] *

Institute of Biotechnology, Yeungnam University, 214-1, Dae-dong, Gyeongsan 712-749, Korea

ABSTRACT : Zinc (Zn) is a micromineral and functions as a cofactor of many enzymes and its deficiency induces retardation of growth and dysfunction of the immune system in animals. This study was conducted to determine lipogenic activity of Zn in bovine intramuscular adipocytes. Preadipocytes were isolated from intramuscular fat depots of 26 month old Korean (Hanwoo) steers and cultured in media containing Zn. At confluence, the cells were treated with insulin, dexamethasone, and 1-methyl-3-isobutyl-xanthine to induce differentiation (accumulation of lipid droplets in cells). The sources of Zn were zinc chloride ($ZnCl_2$) and zinc sulfate ($ZnSO_4$), and the final concentrations of both Zn sources were 0, 5, 25, 50 and 100 μM. Glycerol-3-phosphate dehydrogenase (GPDH) activity, an index of adipocyte differentiation, was increased as the concentration of Zn in media increased showing the highest activity (25.74 ng/min/mg protein) at 25 μM of $ZnSO_4$. Supplementation of Zn during differentiation of bovine intramuscular adipocytes tended to decrease the production of nitric oxide (NO). Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) gene expression was increased 10 days after differentiation induction. The current results indicate that Zn has a strong lipogenic activity in cultured bovine intramuscular adipocytes with remarkable suppression of NO production. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 10 : 1378-1382)***

Key Words : Zinc, Intramuscular Adipocytes, Bovine, PPARγ2, Nitric Oxide

INTRODUCTION

Zinc (Zn) is a component of many metalloenzymes such as copper-zinc superoxide dismutase, carbonic anhydrase, alcohol dehydrogenase, carboxypeptidase, alkaline phosphatase and RNA polymerase, and affects the metabolism of carbohydrates, proteins, lipids, and nucleic acids. Typically, foraged-based diets consumed by grazing ruminants contain inadequate concentrations of copper (Cu) and zinc (Zn) to meet the animals requirement (Vierboom et al., 2003).

Recently, it has been found that Zn is involved in adipose metabolism, insulin resistance and increasing obesity. *In vitro* addition of Zn enhanced insulin-stimulated conversion of glucose into lipids of adipocytes from rats and mice (Shisheva et al., 1992; Chen et al., 1996). Tang and Shay (2001) reported that Zn could induce an increase in glucose transport into cells, probably acting through the insulin signaling pathway. Zn supplementation may increase total carcass body fat in ob/ob mice (Chen et al., 1998) and mice fed a high fat diet (Begin-Heick et al., 1985).

Zn methionine, an organic form of Zn, was reported to improve growth performance and carcass characteristics of feedlot cattle (Brethour, 1984; Spears, 1989). On the other hand, stress induction produced no differences ($p>0.05$) in apparent absorption or retention of Zn between organic and inorganic Zn sources (Nockels et al., 1993). Marbling score was increased ($p<0.05$) for steers fed Zn methionine compared to those in the control group and those receiving Zn oxide. Steers fed Zn methionine tended to have more external fat than steers fed the control diet (Greene et al., 1988). Supplemental zinc concentration in finishing diets did not influence feedlot performance and had a minimal impact on carcass quality in finishing beef steers (Malcolm-Callis et al., 2000).

Zn stimulates the differentiation of bovine preadipocytes through inhibition of nitric oxide (NO), an important paracrine factor that suppresses adipogenesis (Hino et al., 2001).

Taken together, these observations raise questions about mechanisms underlying actions of Zn on lipogenic activities of bovine adipocytes, especially intramuscular adipocytes. Thus, the objective of this study was to investigate the effects of Zn supplementation into media for adipocytes isolated from bovine intramuscular fat depots in terms of morphological changes, glycerol-3-phosphate dehydrogenase (GPDH) activity, NO production, and the expression of peroxisome proliferator-activated receptor gamma 2 (PPARγ2).

MATERIALS AND METHODS

Preparation of intramuscular adipocytes

Intramuscular adipocytes used for all assays were obtained from 26 month old Korean (Hanwoo) steers. Approximately 100 g of *M. longissimus dorsi* was taken from the 13th rib area and was immediately placed in 40°C Hank's solution. In the laboratory hood, fatty pads in *M. longissimus dorsi* were dissected with scissors, and collagenase digestion was performed for an hour. After

* Corresponding Author: Chang-Bon Choi. Tel: +8253-810-2932, Fax: +82-53-816-3637, E-mail: cbchoi@yu.ac.kr

[1] Department of Animal Science, Yeungnam University, 214-1, Dae-dong, Gyeongsan 712-749, Korea.

Received January 26, 2004; Accepted June 16, 2004

centrifugation, the suspension was filtered through a 250 μm nylon mesh in order to remove undigested tissues and other debris. Stromal-vascular (SV) cells in the pellets after centrifugation (1,500 rpm, 5 min) were suspended and washed with Dubelcco's modified Eagle's medium (DMEM) with 5% FBS and the cells were counted and seeded.

Cell culture

SV cells were seeded into 12 well or 6 well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a density of 1×10^4 cells/ml. The cells were cultured in DMEM containing 5% FBS, penicillin-streptomycin (penicillin G sodium 10,000 unit/ml and streptomycin sulfate 10,000 μg/ml), and amphotericin B (250 ng/ml) in a humidified atmosphere with 5% CO_2 and 95% air. After reaching confluence, cells were treated with 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutyl-xanthine and 10 μg/ml insulin to induce differentiation. Forty-eight hours later, cells were cultured in DMEM containing 5% FBS, 10 μg/ml insulin, and Zn for 10 days. Preliminary studies with several harvesting times of 0, 2, 4, 6, 8, 10 and 12 days showed cells were fully differentiated at day 10 of differentiation induction. The sources of Zn were zinc chloride ($ZnCl_2$) and zinc sulfate ($ZnSO_4$) and the final concentrations for each Zn source were 0, 5, 25, 50 and 100 μM. The media were changed at 48 h intervals.

Oil Red O staining and glycerol-3-phosphate dehydrogenase (GPDH) activity assay

Differentiated adipocytes were identified by the presence of lipid droplets in cytoplasm. Cells were fixed with 10% formalin in PBS and stained with 0.3% Oil Red O. Droplets of triglycerides were visualized under the microscope after staining.

To analyze GPDH activities, cells were washed twice with DMEM and lysed with homogenizing buffer containing 0.25 M sucrose, 1 M Na_2 EDTA·2 H_2O, 5 mM Tris-base, and 1 mM dithiothreitol (pH 7.4). The lysates were centrifuged at 12,500 rpm for 10 min at 4°C. The reaction mixture contained 100 mM triethanolamine-EDTA premix, 0.1 mM β-mercaptoethanol, 0.176 mM NADH, and 0.8 mM dihydroacetone phosphate. One unit of activity is expressed as the amount of enzyme causing the oxidation of 1 μmol NADH per min.

NO^-_2/NO^-_3 determinations

NO^-_2/NO^-_3 was determined by a modified method of Conrad et al. (1993). Briefly, at day 10 of differentiation, 200 μl of media from each treatment well was incubated for 1 h at 25°C with 100 μl of nitrate reductase (90 milliunits/ml), 100 μl of NADPH (0.28 mM), 100 μl of FAD (35 μM), and 200 μl of potassium phosphate buffer (0.1 M, pH 7.5). The reaction was stopped by boiling for 3 min. An equal volume (700 μl) of Griess reagent (1:1 mixture of 2% sulfanilamide in 5% H_3PO_4 and 0.2% N-(1-naphthyl) ethylenediamine dihydrochloride in water) was then added to the reduced samples and incubated at 60°C for 10 min. The NO^-_2 reacts with the Griess reagent to form a chromophore and its absorbance at 546 nm was measured in a spectrophotometer.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA from cultured cells was extracted using RNAzol-B solution (Tel-test Inc., Frendswood, USA). Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed using TGRADIENT (Biometra, T-Gradient, Germany) employing β-actin as a negative control. The synthesized cDNA was amplified by a PCR reaction using bovine primers for peroxisome proliferator-activated receptor gamma 2 (PPARγ2). The PCR primer sequences for β-actin were: 5′-ATCTGGCACCACACCTTCTACA ATGAGCTGCG-3′ (+294 to +325; gene bank X00351) and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′(+ 1,070 to +1,131; gene bank X00351)(Ponte et al., 1984) and for PPARγ2 were: 5′-GCATTTCCACTCCGCACTAT-3′ (+394 to +413; gene bank Y12420) and 5′-TATGAGAC ATCCCCACGCA-3′(+777 to +796; gene bank Y12320) (Sundvold et al., 1997). The reaction conditions were 94°C for 3 min for 1 cycle, 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min for 32 cycles, 72°C for 10 min for 1 cycle. Products were visualized with ethidium bromide on 1.2% agarose gel.

Statistical analysis

Data for GPDH were analyzed using the general linear model (GLM) of SAS (copyright 1999-2000 SAS Institute Inc. Cary, NC, USA). The significances among treatment groups were tested with statistical probabilities expressed as $p<0.005$.

RESULTS AND DISCUSSION

Supplementation of Zn in media of bovine intramuscular adipocytes caused remarkable morphological changes determined by increases in the number and size of Oil Red O stained lipid droplets in cytosol (Figure 1). Both $ZnCl_2$ and $ZnSO_4$ showed stimulatory effects on triglyceride synthesis in bovine intramuscular adipocytes.

Regardless of its source, supplementation of Zn into media of bovine intramuscular adipocytes during differentiation significantly ($p<0.05$) increased GPDH activities (Figure 2), especially at 50 μM concentration of

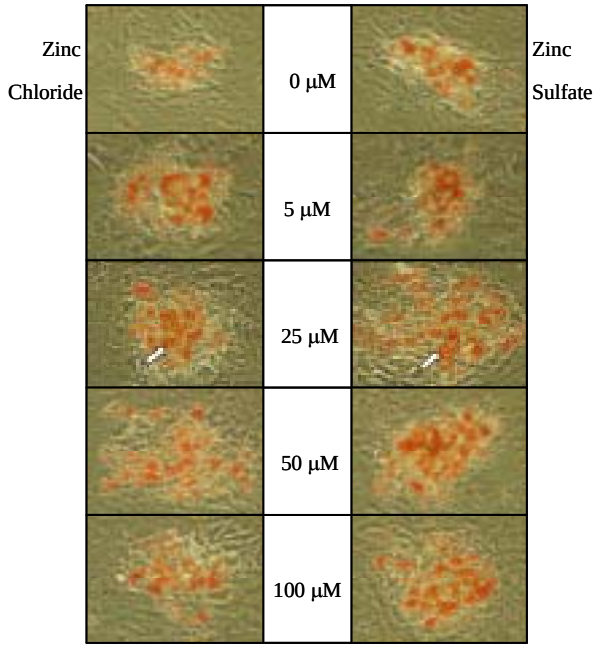

Figure 1. Changes in morphology in adipocytes isolated from intramuscular fat depots of Korean (Hanwoo) cattle and induced to differentiate with insulin, dexamethasone, and 1-methyl-3-isobutyl-xanthine for 10 days. Zinc chloride and zinc sulfate were added at the concentrations of 0, 5, 25, 50 and 100 μM, respectively. The cells were fixed in 10% formalin and stained with 0.3% Oil Red O. Arrows indicate representative lipid droplets (×200).

$ZnCl_2$ and at 25 μM concentration of $ZnSO_4$, respectively.

Zn has some insulin-like effects, such as enhancing lipogenesis and glucose transport. Glucose transport assays using 3T3-L1 preadipocyte revealed that Zn could induce an increase in glucose transport into cells (Tang and Shay, 2001). Hino et al. (2001) reported that Zn was able to enhance insulin-induced adipogenesis in 3T3-L1 and bovine stromal-vascular (SV) cells prepared from perirenal adipose tissue.

Zn might express its lipogenic activity via suppression of NO production. In the current study, the concentration of NO at 10 days after differentiation induction of bovine intramuscular adipocytes tended to reduce as the concentration of both $ZnCl_2$ and $ZnSO_4$ in media increased (Figure 3).

These insulin-like effects of Zn resulted from an insulin-independent mechanism, because Zn does not inhibit lipolysis in isolated adipocytes (Shisheva et al., 1992). Hino et al. (2001) suggested an alternative mechanism underlying stimulatory effects of Zn on lipogenesis by showing reduced NO concentration in the media during culture of bovine SV cells with supplementation of Zn.

The results in this study indicate that Zn expresses its stimulatory effects on lipogenesis of bovine intramuscular adipocytes by suppressing NO production (May and

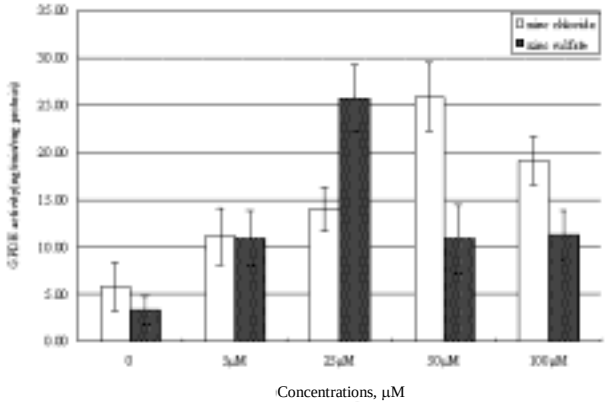

Figure 2. Effect of zinc on glycerol-3-phosphate dehydrogenase activity in adipocytes isolated from intramuscular fat depots of Korean (Hanwoo) cattle and induced to differentiate with insulin, dexamethasone, and 1-methyl-3-isobutyl-xanthine for 10 days. Zinc chloride and zinc sulfate were added at the concentrations of 0, 5, 25, 50 and 100 μM, respectively. Values are mean±SE (n=5). [a, b] Bars with different alphabet in either zinc chloride or zinc sulfate group are significantly ($p<0.05$) different.

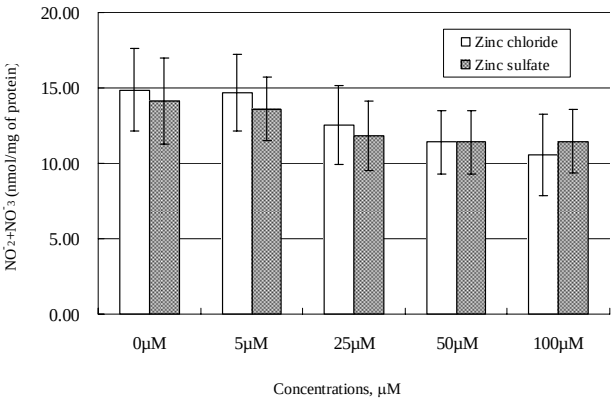

Figure 3. Effect of zinc on the production of NO^-_2 and NO^-_3 in adipocytes isolated from intramuscular fat depots of Korean (Hanwoo) cattle and induced to differentiate with insulin, dexamethasone, and 1-methyl-3-isobutyl-xanthine for 10 days. Zinc chloride and zinc sulfate were added at the concentrations of 0, 5, 25, 50 and 100 μM, respectively. Values are mean±SE (n=5). $p>0.05$.

Contoreggi, 1982; Nisoli et al., 1998).

Changes occur in the expression of transcriptional factor, PPARγ2, during differentiation of bovine intramuscular adipocytes treated with either $ZnCl_2$ or $ZnSO_4$. Both $ZnCl_2$ and $ZnSO_4$ enhanced PPARγ2 expression when these Zn sources were added to differentiation media at 50 and 100 μM of concentration (Figure 4). These changes indicate that Zn may cause the increase in lipid accumulation in the cytosol by enhancing the expression of transcription factor and then genes responsible for the synthesis of triglyceride

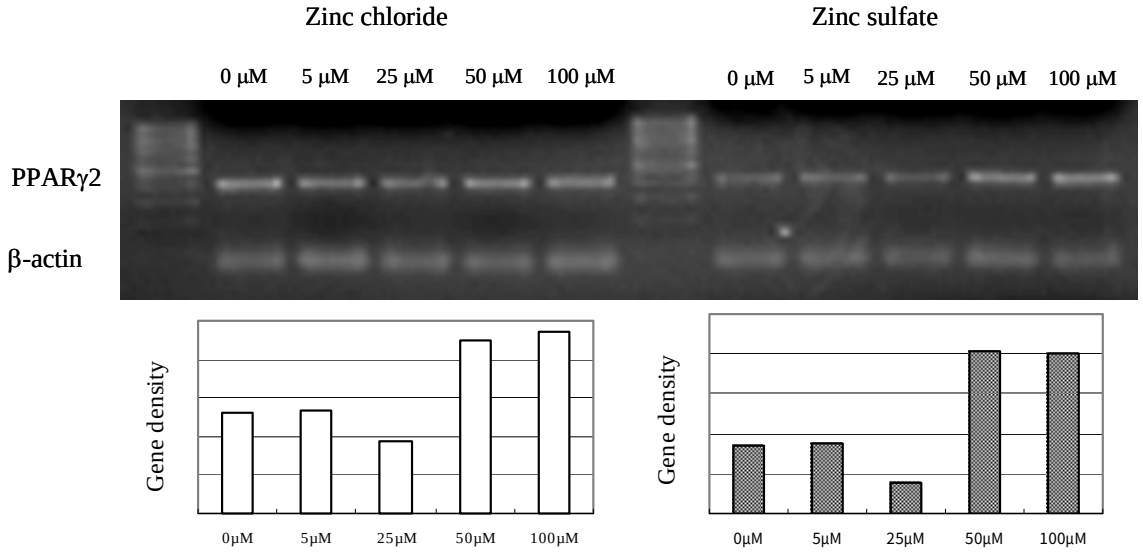

Figure 4. Changes in the expression of peroxisome proliferator-activated receptor gamma 2 (PPARγ2) gene in adipocytes isolated from 26 months old Korean (Hanwoo) cattle and induced to differentiate with insulin, dexamethasone and 1-methyl-3-isobutyl-xanthine for 10 days. Zinc chloride and zinc sulfate were added at concentrations of 0, 5, 25, 50 and 100 μM, respectively. Total RNA was extracted from differentiated adipocytes and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed according to "Materials and methods".

such as lipoprotein lipase.

It has been known for years that PPAR activators, such as fibrates and fatty acids, induce adipocyte differentiation, and recent results show that PPARγ2 regulate several highly specialized proteins during adipocyte differentiation (Tontonoz et al., 1994,1995). PPARγ2 agonist-mediated differentiation includes lipid accumulation and expression of many endogenous genes characteristic of adipocyte (Hu et al., 1995).

ACKNOWLEDGEMENTS

This study was supported by the Yeungnam University special research grants in 2003.

REFERENCES

Begin-Heick, N., M. Dalpe-Scott, J. Rowe and H. M. Heick. 1985. Zinc supplementation attenuates insulin secretory activity in pancreatic islets of the ob/ob mouse. Diatetes. 34:179-184.

Brethour, J. R. 1984. Zinc methionine in steer finishing rations. Report of Progress No. 452. Hays Branch, Kansas State University. p. 11.

Chen, M., P. Lin, V. Cheng and W. Lin. 1996. Zinc supplementation aggravates body fat accumulation in genetically obese mice and dietary-obese mice. Biol. Trace Elem. Res. 52:125-132.

Chen, M. D., S. J. Liou, P. Y. Lin, V. C. Yang, P. S. Alexander and W. H. Lin. 1998. Effects of zinc supplementation on the plasma glucose level and insulin activity in genetically obese (ob/ob) mice. Biol. Trace Elem. Res. 61:303-311.

Conrad, K. P., G. M. Joffe, H. Kruszyna, R. Kruszyna, L. G. Rochelle, R. P. Smith, J. E. Chavez and M. D. Mosher. 1993. Identification of increased nitric oxide biosynthesis during pregnancy in rats. FASEB J. 7(6):566-571.

Greene, L. W., D. K. Lunt, F. M. Byers, N. K. Chirase, C. E. Richmond, R. E. Kuntson and G. T. Schelling. 1988. Performance and carcass quality of steers supplemented with zinc oxide or zinc methionine. J. Anim. Sci. 66:1818-1823.

Hill, G. M., G. L. Cromwell, T. D. Crenshaw, C. R. Dove, R. C. Ewan, D. A. Knabe, A. J. Lewis, G. W. Libal, D. C. Mahman, G. C. Shuron and T. L. Veum. 2000. Growth promotion effects and plasma changes from feeding high dietry concentrations of zinc and copper to weaning pigs (regional study). J. Anim. Sci. 78:1010-1016.

Hino, N., S. Tanaka, T. Matsui and H. Yano. 2001. Zinc enhances bovine preadipocyte differentiation through inhibition of nitric oxide production. Reproductive Biotechnology 452:385-389.

Hu, E., P. Tontonoz and B. M. Spiegelman. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factor PPARγ and C/EBPα. Proc. Natl. Acad. Sci. 21:950-958.

Malcolm-Callis, K. J., G. C. Duff, S. A. Gunter, E. B. Kegley and D. A. Vermeire. 2000. Effects of supplemental zinc concentration and source on performance, carcass characteristics, and serum values in finishing beef steers. 78:2801-2808.

May, J. M. and C. S. Contoreggi. 1982. The mechanism of the insulin-like effects of ionic zinc. J. Biol. Chem. 257:4362-4368.

Nisoli, E., E. Clementi, C. Tonello, C. Sciorati, L. Briscini and M. O. Carruba. 1998. Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures. Br. J. Pharmacol. 125:888-894.

Nockels, C. F., J. DeBonis and J. Torrent. 1993. Stress induction affects copper and zinc balance in calves fed organic and inorganic copper and zinc sources. J. Anim. Sci. 71:2539-2545.

Ponte, P., S. Y. Ng, J. Engle, P. Gunning and L. Kedes. 1984. Evolutionary Conservation in the Untranslated Regions ofo Action mRNAs:DNA Sequence of a Human Beta-action cDNA.

Shisheva, A., D. Gegel and Y. Shechter. 1992. Insulin-like effects of zinc ion *in vitro* and *in vivo*. Preferential effects on desensitized adipocytes and induction of normoglycemia in streptozocin-induced rats. Diabetes. 41:982-988.

Spears, J. W. 1989. Zinc methionine for ruminants: relative bioavailability of zinc in lambs and growth and performance of growing heifers. J. Anim. Sci. 67:835-843.

Sundvold, H., A. Brzozowska and S. Lien. 1997. Characterisation of bovine peroxisome proliferator-activated receptor γ1 and γ2: genetic mapping and differential expression of the two isoforms. Biochem. Biophy. Res. Commun. 239:857-861.

Tang, X. and N. F. Shay. 2001. Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes. J. Nutr. 13:1414-1420.

Tontonoz, P., E. Hu and B. M. Spiegelman. 1994. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. 79:1147-1156.

Tontonoz, P., E. Hu, J. Devine, E. G. Beale and B. M. Spiegelman. 1995. PPAR-γ-2 regulates adoipose expression of the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. 15:351-357.

Vierboom, M., T. E. Engle and C. V. Kimberling. 2003. Effects of gestational staus on apparent absorption and retention of copper and zinc in mature angus cows and suffolk ewes. Asian-Aust. J. Amin. Sci. 16(4):515-518.